Pre-Conceptional Exposure to Glyphosate Affects the Maternal Hepatic and Ovarian Proteome

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ABSTRACT

Exposure to glyphosate (GLY), a commonly used herbicide, is supported by urinary detection and associated with shortened gestation in women. This study tested the hypothesis that chronic low-dose pre-conceptional GLY exposure would affect maternal ovarian function mid- and post-gestation. Mice (C57BL/6; $n = 40$) were exposed *per os* to saline vehicle control (CT; $n = 20$) or GLY (2 mg/kg; $n = 20$) daily for 10 weeks starting at 7 weeks of age. Post-exposure, females were impregnated and euthanized at gestation day 14 (GD14) or post-weaning (PW). Pregnancy success was reduced from 75% to 55% by GLY exposure. No treatment effect ($p > .05$) on body weight, maternal serum 17β-estradiol, or litter size was noted. Ovarian weight was unaffected or reduced ($p < .05$) by GLY in GD14 and PW dams, respectively. Exposure to GLY decreased ($p < .05$) PW ovarian secondary follicle number with no other follicle composition impacts. Protein abundance analysis by LC-MS/MS identified that GLY altered ($p < .05$) 26 ovarian and 41 hepatic proteins in GD14 dams and 39 hepatic proteins in PW dams. In GD14 dams, GLY increased ovarian protein abundance of SEC16A ($p < .05$; 29-fold) and hepatic RPS27L and GM4952 ($p < .05$; ~4-fold). In both GD14 and PW dams, GLY exposure increased ($p < .05$) hepatic RPS4 and decreased ($p < .05$) ECHDC3. Pathway analysis using DAVID identified 10 GLY hepatic pathway targets with FDR ≤ 0.07 in GD14 dams.

Key words: glyphosate; ovary; proteome; pregnancy.

Ovotoxicant exposures compromise the ovary in a variety of ways including but not limited to, depletion of primordial germ cells (Borman *et al.*, 2000; Hoyer and Keating, 2014; Mattison and Schulman, 1980), primordial follicle growth hyperactivation (Keating *et al.*, 2011), induction of DNA damage (Ganesan *et al.*, 2013), and alteration to follicular growth and viability (Hoyer, 2001) at each stage of folliculogenesis. In addition, ovotoxicants can affect steroidogenesis and result in premature follicular loss (Krarup, 1969) and early menopause as a consequence (Hoyer, 2001; Hoyer and Keating, 2014). Additionally, negative impacts on pre- and peri-pubertal sexual maturation in exposed animals are documented (Hatch *et al.*, 2011; Özen and Darcan, 2011; Yang *et al.*, 2016). Further, ovotoxicant exposure during fetal development can harm offspring germ cell number and quality (Brieño-Enríquez *et al.*, 2015; Johansson *et al.*, 2016).

Glyphosate (GLY) is a widely used herbicide in urban and rural environments (Gillezeau *et al.*, 2019). Since the mid-1990s,

the introduction of GLY-resistant crops resulted in a dramatic increase in GLY agricultural usage for weed control (Benbrook, 2016). Commercially, GLY salts are mixed with surfactants to increase penetrative capacity in plants (Benbrook, 2016) and referred to as GLY-based herbicides (GBHs). Both GLY and GBHs are also broadly utilized for weed control in home gardens, railways, and roadways.

The presence of GLY residues in food stuffs (Bøhn *et al.*, 2014; Granby *et al.*, 2003; Zoller *et al.*, 2018, 2020) and in human urine (Lemke *et al.*, 2021; Mills *et al.*, 2017; Soukup *et al.*, 2020) supports human GLY exposure. A review of human GLY studies from Europe and the United States concluded lack of GLY health risk based upon the level of exposure being lower than the proposed acceptable daily intake established by the European Food Safety Authority (0.5 mg/kg/body weight) (Niemann *et al.*, 2015). The level of GLY and/or the main metabolite aminomethylphosphonic acid (AMPA) concentrations in human urine samples

relative to known ingested concentrations determined that approximately 1%–6% of ingested GLY was recoverable in urine (Faniband et al., 2021), whereas GLY is readily excreted through feces and urine (Williams et al., 2000).

In rodents, GLY exposure increased atretic ovarian follicle number and decreased antral follicle number at doses of 126 or 315 mg/kg/day (Hamdaoui et al., 2018). Increased granulosa and theca cell proliferation and decreased follicle-stimulating hormone receptor and growth and differentiation factor 9 mRNA level at a GLY dose of 2 mg/kg in lambs has been observed (Alarcón et al., 2019). In contrast, no observable effects of 5 or 10 weeks of 2 mg/kg GLY exposure on follicle number or other ovarian endpoints have been reported in mice (Ganesan et al., 2020). Further, a GLY dose response study determined no effect of 20 weeks of GLY (0.25–1.5 mg/kg) exposure on follicle number with the exception of the upper 2 mg/kg dose, in which increased follicle number relative to control treated mice was noted, in the absence of an effect on estrous cyclicity in mice (Ganesan and Keating, 2020). Exposure to 500 µM GLY impaired oocyte maturation through reduced germinal vesicle breakdown proposed to be due to increased reactive oxygen species generation (Zhang et al., 2019). Interestingly, exposure to 0.25–2 mg/kg GLY altered ovarian proteins with mitochondrial and oxidative stress response functions (Ganesan and Keating, 2020). The F2 offspring of F1 rats exposed both perinatally and during lactation to GBH (2 or 200 mg/kg) had delayed fetal growth and congenital anomalies at the higher GBH dose (Milesi et al., 2018). In humans, GLY urinary concentration of pregnant women was correlated with shortened gestation length (Lesseur et al., 2022; Parvez et al., 2018). Thus, there is rationale for consideration of GLY as a reproductive toxicant.

Based upon evidence of GLY-induced alterations to ovarian and reproductive endpoints and the recognized ability of toxicant exposures to have multi- and trans-generational effects (Milesi et al., 2021; Patisaul, 2021), this study investigated the hypothesis that GLY exposure would affect ovarian endpoints in dams due to pre-conceptional exposure. An exposure level of 2 mg/kg/day was chosen, based upon lack of observable effects of this dose on reproductive and developmental endpoints by the Environmental Protection Agency (ATSDR, 2020). An exposure duration of 70 days was conducted to encompass an entire round of folliculogenesis (Hoyer, 2001) to evaluate if GLY exposure affected ovarian follicular composition and the maternal liver and ovary proteome.

MATERIALS AND METHODS

Reagents. GLY (CAS no. 1071-83-6), Eosin Y, EDTA, paraformaldehyde (PFA), Tween20, 2-β-mercaptoethanol, Tris base, Tris HCL, and sodium chloride were purchased from Sigma-Aldrich Inc. (St Louis, Missouri). Mayer's Hematoxylin (F380-2) was purchased from Rowley Biochemical Inc. (Danvers, Massachusetts). Sodium citrate, glycerol, citric acid, and Pierce BCA protein assay kit were from Thermo Fisher Scientific.

Animal dosing and breeding. All animal experiments were approved by the Institutional Animal Care and Use Committee at Iowa State University. Female C57Bl/6 mice were purchased from Jackson Laboratories aged 6 weeks. After 1 week of acclimation, mice were randomly split into 2 groups: one group received saline *per os* from a pipette tip as vehicle control (CT; $n = 20$) and the other group received GLY (2 mg/kg; $n = 20$) diluted in saline once per day for 10 weeks by the same route. Mice were maintained under controlled room temperature (21°C–22°C) and 12 h light: dark cycles with *ad libitum* access to food and water.

Food intake and body weight were measured weekly. Food consumption was calculated as the mean chow (g) eaten per cage per week.

After 10 weeks of CT or GLY exposure, females were housed with C57Bl/6 males for approximately 5 days and the appearance of a vaginal plug was monitored daily. The monitoring of the vaginal plug observance was designated as gestational day 1 (GD1). The pregnancy success for each group was calculated as: the number of pregnant dams as a percent of the total number of females housed with males. At GD14, CT and GLY females ($n = 10$ per treatment) were euthanized for tissue collection and embryo enumeration and designated as GD14. The remainder of the pregnant dams ($n = 10$ per treatment) completed pregnancy and lactation and were euthanized 1-week post-weaning and designated as PW. All animals in the study were euthanized by CO_2 asphyxiation followed by cervical dislocation.

Tissue collection. Liver, spleen, and ovaries were collected from GD14 and PW dams, weighed and the liver and ovary were immediately either snap frozen in liquid nitrogen or fixed in 4% PFA overnight at 4°C for histological analysis. One ovary was frozen and stored at −80°C for protein analysis, and another was fixed in 4% PFA for hematoxylin and eosin staining. Blood was collected by cardiac puncture, stored on ice and centrifuged for 15 min at 10 000 rpm for serum separation.

Serum 17β-estradiol and progesterone hormone level quantification. The level of 17β-estradiol and progesterone in serum was quantified by the Ligand Assay & Analysis Core of the Center for Research in Reproduction, University of Virginia with 2 technical replicates per sample.

Histological analysis. Ovaries were fixed in 4% PFA for 24 h, transferred to 70% ethanol, and then paraffin embedded. One ovary from each mouse was sectioned (5 µM thickness) and every 6th section was mounted and stained with hematoxylin and eosin. Oocyte-containing follicles and corpora lutea (CLs) were identified and counted in every 12th section. Healthy follicles were classified and counted as described previously (Flaws et al., 1994). Atretic follicles were differentiated from healthy follicles by the appearance of pyknotic bodies and intense eosinophilic staining of oocytes.

LC-MS/MS analysis. Ovarian and liver tissue homogenates were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS). Briefly, protein extracts were reduced with dithiothreitol, with the cysteine groups modified with iodoacetamide, and digested overnight with trypsin/Lys-C. The addition of formic acid stopped digestion before drying down samples in a SpeedVac. Samples were desalted using C18 MicroSpin Columns (Nest Group SEM SS18V) before drying again in a SpeedVac. Peptide retention time calibration (PRTC) standard (Pierce part no. 88320) was spiked into the sample to serve as an internal control. The peptides were then separated by liquid chromatography and analyzed by MS/MS by fragmenting each peptide using a Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific). The resulting intact and fragmentation pattern was compared with a theoretical fragmentation pattern (from either MASCOT or Sequest HT) for protein identification. Label-free quantification technique used the Minora Feature Detector to detect and quantify isotopic clusters. The PRTC areas were used to normalize the data between samples. Proteins were identified by searching databases with both MS and MS/MS data using Mascot software as described previously (Xia and Wishart, 2016).

Statistical analysis. Statistical analysis was conducted by unpaired *t* test using Prism 9.0.1 software (GraphPad Prism). Statistical significance was defined as $p < .05$. For the pathway analyses based on LC-MS/MS results, proteins that differed from CT at $p < .1$ were included. Biological pathways altered by GLY exposure were predicted using DAVID v 6.8 software. The number for "Gene Found" represented the number of genes identified from proteomics analyses. The number for "Gene Pathway" represented the total number of genes in the particular pathway as found in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database. The false discovery rate (FDR) for every presented pathway was also calculated using DAVID v 6.8 software. The results in barcharts are represented mean ± standard error of mean (SEM).

Samples identification methods. The sample identity for follicle counting, 17β-estradiol, progesterone, and proteomic analyses was not known to the investigator conducting the analyses.

RESULTS

Influence of GLY Pre-Conceptional Exposure on Body Weight

The mean body weight for females at the end of 10 weeks of dosing before mating with males did not differ for GLY-exposed females ($p > .05$; CT = $20.25 ± 0.3$ g; GLY = $21 ± 0.4$ g). The food eaten per cage per week during the dosing period also did not differ between CT and GLY groups. Pre-conceptional GLY exposure did not affect body weight at GD14 ($p > .05$; CT = $29.07 ± 1.1$ g; GLY = $31.07 ± 0.5$ g; Figure 1A) or PW ($p > .05$; CT = $24.30 ± 0.5$ g, GLY = $23.73 ± 1.1$ g; Figure 1B).

Effect of Pre-Conceptional GLY Exposure on Pregnancy Success

Pregnancy success in CT mice was 75% with 15 out of 20 females conceiving and delivering offspring (data not shown). In GLY-exposed mice, pregnancy success was reduced to 55% with 11 of 20 females delivering offspring (data not shown).

Impact of Pre-Conceptional GLY Exposure on Organ Weight

There was no difference ($p > .05$) in liver or spleen weight in GLY exposed relative to CT mice at GD14 (Figs. 2A and 2B) or PW (Figs. 2C and 2D). Ovarian weight did not differ between treatment at GD14 ($p > .05$; CT = $0.013 ± 0.001$ g; GLY = $0.011 ± 0.001$ g; Figure 3A). Ovarian weight was lower in GLY-exposed PW dams ($p < .05$; CT = $0.013 ± 0.0008$ g; GLY = $0.009 ± 0.0007$ g; Figure 3B).

Impact of Pre-Conceptional GLY Exposure on Circulating Serum 17β-Estradiol and Progesterone Levels

The levels of 17β-estradiol and progesterone were measured only in the serum obtained from the GD14 dams due to a low volume of serum obtained from PW dams. There was no treatment effect on serum 17β-estradiol ($p < .05$; CT = $12.14 ± 3.2$ pg/ml; GLY = $10.61 ± 1.4$ pg/ml; Figure 4A) or progesterone ($p < .05$; CT = $12.7 ± 3.2$ ng/ml; GLY = $15.5 ± 2.5$ ng/ml; Figure 4B) at GD14.

Effect of Pre-Conceptional GLY Exposure on Litter Size

The mean number of embryos per litter was similar for GD14 GLY exposed compared with CT dams ($p > .05$; CT = $8.6 ± 0.5$; GLY = $7.3 ± 0.7$; Figure 5A). The mean number of pups born alive per litter did not differ between CT and GLY exposed dams ($p > .05$; CT = $7.3 ± 0.6$; GLY = $5.8 ± 0.9$; Figure 5B).

Influence of Pre-Conceptional GLY Exposure on Ovarian Follicle Number and Classification

The number of CL and atretic follicles were counted in GD14 ovaries. There was no effect of pre-conceptional GLY exposure on the number of CL ($p > .05$; CT = $3.6 ± 0.3$; GLY = $3.8 ± 0.4$; Figure 6A) or atretic follicles ($p > .05$; CT = $61.4 ± 14.5$; GLY = $73.0 ± 14.1$; Figure 6B). In PW dams, the number of primordial (Figure 7A), primary (Figure 7B), pre-antral (Figure 7D), antral (Figure 7E), CL (Figure 7F), and atretic follicles (Figure 7G) did not differ ($p > .05$) between CT and GLY exposed dams. The number of secondary follicles (Figure 7C) were lower ($p < .05$; CT = $9 ± 0.8$; GLY = $5 ± 0.6$) in the ovaries of GLY exposed relative to CT dams.

Effect of Pre-Conceptional GLY Exposure on GD14 Ovarian and Hepatic Proteome

In GD14 mice, GLY exposure altered ($p < .05$) the abundance of 26 ovarian proteins, relative to CT mice (Table 1) with an FDR > 0.05. Out of those, the abundance of 2 proteins was increased and 4 proteins was decreased with a fold change > 2 in GLY compared with CT dams (Figure 8A). Protein transport protein sec16 (SEC16A) was increased 29-fold by GLY exposure. Pathway analyses of the ovarian altered proteins performed with DAVID did not identify possible targets with FDR < 0.1.

In the liver, GLY exposure altered ($p < .05$) the level of 41 proteins relative to CT mice (Table 2) with FDR > 0.05. A hepatic steroidogenic protein, 17-beta-hydroxysteroid dehydrogenase 13 (HSD17B13) was decreased by 0.6-fold. Proteins involved in xenobiotic metabolism were altered in livers of GLY-exposed dams and included glutathione S-transferase alpha (GSTA5; 1.6-fold increase), cytochrome P450 isoform 2C40 (CYP2C40; 0.8-fold decrease), sulfotransferase 1A1 (SULT1A1; 1.3-fold increase), cytochrome P450 isoform 2C37 (CYP2C37; 0.5-fold decrease), and cytochrome P450 isoform 2D10 (CYP2D10; 0.9-fold decrease; Table 2). Within the proteins that were altered by GLY exposure, hepatic protein abundance of 4 proteins increased and 4 decreased with a fold change >2 compared with CT dams (Figure 8B). Both 40S ribosomal protein S27-like (RPS27) and Glycine N-acyltransferase-like (GM4952) proteins were increased around 4-fold compared with CT dams.

For the GD14 hepatic proteome changes induced by GLY exposure, enrichment analysis identified 10 pathways with FDR < 0.07: chemical carcinogenesis, linoleic acid metabolism, steroid hormone biosynthesis, retinol metabolism, arachidonic acid metabolism, ribosome, inflammatory mediator regulation of TRP channels, metabolic pathways, serotonergic synapse, and Parkinson's disease (Table 3).

Impact of Pre-Conceptional GLY Exposure on the Hepatic Proteome of PW Dams

GLY-exposed PW dams had alterations ($p < .05$) to the abundance of 39 hepatic proteins relative to CT mice (Table 4; FDR > 0.05), including the xenobiotic biotransformation proteins cytochrome P450 isoform 2D22 (CYP2D22; 0.8-fold decrease) and dimethylaniline monooxygenase 1 (FMO1; 0.9-fold decrease). The abundance of 4 proteins were increased and 3 proteins were decreased by GLY exposure with a fold change > 2 (Figure 9). Pathway analyses of the hepatic proteome performed with DAVID platform did not identify targets with FDR < 0.1.

DISCUSSION

The potential risks of GLY chronic pre-conceptional exposure on ovarian follicle composition and the proteome of the ovary and liver both during and after pregnancy was investigated in
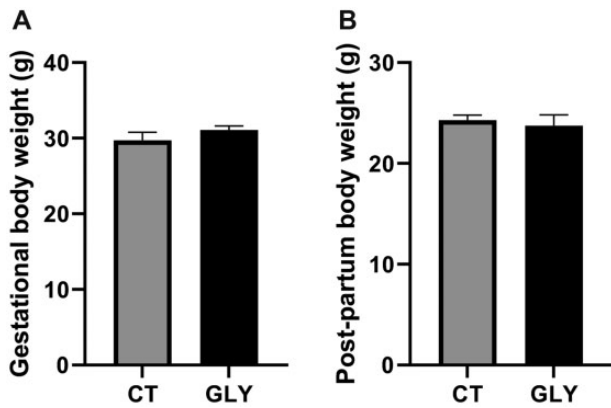
Figure 1. Impact of pre-conceptional GLY exposure on GD14 and PW female body weight. Female C57Bl/6 mice were exposed to saline vehicle control (CT) or GLY (2 mg/kg) pre-conceptionally for 10 weeks daily *per os*. A, GD14 dam weight ($n = 7$ for both CT and GLY). B, PW dam body weight ($n = 8$ for CT, $n = 3$ for GLY). Data points represent mean ± SEM.
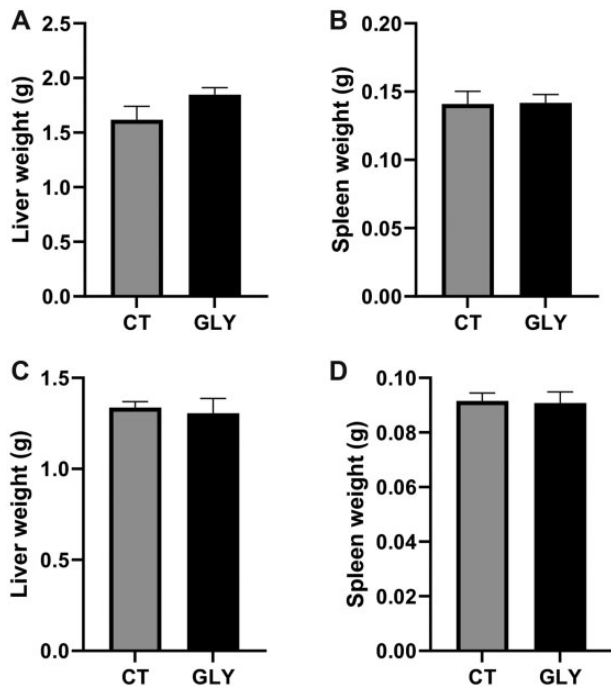

Figure 2. Impact of pre-conceptional GLY exposure on female organ weight. Female C57Bl/6 mice were exposed to saline vehicle control (CT) or GLY (2 mg/kg) pre-conceptionally for 10 weeks daily *per os*. (A) Liver and (B) spleen weight in GD14 dams ($n = 7$ for both CT and GLY). (C) Liver and (D) spleen weight in PW dams ($n = 8$ for CT, $n = 3$ for GLY). Data points represent mean ± SEM.
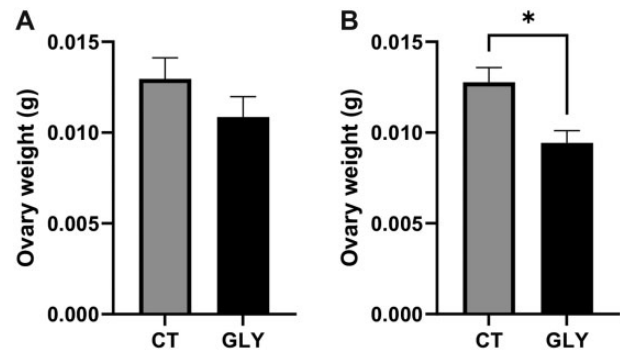

Figure 3. Impact of pre-conceptional GLY exposure on GD14 and PW ovarian weight. Female C57Bl/6 mice were exposed to saline vehicle control (CT) or GLY (2 mg/kg) pre-conceptionally for 10 weeks daily *per os*. Ovarian weight of (A) GD14 ($n = 7$ for both CT and GLY) and (B) PW dams ($n = 8$ CT, $n = 3$ for GLY). Data points represent mean ± SEM. Asterisk represents difference from CT; $p < .05$.
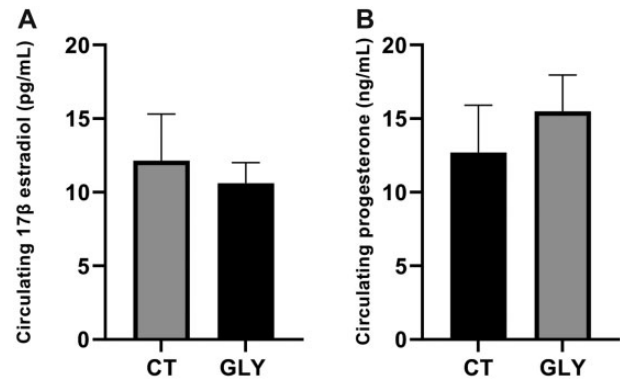

Figure 4. Impact of pre-conceptional GLY exposure on circulating 17β-estradiol and progesterone in GD14 dams. Female C57Bl/6 mice were exposed to saline vehicle control (CT) or GLY (2 mg/kg) pre-conceptionally for 10 weeks daily *per os*. (A) 17β-estradiol ($n = 7$ for both CT and GLY) and (B) progesterone level ($n = 3$ for CT, $n = 5$ for GLY). Data points represent mean ± SEM.
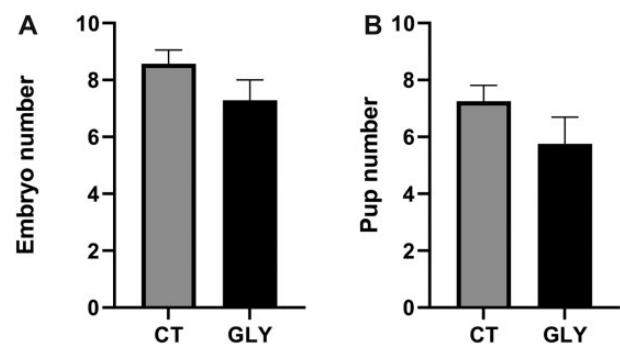

Figure 5. Effect of pre-conceptional GLY exposure on litter size. Female C57Bl/6 mice were exposed to saline vehicle control (CT) or GLY (2 mg/kg) pre-conceptionally for 10 weeks daily *per os*. A, The mean number of embryos per litter at GD14 ($n = 7$ for both CT and GLY). B, The mean number of pups born alive per litter ($n = 8$ for CT, $n = 4$ for GLY). *N* represents the litter number. Data points represent mean ± SEM.

this study. GLY's widespread agricultural usage became possible due to development of GLY-resistant crops in the early 1990s (Benbrook, 2016). By 2014, approximately 240 million pounds were sprayed annually on U.S. agricultural fields (Benbrook, 2016). Excretion of GLY and its main metabolite, AMPA, have been detected in human urine (Connolly *et al.*, 2018; Curwin *et al.*, 2007; Faniband *et al.*, 2021; Knudsen *et al.*, 2017). In commercial formulations, GLY is mixed with surfactants to increase its penetrative qualities to inhibit weed growth (Mesnage *et al.*, 2019). The reproductive risks of GBHs have been assessed in human (Arbuckle *et al.*, 2001; Bell *et al.*, 2001; Farr *et al.*, 2004; Garry *et al.*, 2002; Savitz *et al.*, 1997),

rodent (Dallegrave *et al.*, 2003; Guerrero Schimpf *et al.*, 2017; Kubsad *et al.*, 2019; Lorenz *et al.*, 2019, 2020; Ren *et al.*, 2018), and ruminant (Alarcón *et al.*, 2019) studies, though the dosage of GLY or GBHs varies widely between studies. Lack of overt ovotoxicity of GLY at a concentration of 2 mg/kg for 5 or 10 weeks of exposure has been reported (Ganesan *et al.*, 2020).

However, the same dose impacted the ovarian proteome after a longer exposure paradigm of 20 weeks with particular effects on mitochondrial and oxidative stress proteins. Additionally, a higher number of ovarian follicles present within the ovary were noted implicating the potential of GLY to affect ovarian function (Ganesan and Keating, 2020). Whether these impacts resulted in fertility consequences remain unclear.

In this study, the effects of a chronic pre-conceptional exposure of GLY on maternal ovarian follicle number and the ovarian and hepatic proteome were investigated. The administration route chosen mimicked both oral and chronic exposure. The dose was chosen based upon previous studies by our group

and others (Alarcón et al., 2019; Ganesan and Keating, 2020; Ganesan et al., 2020) and the duration of 70 days encompassed an entire round of folliculogenesis (Butcher et al., 1974; Rajkovic et al., 2006).

Though previous studies report a negative impact of GLY exposure on body weight (Ait Bali et al., 2017; Jasper et al., 2012; Yousef et al., 1995), a difference in the weight gain between CT and GLY exposed mice after 10 weeks of exposure was not demonstrated. A similar lack of an effect of 2 mg/kg GLY on female body weight after 5 or 10 weeks of exposure was also shown in our previous studies (Ganesan et al., 2020). Additionally, there was no impact of GLY exposure on food intake, with the caveat that the food intake measurements were per cage rather than total caloric intake per individual mouse. Thus, no evidence of an impact of glyphosate exposure on food consumption or weight gain was observed.

A reduction in pregnancy was noted due to GLY exposure. The mice were approximately 17 weeks of age at the time of breeding, thus, this age could influence pregnancy outcomes independent of chemical exposure. Although difficult to conclude if pregnancy loss was increased from this study design, a negative impact on uterine morphology and decidualization was reported previously in female rats that were exposed to 2 mg/kg of GBH neonatally (Ingaramo et al., 2016), potentially affecting pregnancy success. An association between urinary GLY level and shortened gestation (Lesseur et al., 2022; Parvez et al., 2018) and oxidative stress (Eaton et al., 2022) has also been reported in women. Previous studies reported a GLY impact on maternal weight (Ren et al., 2018; Teleken et al., 2020), however this was not noted in the current study in either the mid-gestation or post-partum mice. There was also no impact of GLY on liver or spleen weight. Interestingly, ovarian weight was reduced in PW mice due to pre-conceptional GLY exposure but this effect was not observed in
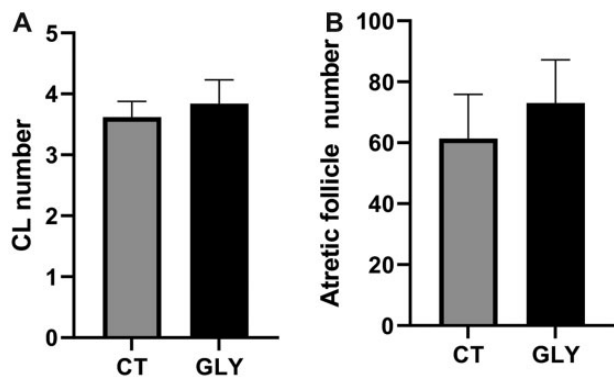
Figure 6. Effect of pre-conceptional GLY exposure on follicle number in GD14 dams. Female C57Bl/6 mice were exposed to saline vehicle control (CT) or GLY (2 mg/kg) pre-conceptionally for 10 weeks daily *per os*. A, The number of CL in the ovaries from GD14 dams (n = 5 for both CT and GLY). B, The number of atretic follicles in ovaries of GD14 dams (n = 5 for both CT and GLY). N represents the number of dams. Data points represent mean ± SEM.
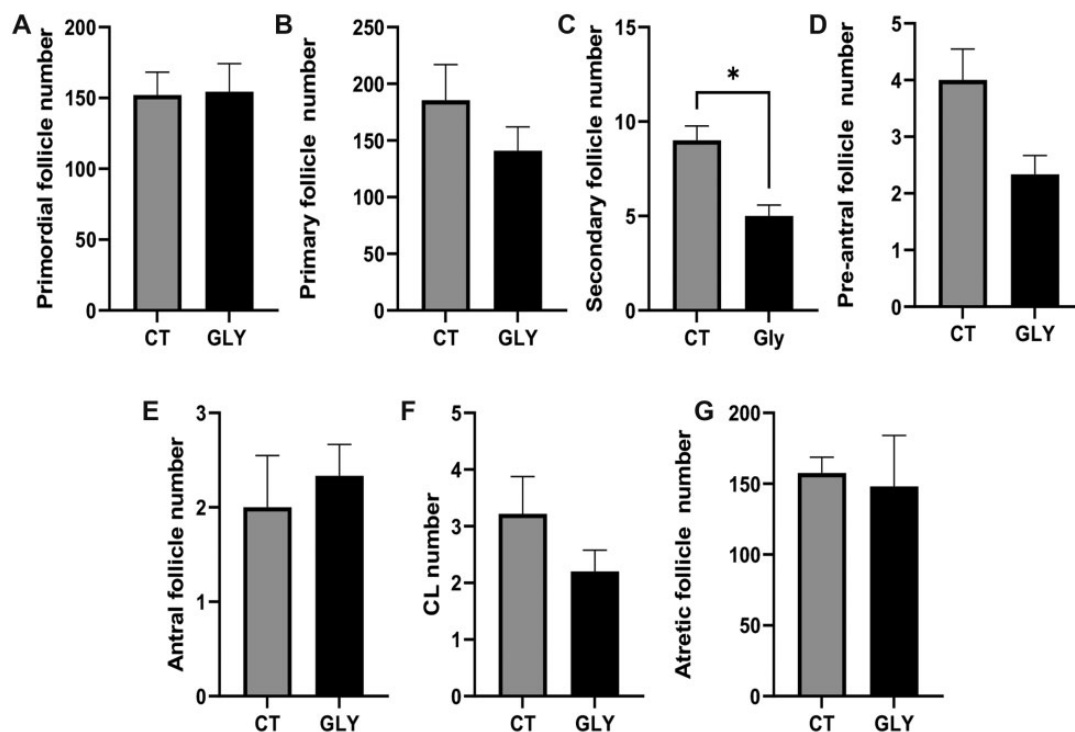
Figure 7. Effect of pre-conceptional GLY exposure on ovarian follicle number in PW dams. Female C57Bl/6 mice were exposed to saline vehicle control (CT) or GLY (2 mg/kg) pre-conceptionally for 10 weeks daily *per os*. (A) Primordial, (B) primary, (C) secondary, (D) pre-antral, and (E) antral follicle number, (F) CL number, and (G) atretic follicle number in PW dams (n = 5 for CT, and n = 3 for GLY). N represents the number of dams. Data points represent mean ± SEM. Asterisk represents difference from CT; p < .05.

Table 1. Ovarian proteins altered by GLY exposure in GD14 dams

Protein name	log2(FC)	p value
Protein transport protein Sec16A	4.8591	.035915
Pyridoxal-dependent decarboxylase domain-containing protein 1	1.5537	.013687
Nup214 protein	1	.000487
ADP-ribose glycohydrolase MACROD1	0.69986	.033962
Nonspecific serine/threonine protein kinase	0.63402	.035074
YTH domain-containing family protein 3	0.5125	.034148
Eukaryotic translation initiation factor 1A, X-chromosomal	0.48301	.012895
40S ribosomal protein S21	0.4387	.027007
Receptor expression-enhancing protein	0.32646	.012106
Polyadenylate-binding protein 1	0.25679	.013489
Importin N-terminal domain-containing protein R	0.18224	.007486
Heat shock 70 kDa protein 4	0.11288	.046343
Thyroid hormone receptor-associated protein	−0.20908	.03274
Proteasome (Prosome, macropain) 28 subunit, alpha	−0.22259	.044676
Heterogeneous nuclear ribonucleoprotein	−0.22832	.012654
cAMP-dependent protein kinase type II-beta regulatory subunit	−0.24806	.030404
Hemopexin	−0.25051	.011695
Ribosomal protein L19	−0.35551	.014565
40S ribosomal protein S13	−0.43121	.026505
Tensin-3	−0.5652	.033498
Long-chain-fatty-acid–CoA ligase 1	−0.97356	.029265
Guanylate-binding protein 2	−0.97623	.043476
Histidine–tRNA ligase	−1.1861	.013373
MOB1, Mps one binder kinase activator-like 1A	−1.4189	.024608
Elongation factor 1-alpha 2	−1.5525	.021982
Protease, serine 1 (trypsin 1)	−1.7669	.021208

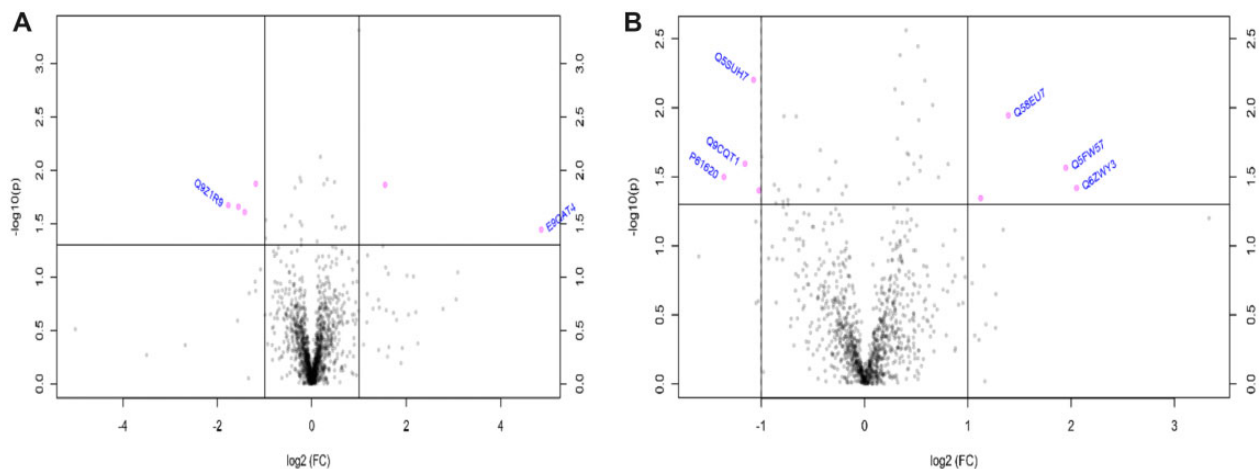**Figure 8.** Impact of pre-conceptional GLY exposure on ovarian and hepatic proteome of GD14 dams. Female C57Bl/6 mice were exposed to saline vehicle control (CT) or GLY (2 mg/kg) pre-conceptionally for 10 weeks daily *per os*. (A) Ovary (n = 5 for CT, and n = 4 for GLY) and (B) liver (n = 5 for CT, and n = 4 for GLY) were collected at GD14 and proteins analyzed by LC-MS/MS. Bioinformatic analyses were performed to determine differences in protein abundance due to GLY exposure relative to CT. The pink dots in the volcano plots above the horizontal dotted line indicate proteins that differed in abundance with fold change > 2 and $p < .05$.

GD14 mice. These findings could indicate an effect of GLY on ovarian composition intensified by ovarian aging and longer term studies are warranted.

The ovarian and placental hormones progesterone and 17β-estradiol are important for maintaining pregnancy, and dysregulation of their levels could result in a spectrum of pregnancy complications including miscarriage (Berkane *et al.*, 2017; Kumar and Magon, 2012). Several previous studies demonstrated the potential of GBHs to act as endocrine disruptors impacting the circulating levels of the progesterone and/or 17β-estradiol (Perego *et al.*, 2017; Ren *et al.*, 2018; Wrobel, 2018). However, previous studies from our group did not observe changes in estrous cyclicity or in circulating levels of 17β-estradiol or progesterone in female mice after 10 and/or 20 weeks of 2 mg/kg of GLY exposure (Ganesan and Keating, 2020). In the current study, circulating levels of 17β-estradiol and progesterone were similar between treatments in pregnant dams in contrast with studies documenting 17β-estradiol and progesterone effects during pregnancy when exposed to higher concentrations of GBHs (Ren *et al.*, 2018). Thus, pre-conceptional GLY exposure did not result in observable changes to 17β-estradiol and progesterone during gestation in this paradigm.

Our previous studies on the impact of GLY exposure at 2 mg/kg for 5 or 10 weeks on ovarian follicle number did not reveal

Table 2. Hepatic proteins altered in GLY-exposed GD14 dams

Protein name	log2(FC)	p value
40S ribosomal protein S27-like	2.0531	.038138
Glycine N-acyltransferase-like protein	1.9495	.027228
Rbp1 protein	1.3924	.011364
Proteasome subunit beta type-5	1.125	.04518
Actin-related protein 2/3 complex subunit 4	0.8095	.025579
Glutathione transferase	0.71643	.031901
Serine/arginine-rich splicing factor 1	0.66019	.0095619
40S ribosomal protein S26	0.58371	.0063546
Calmodulin-2	0.54516	.022671
Cytochrome c oxidase subunit 7C, mitochondrial	0.53707	.028641
Signal recognition particle subunit SRP72	0.52603	.012295
40S ribosomal protein S28	0.51754	.0035913
Ribosomal protein S10	0.47892	.025479
Parkinson disease protein 7 homolog	0.42685	.021807
Sulfotransferase	0.42095	.032665
Ribosome-binding protein 1	0.4121	.021396
General vesicular transport factor p115	0.40249	.0027542
Glucosidase 2 subunit beta	0.36828	.0092784
Uncharacterized protein	0.34407	.0041649
Coatomer subunit alpha	0.3376	.035259
40S ribosomal protein S4	0.31858	.016734
Receptor of activated protein C kinase 1	0.29557	.0073505
Protein NDRG2	−0.17772	.037023
Cytochrome P450 2D10	−0.21536	.042145
Proteasome subunit alpha type	−0.27902	.024665
Cytochrome P450 2C40	−0.34197	.032644
Enoyl-CoA hydratase domain-containing protein 3, mitochondrial	−0.4281	.020302
Basigin	−0.44971	.039483
Sodium/bile acid cotransporter	−0.62562	.036768
Dihydropyrimidinase-related protein 2	−0.66024	.011562
26S proteasome non-ATPase regulatory subunit 5	−0.74082	.046609
17-beta-hydroxysteroid dehydrogenase	−0.78023	.011526
Rho GTPase-activating protein 29	−0.78841	.047922
Very-long-chain (3R)-3-hydroxyacyl-CoA dehydratase 3	−0.8298	.039349
Uncharacterized protein	−0.85731	.049258
MSP domain-containing protein	−0.87332	.036146
Cingulin	−0.99599	.022682
Cytochrome P450 2C37	−1.0215	.039765
Clathrin interactor 1	−1.0733	.0062831
Methylthioribose-1-phosphate isomerase	−1.1561	.025484
Protein transport protein Sec61 subunit alpha isoform 1	−1.3603	.031718

Table 3. KEGG pathways altered in liver of GLY-exposed GD14 dams

Pathway	Gene_Found	Gene_Pathway	FDR
Chemical carcinogenesis	9	84	5.94E-05
Linoleic acid metabolism	6	50	0.001856714
Steroid hormone biosynthesis	7	92	0.001856714
Retinol metabolism	7	97	0.001856714
Arachidonic acid metabolism	6	86	0.014565451
Ribosome	7	179	0.017561422
Inflammatory mediator regulation of TRP channels	6	127	0.045517365
Metabolic pathways	20	1572	0.045517365
Serotonergic synapse	6	131	0.046510328
Parkinson's disease	6	264	0.069861538

changes in the number of follicles at any stage of development compared with control treated females (Ganesan et al., 2020). However, after 20 weeks of the identical chronic GLY exposure as in the current study, females had a greater number of ovarian primordial and antral follicles compared with control mice (Ganesan and Keating, 2020). We did not observe a difference in the number of CL or atretic follicles in pre-conceptional GLY-exposed GD14 compared with CT mice. Contradictory to our findings, mice exposed to GLY dissolved in drinking water (0.5%) had higher numbers of atretic follicles (Ren et al., 2018). In

Table 4. Hepatic proteins altered in GLY-exposed PW dams

Protein name	log2(FC)	p value
Tubulin beta chain	1.7202	.027847
Tubulin beta-4A chain	1.6954	.045874
Nucleoside diphosphate-linked moiety X motif 19	1.4978	.016522
Coatomer subunit delta	1.2332	.023293
Eukaryotic translation initiation factor 4 gamma 1	0.88856	.017282
Calumenin	0.79541	.038917
Clustered mitochondria protein homolog	0.75834	.011274
Dimethyladenosine transferase 1, mitochondrial	0.59067	.0065102
Mitochondrial antiviral-signaling protein	0.54541	.018431
Elongation factor Tu, mitochondrial	0.44876	.020531
Inorganic pyrophosphatase 2, mitochondrial	0.41068	.04521
Lipoamide acyltransferase component of branched-chain alpha-keto acid dehydrogenase complex, mitochondrial	0.37084	.028903
Inorganic diphosphatase	0.36458	.013001
40S ribosomal protein S4	0.27893	.013402
AB hydrolase-1 domain-containing protein	0.23006	.045266
3-hydroxyisobutyryl-CoA hydrolase, mitochondrial	0.22328	.028083
Fructose-bisphosphate aldolase	0.1679	.025904
Delta-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial	−0.16064	.037779
Cytochrome c oxidase subunit 5A, mitochondrial	−0.16111	.034132
Translationally-controlled tumor protein	−0.18	.047163
Dimethylaniline monooxygenase [N-oxide-forming] 1	−0.18419	.039827
Enoyl-CoA hydratase domain-containing protein 3, mitochondrial	−0.25261	.025276
Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, beta polypeptide	−0.27372	.035466
NADH dehydrogenase [ubiquinone] flavoprotein 1, mitochondrial	−0.29884	.038423
Cytochrome P450, family 2, subfamily d, polypeptide 22	−0.30752	.032319
Rap1A-retro1	−0.35868	.042217
Glycogen phosphorylase, liver form	−0.38031	.012002
Proteasome subunit alpha type	−0.40282	.010637
60S ribosomal protein L28	−0.40588	.045524
Dehydrogenase/reductase SDR family member 1	−0.46466	.00045012
High mobility group nucleosome-binding domain-containing protein 5	−0.47242	.043406
Apolipoprotein A-II	−0.48597	.0062553
Atlastin-3	−0.48807	.011552
Persulfide dioxygenase ETHE1, mitochondrial	−0.5115	.028812
Predicted gene 884	−0.65678	.04312
Coronin	−0.75583	.036708
Tripeptidyl-peptidase 2	−1.0908	.011161
Magnesium transporter NIPA3	−1.155	.021219
Methylglutaconyl-CoA hydratase, mitochondrial	−1.589	.033778

PW dams, decreased secondary follicle number was observed due to GLY exposure, which may be explanatory for the reduced ovarian weight observed. These divergent GLY effects are likely explained by difference in dosing paradigms between studies.

To identify modes of action of pre-conceptional GLY exposure on the ovary and liver, a non-targeted approach was taken and whole ovarian or hepatic protein was analyzed by LC-MS/MS. The impact of GLY on the maternal GD14 ovary included changes to 26 proteins with the abundance of SEC16A being increased by 29-fold. SEC16A is critical in its regulatory role of several proteins' endoplasmic reticulum conventional and unconventional secretion (Piao et al., 2017; Supek et al., 2002; Watson et al., 2006), and is involved in nutrient-induced autophagy (Zahoor and Farhan, 2018). Such a dramatic increase due to GLY exposure in pregnancy could be a sign of dysregulation of metabolic processes. The level of ADP-ribose glycohydrolase MACROD1, that participates in post-translational modifications of target proteins, was increased 1.6-fold in the ovaries of GD14 dams exposed to GLY. This protein is activated in response to environmental triggers (Feijs et al., 2013) and involved in DNA damage response mechanisms (Yang et al., 2020). Expressed in many tissues, MACROD1 abundance is elevated in several types of cancers (Feijs et al., 2020). In addition, among the proteins demonstrating increased abundance in the ovarian tissues from the pregnant dams exposed to GLY, heat shock 70 kDa protein 4 (HSPA4) was increased (1.1-fold). Increased activity of HSPA4 and other heat shock proteins of the HSP110 family was implicated in carcinogenesis and drug resistance (Gotoh et al., 2004; Kimura et al., 2016). The increase in abundance of these proteins in the ovaries from GLY-exposed dams could indicate that, even at low concentrations, ovarian molecular changes are observed due to pre-conceptional GLY exposure. It is important to note that these findings represent a snapshot of the ovarian impacts and whether these changes could influence gestational outcomes or long-term fertility cannot be concluded.

There were changes to the abundance of 41 and 39 hepatic proteins in the GD14 and PW livers, respectively. The liver, as an important metabolic organ, serves as the major site for xenobiotic biotransformation with dramatically higher levels of Phase I and Phase II biotransformation enzymes than other organs (Gu and Manautou, 2012). This study identified a preconceptional GLY impact on the level of maternal hepatic
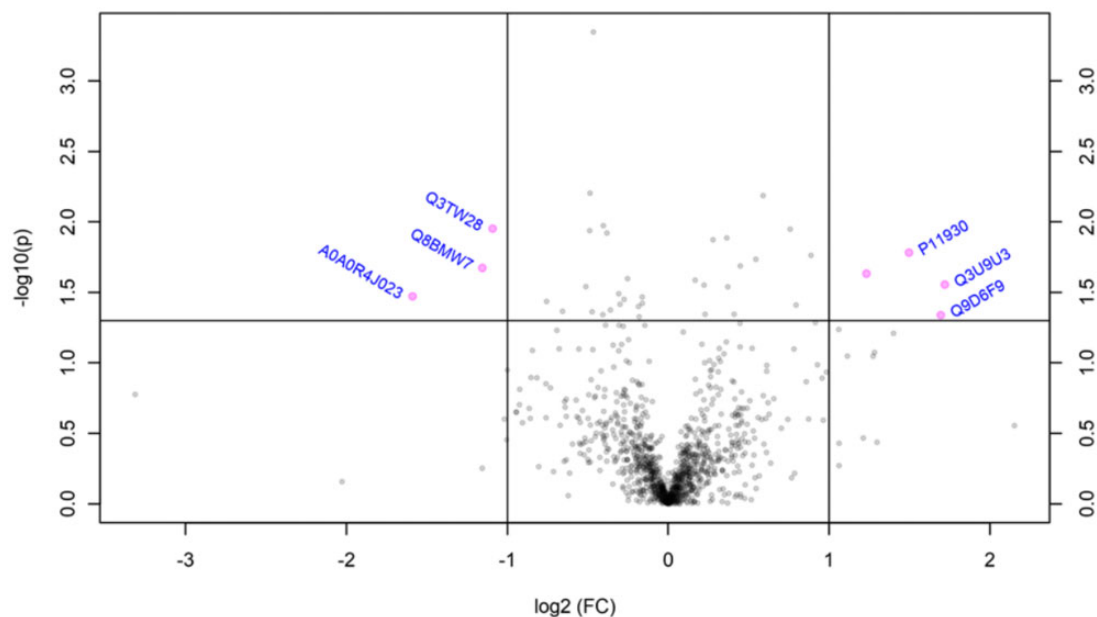
Figure 9. Impact of pre-conceptional GLY exposure on the hepatic proteome of PW dams. Female C57Bl/6 mice were exposed to saline vehicle control (CT) or GLY (2 mg/kg) pre-conceptionally for 10 weeks daily *per os*. Livers ($n = 4$ for CT, and $n = 3$ for GLY) were collected from PW dams and proteins analyzed by LC-MS/MS. Bioinformatic analyses were performed to determine differences in protein abundance due to GLY exposure relative to CT. Pink dots in volcano plots above the horizontal dotted line indicate proteins that differed in abundance with fold change > 2 and $p < .05$.

xenobiotic Phase I biotransformation enzymes including CYP2C40, CYP2C37, and CYP2D10, and the Phase II biotransformation proteins SULT1A1 and GSTA5. In the livers of PW mice, two Phase I biotransformation enzymes, CYP2D22 and FMO1, were decreased due to GLY exposure. Interestingly, in both GD14 and PW dams, hepatic 40S ribosomal protein S4 (RPS4) level was increased and Enoyl-CoA hydratase domain-containing protein 3, mitochondrial (ECHDC3) abundance was decreased. This similar pattern of RPS4 and ECHDC3 proteins at both GD14 and PW may indicate a persistent influence of GLY exposure.

Hepatic proteomic pathway analyses demonstrated that pre-conceptional GLY exposure changed proteins that represented specific pathways during pregnancy. Among the potential pathway targeted by GLY at GD14 with FDR < 0.07 were chemical carcinogenesis, steroid hormone biosynthesis, retinol metabolism, and metabolic pathways, all of which are important for maintaining pregnancy.

Taken together, lack of observed impacts of GLY exposure on body weight, liver or spleen weight, food intake, GD14 ovary weight, GD14 ovarian follicle composition, GD14 17β-estradiol and progesterone levels suggest an absence of endocrine disruption and overt toxicity. Reduced PW secondary follicle number and ovarian weight and an altered ovarian and hepatic proteome suggest that pre-conceptional chronic GLY exposure may affect maternal ovarian and hepatic function. Further studies to evaluate any long term impacts of the changes observed herein are needed.

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