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Absence of glyphosate-induced effects on ovarian folliculogenesis and steroidogenesis

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ABSTRACT

Glyphosate (**GLY**) is an herbicidal active ingredient and both *in vitro* and *in vivo* studies suggest that GLY alters ovarian function. To determine if a chronic GLY exposure model affected steroidogenesis or folliculogenesis *in vivo*, postnatal day 42 C57BL6 female mice were orally delivered vehicle control (saline) or GLY (2 mg/Kg) from a pipette tip five days per week for either five or ten weeks. Mice were euthanized at the pro-estrus stage of the estrous cycle. GLY exposure did not impact body weight gain, organ weights, or healthy follicle numbers. In addition, GLY exposure did not affect abundance of ovarian mRNA encoding kit ligand *(Kitlg)*, KIT proto-oncogene receptor tyrosine kinase (c-*Kit)*, insulin receptor (*Insr)*, insulin receptor substrate (*Irs1* or *Irs2)* and protein thymoma viral proto-oncogene 1 (AKT) or phosphorylated AKT. Ovarian mRNA or protein abundance of *Star*, 3β-hydroxysteroid dehydrogenase (*Hsd3b1), Cyp11a1* or *Cyp19a* were also not altered by GLY. Circulating 17β-estradiol and progesterone concentration were unaffected by GLY exposure. In conclusion, chronic GLY exposure for five or ten weeks did not affect the ovarian endpoints examined herein.

1. Introduction

The herbicide glyphosate (GLY) has broad US and global usage. With the development of GLY-tolerant soybeans, corn and cotton in 1996, GLY application dramatically increased in the past two decades, now accounting for 50 % of total herbicide applied, with almost 100 % of wheat, corn, soybean, cotton and potato cultivation receiving GLY treatment (USDA, 2014). This usage does not account for urban utilization - outside of agriculture, GLY is extensively utilized for weed control in home gardens, recreation spaces, railways and roadways [1]. The detection of GLY residues in food stuffs [2] and in human urine [3–7] has raised concern regarding GLY's health effects, although urinary GLY presence is not necessarily indicative of a health risk, since GLY is readily excreted, through feces and urine [8].

The Ontario Farm Family Health study reported that GLY exposure during late pregnancy was correlated with spontaneous abortion in women [9]. An association between GLY exposure and a shortened gestational length was also reported recently in a birth cohort from Indiana [10]. *In vitro* experiments implicated GLY as a potential endocrine disruptor through effects on steroidogenic acute regulatory protein (STAR) [11] and cytochrome P450 19A (CYP19A1) [12,13] - ovarian proteins that catalyze cholesterol conversion to 17β-estradiol (E_2). Enhanced growth of estrogen receptor positive breast cancer cells due to GLY exposure has been reported (Thongprakaisang et al., 2013) and GLY decreased E_2 but increased proliferation in a dose-dependent manner in bovine granulosa cells [14]. Reduced E_2, increased atretic follicle number, and reduced surface area of antral follicles were demonstrated *in vivo* in rats exposed to two high doses of GLY (126 or 315 mg/kg/d) for 60 days [15], but these exposure levels were extremely high and likely irrelevant to human exposure levels. Despite this, there is a growing body of evidence implicating GLY as a female reprotoxicant.

However, in contrast to the aforesaid, risk assessment studies have found no effect of GLY on E_2 production [16], nor was there any impact of GLY exposure on gross phenotypic reproductive measures or the endocrine system in acute and chronic exposure studies [8]. Consequently, the documentation of how (and if) GLY affect female reproduction are equivocal and the exact reasons remain ambiguous.

There is lack of consistency in the GLY dosage investigated across studies and their questionable human exposure relevance. In addition, many studies have used a GLY based herbicide (GBH) exposure rather than GLY specifically, a scenario which obviously introduces confounding effects of the adjuvants and surfactants within those formulations. In order to circumvent some of these limitations, this study investigated the hypothesis that chronic, oral GLY exposure at a dose considered non-hazardous (2 mg/Kg/d; [17] would alter estrous

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cyclicity, follicle number, circulating ovarian steroid hormone levels and ovarian intracellular signaling in adult female mice.

2. Materials and methods

2.1. Reagents

Glyphosate (CAS # 1071-83-6), 2-β-mercaptoethanol, Tris base, Tris HCL, Sodium chloride, EDTA, SDS, NaF, HEPES and Tween-20 were purchased from Sigma-Aldrich Inc. (St Louis, MO). Ponceau S, Invitrogen iBlot 2NC regular stacks, Pierce BCA protein assay kit, Glycerol, Citric acid, Saline, DAPI nuclear stain and Sodium citrate were obtained from Thermo Fisher Scientific. Mini-PROTEAN TGX™ precast protein gels and protein size markers were purchased from BioRad. RNeasy Mini kit, QIA shredder kit, RNeasy Min elute kit, and Quantitect ™ SYBR Green PCR kit were purchased from Qiagen Inc (Valencia, CA). All primers were purchased from the Iowa State University DNA facility (Ames, IA). Antibodies directed against AKT, phosphorylated AKT, CYP11A1, anti-mouse Alexa flour 488 and SignalFire ECL plus chemical luminescence detection kit were from Cell Signaling (Danvers, MA). Antibodies directed against ERα, STAR and donkey anti-rabbit IgG-FITC were from Abcam. Novus Biologicals (Centennial, CO) was the source of antibodies directed against HSD3β and CYP19A1. ELISA kits to measure E_2 and progesterone (P_4) were from DRG International Inc (Springfield, NJ).

2.2. Animals

The Iowa State University Institutional Animal Care and Use Committee approved all animal experiments in accordance with NIH guidelines. Postnatal day (PND) 42 C57BL/6 J female mice received vehicle control (saline; n = 20) or glyphosate (GLY; 2 mg/Kg/day; n = 20) *per os* for five days per week for a duration of 5 (n = 10 control; n = 10 GLY) or 10 weeks (n = 10 control; n = 10 GLY). Mice were housed in groups of 3–4 and were provided with *ad libitum* access to food and drinking water. Body weight was obtained weekly. Vaginal cytological analysis was performed for 10 days in the 5-week exposure study and for 21 days in the 10-week study prior to euthanasia and the length spent in each stage of the estrous cycle calculated as a percentage of the time analyzed. Mice were euthanized at the pro-estrus phase of their estrous cycle. Weights of the ovaries, uterus, liver, kidneys and heart were recorded. Blood samples were collected post-euthanasia by cardiac puncture.

2.3. Histological analysis

One ovary per animal (n = 10 per treatment) was fixed in 4% paraformaldehyde for 24 h and transferred to 70 % ethanol and paraffin embedded. Ovaries were sectioned (5 μM thickness) and every 6th section was mounted and stained by hematoxylin and eosin. Healthy oocyte-containing follicles were identified and counted in every 12th section. Unhealthy follicles were distinguished from healthy follicles by the appearance of pyknotic bodies and intense eosinophilic staining of oocytes. Follicles were classified and enumerated as previously described [18].

2.4. Quantitative reverse transcriptase polymerase chain reaction

Approximately 25 % of each ovary was used for RNA isolation and two ovarian samples were combined (n = 10 ovaries; 5 RNA samples). RNA was isolated using a RNeasy Mini kit and the RNA concentration determined using a Nano Drop spectrometer (λ = 260/280 nm; ND 1000; Nanodrop Technologies Inc., Wilmington, DE). RNA (200 ng) was reverse transcribed to cDNA, followed by quantitative PCR. Primers for specific genes of interest were designed by Primer 3 Input Version (0.4.0) (listed in Table 1). The regular cycling program consisted of a 15 min hold at 95 °C and 45 cycles of denaturing at 95 °C for 15 s, annealing at 58 °C for 15 s, and extension at 72 °C for 20 s at which point data were acquired. Each sample was normalized to 18 s RNA before quantification. Quantification of fold-change in gene expression was performed using the $2^{-\Delta\Delta Ct}$ method [19,20].

Table 1
Primer Sequences.

Gene	Forward Primer	Reverse Primer
c-Kit	ttctccttaggaagcagccc	cctgcttgaatgttggcctt
Kit-lg	tcagtcatagattggagtttgca	tgtatcaaaagggtcgggaca
Akt	tttgttgctgtgtcccatgc	caagtgctaggagaagggct
Star	atgttcctcgctacgttcaag	cccagtgctctccagttgag
Hsd3b1	gctggaaactgtgagcttcc	tgcttcctcccagttgacaa
Cyp11a1	aggtccttcaatgagatcccctt	tccctgtaaatggggccatac
Cyp19a1	atgttcttggaaatgctgaaccc	aggacctggtattgaagacgac
ERa	aattctgacaatcgacgccag	gtgcttcaacattctccctcctc
Erb	tgctccaagggtaggatggac	ctgtgcctcttctcacaagga
Insr	tgtcatcaatgggcagtttg	atcaggttccgaacagttgc
Irs1	ctatgccagcatcagcttcc	ggaggatttgctgaggtcat
Irs2	gaagcggctaagtctcatgg	gacggtggtggtagaggaaa

2.5. Western blot analysis

Ovaries (n = 10 per treatment) were homogenized in tissue lysis buffer (containing protease and phosphatase inhibitors) and centrifuged at 10,000 rpm twice for 15 min. The protein concentration of the supernatant was measured using a bicinchoninic acid assay and stored at −80 °C until further use. Protein was separated by SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were blocked for 1 h in 5% milk in Tris-buffered saline containing tween 20. Membranes were incubated in primary antibodies directed against protein kinase B (AKT; primary -1:200; secondary - 1:500), phosphorylated AKT (pAKT; primary - 1:200; secondary -1:200), estrogen receptor alpha (ERα; primary - 1:500; secondary - 1:500), estrogen receptor beta (ERβ; primary - 1:500; secondary - 1:200), steroidogenic acute regulatory protein (STAR; primary - 1:500; secondary - 1:200); 3-β hydroxysteroid dehydrogenase (HSD3β; primary - 1:1000; secondary - 1000), cytochrome P450 isoform 11A1 (CYP11A1; primary - 1:500; secondary - 1:500) and cytochrome P450 isoform 19A1 (CYP19A1; primary - 1:500; secondary - 1:500) overnight at 4 °C with rocking. Following three washes in TTBS (1X) membranes were incubated with species-specific secondary antibody for 1 h at room temperature. Autoradiograms were developed on X-ray films in a dark room following 10 min incubation of membranes with 1X SignalFire™ ECL reagent. Densitometry of the appropriate-sized bands was measured using Image Studio Lite Version 3.1 (LI-COR Biosciences, Lincoln, NE) which eliminates background noise. The sum of the gray values of all the pixels in the selection divided by the number of pixels, or mean gray value was quantified for each membrane using ImageJ software. Membranes were normalized to Ponceau S protein staining in which the entire lane of the transferred protein was quantified to account for loading variation. To ensure antibody specificity, negative control blots for each antibody used were performed in which the membranes were incubated with primary antibody only, secondary antibody only, or normal IgG in place of primary antibody with the inclusion of the appropriate secondary antibody. No protein bands were observed on these control blots indicating the specificity of the protein bands detected and analyzed.

2.6. Immunofluorescence staining

Paraffin embedded ovaries (n = 5 per treatment) from the 10 week exposure group were serially sectioned (5 μM thick) and every 10th section was mounted. Sections were deparaffinized in xylene and rehydrated with subsequent washes in ethanol. Antigen retrieval was carried out by microwaving sections for 7 min in sodium citrate buffer

(1 M, pH 6.1). Sections were blocked in 5% BSA for 1 h at room temperature. Sections were incubated with primary antibodies directed against pAKT (1:50 dilution), STAR (1:500 dilution), HSD3β (1:500 dilution), CYP11A1 (1:100 dilution), CYP19A1 (1:100 dilution) and ERα (1:200 dilution) overnight at 4 °C. After washing in 1% PBS, sections were incubated with the appropriate donkey anti-rabbit IgG-FITC (1:200 to 1:500 dilutions) or anti-mouse Alexa flour 488 (1:200 dilution) secondary antibody for 1 h. Slides were counterstained with 4-6-diamidino-2-phenylindole (DAPI) for 5 min. Negative technical controls incubated the ovarian sections omitting both the primary and secondary antibody to ensure specificity of the antibodies. Images were captured using a Leica DMI3000 B fluorescent microscope. Raw internal density of staining was analyzed using Image J software. For analysis, 5 ovaries per treatment were assessed with three sections per ovary and 7–10 large follicles per slide analyzed.

2.7. Steroid hormone quantification

Serum (25 μL; n = 10 per treatment) was added in duplicate per sample to an ELISA plate to measure E_2 or P_4. Plates were incubated for 60 − 90 min after adding the enzyme conjugate (100 − 200 μl). The wells were rinsed with wash solution three times, substrate solution added (100 − 200 μl) and incubated for 15 − 30 min. The enzymatic reaction was stopped by adding stop solution (50 − 100 μl) and the signal determined within 10 min using a plate reader at 450 nm absorbance.

2.8. Sample identity blinding

The identity of samples used for ELISA and follicle counting were not known to the investigator performing the analysis. In addition, mRNA for PCR and protein samples from mice treated for 10-weeks for western blotting were double blinded – the identity of the samples was unknown to the investigator and after western blots were completed, they were then identified by group for the purpose of statistical analysis. Only after the statistical analysis was completed was group identity revealed.

2.9. Statistical analysis

Raw data were analyzed by unpaired *t*-test. All statistical analysis was performed using Prism 5.04 software (GraphPad Software). Statistical significance (*) was defined as $P < 0.05$. A tendency for a statistically meaningful difference was considering if the *P*-value was between 0.05 − 0.1.

3. Results

3.1. Impact of GLY exposure on body weight gain

Body weight gain was determined from the onset to the completion of the dosing period which was either a duration of 5 or 10 weeks. As anticipated, mice gained weight with aging, however, there was no impact of GLY exposure on body weight gain at either time point (Fig. 1).

3.2. Effect of GLY exposure on the estrous cyclicity

In mice exposed to GLY for either duration, no impact of GLY exposure on the percentage time spent at any stage of the estrous cycle was observed, though there was a tendency ($P < 0.1$) for increased time spent in estrus and reduced time spent in metestrus/diestrus after 5 weeks of GLY exposure (Fig. 2).

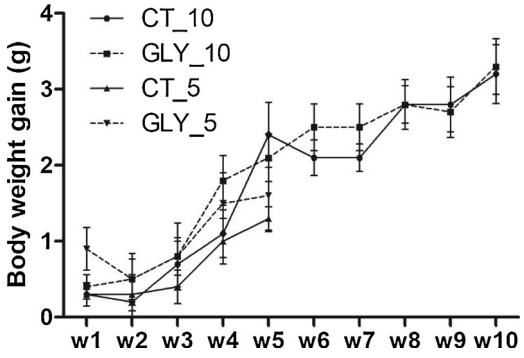

Fig. 1. Effect of glyphosate on body weight.
Female C57Bl/6 mice were exposed to saline vehicle control (CT) or GLY (2 mg/Kg) *per os* from a pipette tip five days per week for 5 or 10 weeks. Body weight was measured from day 0 of dosing to end of 5 or 10 weeks of dosing. Values are expressed as average body weight gained every week ± SEM; n = 10. Mice exposed to saline (CT) for 5 weeks indicated by solid line with triangle data point marker; Mice exposed to GLY for 5 weeks indicated by dashed line with triangle data point marker; Mice exposed to saline (CT) for 10 weeks indicated by solid line with circle data point marker; Mice exposed to GLY for 10 weeks indicated by dashed line with square data point marker.

3.3. Endocrinological impact of GLY exposure

Mice were euthanized at the pro-estrus stage of the estrous cycle, thus E_2 (Fig. 3A) levels were higher than P_4 (Fig. 3B). There was no effect of 5 or 10 weeks of GLY exposure on circulating E_2 (Fig. 3A). No impact of GLY exposure on serum P_4 was noted at either timepoint (Fig. 3B).

3.4. Relative organ weight effects of GLY exposure

There was no effect of either duration of GLY exposure on the relative weight of the heart (Fig. 4A,F), liver (Fig. 4B,G), kidneys (Fig. 4C,H), or uterus (Fig. 4D,I). In addition, neither 5 weeks (Fig. 4E) or 10 weeks of GLY exposure (Fig. 4J) affected ovarian weight, although a tendency ($P < 0.1$) towards a reduction in ovarian weight after 10 weeks of exposure was observed.

3.5. Impact of GLY exposure on ovarian follicle number

GLY exposure for 5 (Fig. 5A) or 10 weeks (Fig. 5B) did not influence the number of ovarian primordial, primary, secondary or antral follicles. There was also not a different between CT and GLY treated mice at either 5 or 10 weeks in total follicle number or the percentage of follicles per developmental stage within the ovary. Corpora lutea were evident, thus, there were not a defect in ovulation noted and there was no different in the number of CL per ovary due to GLY exposure (data not shown and Fig. 5C-F).

3.6. Effect of GLY exposure on ovarian mRNA encoding genes involved in ovarian folliculogenesis and steroidogenesis

GLY exposure did not impact abundance of ovarian mRNA encoding the PI3K members kit ligand (*Kitl*), KIT proto-oncogene receptor tyrosine kinase (c-*Kit*), or protein kinase b (*Akt*) after 5 (Fig. 6A) or 10 (Fig. 6D) weeks. GLY exposure also did not alter ovarian mRNA abundance of steroidogenic acute regulatory protein (*Star*), 3β-hydroxysteroid dehydrogenase (*Hsd3b*), cytochrome P450 (*Cyp*) *11a1* and *Cyp19a* after 5 (Fig. 6B) or 10 weeks (Fig. 6E) of exposure. In addition, there was no effect of GLY exposure on mRNA encoding the ovarian insulin receptor (*Insr*), insulin receptor substrate 1 (*Irs1*) or *Irs2,* estrogen receptor (*Er*) alpha (*Erα*) or *Er* beta (*Erβ*) mRNA abundance at either 5-weeks (Fig. 6C) or 10-weeks (Fig. 6F) post-exposure.

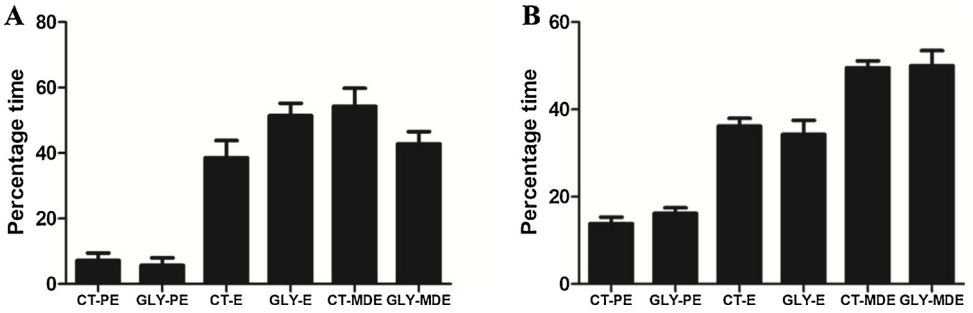

Fig. 2. Effect of GLY exposure on percentage time spent at stages of estrous cycle. Female C57Bl/6 mice were exposed to saline vehicle control (CT) or GLY (2 mg/Kg) *per os* from a pipette tip five days per week for (A) 5 or (B) 10 weeks. The percentage time spent at stages of estrous cycle were calculated: proestrus = PE; estrus = E; metestrus and diestrus = MDE. Data points represent mean +/− SEM. Statistical analysis was performed on raw data.

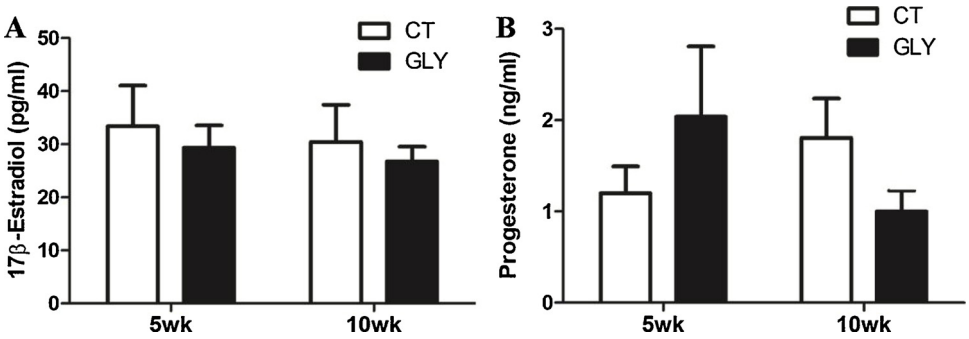

Fig. 3. GLY exposure effect on circulating E_2 and P_4. Female C57Bl/6 mice were exposed to saline vehicle control (CT) or GLY (2 mg/Kg) *per os* from a pipette tip five days per week for 5 or 10 weeks. Circulating (A) E_2 and (B) P_4 were measured by ELISA. Data points represent mean +/- SEM.

3.7. GLY exposure impact on ovarian folliculogenesis and steroidogenesis protein abundance

Using western blot analysis to interrogate changes to ovarian proteins involved in folliculogenesis or steroidogenesis, no impacts of 5 (Fig. 7A,B) or 10 (Fig. 7C,D) weeks of GLY exposure on ERα, ERβ, AKT, pAKT, STAR, HSD3β, CYP11A1, or CYP19A1 protein abundance were detected. Use of immunofluorescence staining in ovaries from the 10 week exposure group identified localization of pAKT (Fig. 8A-C), STAR (Fig. 8D-F), HSD3β (Fig. 8G-I), CYP11A1 (Fig. 8J-L), CYP19A1 (Fig. 8M-O) and ERα (Fig. 8 P–R) but, with the exception of a tendency for a reduction in HSD3B and CYP19A1 ($P < 0.1$); Fig. 9A,B). there was no difference between saline- and GLY-treated mice in the internal density of immunostaining for these proteins.

4. Discussion

Environmental GLY abundance is extensive and GLY accounts for 50 % of U.S. herbicide usage [21]. Despite urinary detection of GLY, human exposure levels remain unclear, though lack of adverse health risks due to chronic dietary GLY intake at 1 mg/Kg/day has been determined. Additionally, a GLY NOAEL for reproductive toxicity has been identified as 2132 mg/Kg/day [8]. While many other studies have investigated dramatically higher GLY exposure levels, the current study sought to determine ovarian impacts of GLY at an exposure level of 2 mg/Kg/day. Additionally, mice drank a GLY solution from a pipette tip to simulate oral exposure and the study lasted 5 or 10-weeks in duration to evaluate chronic exposure. Thus, the dose, duration and route of GLY exposure were low level and chronic and importantly, reflective of human intake. The mice gained weight over the 5 or 10 weeks of dosing and there was no impact of GLY exposure on body weight gain, thus no overt systemic toxicity was observed. Other studies in which substantially higher GLY doses were delivered reported negative impacts of GLY-based herbicides on body weight [22–24], but these exposures were up to 250-fold higher than the dose in this study.

A study in zebrafish reported reduced ovarian weight after GLY exposure (10 mg/L) [25] and GBH (500 mg/Kg) reduced ovarian weight and the number of corpora lutea in rats [26]. Also, *in vitro* GLY (0.5–5 μg/mL) reduced proliferation of bovine granulosa cells [27]. In pregnant mice exposed to GLY or GBH (0.5 % in solution) from

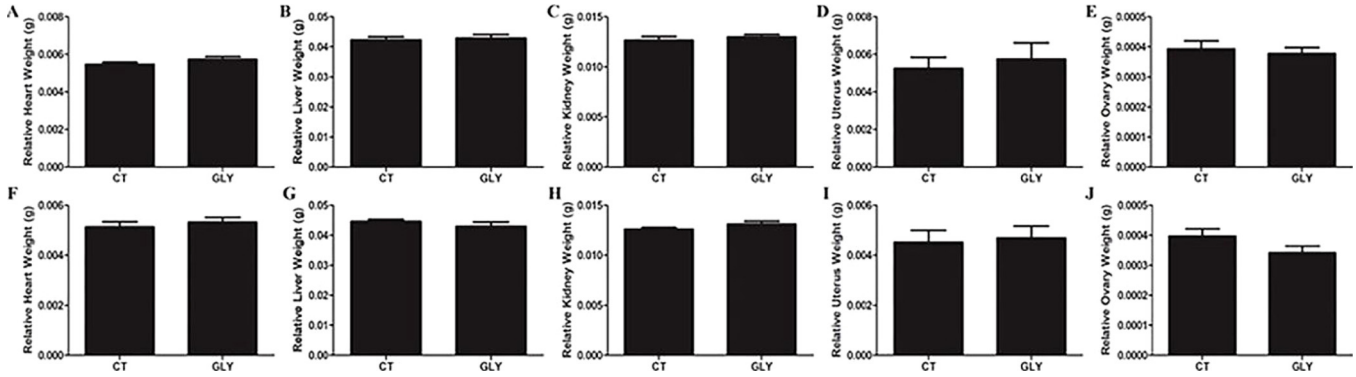

Fig. 4. Relative organ weight impacts of GLY exposure. Female C57Bl/6 mice were exposed to saline vehicle control (CT) or GLY (2 mg/Kg) *per os* from a pipette tip five days per week for (A-E) 5 or (F-J) 10 weeks. Organ weights (g) were collected post-euthanasia and normalized to body weight: (A,C) Heart, (B,G) Liver, (C,H) Kidney, (D,I) Uterus and (E,J) Ovary weight. Data points represent mean +/- SEM.

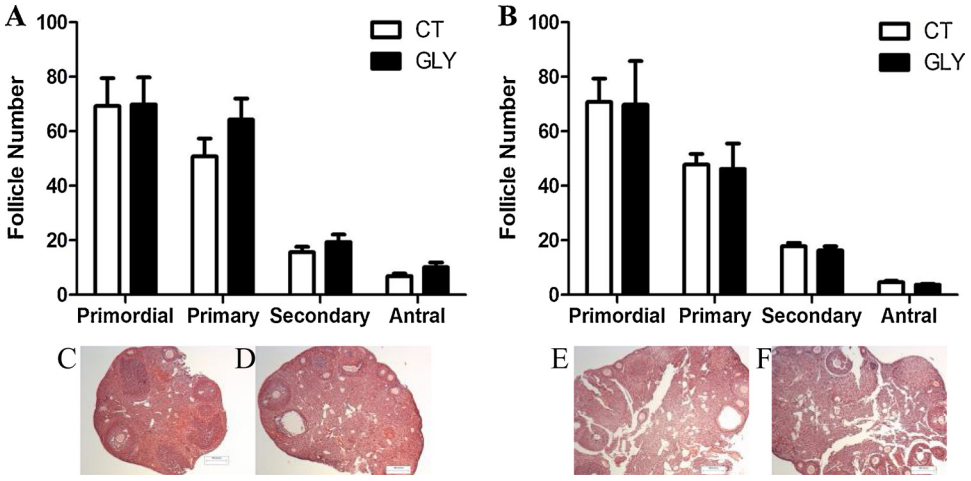

Fig. 5. Effect of GLY exposure on ovarian follicle number. Female C57Bl/6 mice were exposed to saline vehicle control (CT) or GLY (2 mg/Kg) *per os* from a pipette tip five days per week for (A) 5 or (B) 10 weeks. Ovaries were sectioned, stained with haemotoxylin and eosin and follicular stages classified and counted as primordial, primary, secondary, and antral. Data points represent mean +/- SEM. Representative (C) CT 5 week, (D) GLY 5 week, (E) CT 10 week and (F) GLY 10 week ovaries are provided.

gestation day 1–19, reduced body and ovarian weight was noted [28]. However, herein, there was no effect of GLY exposure for 5- or 10-weeks on heart, liver, kidney, or uterus weight, indicating lack of overt toxicity of the exposure and lack of any specific gross effect on the organs examined. There was a tendency for ovarian weight to be reduced with the 10 week exposure paradigm, potentially indicating initiation of alterations to ovarian weight that could have occurred with a longer exposure.

Oral exposure to GBH (126–315 mg/kg) for 60 days increased atretic follicle number and reduced surface area of antral follicles in rats [15] and necrotic cells, vacuolization, dissociation of oocyte and granulosa cells and atretic follicles were observed in the ovaries [15]. Gestational exposure to GLY or GBH (0.5 % in solution) from gestation day 1–19 also increased atretic follicle numbers and reduced the number of larger follicles [28]. Further, neonatal exposure to GBH (2 mg/Kg GLY) reduced follicle numbers in exposed lambs [29]. The exposure paradigm described herein did not affect the number of primordial, primary, secondary or antral follicles. In addition, while there was a tendency for increased time spent in estrus and decreased duration of diestrus/metestrus, this observation was absent after 10 weeks of exposure. Considering the variation in magnitude of GLY exposure across the published literature to date, there could be a dose-dependent effect of GLY on ovarian follicular viability, such that extremely high levels cause ovarian damage. Our results suggest that at levels relevant to human exposure, ovarian follicle dynamics are unaffected by GLY.

Although a follicle might be ostensibly visually healthy, it may be undergoing molecular alterations that could compromise its viability. Consequently, in order to further explore the impacts of GLY exposure on a molecular pathway that is documented to impact ovarian follicular health and maturation, the abundance of PI3K signaling pathway members was determined. Oocyte viability [30], follicular development and steroidogenesis [31,32] are regulated by PI3K signaling and activation of primordial follicle growth is impacted by PI3K inhibition [33]. Further, ovarian toxicants that impact ovarian follicular number and viability have been demonstrated to affect PI3K signaling members [33–35]. Specifically, 4-vinylcyclohexene diepoxide (VCD) destroys primordial and primary follicles [18,33,36,37], and the mode of action is *via* the PI3K member c-KIT [38]. Additionally, 7,12-dimethylbenzanthracene (DMBA) destroys all stage follicles [39] and inhibiting PI3K completely prevents ovotoxicity induced by VCD but conversely dramatically accelerates DMBA-induced follicle loss [33]. Thus, the PI3K pathway is sensitive to chemical exposures and this responsivity has been documented using the techniques used in the current study.

In agreement with the lack of any impact of GLY on ovarian follicle number, we did not detect an effect on the PI3K members analyzed. In fact, there was no impact of either duration of GLY exposure on mRNA encoding *c-kit*, *Kitlg*, or *Akt*. Activating the insulin receptor (*Insr*) is documented to an upstream of the PI3K pathway, thus, we determined any potential impact thereon. However, there was also no impact of

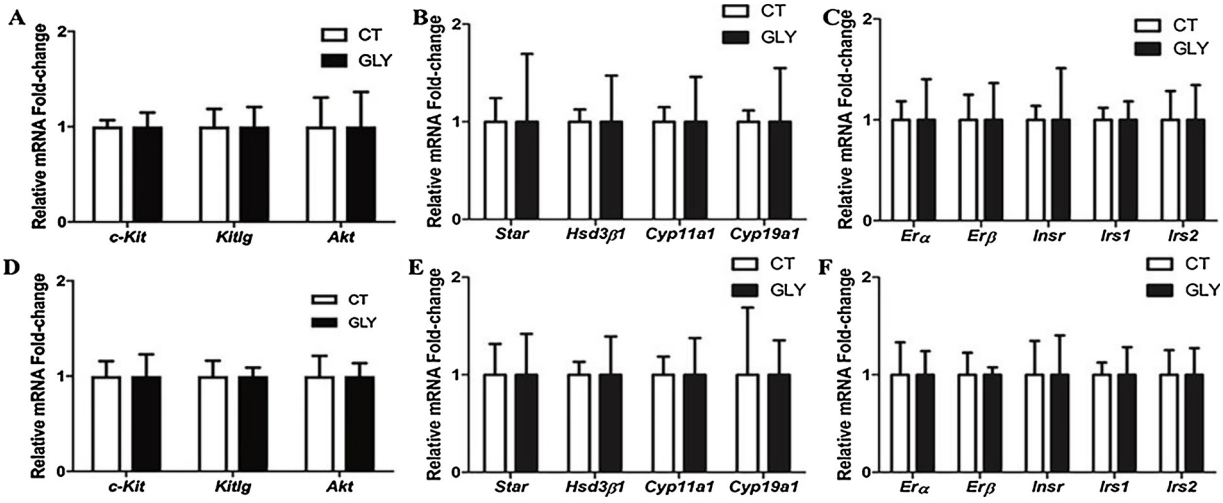

Fig. 6. Impact of GLY exposure on ovarian mRNA abundance. Female C57Bl/6 mice were exposed to saline vehicle control (CT) or GLY (2 mg/Kg) *per os* from a pipette tip five days per week for (A-C) 5 or (D-F) 10 weeks. Ovaries were homogenized and mRNA isolated for qRT-PCR to analyze relative abundance of genes involved in (A,D) PI3K signaling; (B,E) Steroidogenesis; and (C,F) Endocrine signaling. Data points represent fold change relative to CT treated ovaries +/− SEM.

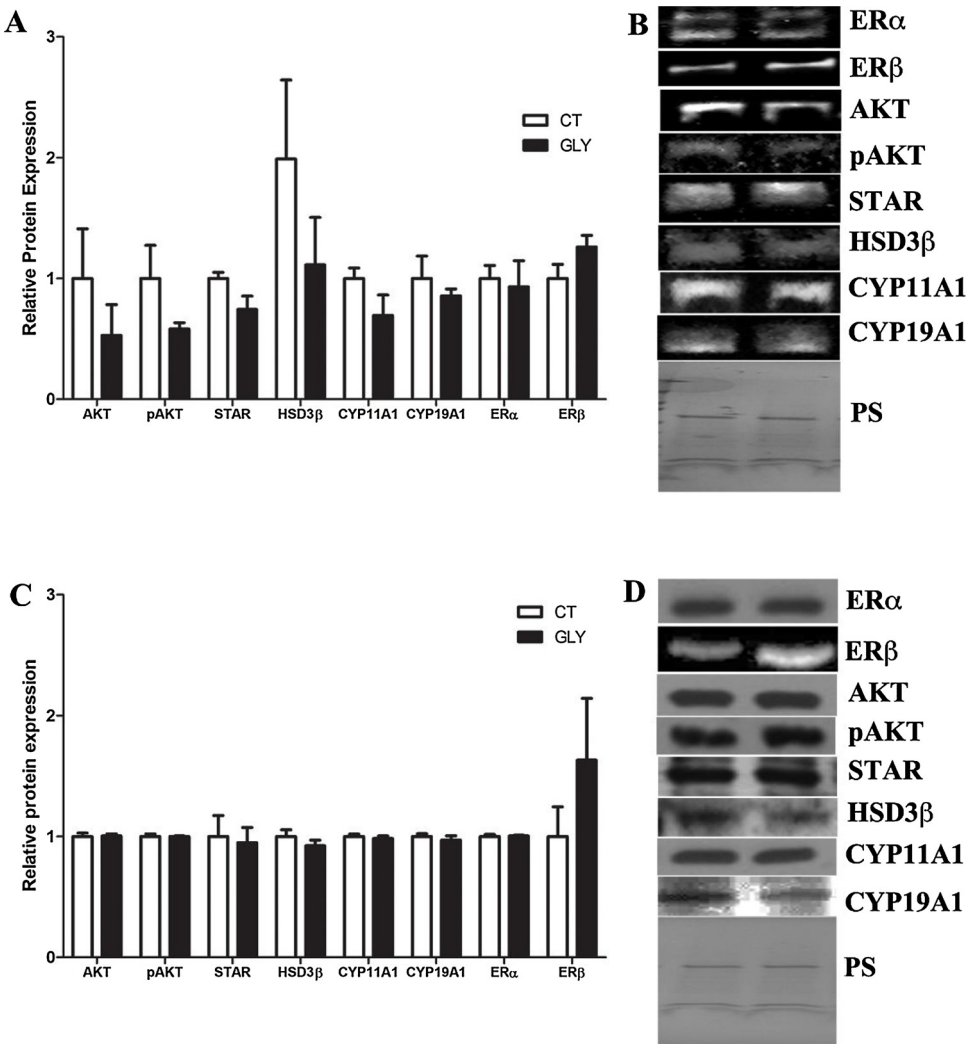

Fig. 7. Consequence of GLY exposure on ovarian protein abundance. Female C57Bl/6 mice were exposed to saline vehicle control (CT) or GLY (2 mg/Kg) *per os* from a pipette tip five days per week for (A,B) 5 or (C,D) 10 weeks. Ovaries were homogenized and western blotting performed to quantify protein abundance of AKT, pAKT, STAR, HSD3B, CYP11A1, CYP19A1, ERA, and ERB. (A,C) Bars represent mean +/- SEM. (B,D) Representative images of western blots for each protein with ponceau S staining represented as PS.

GLY on *Insr*, or the insulin receptor substrates 1 (*Isr1*) or 2 (*Isr2*). There is potential for mRNA to not differ but to have altered protein abundance in response to a cellular stress. Thus, the impact of GLY exposure on ovarian PI3K proteins was also assessed by western blotting and/or immunofluorescence staining. There was no impact of GLY on the abundance of AKT or pAKT as determined by western blotting and immunofluorescence staining determined localization of pAKT in theca cells and oocyte of large follicles but there was no observable effect of GLY on pAKT. Thus, despite other study's findings of altered folliculogenesis [28,29,40], in the paradigm explored in this study, there was no evidence that GLY has negative consequences for ovarian PI3K signaling.

STAR protein facilitates cholesterol translocation from the mitochondrial outer to inner membrane while CYP19a catalyzes conversion of testosterone to E_2 in granulosa cells. The potential for GLY to represent an endocrine disruptor was initially supported by *in vitro* studies: GLY exposure reduced STAR protein in Leydig cells [11], and reduced CYP19a activity in both a placental cell line, JEG3 [12] and an embryonic cell line, 293 [41] suggesting that GLY might alter ovarian steroidogenesis. GLY has been implicated as a potential endocrine disrupting chemical by *in vitro* studies [11,12,41]. Exposure of pregnant mice (gestation day 1–19) to GLY or GBH (0.5 % solution) reduced P_4 and increased estrogen as well as altered hypothalamic and pituitary abundance of *Gnrh* and *Fsh*, respectively [28]. In addition, bovine granulosa cells exposed to GLY (5 μg/mL) produced lower amounts of E_2 than matched controls [27]. In contrast, however, are safety evaluation studies for GLY in which standard endocrinological assessments determined that GLY did not disrupt ovarian-produced hormones [8].

Similar to the lack of a GLY-induced effect on PI3K signaling members, there was no effect of GLY on mRNA encoding *Star*, *Hsd3β1*, *Cyp11a1* or *Cyp19a1* nor was there any impact on the estrogen receptors *Erα* or *Erβ*. There was also no impact of GLY on protein abundance of STAR, HSD3β, CYP11A1, CYP19A1, ER*α* or ERβ after either exposure duration. STAR, HSD3β, ER*α* and CYP11A1 were located in both the granulosa and theca cells, while CYP19A1 was localized to granulosa cells. There was a tendency for reduced abundance of both HSD3B and CYP19A1 as measured by immunofluorescence in large follicles, potentially indicating an onset of altered follicular function at this point, consistent with a reduction in ovarian weight. There was no effect of GLY exposure on abundance as measured by immunofluorescence detection of STAR, CYP11A1, or ERA. This was surprising since reduced ovarian mRNA encoding *Star* and *Hsd3β1* have previously been reported in exposed pregnant mice [28]. Further, increased *Cyp11a1*, *Cyp17a1* and *Cyp19a1* have been reported in pregnant mice exposed to either GLY or GBH [28]. Finally, there was no impact of GLY on E_2 in circulation. There was also no effect of GLY exposure on

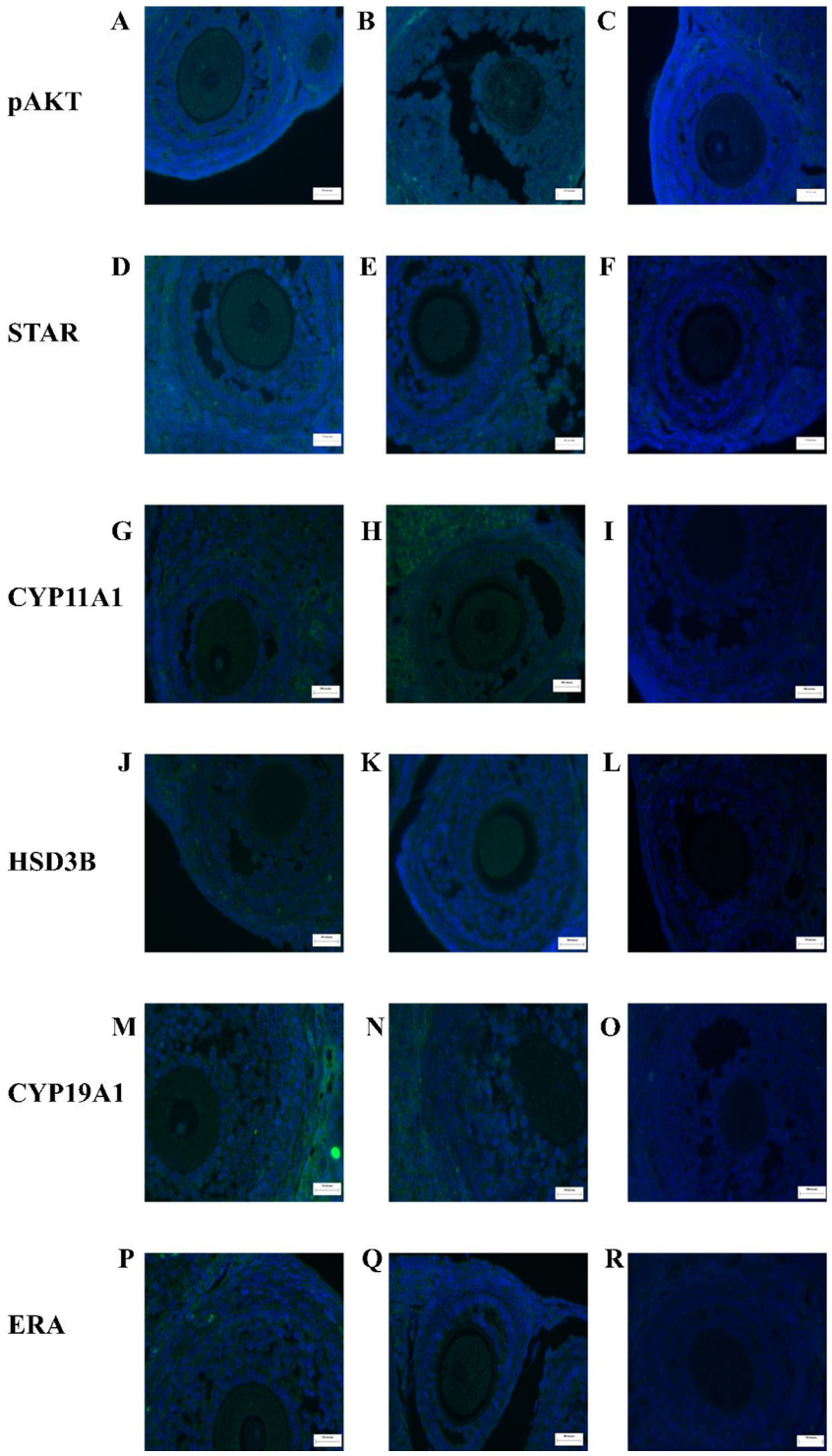

Fig. 8. Effect of GLY exposure on ovarian protein localization in large pre-antral follicles. Female C57Bl/6 mice were exposed to saline vehicle control (CT) or GLY (2 mg/Kg) per os from a pipette tip five days per week for 10 weeks. Immunostaining was performed only in ovaries from the 10 week exposure. Ovaries were prepared for immunofluorescence staining to localize and quantify protein abundance of (A-C) pAKT, (D-F) STAR, (G-I) CYP11A1, (J-L) HSD3B, (M-O) CYP19A1 and (P-R) ERA. Blue staining represents DNA and green staining indicates protein of interest. Scalebar = 100 microns.

circulating P_4 after 5 weeks in contrast with observations in another study [28]. Thus, there was no overt endocrine disrupting impact of GLY on E_2 or P_4 noted. A more optimal paradigm to explore impacts on P_4 would be determining effects in the luteal phase of the estrous cycle. In addition, chronic exposures that are longer in duration may have utility to determine if there are long term ovarian impacts of low level GLY exposure.

In further support of lack of an endocrinological or follicular disruption, while there was a statistical tendency for alterations to estrous cyclicity after 5 weeks of exposure, there was no lasting effect of GLY exposure on the percentage time spent at any stage of the estrous cycle. Thus, all aspects of the endocrine pathway and phenotypic endpoints investigated were largely unaffected by GLY exposure. These data strongly suggest that GLY is not an endocrine disrupting compound at

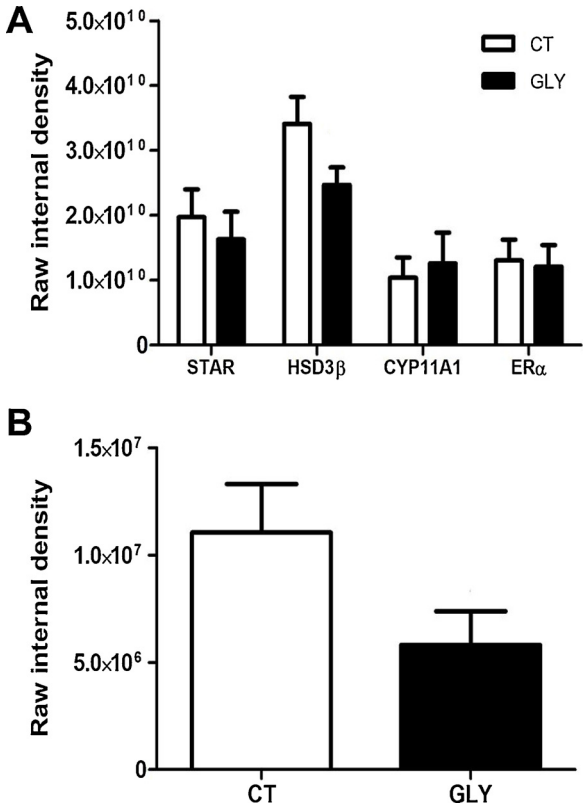

Fig. 9. Effect of GLY exposure on ovarian protein quantification in large pre-antral follicles. Female C57Bl/6 mice were exposed to saline vehicle control (CT) or GLY (2 mg/Kg) *per os* from a pipette tip five days per week for 10 weeks. Ovaries that were immunofluorescence stained were analyzed for abundance of (A) STAR, HSD3B, CYP11A, and ERA. (B) Quantification of CYP19A1. Bars represent mean internal density +/- SEM.

relevant exposure levels and correlate with safety studies on GLY [8] and findings from the standardized H295R steroidogenesis assay [16].

5. Conclusions

Taken together, this study determined absence of GLY-induced alterations to the reproductive endpoints measured. After both 5 and 10 weeks of GLY exposure, relative to the saline vehicle control treated mice, any impacts of GLY on endpoints measured was largely absent. This study involved sample deidentification to remove any unintentional or perceived bias, was chronic in nature and performed at a conservative dosage (relevant to human exposure). In summary, the findings of the current study do not support that chronic, oral GLY at a dose considered non-hazardous alters estrous cyclicity, follicle number, circulating ovarian steroid hormone levels and ovarian intracellular signaling in adult female mice.

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Declaration of Competing Interest

There are no conflicts of interest to declare.

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