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Research Article

Effect of Glyphosate and Roundup on H295R, T47D and Murine Follicular Cell Viability and Estrogen Synthesis *in Vitro*

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ABSTRACT

Humans can be exposed to 21 mM – 2.13 M of Glyphosate in various herbicide formulations (GBH) such as Roundup. GBH decreased the activity of a single estrogen-synthesising enzyme, and stimulated proliferation of Estrogen Receptor (ER)-positive cells under estrogen and serum-free conditions *in vitro*. Study aims were to assess the entire multi-enzyme steroidogenic pathway by using H295R cells and ovarian follicles, and to examine ER-mediated cell proliferation using T47D and OVCAR cells, in the presence of 10% FCS in vitro.

Human T47D, OVCAR and H295R cells were exposed to 0-4.3 M glyphosate or GBH for 4-72 h. Follicles from murine ovaries were exposed to 0, 0.0025 or 0.025 M glyphosate or GBH for 24 h. Secreted estrogen was measured by ELISA, and viable cell numbers by MTT, crystal violet or trypan blue exclusion assay.

Low non-cytotoxic concentrations did not increase ER-positive T47D cell proliferation, nor decrease H295R estrogen synthesis. Primary-derived follicular cells were more resistant to GBH-induced cytotoxicity than the cell lines, and non-cytotoxic concentrations of glyphosate and GBH inhibited follicular estrogen synthesis.

The response of steroidogenic ER-positive follicular cells was different from immortalised cells that expressed either the steroidogenic pathway or ER. Potential endocrine disrupters such as glyphosate and GBH require further assessment *in vivo*.

INTRODUCTION

The development of crops that are resistant to the herbicide glyphosate has expanded the application of Glyphosate-Based Herbicides (GBH) such as Roundup [1,2] .Glyphosate is a competitive inhibitor of an enzyme required for the synthesis of aromatic amino acids in micro-organisms and plants but not animals [3-5]. Glyphosate is soluble in water [6] and has been found at levels of 0.5-5mg/Kg in soil, and 0.1-0.7mg/L in water [2,7]. Williams, et al. claimed that glyphosate does not pose a health or safety risk to humans [4].

The toxicity of GBH derives from the mixture of glyphosate with adjuvants and surfactants [8], which are added to improve the absorption of glyphosate across plant cell walls and lipid plasma membranes [9]. Since these adjuvants are not the active herbicidal agent, they are not subject to toxicological assessment by regulatory authorities [10]. The precise composition of adjuvants classified as inert in commercial herbicides need not be fully disclosed by manufacturers, despite reports demonstrating that the commercial GBH formulations containing adjuvants are more cytotoxic than glyphosate alone [2,11-15]. Commercially available formulations of glyphosate such as Roundup vary the concentration of glyphosate and adjuvants and have been classified as high, medium or low toxicity [13].

Endocrine-Disrupting Chemicals (EDCs) interfere with hormone biosynthesis, metabolism or action [16]. The rate limiting step in the biosynthesis of steroidogenic hormones is the uptake of cholesterol from the outer to the inner mitochondrial membrane, and is mediated by the steroidogenic acute regulatory protein (StAR) [17-19]. Once cholesterol is inside the mitochondria it is rapidly converted to pregnenolone by cytochrome P450 cholesterol side chain cleavage enzyme (CYP450scc, also designated CYP11A1). Pregnenolone can then be converted to 17-hydroxyprogesterone which in turn is converted to Dehydroepiandrosterone (DHEA) and then androstenedione, testosterone and estradiol 17β (E2) [15]. The conversion of androstenedione to E2 is catalysed by the CYP450 aromatase enzyme (CYP19A1) [20].

Richard, et al. exposed human JEG3 cells to non-cytotoxic concentrations of Roundup or glyphosate using serum-free culture conditions with non-physiological *p*H. After 18h exposure, the herbicides were removed and the cells were supplied with androstenedione, the substrate for the CYP450 aromatase enzyme [21]. The synthesised E2 was measured in a radioimmunoassay. This measured the activity of the CYP450 aromatase enzyme but did not examine the complete, intact steroidogenic pathway. In a second assay, aromatase activity was assessed using equine microsomes supplied with tritiated androstenedione. Gasnier, et al. also used a tritiated androgen to estrogen conversion assay in a serum-free culture system, and concluded that GBH are endocrine disrupters in vitro, because they reduced aromatase expression and activity, and Roundup was a more potent inhibitor of CYP450 aromatase activity and mRNA levels than glyphosate alone [8]. Testicular Leydig cells express a steroidogenic pathway and are the male equivalent of ovarian granulosa cells. A preparation of primary-derived rat Leydig cells reduced testosterone production when exposed to non-cytotoxic concentrations of Roundup or glyphosate, whilst glyphosate increased aromatase expression [22]. Although a number of studies [14,23] have reported glyphosate as interfering with estrogen synthesis by inhibiting CYP450 aromatase activity, others found the opposite. Cassault-Meyer, et al. exposed adult rats to Roundup for 8 days, and found the CYP450 aromatase expression increased by approximately 50% in the testes, and that there were no changes in the levels of the estrogen receptors alpha or beta (ERα/ ERβ) [24].

A rapid screening test using the human H295R adrenocortical carcinoma cell line was selected by the US EPA to examine the effects of EDCs on steroid hormone synthesis because the cell line models normal, physiological *in vivo* steroidogenesis [25-27], and can be used to assess an integrated response to putative EDCs [25]. Not only is the entire steroidogenic pathway expressed by the H295R cell line, the steroid hormone biosynthesis also occurs under standard in vitro cell culture conditions of physiological *p*H, and in media supplemented with the weakly estrogenic *p*H indicator phenol red, and 10% Foetal Calf Serum (FCS), which contains approximately 75 pg/mL E2 as well as Sex-Hormone Binding Globulin (SHBG, [28]). Others report 10% FCS as containing 10^{-12} M E2 [10]. The current understanding of EDCs is that they cause a 'deviation from normal homeostatic control or reproduction' [16]. The isolated androstenedione to E2 reaction [8,14,21,23] does not represent the integrated in vivo steroidogenic pathway, and it could be argued that the final step of the steroidogenic biosynthesis pathway that produces E2 should not be used in isolation to discuss endocrine disruption, particularly since the rate limiting step in steroidogenesis is not represented in these assays [17-19].

The H295R assay was standardised in an international inter-laboratory comparison study [29], and validated by noting basal hormone production as well as the effects of 6 model chemicals [27].

In the standardised, validated assay, the H295R cells were exposed to test compounds for 48h at concentrations that did not cause cytotoxicity in an MTT assay. Of the test compounds, forskolin significantly increased E2 production and prochloraz, a specific CYP450 aromatase inhibiter, inhibited E2 production [29]. The US EPA H295R assay [29] is ideal for examining the effects of glyphosate and Roundup on the biosynthesis of steroid hormones [26], but since the cell line does not express ERα or ERβ, a different in vitro model is required to elucidate the effects of GBH on the action of E2.

Fortunati, et al. demonstrated that the combination of SHBG and E2 found in 10% FCS upregulated the transduction molecule, cyclic Adenosine Monophosphate (cAMP), in the human breast cancer cell line MCF7. Dextran Charcoal stripping (DC) reduced the E2 levels in FCS by 97% to 2.2pg/mL, but did not remove SHBG, and DC FCS increased cAMP levels in MCF7 cells [28]. The T 47D cell line was also derived from a human breast carcinoma, and both MCF7 and T47D cell lines express ERα and ERβ. When T47D cells were cultured in 10% DC FCS, glyphosate activated the Estrogen Response Element (ERE), increased expression of ERβ and ERα, and 10^{-9}M glyphosate stimulated T47D cell proliferation by ~30% [30]. Culturing the T47D cells in 10% FCS prevented the proliferative effects of glyphosate, probably because glyphosate is a weak estrogen and was blocked by the E2 present in 10% FCS. Lin & Garry, et al. (2000) found that glyphosate and Roundup stimulated similar levels of proliferation in MCF7 cells that were cultured in DC FCS, and confirmed the observation that glyphosate is a weak estrogen [10]. They also showed that 10^{-5} to 10^{-4}M glyphosate, and 1 to 10µg/mL Roundup with 0.99% glyphosate, stimulated approximately 25% proliferation when the MCF7 cells were cultured in 10% FCS, but there was no significant difference in proliferative activity between glyphosate and the formulation of Roundup that they used. A low 10^{-9}M concentration of glyphosate did not stimulate proliferation of T47D cells cultured in 10% FCS [30], whereas higher 10^{-5} to 10^{-4}M concentrations of glyphosate did increase the proliferation of MCF7 cells [10]. Hormetic effects in which high and low concentrations have different effects have been reported in other endocrine systems, but the effects of glyphosate and Roundup on the proliferation of T47D and MCF7 cells requires further clarification.

The H 295R c ell line can be used to examine the effects of glyphosate or GBH on E2 biosynthesis, and the T47D and MCF7 cell lines can be used to explore the effects of glyphosate and GBH on proliferative effects mediated by ERα or ERβ, but female mammalian E2 biosynthesis in vivo is predominantly a function of the ovarian granulosa cells, which also express ERβ, but not ERα [31]. Steroid hormones synthesised by the granulosa cells bind to steroid hormone receptors expressed by the same cells. Specific activation of ERβ with its natural ligand E2 stimulates granulosa cell proliferation [31], but ER activation also combines with other endocrine signals, such as the Follicle Stimulating Hormone (FSH) mediated upregulation of cAMP, to modulate granulosa cell proliferation. Any effects of glyphosate or Roundup in vivo will be in a context in which two mechanisms of action (E2 biosynthesis and ER activation) occur within one cell. In the ovary in vivo, granulosa cells surround single oocytes in a three-dimensional sphere to form a follicle. The follicular granulosa cells are in turn surrounded by a basal lamina comprised of collagen IV and laminin [32]. We therefore applied a well-established model in which follicles are harvested from adult mouse ovaries and cultured in vitro [19]. The in vitro culture of follicles has the advantage over transformed, continuous cells such as H295R, because not only do follicles consist of primary-derived untransformed cells, follicles in vitro maintain the Three Dimensional (3D) cellular organisation and structure found in vivo [19], and this is likely to facilitate the intercellular signalling associated with the normal regulation of E2 biosynthesis and ER expression.

We hypothesise that when cells are cultured in vitro with physiological pH and 10% FCS with low concentrations of E2, that low non-cytotoxic concentrations of GBH or glyphosate will not stimulate the proliferation of cells expressing ER. Since Cassault-Meyer, et al. 2014 found that Roundup increased CYP450 aromatase expression in vivo, will stimulate E2 synthesis by ovarian follicles in vitro [24].

MATERIALS AND METHODS

Chemicals

All chemicals and reagents used in this study were HPLC grade and obtained from Sigma-Aldrich unless otherwise stated.

Cell Line and Cell Culture

The H295R, T47D, MCF7 and OVCAR cell lines were obtained from the Global Bioresource Centre™ (ATCC) and cultured at 37°C in a humidified atmosphere with 5% CO2 and subcultured every 2-3 days as required [33]. The H295R cells were maintained in DMEM/F12 medium supplemented with 10% heat inactivated Foetal Calf Serum (FCS, Invitrogen Corporation), sodium pyruvate (1mM), HEPES (10 mM), glucose (4.5 g/L), L-glutamine (2mM), sodium bicarbonate (1.5 g/L), penicillin (60 mg/L)and streptomycin (50 mg/L). The T47D cells were cultured in 10.4 g/L RPMI-1640, supplemented with 10% FCS, 1% (v/v) penicillin/streptomycin (Thermo Scientific) and 0.11g/L sodium pyruvate, 2.5 g/L d-Glucose, 2.38g/L HEPES and 1.5 g/L FCS. MCF7 were maintained in EMEM (Thermo Scientific) supplemented with 1.5 g/L sodium bicarbonate, 0.11 g/L sodium pyruvate, 0.01 mg/ml bovine insulin and 10% FCS. For all experiments, exponentially growing cells (80% confluence) were detached from flasks with 0.25% Trypsin-EDTA solution. Cell number and viability was determined using the Trypan Blue exclusion assay on a haemocytometer before each experiment [34].

Glyphosate and Roundup

Glyphosate N-phosphonomethyl glycine (Sigma) was initially dissolved in water then diluted with DMEM/F12 + 10% FCS to 0.05 M, such that the final concentration of DMEM/F12 + 10% FCS was 97% v/v. Roundup Weed Killer Concentrate (Woolworths Pty Ltd.), with 360g/L Glyphosate N-phosphonomethyl glycine + 10% surfactant, was diluted with DMEM/F12 + 10%FCS to 0.05 M, such that the fi nal concentration of DMEM/F12 + 10% FCS was 97% v/v.

The initial 0.05 M stock solutions of Glyphosate and Roundup were acidic, and were adjusted to *p*H 7.4 before further dilution. Each 0.05M stock solution was diluted in DMEM/F12 + 10% FCS medium to generate solutions of $5x10^{-6}$ M, $5x10^{-5}$ M, $5x10^{-4}$ M, $5x10^{-3}$ M, $5x10^{-2}$ M.

ER*α* Protein Detection

Total protein was extracted from a 80-90% confluent T25cm^2 flasks of T47D, MCF7 and OVCAR cell lines using modified RIPA (mRIPA) buffer (50 mM Tris-HCl, *p*H 7.4, 1% NP-40, 0.25% sodium-deoxycholate, 150 mM NaCl, 10 mM EDTA supplemented immediately before use with 1 mM NaF, 1 mM activated Na_3VO_4, 1mM Phenylmethanesulphonylfluoride (PMSF) (solubilised in isopropanol) and 2% Cocktail of Protease Inhibitors (Sigma-

Aldrich)). Cells were rinsed with cold PBS and 500 µl mRIPA was added to each flask on ice for 15 minutes with occasional rocking. The cell lysate was centrifuged at 16000 x g for 15 minutes at 4°C and the supernatant retained. Protein assays were performed using Bovine Serum Albumin (BSA) standards, diluted in mRIPA from 0 to 500 µg/ml. The Bicinchoninic Acid (BCA) Protein Determination Kit (Pierce, Illinois, USA) is a detergent-compatible assay based on the alkaline reduction of Cu^{2+} to Cu^{1+} by proteins, and the formation of a bicinchoninic acid: Cu^{1+} complex [35]. The complex has an absorbance maximum at 562 nm. Each sample was quantified according to the manufacturer's instructions in duplicate using a 96 well plate and an automated microplate reader. Protein (10 µg) from each cell type was boiled for 5 minutes with 3x reducing sample buffer and loaded into a hand-cast 4-12% resolving SDS polyacrylamide gel. 1 µl MagicMark XP (Invitrogen) was used as a protein size marker. BioRad mini PROTEAN 3 cells were used to resolve the protein at 200 V for approximately 40 minutes. MagicMark XPTM protein molecular weight standard was resolved alongside samples.

Detection of ER*α* by Western Analysis

Proteins separated on SDS-PAGE were transferred onto PVDF membrane in Transfer buffer (25 mM Tris, *p*H 8.3, 192 mM glycine, 20% methanol (v/v)) using an ice-cooled mini-Protean II cell (BioRad) for 1.5 hours at 100 V on a magnetic stirrer. The transfer was checked using Ponceau S stain (0.2% w/v) in acetic acid (10% v/v). Membranes were left at 4°C overnight in blocking buffer (40mM Tris pH 7.4, 0.1% (v/v) Tween20, 5% (w/v) skim milk powder). Membranes were rinsed 3 three times in Tris-Tween buffer (40 mM Tris pH 7.4, 0.1% (v/v) Tween20). Membranes were incubated with a 1:500 dilution of rabbit anti-ERα polyclonal antibody (HC-20) (Santa Cruz Biotechnology, Inc., U.S.A.) or 1:200 dilution of rabbit anti-ER*β* polyclonal antibody (H-150) or goat anti-ER*β* polyclonal antibody (L-20) in Blocking buffer for 1 hour at 37°C with gentle rocking. This was followed by 3 rinses in Wash buffer (20 mM Tris, pH 7.4, 150 mM NaCl, and 5% (w/v) skim milk powder). The goat anti-Rabbit HRP secondary antibody (Sigma) was diluted 1:10000 with blocking buffer and incubated with the membrane for 1 hour at 37°C with gentle rocking. After washing in Tris-Tween buffer, ECF substrate (GE Healthcare) was added after Tris-Tween had been drained from the membrane. ECF was added at approximately 1mL per 10 cm^2 membrane. The membrane was left in darkness to develop for 5 minutes. Fluorescence of the HRP bound ECF was visualised on a Typhoon imager (Amersham Biosciences). The blot was repeated on three separate occasions (n = 3) and quantified by pixel count on Image J software [36].

H295R Endocrine Disruption Assay

H295R cells (40000 per well) were incubated for 3h in DMEM/F12 + 10% FCS to facilitate adherence to the culture vessel, these media were discarded, and the cells were exposed to each concentration of glyphosate or Roundup, or estrogen synthesis controls forskolin (10 µm) or prochloraz (3 µm) [26], or a cytotoxic positive control, 70% Methanol (MeOH), in triplicate wells. The 0% control treatments were DMEM/F12 + 10% FCS medium at 97% v/v., in triplicate wells, whereas another six wells contained DMEM/F12 + 10% FCS. The H295R cells were exposed to glyphosate, Roundup or control treatments for 20 h (n = 6 separate occasions) or 48 h (n = 4) before media were collected for estrogen (E2) ELISA, and the mean ± SEM numbers of surviving cells in each well were determined in an MTT assay by comparison with a standard curve generated for each experimental replicate.

MTT Cytotoxicity Assay

Thiazolyl Blue Tetrazolium Bromide (MTT) was dissolved in sterile PBS to give a final concentration of 5mg/mL. The MTT assay was carried out by modification of the original Mosmann (1983) protocol and used a 96 well plate format [37,38]. Standard

curves were generated for each replicate experiment, which consisted of 6 serial dilutions spanning 0-80,000 cells per well in 96 well 'standards' plates, and each cell concentration was examined in 6 replicate wells. H295R cells in the standard curve plates were incubated for 23 h (in the treatment plates cells were incubated for 3h for attachment + 20h exposure) before media were discarded and the number of viable cells per well determined in an MTT assay. The MTT assay was carried out by diluting the stock MTT in DMEM/F12 + 10% FCS to give a working concentration of 0.5 mg/mL. 100 µL MTT was added to each treatment or standard curve well for 1h at 37°C + 5% CO_2. After this, 80 µL of 20% SDS in 0.02M HCl were added to each well to solubilise the purple formazan product, and the cells were incubated for a further 3 h at room temperature [39]. The absorbance was measured at 570 nm, with reference absorbance 630 nm, using an automatic spectrophotometer with KC Junior software.

Crystal Violet Cytotoxicity Assay

This method was adapted from (Bonnekoh et al. 1989, Kueng, Silber, and Eppenberger 1989, Reid et al, 2015). Crystal violet

(Hexamethylpararosaniline chloride) 0.5% w/v in 50% methanol solution was made and stored at room temperature. After cell culture in 96 well plates, media supernatents were removed and 50 µl crystal violet stain was added to each well. Cells were stained and fixed for 10 minutes, gently rinsed with RO water and excess water was removed. Stained cells were left to dry before adding 50 µl 30% acetic acid to solubilise and homogenise the stain. Absorbance of the crystal violet was read at 570 nm with a reference wavelength of 640nm on a µQuant automatic spectrophotometer (Bio-TEK instruments, NSW, Australia) using KC Junior software (Bio-Tek, VT, USA).

Viable cell suspensions of T47D cells were serially diluted in a 96 well plate (Nunc, Denmark) using a multi-channel pipette (80,000 - 1250 cells per well in six replicates) in 100 µl of their respective media. The mean absorbance of the six replicate wells was related to the initial cell number in each well using a linear regression function in Excel. The equation describing the relationship between absorbance and cell number, was then used to convert absorbance to cell number aft e r application of the crystal violet assay to the remaining plates within the assay.

Murine Follicular Granulosa Cell Assay

C57BL mice (n = 12) were culled between 8 and 12 weeks of age because they surplus to requirements for the animal house breeding programme. Th e Flinders Animal Welfare Committee approved our use of ovaries from these mice after cervical dislocation by animal house staff. The ovaries and oviducts were transported in DMEM at 37°C for 10-15 mins before disaggregation. Ovaries were dissected free of the surrounding connective tissue and examined for corpora lutea to indicate previous ovulation. Each ovary was dissected in half and each portion placed in one well of a 24 well plate containing 0.5 mL collagenase IV (2mg/mL). Each half ovary was cut into smaller pieces before incubation for 30 minutes at 37°C with 1 minute agitation every 10 minutes. Following incubation, the ovarian tissue was returned to a plate containing DMEM and mechanically disaggregated for 5 min under a dissecting microscope. The diameters

of intact follicles with undamaged, regular morphology (Figure 1) were measured using a micrometer in the dissection microscope eyepiece, and 24 secondary follicles (78.05-217.5 µm diameter) were selected for each experiment [40].

Follicle culture and exposure for each replicate experiment (n = 4) took place in a round-bottomed 96 well plate. Follicles were cultured in 200 µL of DMEM/F12 + 10% FCS containing the following reagents; glyphosate 0.0025 M and 0.025 M, Roundup 0.0025 M and 0.025 M, 70% ethanol (positive control) and DMEM/F12 + 10% FCS alone as a negative control. Each treatment was examined using 4 secondary follicles in one well, and the experiment was repeated using follicles collected on 4 separate occasions (n = 4). Follicles were incubated for 24 hours at 37°C with 5% CO_2, then the media were collected and stored at -20°C until estrogen concentrations were measured in an ELISA.

The supernatants collected for E2 ELISA were replaced with DMEM/F12, and each group of four follicles was disaggregated by adding 10 µL of 12 mg/ml collagenase IV to give 2 mg/mL working concentration. The follicles were incubated at 37°C for 30 minutes, with agitation after 15 and 30 minutes. 50 µL of 0.05% hyaluronidase were added to each well and the follicles incubated for a further 30 minutes at 37°C with 1 minute agitation every 15 minutes.

The granulosa cells from four follicles (in each treatment) were collected and resuspended in 20 µL of DMEM/F12 and 20 µL Trypan Blue. The numbers of live and dead granulosa cells were determined using a haemocytometer. The granulosa cells from each group of four follicles were assessed in the Trypan Blue exclusion assay by using 3 replicate samples from each granulosa cell suspension [41].

The number of viable granulosa cells in each group of 4 follicles was expressed per follicle, then divided by the average diameter (µm) of the same 4 follicles, to remove the effect of follicle size on granulosa cell numbers. A total of 96 murine follicles were examined in this study, and their diameters were 112 ± 12 µm (mean ± stdev).

Estradiol Enzyme Linked Immunosorbent Assay (ELISA)

In the competitive ELISA used in this study, the primary antibody against Estradiol (E2) was supplied adsorbed to the wells of a 96-well ELISA plate by the manufacturer (Cayman). 'Blank' ELISA control wells contained 50 µL ELISA Immuno Assay (EIA) buffer but no primary antibody, whereas the reference standard wells contained 50 µL purified E2 (0-4000 pg/mL) prepared in DMEM/F12 medium, and the test wells contained 50µL conditioned cell culture medium. 50 µl Glyphosate and Roundup solutions in DMEM/F12 + 10% FCS ($5x10^{-6}$ M, $5x10^{-5}$ M, $5x10^{-4}$ M, $5x10^{-3}$ M, $5x10^{-2}$ M) were also added to test wells to determine if the tested concentrations of the herbicides interfered with the ELISA. The standard and the test wells had 50µl EIA buffer added to them, then all the wells had enzyme conjugated to E2 added before incubation at room temperature for 60 minutes. After washing, 100 µL of substrate were added to each well for 60 minutes. Absorbance was read using an automatic spectrophotometer at primary wavelength 405 nm, and reference wavelength 540nm using KC Junior software. Hormone concentration in test wells was calculated by comparison with the standard curve. The manufacturer states that this ELISA has a detection limit of approximately 20 pg/mL, and the intra- and inter-coefficients of variation in the middle of the standard curve were 13 and 8.2% respectively.

Statistical Analysis

The H295R cell viability and E2 data, and the viable granulosa cell / follicle diameter data, and the E2 concentrations per group of 4 follicles, were analysed by 1-way ANOVA with Tukey post-hoc test. The T47D and OVCAR cell viability data were analysed by analysed by 2-way ANOVA with Bonferoni post-hoc test. The datasets were normally distributed and significance was assigned at $p < 0.05$.

RESULTS

Low concentrations (4.3×10^{-7} to 4.3×10^{-3} M) glyphosate, or glyphosate in Roundup formulation, had no effect on T47D cell viability in the presence of 10% FCS (Figure 2). Exposure to Roundup containing 4.3×10^{-2} M glyphosate however, caused a significant decrease in viability compared to control after 24 h ($p < 0.05$) and 72 h ($p < 0.001$). Glyphosate in Roundup (4.3×10^{-2} M) also caused significantly more cytotoxicity than glyphosate alone after 24 h ($p < 0.001$) and 72h ($p < 0.001$). Glyphosate only caused a significant reduction in viable T47D cell numbers after a 72 h exposure to 4.3 M ($p < 0.001$).

Western blot analysis of protein lysates from each cell line identified the full length 66kDa ERα protein in MCF7 and T47D cells, but not the OVCAR cells (Figure 3). Densitometry of three separate

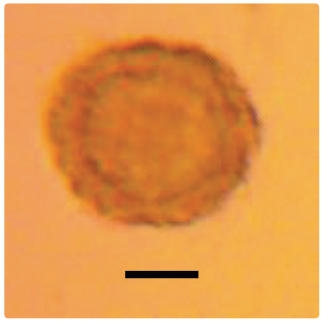

Figure 1: Murine secondary follicle, scale bar 50µm.

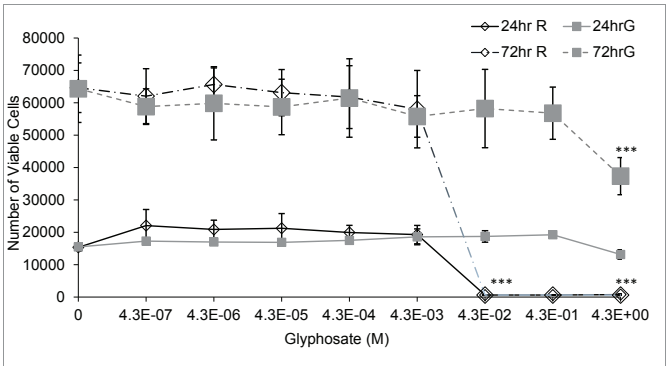

Figure 2: H295R estrogen production and cell viability
H295R cells were exposed in triplicate wells for 20 or 48h on 3 separate occasions. The Estrogen (E2) secreted into the culture media was measured in an ELISA and the numbers of surviving cells in each well were determined in an MTT assay, by comparison with a standard curve generated for each experimental replicate. No E2 was detected after exposure to 70% methanol, the assay positive control. Data are presented as mean ± SEM (n = 3). Medium control DMEM/F12 with 10%FCS. Solvent control 0.1% DMSO in DMEM / F12 with 10%FCS. Forskolin dissolved in DMSO to final concentration of 0.1%. Data analysed by 1-way ANOVA with Tukey post-hoc test, and difference from solvent (forskolin) or medium (prochloraz) control at the same exposure period shown; $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***.

western blots showed the highest expression of ERα in MCF7 (1) relative to T47D (0.74 ± 0.18).

Exposure to low concentrations (4.3 x 10^{-7} to 4.3 x 10^{-4} M) of glyphosate in a Roundup formulation had no effect on OVCAR cell viability (Figure 4), but a 72 h exposure to 4.3 x 10^{-3} M decreased viability ($p < 0.05$), and shorter exposures to 0.43 M glyphosate significantly reduced cell viability after 4 h ($p < 0.05$) and 24 h ($p < 0.001$).

Neither forskolin nor prochloraz were cytotoxic at the concentrations examined in this study (Figure 5). Exposures to forskolin of 20h and 48h caused the H295R cells to increase estrogen production by 3.3 and 3.5-fold respectively. Prochloraz caused a 0.28 and 0.22-fold decrease in estrogen production after 20 and 48 h respectively. Exposure to glyphosate (2.5 x 10^{-6} to 2.5 x 10^{-3} M), or the same concentrations of glyphosate in a Roundup formulation, for 20 or 48 h had no effect on H295R cell viability or E2 production, but the highest concentration tested (2.5 x 10^{-2} M) caused a significant decrease in viable cell numbers ($p < 0.001$) and in E2 synthesis ($p < 0.05$, Figure 6). Lower, non-cytotoxic concentrations of Roundup or glyphosate did not affect estrogen production.

A 24 h exposure to a high (2.5 x 10^{-2} M) concentration of glyphosate, or to glyphosate in a Roundup formulation, had no effect on granulosa cell viability in a 3D follicle culture system (Figure 7), although similar concentrations of glyphosate in Roundup significantly decreased OVCAR (Figure 4) and H295R (Figure 6) cell viability. Non-cytotoxic concentrations (2.5 x 10^{-3} to 2.5 x 10^{-2} M) of glyphosate alone or in Roundup formulation significantly reduced E2 biosynthesis ($p < 0.05$, Figure 7), whereas similar concentrations of glyphosate alone had no effect on E2 biosynthesis by the H295R cells (Figure 6).

DISCUSSION

Estrogenic endocrine disrupting compounds can be agents that interact with the estrogen receptor, or that modulate biosynthesis of the steroid hormone estrogen. We have shown for the first time that although non-cytotoxic concentrations of glyphosate or GBH had no effect on E2 synthesis by a continuous cell line expressing a complete steroidogenic biosynthesis pathway, there were significant reductions in E2 production by primary-derived follicular granulosa cells that expressed both ER and a complete steroidogenic pathway.

Our finding that low concentrations (10^{-7} to 10^{-3} M) of glyphosate did not stimulate T47D cell proliferation in the presence of 10% FCS extends the dose range (10^{-12} to 10^{-6} M) previously reported to have no effect on T47D cell proliferation [30]. We examined the same concentration range of glyphosate in Roundup formulation, and this also failed to stimulate T47D cell proliferation. These results differ from Lin and Garry (2000), who found that 10^{-5} to 10^{-4} M concentrations of

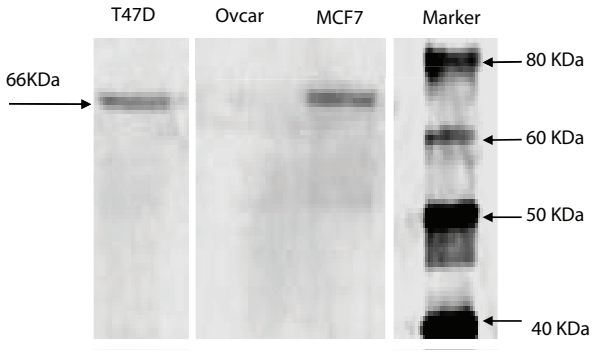

Figure 3: Effect of Glyphosate or Roundup on H295R cell viability and estrogen production
H295 cells were exposed to glyphosate or Roundup in triplicate wells in 96 well plates for 20h (n = 6) or 48h (n = 4). Media were collected for Estrogen (E2) ELISA, and the numbers of viable cells remaining in the wells were determined by MTT assay by comparison with a standard curve generated for each experimental replicate. Data are presented as mean ± SEM, and analysed by 1-way ANOVA with Tukey post-hoc test, and difference from vehicle control (97% DMEM/F12 + 10% FCS) shown; p < 0.05 *, p < 0.01 **, p < 0.001 ***.

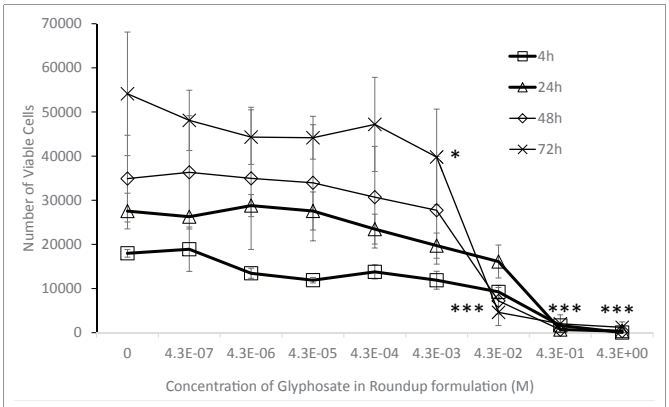

Figure 4: Effect of Glyphosate and Roundup on murine follicle viability and estrogen production
Murine secondary follicles were exposed in groups of 4 follicles to glyphosate or Roundup or DMEM/F12 medium (negative control) or 70% ethanol (positive control) in DMEM/F12 medium for 24h on four separate occasions. A) Follicles were disaggregated, the numbers of viable cells from four follicles were determined in a Trypan Blue exclusion assay, and this value was adjusted to represent the number of cells in one follicle. Since follicles were different sizes, this source of variation was removed by expressing the cell numbers per μm of follicle diameter. Data shown as mean ± stdev (n = 4). B) Estrogen in the media was assessed in an ELISA and shown as mean ± SEM (n = 4). Data analysed by 1-way ANOVA with Tukey post-hoc test, and difference from DMEM/F12 medium control shown; p < 0.05*, p < 0.001***.

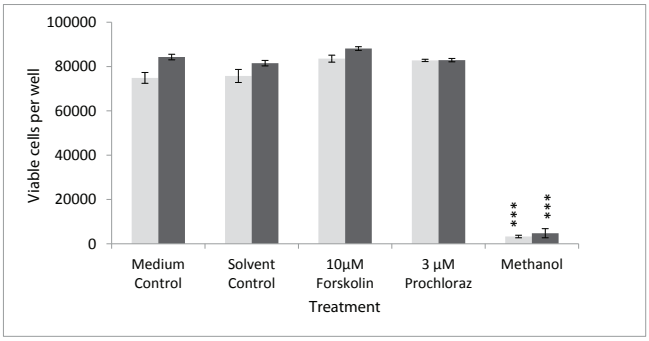

Figure 5: H295R estrogen production and cell viability
H295R cells were exposed in triplicate wells for 20 or 48h on 3 separate occasions. The Estrogen (E2) secreted into the culture media was measured in an ELISA and the numbers of surviving cells in each well were determined in an MTT assay, by comparison with a standard curve generated for each experimental replicate. No E2 was detected after exposure to 70% methanol, the assay positive control. Data are presented as mean ± SEM (n = 3). Medium control DMEM/F12 with 10%FCS. Solvent control 0.1% DMSO in DMEM/F12 with 10%FCS. Forskolin dissolved in DMSO to final concentration of 0.1%. Data analysed by 1-way ANOVA with Tukey post-hoc test, and difference from solvent (forskolin) or medium (prochloraz) control at the same exposure period shown; p < 0.05*, p < 0.01**, p < 0.001***.

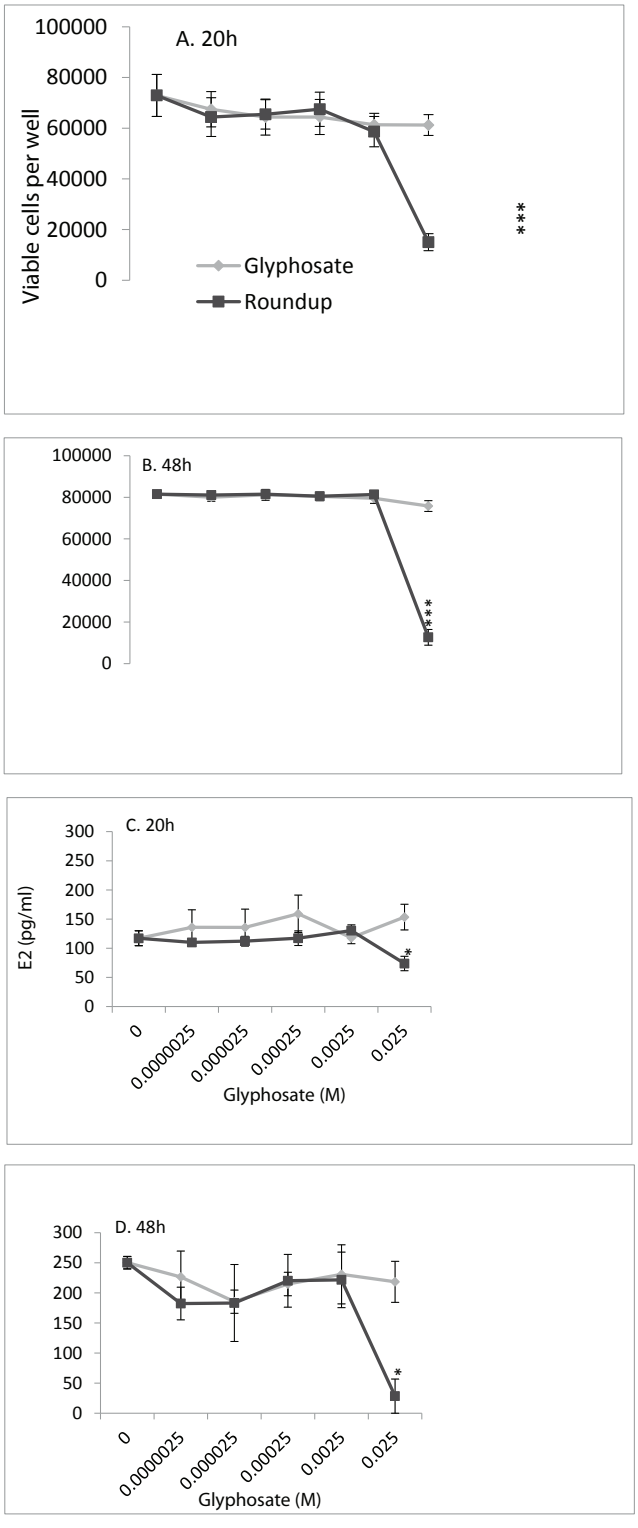

Figure 6: Effect of Glyphosate or Roundup on H295R cell viability and estrogen production

H295R cells were exposed to glyphosate or Roundup in triplicate wells in 96 well plates for 20h (n = 6) or 48h (n = 4). Media were collected for Estrogen (E2) ELISA, and the numbers of viable cells remaining in the wells were determined by MTT assay by comparison with a standard curve generated for each experimental replicate. Data are presented as mean ± SEM, and analysed by 1-way ANOVA with Tukey post-hoc test, and difference from vehicle control (97% DMEM/F12 + 10% FCS) shown; $p < 0.05$*, $p < 0.01$**, $p < 0.001$***.

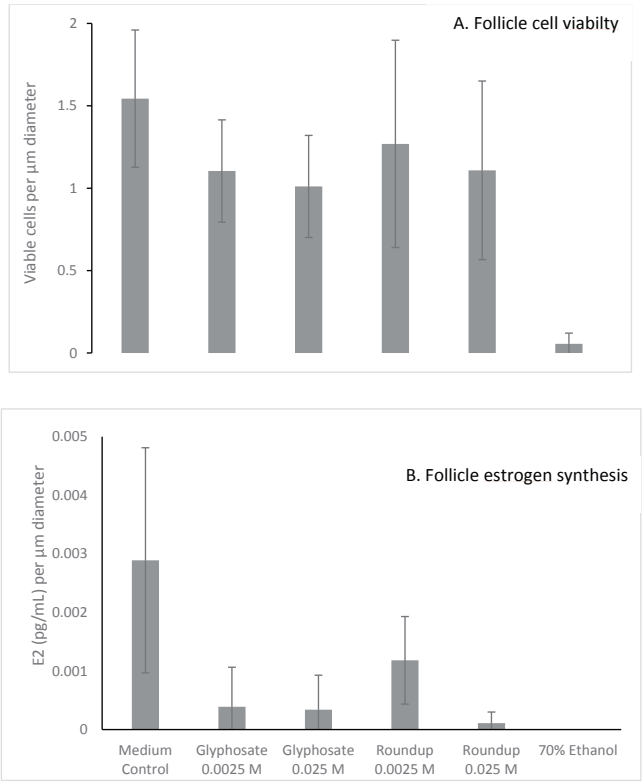

Figure 7: Effect of Glyphosate and Roundup on murine follicle viability and estrogen production

Groups of 4 murine follicles were exposed to glyphosate or Roundup or DMEM/F12 medium (negative control) or 70% ethanol (positive control) in DMEM/F12 medium for 24h on three separate occasions. A) Follicles were disaggregated, the numbers of viable cells from four follicles were determined in a Trypan Blue exclusion assay, and this value was adjusted to represent the number of cells in one follicle. Since follicles were different sizes, this source of variation was removed by expressing the cell numbers per □m of follicle diameter. Data shown as mean ± stdev (n = 3). B) Estrogen in the media was assessed in an ELISA, and this concentration (pg/mL) expressed per µm of follicle diameter, to adjust for different sized follicles. Data shown as mean ± stdev (n = 3). Data analysed by 1-way ANOVA with Tukey post-hoc test, and difference from DMEM/F12 medium control shown; $p < 0.05$ *, $p < 0.001$ ***.

glyphosate, or 1 to 10 µg/mL Roundup with 0.99% glyphosate, both increased MCF7 cell proliferation in the presence of 10% FCS [10]. The MCF7 cells in that study differed from the T47D cells in that they were maintained in low E2 culture conditions without phenol red and with DC FCS before they were exposed to glyphosate or GBH in 10% FCS. Lin and Garry (2000) concluded that the proliferative stimulus was not estrogenic, and was probably caused by other components of the FCS, which supports the possibility that SHBG upregulated cAMP and stimulated MCF7 proliferation [28].

We found that differential cytotoxic effects between glyphosate and the GBH only occurred at higher concentrations; Roundup containing 10^{-2} M glyphosate caused a significant decrease in T47D cell viability after a 24h exposure, whereas glyphosate was cytotoxic only after a 72 h exposure to 4.3 M ($p < 0.001$). The OVCAR cell line was included in this study because it has been reported to express ER [42,43], and could provide an alternative model to the breast cancer cell lines. It was surprising therefore that the OVCAR cell line in our laboratory expressed mRNA for ER alpha but that this was not translated into protein. Although the OVCAR cell line neither synthesised steroid hormones nor expressed ER, the cytotoxic effects

were similar to the steroidogenic H295R cells; glyphosate in Roundup (10^{-2} to 10^{-1} M) was toxic to both cell types after a 24 h exposure, whereas the same concentrations of glyphosate alone had no effect. When steroidogenic Jar cells [42,43] were exposed to the same Roundup formulation as that used in the present study, the 24 h EC50 value was 10^{-6} M, whereas a concentration of glyphosate 100 times higher had no effect on cell viability after a 72 h exposure [15]. The steroidogenic Jar cells appeared to be more sensitive to GBHs than the cell types used in the present study, probably due to the presence of hepatic-like activation enzymes in the Jar cells [44]. However, for most of the cell lines examined, the differential cytotoxicity between glyphosate and GBH has occurred over at least two orders of magnitude, and appears to be unaffected by the presence of ER or steroid hormone synthesis.

The US-EPA developed the H295R assay specifically to characterise the effects of test agents on steroid hormone synthesis

[29]. Our method differed slightly from the inter-laboratory validated method; cells were cultured in 96 well plates instead of 24 well plates, they had an acclimation period of only 3 h as opposed to 24 h, and four of the five validating laboratories extracted hormones using ethyl ether, whereas only one of the laboratories assayed media supernatants directly, as we did. Despite these procedural differences, our forskolin-stimulated estrogen production was in the same range as the validated assay, whereas prochloraz inhibition after 48h was lower; only 0.22-fold decrease as opposed to the validated assay 0.25-fold decrease. The ATCC was unable to tell us what passage age the H295R cells were when we acquired them, and although they were passaged fewer than 10 times before use in our laboratory, it is possible that they were older than 10 passages, and this may account for reduced estrogen production, and reduced inhibitory effects by prochloraz. Nevertheless, we concluded that we reproduced the H295R assay sufficiently well to justify its use in assessing the effects of glyphosate and Roundup on estrogen synthesis.

It has been reported that low, non-toxic concentrations of Roundup or glyphosate inhibited CYP450 aromatase activity, and reduced E2 synthesis in vitro [8,14,21,23] whereas others reported the opposite in vivo: upregulation of aromatase activity (Cassault-Meyer, Seralini, and Galeraud-Denis 2014). In our study, low non-cytotoxic concentrations of GBH or glyphosate had no effect on E2 production by H295R cells in vitro. We applied physiological pH and 10% FCS in our in vitro cell culture model, whereas previous in vitro studies did not [14,45]. Our in vitro conditions were closer, but not identical to in vivo conditions, and our results were closer to the in vivo observations.

Previously, male rat primary-derived steroidogenic cells that were exposed to low non-cytotoxic concentrations of Roundup or glyphosate in vitro reduced testosterone production [22]. Our use of murine follicles brings the in vitro model closer to the in vivo situation, by maintaining intercellular connections and interactions, and the basal lamina, which separates the avascular follicular granulosa cells and oocyte from the environment. Previously, murine antral follicles cultured in vitro Patel et al, 2016. Grew and produced E2 at comparable rates to the smaller secondary follicles in the present study. Although follicular E2 production after 24h culture was relatively low, the concentrations were within the range of measurement of the ELISA, and the follicles retained their 3D architecture in vitro, whereas longer culture periods resulted in the granulosa cells forming a monolayer. The concentration of GBH (10^{-2} M) that was cytotoxic to continuous cell lines that expressed either the ER (T47D) or a complete steroidogenic pathway (H295R) was not cytotoxic to the primary-derived murine follicles after the same 24h exposure period. Conversely, although the concentrations of GBH and glyphosate that were applied to follicles were not cytotoxic, they did significantly reduce E2 production. This result suggests that CYP450 aromatase expression was decreased, similar to the analogous male primary-derived reproductive cells examined previously [22]. The results from both of these in vitro studies differed from the in vivo study in which 8 days exposure to Roundup increased testicular aromatase expression [24]. The differences may be attributable to the inability of the in vitro models to adequately model the in vivo conditions, or to the longer exposure examined by Cassault-Meyer, et al. 2014. Increasing the exposure of follicles to GBH or glyphosate in vitro will require the application of a more complex gel model to support 3D follicle growth [46], but this may generate useful data regarding the effects of glyphosate on cells that express both ER and an intact steroidogenic pathway.

In summary, when continuous cell lines were cultured in media at physiological pH with 10% FCS and very low levels of E2, neither GBH nor glyphosate stimulated the proliferation of cells that express ER (T47D). We hypothesised that low non-cytotoxic concentrations of GBH or glyphosate would reduce E2 production by cells expressing a complete steroid hormone biosynthesis pathway, but this did not prove to be the case, which implies that CYP450 aromatase expression in H295R cells was not decreased under these in vitro conditions. In contrast, relatively high concentrations of GBH and glyphosate that were cytotoxic to continuous cell lines were not cytotoxic to primary-derived follicular granulosa cells, but the inhibition of E2 production demonstrated that the follicular basal lamina did not exclude the herbicides from the follicular cells. Cassault-Meyer, et al. 2014 reported a Roundup-mediated upregulation of testicular aromatase expression in vivo, but the inhibition of follicular E2 suggests that aromatase was downregulated, and also that the combination of a steroidogenic biosynthesis pathway and ER expression in the same cell manifested a different mechanism of action from cells that express one or the other but not both. Further work is need to clarify this issue; a continuous cell line that both expresses the ER and syntheses E2, such as the KGN granulosa line, should be exposed to GBH or glyphosate in vitro to determine if the results reported in this study were due to the differences between primary-derived and continuous cells, and follicles should be exposed to GBH or glyphosate for longer periods, and their aromatase expression examined in conjunction with E2 synthesis.

It is well known that in vitro culture systems do not reproduce the in vivo conditions of absorption, distribution, metabolism and excretion, but the impact of foetal, post-natal or adult serum, and the proportion of serum, also constitutes a major difference between the in vivo and in vitro environments. The differences have particular relevance when elucidating the actions of a weak estrogen such as glyphosate, and when assessing a system-wide activity such as endocrine disruption. The US Endocrine Society defined endocrine disrupters as being compounds which 'alter the hormonal and homeostatic systems that enable the organism to communicate with and respond to its environment' [47,48], and from this perspective, assessment of the entire steroidogenic pathway is more likely to generate data relating to the whole organism than assessment of a single step in that pathway. In the final analysis though, in vitro studies should be verified by in vivo data before unequivocally classifying a compound as an endocrine disruptor.

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