1. Title: Glyphosate-based herbicide enhances the uterine sensitivity to estradiol in rats

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2. Abstract

In a previous work, we detected that postnatal exposure to a glyphosate-based herbicide (GBH) alters uterine development in prepubertal rats causing endometrial hyperplasia and increasing cell proliferation. Our goal was to determine whether exposure to low-dose of a GBH during postnatal development might enhance the sensitivity of the uterus to an estrogenic treatment. Female Wistar pups were subcutaneously injected with saline solution (control) or GBH using the reference dose (2 mg/kg/day, EPA) on postnatal days (PND) 1, 3, 5, and 7. At weaning (PND21), female rats were bilaterally ovariectomized and treated with silastic capsules containing 17β-estradiol (E2, 1mg/ml) until they were two months of age. On PND60, uterine samples were removed and processed for histology, immunohistochemistry and mRNA extraction to evaluate: i) uterine morphology, ii) uterine cell proliferation by the detection of Ki67, iii) the expression of the estrogen receptors alpha (ESR1) and beta (ESR2), and iv) the expression of WNT7A and β-catenin. GBH-exposed animals showed increased luminal epithelial height and stromal nuclei density. The luminal and glandular epithelium were markedly hyperplastic in 43% of GBH-exposed animals. GBH exposure caused an increase in E2-induced cell proliferation in association with an induction of both ESR1 and ESR2. GBH treatment decreased membranous and cytoplasmic expression of β-catenin in luminal and glandular epithelial cells and increased WNT7A expression in the luminal epithelium. These results suggest that early postnatal exposure to a GBH enhances the sensitivity of the rat uterus to estradiol, and induces histomorphological and molecular changes associated with uterine hyperplasia.

3. Introduction

Estrogens have been implicated as important etiologic agents in cancer of the female reproductive system (Chuffa et al. 2017). Likewise, several epidemiological studies have demonstrated a close correlation between environmental and/or occupational exposure to endocrine disrupting chemicals (EDCs) with estrogenic activity and cancer in women (Cohn et al. 2015; Lerro et al. 2015). Experimental studies performed in rodents have shown that environmental pollutants with estrogenic activity can induce pre-neoplastic uterine lesions as well as endometrial cancer. Yoshizawa et al. (2009) performed an *in vivo* study where female adult Harlan Sprague-Dawley rats orally exposed to chronic administration of dioxin and dioxin-like compounds (polychlorinated biphenyls and furans) showed increased incidence of cystic endometrial hyperplasia, luminal squamous metaplasia and carcinoma in the uterus. Other authors demonstrated that developmental exposure to diethylstilbestrol (DES) and bisphenol A (BPA) increases the incidence and severity of benign, premalignant and neoplastic uterine lesions in aged hamsters, rats, and mice, including atypical hyperplasia and endometrial adenocarcinoma (Leavitt et al. 1981; Newbold et al. 1990; Newbold et al. 2007; Vigezzi et al. 2015, 2016).

Among environmental pollutants, glyphosate-based herbicides (GBHs) have been one of the most intensive used over the last two decades worldwide (Benbrook 2016), resulting in increased environmental, plant and food contamination (Bai & Ogbourne 2016; Primost et al. 2017; Rodrigues & de Souza 2017; Zoller et al. 2017; Van Bruggen et al. 2018). Nonetheless, estimated human exposure from glyphosate maximum residue levels measured in food items and human urine samples has indicated that daily exposure to glyphosate is below the tolerable reference doses currently established by regulatory agencies (Niemann et al. 2015; Solomon 2016; Zoller et al. 2017). However, these tolerable doses of glyphosate are being questioned because they rely on outdated studies and may fail to protect human health or the environment (Vandenberg et al. 2017).

In the last years, it has been controversy and debate regarding the carcinogenic and the endocrine disrupting potential of glyphosate and GBHs. Several bodies of experts have evaluated glyphosate carcinogenicity and drawn different conclusions. The WHO's International Agency for Research on Cancer working group (IARC) concluded "glyphosate is probably carcinogenic to humans (IARC Group 2A)" (IARC 2015), based on the evaluation of the publicly available evidence including data on the active ingredient glyphosate as well as on GBHs. In contrast, the European Food Safety Authority (EFSA) concluded "glyphosate is unlikely to pose a carcinogenic hazard to humans" (EFSA 2015), based on all available data on the active ingredient glyphosate, including industry data (Portier et al. 2016; Tarazona et al. 2017). Similarly, different *in vitro* and *in vivo* studies have revealed possible endocrine-mediated effects of glyphosate and its commercial formulations on hormone-dependent tissues such as testis (Cassault-Meyer et al. 2014), ovary (Perego et al. 2017) and uterus (Guerrero Schimpf et al. 2017; Varayoud et al. 2017). Glyphosate was suggested to have endocrine disrupting properties by inhibiting aromatase activity (Richard et al. 2005) and activating the estrogen receptor alpha (ESR1) and beta (ESR2) in breast cancer cells (Thongprakaisang et al. 2013; Mesnage et al. 2017). However, no evidence of potential interaction of glyphosate with endocrine pathways has been detected in the Endocrine Disruptor Screening Program (EDSP) conducted by the US Environmental Protection Agency (EPA) (US EPA 2015). Thus, the carcinogenic and endocrine disrupting potential of glyphosate and GBHs remains uncertain.

In a previous work, we demonstrated that the exposure to low dose (2 mg/kg of body weight (bw)/day) of a GBH during the first postnatal days (PND) alters the development of the rat uterus by affecting uterine morphology and the expression of proteins that regulate uterine organogenetic differentiation during the neonatal (PND8) and prepubertal (PND21) periods (Guerrero Schimpf et al. 2017). In fact, GBH induced endometrial hyperplasia in association with increased cell proliferation and deregulated different uterine morphoregulatory genes, such as ESR1, progesterone receptor (PR), homeobox A10 and wingless-type MMTV integration site family, member 7A (WNT7A) (Guerrero Schimpf et al. 2017). Alterations in uterine histoarchitecture and disruption of uterine morphoregulatory genes during critical periods of

development might lead to reproductive anomalies during adulthood, such as infertility and early pregnancy loss, and could promote the development of uterine neoplasias (Newbold et al. 1990; Newbold et al. 1997; Varayoud et al. 2008; Varayoud et al. 2014). In this sense, we detected that early postnatal exposure to GBH causes post-implantation embryo loss in adult female rats associated with an altered decidualization response and defective uterine differentiation/proliferation (Ingaramo et al. 2016, 2017). However, less evidence are available about the capability of GBHs to promote uterine neoplasias.

Based on controversial and limited studies, we evaluated whether exposure to low-dose GBH during early stage of development might enhance the sensitivity of the uterus to an estrogenic prepubertal treatment. Female rats postnatally exposed to vehicle or GBH were bilaterally ovariectomized at weaning (PND21) and submitted to a chronic estrogen stimulation until PND60 to evaluate the following end-points: i) uterine morphology, ii) uterine cell proliferation, iii) the expression of the estrogen receptors (ERs) (ESR1 and ESR2), and iv) the expression of WNT7A and β-catenin (*Ctnnb1*), selected as E2-modulated targets that have been implicated in altered uterine E2 responsiveness and neoplasia.

4. Materials and methods

4.1. Animals

All procedures used in this study were performed in accordance with the principles and procedures outlined in the Guide for the Care and Use of Laboratory Animals issued by the US National Academy of Sciences and were approved by the Institutional Ethics Committee of the School of Biochemistry and Biological Sciences (Universidad Nacional del Litoral, Santa Fe, Argentina). Inbred Wistar strain rats were bred at the Department of Human Physiology (Santa Fe, Argentina) and housed in a controlled environment (22°C ± 2°C; lights on from 06:00 to 20:00 h) in stainless steel cages with sterile pine wood shavings as bedding. Rats had free access to pellet laboratory chow (16-014007 Rat-Mouse Diet, Nutrición Animal, Santa Fe, Argentina) and tap water. For more information regarding the food composition, see: Kass et al. 2012, Supplementary data Table 1; and, Vigezzi et al. 2016.

130

131 **4.2. Experimental design**

132 Pups were obtained from timed-pregnant rats housed singly. After delivery (PND0), pups were
133 sexed according to anogenital distance and cross-fostered by distributing the pups of each litter
134 among the mothers. This procedure minimizes the use of siblings and thus avoids potential litter
135 effects. Cross-fostered litters were adjusted to 8 pups, with 8 female pups per litter. When fewer
136 than 8 females were available, an appropriate number of males were retained. Female pups from
137 each foster mother were randomly assigned to one of the following postnatal treatment group:
138 1) control group receiving saline solution, and 2) GBH group receiving a commercial
139 formulation of glyphosate dissolved in saline solution (2 mg of glyphosate/kg bw). The
140 glyphosate formulation used was Roundup FULL II®, a liquid water-soluble formulation
141 containing 66.2% of glyphosate potassium salt (equivalent to 54% w/v of glyphosate acid), as
142 its active ingredient, coadjuvants and inert ingredients. Forty microliters were administered by
143 subcutaneous (sc) injection in the nape of the neck every 48 h from PND1 to PND7. Each
144 treatment day, the dose was calculated based on the concentration of glyphosate acid (54 g of
145 glyphosate per 100 ml of GBH) and the average body weight of the pups. The dose of GBH
146 selected to perform this study is in the order of magnitude of the reference dose (RfD) of 1
147 mg/kg bw/day recently reassigned for glyphosate by the US EPA (2017). Moreover, this dose is
148 representative of the glyphosate residues found in soybean grains (Arregui et al. 2004; Test
149 Biotech, 2013), and is in the order of magnitude of the environmental levels detected in our
150 country (Peruzzo et al. 2008; Bonansea et al. 2017; Primost et al. 2017). Although the RfD for
151 glyphosate is based on oral exposure, the subcutaneous administration enhances delivery of
152 chemical compounds in an early postnatal exposure model (Milesi et al. 2012). The early
153 postnatal model of exposure to endocrine disruptors has been extensively used in our laboratory
154 in both rodents (Ramos et al. 2007, Varayoud et al. 2008; Monje et al. 2009; Milesi et al. 2015;
155 Ingaramo et al. 2016, 2017; Guerrero Schimpf et al. 2017) and lambs (Rivera et al. 2011) and
156 has been demonstrated as a persuasive paradigm to study short- and long-term consequences of
157 neonatal exposure to hormonally active substances (Rivera et al. 2015). As previously reported

158 (Guerrero Schimpf et al. 2017), GBH postnatal treatment (with 2 mg/kg bw/day in pups every
159 48 h from PND1 to PND7) produced no alterations in maternal care and nursing between the
160 experimental groups. No signs of acute or chronic toxicity were observed in the litters, and no
161 differences in weight gain between GBH and control pups were recorded during the experiment.
162 A schematic representation of the experimental design is shown in Fig. 1.
163
164 **4.3. Ovariectomy, estrogen treatment and sample collection**
165 To study the long-term effects of postnatal GBH exposure on uterine sensitivity to E2, we used
166 a model of prepubertal ovariectomized (OVX) rats submitted to chronic estrogen treatment (Fig.
167 1). At weaning (PND21), control (C) and postnatally GBH-exposed female rats were
168 anesthetized with a solution of ketamine:xylazine:acepromazine, 50:5:1 mg/ml. The solution
169 was prepared by mixing 1.0 ml of ketamine hydrochloride (50 mg/ml, Ketamina 50, Holliday-
170 Scott, Buenos Aires, Argentina), 0.25 ml of xylazine hydrochloride (20 mg/ml, Kensol® König,
171 Buenos Aires, Argentina) and 0.1 ml of acepromazine maleate (10 mg/ml, Acedan, Holliday-
172 Scott, Buenos Aires, Argentina). The anesthetic solution was administered intraperitoneally at a
173 dose of 135 µl/100 g bw. Animals were bilaterally OVX to avoid endogenous estradiol (E2)
174 variability. A group of control (C+OVX+E2, n=8) and GBH-exposed (GBH+OVX+E2, n=7)
175 females were submitted to a chronic estrogen treatment, using silastic capsules (inner/outer
176 diameter: 1.98/3.18 mm, 19 mm in length, Dow Corning Corp., Midland, MI, USA) containing
177 1 mg of 17β-estradiol/ml (Sigma-Aldrich, St. Louis, MO, USA) dissolved in corn oil. The
178 capsules were preincubated at 37ºC in phosphate buffer for 24 h to stabilize the release of E2,
179 and then were implanted (one per animal) subcutaneously between the shoulder blades during
180 the anesthesia used at ovariectomy. In order to characterize the uterine response to exogenous
181 E2 treatment, we performed a pilot experiment using OVX prepubertal rats that were not
182 exposed to GBH. For that purpose, another set of control females was implanted with capsules
183 filled with vehicle alone (corn oil) (C+OVX+V, n=5), and the effects were compared against the
184 control group submitted to E2 treatment (C+OVX+E2). The treatment with the E2 implants
185 produces blood levels of E2 that are equivalent to E2 circulating levels during the rat estrous

cycle (Smith et al. 1975; Bottner & Wuttke 2005). This was verified by measuring E2 serum levels (see Section 4.4). After surgery, animals were allowed to recover in a clean cage under a heating lamp. Analgesia (2 mg/kg of flunixin meglumine, Aplonal®, König, Buenos Aires, Argentina) was administered subcutaneously once per day and for three days, starting after capsule implantation. Once the animals were fully conscious and active, they were housed individually and daily monitored for signs of pain or distress for a period of one week. Female rats were sacrificed when they were two months of age (PND60) to collect trunk blood and uterine tissue. Blood samples were centrifuged (3000 rpm, 20 min) and serum was stored at −20 ºC until hormone assay was performed. One uterine horn of each rat was fixed by immersion in 10% buffered formalin for 6 h at 4°C and processed for histology and immunohistochemistry (IHC). The other uterine horn of each rat was immediately frozen in liquid nitrogen and stored at −80 ºC for RNA extraction followed by reverse transcription-polymerase chain reaction (RT-PCR) assay.

4.4. Determination of E2 serum levels

Serum samples stored at −20 °C were thawed and E2 serum levels were assessed using the Ultra-Sensitive Estradiol Radioimmunoassay Kit DSL4800 (Immunotech, Beckman Coulter, Czech Republic) according to the manufacturer's guidelines. All samples were run in duplicate. The assay sensitivity was 2.2 pg/ml. The intra and interassay coefficients of variation were ≤ 8.9 and 12.2 %.

4.5. Histology and morphometry

Uterine samples embedded in paraffin were longitudinally cut (5 µm thick) at three different depths (separated 50 µm from each other), mounted on slides coated with 3-aminopropyl triethoxysilane (Sigma-Aldrich), stained with hematoxylin and eosin (H&E) and examined by light microscope (Olympus BH2 microscope; Olympus, Tokyo, Japan) to analyze uterine morphology. Uterine sections were evaluated for histologic changes by a trained pathologist blinded to the experimental group. We also determined glandular density, stromal nuclei

density, luminal and glandular epithelial heights, and the thickness of the stroma and myometrium. To determine the glandular density, the volume fraction (Vv) of uterine glands was calculated by applying the following formula given by Weibel (1969): $Vv = Pi/P$, where Vv is the estimated volume fraction of the object (glands), Pi is the number of incident points over the glands, and P is the number of incident points over all cells in the studied population (stroma). To obtain the data for this point-counting procedure, a glass disk with a squared grid of 0.8 mm × 0.8 mm was inserted into a Dplan 40× focusing eyepiece (numerical aperture = 0.65, Olympus) (Gundersen et al. 1988; Ramos et al. 2002). The results were expressed as Vv × 100. The Vv of uterine glands was quantified on at least 20 randomly selected fields per section, and three sections per animal (separated 50 µm from each other) were evaluated. The stromal nuclei density was defined as the ratio between the area occupied by stromal nuclei and total subepithelial stromal area (150-µm-wide area adjacent to the epithelium, from the basement membrane toward the outer layer). Areas were quantified using Fiji of Image J, excluding uterine glands, infiltrating cells and blood vessel cells from analysis (Durando et al. 2007). The luminal and glandular epithelial heights, and the thickness of the stromal and myometrial (circular and longitudinal) layers were analyzed by image analysis using Fiji of Image J as previously described (Ramos et al. 2002; Varayoud et al. 2017). Briefly, the images were recorded with a Spot Insight V3.5 color video camera, attached to a microscope (Olympus). To spatially calibrate the software analyzer, square grids from Neubauer's chamber images were captured. At least five fields were recorded in each section using a Dplan 20× objective (numerical aperture = 0.40, Olympus), and three sections per animal (separated 50 µm from each other) were evaluated.

4.6. Reverse transcription and real-time quantitative PCR analysis (qRT-PCR)

4.6.1. RNA extraction and reverse transcription

Individual uterine horn samples from each experimental group were homogenized in TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) and total RNA was extracted according to the manufacturer's instructions. The concentration and purity of total RNA was determined by

measuring the optical density at 260 and 280 nm. All samples were precipitated with ethanol, dissolved in distilled water, and then stored at −80 °C until needed. Equal quantities (1 μg) of total RNA were reverse-transcribed into cDNA with Moloney Murine Leukemia Virus reverse transcriptase (MMLV-RT; 300 units; Promega, Madison, WI, USA) using 200 pmol of random primers (Biodynamics, Buenos Aires, Argentina). Twenty units of ribonuclease inhibitor (RNAout, Invitrogen Argentina, Buenos Aires, Argentina) and 100 nmol of a deoxynucleotide triphosphate mixture were added to each reaction tube at a final volume of 30 μl of 1 × MMLV-RT buffer. Reverse transcription was performed at 37 °C for 90 min and at 42 °C for 15 min. Reactions were stopped by heating at 80 °C for 5 min and cooling on ice. Each reverse-transcribed product was diluted with RNase-free water to a final volume of 60 μl.

4.6.2. qRT-PCR

The mRNA expression levels of total *Esr1*, *Esr2*, *Wnt7a*, *Ctnnb1* and ribosomal protein L19 (*Rpl19*, housekeeping gene) were quantified by qRT-PCR using the Rotor-Gene Q cycler (Quiagen; Tecnolab; Buenos Aires, Argentina). The gene-specific primer sequences are shown in Table 1. For cDNA amplification, 5 μl of cDNA were combined with HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis BioDyne; Biocientífica, Rosario, Argentina) and 10 pmol of each primer (Invitrogen) in a final volume of 20 μl. After initial denaturation at 95 °C for 15 min, the reaction mixture was subjected to successive cycles of denaturation at 95 °C for 15 s, annealing at 56-60 °C for 15 s, and extension at 72 °C for 15 s. Product purity was confirmed by dissociation curves and random samples were subjected to agarose gel electrophoresis. Controls containing no template DNA were included in all assays, yielding no consistent amplification. The cycle threshold (C_T) for each sample was calculated using the Rotor-Gene Q Series Software (Version 1.7, Quiagen; Tecnolab) with an automatic fluorescence threshold setting. The efficiency of PCR reactions was assessed for each target by the amplification of serial dilutions (over five orders of magnitude) of cDNA fragments of the transcripts under analysis. Accordingly, the fold expression over control values was calculated for each target by the relative standard curve methods, which are designed to analyze data from real-time PCR (Cikos

et al., 2007). For all experimental samples, the target quantity is determined from the standard curve, normalized to the quantity of the housekeeping gene and finally divided by the target quantity of the control sample. No significant differences in C_T values were observed for *Rpl19* among the experimental groups.

4.7. Immunohistochemistry

The protein expression of the cell proliferation marker Ki67 and the ERs (ESR1 and ESR2) was analyzed to evaluate the uterine response to exogenous E2 treatment. WNT7A and β-catenin protein immunostaining was evaluated as E2-modulated targets that have been implicated in altered uterine E2 responsiveness and neoplasia. A standard immunohistochemical technique was performed following protocols previously described by our laboratory (Muñoz-de-Toro et al. 1998). Briefly, uterine longitudinal sections (5 μm thick) were deparaffinized and rehydrated in graded ethanol solutions. After microwave pretreatment for antigen retrieval, the endogenous peroxidase activity and non-specific binding sites were blocked. Samples were incubated in a humid chamber with the specific primary antibody (overnight at 4°C) and then with the corresponding biotin-conjugated secondary antibody (30 min at room temperature) (described in Table 2). Reactions were developed using the avidin-biotin-peroxidase method and diaminobenzidine (Sigma-Aldrich) as a chromogen substrate. Each immunohistochemical run included negative controls in which the primary antibody was replaced by non-immune horse serum (Sigma-Aldrich). For Ki67 immunodetection, samples were counterstained with Mayer's hematoxylin (Biopur, Rosario, Argentina). Samples were dehydrated and mounted with permanent mounting medium (Eukitt, Sigma-Aldrich).

4.8. Quantification of cell proliferation

Tissue sections (three per animal, separated 50 μm from each other) were evaluated using the Olympus BH2 microscope with the Dplan 40× objective (numerical aperture = 0.65; Olympus). The proliferation rate was assessed in the luminal and glandular epithelium as a percentage of Ki67-positive cells on a total of 2000 cells per compartment in each uterine section. In the

298 subepithelial stroma, the proliferation rate was obtained considering the Vv of the Ki67-positive
299 cells, as previously described in section 4.5 of Materials and Methods.
300
301 **4.9. Quantification of protein expression by image analysis**
302 The protein expression of the ERs (ESR1 and ESR2), WNT7A and β-catenin in uterine cells
303 was evaluated by image analysis, using Fiji of Image J as previously described (Ramos et al.
304 2002). In brief, immunostained images were captured with a Dplan 40× objective (numerical
305 aperture =0.65; Olympus) attached to a Spot Insight V3.5 color video camera. After conversion
306 of each image into a gray scale, the integrated optical density (IOD) was measured as a linear
307 combination of the average gray intensity and the relative area occupied by the positive cells
308 (Ramos, et al. 2001; Ramos et al. 2002). Because the IOD is a dimensionless parameter, the
309 results were expressed as arbitrary units. Protein quantification was performed in duplicate in
310 the luminal and glandular epithelium, and in the subepithelial stroma (150-µm-wide area
311 adjacent to the epithelium, from the basement membrane toward the outer layer) on two uterine
312 sections, separated 50 µm from each other. Moreover, in each uterine section quantification was
313 performed on at least 10 randomly selected fields.
314
315 **4.10. Statistical analysis**
316 Results are expressed as the mean ± SEM. All data were analyzed using a Mann-Whitney *U* test
317 (GraphPad Prism Version 5.03). Values with $p<0.05$ were accepted as significant.
318
319 **5. Results**
320 **5.1. Estradiol serum levels**
321 In order to know the serum levels of 17β-estradiol reached by E2 implants, the concentration of
322 E2 was measured in the serum of C+OVX+V, C+OVX+E2 and GBH+OVX+E2 female rats. As
323 expected, E2 serum levels were below the detection limit of the assay in OVX rats treated with
324 vehicle (C+OVX+V group). In C+OVX+E2 females, E2 implants raised E2 serum
325 concentrations to around 30 pg/ml. No differences were observed in control and postnatally

GBH-exposed animals treated with E2 (C+OVX+E2: 33.1 ± 3.3 pg/ml; GBH+OVX+E2: 31.6 ± 2.0 pg/ml, p=0.90) (Table 3).

5.2. Characterization of uterine responses to E2 treatment

Our first aim was to characterize the uterine response to estrogenic treatment between C+OVX+V and C+OVX+E2 by comparing uterine morphology, cell proliferation and the expression of both ESR1 and ESR2. As expected, uterine morphology was clearly different between vehicle- and E2-stimulated rats. The larger longitudinal section area of the E2-exposed uteri was evident at low magnification (Fig. 2A *vs* 2B). Uteri from C+OVX+V females revealed an atrophic endometrium with simple cuboidal epithelium in the lumen and glands as well as densely-packed stromal cells (Fig. 2C). In contrast, the luminal epithelium in E2-exposed uteri (C+OVX+E2 group) was hypertrophic (pseudostratified columnar cells) to low-grade hyperplastic. Additionally, the subepithelial stroma showed signs of water imbibition and was heavily infiltrated by inflammatory leukocytes (Fig. 2D).

Uterine response to E2 involves the differential modulation of the ERs and changes in cell proliferation. In our model of exposure, E2 administration resulted in downregulation of *Esr1* mRNA levels (Fig. 3A; $p<0.05$) and upregulation of the *Esr2* mRNA levels (Fig. 3B; $p<0.05$). Immunohistochemical analysis of protein expression showed that E2-treated animals exhibited down-regulation of ESR1 (Fig. 3C and D *vs*. E; $p<0.05$) and upregulation of ESR2 (Fig. 3C and F *vs*. G; $p<0.05$) in all uterine regions evaluated. In addition, a dramatic increase in cell proliferation in both luminal and glandular epithelial cells was detected after E2 treatment (Fig. 3C and H *vs*. I; $p<0.01$).

5.3. Long-lasting effects of GBH on uterine sensitivity to E2

5.3.1. Uterine histomorphology

Histological features of uterine sections are illustrated in Fig. 4. As previously mentioned, E2 stimulation prompted hypertrophy (pseudostratified columnar cells) and low-grade hyperplasia in the uterine luminal epithelium (C+OVX+E2 group) (Fig. 4A and D). We observed that uteri

of female rats postnatally exposed to GBH and treated with E2 (GBH+OVX+E2 group) showed histomorphological alterations. In fact, the luminal and glandular epithelium were markedly hyperplastic (43% of animals). GBH+OVX+E2 rats showed areas of endometrium with stratification of the luminal epithelium, hypochromic nuclei and mitotic figures (Fig. 4B and E). Furthermore, in GBH-exposed uteri treated with E2, several abnormal glands were observed that contained cells that were morphologically similar to those of the luminal epithelium (Fig. 4C and F). In addition, GBH-exposed animals showed increased luminal epithelial height and stromal nuclei density (Table 4). The evaluation of other morphological parameters did not show differences between control and postnatally GBH-exposed animals (C+OVX+E2 *vs*. GBH+OVX+E2) (Table 4).

5.3.2. Uterine proliferation

Evaluation of cell proliferation was performed by the assessment of Ki67 expression. An increased proliferative response to E2 treatment was detected in the postnatally GBH-exposed uteri. The evaluation of cell proliferation in the luminal epithelial compartment indicated that in GBH+OVX+E2 females the Ki67 expression was higher (29.07 ± 3.89%, $p<0.05$) than in the C+OVX+E2 group (16.36 ± 1.10%) (Fig. 5A). Photomicrographs illustrating these results are shown in Fig. 5B. Glandular epithelial cells showed similar changes, but the induction of cell proliferation was not different between control and GBH-exposed females (C+OVX+E2: 16.32 ± 1.42%; GBH+OVX+E2: 20.16 ± 3.42%, $p=0.62$) (Fig. 5A). Finally, the stromal cell proliferation was very low showing no changes between C+OVX+E2 and GBH+OVX+E2 treated animals (data not shown).

5.3.3. Expression of the estrogen receptors

Uterine expression of the ESR1 and ESR2 in control and GBH-exposed animals that received E2 treatment was evaluated at mRNA and protein levels. As previously mentioned, E2 stimulation induced downregulation of *Esr1* mRNA (C+OVX+E2 *vs*. C+OVX+V rats, Fig. 3A). We found that when rats were postnatally exposed to GBH, the *Esr1* mRNA downregulation

was accentuated (Fig. 6A; $p<0.05$). Likewise, the induction of *Esr2* mRNA detected in C+OVX+E2 animals, was enhanced in the uteri from GBH+OVX+E2 group (Fig. 6B; $p<0.05$). A similar response was detected when we analyzed the protein expression of ESR2. Postnatal exposure to GBH enhanced the ESR2 increase caused by E2 treatment in all uterine regions evaluated (luminal, glandular and stromal) (Fig. 6C; $p<0.05$). For ESR1, a strong induction was detected in the luminal epithelial cells of GBH-exposed animals (Fig. 6C, $p<0.05$), without changes in the subepithelial stroma or the glandular epithelium. Based on microscopic examination, increased levels of epithelial ESR1 expression were related not only to an increased in cell number but also to changes in expression levels within cells (the intensity of immunostaining). Representative images illustrating these results are shown in Fig. 6D.

5.3.4. Expression of E2-modulated targets that have been implicated in altered uterine E2 responsiveness and neoplasia

Uterine expression of WNT7A and β-catenin in control and GBH-exposed animals that received E2 treatment was evaluated at mRNA and protein levels. Treatment with GBH increased the expression of *Wnt7a* mRNA relative to control rats (Fig. 7A; $p<0.05$), while induced downregulation of *Ctnnb1* mRNA levels (Fig. 7B; $p<0.05$). Considering the differential role of these molecules according to their subcellular localization, we evaluated the spatial pattern of WNT7A and β-catenin expression in uteri of control and GBH-exposed rats by IHC. Both groups displayed similar expression patterns. WNT7A showed nuclear expression in the subepithelial stroma, and nuclear and cytoplasmic expression in uterine epithelial (luminal and glandular) cells. β-catenin displayed membranous and cytoplasmic pattern in the luminal and glandular epithelium with negative immunoreaction in the stroma. Despite no changes in the spatial pattern of WNT7A and β-catenin were observed, differences in immunostaining intensity were found between the experimental groups. Female rats postnatally exposed to GBH exhibited induction of WNT7A in the luminal epithelium (Fig. 7C; $p<0.05$). Surprisingly, β-catenin expression was notably decreased in the luminal and glandular epithelium of GBH-

409 treated animals (Fig. 7C; $p<0.05$). Representative images illustrating these results are shown in
410 Fig. 7D.
411
412 **6. Discussion**
413 Prior data obtained from siblings of the animals used in the present study revealed that the
414 exposure to low dose (2mg/kg b.w/day) of a GBH during the first week of life resulted in
415 alterations of uterine organogenetic and functional differentiation that caused subfertility later in
416 life. In this line, GBH exposure elicited endometrial hyperplasia in prepubertal rats together
417 with increased cell proliferation and altered expression of proteins involved in uterine
418 organogenetic differentiation (Guerrero Schimpf et al. 2017). Long-term uterine effects included
419 alterations in endocrine-dependent mechanisms involved in decidualization that leaded to post-
420 implantation embryo loss. These alterations encompassed a dysregulation of ESR1-PR signaling
421 (Ingaramo et al. 2016) and Wnt pathways (Ingaramo et al. 2017).
422
423 In the present study, we used a model in which animals were OVX on PND21 (prepubertal
424 period). We hypothesized that GBH exposure during early stage of development might enhance
425 the sensitivity of the rat uterus to E2. To test this hypothesis, different end-points were
426 evaluated to determine the long-term uterine response to E2: i) uterine morphology, ii) uterine
427 cell proliferation, iii) the expression of ESR1 and ESR2, and iv) the expression of WNT7A and
428 β-catenin, selected as E2-modulated targets that have been implicated in altered uterine E2
429 responsiveness and neoplasia.
430
431 The uterine responsiveness to E2 stimulation in OVX rodents has been extensively studied
432 using different models. We chose chronic E2 treatment since it has been shown that prolonged
433 E2 stimulation plays an important role in the development of endometrial pathologies such as
434 hyperplasia and carcinoma (Chufa et al. 2010). In this model, serum concentrations of E2 in E2-
435 implanted rats were equivalent to circulating levels during the rat estrous cycle (Smith et al.
436 1975). No differences in E2 serum levels between control and GBH-exposed rats were detected,

437 indicating that all animals were exposed to the same levels of E2. Some studies have shown that
438 GBH exposure can disrupt aromatase (Richard et al. 2005; Cassault-Meyer et al. 2014; Defarge
439 et al. 2016), the key enzyme responsible for the conversion of androgens into estrogens. It is
440 worth noting here that, since we worked with OVX rats that received exogenous E2 treatment,
441 the contribution of extragonadal aromatization to E2 serum levels would be negligible.
442 However, taking into account that aromatase is an estrogen sensitive-gene and is related to
443 uterine disorders (Bulun et al. 2005), we cannot discard that aromatase expression could be
444 affected in GBH-exposed animals.
445
446 Using this model, we detected that when OVX animals were chronically exposed to E2, uterine
447 histomorphology showed signs of higher sensitivity to E2 in the postnatally GBH-treated group.
448 The results showed that the luminal and glandular epithelium were markedly hyperplastic.
449 Different morphological changes were detected in GBH-exposed animal, such as increase of
450 luminal epithelial height and higher stromal nuclei density, both histological features of simple
451 endometrial hyperplasia (Dixon et al. 2014; Sanderson et al. 2017). Similar alterations in both
452 uterine regions were observed at the neonatal period (PND8) (Guerrero Schimpf et al. 2017),
453 suggesting that GBH exposure permanently alters uterine histoarchitecture. Interestingly, DES-
454 exposed mice and hamsters that exhibited endometrial hyperplasia (Hendry et al. 1997;
455 Yamashita et al. 2006) developed uterine adenocarcinoma later in life (Leavitt et al. 1981;
456 Newbold et al. 1990). Considering that endometrial hyperplasia has the potential to progress to
457 endometrial cancer (Sanderson et al. 2017), our present findings provide the first evidence of a
458 link between developmental exposure to GBH and uterine development of pre-neoplastic
459 lesions after prolonged E2 exposure.
460
461 In addition, we observed a dramatic increase in uterine epithelial cell proliferation in association
462 with a strong induction of ESR1 in postnatally GBH-exposed females. ESR1 plays a crucial role
463 in uterine cell proliferation stimulated by E2. *In vivo* studies have demonstrated that both
464 stromal and epithelial ESR1 expression is important to induce uterine proliferation and

epithelial cell growth. Winuthayanon and colleagues (2014) have shown that epithelial ESR1 is essential for long-term uterine responsiveness to E2. Its expression induces gene products involved in mitosis and cell cycle progression as well as inhibition of apoptosis. These authors suggest that epithelial ESR1 may generate autocrine signals or provide a positive feedback to stromal cells to stimulate epithelial cell proliferation (Winuthayanon et al. 2010, 2014). Hence, the epithelial hyperplasia of GBH-exposed animals could be attributed, at least in part, to an increased expression of epithelial ESR1 that generates a maximal mitogenic signal. Moreover, our results are in concordance with the findings of Tica et al. (2016) who showed that ESR1 expression is increased in normal proliferative and benign hyperplastic epithelial cells. It is worth noting the differences in responsiveness to estrogens in relation to proliferative activity between luminal and glandular epithelial cells. In fact, luminal epithelial cells in GBH-exposed animals were more sensitive to E2-induced cell proliferation. Taking into account the important role of ESR1 over cell proliferation control, higher luminal epithelial proliferation might be related to higher ESR1 expression in this compartment. Contrary, in the glandular epithelium neither proliferation nor ESR1 expression were affected.

As previously mentioned, in our model of exposure, E2 administration induced downregulation of *Esr1* mRNA levels. In addition, GBH-exposed animals showed decreased expression of *Esr1* mRNA. However, a similar decrease in ESR1 protein expression was not observed. In fact, GBH treatment increased ESR1 in the luminal epithelium. In this context, we propose that GBH could affect protein turnover due to lower activity of the ubiquitin-proteasome pathway. It has been reported that proteasome-mediated proteolysis modulates the cellular concentration of ESR1 in a process that requires ligand binding to the receptor (Preisler-Mashek et al. 2002). Moreover, it has been demonstrated that the extent to which the overall ESR1 levels are affected depends on the ligand and is not related to ligand-binding affinity or activation of transcription (Preisler-Mashek et al. 2002).

ESR2 is important for differentiation and growth of the uterine epithelium. In addition, loss of ESR2 function results in an enhanced uterine responsiveness to E2 in the neonatal and adult uterus, highlighting its role in the inhibition of epithelial cell proliferation (Wada-Hiraike et al. 2006; Nakayima et al. 2015). Besides this protective action in normal endometrial tissue, a completely different role has been proposed in benign and malignant proliferative endometrial pathologies. Increased levels of ESR2 found in high-grade and advanced stage endometrial carcinomas as well as in endometriotic stromal cells suggest an involvement in aberrant cell proliferation and tumor-promoting activity (Bulun et al. 2010; Hapangama et al. 2015). Here, we found an increase in ESR2 uterine expression at mRNA and protein levels in GBH-exposed uteri after E2 administration. These results support the concept that alterations in ESR2 expression are important in the development and progression of uterine proliferative lesions.

In the endometrium, Wnt/β-catenin signaling pathway is implicated in different processes such as uterine development and epithelial cell proliferation in response to E2 (Van der Horst et al. 2012). β-catenin plays a dual role as an adhesion molecule in adherens junctions at the plasma membrane and as a key intermediate in the canonical Wnt signaling pathway (Brembeck et al. 2006). WNT7A is a member of the Wnt protein family that is able to directly stimulate canonical Wnt signaling. We were interested in examining WNT7A expression because previous studies have shown that this protein is up regulated by E2 (Wang et al. 2009) and overexpressed in uterine disorders such as endometriosis and endometrial cancer (Gaetje et al. 2007; Liu et al. 2013). In our model, GBH treatment increased WNT7A mRNA and protein expression in the luminal epithelium, suggesting enhanced Wnt/β-catenin signaling. However, the pattern of changes in β-catenin differed from those in WNT7A expression. Decreased membranous and cytoplasmic β-catenin expression without nuclear immunoreaction was detected in the luminal and glandular epithelium of GBH-exposed rats. In accordance with these results, decreased β-catenin expression was observed in epithelial cells from hyperplastic uterine lesions in mice treated whit lithium and E2 (Gunin et al. 2004). Despite nuclear β-catenin accumulation is a well-known alteration in uterine hyperplasia and neoplasia, it has been

520 demonstrated that β-catenin cell membrane expression is decreased in these types of lesions
521 (Saegusa et al. 2001; Florescu et al. 2016). Loss of β-catenin expression on the cell surface
522 destabilizes and weakens cell-cell adhesion favoring cell dissociation (Guarino et al. 2007).
523 Taking into account morphologic changes induced by GBH-exposure, alterations in intercellular
524 contacts and continuous activation of Wnt/β-catenin signaling pathway may contribute to the
525 development of endometrial cancer.
526
527 Few studies have shown that glyphosate and its formulations can interact with the ERs. Gasnier
528 et al. (2009) showed inhibition of ESR1 and ESR2 transcriptional activities by different GBH
529 formulations in human liver HepG2 cells transfected with an estrogen response element
530 construct and the ERs. On the contrary, other studies showed that glyphosate (the active
531 ingredient of formulations) promotes cell proliferation in hormone-dependent breast cancer cells
532 by activating the ESR1 (Thongprakaisang et al. 2013; Mesnage et al. 2017). Similarly, we and
533 other authors have shown that glyphosate and GBHs can modulate the expression of both ER
534 subtypes in the rat uterus (Varayoud et al. 2017) as well as induce ESR1 and ESR2 protein
535 expression in T47D breast cancer cells (Thongprakaisang et al. 2013). In the present study, we
536 observed that postnatal exposure to GBH enhanced ESR1 and ESR2 expression in the rat uterus
537 after E2 treatment. However, we found some differences in the expression pattern of both ERs
538 at mRNA level. The results indicate different sensitivity between *Esr1* and *Esr2* in response to
539 GBH exposure. While downregulation of *Esr1* caused by GBH exposure was similar from that
540 induced by E2 treatment alone, the enhancement of *Esr2* with GBH was lower than E2
541 treatment alone. Similar to our findings, other study showed that the expression of both ERs in
542 human breast cancer cells *in vitro* was differentially modulated after glyphosate treatment
543 (Thongprakaisang et al. 2013). Taken together, the above-mentioned results contribute to a
544 growing body of evidence supporting the potential interaction of GBHs with the estrogen
545 signaling pathways.
546

In the last years, one matter of concern has focused on toxicological properties of chemicals used in commercial formulations of GBHs. GBHs are commercialized in the form of mixtures consisting of glyphosate (active principle) and different co-formulants (i.e. solvents, penetrating agents, preservatives, surfactants) whose identity is frequently undisclosed because it is considered to be confidential commercial information. Several studies comparing GBHs, co-formulants and glyphosate effects have revealed that GBHs are more toxic than the active ingredient, suggesting that co-formulants can also be a source of toxicity (Defarge et al. 2016; Mesnage & Antoniou 2018). Considering these points, under the condition of our experiment we cannot ascribe the increased uterine sensitivity to E2 only to glyphosate, to co-formulants or both acting together. Further research is required to fully understand glyphosate and co-formulants contribution to GBHs effects.

It is well-recognized that estrogens and their receptors have a profound involvement in uterine pathology. Consistent with this concept, increased sensitivity to E2 may predispose to endometrial dysfunction and/or disease development. Some EDCs have been demonstrated to increase E2 responses in hormone-sensitive tissues and promote the development of tissue lesions and carcinogenesis later in life. Perinatal exposure of female rodents to low doses of BPA has been shown to enhance sensitivity of the mammary gland to E2 at puberty (Muñoz-de-Toro et al. 2005; Wadia et al. 2007) and to elicit the development of mammary carcinomas during adulthood in the absence of any additional treatment (Acevedo et al. 2013). Other authors have also reported that developmental exposure to BPA alters uterine and prostate gland response to E2 in aged rats, causing increased incidence of pre-neoplastic lesions in the uterus (Vigezzi et al. 2015, 2016) and intraepithelial neoplasia in the prostate (Ho et al. 2006). Similarly, mice postnatally exposed to low doses of DES with an enhanced uterine response to E2 at puberty (Newbold et al. 2004) developed uterine endometrial adenocarcinoma later in life (Newbold et al. 1990). In the present study, we found that early postnatal exposure to GBH altered uterine response to E2 in prepubertal rats. These results may indicate that postnatal GBH exposure could predispose to endometrial pathologies such as carcinogenesis in aged rats.

In conclusion, our results showed that postnatal exposure to GBH enhances the sensitivity of the rat uterus to estradiol, and induces histomorphologic and molecular changes associated with uterine hyperplasia. GBH treatment induced morphological, cellular and molecular alterations in the rat uterus that were evidenced by the development of uterine hyperplasia, increase in E2-induced cell proliferation, induction of both ESR1 and ESR2, and deregulation of Wnt/β-catenin signaling pathway. Currently, further experiments are underway to investigate whether subsequent estrogen exposure associated with puberty could induce benign and/or malignant tumors of female reproductive tissues in postnatally GBH-exposed rats.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Figure legends

Fig. 1. Schematic representation of the experimental protocol used to investigate the effects of postnatal exposure to a glyphosate-based herbicide (GBH) on uterine sensitivity to 17β-estradiol (E2). C, control; OVX, ovariectomy; sc, subcutaneous; V, vehicle (corn oil).

Fig. 2. Uterine histology in OVX prepubertal control rats following vehicle treatment (C+OVX+V) or E2 stimulation (C+OVX+E2). Representative images of longitudinal sections of uterine horns of C+OVX+V and C+OVX+E2 animals stained with H&E at low (A and B) and high (C and D) magnification. LE, luminal epithelium; GE, glandular epithelium; SS, subepithelial stroma; MM, myometrium. Scale bar: (A and B) 100 µm; (C and D) 50 µm.

Fig. 3. Uterine responsiveness to E2 stimulation in OVX prepubertal rats: expression of the estrogen receptors alpha (ESR1) and beta (ESR2), and cell proliferation. (A and B) Relative mRNA levels of *Esr1* and *Esr2* in vehicle- (C+OVX+V) and E2-treated (C+OVX+E2) animals were measured by qRT-PCR. Fold expression from C+OVX+V values was calculated by the relative standard curve method. C+OVX+V values were assigned to a reference level of 1 and values are given as mean ± SEM of two independent determinations. Significant effects are depicted with asterisks (* $p<0.05$). (C) Quantification of ESR1 and ESR2 protein immunostaining is expressed as the integrated optical density (IOD). Cell proliferation is shown as a percentage of Ki67-positive cells. Significant effects are depicted with asterisks (* $p<0.05$; ** $p<0.01$). (D-I) Representative images of protein expression of the ESR1 (D and E) and ESR2 (F and G), and cellular proliferative responses by Ki67 immunodetection (H and I) in C+OVX+V and C+OVX+E2 groups. LE, luminal epithelium; GE, glandular epithelium; SS, subepithelial stroma. Scale bar: 50 µm.

Fig. 4. Uterine histological lesions in postnatally GBH-exposed animals after E2 stimulation at low and high magnification. (A and D) Normal uterine histology in OVX control rats after E2 treatment (C+OVX+E2). (B, C, E and F) Representative H&E stained images of postnatally GBH-exposed rats after E2 stimulation (GBH+OVX+E2) demonstrating histological findings. Abnormal endometrial glands (arrows) and areas of endometrium with stratification of the luminal epithelium (asterisk), hypochromic nuclei (asterisk) and mitotic figures (arrowheads)

are indicated. Uterine areas in the insets are magnified in the lower panel. LE, luminal epithelium; GE, glandular epithelium; SS, subepithelial stroma; MM, myometrium. Scale bar: (A, B and C) 50 μm; (D, E and F) 10 μm.

Fig. 5. Effects of GBH postnatal exposure on E2-induced cell proliferation. (A) Ki67 protein expression was quantified in luminal and glandular epithelial uterine cells. Values in bar graphs are the mean ± SEM. The asterisk indicates statistical significance compared with the control (* $p<0.05$). (B) Representative images of immunohistochemical detection of Ki67 on uterine sections. The asterisk indicates the increase of Ki67 expression. LE, luminal epithelium; GE, glandular epithelium; SS, subepithelial stroma. Scale bar: 50 μm.

Fig. 6. Effects of GBH postnatal exposure on uterine expression of the estrogen receptors alpha (ESR1) and beta (ESR2). (A and B) Relative mRNA levels of *Esr1* (A) and *Esr2* (B) in control and postnatally GBH-exposed animals were measured by qRT-PCR. Fold expression from C+OVX+E2 values was calculated by the relative standard curve method. C+OVX+E2 values were assigned to a reference level of 1 and values are given as mean ± SEM of two independent determinations. Significant effects are depicted with asterisks (* $p<0.05$). (C) Quantification of ESR1 and ESR2 protein immunostaining in uterine regions is expressed as the integrated optical density (IOD). Values in bar graphs are the mean ± SEM. Asterisks indicate statistical significance compared with the control (* $p<0.05$). (D) Representative images of immunohistochemical staining for ESR1 and ESR2 on uterine sections. Significant effects are depicted with asterisks. LE, luminal epithelium; GE, glandular epithelium; SS, subepithelial stroma. Scale bar: 50 μm.

Fig. 7. Effects of GBH postnatal exposure on the expression of E2-modulated targets that have been implicated in altered uterine E2 responsiveness and neoplasia. (A and B) Relative mRNA levels of *Wnt7a* (A) and *Ctnnb1* (B) in control and postnatally GBH-exposed animals were measured by qRT-PCR. Fold expression from C+OVX+E2 values was calculated by the relative standard curve method. C+OVX+E2 values were assigned to a reference level of 1 and values are given as mean ± SEM of two independent determinations. Significant effects are depicted with asterisks (* $p<0.05$). (C) Quantification of WNT7A and β-catenin protein immunostaining

in uterine regions is expressed as the integrated optical density (IOD). Values in bar graphs are the mean ± SEM. Significant effects are depicted with asterisks (* $p<0.05$). (D) Representative images of immunohistochemical staining for WNT7A and β-catenin on uterine sections. Significant effects are depicted with asterisks. LE, luminal epithelium; GE, glandular epithelium; SS, subepithelial stroma. Scale bar: 50 μm.

Table 1

Primers used for qRT-PCR

Gene name	Primer Sense (5' → 3')	Primer Antisense (5' → 3')	Product size (bp)
Esr1	ACTACCTGGAGAACGAGCCC	CCTTGGCAGACTCCATGATC	153
Esr2	TTCTGGGCACCTGTCTCCTT	TAACAGGGCTGGCACAACTG	166
Ctnnb1	GAGCACATCAGGACACCCAGC	GAGGATGTGGAGAGCCCCAGT	116
Wnt7a	CTTACACAATAACGAGGCAGGC	TCTCGGAATTGTGGCAGTGT	126
Rpl19	AGCCTGTGACTGTCCATTCC	TGGCAGTACCCTTCCTCTTC	99

Esr1, estrogen receptor alpha; *Esr2*, estrogen receptor beta; *Ctnnb1*, beta-catenin; *Rpl19*, ribosomal protein L19; *Wnt7a*, wingless-type MMTV integration site family, member 7A.

Table 2

Antibodies used for immunohistochemistry

Antibodies	Dilution	Supplier
Primary		
Anti-Ki67 (clone MIB-5)	1/50	Dako Corp. (Carpinteria, CA, USA)
Anti-ESR1 (clone 6F-11)	1/200	Novocastra (Newcastle upon Tyne, UK)
Anti-ESR2 (clone EMR02)	1/50	Novocastra (Newcastle upon Tyne, UK)
Anti-WNT7A	1/800	Generated and validated in our laboratory (Vigezzi et al. 2016)
Anti-β-catenin (sc-7963)	1/800	Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA)
Secondary		
Anti-mouse (B8774)	1/100	Sigma-Aldrich (St. Louis, MO, USA)
Anti-rabbit (B8895)	1/200	Sigma-Aldrich (St. Louis, MO, USA)

ESR1, estrogen receptor alpha; ESR2, estrogen receptor beta; WNT7A, wingless-type MMTV integration site family, member 7A.

Table 3

Serum levels of 17β-estradiol (E2)

Treatment group	E2 levels (pg/ml)
C+OVX+V	ND
C+OVX+E2	33.1 ± 3.3
GBH+OVX+E2	31.6 ± 2.0

Estradiol levels were measured via RIA. Values are mean ± SEM (n = 5-8 rats/group).

C, control; E2, 17β-estradiol (1 mg/ml); GBH, glyphosate-based herbicide (2 mg of glyphosate/kg bw/day); ND, not detected; OVX, ovariectomized; V, vehicle.

Table 4

Morphometric analysis of uterine sections

Parameters	C+OVX+E2	GBH+OVX+E2
Luminal epithelial height (μm)	32.04 ± 4.03	68.10 ± 13.76 *
Glandular epithelial height (μm)	15.30 ± 1.34	15.42 ± 1.16
Endometrial glands (Vv × 100)	4.63 ± 0.21	5.26 ± 0.40
Stromal nuclei density (%)	19.99 ± 0.97	26.04 ± 1.67 **
Stromal thickness (μm)	571.25 ± 39.44	598.12 ± 42.88
Circular myometrium thickness (μm)	159.42 ± 13.85	198.78 ± 26.48
Longitudinal myometrium thickness (μm)	139.89 ± 12.63	173.33 ± 16.69

Values are means ± SEM (n = 7-8 rats/group).

C, control; E2, 17β-estradiol (1 mg/ml); GBH, glyphosate-based herbicide (2 mg of glyphosate/kg b.w/day); OVX, ovariectomized; Vv, volume fraction. * $p<0.05$, ** $p<0.01$ compared with the control group.