Effects of a Glyphosate-Based Herbicide on the Uterus of Adult Ovariectomized Rats

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Received 18 February 2016; revised 21 June 2016; accepted 2 July 2016

ABSTRACT: Glyphosate is the active ingredient of several herbicide formulations. Different reports suggest that glyphosate-based herbicides (GBHs) may act as endocrine disruptors. We evaluated the potential estrogenic effects of a GBH formulation using the uterotrophic assay. Adult ovariectomized rats were sc injected for 3 consecutive days with: saline solution (vehicle control), 2.10^{-5} g E_2/kg/day (uterotrophic dose; UE_2), 2.10^{-7} g E_2/kg/day (nonuterotrophic dose; NUE_2), or 0.5, 5, or 50 mg GBH/kg/day of the. Twenty-four hours after the last injection, the uterus was removed and weighed and processed for histopathology and mRNA extraction. Epithelial cell proliferation and height and expression of estrogen-responsive genes were evaluated (estrogen receptors, ERα and ERβ; progesterone receptor, PR; complement 3, C3). Uterine weight and epithelial proliferation were not affected by GBH. However, the luminal epithelial cell height increased at GBH0.5. ERα mRNA was downregulated by all GBH doses and E_2 groups, whereas PR and C3 mRNA were diminished by GBH0.5. GBH5-, GBH50-, and UE_2-treated rats showed downregulated ERα protein expression in luminal epithelial cells, while the receptor was upregulated in the stroma. GBH upregulated ERβ (GBH0.5–50) and PR (GBH5) expressions in glandular epithelial cells, similar effect to that of NUE_2 group. These results indicate that, although the uterine weight was not affected, GBH modulates the expression of estrogen-sensitive genes. © 2016 Wiley Periodicals, Inc. Environ Toxicol 00: 000–000, 2016.

Keywords: uterus; glyphosate-based herbicide; estrogen receptor; progesterone receptor

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Contract grant sponsor: Consejo Nacional de Investigaciones Científicas y Técnicas.

Contract grant number: PIP 112-200801-00218

Contract grant sponsor: Agencia Nacional de Promoción Científica y Tecnológica.

Contract grant numbers: PICT 2011-1491 and PICT 2014-1522.

Contract grant sponsor: Universidad Nacional del Litoral.

Contract grant numbers: C.A.I. + D. 2011 501 201101 00423 LI and PAITI 2011.

Published online 00 Month 2016 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/tox.22316

INTRODUCTION

Approximately 800 chemicals are known or suspected to interfere with hormone receptors or hormone metabolism. However, only a few have been investigated by means of tests able to identify overt endocrine effects in intact organisms, and the vast majority of chemicals in current commercial formulations have not been tested at all. This lack of data leads to uncertainties about the extent of risks caused by chemicals that could potentially disrupt the endocrine system (endocrine disrupting chemicals, EDCs) (Bergman et al., 2012).

Glyphosate (*N*-phosphonomethyl glycine) is an active ingredient of broad-spectrum herbicide formulations, whose primary mechanism of action is the inhibition of an enzyme essential for the formation of aromatic amino acids in plants (5-enolpyruvylshikimate 3-phosphate synthase) (Steinrucken and Amrhein, 1980). Glyphosate-based herbicides (GBHs) are the most heavily used herbicide in the world and its usage continues to rise. Since the late 1970s, the volume of GBHs applied has increased ~100-fold (Myers et al., 2016). In several countries, like in Argentina, the constant increase in transgenic glyphosate-resistant soybean single-cropping has been associated with a corresponding increase in herbicide use (Cerdeira et al., 2011). A monitoring study carried out within the main area of soybean sowing showed that the levels of glyphosate range from 0.1 to 0.7 mg/L in surface waters and 0.5 to 5 mg/kg in sediments and soil (Peruzzo et al., 2008; Aparicio et al., 2013).

The United States Environmental Protection Agency (USEPA) has determined a reference dose (RfD) of 2 mg/kg body weight/day of glyphosate based on developmental toxicity studies. The RfD was established on the basis of a non-observed adverse effect level of 175 mg/kg/day and the application of an uncertainty factor of 100 to account for inter- and intraspecies variability (USEPA, http://www.epa.gov/oppsrrd1/REDs/factsheets/0178fact.pdf).

Several researchers have studied the endocrine disrupting effects of glyphosate *in vitro* using rat fresh testicular cells (Clair et al., 2012) and human cell lines (Gasnier et al., 2009; Thongprakaisang et al., 2013). Endocrine disruption was evident for testosterone synthesis (Clair et al., 2012), androgen receptor expression, and aromatase transcription and activity (Gasnier et al., 2009). Proliferative effects in hormone-dependent breast cancer cells and induction of an estrogen response element-dependent gene construct were also described (Thongprakaisang et al., 2013). Glyphosate was reported to inhibit the transcriptional activities of estrogen receptor (ER) α, ERβ, and androgen receptor in human cell lines (Gasnier et al., 2009). Endocrine disrupting effects of GBHs have also been documented *in vivo* although limited to male rats (Dallegrave et al., 2007; Romano et al., 2012; Cassault-Meyer et al., 2014). The results suggest that perinatal exposure to glyphosate alters testicular morphology and function (Dallegrave et al., 2007; Romano et al., 2012). In addition, an acute exposure to GBH in adult rats increases aromatase levels, produces molecular changes in the blood-testis barrier, and decreases normal sperm morphology (Cassault-Meyer et al., 2014).

Due to the limited published *in vivo* data on the effects of GBH on the female reproductive tract, we sought to investigate whether the estrogenic effects observed *in vitro* were also occurring *in vivo*. We used one of the most common assays, the uterotrophic assay (Kanno et al., 2003; Owens and Koëter, 2003; Gelbke et al., 2004).

We chose the adult castrated rat uterotrophic assay because of our previous experience in evaluating the estrogenic effects of endosulfan, a manufactured organochlorine pesticide (Varayoud et al., 2008). We complemented the uterotrophic assay with more sensitive estrogen-dependent endpoints such as uterine epithelial cell proliferation and morphology and hormone receptors expression both at the protein and mRNA levels.

The goal of the present study was to determine whether different doses of GBH cause changes in adult ovariectomized (OVX) rat uteri associated with an uterotrophic effect. Additional estrogen-dependent endpoints, such as luminal epithelial cell height, luminal epithelial proliferation, and estrogen-responsive genes expression, were measured to complement the uterotrophic assay.

MATERIALS AND METHODS

Chemicals

E_2 was purchased from Sigma-Aldrich (Buenos Aires, Argentina). The GBH studied was a liquid water-soluble commercial formulation containing 662 mg/mL of glyphosate potassium salt as its active ingredient, coadjuvants, and inert ingredients. The solutions of GBH were prepared by the addition of appropriate volumes of saline solution.

Animals

All procedures used in this study were approved by the Institutional Ethics Committee of the School of Biochemistry and Biological Sciences (Universidad Nacional del Litoral, Santa Fe, Argentina) and performed in accordance with the principles and procedures outlined in the Guide for the Care and Use of Laboratory Animals issued by the National Research Council of the National Academies (National Research Council of the National Academies, 2011). Sexually mature female rats (90-day old) of an inbred Wistar-derived strain bred at the Instituto de Salud y Ambiente del Litoral (Santa Fe, Argentina) were used. Animals were maintained under a controlled environment (22 ± 2°C; lights on from 06:00 to 20:00 hours) and had free access to pellet laboratory chow (Nutrición Animal, Santa Fe, Argentina) and tap water. The concentration of phytoestrogens in the diet was not evaluated; however, because feed intake was equivalent for control and experimental rats, we assumed that all animals were exposed to the same levels of phytoestrogens (see Kass et al., 2012 for more information regarding food composition). To minimize additional exposures to EDCs, rats were housed in stainless steel cages with wood bedding, and tap water was supplied *ad libitum* in glass bottles with rubber stoppers surrounded by a steel ring.

Experimental Design

All experimental rats (n = 47, 90-day old) were OVX and then allowed to rest for 14 days. Those animals that exhibited at least 7 days of atrophic vaginal smears (Montes and Luque, 1988) were subcutaneously injected for 3 consecutive days with one of the following treatments: (a) saline

TABLE I. Primers used and PCR products for gene expression analysis by qPCR

Gene	Primer Sequence (5′–3′)	Product Size (bp)	Genbank Accession No.
Estrogen receptor alfa (ERA)	Forward: ACTACCTGGAGAACGAGCCC Reverse: CCTTGGCAGACTCCATGATC	153	NM_012689
Progesterone receptor (PR)	Forward: GACCAGTCTCAACCAACTAGGC Reverse: ACACCATCAGGCTCATCCAG	137	L16922
Complement component 3 (C3)	Forward: CTACCCCTTACCCCTCACTC Reverse: GTCTCTTCACTCTCCAGCCG	169	NW_047936
Ribosomal protein L19 (L19)	Forward: AGCCTGTGACTGTCCATTCC Reverse: TGGCAGTACCCTTCCTCTTC	99	NM_031103

solution (control group: 100 µL/animal; $n = 7$), (b) a uterotrophic dose of 2.10^{-5} g/kg/day of E_2 ($n = 8$; UE_2 group), (c) a nonuterotrophic dose of 2.10^{-7} g/kg/day of E_2 ($n = 8$; NUE_2 group), and (d) GBH diluted in saline solution ($n = 8$/dose): 0.5, 5, or 50 mg/kg/day (GBH0.5, GBH5, and GBH50, respectively).

All animals (7–8 rats/group) were sacrificed 24 hours after the last injection and uteri were isolated. One uterine horn from each rat was placed immediately in liquid nitrogen and stored at −80°C for RNA extraction. The other uterine horn (1.5 cm) was weighed and then fixed by immersion in 10% formalin buffer for 6 hours at 4°C, embedded in paraffin, and used for histological studies (morphometric and immunohistochemical analysis).

RNA Extraction and Reverse Transcription

Each experimental group was comprised of 7–8 uterine horns. Individual uterine horns were homogenized in TRIzol® reagent, and total RNA was extracted following the manufacturer's protocol (Invitrogen, Carlsbad, CA). The concentration of total RNA was assessed by A260, and the sample was stored at −80°C until needed.

Equal quantities (4 µg) of total RNA were reverse-transcribed in three independent experiments for 90 minutes at 37°C using 200 pmol of random hexamer primers (Biodynamics, Buenos Aires, Argentina), 100 nmol deoxynucleotide triphosphates, and 300 U Moloney Murine Leukemia Virus reverse transcriptase (MMLV-RT) (Promega, Madison, WI) in a final volume of 30 µL of 1× MMLV-RT buffer. Each reverse-transcribed product was diluted with RNase-free water to a final volume of 60 µL.

Quantitative Real-Time Polymerase Chain Reaction

mRNA expression of ERα, PR, and C3 was quantified by real-time RT-PCR using the Real-Time DNA Step One Cycler (Applied Biosystems, Foster City, CA). These genes were selected as classical targets of estrogen action in the OVX rat uterus (Diel et al., 2000; Varayoud et al., 2008). The ribosomal protein L19 was used to normalize RNA inputs. The gene-specific primer sequences are shown in Table I and were synthesized by Invitrogen.

For cDNA amplification, 5 µL of cDNA was combined with a commercial pre-mix HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis BioDyne, Tartu, Estonia) following the manufacturer's protocol. After initial denaturation at 95°C for 15 minutes, the reaction mixture was subjected to successive cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 15 seconds, and extension at 72°C for 15 seconds. 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 and yielded no consistent amplification. The relative expression levels of each target were calculated based on the cycle threshold (C_T) method (Higuchi et al., 1993). The C_T for each sample was calculated using the Step One™ Software (Applied Biosystems) with an automatic fluorescence threshold (*Rn*) setting. The efficiency of the PCR reactions was assessed for each target by amplification of serial dilutions (over seven orders of magnitude) of cDNA fragments of the transcripts under analysis. Accordingly, fold expression over control values was calculated for each target by the relative standard curve methods, which is designed to analyze data from real-time PCR (PerkinElmer Applied Biosystems, available from: http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_040980.pdf). For all experimental samples, target quantity was determined from the standard curve, normalized by the quantity of the reference gene, and finally divided by the target quantity of the control sample. No significant differences in C_T values were observed for L19 between the different experimental groups.

Immunohistochemistry

Uterine sections (5 µm thick) were deparaffinized and dehydrated in graded ethanol solutions. A standard immunohistochemical technique was used to quantify the expression of steroid receptors (ERα, ERβ, and PR), and the proliferation index, following a previously described protocol (Varayoud et al., 2008). Steroid receptors were immunostained using a mouse antihuman ERα antibody (clone 6F-11, 1:200

TABLE II. Effect of E_2 or GBH on uterine parameters of OVX adult rats

Experimental Group	Dose	Uterine Wet Weight (mg)	Luminal Epithelial Cell Height (μm)	Epithelial Proliferation[a] (%)
Control (vehicle)	–	25.07 ± 1.61	10.41 ± 0.65	0.30 ± 0.07
UE_2 (high dose of E_2)	20 μg/kg/BW day	59.28 ± 4.80*	21.30 ± 1.55*	77.20 ± 5.50*
NUE_2 (low dose of E_2)	0.20 μg/kg/BW day	29.27 ± 2.17	10.24 ± 0.71	0.22 ± 0.12
GBH0.5	0.5 mg/kg/BW day	22.18 ± 0.71	13.27 ± 0.80*	0.51 ± 0.15
GBH5	5 mg/kg/BW day	21.91 ± 1.93	10.59 ± 0.35	1.24 ± 0.39
GBH50	50 mg/kg/BW day	24.74 ± 3.56	11.92 ± 0.45	0.20 ± 0.06

Uterine parameters were evaluated after 3 days of treatment. Values are mean ± SEM (n = 7–8/group). UE, uterotrophic dose; NUE, nonuterotrophic dose.

[a]Cell proliferation was quantified in luminal epithelium.

*Statistically significant differences with the control group ($p < 0.05$).

dilution; Novocastra, Newcastle upon Tyne, UK), mouse antihuman ERβ antibody (clone EMR02, 1:100 dilution; Novocastra), or rabbit antihuman PR (A/B isoforms) antibody (1:500 dilution; Dako Corporation, Carpinteria, CA). Proliferating cells were detected using a mouse antihuman proliferating cell nuclear antigen (PCNA) antibody (clone PC-101, 1:1600 dilutions; Novocastra). Antirabbit/antimouse secondary antibodies (biotin conjugated) were purchased from Sigma-Aldrich. Reactions were developed using a streptavidin–biotin peroxidase method and diaminobenzidine (Sigma-Aldrich) as a chromogen substrate. Samples incubated with anti-PCNA antibodies were counterstained with Mayer's hematoxylin (Biopur, Rosario, Argentina). Negative controls were performed by replacing the primary antibody with non-immune horse serum (Sigma-Aldrich).

Quantification of Cell Proliferation

Tissue sections were evaluated using an Olympus BH2 microscope (illumination: 12-V halogen lamp, 100 W, equipped with a stabilized light source; Olympus, Tokyo, Japan) with the Dplan 40× objective (numerical aperture = 0.65; Olympus). The proliferation index was obtained by considering the percentage of epithelial PCNA-positive cells with a high immunostaining intensity (1000 cells were counted/tissue section and at least three sections per sample were included) (Muñoz de Toro et al., 1998).

Quantification of Steroid Receptors by Image Analysis

The expression of ERα, ERβ, and PR proteins in the uterine cells was evaluated by image analysis, using the Image Pro-Plus 5.0.2.9 system (Media Cybernetics, Silver Spring, MD), as previously described Ramos et al. (2002). Briefly, the images were recorded with a Spot Insight V3.5 color video camera, attached to a microscope (Olympus) and converted to a gray scale. The integrated optical density (IOD) was measured as a linear combination of the average gray intensity and the relative area occupied by the positive cells (Ramos et al., 2001, 2002). Because the IOD is a dimensionless parameter, the results were expressed as arbitrary units. The IODs of ERα, ERβ, and PR were evaluated in the luminal and glandular epithelium of each tissue section, and in the subepithelial stroma (300-μm-wide area adjacent to the epithelium, from the basement membrane toward the outer layers). At least 10 fields of each histological compartment were recorded in each section, and two sections per animal were evaluated. Correction of unequal illumination (shading correction) and calibration of the measurement system were performed with a reference slide.

Measurement of Luminal Epithelial Cell Height

Uterine epithelial cell height was measured in Mayer's hematoxylin-stained uterine sections from the apical (luminal) surface to the basement membrane, as previously described (Varayoud et al., 2008). All measurements were made in areas where luminal folds were not present, and care was taken to avoid measuring sections that were cut obliquely. To spatially calibrate the Image Pro-Plus analyzer, square grids from Neubauer's chamber images were captured as in the above-described experimental conditions.

Data Analysis

All data were calculated as the mean ± SEM. We performed Kruskal–Wallis analysis to obtain the overall significance (testing the hypothesis that the response was not homogeneous across treatments), and the Dunn posttest was applied to compare each experimental group against the control. $p < 0.05$ was accepted as significant.

RESULTS

Uterine Wet Weight

The high dose of E_2 (UE_2) induced a threefold increase in the uterine wet weight compared to the vehicle control

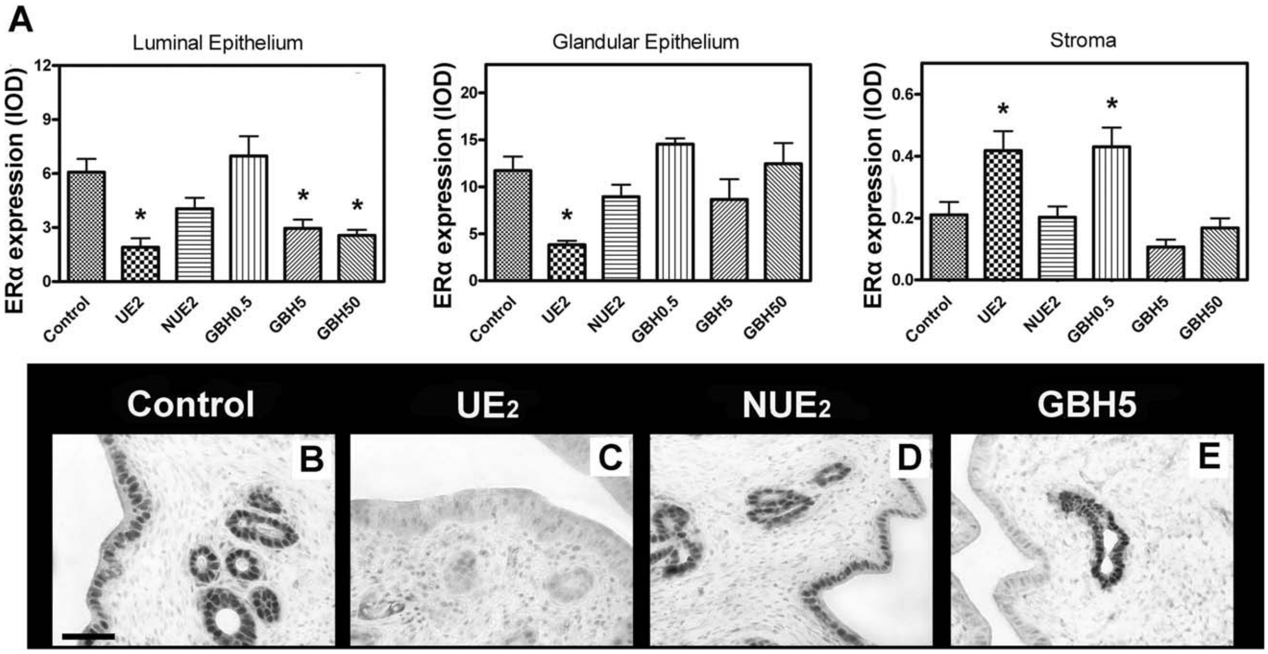

Fig. 1. (A) Quantification of ERα protein expression in the uterine luminal epithelium, glandular epithelium, and stroma. Data are expressed as integral optical density (IOD) as described in the "Materials and methods" section, which is measured as a linear combination between the average immunostained density and the relative area occupied by positive cells in each histological compartment. Statistical significances were tested using Kruskal–Wallis analysis and the Dunn posttest. Each column represents the mean ± SEM of 7–8 rats/group; $^*p < 0.05$. Representative photomicrographs of immunohistochemical detection of uterine ERα in adult OVX rats treated with vehicle (control) (B), UE_2 (uterotrophic dose) (C), NUE_2 (nonuterotrophic dose) (D), or GBH (E). Control rats showed a highly constitutive expression of ERα both in the luminal and the glandular epithelium (B); in contrast, UE_2-treated rats showed a downregulation of ERα protein in epithelial cells (luminal and glandular) and an upregulation in the stroma (C); ERα protein expression in uterine sections of NUE_2-treated rats (D) was not different from control; GBH (E) exhibited ERα downregulation in the luminal epithelium. Immunostaining reactions were developed using DAB as chromogen substrate. Scale bar: 50 μm.

($p < 0.05$). Neither the low dose of E_2 (NUE_2) nor the three doses of a GBH (GBH0.5, GBH5, and GBH50) showed statistically significant difference in uterine wet weight with the vehicle control (Table II).

Luminal Epithelial Cell Height and Luminal Epithelial Proliferation

The luminal epithelial cell height (LECH) increased almost twofold in the UE_2-treated animals compared to controls ($p < 0.001$, Table II). A significant increase in LECH was seen in the GBH0.5 compared to vehicle group ($p < 0.05$), although this effect was less than twofold. The NUE_2, GBH5, and GBH50 groups did not show differences in LECH compared to vehicle group (Table II).

PCNA immunostaining was used to assess the percentage of proliferating luminal epithelial cells (Table II). Vehicle control animals showed a low proliferative activity (0.30 ± 0.07%), whereas UE_2-treated rats showed a significant increase in the proliferative activity of luminal epithelial cells (77.20 ± 5.50%). The expression of PCNA protein in the NUE_2- and GBH-treated animals did not differ from that in vehicle control animals.

Steroid Receptor Protein Expression in Uterine Compartments

Changes in the expression of ERα, ERβ, and PR proteins were observed in all GBH-treated animals and both estrogen-treated groups. Differences in the intensity of protein expression and tissue compartment (i.e., epithelium and stroma) localization were observed between groups. The highest ERα expression was found in uterine luminal and glandular epithelial cells of vehicle control animals (Fig. 1). ERα immunostaining was significantly decreased ($p < 0.05$) in luminal epithelium after treatment with UE_2, GBH5, and

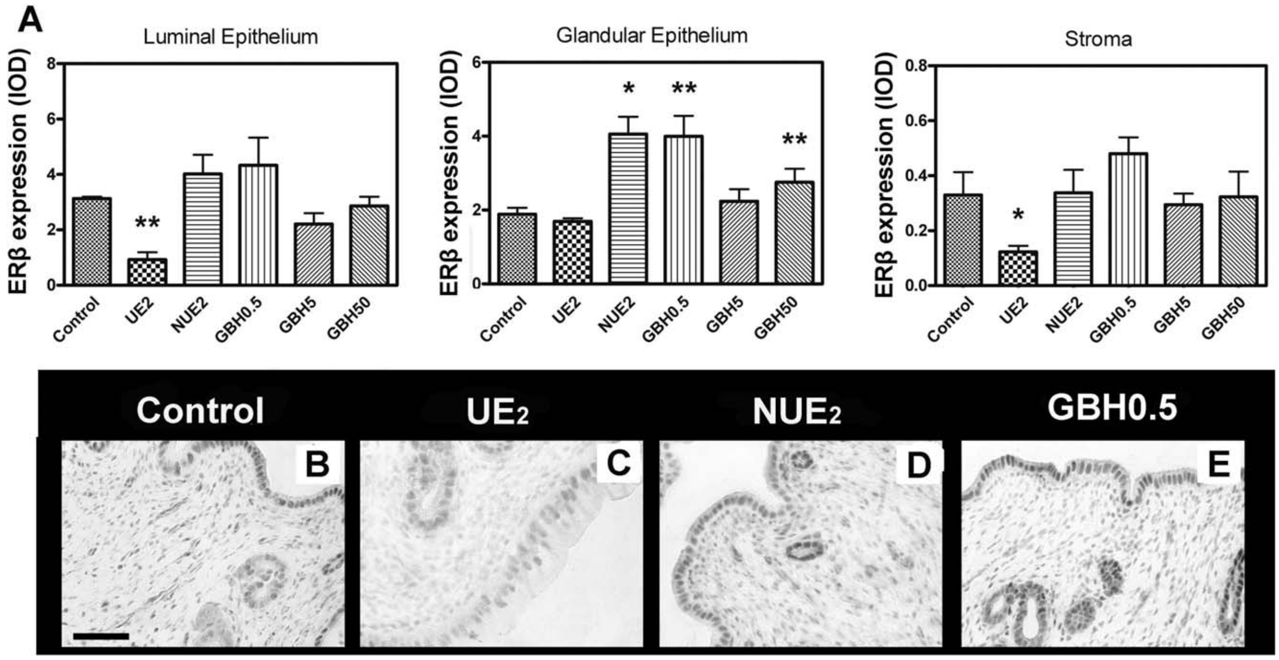

Fig. 2. (A) Quantification of ERβ protein expression in the uterine luminal epithelium, glandular epithelium and stroma. Data are expressed as IOD. Statistical significances were tested using Kruskal–Wallis analysis and the Dunn posttest. Each column represents the mean ± SEM of 7–8 rats/group; *$p < 0.05$; **$p < 0.01$. Representative photomicrographs of immunohistochemical detection of uterine ERβ in adult OVX rats treated with vehicle (control) (B), UE_2 (uterotrophic dose) (C), NUE_2 (nonuterotrophic dose) (D), or GBH (E). Expression of ERβ protein was downregulated by UE_2 treatment both in the luminal epithelium and stroma (C); in contrast, NUE_2 treatment increased ERβ expression in the glandular epithelium (D). This induction in ERβ was also observed with GBH (E) treatment. Immunostaining reactions were developed using DAB as chromogen substrate. Scale bar: 50 μm.

GBH50 (Fig. 1). In contrast, none of the GBH doses significantly reduce ERα expression in glandular epithelial cells while UE_2 treatment did significantly reduce it ($p < 0.05$). In the uterine stromal compartment, ERα expression was induced in the UE_2 and GBH0.5 groups ($p < 0.05$, Fig. 1).

High expression of ERβ was observed in luminal and glandular epithelial cells of controls animals, whereas the stromal cells showed a low expression (Fig. 2). UE_2 treatment elicited a downregulation of ERβ protein in the luminal epithelium and the stroma ($p < 0.05$). In contrast, the NUE_2 group showed an increased expression of ERβ in epithelial cells, with statistical differences specifically in the glandular compartment (Fig. 2). GBH showed a similar effect to NUE_2 group, increasing the expression of ERβ in the glandular epithelium in GBH0.5- and GBH50-treated animals (Fig. 2).

PR expression was strongly induced in the stroma of UE_2-treated animals, this contrasted with a downregulation in the luminal and glandular epithelial cells ($p < 0.05$, Fig. 3). In NUE_2-treated animals, PR expression was significantly increased in glandular epithelial cells, without changes in the luminal epithelium and stroma ($p < 0.05$, Fig. 3). Only GBH5 group showed an increased in PR expression and it was located in the glandular cells ($p < 0.05$ Fig. 3), this is similar to the NUE_2 group.

Regulation of Estrogen-Responsive Genes

Relative changes in uterine gene expression were determined using real-time RT-PCR analysis. L19 was selected as internal control since RNA concentrations were similar across groups. Treatment with all doses of GBH and E_2 led to a downregulation of ERα mRNA (Fig. 4). The expression of C3 mRNA was enhanced by the high E_2 dose (UE_2 group). The administration of GBH0.5 decreased C3 mRNA expression, whereas NUE_2, GBH5, and GBH50 showed no changes in C3 mRNA expression compared to control. The expression of PR mRNA in the uterus was slightly induced by the high dose of E_2. A significant decrease in PR mRNA expression was observed in the GBH0.5 group, although no changes were observed in other GBH treated groups (Fig. 4).

DISCUSSION

EDCs have been shown to cause adverse effects in a broad spectrum of organs, biological systems, and endpoints at a wide range of concentrations or doses (vom Saal and Welshons, 2006; Welshons et al., 2006). Here, we show that GBH did not increase the wet weight of the uterus but it did alter estrogen-dependent gene and protein expression. These changes were not dose dependent and vary based on the

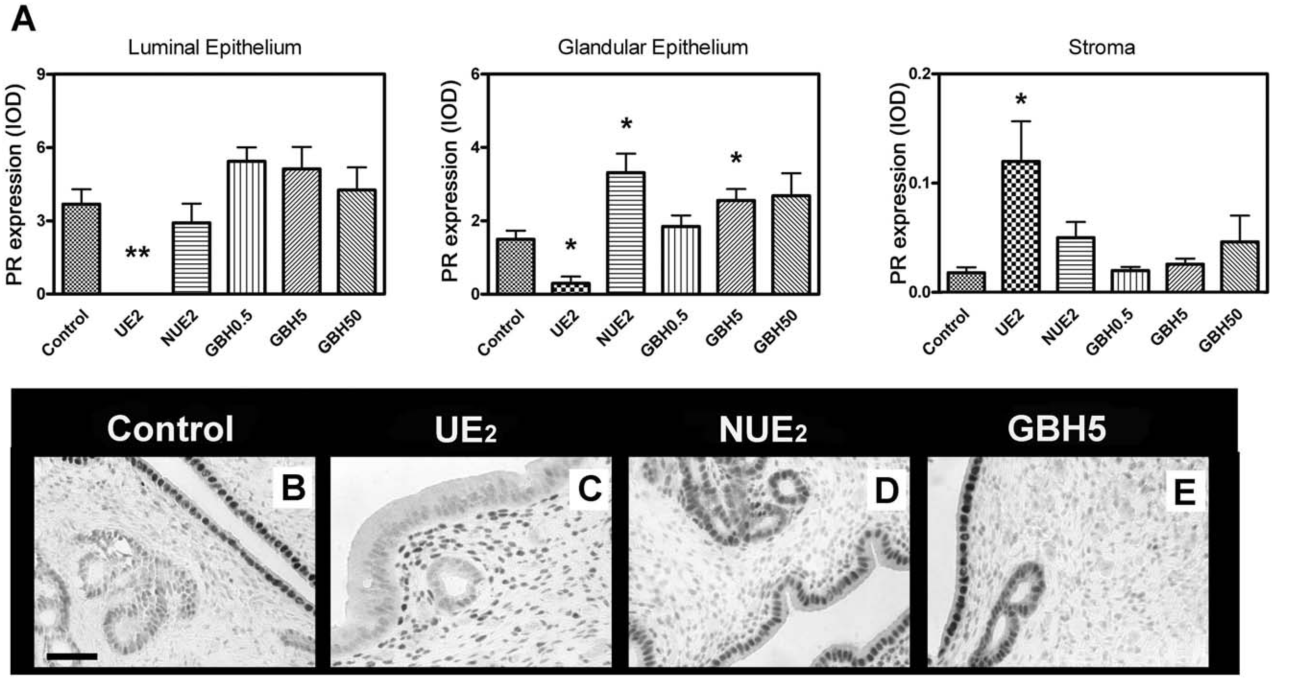

Fig. 3. (A) Quantification of PR protein expression in the uterine luminal epithelium, glandular epithelium, and stroma. Data are expressed as IOD. Statistical significances were tested using Kruskal–Wallis analysis and the Dunn posttest. Each column represents the mean ± SEM of 7–8 rats/group; *$p < 0.05$; **$p < 0.01$. Representative photomicrographs of immunohistochemical detection of uterine PR in adult OVX rats treated with vehicle (control) (B), UE_2 (uterotrophic dose) (C), NUE_2 (nonuterotrophic dose) (D), or GBH (E). PR protein was strongly induced in the stroma of UE_2-treated rats while the luminal and glandular epithelium showed a clear downregulation compared to controls (B *vs*. C). The NUE_2 (D) and GBH (E) groups showed a significant increase in PR in glandular epithelial cells. Immunostaining reactions were developed using DAB as chromogen substrate. Scale bar: 50 μm.

uterine compartment (i.e., luminal epithelium, glandular epithelium, and stroma).

The uterotrophic assay is a classical *in vivo* test to evaluate estrogenic activity at a level of organization that includes the whole organ. The evaluation of morphological and molecular changes in combination with increased uterine weight response provides additional information regarding the molecular mechanisms of the action of the EDCs. However, the sensitivity of the uterotrophic assay has been questioned (Diel et al., 2000; Newbold et al., 2001) since the assay has been shown to be negative for several well-known estrogen-mimics including bisphenol A, genistein, endosulfan, and kepone (Newbold et al., 2001; Moller et al., 2010). Limiting the assessment of a potential EDC to solely the uterotrophic response could result therefore in a potential false-negative result. On the contrary, complementing the assay with histopathological evaluation and molecular targets offers the opportunity to increase the likelihood of identifying estrogenic effects that could have been missed.

Although GBH did not produce a positive uterotrophic assay, the lowest dose of GBH increased the height of luminal epithelial cells which is a well-recognized morphological estrogenic uterine response (Padilla-Banks et al., 2001). This result has an important relevance due to the stimulation of the uterine luminal epithelium height that has been associated with uterine disorders, such as endometriosis and endometrial carcinoma (van Leeuwen et al., 1994).

Additionally, we found that GBH is able to modulate uterine estrogen-sensitive genes at the mRNA and protein levels (molecular changes). To our knowledge, this is the first study to show that GBH is able to regulate *in vivo* ERα, ERβ, and PR expression in the rat uterus. The main changes were observed at protein level, a physiological endpoint to evaluate endocrine disruption effects on estrogen-sensitive genes (Diel et al., 2000). The uterus is the primary target organ for estrogen, which exerts its effects via two main classical ER isoforms: ERα and ERβ. The ERα is the most studied, and its role is more extensive evaluated. The expression of ERα in luminal uterine epithelium is a useful tool to assess estrogenic activity (Nephew et al., 2000). In the case of ERβ, it has been proposed with a protective role from the undesired effects induce through ERα. Almost all benign and malignant endometrial proliferative diseases show changes in the ERβ expression (Nakajima et al., 2015), highlighting its protective role.

At protein level, we evaluated ERα, ERβ, and PR in different uterine compartments. We demonstrated that GBH (5 and 50) downregulated the ERα expression in luminal

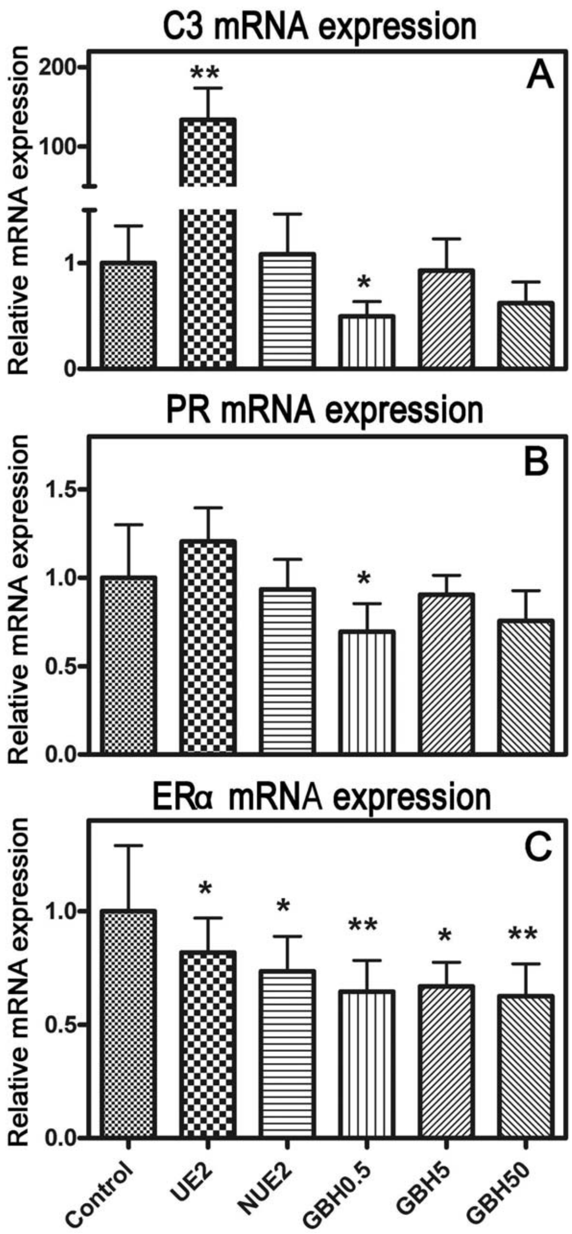

Fig. 4. Real-time RT-PCR analysis was done to determine uterine expression levels of C3 (a), PR (b), and ERα (c) after treatment. The vertical axis corresponds to the relative mRNA level of each target gene normalized to L19 expression. The mRNA level of the control group is expressed as 1. Statistical significances were tested using Kruskal–Wallis analysis and the Dunn posttest. Values are showed as mean ± SEM (7–8 rats/group) and significant effects are depicted with asterisks (*$p < 0.05$; **$p < 0.01$).

epithelial cells, but GBH0.5 upregulated the ERα expression in the stroma. These changes are in agreement with those induced by the UE_2 dose. Regarding effect of GBH on ERα expression in luminal epithelial cells, similar results were reported when genistein and DES were used (Newbold et al., 2004; Diel et al., 2006). Regarding ERβ, GBH increased its expression in glandular epithelial cells. Our results indicate that *in vivo* treatment with GBH affects the expression of uterine ERα and ERβ in a different manner, and results are affected by the dose of GBH and the uterine cell type studied. Using *in vitro* systems two studies have demonstrated that ERs are targets of the herbicide (glyphosate or GBH); nevertheless, different responses were detected between them (Gasnier et al., 2009; Thongprakaisang et al., 2013). Thongprakaisang et al. (2013) showed that GBH affects ER expression in mammary cells, producing an induction of ERα and ERβ in the human T47D hormone-dependent breast cancer cell line. They also found that patterns of ERα and ERβ induction by glyphosate were different, characterized by a quick activation of ERβ, and a slower but prolonged induction of ERα. The authors hypothesized that glyphosate may behave like a weak xenoestrogen and may activate both ER subtypes but with a different time course (Thongprakaisang et al., 2013). Gasnier et al. (2009) showed inhibition of the transcription activities of ERα and ERβ in HepG2 cells by a GBH formulation. Keeping in mind that both ERs are fundamental for a normal uterine function and that ERα deregulation has been associated with uterine disorders (endometriosis or endometrial carcinoma) (van Leeuwen et al., 1994); more studies are needed to assess whether GBH exposure could have a negative impact on reproductive performance or a higher predisposition to those pathologies. PR is a nuclear receptor for progesterone, a classical estrogen-regulated protein, and a transcription factor. In this study, we found that GBH5 causes an increase in PR expression in the uterine glandular compartment. A similar change was observed in the NUE_2 group. Previously, studies of neonatal exposure to other EDCs, such as, DES, BPA, and endosulfan, have shown effects on uterine PR expression during postnatal development with consequences in the adulthood (Varayoud et al., 2011; Milesi et al., 2012, 2015). Based on our results, we suggest that GBH effects on glandular PR expression could affect the normal uterine development and the uterine functional differentiation, which could potentially affect fertility.

At mRNA level, ERα showed a downregulation with both E_2 treatments (UE_2 and NUE_2) and with all GBH doses. The studies conducted both in OVX and in immature animals demonstrate that estrogens and antiestrogens downregulate ERα in the uterus (Medlock et al., 1992; Branham et al., 1996; Wang et al., 1999; Diel et al., 2000; Kummer et al., 2007). Therefore, this parameter might reflect both estrogenic and antiestrogenic effects and should be interpreted with caution. Downregulation of ERα might reflect different modes of action: degradation of ERα upon binding of both estrogens and antiestrogens (Nawaz et al., 1999) or activation of molecules that could promote ERα degradation, possibly through its E3 ubiquitin ligase activity (Ohtake et al., 2007). A downregulation of PR and C3 mRNA was detected with GBH0.5, a result that correlates with previous results (Diel et al., 2002). It has been suggested that an induction of PR and C3 mRNA may be the result of an estrogenic classical response (Diel et al., 2002). Similar to GBH, a downregulation of PR mRNA was also observed following administration of BPA and DDT and has been described as response to its own ligand, to pure antiestrogens and to androgens (Diel et al., 2000). In relation to C3 uterine expression, other EDCs have shown estrogenic actions without modifications in C3 expression (i.e., endosulfan and kepone) (Newbold et al., 2001). The results described here regarding different changes at mRNA level of estrogen-related genes might indicate that GBH could act as an endocrine disruptor, with potentially more than one mechanism of action. Future *in vivo* studies comparing GBH with hormones (androgen, progesterone, and antiestrogen) could help determining the mechanisms of action of the herbicide formulation on the uterus.

Since we have used an OVX rat model, we do not know if neonatal GBH exposure could produce alterations in different physiological reproductive situations (i.e., estrus cycles, pregnancy, and aging). However, since ERα, ERβ, and PR are crucial molecules in the endocrine control of many reproductive organs and GBH is widely disseminated in the environment, our present results stress the significance to perform *in vivo* experiments to investigate the effects of low doses of GBH in the development and function of the female reproductive tract.

There is a growing evidence to indicate that the guiding principle of traditional toxicology that "the dose makes the poison" may not always be the case because some EDCs do not induce the classical dose–response relationships (Schulte-Oehlmann et al., 2006; Vandenberg et al., 2012). In addition, it has been emphasized that when nonmonotonic dose–response curves (NMDRCs) occur, the effects of low doses cannot be predicted by the effects observed at high doses (Vandenberg et al., 2012). NMDRCs are often U-shaped (with maximal responses of the measured endpoint observed at low and high doses) or inverted U-shaped (with maximal responses observed at intermediate doses). Nonmonotonicity is not synomymous with low dose, because there are low-dose effects that follow monotonic dose-response curves. Thus, it is not required that a study include doses that span from the true low-dose range to the high toxicological range to detect nonmonotonicity. The consequence of NMDRCs for toxicity testing is that a safe dose determined from high doses does not guarantee safety at lower, untested doses that may be closer to current human exposures. In future studies, the evaluation of other doses of GBH may define if GBH follows a NMDRC pattern. Taking into account these concepts, we and others propose that fundamental changes in chemical testing and safety determination are needed to protect human health (Vandenberg et al., 2012).

We are grateful to Mr. Juan Grant and Mr. Juan C. Villarreal for technical assistance and animal care.

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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