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Neonatal exposure to a glyphosate based herbicide alters the development of the rat uterus

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HIGHLIGHTS

- Neonatal exposure to GBH lead to endometrial hyperplasia and increase proliferation
- GBH disrupts proteins involved in uterine organogenetic differentiation
- GBH exposure induced persistent increase of PR and Hoxa10 proteins

ABSTRACT

Glyphosate-based herbicides (GBHs) are extensively used to control weeds on both cropland and non-cropland areas. No reports are available regarding the effects of GBHs exposure on uterine development. We evaluated if neonatal exposure to a GBH affects uterine morphology, proliferation and expression of proteins that regulate uterine organogenetic differentiation in rats. Female Wistar pups received saline solution (control, C) or a commercial formulation of glyphosate (GBH, 2 mg/kg) by sc injection every 48 h from postnatal day (PND) 1 to PND7. Rats were sacrificed on PND8 (neonatal period) and PND21 (prepubertal period) to evaluate acute and short-term effects, respectively. The uterine morphology was evaluated in hematoxylin and eosin stained sections. The epithelial and stromal immunophenotypes were established by assessing the expression of luminal epithelial protein (cytokeratin 8; CK8), basal epithelial proteins (p63 and pan cytokeratin CK1, 5, 10 and 14); and vimentin by immunohistochemistry (IHC). To investigate changes on proteins that regulate uterine organogenetic differentiation we evaluated the expression of estrogen receptor alpha (ERα), progesterone receptor (PR), Hoxa10 and Wnt7a by IHC. The GBH-exposed uteri showed morphological changes, characterized by an increase in the incidence of luminal epithelial hyperplasia (LEH) and an increase in the stromal and myometrial thickness. The epithelial cells showed a positive immunostaining for CK8, while the stromal cells for vimentin. GBH treatment increased cell proliferation in the luminal and stromal compartment on PND8, without changes on PND21. GBH treatment also altered the expression of proteins involved in uterine organogenetic differentiation. PR and Hoxa10 were deregulated both immediately and two weeks after the exposure. ERα was induced in the stromal compartment on PND8, and was downregulated in the luminal epithelial cells of gyphosate-exposed animals on PND21. GBH treatment also increased the expression of Wnt7a in the stromal and glandular epithelial cells on PND21. Neonatal exposure to GBH disrupts the postnatal uterine development at the neonatal and prepubertal period. All these changes may alter the functional differentiation of the uterus, affecting the female fertility and/or promoting the development of neoplasias.

Abbreviations

CK, cytokeratin; DES, diethylstilbestrol; EDCs, endocrine-disrupting chemicals; ERα, estrogen receptor alpha; GBHs, glyphosate-based herbicides; IARC, International Agency for Research on Cancer; IHC, immuhistochemistry; IOD, integral optical density;LEH, luminal epithelial hyperplasia; PND, postnatal day; PR, progesterone receptor;RfD, reference dose;U.S.EPA, United StatesEnvironmental Protection Agency;Vv, Volume fraction.

Keywords: Glyphosate based herbicide, Uterus, Luminal epithelial hyperplasia, Progesterone receptor, Hoxa10, Estrogen receptor alpha.

1. INTRODUCTION

Glyphosate (N-phosphonomethyl glycine) is the active ingredient of a number of broad-spectrum herbicide formulations, widely used all over the world to control weeds on both cropland and non-cropland areas (Baylis, 2000; Woodburn, 2000; Cerdeira et al., 2007; Duke and Powles, 2008). Commercial formulations of glyphosate include other chemical compounds that act as solvents, adjuvants, preservatives or surfactants. Although these substances are classified as inert compounds, it has been demonstrated that the formulations of glyphosate are more toxic than the compound in its technical grade (Richard et al., 2005; BenachourandSeralini, 2009; Mesnage et al., 2014). In Argentina, the areas of lands in transgenic glyphosate-resistant soybean production have extensively increased, and that has been accompanied by an increase in the herbicide use (Cerdeira et al., 2011). To date, more than 200 million litters of GBHs are applied every year in our country (Aparicio et al., 2013).

Although glyphosate has been considered to have low persistency, the magnitude of environmental impact depends on the rate and frequency of glyphosate application (Mamy et al., 2010). In Argentina, a monitoring study carried out within the main area of soybean production, revealed 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). Other studies reported the presence of glyphosate residues in pre-harvest soybean (Arregui et al., 2004; Test Biotech, 2013) and in crops at harvest (Agricultural Marketing Service - U.S. Department of Agriculture, 2013). In addition, Curwin et al. (2007a,b) reported glyphosate detection in the urine of families living in farms and nonfarm households, although the estimated exposure levels to glyphosate were several orders of magnitude below thereference dose (RfD) proposed by the U.S. Environmental Protection Agency (U.S. EPA, 1993).

In a recent report, a consensus statement analyzeddifferent results related to GBHs (Myers et al., 2016).Some studies indicate that GBHs disrupt endocrine-signalling systems *in vitro*(Richard et al., 2005; Gasnier et al., 2009; Thongprakaisang et al., 2013;Defarge et al., 2016). Few *in vivo* studies have dealt with the effects of GBHs, and no reports are available regarding the consequence of GBHs exposure during critical periods of developmentonthe female reproductive tract.

The female reproductive tract and particularly the uterus are highly sensitive to developmentally disruptive effects of hormonal steroids and natural or synthetic endocrine-disrupting chemicals (EDCs) (Spencer et al., 2012; Varayoud et al., 2014). Transient disruption of the normal developmental program has long-term adverse consequence for uterine function and reproductive health (Varayoud et al., 2008; Varayoud et al., 2011; Milesi et al., 2012; Milesi et al., 2015). In the present work we hypothesized that early postnatal exposure to a GBH might interfere with normal uterine development and differentiation. We evaluated the effects of neonatal exposure to a low dose of a GBH on the uterine morphology, the cell proliferation and the expression of proteins involved in uterine organogenetic differentiation, such as, ERα, PR, Hoxa10 (a member of the Hox gene family) and Wnt7a (a member of the Wnt gene family). The effects were determined at two time points: i) shortly after the end of the exposure period (PND8, neonatal period) to evaluate the acute response to GBH exposure, and ii) two weeks after the end of the exposure period (PND21, prepubertal period), to investigate whether the effects persisted and/or were manifested in a stage distant from the GBH exposure. The selection of proteins to be evaluated was based on their role in uterine organogenetic differentiation. Hoxa10 and Wnt7a, regulate several developmental pathways that guide uterine growth and differentiation during embryogenesis and postnatal development (Benson et al., 1996; Miller and Sassoon, 1998; Spencer et al., 2012). These molecules are also dynamically expressed in adult endometrium, where they play a pivotal role on embryo implantation (Bagot et al., 2000; Dunlap et al., 2011). Because of many EDCs exert their actions through the interaction with sex steroid hormone receptors (Roy et al., 2009), we postulate that uterine ERα and PR proteins could be affected by a GBH developmental exposure.

2. MATERIALS AND METHODS

2.1. Animals

All procedures used in this study were approved by the Institutional Ethic Committee of the School of Biochemistry and Biological Sciences (Universidad Nacional del Litoral, Santa Fe, Argentina), and were performed in accordance with the principles and procedures outlined in the Guide for the Care and Use of Laboratory Animals issued by the U.S. National Academy of Sciences. Inbred Wistar strain rats were bred at the Department of Human Physiology (Santa Fe,

Argentina) and housed under a controlled environment (22°C ± 2°C; lights on from 06:00 to 20:00 h) with 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) and Andreoli et al. (2015). To minimize additional exposure to EDCs, rats were housed in stainless steel cages with sterile pine wood shavings as bedding, and tap water was supplied in glass bottles with rubber stoppers surrounded by a steel ring.

2.2. Experimental design

Pups were obtained from 8-10 timed-pregnant Wistar rats per group housed singly. After parturition (PND0), pups were sexed according to anogenital distance and litters of eight pups (preferably four males and four females) were left per mother. Female pups from each mother were randomly assigned to the following neonatal treatment groups: 1) control group receiving saline solution, and 2) GBH group receiving a commercial formulation of glyphosate dissolved in saline solution (2 mg/kg b.w). The glyphosate formulation used was Roundup FULL II®, a liquid water-soluble formulation containing 66.2% of glyphosate potassium salt, as its active ingredient, coadjunvants and inert ingredients. Substances (40 µl) were administered by s.c. injection in the nape of the neck every 48 h from PND1 to PND7. Each treatment day, the dose was calculated based on the average body weight of the pups. The dose of GBH was selected based on the reference dose (RfD)for glyphosate proposed by the U.S. Environmental Protection Agency (U.S. EPA, 1993) Although the RfD for glyphosate is based on oral exposure, the subcutaneous via is the unique administration route that warrants the whole incorporation of a chemical compound when an early postnatal exposure model is used.Eight rats from each neonatal treatment group were weighted and sacrificed by decapitation on PND8 and PND21 to evaluate acute and short-term effects, respectively. Uterine horns were removed, fixed by immersion in 4% paraformaldehyde buffer for 6 h at 4°C and processed for histology and IHC.

2.3. Histological analysis

Uterine longitudinal sections (5 µm thick) were stained with hematoxylin and eosin and examined by light microscope (Olympus BH2 microscope; Olympus, Tokyo, Japan) to analyze the uterine morphology. Three sections per animal separated 25 µm from each other were evaluated. First, we quantified the number of luminal epithelial layers using a Dplan

40× objective (numerical aperture = 0.65; Olympus) on PND8 and 20× objective (numerical aperture = 0.40; Olympus) on PND21. Luminal epithelial hyperplasia (LEH) was established as a luminal epithelium with more than four cellular layers. A total of 10 fields were evaluated/section and the results were expressed as % of incidence of LEH. The number of uterine glands was determined on 10 randomly selected fields using a Dplan 20× objective. Finally, the thickness of the subephitelial stroma and myometrium layers was analyzed by 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). To spatially calibrate the Image Pro-Plus analyzer, square grids from Neubauer's chamber images were captured. At least 10 fields were recorded in each section using a Dplan 40× objective (numerical aperture = 0.65; Olympus).

2.4. Immunohistochemistry

In order to determine the immunophenotype of uterine cells we evaluated the expression of cytokeratins (molecular markers for different types of epithelial differentiation). We evaluated the expression of CK8 (indicative of simple epithelium) and p63 and panCKs-CK1, 5, 10 and 14- (indicative of stratified epithelium). To characterize the immunophenotype of the stromal cells we used an antibody against vimentin, a cytoskeletal protein expressed in mesenchymal-derived cells. Ki67 was used as a proliferation marker of cells in G1, G2, S and M cell cycle stages. Primary antibodies against steroid receptors (ERα and PR), Hoxa10 and Wnt7a were used to evaluate uterine organogenetic differentiation. Antibodies used for IHC were described in Table 1. For Wnt7a immunodetection we used a rabbit polyclonal antibody generated and tested in our laboratory, according to previously described protocols (Rey et al., 2006;Varayoud et al., 2008). The Wnt7a antigen included a region corresponding to amino acids 194-283 of the rat sequence (accession no.EDL91365.1), and the antiserum was purified using antigen-linked affinity chromatography (Hi-Trap NHS activated HP column; GE Healthcare, Buenos Aires, Argentina). The specificity of the antibodies was determined using validation tests. First, 1 μg of Wnt-7a antibody was adsorbed for 24 h at 4 °C with 10–20 μg of the antigenic peptide used to generate the antibody. No staining of positive control tissues was observed by immunohistochemical assays using the antibody-antigen complexes. In addition, the specificity of the antiserum was tested via Western blotting.

A standard immunohistochemical technique, following protocols previously described by our laboratory (Muñoz-de-Toro et al., 1998), was performed. Briefly, uterine longitudinal sections (5 µm thick) were deparaffinized and rehydrated in graded ethanol. 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 1). Reactions were developed using the avidin-biotin-peroxidase method and diaminobenzidine (DAB) (Sigma-Aldrich) as a chromogen substrate. Samples were dehydrated and mounted with permanent mounting medium (Eukitt, Sigma-Aldrich). For Ki67 immunodetection, the samples were counterstained with Mayer hematoxylin (Biopur, Rosario, Argentina). Each immunohistochemical run included negative controls in which the primary antibody was replaced by non-immune horse serum (Sigma-Aldrich).

2.5. Quantification of cell proliferation

Cell proliferation was evaluated in all uterine compartments using the Olympus BH2 microscope with a Dplan 100× objective (numerical aperture = 1.25; Olympus). In the luminal epithelium,the proliferation rate was assessed as a percentage of Ki67-positive cells on a total of 2000 cells/section. In the subepithelial stroma and myometrium,the proliferation index was obtained considering the volume fraction (Vv) of the Ki67-positive cells, calculated by applying the followingformula by Weibel (1969): $Vv = Pi/P$, where *Vv* is the estimated volume fraction of the object, *Pi* is the number of incident points over the positive cells, and *P* is the number of incident points over all the cells in the studied population.To obtain the data for the point-counting procedure, a glass disk with a squared grid of 0.8 mm × 0.8 mm was inserted into a focusing eyepiece (Gundersen et al., 1988; Ramos et al., 2002).The results were expressed as Vv × 100. The cell proliferation in the subepithelial stroma and myometrium was quantified on at least 10 randomly selected fields per section, and two sections per animal (separated 25 µm from each other) were evaluated.

2.6. Quantification of protein expression by image analysis

The expression of ERα, PR, Hoxa10 and Wnt7a proteins in all tissue compartments of the uterus was evaluated by image analysis, using the Image Pro-Plus 5.0.2.9 system (Media Cybernetics) as previously described (Ramos et al., 2002). Immunostained images were captured with a Dplan 40× objective (numerical aperture =0.65; Olympus) attached to a Spot Insight V3.5 color video camera. After convert each image into 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; Ramos et al., 2002). Because the IOD is a dimensionless parameter, the results were expressed as arbitrary unit. In the subephitelial stroma and myometrium, quantification was performed on at least 10 randomly selected fields per section, and two sections per animal (separated 25 μm from each other) were evaluated. In the luminal epithelium, quantification was performed in areas where luminal folds were not present, while in the glandular epithelium, protein expression was measured on at least 10 endometrial glands of each uterine sample. Because uterine gland formation in the rat occurs on PND9 (Branham et al., 1985), quantification in the glandular epithelium was only performed on PND21.

2.7. Statistics

All data are expressed as the mean ± SEM. The incidence of LEH was analyzed by the Fisher's exact test. In order to analyzethe other variables weselecteda Mann-Whitney testdue to the small sample size (n=8) and the impossibility to know the distribution of our variables under study (Fay et al., 2010). $p<0.01$ (**) and $p<0.05$ (*) were accepted as significant.

3. RESULTS

No alterations in maternal care and nursing were detected between the experimental groups. No signs of acute or chronic toxicity were observed in the litters, and no significant differences in weight gain between treated and control pups were recorded during the experiment. At the end of both the neonatal (PND8) and the prepubertal period (PND21) pup´sbody weights were similar between the GBH-exposed animals (PND8: 14.28 ± 0.20 g and PND21: 35.56 ± 0.59 g) and control animals (PND8: 14.22 ± 0.30 g and PND21: 36.55 ± 0.58 g).

3.1. GBH exposure modified the uterine morphology

The uterus of control animals revealed typical morphological features with a simple columnar luminal epithelium supported by stromal cells, and two thin layers of smooth muscle on PND8 (Fig. 1A). Two weeks later (PND21), the uterus showed well developed morphological features: columnar luminal epithelium, simple tubular glands lined with simple cuboidal epithelium, surrounded with a more stratified endometrial stroma and a thicker myometrium than those on PND8. The neonatal GBH-exposed uteri exhibitedmorphological changes. We observed that the 75% (6/8) of female pups showed LEH ($p<0.05$, Fig. 1B) in association with an increase in the thickness of subepithelial stroma and circular myometrium on PND8 ($p<0.05$) (Table 2). The 37.5% (3/8) of the GBH-exposed animals exhibited LEH on PND21; however, differences were not significant (Fig. 1B). The thickness of the subepithelial stroma of PND21 GBH-treated animals showed a trend to persist increased ($p = 0.053$, Table 2). Neither significant differences relative to the thickness of the myometrium nor the number of glands were recorded on PND21 (Table 2).

3.2. Uterine epithelial and stromal immunophenotypes in GBH-exposed animals

Then, we determined if the epithelial and stromal cells showed a normal uterine immunophenotype in GBH-treated animals. Normal uterine epithelium expresses CK8 (simple epithelium) and normalsubepithelial stroma expresses vimentin (Fig.2). If GBH treatment affects uterine development, the immunphenotype of uterine compartments could be modified.GBH exposure did not affect the immnuphenotype of the uterine compartments neither shortly after the end of the exposure(PND8) nor two weeks after the end of the exposure (PND21). The epithelial cells were immunoreactive for CK8 (simple epithelium) (Fig. 2) and were negative for basal CKs (CK1, 5, 10 and 14) and p63 (data not shown). These expression profiles indicate the absence of squamouscell metaplasia, i.e., change in columnar epithelial cells tostratified squamous epithelium..

3.3. GBH exposure increased epithelial and stromal proliferation on PND8

The evaluation of Ki67 expression indicated that uterine epithelial and stromal proliferation was robust in control animals on PND8. Neonatal GBH exposure elicited an increase in cell proliferation in the epithelial (C: 28.34 ± 1.30%; GBH: 41.28 ± 2.45%, $p<0.01$) and the stromal compartments (C: 5.31 ± 0.40; GBH: 8.55 ± 0.80, $p<0.05$) (Fig. 3, A and B). Endometrial cell

proliferation decreased markedly by PND21, reaching very low values (i.e. the percentage of Ki67-positive cells for the epithelium was less than 0.5%), without changes between control and GBH-treated animals (data not shown).

3.4. Expression of proteins involved in uterine organogenetic differentiation

3.4.1. ERα

The quantification of uterine ERα on PND8 (neonatal period) and PND21 (prepubertal period) of control and GBH-treated animals is presented in Fig. 4A. Female pups neonatally exposed to the herbicide displayed an induction of ERα in the subepithelial stroma on PND8 ($p<0.05$, Fig. 4A). Although GBH-induced changes reverted two weeks after the end of treatment (PND21), a downregulation of ERα expression was detected in the luminal epithelium ($p<0.05$, Fig. 4A). The ERα expression in the myometrium did not show statistically differences between control and GBH-treated animals at both periods (Fig. 4A). Representative photomicrographs of these results are shown in Fig. 4B.

3.4.2. PR

Fig. 5A shows the results of PR quantification in control and GBH-exposed animals. Surprisingly, on PND8, GBH treatment notably increased PR expression in both the luminal epithelium and the stromal compartments ($p<0.01$, Fig. 5A). The deregulation of PR in the subepithelial stroma persisted up to the prepubertal period (PND21, $p<0.05$). The quantification of PR on myometrium was not performed because the detection of PR was weak on PND8 and PND21, in both control and GBH-treated animals. Representative photomicrographs of these results are shown in Fig. 5B.

3.4.3. Hoxa10

No immunostaining for Hoxa10 was detectable in the luminal nor glandular epithelium at any stage examined.In contrast, strong nuclear immunostaining for Hoxa10 was observed in the subepithelial stroma and myometrium from PND8 animals.Fig. 6A shows the Hoxa10 quantification in the uteri of control and GBH-treated animals. On PND8, an up regulation of Hoxa10 was observed in both the subepithelial stroma and the myometrium of GBH-exposed animals ($p<0.05$, Fig. 6A). The changes observed in the stromal compartment persisted up to

PND21 ($p<0.05$), while the myometrial expression was similar to control rats (Fig. 6A). Representative photomicrographs of these results are shown in Fig. 6B.

3.4.4. Wnt7a

No changes in Wnt7a expression were observed between the GBH-exposed female pups and control animals in any of the uterine compartments on PND8. However, on PND21,an induction of Wnt7a protein was evident in the stromal and glandular cells ($p<0.01$, Fig. 7A) of GBH-treated animals. Representative photomicrographs of these results are shown in Fig. 7B.

4. DISCUSSION

To our knowledge, this is the first study showing that postnatal exposure to a GBH affects the uterine morphology and the expression of proteins that regulate uterine organogenetic differentiation in neonatal and prepubertal rats. The most relevant effects were incidence of LEH (75% of animals), increase in stromal and circular myometrium thickness, increase in epithelial and stromal proliferation, and induction of Hoxa10, PR and ERα on PND8. Two weeks after the end of the GBH-exposure, some changes were remained, such as the deregulation of Hoxa10, PR and ERα expression. During this period, a deregulation of Wnt7a uterine expression was also observed.

The organogenetic development and differentiation of most reproductive tract organs is completed during the fetal period; however, the uterus is not fully developed or differentiated at birth. Establishment of tissue-specific histoarchitectureis completed postnatally in laboratory rodents, domestic animals and presumably humans (Cunha, 1976; Bartol et al., 1999; Kurita and Nakamura, 2008; Spencer et al., 2012). The functional capacity of the adult uterus is established by developmental events associated with ‘programming’ of uterine tissues during prenatal and postnatal life (Sassoon, 1999; Kobayashi and Behringer, 2003; Crain et al., 2008; Walker, 2011).

The postnatal development of the uterus is highly sensitive to a brief exposure to different substances: hormonal steroids (estradiol, testosterone, progesterone) and others, in general classified as EDCs. A brief exposure to substances with hormonal activity disrupts the uterine development in the prepubertal period with consequences at adulthood (Varayoud et al., 2008;

Varayoud et al., 2011; Milesi et al., 2015). In the present study, we selected a model of exposure during the first week of age, to evaluate the effects of GBH on postnatal uterine development. Regarding morphological evaluation, GBH-exposed uteri showed LEH with a higher proliferation rate on PND8. The immunophenotype of the epithelial cells indicates a normal phenotype (CK8 immunoreactive cells, indicative of normal uterine epithelium). In addition, an increase thickness and a higher proliferation rate were detected in the subepithelial stroma of GBH-treated animals. Again, the stromal cells showed the typical immunophenotype of uterine stroma (vimentin-immunoreactive cells, indicative of fibroblastic cells). Clearly, the increased epithelial and stromal proliferation induced by neonatal GBH treatment was temporary and reversible. The epithelial and stromal proliferation rate was very low on PND21, both in control and GBH-exposed animals. However, the 37.5% of animals showed a hyperplastic epithelium on PND21. All these results could indicate an increase susceptibility to uterine dysfunctions, such as development of uterine carcinoma. Some evidences could indicate an association between GBH and carcinogenesis. Glyphosate exposure to hormone-dependent breast cancer cells *in vitro* resulted in increased cell proliferation (Thongprakaisang et al. 2013), while an *in vivo* study suggested that a glyphosate formulation has tumor promoting potential in skin carcinogenesis in mice (George et al., 2010). A recent report suggest an augmented risk of cutaneous melanoma among subjects with exposure to pesticides (glyphosate, mancozeband maneb), in particular among those exposed to occupational sun exposure (Fortes et al, 2016). A recent report showed that there are controversial results related to the classification of the herbicide glyphosate as a "probably carcinogenic to humans" (Portier et al, 2016). The International Agency for Research on Cancer (IARC, World Health Organization)concluded that glyphosate is a 'probable human carcinogen', putting it into IARC category 2A due to *sufficient evidence* of carcinogenicity in animals, *limited evidence* of carcinogenicity in humans and *strong* evidence for two carcinogenic mechanisms(IARC, 2015). However, the European Food Safety Authority (EFSA) concluded that 'glyphosate is unlikely to pose a carcinogenic hazard to humans and the evidence does not support classification with regard to its carcinogenic potential' (European Food Safety Authority, 2015). The authors concluded that owing to the potential public health impact of glyphosate, it is essential that all scientific evidence relating to its possible carcinogenicity is publicly accessible and reviewed transparently in accordance with established scientific criteria (Portier et al, 2016).

Subsequently, we investigated whether early postnatal exposure to a GBH induced changes in the expression of proteins that regulate uterine organogenetic differentiation in neonatal (PND8) and prepubertal (PND21) periods. Specifically, we found that the GBH exposurealtered the expression of PR and Hoxa10 both, immediately and two weeks after the exposure.Both PR and Hoxa10 are two key genes during embryo implantation and decidualization. Previously we detected that when an EDC disrupts the Hoxa10 and PR expression during development the animals showed a lower number of implantation sites during pregnancy (Varayoud et al, 2008, 2011; Milesi et al, 2012, 2015). Taking into account our and other results we could suggest that GBH-postnatally exposed rats could show long-term effects such as subfertility. In addition, the timing, nature, and severity of endocrine system impacts will vary depending on the levels and timing of GBH exposures, the age and health status of exposed organisms. Exposures can trigger a cascade of biological effects that may culminate later in chronic diseases (Myers et al, 2016).

ERα was induced in the stromal compartment on PND8, and was down-regulated in the luminal epithelial cells of GBH-exposed animals on PND21. GBH treatment also increased the expression of Wnt7a in the stromal and glandular epithelial cells on PND21. Several agents with hormonal-like activity have been shown to disrupt the expression of developmental-related genes. Similar to the effects observed in this study, female pups neonatally exposed to endosulfan, an organochlorine pesticide recently banned in our country, showed an increased Hoxa10 uterine expression on PND8 and PND21 (Milesi et al., 2012). Other authors reported a dose-responsive increase in uterine Hoxa10 expression in 2-week-old mice following *in utero* BPA exposure (Smith and Taylor, 2007). As for Wnt7a, a down-regulation in its expression has been reported in 6-day-old female mice exposed to DES and Aroclor 1254 from PND1 to PND5 (Ma and Sassoon, 2006). Similar to the changes observed in the postnatal exposure model, when mice were exposed *in utero* to a high dose of DES, low Wnt7a expression was detected at birth that normalized at 5 days after delivery (Sassoon, 1999). In our study, the treatment with a GBH increased the expression of Wnt7a in the stromal and glandular epithelial cells on PND21. It has been reported that Wnt7a differentially regulates the uterine expression of Hoxa10 during embryogenesis and adulthood (Miller and Sasson, 1998; Kitajewski and Sassoon, 2000). The deregulation of both Wnt7a and Hoxa10 detected in our study, suggests a possible mechanistic interaction in the GBH's uterine disruption. In our study, PR and ERα were affected by GBH exposure. A previous report of Thongprakaisang et al. (2013) showed that GBH affects ER

expression in mammary cells, producing an induction of ERα in the human T47D hormone-dependent breast cancer cell line. The authors hypothesized that glyphosate may act as a weak xenoestrogen, activating ERα (Thongprakaisang et al., 2013). In accordance with these authors we detected an induction of ERα after GBH exposure. Taken together, these findings confirm previous evidence that steroid receptors, Hoxa10 and Wnt7a are common targets of endocrine disruption.

Even though the U.S. EPA has recently concluded that the weight of the evidence is not sufficient to classified glyphosate as EDC (EPA's conclusions for Glyphosate Tier 1 battery screening, June 2015), different studies have shown a disruption of endocrine-signalling systems in vitro(Thongprakaisang et al., 2013; Gasnier et al, 2009) . The present study shows for the first time, endocrine disrupting effects of a GBH on the uterus in neonatal and prepubertal rats, supporting the fact that GBHs might act as an EDC. At this moment, we began to study in new experiments if a long-term exposure could affect the uterine functional differentiation, with the intention to mimic the human exposure. More comprehensive toxicity experiments are needed including those using "two hit" study designs, which examine early life exposures to GBHs followed by later-life exposures to chemical or other environmental stressors (Myers et al, 2016).

As above-mentioned, chemical mixtures in formulations can have effects that are not predicted from tests of single compounds(Rajapakse et al., 2002; Silva et al., 2002). GBHs themselves are chemical mixtures; in addition to the inclusion of glyphosate (the active ingredient), these herbicides include adjuvants such as surfactants, which can make GBH-product formulations more toxic than glyphosate alone (Mesnage et al., 2014). *In vitro*studies have demonstrated toxic effects at low doses of GBHs in embryonic, fetal, placental and testicular cells (Richard et al., 2005;Benachourand Seralini, 2009; Clair et al., 2012; Mesnage et al., 2013).

Uterine morphogenesis is governed by several hormonal, cellular and molecular mechanisms. As previously stated, disruption of the normal developmental program by neonatal exposure to EDCs might induce permanent changes in the structure and function of tissues and organs (Newbold et al., 1990; Kitajewski and Sassoon, 2000; Bosquiazzo et al., 2013). Hoxa10 and Wnt7a regulate anterior-posterior and radial patterning of the müllerian duct. Specifically, Wnt7a plays a pivotal role in postnatal uterine myometrial and epithelial morphogenesis. It has been

reported that a deregulation in Wnt7a expression lead to abnormal multilayered epithelium (Carta and Sassoon et al., 2004) and hyperplastic and disorganized myometrium (Miller and Sassoon, 1998; Miller et al., 1998a). Altered expression of Hoxa10 and Wnt7a during development results in uterine anomalies, as seen in mice exposed prenatally to DES (Miller et al., 1998a,b; Block et al., 2000). Mice exposed to DES showed many uterine malformations, characterized by luminal and glandular squamous metaplasia, endometrial hyperplasia and increased risk of leiomyomas (Kitajewski and Sassoon, 2000). Similar to DES, in our work, a brief exposure to a GBH during development lead to endometrial hyperplasia. These organizationally induced alterations in uterine histoarchitecture might lead to reproductive anomalies, such as infertility and early pregnancy loss, and could promote the development of uterine neoplasias (Newbold et al., 1990; Newbold et al., 1997).

5. CONCLUSION

In summary, our results show that early postnatal exposure to a GBH, at dose similar to the RfD, alters uterine morphology and the expression of proteins involved in uterine development and differentiation. We considered that more studied should be conducted to fully understand the effects of GBH on the female reproductive health, as well as, its molecular mechanism of action. Currently, further experiments are underway to investigate whether the deregulation of steroid receptors, Hoxa10 and Wnt7a following postnatal exposure to a GBH could affect the proper uterine function along pregnancy and/or promote uterine neoplasias.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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FIGURE LEGENDS

Fig. 1 Incidence of uterine luminal epithelial hyperplasia (LEH) on neonatal (PND8) and prepubertal (PND21) control and GBH-exposed animals. (A) Representative photomicrographs illustrating the morphological changes in hematoxilin and eosin stained uterine sections. LE, luminal epithelium; GE, glandular epithelium; SS, subepithelial stroma. Scale bar: 50 µm. (B) The graphs represent the incidence of LEH on PND8 and PND21. Data were analyzed using Fischer's exact Test (*p<0.05).

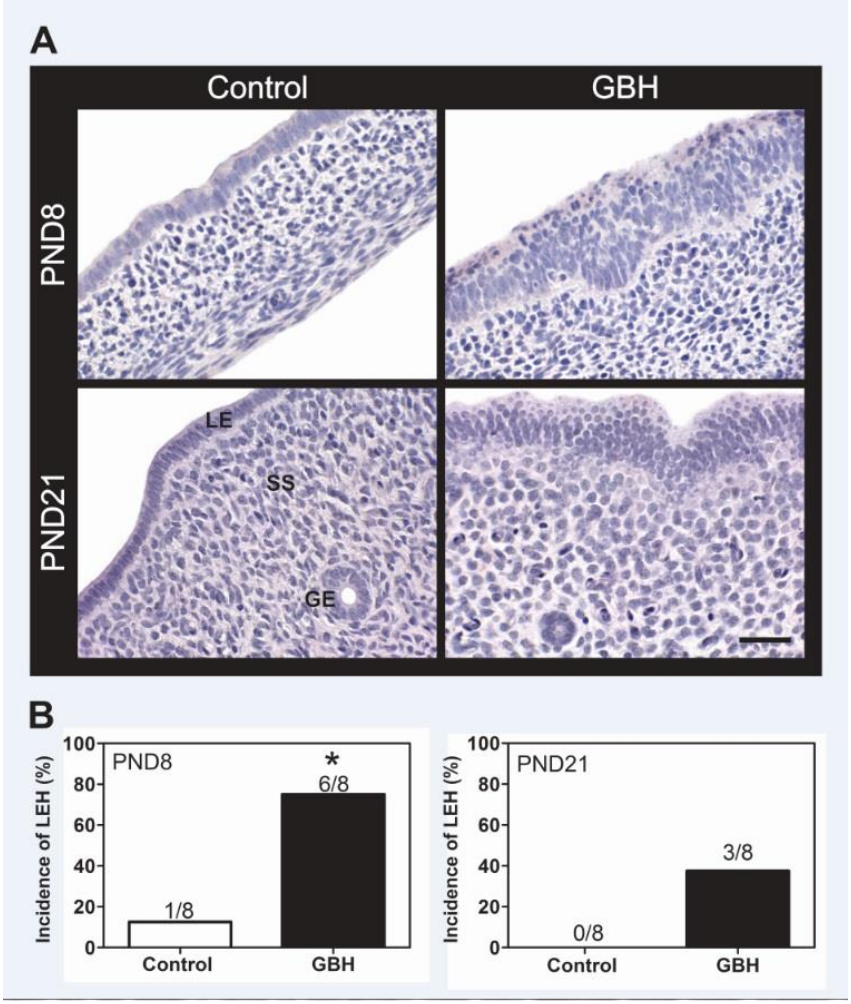

Fig. 2 Epithelial and stromal immunophenotypes of control and GBH-exposed animals on neonatal period (PND8). Representative photomicrographs show luminal cytokeratin 8 (CK8) and vimentin immunoreactions in luminal epithelium and subepithelial stroma, respectively. Positive and negative controls of each immunohistochemical run are shown. Positive controls for p63 and panCKs were uterine sections from one-year-old rats that exhibit glands with squamous metaplasia. LE, luminal epithelium; SS, subepithelial stroma. Scale bar: 50 µm.

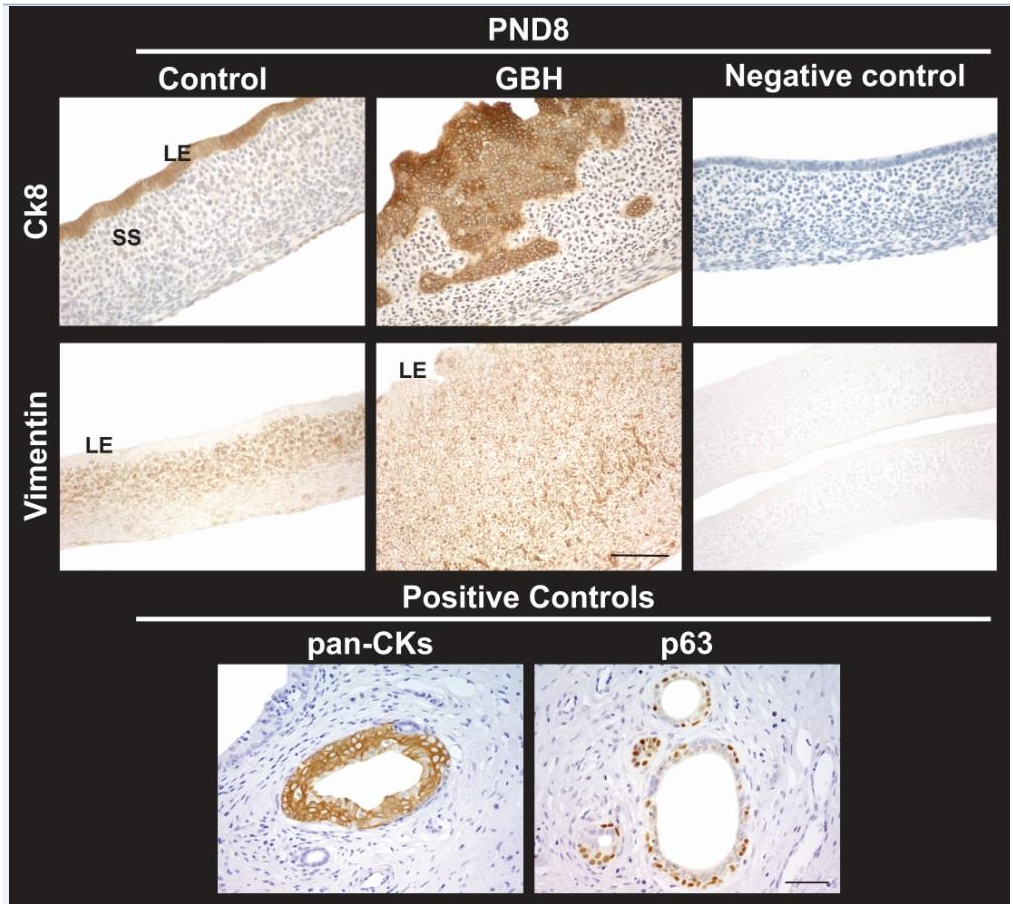

Fig. 3 Cell proliferation by Ki67 immunodetection on control and GBH-exposed animals on PND8. (A) Ki67 protein was expressed and quantified in all uterine compartments. Values in bar graphs are the mean ± SEM (8 rats/group). Data were analyzed using Mann Whitney Test (**p<0.01; *p<0.05). Vv, volume fraction. (B) Representative photomicrographs of immunohistochemical detection of Ki67 on uterine sections. Negative control of the immunohistochemical run is shown. Asterisks indicate the increase of Ki67 expression. LE, luminal epithelium; SS, subepithelial stroma; M, myometrium; Scale bar: 50 µm.

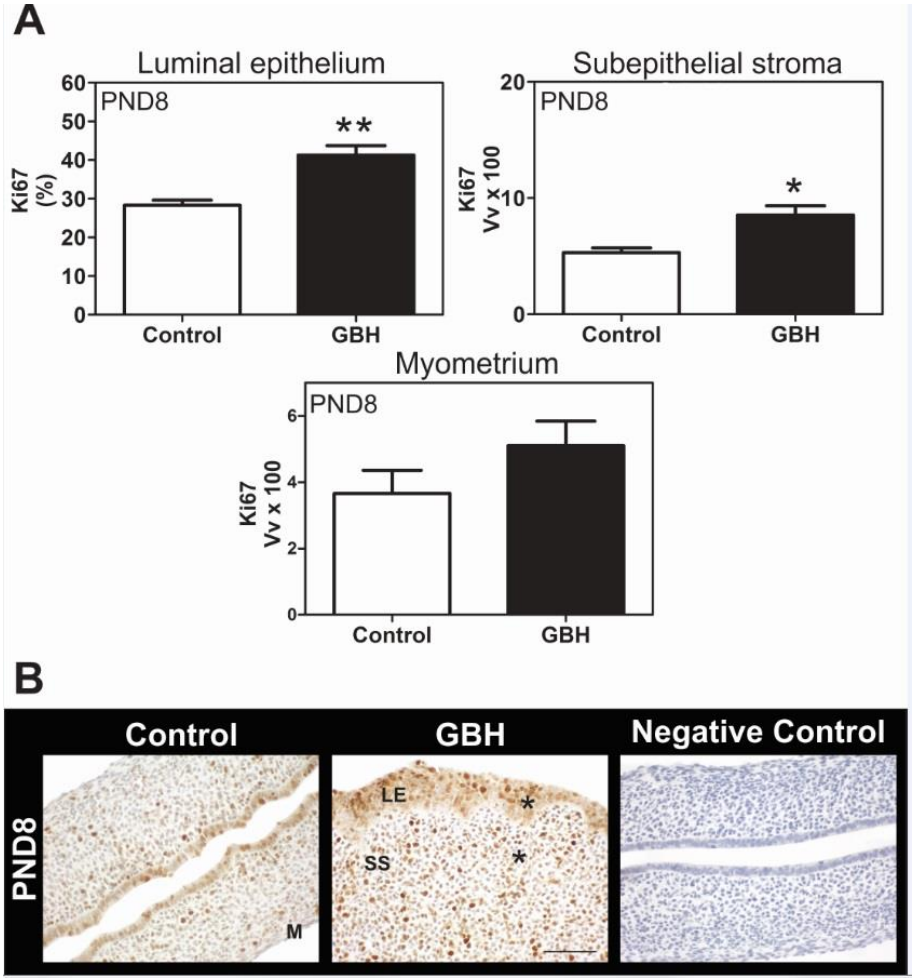

Fig. 4 Uterine ERα protein expression in control and GBH-treated animals. (A) Quantification of ERα protein expression in all uterine compartmentson rats on PND8 and PND21. Results were expressed astheintegrated optical density (IOD), which consists of a linear combination of the average immunostained density and the relative area occupied by positive cells. Values in bar graphs are the mean ± SEM (8 rats/group). Data were analyzed with the Mann Whitney Test (*$p<0.05$). (B) Representative photomicrographs of immnuhistochemical detection of ERα on uterine sections. Negative control of the immunohistochemical run is shown. Asterisk indicates the increase of ERα expression and the arrow indicates a decrease in its expression. LE, luminal epithelium; GE, glandular epithelium; SS, subepithelial stroma; M, myometrium. Scale bar: 50 µm.

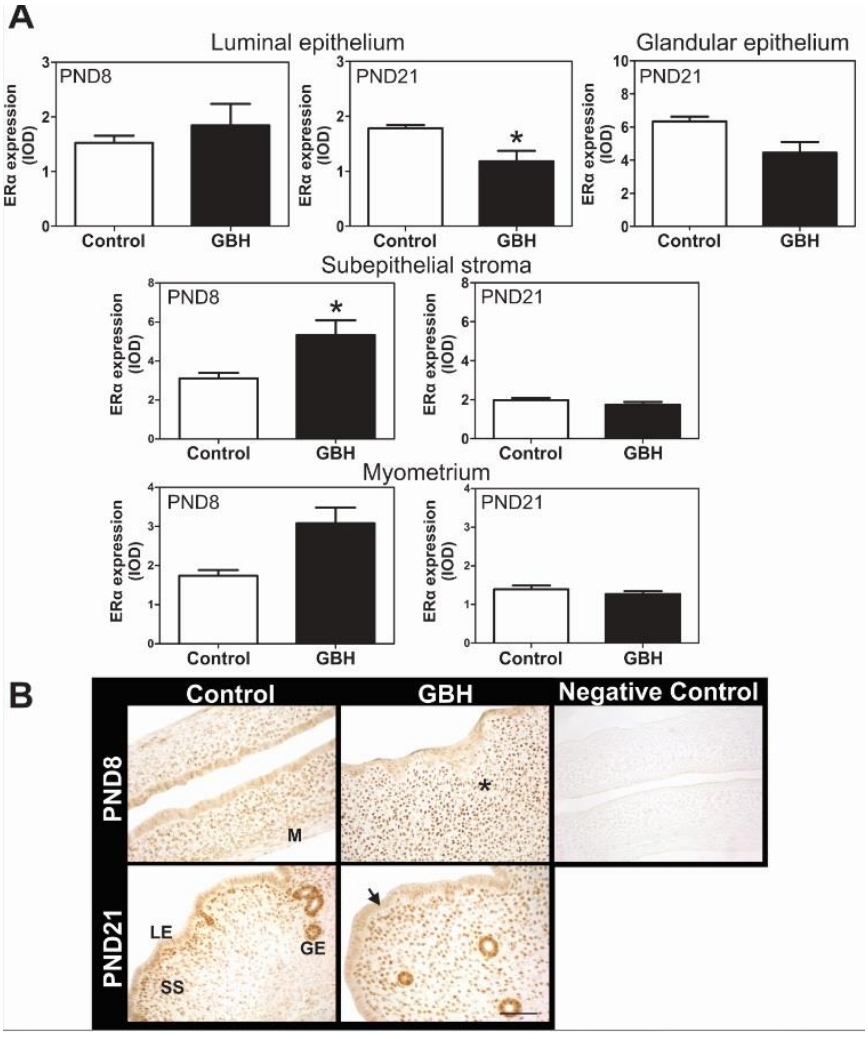

Fig. 5 Uterine PR protein expression in control and GBH-treated animals. (A) Quantification of PR protein expression in the uterine epithelial and stromal compartments of rats on PND8 and PND21. The results were expressedastheintegrated optical density (IOD). Values in bar graphs are the mean ± SEM (8 rats/group). Data were analyzed using Mann Whitney Test (**p<0.01; *p<0.05)(B) Representative photomicrographs of immnuhistochemical detection of PR on uterine sections. Negative control of the immunohistochemical run is shown. Asterisks indicate the increase of PR expression. LE, luminal epithelium; GE, glandular epithelium; SS, subepithelial stroma. Scale bar: 50 µm.

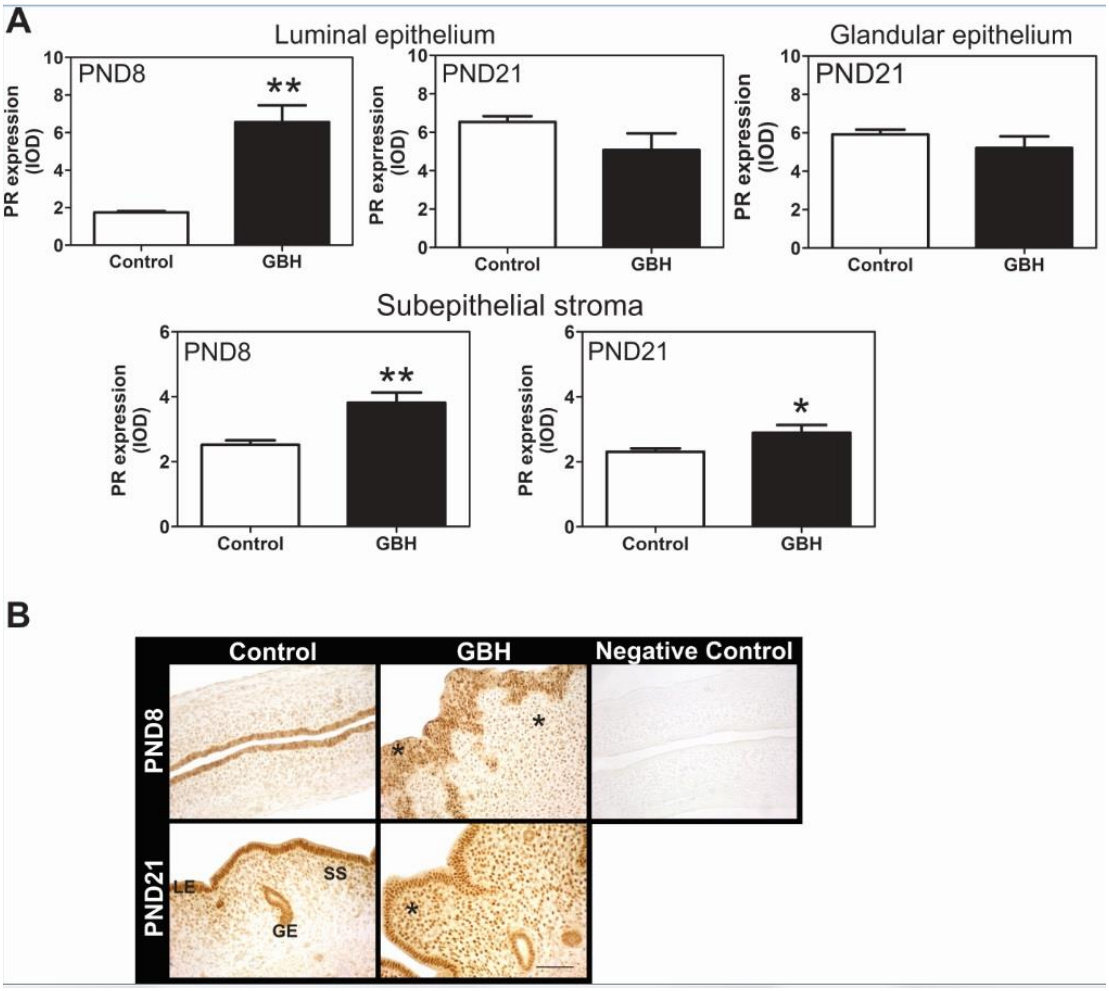

Fig. 6 Uterine Hoxa10 protein expression in control and GBH-treated animals. (A) Quantification of Hoxa10 protein expression in the uterine subepithelial stroma and myometrium of rats on PND8 and PND21. Results were expressed as the integrated optical density (IOD). Values in bar graphs are the mean ± SEM (8 rats/group). Data were analyzed using Mann Whitney Test (**p<0.01; *p<0.05) (B) Representative photomicrographs of immnuhistochemical detection of Hoxa10 on uterine sections. Negative control of the immunohistochemical run is shown. Asterisks indicate the increase of Hoxa10 expression. SS, subepithelial stroma; M, myometrium. Scale bar: 50 µm.

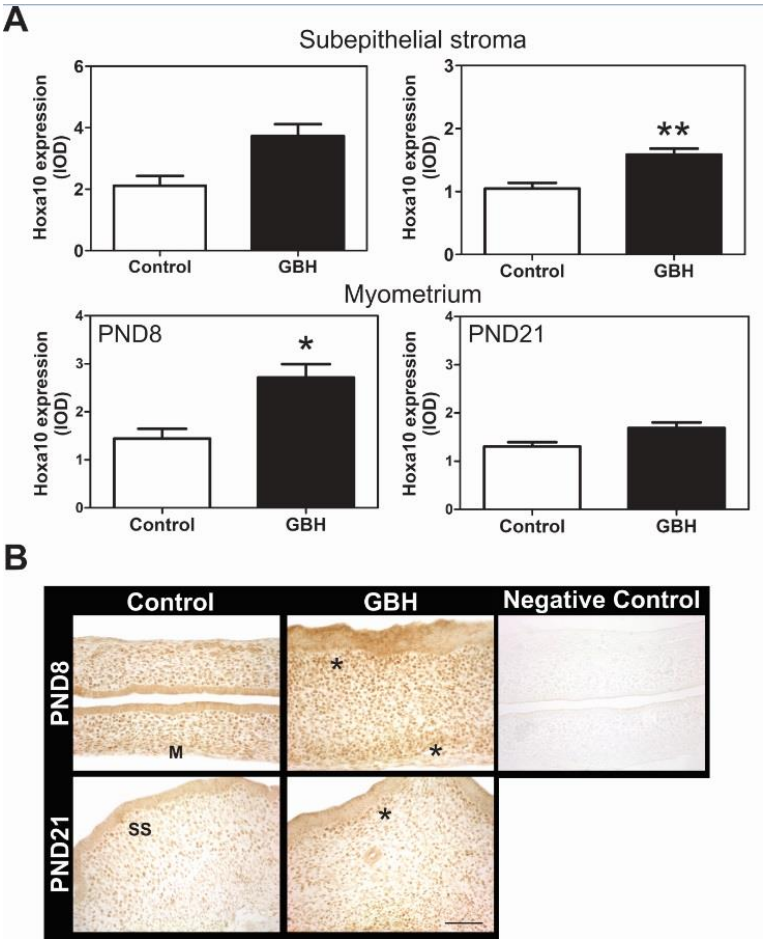

Fig. 7 Uterine Wnt7a protein expression in control and GBH-treated animals. (A) Quantification of Wnt7a protein expression in all uterine compartments of rats on PND8 and PND21. Results were expressedastheintegrated optical density (IOD). Values in bar graphs are the mean ± SEM (8 rats/group). Data were analyzed using Mann Whitney Test (**p<0.01). (B) Representative photomicrographs of immnuhistochemical detection of Wnt7a on uterine sections. Negative control of the immunohistochemical run is shown. Asterisks indicate the increase of Wnt7a expression. LE, luminal epithelium; GE, glandular epithelium; SS, subepithelial stroma; M, myometrium. Scale bar: 50 µm.

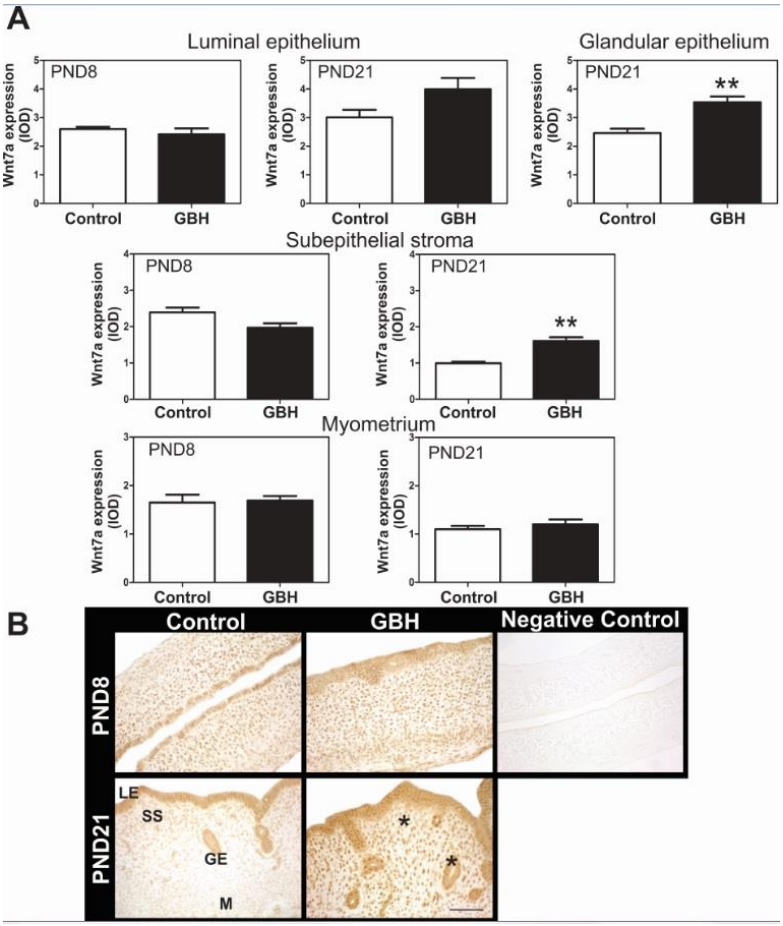

Table 1
Antibodies used for immunohistochemistry.

Antibodies	Dilution	Supplier
Primary		
Anti-Ki67 (clone MIB-5)	1/25	Dako Corp. (Carpinteria, CA, USA)
Anti-CK8 (PH192)	1/1600	The Binding Site Limited (Birmingham, UK)
Anti-pan-CK basal (clone 34βE12)	1/100	Novocastra (Newcastle upon Tyne, UK)
Anti-p63 (clone 4A4)	1/100	Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA)
Anti-Vimentin (clone V9)	1/100	Novocastra (Newcastle upon Tyne, UK)
Anti-ERα (clone 6F-11)	1/200	Novocastra (Newcastle upon Tyne, UK)
Anti-PR (clone A0098)	1/100	Dako Corp. (Carpinteria, CA, USA)
Anti-Hoxa10 (sc-17159)	1/400	Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA)
Anti-Wnt7a	1/800	Generated and validated in our laboratory (Vigezzi et al., 2016)
Secondary		
Anti-mouse (B8774)	1/100	Sigma (St. Louis, MO)
Anti-rabbit (B8895)	1/200	Sigma (St. Louis, MO)
Anti-sheep (B7390)	1/200	Sigma (St. Louis, MO)
Anti-goat (sc-2042)	1/200	Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA)

Table 2

Analysis of uterine morphology of control or GBH-treated rats

Values are means ± SEM (n=8 rats/group). Asterisk indicates statistically significant differences ($p<0.05$). GBH: glyphosate-based herbicide (dose: 2 mg of glyphosate/Kg/d). N/D: no detected.

	PND8		**PND21**	
Parameters	Control	GBH	Control	GBH
Subepithelial stroma thickness (µm)	84.48 ± 6.23	118.37 ± 9.45 *	164.75 ± 1.51	182.24 ± 10.22
Circular myometrium thickness (µm)	22.86 ± 1.27	28.15 ± 1.58 *	48.18 ± 1.21	49.30 ± 2.66
Longitudinal myometrium thickness (µm)	19.70 ± 0.72	21.36 ± 1.81	36.78 ± 1.91	45.22 ± 4.64
Endometrial glands (number/field)	N/D	N/D	10.58 ± 0.73	10.84 ± 0.54