Reproductive Toxicology 73 (2017) 87–95

Contents lists available at ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox

# Neonatal exposure to a glyphosate-based herbicide alters uterine decidualization in rats

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ARTICLE INFO

*Article history:*
Received 17 May 2017
Received in revised form 25 July 2017
Accepted 27 July 2017
Available online 2 August 2017

*Keywords:*
Decidualization
Glyphosate-based herbicide
Implantation sites
Wnt5a
Wnt7a
β-catenin
Dkk1
sFRP4

ABSTRACT

We investigated whether defective modulation of uterine signaling may cause decidualization failure in rats neonatally exposed to a glyphosate-based herbicide (GBH). Female pups received vehicle or 2 mg/kg of GBH from postnatal day (PND) 1 to PND7. On PND8 and PND21, Wnt5a and β-catenin expression was evaluated in uterine samples. On gestational day (GD) 9, Wnt5a, Wnt7a and β-catenin expression and Dkk1 and sFRP4 mRNA were evaluated on implantation sites. On PND8, GBH-exposed rats showed increased Wnt5a and β-catenin expression in luminal epithelium (LE), whereas on PND21, they showed increased Wnt5a and β-catenin expression in subepithelial stroma but decreased β-catenin expression in glandular epithelium. On GD9, GBH-exposed rats showed decreased Wnt5a and Wnt7a expression in the antimesometrial zone and LE respectively, without changes in β-catenin expression, while Dkk1 and sFRP4 were up- and down-regulated respectively. We concluded that neonatal GBH exposure may lead to embryo losses by disturbing uterine signaling.



## 1. Introduction

Over the past few years, the use of pesticides has significantly increased mainly associated with agriculture, causing environmental pollution [1,2]. Specifically, the use of glyphosate, one of the most widely applied pesticides worldwide, increased more than 12-fold, from 67 million kg in 1995 to 826 million kg in 2014 [3]. Studies from laboratory animals suggest that exposure to glyphosate can induce adverse health outcomes [4–9]. Moreover, it has been demonstrated that low and environmentally relevant concentrations of glyphosate may possess estrogenic activity, suggesting that this pesticide may be classified as an endocrine-disrupting chemical (EDC) [10–13]. EDCs interfere with the action of hormones and may alter physiology during the whole life span of an individual. If an EDC alters hormone actions during ontogenesis, these effects may be permanent and affect organ development and function [14].

In previous works, we demonstrated that exposure of rats to a low dose (2 mg/kg/day) of a commercial formulation of a glyphosate-based herbicide (GBH) during the first postnatal days (PND) alters their uterine development [8]. We also observed a deregulation of the uterine expression of wingless-type MMTV integration site family, member 7A (Wnt7a), Homeobox A10 (Hoxa10), progesterone receptor (PR) and estrogen receptor alpha (ERα) on PND21 [8]. We have also found that GBH-exposed rats show a normal rate of pregnancy, number of corpora lutea and steroid hormone levels, but an increased number of resorption sites, indicating reproductive failures [9]. In addition, the GBH-treated rats showed a decreased expression of the PR-COUP-TFII-Bmp2 pathway and an increased expression of Hoxa10, with changes in the proliferation of decidualized uterine cells [9]. We propose that these findings could partially explain the mechanism of GBH-induced post-implantation embryo loss.

Wnt5a and Wnt7a play a significant role during uterine morphogenesis, being important for blastocyst implantation and female

*Abbreviations:* Wnt, wingless-type MMTV integration site family; β-catenin, beta-catenin; Dkk1, dickkopf-related protein 1; sFRP4, secreted frizzled-related protein 4; PR, progesterone receptor; ERα, estrogen receptor alpha; Hoxa10, Homeobox A10; COUP-TFII, COUP transcription factor 2; Bmp2, bone morphogenetic protein 2; L19, ribosomal protein L19; ROR, retinoid-related orphan receptors; dNTP, deoxynucleotide triphosphate; cDNA, complementary DNA; GBH, glyphosate-based herbicide; GD, gestational day; PND, postnatal day; IS, implantation sites; EDCs, endocrine-disrupting chemicals; M, mesometrial; AM, antimesometrial; IOD, integral optical density; IHC, immunohistochemistry; $C_T$, cycle threshold; SEM, standard error of the mean; LE, luminal epithelium; GE, glandular epithelium; SS, subepithelial stroma.

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http://dx.doi.org/10.1016/j.reprotox.2017.07.022
0890-6238/

fertility [15–18]. Indeed, Wnt5a-dysregulated female mice have been found to show increasing numbers of resorption sites on days 10 and 12 of gestation [19]. β-catenin has also been shown to be an important component of normal uterine function and its deregulation in mutant mice results in defects in glandular formation, decidualization and fertility [20]. Dkk1 and sFRP4 are two proteins associated with the Wnt signaling pathway, which are expressed in the decidual compartment [21–23]. Dkk1 could promote trophoblast cell invasion during murine placentation [23], whereas sFRP4 expression has been reported to be up-regulated in the pregnant uterus, reaching a peak on day 12 of pregnancy and restricted to decidual cells [22].

Based on the above, we hypothesized that early postnatal exposure to GBH alters the uterine development and function by a deregulation of the Wnt5a, Wnt7a and β-catenin signaling pathway. To test this hypothesis, we investigated the effects of a brief neonatal exposure to a low dose of GBH in the uteri of female rats at the following three time points: i) immediately after the end of the exposure period (PND8, neonatal period) to evaluate the acute response to GBH; ii) two weeks after the end of the exposure period (PND21, prepubertal period) to investigate the short-term response; and iii) during pregnancy on GD9 to evaluate long-term effects on uterine decidualization. In addition, the postnatal ontogenetic pattern and cellular distribution of uterine Wnt5a were evaluated in unexposed rats (between PND1 and PND35) and unexposed pregnant rats on GD3, 4 and 5.

## 2. Material and methods

### 2.1. Animals

All the 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 U.S. National Academy of Sciences. Rats of an inbred Wistar-derived strain from the Department of Human Physiology (Universidad Nacional del Litoral, Santa Fe, Argentina) were used. The animals were maintained under a controlled environment (22 ± 1 °C; lights on from 0600 to 2000 h) 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 food intake of control and GBH-treated rats was equivalent, we assumed that all animals were exposed to the same levels of phytoestrogens [see Kass et al. [24] 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 in glass bottles with rubber stoppers surrounded by a steel ring.

### 2.2. Experimental design

Adult female rats (90 days old) were housed with males of proven fertility. Pregnant rats were housed singly, and, at delivery, pups were sexed according to the anogenital distance. To minimize the use of siblings and avoid potential litter effects, offspring of the same litter were distributed between different mothers. Cross-fostered litters were adjusted to eight pups, prioritizing a maximum of eight female pups per litter when possible. When fewer than eight females were available, an appropriate number of males were retained. Female pups were assigned to two neonatal treatment groups: 1) the control group, receiving saline solution (n = 34); and 2) the GBH group, receiving a commercial formulation of glyphosate dissolved in saline solution at 2 mg/kg (n = 38). The glyphosate formulation used was a liquid water-soluble formulation containing 66.2% of glyphosate potassium salt, as its active ingredient, coadjuvants and inert ingredients. The amount of equivalent glyphosate acid is 54%. To calculate the dose of 2 mg/kg, we took into account that 100 ml of GBH contains 54 g of glyphosate. Therefore, according to the weight (6–7 g per pup on PND1), the rats were injected once a day with an herbicide solution containing approximately 0.013 mg of glyphosate per injection. Treatments were given on PND 1, 3, 5, and 7 by sc injections in the nape of the neck. The dose of GBH was selected based on our previous reports [8,9]. After the end of the treatment, two groups of female pups were sacrificed by decapitation on PND8 (neonatal period, 8 pups/group) and PND21 (prepubertal period, 8 pups/group), and uterine tissues were collected. Another group of female rats were weaned on PND21, housed at four per cage, and held without further treatment until adulthood. On PND90, female rats neonatally exposed to GBH or vehicle were housed for two consecutive weeks with sexually mature untreated males of the same strain and of proven fertility to allow several possible matings. Every morning, vaginal smears were obtained to check for the presence of spermatozoa [25]. The first day on which a sperm-positive smear was detected was considered GD1. In the morning of GD9 (post-implantation period), the control (n = 8) and GBH-exposed (n = 8) pregnant rats were sacrificed, and uterine samples were collected. For each rat, all implantation sites (IS) were collected, weighed and randomly distributed to be processed for different experimental purposes. The samples were fixed in 10% buffered formalin for 6 h at room temperature and embedded in paraffin and processed for immunohistochemical assays. For RNA extraction, the dissected IS were immediately frozen in liquid nitrogen and stored at −80 °C. Fig. 1 shows a schematic representation of the experimental groups designed to investigate the effects of neonatal GBH exposure.

In addition, to better understand the function of Wnt5a, we assessed spatiotemporal uterine protein expression before and during pregnancy. To determine the postnatal ontogenic patterns and cellular distribution of Wnt5a, uterine samples of female rats (6 per time point) were obtained on PND1, 8, 21, 35. To evaluate Wnt5a protein expression during pregnancy, we selected critical periods in which Wnt5a is involved. Since it has been demonstrated that Wnt5a expression in mice on days 3 and 4 of pregnancy is associated with uterine receptivity and that Wnt5a expression on GD5 is localized in the mesometrial (M) zone of IS [19], uterine samples of female rats on GD3, 4 and 5 were processed for immunohistochemical analysis.

### 2.3. Immunohistochemistry

#### 2.3.1. Antibodies

A primary mouse monoclonal antibody against β-catenin was purchased from Santa Cruz Biotechnology Inc. (sc-7963; 1:800 dilution; Santa Cruz, CA, USA). A validation test was performed to verify the specificity of the commercial anti-β-catenin antibody as previously described [26]. For Wnt7a and Wnt5a immune-detection, we used rabbit polyclonal antibodies (1:800 dilution) generated and tested in our laboratory, according to previously described protocols [27]. The Wnt5a antigen included a region corresponding to amino acids 207 and 300 of the Wnt5a rat sequence (accession number Q9QXQ7), whereas the Wnt7a antigen included a region corresponding to amino acids 194–283 of the rat sequence (accession number EDL91365.1). 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 Wnt7a and 1 μg of Wnt5a antibodies were adsorbed for 24 h at 4 °C with 10–20 μg of the antigenic peptides used to generate the antibodies. Then, the antibody-antigen complexes were applied to

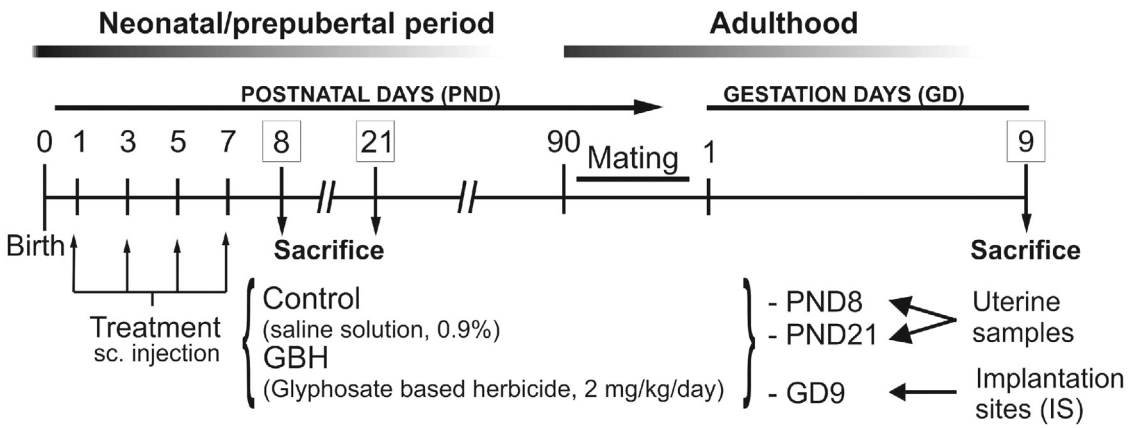


**Fig. 1.** Schematic representation of the experimental protocol used to investigate the effects of neonatal exposure to a glyphosate-based herbicide (GBH) on female rats along prepubertal development and on gestational day (GD) 9. PND: postnatal day; sc: subcutaneous.

sections of control uteri in immunohistochemical assays. In addition, the specificity of the antiserum was tested by Western blot [27].

#### 2.3.2. Assay

Immunohistochemistry was performed to evaluate protein expression of Wnt7a, Wnt5a and β-catenin. At least three cross-sections paraffin-embedded (5 μm thickness) at different depths at the middle point from each IS were immunostained. Sections were mounted on 3-aminopropyl triethoxy-silane (Sigma-Aldrich, Buenos Aires, Argentina)-coated slides and microwave pretreatment for antigen retrieval was performed [28]. The endogenous peroxidase activity and non-specific binding sites were blocked. The samples were incubated in a humid chamber first with the specific primary antibody (for 14–16 h at 4 °C) and then with the corresponding biotin-conjugated secondary antibody (for 30 min at room temperature): anti-rabbit (1:200 dilution, B8895) and anti-mouse (1:100 dilution, B8774), both purchased from Sigma. The reactions were developed using a streptavidin-biotin peroxidase method and diaminobenzidine (Sigma) as a chromogenic substrate. Each immunohistochemical run included positive controls (sections from tissues known to express the proteins of interest) and negative controls (in which the primary antibody was replaced by non-immune serum of the species used to generate the primary antibody [26]). Samples were mounted with permanent mounting medium (Eukitt, Sigma-Aldrich).

### 2.4. Quantification of protein expression

The expression of Wnt5a, Wnt7a and β-catenin in the uterine samples was evaluated by image analysis using the Image Pro-Plus 5.0.2.9 system (Media Cybernetics, Silver Spring, MD, USA), as previously described [29,30]. Briefly, to perform the quantification, at least 10 images at 400× magnification per sample were recorded with a Spot Insight V3.5 color video camera attached to an Olympus BH2 microscope (illumination: 12 V halogen lamp, 100 W, equipped with a stabilized light source; Olympus, Tokyo, Japan) using an objective A-Plan 20×/0.45 (Carl Zeiss Microscopy, LLC, USA) and converted to gray scale. The integrated optical density (IOD) was measured as a linear combination of the average gray intensity and the relative area occupied by positive cells. Because the IOD is a dimensionless parameter, the results are expressed as arbitrary units [29,30]. This standardized quantification method allowed quantitatively evaluating protein expression relative to the histological distribution pattern of protein localization. The proper control of each IHC step assures the reliability and reproducibility of the results of protein expression. In samples of PND8 and PND21, protein expression was quantified in the subepithelial stroma and luminal and glandular epithelia. At least 10 randomly selected fields per section and two sections per rat (separated by 50 μm from each other) were assessed. In samples of GD9, protein expression was quantified in the antimesometrial (AM) and M zones of the IS, as described in [9]. The AM zone was delimited by the myometrium and the adjacent M zone. In the M zone, each compartment was differentially delimited and quantified: luminal and glandular epithelium and subepithelial stroma (a 200-μm-wide area adjacent to the epithelium, from the basement membrane toward the outer layers). The protein expressions in the AM or M zones were quantified on at least ten fields per section and two sections per rat (separated 50 μm from each other).

### 2.5. Reverse transcription and real-time quantitative PCR analysis

#### 2.5.1. RNA extraction and reverse transcription

Total RNA was individually extracted using TRIZOL reagent (Life Technologies, Grand Island, NY, USA) 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, and then dissolved in distilled water, and their quality was verified by gel electrophoresis. Equal quantities (1 μg) of total RNA were reverse-transcribed into cDNA with Moloney Murine Leukemia Virus reverse transcriptase (300 units; Promega, Madison, WI, USA) using 200 pmol of random primers (Promega). Twenty units of ribonuclease inhibitor (RNAout; Invitrogen Argentina, Buenos Aires, Argentina) and 100 nmol of a deoxynucleotide triphosphate (dNTP) mixture were added to each reaction tube at a final volume of 30 μl of 1x reverse transcriptase 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.

#### 2.5.2. Real-time quantitative PCR

Each reverse-transcribed product was diluted with ribonuclease-free water to a final volume of 60 μl and further amplified using the Real-Time Rotor-Gene Q (Quiagen; Tecnolab; Buenos Aires, Argentina). Ribosomal protein (L19) was used as housekeeping gene. The primer sequences for L19 were 5′-AGCCTGTGACTGTCCATTCC-3′ (Forward) and 5′-TGGCAGTACCCTTCCTCTTC-3′ (Reverse). The primer sequences used for Dkk1 were: 5′-GCTCTGTCTGCCTCCGATCA-3′ (forward) and 5′-GCCTTTCCTCCTGTGCTTGG-3′ (reverse) and the primer sequences used for sFRP4 were 5′-TTAGTTGAGAAATGGAGAGA-3′ (forward) and 5′-CTTTGATGTTCTTCTTAGGA-3′ (reverse). For cDNA

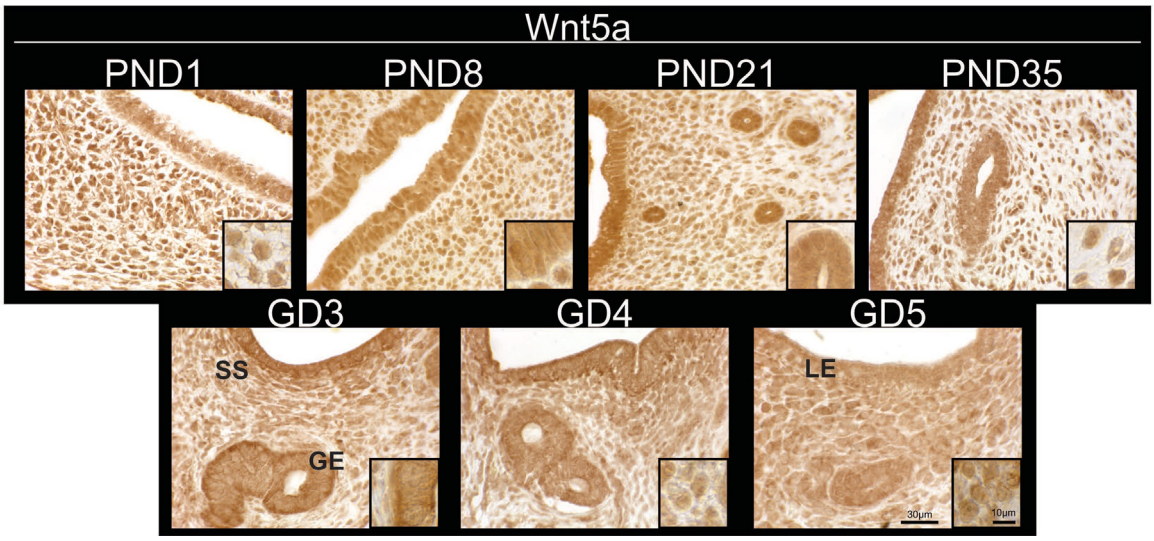


**Fig. 2.** Tissue and cellular pattern of Wnt5a protein expression in the rat uterine compartments during prepubertal development and early pregnancy. Representative photomicrographs show nuclear immunostaining for Wnt5a in the subepithelial stroma (SS) and cytoplasmic/nuclear immunostaining at the luminal epithelium (LE) and glandular epithelium (GE). Differences in immunostaining intensity were observed depending on the evaluation periods. The insets in the figure show the distribution of Wnt5a staining at higher magnification.

amplification, 5 µl of cDNA was 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 60 °C (L19), 56 °C (Dkk1) or 53 °C (sFRP4) 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. Relative gene expression data were calculated using the comparative cycle threshold ($C_T$) method [31]. For each sample, $C_T$ was calculated as the difference in $C_T$ between target mRNA and L19 mRNA. The $C_T$ for each sample was calculated using the Rotor-Gene Q-Pure Detection software (Version 1.7, Quiagen; Tecnolab). Accordingly, the fold expression over control values was calculated for each target by relative standard curve methods, which are designed to analyze data from real-time PCR [32]. For all experimental samples, the relative target quantity was determined from the standard curve, normalized to the relative quantity of the reference gene and finally divided by the normalized target value of the control sample. No significant differences in $C_T$ values were observed for the ribosomal protein L19 between the different experimental groups.

### 2.6. Statistical analysis

Data are expressed as the mean ± SEM. The Mann–Whitney *U* test was applied for the analysis of variables. Differences were considered significant at a $P < 0.05$.

## 3. Results

### 3.1. Postnatal ontogenetic patterns of Wnt5a protein expression in uterine compartments

Fig. 2 shows the patterns of Wnt5a protein expression throughout postnatal development and differentiation at four time points: PND1, 8, 21 and 35; and during three times along early pregnancy: GD3, 4 and 5. Given that the animals were not treated, changes in the protein levels were associated with age or gestation stage. Wnt5a was expressed in the nuclei of stromal cells and in the cytoplasm and nuclei of epithelial (luminal and glandular) uterine cells, and the expression showed no significant changes from PND1 to PND35. During the gestation period evaluated, the cellular localization of Wnt5a expression was similar to that observed in non-pregnant uteri, without changes between GD3 and GD5. Interestingly, cytoplasmic immunostaining was visible in stromal uterine cells toward GD5.

### 3.2. Effects of postnatal exposure to GBH on Wnt5a and β-catenin protein expression during neonatal and prepubertal periods

Fig. 3A shows the results of Wnt5a and β-catenin expression quantification on PND8 (neonatal period) in control and GBH-treated rats. The female rats neonatally exposed to GBH exhibited induction of both proteins in the luminal epithelium on PND8 showing an acute response to the treatment. No changes were observed in the stromal compartment. Representative photomicrographs illustrating these changes are shown in Fig. 3B.

Then, we measured the expression of both proteins 2 weeks after the end of the treatment during the prepubertal period (PND21). Wnt5a and β-catenin expression were notably increased in the subepithelial stroma of the GBH group (Fig. 4A). In addition, β-catenin expression showed a decrease in the glandular epithelium of the GBH-treated group. Representative photomicrographs illustrating these changes are shown in Fig. 4B.

### 3.3. Effects of postnatal exposure to GBH on Wnt5a, Wnt7a and β-catenin protein expression in the post-implantation period

Fig. 5 shows the results of Wnt5a, Wnt7a and β-catenin expression quantification in the AM and M zones of the IS on GD9. GBH affected the expression of both Wnt proteins. Wnt5a expression decreased in the AM zone, whereas Wnt7a expression decreased in the luminal epithelium of the M zone. No difference was detected in β-catenin expression in the AM and M zones of the IS. Representative photomicrographs illustrating these changes are shown in Fig. 6.

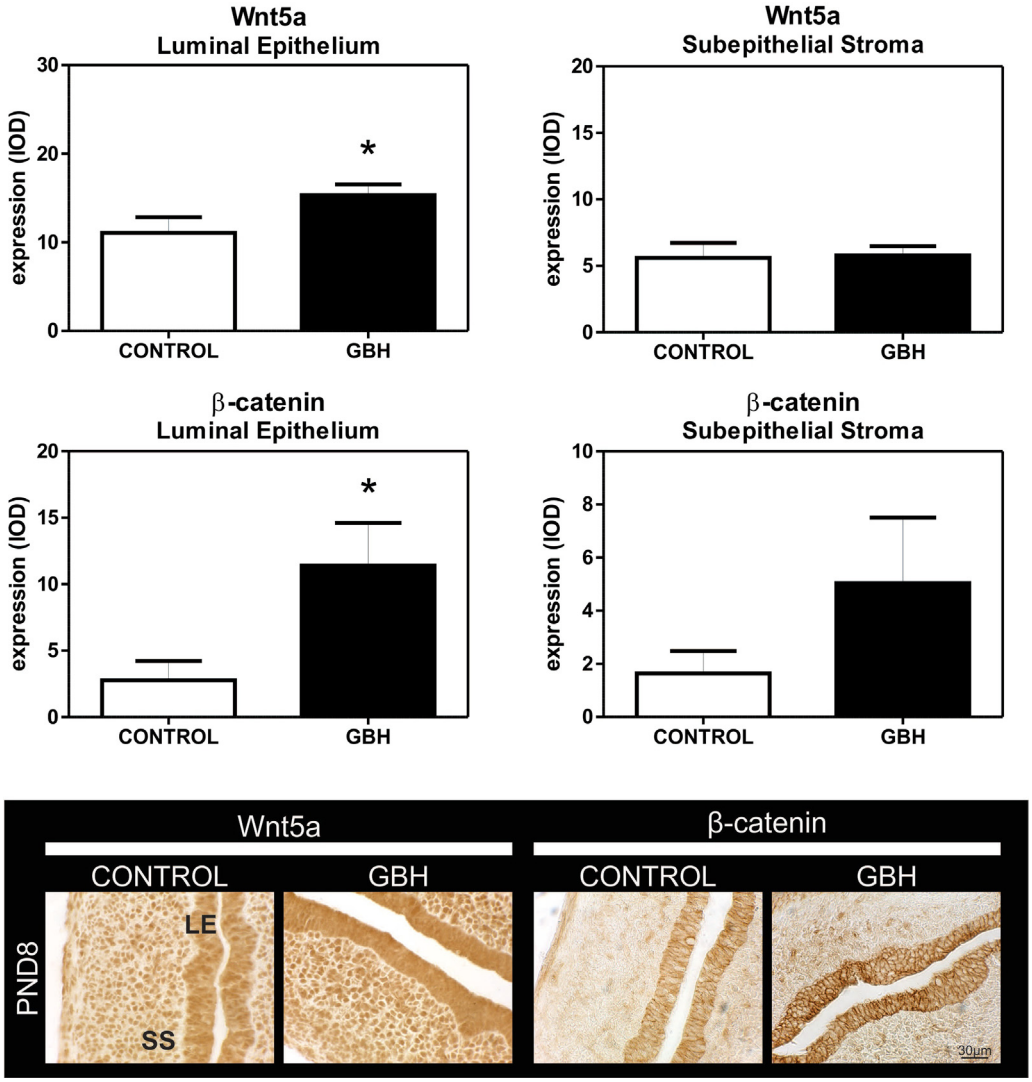


**Fig. 3.** Effects of neonatal exposure to a GBH on Wnt5a and β-catenin expression in the rat uterine tissue compartment on PND8. (A) Quantification of Wnt5a and β-catenin protein immunostaining is expressed as the integrated optical density (IOD). Each column represents the mean ± SEM of 8 rats/group (*P < 0.05 vs. control). The LE showed an increase in Wnt5a and β-catenin expression in GBH-treated rats. (B) Representative photomicrographs of Wnt5a and β-catenin immunostaining.

### *3.4. Effects of postnatal exposure to GBH on the mRNA levels of genes associated with the Wnt signaling pathway*

mRNA expression of Dkk1 and sFRP4 was quantified to find out whether the decrease in Wnt5a and Wnt7a protein expression in the IS of GD9 was associated with defective modulation of these two genes. Fig. 7 shows the relative mRNA expression of Dkk1 and sFRP4 between GBH and control animals. Interestingly, we found an increase in Dkk1 mRNA level and a decrease in sFRP4 mRNA levels.

## 4. Discussion

In the present study, we demonstrated that neonatal exposure to a GBH affected the expression of uterine Wnts (Wnt5a and Wnt7a) and β-catenin in prepubertal and adult pregnant rats. Recently, we have shown long lasting effects of neonatal exposure to a GBH that increases the incidence of resorption sites on adults pregnant rats [9]. Although we do not know the time at which the resorption of IS occurs, on GD9 we did not observe resorption sites, and consequently assumed that we were close to the beginning of the reabsorption process. GD9 was considered a precise time point to study molecules associated with decidualization since it is the day before AM decidua achieve maximum development [33]. The results of the present study indicate that GBH may induce impaired fertility by affecting the expression of Wnts and β-catenin in decidualized uterine cells. Moreover, in a previous study we also demonstrated that progesterone receptor was decreased in IS on GD9 in rats that were neonatally exposed to GBH [9], thus numerous processes and signaling pathways can be disrupted [34,35]. However, we could not determine whether the impact of neonatal exposure to GBH leads to primary failure of implantation chamber remodeling or secondary to disruption of chorionic development or placentation.

An early acute effect of the GBH treatment was the significantly increased expression of Wnt5a and β-catenin in the luminal epithelium on PND8. Previously, we evaluated Wnt7a expression in the uterine compartment on PND8 and found an induction in stromal and glandular cells after GBH treatment [8]. Hou et al. [36] described differential cell-specific up-regulation of Wnt5a mRNA after injection of estradiol (E2) in wild-type or ovariectomized ER (-/-) mice, suggesting that estrogen up-regulates canonical Wnt signaling in the uterus, in an ER-independent manner. Taking into account that several works have attributed estrogenic effects to

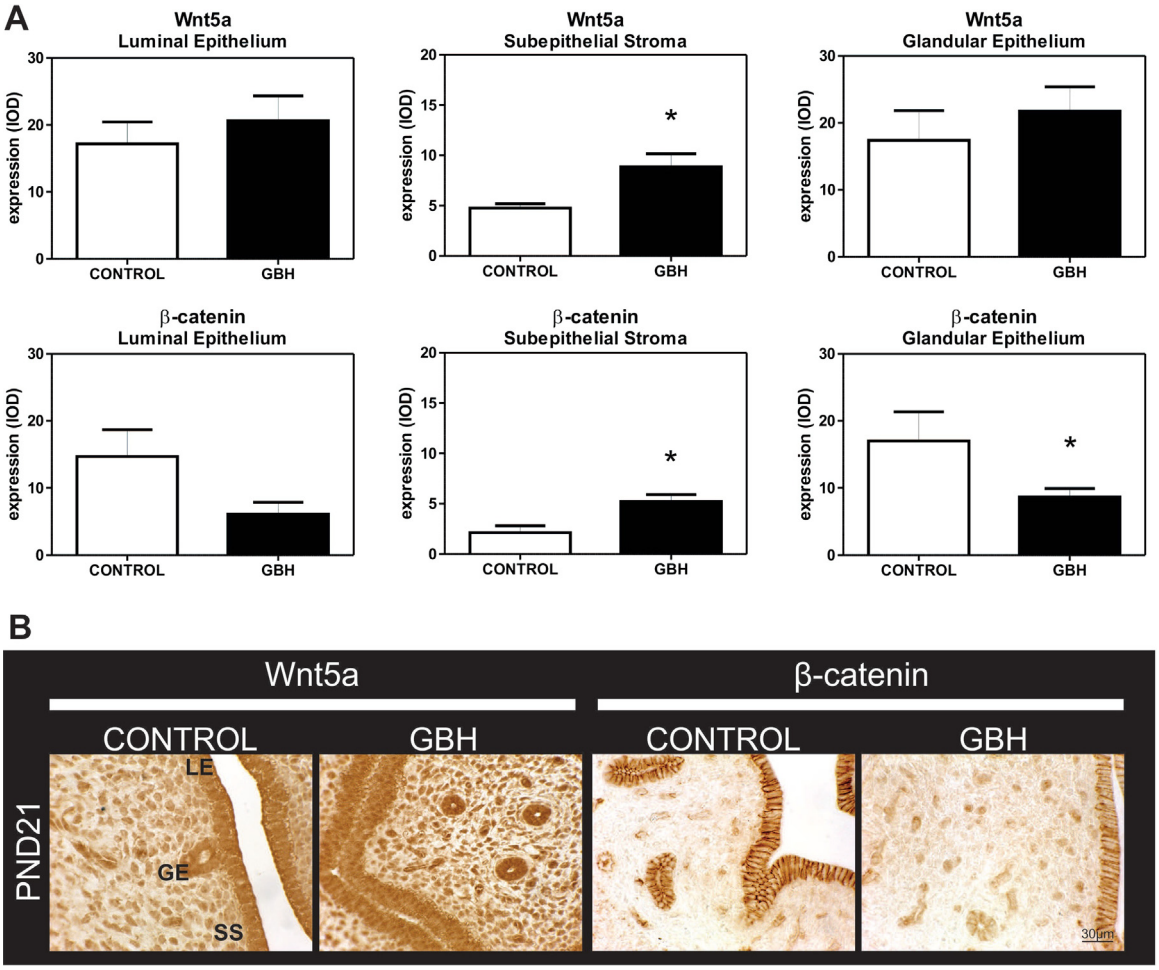


**Fig. 4.** Effects of neonatal exposure to a GBH on Wnt5a and β-catenin expression in the rat uterine tissue compartment on PND21. (A) Quantification of Wnt5a and β-catenin protein immunostaining is expressed as the integrated optical density (IOD). Each column represents the mean ± SEM of 8 rats/group (*P < 0.05 vs. control). The GE showed an increase in β-catenin expression in GBH-treated rats. In the SS, both Wnt5a and β-catenin expressions were increased in GBH-treated rats. (B) Representative photomicrographs of Wnt5a and β-catenin immunostaining.

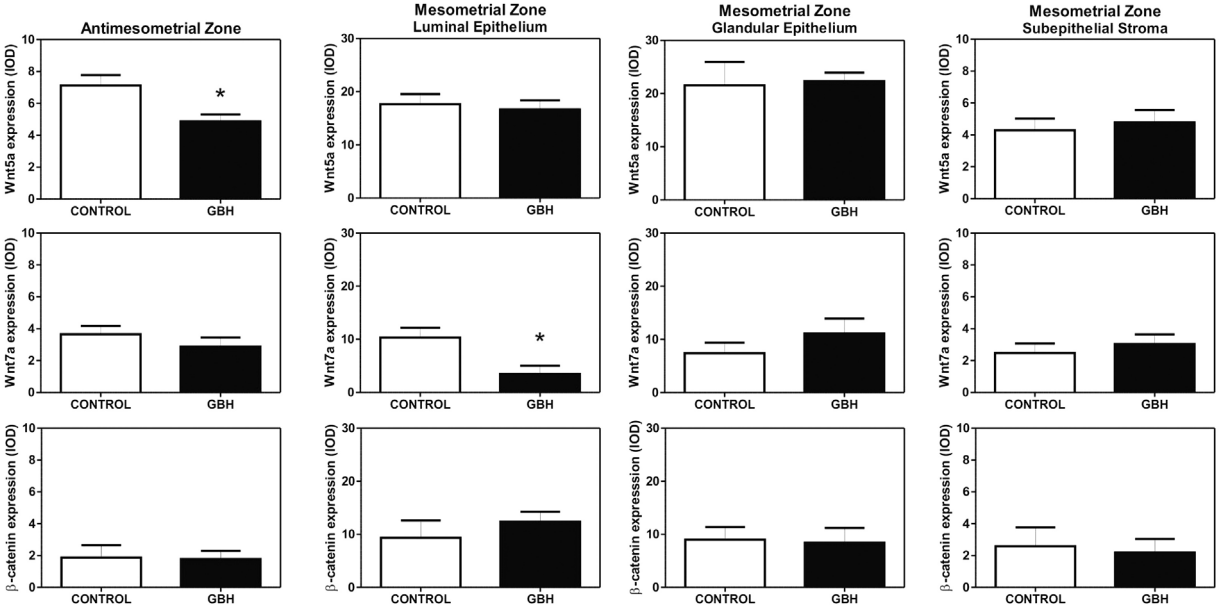


**Fig. 5.** Effects of neonatal exposure to a GBH on Wnt5a, Wnt7a and β-catenin expression in rat implantation sites (IS) on GD9. Quantification of protein immunostaining in the mesometrial (M) and antimesometrial (AM) zones is expressed as the IOD. A decrease in Wnt5a expression was observed in the AM zone of the GBH group, whereas an increase in Wnt7a expression was observed in the LE of the GBH group. No changes were observed in β-catenin expression between the experimental groups. Each column represents the mean ± SEM of 8 rats/group (*P < 0.05 vs. control).

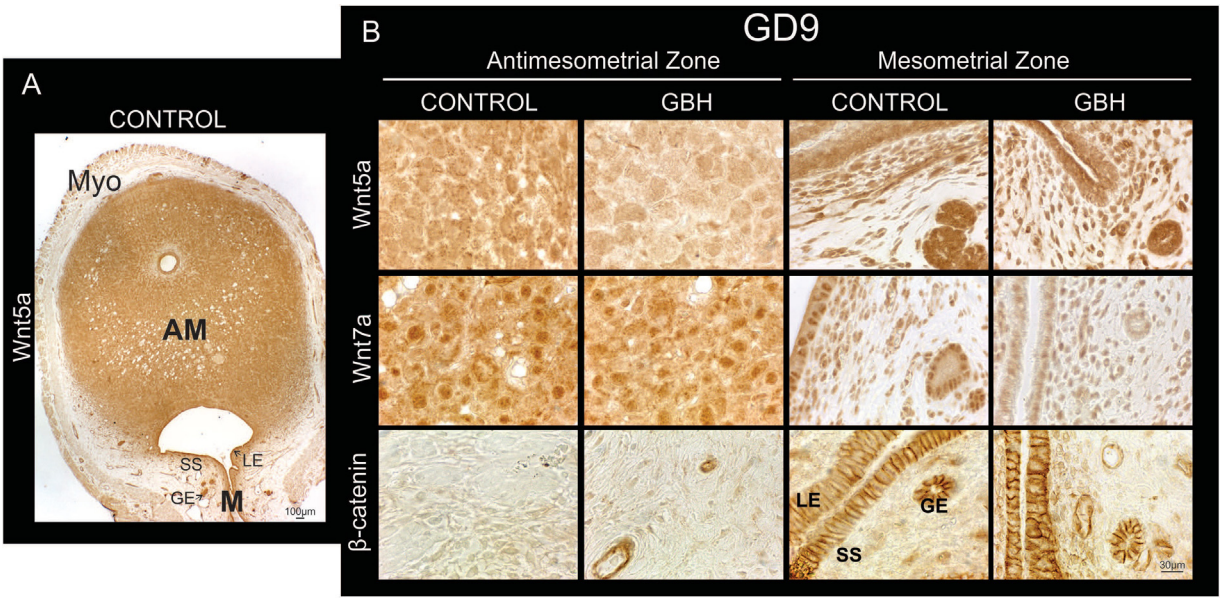


**Fig. 6.** Representative photomicrographs of the expression of uterine markers in control and GBH-exposed animals in the IS on GD9. (A) Low-magnification photomicrographs of representative control IS of pregnant rats on GD9 immunostained for Wnt5a. Image showing different decidualized zones. The antimesometrial (AM) zone, smooth muscle (Myo), and mesometrial zone (M) are shown. In the mesometrial (M) zone, it is possible to observe the luminal epithelium (LE), the subepithelial stroma (SS) and the glandular epithelium (GE). (B) Images show the changes in Wnt5a and Wnt7a immunostaining. Wnt5a decreased in the AM zone, whereas Wnt7a decreased in the LE of GBH-treated rats.

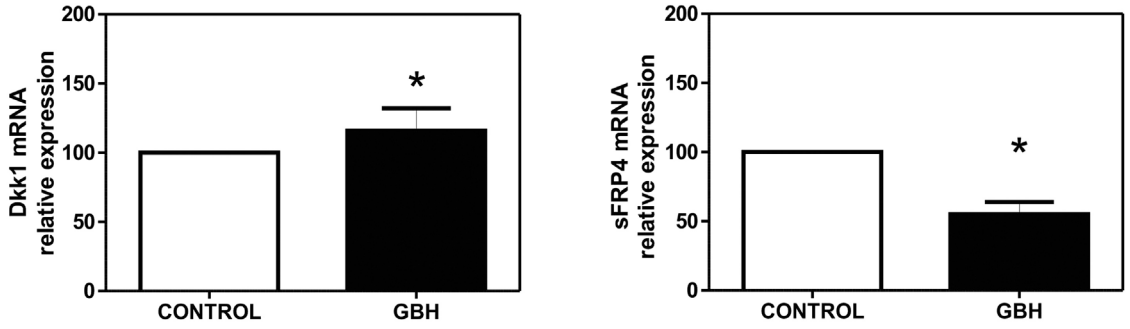


**Fig. 7.** Effects of neonatal exposure to a GBH on the mRNA levels of Dkk1 and sFRP4 in the IS of pregnant rats on GD9, quantified by real-time RT-PCR. Control values were assigned to a reference level of 100 and values of the GBH group are given as mean ± SEM. The ribosomal protein L19 was used as an internal control. Each column represents the mean ± SEM (n = 8 per group). *, $P < 0.05$ vs. the control group.

glyphosate [10–13], we suggest that the increase in Wnt5a and β-catenin expression in the luminal epithelium on PND8 may be due to the GBH treatment.

In previous works, we described that GBH increases Wnt7a expression in prepubertal rats on PND21 [8], without changes on PND8. Interestingly, the present results showed that the expression of both Wnt5a and β-catenin was increased in the stromal compartment of GBH-treated rats on PND21. During postnatal differentiation, Wnt5a genes are required for normal glandular genesis and for the uterine responses to exogenous estrogens [15,37]. Published studies suggest that Wnt7a is locally repressed to allow the luminal epithelium to regulate uterine gland formation and that Wnt5a provides a permissive environment for proper regulation of Wnt7a [15]. In our animals, the fact that the number of glands was not affected by the deregulation of Wnts during development may be related to problems of subfertility during adulthood. Indeed, it has been demonstrated that, in adult females, the uterine overexpression of Wnt5a interferes with critical aspects of embryo implantation leading to reduced fertility [19].

In the present study, GBH-treated rats showed defective expression of β-catenin in the uterine glandular epithelium on PND21. A similar result has been previously detected in rats exposed to endosulfan [26]. Different studies have reported that defective uterine expression of β-catenin leads to compromised fertility [18,20,38]. In the case of endosulfan, we detected subfertility, evidenced by a decrease in the pregnancy rates and number of implantation sites [26,30]. In the case of GBH, we detected a high number of resorption sites [9]. All these results could indicate that the deregulation of Wnt proteins during development affects the normal reproduction of rats exposed to both pesticides, although affecting different aspects of the reproductive mechanism.

In adulthood, the levels and the uterine spatial pattern of Wnt5a expression fluctuate during the estrous cycle, suggesting a continued role in the adult [15,37]. Wnt signaling is essential for decidualization and has been observed that different Wnt ligands and β-catenin are dynamically expressed during the decidual process in mice [39]. During decidualization, the interactions among these molecules regulate the proliferation, differentiation, and intense tissue remodeling [39]. Indeed, Cha et al. [19] demonstrated that Wnt5a-ROR signaling is active in the adult uterus to confer appropriate luminal epithelium organization for implantation and that deregulation of this pathway leads to aberrant crypt formation, inappropriate embryo spacing, abnormal decidual growth and impaired implantation.

As mentioned before, we have previously found that neonatal treatment with GBH increases the number of resorptions in pregnant rats, without altering the ovarian E2 or P levels on GD9 [9]. In the present study, we propose that this increase in the number of resorptions may be due to the disruption of the PR-COUPTFII-Bmp2 pathway. Here, we detected that, in GBH-treated rats, Wnt5a expression has a different pattern of cellular and tissue immunostaining along development as well as in the IS during pregnancy. Cha et al. [19] demonstrated that both the uterine inactivation and overexpression of Wnt5a lead to defective implantation and severe subfertility. These authors also noted that Wnt5a-dysregulated females showed an increase in the number of resorption sites on days 10 and 12 of gestation [19]. In accordance with these authors, we suggest that another potential mechanism triggering resorptions in the GBH group could be an imbalance in Wnt5a expression in the IS of pregnant rats.

In the present study, we also investigated the expression of Wnt7a in the IS on GD9. Previously, we described a high expression of Wnt7a in the stromal compartment of GBH-treated rats on PND21 [8]. During adulthood, we detected a decreased immunostaining of Wnt7a in the luminal epithelium of the M zone in the GBH group. It is known that Wnt7a epithelial expression is a critical factor for the blastocyst-uterine interactions and for the regulation of the initiation of the decidualization process through canonical Wnt/β-catenin signaling [40]. Thus, the decreased expression of Wnt7a in the GBH group on GD9 suggests that Wnt7a is also involved in decidualization failure.

Despite the active role of Wnt proteins, it is interesting to observe that the expression of β-catenin on the IS of GD9 in GBH-treated rats showed no differences between the experimental groups. In that sense, it has been reported that the inhibition of the β-catenin signaling pathway would play a very important role in regulating trophoblast invasion and that its regulation would be related to Dkk1 [23].

To further study the expression pattern of Wnt signaling pathway modulators, Dkk1 and sFRP4 were evaluated on the IS of GD9. Decidualization is associated with increased expression of genes, including Dkk1 and sFRP4 [22,23]. Although we could not infer which is the tissue compartment of these molecules at the IS, in situ hybridization revealed that sFRP4 transcripts are specifically distributed in the decidua of rats on GD12 [22] and that Dkk1 is highly expressed in decidua of days 6 and 7 of mouse pregnancy [41]. It has been suggested that sFRP4 could modulate signals of Wnt genes in pregnant rats via binding to Wnt genes such as Wnt-4, Wnt-5a and Wnt-7a [22]. In the present study, the expression of the Dkk1 gene was significantly increased in the GBH group compared the control group, whereas that of sFRP4 was decreased. Peng et al. [23] reported that the Dkk1 secreted by decidual cells could increase the invasive ability of trophoblasts during placentation. Since the process is strictly regulated, either too little or an excess of invasion could result in pregnancy abnormalities [23]. It has been reported that the sFRP4 gene is up-regulated during the late phase of proliferation in decidual cells and would be involved in the initiation of decidual apoptosis by modulating some Wnt signals [22]. Previously, we reported an increased proliferation in decidual cells on GD9 of GBH-treated rats together with no changes in the percentage of apoptosis [9]. Consequently, it may be suggested that the decrease in sFRP4 in GBH-treated rats described here affects the apoptosis/proliferation balance of decidual cells.

In conclusion, taken together, the results of the present study evidence a deregulation of the Wnt pathways that regulate uterine decidualization when rats are neonatally treated with a low dose of GBH. In a critical period of gestation like the decidualization process, an imbalance of regulatory molecules as Wnt/β-catenin could cause gestation failure. Our findings may be important for women at risk of low fertility, given that it has been demonstrated that Wnt-5 is relevant for normal decidualization in humans, and thus for trophoblast invasion and implantation [40]. Moreover, the deficiency in Wnt-5 may be involved in the development of preeclampsia [42]. Although the rat is an appropriate model to study the mechanisms of decidualization [43], further investigations are needed to translate these data to humans.

## 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.

## Funding

This work was supported by Universidad Nacional del Litoral (Santa Fe, Argentina) (CAI+D program), Argentine Council for Scientific and Technological Research (CONICET), and the Argentine Agency for the Promotion of Science and Technology (ANPCyT).

## Acknowledgements

PII, MMM, JV and EHL are Career Investigators, MGS and RA are fellows of the CONICET.

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