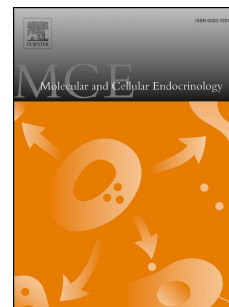


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Epigenetic disruption of estrogen receptor alpha is induced by a glyphosate-based herbicide in the preimplantation uterus of rats

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Abstract

Previously, we have shown that perinatal exposure to a glyphosate-based herbicide (GBH) induces implantation failures in rats. Estrogen receptor alpha (ER α) is critical for successful implantation. ER α transcription is under the control of five promoters (E1, OT, O, ON, and OS), which yield different transcripts. Here, we studied whether perinatal exposure to a GBH alters uterine ER α gene expression and prompts epigenetic modifications in its regulatory regions during the preimplantation period. Pregnant rats (F0) were orally treated with 350 mg glyphosate/kg bw/day through food from gestational day (GD) 9 until weaning. F1 females were bred, and uterine samples were collected on GD5 (preimplantation period). ER α mRNA levels and its transcript variants were evaluated by RT-qPCR. Enzyme-specific restriction sites and predicted transcription factors were searched *in silico* in the ER α promoter regions to assess the methylation status using the methylation-sensitive restriction enzymes-PCR technique. Post-translational modifications of histones were studied by the chromatin immunoprecipitation assay. GBH upregulated the expression of total ER α mRNA by increasing the abundance of the ER α -O transcript variant. In addition, different epigenetic changes were detected in the O promoter. A decrease in DNA methylation was observed in one of the three sites evaluated in the O promoter. Moreover, histone H4 acetylation and histone H3 lysine 9 trimethylation (H3K9me3) were enriched in the O promoter in GBH-exposed rats, whereas H3K27me3 was decreased. All these alterations could account for the increase in ER α gene expression. Our findings show that perinatal exposure to a GBH causes long-term epigenetic disruption of the uterine ER α gene, which could be associated with the GBH-induced implantation failures.

Keywords:

Glyphosate-based herbicide; uterus; estrogen receptor alpha; epigenetic modifications;
preimplantation period.

Abbreviations

5'UTRs: 5'untranslated regions
ChIP: chromatin immunoprecipitation
CpG: cytosine-phosphate-guanine dinucleotide
CT: cycle threshold
EDCs: endocrine-disrupting chemicals
ER α : estrogen receptor alpha
F0: dams directly exposed to the glyphosate-based herbicide formulation
F1: F0's offspring
GBHs: glyphosate-based herbicides
GD: gestational day
IC: internal control
IPs: immunoprecipitated complexes
LD: lactation day
NOAEL: no-observed adverse effect level
PND: postnatal day
PTMs: histone post-translational modifications
qRT-PCR: real time RT-PCR
TFs: transcription factors

1. Introduction

In South America, a large number of residents in rural areas and in the vicinity of
cultivated fields are at risk of being exposed to agricultural pesticides. Adverse birth

outcomes and altered placental biomarkers have been associated with pesticide exposure in rural populations in our region (Rezende Chrisman et al., 2016; Rivero Osimani et al., 2016). GBHs (glyphosate-based herbicides) are the most heavily applied pesticides in the world, including in South America. The increment of GBH-tolerant crop varieties and their cultivated areas have contributed to increasing environmental loads and human exposures to GBH (Benbrook, 2016), not only through residence near sprayed areas but also through use at home, and in the diet (Bai and Ogbourne, 2016).

Several studies have revealed that GBHs have endocrine-disrupting properties using different experimental approaches, both with cell cultures and murine models (Dallegrave et al., 2007; Mesnage et al., 2017; Romano et al., 2012). The uterus is an organ that is extremely sensitive to the developmentally disruptive effects of environmental chemicals which are associated with reduced fertility (Ingaramo et al., 2016; Milesi et al., 2015; Varayoud et al., 2011) and the occurrence of uterine cancer and fibroids later in life (Katz et al., 2016; Morgan et al., 2016). Previously, we detected that GBH modifies the uterine expression of estrogen-sensitive genes using the uterotrophic assay (Varayoud et al., 2017). In addition, we reported that postnatal exposure to a GBH alters uterine morphology and the expression of key proteins for uterine development and differentiation in rats (Guerrero Schimpf et al., 2017).

Environmental factors such as endocrine-disrupting chemicals (EDCs) are able to produce epigenetic marks leading to changes in gene expression and inheritance of potentially adverse alterations underlying the genome (Guerrero-Bosagna et al., 2013). These epigenetic marks include alterations in the level of DNA methylation, histone post-translational modifications (PTMs), and noncoding RNAs. In DNA methylation, a methyl group is enzymatically added to the cytosine base in a cytosine-phosphate-guanine dinucleotide (CpG) context in CG-rich areas, named CpG islands, which are

predominantly associated with regulatory elements such as promoters (Jacobs et al., 2017). Methylated DNA is generally linked to a decreased transcription factor binding capacity that diminishes transcriptional expression of the corresponding gene (Schübeler, 2015). On the other hand, histones can undergo multiple enzymatic modifications, such as acetylation, methylation, phosphorylation, etc. These PTMs can change the chromatin landscape by altering the charge between nucleosomes and DNA and the accessibility of DNA to transcription factors (Venkatesh and Workman, 2015).

Estrogens are critical hormones to prepare uterus for embryo implantation, and the secretion of 17 β -estradiol is strictly regulated during the window of uterine receptivity (Ma et al., 2003). In the uterus, estrogen signaling is mainly mediated through the classic nuclear receptor, estrogen receptor alpha (ER α) (Wang and Dey, 2006). ER α expression is under a very strict hormonal and temporal control, indicative of spatio-temporal roles during implantation (Vasquez and DeMayo, 2013). The rat ER α gene has five promoters that control ER α transcription initiation and that result in transcripts with different 5' untranslated regions (5'UTRs) derived from exons OS, ON, O, OT, and E1 (Monje et al., 2007). This multiple promoter system is involved in developmental stage- and tissue-specific regulation of ER α gene expression (Ishii et al., 2010).

A previous study revealed that glyphosate activates ER α by a ligand-independent mechanism combining *in vitro* and *in silico* tools (Mesnage et al., 2017). Recent results from our lab showed that perinatal exposure to a GBH impairs reproductive performance by increasing the rate of preimplantation embryo loss (Milesi et al., 2018). Considering the key role of uterine ER α in embryo implantation, in the present work, we investigated whether perinatal exposure to a GBH alters the transcriptional regulation of the ER α gene in the uterus of F1 dams during the preimplantation period. We assessed total ER α mRNA levels and the relative abundance of ER α transcripts with

alternative 5'UTRs. Moreover, we analyzed the methylation status and histone PTMs in the regulatory region of the ER α gene as potential epigenetic marks induced by GBH exposure.

2. Materials and methods

2.1. Chemicals

The glyphosate formulation used in this study was MAGNUM SUPER II marketed in Argentina by Grupo Agros S.R.L. It is a liquid water-soluble formulation containing 66.2% of glyphosate potassium salt (equivalent to 54% w/v of glyphosate acid), as its active ingredient, coadjuvants and inert ingredients. We chose MAGNUM SUPER II formulation based on the fact that it is made and marketed in our country and also, it is representative of formulations with high content of glyphosate indicated against weeds difficult to eradicate.

2.2. Animals

The procedures used in this study were approved by the Institutional Ethics Committee of the Facultad de Bioquímica y Ciencias Biológicas (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 United States National Academy of Sciences.

We used inbred Wistar-derived strain rats that were bred in the Department of Human Physiology (Universidad Nacional del Litoral), and housed in a controlled environment (22 ± 2 °C; cycle of 14 h light/24 h day) in stainless steel cages with wood bedding. We selected an inbred strain as it provides a homogeneous animal model with minimal polymorphisms (Festing, 1979; 1993), which facilitates the detection of

treatment effects and allows making comparisons between experiments performed in any laboratory.

2.3. Experimental design

At the proestrus stage, nulliparous female rats with sexually receptive behavior were caged overnight with males of proven fertility. As animals are exposed to lights from 06:00 to 20:00 h, ovulation occurs approximately at 02:00 am in our colony (Labhsetwar, 1970), which was confirmed through vaginal smears (unpublished results from our lab). Every morning, vaginal smears were performed to check for the presence of spermatozoa (Montes and Luque, 1988). The first day on which a sperm-positive smear was detected, it was considered gestational day 1 (GD1). Pregnant females (F0) were housed singly and randomly assigned to one of the following oral treatment groups: control group (n= 8) provided with a laboratory pellet chow-based paste, and GBH group (n= 8) provided with paste supplemented with GBH in a dose of 350 mg of glyphosate/kg bw/day. We chose a dose lower than the no-observed adverse effect level (NOAEL) of 1000 mg/kg bw/day for maternal and developmental toxicity in rats (Williams et al., 2000). Previous works indicated that similar doses to the dose we used in the present study cause deleterious effects, specifically on reproductive system of F1 rats using models of perinatal exposure (Dallegrave et al., 2007; Milesi et al., 2018). The laboratory chow-based paste was prepared for each experimental group according to Milesi et al., (2018). Briefly, optimized quantities of pellet chow (Nutrición Animal, Santa Fe, Argentina) and water were blend. For GBH treatment, a glyphosate commercial formulation was added to the water according to the above described dose. The mixture was covered and stood overnight, after that it was homogenized to form a paste and chow balls were prepared to control and GBH groups. The pellet-based paste

for both groups was prepared freshly every three days i.e., the same day the food was replaced. Tap water was supplied *ad libitum* in glass bottles with rubber stoppers surrounded by a steel ring. F0 pregnant females received the oral treatment from GD9 until the end of weaning (on lactational day (LD) 21). As embryo implantation occurs in the evening of GD5 in our colony (Milesi et al., 2015), we began the oral treatment of F0 dams on GD9 to avoid potential GBH-induced implantation failures. The dose of glyphosate achieved was calculated based on the average body weight and food intake during the treatment period, as previously reported in Milesi et al., (2018).

After delivery (postnatal day (PND) 0), F1 pups were weighed and sexed according to the anogenital distance, and litters of eight pups (preferably four males and four females) were left with F0 lactating mothers. No alterations in maternal care or signs of acute toxicity were detected between groups. At weaning (PND21), female offspring were housed in groups of four rats according to the treatment group (control or perinatally GBH-exposed) with free access to pellet laboratory chow and tap water. Male offspring were used in other experiments. Regarding F1 females, no significant differences were recorded in weight gain between control and GBH-exposed rats during the experiment, as reported in Milesi et al., (2018). On PND90, F1 females were housed with untreated males of the same strain and of proven fertility. The first day on which a sperm-positive smear was detected, it was considered the GD1 (Montes and Luque, 1988). Control (n= 8) and GBH-exposed pregnant F1 rats (GBH, n= 8) were sacrificed on the morning of GD5 (preimplantation period). Uterine tissue was collected and snap-frozen in liquid nitrogen and stored at -80 °C for RNA, DNA and chromatin extraction. Fig. 1 shows a schematic representation of the experimental design.

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

An optimized PCR protocol was employed to analyze the relative expression levels of total ER α mRNA and the ER α transcript variants containing alternative 5'UTRs OS, ON, O, OT, and E1. Fig. 2 shows the genomic organization of the promoter region of the rat ER α gene. Uterine samples from each experimental group were individually homogenized in TRIzol (Invitrogen, Carlsbad, CA, USA), and RNA was extracted according to the manufacturer's protocol. The concentration of total RNA was assessed by A₂₆₀, and the samples were stored at -80 °C until later analysis. Equal quantities (1 μ g) 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, Madison, WI). Twenty units of ribonuclease inhibitor (RNasin) (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 μ l of 1 \times 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.

Each reverse-transcribed product was diluted with RNase free water to a final volume of 60 μ l and further amplified in duplicate using the Real-Time DNA Step One Cyclor (Applied Biosystems Inc., Foster City, CA, USA). Primer pairs used for amplification of ribosomal protein L19 (housekeeping gene), total ER α , ER α 5'UTRs cDNA's were designed with the software Vector NTI Suite Version 6.0 (InforMax Inc, North Bethesda, MD) and are shown in Table 1. For cDNA amplification, 5 μ l of cDNA was combined with HOT FIREPol EvaGreen® qPCR Mix Plus (Solis BioDyne; Tartu; Estonia) and 10 pmol of each primer (Invitrogen, Carlsbad, CA) to a final volume of 20 μ l. Each sample was quantified in triplicate. 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 55-60 °C for 15 s, and extension at 72 °C for 15 s. Product purity was confirmed by dissociation curves, and random samples were subjected to agarose gel electrophoresis. Controls containing no template DNA were included in all assays, and these reactions did not yield any consistent amplification. The relative expression levels of each target were calculated based on the cycle threshold (CT) method (Higuchi et al., 1993). The CT for each sample was calculated using the Step One Software (Applied Biosystems Inc.) with an automatic fluorescence threshold (Rn) setting. The efficiency of PCR reactions was assessed for each target by the amplification of serial dilutions (over six orders of magnitude) of cDNA fragments of the transcripts under analysis. Accordingly, the fold expression over control values was calculated for each target by the relative standard curve method, which is designed to analyze data from real-time PCR (Čikoš et al., 2007). For all experimental samples, the target quantity is determined from the standard curve, normalized to the quantity of the housekeeping gene and finally divided by the target quantity of the control sample. No significant differences in CT values were observed for L19 between the experimental groups.

2.5. Bioinformatics

The ER α promoter regions were analyzed for CpG islands using the Methyl Primer Express Software v1.0 (Applied Biosystems, Foster City, CA). A CpG island was defined as a DNA sequence of 200 bp with a calculated percentage of CpGs of more than 50% and a calculated versus expected CpG distribution higher than 0.65. These regions also were checked for restriction sites for *Bst*UI and *Mae*II enzymes to evaluate the number of methylation-sensitive sites. To recognize the putative binding sites for transcription factors, we used the PROMO program (<http://alggen.lsi.upc.es/cgi->

bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) (Farré et al., 2003). PCR primers were designed with the software Vector NTI Suite Version 6.0 (Infomax Inc., North Bethesda, MD, USA).

2.6. Methylation-Sensitive Restriction Enzymes-PCR analysis

We investigated the methylation status of the ER α promoters in the experimental groups using a combination of digestions with methylation-sensitive restriction enzymes and subsequent real-time PCR analysis (Milesi et al., 2017; Rossetti et al., 2015). Uterine DNA from each group was individually prepared using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI). The concentration of total DNA was assessed by A₂₆₀, and DNA was stored at 2-8 °C until needed. Equal quantities (1 µg) of total DNA were digested with 5 units of *Hind*III (Promega, Madison, WI) for 3 h at 37 °C to reduce the size of the DNA fragments and then purified with the Wizard SV gel and PCR Clean-Up System Kit (Promega, Madison, WI). Then, 1 µg of *Hind*III-cleaved DNA was incubated for 1 h with 1 unit of *Mae*II (Roche Applied Science, Indianapolis, IN, USA) or 1 unit of *Bst*UI (New England BioLab, Beverly, MA, USA) and 1X enzyme buffer at 50 °C or 60 °C respectively, in a covered water bath (Tecno Dalvo, Santa Fe Argentina) following the manufacturer's instructions. The products of digestion were purified with the Wizard SV gel and PCR Clean-Up System Kit according to the manufacturer's protocol (Promega, Madison, WI). An optimized PCR protocol was employed to analyze the relative expression levels of various regions of the ER α promoters. Primer pairs were designed with the software Vector NTI Suite Version 6.0 and are shown in Table 2. The relative expression level of the different DNA regions was analyzed by real-time PCR (see section 2.4 from Materials and Methods). Each sample was quantified in triplicate. A region devoid of *Bst*UI and *Mae*II

restriction sites was amplified as an internal control (IC). The methylation restriction enzymes *MaeII* and *BstUI* are unable to cut at methylated sites, allowing amplification of the fragment. In contrast, if the CpG-rich site is not methylated, *MaeII* or *BstUI* cleaves the DNA and prevents amplification of the fragment. The relative degree of promoter methylation was calculated by CT values plotted against the log input DNA, yielding standard curves for the quantification of unknown samples (Čikoš et al., 2007). For all experimental samples, the target quantity is determined from the standard curve, normalized to the quantity of the IC region (not sensitive to enzyme digestion) and finally divided by the target quantity of the control sample.

2.7. Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) analysis was performed according to a modified protocol (Altamirano et al., 2017; Kazi and Koos, 2007). Briefly, ~50 mg of frozen uterine sample was dissociated into small pieces and immersed in 1% formaldehyde solution for 15 min. Cross-linking was stopped by adding 1.5 ml of 1 M glycine for 5 min. The tissue pellet was homogenized in 600 µl of RIPA lysis buffer [50 mM Tris-HCl (pH 7.4), 1% Nonidet P40, 0.5% sodium deoxycholate, 1 mM EDTA with 1X protease inhibitor (Complete Mini, Protease Inhibitor Cocktail Tablet, Roche Diagnostics GMBH, Germany) and phosphatase inhibitor (Phos-STOP, Phosphatase Inhibitor Cocktail Tablets, Roche Diagnostics GMBH) in PBS, pH 7]. Homogenates were centrifuged at 12,000 rpm for 5 min at 4 °C and the supernatants removed and discarded. The separated nuclei were lysed in SDS lysis buffer [50 mM Tris-HCl (pH 8.1), 5 mM EDTA, 0.1% SDS] containing proteases and phosphatase inhibitors (as above), and incubated on ice for 20 min. Then, samples were sonicated on ice for 40x 2 s cycles, followed by 1 min cooling interval between each cycle, using a Sonic Vibra-

Cell™ VCX750 (Sonics & Materials, Newtown, CT, USA) at 30% of power. The resulting DNA fragment size was about 0.5-1.0 kpb. After sonication, the samples were centrifuged at 14,000 rpm for 10 min at 4 °C. The supernatants were then collected and stored at -80 °C. Then, 50 µl of Dynabeads® Protein A (Invitrogen) was incubated with 2.5 µl of rabbit polyclonal antibody Anti-Acetyl-Histone H3 (H3Ac) (Upstate Biotechnology, Lake Placid, NY, USA) or Anti-Acetyl-Histone H4 (H4Ac) or Anti-trimethyl-Histone H3 (Lys9) (H3K9me3) or Anti-trimethyl-Histone H3 (Lys27) (H3K27me3) (EMD Millipore, Darmstadt, Germany) for 10 min. For the nonspecific antibody control, an equal volume of non-immune rabbit serum was substituted for the specific antibody (negative control). Sonicated sample aliquots were thawed on ice and diluted 1:10 with dilution buffer [20 mM Tris-HCl (pH. 8.1), 150 mM NaCl, 2 mM EDTA, 1% triton X-100, and protease inhibitors (as above)]. The samples were incubated with the Dynabeads® Protein A-antibody complex overnight at 4 °C with rotation. The immunoprecipitated complexes (IPs) were washed sequentially three times with PBS solution and once with TE buffer (pH 8.0). Protein-DNA complexes were eluted from the Dynabeads® Protein A by incubation in 100 µl of elution buffer [100 mM NaHCO₃ and 1% SDS], and 0.5 µl of proteinase K 20 mg/ml (Sigma-Aldrich) at 65 °C for 2 h to remove protein. The cross-linking was reversed with incubation at 95 °C for 10 min. Ten percent of the total sonicated supernatant was saved as an input control and further processed in parallel with the eluted IPs at the cross-linking reversal step. DNA was purified with a PureLink™ Quick Gel Extraction & PCR Purification Combo kit (Invitrogen). The recovered DNA was then quantified by real-time PCR (see 2.4 section from Materials and Methods). Primers were used to amplify genomic sequences at the O promoter of ERα gene (Table 2). The relative amounts of IPs and input DNA were determined by comparison to a standard curve generated by serial

dilutions of input DNA. Both experimental IPs and input DNA were run in triplicate. IPs with specific antibodies was normalized by first subtracting the signals obtained with the nonspecific antibody control, and then expressing the normalized value as a ratio to input DNA and to the normalized value of the control samples.

2.8. Statistical analysis

All data are expressed as the mean \pm SEM. Differences in control (n= 8) and GBH-exposed group (n= 8) were analyzed using Mann-Whitney test due to the small sample size and the impossibility to know the distribution of our variables under study (Fay and Proschan, 2010). All statistical analyses were performed using GraphPad Prism version 5.03 (GraphPad Software, San Diego, CA, USA). Differences were considered significant at $p < 0.05$.

3. Results

3.1. Perinatal exposure to GBH alters the expression of ER α mRNA and the relative abundance of its ER α -O transcript variant in the uterus during the preimplantation period

In order to know the long-term effects of perinatal exposure to GBH on ER α expression, we evaluated total ER α mRNA levels in uterine tissue collected during the preimplantation period. GBH exposure increased the expression of ER α mRNA relative to control rats (Fig. 3A). To determine whether the increase in ER α mRNA expression was associated with changes in transcriptional promoter usage, relative expression levels of exons encoding 5'UTR OS, ON, O, OT, and E1 of the rat ER α gene were studied by real-time PCR. The 5'UTR ON variant was detected neither in the controls nor in the GBH-exposed group under our experimental conditions. We observed that

ER α gene transcription is regulated by means of promoters associated with the 5'UTRs exons OS, O, OT and E1 in control and in GBH-exposed rats during the preimplantation period. We detected that GBH increased total ER α mRNA which was associated with an increased expression of the ER α -O transcript variant (Fig. 3B).

3.2. *In silico* analysis of candidate sites of DNA methylation and potential transcription factor binding sites in the rat ER α -O promoter

Based on the above results, we decided to investigate whether the ER α -O transcript variant is epigenetically regulated in the uterus as a consequence of GBH exposure. To search for potential sites of DNA methylation, we analyzed the ER α -O promoter region for CpG islands and checked for restriction sites for *Bst*UI and *Mae*II enzymes. Moreover, we searched for transcription factors that regulate these putative sites of DNA methylation. Fig. 4A shows a schematic representation of the ER α -O promoter, the binding sites for transcription factors and methylation-targeted CG areas. We identified one CpG island (-149 to +149) with two restriction sites for *Bst*UI associated with the E47 and E2F-1 transcription factors and another CpG island (-1921 to -1706) with one restriction site for *Mae*II associated with the GATA-1 transcription factor.

3.3. GBH exposure modifies the methylation status of the ER α -O promoter during the preimplantation period

To elucidate whether altered transcript levels of the ER α -O variant are associated with differential DNA methylation due to GBH exposure, we determined the methylation status of the transcriptionally active promoter (O promoter) and its surrounding DNA areas. Genomic DNA extracted from the uterus during the preimplantation period was incubated with the *Bst*UI and *Mae*II restriction enzymes,

and the targeted DNA regions were studied by real-time PCR. An internal control designed within the promoter region was used as a PCR control for quantitative analysis. In the O promoter, a decrease in the methylation status was detected at the *BstUI*-1 site in the F1 female rats exposed to GBH. No changes were found in DNA methylation at the *BstUI*-2 and *MaeII* sites (Fig. 4B).

3.4. GBH exposure induces changes in the pattern of histone post-translational modifications during the preimplantation period

To assess whether perinatal exposure to GBH induced changes in histone post-translational modifications (lysine acetylation and methylation) in the ER α -O promoter, we performed ChIP analyses using uterine tissue samples on GD5. The levels of histone acetylation (H3Ac and H4Ac) and methylation at different lysine residues (H3K9me3 and H3K27me3) in three different regions within the ER α -O promoter were measured by ChIP assays (Fig. 5). In all the regions analyzed, ChIP data showed an increased level of H4Ac in the GBH-exposed group (Fig. 5B; $p < 0.05$). Regarding H3Ac, only decreased acetylation at the *MaeII* site was detected (Fig. 5A; $p < 0.05$). On the other hand, histone methylation analysis revealed increased H3K9me3 in most of the sites studied in GBH-exposed rats (Fig. 5C; $p < 0.05$). In addition, a lower H3K27me3 level was found in GBH-exposed rats compared to controls (Fig. 5D; $p < 0.05$).

4. Discussion

ER α is the most important mediator of estrogen signaling during early pregnancy and it is necessary for successful implantation (Lee et al., 2012). Previously, we reported that perinatal exposure to a GBH increases the rate of preimplantation embryo loss as a consequence of a decrease in the number of implanted embryos (Milesi et al.,

2018). In the present work, we showed that ER α mRNA expression increases in the uterus of perinatally GBH-exposed female rats during the preimplantation period and that different epigenetic mechanisms are associated with the upregulation of ER α gene expression.

Embryo implantation is a rate-limiting stage of mammalian pregnancy, and it is a hormonally controlled process involving the synchronized readiness of a blastocyst and a receptive state in the uterine endometrium (Padmanabhan and Laloraya, 2016). The uterus undergoes structural and functional changes that enable the blastocyst to attach and initiate the process of implantation (Zhang et al., 2013). ER α plays a critical role in preparing endometrium for blastocyst attachment (Wang and Dey, 2006). In our work, we found that perinatally GBH-exposed F1 female rats exhibited high uterine levels of ER α mRNA in the preimplantation period. The uterus has proven to be a sensitive target of different environmental chemicals. Similar to our results, early life exposure to bisphenol A (BPA), diethylstilbestrol and the insecticide endosulfan altered the reproductive performance of female rats and induced implantation failure by, at least in part, disrupting uterine ER α gene expression during the preimplantation period (Milesi et al., 2015; 2017; Varayoud et al., 2011). In addition, high levels of ER α at the preimplantation stage were observed in women with different gynecological disorders, such as polycystic ovarian syndrome (Quezada et al., 2006), endometriosis (Lessey et al., 2006) and unexplained infertility (Dorostghoal et al., 2018), conditions which are all associated with detrimental effects on fertility.

Many of the EDCs present in the environment or diet mimic the endogenous estrogen functions or interfere with estrogen signaling pathways, which are mediated by estrogen receptors (Shanle and Xu, 2011). Despite the fact that the Endocrine Disruptor

Screening Program (EDSP) conducted by the US Environmental Protection Agency (EPA) concluded that there was no convincing evidence to classify glyphosate as EDC (EPA, 2015), several studies have shown that glyphosate and its commercial formulations disrupt endocrine-signaling systems *in vitro* (Gasnier et al., 2009; Richard et al., 2005) and in mammalian and nonmammalian species (Altamirano et al., 2018; Armiliato et al., 2014; de Souza et al., 2017). In accordance with our results, previous studies performed in our lab reported that uterine ER α expression is altered by GBH when using different experimental models in rats (Guerrero Schimpf et al., 2017; Ingaramo et al., 2016; Varayoud et al., 2017). Varayoud et al., (2017) investigated the potential estrogenic effects of a GBH formulation by the uterotrophic assay. They used ovariectomized rats which were subcutaneously injected with a GBH formulation in doses of 0.5, 5, or 50 mg glyphosate/kg bw/day. In the present work, we assessed the effects of perinatal exposure to a GBH formulation in a dose of 350 mg glyphosate/kg bw/day administrated by oral route. In Varayoud et al., (2017) GBH induced a decrease in ER α mRNA expression levels, whereas in this work, we detected an increase in ER α mRNA levels. The experimental conditions and animal models could account for the differences observed. Moreover, distinct effects on ER α mRNA levels were reported after glyphosate exposure using *in vitro* assays. Some authors (Thongprakaisang et al., 2013; Mesnage et al., 2017) showed an ER α activation in T47D human breast cancer cells, while Gasnier et al., (2009) found an inhibition of the transcription activity of ER α in HepG2 human hepatic cells. Overall, these findings suggest that GBH formulations modulate in different ways the expression of ER α depending on the experimental model. In addition, we could conclude that ER α is a molecular target affected by GBH in different conditions.

Our findings show a deregulation of ER α that could be associated with GBH-induced implantation failures. However, other key endocrine pathways, such as those regulated by progesterone receptor are critical for blastocyst implantation in the uterus (Vasquez and DeMayo, 2013). It is well known that female mice lacking progesterone receptor are infertile with many defects in uterine functions (Lydon et al., 1995). Moreover, additional factors, such as delayed maturity of ooplasm, chromosomal aberration of oocytes, aneuploidy of embryos, or defects in communication between the embryo and the endometrium, are able to cause implantation failures (Yuan et al., 2018). Therefore, we can not exclude the possibility that these alterations also occurred in the GBH-exposed rats.

Alternative promoter usage enables the generation of multiple mRNA transcripts from a single gene. This alternative promoter system confers tissue-specific and temporal expression on the rat ER α gene (Ishii et al., 2010). When analyzing the transcriptional control of the ER α gene, we detected that transcript variants from exons E1, OT, O and OS are involved in its regulation in the uterus of control animals during the preimplantation period. The ER α -ON transcript variant was not detected in the uterus of control or GBH-exposed rats under the conditions of our study. This result is in concordance with Hattori et al., (2015) who reported that the ER α -ON variant in rats requires a higher number of PCR cycles than the other variants to be detected. The expression of the rat ER α promoters in reproductive organs (uterus, ovary, testis) is due to mainly to the utilization of O promoter, while OS and ON promoters contribute to the wider distribution (Ishii et al., 2010). In our lab, we have detected changes in the promoter usage in the rat uterus at different physiological conditions, such as estrous cycle, pregnancy and age. Monje et al., (2007) showed that the five promoters are

expressed in cycling rats, while in pregnant (Milesi et al., 2017) and aged rats (Vigazzi et al., 2016) the predominant variants are ER α -OS, ER α -O, ER α -OT, and ER α -E1.

In the present study, GBH-exposed rats showed that the mRNA levels of the ER α -O variant were higher than those in the control group. Selective promoter usage of the ER α gene in the presence of various EDCs (arsenic, BPA, endosulfan) has also been demonstrated in hormone-dependent organs in rats (Milesi et al., 2017; Parodi et al., 2015; Vigazzi et al., 2016). Furthermore, some authors (Hamada et al., 2005; Shimogawa et al., 2014) have proposed that whereas some promoters are dominantly activated to maintain the ER α expression level under normal conditions, specific promoters are activated under pathological conditions. Particularly, it was observed an increase in total ER α mRNA levels that was accompanied by an increase in ER α -O variant in rats with localized brain injury (Shimogawa et al., 2014). Our results suggest that specific activation of the ER α -O promoter in GBH-exposed rats might be related to the induction of ER α expression.

Experimental and human cohort studies have shown that altered epigenetic marks such as changes in DNA methylation and histone PTMs, induced by EDCs in early development, can persist into later life (Casati et al., 2015; Jacobs et al., 2017). In addition, certain health outcomes are detected after birth, but some of them are manifested later or in combination with an additional stressor (Jacobs et al., 2017). DNA methylation status of ER α promoter regions and the corresponding ER α expression levels are markedly modified across development and between distinct tissues as a consequence of normal physiological changes (Fürst et al., 2012; Westberry et al., 2010). However, numerous studies have indicated that aberrant methylation

within the ER α promoter regions is associated with ER α expression deregulation in pathological conditions such as breast and colon cancer and uterine leiomyomas (Agrawal et al., 2007; Asada et al., 2008).

To evaluate the methylation status of ER α -O promoter, we decided to study changes within CpG islands. Nearly 90% of CpG dinucleotides outside a CpG island are methylated, however, CpGs within an island are predominantly unmethylated, allowing for specific methylation and subsequently differential expression of genes (Jaenisch and Bird, 2003). Our findings showed a decreased methylation status at the *Bst*UI-1 site in the ER α -O promoter that correlated with higher ER α mRNA levels in the GBH-exposed rats. These findings are consistent with a recent study from our laboratory (Milesi et al., 2017), in which predominant hypomethylation in the E1, O, and OT promoters of the ER α gene in the preimplantation uterus was accompanied by an increase in ER α expression after neonatal exposure to endosulfan. In addition, neonatal BPA exposure induced hypermethylation of the ER α promoter region in the testes of adult male rats (Doshi et al., 2011). All these data suggest that changes in the DNA methylation level of the ER α promoter region is an epigenetic mechanism sensitive to environmental factors, especially in early life.

Transcriptional regulatory mechanisms are mediated by a set of transcription factors (TFs), which are proteins that have the ability to bind to a specific upstream regulatory sequence on genes and to regulate their transcription (Tapia et al., 2011). Methylation of gene promoter regions may influence mRNA expression by regulating the binding of TFs. Methylation has the potential to block TF binding through the interference of base recognition or recruitment of methylation-specific binding proteins (Tate and Bird,

1993). Interestingly, we identified a hypomethylated site in the GBH-exposed group, which is a potential binding site for the TFs E2F-1 and E47. Both TFs were predicted to be key regulators of endometrial receptivity in humans (Tapia et al., 2011). More recently, E2F-1 was also associated with embryo implantation in hamsters (Huang et al., 2018). Hence, these TFs might participate in the transcriptional upregulation of ER α in GBH-exposed rats.

Histone PTMs on specific amino acid residues change the structure of chromatin, leading to activation or suppression of transcription. Among these modifications, we evaluated the acetylation and methylation of lysine residues. Generally, lysine acetylation is associated with relaxation of chromatin, granting access for transcription factors and initiation of gene transcription (Santangeli et al., 2017). In our work, the H4Ac level was increased in all the sites studied within the ER α -O promoter in GBH-exposed rats, whereas H3Ac was only decreased in one of these sites. This suggests a rather relaxed chromosomal structure due to a predominant local effect of H4Ac, which might be related to the higher expression of ER α mRNA. On the other hand, lysine methylation of histones creates specific and unique signals depending on the residue modified (Lachner et al., 2003). The H3K27me3 mark, strongly associated with silencing of transcription (Bhan et al., 2014), was decreased in all the sites evaluated within the ER α -O promoter in GBH-exposed rats. Additionally, H3K9me3 was increased in 2 out of 3 sites evaluated and particularly interesting, at the putative TF binding site (*Bst*UI-1) in the GBH group. H3K9me3 has a well-established role in condensed chromatin formation (Peters et al., 2003). However, Vakoc et al., (2005) found that it is also associated with the transcribed regions of active genes in mammals, playing dual roles in both compact chromatin maintenance and transcription elongation.

Taken together, these findings indicate that the changes in histone PTMs in the ER α -O promoter induced by GBH could explain the increased expression of the ER α gene. Similar to our results, a globally altered pattern of histone PTMs was observed by early exposure to EDCs, such as polychlorinated biphenyls and the estrogenic chemical diethylstilbestrol in the liver and uterus of murine models, respectively (Casati et al., 2012; Jefferson et al., 2018). Some evidence supports that DNA methylation and histone PTMs pathways could regulate gene transcription acting together (Cedar and Bergman, 2009; Razin, 1998). In accordance with this idea, in our work, GBH-induced epigenetic changes in the regions studied were associated with alterations in both DNA methylation and histone PTMs. However, we could not discard that other regions of the ER α gene might be epigenetically modified by GBH exposure.

Several recent works have reported the levels of glyphosate in water, soil and dust (Bonansea et al., 2017; Mendez et al., 2017; Primost et al., 2017; Ronco et al., 2016). In Argentinian farms, glyphosate was reported in the water that is consumed by livestock (21.2 μ g/L glyphosate) (Demonte et al., 2018) and in genetically modified soybean (1.8 mg/kg) and plants (4.4 mg/kg) (Arregui et al., 2004). This evidence suggests that there is a risk of GBHs exposure, and stress concerns about their effects on human and animal health. Some studies carried out in rats have shown that glyphosate formulations cause detrimental reproductive effects at doses in the order of magnitude of the dose we currently studied and below the NOAEL. Dallegrave et al., (2007) and Romano et al., (2012) reported that GBH promoted behavioral, histological and endocrine changes in reproductive parameters in male rats, while in previous works, we showed that GBH impaired female reproductive performance (Ingaramo et al., 2016; Milesi et al., 2018). In addition, human biomonitoring studies detected glyphosate in serum and urine samples from pregnant women. Glyphosate concentrations were associated with

occupational and residence exposure (Kongtip et al., 2017; Parvez et al., 2018). More importantly, Parvez et al., (2018) found that higher glyphosate urine levels were correlated with shortened pregnancy. All these works support the fact that exposure to GBH formulations during critical periods of development cause detrimental effects on reproduction, and alert population about potential effects on human reproductive health.

To our knowledge, this is the first study reporting that a GBH formulation induces epigenetic modifications in adult female rats following *in utero* and lactational exposure. Here, we found that GBH upregulates uterine ER α mRNA expression and its ER α -O transcript variant during the preimplantation period. We propose that hypomethylation in association with altered patterns of histone PTMs in the O promoter of the ER α gene could explain ER α transcriptional deregulation in the uterus of GBH-exposed female rats. These results contribute to elucidate the GBH-induced implantation failures. Finally, this work increases evidence supporting the potential role of GBHs as EDCs.

Declaration of interest

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

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Figure legends

Fig. 1. Schematic representation of the experimental protocol used to study the effect of perinatal (gestation plus lactation) exposure to a GBH on the uterus of F1 female rats during the preimplantation period. GD: gestational day; PND: postnatal day.

Fig. 2. Schematic representation of the genomic organization of the promoter region of the rat ER α gene. The ER α gene comprises five alternative promoters: E1, OT, O, ON and OS, indicated by grey boxes. The common region to all the alternative transcripts of ER α is exon 1, indicated by a white box. Relative positions and orientations of promoter-specific real-time PCR primers are indicated by black arrows. Transcription initiation sites are indicated by +1 and ATG codon shows the translation start site.

Fig. 3. Effect of perinatal exposure to GBH on ER α transcription and the relative abundance of ER α transcripts with alternative 5'UTRs in the rat uterus during the preimplantation period. **(A)** mRNA relative expression of ER α **(B)** and the promoters ER α -OS, ER α -O, and ER α -OT and ER α -E1. The mRNA expression levels were measured by real-time RT-PCR and fold expression relative to control values was calculated by the relative standard curve method. Control values were assigned to a reference level of 1. Each column represents the mean \pm SEM (three determinations per animal of 8 animals per group). Asterisks indicate statistical significance compared to the control (*P < 0.05 vs. control).

Fig. 4. Effect of perinatal exposure to GBH on the methylation status of ER α -O promoter in the rat uterus during the preimplantation period. **(A)** Schematic representation of the O promoter, its binding proteins and methylation targeted CG areas. Predicted binding sites for transcription factors GATA-1, E47 and E2F-1 and CpG islands are shown. CG target sites for digestion by the methylation sensitive restriction enzymes *Bst*UI (CGCG) and *Mae*II (ACGT) are indicated by filled circles. Positions and orientations of PCR primers are indicated by black and grey arrows for the restriction enzyme sites and internal control, respectively. The transcription initiation site (TIS) is indicated by +1. **(B)** Methylation analysis of the ER α -O promoter using methylation-sensitive restriction enzymes. The relative methylation status in GBH-exposed rats is indicated as relative values versus those of control rats. Control values were assigned to a reference level of 1. Each column represents the mean \pm SEM (three determinations per animal of 8 animals per group). Asterisks indicate statistical significance compared to the control (* P < 0.05 vs. control).

Fig. 5. Analysis of histone post-translational modifications in ER α -O promoter in the uterus of GBH-exposed F1 rats during the preimplantation period. **(A, B)** Histone H3 and H4 acetylation **(C, D)** and histone H3 lysine trimethylation (H3K9 and H3K27 methylation) were evaluated in ER α -O promoter by ChIP assays. The samples values were normalized to INPUT expression and to the control animals. Control values were assigned to a reference level of 1. Each column represents the mean \pm SEM (three determinations per animal of 8 animals per group). Asterisks indicate statistical significance compared to the control (* P < 0.05 vs. control).

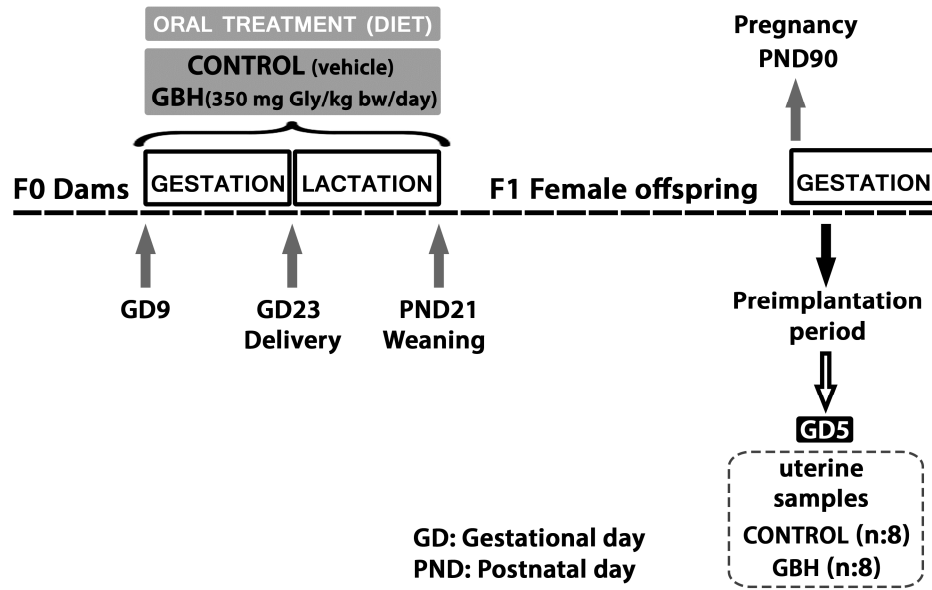
Table 1. Primers and PCR products for real-time RT-PCR experiments.

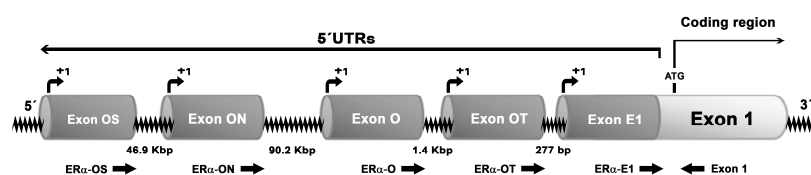
Gene	Primer sense (5'-3')	Primer antisense (5'-3')	Product size (bp)	Annealing temperature (°C)
L19	AGCCTGTGACTGTCCATTCC	TGGCAGTACCCTTCCTCTTC	99	60
ER α	ACTACCTGGAGAACGAGCCC	CCTTGGCAGACTCCATGATC	153	60
ER α -OS	CCCTCCTCTGCCATTGTCTA		166	58
ER α -ON	TCTGGGGCATCTCCTTCAA		193	57
ER α -O	AGCACATTCCTTCCTTCCG		196	58
ER α -OT	TCCAGCAGGTTTGCGATGT		164	59
ER α -E1	TAACCTCGGGCTCTACTCTT		133	57
Exon 1		ATTCCCGAGGCTTTGGTGT	-	-

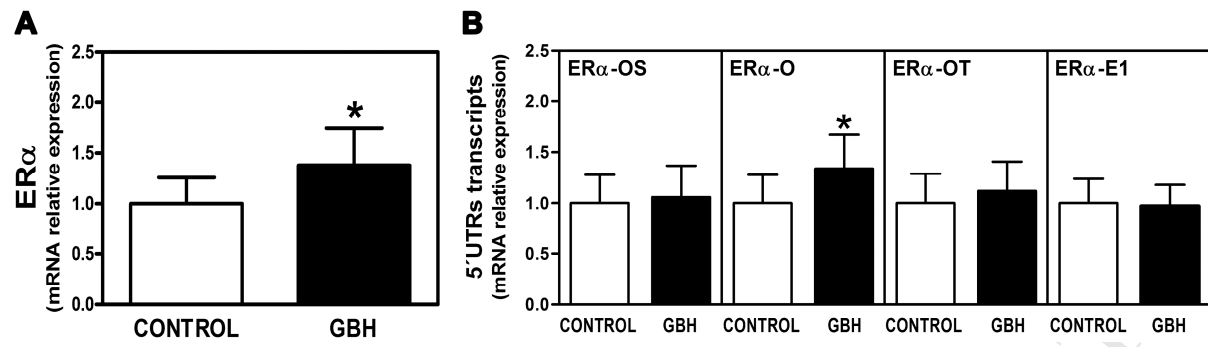
Table 2. Primers and PCR products for methylation-sensitive and ChIP analyses.

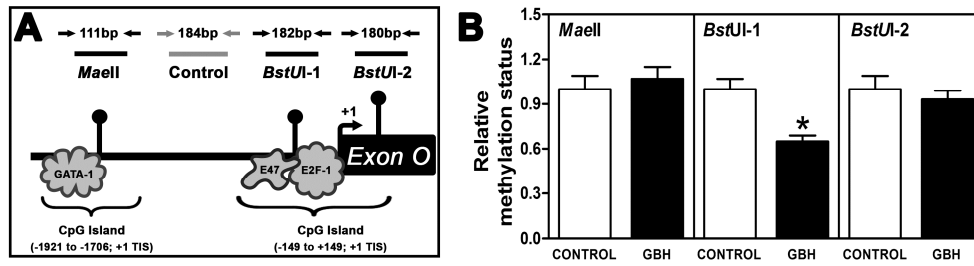
Gene	Primer sense (5'-3')	Primer antisense (5'-3')	Product size (bp)	Annealing temperature (°C)
IC ER α -O	TGGCTAGAGCAGTGGGGTTG	GGGGACTTTGGCTCTGGAGA	184	57
<i>Bst</i> UI- ER α -O	GGAATGCTGATTCTAGTGGT	TGTGTTTGTATGTGGAGTGG	182	57
<i>Bst</i> UI-2 ER α -O	GAGAGTCCCTGCCACTCCACAT	CCGATCCTACCCTGCTGGTT	180	57
<i>Mae</i> II ER α -O	AGAAGGCAAAGGAGTGTCAG	CCATCTTCATTCTCTCTCCG	111	53

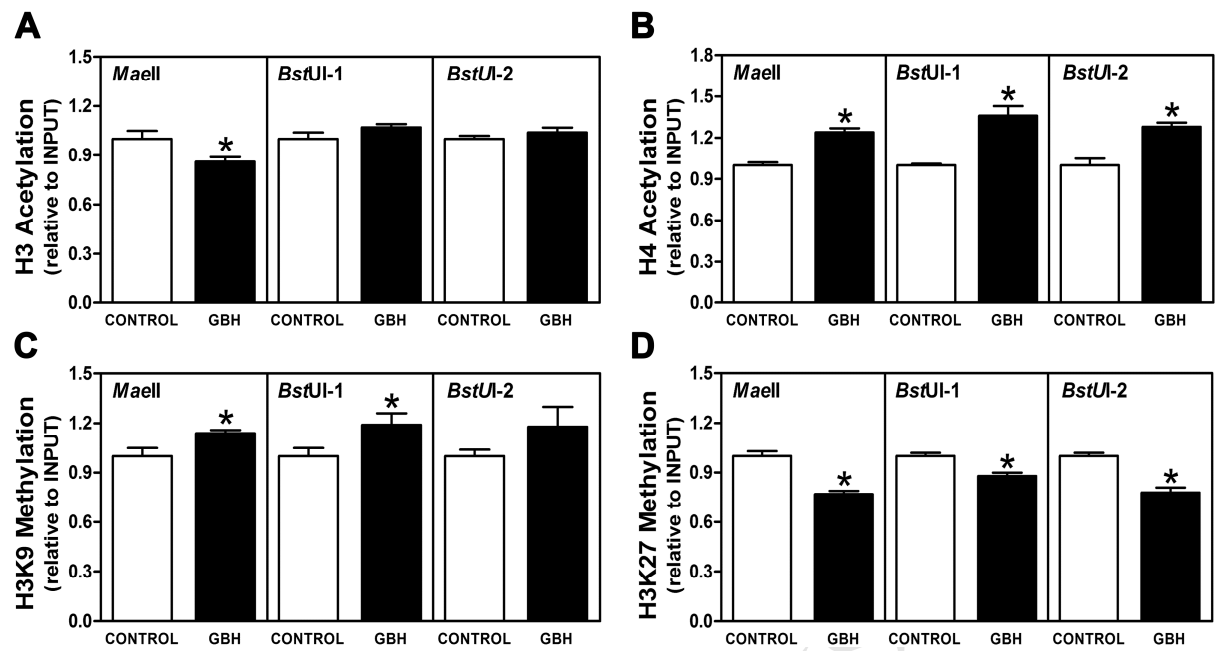
IC: Internal control











Highlights

- Perinatal exposure to a GBH upregulates uterine ER α mRNA during the preimplantation period.
- GBH increases the relative abundance of ER α -O transcript variant.
- GBH modifies methylation status and histone PTMs in the O promoter of ER α gene.
- These alterations might account for the GBH-induced implantation failures.