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Uterine ERα epigenetic modifications are induced by the endocrine disruptor endosulfan in female rats with impaired fertility

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ABSTRACT

High ERα activity may disrupt the window of uterine receptivity, causing defective implantation. We investigated whether implantation failures prompted by endosulfan are associated with aberrant ERα uterine expression and DNA methylation status during the pre-implantation period. ERα-dependent target genes that play a crucial role in the uterine receptivity for embryo attachment and implantation were also investigated. Newborn female rats received corn oil (vehicle, Control), 6 μg/kg/d of endosulfan (Endo6) or 600 μg/kg/d of endosulfan (Endo600) on postnatal days (PND) 1, 3, 5, and 7. On PND90, females were made pregnant and on gestational day 5 (GD5, pre-implantation period) uterine samples were collected. ERα expression was assessed at protein and mRNA levels by immunohistochemistry and real time RT-PCR, respectively. ERα transcript variants mRNA containing alternative 5'-untranslated regions (5′UTRs) were also evaluated. We searched for predicted transcription factors binding sites in ERα regulatory regions and assessed their methylation status by Methylation-Sensitive Restriction Enzymes-PCR technique (MSRE-PCR). The expression of the ERα-dependent uterine target genes, i.e. mucin-1 (MUC-1), insulin-like growth factor-1 (IGF-1), and leukemia inhibitory factor (LIF), was assessed by real time RT-PCR. Both doses of endosulfan increased the expression of ERα and its transcript variants ERα-OS, ERα-O, ERα-OT and ERα-E1. Moreover, a decreased DNA methylation levels were detected in some ERα regulatory regions, suggesting an epigenetic up-regulation of it transcription. ERα overexpression was associated with an induction of its downstream genes, MUC-1 and IGF-1, suggesting that endosulfan might alter the uterine estrogenic pathway compromising uterine receptivity. These alterations could account, at least in part, for the endosulfan-induced implantation failures.

1. Introduction

Endocrine-disrupting chemicals (EDCs) are synthetic or natural compounds in the environment that can interfere with endocrine functions (De Coster and van Larebeke, 2012; Roy et al., 2009; Schug et al., 2011). The primary targets of EDCs are the reproductive organs, and exposure during fetal or early postnatal life can induce developmental and reproductive disturbance (Miyagawa et al., 2011). Among these chemicals, organochlorine pesticides are of great concern as they accumulate in the human tissues because of their high lipophilicity and resistance to metabolism (Stoker et al., 2011). Endosulfan, an organochlorine pesticide recently banned but with long persistence in the environment, has been found in human placenta, adipose tissue, cord blood and breast milk samples (Muñoz-de-Toro et al., 2006; Shen et al., 2008; Silva and Gammon, 2009). Human and animal studies have reported developmental and reproductive disorders caused by endosulfan exposure (Dalsenter et al., 1999; Milesi et al., 2012, 2015; Saiyed et al., 2003; Silva and Gammon, 2009; Singh et al., 2007; Sinha et al., 2001).

Environmental factors can alter the progression of epigenetic programming during fetus development, and these aberrant marks might lead to abnormalities and diseases later in life (Singh and Li, 2012). One of the best known epigenetic modifications is DNA methylation. DNA methylation involves the covalent addition of a methyl group to a cytosine residue of a CpG dinucleotide. CpG-dense DNA regions, known as CpG islands, are located in particular gene promoters (or theirs surrounding areas), and are associated with regulatory functions in gene transcription (Kanherkar et al., 2014). Methylation of CpG islands promotes the recruitment of methyl CpG binding proteins (MBDs) which interact with transcription repressors and chro-

Abbreviations: Endo6, female rat group treated with 6 μg/kg/d of endosulfan; Endo600, female rat group treated with 600 μg/kg/d of endosulfan; EDCs, endocrine-disrupting chemicals; PND, postnatal day; GD, gestational day; RfD, reference dose; NOEL, no observed effect level; ERα, estrogen receptor alpha; 5′UTRs, 5'-untranslated regions; MUC-1, mucin-1; IGF-1, insulin-like growth factor-1; LIF, leukemia inhibitory factor; IOD, integral optical density; IC, internal control; MSRE-PCR, Methylation-Sensitive Restriction Enzymes-PCR technique

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matin remodeling factors (Bird, 2002; Li, 2002). These changes in DNA methylation and chromatin organization induce gene silencing by interrupting the recognition and binding of transcription factors (Shiota, 2004).

Endosulfan has been classified as a xenoestrogen, and both *in vitro* and *in vivo* studies have demonstrated that its estrogenic actions are mediated mainly via ER signaling pathways (Lemaire et al., 2006; Soto et al., 1994; Varayoud et al., 2008). ERα is a member of the nuclear receptor superfamily and specifically binds to estrogen or estrogen like chemicals regulating transcription of target genes. In the uterus, ERα is the main mediator of estrogen action, playing a key role on development and reproduction. It has been reported that multiple promoters might be a common feature of steroid hormone receptors (Kos et al., 2001). In the rat, it has been identified five promoters called OS, ON, O, OT, and E1 which control ERα transcription initiation yielding different transcripts with alternative 5′UTRs (Monje et al., 2007). Splicing of 5′UTRs exons to the acceptor splice site in the first coding exon results in multiple transcripts encoding the same full-length protein. These mRNA variants differ only in their 5′UTRs (Kos et al., 2001). Little is known about the precise function of multiple ERα promoters; however, it has been demonstrated that they confer tissue-specific expression, as well as, developmental stage-specific regulation of ERα gene in many organs (Kato et al., 1998; Hamada et al., 2005). Aberrant methylation of CpG islands of the ERα promoters has been associated to increased incidence of neoplasia and reproductive disorders (Doshi et al., 2011; Issa et al., 1994, 1996).

In a previous study we demonstrated that low doses of endosulfan (doses similar to the no observed effect level (NOEL) and the reference dose (RfD)), induces subfertility in female rats, characterized by a drop in the pregnancy rate and in the number of implanted embryos (Milesi et al., 2015). These alterations were associated with a decrease in the uterine stromal cell proliferation at the pre-implantation period and a disruption of a progesterone-mediated pathway involved in its control. Besides progesterone, uterine receptivity is regulated by E_2, and the coordinate effects of both hormones determine the "window" for blastocyst implantation (Cha et al., 2012). It has been demonstrated that high E_2 levels and/or ERα activity in early pregnancy may disrupt the window of uterine receptivity, causing defective implantation (Ma et al., 2003; Lee et al., 2010). Taking into account the crucial role of uterine ERα on embryo implantation, in the present study we evaluated the long-term effects of early postnatal endosulfan exposure over the control of ERα transcription and translation, investigating the relative abundance of ERα transcripts with alternative 5′UTR exons and the methylation status of ERα gene promoters during the pre-implantation period. We also assessed the expression of ERα-dependent target genes, i.e. MUC-1, IGF-1, and LIF, which play a crucial role in the uterine receptivity for embryo attachment and implantation (Kawagoe et al., 2012).

The results showed that neonatal exposure to endosulfan increases ERα at transcription and translation levels in the pre-implantation uterus, and induces changes in the relative abundance of ERα transcripts with alternative 5′UTRs. We also detected hypomethylation in several CpG sites in CpG islands of ERα promoters, indicating that an alteration in DNA methylation could be a mechanism of endosulfan-altered ERα transcription. We also detected aberrant expression of ERα-dependent target genes associated with the implantation process, which may compromise uterine receptivity.

2. Materials and methods

2.1. 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. The rats, which were of an inbred Wistar-derived strain bred in the Department of Human Physiology (Universidad Nacional del Litoral), were housed in a controlled environment (22 ± 2 °C; lights on from 06:00 to 20:00 h) with 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 was equivalent for the control and experimental rats (our unpublished observations), we assumed that all the animals were exposed to the identical levels of phytoestrogens. To minimize additional exposure to EDCs, the rats were housed in stainless steel cages with wood bedding, and tap water was supplied *ad libitum* in glass bottles with rubber stoppers surrounded by a steel ring.

2.2. Experimental design

The pups were obtained from timed-pregnant Wistar rats housed singly. After delivery (PND0), the pups were sexed according to the anogenital distance and cross fostered by distributing the pups of each litter among the mothers. This procedure allowed us to minimize the use of siblings and thus avoid potential litter effects. Cross-fostered litters were adjusted to 10 pups, with 10 female pups per litter, if possible. When fewer than 10 females were available, an appropriate number of males were retained. As litter sex ratio is an important variable in both physiological and behavioral outcomes (Crews et al., 2009), we checked that "litter effect" did not affect outcomes by considering it as a random co-variate (data not shown). The female pups from each foster mother were assigned to one of the following neonatal treatment groups: 1) the control group that received corn oil vehicle alone 2) endosulfan (with 98% purity; Chem Service, West Chester, PA, USA) at 6 μg/kg (Endo6), or 3) endosulfan at 600 μg/kg (Endo600). The treatments were administered directly to the pups by s.c. injections in the nape of the neck every 48 h from PND1 to PND7. All female pups within a cross-fostered litter received the same treatment. The low dose of endosulfan used was similar to the RfD established for this pesticide, whereas the high dose was 100-fold greater than the RfD and equal to the NOEL. The NOEL established for endosulfan was determined on the basis of several adverse effects such as decreased female body weight, hematological changes and kidney pathology observed in different animal models (ATSDR, 2000; Marshall and Rutherford, 2003). However, and taking into account that the NOEL is a rather random regulatory threshold, it could not rule out the possibility that below it other adverse effects not detected in the classical evaluations may occur. Neither signs of acute or chronic toxicity nor significant differences in weight gain between the endosulfan-exposed and control pups were recorded during the experiment (data not shown). No alterations in maternal care were detected between the different experimental groups.

Female rats were weaned on PND21, and then four were housed in each cage and held without further treatment. On PND90, the female rats were housed for two consecutive weeks with sexually ma-

ture untreated males of the same strain and of proven fertility to allow several possible matings. 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 was considered the GD1. Control (n = 12) and endosulfan-exposed pregnant female rats (Endo6, n = 12; Endo600, n = 12) were sacrificed on the morning of GD5 (pre-implantation period), and uterine tissue was collected. One uterine horn from each rat was fixed by immersion in a 4% paraformaldehyde buffer for 24 h at 4 °C, embedded in paraffin and processed for the immunohistochemical assay. The other uterine horn was placed immediately in liquid nitrogen and stored at −80 °C for RNA or DNA extraction.

2.3. Immunohistochemistry

A standard immunohistochemical (IHC) technique (avidin-biotin-peroxidase) was performed to evaluate the expression of ERα in the uterus from control and endosulfan-treated rats, using previously described protocols (Muñoz-de-Toro et al., 1998). Briefly, longitudinal uterine sections (5-μm thick) were deparaffinized and dehydrated in graded ethanol, and then subjected to a microwave pretreatment for antigen retrieval. The endogenous peroxidase activity and non-specific binding sites were blocked. The samples were incubated in a humid chamber first with the mouse monoclonal anti-ERα antibody (clone 6F-11, 1/400 dilution, Novocastra, Newcastle upon Tyne, UK) for 14–16 h at 4 °C and then with the anti-mouse secondary antibody (biotin conjugate, B8774, 1/100 dilution, Sigma, St. Louis, MO) for 30 min at room temperature. The reactions were developed using the streptavidin-biotin peroxidase method and tablets of diaminobenzidine (Sigma) as a chromogenic substrate. The samples were dehydrated and mounted with a permanent mounting medium (Eukitt, Sigma). Immunohistochemical assays were performed in two runs, each one including samples from the different experimental groups (i.e. controls and samples of treated groups), as well as, positive and negative controls.

2.4. Quantification of ERα protein expression by image analysis

The expression of ERα protein in the subepithelial stroma was evaluated by image analysis using the Image Pro-Plus 5.0.2.9 system (Media Cybernetics, Silver Spring, MD, USA), as previously described (Ramos et al., 2002). In brief, the images were recorded with a Spot Insight V3.5 color video camera attached to an Olympus BH2 microscope (Olympus Optical Co., Ltd., Tokyo, Japan) with a Dplan 40 × objective (numerical aperture ± 0.65; Olympus) and converted to a gray scale. The integrated optical density (IOD) was measured as a linear combination of the average gray intensity and the relative area occupied by the positive cells (Ramos et al., 2001, 2002). Since the IOD is a dimensionless parameter, the results were expressed as arbitrary units. Taking into account the heterogeneity of uterine stroma, IHC analysis and protein quantification were performed by duplicated on two uterine sections, separated 50 μm from each other. For that purpose, longitudinal uterine sections were collected once the lumen was reached all along the tissue. Moreover, in each uterine section protein quantification was performed on at least 10 randomly selected fields with a magnification of ×400, which allowed covering a vast area of the tissue.

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

An optimized PCR protocol was employed to analyze the relative expression levels of total ERα mRNA, the ERα transcript variants containing alternative 5'-untranslated regions OS, ON, O, OT, and E1, and the ERα-dependent uterine target genes, MUC-1, IGF-1, and LIF. Fig. 1 shows the genomic organization of the promoter region of the rat ERα gene. Uterine samples from each experimental group were individually homogenized in TRIzol (Invitrogen, Carlsbad, CA, USA), and RNA was prepared according to the manufacturer's protocol. The concentration of total RNA was assessed by A_{260}, and the samples were stored at −80 °C until later analysis. 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, Madison, WI). 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 1 × 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 μl and further amplified in triplicate using the Real-Time Rotor-Gene Q Cycler (Qiagen; Hilden; Germany). Primer pairs used for amplification of ribosomal protein L19 (L19, housekeeping gene), total ERα, ERα 5′UTRs, MUC-1, IGF-1, and LIF 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 μl 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 μl. Each sample was quantified in duplicate. 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, an-

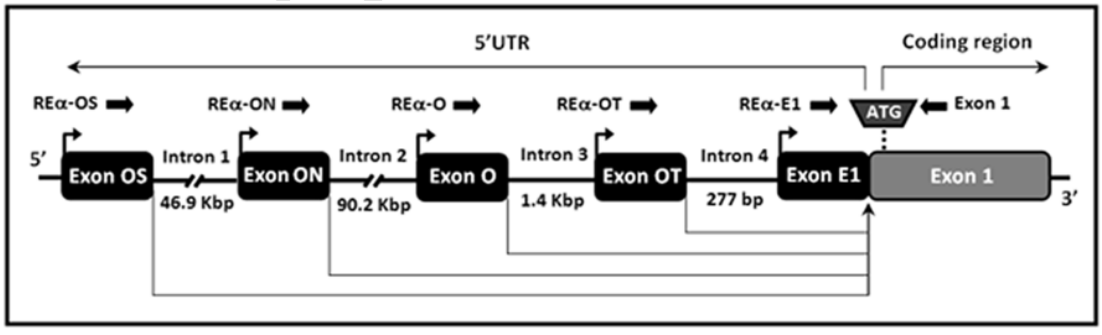

Fig. 1. Schematic representation of the genomic organization of the promoter region of the rat ERα gene. Relative positions and orientations of promoter-specific real-time PCR primers are indicated by black arrows. The common region to all the alternative 5′UTRs of ERα is indicated by a solid grey box.

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

Target	Primer sequence (5′-3′)	Product size (bp)
L19	Forward: GAAATCGCCAATGCCAACTC Reverse: ACCTTCAGGTACAGGCTGTC	290
ERα	Forward: ACTACCTGGAGAACGAGCCC Reverse: CCTTGGCAGACTCCATGATC	153
ERα-E1	Forward: TAACCTCGGGCTCTACTCTT	133
ERα-OT	Forward: TCCAGCAGGTTTGCGATGT	164
ERα-O	Forward: AGCACATTCCTTCCTTCCG	196
ERα-ON	Forward: TCTGGGGCATCTCCTTCAA	193
ERα-OS	Forward: CCCTCCTCTGCCATTGTCTA	166
Exon 1	Reverse: ATTCCCGAGGCTTTGGTGT	–
MUC-1	Forward: CACTCACGGACGCTATGTGC Reverse: CGCTACTGCCATTGCCTGTC	80
IGF-1	Forward: CTCAAGGATGGCGTCTTCAC Reverse: GAACTTGCTCGTTGGACAGG	137
LIF	Forward: AGAGTCAACTGGCTCAACTC Reverse: CTTATCCACGTTGTTGGGAA	95

nealing 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 Rotor-Gene Q – Pure Detection software (Version: 1.7, Quiagen; Tecnolab). The efficiency of PCR reactions was assessed for each target by the amplification of serial dilutions (over five orders of magnitude) of cDNA fragments of the transcripts under analysis. Accordingly, the fold expression over control values was calculated for each target by the relative standard curve methods, which are designed to analyze data from real-time PCR (Cikos et al., 2007). For all experimental samples, the target quantity is determined from the standard curve, normalized to the quantity of the housekeeping gene and finally divided by the target quantity of the control sample. No significant differences in CT values were observed for L19 among the experimental groups.

2.6. Bioinformatics

The ERα 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 *BstU*I enzyme to evaluate the number of methylation-sensitive sites. To recognize the putative binding sites for transcription factors, we used the TFSEARCH program (http://www.cbrc.jp/research/db/TFSEARCH.html). PCR primers were designed with the software Vector NTI Suite Version 6.0.

2.7. Methylation-sensitive analysis

We investigated the methylation status of the ERα promoters in experimental groups using a combination of digestions with methylation-sensitive restriction enzymes and subsequent real-time PCR analysis (Bruce et al., 2008; von Kanel et al., 2010; Rossetti et al., 2016). 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 ADN 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) 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). A 130 ng sample of *Hin*dIII-cleaved DNA was digested overnight with 2 units of *BstU*I (New England BioLabs, Beverly, MA) and 1× enzyme buffer at 60 °C, in a covered water bath (Tecno Dalvo, Santa Fe, Argentina) to ensure complete digestion. 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 ERα promoters. For DNA amplification, 5 μl of DNA was combined with HOT FIREPol EvaGreen qPCR Mix Plus and 10 pmol of each primer (Invitrogen, Carlsbad, CA) to a final volume of 20 μl. Each sample was quantified in duplicate. 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 50–60 °C for 15 s, and extension at 72 °C for 15 s. The product purity was confirmed by dissociation curves, and random samples were subjected to agarose gel electrophoresis. The CT for each sample and the PCR reaction efficiencies were calculated as described in section 2.3. A region devoid of *BstU*I restriction sites was amplified as an internal control (IC). Primer pairs were designed with the software Vector NTI Suite Version 6.0 and are shown in Table 2.

When a CpG-rich site is methylated, enzymatic digestion with *BstU*I is not possible, allowing amplification of the fragment. In contrast, if the CpG-rich site is not methylated, *BstU*I 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 (internal control), yielding standard curves for the quantification of unknown samples (Cikos et al., 2007).

2.8. Statistical analysis

Differences in protein, mRNA and DNA levels between control and endosulfan-treated groups were analyzed using Kruskal-Wallis test followed by Dunn's method for the multiple comparisons. The statistical software package GraphPad Prism Version 5.03 was used with a significance level of 0.05. All results are expressed as the mean ± S.E.M.

Table 2
Primers and PCR products for real-time quantitative RT-PCR experiments.

Target	Primer sequence (5′-3′)	Product size (bp)
IC Promoter E1	Forward: TTCACACCAAAGCCTCGGGA Reverse: ATCTTGAGCTGCGGGCGATT	85
*BstU*I Promoter E1	Forward: CTCTTTTAACCTCGGGCTCT Reverse: GAGGTACAGATTGGCTTCCC	87
IC Promoter OT	Forward: GTTTGAGAAAGCAACTTACC Reverse: GAATCCTACAAGTCCAGAAA	142
*BstU*I Promoter OT	Forward: CCAGGAAAGTTAAGTTCAGG Reverse: GTTCTTAGACATCGCAAACC	127
IC Promoter O	Forward: TGGCTAGAGCAGTGGGGTTG Reverse: GGGGACTTTGGCTCTGGAGA	184
*BstU*I (a) Promoter O	Forward: GGAATGCTGATTCTAGTGGT Reverse: TGTGTTTGTATGTGGAGTGG	182
*BstU*I (b) Promoter O	Forward: GAGAGTCCCTGCCACTCCACAT Reverse: CCGATCCTACCCTGCTGGTT	180

3. Results

3.1. Effects of neonatal exposure to endosulfan on uterine ERα expression during the pre-implantation period

The long-term effects of neonatal endosulfan exposure on ERα expression were assessed via analysis of protein and mRNA levels in uterine tissue collected during the pre-implantation period (GD5). Treatment with both doses of endosulfan increased the expression of ERα mRNA relative to control rats, while only the Endo600 group elicited an increase in the expression of ERα at protein level in the subepithelial stroma (Fig. 2A–B). No changes were observed in ERα protein expression in the glandular epithelium, and no immunostaining was detected in the luminal epithelium (data not shown). Based on microscopic examination, we observed that changes in ERα protein expression in the E600 group were related to changes in level of expression within cells (the intensity of immunostaining). Photomicrographs illustrating these results are shown in Fig. 2 C.

3.2. Endosulfan modifies the relative abundance of ERα transcripts with alternative 5'UTRs

To determine whether the increase in total ERα mRNA expression was associated with changes in transcriptional promoter usage, relative expression levels of all exons encoding 5′UTR of the rat ERα gene were studied using a real-time PCR approach. We determined that ERα gene transcription in the receptive uterus is regulated by means of promoters associated with the 5′UTRs exons OS, O, OT and E1, regardless of treatment group. Under the experimental conditions of our study, the expression of transcripts containing the ON exon could not be detected in the uterine samples. We also found that exposure to both doses of endosulfan modified the abundance of the active transcript variants. In fact, female rats from Endo6 and Endo600 groups showed an increase in the expression of the ERα-OS, ERα-O, ERα-OT and ERα-E1 transcript variants (Fig. 3A–D).

3.3. In silico analysis of candidate sites of DNA methylation and potential transcription binding sites in the rat ERα promoter regions

In light of these results, our next aim was to investigate whether the ERα active transcript variants in the uterus are epigenetically regulated. In order to search potential sites for DNA methylation we analyzed their promoter regions for CpG islands and checked for restriction sites for *BstU*I enzyme. In addition, we searched for transcription factors that regulate the putative sites studied of DNA methylation. Fig. 4 shows the maps of E1 (A), OT (B), and O (C) associated promoters, their binding site for transcription factors and methylation-targeted CG areas. In the E1 promoter and Exon 1 we identified one CpG island, located at −11 to +440, where one restriction site for *BstU*I was found, which is potentially regulated by Egr-2 and Egr-3 transcription factors. In the OT promoter we found one CpG island, located at +350 to +573, with three restriction sites for *BstU*I associated with Stat-x and Gata (1, 2 and 3) transcription factors. As for O promoter, we identified one CpG island (−149 to +149) with two restriction sites for *BstU*I, associated with the E47 transcription factor. Neither CpG islands nor CG target sites for digestion by *BstU*I were found in OS promoter.

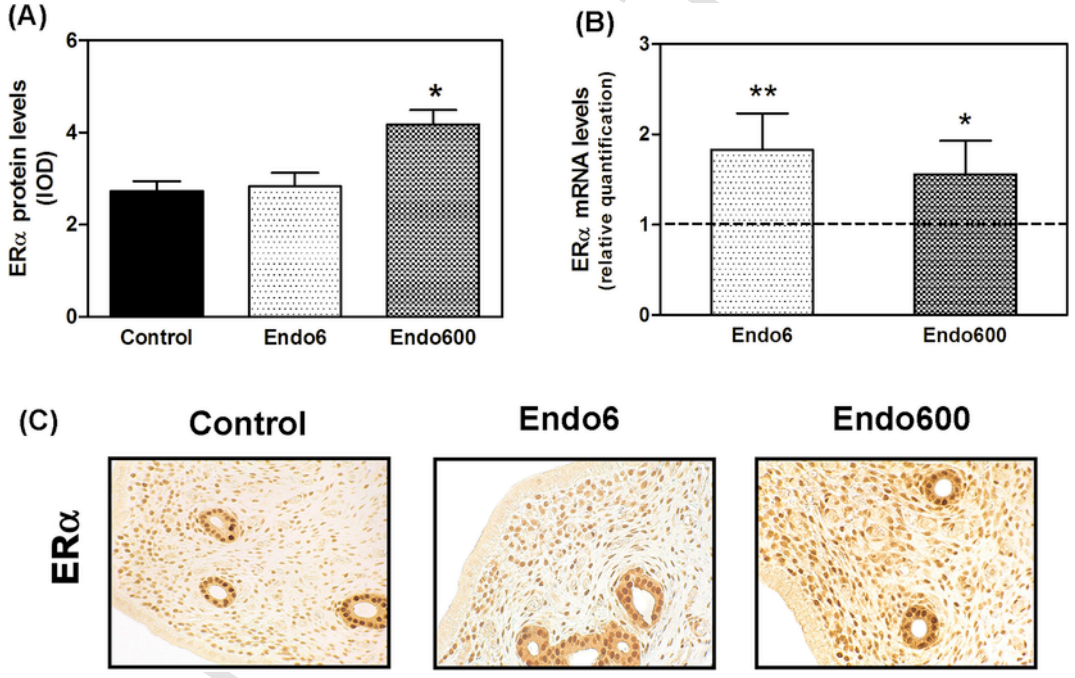

Fig. 2. Effect of neonatal endosulfan exposure on ERα transcription and translation in the rat uterus during the pre-implantation period. (A) Protein levels were analyzed by immunohistochemistry and the results are expressed as IOD. Each column represents the mean ± SEM of two sections per animal. (B) Relative mRNA levels were measured by real-time RT-PCR and fold expression from control values were calculated by the relative standard curve method. Control values were assigned to a reference level of 1 and values are given as mean ± SEM of two independent determinations. *Asterisks* indicate statistical significance compared with the control (*P < 0.05; **P < 0.01 *vs.* control). (C) Representative photomicrographs of uterine ERα protein expression of control and endosulfan-treated rats. LE, luminal epithelium; GE, glandular epithelium; ST, subepithelial stroma. Original magnification, × 400.

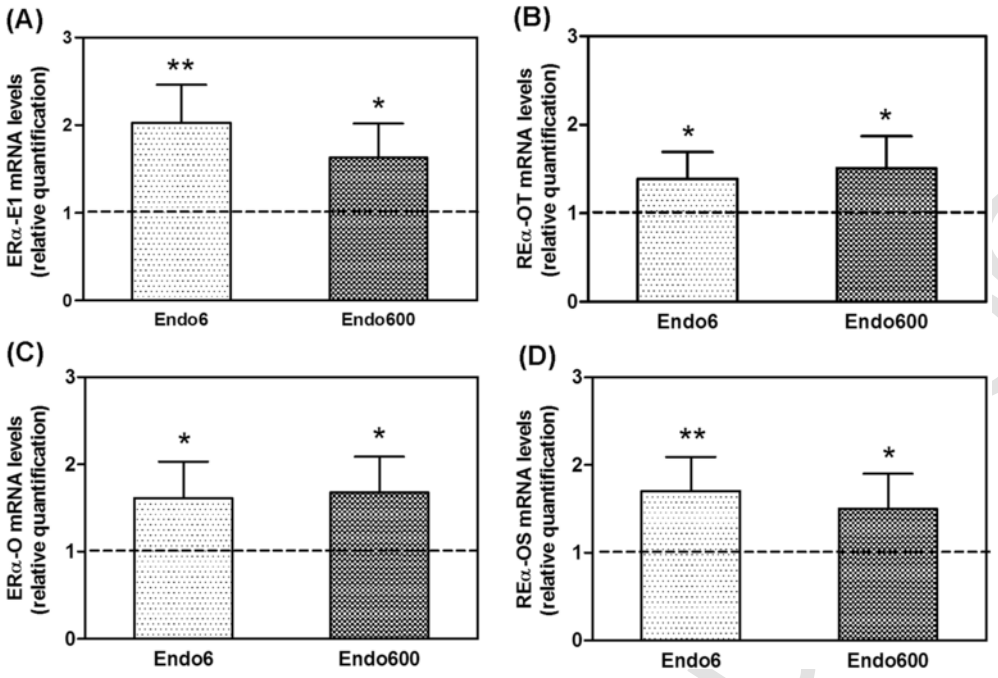

Fig. 3. Effect of neonatal endosulfan exposure on the relative abundance of ERα transcripts with alternative 5′UTRs in the rat uterus during the pre-implantation period. Relative mRNA levels of the promoters ERα-E1 (A), ERα-OT (B), and ERα-O (C) and ERα-OS (D) were measured by real-time RT-PCR and fold expression from control values were calculated for each target by the relative standard curve method. Control values were assigned to a reference level of 1 and values are given as mean ± SEM of two independent determinations. *Asterisks* indicate statistical significance compared with the control (*P < 0.05; **P < 0.01 *vs.* control).

3.4. Neonatal exposure to endosulfan alters the methylation status of ERα transcriptional promoters during the pre-implantation period

To elucidate if endosulfan-induced changes in the O, OT and E1 transcript variants levels are associated to differential DNA methylation, we determined the methylation status of the transcriptionally active promoters and surrounding DNA areas. Genomic DNA extracted from the uterus during the pre-implantation period was incubated with the *BstU*I restriction enzyme, and the targeted DNA regions were studied by real-time PCR. An internal control designed within the promoter region or surrounding areas was used as a PCR control for quantitative analysis. In the E1 promoter, a decrease in the methylation status was detected at the *BstU*I site in the female rats exposed to both doses of endosulfan, when compared with the controls (Fig. 5 A). Similar results were obtained at the *BstU*I site of OT promoter in the Endo6 group (Fig. 5 B). As for O promoter, DNA methylation pattern varied depending on the endosulfan dose and the restriction site studied. At the *BstU*I site (a) both doses of endosulfan decrease the methylation status respect to the control (Fig. 5 C). The same result was found at the *BstU*I site (b) for the Endo6 group, while the Endo600 group showed an increase in the methylation status (Fig. 5 D).

3.5. Aberrant ERα expression disrupted the expression of ERα-target genes that regulate uterine receptivity

To elucidate a possible mechanism underlying the implantation failure, we investigated whether the aberrant expression of ERα was associated with abnormal expression of MUC-1, IGF-1, and LIF, all ERα-dependent target genes that play a crucial role for uterine receptivity in early pregnancy. Both Endo6 and Endo600 groups showed increased expression of MUC-1 and IGF-1 mRNAs (Fig. 6A and B). No changes were observed in LIF mRNA expression between groups (Fig. 6C).

4. Discussion

In the present work we showed for the first time that neonatal exposure to endosulfan increases the expression of ERα and its transcript variants, ERα-OS, ERα-O, ERα-OT and ERα-E1, during the pre-implantation period. Our results also provide novel evidence about the mechanism trough which endosulfan might alter ERα expression, by finding DNA hypomethylation in several CpG sites in CpG islands of ERα promoters in endosulfan-exposed rats. We also demonstrated that aberrant ERα expression is associated with a disruption of ERα-dependent uterine target genes that play a crucial role for embryo implantation.

Embryo implantation is a complex process that requires the interaction between developmentally competent blastocysts and a receptive uterus. The establishment of uterine receptivity to support blastocyst implantation primarily depends on the coordinated effects of estrogen and progesterone. Prior to implantation, ovarian steroids acting trough theirs nuclear receptors, activate the transcription of genes that stimulate uterine proliferation and differentiation (Review in Varayoud et al., 2014). In a previous work, we demonstrated that neonatal exposure to endosulfan induced subfertility, characterized by a drop in the pregnancy rates and pre-implantation embryo loss (Milesi et al., 2015). These alterations were associated with a defective uterine stromal cell proliferation and a disruption of one of the main progesterone-dependent endocrine pathways that regulate this process, i.e., progesterone receptor/coregulators/Hoxa10. Overall, much interest has been focused in exploring the progesterone-mediated pathways involved in the control of uterine decidualization; however, until recently the role of ERα in this process remained poorly understood. Using ERα knockout models, Pawar et al. (2015) demonstrated that epithelial ERα directs differentiation of uterine

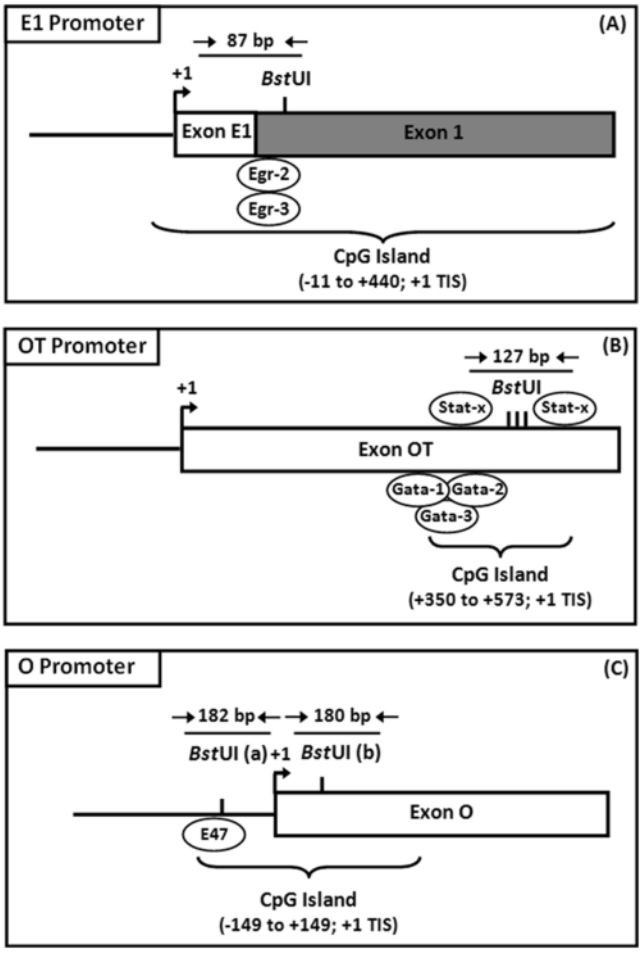

Fig. 4. Maps of E1 (A), OT (B) and O (C) promoters, their binding proteins and methylation-targeted CG areas. Predicted binding sites for transcription factors Egr, Stat, Gata, and E47 are shown as circles. CpG islands and CG target sites for digestion by the *BstU*I (CGCG) methylation-sensitive restriction enzyme are indicated. Positions and orientations of PCR primers are indicated by black arrows. +1 TIS: Transcription initiation site.

stromal cells via a paracrine mechanism during early pregnancy. The authors reported that a deregulation of uterine ERα signaling during pregnancy may impair epithelial-stromal dialogue, and contributes to endometrial dysfunction and early pregnancy loss. In the present work, we found that implantation failures prompted by early postnatal endosulfan exposure is also associated with a uterine disruption of the endocrine pathway regulated by ERα during the pre-implantation period. The endosulfan selected doses produced different effects on ERα expression; Endo6 increased the ERα mRNA, but not ERα protein, while Endo600 increased both mRNA and protein expression. In this context, we propose that Endo6 could differentially response to the uterine hormonal milieu on day 5 of pregnancy, leading to a high protein turnover due to higher activity of the ubiquitin-proteosome pathway. It has been reported that proteasome-mediated proteolysis modulates the cellular concentration of ERα in a process that requires ligand binding to the receptor (Preisler-Mashek et al., 2002). Moreover, it has been demonstrated that the extent to which the overall ERα levels are affected depend on the ligand and is not related to ligand-binding affinity or activation of transcription (Preisler-Mashek et al., 2002).

Our results also demonstrated a deregulation of ERα-dependent target genes associated with the implantation process in endosulfan-exposed females. In fact, both doses of endosulfan increased the uterine expression of MUC-1 and IGF-1 during early pregnancy. MUC-1 is a glycoprotein line the apical surface of epithelial cells which acts as an antiadhesive masking molecule. During the pre-implantation period, MUC-1 must be timely down-regulated in the luminal epithelium throughout the uterus to allow embryo attachment (reviewed in Dey et al., 2004). Based on our results, the increased MUC-1 might cause blastocyst attachment failure in endosulfan exposed females with impaired implantation. As for IGF-1, it is an important growth factor that modulates steroid hormone actions in the endometrium stimulating proliferation and differentiation. It is well known that the deregulation of the insulin-like growth factor system may compromise uterine receptivity (reviewed in Dey et al., 2004). Taken together, the present and previous results (Milesi et al., 2015; Ingaramo et al., 2016) show different uterine defects that could be associated with the endosulfan-induced implantation failures. However, we cannot rule out that other failures such as reduction of fertilization rate of the oocytes, defects in tubal transport and/or embryo development may occur in the same animals.

As for the ERα gene transcriptional regulation, we found that transcript variants derived from exons E1, OT, O and OS are implicated in its control in the uterus during the pre-implantation stage, despite treatment group. Under the condition of our study, ERα-ON transcript variant was not detected in the uterus of control and endosulfan-treated rats. To date, the function of multiple promoters in the ER gene has not been completely elucidated. It has been demonstrated that a mechanism of promoter selection is involved in the complex stage- and region-specific regulation of ERα gene expression in many organs (Kato et al., 1998; Hamada et al., 2005). That means that different tissues use different promoters, and that different promoters are used in different physiological or developmental stages (Kos et al., 2001). Osada et al. (2001) reported that the levels of ERα mRNA containing the 5′ UTR OT exon was highest in the classical estrogen target tissues such as the anterior hypophysis and uterus. Ishii et al. (2010) found that the expression of ERα-O mRNAs is detected preferentially in the reproductive organs, while the ERα-OS mRNAs are distributed extensively in reproductive and non-reproductive organs. These authors also reported that the levels of ERα containing the ON exon exhibit similar expression patterns to the ERα-OS mRNA, but that its expression is too low, which is agreement with our findings. On the other hand, previous results from our lab showed that unlike pregnant females, cycling adult females express the transcript variant derived from exon ON in the uterine tissue, suggesting that this transcript variant is differentially expressed according to the physiological stage (Monje et al., 2007). Several studies have demonstrated that some promoters are active, albeit to different extents, in several tissues. It has been suggested that these promoters result in basal levels of ER mRNA and that the tissue-specific promoters refine the level of ER expression according to the requirements of the cell (Kos et al., 2001).

Our results also showed that neonatal exposure to endosulfan affects the relative abundance of the active alternative 5′UTRs ERα transcripts in the receptive uterus. In fact, the increased expression of ERα mRNA levels in endosulfan-treated rats was at the expense of the upregulation of ERα-E1, ERα-OT, ERα-O and ERα-OS transcript variants. In a previous work performed in our laboratory, it has been demonstrated that bisphenol A (an organic compound used in the manufacture of polycarbonate plastic and epoxy resins), differentially activates the ERα gene promoters in the hypothalamus in a dose-sensitive manner inducing different effects in gene transcription and translation (Monje et al., 2007). It has been reported that regulatory elements and short open reading frames in the 5′ UTR can control the

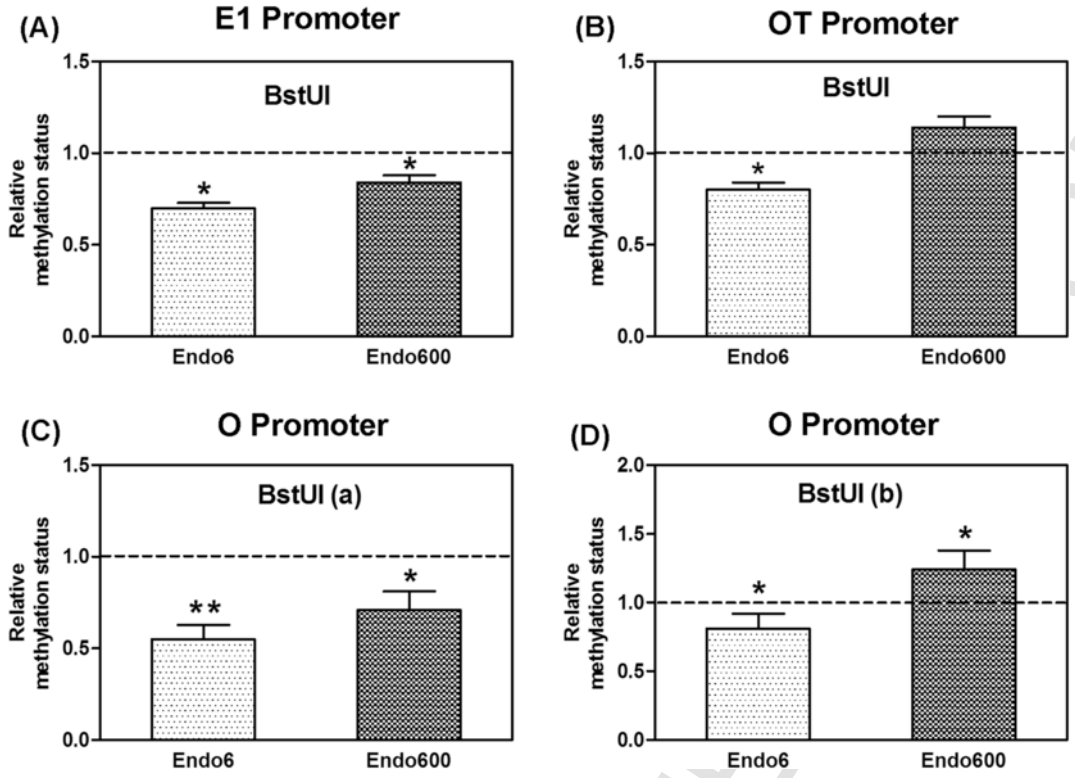

Fig. 5. Effect of neonatal endosulfan exposure on the methylation status of ERα gene promoters in the rat uterus during the pre-implantation period. Methylation-sensitive restriction sites of the E1 (A), OT (B) and O (C–D) promoters were studied. The relative methylation status in endosulfan-exposed rats is indicated as relative values versus those of control rats. Control values were assigned to a reference level of 1 and values are given as mean ± SEM of two independent determinations. *Asterisks* indicate statistical significance compared with the control (*P < 0.05; **P < 0.01 *vs.* control).

translation of mRNA (Gray and Wickens, 1998). Several regulatory roles have been assigned to these 5'-upstream open reading frame, such as limiting the protein expression by a translation reinitiation mechanism (Kos et al., 2002; Blaschke et al., 2003). In the present work both doses of endosulfan alters the abundance of ERα transcript variants in the same manner probably inducing adverse molecular and functional uterine effects.

Modification of DNA methylation under physiological conditions plays a crucial role in the regulation of several biological processes such as, cell fate-specific expression changes and cellular proliferation and differentiation, both in embryonic and adult tissues (Kanherkar et al., 2014). This epigenetic event remodels the chromatin structure and determines the state of gene transcription, not only permanently, but also transiently under certain stimuli such as hormonal changes during the estrous cycle and pregnancy (Ghabreau et al., 2004; Yamagata et al., 2009). It has been demonstrated that DNA methylation is dynamically altered in the pre-implantation uterus, and that maternal regulation of DNA methylation specifically controls uterine decidualization in early pregnancy (Gao et al., 2012). Additionally, aberrant DNA methylation in the endometrium may be associated with implantation failure and early pregnancy loss, as well as, with other endometrial pathologies such as endometriosis and endometrial carcinoma (Ghabreau et al., 2004; Logan et al., 2010; Xue et al., 2007). In our work, we found that in the endosulfan-exposed rats the increased ERα expression was correlated with a predominant hypomethylation of CpG islands of the ERα promoters O, OT and E1 in the uterus during the pre-implantation period. In the Endo600 group differential methylation was detected at both *BstU*I sites studied of the O promoter. While *BstU*Ia site was hypomethylated, *BstU*Ib resulted hypermethylated. These findings, suggest that this site might be particularly related to the increased expression of ERα at the transcriptional level. It has been reported that aberrant DNA methylation, including both hyper- and hypomethylation, appears to play a complementary role in the developing of several diseases, like mental disorders or cancer, and that the balance between both stages determines the global or predominant DNA methylation status (Chan et al., 2005; Lee and Huang, 2016). Overall, findings of our work suggested methylation-mediated epigenetic changes as one of the possible mechanisms of endosulfan induced subfertility. Modifications of DNA methylation status of ERα promoters have been reported for others EDCs, and have been associated with reproductive pathologies and/or tumour development (Asada et al., 2008; Berger and Daxenbichler, 2002; Doshi et al., 2011). In a human breast cancer MCF7 cells, treatment with the industrial plasticizer butyl benzyl phthalate, increased ERα mRNA expression levels and led to demethylation of ERα promoter-associated CpG islands (Kang and Lee, 2005). In another study, neonatal exposure of male rats to bisphenol A led to hypermethylation of the promoter region of ERα in testis (Doshi et al., 2011). The authors suggested that aberrant DNA methylation is responsible for the BPA induced adverse effects on spermatogenesis and fertility. Additionally, a link between uterine leiomyomas and ERα expression has been suggested, as ERα is more highly expressed in uterine leiomyomas than in normal myometrium (Kovács et al., 2001). Further analysis of the DNA methylation status revealed that ERα promoter regions are hypomethylated in uterine leiomyomas (Asada et al., 2008).

It is well known that estrogen regulates expression of genes through activation of many signaling pathways involving cytokines, growth factors and transcription factors (Singh et al., 2011). In our work, *in silico* analysis of ERα promoters revealed that the sites that

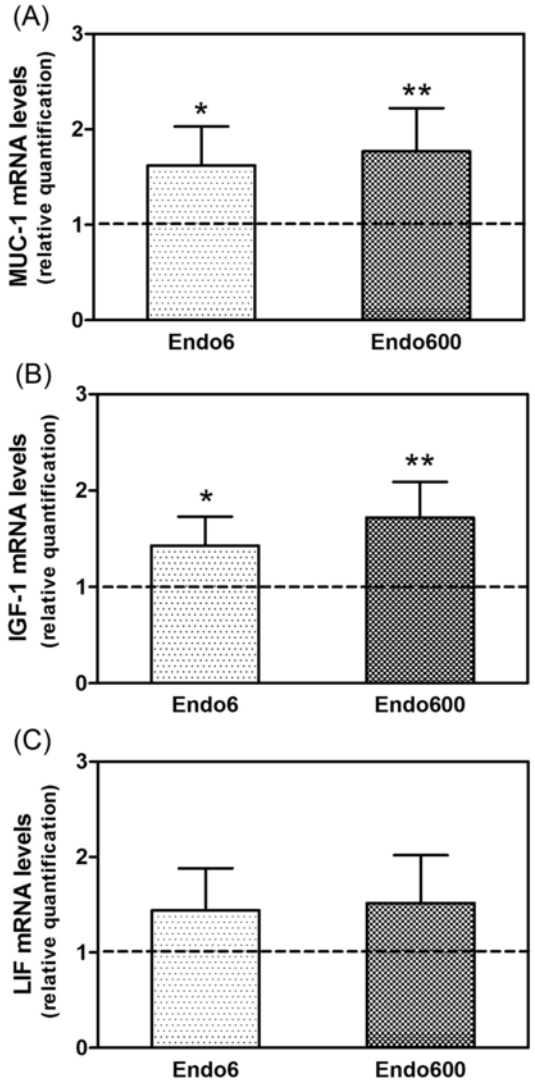

Fig. 6. Effect of neonatal endosulfan exposure on the expression of ERα-dependent target genes in the rat uterus during the pre-implantation period. Relative mRNA levels of MUC-1 (A), IGF-1 (B) and LIF (C), were measured by real-time RT-PCR and fold expression from control values were calculated by the relative standard curve method. Control values were assigned to a reference level of 1 and values are given as mean ± SEM of two independent determinations. *Asterisks* indicate statistical significance compared with the control (*P < 0.05; **P < 0.01 *vs.* control).

showed hypomethylation of DNA are potentially regulated by the transcription factors Stat, Egr, Gata, and E47. Experimental studies have demonstrated that these transcription factors are associated with the control of the implantation process (Sun et al., 2013). Conditional deletion of uterine Stat3 in mice shows that it is critical for uterine receptivity and implantation. Implantation failure in Stat3 knockout mice was associated with higher uterine estrogenic responses prior to the time of implantation, and with altered luminal epithelium differentiation, which is essential for embryo attachment (Sun et al., 2013). Other authors reported that transient and local suppression of Stat-3 by Stat-3 decoy transfer into the uterine cavity during implantation, resulted in <30% implantation caused by suppression of decidualization (Nakamura et al., 2006). Egr, are zinc-finger transcription factors that regulate cell growth, differentiation and apoptosis in the uterus. It has been shown that injection of Egr1 siRNA into the mouse uterine horn reduces the number of implanted embryos and affects the uterine vascular permeability (Guo et al., 2014). Liang et al. (2014) demonstrated that Egr1 is regulated by estrogen as a downstream target through LIF (leukemia inhibitory factor)/Stat3 signaling pathway in mouse uterus, and that it is critical for endometrial decidualization. Gata, another zinc-finger family of transcription factors, have been identified as important regulators of decidualization of the uterine stroma. In human endometrial stromal cells, Gata-3 gene expression was demonstrated to be under hormonal conditions mimicking decidualization (Lu et al., 2013). Moreover, Rubel et al. (2012) showed that Gata2 is expressed during critical phases of early pregnancy, and that it may play a major role in mediating progesterone signaling in the mouse uterus. Finally, the E47 transcription factor has been also suggested as a potential regulator of genes associated to endometrial receptivity, by means of bioinformatic analyses (Tapia et al., 2011).

Overall, in the present work we demonstrated that neonatal exposure to endosulfan increased the expression levels of ERα and its transcript variants during the pre-implantation period. Moreover, a decreased DNA methylation levels were detected in some ERα regulatory regions, suggesting an epigenetic up-regulation of it transcription. ERα overexpression was associated with a deregulation of ERα-dependent uterine target genes that regulate embryo implantation, suggesting that endosulfan might alter the estrogenic signaling pathway compromising uterine receptivity. These alterations could account, at least in part, for the endosulfan-induced implantation failures.

Declaration of interest

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

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