Molecular and Cellular Endocrinology 401 (2015) 248–259

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

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

Neonatal exposure to low doses of endosulfan induces implantation failure and disrupts uterine functional differentiation at the pre-implantation period in rats

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ARTICLE INFO

Article history:
Received 12 July 2014
Received in revised form 26 November 2014
Accepted 28 November 2014
Available online 5 December 2014

Keywords:
Endosulfan
Uterus
Implantation
Estrogen receptor alpha
Progesterone receptor
Hoxa10

ABSTRACT

We investigated whether neonatal exposure to low doses of endosulfan affects fertility and uterine functional differentiation at pre-implantation in rats. Newborn female rats received the vehicle, 0.2 μg/kg/d of diethylstilbestrol (DES), 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, the rats were mated to evaluate their reproductive performance on gestational day (GD) 19 and their ovarian steroid serum levels, endometrial proliferation and implantation-associated proteins on GD5. DES and endosulfan decreased the pregnancy rate and the number of implantation sites. On GD5, DES and endosulfan did not change the serum levels of 17β-estradiol (E2) and progesterone (P); the endometrial proliferation decreased, which was associated with silencing of Hoxa10 in the Endo600-treated rats. Both doses of endosulfan increased the progesterone receptor (PR) expression, whereas the higher dose led additionally to an increase in estrogen receptor alpha (ERα). In the Endo600-treated rats, the down-regulation of Hoxa10 was associated with a deregulation of the steroid receptor coregulators. Alterations in endometrial proliferation and the endocrine pathway of Hoxa10/steroid receptors/coregulators might be the mechanism of endosulfan-induced implantation failure.

1. Introduction

Over the past few decades, female reproductive disorders have notably increased and have become an emerging women's health concern (Crain et al., 2008). Epidemiological studies have focused on the association between developmental exposure to endocrine-disrupting chemicals (EDCs) and human female reproductive disorders. These studies found a close correlation between occupational and nutritional exposure to organochlorine pesticides and the incidence of fertility disorders, such as spontaneous abortion (Bretveld et al., 2008) and decreased fertility rates (Rosano et al., 2009).

Until recently, one of the most widely used organochlorine compounds for agricultural purposes all over the world was the pesticide, endosulfan. The use of endosulfan has been restricted and banned in most countries owing to its high toxicity (The Commission of the European Communities, 2005). In Argentina, the manufacture, formulation, commercialization and use of products containing this active principle have been recently banned (http://www.senasa.gov.ar). However, because of its persistence and high lipophilicity, large quantities of endosulfan continue to contaminate the environment. Endosulfan bioconcentrates in the biota and biomagnifies through the food chain, accumulating in the fatty tissues of living organisms (Naqvi and Vaishnavi, 1993; Stoker et al., 2011).

Many experimental studies performed in rodents have associated early exposure to endosulfan during development with male reproductive toxicity (Dalsenter et al., 1999; Silva and Gammon, 2009; Sinha et al., 2001). In another study, Saiyed et al. (2003) associated developmental exposure to endosulfan with reproductive

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; E2, 17β-estradiol; DES, diethylstilbestrol; MXC, methoxychlor; EDCs, endocrine-disrupting chemicals; P, progesterone; ERα, estrogen receptor alpha; PR, progesterone receptor; SRC-1, steroid receptor coactivator 1; SRC-3, steroid receptor coactivator 3; SMRT, silencing mediator for retinoic acid and thyroid hormone receptor; α-SMA, alpha smooth muscle actin; BrdU, bromodeoxyuridine; CKIs, cyclin-dependent kinase inhibitors; CLs, corpora lutea; PND, postnatal day; GD, gestational day; RfD, reference dose; NOEL, no observed effect level; IOD, integral optical density.

Grant support: This study was supported by grants from the CONICET (PIP 112-200801-00218), the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT, PICT 2011-1491) and the Universidad Nacional del Litoral (CAI + D 2011, 501 201101 00423 LI). MMM, JGR, EHL and JV are Career Investigators of the CONICET.

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http://dx.doi.org/10.1016/j.mce.2014.11.028
0303-7207/

effects in male children and adolescents. In this study, male children from a village located at the foothills of cashew plantations, where endosulfan had been aerially sprayed for more than 20 years, showed a delay in sexual maturity and an alteration in sex hormone synthesis. Although endosulfan toxicity to the male reproductive system is well documented, little is known regarding its effects on female reproduction.

One of the most frequent causes of female infertility is implantation failure (Adamson and Baker, 2003; Sharkey and Macklon, 2013). Successful implantation requires the development of the embryo to the blastocyst stage and an intricate program of uterine preparation (reviewed in Varayoud et al. 2014). The establishment of a receptive uterine environment to support blastocyst implantation primarily depends on the coordinated effects of estrogen and P. Prior to implantation, ovarian steroids activate the transcription of genes that stimulate proliferation and differentiation of the uterine epithelium and stroma. Hence, impaired endometrial growth and differentiation might be a significant factor contributing to infertility (Adamson and Baker, 2003).

One gene that is essential for female fertility in humans and rodents is the homeobox gene, Hoxa10. Adult female mice with a targeted disruption of Hoxa10 ovulate normally; however, they do not support embryo implantation because of defective uterine decidualization, which results in recurrent pregnancy loss and infertility (Benson et al., 1996). The repression of Hoxa10 by transferring Hoxa10 antisense oligonucleotides into the uterine lumen results in a significant decrease in embryo implantations (Bagot et al., 2000). The main role of Hoxa10 is the stimulation of endometrial proliferation previous to embryo implantation. Adult Hoxa10 gene expression is regulated by sex steroid hormones, particularly E2 and P, and their cognate receptors from the nuclear receptor superfamily. Other proteins designated as coregulators act with steroid receptors as part of the transcription machinery (coactivators and coregulators) (Smith and O'Malley, 2004). As Hoxa10 acts downstream of the sex steroid hormones to regulate uterine functional differentiation, an aberrant expression of steroid receptors, coregulator proteins or Hoxa10 might be associated with endometrial functional deficiency manifested clinically as implantation failures (Daftary and Taylor, 2006; Varayoud et al., 2011).

In a previous work, we found that early postnatal exposure to low doses of endosulfan alters the expression of the estrogen-dependent genes that regulate uterine development and differentiation (Milesi et al., 2012). We detected deregulation of estrogen receptor alpha (ERα), progesterone receptor (PR), alpha smooth muscle actin (α-SMA) and Hoxa10 during the neonatal and prepubertal periods (Milesi et al., 2012). The disruption of uterine morphoregulatory genes during critical periods of development might reprogram the normal physiological responses to sex steroid hormones in adulthood, with lasting consequences for reproductive health (Varayoud et al. 2008a, 2014).

In this study, we postulated that neonatal exposure to environmentally relevant doses of endosulfan could affect the endocrine Hoxa10 signaling pathway in the peri-implantation period, causing failures in the implantation process at adulthood. By using female rats exposed early to xenoestrogens [endosulfan or diethylstilbestrol (DES)], we evaluated the effects of the exposure on the following: *i)* the reproductive performance, by determining the pregnancy rates, the number of corpora lutea (CLs), and the implantation and resorption sites on gestational day 19 (GD19), *ii)* the uterine functional differentiation, by measuring the endometrial cell proliferation and Hoxa10 protein expression on GD5 (pre-implantation period), and *iii)* the endocrine pathways involved in the control of endometrial cell proliferation during the pre-implantation period, by determining the serum levels of the ovarian steroid hormones (E2 and P) and the expression of ERα, PR, and their coregulator proteins. Because low doses of classical estrogens are recommended as a control when comparing the effects of weak xenoestrogenic compounds (Newbold, 2004), a low dose of the synthetic estrogen, DES (0.2 μg/kg/d), was used as an endocrine disruptor control.

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. The female pups from each foster mother were assigned to one of the following neonatal treatment groups: *i)* the control group that received corn oil vehicle alone *ii)* DES (Sigma, St. Louis, MO, USA) at 0.2 μg/kg, *iii)* endosulfan (with 98% purity; Chem Service, West Chester, PA, USA) at 6 μg/kg (Endo6), or *iv)* endosulfan at 600 μg/kg (Endo600). The treatments were administered by s.c. injections in the nape of the neck every 48 h from PND1 to PND7. The low dose of endosulfan used was similar to the reference dose (RfD) established for this pesticide, whereas the high dose was 100-fold greater than the RfD and equal to the no observed effect level (NOEL).

Neonatal DES exposure over a wide dose range has been used to predict potential adverse effects on the reproductive tract (Newbold et al., 2004). According to previous results, low doses of classical estrogens, similar to that used in our work, are recommended to compare the effects of weak environmental estrogens (Newbold, 2004). Neither signs of acute or chronic toxicity nor significant differences in weight gain between the xenoestrogen-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. The female rats exposed to xenoestrogens did not exhibit advanced puberty, measured as early vaginal opening compared with the controls (data not shown). On PND90, the female rats neonatally exposed to xenoestrogens were housed for two consecutive weeks with sexually mature untreated males of the same strain and of proven fertility to allow several possible matings. Every morning, vaginal

smears were 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 GD1. Pregnant female rats were assigned to the following different experiments: *i)* assessment of reproductive performance by determining the pregnancy rates, the number of CLs, and the number of implantation and resorption sites on GD19; *ii)* assessment of the endometrial cell proliferation, Hoxa10 expression, ovarian steroid serum levels and expression of proteins associated with the endocrine regulation of Hoxa10 [ERα, PR and the coregulators: SMRT (the silencing mediator for the retinoic acid and thyroid hormone receptor) and SRC-1/SRC-3 (steroid receptor coactivators 1 and 3)] on GD5.

2.3. Evaluation of reproductive performance

The control (n = 20) and xenoestrogen-treated female rats (DES, n = 22; Endo6, n = 20; Endo600, n = 22) with a sperm-positive smear were housed separately, and their reproductive performance was evaluated on GD19. The pregnancy rate was calculated as the number of pregnant females/number of females housed with a male × 100. The ovaries from the pregnant rats were dissected, and the number of profusely irrigated CLs was counted by direct visualization using a stereomicroscope (Leica Corp., Buffalo, NY, USA). The two-horned uteri were removed and visually inspected to identify the number of resorption sites and implantation sites. Resorption sites were defined as endometrial sites with an appended amorphous mass without a fetus. The number of implantation sites was defined as the result of the total number of placentas with fetuses plus the total number of resorption sites (Barreto et al., 2004).

2.4. Assessment of steroid hormones, endometrial cell proliferation and uterine protein expression

The other control rats (n = 12) and the xenoestrogen-exposed pregnant female rats (DES, n = 8; Endo6, n = 12; Endo600, n = 12) were sacrificed on the morning of GD5, and trunk blood was collected for the hormone assays. In our colony, the embryo implantation process occurred in the evening of GD5; the samples were collected in the late pre-implantation period. Uterine tissue was collected, fixed by immersion in a 4% paraformaldehyde buffer for 24 h at 4 °C, embedded in paraffin and processed for the immunohistochemical and immunofluorescence assays. Four hours previous to sacrifice, each rat was injected (i.p.) with the thymidine analog, bromodeoxyuridine (BrdU; 60 mg/kg; Sigma), to evaluate the endometrial cell proliferation.

2.4.1. Hormone assays

The serum samples stored at –20 °C were thawed, and the serum levels of E2 and P were determined by RIA after ethyl ether and hexane (Merck, Buenos Aires, Argentina) extraction, respectively (Bosquiazzo et al., 2007). The antibodies were provided by G. D. Niswender, and the labeled hormones were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA, USA). The assay sensitivities were 1.6 pg/ml and 1.2 ng/ml for E2 and P, respectively. The intra- and interassay coefficients of variation were 3.6 and 11% for E2 and 9 and 14.3% for P, respectively.

2.4.2. Immunohistochemistry with the streptavidin–biotin peroxidase method

An immunohistochemistry analysis was performed to evaluate the endometrial cell proliferation by means of the BrdU incorporation technique, and the expression of the proteins associated with the implantation process, i.e., Hoxa10, ERα, PR (A/B isoforms), and their coregulators, SMRT, SRC-1 and SRC-3.

Uterine sections (5-μm thick) were deparaffinized and dehydrated in graded ethanol. BrdU incorporation to detect the cells in the S phase of the cell cycle was evaluated as previously described (Kass et al., 2000). The endogenous peroxidase activity and non-specific binding sites were blocked. The samples were incubated in a humid chamber first with the specific primary antibody (for 14–16 h at 4 °C) and then with the corresponding biotin-conjugated secondary antibody (for 30 min at room temperature) (Table 1). The reactions were developed using the streptavidin–biotin peroxidase method and diaminobenzidine (Sigma) as a chromogenic substrate. The samples were dehydrated and mounted with a permanent mounting medium (Eukitt, Sigma). For the BrdU immunodetection, the samples were counterstained with Harris hematoxylin (Biopur, Rosario, Argentina). Each immunohistochemical run included positive controls (a section from a tissue known to express the protein of interest) and negative controls (in which the primary antibody was replaced by the non-immune serum of the species used to generate the primary antibody). The negative controls for BrdU immunodetection were samples from the animals that had not received BrdU. The specificity of the commercial primary antibodies used was determined by the suppliers. For the antibodies (SRC-3 and SMRT) produced in our laboratory, specificity validation tests were applied. First, 1 μg of SRC-3 or SMRT antibodies was reabsorbed for 24 h at 4 °C with 10–20 μg of the antigenic peptides used to generate the antibodies. No staining was observed when the antibody–antigen complexes were used to perform immunohistochemical assays in the positive control tissues. In addition, the specificity of the antiserum was tested via Western blot (Varayoud et al., 2008a).

Table 1
Antibodies used for immunohistochemistry.

Antibodies	Dilution	Supplier
Primary		
Anti-BrdU (clone 85-2C8)	1/100	Novocastra (Newcastle upon Tyne, UK)
Anti-ERα (clone 6F-11)	1/400	Novocastra (Newcastle upon Tyne, UK)
Anti-PR (clone A0098)	1/400	Dako Corp. (Carpinteria, CA)
Anti-Hoxa10 (sc-17159)	1/50	Santa Cruz Biotechnology Inc. (Santa Cruz, CA)
Anti-SRC-1 (128E7)	1/800	Cell Signaling Technology, Inc. (Danvers, MA)
Anti-SRC3	1/50	Produced in our lab (Varayoud et al., 2008a)
Anti-SMRT	1/50	Produced in our lab (Varayoud et al., 2008a)
Secondary		
Anti-rabbit (B8895)	1/200	Sigma (St. Louis, MO)
Anti-mouse (B8774)	1/100	Sigma (St. Louis, MO)
Anti-goat (sc-2042)	1/200	Santa Cruz Biotechnology Inc. (Santa Cruz, CA)

2.4.3. Quantification of cell proliferation and protein expression by image analysis

The tissue sections were evaluated using an Olympus BH2 microscope (Olympus Optical Co., Ltd., Tokyo, Japan) with a Dplan 40× objective (numerical aperture = 0.65; Olympus). The incorporation of BrdU was quantitatively analyzed in the uterine subepithelial stromal cells. The proliferation index was obtained considering the volume fraction (Vv) of the positive cells, calculated by applying the following formula by Weibel (1969): $Vv = Pi/P$, where Vv is the estimated volume fraction of the object, Pi is the number of incident points over the positive cells, and P is the number of incident points over all the cells in the studied population. To obtain the data for the point-counting procedure, a glass disk with a squared grid was inserted into a focusing eyepiece (Gundersen et al., 1988; Ramos et al., 2002). The vessel cells and infiltrating inflammatory cells, e.g., neutrophils, macrophages, and eosinophils, were excluded in all the analyses.

The expression of ERα, PR, and Hoxa10 and the SRC-1, SRC-3 and SMRT proteins in the subepithelial stromal cells 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). Briefly, the images were recorded with a Spot Insight V3.5 color video camera, attached to a microscope (Olympus) and converted to a gray scale. The subepithelial stromal compartment was delimited (a 300-μm-wide area adjacent to the epithelium, from the basement membrane toward the outer layers). The integrated optical density (IOD) was measured as a linear combination of the average gray intensity and the relative area occupied by the positive cells (Ramos et al., 2001, 2002). Because the IOD is a dimensionless parameter, the results were expressed as arbitrary units. The cell proliferation and protein expression in the subepithelial stroma were quantified on at least 30 and 10 randomly selected fields per section, respectively, and two sections per rat (separated 50 μm from each other) were evaluated.

2.4.4. Dual immunofluorescence staining

Dual immunofluorescence staining was performed to evaluate the colocalization of ERα/PR, ERα/SRC-1, ERα/SMRT, Hoxa10/SRC-1 and Hoxa10/SMRT in the uterine subepithelial stroma. The uterine sections were deparaffinized, rehydrated, and subjected to microwaves for the antigen retrieval. The sections were blocked with sodium borohydride for 40 min to reduce the autofluorescence and with normal donkey serum (Hoxa10) or goat serum (ERα, PR, SMRT, SRC-1) (Sigma) for 1 h to minimize the nonspecific background. The incubation with primary antibodies (described in Table 1) was performed overnight at 4 °C. The secondary antibodies were incubated for 1 h, and then the sections were washed for a total of 45 min in three changes of PBS. The following secondary antibodies were used: tetramethylrhodamine isothiocyanate (TRICT)-conjugated goat anti-rabbit (016-020-084, red, 1:200 dilution; Jackson ImmunoResearch, West Grove, PA, USA), TRICT-conjugated goat anti-mouse (115-025-003, red, 1:200 dilution, Jackson ImmunoResearch), Alexa Fluor 488 goat anti-rabbit (A-11034, green, 1:100 dilution; Invitrogen Molecular Probes, Eugene, OR, USA), and Cy2-conjugated goat anti-mouse (200-222-037, green, 1:100 dilution, Jackson ImmunoResearch).

For the Hoxa10 detection, anti-goat secondary antibodies (1:200 dilution, biotin conjugate) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) followed by incubation with TRICT-conjugated streptavidin (016-020-084, 1/150 dilution; Jackson ImmunoResearch) were used. Finally, all the sections were mounted in Prolong Gold fluorescent mountant (Invitrogen) with 4′,6-diamidino-2-phenylindole dihydrochloride (Fluka; Sigma) and stored in the dark at room temperature. The negative controls included uterine sections incubated using primary antibody buffer solution (3% BSA, 0.1% Tween 20 in PBS) instead of the primary antibody to control for nonspecific staining. All the immunostained sections were examined using an Olympus BX-51 microscope equipped for epifluorescence detection with the appropriate filters (Olympus). The images were recorded using a high-resolution USB 2.0 digital color camera (QImaging Go-3; QImaging, Surrey, British Columbia, Canada).

2.5. Statistics

All the results are expressed as the mean ± SEM. The pregnancy rates were analyzed using Fisher's exact test. The data from the number of implantation sites and CLs were analyzed using one-way ANOVA followed by Dunnett's test for the multiple comparisons (after Bartlett's test for the homogeneity of the variance). The number of resorption sites was analyzed using a generalized linear model with a negative binomial response, using the glm.nb function of the R statistical software (The R Foundation for Statistical Computing). For the other variables, a Kruskal–Wallis test followed by Dunn's method of multiple comparisons was applied. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Postnatal exposure to DES and endosulfan impairs fertility

Female rats exposed to xenoestrogens during the first postnatal week showed a decrease in pregnancy rates in adulthood. No pregnancies occurred in 36% of the DES- and 23% of the Endo600-treated rats, whereas the pregnancy rate in the controls was 100% (Fig. 1A). A similar trend was observed for the Endo6 group; however, the differences were not significantly different. When pregnancies were established, no differences in the number of CLs were observed (CLs/rat: 11–13) between the groups (Fig. 1B). Although the number of resorption sites was similar, regardless of the postnatal treatment (Fig. 1C), differences were detected in the number of implantation sites between the groups. The treatment with DES and both doses of endosulfan elicited a reduction in the number of implantation sites (Fig. 1D).

3.2. Ovarian steroid levels

The serum levels of E2 and P in the control and xenoestrogen-exposed rats were measured on the morning of GD5 (the pre-implantation period). No differences in the serum concentrations of E2 and P were found among all the experimental groups (the E2 serum levels expressed in pg/ml as follows: Control, 88.7 ± 13.6; DES, 106.1 ± 15.5; Endo6, 103.3 ± 11.7; Endo600, 87.6 ± 25.9; $P > 0.05$; and P serum levels expressed in ng/ml: as follows: Control, 32.4 ± 1.8; DES, 36.9 ± 5.5; Endo6, 39.5 ± 3.3; Endo600, 36.9 ± 2.8; $P > 0.05$).

3.3. Stromal cell proliferation and Hoxa10 expression

The uterine stromal cell proliferation, measured as the proportion of cells that incorporated BrdU on GD5, is shown in Fig. 2A. A significant decrease in the stromal cell proliferation was detected in the pre-implantation uterus from the DES- and endosulfan-exposed female rats. In fact, the incorporation of BrdU into the subepithelial stromal cells (expressed as Vv × 100) in the DES, Endo600 and Endo6 groups was 3-, 2-, and 1.5-fold lower than in the control group, respectively. Photomicrographs illustrating these results are shown in Fig. 2B. As for Hoxa10, high expression was detected in the stromal compartment of the control rats on GD5 (Fig. 3). The Hoxa10 expression was down regulated in the stroma of the Endo600-treated rats.

3.4. PR and ERα expression in the pre-implantation uterus

The female rats exposed neonatally to endosulfan showed a deregulation of the steroid hormone receptors in the pre-implantation uterus (Fig. 4). Treatment with both doses of endosulfan increased the PR protein expression in the subepithelial stroma, whereas that with Endo600 increased ERα expression significantly in the same uterine compartment.

3.5. Expression of steroid receptor coregulators

To address the molecular mechanisms involved in the deregulation of the steroid receptors and Hoxa10 proteins found in the Endo600 group, the expression of the steroid hormone receptor coregulators in the control and Endo600-treated rats was assessed by immunohistochemistry and quantitative image analysis. SMRT and SRC-1/SRC-3 were selected as representative members of the corepressor and coactivator families, respectively. The female rats neonatally exposed to the high dose of endosulfan

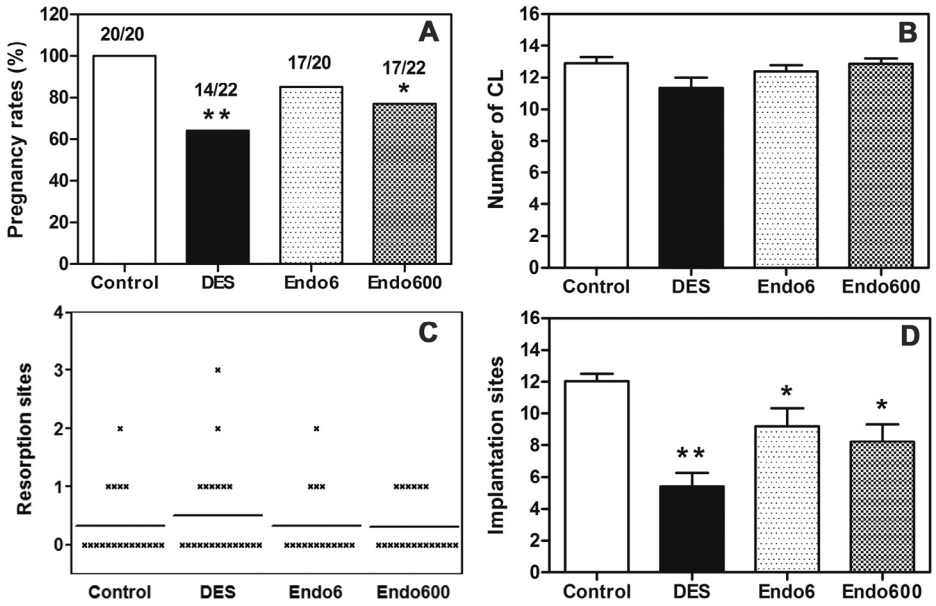

Fig. 1. Reproductive performance in control and neonatally xenoestrogen-treated rats recorded on gestational day 19 (GD19). (A) The pregnancy rate was calculated by the average number of females that were pregnant and the number of females housed with a fertile male. For each experimental group, the number of corpora lutea (CLs) (B) and the number of implantation sites (D) are expressed as the mean ± SEM (Control, n = 20; DES, n = 22; Endo6, n = 20; Endo600, n = 22). The numbers of resorption sites (C) in each individual pregnant rat were plotted, and the *horizontal lines* are the mean for each experimental group (n ≥ 20 per group). *Asterisks* indicate statistical significance compared with the control (*$P < 0.05$; **$P < 0.01$ *vs.* control).

exhibited deregulation in the expression of two coregulators in the uterine subepithelial stroma (Fig. 5). These rats showed a significant increase in SMRT and a decrease in SRC-1 in the pre-implantation uterus. No differences were observed in the SRC-3 protein expression between the vehicle- and Endo600-treated female rats.

3.6. Colocalization of ERα, PR, Hoxa10, and the coregulators, SRC-1 and SMRT

Dual immunofluorescence staining was performed to determine whether the proteins studied were colocalized in the uterine subepithelial cells. The comparative expression pattern of ERα/PR,

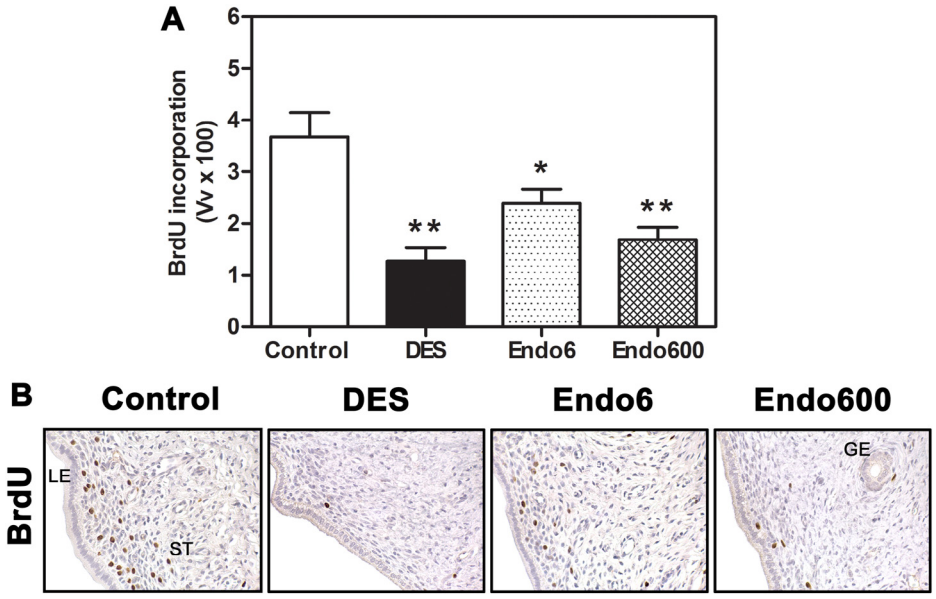

Fig. 2. Effect of xenoestrogen exposure on uterine stromal cell proliferation on GD5. (A) Stromal proliferative activity is expressed as volume fractions (Vv × 100). Each column represents the mean ± SEM (Control, n = 12; DES, n = 8; Endo6, n = 12; Endo600, n = 12). *Asterisks* indicate statistical significance compared with the control (*$P < 0.05$; **$P < 0.01$). (B) Photomicrographs show a significant decrease in the incorporation of BrdU in the uterine subepithelial stroma of DES-, Endo6- and Endo600-treated rats. LE, luminal epithelium; GE, glandular epithelium; ST, subepithelial stroma. Original magnification, × 400.

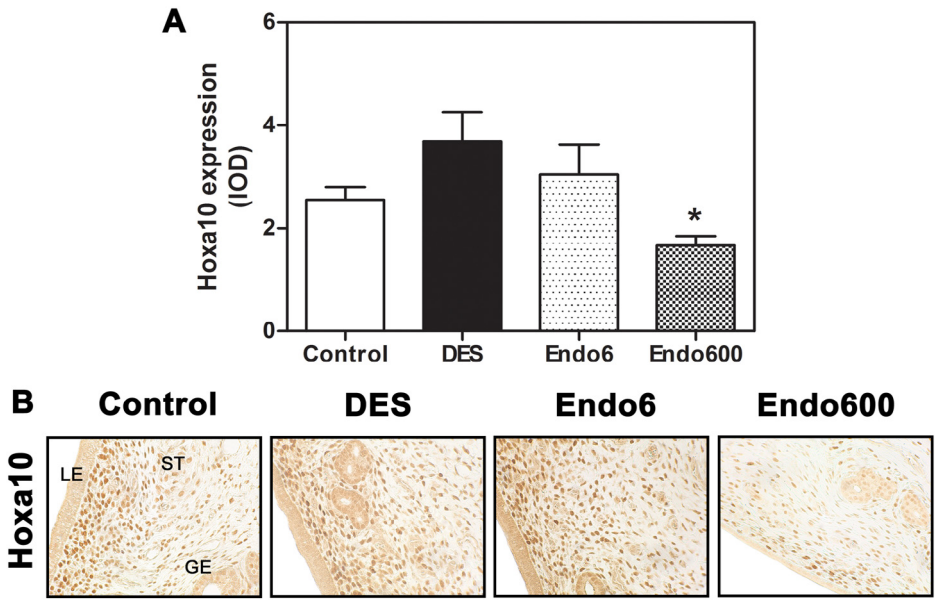

Fig. 3. Effects of neonatal xenoestrogens exposure on Hoxa10 protein expression in the uterine subepithelial stroma on GD5. (A) Hoxa10 immunostaining in the subepithelial stroma is expressed as the integrated optical density (IOD), which consists of a linear combination between the average of immunostaining intensity and the relative area occupied by positive cells. Each column represents the mean ± SEM (Control, n = 12; DES, n = 8; Endo6, n = 12; Endo600, n = 12). *Asterisks* indicate statistical significance compared with the control (*$P < 0.05$). (B) Representative photomicrographs of Hoxa10 protein expression in uterine samples of control and xenoestrogen-treated rats. LE, luminal epithelium; GE, glandular epithelium; ST, subepithelial stroma. Original magnification, × 400.

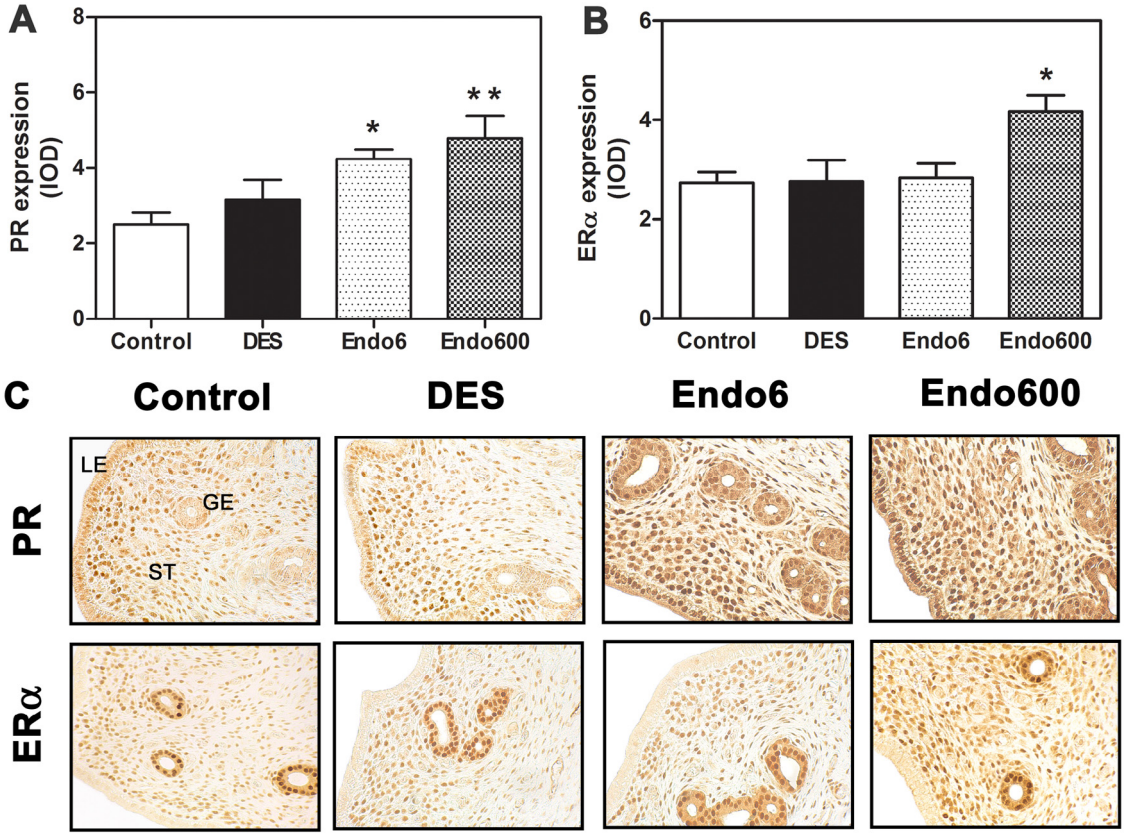

Fig. 4. Effects of neonatal xenoestrogen exposure on steroid receptor protein expression in the uterine subepithelial stroma on GD5. (A) PR and (B) ERα immunostaining in the subepithelial stroma is expressed as IOD. Each column represents the mean ± SEM (Control, n = 12; DES, n = 8; Endo6, n = 12; Endo600, n = 12). *Asterisks* indicate statistical significance compared with the control (*$P < 0.05$; **$P < 0.01$ *vs.* control). (C) Representative photomicrographs of uterine steroid receptor expression of control and xenoestrogen-treated rats. LE, luminal epithelium; GE, glandular epithelium; ST, subepithelial stroma. Original magnification, × 400.

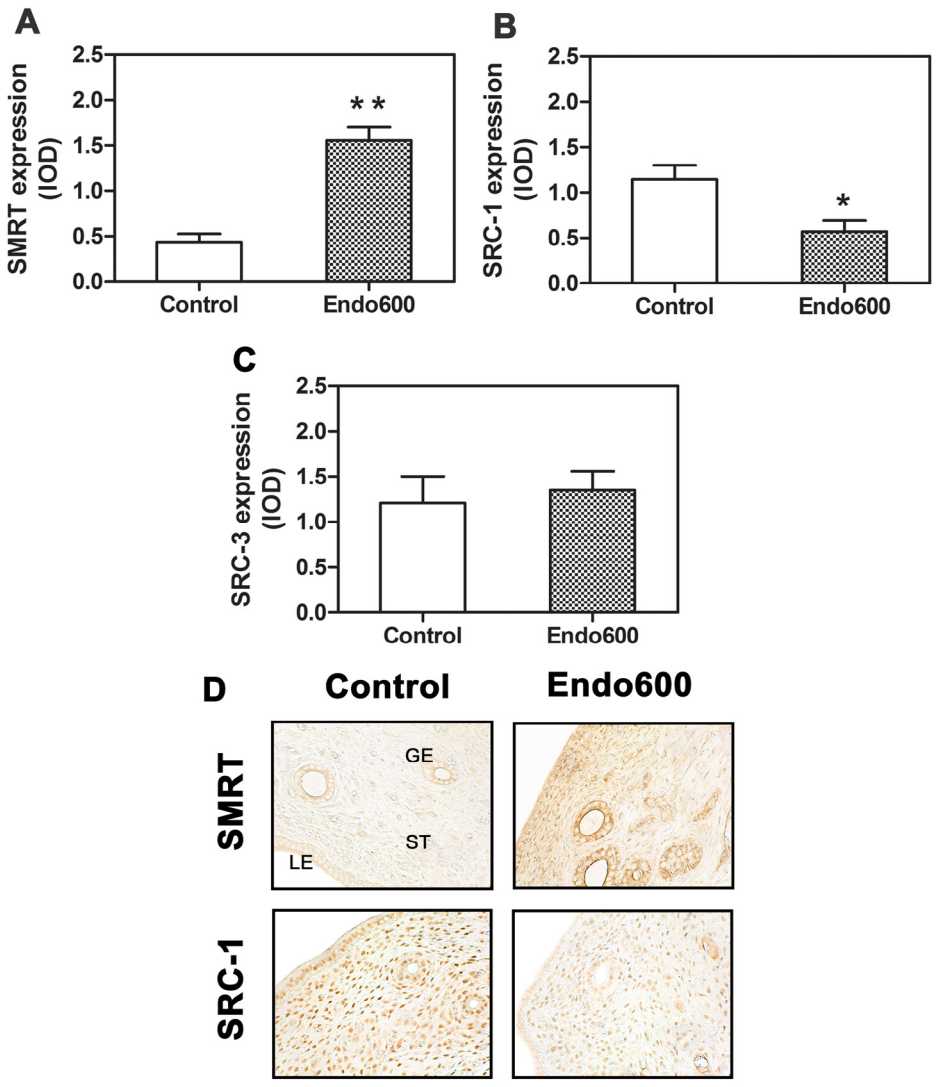

Fig. 5. Expression of steroid receptor coregulator proteins in the uterine subepithelial stroma of control and Endo600-treated rats on GD5. SMRT(A), SRC-1 (B) and SRC-3 immunostaining (C) in the subepithelial stroma is expressed as IOD. Each column represents the mean ± SEM (Control, n = 12; DES, n = 8; Endo6, n = 12; Endo600, n = 12). *Asterisks* indicate statistical significance compared with the control (*$P < 0.05$; **$P < 0.01$ *vs.* control). (D) Representative photomicrographs of uterine steroid receptor coregulator expression of control and Endo600-treated rats. LE, luminal epithelium; GE, glandular epithelium; ST, subepithelial stroma. Original magnification, × 400.

ERα/SRC-1 and ERα/SMRT between the uterine tissues of the controls and the Endo600-treated rats is shown in Fig. 6. An intense nuclear colocalization of the ERα/PR proteins was observed in the control and Endo600-treated rats (Fig. 6A). Similar to the results of the immunohistochemical staining, the expression levels of both steroid receptors were higher in the Endo600 group than in the control group. Similarly, ERα and SRC-1 colocalized in the nucleus of the subepithelial cells in both groups, and a lower expression of SCR-1 was detected in the Endo600-treated rats (Fig. 6B). The high levels of SMRT expression detected in the cytoplasm of the subepithelial stromal cells of the Endo600-treated rats were colocalized with the intense nuclear expression of ERα (Fig. 6C). Regarding Hoxa10, a high expression was observed in the control animals, predominantly in the nucleus of the subepithelial cells, which colocalized with a high expression of SRC-1 (Fig. 7A) and low expression of SMRT (Fig. 7B). An inverted pattern of expression was detected in the Endo600 group. The female rats treated with the high dose of endosulfan showed a low expression of Hoxa10 associated with a low expression of SRC-1 (Fig. 7A) and high expression of SMRT (Fig. 7B).

4. Discussion

This study aimed to investigate the effects of neonatal exposure to low doses of endosulfan on the reproductive performance and uterine functional differentiation of female rats in the preimplantation period. Uterine organogenic differentiation is sensitive to exposure to EDCs during critical periods of development, and these compounds might interfere with the physiology of normal endocrine-regulated events, leading to adverse effects later in life (Varayoud et al., 2014). In a previous work, we found that exposure to low doses of endosulfan during the first postnatal week alters the expression of the proteins that regulate uterine development and differentiation, such as the steroid receptors, Hoxa10 and α-SMA (Milesi et al., 2012). In this study, we demonstrated that the disruption of these developmental regulatory proteins early in life alters the normal uterine physiological responses to the hormonal stimuli of gestation, leading to reproductive disorders in adulthood. We found that female rats neonatally exposed to endosulfan exhibited subfertility, characterized by a drop in the pregnancy rates and in the number of implantation sites. In addition, we detected

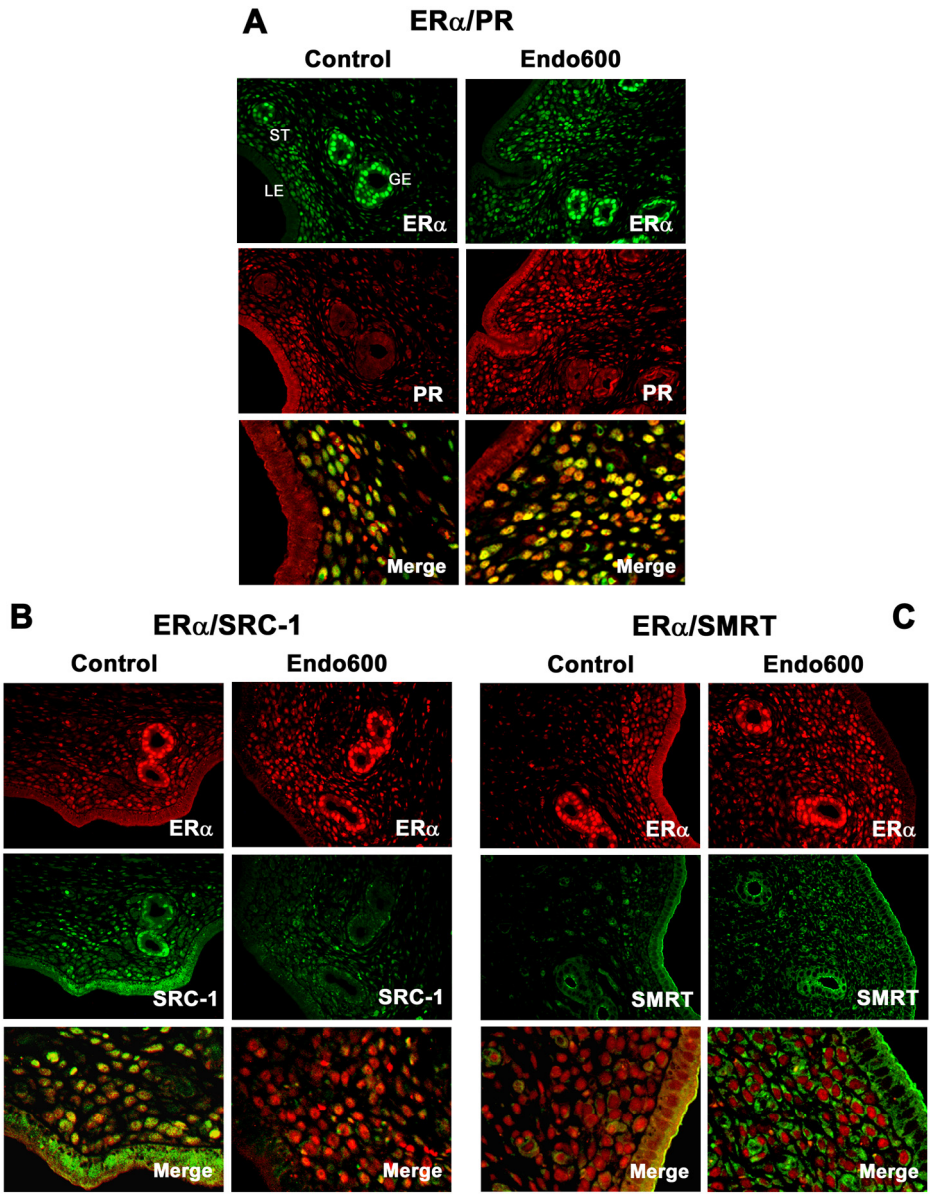

Fig. 6. Representative photomicrographs of dual immunofluorescence staining for ERα/PR (A), ERα/SRC-1 (B), and ERα/SMRT (C) in the uterus of control and Endo600-treated rats on GD5. ERα and PR proteins were significantly increased in the uterine subepithelial stroma of the Endo600 group. An intense nuclear colocalization of ERα and PR in the subepithelial stromal cells, evidenced as yellow nuclei in merge images, was detected in both control and Endo600-treated rats (A). Endo600-treated rats showed lower expression of SRC-1 in the subepithelial stromal cells, and coexpression of this steroid receptor coactivator with ERα in the nucleus of the uterine subepithelial stroma cells was observed in the control and Endo600 groups (B). The high levels of SMRT expression detected in the cytoplasm of the subepithelial stromal cells of Endo600-treated rats were colocalized with the intense nuclear expression of ERα (C). LE, luminal epithelium; GE, Glandular epithelium; ST, subepithelial stroma. Original magnification, × 400; Merge, × 1000.

failures in the uterine functional differentiation at the peri-implantation period, manifested as a disruption of the endocrine pathways that regulate the proliferation of the endometrial cells and the expression of implantation-associated proteins.

We found that both DES and the high dose of endosulfan elicited a drop in the pregnancy rate. Similar results were reported in a previous work of our lab, in which we studied the effects of bisphenol A (Varayoud et al., 2011). Additionally, we found a lower number of implantation sites in the rats treated with DES and both doses of endosulfan, suggesting that these xenoestrogens could cause intrinsic defects at the uterine level that precede the embryo arrival. In a different model of exposure, Hiremath and Kaliwal (2002) studied the effects on implantation, of different doses of endosulfan (1, 2, 3 or 4 mg/kg/d) administered to pregnant mice for 7 consecutive days. These authors reported that only the highest dose of endosulfan (4 mg/kg/d) induced failures in the implantation process. In our study, the number of CLs recorded in the ovaries showed no differences between the experimental groups, suggesting that neither the ovulation rate nor the CL "activation" were altered as a consequence of neonatal exposure to DES or endosulfan. Similarly, no changes in the number of resorption sites were detected between the control and the xenoestrogen-treated rats, ruling out a post-implantation failure. Exposure to EDCs during critical periods of development has been postulated to contribute to declining conception rates and an increased incidence of female reproductive disorders, such as altered cyclicity, endometriosis, fetal growth retardation, and pregnancy loss, among others (Crain et al., 2008). To our knowledge, this is the first report showing that

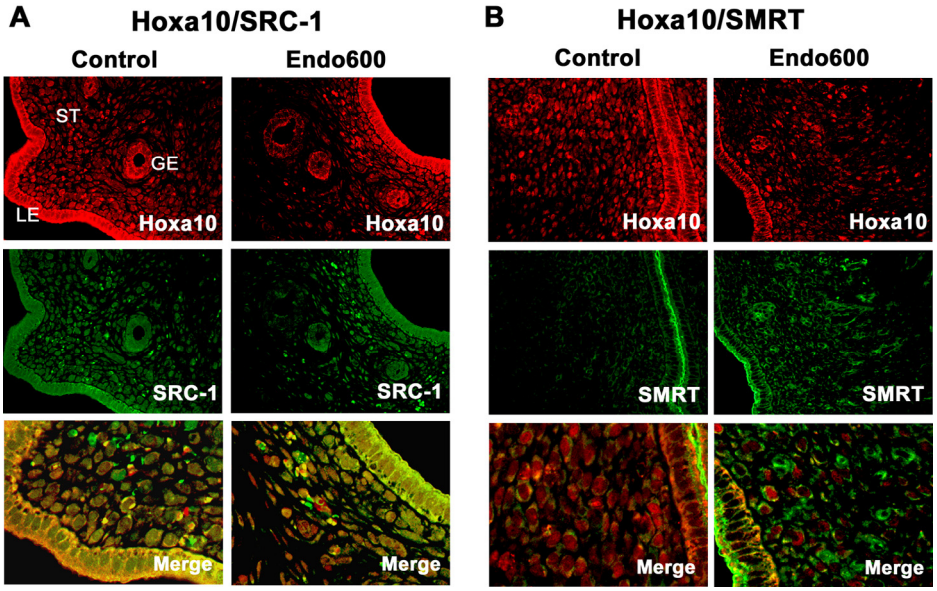

Fig. 7. Representative photomicrographs of dual immunofluorescence staining for Hoxa10/SRC-1 (A) and Hoxa10/SMRT (B) in the uterus of control and Endo600-treated rats on GD5. The lower expression of Hoxa10 in the uterine subepithelial stroma of Endo600-treated rats is coincident with a lower expression of SRC-1 and a higher expression of SMRT in the same compartment. Co-expression of Hoxa10 with SRC-1 in the nucleus and with SMRT in the cytoplasm was detected in the control and Endo600 groups. LE, luminal epithelium; GE, Glandular epithelium; ST, subepithelial stroma. Original magnification, × 400; Merge, × 1000.

exposure to low doses of endosulfan during the first postnatal week negatively affects female reproductive health, causing subfertility.

Subsequently, we investigated whether the implantation failures were associated with defects in the uterine functional differentiation in the peri-implantation period. For that purpose, we evaluated the endometrial cell proliferation and Hoxa10 expression on GD5. The treatment with DES and both doses of endosulfan elicited a significant decrease in the proliferation of subepithelial stromal cells in the pre-implantation period. In the Endo600 group, the impaired proliferation was associated with a silencing of the Hoxa10 protein in the subepithelial compartment. Yao et al. (2003) reported that mutant mice lacking normal Hoxa10 expression show defective P-dependent uterine stromal cell proliferation. Additionally, these authors found that Hoxa10 mutants exhibited an alteration in the expression of two cyclin-dependent kinase inhibitors (CKIs), p57 and p15, which are the main cell cycle regulators in the pre-implantation uterine stroma. The authors suggested that the decrease in stromal cell proliferation in the Hoxa10 mutants might be because of cell cycle arrest during early Go/G1 caused by higher levels of expression of the afore-mentioned CKIs as a consequence of the Hoxa10 repression. During the pre-implantation period, Hoxa10 is required for the proliferation of the stromal cells and their subsequent differentiation into decidual cells, which are critical events for endometrial receptivity and embryo implantation (Lim et al., 1999). We suggest that decreased Hoxa10 expression might be related to the disruption of endometrial proliferation and the implantation failure detected in pregnant female rats neonatally exposed to endosulfan. Similar results were found with other organochlorine pesticides. It has been demonstrated that prenatal or postnatal exposure to methoxychlor (MXC) in mice blocks the decidual cell response, inhibiting implantation and support of the early embryo (Hall et al., 1997). Fei et al. (2005) reported that neonatal MXC treatment induces a permanent decrease in the Hoxa10 expression that persists into adulthood and suggested that the mechanism by which this agent disrupts uterine function is by suppression of the Hoxa10 expression.

The effects of a compound with proven estrogenic activity, such as endosulfan, might differ from those elicited by DES (Arase et al., 2011; Khurana et al., 2000; Milesi et al., 2012). In a previous work, we demonstrated that even when E2 is administered, different responses in the uterotrophic assay and the expression of estrogen-sensitive genes are observed that are dose dependent (Varayoud et al., 2008b). In that study, we demonstrated that endosulfan mimics the effects of a non-uterotrophic low dose of E2 and fails to reproduce the effects observed after the injection of a high, uterotrophic dose of the hormone. In our study, the long-term effects of endosulfan and DES are partially coincident. We detected implantation failure in association with a lower stromal proliferation with DES and the two doses of endosulfan. Only Endo600 showed a disruption of the Hoxa10 expression. Considering these particular responses (according to the EDC or the dose of endosulfan), we could postulate that Endo6 and DES might induce the alteration of uterine functional differentiation by affecting other pathways of endometrial proliferation control. Different studies have shown that endometrial cell proliferation is a complex process in which many signaling pathways are involved, such as Wnt-β-catenin signaling (Hayashi et al., 2009; Mohamed et al., 2005) or Ihh–COUP-TFII signaling (Kurihara et al., 2007; Paria et al., 2001).

In accordance with our previous results (Milesi et al., 2012) and the results reported here, postnatal endosulfan exposure affects the prepubertal and the adult expression of Hoxa10 in a different manner. Whereas during the prepubertal period Hoxa10 increased, an opposite change was detected during the pre-implantation period. We postulated that the deregulation of Hoxa10 during postnatal development could affect the Hoxa10 response to E2 and P in the adult uterus. Some authors have postulated that epigenetic mechanisms, such as DNA methylation, could explain the disruption of Hoxa10 in prepuberty with consequences in adulthood (Bromer et al., 2009, 2010). In future studies we will determine whether epigenetic mechanisms could explain the alterations of Hoxa10 expression at different ages.

During the mid-secretory phase of the menstrual cycle, the Hoxa10 levels show a dramatic increase, when P levels are high (Taylor et al., 1998). In addition, the E2 regulation of Hoxa10 has been associated with the detection of ER binding of two putative estrogen response elements in the 5′ regulatory region of Hoxa10

(Akbas et al., 2004). The transcriptional regulation of Hoxa10 by P is mediated through PR and therefore blocked by RU486 (Ma et al., 1998). We next evaluated the endocrine pathways involved in the control of endometrial cell proliferation in the pre-implantation period, by determining the serum levels of ovarian steroid hormones and the expression of ERα, PR (A/B isoforms), and its coregulator proteins in the uterine subepithelial stroma on GD5. Additionally, we evaluated whether these proteins and Hoxa10 co-localize in uterine subepithelial cells. Neonatal exposure to endosulfan induced a deregulation in the expression of both steroid receptors, without changes in the serum levels of E2 and P. These results rule out a functional insufficiency of the CLs as a cause of Hoxa10 silencing.

Up-regulation of the PR expression was observed in the subepithelial stroma of females neonatally exposed to both doses of endosulfan. In humans, as in rodents, PR exists as two functionally distinct isoforms, PR-A and PR-B (Brosens et al., 1999; Wang et al., 1998). It has been reported that during decidualization, PR-B is down regulated in the epithelial and stromal compartments, whereas PR-A expression persists in the stromal compartment, suggesting that PR-A is the main mediator of uterine decidual transformation (Brosens et al., 1999; Wang et al., 1998). Additional studies have demonstrated that selective ablation of PR-A in mice, and not of PR-B, induces severe abnormalities in ovarian and uterine function, leading to female infertility (Conneely et al., 2001, 2003). Considering that PR-A is the predominant functional isoform in the uterus, we suggest that this isoform might be the most affected by postnatal treatment with endosulfan. The Endo600-treated rats exhibited higher levels of ERα expression in the same compartment as well. The Hoxa10 uterine regulation at the pre-implantation period is primary up-stream PR-signaling. Therefore, the apparent paradoxical results between the levels of Hoxa10 and the PR expression in the endosulfan-treated rats might be explained by taking into account the ability of the steroid receptor coregulators to mediate the transcriptional activity of the steroid receptors. In this regard, we detected that postnatal exposure to Endo600 affected the uterine stromal SMRT and SRC-1 expression, showing a clear up-regulation of SMRT and down-regulation of SRC-1 in the subepithelial compartment. Previously, we detected that SMRT, SRC-3 and SRC-1 are targets of EDC in the uterus, hypothalamus and mammary glands of rats neonatally exposed to the chemicals (Bosquiazzo et al., 2010; Durando et al., 2011; Monje et al., 2009; Varayoud et al., 2008a). In addition, female mice with a disruption of the SRC-1 gene exhibit a decrease in the ability of uterine endometrial stromal cells to undergo decidual transformation (Xu et al., 1998), indicating that this coactivator plays a critical role in uterine events that are dependent on the steroid hormone signaling pathway. SMRT is a limiting factor that inhibits the transcriptional activity of the steroid hormone receptors by recruiting histone deacetylases and disrupting the receptor dimmer interactions (Privalsky, 2004). In our experiment, the higher expression of SMRT and lower expression of SCR-1 in the Endo600-treated animals were accompanied by a lower expression of the Hoxa10 gene in the same cells. These coregulator changes might provide a possible explanation, which is associated with the alteration of the endocrine-related control of stromal proliferation during the pre-implantation period.

The immunofluorescence results showed a co-localization of the Hoxa10/steroid receptors and coregulators. This information is important considering the complex regulation of Hoxa10 by E2 and P in the uterus. Previous results using *in vivo* and *in vitro* models showed that ERα and PR are implicated in the regulation of Hoxa10 expression. Using these models, the results show that a combination of E2 and P treatment increases the Hoxa10 expression to higher levels than those obtained after treatment with either hormone alone (Gui et al., 1999; Taylor et al., 1998).

In utero and early postnatal exposure to EDCs could interfere with the action of the steroid hormones essential to the development of the reproductive tract, resulting in permanent alterations in gene expression and organ function. Although some of these alterations might be detected in fetuses or newborns, others, such as failures in the functionality of the reproductive organs, might only become apparent upon sexual maturation (Chen et al., 2010). A classic case of endocrine disruption *in utero* that leads to adult disease is that of prenatal exposure to DES. The daughters of women given DES while pregnant were early shown to have rare cervicovaginal cancers (Barclay, 1979; Herbst et al., 1971), decreased fertility and an increased rate of ectopic pregnancies (Goldberg and Falcone, 1999), and early menopause (Hatch et al., 2006). Later, experimental studies on animal models exposed to DES during gestation supported these antecedents (Kim et al., 2009; Miyagawa et al., 2011; Newbold, 2008; Newbold et al., 2007). In accordance with these observations, we found that the female rats neonatally exposed to a low dose of DES exhibited uterine functional abnormalities that compromised fertility. Many organochlorine pesticides have been demonstrated to possess estrogenic properties resulting in adverse effects on the reproductive system in animal models (Cummings, 1997; Gellert, 1978; Kupfer, 1975). In a previous study, we demonstrated that endosulfan mimics the action of a non-uterotrophic dose of E2, causing a deregulation in the uterine expression of E2-dependent genes in ovariectomized adult female rats (Varayoud et al., 2008a). In this work, we demonstrated that exposure to endosulfan during the developmental period induced adverse effects on female reproductive health later in life, by altering the uterine functional differentiation at the pre-implantation period. As it was previously stated, the synchronized development of the embryo to the blastocyst stage and differentiation of the uterus to the receptive state are essential to successful implantation. In light of that, we could not rule out the possibility that implantation failures in the xenoestrogen-exposed rats are caused by post-ovulation problems as well, such as a reduction of the fertilization rate of the oocytes or embryo development failure before implantation.

Our results show that neonatal exposure to endosulfan induces implantation failure, causing subfertility. Alterations in the stromal cell proliferation and in the uterine expression of Hoxa10 might affect embryo implantation. In light of this, we propose that exposure to xenoestrogens during critical periods of perinatal life changes the uterine hormonal response during adulthood by disrupting the assembly of the PR- and ER-dependent genes by the transcription machinery. Studies are underway to investigate whether neonatal exposure to endosulfan affects the transcription factor assembly in the Hoxa10 promoter region.

Acknowledgements

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

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