Reproductive Toxicology 33 (2012) 85–93

Contents lists available at SciVerse ScienceDirect

Reproductive Toxicology

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

Neonatal exposure to low doses of endosulfan disrupts the expression of proteins regulating uterine development and differentiation

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ARTICLE INFO

Article history:
Received 3 May 2011
Received in revised form
23 November 2011
Accepted 6 December 2011
Available online 17 December 2011

Keywords:
Endosulfan
Uterus
Hoxa10
Estrogen receptor alpha
Progesterone receptor
Alpha-smooth muscle actin

ABSTRACT

This study investigates the effects of neonatal exposure to low doses of endosulfan on the expression of proteins regulating uterine development and differentiation. Female pups received vehicle, endosulfan (Endo6: 6 μg/kg, Endo600: 600 μg/kg) or diethylstilbestrol (DES: 0.2 μg/kg) from postnatal day 1 (PND1) to PND7. The uterine expression of estrogen receptor alpha (ERα), progesterone receptor (PR), Hoxa10 and alpha smooth muscle actin (α-SMA) was detected by immunohistochemistry on PND8 (neonatal period) and PND21 (prepubertal period), to evaluate acute and short-term responses. ERα, Hoxa10 and α-SMA were induced in the Endo600 group in both ages, while a striking decrease in PR expression was detected in the prepubertal rats following each dose of endosulfan. DES treatment deregulated ERα and Hoxa10 uterine expression at each age. Studies are currently underway to investigate whether the dysregulation of steroid receptors, Hoxa10 and α-SMA observed following neonatal exposure to endosulfan affect uterine functions in adulthood.

1. Introduction

Endosulfan is an organochlorine pesticide used in large-scale agriculture for controlling a variety of insects and mites that attack food (i.e., vegetables, fruits, tea, coffee, cocoa, grains) and non-food (i.e., tobacco and cotton) crops. Endosulfan bioaccumulates and biomagnifies in food chains and persists for lengthy periods of time in the environment [1]. Food contaminated with endosulfan residue is the main source of animal and human exposure [1]. Although its use has been banned in more than 60 countries owing to its high toxicity [2], it remains in use in several other countries, like Argentina, India and China.

Studies performed in rodent experimental models have associated environmental exposure to endosulfan in early life with reproductive and developmental abnormalities [3]. Permanent adverse effects of endosulfan on the reproductive ability of male-offspring rats (i.e., decreases in the daily sperm production and the percentage of seminiferous tubules with complete spermatogenesis) were observed after *in utero* and lactational exposure to 3 mg/kg-d of endosulfan [4]. The administration of 1 mg/kg of endosulfan to pregnant rats from gestational days 6 to 20 increased fetal resorption and induced gross fetal anomalies [5].

The no observed effect level (NOEL) established for endosulfan is 600 μg/kg-d and it 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 [6]. The acceptable oral reference dose (RfD) and the acceptable daily intake (ADI) have been set by various regulatory agencies as 100 times smaller than the NOEL (i.e., 6 μg/kg-d) to account for inter- and intra-species variability [6,7]. Recently, using an ovariectomized adult rat model, we demonstrated that endosulfan acts as an estrogen-like endocrine disruptor at doses similar to both the NOEL and the ADI levels [8]. These findings stress concerns about the potential effects of environmentally relevant doses of this pesticide on the development and function of the female reproductive tract.

Thus far, the majority of studies aimed at investigating the endocrine action of endosulfan have been focused on the reproductive effects of the chemical on male rats that have been exposed *in utero* and during lactation. In addition, most of the previous work has tested doses several orders of magnitude over the RfD or the NOEL. To our knowledge, no report is available on the effects on the female reproductive system of neonatal exposure to low doses of endosulfan.

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doi:10.1016/j.reprotox.2011.12.003

It is well documented that exposure to endocrine-disrupting chemicals (EDC) during critical periods of development (i.e., *in utero* or during the early postnatal period) may adversely affect the morphology and function of reproductive organs by interfering with the synthesis, metabolism, binding or cellular responses of natural estrogens [9,10]. Given that the uterus is a major target organ for circulating sex steroid hormones, we hypothesized that early postnatal exposure to endosulfan might interfere with normal uterine development and differentiation. In this work, we evaluated the effects of neonatal exposure to low doses of endosulfan on the expression of ERα, PR, Hoxa10 and α-SMA, in the uteri of female rats at two time points: female rats were studied shortly after the end of the exposure period (PND8, neonatal period), to evaluate the acute response to the EDC exposure, and 2 weeks after the end of the exposure period (PND21, prepubertal period), to investigate whether the effects persisted and/or were manifested in a stage distant from the EDC exposure. The selection of proteins to be evaluated was based on their role in uterine development/differentiation and responsiveness to other endocrine active compounds. Hoxa10, a member of the HOX gene family, directs embryonic uterine development and is also dynamically expressed in adult endometrium, where it is necessary for embryo implantation [11]. α-SMA is used as a myocyte differentiation marker and its pattern of expression is developmentally regulated [12]. Previous results suggest that the development of myometrium is particularly sensitive to estrogenic compounds, and can be affected by steroid receptors modulation [12]. Taking into account that many EDC may adversely impact hormonal signalling through the interaction with sex steroid hormone receptors, we postulate that uterine ERα and PR expression could be affected by endosulfan's developmental exposure [9].

Because low doses of classical estrogens are recommended as a control when comparing the effects of weak xenoestrogenic compounds [13], a low dose of the synthetic estrogen diethylstilbestrol (DES; 0.2 μg/kg-d) was used as an endocrine disruptor control. In addition to characterize the effects of EDC exposure, we investigated the postnatal ontogenic pattern and cellular distribution of ERα, PR, Hoxa10 and α-SMA proteins in the normally developing uteri of unexposed rats. The results showed that early postnatal exposure to low doses of endosulfan disrupt the expression of estrogen-dependent genes that regulate uterine development and differentiation. In addition, our results suggest that endosulfan might exhibit estrogenic activity, as it mimics some of the effects caused by DES exposure.

2. Materials and methods

2.1. Animals and experimental design

All procedures used in this study were approved by the Institutional Ethic Committee of the School of Biochemistry and Biological Sciences (Universidad Nacional del Litoral, Santa Fe, Argentina) and were performed in accordance with the principles and procedures outlined in the Guide for the Care and Use of Laboratory Animals issued by the U.S. National Academy of Sciences. Pups were obtained from timed-pregnant Wistar rats bred at the Department of Human Physiology (Santa Fe, Argentina) and housed under 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 mothers' diet was not evaluated; however, because food intake was equivalent for control and experimental rats (unpublished observations), we assumed that all animals were exposed to the same levels of phytoestrogens. To minimize additional exposures to endocrine-disrupting chemicals, rats were maintained in stainless steel cages with wood bedding, and tap water was supplied in glass bottles with rubber stoppers surrounded by a steel ring. After delivery (PND0), pups were sexed according to anogenital distance and cross-fostered to minimize the grouping of siblings and, thereby, avoid potential litter effects. Litter size was adjusted to 12 female offspring per mother whenever possible. If fewer than 10 females were available, an appropriate number of males was retained. Female pups from each foster mother were assigned to one of the following neonatal treatment groups (8–12 pups per group): (1) control group receiving corn oil vehicle alone and treatment groups receiving (2) DES (Sigma, St. Louis, MO) at 0.2 μg/kg (DES), (3) endosulfan (98% of purity; Chem Service, West Chester, PA, USA) at 6 μg/kg (Endo6), or (4) endosulfan at 600 μg/kg (Endo600). Substances were dissolved in corn oil, and then 40 μl was administered to the pups by s.c. injection in the nape of the neck every 48 h from PND1 to PND7. The low dose of endosulfan used in our study was similar to the RfD established for this pesticide, while the high dose was 100-fold greater than the RfD and equal to the NOEL. Although the RfD and NOEL for endosulfan are based on oral exposure, the subcutaneous via is the unique administration route that warrants the whole incorporation of a chemical compound when an early postnatal exposure model is used. No signs of acute or chronic toxicity were observed, and no significant differences in weight gain between treated and control pups were recorded during the experiment (data not shown). No alterations in maternal care were detected between the different experimental groups. Pups were sacrificed at PND8 and PND21 by decapitation, and a portion of the uterine horn (1.5 cm) was removed. In addition, in order to determine the postnatal ontogenic pattern and cellular distribution of steroid hormone receptors, Hoxa10 and α-SMA, female rats (6 per time point) were sacrificed at PND1, 8, 21 and 35 and the uterine samples were obtained. Uterine samples were fixed by immersion in 4% paraformaldehyde buffer for 6 h at 4 °C, embedded in paraffin, and processed for histological and immunohistochemical analysis.

2.2. Histological analysis

Uterine sections were stained with hematoxylin and eosin, and examined by light microscope (Olympus BH2 microscope; Olympus, Tokyo, Japan) to compare the uterine morphology of control and treated rats. The thickness of myometrium and subephitelial stroma layers and the height of lumen epithelial cells were analyzed by Image Pro-Plus 5.0.2.9 system (Media Cybernetics, Silver Spring, MD). The number of uterine glands was also counted. Three sections per animal (5 μm in thickness) separated 25 μm from each other were evaluated, and for each section quantification was performed on 10 randomly selected fields.

2.3. Antibodies

The primary antibodies used were as follows: (1) mouse monoclonal antibody to ERα (clone 6F-11, 1:800 dilution; Novocastra, Newcastle upon Tyne, UK), (2) rabbit polyclonal anti-PR (A/B isoforms, A0098, 1:50 dilution; Dako Corp., Carpinteria, CA), (3) goat polyclonal antibody to Hoxa10 (sc-17159, 1:200 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA), and (4) mouse monoclonal antibody for α-SMA (clone α-sm-1, 1:100 dilution; Novocastra). Anti-rabbit and anti-mouse secondary antibodies (biotin conjugate, B8895/B8774) were purchased from Sigma. An anti-goat secondary antibody (biotin conjugate, sc-2042) was purchased from Santa Cruz Biotechnology Inc.

2.4. Immunohistochemistry

A standard immunohistochemical technique (avidin-biotin-peroxidase) was used to visualize ERα, PR, Hoxa10 and α-SMA immunostaining intensity and distribution using previously described protocols [14]. Briefly, uterine sections 5 μm thick were de-paraffinized and rehydrated in a series of xylene and ethanol washes and then subjected to a microwave pretreatment for antigen retrieval. After blocking endogenous peroxidase activity and non-specific binding sites, samples were incubated in a humid chamber first with the specific primary antibody (14–16 h at 4 °C) and then with the corresponding biotin-conjugated secondary antibody (30 min at room temperature). Negative controls were obtained by substituting the primary antibody with the non-immune sera (Sigma) of the species used to generate the primary antibody. The reaction was developed using the avidin-biotin-peroxidase method and diaminobenzidine (DAB) (Sigma) as a chromogen substrate. Samples were dehydrated and mounted with permanent mounting medium (PMyR, Buenos Aires, Argentina).

2.5. Quantification of protein expression by image analysis

The expression of ERα, PR, Hoxa10, and α-SMA proteins in all uterine compartments (luminal epithelium, glandular epithelium, subepithelial stroma and myometrium) was evaluated by image analysis. The integrated optical density (IOD) was measured as a linear combination of the average grey intensity and the relative area occupied by positive cells [15,16]. This linear combination is proportional to the protein content of each histological compartment [17]. Image analysis was performed using the Image Pro-Plus 5.0.2.9 system (Media Cybernetics, Silver Spring, MD), as previously described [16]. In brief, the images were recorded using a Spot Insight V3.5 color video camera attached to an Olympus BH2 microscope (illumination, 12 V halogen lamp and 100 W, equipped with a stabilized light source; Olympus, Tokyo, Japan) using a Dplan 40× objective (numerical aperture = 0.65; Olympus). The microscope was configured for Koehler illumination. The correction of unequal illumination (shading correction) and the calibration of the measurement system were performed using a reference slide. The images of the immunostained slides were converted to grey scale, and the different uterine compartments were delimited (subepithelial stroma: 30–40 μm from the basement membrane toward the outer layer; myometrium: 40–60 μm from the outer layer toward the basement membrane). Although the myometrium can be discerned from the subepithelial

Table 1
Morphometric analysis of uterine sections from 21-day-old rats exposed neonatally to vehicle (Control), 0.2 μg/kg-d of diethylstilbestrol (DES), 6 (Endo6) or 600 (Endo600) μg/kg-d of endosulfan.

	Luminal epithelium height (μm)	Endometrial glands (number/10 fields)	Subepithelial stroma thickness (μm)	Circular miometrium thickness (μm)	Longitudinal miometrium thickness (μm)
Control	17.99 ± 1.01	21.00 ± 1.16	201.40 ± 18.58	66.20 ± 1.79	55.73 ± 4.13
DES	19.27 ± 0.77	20.75 ± 1.38	177.30 ± 9.96	57.72 ± 3.24	59.19 ± 4.08
Endo6	17.68 ± 1.06	19.25 ± 1.89	164.10 ± 15.52	57.46 ± 1.41	44.12 ± 4.25
Endo600	18.73 ± 0.68	21.67 ± 2.40	190.60 ± 17.56	60.88 ± 1.91	54.13 ± 3.24

Measurements were done in 3 uterine sections per animal, 8 rats in each group. Results are expressed as media ± SEM.

stroma by the general morphology of the uterine cells, immunostaining for α-SMA allowed us to accurately establish the limit between both compartments. In the subephitelial stroma and in the myometrium, quantification was performed on at least 10 randomly selected fields per section, and two sections per animal (separated 50 μm from each other) were evaluated. In the luminal epithelium, quantification was performed in areas where luminal folds were not present, whereas in the glandular epithelium, protein expression was measured on at least 10 endometrial glands of each uterine sample. Because uterine gland formation in the rat occurs on PND9 [18], quantification in the glandular epithelium was only performed on PND21.

2.6. Data analysis

All values represent the mean ± SEM. A Kruskal–Wallis analysis was performed to determine the overall significance (testing the hypothesis that responses were not homogeneous across treatments), and the Dunn post-test was applied to compared each experimental group against the control. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Morphometric analysis

As shown in Table 1, neonatal exposure to endosulfan or DES did not result in significant differences relative to control group in any of the morphological uterine features evaluated (i.e.: thickness of myometrium and subepithelial stroma, luminal epithelial height, number of glands).

3.2. Proteins expression by IHQ

3.2.1. ERα

The postnatal ontogenic pattern of ERα protein in the developing rat uterus is illustrated in Fig. 1. ERα immunostaining was restricted to the nuclei of the uterine cells. Uterine epithelial cells showed no ERα immunoreaction at PND1, but the signal became increasingly apparent in the later time points, up to 35 days. The staining intensity of stromal ERα increased from PND1 to PND8 but then was slightly decreased on PND21. From PND21 to PND35, an increase in ERα immunostaining was verified in the stromal compartment.

Figs. 2 and 3 show the expression of ERα in the uteri of vehicle- and EDC-treated rats. Treatment with the highest dose of endosulfan elicited an induction of ERα expression in the subepithelial stroma and the myometrium on PND8 (acute response). Although changes in the subepithelial stroma and myometrium soon reverted, an increase in ERα was observed in the epithelium (luminal and glandular) of the Endo600-treated group only on PND21 (Fig. 3). Neonatal exposure to DES did not induce changes in the expression of ERα on PND8, but noticeable differences were detected at PND21. In fact, ERα was induced in the subepithelial stroma and in the luminal and glandular epithelium of DES-treated rats on PND21. In addition, no differences in ERα expression were observed between Endo6- and vehicle-treated animals in any of the uterine compartments, either on PND8 or PND21.

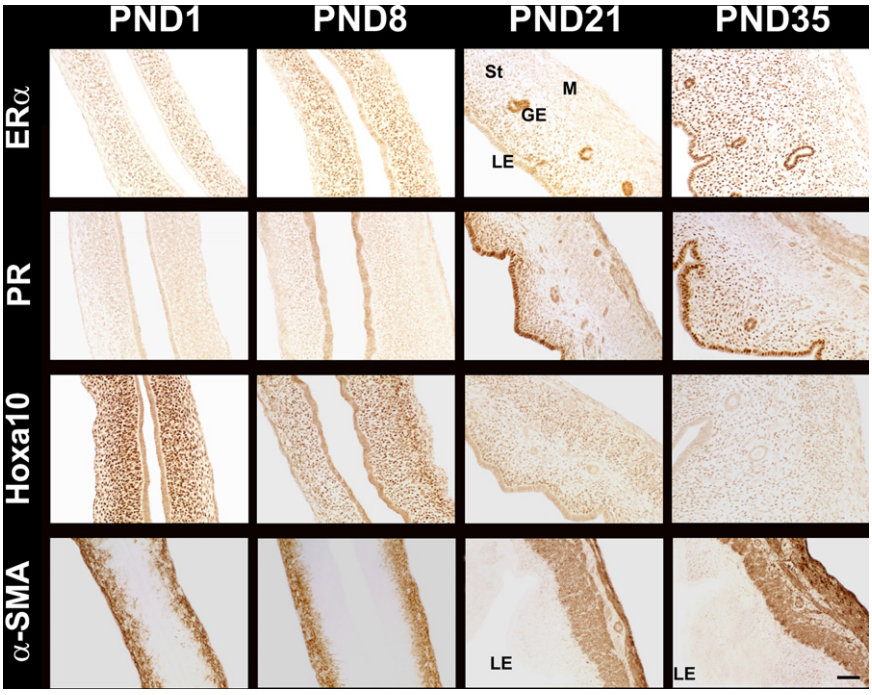

Fig. 1. Representative photomicrographs illustrating the postnatal ontogenic pattern and cellular distribution of ERα, PR, Hoxa10 and α-SMA protein expression in the developing rat uterus. PND, postnatal day; LE, luminal epithelium; GE, glandular epithelium; St, subepithelial stroma; M, myometrium. Scale bar: 50 μm.

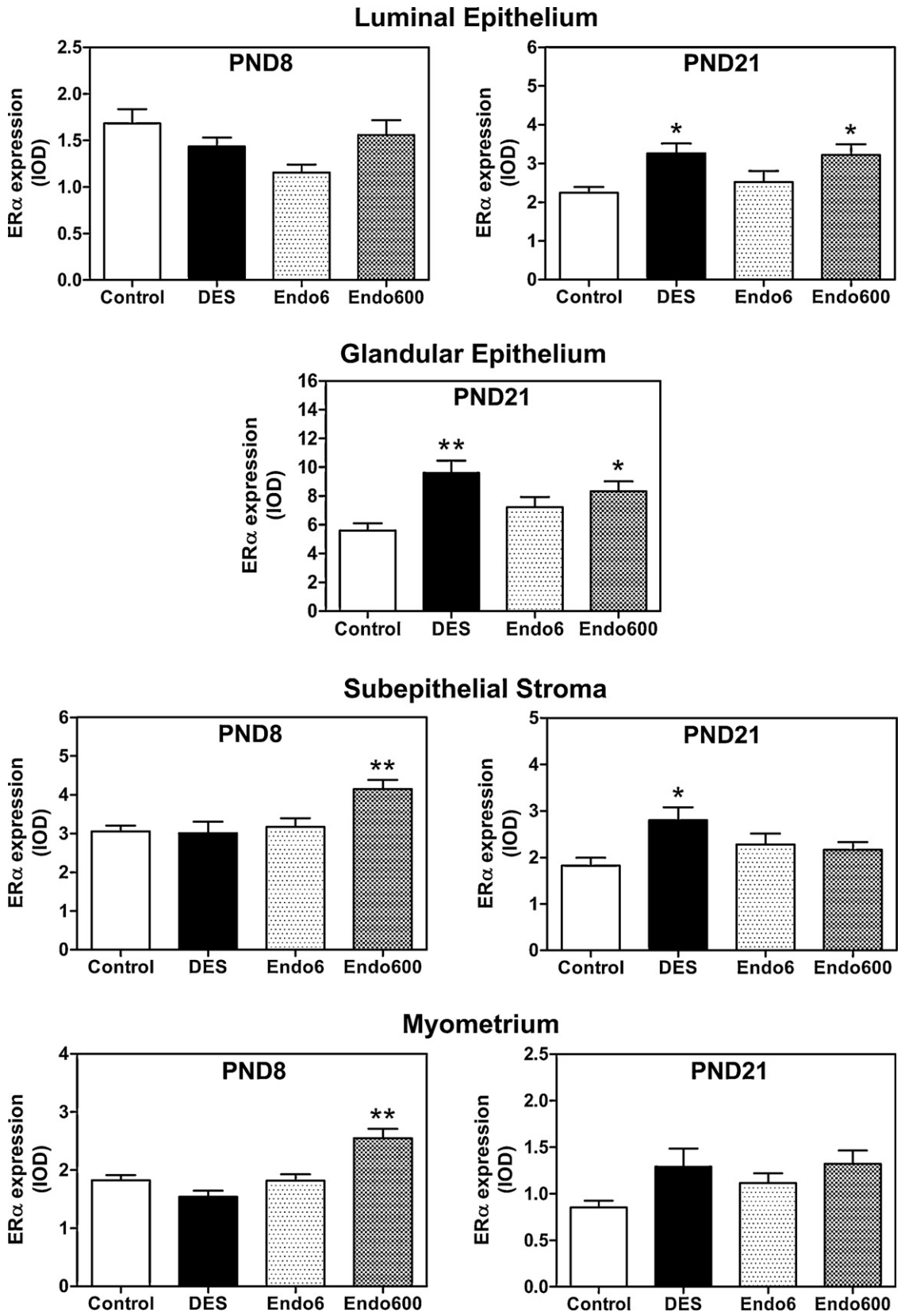

Fig. 2. Quantification of ERα protein expression in the uterine luminal epithelium, glandular epithelium, subepithelial stroma and myometrium of vehicle- or EDC-treated rats on PND8 and PND21. Data were expressed as IOD, which consists of a linear combination of the average immunostained density and the relative area occupied by positive cells. Values are the mean ± SEM of 8–12 rats/group (*$P < 0.05$; **$P < 0.01$ *vs.* control).

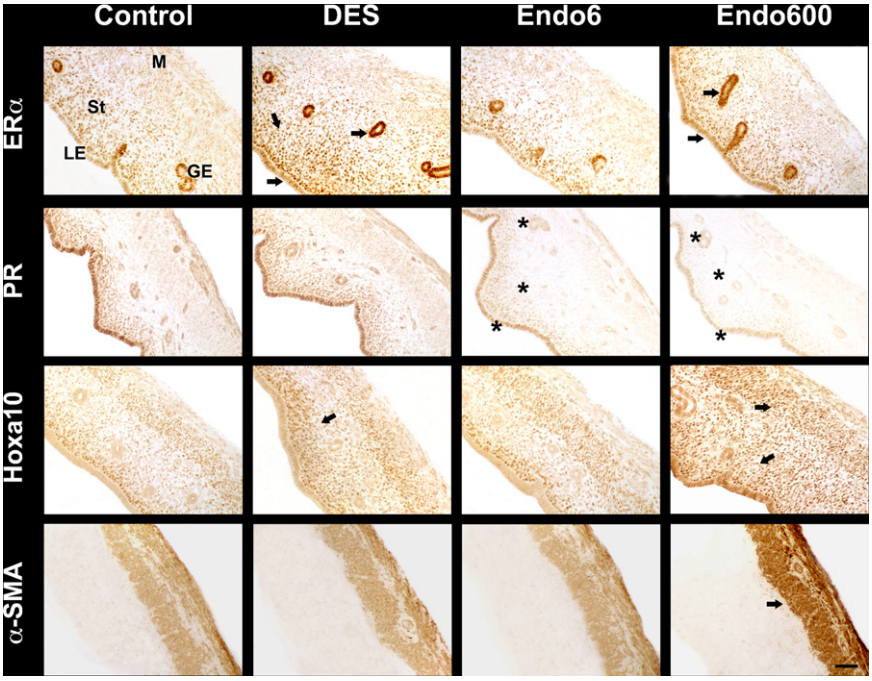

Fig. 3. Representative photomicrographs of uterine ERα, PR, Hoxa10 and α-SMA protein expression of vehicle- or EDC-treated rats on PND21. The arrows indicate increase *vs.* control, and the asterisks indicate decrease *vs.* control. LE, luminal epithelium; GE, glandular epithelium; St, subepithelial stroma; M, myometrium. Scale bar: 50 μm.

3.2.2. PR

As shown in Fig. 1, the immunostaining signal for PR was nearly negligible on PND1 and PND8 in all uterine compartments, but it noticeably increased as the rats aged. Similar to ERα, the PR immunostaining was circumscribed on the uterine cell nuclei.

The quantification of uterine PR in the control and EDC-exposed rats is presented in Fig. 4. Although PR expression on PND8 was comparable in all experimental groups, changes were detected on PND21 between the control and endosulfan-treated rats (Fig. 3). Indeed, treatment with either dose of endosulfan decreased the expression of PR in the subepithelial stroma, glandular and luminal epithelium. On the contrary, no significant differences were observed between DES- and vehicle-treated animals, either at PND8 or PND21. No quantification of PR was performed in the myometrium, as the immunostaining was weak and was similar in all 8- and 21-day-old rats.

3.2.3. Hoxa10

The ontogenic Hoxa10 expression in the rat uterus from PND1 to PND35 is presented in Fig. 1. No immunostaining for Hoxa10 was detectable in the luminal or glandular epithelium at any stage examined. In contrast, intense nuclear staining for Hoxa10 was observed in the subepithelial stroma and myometrium on PND1, but it markedly decreased throughout development.

Figs. 3 and 5 show the Hoxa10 quantification in the uteri of vehicle- and EDC-treated rats. On PND8, the induction of Hoxa10 was evident in the myometrium of Endo600-treated rats. Although no significant differences were detected in the subepithelial stroma, a tendency for higher levels of Hoxa10 expression was observed in the Endo600 group. The changes observed in the myometrium persisted up to PND21, when increased expression of Hoxa10 was also detected in the subepithelial stroma. Unlike the Endo600 group, Hoxa10 was repressed in the subepithelial stroma and myometrium of DES-treated animals on PND8. The acute effect of DES treatment on the myometrium reverted at PND21, as no significant differences were detected 2 weeks after the end of exposure. In contrast, in the subepithelial stroma DES elicited an induction of Hoxa10 by PND21. Endo6-treated animals paralleled the patterns of Hoxa10 expression in control animals.

3.2.4. α-SMA

α-SMA was expressed in the cytoplasm of uterine myocites, and its immunostaining increased from PND1 to PND35 (Fig. 1).

The quantification of α-SMA in 8- and 21-day-old control and EDC-treated animals is shown in Figs. 3 and 6. Rats neonatally exposed to the high dose of endosulfan exhibited an induction of α-SMA on both PND8 and PND21. Contrarily, neither DES nor Endo6 elicited changes in the expression of α-SMA at each age.

Based on microscopic examination, in all uterine compartments, differences in the immunohistochemical expression of the studied proteins between groups were due to changes in level of expression within cells (the intensity of expression).

4. Discussion

The findings of the present study provide evidence that neonatal exposure to endosulfan disrupts the expression of proteins regulating uterine development and differentiation, including ERα, PR, Hoxa10 and α-SMA. In addition, we described the postnatal ontogenetic pattern and cellular distribution of these proteins in the developing rat uterus.

Although the expression of ERα and PR has been extensively studied in the adult and fetal reproductive tract of the rat [19–22], little is known about the extent and patterns of expression of these steroid receptors during early postnatal development. In accordance with the findings of Ohta et al. [23], we observed no expression of ERα or PR in the uterine epithelial cells on PND1. Yamashita et al. [24] and Sato et al. [25] investigated the distribution of ERα in the female mouse genital tract and found no expression in the uterine epithelium until PND4 and PND5, respectively. Moreover, similar to that observed by other authors [23,26], we detected increased ERα and PR immunostaining in the nuclei of all uterine cells during development, with the exception of PR in the myometrium. In the myometrium, a low level of expression of PR

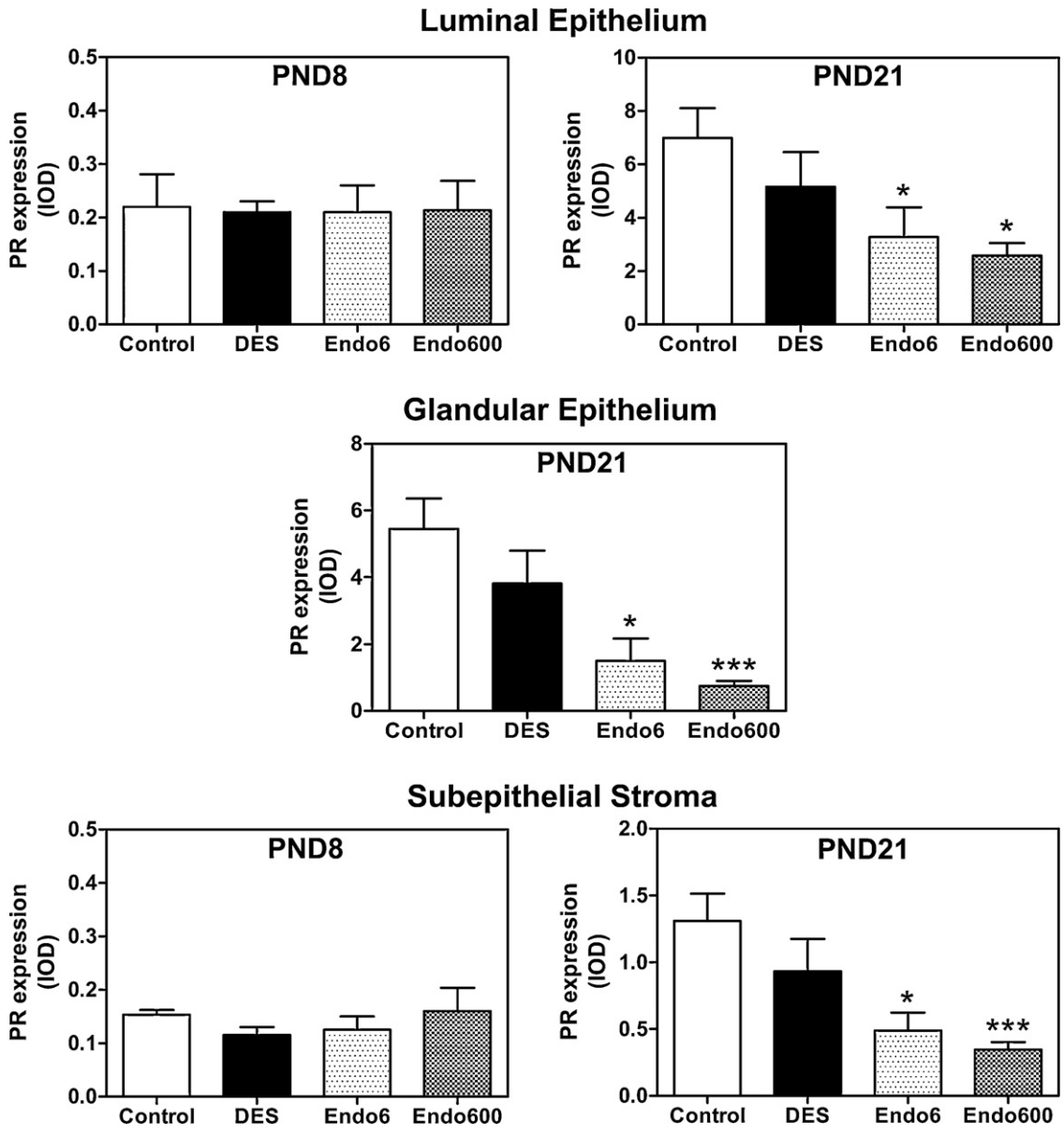

Fig. 4. Quantification of PR protein expression in the uterine luminal epithelium, glandular epithelium and subepithelial stroma of vehicle- or EDC-treated rats on PND8 and PND21. Data were expressed as IOD, which consists of a linear combination of the average immunostained density and the relative area occupied by positive cells. Values are the mean ± SEM of 8–12 rats/group (*$P < 0.05$; ***$P < 0.001$ *vs.* control).

was detected from birth to PND35, which agrees with the results of Ohta et al. [23] who reported that smooth muscle cells showed no definite positive staining of PR until PND20. To our knowledge, no report is available about the ontogeny and cellular distribution of Hoxa10 in the rat uterus during early postnatal development. Here, we observed that Hoxa10 expression declined with age and was detected both in the myometrium and in the subepithelial stroma but not in the epithelium (luminal and glandular). The absence of expression in the epithelium is in agreement with the results of Hu et al. [27] who found no Hoxa10 expression in the epithelium of the mouse uterus between PND3 and PND15 using *in situ* hybridization analyses. Moreover, we found that the α-SMA expression increased from PND1 to PND35. This result is in agreement with a previous study that revealed an age related increase in α-SMA expression in the myometrium of the mouse uterus [27].

Subsequently, we studied the effect of neonatal endosulfan exposure on the rat uterus at two time points, i.e., close to the end of treatment (PND8), to evaluate the acute response to the exposure, and 2 weeks after the end of treatment (PND21), to investigate whether the effects persisted and manifested in a stage distant from the exposure period. Further, the effects of endosulfan were compared with those of DES.

Regarding morphological evaluation, neither DES nor endosulfan altered the uterine tissue structure at the doses tested. The postnatal exposure to DES induces uterine morphological changes but using higher doses [28] relative to this study. It is well established that the exposure to low doses of environmental chemicals that cause endocrine disruption during developmental periods may promote subtle morphological effects.

As previously stated, little is known about the deleterious effects of endosulfan on female reproductive health. Recently, we demonstrated that endosulfan, at doses similar to ADI and NOEL, mimics the action of a non-uterotrophic dose of E2, causing a deregulation of ERα and PR uterine expression in ovariectomized adult female rats [8]. The present study shows that early postnatal exposure to low doses of endosulfan induced changes in the expression of

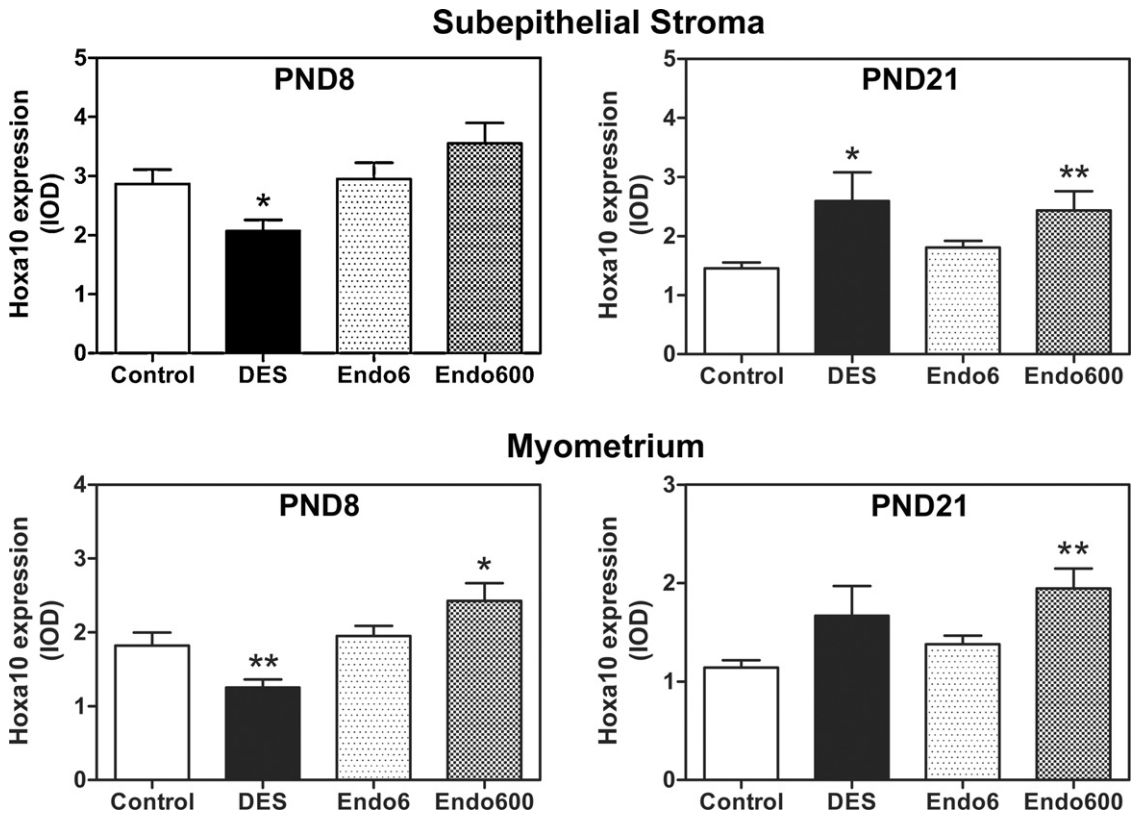

Fig. 5. Quantification of Hoxa10 protein expression in the uterine subepithelial stroma and myometrium of vehicle- or EDC-treated rats on PND8 and PND21. Data were expressed as IOD, which consists of a linear combination of the average immunostained density and the relative area occupied by positive cells. Values are the mean ± SEM of 8–12 rats/group (*$P < 0.05$; **$P < 0.01$ *vs.* control).

proteins that regulate uterine development and differentiation in neonatal and prepubertal female rats, findings which show coincidences with the effects caused by DES. Specifically, we found that the highest dose of endosulfan (Endo600) altered the expression of ERα, PR, Hoxa10 and α-SMA both immediately after treatment ended and 2 weeks after the exposure. Although ERα, Hoxa10 and α-SMA were induced in response to Endo600 treatment on PND21, a striking decrease in PR expression was detected in all uterine compartments. The lowest dose of endosulfan (Endo6) also resulted in the repression of PR in the uteri of neonatal and prepubertal female rats. Neonatal DES exposure also elicited a dysregulation of ERα and Hoxa10 uterine expression, both on PND8 and PND21, but PR and α-SMA were not modified in response to the treatment. In coincidence with Endo600 group, DES increased the uterine expression of ERα and Hoxa10 in 21-day-old prepubertal female rats. Recently, it has been pointed out the importance of including an appropriated positive control to test the responsiveness of the animal model to endocrine-active agents [29]. In the present work, we found a repression in Hoxa10 uterine expression shortly after DES treatment (PND8), which agree with previous reports [30,31]. These findings supported the choice of the animal model used as sensitive to EDC.

Several agents with estrogen-like activity have been shown to disrupt the expression of developmental-related genes. Similar to the effects observed in this study, a reduction in PR and an increase in ERα uterine expression were reported in adult female rats exposed *in utero* to the EDC polybrominated diphenylether PBDE 99 (a flame retardant chemical used in plastic, textiles, foams and

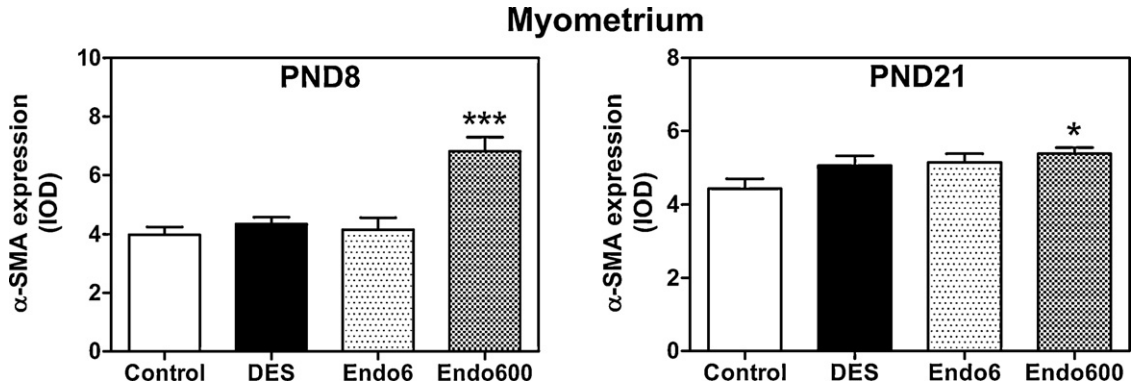

Fig. 6. Quantification of α-SMA protein expression in the uterine myometrium of vehicle- or EDC-treated rats on PND8 and PND21. Data were expressed as IOD, which consists of a linear combination of the average immunostained density and the relative area occupied by positive cells. Values are the mean ± SEM of 8–12 rats/group (*$P < 0.05$; ***$P < 0.001$ *vs.* control).

electronic devices) at doses devoid of general toxicity [32]. Unlike endosulfan, neonatal exposure to methoxychlor (an organochlorine pesticide with estrogenic activity) decreased Hoxa10 uterine expression in 2-week-old CD-1 mice [33]. Similarly, in a recent report we found that neonatal exposure to bisphenol A, an organic compound used in the manufacture of polycarbonate plastic and epoxy resins, reduced Hoxa10 levels in the uteri of prepubertal rats [34]. Other authors, however, reported a dose-responsive increase in uterine Hoxa10 expression in 2-week-old mice following *in utero* BPA exposure [35]. Taken together, these findings confirm previous evidence that ERα, PR and Hoxa10 genes are common targets of endocrine disruption and suggest that their dysregulation might be involved in the reproductive tract anomalies related to EDC exposure. The results of the current study additionally suggest that endosulfan may exhibit estrogen agonist activity, as it acts as an inducer of estrogen-dependent genes in the rat uterus. It has been reported that endosulfan acts as an EDC by binding to estrogen receptors [9]. Lemaire et al. [36] demonstrated that endosulfan competes with estradiol (E2) for binding to ERα, and that it is able to trans-activate this receptor and induce the transcription of an estrogen response element (ERE)-dependent gene construct in an ERα-transfected HELN cell line.

It is well recognized that steroid receptors and Hoxa10 play a key role in embryonic morphogenesis and differentiation. Hoxa10 functions as a transcriptional factor regulating downstream genes necessary for the development and differentiation of the reproductive tract. Some studies have demonstrated that Hoxa10 is an estrogen and progesterone responsive gene in the uterine endometrium [37–39]. The transcriptional activation of Hoxa10 by E2 is mediated through its nuclear receptors [40,41]. Sequence analysis of the 5′ regulatory region of HOXA10 revealed the presence of two putative estrogen response elements (EREs) that bind the estrogen receptors ERα and ERβ [40]. On the other hand, α-SMA plays an important role in uterine development and differentiation and its expression in the rat uterus was found to be regulated by estradiol; however, the molecular mechanisms underlying this regulation remain to be elucidated [42]. These findings allow us to suggest that the increased expression of Hoxa10 and α-SMA found in the uterus of the Endo600 group could be linked to the increased ERα protein levels detected in the same group.

Estrogen has been implicated in the transcriptional regulation of PR via its nuclear receptors, but PR expression and distribution in the uterus are dependent on the cell type [43]. Estrogen administrated at a relatively high dose was found to elicit a dual effect on PR expression, inducing its expression in the stromal and glandular epithelial cells and reducing its levels in the luminal epithelium [43–45]. Given that the modulation of PR expression induced by endosulfan differed from that induced by estrogen, the repression of PR observed in the uteri of rats in the Endo6 and Endo600 groups is likely to be mediated by signaling mechanisms different from ER-dependent transcription.

The early postnatal disruption of estrogen-dependent genes that regulate uterine development and differentiation may alter the developmental programming with long-term reproductive consequences [9]. The aberrant expression of steroid receptors and Hoxa10 has been associated with reproductive pathologies, including endometriosis, leiomyomas, and infertility [41,46–48]. Similarly, the deregulation of α-SMA expression may alter proper uterine function during pregnancy, as this structural protein is essential for the support of the growing embryo and for the contraction of the uterine wall during labor [43]. In light of this data, and based on the results obtained in our study, the disruption of the morphoregulatory genes ERα, PR, Hoxa10 and α-SMA caused by low doses of endosulfan exposure during critical stages of differentiation could lead to impaired female reproductive performance. Previous studies have implicated acute exposure to organochlorine pesticides in the incidence of uterine leiomyomas, as women harboring this disease exhibited higher levels of organochlorine in their blood and uterine tissue than unaffected women [49]. Other authors reported that the administration of endosulfan (4 mg/kg-d) to pregnant mice completely inhibits implantation [50]. It is worth pointing out that organochlorine compounds, such as endosulfan, tend to bioaccumulate in fatty tissues and can be transfer to the fetus through the placenta during gestation and to the newborn during lactation [51]. Even though, endosulfan residue and its metabolites have been detected in human placenta, cord blood and breast milk samples [3,51], only one report links reproductive effects in children and adolescents to developmental exposure to endosulfan [52].

In summary, our results show that early postnatal exposure to endosulfan, at doses similar to the ADI and the NOEL, altered the expression of estrogen-dependent genes that regulate uterine development and differentiation. The disruption of uterine morphoregulatory genes during critical periods of development may reprogram the normal physiological responses to sex steroid hormones in adulthood, with lasting consequences for reproductive health [34]. Additional studies are currently underway to investigate whether the dysregulation of steroid receptors, Hoxa10 and α-SMA following postnatal exposure to endosulfan could affect the proper uterine function along pregnancy, labor and delivery in the adult rat.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgements

This work was supported by grants from the Universidad Nacional del Litoral (CAI + D program), the Argentine National Council for Science and Technology (CONICET), and the Argentine National Agency for the Promotion of Science and Technology (ANPCyT). MMM, JV, and EHL are Career Investigators of the CONICET.

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