Journal of Steroid Biochemistry & Molecular Biology 138 (2013) 1–9

Contents lists available at SciVerse ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

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

Perinatal exposure to diethylstilbestrol alters the functional differentiation of the adult rat uterus

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ARTICLE INFO

Article history:
Received 30 November 2012
Received in revised form 15 February 2013
Accepted 18 February 2013

Keywords:
Diethylstilbestrol
Rat
Uterus
Uterine glands
Steroid receptors
p63

ABSTRACT

The exposure to endocrine disrupters and female reproductive tract disorders has not been totally clarified. The present study assessed the long-term effect of perinatal (gestation + lactation) exposure to diethylstilbestrol (DES) on the rat uterus and the effect of estrogen replacement therapy. DES (5 μg/kg bw/day) was administered in the drinking water from gestational day 9 until weaning and we studied the uterus of young adult (PND90) and adult (PND360) females. To investigate whether perinatal exposure to DES modified the uterine response to a long-lasting estrogen treatment, 12-month-old rats exposed to DES were ovariectomized and treated with 17β-estradiol for 3 months (PND460). In young adult rats (PND90), the DES treatment decreased both the proliferation of glandular epithelial cells and the percentage of glandular perimeter occupied by α-smooth muscle actin-positive cells. The other tissue compartments remained unchanged. Cell apoptosis was not altered in DES-exposed females. In control adult rats (PND360), there were some morphologically abnormal uterine glands. In adult rats exposed to DES, the incidence of glands with cellular anomalies increased. In response to estrogens (PND460), the incidence of cystic glands increased in the DES group. We observed glands with daughter glands and conglomerates of glands only on PND460 and in response to estrogen replacement therapy, independently of DES exposure. The p63 isoforms were expressed without changes on PND460. Estrogen receptors α and β showed no changes, while the progesterone receptor decreased in the subepithelial stroma of DES-exposed animals with estrogen treatment. The long-lasting effects of perinatal exposure to DES included the induction of abnormalities in uterine tissues of aged female rats and an altered response of the adult uterus to estradiol.

1. Introduction

Perinatal differentiation of the mammalian genital tract occurs following a complex series of interactions between classical hormone receptors and signaling molecules that ultimately program target cells to respond appropriately to hormonal cues later in life [1]. The uterine luminal and glandular epithelia are derived from the anterior Müllerian epithelium. Differentiation of the uterine epithelium occurs between postnatal days 5 and 7 and is thought to be mediated by signals from the mesenchyme. After postnatal day 7, the fate of the uterine epithelium is determined and cannot be changed by signals from a heterotypic mesenchyme [2]. However, a small number of epithelial cells maintain developmental plasticity in the adult uterus; these may be stem cells and the targets of uterine squamous metaplasia [3].

Transcription factor p63 is the product of the *TP63* gene, which is transcribed into isoforms either containing or lacking the N-terminal transactivation domain: TA or *ΔN* forms respectively [4]. p63 is commonly expressed in cervical and vaginal cells but not in the uterine epithelium [2]. However, normal p63 expression and proper uterine cytodifferentiation can be disturbed by developmental exposure to the synthetic estrogen diethylstilbestrol (DES) [5].

DES is a prototype endocrine disruptor with potent estrogen-like action [6,7]. DES was the first synthetic estrogenic compound orally administered to pregnant women (from 1947 to 1971) in an effort to preserve pregnancy. Although DES is no longer used clinically to prevent miscarriage, a major concern remains because women that were exposed to DES in uterus are now reaching the age at which the incidence of reproductive organ cancers normally increases. Besides this, the use of DES in the past for animal production may be responsible for significant levels

Abbreviations: DES, diethylstilbestrol; GD, gestation day; PND, postnatal day; E2, 17β-estradiol; BrdU, bromodeoxyuridine; α-SMA, smooth muscle α-actin; CK, cytokeratin; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; PR, progesterone receptor; IOD, integrated optical density; CT, cycle threshold.

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http://dx.doi.org/10.1016/j.jsbmb.2013.02.011

present in the environment, mainly related to feed-lot areas [8]. In Argentina, medical formulations containing DES (*e.g.* Gobbibestrol, Gobbi Novag S.A.) are currently approved by the ANMAT (National Administration of Drugs, Foods, and Medical Devices; http://www.anmat.gov.ar/aplicaciones_net/applications/consultas/vademecum/vademecum.asp).

DES exposure in humans has been associated with reproductive tract anomalies such as T-shaped uterine cavity, vaginal adenosis and annular cervical rings [9,10]. In female mice, neonatal DES exposure causes uterine malformations including hypoplasia, stratification of the luminal epithelium, disorganized smooth muscle and reduced endometrial glands [5]. We have previously demonstrated that DES exposure during early postnatal life disrupts uterine expression of *HOXA10*, *HOXA11* and vascular endothelial growth factor, in association with an abnormal responsiveness of uterine stromal cells to sex steroids during adulthood [11,12]. We have also shown long-lasting effects on the number of implantation sites and on implantation-associated gene expression in female rats neonatally treated with DES [13]. Neonatal DES exposure in mice disrupts the mesenchymal signals that specify uterine or vaginal epithelium differentiation [2]. This disruption results in the expression of p63 in the uterine epithelium but in lack of expression in the vaginal epithelium [2]. The molecular mechanisms underlying these anomalies and the disruption of other genes due to developmental exposure to DES are poorly understood.

It is clear that DES has long-term effects that may develop over time [14]. However, no studies have evaluated the effect of birth control pills or hormone replacement therapy in females perinatally treated with DES (DES daughters) [10]. In the present work, we investigated the long-term effects of perinatal (gestation + lactation) DES exposure in adult female rats and in ovariectomized (OVX) adult rats treated with 17β-estradiol (E2). DES was administered by the oral route, the most relevant route of exposure to endocrine disrupting chemicals in the general population.

2. Materials and methods

2.1. Animals

The experimental protocols were designed in accordance with the Guide for the Care and Use of Laboratory Animals issued by the U.S. National Academy of Sciences and approved by the ethics committee of the School of Biochemistry and Biological Sciences, Universidad Nacional del Litoral (Santa Fe, Argentina). Rats of a Wistar-derived strain bred at the Department of Human Physiology (School of Biochemistry and Biological Sciences, Universidad Nacional del Litoral) were kept in a controlled environment ($22 \pm 2\,^{\circ}C$; 14 h of light from 0600 h to 2000 h) with free access to pellet laboratory chow (Nutrición Animal, Santa Fe, Argentina). The concentration of phytoestrogens in the diet was not evaluated; however, because food intake of control and DES-treated rats was equivalent (unpublished data), we assumed that all animals were exposed to the same levels of phytoestrogens. To minimize other exposures to endocrine-disrupting chemicals, rats were housed in stainless steel cages with sterile pine wood shavings as bedding; tap water was supplied *ad libitum* in glass bottles with rubber stoppers.

2.2. Experimental design

Females in proestrus were caged overnight with males of proven fertility. The day on which sperm was found in the vagina was designated day 1 of gestation (GD1). On GD9, which corresponds to the beginning of fetal organogenesis, pregnant rats were weighed and randomly divided into two experimental groups: control (0.001% ethanol) and DES (5 μg/kg bw, Sigma, St. Louis, MO, USA), with 10–12 dams/group. DES was administered in the drinking water from GD9 to weaning (Fig. 1). The dose was calculated on the basis of the average weight of dams and water consumption during pregnancy and lactation. The DES dose used in this experiment was selected based on a pilot experiment (see Section 2.2.1).

After parturition, pups were weighed and sexed according to the anogenital distance; litters of eight pups (preferably four males and four females) were left with lactating mothers until weaning on postnatal day (PND) 21. As an external index of puberty onset, vaginal opening was monitored daily starting on PND30. To evaluate the age-related effects of perinatal exposure to DES on the rat uterus, a group of females were sacrificed in estrus on PND90 (young adults) and PND360 (adults). To evaluate whether perinatal exposure to DES modified the response to a long-lasting treatment with E2, 12-month-old rats from the control and DES groups were ovariectomized (OVX) to avoid endogenous E2 variability and treated with an estrogen replacement therapy ($n = 8$–11 rats/group) for 90 days (Fig. 1). Rats were implanted (sc) with silastic capsules (3.8 mm in diameter; 30 mm in length) filled with 1 mg E2/ml dissolved in sesame oil. In the control group, OVX rats ($n = 5$) were implanted with vehicle (sesame oil)-filled capsules. The treatment with the estrogen implants produces constant blood levels of E2 that are equivalent to circulating levels during the rat estrous cycle [15]. This model is supposed to mimic the estrogen replacement therapy *via* the use of patches in menopausal women [15]. To ensure exposure to constant E2 levels, the implants were changed every 30 days. After the treatment, the animals were sacrificed. No more than two siblings were included in each time point. The remaining females and all males from each litter were assigned to other experiments. Two hours before the autopsy, each rat was injected (ip) with the thymidine analog bromodeoxyuridine (BrdU; 60 mg/kg; Sigma).

2.2.1. Selection of the dose level

A pilot experiment was performed to determine the dose of DES orally administered that result in endocrine disruption, using the modified expression of *HOXA10* mRNA. Previous studies have shown that Hox genes are targets of endocrine disruption [11,16]. The expression of Hoxa10 is regulated by sex steroid hormones. The estradiol regulation of Hoxa10 is associated with the detection of estrogen receptor binding of two putative estrogen response elements in the 5′ regulatory region of Hoxa10. Progestational regulation of Hoxa10 occurs *via* the progesterone receptor and is therefore blocked by RU486 [17].

The 0.05 and 5 μg/kg bw doses of DES used in our pilot study were chosen based on: (a) the limited literature data on oral DES treatment, (b) the interference observed in the development of both the female and the male reproductive tracts in the mouse without evident maternal toxicity or embryotoxicity, and (c) the fact that these doses are in the lower range of human exposure following therapeutic use [10].

2.3. Sample collection

The characteristics of the estrous cycle of our rat strain are similar to those described by vom Saal et al. [18]. At 7/8 months, the length of the estrous cycle begins to increase, showing periods of cornified epithelium interspersed with cycles of 10–14 days (suggestive of a prolonged luteal phase). The mean age of cessation of cyclicity occurs relatively late in life (about 18 months old) and is characterized by persistent anestrus. In the present work, the stage of estrous cycle (proestrus, estrus, metestrus, or diestrus) of each 90- and 360-day-old animal was daily determined by vaginal smears [19] for at least 20 days prior to sample collection. The animals were autopsied in estrus (evaluated by vaginal smears and

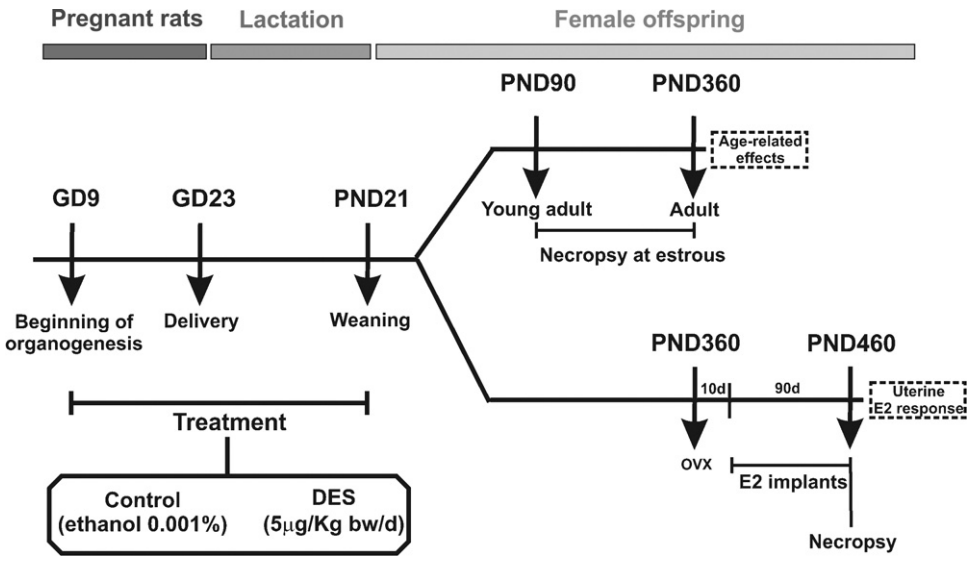

Fig. 1. Experimental design. Schematic representation of the experimental protocol used to study the effects of perinatal (gestation + lactation) exposure to a low dose of diethylstilbestrol (DES) on the uterus of young adult (PND90) and adult (PND360 or 460) females. GD, gestation day; PND, postnatal day; E2, 17β-estradiol; OVX, ovariectomy.

positive lordosis behavior) and uterine tissue was collected and processed for different experimental purposes. For immunohistochemistry, one uterine horn of each rat was fixed in 10% buffered formalin for 24 h at room temperature and embedded in paraffin. For RNA extraction, the other uterine horn of each rat was immediately frozen in liquid nitrogen and stored at −80 °C.

2.4. Histology

Uterine samples embedded in paraffin were cut into 5-μm sections, mounted on slides coated with 3-aminopropyl triethoxysilane (Sigma–Aldrich of Argentina S.A.) and stained with hematoxylin and eosin for light microscopy (Olympus BH2, Tokyo, Japan). Uterine glands were classified using the criteria described by Gunin et al. [20] and McLachlan et al. [21]. To assess the incidence of different gland types, a ratio between animals with at least one gland of the chosen type and the total number of animals per group was established.

2.5. Immunohistochemistry

Uterine sections (5 μm in thickness) were deparaffinized and dehydrated in graded ethanols. BrdU incorporation to detect cells in the S phase of the cell cycle was evaluated as previously described [22]. Endogenous peroxidase activity and nonspecific binding sites were blocked. Primary antibodies against proliferation markers, p63, steroid receptors, basal and luminal cytokeratins, vimentin and smooth muscle α-actin (α-SMA) were incubated overnight at 4 °C (Table 1). The reactions were developed using a streptavidin-biotin peroxidase method and diaminobenzidine (Sigma) as a chromogen substrate. Samples were mounted with permanent mounting medium (Eukitt, Sigma). Each immunohistochemical run included negative controls in which the primary antibody was replaced by non-immune goat serum (Sigma). Negative controls for BrdU immunodetection were samples from animals that did not receive BrdU.

2.6. In situ detection of apoptosis

To evaluate apoptosis, sections were analyzed for *in situ* detection of cells with DNA strand breaks using the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) technique (ApopTag; Intergen Co., Purchase, NY, USA) as previously described [23]. Sections were incubated with a mixture containing digoxigenin deoxynucleotide triphosphate, unlabeled deoxynucleotide triphosphate, and terminal dideoxy transferase (TdT). Subsequently, slides were incubated with antidigoxigenin-peroxidase and substrate-chromogen mixture (DAB; Sigma). Samples were counterstained with Mayer's hematoxylin and mounted with permanent mounting medium. Negative control slides were run using exactly the same procedures, except that distilled water was added instead of TdT. As positive control, an involuting rat prostate after the second day of castration was processed in the same way as the experimental samples.

Table 1
Antibodies used for immunohistochemistry.

Antibodies	Dilution	Supplier
Primary		
Anti-PR (clone A0098)	1/500	Dako Corp. (Carpinteria, CA)
Anti-ERα (clone 6F-11)	1/200	Novocastra (Newcastle upon Tyne, UK)
Anti-p63 (clone 4A4)	1/100	Santa Cruz Biotechnology Inc. (Santa Cruz, CA)
Anti-pan-CK basal (clone 34βE12)	1/100	Novocastra (Newcastle upon Tyne, UK)
Anti-CK8	1/1600	The Binding Site Limited (Birmingham, UK)
Anti-BrdU (clone 85-2C8)	1/100	Novocastra (Newcastle upon Tyne, UK)
Anti-ERβ (clone EMRO2)	1/100	Novocastra (Newcastle upon Tyne)
Anti-vimentin (clone V9)	1/50	Novocastra (Newcastle upon Tyne)
Anti-smooth muscle α-actin (α-SMA clone αsm-1)	1/100	Novocastra (Newcastle upon Tyne)
Secondary		
Anti-mouse	1/80	Sigma (St. Louis, MO)
Anti-goat	1/200	Sigma (St. Louis, MO)
Anti-sheep	1/200	Sigma (St. Louis, MO)

2.7. Image analysis and morphometry

2.7.1. Cell proliferation and apoptosis

Immunostained tissue sections were evaluated using an Olympus BH2 microscope (Olympus Optical Co. Ltd, Tokyo, Japan), with a Dplan 100× objective (numerical aperture = 1.25; Olympus). The incorporation of BrdU and apoptosis were quantitatively analyzed in all tissue compartments of the uterus. Proliferative and apoptotic indices were obtained considering either the percentage of positive cells (for epithelial cells, by counting 2000 cells/tissue section) or the volume fraction of positive cells (for stromal and muscle cells) calculated by applying the formula given by Weibel [24].

2.7.2. Steroid receptors

The images of tissue sections were captured with a Spot Insight version 3.5 color video camera using a BH2 microscope with a Dplan 20× objective (numerical aperture, 0.65; Olympus). To measure the integrated optical density (IOD) of ERα, ERβ and PR immunostaining in the subepithelial stroma, images were analyzed using the Image Pro-Plus 5.0 system (Media Cybernetics, Silver Spring, MD, USA) as previously described [23]. At least 10 fields were recorded in each section, and three sections per animal were evaluated. The subepithelial–stromal compartment was delimited (a 300-μm-wide area adjacent to the epithelium from the basement membrane toward the outer layers). The stromal areas and the IOD were measured as a linear combination between the average gray intensity and the relative area occupied by positive cells. Because the IOD is a dimensionless parameter, the results were expressed as arbitrary units.

2.7.3. Vimentin and α-SMA

The images were recorded with a Spot Insight version 3.5 color video camera (Diagnostic Instruments), using a BH2 microscope with a Dplan 20× objective. The expression of vimentin was quantified in the periglandular stroma zone defined as 10-μm-wide area around the glands (from the basement membrane towards the outer layers), and the relative area occupied by vimentin-positive cells was determined [25]. α-SMA expression was measured as the proportion of the glandular perimeter occupied by cytoplasmic projections of α-SMA-positive cells (linear density) [26].

2.7.4. p63 and cytokeratins

Three uterine sections were qualitatively evaluated to assess the distribution of positive cells in the luminal and glandular epithelium.

2.8. Reverse transcription and real-time quantitative PCR analysis

2.8.1. RNA extraction and reverse transcription

Individual uterine horn samples were 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 A260, and the samples were stored at −80 °C until needed. Equal quantities (4 μg) of total RNA were reverse-transcribed into cDNA with Moloney Murine Leukemia Virus reverse transcriptase (10 units; Promega, Madison, WI, USA) using 200 pmol of random primers (Promega). Twenty units of ribonuclease inhibitor (RNAout; Invitrogen Argentina, Buenos Aires, Argentina) and 100 nmol of a deoxynucleotide triphosphate (dNTP) mixture were added to each reaction tube in 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.

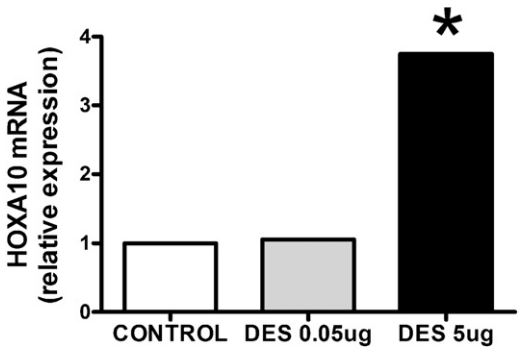

Fig. 2. Effect of perinatal exposure to low doses of DES on *HOXA10* mRNA in the uterus of prepubertal rats (PND8). Relative *HOXA10* mRNA levels were measured by real-time RT-PCR in control and DES (0.05 and 5 μg/kg bw) rats. Samples were normalized to 18S expression and to control animals; a value of 1 was assigned to the control group (*$p < 0.05$, REST 2009 software).

2.8.2. Real-time quantitative PCR

Each reverse-transcribed product was diluted with ribonuclease-free water to a final volume of 60 μl and further amplified in triplicate using the Real-Time Rotor-Gene Q (Quiagen; Tecnolab; Buenos Aires, Argentina). Primer sequences used for amplification of *HOXA10*, *TAp63*, *ΔNp63*, and ribosomal subunit 18S (housekeeping gene) cDNAs are described in Table 2. For cDNA amplification, 5 μl of cDNA was combined with HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne; Biocientífica, Rosario, Argentina), and 10 pmol of each primer (Invitrogen) in a final volume of 20 μl. After initial denaturation at 95 °C for 15 min, the reaction mixture was subjected to successive cycles of denaturation at 95 °C for 15 s, annealing at 54 °C (*ΔNp63*), 55 °C (*TAp63*), 57 °C (*HOXA10*) and 60 °C (18S) for 20 s, and extension at 72 °C for 20 s. Product purity was confirmed by dissociation curves, and random samples were subjected to agarose gel electrophoresis. Controls containing no template DNA were included in all assays, yielding no consistent amplification. The relative expression levels of each target were calculated based on the cycle threshold (CT) method [27]. The CT for each sample was calculated using Rotor-Gene Q-Pure Detection software (Version 1.7, Quiagen; Tecnolab). Accordingly, fold expression over control values was calculated for each target by Relative Expression Software Tool V2.0.13 (REST; Quiagen; Tecnolab), which is specifically designed to analyze data from real-time PCR. No significant differences in CT values were observed for 18S between the different experimental groups.

2.9. Statistical analysis

All data were expressed as the mean ± SEM. The incidence of uterine lesions was analyzed by Fisher's exact test. The other variables were analyzed using the t test. $p \leq 0.05$ was accepted as significant.

3. Results

3.1. DES dose

Results from the pilot study performed to select the DES dose showed that exposure of animals to 5 μg/kg bw of DES induced uterine *HOXA10* mRNA on PND8 (Fig. 2). Based on these results, all the experiments were performed using 5 μg/kg bw of DES.

The treatment with DES did not produce signs of maternal toxicity, abnormal behavior or changes in the dam body weight gain or water consumption. The gestation length was unaltered since all dams delivered on gestational day (GD) 23. No gross malformations were observed in pups on PND1; the average number of

Table 2
Primers and PCR products for real time quantitative PCR.

Genes	Sense primer	Antisense primer	Product size (bp)
HOXA10	5′-AACAGTAAAGCCTCTCCGGA-3′	5′-TGCTTCGTGTAAGGGCAGC-3′	113
TAp63	5′-AGCACCCAGACAAGTGAGTTC-3′	5′-GGTCACTGAGGTCTGAGTCTTG-3′	190
ΔNp63	5′-GTACCTGGAAAGCAATGCC-3′	5′-GTAGATGAGGAGCCGTTCTG-3′	105
18S	5′-TAAGTCCCTGCCCTTTGTACACA-3′	5′-GATCCGAGGGCCTCACTAAAC-3′	71

live pups/litter was not significantly different. The litter sex ratio showed no differences and was within the normal range (50% females and 50% males). Litter survival and weight gained during lactation was unaltered. The onset of puberty in female pups showed no significant differences between groups. Most females perinatally exposed to DES exhibited regular estrous cycles (5 days) when examined at 3 months of age (PND90). At 12 months (PND360), the vaginal smears of some animals revealed intermittent extended periods of diestrus, whereas others exhibited extended periods of proestrus/estrus. The patterns of estrous cycles did not differ between treated and control groups (data not shown).

3.2. Uterine evaluation in young adult (PND90) and adult (PND360) rats perinatally exposed to DES

3.2.1. Young adults (PND90)

Uterine histology showed no changes in DES-treated rats as compared with that of controls. In the luminal epithelium, subepithelial stroma and muscular region, BrdU incorporation and apoptotic indexes showed no differences between control and DES-exposed animals, whereas in the glandular epithelium, a significant decrease in proliferative activity was observed in animals exposed to DES (Fig. 3). Next, to know whether the stromal compartment surrounding the glandular epithelium was modified, we investigated the expression of α-SMA, vimentin and ERα in the stromal cells neighboring the glandular epithelium. The results revealed a significant reduction in the percentage of glandular perimeter occupied by α-SMA-positive cells in the DES-exposed group (Fig. 4A–C). No differences were observed in the areas occupied by vimentin- or ERα-positive cells between control and DES-exposed groups (Fig. 4D and E).

3.2.2. Adults (PND360)

Different morphological types of uterine glands were observed at estrus in controls: (a) *normal glands* (round, oval or elongated shape with simple cuboidal epithelium) (Fig. 5A, arrows), (b) *glands with squamous metaplasia* (two or three layers of cells, constituting a stratified epithelium) (Fig. 5A, arrowhead), (c) *glands with cellular anomalies* (cylindrical epithelium, low nuclei/cytoplasm ratio, undefined cytoplasmic borders, or cells with dispersed chromatin and atypical arrangement) (Fig. 5B and C), and (d) *cystic glands* (usually large size, enlarged lumen and flat epithelium) (Fig. 5D). In DES-treated rats, only the incidence of glands with cellular anomalies was increased (control 31.3% *vs* DES 69.2%; Table 3).

Table 3
Incidence of histologically diagnosed uterine abnormalities in adult rats (PND360) perinatally exposed to DES.

	Treatment	
	Control	DES
Glands with squamous metaplasia	9/16 (56.25%)	3/13 (23.07%)
Glands with cellular anomalies	5/16 (31.25%)	9/13 (69.23%)[a]
Cystic glands	10/16 (62.5%)	9/13 (69.23%)

[a] $p < 0.05$ compared with the control group.

3.2.2.1. Immunophenotype of uterine glands.

Next, in uterine samples from adult rats, we studied the immunophenotype and proliferative activity of uterine gland cells by evaluating the expression of cytokeratins (molecular markers for different types of epithelial differentiation), p63, steroid hormone receptors and BrdU incorporation. The expression pattern of the molecules evaluated was not affected by DES exposure. All types of glands were immunoreactive for ERα, CK8 (simple epithelium) and immunonegative for PR, basal cytokeratin (CK34βE12) and p63, except for glands with squamous cell metaplasia, which exhibited a different pattern of cytokeratin markers. Glands with squamous metaplasia expressed both basal and luminal CK. CK34βE12 was evident in basal cells, while CK8 was restricted to luminal cells. The expression of p63 in squamous metaplasia was observed in more than one cellular layer, mainly in basal cells, which expressed ERα (data not shown).

3.3. Uterine response to estrogen of adult rats perinatally exposed to DES

To assess the effects of constant exposure to estrogens in adult rats perinatally exposed to DES or vehicle, a group of rats were ovariectomized on PND360 and implanted with silastic capsules filled with E2 (as described in material and methods). The histoarchitecture of uterine horns of the vehicle-implanted rats exhibited signs of atrophy. The subepithelial stroma presented numerous cells with very narrow cytoplasm and round, markedly stained nuclei surrounding normal glands (data not shown). In response to estrogen, the incidence of glands with cellular anomalies and of glands with squamous metaplasia increased slightly in the DES group, although this increase was not statistically significant (Table 4). The percentage of rats with uterine cystic glands in the DES + E2 group (100%) was significantly higher than that of the control + E2 group (64.3%) ($p < 0.05$) (Table 4). Very remarkably, on PND460, we observed *glands with daughter glands*; this type has various shapes – round, elongate, tortuous – and sizes, and has formed daughter glands inside the epithelium or inside the mother gland lumen or on the outer surface of the mother gland, like budding gland (Fig. 5E, Table 4). On PND460, we also observed *conglomerates of glands* with complex architecture, in which individual glands were close to each other almost without intervening stroma (Fig. 5F). This type of conglomerate may develop from glands with daughter glands. Interestingly, the presence of glands with daughter glands and conglomerates of glands on PND460 were observed in animals treated with E2 independently of the perinatal exposure to DES (Table 4).

Table 4
Incidence of histologically diagnosed uterine abnormalities in adult rats (PND460) perinatally exposed to DES and treated with E2.

	Treatment	
	Control + E2	DES + E2
Glands with cellular anomalies	9/14 (64.28%)	9/11 (81.81%)
Glands with squamous metaplasia	10/14 (71.42%)	10/11 (90.9%)
Cystic glands	9/14 (64.28%)	11/11 (100%)[a]
Glands with daughter glands	2/14 (14.28%)	2/11 (18.18%)

[a] $p < 0.05$ compared with the control group.

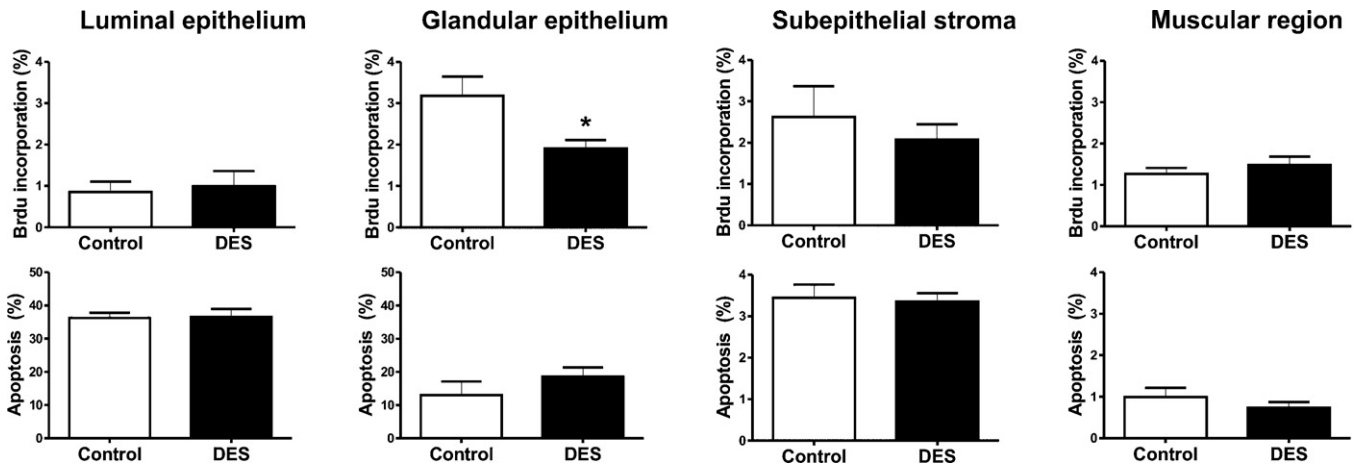

Fig. 3. Effect of perinatal exposure to DES on cellular turnover in the uterus of rats on PND90. The bar graphs show the proliferative rate and apoptosis rate quantified by immunohistochemistry and TUNEL respectively, in different regions of the uterus. Each column represents the mean ± SEM ($n > 8$ per group). Asterisks denote $p < 0.05$ *versus* the control group.

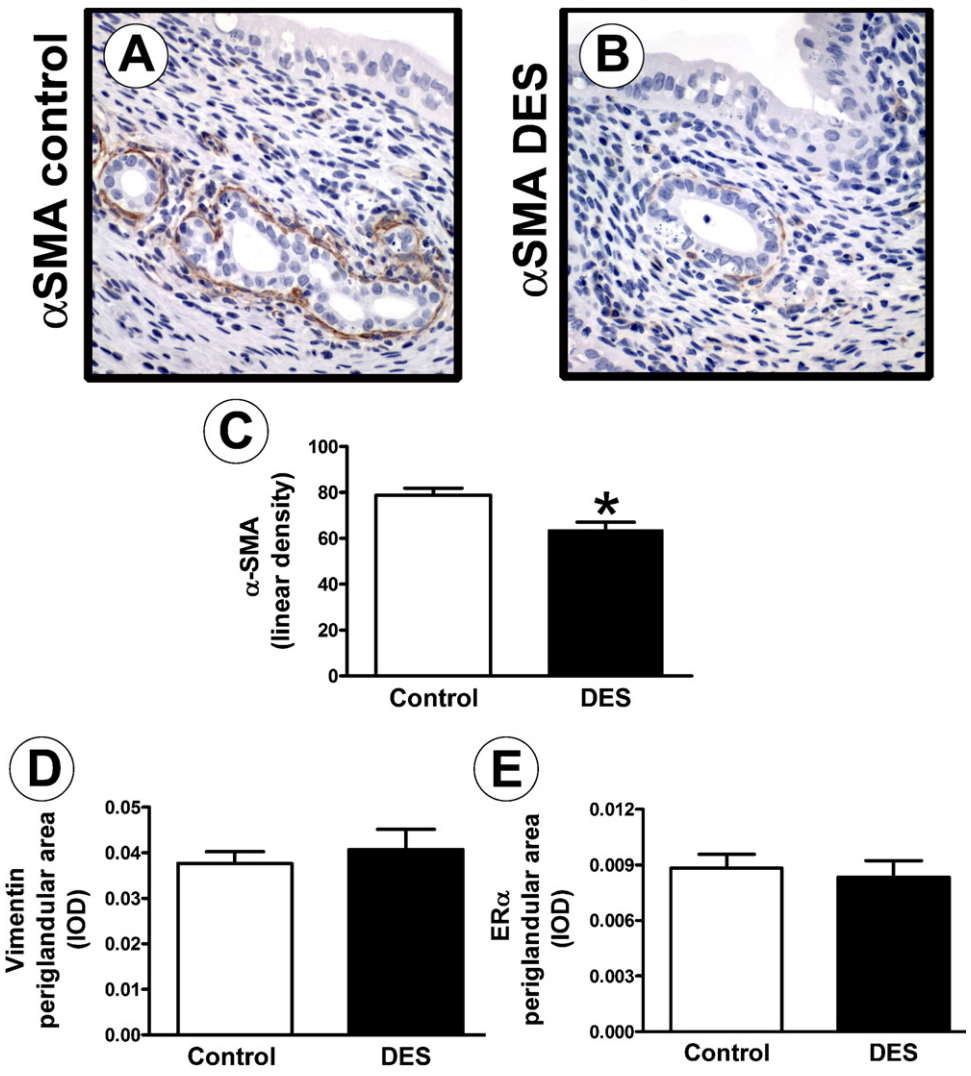

Fig. 4. Effects of perinatal DES exposure on the uterine periglandular stroma of young adult (PND90) rats. Representative photomicrographs of immunohistochemical detection of αSMA (A and B) on uterine sections. Scale bar: 50 μm. (C, D and E) Quantification of αSMA, vimentin and ERα immunostaining in the uterine periglandular stroma. The positive staining of αSMA is expressed as linear density (C). Vimentin (D) and ERα (E) immunostaining are expressed as the integrated optical density (IOD), which is a linear combination between the average of immunostaining intensity and the relative area occupied by positive cells. Each column represents the mean ± SEM ($n > 8$ per group). Asterisks denote $p < 0.05$, *versus* the control group.

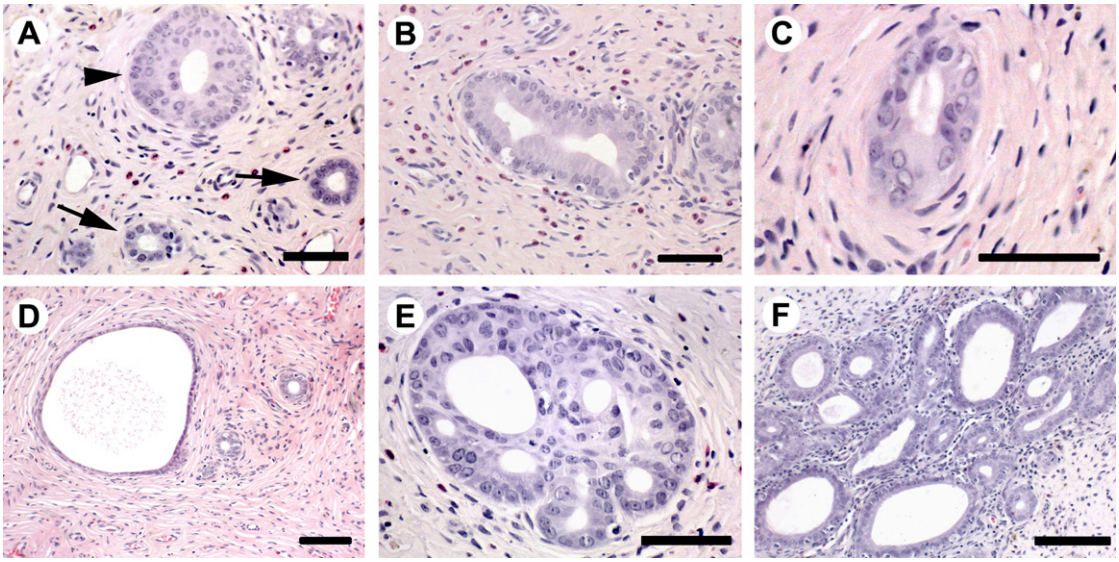

Fig. 5. Representative photomicrographs demonstrating different types of endometrial glands observed in adult rats. (A) Glands with squamous metaplasia (arrowhead) and normal gland (arrow), (B) hypertrophic gland, (C) gland with cellular anomalies, (D) cystic gland, (E) gland with daughter glands and (F) glands forming conglomerates. Scale bar: (A–C and E) 50 μm; (D and F) 75 μm.

3.3.1. Immunophenotype and steroid receptor expression in uterine glands

The immunophenotype and proliferative activity of uterine gland cells were the same as those described on PND360 and not affected by E2 treatment (data not shown).

ERα, ERβ and PR, key mediators of steroid-hormone action, were evaluated in the subepithelial stroma in response to the estrogen treatment (Fig. 6). In DES-exposed rats (DES + E2), the PR subepithelial expression was lower than in controls (control + E2) (Fig. 6A, D, and G). Regarding subepithelial ERα and ERβ expression, no differences were observed between DES-exposed females and controls in response to E2 (Fig. 6).

3.3.2. Expression of p63 mRNA in uterine tissue

PCR results demonstrated that *ΔN-* and *TA-p63* isoforms were expressed without changes on PND460 (data not shown).

4. Discussion

In the present study, we demonstrated that perinatal oral exposure to a low dose of DES altered the development of the uterine tissue, effects observable in sexually mature adult rats. We also showed that exposure to DES during critical stages can reprogram the development of the uterus and consequently, the uterus alters its response to an estrogen replacement therapy later in life.

Considerable evidence from *in vivo* studies has demonstrated that the uterine tissue is one of the targets of xenoestrogenic chemicals [11] and that early DES exposure induces important changes in the rodent reproductive female tract long after the exposure period has ended [28]. Most studies in rodents have been performed by subcutaneous treatment, whereas DES is readily bioavailable upon oral intake. Comparatively, limited data are available concerning oral exposure, the most relevant route of endocrine-disrupting chemical intake in the general population. We have shown that the DES dose orally administered during pregnancy and lactation results in a known endocrine disruption, evidenced by increased uterine *HOXA10* expression in female pups. Smith and Taylor [16] reported a dose–responsive increase in *HOXA10* expression in uterine stromal cells in 2- and 6-week-old mice in uteri exposed to bisphenol A (BPA), whereas Varayoud et al. [11] showed that postnatal exposure to BPA decreases uterine *HOXA10* expression. These studies and our present results show that Hox genes are targets of endocrine disruption and suggest that exposure during different developmental periods could lead to different effects.

DES exposure during the perinatal period did not modify cell apoptosis but reduced glandular cell proliferation in the rat uterine tissue on PND 90 (young adults). Moreover, in the same experimental group, we demonstrated a decreased expression of α-SMA in stromal cells surrounding the uterine glandular epithelium, without changes in vimentin and ERα expression. This decrease could be due to the altered tissue organization in the surrounding stroma. Thus, the epithelial–stromal communication could be affected by DES exposure. These observations are in agreement with those reported in prostate tissue of rats neonatally exposed to the xenoestrogen bisphenol A (BPA), where a reduced number of smooth muscle cells in the prostate periductal stroma of BPA-treated rats and an altered glandular cell function were observed [25]. Signals from the stroma are believed to be critical in determining the decision of epithelial cells to undergo proliferation, apoptosis, or differentiation [29].

On PND 360 (adult rats), control animals showed uterine tissue abnormalities, including: cystic glands, glands with cellular anomalies, and glands with squamous metaplasia. Therefore, these uterine changes occur during the normal female aging process. In accordance with these observations, Khalyfa et al. [30] demonstrated age-related changes in the expression of estrogen target genes in the mouse uterus, suggesting that the modified expression of genes may play a role in reproductive senescence and explain the decline in reproductive function in old animals. Although the abnormal structure of uterine glands was demonstrated as an age-related change, the incidence of glandular abnormalities was higher in DES-exposed animals. Therefore, the incidence of abnormalities in uterine glands can be used as an endpoint of endocrine disruption.

Rats exposed to DES and treated with estrogen replacement therapy showed an increase in the occurrence of uterine lesions, and the incidence of cystic glands reached statistical significance. Our present results suggest that perinatal exposure to DES increases the sensitivity of the developing uterus to estrogens, thereby creating a permissive state that could lead to the formation of lesions. Perinatal exposure to DES would exert a priming effect on the

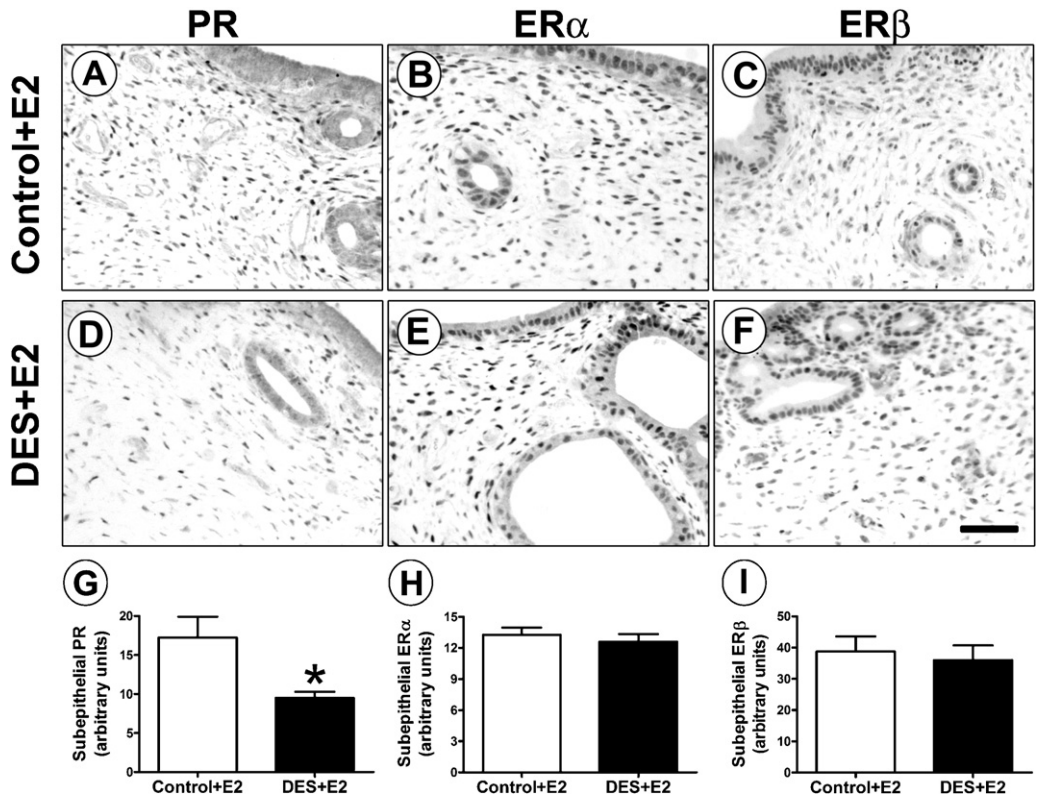

Fig. 6. Effects of perinatal DES exposure on the expression of steroid receptors in the uterus of rats (PND460) treated with E2. Representative photomicrographs of immunohistochemical detection of PR (A and D), ERα (B and E), and ERβ (C and F) on uterine sections. These images were obtained from sections without hematoxylin counterstaining. Scale bar: 50 μm. (G–I) Immunostaining of steroid receptors in the subepithelial stroma was quantified by image analysis by evaluating the integrated optical density (IOD). Results are expressed as relative units. Each column represents the mean ± SEM ($n > 8$ per group). Asterisks denote $p < 0.05$ *versus* the control group.

later exposure to estrogens. Previous studies have demonstrated that exposure to xenoestrogens results in an increased number of pre-neoplastic lesions in the mammary gland, inferring that it may be more sensitive than the mammary gland of unexposed animals [31,32].

As a consequence of the E2 treatment, the uterine samples from the estrogen replacement therapy group showed the presence of glands with daughter glands and conglomerates of glands independently of the perinatal treatment with DES. Gunin et al. [20] have previously postulated that if epithelial cells of a gland divide perpendicularly to the basement membrane, they will probably form stratified epithelium and/or branches, papillae, and/or daughter glands, and/or conglomerates of glands. These authors showed a correlation between estradiol-induced changes in the architecture of glands and mitosis orientation, suggesting that mitoses perpendicular to the basement membrane are responsible for precancerous changes. Mitosis orientation may be more important than estrogen-dependent changes in proliferation [20].

In adult tissues, p63 is expressed in stratified epithelia, but not in single-layered epithelia, suggesting a role for p63 in the development and maintenance of stratified epithelia [33]. Two classes of *p63* transcripts, *ΔNp63* and *TAp63*, arise from the use of alternative promoters and transcription start sites. The roles of these isoforms are poorly known. *In vitro* data suggest that *ΔNp63* has a dominant-negative function toward *TAp63* [34]; however, *ΔNp63* has also been shown to be able to transactivate target gene expression [35]. Koster et al. [33] demonstrated that *TAp63* is the first *p63* isoform expressed during embryogenesis and that it is required for initiation of epithelial stratification. Furthermore, ectopic expression of *TAp63* in a simple epithelium *in vivo* results in the induction of squamous metaplasia, confirming the role of p63 as a master molecular switch [33]. Similarly, our results demonstrate that only uterine glands with squamous metaplasia expressed p63 protein and mRNA of both *p63* isoforms. These p63-positive cells showed expression of basal cytokeratin and ERα. Comparable results have also been observed in DES-induced metaplastic foci on the luminal epithelium of the mouse uterus [36].

The stroma-epithelial communications are critical for mediating the effects of hormonal agents [29]. On PND460, the decreased expression of PR in uterine stromal cells in rats treated with DES + E2 may be affecting the hormonal signaling pathway. Therefore, a higher incidence of glandular lesions could be due to the disruption of communications among stroma and glandular epithelial cells. From the tissue organization field theory perspective, carcinogenesis may result from the disruption of the cell-to-cell communication involving the parenchyma and its stroma [37].

Regarding ERβ protein expression in the rat uterus, there are conflicting reports. Pelletier et al. [38] and Wang et al. [39] have shown very low ERβ protein expression in the rat uterus; however, other studies have demonstrated a different uterine expression of this protein [40,41]. In the present study, we used a monoclonal antibody against ERβ that has been shown to have an enhanced sensitivity in paraffin-embedded rodent tissues [42–44]. Our results showed a high ERβ protein expression in the rat uterine tissue.

In summary, here we found abnormalities in uterine tissues of aged female rats and their increased incidence in rats perinatally exposed to DES by the oral route. We also found that the response of the adult uterus to estrogen was altered by exposure to an estrogenic chemical during fetal and neonatal life. Exposure to DES during critical stages of development can reprogram the uterus, thus changing the response to estrogen later in life. New data on the regulation of estrogen target gene expression in the uterus of aging and xenoestrogen-exposed rats might provide clues to

better understand why hormonal replacement therapy increases the incidence of uterine cancer.

Grant support

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

Acknowledgements

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

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