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LONG-TERM EFFECTS OF POSTNATAL EXPOSURE TO DIETHYLSTILBESTROL ON UTERINE ESTROGEN RECEPTOR AND GROWTH

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Summary—Diethylstilbestrol (DES) treatment of female rats on postnatal days (PND) 1–5 reduces uterine growth, estrogen receptor (ER) level and gland number by PND 25, while daily DES treatment on PND 1–25 increases uterine growth 4-fold, further reduces ER level and completely suppresses gland formation. We now report the persistence of these effects in adults. By PND 60, uterine weight was 70% of controls in rats injected with DES on PND 1–5 but only 10% of controls in rats injected PND 1–10 or longer. In fact, uterine weights were the same on PND 10 and 60. Uterine gland numbers were reduced to 30% of controls in all DES-treated rats regardless of exposure length; however, luminal and glandular epithelial cell heights were reduced to < 50 and 70%, respectively, of controls when DES was given on PND 1–25 but not when given on PND 1–5. Ovariectomy 7 days prior to sacrifice on PND 60 reduced uterine weight in controls by 67% and in rats injected with DES on PND 1–5 by 53%, but had no effect in rats injected with DES on PND 1–10. DES exposure at either PND 1–5 or 1–10 lowered ER levels by 35–50% at both 60 and 90 days. Treatment with a high dose of estradiol (E_2) 1 week before sacrifice significantly down-regulated ER to the same concentration in all treatment groups at PND 60 and 90. Following E_2 treatment, all groups also showed increased uterine weight at PND 60 and 90. These data show there is a short period of development (PND 5–10) in which further DES exposure indirectly inhibits uterine growth.

INTRODUCTION

Exposure of human fetuses to the synthetic estrogen diethylstilbestrol (DES) has resulted in a variety of long-term adverse effects in the reproductive tract [1, 2]. The neonatal rodent has been proposed as an appropriate model for examining the potential adverse effects of chemicals on the developing reproductive tract [3, 4]. This proposal was based in part on the knowledge that the stage of development seen in the rodent shortly after birth is about equivalent to the stage of development seen in humans at the end of the first trimester of pregnancy [3]. However, unlike humans, rats treated with DES in the first 5 days after birth were found to be acyclic as adults [5]. When rats were treated neonatally with DES and later ovariectomized and treated with estradiol (E_2) for various periods, the development of squamous metaplasia in both the luminal and glandular epithelium was observed in a time-dependent manner [6]. Mice treated neonatally with estrogens subsequently had vaginal abnormalities which were ovary-independent after high doses and ovary-dependent after low doses [7]. Neonatal exposure of rats to DES significantly decreased estrogen receptor (ER) levels (to 40% of control), while increasing uterine weight 2-fold on postnatal day (PND) 5, and elicited a premature onset of uterine gland genesis on PND 8 [8]. However, on PND 26, both ER levels and gland number were 60–70% and uterine weight was 50% of control values [8]. Rats given a high dose of estradiol benzoate on PND 3 showed a reduced uterine responsitivity on PND 21 along with reduced cytosolic ER levels [9]. Daily exposure to DES resulted in ER concentrations that were 15% of control levels, the absence of uterine glands and uterine weight that was four times greater than controls at 25 days of age [8]. Pharmacological doses of E_2 implanted in ovariectomized adult rats also caused ER down-regulation (~50%) with a

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concomitant 4-fold increase in uterine weight [10]. These effects can be elicited within 3–6 h after implantation with return to control values being seen within 1 week after removal of the estrogen source [10, 11].

These data are consistent with the suggestion that neonatal exposure to estrogens causes a permanent perturbation in either the developing hypothalamo–hypophyseal–ovarian axis or in the reproductive tract itself [6, 7]. In this study we examined adult rats exposed to DES as neonates for the persistence of the DES effect on uterine ER concentration, weight and gland number. As well, we examined the DES effect on ovarian dependence of uterine weight, responsiveness of glandular and luminal epithelium of E_2 and ER down-regulation by E_2.

EXPERIMENTAL

Offspring from date-mated Sprague–Dawley rats were culled according to sex, and the females were randomly distributed to dams (6–7 pups/dam) within 24 h of birth (PND 1). Groups of pups were injected s.c. with 10 μg of DES per animal in the middorsal region, on each of PND 1–5, 1–10, 1–15, 1–20 or 1–25 and sacrificed at either 60 or 90 days. Control animals were untreated.

Intact animals from both treated and control groups were sacrificed on day 60 to assess the persistence of treatment effects. In these and subsequent experiments, animals were weighed and then killed by cervical dislocation after light ether anesthesia. Uteri were dissected free from connecting mesentery, weighed and then processed histologically as described previously [12] for uterine gland counts and luminal epithelium (LE) and glandular epithelium (GE) height measurements.

Other groups of animals, controls as well as those DES-treated on PND 1–5 or 1–10, were ovariectomized 1 week before sacrifice on either day 60 or 90. Some of these animals were implanted with a silastic capsule containing 5.0 mg E_2/ml of sesame oil [13]. At sacrifice 7 days later, the uteri were removed, weighed and homogenized in cold TE buffer (10 mM Tris, 1.5 mM EDTA, pH 7.4) at a concentration of 30 mg tissue/ml of buffer. The homogenate was processed as described previously with nuclear and cytosolic ER levels being determined by Scatchard plots of data from the [^{3}H]E_2 exchange assay [10].

Statistical analyses were conducted using a two-way ANOVA. Results are presented as means ± SEM with a level of significance of $P < 0.05$ (significance was determined by Duncan's multiple range test). Statistical evaluation of neonatal weight ratios was done using the propagation of error technique [14].

RESULTS

At 60 days, intact rats injected with DES on PND 1–5 had uterine weights which were 60% that of controls (Fig. 1). Treatment for 10 days or more resulted in much lower uterine weights (about 10% of controls). In rats injected with DES on either PND 1–5 or 1–25, gland numbers were reduced to 30% of controls (Figs 2 and 3). Treatment with E_2 for 1 week before sacrifice in rats treated with DES on PND 1–25 had no effect on uterine gland number (Figs 2 and 3). Exposure to DES on PND 1–5 did not alter LE height on day 60 although there was a 50% increase in GE height. However, treatment on PND 1–25 decreased LE height (to < 50% of controls) and GE height (to < 70% of controls) (Figs 2 and 3). Despite the almost 2-fold difference between LE and GE height in controls, the heights were the same in these two cell types following DES treatment on PND 1–25. LE and GE height increased to values significantly greater than in untreated controls when the rats were implanted with E_2 1 week before sacrifice (Figs 2 and 3). This treatment also restored the

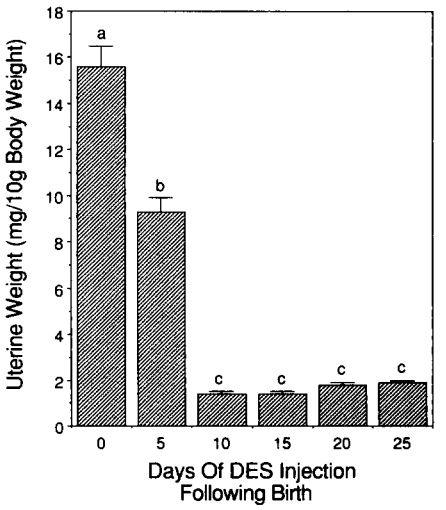

Fig. 1. Uterine weights normalized to body weights in intact 60 day rats, untreated controls or rats treated with 10 μg DES on PND 1–5, 1–10, 1–15, 1–20 or 1–25. Values are expressed as the means ± SEM. Values with different letters are significantly different at $P \leq 0.05$.

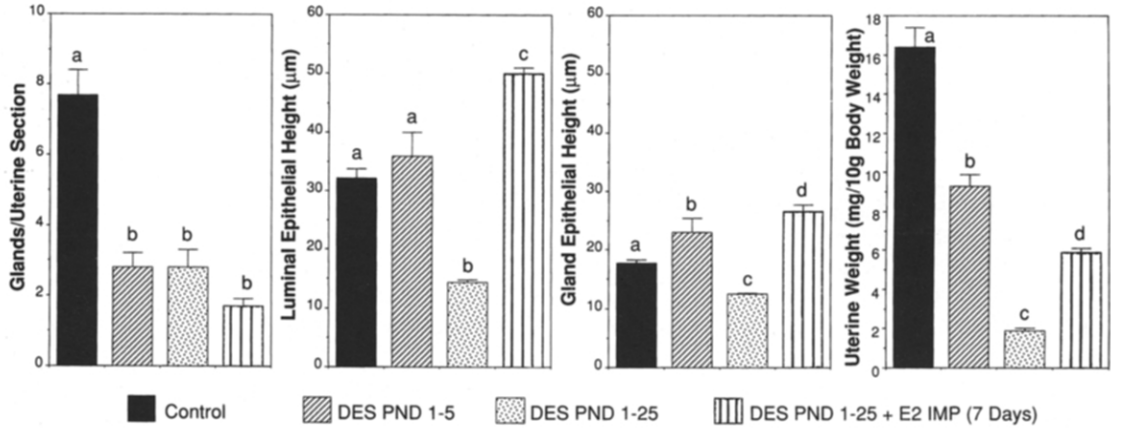

Fig. 2. Assessment of uterine growth and morphological parameters in intact rats on day 60 following neonatal exposure to 10 μg DES on PND 1–5 and 1–25. Values are expressed as the means $\pm$ SEM. Values with different letters are significantly different at $P \leq 0.05$.

almost 2-fold difference in LE and GE height. Uterine weight in rats treated on PND 1–25 increased 3-fold after E_2 implantation (Fig. 2) but still remained well below both the untreated control and DES 1–5 values.

Rats were ovariectomized for 1 week to determine the ovarian effect on uterine weight. Ovariectomy caused a significant decrease in uterine weight in the control rats and those injected on PND 1–5 when compared to intact animals (65 and 50%, respectively), while in rats injected on PND 1–10, no further decrease in uterine weight was seen after ovariectomy at either 60 or 90 days (Table 1, Fig. 4). The normalized uterine weights of rats injected on PND 1–10 were also significantly reduced compared to ovariectomized controls at both 60 and 90 days (Table 1, Fig. 4).

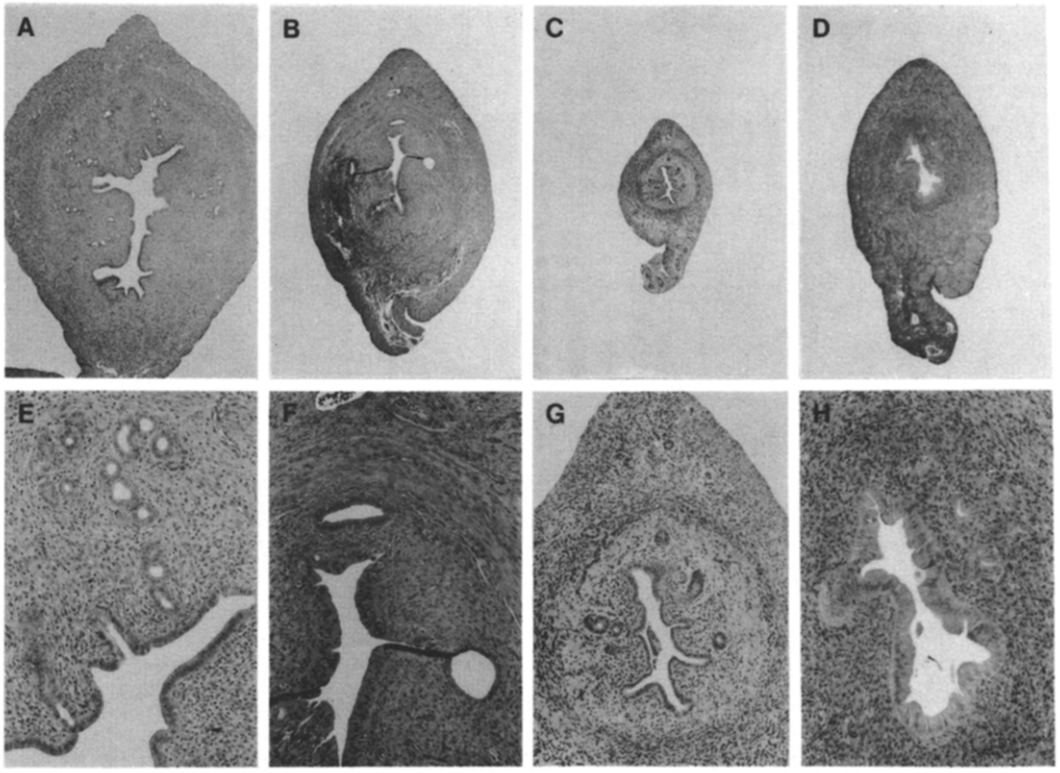

Fig. 3. Photomicrographs of uteri from control rats (A, E), rats injected with 10 μg DES on PND 1–5 (B, F), PND 1–25 (C, G) or PND 1–25 and then implanted with E_2 (5.0 mg/ml) 1 week before sacrifice (D, H). Magnifications for A–D and E–H are 25 × and 100 ×, respectively.

Table 1. Comparison of uterine weights (mg/10 g body wt) at day 60 from controls or rats exposed to 10 μg DES on PND 1–5 or 1–10

Treatment	Uterine Wt (Intact)	Uterine Wt (OVX)	Fold-Decrease (Intact/OVX)	Uterine Wt (OVX + E_2)	Fold-Increase (OVX + E_2/OVX)
Control	15.6 ± 0.9	5.4 ± 0.2	2.89	21.6 ± 0.6	3.98
DES (PND 1–5)	9.3 ± 0.6	4.5 ± 0.1	2.07*	11.4 ± 0.6	2.53*
DES (PND 1–10)	1.4 ± 0.1	1.8 ± 0.2	0.78*	5.6 ± 0.3	3.11*

A subset of the rats from each treatment group were ovariectomized (OVX) on PND 53 with some receiving an implant containing 5.0 mg E_2/ml oil at the same time. Values are expressed as the means ± SEM. * = $P \leq 0.05$ compared to control value.

To determine the reversibility of these effects, ovariectomized control and DES-treated rats were implanted with E_2 for 1 week before sacrifice. While all groups showed a substantial increase in uterine weight, neonatal exposure to DES significantly reduced the ability of the uteri to respond to the estrogen stimulus even when expressed as the fold increase above controls (Table 1).

Total uterine ER concentrations (nuclear plus cytosolic fractions) in ovariectomized rats exposed to 10 μg DES on PND 1–5 and 1–10 were significantly lower than controls at both 60 and 90 days, with the loss occurring entirely from the cytosolic fraction (Fig. 5). The overall levels of ER for all treatments were somewhat lower at 90 days than at 60 days. However, exposure to DES on PND 1–5 caused a greater suppression of ER at 60 and 90 days than did exposure on PND 1–10 (Fig. 5). The E_2 implant down-regulated uterine ER to the same minimum value at both sacrifice times regardless of previous neonatal treatment. The same trend in ER reduction was observed at 180 days (data not shown).

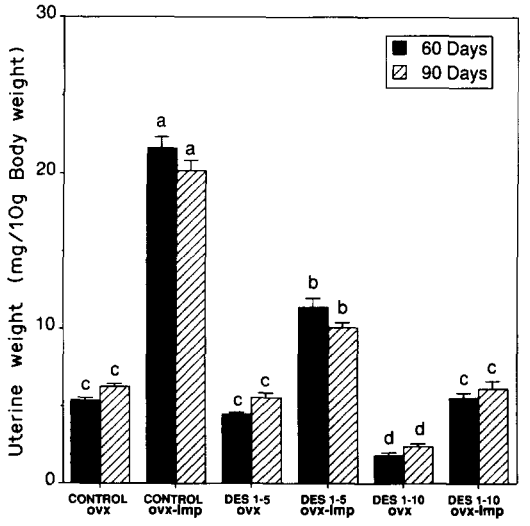

Fig. 4. Uterine weights normalized to body weight in 60-day and 90-day old rats that were either untreated (controls) or exposed to 10 μg DES on PND 1–5 or 1–10. 1 week before sacrifice (PND 53 and 83) all rats were ovariectomized and some animals in each treatment group were implanted with silastic implants containing 5.0 mg E_2/ml sesame oil. Values are expressed as the means ± SEM. Values with different letters are significantly different at $P \leq 0.05$.

DISCUSSION

These data demonstrate that the uterus only partially recovers from the morphological abnormalities and other effects elicited by neonatal exposure to DES. In intact rats at day 60,

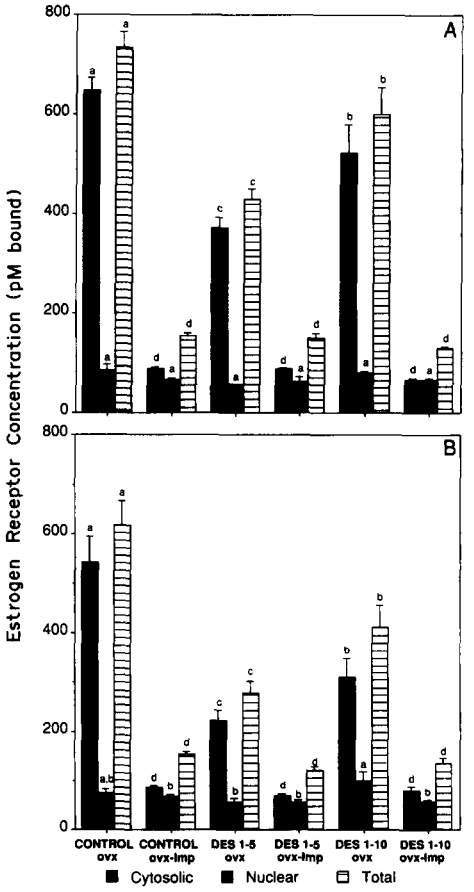

Fig. 5. Cytosolic, nuclear and total ER concentrations (expressed as pM E_2 bound) in 60-day (A) and 90-day (B) old rats either untreated or neonatally exposed to 10 μg DES on PND 1–5 or 1–10. All rats were ovariectomized on day 53 with part of each group being implanted with 5.0 mg E_2/ml oil at the same time. Values are expressed as the means ± SEM. Statistical comparisons were made within each category (cytosolic, nuclear, or total) of ER. Values with different letters are significantly different at $P \leq 0.05$.

uterine weight following DES exposure on PND 1–5 was reduced when compared to controls. The percent reduction was the same as seen previously at 25 days for these treatment groups [8]. Thus, the smaller uterus in DES-treated rats grew to the same extent, proportionally, as in the controls from days 25–60. The decreased uterine weight could be due to either an altered hypothalamo–hypophyseal–ovarian axis resulting in lowered estrogen secretion, or to an impaired ability of the uterus to respond to normal levels of circulating estrogens. Ovariectomy 1 week before sacrifice (day 53) caused a decrease in uterine weight in the PND 1–5 DES-exposed rats which was proportionally smaller than in controls; additionally, E_2 implants increased (on a % basis) uterine weights slightly less than seen in controls. This demonstrates that the uteri are responding less extensively to ovariectomy or E_2 treatment. After DES exposure on PND 1–10, ovariectomy did not further decrease uterine weight, suggesting either that the ovaries were producing no E_2 or that the uterus was resistant to estrogen stimulation. These uteri weighed the same on PND 10 and 60; in an earlier study [8] uterine weight was 40.2 ± 1.3 mg on PND 10 while here the value on PND 60 was 39.5 ± 3.7 mg. While this is most likely due to a failure of ovarian estrogen secretion, a direct growth-inhibiting effect of PND 1–10 DES treatment on the uterus cannot be excluded. E_2 implants increased uterine weight in the DES 1–10 group but proportionally less so than in controls similar to results from the DES 1–5 group. This demonstrates that the uterus is only partially resistant to E_2 and suggests that a failure of ovaries to produce sufficient estrogen appears responsible for the bulk of the effect. Likewise, the 3–4-fold increase in LE height seen in intact rats injected on PND 1–25 and implanted with E_2 1 week before sacrifice demonstrates a normal uterine epithelial cell response [15]. Earlier experiments showed a 50% reduction in gland number on PND 25 following PND 1–5 DES treatment and almost complete inhibition of gland appearance on the last day of the PND 1–25 DES treatment [8]. Here, gland numbers were about one-third of controls by PND 60, demonstrating that the inhibition observed following PND 1–5 DES treatment was not reversed, but that recovery was seen from the effects of the longer treatment. This suggests that glands cannot form until the tissue DES concentration decreases and that the normal developmental period for gland genesis (days 10–14) can be delayed at least until PND 25, an effect similar to that of E_2 [12]. Similar inhibition of gland genesis has previously been suggested to be due to hypertrophy of the LE, which could inhibit the epithelial invagination process necessary for gland formation [12, 15]. However, the DES-induced and ongoing ovarian defect in E_2 production was probably not the cause of the inhibition of uterine gland genesis. Experiments in which rats were bilaterally ovariectomized and/or adrenalectomized on PND 6, which would result in complete ablation of E_2, demonstrated a normal pattern of gland genesis (unpublished observations). Additionally, lowered uterine gland number is a developmental defect appearing prior to pubertal ovarian function [15].

The effect of neonatal exposure to DES on ER concentration at both 60 and 90 days was dependent on the length of exposure. The complex relationship between exposure and subsequent alterations is exemplified by the relationship between ER concentration and uterine weight. After ovariectomy, rats previously exposed to DES on PND 1–10 had uterine weights which were $< 10\%$ of the ovariectomized controls and $< 40\%$ of the ovariectomized PND 1–5 DES-treated rats, yet the ER concentration in these very small uteri was significantly elevated compared to the PND 1–5 group at both 60 and 90 days. The increase in uterine weight observed in both DES-treated groups following E_2 implantation on day 53, coupled with a simultaneous decrease in ER concentrations, demonstrates that the uteri of these animals were capable of responding to estrogens by down-regulating ER [10]. The fact that the control group and both DES-treated groups had comparably low ER concentrations following E_2 implantation implies that there is a lower limit to the extent of down-regulation of ER by pharmacological E_2 doses.

The data suggest that neonatal (PND 1–5 or 1–10) exposure to DES perturbed the hypothalamo–hypophyseal–ovarian axis resulting in reduced ovarian secretion of estrogen. For the DES 1–25 group, estrogen production must have been below the response threshold, since ovariectomy failed to reduce uterine weight. This is supported by reports that neonatal estrogen exposure causes decreased circulating serum E_2 levels [16] as well as decreased E_2 responses in adults [9, 17–19]. While Clark and Gorski [20] have shown that the ontogeny of

uterine ER is not dependent on a functioning ovary, we have suggested that endogenous estrogen secretion by the neonate does play a role in end organ conditioning resulting in a normal functioning uterus [8].

We conclude that the uterus can partially recover from the inhibitory effects caused by neonatal exposure to DES as evidenced by increased ER concentration and gland number at PND 60 and 90 when compared to identically dosed rats measured at PND 25. These effects appear to be due to a permanent alteration in the hypothalamo–hypophyseal–ovarian axis and, to a lesser extent, to a lowered ability of the uterus to respond to estrogens.

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