Human Reproduction Vol.16, No.5 pp. 988–996, 2001

Altered prostate growth and daily sperm production in male mice exposed prenatally to subclinical doses of 17α-ethinyl oestradiol

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Approximately 2 million women in the USA and Europe continue taking oral contraceptives each year during undetected pregnancy due primarily to non-compliance and also to individual variation in sensitivity to hormones in the contraceptives. Prenatal exposure to oral contraceptives containing 17α-ethinyl oestradiol (EE) has generally not been associated with an increased incidence of externally observable malformations at birth. The purpose of this study was to assess effects on reproductive organs in adult male mice that had been exposed during gestation day 0 through 17 (equivalent to gestation week 16 in humans) to clinically relevant (~0.5 μg/kg/day) and lower doses of EE. Doses used in this study ranged from 0.002 to 2 μg/kg/day. By 5 months of age, prostate weight was significantly ($P < 0.05$) higher than controls in most treatment groups of EE (0.02–2 μg/kg). Prostatic androgen receptor populations were significantly elevated only in the 0.02 μg/kg group, suggesting different mechanisms for the increase in prostate weight at different doses. Daily sperm production (DSP) and DSP per gramme of testis were reduced in all treatment groups during adolescence, but not later in adulthood. These findings are consistent with prior studies showing that prenatal exposure of mice to very low doses of a number of oestrogenic chemicals can alter the adult male reproductive system without causing gross external malformations.

Key words: endocrine disruption/fetus/oral contraceptive/prostate/testes

Introduction

In the United States of America and Europe, it is estimated that between 2 and 5% of the 55 to 60 million women who take oral contraceptives (OC) continue use during undetected early pregnancy (Smithells, 1981), resulting in unintended in-utero exposure to reproductive steroidal hormones in the offspring. The high incidence of pregnancy is presumed to be due to non-compliance and, in a small number of cases, individual variation in sensitivity to contraceptive hormones (Hite *et al.*, 1999). Use of OC in unrecognized pregnancy often continues well into the first trimester, and may extend into the fourth month of gestation (Kallen *et al.*, 1991; Li *et al.*, 1995), encompassing a critical period for disruption of reproductive organ development (DeSesso, 1997).

In 1993, the American College of Obstetrics and Gynecology concluded that oral contraceptive use during early pregnancy or shortly before pregnancy is not associated with increased risk of fetal malformation (American College of Obstetrics and Gynecology, 1993). Even when malformations are limited specifically to those of the genitourinary tract, where there are tissues known to be sensitive to reproductive steroidal hormones, the association between OC use in early pregnancy and malformations is still generally considered absent (Kallen *et al.*, 1991; Raman-Wilms *et al.*, 1995; Martinez-Frias *et al.*, 1998). However, there have also been reports to the contrary (Li *et al.*, 1995; Li, 1998). Despite the general lack of positive epidemiological data linking OC use during pregnancy with malformations at birth, concern remains because prenatal exposure to exogenous sex hormones is known to cause adverse effects other than externally observable malformations in both humans and animals (Gray, 1992; Newbold, 1995). In addition, OC used in the USA contain 17α-ethinyl oestradiol (EE), a synthetic oestrogen with an oestrogenic potency very similar to that of diethylstilbestrol (DES), another synthetic oestrogen known to be a reproductive teratogen.

DES is a non-steroidal synthetic oestrogen that was given to more than 3 million women in the United States during the 1950s and 1960s, primarily to prevent miscarriage and

premature births. Use for this purpose of DES was banned in the USA in 1971 after the chance discovery that DES-exposed daughters were at an increased risk of developing clear-cell adenocarcinoma of the vagina and cervix (Herbst, 1981; Mittendorf, 1995). Further follow-up of DES-exposed daughters found they were also at increased risk for developing a variety of reproductive tract abnormalities. These ranged from vaginal epithelial cell changes, cervicovaginal, uterine and Fallopian tube structural anomalies, increased incidence of infertility, ectopic pregnancies, premature births, and possibly increased incidence of immune and behavioural disorders, such as depression (Bibbo *et al.*, 1977; Herbst, 1981; Stillman, 1982; Blair *et al.*, 1992; Hines, 1992; Giusti *et al.*, 1995; Newbold, 1995). Damage to developing reproductive organs in women was typically found if exposure to DES occurred prior to week 18 of pregnancy (Herbst *et al.*, 1979).

In males, in-utero DES exposure has also been associated with a variety of reproductive abnormalities, including increased incidence of epididymal cysts, cryptorchidism, hypospadias, and possibly sperm abnormalities (Bibbo *et al.*, 1977; Stillman, 1982; Giusti *et al.*, 1995). However, studies of DES effects in men have been limited to cohorts of only a few hundred exposed males, so the effects of DES on human males are less certain than in females. Of particular importance is whether exposure began prior to the end of the first trimester and continued into the second trimester of pregnancy, since it is during this time that the greatest damage to reproductive organs would occur (Wilcox *et al.*, 1995). It is unknown whether DES-exposed daughters and sons are at increased risk for other hormonally mediated cancers, such as breast or prostate cancer, as they are just approaching the age when these types of cancers are more likely to occur.

DES and EE have similar potencies as assessed by their effect on uterine growth, a well-established oestrogen bioassay, when administered subcutaneously to prepubertal rats (Branham *et al.*, 1988). However, in a separate set of experiments on immature rats, the relative potencies of DES and EE varied depending upon whether administration was oral or subcutaneous, with these oestrogenic chemicals being more similar during oral administration (Reel *et al.*, 1996). In any event, both DES and EE are more potent stimulators of uterine growth than 17β-oestradiol, the primary endogenous oestrogen (Branham *et al.*, 1988; Reel *et al.*, 1996). In addition, EE has been shown recently to cause similar incidence of uterine adenocarcinomas as DES in adult mice following neonatal exposure. An estimated 3% of DES administered to pregnant mice reaches the fetus, primarily concentrating in the reproductive tract (Shah and McLachlan, 1976). While metabolism of EE by the maternal liver does occur, unconjugated EE reaches the fetus (Slikker *et al.*, 1982), although uptake of EE into fetal tissues after administration to pregnant mice has not been reported.

One basis for the high oestrogenic activity of both DES and EE relative to oestradiol is that DES and EE show little binding to oestrogen-binding plasma proteins, alpha-fetoprotein in rodents and sex hormone binding globulin in humans (Akpoviroro and Fotherby, 1980; Branham *et al.*, 1988). These plasma oestrogen-binding proteins modulate the levels of free, bioavailable oestrogen in serum (Nagel *et al.*, 1998).

Despite the similarity in oestrogenic potency of DES and EE, it appears that lessons learned from DES have not been applied to the evaluation of risks resulting from fetal exposure to EE (American College of Obstetrics and Gynecology, 1993). One reason for this is that the high-dose exposures of DES (1.5–150 mg/day) (Bern *et al.*, 1987) are regarded as irrelevant to the low doses of EE (35–50 μg/day) found in oral contraceptives. Importantly, even the very high doses of DES that were once used did not result in external malformations in most offspring, which is why millions of women were administered DES over two decades. This clearly shows that external malformations are not an expected outcome of fetal exposure to oestrogenic chemicals, except in a very small proportion of cases, even with very high doses. These findings also clearly demonstrate that the absence of malformations at birth does not preclude reproductive dysfunction later in life due to fetal exposure to oestrogenic chemicals (Gill *et al.*, 1976).

Following the initial observation of increased vaginal adenocarcinoma in DES-exposed daughters, a re-evaluation of the animal literature on DES suggested that reproductive disorders in exposed offspring should have been expected. The animal literature regarding the adverse effects of developmental exposure to DES extends back at least to the early 1960s (Dunn and Green, 1963; Takasugi and Bern, 1964), but these findings were ignored, as many physicians and scientists apparently believed that the animal data were irrelevant to human health. Subsequent experiments with mice and rats have resulted in findings that were highly consistent with findings in humans (Newbold, 1995; Swan and vom Saal, 2001). The literature on DES has contributed to a general awareness regarding the high level of conservation of the hormonal mechanisms mediating differentiation of the reproductive organs in mammals (Kavlock and Ankley, 1996; National Research Council, 1999).

While there is now an abundance of data concerning the consequences of developmental exposure to DES, there have been relatively few studies evaluating the long-term effects resulting from prenatal exposure to EE. A MEDLINE January 2001 search for diethylstilbestrol/prenatal and ethinyl oestradiol/prenatal found 488 and 14 references respectively. Prenatal exposure to EE has been shown to disrupt reproductive function in both male and female mice at higher than clinically relevant doses, while effects at doses within the clinical range are unexplored. In male mice, fetal exposure to EE results in an increased incidence of cryptorchidism (Walker *et al.*, 1990; Yasuda *et al.*, 1985b), epididymal azoospermia (Yasuda *et al.*, 1988), atrophy of seminiferous tubules, and altered Sertoli and Leydig cell differentiation (Yasuda *et al.*, 1985a, 1986a,b). Female mice exposed to EE prenatally develop follicular cell hyperplasia (Yasuda *et al.*, 1987), ovarian hypoplasia, and increased degeneration of primordial follicles (Yasuda *et al.*, 1985b). However, these histological and functional responses to prenatal EE have only been studied at maternal doses of 20 μg/kg/day and above, with alterations typically occurring at 200 μg/kg/day. The clinically relevant dose for EE in most

OC is ~0.5 μg/kg/day; this is based on use of an OC with 35 μg EE by a woman who weighs 68 kg.

Since there is little information as to whether exposure to clinically-relevant doses of EE during prenatal life can disrupt the development of reproductive organs in experimental animals, the goal of the current study was to investigate the effects on adult male reproductive organs following in-utero exposure to doses of EE that were at or below the clinically-relevant dose range for EE found in oral contraceptives.

Materials and methods

Animals and housing

CF-1 mice (*Mus musculus domesticus*) were obtained from Charles River Laboratories (Wilmington, MA, USA) in 1979 and maintained as an outbred colony in a closed facility since that time. All animals were housed in standard (30×20×12 cm) polypropylene cages on corn cob bedding. Pregnant and lactating mice were fed Purina breeder chow (#5008; Purina, Richmond, IN, USA) and, after weaning, males were maintained on Purina #5001 standard chow. Rooms were maintained at 23°C on a 12:12 light:dark cycle, with lights on at 10:00 h, so that timed-mating occurred at the end of the dark phase and examination for seminal plugs occurred after the onset of the light phase of the light:dark cycle.

Treatment of pregnant females with 17α-ethinyl oestradiol

Virgin females (aged 3–4 months) were time-mated by being placed daily with a stud male for 4 h beginning 2 h before the end of the dark phase of the light:dark cycle. Mating was verified by the presence of a seminal plug (day 0 of pregnancy). Plug-positive mice were either left unhandled or were given (orally) an average dose of 0, 0.002, 0.02, 0.2, 2, 20 or 200 μg/kg EE dissolved in 30 μl of tocopherol-stripped corn oil (ICN, Aurora, OH, USA) once daily during gestational days 0–17. Dams were weighed every 3 days throughout gestation and allowed to deliver normally on gestation day 19. The corn oil was administered by electronic micropipette (Rainen Instruments, Woburn, MA, USA) because mice readily consume corn oil that is pipetted into their mouth, and this procedure is not as stressful as gavage (force-feeding by stomach tube). No plug-positive mice dosed with 20 or 200 μg/kg of EE remained pregnant, and only offspring from the 0.002, 0.02, 0.2 and 2 μg/kg groups were generated. Each group consisted of 10–12 dams.

Reproductive tissue collection in males

Total litter size ranged from 6 to 18 [mean 10.9 ± 2.2 (SD)]. The number of males in each litter ranged from 2 to 10 (mean 5.5 ± 1.7). Female offspring were removed at weaning, and males remained in litter groups of two to five males per cage. No more than five males were kept for any litter. For males killed when 50 days old, a randomly selected male was individually housed at weaning. For males killed when 5 months old, a randomly selected male was individually housed, beginning at 4 months of age. The reason that males were individually housed is that when male mice are housed in groups, a non-linear hierarchy is often observed where there is one dominant male and the remaining males are subordinate: this can have marked effects on reproductive organs and behaviour in CF-1 mice. However, individual housing for 1 month eliminates the prior effects of subordination in CF-1 mice (unpublished observation). Following weaning when one male per litter was individually housed, the remaining males from the same litter continued to be housed together. Similarly, when another male per litter was removed at 4 months of age, the remaining animals continued to be housed together until used for additional, separate experiments. At both 50 days and 5 months of age, animals were weighed and euthanized. The coagulating glands, seminal vesicles, prostate and one testis and epididymis were then removed and weighed. Fluid was removed from the seminal vesicles and coagulating glands by blotting prior to being weighed. Prostate and testes were immediately frozen in liquid nitrogen, and then stored at −70°C.

Daily sperm production

Daily sperm production (DSP) was determined using a frozen right or left testis from control and treated males by a procedure that has been previously described (Robb *et al.*, 1978; Cooke *et al.*, 1991; Joyce *et al.*, 1993). Briefly, after being removed and weighed, the tissues were placed in liquid nitrogen, and subsequently kept at −70°C until being examined. Testes were then homogenized for 3 min in 25 ml of physiological saline containing 0.05% (v/v) Triton X-100 (Sigma, St Louis, MO, USA) using a semimicro Waring container (PGC # 77-8549) on a Waring blender (Fisher # 14-509-10). Step 14–16 spermatids (stage II–VIII) survive this homogenization, and their nuclei can then be counted using a haemocytometer (Fisher # 02-671-10). To count the spermatids, a 200 μl sample of homogenate was diluted with 300 μl of saline and 500 μl of 4% Trypan blue, which stains spermatids and facilitates counting (Cooke *et al.*, 1991). Sample aliquots of 5.5 μl were then placed on the haemocytometer and counted twice at 100× magnification under a microscope to determine the average number of spermatids per sample. These values were used to obtain the total number of spermatids per testis, and this number was then divided by the testis weight to give spermatids per gramme of testes. Developing spermatids spend 4.84 days in steps 14–16 during spermatogenesis in the mouse. Thus, the values for the number of spermatids per testis and spermatids per gramme of testis were divided by 4.84 to obtain daily sperm production and efficiency of sperm production (per gramme of testis) respectively (Robb *et al.*, 1978; Joyce *et al.*, 1993).

Prostate androgen receptor assay

In addition to measuring androgen receptors (AR), protein was measured as a reference for expressing AR content, and the amount of DNA was also measured to discern between hypertrophy and hyperplasia. Prostates were homogenized on ice in running buffer [13% glycerol and 83 mmol/l Tris–HCl (pH 6.8) with 0.67 mg/ml sodium dodecyl sulphate and 21 mg/ml dithiothreitol], and boiled at 100°C for 3 min. After samples were taken for DNA and protein assay (see below) the homogenates were centrifuged at 10 000 r.p.m. (9300 *g*) for 5 min at 4°C. Homogenization of prostates in any buffer other than the denaturing running buffer was accompanied by losses in the labile AR protein.

To determine total protein and DNA concentrations directly in this homogenate, the following procedure (Brown *et al.*, 1989, modified) was used. A 50 μl aliquot of prostate homogenate was added to 440 μl of double-distilled water, after which 50 μl of 0.15% (w/v) sodium deoxycholate was added and mixed. After incubation for 10 min at 25°C, 60 μl of 80% (w/v) trichloroacetic acid (TCA: final concentration 8.0%) were then added, the tubes were vortexed, and centrifuged at 3000 *g* for 15 min at 25°C. The supernatants were discarded and the pellets washed with 500 μl 8% TCA, centrifuged, and supernatants were carefully removed again. The pellets were dissolved by brief vortexing or sonication in 462.5 μl of added 10 mmol/l EDTA (pH 12.4), and then neutralized to pH 7.2 with 37.5 μl of 0.77 mol/l KH_2PO_4 for a total volume of 500 μl. Protein and DNA concentrations in this solution were determined in 50 μl aliquots by using the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA) and by using the Hoechst dye 33258 fluorimetric

DNA assay (Labarca and Paigen, 1980; Taylor *et al.*, 1995). The standard curve samples for the assays [50 µl protein, or 50 µl DNA with 50 µl 5% bovine serum albumin (BSA) carrier] were subjected to the same precipitation and extraction procedure as were homogenate samples.

After determination of protein, each sample was diluted to a constant protein concentration (µg protein/µl) with loading buffer containing bromophenol blue dye at 0.25 mg/ml. Samples were then loaded onto 8% SDS–polyacrylamide mini-gels (20 µg protein in 20 µl buffer per lane), electrophoresed for 2 h at 10 mA/gel, and electroblotted onto nitrocellulose membranes using a semi-dry transfer apparatus (BioRad, Hercules, CA, USA) for 45 min at 12 V. Each gel contained a sample from one prostate of each treatment group, and a pooled standard of prostates from five to seven month-old animals was loaded in two lanes on each gel as a reference standard for comparisons between gels.

The membrane was blocked with 5% BSA (fraction IV) in 10 mmol/l Tris (pH 7.5) at room temperature for 1 h, washed in Tris/Tween (0.02% Tween, 10 mmol/l Tris), then incubated with 1:10 000 monoclonal rat anti-androgen receptor (Affinity BioReagents, Neshanic Station, NJ, USA) in 10 mmol/l Tris (pH 7.5) overnight at room temperature. The membrane was washed in Tris/Tween for 30 min and incubated in 5% normal rabbit serum (Sigma) at room temperature for 90 min, washed, and incubated in rabbit anti-rat IgG horseradish peroxidase conjugate (Sigma), washed for 1 h and reacted with Lumi-glo chemiluminescence kit (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). Membranes were exposed to film, and films were scanned and analysed for band density using Kodak 1D imaging software. Band densities for each lane of a gel were divided by the average density of the two pooled standard bands, and then expressed for each group as a percentage of the pooled standard control values run in duplicate on each gel (band density % of standard per µg protein). Since these data were based on an equal amount of loaded protein per lane, the band densities could be multiplied by total protein per prostate to express as total AR per prostate (band density % standard per prostate), as well as AR per µg protein. Total AR per prostate were divided by µg DNA per prostate to yield AR per µg DNA (band density % of standard per µg DNA; roughly proportional to receptors per cell). Periodically, an assay involving serial dilution of the control samples was conducted to verify that the assay was operating within its linear range.

Statistical analyses

Statistical analyses were conducted using the general linearized model (GLM) procedure in the Statistical Analysis System (SAS). Since only one animal from each litter was used in an experiment, correction for litter effects was not necessary. Homogeneity of variance between groups was assessed using Levene's test. For those variables showing heterogeneous variance, a logarithmic transformation eliminated the heterogeneity, and thus the statistical analysis was performed on the log-transformed data. If body weight was significantly ($P < 0.05$) correlated with organ weight, analysis of co-variance (ANCOVA) was conducted, with body weight as the co-variate, in order to assess group differences. If body weight was not significantly correlated with organ weight, then the data were analysed using analysis of variance (ANOVA).

Planned pairwise comparisons were made following a significant ($P < 0.05$) omnibus ANOVA (or ANCOVA) by the least significant difference (LSD) test. In presenting the results, the results of the overall ANOVA (or ANCOVA) are reported first, followed by results of planned comparisons. To control the experiment wise error rate, the statistical significance of the pairwise differences was limited to the significance level of the overall test. The primary comparisons of interest were between the control and experimental (EE-exposed) groups.

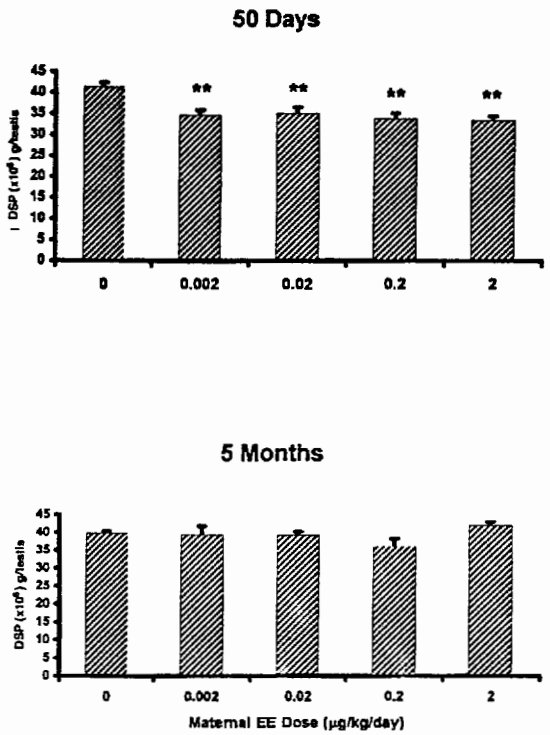

Figure 1. Mean (± SEM) daily sperm production ($\times 10^6$) per gram testis in CF-1 mice exposed as fetuses to ethinyl oestradiol (EE) via maternal administration on days 0–17 of gestation. Testis were examined when the males were 50 days old or 5 months old (see Tables I and II for animal numbers per group). $^{**}P < 0.01$; $^{*}P < 0.05$.

Results

No group differences were detected in dams with regard to either overall weight gain during pregnancy or weight gain corrected for the number of pups. No offspring were obtained with dams treated with either the 20 or 200 µg/kg doses as these treatments caused 100% pregnancy loss. Data from unhandled and oil control males were combined to form one control group because they did not differ in any response measured (at both 50 days and 5 months). All results are presented as means (± SEM).

DSP and DSP/g testis

Daily sperm production was analysed both without correction for testis weight (DSP) and after correction for testis weight (DSP/g testis). At 50 days of age, neither value correlated significantly with body weight. All doses of EE at 50 days of age resulted in significantly ($P < 0.01$) reduced DSP and DSP/g testis relative to controls, although these effects were not more severe at higher doses (Figure 1; Table I). At 5 months, both DSP and DSP/g testis were significantly

Table I. Summary data for prenatally ethinyl oestradiol (EE)-treated males at 50 days of age

Parameter	Control[a]	Ethinyl oestradiol dose group (μg/kg)			
		0.002	0.02	0.2	2
Body weight (g)	28.7 ± 0.3	29.0 ± 0.5	29.2 ± 0.4	28.9 ± 0.3	29.1 ± 0.6
	(24)	(12)	(11)	(11)	(9)
DSP ($\times 10^6$)	3.92 ± 0.11	3.39 ± 0.18**	3.40 ± 0.18**	3.19 ± 0.17**	3.27 ± 0.12**
$r = -0.01$	(23)	(9)	(11)	(9)	(9)
DSP/g testis ($\times 10^6$)	41.2 ± 1.2	34.4 ± 1.5**	34.7 ± 1.8**	33.5 ± 1.7**	33.2 ± 1.4**
$r = -0.11$	(23)	(9)	(11)	(9)	(9)
Testis (mg)	95.9 ± 1.4	97.6 ± 3.2	98.4 ± 2.1	93.2 ± 3.5	98.9 ± 2.8
$r = -0.05$	(24)	(12)	(11)	(11)	(9)
Epididymis (mg)	28.7 ± 0.4	30.0 ± 0.8	29.3 ± 0.8	29.3 ± 1.0	30.0 ± 1.0
$r = 0.15$	(24)	(12)	(10)	(11)	(9)
Prostate (mg)	23.7 ± 0.7	26.3 ± 1.4+	28.6 ± 1.3**	26.3 ± 0.8‡	27.8 ± 1.4**
$r = 0.10$	(24)	(12)	(11)	(11)	(9)
Seminal vesicles (mg)	28.5 ± 0.8	31.1 ± 1.3	29.2 ± 1.4	30.1 ± 1.4	31.0 ± 2.2
$r = 0.08$	(24)	(12)	(11)	(11)	(9)
Coagulating glands (mg)	12.5 ± 0.4	13.7 ± 0.7	12.9 ± 0.7	13.1 ± 0.4	12.5 ± 0.9
$r = 0.21$	(24)	(12)	(11)	(11)	(8)

Summary data in 50-day-old mice exposed as fetuses to EE via maternal administration of 0.002–2 μg/kg/day on days 0–17 of gestation. Means (± SEM) are either adjusted or unadjusted for body weight, depending upon whether body weight was significantly correlated with response. Pearson's correlation coefficient (r) between response and body weight was calculated for all animals without regard to dose. Testis and epididymis weights are based on one per animal. Number of animals in each dose group are in parentheses.
Statistical analyses were based on comparison of treated groups with control animals: $^{**}P \leq 0.01$; $^{*}P \leq 0.05$; $^{+}P < 0.07$; $^{\ddagger}P < 0.08$.
[a]Control animals included 10 mice that were unhandled and 13 or 14 given corn oil.

($P < 0.05$) correlated with body weight. However, no significant group differences in DSP or DSP/g testis at 5 months were detected.

Prostate weight and prostatic androgen receptors

Prostate weight at 50 days was not significantly correlated with body weight, but the correlation between these two variables was significant ($P < 0.05$) at 5 months. Prostate weight was increased in all four dosed groups at both 50 days and at 5 months of age (Tables I and II; Figures 2 and 3). For most dosed groups in 5-month-old males, the increase was statistically significant ($P < 0.05$ or $P < 0.01$), whereas for the other dosed groups the increased prostate weights were not significant. These increases were seen for a wide range of doses: 0.002 to 2 μg/kg/day.

Androgen receptors (AR) were measured only from prostates collected from 5-month-old males, and were obtained from ~10 males from each group. Summary data for all prostates collected from animals are shown in Table II and Figure 2. However, summary data only from prostates used in the AR assay are presented in Figure 3 along with AR per protein. Other results related to AR, total protein and total DNA per prostate are shown in Table II. Although prostate weights used for AR analysis were increased by 13–17% in the 0.02, 0.2 and 2 μg/kg dose groups, changes in AR-related measures (total AR, AR per DNA, AR per protein) were generally not significant. Since previous studies had shown that the dose–response relationship for prostate size following fetal exposure to oestrogenic chemicals is not monotonic with increasing dose (vom Saal *et al.*, 1997), both a linear and a quadratic model were evaluated for the three AR variables.

Although these three AR variables were highly correlated ($r = 0.92$–0.98, $P < 0.001$) and showed generally similar

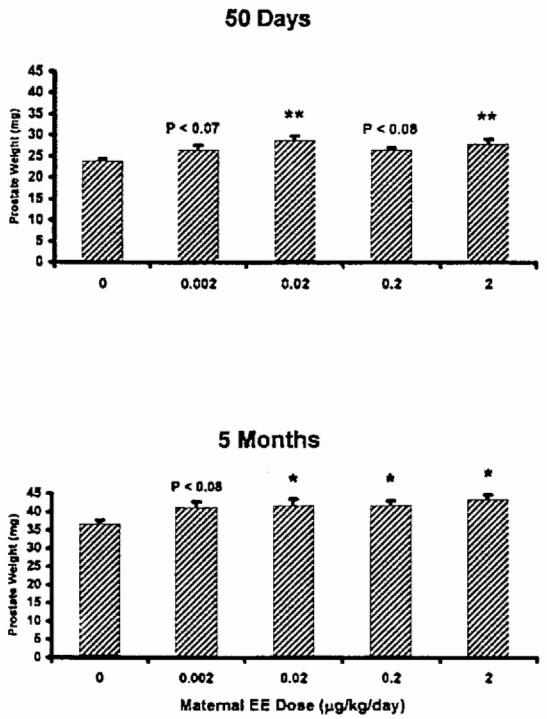

Figure 2. Mean (± SEM) prostate weight (mg) in CF-1 mice exposed as fetuses to ethinyl oestradiol (EE) via maternal administration on days 0–17 of gestation. Prostates were weighed when the males were 50 days old or 5 months old (see Tables I and II for animal numbers per group). $^{**}P < 0.01$; $^{*}P < 0.05$.

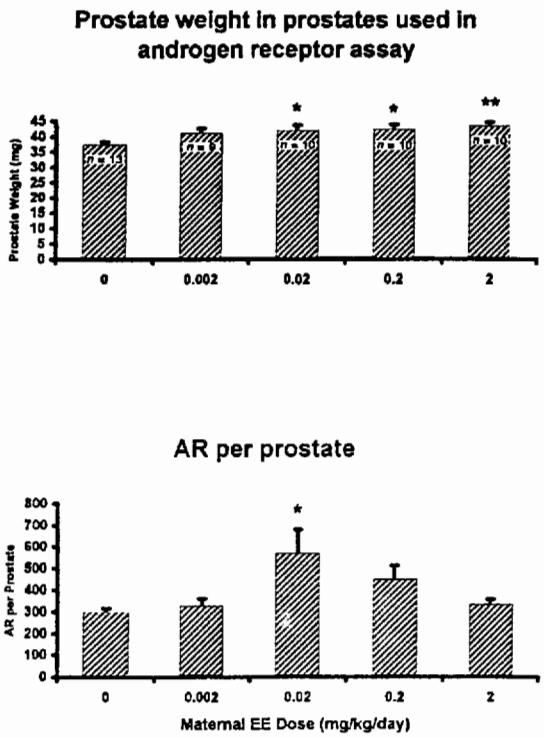

Figure 3. Mean (± SEM) prostate weight (mg) for prostates used in the androgen receptor (AR) assay, and AR per prostate (expressed as band density (% standard) per prostate] in CF-1 mice exposed as fetuses to ethinyl oestradiol (EE) via maternal administration on days 0–17 of gestation. The number of animals tested per group was the same for all assays, and is identified in the top panel. $^{**}P < 0.01$; $^{*}P < 0.05$.

dose-related patterns of response, a significant ($P = 0.04$) quadratic term was found only for AR per prostate. A follow-up LSD comparison indicated that the 0.02 µg/kg group had a significantly ($P < 0.05$) increased number of AR per prostate (~50%) relative to controls (Figure 3). Slight elevations in AR per DNA and AR per protein were seen in the 0.02 µg/kg/day group relative to controls, although these increases were not statistically significant. Moreover, total protein and total DNA per prostate were also somewhat, but not significantly, elevated in the 0.02 µg/kg/day group (Table II).

Body weight and weights of testis, epididymis, seminal vesicle, and coagulating gland

Analysis of variance indicated no difference in body weight between groups at 50 days of age. Similarly, there were no differences in other reproductive organ weights in 50-day-old animals, none of which was significantly correlated with body weight (Table I). At 5 months of age, all treatment groups showed some increase in body weight compared with controls, but the increases were only statistically significant ($P < 0.05$) in the 0.002 and 2 µg/kg/day groups (Table II). Similar to findings in 50-day-old males, there were no overall differences at 5 months between groups in seminal vesicle, coagulating gland, epididymal or testis weights (Tables I and II).

Discussion

Developmental studies in rats that assessed fetal mortality, incidence of malformation and fertility following in-utero exposure to OC preparations containing EE and the progestin norethisterone have led to the conclusion that a dose of 1.75 µg/kg for EE produces no effect; this is referred to as the no observed effect level (NOEL) (Harada *et al.*, 1991a,b). However, in the current study it was found that exposure to a dose as low as 0.002 µg/kg/day EE during pregnancy (which is substantially lower than the ~0.5 µg/kg dose level used in OC by women) transiently reduced daily sperm production and permanently increased prostate size and number of prostatic AR. It is of considerable importance that the changes observed would not be detected if only externally visible malformations or a decrease in fertility were being sought. Specifically, at doses of 0.02 to 2 µg/kg/day EE, adult prostate weight was increased. In addition, at doses of 0.002 to 2 µg/kg/day there was a transient decrease in the levels of sperm production each day. The effect on sperm production occurred at an almost 1000-fold lower dose than the previously reported NOEL, and at a dose 250-fold lower than those contained in OC commonly used by women.

Because the total number of Sertoli cells accounts for most of the variability (<85%) in DSP in adult rats (Berndtson and Thompson, 1990), one hypothesis is that prenatal exposure to oestrogen may permanently reduce Sertoli cell numbers, resulting in subsequent decreases in adult sperm output (Sharpe and Skakkebaek, 1993). Sertoli cell proliferation begins on embryonic day 14 (Vergouwen *et al.*, 1991) and ends early in postnatal life in the mouse (postnatal days 12–17, depending on the strain) with no additional proliferation in adulthood (Kluin *et al.*, 1984; Vergouwen *et al.*, 1991). Therefore, a decrease in DSP resulting from a decrease in Sertoli cells is predicted to be a permanent effect. However, in the current study, DSP was decreased by EE at 50 days of age, but was not different from controls when males in most dose groups were 5 months old. This finding suggests that the effects of EE during fetal life on DSP are not due to a decreased Sertoli cell numbers.

Our finding that a specific very low maternal dose of EE produced an 'imprinted' increase in prostatic AR in male offspring was not unexpected. It has been reported previously that circulating oestradiol in male mouse fetuses is positively correlated with adult prostate size and numbers of prostatic AR (Nonneman *et al.*, 1992), which was confirmed when prostate size, number of prostatic glands and prostatic AR were increased in male mice in response to a very small experimental increase in circulating oestradiol during fetal life (while testosterone concentrations remained constant) (vom Saal *et al.*, 1997). This finding has recently been confirmed (Gupta, 2000). Interestingly, in the present study an increase in AR levels was only observed in the 0.02 µg/kg/day EE dose group, despite increased prostate weight at all doses examined. These observations suggest qualitative differences in mechanisms contributing to the increased prostate weight across the dose range examined, although further research is needed to test this hypothesis.

Table II. Summary data for 5-month-old males treated prenatally with ethinyl oestradiol (EE)

Parameter	Control[a] (unh+oil)	Ethinyl oestradiol dose group (μg/kg)			
		0.002	0.02	0.2	2
Body weight (g)	34.7 ± 0.4	36.6 ± 0.6*	35.6 ± 0.6	35.7 ± 0.3	36.6 ± 0.7*
	[24 (10+14)]	(12)	(10)	(12)	(10)
DSP ($\times 10^6$)	4.50 ± 0.16	4.51 ± 0.40	4.31 ± 0.12	3.89 ± 0.28	4.90 ± 0.21
$r = 0.33^{**}$	[15 (5+10)]	(11)	(10)	(11)	(10)
DSP/g testis ($\times 10^6$)	39.5 ± 0.9	38.9 ± 2.9	38.7 ± 1.4	35.5 ± 2.6	41.4 ± 1.5
$r = 0.26^{*}$	[15 (5+10)]	(11)	(10)	(11)	(10)
Testis (mg)	112.5 ± 1.8	114.0 ± 4.4	111.9 ± 2.3	110.5 ± 1.6	118.4 ± 2.6
$r = 0.17$	[24 (10+14)]	(12)	(10)	(12)	(10)
Epididymis (mg)	41.6 ± 0.5	43.5 ± 1.4	43.8 ± 0.8	42.0 ± 0.8	45.1 ± 1.4
$r = 0.31^{**}$	[24 (10+14)]	(12)	(10)	(12)	(10)
Prostate (mg)	36.6 ± 1.1	41.1 ± 1.8^{+}	41.7 ± 1.9*	41.7 ± 1.4*	43.2 ± 1.5*
$r = 0.30^{**}$	[23 (9+14)]	(12)	(10)	(12)	(10)
Seminal vesicles (mg)	48.4 ± 1.5	48.8 ± 2.8	47.7 ± 2.6	49.7 ± 1.5	46.5 ± 3.6
$r = 0.28^{*}$	[24 (10+14)]	(12)	(10)	(12)	(9)
Coagulating glands (mg)	19.5 ± 0.8	21.5 ± 1.5	19.9 ± 0.9	21.8 ± 0.7	20.7 ± 1.5
$r = 0.15$	[24 (10+14)]	(11)	(10)	(12)	(10)
Protein per prostate (μg)	3002 ± 187	3207 ± 206	3691 ± 271	3588 ± 182	3495 ± 163
	(13)	(9)	(10)	(10)	(10)
DNA per prostate (μg)	79.2 ± 4.4	87.9 ± 7.7	96.5 ± 8.0	86.1 ± 4.3	90.4 ± 5.4
	(13)	(9)	(10)	(10)	(10)
AR per protein	99.8 ± 10.5	100.6 ± 12.7	147.0 ± 27.9	119.1 ± 16.4	91.8 ± 9.2
	(13)	(9)	(10)	(10)	(10)
AR per DNA (~cell)	3750 ± 390	3658 ± 404	5818 ± 1276	5010 ± 717	3623 ± 415
	(13)	(9)	(10)	(10)	(10)

Summary data in 5-month-old mice exposed as fetuses to EE via maternal administration of 0.002–2 (g/kg/day on days 0–17 of gestation. Means (± SEM) are either adjusted or unadjusted for body weight, depending upon whether body weight was significantly correlated with response. Pearson's correlation coefficient (r) between response and body weight was calculated for all animals without regard to dose. Testis and epididymis weights are based on one per animal. Androgen receptors (AR) per protein are expressed as band density (% standard) per μg protein. Similarly, AR per DNA (~cell) is expressed as band density (% standard) per μg DNA. Numbers of animals in each dose group are shown in parentheses. Statistical analyses were based on comparison of treated groups with control animals: $^{**}P \leq 0.01$; $^{*}P \leq 0.05$; $^{+}P < 0.08$.

[a]Control animal group comprised unhandled (unh) and corn oil-treated mice as indicated.

It is generally accepted that in moving from very low to much higher doses of a hormone, the hormone can potentially interact with response systems for other hormones, thus leading to qualitatively different, rather than just quantitatively different, outcomes (Kavlock and Ankley, 1996).

The elevated body weight in the 5-month EE-dosed groups was a potentially complicating factor in the evaluation of prostate and other organ weight. Although alternative approaches are possible (e.g. organ/body weight ratios), it is felt that the most appropriate method for adjusting organ weight for the influence of body weight is ANCOVA, because the correction for body weight is based on the observed relationship calculated from the data rather than on the use of a specific, proportional (one-to-one) relationship between organ and body weight. Moreover, for those organ weights showing no association with body weight, body-weight adjustment is neither needed nor appropriate.

Although there have been virtually no prior experimental animal studies to determine whether exposure to clinically relevant doses of EE during prenatal life can disrupt the development of reproductive organs, several studies have shown that prenatal exposure to DES, at the same very low doses examined here, produce similar effects on prostatic growth described here for EE (vom Saal *et al.*, 1997; Gupta, 2000). This is not surprising, since EE and DES are very similar in their oestrogenic activity in rodents (Branham *et al.*, 1988). For example, oral doses of 0.02, 0.2 and 2.0 μg/kg/day of DES during days 11–17 of pregnancy in mice resulted in a permanently enlarged prostate in male offspring that were examined in adulthood (vom Saal *et al.*, 1997). An important additional finding is that administration of a 200 μg/kg/day dose of DES to pregnant female mice resulted in an inhibition of normal prostate development (vom Saal *et al.*, 1997), revealing that low doses of oestrogenic chemicals exert a stimulating effect, while high doses have an opposite, inhibitory, effect on prostate development in mice. These findings concerning the effects of low versus high doses of DES on prostate development have recently been confirmed in CD-1 mice with administration to pregnant females and with direct application of DES to urogenital sinus explants, demonstrating direct effects of oestrogen on prostate development independent of effects on the mother or placenta (Gupta, 2000).

Female offspring are also affected by maternal administration of these low doses of DES: administration of a 0.01 μg/kg/day dose of DES to pregnant mice was associated with subfertility and a decrease in the number of ovulated oocytes in response to a superovulating dose of gonadotrophins in female offspring (McLachlan *et al.*, 1982). In contrast to the inhibitory effects that prenatally administered very low doses of DES or EE have on the gonads (ovaries or testes), we and others (Newbold *et al.*, 1999), have found that similar to the prostate, the response

of the uterus to oestrogen stimulation in female offspring is enhanced by prenatal exposure to very low doses of DES (0.001–0.1 μg/kg/day). In sharp contrast, normal development of both the uterus in female offspring and prostate in male offspring is inhibited by maternal administration of high doses of DES (100 μg/kg/day) (vom Saal *et al.*, 1997; Alworth *et al.*, 1999; Newbold *et al.*,1999). In the present study, only male offspring were examined, but based on these recent findings with DES, future studies focusing on the effects of maternal administration of EE on female offspring are warranted.

In summary, in the present study it was found that prenatal exposure to low doses of the oestrogen used in OC in the USA, ethinyl oestradiol, can alter the development of the prostate and testes in male mice. Characterizing the effects of low-dose exposure to oestrogen is not only of interest to clinicians, but has become of significant issue to those in the environmental community. Over the past several years there has been increasing concern that certain chemicals in the environment can mimic oestrogen action, and if exposure occurs during critical periods in organ development, these might potentially contribute to a variety of human health problems (Colborn and Clement, 1992). These chemicals are termed oestrogenic endocrine disrupting chemicals (EEDC), and have been discussed in the context of being a potential factor contributing to human epidemiological findings of decreases in sperm concentration, increases in hypospadias, cryptorchidism, and hormonally mediated cancers, such as prostate and testicular cancer (Colborn and Clement, 1992; Toppari *et al.*, 1996; Swan *et al.*, 1997; Paulozzi, 1999). One of the most controversial aspects of the EEDC debate is whether exposure to low doses of chemicals can disrupt development. The data presented here show that effects of EE occur at doses considerably below those previously assumed to be without effect.

Hence, it is proposed that the risk to offspring exposed *in utero* to EE (as well as progestins in OC) should be further explored. One of the most important lessons learned from DES, that the absence of observable malformations at birth did not serve as a predictor of reproductive system (and other) abnormalities later in life, should be applied to the assessment of risk associated with in-utero exposure to EE in OC. Until there are appropriate human studies with outcomes other than just malformations to assess the actual risk to human fetuses of exposure to the low doses of EE in OC, an emphasis on education and public awareness is suggested. That is, increased efforts should be devoted to educating the millions of women using OC with regard to the potential risks (as suggested by animal studies) that are associated with exposing their fetuses to even very low, subclinical doses of steroids used in OC. This should also raise awareness of the need for appropriate human studies to provide more definitive data to determine the actual risks.

Acknowledgements

These studies were supported by NIH grant ES08293 to F.vom S., NIH grant CA50354, and University of Missouri VMFC0018 to W.V.W. K.A.T. was supported by an EPA predoctoral fellowship (U-914991) and a grant from the W.Alton Jones Foundation.

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Received on August 15, 2000; accepted on February 14, 2001