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 (CSP) 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 disruptor, oestrogen, reproductive, 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 steroid 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 (Hill 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., 1993), encompassing a critical period for disruption of reproductive organ development (DeLesso, 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 issues known to be sensitive to reproductive steroid hormones, the association between OC use in early pregnancy and malformations is still generally considered weak (Kallen et al., 1991; Raman-Wilms et al., 1995; Martinez-Prieto et al., 1998). However, there have also been reports to the contrary (Li et al., 1993; 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 diethylstilboestrol (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
prostate birth. 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, 1961; Minardello, 1995). Further follow-up of DES-exposed daughters found they were also at increased risk for developing a variety of other reproductive abnormalities. These include vaginal epithelial cell changes, cervicovaginal, uterine and Fullhagen tube structural anomalies, increased incidence of infertility, ectopic pregnancies, premature births, and possibly increased incidence of immune and behavioural disorders, such as depression (Bithlo et al., 1979; Herbst, 1981; Stillman, 1986; Boiler et al., 1992; Hines, 1992; Gurski 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, cystic mediolysis, hypospadias, and possibly spermatogenic abnormalities (Bithlo et al., 1979; Stillman, 1982; Gurski et al., 1985). 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 clear 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 (Wikel 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 gestational dosages as assessed by their effect on the same genetic, a well-established estrogen bioassay, when administered scatereously to pregnant rats (Bratton 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 estrogenic chemicals being more similar during oral administration (Redel et al., 1996). In any event, both DES and EE are more potent stimulators of uterine growth than 17β-estradiol, the primary endogenous estrogen (Bratton et al., 1988; Redel et al., 1996). In addition, EE has been shown recently to cause similar incidence of uterine adenocarcinomas in DES in a chronic, lifelong non-neonatal exposure. An estimated 3% of US DES administered to pregnant mice reaches the fetus, primarily concentrating in the reproductive tract (Shah and McLaughlin, 1976). While mechanism of EE by the maternal liver does occur, unconjugated EE reaches the fetus (Sticker et al., 1983), although uptake of EE into fetal tissues and administration to pregnant mice has not been reported.

One basis for the high estrogenic activity of both DES and EE is that, in contrast to estradiol, DES and EE show little binding to estrogen-binding plasma proteins, alphafetoprotein in rodents and sex hormone binding globulin in humans (Agnihotri and Forthoff, 1969; Bratton et al., 1988). These plasma estrogen-binding globulin levels the levels of free, bioavailable oestrogens in serum (Nagel et al., 1998).

Despite the similarity in estrogenic 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 Obstetricians and Gynecologists, 1993). One reason for this is the high-dose exposures of DES (1-150 mg/kg) (Bern et al., 1987) are regarded as not relevant to the low doses of EE (35-50 mg/kg) 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 estrogenic 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 estrogenic 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 be expected. The animal literature regarding the adverse effect of developmental exposure to DES extends back at least to the early 1960s (Dunn and Green, 1963; Taliani and Barn, 1964); but these findings were ignored, as early physicians and scientists apparently believed that the animal data was 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, 2003). The literature on DES has contributed to a general awareness regarding the high level of conservation of the hormonal mechanisms mediating differences of the reproductive organs in mammals (Kavlock and Ausley, 1966; 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 died, stillborn/early and ethanol oesophagostomy/ontogeny 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 unexplained. In male mice, adult exposure to EE results in an increased incidence of cryptorchidism (Walker et al., 1990). Yasuda et al., (1958) showed that esophagus and genitalia of fetal rats and Leydig cell differentiation (Yasuda et al., 1957, 1960a, b). Female mice exposed to EE prenatally develop vaginal cell hypertrophy (Yasuda et al., 1987), vaginal hypoplasia, and increased degeneration of primordial follicles (Yasuda et al., 1958). However, these histological and functional responses in prenatal EE have only been studied at maternal doses of 20 mg/kg/day and above, with alterations typically occurring at 30 mg/kg/day. The clinically relevant dose for EE in man
OC is -0.5 μg/kg/day; this is based on use of an OC with a production weight of 68 kg. Since there is little information as to whether exposed to clinically-relevant doses of EE during prenatal life can disrupt the development of reproductive organs in experimental animals. In the current study it was investigated the effects on adult male reproductive organs following in vivo 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

CP-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 (30x20x12 cm) polypropylene cages in a core cub building. Premen and lactating mice were fed Purina breeding chow (#5005); Purina, Richmond, IN, USA until, after weaning, males were maintained on Purina #5007 standard chow. Rooms were maintained at 23°C on a 12:12 light-dark cycle, with lights on at 10:00 h, so that rumination 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 17β-estradiol estramustine

Virgin females (aged 3-4 months) were timed-mated by being placed daily with a stud male for 4 beginning 2 h before the end of the dark phase of the light-dark cycle. Matings were verified by the presence of a seminal plug (day 0 of pregnancy). Play-positive mice were either left unhandled or were given (orally) an average dose of 0.0002, 0.02, 0.2, 2, 20 or 200 μg/kg EE dissolved in 30 μl of isooctylated-stripped corn oil (ICN, Aurora, OH, USA) once daily during gestation 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 microprobes (Rapidloc). Naloxone, MA, USA [naloxone methoxymristate (Rapidloc)] to the mice immediately before conception oil on the day before and on day 10, 11, 13, 15, 17, 19, and 29 of pregnancy, and on day 1 of the estrus cycle. No play-positive mice were administered with 20 or 200 μg/kg of EE reared 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 1 to 10 (mean 5 ± 1.7). Female offspring were removed at weaning, and males remained in litter groups of two to five mice per cage. No more than five males were kept for any litter. By day 11-12 of life, a randomly selected male was individually housed in a wire-bottomed cage. For males killed when 5 months old, a randomly selected male was individually housed in a wire-bottomed cage. The reason this 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 subordinates; this can have masked effects on reproductive organ and behaviour in CP-1 mice. However, individual housing for 1 month eliminates the poor effects of subordination in CP-1 mice (unpublished observations). Following weaning, when one or more males were 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 1 month of age, animals were weighed and euthanized. The castrating glands, seminal vesicles, prostate and testes and epididymis were then removed and weighed. Fluid was removed from the seminal vesicles and castrating glands by blushing 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; Crooke et al., 1991; Joyce et al., 1991). Briefly, after being removed and weighed, the tissues were placed in liquid nitrogen, and subsequently kept at -70°C until being examined. Tissue were then homogenized for 3 min in 25 ml of physiological saline containing 0.5% (w/v) Triton X-100 (Sigma, St Louis, MO, USA) using a Bismark Waring Blendor (Fisher #1-909-10). Step #14-16 spermatozoa (stage II-VIII) survived this homogenization, and their nuclei can thus be observed using a phase-contrast microscope. A microscopic magnification on a microscope to determine the average number of spermatozoa per testis. These values were used to obtain the total number of spermatozoa per testis and this number was then divided by the tissue weight to give spermatozoa per gram of testes. Developing spermatozoa were counted in four 1 mm square fields for 14-16 different measurements of light microscopy. Thus, the values for the number of spermatozoa per testis and spermatozoa per gram of testes were divided by 484 to obtain daily production and efficiency of sperm production (as a percentage of testes) respectively (Robb et al., 1978; Joyce et al., 1993).

Prostate androgen receptor assay

In addition to measuring prostate receptor capacity (AR), protein was measured as a reference for expressing AR content, and the amount of DNA was also measured to discern between hypotrophy and hyperplasia. Prostate were homogenized on ice in running buffer (130 mM NaCl and 3 mM Tris-HCl pH 7.4 with 0.07 mg/ml sodium deoxycholate and 21 mM sodium dodecyl sulfate) 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 rpm (2000 g) for 5 min at 4°C. The supernatant of these aliquots, other than the detraining running buffer was accompanied by bovine in the final AR protein.

To determine total protein and DNA concentrations directly in this homogenate, the following procedure (Brown et al., 1989, modified) was used. Each 0.4 ml aliquot of prostate homogenate was added to 440 ml of double-distilled water, after which 50 0.1% (v/v) sodium deoxycholate was added and mixed. After incubation for 10 min at 20°C, 60 ml of 80% (v/v) trichloroacetic acid (TCA, final concentration 8%), were then added, the tubes were vortexed, and centrifuged at 3000 g for 10 min at 4°C. The supernatant were discarded and the pellets washed with 500 ml 95% TCA, centrifuged, and supernatants were carefully removed again. The pellets were dissolved by brief vortexing or sonication in 4.5 ml of 10% trichloroacetic acid (pH 1.2) and, then reconstituted to 7.2 ml with 37.5 ml of 0.77 ml KCl/NaCl for a final volume of 50 ml. Protein and DNA concentrations in this solution were determined in 50 ml aliquots of using the Biochromsphar 180 (Biochrom, Richmond, IL, USA) and by using the Hoech-ers 35268 Hammarstrom
DNA assay (Labarca and Palenik, 1980; Taylor et al., 1995). The standard curve samples for the assay (50 μl protein or 50 μl DNA with 50 μl 3% 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 0.3 mg/ml. Samples were then loaded onto 12% SDS-polyacrylamide mini-gels (20 μg protein in 20 μl buffer per lane), electroblotted for 2 h in 50 mM Tris, and electroblotted onto nitrocellulose membranes using a semi-dry transfer apparatus (BiRad, Hercules, CA, USA) for 45 min at 12 V. Each gel contained a sample from one point of each treatment group, and a pooled standard of proteins 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 soaked with 5% BSA (fraction IV) in 10 mM Tris (pH 7.5) at room temperature for 1 h, washed in Tris-Tween (0.05%) Tween, 10 mM Tris, then incubated with 1:10,000 monoclonal anti-anti-ergosterol (Affinity BioReagents, Northlake-Station, XI, USA) in 10 mM Tris (pH 7.5) containing 5% BSA 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 1 h, washed, and incubated in rabbit anti-rat IgG horseradish peroxidase conjugate (Sigma), washed for 1 h, and reacted with Lumi-glo chemiluminescence kit (Eckerdag & Perry Laboratories, Guilderland, MD, USA). Membranes were exposed to film, and films were scanned and analyzed 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 triplicate 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 protease to express as total AR per protease (band density % standard per protein), as well as AR per μg protein. Titral AR per protease were divided by μg DNA per protease to yield AR per μg DNA (band density % 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 linear model (GLM) procedure in the Statistical Analysis System (SAS). Since only one animal from each fish 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 homogeneity of variance, a log transformed ANOVA was used to evaluate the effects of treatment groups. For those variables that were not homogenous, a non-parametric test was used to test for differences between the groups. If there were significant differences, the data were analyzed using a Student t-test.

Planned pairwise comparisons were made following a significant (P < 0.05) omnibus ANOVA test by applying a Tukey's HSD test. In presenting the results, the results of the overall ANOVA (or ANOVA) are reported first, followed by results of planned comparison. To control the experiment-wise error rate, the statistical significance of the pairwise differences was calculated using a post hoc test.

Figures

Figures 1 and 2: Daily sperm production (μg) per gram tissue was measured at 50 days of age and 5 months of age (see Tables 1 and 2 for arithmetic values per group). These data were presented as mean ± SEM.

Results

No group differences were detected in dams with regard to overall weight gain during pregnancy or weight gain corrected for the number of pups. No offsprings were obtained from dams treated with either the 20 or 200 mg/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 DSPg tests

Daily sperm production was analysed both without correction for tests weights (DSP) and after correction for tests weights (DSPg tests). At 50 days of age, neither value correlated significantly with body weight. All doses of EE at 50 days of age assayed in significantly (P < 0.01) reduced DSP and DSPg tests relative to controls, although these effects were not more severe at higher doses (Figure 1; Table 1). At 5 months, both DSP and DSPg tests were significantly
Table 1. Summary data for pentylenetetrazol (PTZ)-treated males at 30 days of age

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control*</th>
<th>Ethyl methyl dioxime group (μg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.002</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>28 ± 0.3</td>
<td>28.2 ± 0.3</td>
</tr>
<tr>
<td>DPP (μL)</td>
<td>0.60 ± 0.01</td>
<td>3.29 ± 0.18*</td>
</tr>
<tr>
<td>r = 0.1</td>
<td>0.42 ± 0.2</td>
<td>0.44 ± 0.5**</td>
</tr>
<tr>
<td>Testis (mg)</td>
<td>95.8 ± 1.4</td>
<td>97.4 ± 3.2</td>
</tr>
<tr>
<td>Epididymis (mg)</td>
<td>76.1 ± 0.4</td>
<td>76.2 ± 0.8</td>
</tr>
<tr>
<td>Prostate (mg)</td>
<td>23.7 ± 0.7</td>
<td>26.2 ± 1.4</td>
</tr>
<tr>
<td>r = 0.03</td>
<td>0.6 ± 0.04</td>
<td>0.6 ± 0.8</td>
</tr>
<tr>
<td>Seminal vesicles (mg)</td>
<td>28.3 ± 0.8</td>
<td>31.2 ± 1.3</td>
</tr>
<tr>
<td>r = 0.08</td>
<td>0.12 ± 0.07</td>
<td>0.12 ± 0.7</td>
</tr>
<tr>
<td>Corporating glands (mg)</td>
<td>12.3 ± 0.6</td>
<td>12.3 ± 0.6</td>
</tr>
</tbody>
</table>

Summary data in 30-day-old mice exposed to a single injection of PTZ in the food at a dosage of 0.002-2 μg/kg daily on day 0-17 of gestation. Means ± SEM are referent adjusted or unadjusted for body weight, depending upon whether body weight was significantly correlated with outcome. Parameters' covariance coefficients for between group and body weight via calculation for all animals without regard in dose. Tests and epididymis weights are based on a single per animal. Number of animals in each dose group is in parentheses. Statistical analysis were based on comparison of treated groups with control animals. **P < 0.01; *P < 0.05; *P < 0.05; **P < 0.01.

*Control animals included 10 mice that were euthanized at 3 or 14 days post birth.

(P < 0.05) correlated with body weight. However, no significant group differences in DPP or DPPs was seen at 5 months of age.

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 dose groups at both 30 days and at 5 months of age (Tables 1 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 dose 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 prostate sections from 5-month-old males, and were obtained from 10 males from each group. Summary data for all prostate 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 prostate. Other results related to AR, total protein and total DNA per prostate are shown in Table II. Although prostate weights were selected for AR analysis were increased by 13-17% in the 0.002, 0.2 and 2 μg/kg dose groups, changes in AR-related measures (total AR, AR per DNA, AR per prostate) were generally not significant. Since previous studies had shown that the dose-response relationship for prostate size following fetal exposure to xenobiotic chemicals is not monochron with increasing dose (von 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 trends, the determination coefficients for the regression analyses were low for these AR variables.

Figure 2. Mean (± SEM) prostate weight (mg) in CF-1 mice exposed to pentylenetetrazol (PTZ) via maternal administration on day 0-17 of gestation. Prostates were weighed when the males were 30 days old or 5 months old (see Tables I and II for animal numbers per groups. **P < 0.01; *P < 0.05.

902
Prostate weight in prostates used in androgen receptor assay

![Graph showing prostate weight vs. AR per prostate](image)

**Figure 3.** Mean (+ SE) prostate weight (mg) for prostates used in androgen receptor (AR) assay, and AR per prostate, expressed as androgen independent prostate (EPI) mice exposed to ethynl estradiol (EE) via IP delivery on days 10-17 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 (~10%) 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

**Table I.** 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 0.02 and 2 μg/kg/day groups (Table III). Similar to findings in 50-day-old males, there were no overall differences at 5 months between groups in seminal vesicle, coagulating gland, epididymis or testis weights (Tables I and III).

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 progesterone nonsteroidals 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, all 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 250-64 fold lower than those contained in OC communities used by women.

Because the total number of Sertoli cells accounts for most of the variability (~85%) in DSP in adult rats (Bardan and Thompson, 1990), one hypothesis is that perinatal exposure to estrogen may permanently reduce Sertoli cell numbers, resulting in subsequent decreases in adult sperm output (Sharpe and Skakkebæk, 1997). Sertoli cell proliferation begins on embryonic day 14 (Dempowicz 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 (Kniah et al., 1994; Yegashwini 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 control 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 "impaired" increase in prostatic AR in male offspring was not unexpected. It has been reported previously that circulating estradiol in vivo is positively correlated with adult prostate size and numbers of prostatic AR (Nieminen et al., 1992), which was confirmed when prostate size, number of prostatic glands and prostatic AR were increased in male mice in response to very small experimental increases in circulating estrogen during fetal life (while testosterone concentrations remained constant) (von 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.

993
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 (Karlin and Atchley, 1997). The elevated body weight in the 5-month EE-dosed group 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, proportionate (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 (van Saal et al., 1997, Gupta, 2005). This is in opposition to estradiol and E2 doses are very similar in their oestrogenic activity in rodents (Bratton 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 (van Saal et al., 1997). An important additional finding is that administration of a 2.0 μg/kg/day dose of DES to pregnant females resulted in an inhibition of normal prostate development (van Saal et al., 1997), suggesting 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 uterine tissues 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/g/day dose of DES to pregnant mice was associated with feminization and a decrease in the number of ovulated oocytes in response to superovulating dose of gonadotrophins in female offspring (McLauchlin et al., 1992). In contrast to the inhibitory effects that prenatally administered very low doses of DES or EE have on the gonadal ovaries or testes, we and others (Moshref 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 (0.00003-0.3 ng/day). In sheep, control, normal development of DES: both the uterus in female offspring and prostate in male mice. Characterizing the effects of low-dose exposure to oestrogen is not only of interest to clinicians, but has become of significant importance in the environment. Over the past several years there has been increasing concern that certain chemicals in the environment may be oestrogenic, and if exposure occurs during critical periods in organ development, these effects may contribute to a variety of human health problems (Colborn and Clement, 1992). These compounds are termed endocrine disruptor chemicals (EDCs) and have been discussed in the context of being a potential factor contributing to human epidemiological findings of decreases in sperm concentration, increases in hypothyroidism, and hormonally mediated cancers, such as prostate and testicular cancer (Colborn and Clement, 1992; Toppard et al., 1996; Swann et al., 1997; Pimentel, 1999). One of the most controversial aspects of the EEDC debate is whether exposure to low doses of chemicals can disrupt development. The question asked is whether exposure to these chemicals occurs at all, and if so, are these exposures to EDCs in utero. There are appropriate human studies with outcomes other than malformations to assess the actual risk to human fetuses with exposure to the low doses of DES in rats, it has been suggested that such studies are not being conducted. Th is might also raise awareness of the need for appropriate human studies to provide more definitive data to determine the actual risks.

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1. Introduction What does oestrogen stimulation in female offspring mean? What is the role of the uterus in female offspring and prostate in male mice? How can the effects of low-dose exposure to oestrogen be studied? How do chemicals in the environment contribute to human health problems? What are endocrine disruptor chemicals (EDCs), and why are they important? What are some potential effects of low doses of certain chemicals? What are the controversies surrounding the EEDC debate? What is the question about exposure to low doses of chemicals? What are some appropriate human studies that could assess actual risks to human fetuses? What is another potential way to raise awareness about these issues? How can human studies provide more definitive data? 

References


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