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Abnormalities in Functional Development of the Sertoli Cells in Rats Treated Neonatally with Diethylstilbestrol: A Possible Role for Estrogens in Sertoli Cell Development[1]

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ABSTRACT

Diethylstilbestrol (DES) was administered neonatally (Days 2–12; 10 μg on alternate days) to rats, and developmental changes in Sertoli cell function were evaluated at 18, 25, and 35 days of age and compared to those observed in rats administered a GnRH antagonist (GnRHa; Days 2 and 5; 10 mg/kg) or a vehicle (controls). DES and GnRHa treatments resulted in similar reductions in both Sertoli cell numbers (40% for DES, 48% for GnRHa) and suppression of testicular growth at 18 and 25 days, though by 35 days the suppression was more pronounced ($p < 0.001$) in DES-treated animals. Plasma FSH levels were suppressed markedly at 18 and 25 days, but not at 35 days, in GnRHa-treated rats, whereas in DES-treated rats the FSH levels were suppressed significantly only at 35 days. Both treatments suppressed plasma levels of inhibin B, though this was more pronounced ($p < 0.05$) in DES- than in GnRHa-treated rats. In controls, Sertoli cell immunoexpression of inhibin α, sulfated glycoprotein-1 (SGP-1), and androgen receptor (AR) increased in intensity and changed to an adult, stage-dependent pattern by 25 days. In GnRHa-treated rats these changes were reduced in intensity but were similar to those in controls at 35 days. In DES-treated rats, the increase in intensity and stage-dependent pattern of immunoexpression of inhibin α, SGP-1, and AR were virtually absent at 25 days but were present by 35 days. Germ cell volume per Sertoli cell was reduced in GnRHa- and DES-treated rats compared with controls at 18 and 25 days but was significantly greater ($p < 0.001$) in DES- than in GnRHa-treated rats at 35 days. The proportion of apoptotic to viable germ cells was increased ($p < 0.01$) in GnRHa- and DES-treated rats compared with controls at 18 and 25 days; but at 35 days, values in GnRHa-treated rats had declined to control values whereas those for DES-treated rats remained 10-fold elevated ($p < 0.001$).

In adulthood, testis weight and daily sperm production were reduced by 43% and 44%, respectively, in GnRHa-treated rats, but spermatogenesis was grossly normal. Comparable changes were observed in ~25% of DES-treated rats, but the majority exhibited > 60% reduction in testis weight with many Sertoli cell-only tubules and very low daily sperm production. Taken together, these data are interpreted as providing evidence for direct modulation of Sertoli cell (maturational) development by DES.

INTRODUCTION

Many reports in the literature demonstrate that exposure of the fetal/neonatal male rodent and/or human to exogenous estrogenic compounds (estradiol, ethinyl estradiol, or diethylstilbestrol) can cause a range of abnormalities of the reproductive system including atrophic/small testes, epididymal cysts, abnormalities of the rete testis, delayed puberty, etc. [1–4]. Because estrogen administration neonatally can suppress FSH (and probably LH) secretion at a time when this hormone is playing an important role in testicular development, it has generally been concluded that this indirect pathway of action is responsible for the adverse effects of neonatal estrogen exposure on the testis [1, 5]. However, direct effects of estrogens within the testis or male reproductive tract have not been excluded. This latter possibility has been strengthened by two recent discoveries. One is the finding that male transgenic (ERKO) mice in which the α form of the estrogen receptor (ERα) is inactivated are infertile [6], an effect that appears to result from impaired fluid resorption from the efferent ducts [7] and/or to occur because of other uncharacterized defects in sperm fertilizing ability [6]. The second discovery was the β form of the estrogen receptor, ERβ [8, 9], which is expressed in Sertoli cells, some germ cells, the epididymis, and accessory sex organs and to some extent in the efferent ducts [8–11]. In contrast, the expression of ERα is confined to Leydig cells (and possibly their precursors) and to the efferent ducts [12–14]. The widespread distribution of ERs in the male reproductive system is not just a feature of the adult male; available evidence suggests a similar distribution of ERs in the fetus/neonate and throughout development ([11, 13] and unpublished data). This would seem to indicate that estrogens could play multiple roles in the development and/or function of the male reproductive system.

These discoveries have come at a time of increasing concern about the potential impact of ''environmental estrogens'' on reproductive development and function of the male and about the possibility (unproved) that exposure of males to such chemicals could have contributed to reported adverse changes in human male reproductive health [4, 15]. One of the major obstacles to addressing this broad and complex issue is the lack of understanding of the physiological roles of estrogens in the male.

The objective of the present studies was to seek to establish whether estrogens can modulate Sertoli cell function in the developing testis. This was done by assessing the effects of neonatal diethylstilbestrol (DES) administration on the functional development of the rat testis in early puberty, with emphasis on maturational changes in Sertoli cell immunoexpression of three key proteins and the capacity of the Sertoli cell to support germ cell development. The changes observed were related to the subsequent normality

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or otherwise of the adult testis. Previous studies have shown that perinatal treatment of rodents with DES induces adverse changes similar to those that occur after administration of estradiol-17β or ethinyl estradiol [1–4]. In an attempt to discriminate between direct and pituitary-mediated effects of DES on the developing testis, results for DES-treated rats were compared with those for animals in which FSH (and LH) secretion were suppressed neonatally by administration of a GnRH antagonist (GnRHa). The latter treatment has been shown to retard puberty and reduce Sertoli cell numbers (and thus final testis size) in rats, though otherwise the rats exhibit grossly normal spermatogenesis in adulthood [16, 17].

MATERIALS AND METHODS

Animals and Treatments

Wistar rats bred in our own animal house were maintained under standard conditions. Beginning on postnatal Day 2 (day of birth = Day 1), rats were subjected to one of the following treatments: 1) s.c. injection of DES (Sigma Chemical Co., St. Louis, MO) at a dose of 10 μg in 20 μl corn oil on Days 2, 4, 6, 8, 10, and 12; 2) s.c. injection of 10 mg/kg of a long-acting GnRH antagonist (Antarelix; Europeptides, Argenteuil, France) in 20 μl 5% mannitol on Days 2 and 5; 3) injection with the appropriate vehicle (controls); because oil-injected and 5% mannitol-injected control rats did not differ significantly for any of the parameters measured in initial studies, data for these animals were pooled, and in all subsequent experiments all controls were injected with corn oil as above. Groups of 5–13 rats from the three treatment groups were subsequently killed on Days 18, 25, and 35, to study functional development of the testis in early puberty, and on Day 90–100 to assess the final outcome of these treatments on the gross morphology of the testis. Further groups of 5 animals were evaluated at 18 days of age for determination of Sertoli cell numbers as described below. For all animals, body and testis weights were recorded at death. In a preliminary experiment, groups of 5 control and 4 DES-treated rats were killed on Day 10 to enable measurement of blood FSH levels during the period of DES treatment.

Experimental Design

When the present studies were started, the initial premise was that neonatal DES exposure would reduce Sertoli cell numbers permanently via the suppression of FSH secretion. Treatments were therefore designed and tested in preliminary experiments to encompass the period when Sertoli cells are replicating postnatally (i.e., Days 1–15, although most of the replication occurs up to Days 10–12; [17–19]). Hence DES treatment spanned Days 2–12, with injections administered on alternate days in oil to prolong the absorption/exposure period. Similarly, preliminary experiments had established that the dose and treatment period for GnRHa injection would result in pronounced suppression of FSH levels throughout the first 2–3 wk postnatally. Although the two treatments were not matched in terms of the degree of suppression of FSH levels achieved, it was considered that the reduction in FSH levels achieved by GnRHa treatment represented near-maximal suppression (see below) and that this was therefore a useful group against which to compare the effects of DES treatment.

Collection of Samples, Processing of Tissues, and Measurement of Daily Sperm Production

On the day of sampling, animals were anesthetized with halothane, and blood was collected from the heart into a heparinized syringe; animals were then killed by cervical dislocation. Plasma samples were stored at −20°C until used for hormone analysis. In animals sampled at 18 and 25 days of age, the testes were removed and immersion fixed in Bouin's fluid for 5.5 h at room temperature before being transferred into 70% ethanol. Animals sampled at 35 days of age or in adulthood were perfusion fixed for assessment of testicular morphology, or (in adults only) their testes were removed and weighed for measurement of daily sperm production after fixation in collidine as described previously [20]. Control and treated adult rats and groups at 35 days of age were perfusion fixed with Bouin's as described previously [13, 21]. Before processing, Bouin's-fixed testicular tissue from all age groups was cut into 2–6 pieces with a razor blade. Fixed tissue was then processed for 17.5 h in an automated Shandon (Sewickley, PA) processor and embedded in paraffin wax. Sections of 5-μm thickness were cut and floated onto coated slides (2% 3-aminopropyltriethoxy-saline; Sigma) and dried at 50°C overnight before being used for immunocytochemistry as described below. As DES but not GnRHa treatment resulted in morphological abnormalities to the testis in adulthood, representative control and DES-treated adult rats were perfusion fixed with cacodylate-buffered 3% glutaraldehyde, and tissue blocks were processed into plastic as detailed elsewhere [22] to allow more accurate evaluation of the induced changes.

Measurement of Sertoli Cell Numbers

Testis tissue from the three groups of animals at 18 days of age were immersion fixed in Bouin's for 24 h. Testes were then sampled in a random systematic manner: they were sliced transversely into 4 pieces using a razor blade, and then either slices 1+3 or slices 2+4 were processed through graded ethanols before infiltration with JB4 resin (TAAB, Berkshire, UK). After polymerization, 20-μm sections were cut on a Reichart 2050 microtome (Reichart Jung, Nossloch, Germany) using a Diatome Histoknife (Reichart Jung), mounted onto glass slides, and stained with Harris hematoxylin. Sertoli cells were then counted using the optical dissector method as described by Wreford [23].

Antibodies Used for Immunohistochemistry

Immunolocalization of inhibin α utilized a monoclonal antibody (173/9K), described previously [24, 25], at a concentration of 2 μg/ml. Immunolocalization of sulfated glycoprotein-1 (SGP-1) utilized a rabbit polyclonal antibody, kindly provided by Dr. Steven Sylvester (University of Washington, Vancouver), at a dilution of 1:1000. The androgen receptor (AR) was immunolocalized using a rabbit polyclonal antibody (Novocastra, Newcastle-upon-Tyne, England) raised against the first 17 amino acids of the N-terminus of AR, as described previously [26]; it was used at a dilution of 1:20.

Immunohistochemistry

Sections were dewaxed in Histoclear (National Diagnostics, Fleet Business Park, Hull, England), rehydrated in graded ethanols, and washed in water and Tris-buffered saline (TBS; 0.05 M Tris-HCl, pH 7.4. 0.85% NaCl). For

immunolocalization of inhibin α and SGP-1, endogenous peroxidase activity was blocked at this stage by incubating sections in 3% hydrogen peroxide in methanol for 30 min. For inhibin α and AR, an antigen retrieval step [25, 26] was performed at this stage. Thus, sections were microwaved in 0.01 M citrate buffer (pH 6.0) at full power (750 watts) for 20 min and then left to stand, undisturbed, for 20 min, before being washed twice (5 min each) in TBS. To block nonspecific binding sites, all sections were incubated for 20 min with the appropriate normal serum, either normal rabbit serum or normal swine serum (Scottish Antibody Production Unit, Carluke, Scotland). Primary antibodies were added to the sections at the appropriate dilution either in normal rabbit serum:TBS (1:5, v:v) or in normal swine serum:TBS (1:5, v:v) and, with the exception of AR immunohistochemistry, incubated under plastic coverslips overnight at 4°C in a light-proof box. For AR, incubation was for 2 h at room temperature without coverslips. When used, the latter were removed and sections washed twice (5 min each) in TBS before incubation for 30 min with a linking antibody, namely a 1:500 dilution in TBS of either biotinylated rabbit anti-mouse immunoglobulins (Dako, High Wycombe, UK) or biotinylated swine anti-rabbit immunoglobulins (Dako). After two 5-min washes in TBS, sections were incubated for 30 min with avidin-biotin conjugated to either alkaline phosphatase (AR) or horseradish peroxidase (inhibin α, SGP-1) (both from Dako). After two further 5-min washes in TBS, when horseradish peroxidase was used, a diaminobenzidine-based chromogen was added to slides, leaving a brown-colored precipitate at the sites of antibody localization. Alternatively, when alkaline phosphatase was used, after two 5-min washes in TBS the slides were given a final wash in 100 mM Tris-MgCl buffer (100 mM NaCl and 50 mM MgCl; pH 9.5) before the addition of nitro blue tetrazolium (337.5 μg/ml), 5-bromo-4 chloro-3-indolylphosphate (175 μg/ml), and 0.001% levamisole in 10 ml Tris-MgCl buffer to develop color (blue) at the sites of antibody localization. The slides were developed until the color reached the required intensity in controls, when the reaction was stopped by immersion of the slides in distilled water. Sections were then counterstained with hematoxylin before being dehydrated either in graded ethanols (diaminobenzidine procedure) or in absolute ethanol (nitro blue tetrazolium procedure) and cleared in xylene. Finally, slides were coverslipped using Pertex mounting medium (CellPath plc, Hemel Hempstead, UK). As negative controls, slides were processed as above except that the appropriate normal serum was substituted for the primary antibody.

Comparative Evaluation of Immunostaining

Sections from control animals and the two treatment groups were processed in parallel for comparison of immunostaining on at least two separate occasions to ensure reproducibility of results. On each occasion, sections from 5–8 animals in each group at the three time points were evaluated for immunostaining. After detailed evaluation of immunostaining, confirmation of findings was obtained by mounting representative sections from control, DES-, and GnRHa-treated animals on the same slide and then repeating the immunostaining. These slides were then examined and photographed using an Olympus Provis microscope (Olympus Optical, Honduras Street, London, UK) fitted with a Kodak DCS420 camera (Eastman Kodak, Rochester, NY). Captured images were stored on an 8100 PowerPC computer (Apple MacIntosh, Cupertino, CA) and compiled using Photoshop 3.0 (Adobe, Mountain View, CA) before printing on a Kodak XLS 8600 PS printer.

Quantification of Apoptotic and Nonapoptotic (Viable) Germ Cells as a Measure of Sertoli Cell Functional Development

As an overall indicator of functional maturation of Sertoli cells, their ability to support germ cell development during the pubertal expansion of spermatogenesis was assessed by measuring 1) the total volume of germ cells supported by each Sertoli cell and 2) the volume ratio of apoptotic to nonapoptotic (viable) germ cells per testis. The latter measure was included as it was considered that impairment of Sertoli cell functional development might manifest itself in a relative inability to sustain germ cells through their development. DNA fragmentation was detected by in situ DNA 3′ end-labeling in histological sections using a nonradioactive labeling method that resulted in a high degree of specificity and low background staining. The procedure for labeling of apoptotic cells was optimized and validated using testicular sections from adult rats that had been treated with either 1) 650 mg/kg methoxyaceticacid 24 h earlier to cause widespread degeneration of pachytene primary spermatocytes [27], or 2) 75 mg/kg ethane dimethane sulfonate 6 days earlier to induce Leydig cell loss and, consequently, selective degeneration of a small proportion of germ cells at stages VII–VIII of the spermatogenic cycle [22, 28]. For both treatments we have previously quantified the numbers of degenerating germ cells using established morphological criteria [22, 27, 28], and in the present studies it was confirmed that the cell-labeling procedure resulted in a number of apoptotic cells per seminiferous-tubule cross section at specific stages of the spermatogenic cycle that was similar to the number obtained using morphological criteria.

Testis sections (5 μm) were mounted on coated slides, deparaffinized, and rehydrated. Endogenous peroxidase activity was blocked by immersion in 3% (v:v) H_2O_2 in methanol for 30 min. After two washes (5 min each) in PBS (0.01 M, pH 7.4), slides were placed on an ice-cold plate, and 5 nM digoxigenin-11-dUTP and 50 U/ml terminal deoxynucleotidyl transferase (TdT) (both from Boehringer Mannheim GmbH, Mannheim, Germany) were added in enzyme buffer comprising 30 mM Tris-HCl, pH 7.2, 140 mM sodium cacodylate (Agar Scientific Ltd., Stansted, UK), and 1.5 mM $CoCl_2$. For negative control slides, only the enzyme buffer lacking TdT was added. The slides were immediately coverslipped using GelBond (FMC BioProducts, Rockland, ME), sealed with a rubber solution (Cowgum; Cow Proofing Ltd., Slough, UK) in hexane, and incubated at 37°C for 30 min. The coverslips were then removed and the slides washed twice (5 min each) in PBS, followed by blocking for 10 min at room temperature with normal rabbit serum (NRS) in PBS (1 part NRS:4 parts PBS). Sections were then incubated for 90 min at room temperature in a humidified chamber with sheep anti-digoxigenin antibody (Boehringer Mannheim) diluted 1:100 in the blocking solution. After a further two washes in PBS, the slides were incubated for 30 min with biotin-conjugated rabbit anti-sheep immunoglobulins (Vector Laboratories, Peterborough, UK) diluted 1:500 in TBS containing NRS (1 part NRS:4 parts TBS) at room temperature in a humid chamber. Two washes in TBS (5 min each) were followed by incubation for 30 min at room temperature with horse-

radish peroxidase-avidin biotin complex (Dako) diluted in 0.05 M Tris-HCl, pH 7.4, according to the manufacturer's instructions. Sections were again washed twice in TBS and then visualized with 0.05% (w:v) 3,3′-diaminobenzidine (Sigma) in 0.05 M Tris-HCl, pH 7.4, and 0.01% H_2O_2. Finally, sections were washed in distilled water, lightly counterstained with hematoxylin, dehydrated in graded ethanols, cleared in xylene, and coverslipped using Pertex mounting medium (CellPath plc).

Apoptotic cells in cross sections of testes from 3–7 rats in each group at 18, 25, and 35 days of age were evaluated and examined under oil immersion using a Leitz 63x plan apo objective (Leitz Wetzlar GBH, Wetzlar, Germany) fitted to a Leitz laborlux microscope fitted with a 121-point eyepiece graticule. Using a systematic clock-face sampling pattern from a random starting point, 16 fields were counted that avoided immersion-fixation artifacts. Points falling over Sertoli cell or germ cell nuclei (apoptotic or nonapoptotic) were scored and expressed as a percentage of the 121 points possible; nonapoptotic spermatogonia, spermatocytes, and round spermatids were distinguished (when present), but their total volumes were combined for analysis in the current studies. The values for percentage volume were converted to absolute volumes per testis by reference to testis volume (i.e., weight). For each animal, the total germ cell volume per testis (apoptotic+nonapoptotic) was expressed relative to total Sertoli cell volume per testis; correction for Sertoli cell volume was applied as Sertoli cell numbers were reduced in both GnRHa- and DES-treated rats as compared to controls (see *Results*). To provide some indication of the qualitative support of germ cell development by Sertoli cells, the volume ratio of apoptotic to total germ cells was also calculated for each animal.

Hormone Measurements

Plasma levels of FSH were measured by RIA using materials generously supplied by NIDDK (Rockville, MD). Results have been expressed in terms of the rat FSH RP-2 standard. In this assay, plasma FSH levels measured in hypophysectomized rats are in the range 1.2–2.3 ng/ml, and values in this range are therefore considered to be baseline. Plasma levels of inhibin B were measured using a two-site enzyme-linked immunoassay that utilizes a capture antibody directed against the C-terminal portion of the human βB subunit and the F(ab) fraction of a mouse monoclonal antibody (R1) to the N-terminal portion of the inhibin α subunit conjugated to alkaline phosphatase [29]. The assay has been previously validated for measurement of inhibin B in the rat [29], and in the present studies it was confirmed that rat plasma diluted in parallel with the inhibin B standard.

Statistics

Comparison of body weights, testis weights, and hormone and Sertoli and germ cell number data for the three groups at each age point was made using ANOVA, and where significant differences between groups were indicated, subgroup comparisons utilized the same test with the variance from the experiment as a whole as the measure of error. Data that were not normally distributed were logarithmically transformed prior to analysis as above (inhibin B).

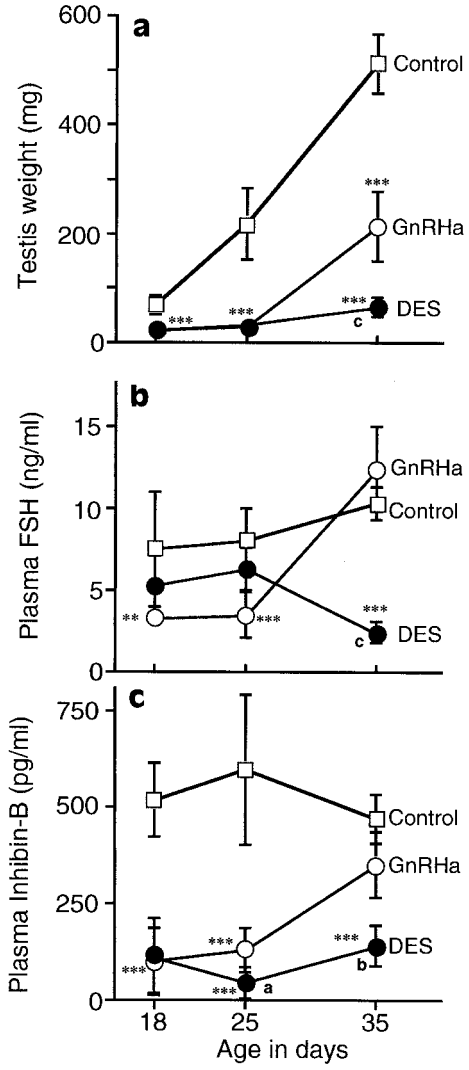

FIG. 1. Effect of neonatal treatment with vehicle (controls), DES, or GnRHa on testis weight (**a**) and the plasma levels of FSH (**b**) and inhibin B (**c**) at 18, 25, and 35 days of age. Each point is the mean ± SD for 4–13 rats. $^{**}p < 0.01$, $^{***}p < 0.001$ in comparison with respective control value. $^{a}p < 0.05$, $^{b}p < 0.01$, $^{c}p < 0.001$, in comparison with respective GnRHa value.

RESULTS

Effect of Treatments on Testis Weight and Gross Morphology of the Testis at 18–35 Days of Age

In DES- and GnRHa-treated rats, testis weight was reduced comparably at Days 18 and 25, to ~33% and 14%, respectively, of control levels (Fig. 1a). At 35 days of age, testis size in GnRHa-treated animals was still < 50% of control values, but pubertal testis growth had begun. In contrast, testes in DES-treated rats increased only marginally in size from 25 to 35 days and were significantly ($p < 0.001$) smaller than in GnRHa-treated animals at 35 days (Fig. 1a). Testicular descent was delayed in DES-treated rats at 25 days, compared with controls, as testes were still inguinally located, though by 35 days, testes were scrotally positioned (data not shown). A proportion of GnRHa-treated rats also exhibited inguinally located testes at 25 days but not at 35 days (data not shown). Morphologically, the testes of DES- and GnRHa-treated rats were similar at 18 and 25 days, with both exhibiting delay in development of

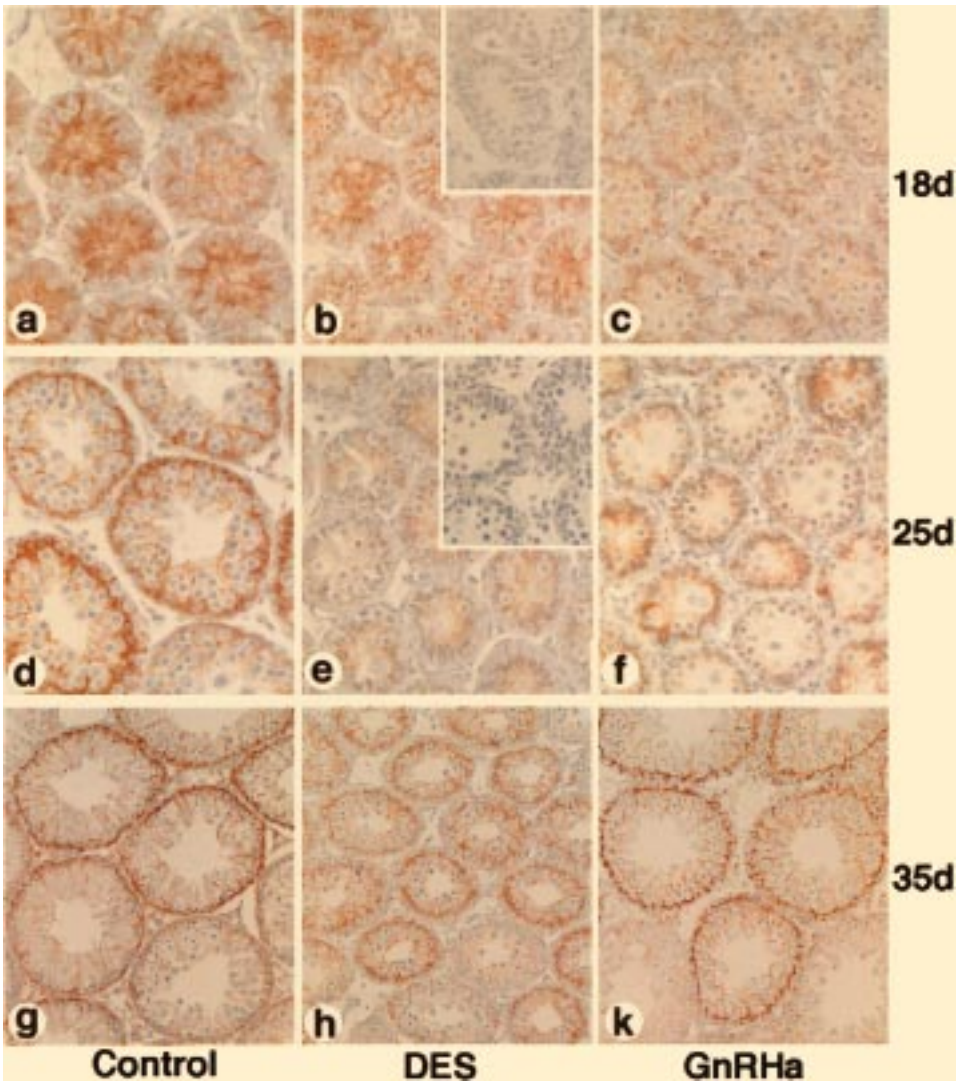

FIG. 2. Immunoexpression of inhibin α in the testis at 18, 25, and 35 days of age in rats treated neonatally with vehicle (controls), DES, or GnRHa. Insets in **b** and **e** show negative controls. Note that in control testes there is an increase in immunoexpression of inhibin α from 18 (**a**) to 25 days (**d**) as well as a change to a stage-dependent pattern of expression. This change is less marked in GnRHa-treated animals at 25 days of age and is completely absent in DES-treated rats, though in both groups immunoexpression is similar to that in controls at 35 days. ×400 (top 2 rows) or ×200 (bottom row) (reproduced at 75%).

normal spermatogenesis and in the formation of seminiferous tubule lumens, though the latter defect was more pronounced at 25 days in the DES-treated than in the GnRHa-treated animals (see Figs. 2–4). At 35 days, the gross morphology of the testes in GnRHa-treated rats was essentially normal whereas DES-treated rats exhibited variable degrees of delay in expansion of spermatogenesis and occasional germ cell-depleted tubules (see Figs. 2–4).

Effect of Treatments on Blood Hormone Levels

In GnRHa-treated animals, the pattern of change in plasma levels of FSH (Fig. 1b) and inhibin B (Fig. 1c) paralleled the change in testis weight (Fig. 1a); i.e., levels were reduced markedly compared with control levels at 18 and 25 days of age and then increased substantially toward control values at 35 days. At 18 and 25 days of age, plasma FSH levels in GnRHa-treated rats were reduced by 56–65% and were at levels just above baseline (i.e., the range found in hypophysectomized rat plasma). DES-treated animals showed a more complex pattern of change. FSH levels were only reduced marginally, when compared with control values, at 18 and 25 days of age but then decreased—rather than increasing as was the case in GnRHa-treated rats—at 35 days to levels significantly ($p < 0.001$) lower than in either control or GnRHa-treated animals (Fig. 1b). In a preliminary study it had been established that at 10 days of age when rats were still on DES treatment, plasma FSH was reduced by 70% ($p < 0.001$) to levels close to baseline (DES: 2.1 ± 0.4 ng/ml; control: 7.1 ± 1.7 ng/ml; means ± SD, N = 4–5). Plasma levels of inhibin B in DES-treated rats were reduced below control levels at all times, and the magnitude of this suppression was significantly greater ($p < 0.05$) than in the GnRHa-treated group at 25 and 35 days of age (Fig. 1c). As in GnRHa-treated animals, the pattern of change in plasma levels of inhibin B from 18 to 35 days of age in DES-treated rats more or less paralleled the change in testis size (Fig. 1, a and c).

Effect of Treatment on Sertoli Cell Number

Treatment with DES or GnRHa resulted in reductions of 40% ($28.1 \pm 5.7 \times 10^6$) and 48% ($24.6 \pm 4.4 \times 10^6$), respectively, in Sertoli cell numbers per testis when compared with values in control rats ($46.7 \pm 6.9 \times 10^6$) at 18 days of age (means ± SD, N = 5; both treatments $p < 0.01$ in comparison with control). These decreases closely matched the percentage change in Sertoli cell volume measured at the various ages by point-counting (data not shown).

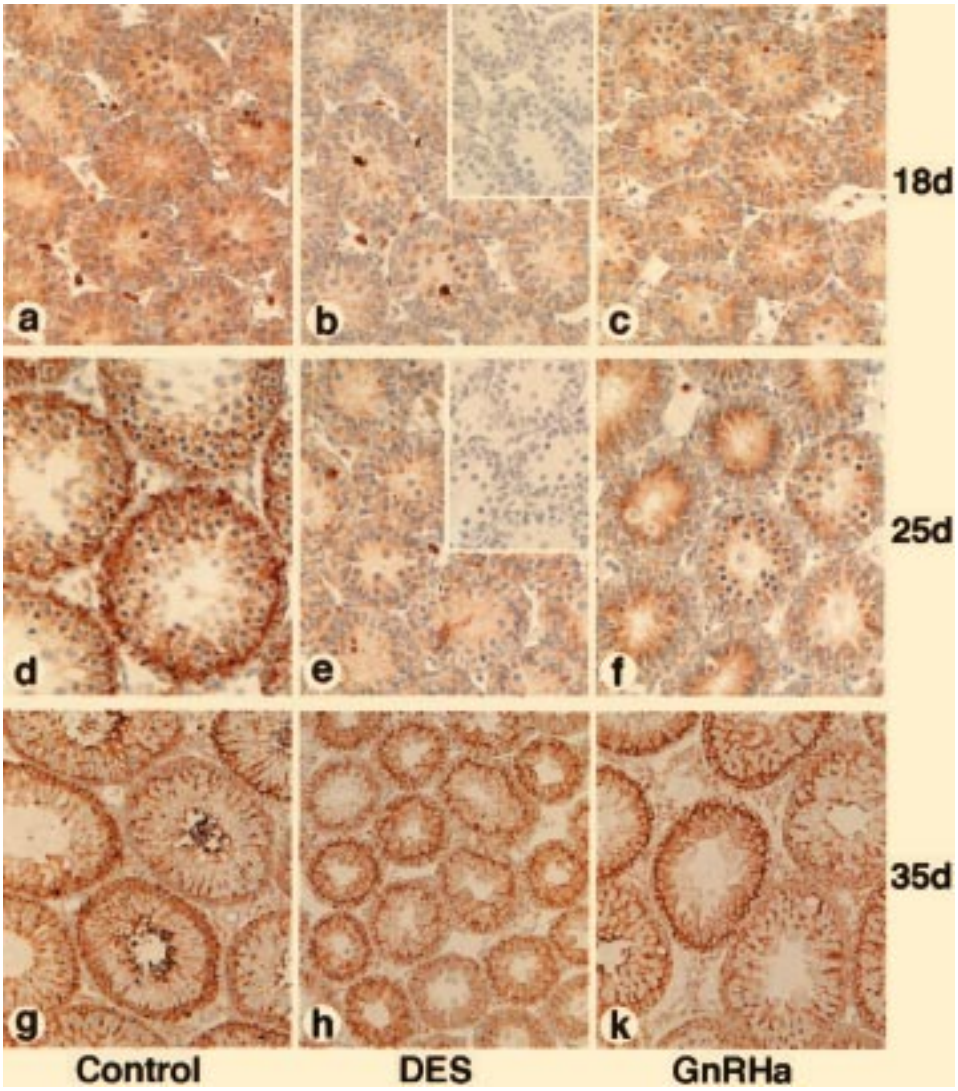

FIG. 3. Immunoexpression of SGP-1 in the testis at 18, 25, and 35 days of age in rats treated neonatally with vehicle (controls), DES, or GnRHa. Insets in **b** and **e** show negative controls. Note that in control testes there is a marked increase in immunoexpression of SGP-1 from 18 (**a**) to 25 days (**d**). This change is less marked in GnRHa-treated animals (**c, f**) and is completely absent in DES-treated rats (**b, e**), though in both groups immunoexpression is similar to that in controls at 35 days. ×400 (top 2 rows) or ×200 (bottom row) (reproduced at 75%).

Effect of Treatment on Maturational Development of the Sertoli Cells

On the basis of previously published [25, 26] and unpublished data of our own, we were aware that Sertoli cell immunoexpression of three proteins (inhibin α, SGP-1, and AR) showed important maturational changes between 18 and 25 days of age. These changes were confirmed in control rats in the present studies and are illustrated in Figures 2–4. For all three proteins, the intensity of immunoexpression increased markedly from 18 to 25 days of age and became expressed in a stage-dependent pattern typical of the mature adult Sertoli cell; from 25 to 35 days of age there was little change in immunoexpression of these proteins. GnRHa-treated animals also exhibited this pattern of development in immunoexpression of the three proteins, although the changes from 18 to 25 days were muted compared with those in controls. By 35 days, the pattern and intensity of immunoexpression of SGP-1 and inhibin α in GnRHa-treated rats was similar to that for controls (Figs. 2 and 3), though immunoexpression of AR was of lower intensity in some animals at this age (Fig. 4). DES-treated rats exhibited more pronounced delay in maturational development of an adult pattern of immunoexpression of the three proteins compared with GnRHa-treated rats. Thus, between 18 and 25 days of age there was virtually no change in immunoexpression pattern or intensity as occurred in controls, although by 35 days of age a reasonably normal pattern of immunoexpression of the three proteins was evident in DES-treated rats (Figs. 2–4). In contrast to the treatment-induced differences in immunoexpression of AR in Sertoli cells, expression of AR in peritubular myoid cells was evident in all treatment groups at all time points and showed only marginally lower intensity in DES-treated compared with the other two groups (Figs. 2–4).

Effect of Treatment on Maturational Changes in Sertoli Cell Support of Germ Cell Development

As the total number of Sertoli cells per testis was reduced in DES- and GnRHa-treated rats when compared with controls, and as Sertoli cell volume data showed a similar decrease at each age, data for germ cell volume were expressed relative to Sertoli cell volume. At 18 days of age, treatment with GnRHa or with DES had only minor effects on germ cell volume/Sertoli cell, although the ratio of apoptotic to viable germ cells was increased significantly (Fig. 5). The increased incidence of apoptotic germ cells persisted at 25 days of age in treated rats and was associated with a major reduction ($p < 0.001$) in the volume of germ cells per Sertoli cell in comparison with control values. However, by 35 days in GnRHa-treated rats the inci-

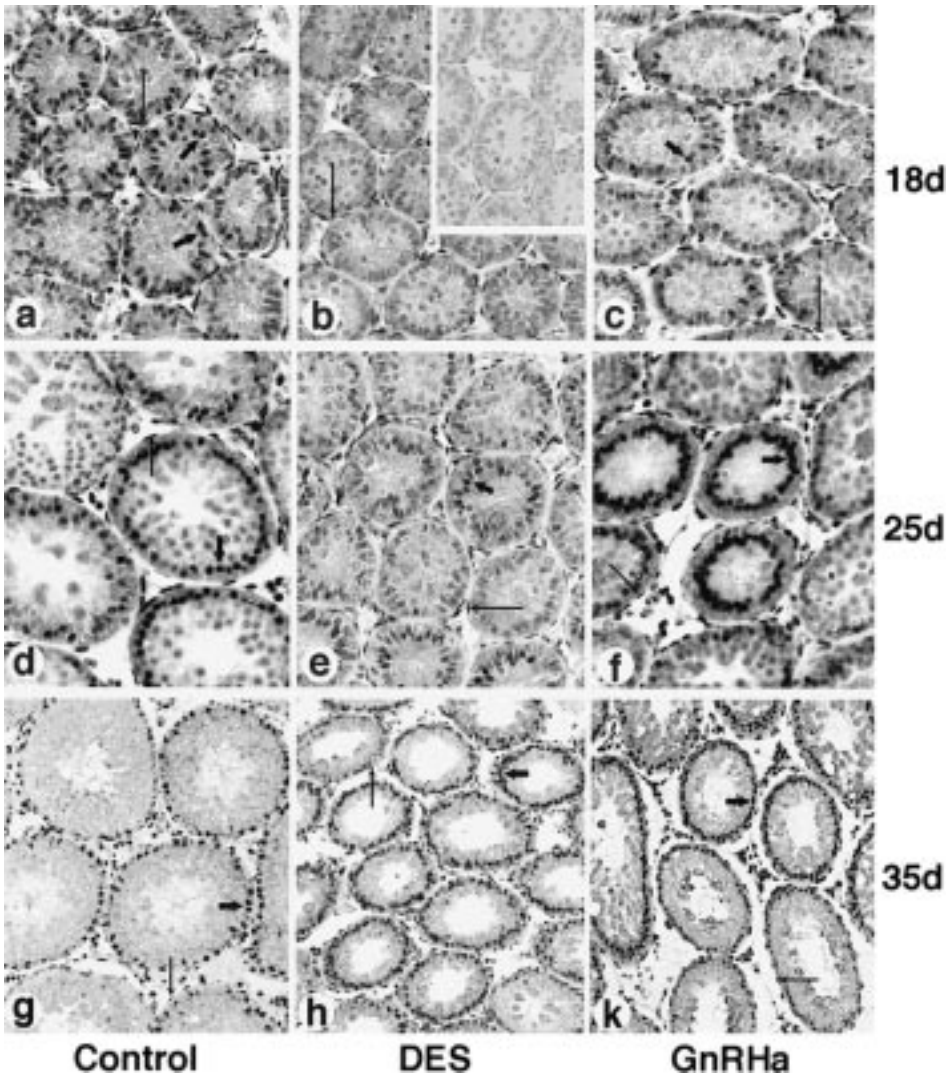

FIG. 4. Immunoexpression of AR in the testis at 18, 25, and 35 days of age in rats treated neonatally with vehicle (controls), DES, or GnRHa. Inset in **b** shows a negative control. Note the presence of variable immunostaining for AR in Sertoli cells (short arrows) in the control at 18 days (**a**), which increases in intensity by 25 days and becomes stage-dependent (**d**). This change is evident at lower intensity in GnRHa-treated animals (**c, f**) but is completely absent in DES-treated rats in which Sertoli cell immunoexpression of AR is generally absent (**b**) or sporadic (**e**); by 35 days, immunoexpression of AR in both treatment groups is more similar to that in controls. Note that immunoexpression of AR in peritubular cells (thin arrows) is evident to some extent in all groups at all time points. ×400 (top 2 rows) or ×200 (bottom row) (reproduced at 75%).

dence of apoptotic germ cells had decreased to near control levels, and, correspondingly, the germ cell volume per Sertoli cell had increased to ~70% of normal control levels. In contrast, in DES-treated rats at 35 days, the germ cell volume/Sertoli cell remained grossly subnormal and the proportion of apoptotic to viable germ cells remained elevated at levels comparable to those observed at 25 days of age (Fig. 5).

Effect of Treatment on Body Weight, Testis Weight and Morphology, and Daily Sperm Production in Adulthood

Animals treated neonatally with DES or GnRHa were significantly smaller at 90–100 days of age than were the corresponding controls, a difference that was most marked for the DES-treated males (Table 1). In GnRHa-treated rats, mean testis weight was reduced by 43% (Table 1), but gross testicular morphology was essentially normal (Fig. 6, e and f) and daily sperm production was reduced (by 44%) in parallel with the decrease in testis weight and with the decrease (48%) in Sertoli cell numbers reported above. In DES-treated rats, mean testis weight was reduced by 58% and daily sperm production by 62% (Table 1); these reductions were disproportionately greater than the reduction (40%) in Sertoli cell numbers reported above. However, there was considerable variation between animals. In ~25% of DES-treated animals, testis weight and daily sperm production were reduced to levels comparable to those observed in GnRHa-treated rats, and such testes also exhibited grossly normal testicular morphology (Fig. 6b); it was presumed that this change was most logically explained just by a decrease in Sertoli cell numbers. However, ~60% of the DES-treated rats had considerably smaller testes (460–736 mg) and much lower daily sperm production (0.5–5.4 $\times$ 10^6 per testis); and morphologically, many of the seminiferous tubules in such testes had reduced germ cell numbers and/or exhibited a Sertoli cell-only phenotype (Fig. 6c). The remaining DES-treated rats had necrotic testes (Fig. 6d), which had fused with the peritoneal membranes in the inguinal canal and which were not weighed or assessed for daily sperm production.

DISCUSSION

The main aim of the present study was to compare the functional development of Sertoli cells in animals treated neonatally either with GnRHa or with an estrogenic chemical (DES) in order to establish whether the adverse effects of the latter on the onset and expansion of spermatogenesis could be accounted for simply by the suppression of FSH (and possibly LH) secretion that occurs during neonatal estrogen treatment [1, 5, 30, 31]. It was considered that dif-

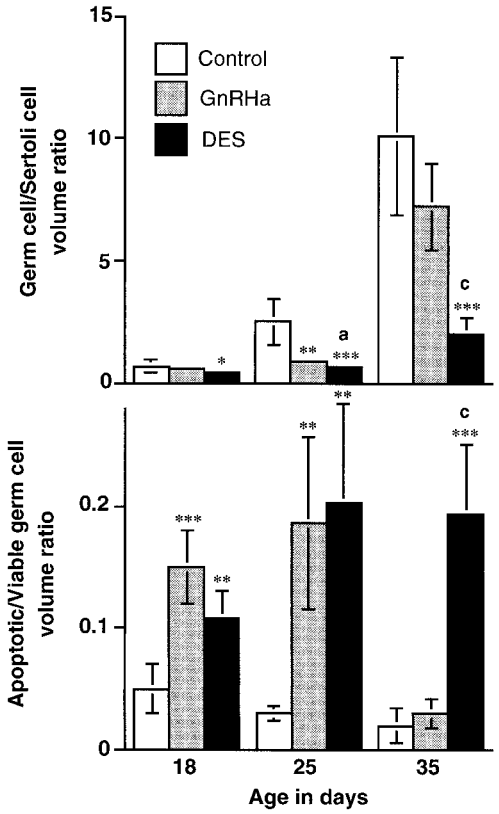

FIG. 5. Effect of neonatal treatment with vehicle (controls), DES, or GnRHa on germ cell volume per Sertoli cell (top) and the volume ratio of apoptotic to nonapoptotic germ cells (bottom) at 18, 25, and 35 days of age. Each point is the mean ± SD for 3–7 rats. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ in comparison with respective control value. $^{a}p < 0.05$, $^{c}p < 0.001$, in comparison with respective GnRHa value.

ferences observed between these two treatments would provide a means of establishing whether the developing Sertoli cell is an important target for estrogen action. Both treatments caused grossly similar reductions in Sertoli cell numbers and delay in the normal expansion of spermatogenesis at 18 and 25 days of age, but at 35 days, testes from DES-treated rats remained suppressed in their development whereas GnRHa-treated animals exhibited pronounced expansion of spermatogenesis. These differences persisted through to adulthood, when the majority of DES-treated rats exhibited disorders of spermatogenesis whereas GnRHa-treated rats exhibited grossly normal spermatogenesis. Comparison of functional development of the Sertoli cells at 18, 25, and 35 days of age, based on immunoexpression of inhibin α, SGP-1, and AR and on the viability and volume of germ cells supported per Sertoli cell, revealed clear and consistent differences between DES- and GnRHa-treated rats. Whereas the GnRHa-treated rats showed changes from control that were consistent simply with delay in the functional maturation of Sertoli cells (consequent to FSH suppression), DES-treated rats exhibited changes that were more consistent with (permanent) impairment of functional maturation. These findings indicate that endogenous estrogens could play a physiological role in the maturational development of Sertoli cells.

Numerous reports in the literature show that neonatal administration of estrogens (estradiol-17β, ethinyl estradiol, DES) to rodents invariably results in delayed development and/or permanent impairment of spermatogenesis [1–5]. The mechanisms underlying these effects have not been

TABLE 1. Effects of neonatal treatment with vehicle (control), DES, or GnRHa on body and testis weights, plasma FSH levels, and DSP in adulthood (means ± SD; range in parentheses).

Treatment group	Body weight (g)	Testis weight (mg)	DSP (10^6/testis)
Control	496 ± 40 (410–573) n = 18	1783 ± 229 (1445–2178) n = 18	16.53 ± 1.52 (14.3–18.0) n = 8
DES	412 ± 37*** (358–477) n = 11	751 ± 240***[a] (460–1208)[‡] n = 8[‡]	6.35 ± 3.80***[‡] (0.53–9.4)[‡] n = 5[‡]
GnRHa	444 ± 47** (367–508) n = 8	1012 ± 99*** (888–1142) n = 8	9.75 ± 0.82*** (8.3–10.5) n = 6

** $p < 0.01$, *** $p < 0.001$, in comparison with respective value for control group.
[a] $p < 0.05$, in comparison with respective value for GnRHa-treated group.
[‡] Excludes animals with necrotic testes.

clearly identified. Because of the well-described ability of neonatal estrogen treatment to suppress FSH secretion [1, 5, 30] at a time when this hormone is playing an important role in both Sertoli cell development and the expansion of spermatogenesis, it has generally been concluded that this suppression explains the consequences of neonatal estrogen treatment [1, 5]. In this respect, it is clear that suppression of FSH will result in fewer Sertoli cells due to reduction in their mitotic frequency in neonatal life [16, 17], and this explains why Sertoli cell number was reduced by 48% in GnRHa-treated rats in the present study. A similar explanation could account for the 40% reduction in Sertoli cell numbers in DES-treated rats in view of the observed reduction in FSH levels at 10 days of age during treatment. However, as Sertoli cells also express ERβ [11], the possibility of a direct effect of the DES on Sertoli cell multiplication cannot be excluded. In the present studies, FSH levels were grossly suppressed in GnRHa-treated animals at 18 and 25, but not at 35, days of age, whereas after cessation of treatment, DES-treated rats exhibited significant suppression of FSH levels only at 35 days of age, which could reflect reprogramming effects of DES on the hypothalamus [1]. Although the different pattern of suppression of FSH levels in the two treatment groups complicates interpretation of the findings, it is clear from comparison of DES- and GnRHa-treated rats at 25 days of age that the former were maturationally retarded to a greater degree than were the latter, despite the fact that GnRHa-treated animals still exhibited gross suppression of FSH levels at this age whereas DES-treated rats did not. Indeed, the fact that plasma levels of inhibin B (which derives from the Sertoli cells) were consistently lower in DES-treated than in GnRHa-treated rats, irrespective of the FSH levels, again argues for a fundamental difference in the function of Sertoli cells in the two groups. This conclusion is also supported by the finding that FSH levels in adult rats in the two treatment groups are comparable (unpublished data) whereas spermatogenesis is impaired only in DES-treated rats. Considering all of the findings mentioned, it is difficult to account for the additional changes observed in DES-treated rats by suppression of FSH levels. Other possible effects of DES treatment, including direct effects of the DES on Sertoli cells, are therefore presumably involved.

There is a surprising lack of published studies assessing the effects of estrogens in vitro on Sertoli cells from neonatal/prepubertal rats, though there is good evidence that immature Sertoli cells can bind radiolabeled estrogens in

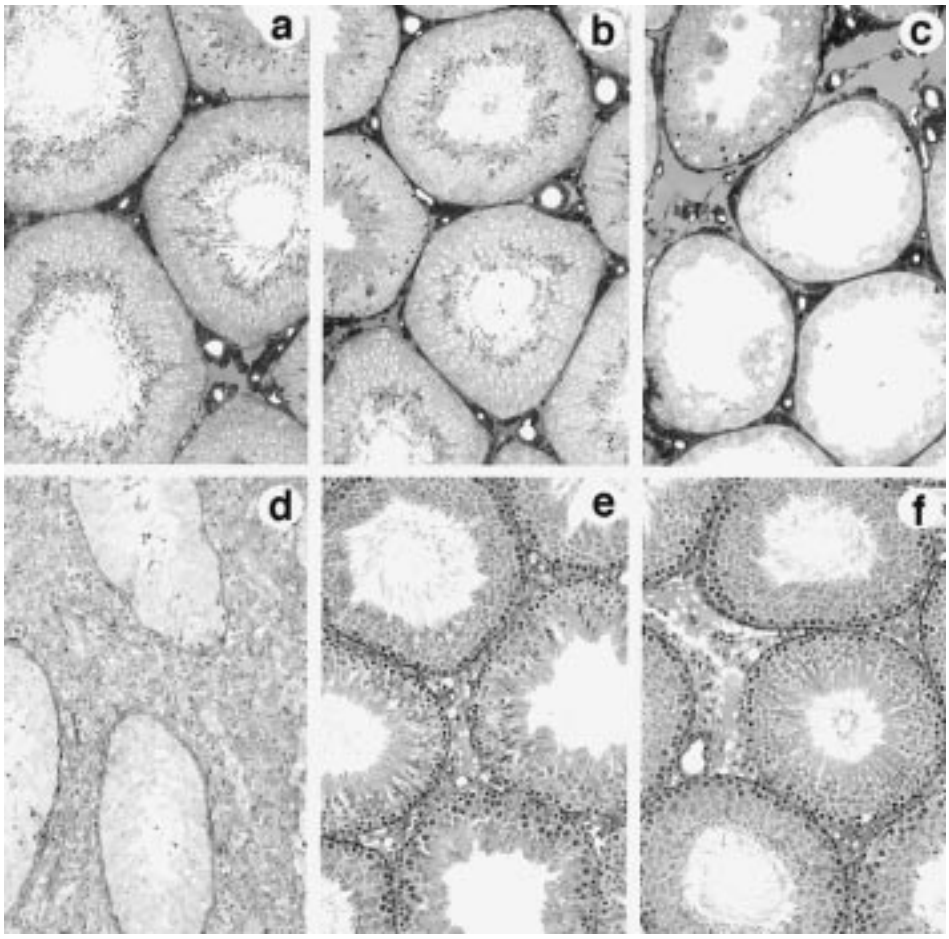

FIG. 6. Representative testicular morphology in adult rats that had been treated neonatally with vehicle (**a, e**), with DES (**b–d**), or with GnRHa (**f**). Note that the latter group and ~25% of DES-treated rats had similar-sized testes, which were about 40% smaller than controls but which exhibited grossly normal testicular morphology (compare **a**, **b**, and **e**). However, the majority of DES-treated rats had considerably smaller testes showing various degrees of impairment of spermatogenesis and including some Sertoli cell-only tubules (**c**). A small proportion of DES-treated rats had necrotic testes (**d**) that were fused to peritoneal membranes within the inguinal canal. ×200 (reproduced at 79%).

vitro [32] and in vivo [33]. Although ERα expression is lacking in Sertoli cells in the rat throughout life [13], the discovery of ERβ and its immunolocalization to Sertoli cells in both the adult [10] and the fetal/neonatal rat [11] now offers a logical mechanistic pathway for estrogen action on the Sertoli cells. In this respect, the fact that immature, undifferentiated Sertoli cells are themselves a source of estrogen in the neonatal period [34] may mean that this estrogen physiologically exerts autocrine effects on the Sertoli cells to prevent their differentiation. Additionally, either signals involving the thyroid axis [32, 35, 36], or sources such as developing germ cells [37] or androgens from the developing Leydig cells [34], could individually or collectively signal to shut down aromatase activity in the Sertoli cells as a prelude to their differentiation. If this reasoning is correct, it would provide an explanation for the present finding that exogenous administration of DES during neonatal life was able to ''short-circuit'' this cascade by prolonging exposure of the Sertoli cells to estrogen and thus impairing or delaying their functional maturation. In this context, it would seem that any contribution to such a delay from the absence of germ cells [37] in DES-treated rats must be small, as similar delays in germ cell development were also evident in GnRHa-treated rats up to 25 days of age, yet these animals did not exhibit such pronounced delay in maturational development of their Sertoli cells. Though such contrasts are consistent with direct effects of DES on Sertoli cells, they do not exclude an influence of FSH, as this hormone is clearly involved in positively regulating aromatase activity in immature Sertoli cells [34, 36]. Suppression of FSH levels and coincident elevated estrogen exposure due to DES treatment could be important interacting factors in suppressing normal maturational development of the Sertoli cells.

In seeking to explain the differences observed between the effects of treatment with GnRHa and DES, other possibilities also exist. These include direct effects of the DES on Leydig cells, on germ cells, on testicular descent, or on the functional development of the excurrent duct system of the testis. Leydig cells from both the fetal and adult generation express ERs [11, 13], and there is published evidence that estrogens can suppress Leydig cell development and/or steroidogenesis [17, 38, 39]. However, gross comparison of Leydig cell numbers and expression of 3β-hydroxysteroid dehydrogenase in DES- and GnRHa-treated rats at the various ages revealed parallel suppression in both groups compared with controls (unpublished data), although we cannot exclude differences in the types or levels of androgens produced. Recent immunohistochemical data have shown that spermatogonia and spermatocytes express ERβ [11], raising the possibility that DES could have acted directly on these cells during the treatment period. As the role of estrogens in these germ cells is unknown, it is difficult to evaluate this possibility, although it is perhaps unlikely that such effects would have persisted throughout puberty and into adulthood.

Fetal/neonatal exposure to exogenous estrogens can interfere with normal descent of the testes into the scrotum, and the consequently higher temperature of the abdominally/inguinally located testes would result in impaired expansion of spermatogenesis [1, 2, 40]. Testicular descent in the rat is not complete until Days 21–23; and in the DES-treat-

ed rats, testicular descent was delayed at Day 25, when compared with that in controls, with testes being located at the inguinal ring. However, testes were normally descended at 35 days in DES-treated rats; and this finding, coupled with the fact that testicular descent in some of the GnRHa-treated rats was also delayed at 25 days, suggests that delayed testicular descent is unlikely to explain the differences in Sertoli cell functional maturation between DES- and GnRHa-treated rats. However, the finding of necrotic testes in a small proportion of adults that had been treated neonatally with DES, and their fusion with peritoneal membranes within the inguinal canal, imply that for at least a proportion of DES-treated rats, problems with testicular descent may be of overriding importance.

Although not reported in the present paper, marked distension of the rete testis was observed in DES-treated rats, but not in GnRHa-treated or control rats, at all ages from 10 days through to adulthood [41]. This distension occurred despite the virtual absence of lumens from the seminiferous tubules of the DES-treated animals at 18 and 25 days of age and may be a consequence of impaired fluid resorption from the efferent ducts [41], which are established as an important site of estrogen action [7, 12–14]. As distension of the rete persists through to adulthood in DES-treated rats, it may be that the underlying abnormalities that this reflects or causes may interfere with normal expansion of spermatogenesis via perturbation of the normal fluid dynamics of the developing testis [42].

In the present studies there was considerable variation in response of individual animals to the DES treatment. This variability may reflect the multiple possible sites at which DES could act as an estrogen, as outlined above. In addition, the effect of DES on body weight (mechanism unknown, but the gut is a target for estrogens; [11]) could be a contributory factor, though it should be noted that GnRHa-treated rats also showed a significant reduction in body weight. In this regard, we have also observed similar or more marked variation between animals from the same litter in their response to administration of doses of DES that do not affect body weight or in the response of animals to administration of an aromatase inhibitor (unpublished data). At present we have no explanation for this variable response.

Potentially one of the most important of the present findings in DES-treated rats was that effects were evident many weeks after the last administration of DES. As evidenced by studies in the mouse [43], DES does not have a particularly long half-life in the circulation, although it may have persisted in treated rats for several days after its last administration in the present studies due to the use of corn oil as a vehicle. This seems unlikely to explain effects observed at 25 days of age and later. The effects of DES at these ages, and perhaps also at Day 18, might therefore reflect permanent reprogramming/faulty programming of reproductive development as a consequence of inappropriate estrogen exposure neonatally; this appears to be the case for the permanent DES-induced changes in structural development of the efferent ducts and rete testis [41], and similar effects in the brain are well established [1]. The present data raise the possibility of a similar permanent effect on Sertoli cell maturational/functional development, and this should be a focus for future studies to confirm.

In the context of environmental estrogens and the shortage of endpoints of estrogen action in the male to employ in risk assessment studies, the present findings have identified some potentially important leads. Though the usefulness of the parameters employed in the present studies is complicated by the possibility of estrogen action at several levels (pituitary gland, Sertoli cell, rete testis, and efferent ducts, testicular descent), their use in future studies involving the neonatal administration of a range of doses of DES and candidate environmental estrogens should enable important strides forward to be made. Such studies are in progress.

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