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Neonatal Estrogen Exposure of Male Rats Alters Reproductive Functions at Adulthood[1]

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ABSTRACT

The effects of neonatal exposure to different doses of diethylstilbestrol (DES) on the reproductive functions of male rats at adulthood were evaluated. Sprague-Dawley rats (5–8/group) received sc injections of 25 μl olive oil containing DES (Sigma Chemical Co., St. Louis, MO) at a dose of 10 μg, 1 μg, 100 ng, 10 ng, or 1 ng per rat on alternate days from Postnatal Days 2–12. Control animals received olive oil only. All animals were allowed to develop until 83–91 days of age; however, when they were 70 to 80 days old, four male rats each from the 10 μg, 1 μg, 100 ng, and control groups were cohabited with untreated 60- to 70-day-old females (1:1) for 12 days. At the end of cohabitation, both mated and unmated male rats were weighed, and blood and tissue samples were collected and processed. Results revealed that although sperm motility patterns and sperm morphology were adversely affected in the 10-μg group, other reproductive parameters, including 1) daily sperm production (DSP)/testis; 2) absolute and relative weights of the testis, epididymis, and seminal vesicle; and 3) sperm numbers in both regions of the epididymis declined significantly in a dose-dependent manner in the 10- and 1-μg groups. Conversely, in the <1-μg groups, none of these parameters (except DSP/testis and weight of the epididymis in the 100-ng group, and sperm numbers in the epididymis of the 100- and 10-ng groups) was different from controls. Generally, plasma testosterone levels decreased in the 10- and 1-μg groups, FSH level increased in the 10-μg group, and prolactin and LH levels were unaltered. In the fertility study, although each male in the 1-μg, 100-ng, and control groups produced a copulatory plug and impregnated a female, none could do so in the 10-μg group. The mean number of pups per litter was reduced to eight in the 1-μg group, in contrast to 15 each in the 100-ng and control groups. In conclusion, exposure of neonatal male rats to DES altered sperm motility patterns, sperm fertility (as evident from the reduced number of pups in the 1-μg group), and sexual behavior (as evident from the absence of copulatory plugs in the 10-μg group) and reduced weights of reproductive organs, DSP/testis, and sperm numbers in the epididymis. Whether these alterations/reductions persist in older rats (6–8 mo of age) is under investigation.

epididymis, estradiol, sperm, sperm motility and transport, testis

INTRODUCTION

Experimental studies involving ablation and/or x-irradiation of the fetal testis [1], orchidectomy/hypophysectomy with or without testosterone substitution [2, 3], ligation of efferent ductules [4, 5], and in vitro culture [6] have established that androgens are essential for development of male reproductive organs, sperm production, and sperm maturation. Conversely, the role of estrogen in male reproduction remains unclear, although it is known that the rete fluid contains a high concentration of estrogens [7] and that both estrogen receptors (ER), ERα and ERβ, are widely distributed in male reproductive organs of various species [8, review], including rats [9–11]. Recent studies reporting infertility in genetically altered mice lacking the aromatase enzyme [12, 13] or estrogen receptor α [14] and describing poor semen quality in male patients with a defective ERα gene [15] or aromatase gene [16] have indicated that deprivation of estrogen or its receptors impairs male reproductive functions. Similarly, an excessive exposure of estrogen leads to reproductive abnormalities. A higher incidence of retained testes, atrophic testes, sperm abnormalities, and epididymal cysts has been reported in some male offspring of women who were exposed to estrogenic compounds during pregnancy [17]. Similar disorders, as well as lower fertility, have been found in laboratory animals that were treated neonatally/perinatally with estrogens [18–21].

It is interesting that both deprivation of and excessive exposure to estrogens can lead to reproductive disorders, including infertility. Adding to the interest are recent observations that estrogens at very low doses can enlarge the prostate gland, whereas higher doses have the opposite effect [22, 23]. This interest is further heightened by reports of decreasing sperm counts in men over a period of decades [24, 25] and of a possible link between an exposure to estrogenic chemicals and an increase in the incidence of reproductive abnormalities in men [21] and wildlife [26, 27].

Hence it is clear from the above review that estrogens play an important role in male reproduction, but their mechanisms of action in reproductive physiology or in inducing reproductive abnormalities remain unclear. By treating Wistar rats neonatally with different doses of diethylstilbestrol (DES), Sharpe et al. [28] and others [29–31] have reported a dose-dependent decrease in testis weight and germ cell volume/testis and an increase in apoptotic index of germ cells at adulthood, implying a long-lasting effect of neonatal exposure to estrogens. Furthermore, fertility in these rats was reduced to zero with higher doses (10 μg and 1 μg rat^{-1} day^{-1} on alternate days from Postnatal Days 2–12) and to 50% with the lowest dose (10 ng), the dose that had a stimulatory effect on spermatogenesis at prepuberty and did not affect testis weight at adulthood [30]. Interest-

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ingly, the latter dose of DES falls within a range that leads to an increase in prostate weight of adult CF-1 mice whose mothers received DES (2 ng/g body weight) from gestation Days 11 to 17 [32].

Considering the potential implications of the above results for human health, the aim of the present study is to determine effects of neonatal estrogen exposure on male reproductive parameters at adulthood in Sprague-Dawley rats, a strain of rat that is commonly used to assess toxicological effects of endocrine disruptors and is genetically less sensitive to estrogens than other strains of rats [33, 34]. In addition, this study broadened its scope by including epididymal parameters, such as numbers, morphology, and motility patterns of sperm in the epididymis, that have not been well characterized following neonatal estrogen exposure.

MATERIALS AND METHODS

Animals and Treatments

Timed-pregnant Sprague-Dawley female rats (Harlan Sprague-Dawley, Indianapolis, IN) were housed individually and observed twice daily for delivery. Animals were maintained under controlled conditions at 22–23°C ambient temperature, 55%–60% relative humidity, 12L:12D cycle, and were supplied with water and feed (Rodent Chow 5001; Purina Mills, St. Louis, MO) ad libitum. Within 24 h of delivery, the litter size was standardized to eight pups per dam, with as many as eight males, if possible. Beginning from the second day (Day 1 is the day of birth) and ending on Day 12, neonatal pups (five to eight males per group, except four males in the 1-ng group) received subcutaneously 25 μl of olive oil containing DES (Sigma Chemical, St. Louis, MO) at a dose of 10 μg, 1 μg, 100 ng, 10 ng, or 1 ng rat^{-1} day^{-1} on alternate days. The control animals were treated the same way except that they received olive oil only. This protocol of treatment is essentially similar to that reported for Wistar rats [29, 30]. Pups were weaned on Postnatal Day 22 and were observed regularly until used for mating at 70–80 days of age and/or necropsied at 83–91 days of age. All animal procedures were approved by the IACUC at Tuskegee University.

Body and Organ Weights

Beginning with Week 1, body weight was recorded every week until the day of necropsy and weight gain/loss for each period was calculated. The weights of the testis, caput and corpus of the epididymis, cauda of the epididymis, and seminal vesicle (including coagulating gland) of both sides were recorded for each animal, and relative weights (weight of the organ/100 g body weight) were calculated. Testes and epididymides were trimmed of fat prior to recording their weights. After weighing, testis and epididymis of the right side were processed for histopathology, and those from the left side were frozen at −80°C until thawed for sperm counts.

Sperm Numbers in the Testis and the Epididymis

Methodology for determining the number of homogenization-resistant spermatids/sperm in the testis, caput and corpus of the epididymis, and cauda of the epididymis was previously described from our laboratory [35]. Briefly, the testis was thawed, the capsule was detached and weighed, and the parenchyma was homogenized in 50 ml of PBS using a Waring laboratory blender (Fisher Scientific, Pittsburgh, PA) that was set at low speed. The homogenate was filtered through a metal sieve, and a 200 μl aliquot of the filtrate was mixed with 100 μl of PBS and 200 μl of 0.6% trypan blue, which stains elongated spermatids. An 8.5 μl aliquot of the latter mixture was then used to determine the average number of homogenization-resistant spermatids/sperm in each sample in duplicate using a hemocytometer. Daily sperm production (DSP) was calculated by dividing the total number of spermatids/sperm per testis or per gram of testicular parenchyma (testis weight minus weight of the capsule) by 6.1 days, the duration of step 19 spermatids in the seminiferous epithelial cycle [36].

Sperm Motility Parameters

Methodologies for collection of sperm from the cauda of the right epididymis and for analyzing sperm motion parameters using a computer-assisted semen analysis system (Integrated Visual Optical Systems, Hamilton Thorn Research, Beverly, MA) were the same as previously described from our laboratory [35]. Briefly, 200 sperm from each animal were analyzed for the following motion parameters: percentage of motile sperm (MSP), percentage of progressively motile sperm (PMP), average path velocity (VAP), straight line velocity (VSL), curvilinear velocity (VCL), lateral head displacement (ALH), straightness (STR) ([straight line velocity/average path velocity] × 100), and linearity (LIN) ([straight line velocity/curvilinear velocity] × 100). Prior to recordings of sperm motility parameters, the instrument was optimized and the analysis was done using the settings described previously from our laboratory [35]. Sperm were considered motile if average path velocity exceeded 20 μm/sec and considered progressively motile if average path velocity exceeded 100 μm/sec. All procedures were performed at 37°C, and all equipment that came in contact with sperm was prewarmed to and maintained at 37°C.

Sperm Morphology

An aliquot of sperm suspension used for motility analysis was diluted with formaldehyde, and a 10 μl of the diluted sample was examined with a phase contrast microscope. Two hundred sperm from each animal were evaluated and classified as follows: normal, head defect, middle piece defect, principal piece defect, proximal droplet, distal droplet, and detached head. In sperm with two or more defects, only the defect affecting its more proximal portion was recorded [35].

Hormonal Measurement

One blood sample was collected from the heart of each animal prior to necropsy, and plasma was frozen at −20°C until assayed. LH, FSH, and prolactin were measured using materials obtained through NHPP, NIDDK, and Dr. A.F. Parlow (antibodies: NIDDK-anti-rLH-S-11, NIDDK-anti-rFSH-S-11, NIDDK-anti-rPRL-S-9; reference standards: NIDDK-rLH-RP-3, NIDDK-rFSH-RP-2, NIDDK-rPRL-RP-3; tracers: NIDDK-rLH-I-10, NIDDK-rFSH-I-9, NIDDK-rPRL-I-6). The sensitivities of the assays were calculated to be 0.3, 2.3, and 0.4 ng/ml for LH, FSH, and prolactin, respectively. Testosterone was measured using a Coat-a-Count testosterone radioimmunoassay (Diagnostic Products Corporation, Los Angeles, CA) according to the manufacturer's protocol. The sensitivity of the assay was calculated to be 0.2 ng/ml. All samples were quantified in a single assay for each hormone with intraassay coefficients of variation of 6%, 9%, 7%, and 7% for LH, FSH, prolactin, and testosterone, respectively.

Histopathology of the Testis, Rete Testis, Efferent Ductules, and Epididymis

Testis. Three- to 5-mm thick slices of tissues from the middle region of the right testis were fixed for 24 h in 4% (w:v) paraformaldehyde in PBS (pH 7.4). The reason for selecting paraformaldehyde, instead of Bouin fluid, as a fixative was to use these tissues not only for histology but also for immunocytochemistry of steroid receptors (topic of another manuscript), which, in our hands, is better illustrated by fixing tissues in paraformaldehyde [37]. Tissues were embedded in paraffin, cut into 5 μm thickness, stained with periodic acid-Schiff (PAS), counterstained with hematoxylin, and examined with a light microscope. In two to three random paraffin sections from each animal, 20 cross-sections of stages VII and VIII seminiferous tubules each from the control, 10-μg, and 1-μg groups ($n = 5$ for each group) was measured blindly for tubular diameter (basal lamina to basal lamina), epithelial height (basal lamina to neck of elongated spermatids), and luminal diameter using a computer-assisted image analysis system.

Rete testis, efferent ductules, and epididymis. The proximal end of the testis containing rete testis (2–3 mm thick); the entire efferent ductules (interposed between the testis and the epididymis); and the caput, including initial segment (cut in the middle into two halves), and cauda (cut in the middle into two halves) regions of the epididymis were fixed and processed as described above for the testis, except that the proximal testis from the control, 10-μg, and 1-μg groups was serially sectioned. Every 20th serial section, with as many as 15 sections spanning a distance of 1.5–2 mm, was examined for changes in luminal diameter of the rete testis, since it is known that neonatal estrogen exposure causes distension of its lumen [31, 38, 39]. In addition, in two to three random paraffin sections from each animal, 15 tubular cross-sections each from the proximal and distal regions of the efferent ductules and the initial segment, caput, and cauda regions of the epididymis were measured for tubular diameter (basal lamina to basal lamina), epithelial height, and luminal diameter using a

TABLE 1. Effect of neonatal DES treatment on body weight (BW) and paired relative weight (mg/100 g BW) of reproductive organs at adulthood (83–91 day) in rats.[a]

DES	BW (g)	Testis	EP caput and corpus	EP cauda	Seminal vesicle
Control (n = 7)	398 ± 7	919 ± 9	167 ± 2	104 ± 2	327 ± 17
10 μg (n = 6)	355 ± 7*	689 ± 22*	97 ± 3*	53 ± 4*	50 ± 5*
1 μg (n = 5)	410 ± 7	698 ± 32*	146 ± 6*	88 ± 3*	257 ± 21*
100 ng (n = 8)	401 ± 6	843 ± 18*	147 ± 4*	95 ± 3*	306 ± 10
10 ng (n = 5)	407 ± 10	892 ± 23	167 ± 4	105 ± 2	353 ± 14
1 ng (n = 4)	384 ± 10	1020 ± 35*	172 ± 4	120 ± 3*	334 ± 4

[a] Data are expressed as mean ± SEM. EP, Epididymis.
* Significantly ($P \leq 0.05$) different from control within a row.

computer-assisted image analysis system. These measurements were made in the control, 10-μg, and 1-μg groups only (n = 5 for each group) since the lower dose groups revealed minimal to no adverse effects on reproductive parameters studied here. Digital images of PAS-stained paraffin sections were captured using a Leitz Orthoplan microscope (Vashaw Scientific, Inc., Norcross, GA) and Kodak Microscopy Documentation System 290 (Eastman Kodak Company, Rochester, NY), and were assembled using Adobe Photoshop 6.0 (MicroWarehouse, Norwalk, CT).

Fertility. Four 70- to 80-day-old male rats each from the 10 μg, 1 μg, 100 ng, and control groups were transferred to a mating cage floored with a wire mesh grid and cohabited with untreated, 60- to 70-day-old female rats (1:1) for 12 day. Cages were checked for the presence of copulatory plugs, and vaginal washings were evaluated for the presence of sperm everyday. Females with positive plugs and/or sperm were separated from males and were killed at 15–18 day of pregnancy. Both uteri were removed and examined for the number of live fetuses and implantations. In addition, both ovaries were removed and the number of corpora lutea was counted. Data were analyzed to determine the effects of neonatal estrogen exposure on the number of live embryos per litter, preimplantation loss (difference between the number of corpora lutea and the number of implantation sites expressed as per number of corpora lutea), and postimplantation loss (difference between the number of implantation sites and the number of live fetuses expressed as per number of implantation sites). In addition, the conception time, the interval between the first day of cohabitation and the day of plug and/or sperm, was recorded for each female.

Statistics

Statistical analyses were performed using Sigma Stat statistical software (Jandel Scientific, Chicago, IL). One-way analyses of variance were utilized on all parameters, except the weekly body weight, which was analyzed by two-way analysis of variance for repeated measures. Treatment groups with means significantly different ($P \leq 0.05$) from controls were identified using the Dunnett test. When data were not distributed normally, or heterogeneity of variance was identified, analyses were performed on transformed data or ranked data; however, arithmetic means and SEM are presented in tables and figures.

RESULTS

Body Weight

The final body weight at Postnatal Week 12–13 was similar in the ≤1-μg groups and controls, but it was almost 7% lower ($P \leq 0.05$) in the 10-μg group than in controls (Table 1). Similarly, the mean weekly body weight was lower throughout development in the 10-μg group only, but the decrease was not significant ($P \leq 0.05$) during the first 5 wk of age (Fig. 1). A spurt in growth, characterized by a steep gain in weekly body weight, started at 5 wk and continued through 9 wk of age, regardless of the treatment. During this period, a control pup gained an average of 195 g of weight, in contrast to 175 g in the 10-μg group (Fig. 1). The two-way analysis of variance for repeated measures identified significant effects of age, treatment, and a treatment by age interaction.

Organ Weight

Both absolute (Fig. 2) and relative weights (Table 1) of the testis, caput and corpus of the epididymis, cauda of the epididymis, and seminal vesicle responded in a dose-dependent manner, with no difference from control observed in the 10-ng group and the maximum decrease observed in the 10-μg group. Conversely, the relative weight of the testis and the cauda of the epididymis was significantly ($P \leq 0.05$) higher in the 1-ng group (Table 1). Within the 10-μg group, the level of weight loss differed among organs. It was much more pronounced in the seminal vesicle (85%) and both regions of the epididymis (42%–50%) than in the testis (25%).

Number of Sperm in the Testis and Epididymis

The total number of homogenization-resistant sperm per testis was significantly reduced, in a dose-dependent manner, by almost 48%, 35%, and 24% in the 10-μg, 1-μg, and 100-ng groups, respectively (Fig. 3). However, the 14% reduction in the mean observed in the 10-ng group was not significantly different from controls. Unlike sperm numbers per testis, the DSP per gram of testicular parenchyma (in-

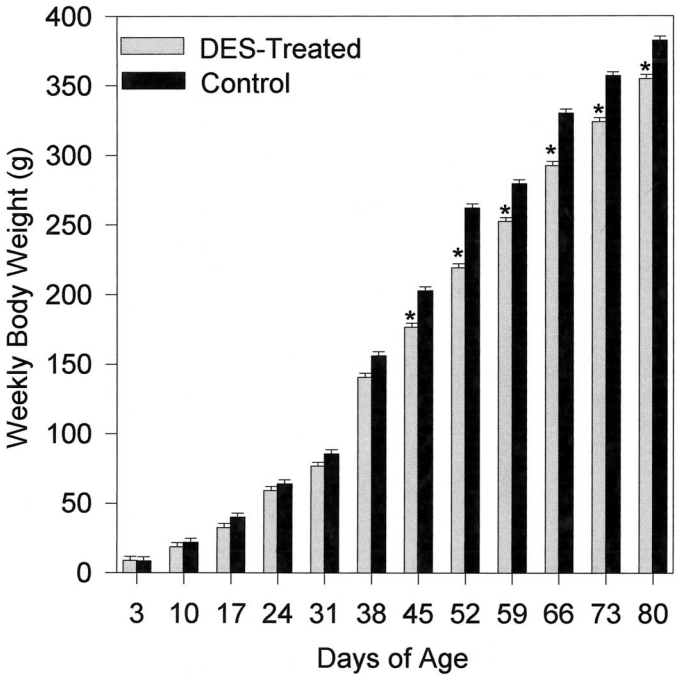

FIG. 1. Mean (±SEM) weekly body weight in Sprague-Dawley rats treated neonatally with 10 μg of DES. *Significant differences from controls ($P \leq 0.05$). Control, n = 7; 10 μg, n = 6.

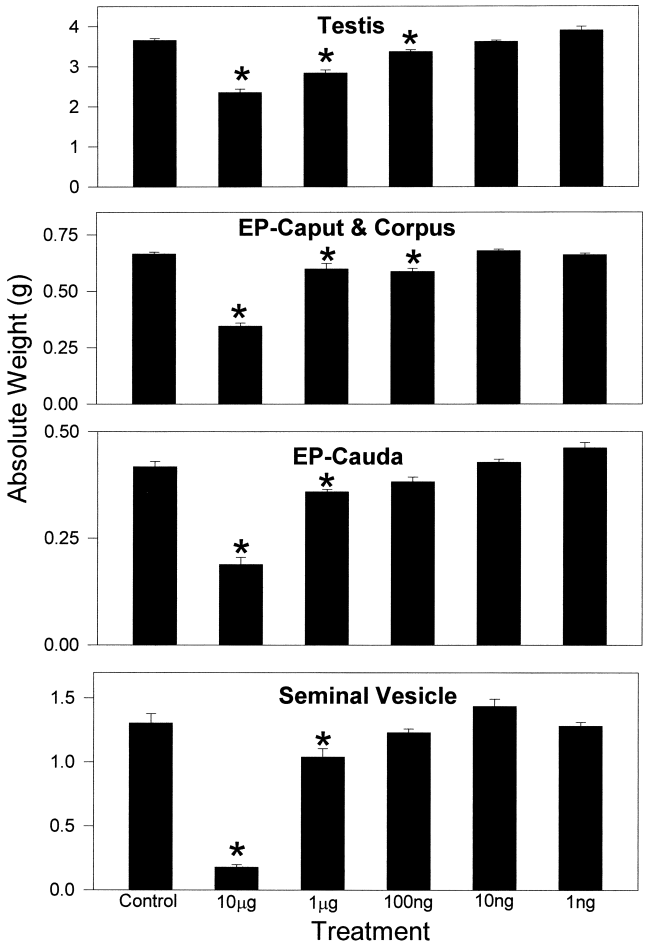

FIG. 2. Mean (±SEM) paired absolute weight of reproductive organs at adulthood (83–91 days of age) in Sprague-Dawley rats treated neonatally with different doses of DES. *Significant differences from controls ($P \leq 0.05$). Control, n = 7; 10 μg, n = 6; 1 μg, n = 5; 100 ng, n = 8; 10 ng, n = 5; 1 ng, n = 4.

dicator for efficiency of spermatogenesis) was not altered in the 10-μg to 10-ng groups (Fig. 3). Both mean values for sperm numbers per testis and DSP per gram of testicular parenchyma were higher in the 1-ng group (114% and 107% of controls, respectively), but they could not be distinguished from controls.

Sperm numbers in the epididymis were dramatically low in the 10-μg group and, comparatively, the decrease was much higher in the cauda (92%) than in the caput and corpus (72%; Fig. 4). Conversely, reductions in the 1-μg to 10-ng groups, although significant, were similar for both regions of the epididymis and ranged from 20% to 40% depending on the dose. Unlike in the higher dose groups, sperm numbers in the 1-ng group did not significantly differ from that of controls in either region of the epididymis.

Computer-Assisted Sperm Motility Parameters

Among various motility parameters, MSP, PMP, STR (percentage), and LIN (percentage) were significantly decreased in the 10-μg group (Fig. 5). Of all these parameters, the maximum effect was observed in PMP, which was reduced to 35% of controls. None of the motility parameters was different from controls in the ≤1-μg groups, except VCL (micrometers per second), which was significantly higher in the 1-μg group (Fig. 5).

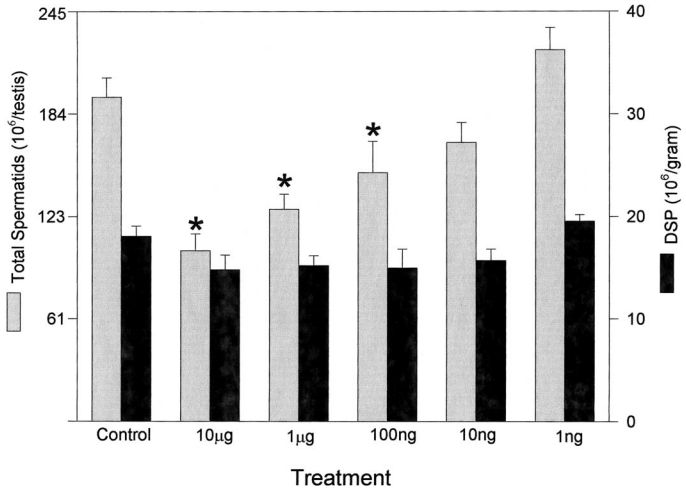

FIG. 3. Mean (±SEM) number of homogenization-resistant spermatids/sperm in the testis at adulthood (83–91 days of age) in Sprague-Dawley rats treated neonatally with different doses of DES. *Significant differences from controls ($P \leq 0.05$). Control, n = 7; 10 μg, n = 6; 1 μg, n = 5; 100 ng, n = 6; 10 ng, n = 5; 1 ng, n = 4.

Sperm Morphology

The incidence of abnormal sperm was similar in the ≤1-μg groups and controls, but it was significantly higher in the 10-μg group (Table 2). Of various sperm abnormalities affecting the head, middle piece, or principal piece, the misshapen head and bent middle piece were most frequently encountered and constituted 78% and 10% of abnormal sperm, respectively, in the 10-μg group. Conversely, the incidence of sperm with retained distal cytoplasmic droplet was significantly lower in the 10-μg group (Table 2).

Hormones

The mean plasma concentration of testosterone was significantly lower in the 10-μg and 1-μg groups (25%–30% of controls), but was not different from controls in the lower dose groups (Fig. 6). Conversely, the mean FSH level was almost fourfold higher in the 10-μg group but could not be distinguished from controls in the other groups. Neither prolactin nor LH was significantly altered in any of the treated groups (Fig. 6).

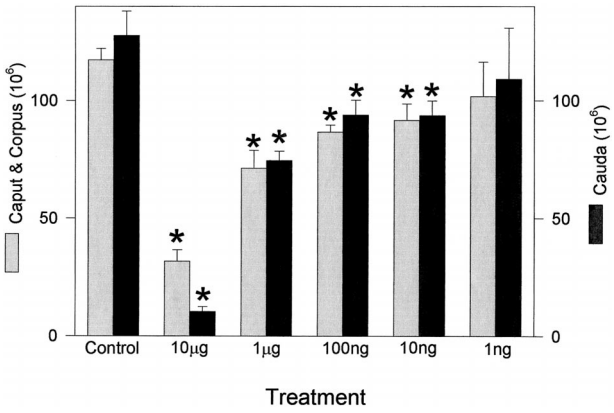

FIG. 4. Mean (±SEM) number of homogenization-resistant sperm in the caput and corpus and the cauda of the epididymis at adulthood (83–91 days of age) in Sprague-Dawley rats treated neonatally with different doses of DES. *Significant differences from controls ($P \leq 0.05$). Control, n = 7; 10 μg, n = 6; 1 μg, n = 5; 100 ng, n = 6; 10 ng, n = 5; 1 ng, n = 4.

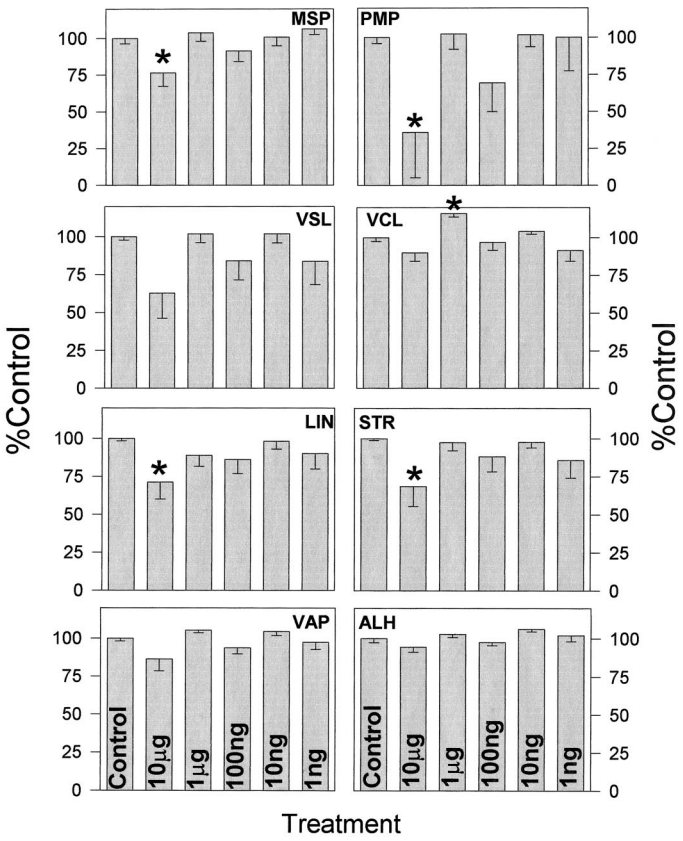

FIG. 5. Motility patterns of sperm from the cauda of the epididymis at adulthood (83–91 days of age) in Sprague-Dawley rats treated neonatally with different doses of DES. MSP, Percentage of motile sperm; PMP, percentage of progressively motile sperm; VSL, straight line velocity; VCL, curvilinear velocity; LIN, linearity; STR, straightness; VAP, average path velocity; ALH, lateral head displacement. Data are expressed as percentage of controls. *Significant differences from controls ($P \leq 0.05$). Control, n = 6; 10 μg to 10 ng, n = 5 in each group; 1 ng, n = 4.

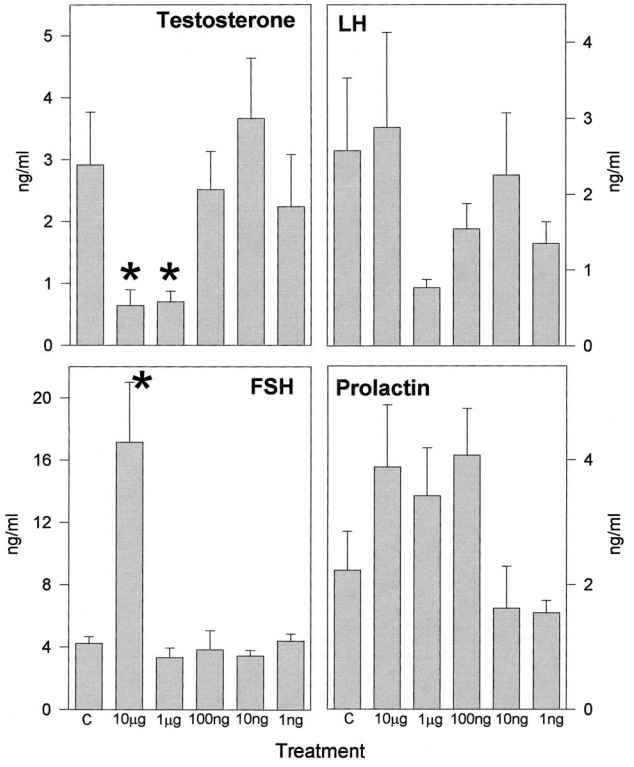

FIG. 6. Mean (±SEM) plasma concentration of testosterone, LH, FSH, and prolactin at adulthood (83–91 days of age) in Sprague-Dawley rats treated neonatally with different doses of DES. *Significant differences from controls ($P \leq 0.05$). Control, n = 7; 10 μg, n = 6; 1 μg, n = 5; 100 ng, n = 6; 10 ng, n = 5; 1 ng, n = 4.

Histopathology of the Testis, Rete Testis, Efferent Ductules, and Epididymis

Histological examination of the testis did not reveal any evidence of degeneration of germ cells and/or retention of spermatids in the seminiferous epithelium, regardless of the dose of DES or the stage of seminiferous epithelial cycle (Fig. 7, A and B). With the exception of seminiferous epithelial height in the 10-μg group, mean measurements for the epithelial height, luminal diameter, and tubular diameter of stages VII and VIII tubules did not significantly differ between controls and treated groups (Table 3).

An examination of serial sections of the rete testis revealed that it consisted of a sac-like structure interrupted by trabeculae, which gave the rete an appearance of a maze of interconnecting tubules. The intratesticular sac of the rete was up to 1.7 mm deep under the capsule of the testis, and its luminal diameter, along the length, was highly variable within, as well as between, animals both in the control and treated groups, varying from 20 μm in some sections to 400 μm in other sections. Compared with controls, there was no morphological evidence of dilation of lumen or accumulation of sperm in any of the treated groups. On the other hand, the mean luminal diameter of the distal efferent ductules was increased by 60% ($P \leq 0.05$) and that of the proximal efferent ductules by 30% in the 10-μg group, although the latter increase was not significantly different from controls (Table 3). However, it should be noted that the luminal diameter of proximal tubules varied widely not only among tubules within the same animal, but also along the length. Generally, tubules nearer their origin from the extratesticular rete tend to have a larger luminal diameter than those present distally. The general morphology of ef-

TABLE 2. Effect of neonatal DES treatment on sperm morphology at adulthood (83–91 day) in rats.[a]

DES	Normal (%)	Defective (%)				
		Head	Midpiece	Principal piece	Distal droplet	Detached head
Control (n = 7)	85.1 ± 4.6	4.86 ± 1.7	1.14 ± 0.5	0 ± 0	5.3 ± 1.2	3.6 ± 2.8
10 μg (n = 6)	66.7* ± 6.0	25.8* ± 5.3	3.3* ± 1.0	0.67 ± 0.3	0.167* ± 0.2	3.0 ± 6.0
1 μg (n = 5)	89.2 ± 1.2	4.6 ± 2.1	0.4 ± 0.2	0.4 ± 0.4	2.6 ± 0.2	2.8 ± 4.2
100 μg (n = 5)	84.2 ± 4.6	5.6 ± 2.0	1.2 ± 1.2	0.4 ± 0.4	5.8 ± 1.1	2.8 ± 1.5
10 ng (n = 5)	84.4 ± 2.6	7.2 ± 2.6	1.2 ± 0.2	0 ± 0	7.2 ± 0.6	0.6 ± 0.4
1 ng (n = 4)	89.3 ± 3.1	4.5 ± 1.7	0 ± 0	0 ± 0	5.0 ± 1.4	1.3 ± 0.8

[a] Data are expressed as mean ± SEM.
* Significantly ($P \leq 0.05$) different from control.

TABLE 3. Epithelial height (EH), luminal diameter (LD), and tubular diameter (TD) in different regions of the reproductive tract of adult male rats treated neonatally with DES.[a]

	10 μg DES (n = 5)			1 μg DES (n = 5)		
	EH (μ)	LD (μ)	TD (μ)	EH (μ)	LD (μ)	TD (μ)
Testis (stages VII–VIII), tubule[b]	66.4 ± 2.4*	139.8 ± 6.9	262 ± 8.9	58.1 ± 1.0	139.6 ± 5.3	247.9 ± 5.0
Rete testis[c]	5.8 ± 0.45	80.8 ± 14.1	92.6 ± 14.7	5.8 ± 0.21	76.5 ± 10.9	86.5 ± 11.2
Efferent ductules, proximal[c]	21.0 ± 1.3	130.1 ± 12.7	173.3 ± 13.3	25.8 ± 1.3	130.4 ± 4.6*	179.7 ± 2.9*
Efferent ductules, distal[c]	23.0 ± 2.3	87.5 ± 6.1*	131.1 ± 8.8*	27.1 ± 1.2	70.6 ± 7.7	123.2 ± 8.9*
Epididymis, initial segment[c]	36.4 ± 3.1	98.1 ± 4.2	168.3 ± 4.9	35.2 ± 3.5	90.0 ± 4.7	160.6 ± 12.1
Epididymis, caput[c]	19.3 ± 1.1	225.8 ± 8.4	262.8 ± 6.8	18.2 ± 1.9	232.0 ± 18.6	264.8 ± 16.6
Epididymis, cauda[c]	27.7 ± 1.6*	164.3 ± 15.0*	218.8 ± 13.6*	21.7 ± 1.5	226.9 ± 13.7	267.6 ± 11.0

[a] Data are expressed as mean ± SEM.
[b] Twenty tubules per animal.
[c] Fifteen tubules per animal.
* Significantly ($P \leq 0.05$) different from control within a row.

ferent ductules, including epithelial height, did not alter as a result of neonatal estrogen treatment except in the 10-μg group, where three of the six animals had focal, mononuclear infiltration in the epithelium, peritubular tissue, and/or lumen of some of the ductules. In two of the latter three animals, the epithelium in some tubules was hypertrophied and the lumen contained PAS-positive material. In addition, the lumen, at certain points along the length, was partially obstructed by a cellular, tongue-like structure. The latter, in serial sections, was found to be continuous with the lining epithelium (Fig. 7, C–F). Neither lymphocytic infiltration nor epithelial hypertrophy was observed in the efferent

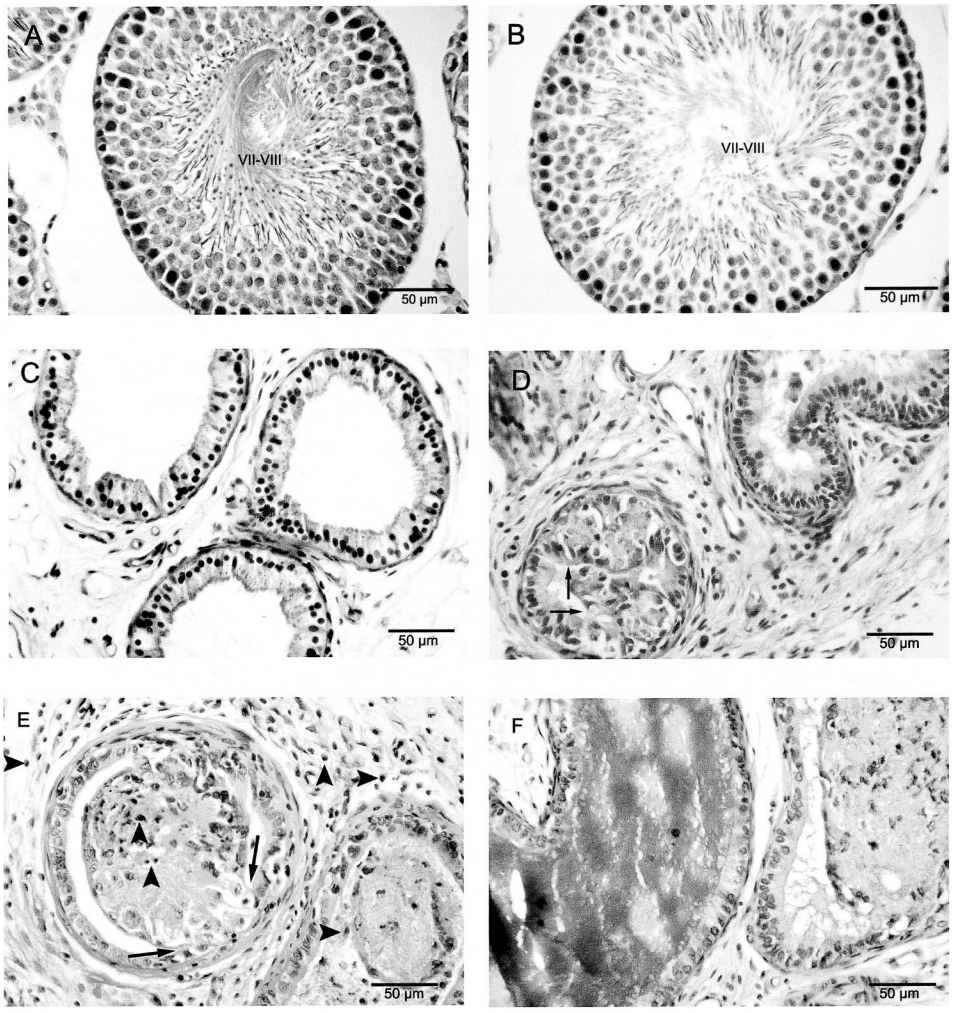

FIG. 7. **A**, **B**) In these micrographs of stage VII to VIII seminiferous tubules from the control (**A**) and the 10-μg group (**B**), the morphology of seminiferous epithelium is essentially similar. **C**–**F**) Efferent ductules from the control (**C**) and the 10-μg DES group (**D**–**F**). **D**) Hypertrophied epithelium projecting into the lumen of the ductule (arrows). **E**) Arrows show continuity of hypertrophied epithelium with the lining epithelium, and arrowheads indicate lymphocytes that are infiltrated in the intertubular tissue as well as in the lumen of ductules. **F**) Note PAS-positive material in the lumen of the left ductule and a cellular structure obstructing the lumen in the right ductule.

TABLE 3. Extended.

	Control (n = 5)		
	EH (μ)	LD (μ)	TD (μ)
Testis (stages VII–VIII), tubule[b]	59.4 ± 1.3	141.1 ± 5.2	247.5 ± 5.5
Rete testis[c]	5.4 ± 0.05	101.5 ± 26.2	111.7 ± 26.7
Efferent ductules, proximal[c]	26.4 ± 1.5	100.9 ± 11.3	152.2 ± 12.7
Efferent ductules, distal[c]	23.8 ± 0.99	54.8 ± 4.4	101.2 ± 3.5
Epididymis, initial segment[c]	42.7 ± 2.8	102.9 ± 8.7	188.0 ± 13.0
Epididymis, caput[c]	17.4 ± 1.2	234.1 ± 9.4	266.1 ± 8.0
Epididymis, cauda[c]	20.4 ± 1.5	227.6 ± 6.5	267.2 ± 4.5

ductules of control or ≤1-μg groups (n = 5 for each group, except n = 4 in the 1-ng group).

None of the morphometric features in the initial segment and caput of the epididymis was different from that of controls in the 10-μg or 1-μg groups (Table 3). However, both luminal and tubular diameters were significantly lower and epithelial height was significantly higher in the cauda epididymis of the 10-μg group (Table 3).

Fertility

Fertility-related parameters studied were fertility index, presence of copulatory plugs and/or sperm in vaginal washing, pups per litter, and pre- and postimplantation losses. Although all females mated with males in the control, 1-μg, or 100-ng groups had copulatory plugs and produced pups (fertility index 100%), neither copulatory plugs nor pups were present in females cohabited with males of the 10-μg group (fertility index 0%; Table 4). The mean number of live pups per litter was 14.5 and 14.8 in the control and 100-ng groups, respectively, in contrast to 7.8 in the 1-μg group. The mean preimplantation loss (difference between the number of corpora lutea and the number of implantation sites expressed as per number of corpora lutea) was 49% for the 1 μg group, in contrast to 12% and 6% for the 100-ng and control groups, respectively. The mean values for both of the above parameters in the 1-μg group reflect heterogenous responses among animals. For example, although females mated with two males of the group had very few pups (2 or 3 per litter), those mated with the other two males of the group had as many pups as in the control and lower dose groups (13 per litter). Similarly, the preimplantation loss for the first two females was exceedingly high, whereas that for the latter two females was essentially similar to that of the control and lower dose groups. The mean postimplantation loss (difference between the number of implantation sites and the number of live fetuses expressed as per number of implantation sites) for the 1-μg, 100-ng, and control groups was 9.5%, 3.6%, and 3.3%, respectively. The mean conception time (interval between the day of cohabitation and the day of presence of plug/sperm) was 3.5, 3.0, and 5.3 days in the control, 100-ng, and 1-μg groups, respectively (Table 4).

DISCUSSION

This study, using different dose regimens of DES (10 μg, 1 μg, 100 ng, 10 ng, and 1 ng) determined the effects of neonatal estrogen exposure on adult reproduction in male Sprague-Dawley rats and compared them with those previously reported in Wistar rats (28–30]. Results of the study provided clear evidence for the first time that neonatal estrogen exposure compromised sperm morphology, sperm motility patterns, and sperm numbers in the adult epididymis. In addition, they confirmed earlier reports that indicated significant changes in weights of the testis, epididymis, and seminal vesicle; sperm production; plasma testosterone concentration; and/or fertility at maturity following neonatal and/or prenatal exposure to estrogenic compounds in rats [28, 40–43], mice [18, 19, 44], hamsters [45], and rabbits [46].

Generally, adverse effects observed in Sprague-Dawley rats were maximal in the 10-μg group, moderate in the 1-μg group, and minimal to absent in the lower dose groups. Similar dose-dependent effects were also reported in Wistar rats, but the level of effects on the testis and fertility differed markedly between the two strains of rats. For example, Wistar rats treated neonatally with 10 μg of DES had degeneration of germ cells and Sertoli cell-only seminiferous tubules in 60% of animals, and atrophic, necrotic, and/or cryptorchid testis in 15% of animals at adulthood [28]. Conversely, none of these phenotypes was observed in Sprague-Dawley rats, regardless of the dose. Although none of the Wistar rats in the 1-μg group and only 50% in the 10-ng group sired pups [30], all Sprague-Dawley rats did so in the 1-μg and 100-ng groups. Hence, these comparisons suggest that Sprague-Dawley rats may be less sensitive than Wistar rats to disruptive actions of estrogens on reproductive functions. Previous studies have also shown that Sprague-Dawley rats and/or CD-1 mice are much more resistant to estrogenic compounds than other strains [33, 34, 47]. These differences in susceptibility to estrogenic compounds signify the importance of understanding genetic mechanisms that are responsible for differential responses.

TABLE 4. Effect of neonatal DES treatment on fertility at adulthood (83–91 day) in rats.[a]

	Parameter				
DES	Pregnant/mated	Pups/litter	Implantation sites	Number of corpora lutea	Interval between cohabitation and plug/sperm (day)
Control	4/4	14, 16, 14, 14 (14.5)	15, 16, 14, 15 (15)	16, 16, 16, 16 (16)	3, 1, 2, 8 (3.5)
10 μg	0/4	N/A	N/A	N/A	N/A
1 μg	4/4	13, 2, 13, 3 (7.8)	15, 2, 13, 4	21, 14, 14, 16 (16.3)	8, 6, 5, 2 (5.3)
100 ng	4/4	13, 11, 20, 15 (14.8)	13, 12, 20, 16 (15.3)	20, 13, 20, 17 (17.5)	1, 5, 3, 3 (3)

[a] Individual data are presented; means are listed within parentheses. N/A, Not applicable.

An important finding of the study was the marked differences in weight loss among reproductive organs in the 10-μg group. Whereas the relative weight decreased by 25% in the testis, it decreased by almost 50% in the caput and corpus or the cauda of the epididymis and by 85% in the seminal vesicle. These results are in agreement with those of a recent study that reported an 80% reduction in the weight of the seminal vesicle, in contrast to 20% in the testis, of adult Sprague-Dawley rats that were treated neonatally with estradiol benzoate at a rate of 1500 μg/kg body weight (similar to 10 μg/rat in the present study) [47]. Interestingly, similar differential response in weight loss of reproductive organs was also observed in rats following exposure to estrogenic compounds during adulthood in rats [35, 48–50]. Reasons for this differential response are not clear, but differences in susceptibility to estrogens and/or testosterone may play some role since both estrogen and androgen receptors are present in the testis, epididymis, and seminal vesicle [10, 51–55], and testosterone is reduced in adult animals following neonatal estrogen exposure [28, 29, 40].

It is well established that prenatal and/or neonatal exposure to estrogenic compounds leads to smaller testes and lower sperm production in rodents [21, 28, 41, 56]. Our observations of significant, dose-dependent reductions in testis weight and DSP per testis (total number of sperm per testis/6.1 day) in the 10-μg, 1-μg, and 100-ng groups are in agreement with the above concept. However, despite a dose-dependent decrease in DSP per testis, the DSP per gram of testicular parenchyma did not differ between the control and treated animals or between treated groups. These results imply that neonatal estrogen exposure did not perturb efficiency of spermatogenesis in Sprague-Dawley rats, an interpretation supported by the lack of any morphological evidence of degeneration of germ cells, retention of spermatids, and/or phagocytosis of spermatids in the seminiferous epithelium, regardless of the stage of tubules or the dose of DES. This is further supported by no adverse effect on seminiferous epithelial height, luminal diameter, or tubular diameter of stages VII and VIII tubules, the stages where spermiation occurs and which are testosterone-dependent [57, 58]. Contrary to these results, Wistar rats treated neonatally with DES at a rate of 10 μg/rat had more than a fourfold increase in germ cell apoptosis and more than a twofold increase in the lumen volume of seminiferous tubules than controls at adulthood [29]. According to these authors, the retrograde buildup of testicular fluid in the lumen of seminiferous tubules, as a consequence of maldevelopment of the rete testis and efferent ductules [31, 39], may account for increased germ cell degeneration and thus reduced efficiency of spermatogenesis in adult Wistar rats treated neonatally with estrogens. If this were true, then the lack of morphological evidence of dilation of the rete testis and/or lumen of the seminiferous tubules in the present study goes hand in hand with the normal efficiency of spermatogenesis at adulthood in neonatally estrogenized Sprague-Dawley rats and thus again points to a genetic difference in susceptibility to estrogens between these two strains of rats.

The present observations of reduced testosterone are in agreement with those of previous studies, which also reported lower testosterone in adult animals as a consequence of estrogen treatment of neonatal animals [28–30, 40, 59]. Interestingly, this decline occurred without any alteration in LH concentration and thus suggests permanent changes in functions of Leydig cells, which may include reductions in LH receptor concentration, sensitivity of Leydig cells to LH, and/or enzymes associated with testosterone synthesis. Testosterone decline not involving the negative feedback inhibition of LH was also described in rats exposed to estrogens during adulthood [35, 42, 50] and may be attributed to a direct inhibitory effect of estrogens on enzymes involved in the production of testosterone [60, 61].

Another important finding of the present study was the significant, dose-dependent reductions in sperm numbers in the epididymis, which can be partly attributed to lower daily sperm production. However, comparatively the level of reduction in epididymal sperm numbers was disproportionately higher in the 10- and 1-μg groups, especially the former. For example, although daily sperm production per testis was decreased by 48%, sperm numbers were decreased by 72% in the caput and corpus and by 92% in the cauda of the epididymis in the 10-μg group. A similar disproportionately greater decline in epididymal sperm numbers was also previously reported in rats [35] and mice [62] exposed to estrogen during adulthood and was attributed to accelerated sperm transport in the epididymis [62].

Despite decreased sperm production and epididymal sperm numbers in the 10-μg, 1-μg, and 100-ng groups, morphology and motility patterns of sperm were adversely affected only in the 10-μg group, suggesting higher resistance of these phenotypes in lower dose groups. Since the higher percentage of abnormalities affected the head and middle piece of sperm, it is likely that these defects originated during the metamorphic stage of spermatogenesis because this stage involves modeling of the head and tail of spermatozoa [57]. There are few studies that have examined effects of estrogen on motility and/or morphology of sperm. The percentage of motile sperm was significantly reduced in adult rats treated with ethinyl estradiol after 1 wk of treatment [50]. A higher percentage of decapitated and/or abnormal sperm was reported after estradiol administration to adult rats [49, 63, 64]. Recently, we reported that of all motility parameters, the percentage of sperm with progressive motility was the most sensitive parameter to estrogen exposure in adult animals. It was reduced to less than 20% of controls in rats treated with DES at a rate of 40 μg to 1 mg rat^{-1} day^{-1} for 12 days and to 40% of controls in the 8-μg group [35]. Similarly, in the present study progressive forward motility was most severely affected and was reduced to 35% of controls in the 10-μg group.

Unlike inhibitory effects observed in the testis with higher doses of DES, the relative weight of the testis was significantly increased by 10% in the 1-ng group, indicating a stimulatory effect of the lowest dose of DES used in the present study. This stimulatory effect was coupled with a similar percentage increase in DSP per testis or per gram of testicular parenchyma, but both increases were not significantly different from controls. These results are essentially in accord with those of a recent study in which Sprague-Dawley rats treated neonatally with estradiol benzoate had larger testes at postnatal Days 35 and 90 with lower doses, whereas the reverse was the case with higher doses [47]. Similarly, another study reported stimulatory effects of estrogen exposure on prepubertal spermatogenesis, where spermatocyte nuclear volume per Sertoli cell nuclear volume was significantly increased in rats treated neonatally with low doses of DES [30]. Comparable stimulatory effects were also reported on the prostate weight of rats treated with low doses of estrogens neonatally [23] or prenatally [22, 32]. The low-dose stimulatory effects of estrogen on adult testes of rats estrogenized neonatally can

result from a direct effect of estrogen on Sertoli cells and/or germ cells since both cell types have been shown to express estrogen receptors at all stages of development [65–67].

It is well established that more than 90% of testicular fluid in rodents is absorbed in the efferent ductules [68], and a disturbance in this function can lead to dilation of the efferent ductules and rete testis, which is probably the cause of infertility seen in mutant mice lacking ERα [8]. Several studies from Hess et al. [9, 10] have demonstrated that estrogens are important in testicular fluid absorption because efferent ductules contain higher concentration of ERα compared with other segments of the male reproductive tract. In addition, epithelial cells lining the efferent ductules are shorter and contain less developed endocytotic apparatus (shorter microvilli and fewer pinocytotic vesicles and canaliculi) in ERα knockout mice than in wild mice of the same age [69, 70], and antiestrogen ICI 182 780 causes dilation of the rete testis and efferent ductules [71]. Ironically, an overexposure of estrogen during the neonatal period is also shown to cause dilation of the rete testis and/or efferent ductules in Wistar rats [31, 38, 39]. Considering the potential functional significance of these findings for human health, we wanted to determine whether neonatally estrogenized Sprague-Dawley rats also show dilations of the intratesticular rete testis or efferent ductules at adulthood. In an examination of serial sections, the luminal diameter of the rete testis varied widely from 20 to 400 μm within the same animal, as well as between animals, but there was no evidence of its unusual dilation in any of the treated groups.

Unlike the rete testis, efferent ductules, especially distal ductules, had an increased luminal diameter in the 10-μg group compared with controls, suggestive of a defect in the absorption of testicular fluid. It will be of interest to investigate whether dilation of the efferent ductules will accentuate and progressively lead to distension of the lumen in the rete testis, with time, in older animals. Regardless, the above alterations in efferent ductules, along with observations of leukocytic infiltration in three of the six animals of the 10-μg group, indicate efferent ductules as the primary site for estrogen action in the male reproductive tract. A similar increase in the luminal diameter was also reported in the efferent ductules of adult Wistar rats that were treated with estrogens neonatally [31]; however, Fisher et al. [31], to our knowledge, did not make any distinction between proximal and distal regions of the efferent ductules.

The final parameter studied was the effect of neonatal DES treatment on fertility-related parameters and their relationships, if any, with testicular, epididymal, and/or behavioral disorders. Observations that all males in the 1-μg and 100-ng groups sired pups, but none of the males in the 10-μg group did, indicate that neonatal DES exposure at the highest dose used in this study was effective in inducing infertility in 100% of males. The absence of copulatory plugs or sperm in vaginal washings in females mated with these males suggests that infertility probably resulted from a compromise in sexual behavior. Additional observations of fewer pups per litter and higher preimplantation loss in the 1-μg group suggest compromised sperm fertility, although this interpretation deserves caution since two of the four males sired normal numbers of pups per litter (13 each). Similar to our results, Wistar male rats treated neonatally with 10-μg of DES were infertile, and females mated with them did not have copulatory plugs; however, unlike our results, all Wistar male rats in the 1-μg group did not mate [30]. The latter authors did not measure fertility in the 100-ng group, but 50% of males in the 10-ng group did not mate. These similarities and differences in fertility between Wistar and Sprague-Dawley rats indicate that neonatal estrogen exposure can change the sexual behavior of rats, but the threshold for this change is lower in Wistar rats than Sprague-Dawley rats.

The estrogen-induced depressed sexual behavior, as evident from the absence or reduced number of copulatory plugs, has been reported previously in rats treated with estrogens neonatally [40] or during adulthood [35, 48]. Whether the observed decline in plasma testosterone level in the 10-μg group played a role in altering sexual behavior is doubtful since a similar decline (reduced to 30% of controls) was also observed in the 1-μg group, which exhibited normal sexual behavior. Hence it appears that neonatal DES treatment in the 10-μg group altered the sexual masculinization of the central nervous system that is necessary for adult male sexual behavior [72].

In conclusion, this study provides compelling evidence of altered reproductive functions, including motility and morphology of sperm, fertility, and sexual behavior, in adult Sprague-Dawley rats that were neonatally treated with DES. Further, a comparison of present results with those already published in Wistar rats, using similar dose regimens, suggests genetic differences in susceptibility to estrogens between strains of rats.

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