BIOLOGY OF REPRODUCTION **70,** 1504–1517 (2004)
Published online before print 28 January 2004.
DOI 10.1095/biolreprod.103.026328

Abnormal Morphology of the Penis in Male Rats Exposed Neonatally to Diethylstilbestrol Is Associated with Altered Profile of Estrogen Receptor-α Protein, but Not of Androgen Receptor Protein: A Developmental and Immunocytochemical Study[1]

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ABSTRACT

Objectives of the study were to determine developmental changes in morphology and expression of androgen receptor (AR) and estrogen receptor (ER)α in the body of the rat penis exposed neonatally to diethylstilbestrol (DES). Male pups received DES at a dose of 10 μg per rat on alternate days from Postnatal Day 2 to Postnatal Day 12. Controls received olive oil vehicle only. Tissue samples were collected on Days 18 (prepuberty), 41 (puberty), and 120 (adult) of age. DES-induced abnormalities were evident at 18 days of age and included smaller, lighter, and thinner penis, loss of cavernous spaces and associated smooth muscle cells, and increased deposition of fat cells in the corpora cavernosa penis. Fat cells virtually filled the entire area of the corpora cavernosa at puberty and adulthood. Plasma testosterone (T) was reduced to an undetectable level, while LH was unaltered in all treated groups. AR-positive cells were ubiquitous and their profile (incidence and staining intensity) did not differ between control and treated rats of the respective age groups. Conversely, ERα-positive cells were limited to the stroma of corpus spongiosus in all age groups of both control and treated rats, but the expression in treated rats at 18 days was up-regulated in stromal cells of corpora cavernosa, coincident with the presence of morphological abnormalities. Hence, this study reports for the first time DES-induced developmental, morphological abnormalities in the body of the penis and suggests that these abnormalities may have resulted from decreased T and/or overexpression of ERα.

androgen receptor, estradiol, estradiol receptor, penis, toxicology

INTRODUCTION

It is well established that testosterone (T) and/or dihydrotestosterone are essential for differentiation, growth, and maintenance of both structure and function of male reproductive organs, including the penis [1, 2]. The role of estrogen in mediation of reproductive development and function is less clear. However, it is known that receptors for estrogen are widely distributed in the male reproductive tract of various species [3], including rats [4]. Moreover, mice lacking the capacity to express genes for estrogen receptor (ER)α [5], both ERα and ERβ [3], or aromatase enzyme [6, 7] are infertile. In addition, it is also known that lab animals exposed to estrogenic compounds during critical periods of development display reproductive abnormalities, including epididymal cysts, retention of testes, smaller testes, microphallus, and hypospadias [8, review]. Similarly, disorders of reproductive tract development in wildlife [9] and higher incidences of testicular cancer and hypospadias in men from certain parts of the world [8] have been linked to inappropriate exposure to environmental estrogens. Higher incidences of reproductive abnormalities have also been reported in male offspring of women treated with DES during pregnancy [10, review]. Thus, while estrogen action may be essential for reproductive functionality in the male, untimely exposure to estrogen or related xenobiotics during critical developmental periods can have lasting and often negative consequences for male reproductive health and fertility later in life.

Considering the potential hazardous effects of estrogenic compounds in animal and human health, our long-range goal is to understand the mechanisms through which estrogens affect organizationally and functionally critical developmental processes in the male reproductive tract. We previously reported that exposure of neonatal [11] or adult [12] male rats to estrogen led to infertility. Interestingly, the loss of fertility in the former, but not the latter, appeared to be permanent and most likely due to the replacement of cavernous spaces by adipose cells in the corpora cavernosa penis [13]. Realizing that these novel findings may have significance in erectile dysfunction, the first objective of the study was to determine effects of neonatal DES exposure on patterns of penile development in the rat.

The second objective of the study was to determine whether estrogen-induced alterations in the rat penis during postnatal development are associated with changes in the profile (incidence and staining intensity) of androgen receptor (AR)- and/or ERα-positive cells. The concentration of AR and ER protein and/or mRNA in the rat penis has been shown to decline from a peak level at or before puberty to barely detectable levels in adulthood [14–16]. There is only one report in the literature that describes im-

[1]Supported by NIH grants MBRS-5-S06-GM-08091 (to H.G.) and RCMI-5-G128803059 and by USDA grant CSR-EES-ALX-TU-CTIF.
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Received: 5 December 2003.
First decision: 25 December 2003.
Accepted: 13 January 2004.

ISSN: 0006-3363. http://www.biolreprod.org

munolocalization of AR at the cellular level during different stages of development in the rat penis [17], and there is no information on the expression of penile ERα during development. Because we [11] and others [18, 19] have shown that neonatal exposure to estrogen dramatically decreases plasma T and because AR and ERα expression is hormonally regulated in male reproductive end organs [20–22], it is possible that estrogen-induced penile abnormalities may have resulted from altered profile of AR and/or ERα.

MATERIALS AND METHODS

Animals and Treatments

Neonatal and/or adult Sprague-Dawley male and female rats (Harlan Sprague Dawley, Indianapolis, IN) were maintained at 22–23°C ambient temperature, 55–60% relative humidity, and 12L:12D cycle, and had free access to food (Rodent Chow 5001; Purina Mills, St. Louis, MO) and water for 24 h. The Institutional Animal Care and Use Committee at Tuskegee University approved all animal procedures, and animals were handled in accordance with the guidelines of the National Institutes of Health Guiding Principles for the Care and Use of Animal Research.

Timed-pregnant Sprague-Dawley female rats were housed individually. Within 24 h of delivery, the litter size was adjusted to eight pups per litter, with as many as eight males, if possible. Pups (5–8 males/group, all pups within a group were littermates) received subcutaneous injections of 25 μl of olive oil containing DES (Sigma, St. Louis, MO) at a dose of 10 μg per rat (approximately 0.5–1 mg/kg), per day, on alternate days, from Postnatal Day 2 to Postnatal Day 12. This dose regimen was selected based on our previous study, where it caused 100% infertility in male rats at adulthood and the loss of fertility was associated with the loss of cavernous spaces and accumulation of fat cells in the body of the penis [13]. The control animals received olive oil only. The experiment was performed between October and February 2003, and pups were observed for development weekly until killed, following approved procedures, for examination of the penis and reproductive hormones at 18 (prepuberty), 41 (puberty), and 120 (adult) days of age. Because penises of these animals were not measured for weight, length, and diameter, these measurements were made in animals of another experiment performed between July and October 2003, using the same treatment protocol as in experiment 1.

Examination of Penis

The penis was grossly examined for its length, diameter, and weight. The stretched length was measured from the tip of the glans penis to the midpoint of the ischial arch (the point of origin of the root of the penis) and the diameter from the middle of the body of the penis with a caliper (calibrations up to 0.1 mm). After removing the free, loose connective tissue, the entire penis was weighed and then its body was processed for histopathological, histochemical, and immunocytochemical analyses. In addition, for evaluating the development of the os penis, one penis from each group was radiographed using a cabinet radiographic system (Faxitron series; Hewlett-Packard, McMinnville, OR) and exposing samples to a tube voltage of 25 Kvp (peak anode tube kilovolts) for 3 sec. The x-ray film was developed using an automated processor (Series VI Rapid Processor, Rapid Processing X-OMAT Processor; Eastman-Kodak Co., Rochester, NY).

For all three analyses, 3–5-mm-long sections of tissues from the middle of the body of the penis were fixed in 10% formaldehyde for 24–48 h. For histopathology (n = 5/group), tissues were embedded in paraffin, cut at 5-μm thickness, and stained with hematoxylin-eosin. In addition, 1-mm-thick sections of tissues were fixed in glutaraldehyde, postfixed in osmium tetroxide, and embedded in epoxy, as described previously from our laboratory [23]. One micrometer-thick epoxy sections were stained with 1% toluidine blue in 1% borax.

For histochemical demonstration of fat (n = 5/group), tissues were fixed for 24 h in 10% formaldehyde, followed by en bloc staining of fat for 8 h with 1% osmium tetroxide dissolved in 2.5% potassium dichromate solution [24], and then processed for paraffin embedding. Sections were cut at 5-μm thickness and unparaffinized sections were examined using light microscopy. The adjacent serial sections were stained with hematoxylin-eosin to allow for examination of histological details. In addition, frozen sections from formaldehyde-fixed tissues were stained for fat with Sudan Black [24].

Methodologies for immunocytochemistry of AR and ERα (n = 5/group) are explained below. The prostate gland and the distal part of the ductus deferens were included as a positive control for AR [25] and ERα [26], respectively.

Immunocytochemistry of AR

Androgen receptor protein was immunolocalized using the PG-21 rabbit anti-rat/human AR polyclonal antibody (PG-21), at a concentration of 2 μg/ml, according to methods described by Prins et al. first for frozen sections [25] and then modified for paraffin sections [27]. Briefly, sections were subjected to Decloaking chamber pressure cooker (Biocare Medical, Walnut Creek, CA) heating for 5 min in 0.01 M citrate buffer, pH 6.0, cooled for 10 min, rinsed in deionized water, treated for 10 min with 3% hydrogen peroxide to remove endogenous peroxidases, and incubated for 30 min with Superblock blocking buffer in PBS (Pierce Biotechnology, Rockford, IL). While one section on each slide served as a control and received normal rabbit IgG (Vector Laboratories, Inc., Burlingame, CA), the other section on the same slide received primary antibody and then slides were incubated overnight at 4°C in a humidified chamber. The primary antibody was reacted with a species-specific biotinylated secondary antibody, and the biotin was detected with an avidin-biotin peroxidase kit (ABC-Elite; Vector Laboratories) using diaminobenzidine tetrachloride as a chromogen. Developed sections were rinsed in tap water, dehydrated in ethanol, cleared in xylene, and coverslipped using Permount (Fisher Scientific, Suwanee, GA).

Immunocytochemstry of ERα

Immunolocalization of ERα was achieved using the prediluted, ready-to-use, mouse anti-human ERα monoclonal antibody (6F11; Zymed Laboratories Inc., San Francisco, CA), and the Vectastain Elite ABC Kit (Vector Laboratories). This antibody has been shown to recognize ERα protein in formaldehyde-fixed paraffin-embedded reproductive tissues in male rats [28]. To unmask ERα, sections were subjected to a domestic pressure cooker heating for 3 min (after start of steam emission) in 0.01 M citrate buffer. After cooling the pressure cooker, slides were removed and cooled for 15 min and then washed in PBS for 5 min. The subsequent procedures were essentially identical to those suggested in the kit instructions. Briefly, sections were incubated with the diluted protein blocker reagent for 20 min, followed by treatments with ERα-specific antibody on one section (test) and with mouse isotype control fluid (Zymed Laboratories Inc.) on the other section (control) in the same slide. Slides were incubated overnight at 4°C in a humidified chamber, brought to room temperature, and incubated with biotinylated secondary antibody for 40 min at room temperature in a humidified chamber. Slides were rinsed in PBS for 5 min, placed in 3% hydrogen peroxide for 5 min to block endogenous peroxidase, rinsed in tap water, and rinsed in fresh PBS for 5 min. Following application of the avidin-biotinylated peroxidase complex for 40 min at room temperature, color was developed using diaminobenzidine tetrachloride, as described previously from our laboratory [29].

Evaluation of Immunostaining

Groups of slides representing tissues from the control and treatment groups (n = 4–5 for each group) were processed together. Sections were not counterstained with a nuclear stain to avoid masking of the immunostain and to allow direct comparisons of differences in staining intensities. However, adjacent serial sections were stained with hematoxylin-eosin for examination of histological details. On the basis of visual examination, nuclear staining intensity was designated as negative (absent), weak, moderate, or strong. An individual knowledgeable about immunocytochemistry but unaware of the experimental protocol did the evaluations. Nuclei were designated negative if the staining intensity did not differ visibly from that of negative control sections on a within-slide basis. In contrast, staining observed consistently for AR in epithelial acinar cells of the prostate was designated as strong.

Digital images of histopathological, histochemical, and immunocytochemical sections, as well as gross specimens of the penis, were captured using Leitz Orthoplan microscope (Vashaw Scientific, Inc., Norcross, GA, and Kodak Microscopy Documentation System 290; Eastman Kodak Company), and were assembled using Adobe Photoshop 7.0 (Micro-Warehouse, Norwalk, CT).

Hormonal Measurement

One blood sample was collected from the heart of each animal before necropsy, and plasma was frozen at −20°C until assayed. LH was mea-

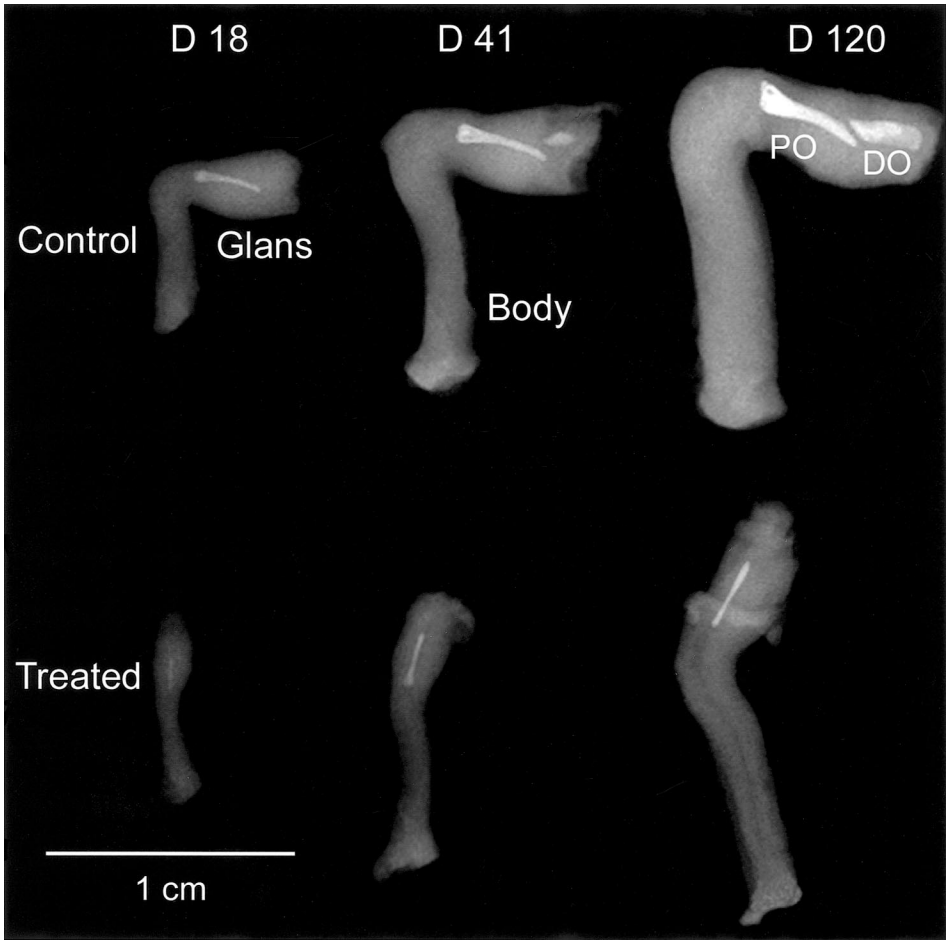

FIG. 1. Radiographs of the penis at 18, 41, and 120 days of age in rats treated with oil (control) or DES neonatally. Note reductions in length and diameter of the body and glans of the penis, a reduction in thickness of the proximal part of the os penis (P.O.), and the lack of development of the distal part of the os penis (DO), as a result of treatment. Scale bar = 1 cm.

sured using materials obtained through NHPP, NIDDK, and Dr. A.F. Parlow (antibodies: NIDDK-anti-rLH-S-11, reference standards: NIDDK-rLH-RP-3, tracers: NIDDK-rLH-I-10). The sensitivity of the assay was 0.3 ng/ml. 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 0.2 ng/ml. All samples were quantified in a single assay and the intraassay coefficient of variation was 6% and 7% for LH and T, respectively.

Statistics

Statistical analyses were performed using Sigma Stat statistical software (Jandel Scientific, Chicago, IL). One-way analysis of variance was used on body weight, and two-way analyses of variance were used on hormones, penile length, and penile weight. Treatment groups with means significantly different ($P < 0.05$) from controls were identified using Dunnett test. When data were not distributed normally or heterogeneity of variance was identified, analyses were performed on transformed data or ranked data.

RESULTS

Body Weight

The mean (±SEM) body weight in treated animals, in comparison with that in controls, was significantly ($P < 0.05$) lower at Day 18 (31.4 ± 0.7 vs. 44.9 ± 0.8 g), Day 41 (133.0 ± 2.2 vs. 160.7 ± 8.4 g), and Day 120 (425.0 ± 7.4 vs. 470.6 ± 7.1).

Gross Observations of the Penis

Grossly, the penis of the adult rat has a long, cylindrical body (shaft) and a bulbous glans penis, and the transition between the two is characterized by a right angle. The unique feature of the rat penis is a cylindrical bone that extends from the distal end of the body to the tip of the glans penis (Fig. 1). While both proximal (closer to the body) and distal parts of the os penis were present at 41 and 120 days of age, only the proximal part was developed at Day 18 in control animals. All gross parts of the penis, as well as their spatial organization, were identifiable at 18 days of age in both treated and control animals. However, the length, right angle, diameter, and weight of the penis were reduced ($P < 0.05$) as a result of DES treatment, and these reductions persisted at puberty and adulthood (Figs. 1 and 2). In addition, while the distal part of the os penis did not develop, the proximal part developed as a thin bone at Days 41 and 120. The preputial sheath started to separate at 40–42 days of age and was completely free by 50 days of age in control rats. Conversely, it was still partially attached at 120 days of age in treated rats.

Microscopic Observations of the Penis

The body of the adult penis consists of paired corpora cavernosa that are located dorsolaterally and separated by an incomplete septum carrying blood vessels and nerves and a corpus spongiosus that is located ventrally and surrounds the urethra. All of these penile components were not only present but also had similar spatial organization at 18 days of age in both the treated and control rats (Fig. 3, A and E).

The corpus cavernosus penis of control rats at 18 days of age already contained three main components: cavernous spaces, intercavernous stroma, and tunica albuginea (Fig.

3, A–C). The latter formed the capsule of the body of the penis and consisted of an outer fibrous layer and an inner cellular layer, which, in turn, constituted the outer boundary of the cavernous tissue. The cavernous spaces, lined by endothelium and surrounded by smooth muscle cells, were irregular in diameter and mainly concentrated under the cellular layer of tunica albuginea. The intercavernous stroma, probably a continuation of the tunica albuginea, contained both collagen-like fibers and fibroblasts, but the latter predominated. In addition, a substantial number of fat cells, characterized by round, empty spaces in the hematoxylin and eosin-stained paraffin sections and by black, fat droplets in unparaffinized sections stained en bloc with osmium tetroxide, were seen mainly under the cellular layer of tunica albuginea (Fig. 3D).

Conversely, the corpus cavernosus penis of treated rats at 18 days of age lacked both cavernous spaces and the surrounding smooth muscle cells, although some blood vessels were invariably observed (Fig. 3, F and G). The stromal tissue (same as intercavernous stromal tissue in controls, but could not be called so in treated animals because cavernous spaces were absent) was scant in fibers but replete with fibroblasts, which were relatively more abundant here than those in the 18-day control group. In addition, many fat cells (apparently more than in controls) were present throughout the stroma (Fig. 3, G and H), especially numerous under the tunica albuginea, which was reduced to a thin band of cells and fibers.

The corpus cavernosus penis in control rats at Day 41, in comparison with that at Day 18, contained wider cavernous spaces, more fibers, and fewer fibroblasts in the intercavernous stroma and possessed thicker tunica albuginea (Fig. 4, A and B). However, fat cells appeared to be as numerous as in the 18-day-old rats (Fig. 4C). At 120 days of age, the morphology of the corpus cavernosus was essentially similar to that of the pubertal rats, except for an apparent increase in the width of cavernous spaces and density of fibers and an apparent decrease in numbers of fibroblasts in the intercavernous stroma. Only isolated fat cells under the cellular layer of the tunica albuginea were observed in this age group (Fig. 4D).

On the other hand, the corpus cavernosus penis of treated animals at both puberty and adulthood was dominated by wide spaces, the boundaries of which were distinct and demarcated by thin fibers (resembling reticular fibers) in the hematoxylin and eosin-stained sections (Fig. 4, E and F). These spaces contained lipid droplets as revealed by epoxy sections stained with toluidine blue, unparaffinized sections stained en bloc with osmium tetroxide (Fig. 4G), and frozen sections stained with Sudan Black (Fig. 4H). In addition, the tunica albuginea was reduced in thickness and contained fewer fibers and cells, in comparison to controls.

Unlike the corpus cavernosus penis, the corpus spongiosus penis had similar morphology in both the treated and control rats of the respective age groups. It consisted of a urethra that was centrally located and lined by transitional epithelium and of peri-urethral stroma that contained fibroblasts, fibers, blood vessels, and cavernous spaces. The latter were much less developed in the corpus spongiosus than in the corpus cavernosus regardless of the age group (not shown).

Immunocytochemical Analysis of AR and ERα in the Penis

For the purpose of clarity, immunocytochemistry of AR and ERα is described under three morphological components of the body of the penis, corpus cavernosus (Fig. 5, A–L), corpus spongiosus (Fig. 6, A–L), and intercrural septum (Fig. 7, A–L). The age- and/or treatment-related effects on the staining intensity of positive cells are summarized in Table 1. The staining intensity was scored as negative (−), weak (+), moderate (++), or strong (+++), and that for AR in epithelial cells of the prostate gland was used as a benchmark for strong staining (Fig. 5M). No specific staining for AR or ERα was noted in control or treated animals when the adjacent section on the same slide was incubated with irrelevant IgG instead of the primary antibody (Figs. 5, N and O, and 6, M and N). As expected, the ductus deferens, included as a positive control for treated animals, exhibited an altered and enhanced expression of ERα, as a result of neonatal DES treatment (Fig. 6, O and P).

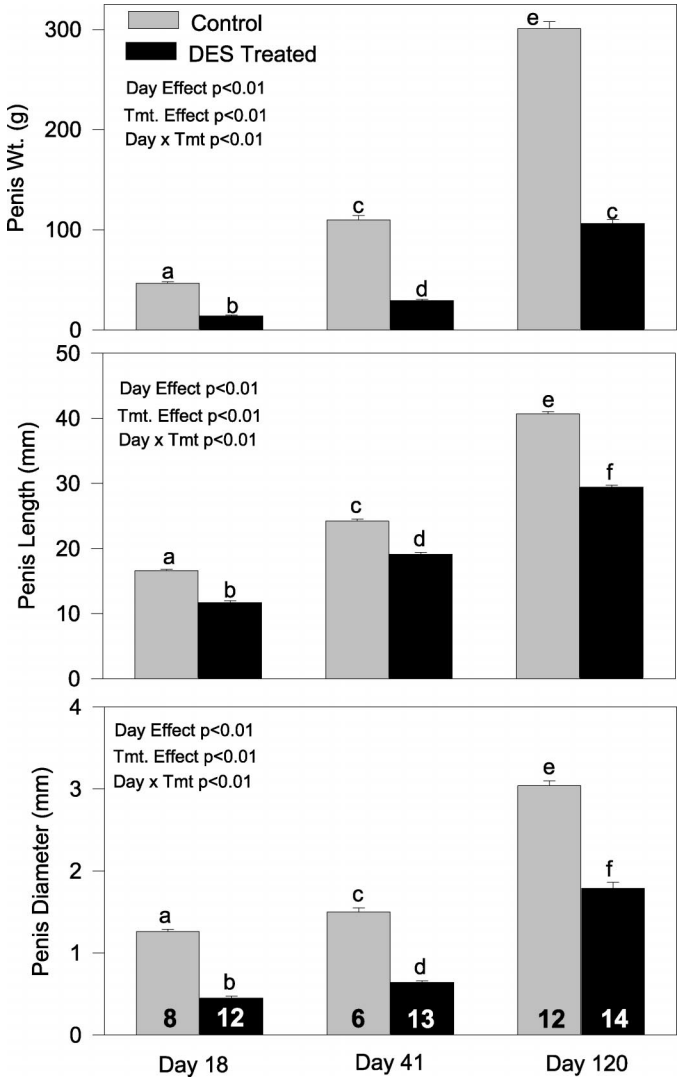

FIG. 2. Effect of exposure of neonatal male rats to DES on the weight, length, and diameter of the penis at 18, 41, and 120 days of age. Data are expressed as mean ± SEM. Bars without a common superscript are significantly different ($P < 0.01$).

Corpus Cavernosus

AR. In controls, all cellular components of the corpus cavernosus, including endothelial and smooth muscle cells of cavernous spaces and fibroblasts of intercavenous stroma

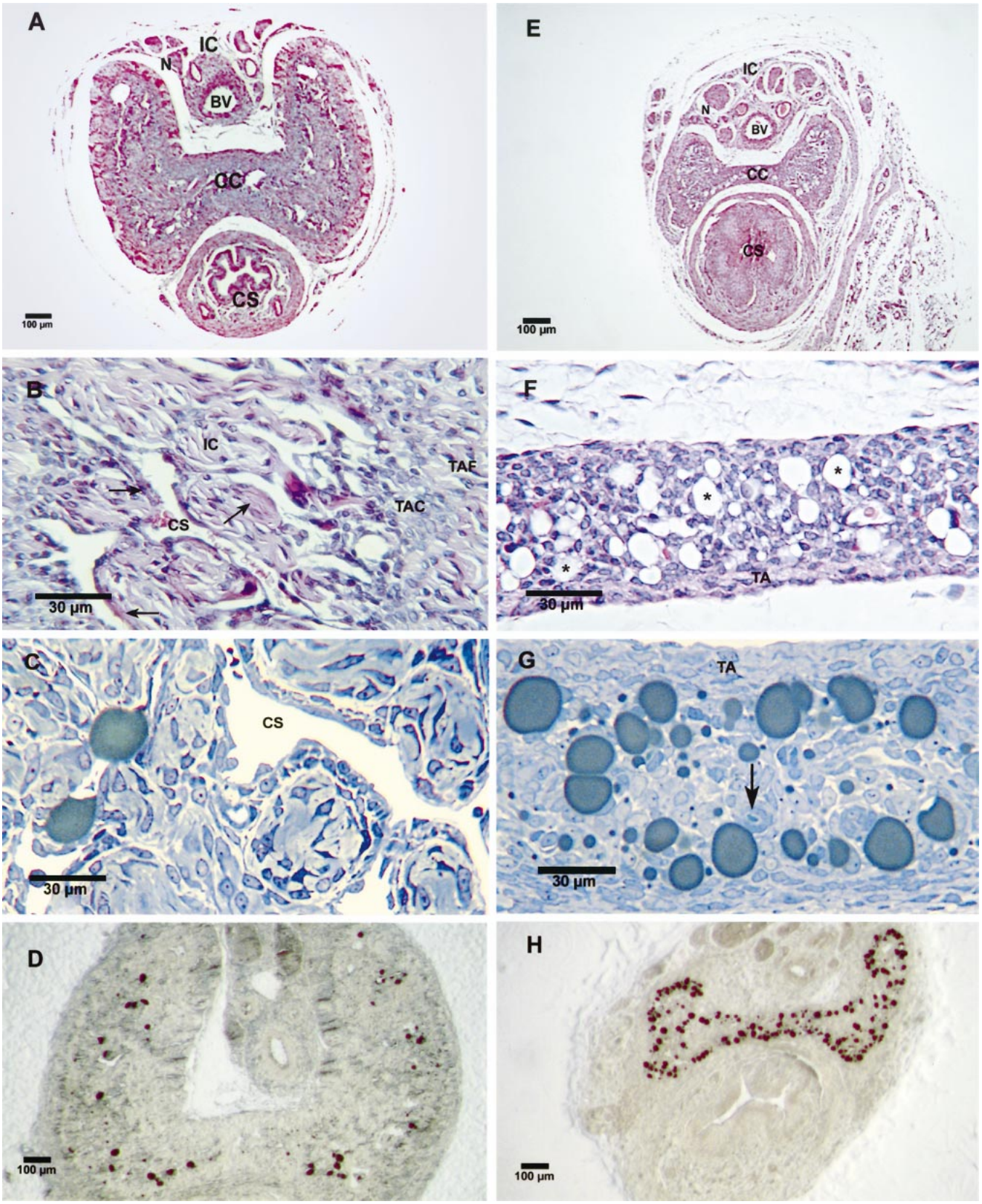

FIG. 3. Micrographs from the body of the penis in control rats (**A**–**D**) and in rats treated with DES neonatally (**E**–**H**) at 18 days of age. **A**, **E**) Note similarity in spatial arrangement of different parts of the body of the penis between control and treated rats: paired corpora cavernosa (CC), corpus spongiosus (CS), and intercrural septum (IC) containing blood vessels (BV) and nerves (N). **B**) Corpus cavernosus penis from the control rat showing tunica albuginea fibrous (TAF), tunica albuginea cellular (TAC), cavernous spaces (CS), and intercavernous septa (IC) containing fibers, stromal fibroblasts, and smooth muscle cells (arrows) under the endothelium of cavernous spaces. **F**) Conversely, in the corpus cavernosum penis of the treated rat, note absence of cavernous spaces, decreased thickness of tunica albuginea (TA), and increased deposition of empty-appearing fat cells (*). **C**, **G**) Epoxy sections of the corpus cavernosum from the control and treated rats. Note increased deposition of fat cells in the treated rat; cavernous spaces (CS), tunica albuginea (TA), small arteriole (arrow). **D**, **H**) In these low-magnification, unparaffinized sections of the body of the penis, note much higher accumulation of fat cells in the corpora cavernosa penis of the treated rat. **A**, **B**, **E**, and **F**) Hematoxylin and eosin, (**C** and **G**) toluidine blue, (**D** and **H**) fat stain. Scale bars = **A**, **D**, **E**, and **H**, 100 μm; **B**, **C**, **F**, and **G**, 30 μm.

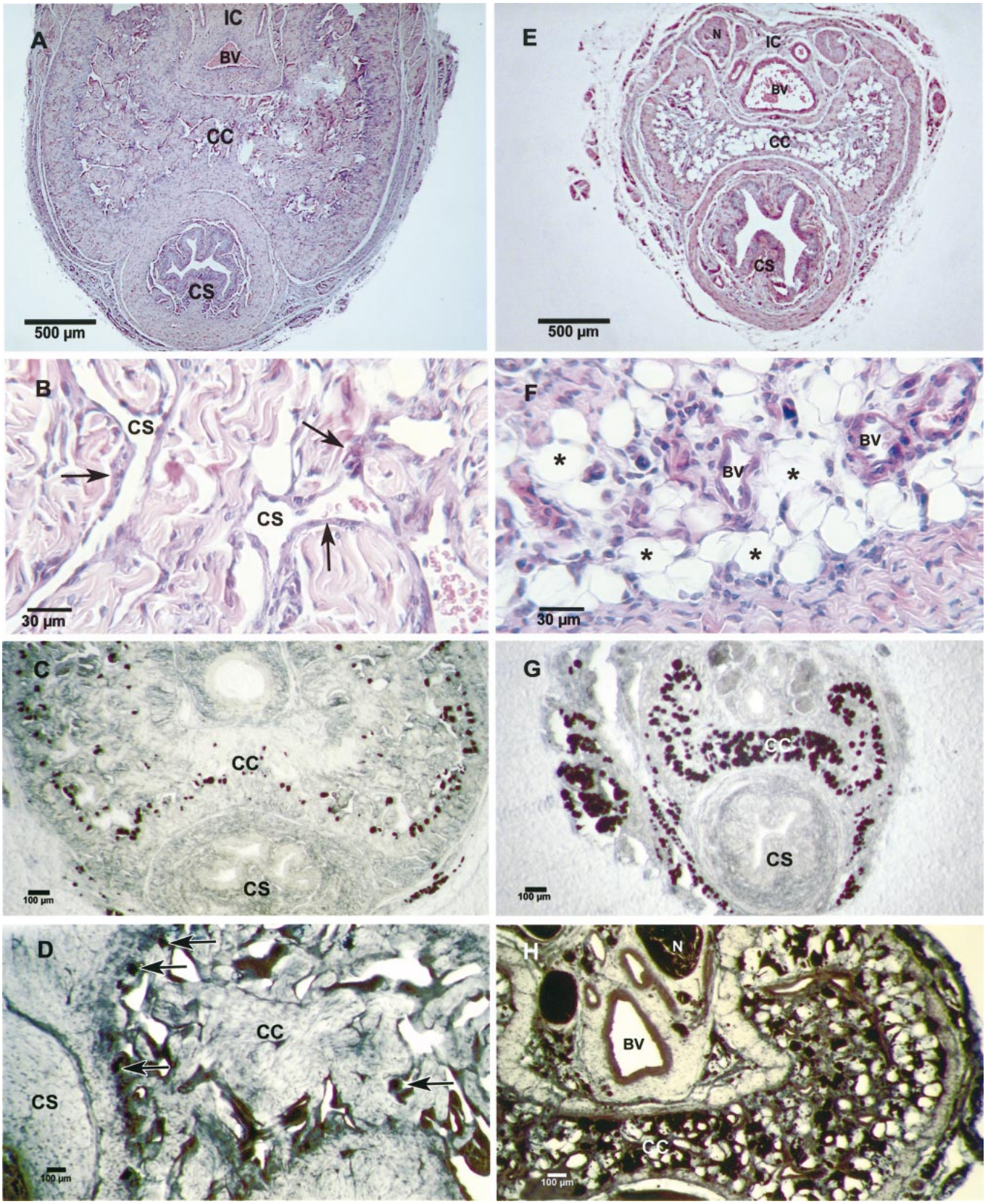

FIG. 4. Micrographs from the body of the penis in control rats (**A**–**D**) and in rats treated with DES neonatally (**E**–**H**) at 41 days (**A**–**C**, **E**–**G**), and 120 days (**D**, **H**) of age. **A**, **E**) Although the spatial arrangement of different parts of the body of the penis, paired corpora cavernosa (CC), corpus spongiosus (CS), and intercrural septum (IC) containing blood vessels (BV) and nerves (N), is similar between control and treated rats; the latter have many more empty-appearing, wider spaces but fewer fibers in the corpora cavernosa. **B, F**) In these micrographs of the corpora cavernosa penis, note cavernous spaces (CS) and smooth muscle cells (arrows) under the endothelium in the control rat, in contrast with accumulation of empty-appearing fat cells (*) in the treated rat. Also, note the presence of blood vessels (BV) in the treated rat. **C**, **G**) In these low-magnification, unparaffinized sections of the body of the penis, note increased deposition of fat cells in the corpora cavernosa penis (CC) of the treated rat. **D**, **H**) Frozen sections of the corpora cavernosa (CC) at 120 days of age. Note a few, isolated fat cells (arrows) adjacent to cavernous spaces in the control rat, in contrast with virtually complete replacement of cavernous spaces by fat cells in the treated rat. Corpus spongiosus, (CS), blood vessels (BV), nerve (N). **A**, **B**, **E**, and **F**, hematoxylin and eosin; **C**, **D**, **G**, and **H**, fat stain. Scale bars = **A** and **E**, 500 μm; **B** and **F**, 30 μm; **C**, **D**, **G**, and **H**, 100 μm.

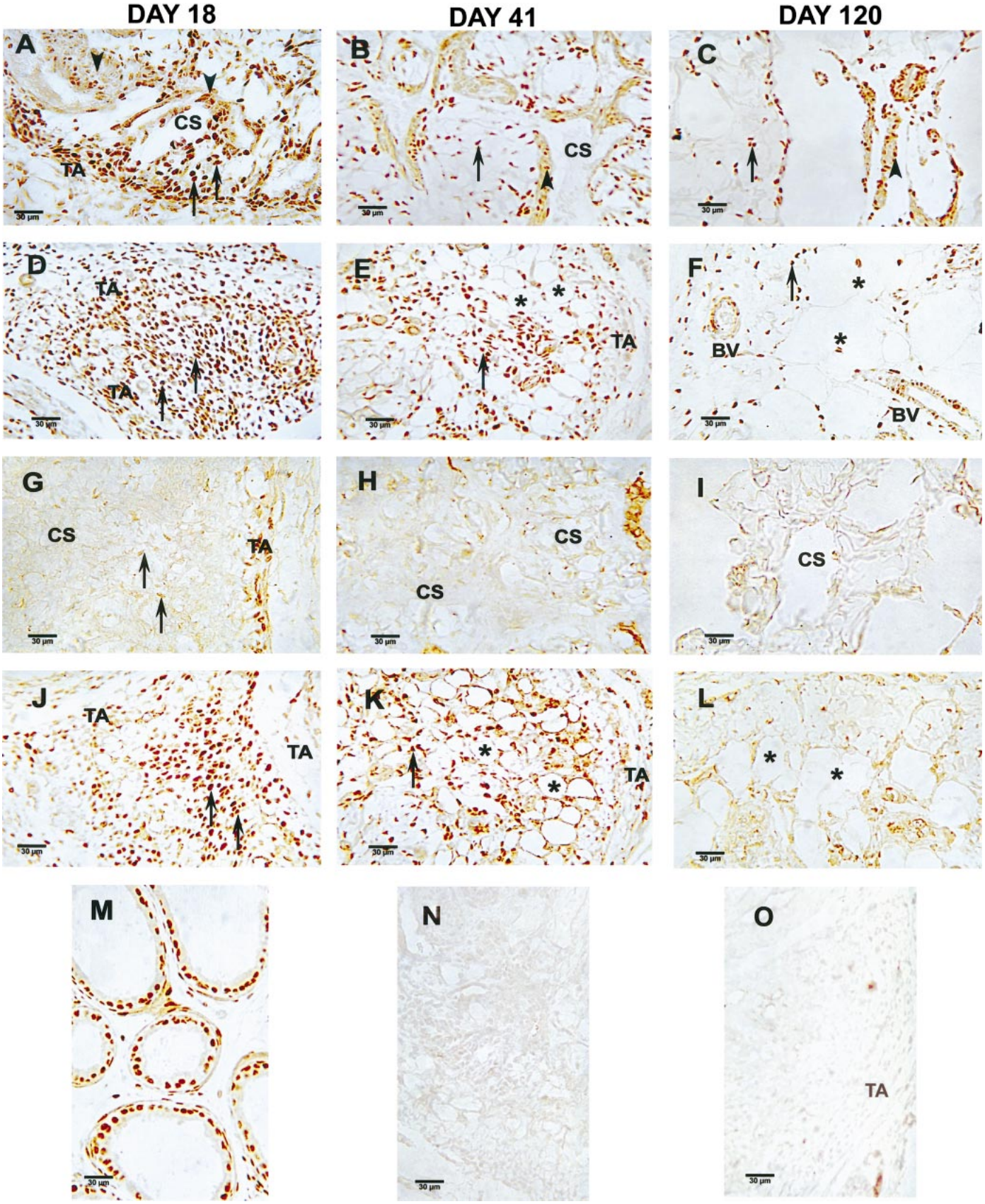

FIG. 5. Immunolocalization of androgen receptor (**A**–**F**) and estrogen receptor α (**G**–**L**) in the corpus cavernosus penis at 18, 41, and 120 days of age in rats treated neonatally with oil (control) (**A**–**C**, **G**–**I**) or DES (**D**–**F**, **J**–**L**). Fibroblasts (arrow), smooth muscle cells (arrowhead), fat cells (star), cavernous spaces (CS), blood vessels (BV), tunica albuginea (TA). **A**–**F**) Note that DES treatment affected neither the incidence nor the staining intensity of androgen receptor-positive cells, regardless of the age (compare **A**–**C** with **D**–**F**). **G**–**L**) Conversely, note a marked increase in the expression of estrogen receptor α-positive cells at Days 18 and 41, especially Day 18 (compare **G**–**I** with **J**–**L**). **M**) Positive control section for androgen receptor from the prostate gland of a mature rat showing a strong nuclear staining in epithelial cells. **N**, **O**) Control sections of the corpus cavernosus penis at Day 18 incubated with blocking serum in place of primary antibody for androgen receptor (**N**) or estrogen receptor α (**O**). Scale bar = 30μm.

and tunica albuginea, exhibited a strong to moderate staining for AR in all age groups (Fig. 5, A–C). The staining was nuclear in all cells, except smooth muscle cells, where the cytoplasm also stained. Generally, the staining intensity of positive nuclei declined from Day 18 to Day 41 but was essentially similar between the latter and Day 120. Similarly, in treated animals, nuclei of all cellular components, including those of fat cells, showed a strong to moderate staining for AR in all age groups (Fig. 5, D–F). Comparatively, AR-positive cells appeared to decrease in numbers with age, especially from Day 18 to Day 41, in both control and treated animals.

ERα. Unlike the ubiquitous presence of AR-positive cells, most cells of the corpus cavernosus in control animals were negative for ERα in all age groups, except a weak to moderate nuclear staining in some cells of the tunica albuginea and a very weak nuclear staining (slightly higher than the background) in some fibroblasts of the intercavernous stroma at Day 18 only (Fig. 5, G–I). Conversely, in treated animals, most nuclei of fibroblasts and fat cells in the stromal tissue and some nuclei in the cellular layer of tunica albuginea at Day 18 and 41 exhibited a strong to moderate staining for ERα, although, comparatively, there were fewer ERα-positive cells at Day 41 (Fig. 5, J and K). Only few fibroblasts and/or fat cells of the stromal tissue were weakly positive at Day 120 (Fig. 5L).

Corpus Spongiosus

AR. In controls, all epithelial cells lining the urethra and stromal cells under the epithelium exhibited a strong to moderate nuclear staining in all age groups (Fig. 6, A–C). Conversely, in treated animals, the nuclear staining in both urethral epithelial and stromal cells was reduced at Days 41 and 120, although the staining intensity in both cell types at Day 18 was similar to that of controls (Fig. 6, D–F). Comparatively, numbers of AR-positive stromal cells appeared to decrease with age, especially from Day 18 to Day 41, in both control and treated animals.

ERα. In controls, while urethral epithelial cells (except occasional basal cells at Day 18), stained negative for ERα in all age groups, stromal cells under the epithelium exhibited a moderate to weak nuclear staining in all age groups (Fig. 6, G–I). On the other hand, in treated animals, although the staining of stromal cells was similar to that of controls, it covered a deeper stromal area under the epithelium in all age groups (Fig. 6, J–L). In addition, most basal epithelial cells in the urethra showed a moderate to weak nuclear staining at Days 18 and 41. Apparently, numbers of ERα-positive stromal cells decreased with age, especially from Day 18 to Day 41, in both control and treated animals.

Intercrural Septum

AR. A moderate nuclear staining in endothelial and smooth muscle cells of blood vessels and fibroblasts of the stroma and a weak nuclear staining in some fibroblasts and/or Schwann cells of the nerve were observed in all age groups in both control (Fig. 7, A–C) and treated (Fig. 7, D and F) rats. In addition, the cytoplasm of smooth muscle cells was also positive in both control and treated animals.

ERα. Except a few nuclei of fibroblast-like cells adjoining blood vessels in some animals at Days 18 and 41, neither endothelial nor smooth muscle cells of blood vessels were positive for ERα in control rats of any age group (Fig. 7, G–I). Conversely, many nuclei in the stroma were weakly positive in treated animals of all age groups (Fig. 7, J–L).

Reproductive Hormones

Testosterone. The mean plasma T concentration in control animals increased from a negligible level at Day 18 to almost 1.0 ng/ml at Day 41 to almost 1.50 ng/ml at Day 120. Conversely, it was negligible at all age groups in treated animals (Fig. 8).

LH. The mean plasma concentration of LH was not different ($P < 0.05$) between control and treated animals, regardless of the age, and ranged from 0.51 to 0.82 ng/ml among groups (Fig. 8).

DISCUSSION

Previously, we reported that adult Sprague-Dawley rats treated neonatally with DES were infertile and the loss of fertility was associated with the loss of cavernous spaces and accumulation of fat cells in the body of the penis [13]. Objectives of this study were to determine time-dependent morphological changes and their association, if any, with developmental changes in the profile (distribution and staining intensity) of AR and ERα in the body of the penis at 18 (prepuberty), 41 (puberty), and 120 (adult) days of age in rats treated with DES neonatally. Results revealed that neonatal DES exposure resulted in abnormal morphology of the penis at 18 days of age, and it was associated with an altered and enhanced expression of ERα, without alterations in patterns of AR distribution. Morphological abnormalities included reductions in the length, weight, right angle, and diameter of the penis, loss of cavernous spaces and adjacent smooth muscle cells, and increased deposition of fat cells in the body of the penis. Not only did these abnormalities persist, but fat cells virtually filled the entire area of the corpora cavernosa penis at both puberty and adulthood. These results provide the first description in the developing rat penis of estrogen-associated gene imprinting in stromal cells that leads to the observed phenotypic abnormalities at puberty and adulthood.

To our knowledge, similar histopathological changes in the penis have not been previously reported, regardless of the dose, length, and/or time (prenatal or postnatal) of the estrogenic exposure in the rat or any other species except the rabbit [30]. These authors reported thickening of the tunica albuginea, subtunical deposition of fat, decreased cavernous spaces, and increased trabecular smooth muscle cells in the body of the penis in New Zealand White rabbits that were treated with bisphenol A for 12 days at 8–12 weeks of age and examined 4–8 weeks after the treatment. Reasons for differences between the two studies may be attributed to differences in the species (rat vs. rabbit), estrogenic compound (DES vs. bisphenol A), and/or the time (neonatal vs. pubertal) of treatment. Regardless of the differences, results of both studies are indicative of potential for erectile dysfunction and point to the cavernous spaces and their smooth muscle cells in the body of the penis as main target sites for estrogen action. In light of these observations, it may be important to investigate in depth the reproductive status, especially the incidence of erectile dysfunctions, in male offspring of women exposed to DES during pregnancy. It is noteworthy that women treated with DES during gestation at Boston Lying-in Hospital received a total median dose of 12 200 mg (equal to 200 mg/kg, based on 60 kg average weight of a pregnant woman) [10], in contrast with a total dose of about 3 mg/kg given to

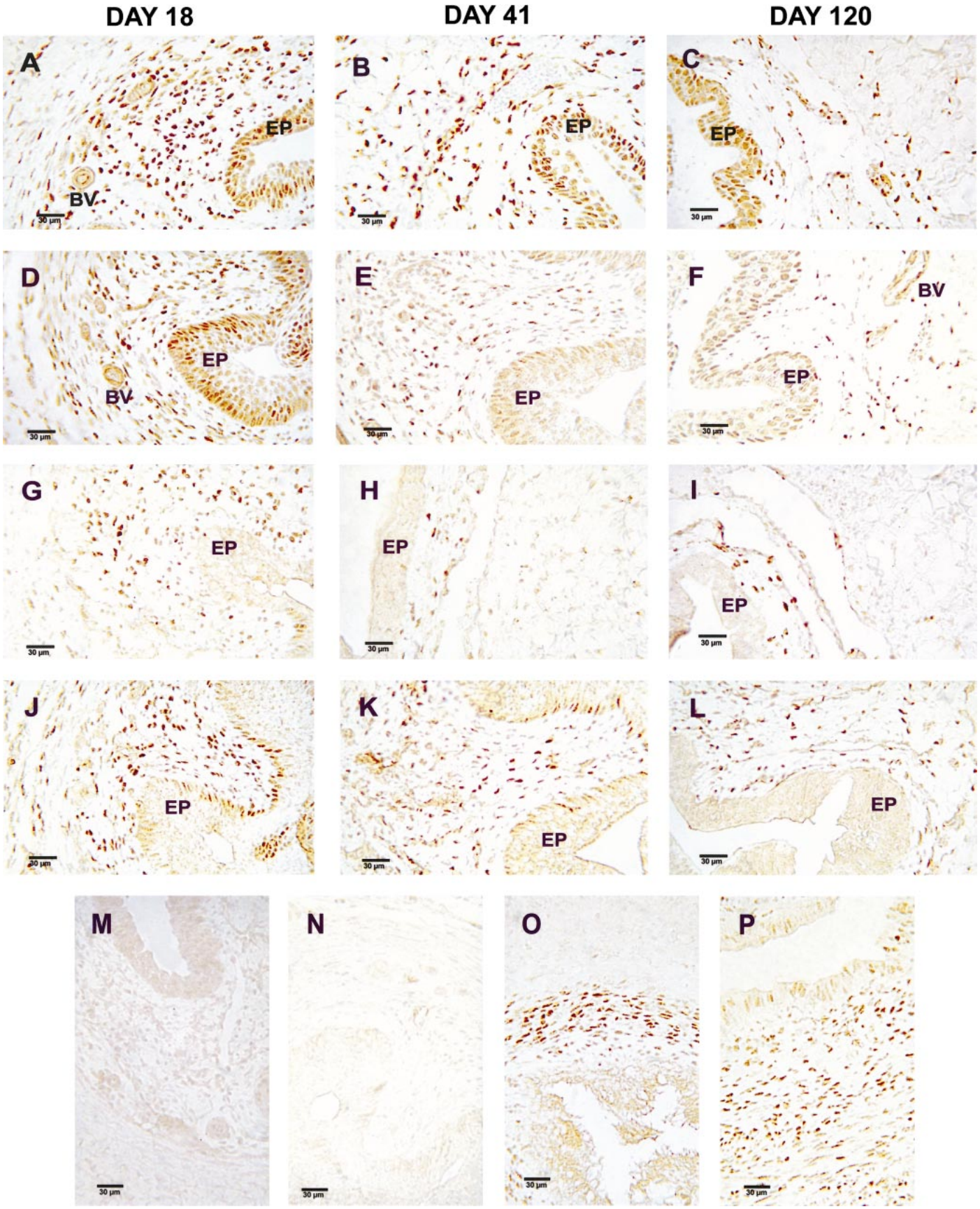

FIG. 6. Immunolocalization of androgen receptor (**A**–**F**) and estrogen receptor α (**G**–**L**) in the corpus spongiosus penis at 18, 41, and 120 days of age in rats treated neonatally with oil (control) (**A**–**C**, **G**–**I**) or DES (**D**–**F**, **J**–**L**). Urethral epithelium (EP), blood vessels (BV). **A**–**F**) Note similarity in androgen receptor-positive cells at Day 18 between control and treated rats (compare **A** with **D**), but a reduction in staining intensity, especially in epithelial cells, in treated rats at Days 41 and 120 (compare **B** and **C** with **E** and **F**). **G**–**L**) Note DES-induced enhanced expression of estrogen receptor α in basal cells of the urethral epithelium at Days 18 and 41 (compare **G** and **H** with **J** and **K**). Also, note a relative increase in numbers of estrogen receptor α-positive stromal cells under

neonatal pups of the present study. Despite the fact that more than two million women were exposed to DES in the United States only [10], to our knowledge, there is no published report on the morphology of penises in the aborted male fetuses or the adult men; however, based on a small cohort of the DES-exposed men studied for fertility, they appeared to be fertile [31].

Barring histopathological changes, the dose-dependent reduction in the length of the penis and delayed separation of preputial sheath were previously reported in rats treated neonatally with estrogens [32]. Similarly, unusually small phalluses in alligators from Lake Apopka in Florida were linked to an excessive spill of estrogenic compounds in the lake [33, 34]. However, the gross abnormality affecting the right angle between the body of the penis and the glans penis that we observed in treated rats has not been reported previously. The reduction in the right angle was almost as apparent at Day 18 as at Days 41 or 120. This may have resulted from the malformation of the os penis, which showed signs of calcification at Day 18 and was fully calcified and developed at Day 41 in control animals but was not calcified at Day 18 and showed only limited development and calcification at Day 41 of the treated animals. The ontogeny of the os penis was shown to be androgen-dependent in both rats and mice [35–37], and neonatal treatment with an antiandrogen decreased its length and calcification [38].

It is well known that the erection of the penis in mammals, including rats, depends on the engorgement of cavernous spaces with blood and the relaxation of smooth muscle cells [39]. Therefore, the absence of both of these components in the corpora cavernosa, but not in the corpus spongiosus, of rats treated neonatally with DES is functionally and clinically important. The reason for their absence, while speculative, may lie in the difference in the blood supply of the two cavernous bodies. Whereas the corpus spongiosus gets its major blood supply from the artery of the bulb (a branch of the artery of the penis), the corpora cavernosa are mainly supplied by the helicine artery (a branch of the deep artery of the penis), which, unlike most other body arteries, has a thick layer of smooth muscle cells under the endothelium [40, 41]. Because both cavernous spaces and adjacent smooth muscle cells are descendents of the helicine artery [39], their absence suggests a selective effect of neonatal estrogen treatment at the level of the helicine artery and beyond.

We hypothesize that, rather than breaking into cavernous spaces, the aberrant helicine artery formed arterioles and capillaries, similar to other body arteries, which provided the needed nutrients to the corpora cavernosa. This would explain the invariant presence of arterioles and capillaries in the corpora cavernosa of all treated animals in this study regardless of age. If this hypothesis is valid, similar effects are less likely to occur if the treatment is initiated after the development of cavernous spaces and smooth muscle cells has taken place in the corpora cavernosa of control animals (approximately Day 18). In this context, it is worth noting that both cavernous spaces and smooth muscle cells were unaltered in adult rats treated with DES at adulthood, although these rats, like the rats treated neonatally with DES and examined at adulthood, had lower fertility and altered sexual behavior [42].

Regardless of the validity of the above hypothesis, it is evident that exposure of neonatal rats to DES led to the loss of cavernous spaces and smooth muscle cells in the corpora cavernosa penis. There could be many unexplained hormonal and/or molecular reasons for their loss, but knowing that postnatal development of the penis is dependent on androgens [2], that penile erection is dependent on androgens [43], and that androgen receptors are present at peak levels in the prepubertal penis of rats [14, 15], it is reasonable to assume that alterations in androgens and/or androgen receptors are involved in the observed effects. Phallus abnormalities reported in rats castrated at birth and their partial restoration upon androgen substitution at the time of castration [44] provide credence to the above reasoning. The coadministration of T with DES prevented most of the gross and histological abnormalities affecting the male reproductive tract, including overgrowth and distention of the rete testis, underdevelopment of epithelium in the efferent ductules and epididymis, and coiling of the vas deferens in Wistar rats treated neonatally with DES [22]. Although it remains to be determined whether neonatal supplementation with T will prevent DES-induced penile abnormalities, the plasma T was almost at an undetectable level in treated rats of all age groups in the present study. Similarly, previous studies also reported a marked reduction in plasma T in rats treated neonatally with estrogens [18, 19, 22, 45].

Despite reduced T in treated animals, the close similarity that we observed in the incidence and staining intensity of AR-positive cells in the body of the penis between control and treated animals in all age groups, especially in stromal cells in the corpora cavernosa penis at 18 days of age, suggests that the presence of AR in the body of the penis is not influenced by an exposure of neonatal rats to DES and/or by reduced T. In as much as the latter is concerned, the present observations cast a doubt on the concept that androgens down-regulate AR in the developing penis [15], but support the concept that androgens are not major regulators of AR during growth of the penis [46, 47]. Nevertheless, the present results, in agreement with those of the previous studies [14, 17], found an age-dependent decline in AR expression, with the strongest staining present at prepuberty. However, it must be noted that the AR decline was more due to a relative decrease in the number of stromal cells than a relative decrease in the staining intensity. The decrease in the number of stromal cells resulted from extacellular stromal expansion of cavernous spaces and connective tissue fibers in the case of control animals and accumulation of fat cells in the treated animals. Contrary to our observations of no alterations in the profile of AR in the rat penis, the neonatal exposure to estrogen caused a reduction in the expression of AR in the rest of the male reproductive tract, including efferent ductules, epididymis, ductus deferens, and seminal vesicle [48, 49], and prostate [20, 49, 50], suggesting an organ-specific effect of estrogen on AR.

Another important finding of the present study, in conjunction with the loss of cavernous spaces and adjacent smooth muscle cells, was the up-regulation of ERα in the body of the penis as a result of neonatal exposure to DES. Interestingly, although the increased staining for ERα appeared to be a general phenomenon of stromal cells, it was particularly marked in stromal cells of the corpora caver-

the epithelium, as a result of the treatment. **M**, **N**) Control sections of the corpus spongiosus penis at Day 18 incubated with blocking serum in place of primary antibody for androgen receptor (**M**) or estrogen receptor α (**N**). **O**, **P**) Positive control sections stained for estrogen receptor α from the ductus deferens of controls (**O**) and treated (**P**) rats. Note enhanced expression of the receptor, as a result of the treatment. Scale bar = 30μm.

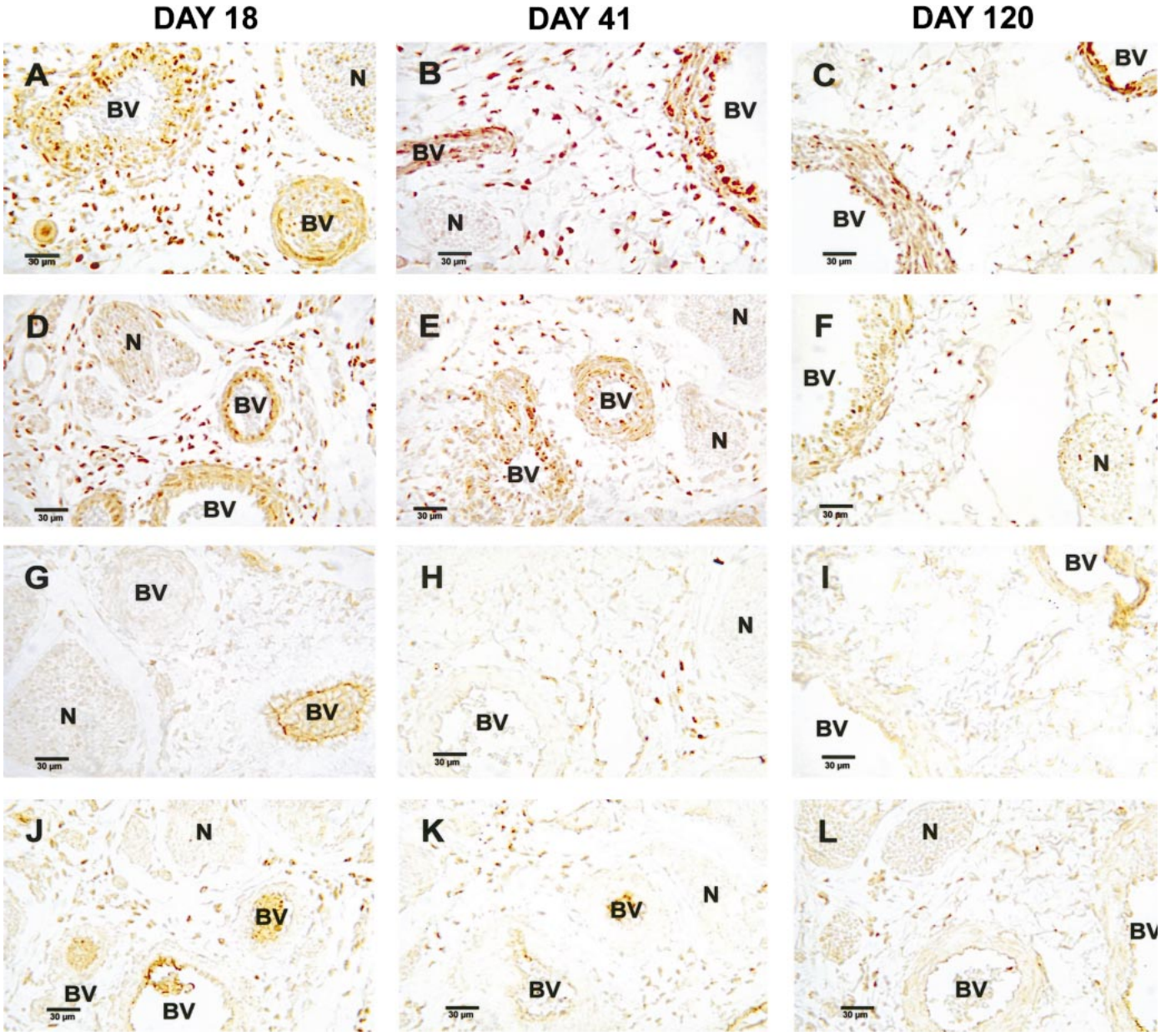

FIG. 7. Immunolocalization of androgen receptor (**A**–**F**) and estrogen receptor α (**G**–**L**) in the intercrural septum of the penis at 18, 41, and 120 days of age in rats treated neonatally with oil (control) (**A**–**C**, **G**–**I**) or DES (**D**–**F**, **J**–**L**). Nerve (N), blood vessels (BV). **A**–**F**) Note similarity in androgen receptor-positive cells between control and treated rats, regardless of the age (compare **A**–**C** with **D**–**F**). **G**–**L**) Note DES-induced enhanced expression of estrogen receptor α-positive stromal cells (compare **G**–**I** with **J**–**L**). Control sections incubated with blocking serum in place of primary antibody were negative (not shown). Scale bar = 30μm.

nosa penis at 18 days of age, the developmental stage when they were the predominant cell type in both control and treated animals and the stage in which signs of abnormal penile morphology were already evident. To our knowledge, this is the first study to show DES-induced enhancement of ERα at the cellular level in the rat penis, although both ERα and ERβ mRNAs and proteins have been identified in the rat penis at Day 1, with ERα being prevalent in the penis spongiosus and ERβ in the penis cavernosus, blood vessels, and nerves [28]. Contrary to our results, these authors, using Western blot analysis, reported a decline in both ER proteins in the 8-wk-old male rats that received 2 μg of DES twice per week for 3 wk. Reasons for differences in results between the two studies may lie in the time of treatment (almost puberty vs. neonatal in our study) and/or the dose of DES. Furthermore, according to these authors, more than 70% reduction in immunodetectable signal of ERα had already occurred between 1 and 8 wk of age in control rats, thus implying that whatever decrease resulted from the DES treatment must be minimal. On the other hand, similar to present findings, neonatal estrogen exposure up-regulated the expression of ERα in the mouse uterus [51], murine male reproductive tract [52], and rat prostate [21, 27]. In this context, observations that ERα-knockout mice of both sexes are infertile [3] and that prostate [27] and female reproductive tract [53] of ERα-knockout mice are resistant to DES-induced developmental abnormalities indicate a special role of ERα in mediating pathophysiology of reproduction. Hence, based on these results and those of the related previous studies, it may be reasonable to speculate that the observed structural changes in the penis resulted from DES-induced decreased T and/

TABLE 1. Comparison of immunostaining of androgen receptor (AR) and estrogen receptor α (ERα) expression in the body of the penis at 18, 41, and 120 days of age in rats treated with DES or oil (control).[a]

	AR						ERα					
	Oil			DES			Oil			DES		
Organ/tissue/cell	Day 18	Day 41	Day 120	Day 18	Day 41	Day 120	Day 18	Day 41	Day 120	Day 18	Day 41	Day 120
Corpus cavernosus												
Endothelium	+++	++	++	++	++	++	−	−	−	−	−	−
Stromal cells	+++	++	++	+++	++	++	+/−	−	−	+++	++	+/−
Smooth muscle	+++	++	++	++	++	++	−	−	−	−	−	−
Corpus spongiosus												
Urethral epithelium	++	++	++	++	+	+	−	−	−	++/−	+/−	−
Stomal cells	+++	++	++	++/+	+	+	++/+	++/+	++/+	++	++/+	+
Intercrural septum												
Endothelium	++	++	++	++	++	++	−	−	−	−	−	−
Stromal cells	++	++	++	++	++	++	+/−	+/−	−	+	+	+
Smooth muscle	++	++	++	++	++	++	−	−	−	−	−	−

[a] The intensity of nuclear staining is based on subjective comparison with AR staining in epithelial cells of the prostate, which is designated strong. (−) Negative, (+) weak, (++) moderate, (+++) strong, (+/−), where all cells of the same type are not positive. The distal part of the ductus deferens was used as a positive control for ERα and the prostate gland for AR.

FIG. 8. Effect of exposure of neonatal male rats to DES on plasma testosterone and LH at 18, 41, and 120 days of age. Data are expressed as mean ± SEM. *, Significantly different ($P < 0.05$) from controls.

or enhanced expression of ERα. To determine whether one or both factors are contributors would warrant additional experiments.

Factors responsible for the selective accumulation of fat cells in the corpora cavernosa penis of treated rats and whether this treatment response is the cause or effect of loss of cavernous spaces and associated smooth muscle cells are issues that remain to be clarified. However, the present observations that fat cells were present in appreciable numbers in the corpora cavernosa of control rats at 18 and 41 days of age and declined to a few cells at 120 days indicate that they are normal components of the corpora cavernosa and may be an additional source of energy at a time of the maximum growth of the penis. These observations also suggest that the precipitous decrease in fat cells from puberty to adulthood in control rats and their dramatic increase from prepuberty to puberty in treated rats may have resulted simply from the expansion of cavernous spaces in the former and the absence thereof in the latter. Alternately, observations may reflect complex interactions of AR and ERα signal transduction systems actions because the present study, as well as previous studies [54, review], have identified receptors for both hormones in fat cells. In this context, it is noteworthy that the absence of ERα in αERKO mice caused hypertrophy and hyperplasia of adipocytes in both sexes [55] and T treatment decreased fat in hypogonadal men [56], implying the significance of both receptors in the regulation of fat cells.

In conclusion, our results provide evidence that exposure of neonatal male rats to DES induces abnormal morphology of the penis, which includes 1) reductions in length, weight, diameter, and right angle between the glans penis and the body of the penis; 2) loss of cavernous spaces and associated smooth muscle cells; and 3) accumulation of fat cells in the corpora cavernosa penis. These structural changes

are already evident at 18 days of age and are associated with reduced plasma T and overexpression of ERα, without AR alterations. This suggests that abnormal penile morphology is due, in part, to DES-induced T suppression and/or enhanced expression of ERα.

ACKNOWLEDGMENTS

Authors acknowledge the technical help of Ms. Barbara Drescher in paraffin sections, Marya Towio-Kinnucan (Auburn University) in epoxy sections, and Dr. John R. Kammermann (Auburn University) in radiographs and thank Dr. Alfonza Atkinson, Dean, for encouragement and support.

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