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Neonatal Exposure of Male Rats to Estradiol Benzoate Causes Rete Testis Dilation and Backflow Impairment of Spermatogenesis

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ABSTRACT

Estrogens administered to perinatal rodents cause spermatogenesis impairment; this study was undertaken to determine the mechanisms by which estrogens exert this effect. Neonatal male Wistar rats received estradiol benzoate (either 0.5 mg/5g BW or 1 mg/5g BW) and were killed at days 10, 22, 33, 45, and 60. Controls received vehicle. In tubule cross-sections of transverse sections of the right testes, 1) tubular diameter (TD) and seminiferous epithelium height (SEH) were measured, 2) normal and impaired spermatogenesis were classified in terms of the most advanced germ cell type present, including tubules lined by Sertoli cells only. A significant dose-dependent rise in the tubule percentage lined by Sertoli cells only at day 60 reflected spermatogenesis impairment. This was evidenced by the presence of multinucleated germ cells in a thin epithelium and sloughed into an enlarged tubular lumen, which was reflected in a significant dose-dependent increase in TD/SEH values from day 22 onward. TD was significantly greater and SEH significantly lower in tubular segments located at the cranial than the caudal halves of rat testes treated with the high (days 22, 33, and 60) and the low dose (day 33). This indicated distension in cranial tubular segments, perhaps due to the fact that these segments were the closest to the dilated rete testis. Consequently, they showed the highest TD/SEH values and the most regressive features of spermatogenesis (tubules lined by Sertoli cells only). In contrast, caudal segments in rat testes treated with the low dose showing TD/SEH values similar to controls displayed a delayed maturation of spermatogenesis coinciding with the late appearance of mature Leydig cells. Anat. Rec. 252:17–33, 1998.

Key words: **Wistar rat; testis; estradiol; tubule; distension; spermatogenesis; impairment**

Perinatal exposure of male rodents to estrogens is useful for understanding the mechanisms involved in the effects on the reproductive tract of men whose mothers either received diethylstilbestrol (DES), a potent estrogen, or were exposed to environmental estrogens during pregnancy (Gill et al., 1979; Newbold et al., 1985; Sharpe and Skakkebaek, 1993).

Spermatogenesis may be impaired in men exposed in utero to DES (Gill et al., 1977), though the results of relevant biopsies have not yet been reported. Impairment of spermatogenesis is a short- and long-term effect linked to perinatal exposure to estrogens (Vitale et al., 1973; McLachlan et al., 1975; Chemes et al., 1976), although the mechanisms by which estrogens exert this effect remain unclear (Giusti et al., 1995).

Estrogen-induced alterations on the hypothalamic-pituitary-gonadal axis lead to different effects on spermatogenesis, for instance: a long-lasting suppression of this

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axis may cause a persistent inhibition of spermatogenesis (Arai et al., 1983; Bellido et al., 1990; Pinilla et al., 1992); a decrease in testosterone secretion can prevent spermatocytes of the first spermatogenesis wave from progressing to the pachytene spermatocytes stage (Chemes et al., 1976); and a decrease in FSH secretion has been related to a delayed maturation of spermatogenesis (Brown-Grant et al., 1975).

A direct action of estrogens on the testis has been related to both a delayed failure of germ cells to proceed beyond the spermatogonia stage (Steinberger and Duckett, 1965) and a permanent impairment of spermatogenesis (Ohta and Takasugi, 1974; McLachlan et al., 1975; Ohta, 1977). Abdominal temperature in estrogen-induced cryptorchid testes may partly prevent normal maturation of spermatogenesis after recovery or administration of gonadotropins (Bugnon et al., 1973; Arai et al., 1983), and may cause irreversible lesions in some tubules (Steinberger, 1971).

Retention of sperm and testicular fluid has been related to impairment of spermatogenesis in adult mice exposed perinatally to DES (Pylkkänen et al., 1991). This might be secondary to deleterious effects on growth and histology of the sperm ducts which have been observed in both perinatally estrogenized rodents (Orgebin-Crist et al., 1983; Newbold et al., 1986, Greco et al., 1993) and men exposed in utero to DES (Griffin and Wilson, 1994).

The aim of this study was to determine whether estradiol benzoate administered to male rats at day 1 of life causes impairment of spermatogenesis, and to determine the mechanisms by which estrogens exert this effect through morphometric assessment of the seminiferous tubules.

MATERIALS AND METHODS

Animals and Treatments

Male Wistar rats were raised in a room under controlled light (12 h light and 12 h darkness) and temperature (20±2° C). The day on which litters were born was taken as day 1 of life. Litter size was adjusted on day 1 to 8–10 male pups per lactating mother up to sacrifice (day 10) or weaning (day 21); male rats were thereafter housed 4–5 per cage. Estradiol benzoate (E_2B) obtained from SIGMA was dissolved in olive oil. On day 1, 70 rats were injected subcutaneously with either 0.5 mg E_2B in 0.1 cc olive oil /5g BW (Group A) or 1 mg E_2B in 0.1 cc olive oil/5g BW (Group B). Controls received 0.1 cc olive oil/5g BW. Administration of a single subcutaneous injection of estradiol benzoate in this work was based on studies showing several degrees of spermatogenesis disruption (Kincl et al., 1963; Brown-Grant et al., 1975). Estradiol benzoate was purposefully administered in high doses to cause impairment of spermatogenesis in order to determine its mechanism of action, according to methodological guidelines provided by Russell (1983). Five rats per Group A, five rats per Group B, and four rats per control Group were killed on postnatal days 10, 22, 33, 45, and 60. Four additional rats were killed on postnatal day 1 for data collection purposes.

This experimental design was based on reports showing the different mechanisms through which estrogens may be involved in several degrees of spermatogenesis disruption: 1) A direct action of perinatal estrogen exposure causes a permanent impairment of spermatogenesis (Ohta and Takasugi, 1974; McLachlan et al., 1975; Ohta, 1977); 2) Estrogen-induced cryptorchidism is linked to both a permanent impairment of spermatogenesis (Steinberger, 1971) and a progressive loss of germ cells leading to tubules lined by Sertoli cells only (Kincl et al., 1963; Bugnon et al., 1973); and 3) retention of sperm may be linked to a mixed population of tubules showing impaired spermatogenesis, including hyalinized tubules, in 60-day-old mice exposed perinatally to estrogens (Pylkkänen et al., 1991). This may be the endpoint of tubular distension, which becomes progressively more pronounced with time (Ilio and Hess, 1994; Nistal and Paniagua, 1997).

The study by Pylkkänen et al. (1991) was the criteria for using the day 60 of age as the termination of this experiment. Rats at postnatal days 10, 22, 33, and 45 were chosen for this study based on several rationales. 1) The earliest effects in spermatogenesis due to a direct action of estrogens have been reported at postnatal day 10 (Ohta and Takasugi, 1974). Plasma estrogen levels are high in rats exposed neonatally to E_2B by this age (Bellido et al., 1985). There are small tubular lumina as early as postnatal day 10, perhaps indicating the initiation of apical fluid secretion by some Sertoli cells (Russell et al., 1989), so testicular fluid retention linked to estrogen exposure (Pylkkänen et al., 1991) might cause tubular distension. 2) Plasma estrogen levels are normalized in 22-day-old rats exposed neonatally to E_2B (Bellido et al., 1985) and, therefore, permanent spermatogenesis impairment due to a direct action of estrogens may be assessed from day 22 onward. Testes of normal and estrogenized rats either reach their scrotal location or remain cryptorchid, respectively, at about this age (Vitale et al., 1973). 3) Leydig cells exhibit mature features in 33-day-old normal rats (Chemes et al., 1979) and the effects on spermatogenesis linked to testosterone suppression due to estrogen-induced disruption in Leydig cells maturation have been reported by this age (Brown-Grant et al., 1975). 4) The effects of neonatal estrogen exposure on spermatogenesis have been assessed in 45-day-old mice (Ohta, 1977), when spermatogenesis is already qualitatively complete in normal rats (Russell et al., 1987). Testes of estrogenized rats remain cryptorchid until about this age (Vitale et al., 1973). At days 10, 22, 33, 45, and 60, animals were weighed and, after decapitation, the abdominal cavity was opened and the location of the testes of each rat was recorded.

Tissue Preparation

Estrogens administered to perinatal mice cause both rete testis dilation (Pylkkänen et al., 1991), which would probably compress the blood vessels entering or leaving the testis through the mediastinum, and thickening of the arterioles (Newbold and McLachlan, 1988). The potential consequences of these effects in the fixation by vascular perfusion are unknown, by which this fixation method was discarded. Right testes with epididymes were removed and immersed in saline solution. Because testicular fluid retention may occur in estrogenized mice (Pylkkänen et al., 1991), the continuity of sperm ducts was preserved down to where the ductuli efferentes opened into the epididymis to prevent fluid loss. Calipers were used to measure cranial-caudal, anterior-posterior, and medial-lateral axes of the testes. Testes were fixed in Bouin-Hollande's solution for 24 hours and their albuginea were pricked several times to increase the penetration of the fixative solution.

They were split perpendicular to their longitudinal axis and both cranial and caudal halves were immersed in Bouin-Hollande's solution for an additional 24 hours. After dehydration, both halves were embedded in paraffin wax. Transverse sections 5 µm thick of both halves of the testis were cut and stained with haematoxylin-eosin (H&E). This type of tissue preparation is commonly sufficient for a determination of major damage or a simple measurement of tubular diameter (Russell, 1983). Consequently, this work only reported the most advanced germ cell type present; the presence of multinucleated germ cells in the epithelium and sloughed into the lumen (McLachlan et al., 1975; Chemes et al., 1976); and measurements of tubular diameter (TD) and seminiferous epithelium height (SEH).

Morphometry

Five randomly selected transverse sections from both halves of each right testis were examined. In 10 randomly selected tubule cross-sections per transverse section, two parameters were evaluated. Firstly, TD and SEH were measured across the minor axis, which was not affected by possible obliquity of the section (Ghosh et al., 1992), using a micrometer eyepiece. No correction factors for lineal changes during fixation and embedding were obtained, therefore, the data of TD and SEH refer only to immersion-fixed testes in Bouin-Hollande's solution and embedded in paraffin. Secondly, spermatogenesis was classified by reference to the most advanced germ cell type present (Hodgen and Sherins, 1973; Brown-Grant et al., 1975) into the following stages: spermatogonia, preleptotene-zygotene spermatocytes, pachytene-secondary spermatocytes, spermatids (steps 1–8), spermatids (steps 9–14) and spermatids (steps 15–19). Tubule cross-sections showing impairment of spermatogenesis were similarly classified. Those showing complete losses of germ cells were classified as "lined by Sertoli cells only." To quantify spermatogenesis, the percentage distribution of all these different stages, including tubules lined by Sertoli cells only, was obtained.

TD and SEH values may reflect the morphological effects linked to the above-mentioned mechanisms through which estrogens not only impair but also delay spermatogenesis. In order to discriminate between their respective effects:

i) The rates of TD and SEH increase in estrogenized rat testes for each age-group were obtained and compared with those of controls to ascertain when these effects occurred and also to assess their strength. These rates for each age group were obtained as the increase in either TD or SEH per rat in a given group with respect to the mean TD or SEH, respectively, of the preceding group. Each increase was divided by the numbers of days elapsing with respect to the preceding age group, and expressed as µm/day. To find the rates of TD and SEH increase by day 10, the mean TD and SEH on day 1 were calculated.

ii) The TD/SEH ratio may reflect the relationship between the TD and the SEH (Hodgen and Sherins, 1973). As early as postnatal day 10 some tubular lumina are open in normal rats (Russell et al., 1989) and continue to grow as the SEH and the TD increase (Hodgen and Sherins, 1973; Mills et al., 1977). Therefore, significant rises in TD/SEH values from day 22 through 60 would be unexpected in control rats, but these values might be altered in rats treated neonatally with estrogens. For example, a prepuberal gonadotropin suppression, as neonatal estrogen exposure does (Brown-Grant et al., 1975), might be reflected in TD/SEH values lower than controls, since it not only inhibits the TD and SEH increase but also the appearance of the lumen (Cattanach et al., 1977; Levin, 1979; Singh et al., 1995). In contrast, tubules showing enlarged lumina lined by thin epithelium due to either a direct action of estrogens on the testis (Ohta and Takasugi, 1974), or estrogen-induced cryptorchidism (Bugnon et al., 1973) or sperm retention (Pylkkänen et al., 1991) might cause increased TD/SEH values.

iii) The cranial/caudal ratios in TD and SEH values as well as in the different stages of spermatogenesis were obtained to assess the potential effects of tubular distension. The rationale for using these ratios was based on the fact that the closest tubular segments to the rete testis are located at the cranial half of the rat testis (Clermont and Huckins, 1961) and are the first and most affected by backflow from the rete (Nykänen and Kormano, 1978). Consequently, their TD values may be increased and their SEH values may be reduced (Ross, 1974) compared to those at the caudal segments. Mean TD and SEH values and the percentage of tubules at different stages of spermatogenesis were recorded for the five cranial and five caudal sections of the testis and related by the following cranial/caudal ratios. 1) cranial/caudal TD ratio (cranial TD divided by caudal TD); 2) Cranial/caudal SEH ratio (cranial SEH divided by caudal SEH); 3) Cranial/caudal sperm ratio, i.e. cranial percentage of a given stage minus caudal percentage of the same stage.

Statistical Analysis

Mean and standard error of the mean (SEM) were calculated for each group, and after a two-way ANOVA statistical significance was evaluated using the Newman-Keuls multiple comparison test. A P of less than 0.05 was selected as the limit of statistical significance.

RESULTS

Gross Findings

In control rats, testes were observed within the scrotum from day 22 onward; in E_2B-treated rats, testes had not descended into the scrotum throughout this study, except the testes of all 60-day-old E_2B (0.5mg)-treated rats. Control rat testes were ovoid in shape and both the anterior-posterior axis and the medial-lateral axis were longest at a midway point. In contrast, the longest anterior-posterior and medial-lateral axes were not recorded at a midway point but at mediastinum level in the testes of all estrogenized rats. The altered-looking of these testes resulted from an expansion of the albuginea at mediastinum level mainly in the posterior direction as well as medially and laterally due to the dilatation of the rete testis, as evidenced by the morphological study (see below).

Morphometric Study

Inflammatory reaction was observed in the right testis of one 33-day-old E_2B (1mg)-treated rat and in the right testis of one 45-day-old E_2B (1mg)-treated rat. All data for both rats were omitted in order to avoid confusion of primary estrogen-induced alterations with secondary

TABLE 1. Effects of Estradiol Benzoate (Group A: 0.5 mg/5 g B.W.; Group B: 1 mg/5 g B.W.) on Testicular Size

Age (days)	Groups	n	Measurements in three dimension of the right testes: Cranial-caudal axis (mm)	Anterior-posterior axis (mm)	Medial-lateral axis (mm)
	Control	4	4.63 ± 0.06	2.93 ± 0.4	2.66 ± 0.06
10	A	5	3.34 ± 0.08**	2.52 ± 0.01	2.27 ± 0.03
	B	5	3.44 ± 0.06**	2.84 ± 0.1	2.5 ± 0.1
	Control	4	9.08 ± 0.61[a]	5.61 ± 0.37[a]	5.21 ± 0.34[a]
22	A	5	4.24 ± 0.1**;[a]	3.48 ± 0.06**;[a]	2.99 ± 0.06**;[a]
	B	5	4.87 ± 0.11**;[a]	4.13 ± 0.14**;[a]	3.57 ± 0.11**;[a]
	Control	4	11.81 ± 0.32[a,b]	8.05 ± 0.24[a–b]	7.31 ± 0.13[a–b]
33	A	5	5.62 ± 0.43**;[a–b]	4.73 ± 0.27**;[a–b]	4.14 ± 0.31**;[a–b]
	B	4	5.7 ± 0.42**;[a–b]	4.85 ± 0.23**;[a,b]	4.17 ± 0.12**;[a,b]
	Control	4	17.62 ± 0.43[a–c]	10.25 ± 0.37[a–c]	9.37 ± 0.27[a–c]
45	A	5	9.76 ± 0.6**;[a–c]	7.54 ± 0.33**;[a–c]	7.05 ± 0.37**;[a–c]
	B	4	8.27 ± 1.45**;[a–c]	6.92 ± 0.73**;[a–c]	6.02 ± 0.85**;[a–c]
	Control	4	22 ± 0.23[a–d]	12.62 ± 0.14[a–d]	11.25 ± 0.28[a–d]
60	A	5	11.12 ± 0.53**;[a–d]	8.86 ± 0.18**;[a–d]	8.21 ± 0.19**;[a–d]
	B	5	10.06 ± 0.69**;[a–d]	8.38 ± 0.63**;[a–d]	7.84 ± 1.51**;[a–d]

Values are expressed as mean ± SEM (n rats/group). The asterisks indicate significant differences with respect to age-matched controls (*, $p < 0.05$; **, $p < 0.01$); ¶ and §, indicate significant dose-dependent differences for $p < 0.05$ and $p < 0.01$ respectively; w, x, y and z represent significant differences ($p < 0.05$) and a, b, c, and d represent significant differences ($p < 0.01$) with respect to the values for groups at 10, 22, 33, and 45 days of age respectively (ANOVA followed by Newman-Keuls multiple comparison test).

changes due to the inflammatory reaction. Consequently, the number of rats in both the 33-day-old E_2B (1mg)-treated Group and 45-day-old E_2B (1mg)-treated Group was 4.

Table 1 shows right testes measurements in three dimensions. All dimensions in estrogenized rats testes progressively increased from day 10 through 60 and were significantly reduced from day 22 onwards compared with controls. There were no significant dose-dependent differences.

Table 2 shows tubular diameter (TD), seminiferous epithelium height (SEH) and the TD/SEH ratio, as well as the rate of TD increase and the rate of SEH increase of the right testes. TD and SEH values in estrogenized rat testes were significantly lower than controls from day 22 onward. There was a significant dose-dependent decrease in SEH from day 33 onward but not in TD, which was significantly greater in 60-day-old rat testes treated with the high than the low dose. There was a significant progressive increase from day 10 through 60 in TD but not in SEH, which underwent a significant reduction from days 10 to 22 in rat testes treated with the high dose. Consequently, the TD/SEH ratio displayed a significant dose-dependent increase from day 22 onwards. At day 60, a significant dose-dependent rise in the rate of TD increase coincided with a significant dose-dependent fall in the rate of SEH increase.

Table 3 shows the percentage distribution of different stages of spermatogenesis of the right testes. The most advanced stages of spermatogenesis significantly decreased in a dose-dependent manner at days 22, 33, and 60. Rat testes treated with the high dose underwent a significant reduction in the percentage of tubules at spermatocytes stages from days 45 to 60. This coincided with a significant percent rise in tubules either at the most advanced stage (Spermatids steps 1–8), as might be expected, or at the less advanced stages (spermatogonia stage and tubules lined by Sertoli cells only), which was considered as a regression of spermatogenesis. Table 4 shows TD, SEH, and TD/SEH ratios for tubule segments in both halves of the right testes.

Cranial Half

TD and SEH values in estrogenized rat testes declined significantly from day 22 onward compared with controls. There was a significant dose-dependent decrease in SEH values from day 45 onward but not in TD values, which were significantly larger in 60-day-old rat testes treated with the high than the lower dose. There was a significant progressive increase from day 10 through 60 in TD values but not in SEH values, which underwent a significant reduction from days 10 to 22. TD/SEH values in rat testes treated with the high dose increased significantly from day 22 onward with respect to controls, and displayed a significant dose-dependent rise from day 45 onward.

Caudal Half

TD and SEH values in estrogenized rat testes were significantly lower than controls at days 22 and 33 and displayed a significant dose-dependent fall from day 45

TABLE 2. Effects of Estradiol Benzoate (Group A: 0.5 mg/5 g B.W.; Group B: 1 mg/5 g B.W.) on Tubular Diameter (TD), Seminiferous Epithelium Height (SEH), and on Their Rates of Increase (Rate of TD Increase and Rate of SEH Increase), as well as on the TD/SEH Ratio (TD Divided by SEH) of the Right Testis

Age (days)	Groups	n	Tubular diameter (TD) (μm)	Rate of TD increase (μm/day)	Seminiferous epithelium height (SEH) (μm)	Rate of SEH increase (μm/day)	TD/SEH ratio
	Control	4	60.3 ± 2.5	1.16 ± 0.18	30.1 ± 1.2	0.58 ± 0.09	2 ± 0
10	A	5	62.2 ± 0.8	1.13 ± 0.09	30.9 ± 0.4	0.55 ± 0.04	2 ± 0.01
	B	5	61.4 ± 0.9	1.04 ± 0.1	30.4 ± 0.4	0.49 ± 0.05	2 ± 0.01
	Control	4	123 ± 5.6[a]	5.22 ± 0.47[a]	44.4 ± 1[a]	1.19 ± 0.08[a]	2.8 ± 0.1
22	A	5	89.6 ± 2.2**;[a]	2.28 ± 0.19**;[a]	26.2 ± 1.3**	−0.39 ± 0.11**;[a]	3.5 ± 0.3[a]
	B	5	98.8 ± 6.2**;[a]	3.11 ± 0.52**;[a]	23 ± 1.9**;[a]	−0.61 ± 0.16**;[a]	4.4 ± 0.5**;¶;[a]
	Control	4	169.3 ± 3.4[a,b]	4.21 ± 0.31[a]	62.2 ± 0.9[a,b]	1.62 ± 0.08[a,b]	2.7 ± 0.03
33	A	5	123.5 ± 7.5**;[a,b]	3.08 ± 0.68**;[a]	34.5 ± 1.4**;[b]	0.76 ± 0.13**;[b]	3.6 ± 0.3*;[a]
	B	4	134.3 ± 9.3**;[a,b]	3.22 ± 0.85[a]	27.9 ± 4.8**;§;[x]	0.44 ± 0.43**;[b]	5.2 ± 1**§;[a,x]
	Control	4	237.4 ± 3.8[a–c]	5.67 ± 0.31[a,y]	76.4 ± 2.4[a–c]	1.1 ± 0.24[a,c]	3.11 ± 0.06[a]
45	A	5	187.1 ± 8.4**;[a–c]	5.3 ± 0.7[a–c]	49.9 ± 4**;[a–c]	1.12 ± 0.23[a,c]	3.79 ± 0.23[a]
	B	4	181.2 ± 12.8**;[a–c]	3.91 ± 1.06**;[a]	34 ± 4.4**;§;[b,y]	0.51 ± 0.36**;§;[b]	5.69 ± 1.32**;§;[a,b]
	Control	4	281.9 ± 4.6[a–d]	2.97 ± 0.31[a,b,y,d]	86.6 ± 3[a–d]	0.68 ± 0.2[x,c,z]	3.26 ± 0.12[a]
60	A	5	232 ± 5.6**;[a–d]	2.99 ± 0.37[a,d]	58.3 ± 6.9**;[a–d]	0.56 ± 0.46[b]	4.11 ± 0.35*;[a]
	B	5	246.8 ± 22.3**;¶;[a–d]	4.37 ± 1.49*;¶;[a,b,y]	36.8 ± 2.4**;§;[a–c]	0.19 ± 0.16**;¶;[w,b]	6.8 ± 0.76**;§;[a–d]

Values are expressed as mean ± SEM (n rats/group). The asterisks indicate significant differences with respect to age-matched controls (*, $p < 0.05$; **, $p < 0.01$); ¶ and §, indicate significant dose-dependent differences for $p < 0.05$ and $p < 0.01$ respectively; w, x, y, and z represent significant differences ($p < 0.05$) and a, b, c, and d represent significant differences ($p < 0.01$) with respect to the values for groups at 10, 22, 33, and 45 days of age respectively (ANOVA followed by Newman-Keuls multiple comparison test).

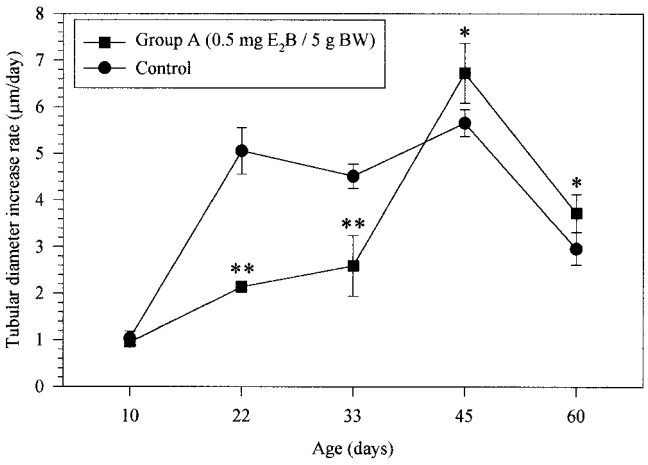

Fig. 1. Effects of the low dose of E_2B (0.5 mg/5g BW) on the rate of TD increase in the tubular segments located at the caudal half of the right testes. Asterisks indicate significant differences with respect to age-matched controls (*, $p<0.05$; **, $p< 0.01$). ANOVA followed by Newman-Keuls multiple comparison test.

onward. TD/SEH values in rat testes treated with the high dose increased significantly from day 22 onward compared with those of controls. In contrast, values in groups receiving the low estrogen dose were quite similar to those of controls throughout the study, perhaps indicating that these caudal segments were not altered by those mechanisms causing increased TD/SEH ratio. However, they displayed a significant decrease in TD from day 22 onward compared to controls, as aforementioned, and in order to determine the strength of this effect throughout the study, their rate of TD increase in each time point was obtained and compared to controls. The results are shown in Figure 1.

Their rate of TD increase significantly fell at days 22 and 33 and significantly rose at days 45 and 60 when compared to respective controls.

The cranial/caudal TD ratio (cranial TD divided by caudal TD) of the right testes is shown in Figure 2.

TD values in control rat testes at no point displayed significant cranial/caudal differences throughout the study. TD values in tubular segments located at the cranial half of 22-, 33-, and 60-day-old rat testes treated with the high dose and the 33-day-old rat testes treated with the low dose were significantly higher than in those located at the caudal half of the same testes. TD values were significantly higher at the caudal than the cranial segments of 45-to-60-day-old rat testes treated with the low dose. TD values in 45-day-old rat testes treated with the high dose did not display significant cranial-caudal differences. The cranial/caudal ratio SEH (cranial SEH divided by caudal SEH) of the right testes is shown in Figure 2.

SEH values in control testes showed no significant cranial/caudal differences throughout the study. SEH values in caudal tubular segments of estrogenized testes were significantly higher than those of cranial segments from day 22 onwards, except in 45-day-old rat testes treated with the high dose, which displayed no significant cranial-caudal differences.

TABLE 3. Effects of Estradiol Benzoate (Group A: 0.5 mg/5 g B.W.; Group B: 1 mg/5 g B.W.) on Spermatogenesis of the Right Testis

			Spermatogenesis stages						
Age (days)	Groups	n	Sertoli cell only	Spermatogonia	Preleptotene-zygotene spermatocytes	Pachytene-secondary spermatocytes	Spermatids (steps 1–8)	Spermatids (steps 9–14)	Spermatids (steps 15–19)
	Control	4	0 ± 0	100 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
10	A	5	0 ± 0	100 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	B	5	0 ± 0	100 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Control	4	0 ± 0	5.7 ± 3.0^{a}	59 ± 1.2^{a}	35.2 ± 3.6^{a}	0 ± 0	0 ± 0	0 ± 0
22	A	5	0 ± 0	49.2 ± 12.4**;^{a}	38.8 ± 9.7**;^{a}	12 ± 3.8**;^{a}	0 ± 0	0 ± 0	0 ± 0
	B	5	0 ± 0	65 ± 5.9**;§;^{a}	35 ± 5.9**;^{a}	0 ± 0**;§	0 ± 0	0 ± 0	0 ± 0
	Control	4	0 ± 0	0 ± 0^{a,b}	6.5 ± 5.2^{b}	54.5 ± 5.2^{a,b}	39 ± 6.9^{a,b}	0 ± 0	0 ± 0
33	A	5	0 ± 0	23 ± 9.8**;^{a,b}	70 ± 6.1**;^{a,b}	7 ± 4.7**	0 ± 0**	0 ± 0	0 ± 0
	B	4	0 ± 0	63 ± 15.0**;§;^{a,b}	36.7 ± 14.8**;§;^{a}	0.2 ± 0.3**;¶	0 ± 0**	0 ± 0	0 ± 0
	Control	4	0 ± 0	0 ± 0^{a,b}	0 ± 0^{b}	0 ± 0^{b,c}	25.5 ± 9.2^{c}	33.5 ± 7$^{a–c}$	41 ± 3.7$^{a–c}$
45	A	5	1.8 ± 0.9	10.2 ± 5.3$^{a–c}$	15.6 ± 8.6**;$^{a–c}$	31.4 ± 2.9**;$^{a–c}$	38 ± 14.3**;$^{a–c}$	1.6 ± 1.3**	0 ± 0**
	B	4	0 ± 0	9.7 ± 3.9$^{a–c}$	62 ± 18.7**;§;$^{a–c}$	22.7 ± 12.5**;$^{a–c}$	5.5 ± 5.2	0 ± 0**	0 ± 0**
	Control	4	0 ± 0	0 ± 0^{a,b}	0 ± 0^{b}	0 ± 0^{b,c}	1.2 ± 0.7^{c}	36.7 ± 1.5$^{a–c}$	62 ± 1.6$^{a–d}$
60	A	5	4.6 ± 2.6^{w}	9.8 ± 5.3$^{a–c}$	4.6 ± 2.1^{b,c,z}	7.8 ± 3.8$^{a–d}$	39.6 ± 11.6**;$^{a–c}$	19.2 ± 7.2**;$^{a–d}$	14.4 ± 8.8**;$^{a–d}$
	B	5	14.2 ± 10**;§;$^{a–d}$	30.2 ± 10.7**;§;$^{a–d}$	15.4 ± 2*;§;$^{a–d}$	13.4 ± 6.5**;$^{a–d}$	26.8 ± 9.7**;§;$^{a–d}$	0 ± 0**;§	0 ± 0**;§

Values are expressed as mean ± SEM of the percentage of tubules for n rats/group. The asterisks indicate significant differences with respect to age-matched controls (*, $p < 0.05$; **, $p < 0.01$); ¶ and § indicate dose-dependent significant differences for $p < 0.05$ and $p < 0.01$ respectively; w, x, y, and z represent significant differences ($p < 0.05$) and a, b, c, and d represent significant differences ($p < 0.01$) with respect to the values for groups at 10, 22, 33, and 45 day of age respectively (ANOVA followed by Newman-Keuls multiple comparison test).

TABLE 4. Effects of Estradiol Benzoate (Group A: 0.5 mg/5 g B.W.; Group B: 1 mg/5 g B.W.) on Tubular Parameters (Tubular Diameter and Seminiferous Epithelium Height) and on the TD/SEH Ratio (TD divided by SEH) in Seminiferous Segments Located in Cranial and Caudal Halves of the Right Testis

			Tubular parameters				TD/SEH ratio	
			Tubular diameter (TD)		Seminiferous epithelium height (SEH)			
Age (days)	Groups	n	Cranial (µm)	Caudal (µm)	Cranial (µm)	Caudal (µm)	Cranial half	Caudal half
10	Control	4	60.1 ± 2.7	60.4 ± 2.7	30.1 ± 1.3	30.2 ± 1.1	2 ± 0	2 ± 0
	A	5	62.8 ± 0.9	61.6 ± 0.7	31.2 ± 0.4	30.6 ± 0.3	2.01 ± 0.01	2.01 ± 0.01
	B	5	61.3 ± 0.9	61.5 ± 1.1	30.2 ± 0.6	28.7 ± 2	2.03 ± 0.02	2.19 ± 0.21
22	Control	4	124.9 ± 5.1[a]	121.1 ± 6[a]	44 ± 1.6[a]	44.8 ± 0.8[a]	2.84 ± 0.15	2.7 ± 0.09[a]
	A	5	90.9 ± 2.9**;[a]	87.2 ± 0.8**;[a]	23.5 ± 1.3**;[a]	30 ± 1**	3.93 ± 0.36[w]	2.91 ± 0.11[a]
	B	5	102.7 ± 6.7**;[a]	92.9 ± 5.7**;[a]	20.8 ± 1.4**;[a]	26.4 ± 3.6**	5.06 ± 0.67*;[a]	3.69 ± 0.45**;§;[a]
33	Control	4	167.9 ± 4.7[a,b]	170.8 ± 2.8[a,b]	62.8 ± 1.3[a,b]	61.7 ± 1.3[a,b]	2.67 ± 0.02	2.77 ± 0.04[a]
	A	5	130.8 ± 7.6**;[a,b]	115.7 ± 7.2**;[a,b]	28 ± 2.2**	41.5 ± 0.9**;[a,b]	4.81 ± 0.55*;[a]	2.8 ± 0.22[a]
	B	4	142.6 ± 8.6**;[a,b]	124.5 ± 10.6**;[a,b]	23.8 ± 3.3**	32.5 ± 6.6**;§;[x]	6.29 ± 1.03**;[a]	4.22 ± 1.04**;§;[a,x]
45	Control	4	236.1 ± 4.1[a–c]	238.6 ± 3.5[a–c]	76 ± 3.2[a–c]	76.7 ± 1.6[a–c]	3.11 ± 0.09	3.11 ± 0.05[a]
	A	5	177.7 ± 10.1**;[a–c]	196.4 ± 7.6**;[a–c]	40.6 ± 5**;[a–c]	59.9 ± 4.4**;[a–c]	4.54 ± 0.48[a]	3.32 ± 0.19[a]
	B	4	181.9 ± 13.2**;[a–c]	180.3 ± 12.5**;§;[a–c]	32.1 ± 4.9**;§;[b,y]	36.2 ± 3.5**;§;[a,b]	6.25 ± 1.77**;¶;[a]	5.17 ± 0.92**;§;[a–c]
60	Control	4	280.7 ± 6[a–d]	283.1 ± 5.2[a–d]	87 ± 2.6[a–d]	86.1 ± 3.7[a–d]	3.23 ± 0.09	3.3 ± 0.2[a]
	A	5	215 ± 4.3**;[a–d]	252.2 ± 6.1**;[a–d]	39 ± 9.2**;[a–c]	82 ± 3.6*;[a–d]	6.46 ± 1.37**;[a,b,y,z]	3.08 ± 0.06[a]
	B	5	257.5 ± 31.5**;§;[a–d]	234.1 ± 12.9**;§;[a–d]	27.2 ± 4.5**;§;[x]	47.3 ± 4.6**;§;[a–d]	10.81 ± 2.85**;§;[a–d]	5.04 ± 0.34**;§;[a–c]

Values are expressed as mean ± SEM (n rats/group). The asterisks indicate significant differences with respect to age-matched controls (*, $p < 0.05$; **, $p < 0.01$); ¶ and §, indicate dose-dependent significant differences for $p < 0.05$ and $p < 0.01$ respectively; w, x, y, and z represent significant differences ($p < 0.05$) and a, b, c, and d represent significant differences ($p < 0.01$) with respect to the values for groups at 10, 22, 33, and 45 day of age respectively (ANOVA followed by Newman-Keuls multiple comparison test).

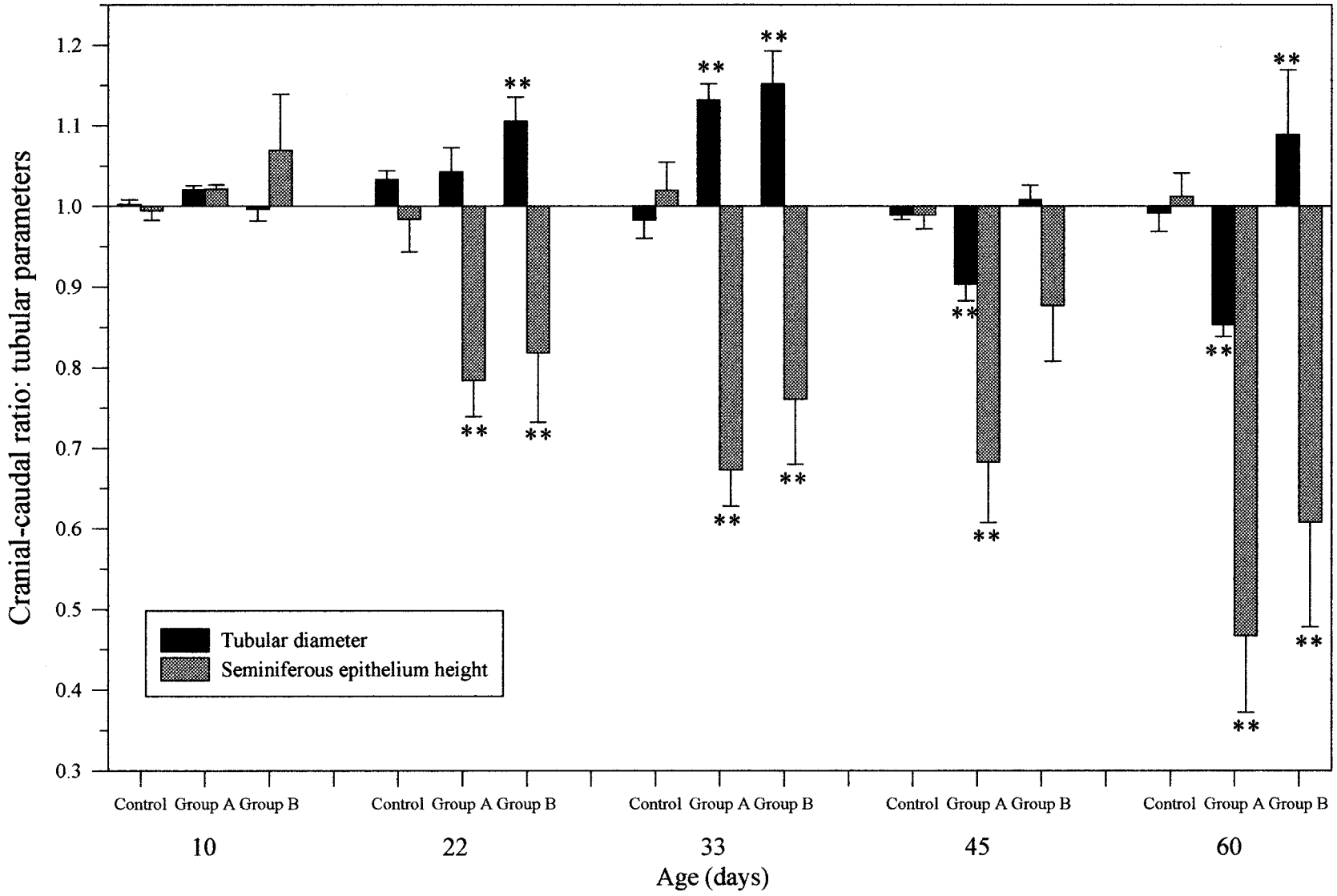

Fig. 2. Effects of E_2B (Group A: 0.5 mg/5gr BW; Group B: 1 mg/5gr BW) on cranial/caudal TD and SEH ratios of the right testes. Asterisks indicate significant differences with respect to age-matched controls (*, $p<0.05$; **, $p< 0.01$). ANOVA followed by Newman-Keuls multiple comparison test.

TABLE 5. Effects of Estradiol Benzoate (Group A: 0.5 mg/5 g B.W.; Group B: 1 mg/5 g B.W.) on the Percentage Distribution of the Different Stages of Spermatogenesis, Including Tubules Lined by Sertoli Cells Only, in Seminiferous Tubule Segments Located in the Cranial Half of the Right Testis

			Spermatogenesis stages						
Age (days)	Groups	n	Sertoli cell only	Spermatogonia	Preleptotene-zygotene spermatocytes	Pachytene-secondary spermatocytes	Spermatids (steps 1–8)	Spermatids (steps 9–14)	Spermatids (steps 15–19)
	Control	4	0 ± 0	100 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
10	A	5	0 ± 0	100 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	B	5	0 ± 0	100 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Control	4	0 ± 0	4.5 ± 3.8[a]	58 ± 0.9[a]	37.5 ± 4.6[a]	0 ± 0	0 ± 0	0 ± 0
22	A	5	0 ± 0	58 ± 17.3**;[a]	31.6 ± 13.4**;[a]	10.4 ± 4.4**	0 ± 0	0 ± 0	0 ± 0
	B	5	0 ± 0	72.4 ± 6.8**;¶;[a]	27.6 ± 6.8**;[a]	0 ± 0**;¶	0 ± 0	0 ± 0	0 ± 0
	Control	4	0 ± 0	0 ± 0[a]	7 ± 7.3	55.5 ± 6.8[a,b]	37.5 ± 8.9[a,b]	0 ± 0	0 ± 0
33	A	5	0 ± 0	21.2 ± 8.3**;[a,b]	70.4 ± 4.9**;[a,b]	8.4 ± 5.3**	0 ± 0**	0 ± 0	0 ± 0
	B	4	0 ± 0	69 ± 11.9**;§;[a]	30.5 ± 11.7**;§;[a]	0.5 ± 0.6**	0 ± 0**	0 ± 0	0 ± 0
	Control	4	0 ± 0	0 ± 0[a]	0 ± 0	0 ± 0[b,c]	25 ± 10.3	32 ± 6.8[a–c]	43 ± 4.7[a–c]
45	A	5	2.8 ± 2.1	19.8 ± 10.1**;[a]	22.4 ± 11.3**;[a]	26.6 ± 10.8**;[a–c]	24.4 ± 13.3[a–c]	0 ± 0**	0 ± 0**
	B	4	0 ± 0	12.2 ± 7.5[a,c]	66 ± 18.7**;§;[a–c]	16.2 ± 11.1**;§;[a–c]	5.5 ± 3.9	0 ± 0**	0 ± 0**
	Control	4	0 ± 0	0 ± 0[a]	0 ± 0	0 ± 0[b,c]	1.5 ± 1.1[c,d]	39 ± 2[a–d]	59 ± 1.7[a–d]
60	A	5	9.2 ± 5.3*;[a–c,z]	19.6 ± 10.6**;[a]	9.2 ± 4.2	14 ± 6.5**;[a]	30 ± 13.7**;[a–c]	9.2 ± 6.8**;[a–d]	8.8 ± 7**;[a–d]
	B	5	22.4 ± 17.7**;§;[a–d]	44 ± 13.3**;§;[a–d]	15.6 ± 6.4*;[a,x,c,d]	8 ± 7.3	10 ± 4.3	0 ± 0**;§	0 ± 0**;§

Values are expressed as mean ± SEM of the percentage of tubules for n rats/group. The asterisks indicate significant differences with respect to age-matched controls (*, $p < 0.05$; **, $p < 0.01$); ¶ and §, indicate dose-dependent significant differences for $p < 0.05$ and $p < 0.01$ respectively; w, x, y, and z represent significant differences ($p < 0.05$) and a, b, c, and d represent significant differences ($p < 0.01$) with respect to the values for groups at 10, 22, 33, and 45 days of age respectively (ANOVA followed by Newman-Keuls multiple comparison test).

TABLE 6. Effects of Estradiol Benzoate (Group A: 0.5 mg/5 g B.W.; Group B: 1 mg/5 g B.W.) on the Percentage Distribution of the Different Stages of Spermatogenesis, Including Tubules Lined by Sertoli Cells Only, in Seminiferous Tubule Segments Located in the Caudal Half of the Right Testis

			Spermatogenesis stages						
Age (days)	Groups	n	Sertoli cell only	Spermatogonia	Preleptotene-zygotene spermatocytes	Pachytene-secondary spermatocytes	Spermatids (steps 1–8)	Spermatids (steps 9–14)	Spermatids (steps 15–19)
	Control	4	0 ± 0	100 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
10	A	5	0 ± 0	100 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	B	5	0 ± 0	100 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Control	4	0 ± 0	7 ± 3.3[a]	60 ± 2.1[a]	33 ± 3.9[a]	0 ± 0	0 ± 0	0 ± 0
22	A	5	0 ± 0	40.4 ± 8.2**;[a]	46 ± 6.3*;[a]	13.6 ± 4.1**;[a]	0 ± 0	0 ± 0	0 ± 0
	B	5	0 ± 0	57.6 ± 7.3**;§;[a]	42.4 ± 7.5**;[a]	0 ± 0**;§	0 ± 0	0 ± 0	0 ± 0
	Control	4	0 ± 0	0 ± 0[a]	6 ± 3.6	53.5 ± 3.6[a,b]	40.5 ± 5.3[a,b]	0 ± 0	0 ± 0
33	A	5	0 ± 0	24.8 ± 12[a,b]	69.6 ± 8.6**;[a,b]	5.6 ± 4.1**;[x]	0 ± 0**	0 ± 0	0 ± 0
	B	4	0 ± 0	57 ± 18.8**;§;[a]	43 ± 18.8**;§;[a]	0 ± 0**	0 ± 0**	0 ± 0	0 ± 0
	Control	4	0 ± 0	0 ± 0[a]	0 ± 0[b]	0 ± 0[b,c]	26 ± 8.4	35 ± 9.5[a–c]	39 ± 6.3[a–c]
45	A	5	0.8 ± 0.9	0.8 ± 0.9[a,b]	8.4 ± 6[b,c]	36.2 ± 9.5**;[a–c]	50.2 ± 15.4**;[a–c]	3.6 ± 3**	0 ± 0**
	B	4	0 ± 0	7.5 ± 6.5[a,b]	57.5 ± 19.8**;§;[a–c]	29 ± 14.6**;[a–c]	6 ± 6.9[§]	0 ± 0**	0 ± 0**
	Control	4	0 ± 0	0 ± 0[a]	0 ± 0[b]	0 ± 0[b,c]	1 ± 0.6[c,d]	34.5 ± 2.5[a–c]	64.5 ± 3.2[a–d]
60	A	5	0 ± 0	0 ± 0[a–c]	0 ± 0[b,c]	1.6 ± 1.8[b–d]	49.2 ± 18.3**;[a–c]	29.2 ± 8.4*;[a–d]	20 ± 11.5**;[a–d]
	B	5	6 ± 4.8	16.4 ± 11.4**;§;[a,b]	15.2 ± 9.1*;§;[a–c]	18.8 ± 9**;§;[a–d]	43.6 ± 15.4**;[a–d]	0 ± 0**;§	0 ± 0**;§

Values are expressed as mean ± SEM of the percentage of tubules for n rats/group. The asterisks indicate significant differences with respect to age-matched controls (*, $p < 0.05$; **, $p < 0.01$); ¶ and §, indicate dose-dependent significant differences for $p < 0.05$ and $p < 0.01$ respectively; w, x, y, and z represent significant differences ($p < 0.05$) and a, b, c, and d represent significant differences ($p < 0.01$) with respect to the values for groups at 10, 22, 33, and 45 days of age respectively (ANOVA followed by Newman-Keuls multiple comparison test).

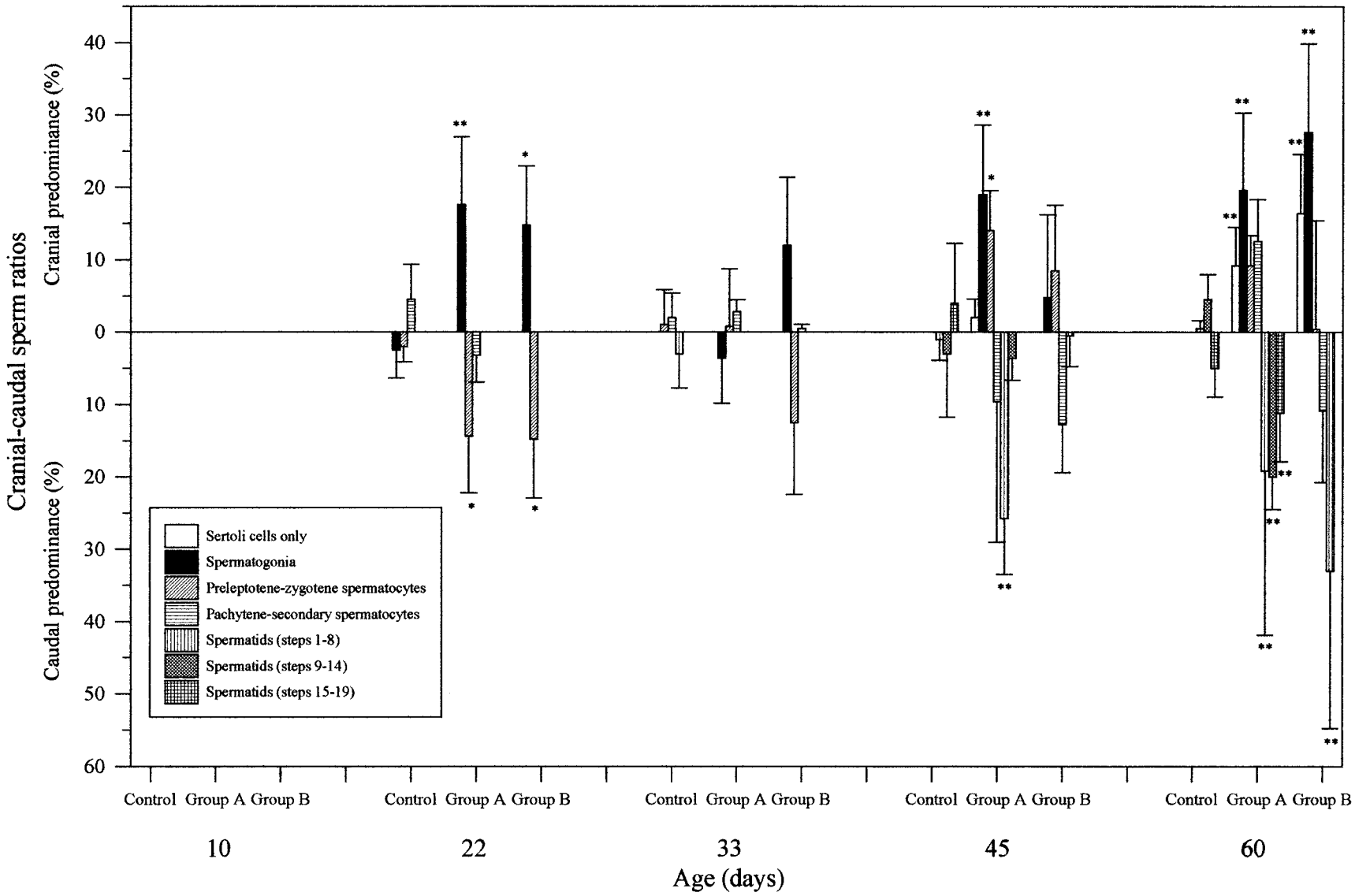

Fig. 3. Effects of E_2B (Group A: 0.5 mg/5g BW; Group B: 1 mg/5gr BW) on the cranial/caudal sperm ratio for each stage of spermatogenesis of the right testes. Asterisks indicate significant differences with respect to age-matched controls (*, $p<0.05$; **, $p< 0.01$). ANOVA followed by Newman-Keuls multiple comparison test.

Table 5 shows the percentage distribution of the different spermatogenesis stages in the cranial half of the right testes. The most advanced stages significantly decreased in a dose-dependent manner from day 22 onward. Rat testes treated with the high dose underwent a significant decrease in the tubule percentage at the preleptotene-zygotene spermatocytes stage from days 45 to 60. Concomitantly, no increase was recorded in the tubule percentage displaying a more advanced stage, but there was a significant rise in the tubule percentage showing either a less advanced stage (spermatogonia) or lined by only Sertoli cells, which seemed to be a severe regression of spermatogenesis. Table 6 shows the percentage distribution of the different spermatogenesis stages in the caudal half of the right testes. The percentage of tubules displaying the most advanced spermatogenesis stages evidenced a significant dose-dependent decline from day 22 onward.

The cranial-caudal sperm ratio (i.e., cranial minus caudal tubule percentage) for each of the different stages of spermatogenesis, including tubules lined by Sertoli cells only, of the right testes are shown in Figure 3.

No spermatogenesis stages displayed significant cranial-caudal differences in control testes at any point in the study. The percentage showing spermatogonia stage in the cranial half of 22-day-old estrogenized testes was significantly higher than that in the caudal half. In 45-day-old rat testes treated with the low dose, a significantly greater percentage of cranial than caudal tubules displayed less advanced stages of spermatogenesis, and in age-matched rat testes treated with the high dose there were no significant cranial-caudal differences at any spermatogenesis stage. The percentage of tubules showing either less advanced stages of spermatogenesis or only Sertoli cells was significantly higher in the cranial than in the caudal half of 60-day-old estrogenized rat testes.

Morphological analysis

Histological sections of right testes. Rete testis in control rats showed a network of channels of varying sizes and shapes (Figures 4A, 4C, and 4F). Dilation of the rete testis, including its extratesticular portion and tubuli recti, was observed in estrogenized rats from day 10 through 60. (Figures 4B, 4D, 4E, 4G, and 4H). Enlarged lumina were seen in some seminiferous tubules near the dilated rete testis as early as postnatal day 10 (Figure 5) and tubules showed no lumen in age-matched controls. Tubule cross-sections of transverse sections at the cranial and caudal halves of the rat testes treated with the high dose and at the cranial half of the rat testes treated with the low dose showed enlarged lumina lined by a thin epithelium from day 22 onwards. This was associated to impairment of spermatogenesis in many tubule cross-sections, as evidenced by the presence of multinucleated germ cells in the epithelium and sloughed into the lumen (Figure 6C). These lesions seemed to be less pronounced in some tubule cross-sections located under the albuginea of the anterior face of the rat testes treated with the low dose (Figure 6A). Neither dilated lumen nor impairment of spermatogenesis were found in the tubule cross-sections of transverse sections at the caudal half of the rat testes treated with the low dose from day 22 onwards (Figures 6B and 6D). Apparently normal fetal Leydig cells were found in both control and estrogenized rat testes at 10 day of age. Developing (day 22) and mature (day 33) Leydig cells were visible in the interstitium of controls, and mature Leydig cells were visible in the interstitium of estrogenized rat testes from day 45 onward.

DISCUSSION

Estradiol benzoate administered to neonatal rats caused dilatation of the rete testis, including its extratesticular portion, throughout this study. It was not surprising to note that as early as day 10 of age, rete testis was dilated and some tubules nearest to it showed enlarged lumina suggesting backflow, since at this age tubular fluid secretion begins and the initiation of fluid flow is not excluded (Russell et al., 1989). Rete testis dilation coincided with tubules showing enlarged lumina and spermatogenesis impairment from day 22 onward, as evidenced by the presence of multinucleated germ cells in a thin epithelium and sloughed into the lumen, in keeping with studies by Vitale et al. (1973) and Chemes et al. (1976). This might be linked to a significant dose-dependent rise in the tubule percentage at the less advanced stages of spermatogenesis from day 22 onward, including tubules lined by Sertoli cells only at day 60. These alterations were also evidenced as a reduction in SEH from days 10 to 22 in rat testes treated with the high dose and a significant dose-dependent decrease in SEH from day 33 onward. However, TD neither decreased from days 10 to 22 nor decreased in a dose-dependent manner from day 33 onward, as might be expected (Russell, 1983), but was significantly larger in rat testes treated with the highest rather than the low dose. Consequently, TD/SEH values significantly increased in a dose-dependent manner from day 22 onward, perhaps reflecting tubular distension. This mechanism has been

Fig. 4. **A**: Light micrograph at mediastinum level. 10-day control rat testis showing rete testis (asterisks) and the extratesticular rete (stars). Double-headed arrows indicate continuity of the lumen between both portions of the rete. Seminiferous tubules (outlined stars) visible. (H&E X47). **B**: Light micrograph at mediastinum level. 10-day rat testis treated with the high estrogen dose showing dilated channels of both the rete testis (asterisks) and the extratesticular rete (stars). Double-headed arrows indicate continuity of the lumen between both portions of the rete. Seminiferous tubule (outlined star) visible between two dilated channels of the rete testis. (H&E X47). **C**: Light micrograph at mediastinum level. Control 22-day testis showing the rete testis (rt-arrow) and extratesticular rete (er-arrow). The continuity between the channels of the rete testis and the extratesticular rete and some cross-sections of ductuli efferentes are seen (arrowhead). (H&E X11). **D**: Light micrograph at mediastinum level. 22-day-old rat testis treated with the low estrogen dose showing dilated channels of both the rete testis (asterisks) and the extratesticular rete (star). Double-headed arrow indicates continuity of the lumen between both portions of the rete. (H&E X11). **E**: Light micrograph at cranial pole. 45 day-day-old rat testis treated with the high estrogen dose. Dilated channels of the rete testis (asterisks) have displaced many seminiferous tubules, some visible (outlined stars). Dilated channels of the extratesticular rete (black stars) are embedded in adipose tissue (AT). (H&E X11). **F**: Light micrograph at mediastinum level. 60-day controls. The rete testis lumen (asterisks) opens into the extratesticular rete (stars). (H&E X10). **G**: Light micrograph at mediastinum level. 60-day-old rat testis treated with the low estrogen dose. Dilated channels of extratesticular rete (stars) are embedded in adipose tissue (AT). Some dilated channels of the rete testis (asterisks) are visible. Sloughed germ cells fill the dilated lumen in some seminiferous tubules. (H&E X10). **H:** Light micrograph at mediastinum level. 60-day-old rat testis treated with the high estrogen dose. Dilated channels of the rete testis (asterisks) have displaced many seminiferous tubules. Dilated channel of the extratesticular rete is seen (star). (H&E X10).

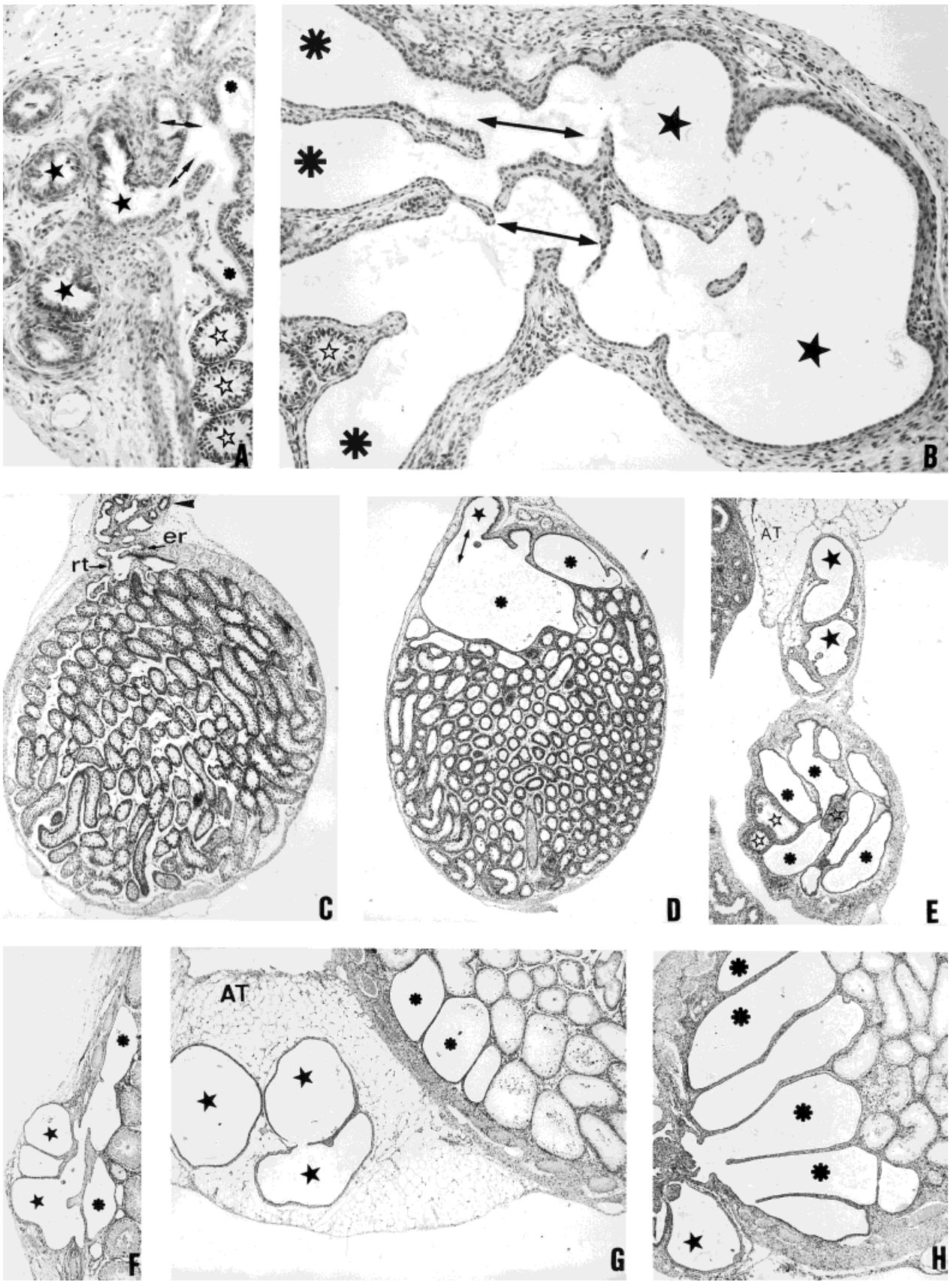

Fig. 4.

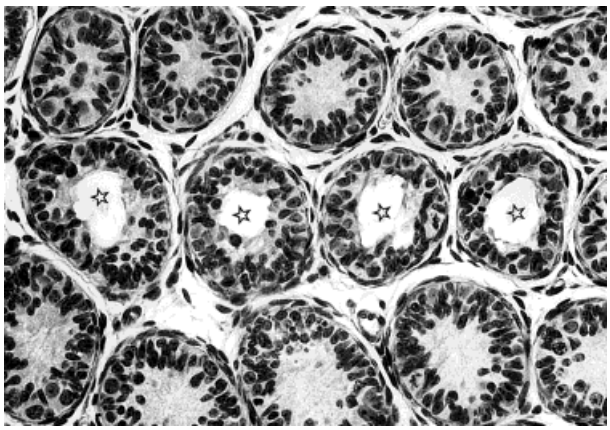

Fig. 5. Four adjacent seminiferous tubule cross-sections of a 10-day-old rat testis treated with the high estrogen dose show enlarged lumina (stars). (H&E X 200)

previously linked to spermatogenesis impairment associated to an increase in the volume density (Vv) of the tubular lumen and a decrease in the Vv of the epithelium in 60-day-old mice exposed neonatally to DES (Pylkkänen et al., 1991). Both these data and the increased TD/SEH values here reflect the occurrence of tubules showing enlarged lumina lined by thin epithelium, which not only may be due to tubular distension but also to a direct action of estrogens (Ohta and Takasugi, 1974) as well as to estrogen-induced cryptorchidism (Bugnon et al., 1973). The discrimination between these mechanisms is not provided in the study by Pylkkänen et al. (1991) and may be inferred from neither their data nor the increased TD/SEH ratio here.

The fact that TD was significantly smaller than controls from day 22 onward might be inconsistent with tubular distension, which would be expected to cause TD larger than controls, as claimed by Ross (1974) and Ross and Dobler (1975). Nevertheless, if tubules displaying delayed growth due to a decrease in E_2B-induced gonadotropin secretion (Brown-Grant et al., 1975; Gaytan et al., 1986) had undergone distension, their TD would probably be smaller than controls. This seemed to occur at day 60, as evidenced by the fact that the rate of TD increase significantly rose in a dose-dependent manner and the rate of SEH increase fell in a dose dependent manner. This coincided with a significant dose-dependent increase in the tubule percentage either at spermatogonia stage or lined by Sertoli cells only due to degeneration and sloughing of

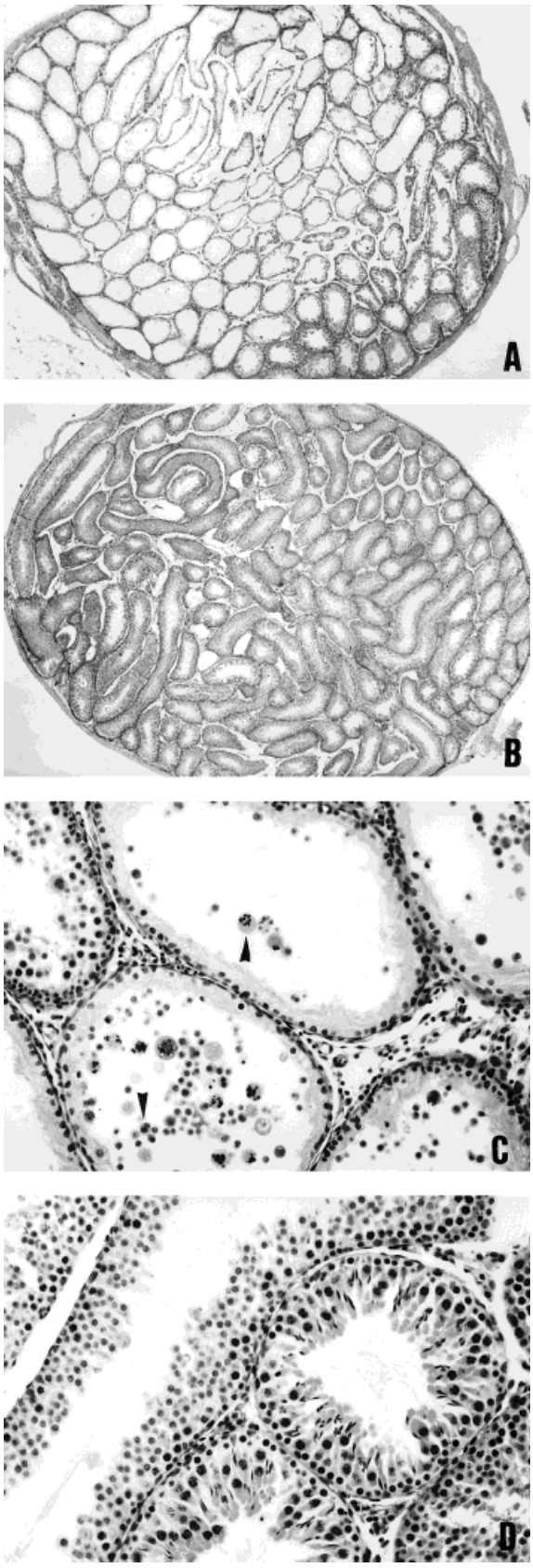

Fig. 6. **A**, **B**, **C**, and **D**. Light micrographs of a 60-day-old rat testis treated with the low dose. A: Transverse section of the cranial half of the testis. Some tubule cross-sections located under the albuginea of the anterior testicular face (bottom right corner) exhibit narrow lumen and thick epithelium compared with those in remaining tubule cross-sections. (H&E X16). B: Transverse section of the caudal half of the same testis. Note the differences in luminal size and thickness of the epithelium in tubule cross-sections compared with those in the most of cross-sections at the cranial half of the same testis which are shown in the preceding figure (Fig. 6A). (H&E X12) C: Cranial tubule cross-sections show impaired spermatogenesis with germ cells sloughed and floating free in dilated tubule lumina (arrows). (H&E X118). D: Caudal tubule cross-section of the same testis exhibiting apparently normal spermatogenesis. (H&E X137).

multinucleated germ cells, which might be consistent with impairment of spermatogenesis due to tubular distension (Neaves, 1973; Ross, 1974; Ilio and Hess, 1994).

The comparative analysis of TD and SEH in the cranial versus the caudal halves of the testes might contribute to support or to refute the occurrence of tubular distension. This is based on the rationale that the closest tubular segments to the rete testis are located at the cranial half of the rat testis (Clermont and Huckins, 1961) and are thus the first and most altered by backflow from the rete (Nykänen and Kormano, 1978). Consequently, their TD would increase and SEH would decrease (Ross, 1974) compared with those at caudal segments. TD was significantly greater and SEH was significantly lower in cranial than in caudal segments of 22-, 33-, and 60-day-old-E_2B (1 mg) treated rat testes and 33-day-old-E_2B (0.5 mg) treated rat testes, reflecting tubular distension in cranial segments. Their TD/SEH values were significantly increased compared with controls, which might be used as an indicator of tubular distension. Thus, a significant dose-dependent increase in TD/SEH values at cranial segments from day 45 onward could indicate a dose-dependent tubular distension. The more pronounced regressive changes in spermatogenesis linked to tubular distension were found in cranial segments of estrogenized rat testes, as might be expected. Thus, degeneration and sloughing of multinucleated spermatocytes leaving spermatogonia as the most advanced germ cell type, as reported elsewhere (Steinberger and Duckett, 1965), might be linked to the fact that the tubule percentage at spermatogonia stage was significantly higher in cranial than in caudal segments of 22-day-old E_2B-treated rat testes. Also, degeneration and sloughing of multinucleated germ cells led to a significant dose-dependent rise in the tubule percentage lined by Sertoli cells only in cranial but not in caudal segments at day 60, which seemed to be a further step in impairment of spermatogenesis (Nistal et al., 1984a). Rat testes treated with the high dose showed TD/SEH values significantly increased in both cranial and caudal segments from day 22 onward compared with controls, indicating generalised tubular distension. No significant cranial/caudal differences were noted in TD and SEH as well as spermatogenesis stages in 45-day-old rat testes, perhaps reflecting a balanced pressure increase throughout their tubules.

Caudal segments in rat testes treated with the low dose displayed TD/SEH values strikingly similar to controls throughout this study and showed no spermatogenesis impairment. This seemed to indicate that they did not undergo tubular distension, perhaps partly due to their lying furthest from the rete (Clermont and Huckins, 1961), but their TD values were significantly lower than controls from day 22 onward. To assess the strength of this delayed growth of the tubules their rate of TD increase at each time point was compared to controls. i) At day 10, their rate of TD increase was similar to controls despite the fact that plasma estrogens levels are high at this age in neonatally estrogenized rats (Bellido et al., 1985). This estrogen failure in preventing initiation of spermatogenesis, in keeping with studies by Steinberger and Duckett (1965), coincided with apparently normal fetal Leydig cells, as noticed elsewhere (Brown-Grant et al., 1975). This seemed to be consistent with normal serum testosterone levels occurring in estrogenized rats during the first weeks of age (Huhtaniemi et al., 1985). Thus, testosterone might initiate spermatogenesis through direct action on the testis, as it does in hypogonadal mice (Singh et al., 1995).

ii) At days 22 and 33, their rate of TD increase was significantly lower than controls. This mainly reflected a failure of germ cells to proceed up to zygotene spermatocytes stage coinciding with the lack of developing (day 22) and mature (day 33) Leydig cells, as reported elsewhere (Brown-Grant et al., 1975). Further, there was a small, but significant, percent fall in tubules at the most advanced stage (pachytene spermatocytes) from days 22 to 33. This might be due to a delayed decrease in testosterone secretion occurring in estrogenized rats (Huhtaniemi et al., 1985), since it causes a rapid loss of pachytene espermatocytes (Russell and Clermont, 1977; Ghosh et al., 1992). However, this germ cell loss did not coincide with a significant decrease in tubular and luminal diameter (luminal diameters are not shown), as might be expected (Russell, 1983; Ghosh et al., 1992), but coincided with a significant increase in TD. Therefore, these data seemed to reflect a retardation of spermatogenesis, in keeping with previous studies (Brown-Grant et al., 1975; Gaytan et al., 1986), rather than a regression. This was also supported by the lack of multinucleate pachytene spermatocytes in the epithelium and sloughed into the lumen, although the presence of degenerating uninucleate pachytene spermatocytes in the epithelium could not be ruled out here because they are difficult to identify in paraffin embedded tissues (Russell et al., 1981).

iii) At days 45 and 60, their rate of TD increase was significantly greater than controls coinciding with the retarded appearance of mature Leydig cells at day 45, as reported elsewhere (Brown-Grant et al., 1975), so their TD and the most advanced stages of spermatogenesis approached control values at day 60. This delayed maturation of spermatogenesis agrees with that linked to abnormalities in testosterone and gonadotropin secretion reported by Brown-Grant et al. (1975) in rats exposed neonatally to E_2B (0.25 mg). However, these authors have not noticed the coexistence of tubular distension, which occurred at the cranial segments of the same testes here, perhaps due to the fact that the level of E_2B exposure in their study was lower than in the present work showing that tubular distension was dose-dependent.

Spermatogenesis is qualitatively normal in the tubular segments at the caudal pole of rat testes exposed neonatally to estrogens and it is impaired in the remaining segments partly due to their ectopic location (Bugnon et al., 1973; Arai et al., 1983). Here, testes of all estrogenized rats remained cryptorchid at 45 day of age, so impairment of spermatogenesis associated to increased TD/SEH values in caudal segments of rat testes treated with the high but not the low dose from days 22 to 45 could not be related to cryptorchidism. This conclusion was underlined further by the occurrence of impaired spermatogenesis both in 60-day-old E_2B (0.5 mg)-treated rat scrotal testes and in age-matched E_2B (1 mg)-treated rat cryptorchid testes, in keeping with studies by McLachlan et al. (1975). However, it should be noted that these authors point out that impaired spermatogenesis in both the scrotal and cryptorchid testes is linked to an additional direct action of perinatal estrogen exposure on the testis. This mechanism is also related to a permanent impairment of spermatogenesis in tubules showing enlarged lumina lined by a thin epithelium (Ohta and Takasugi, 1974; Ohta, 1977). Here, these direct effects might be reflected in increased TD/SEH

values and would not be discriminated from tubular distension. However, a direct action of estrogens could not account for the occurrence of impaired spermatogenesis associated to increased TD/SEH values in cranial but not in caudal segments of rat testes treated with the low dose.

Impairment of spermatogenesis associated with rete testis dilation has been related to sperm retention in 60-day-old mice exposed to DES (100 µg/Kg maternal BW) on the 15th day of gestation (Pylkkänen et al., 1991). Given that this level of DES exposure might have been lower than those in men whose mothers received DES during pregnancy (Gill et al., 1977), impaired spermatogenesis suggested by these authors might be linked to backflow. To our knowledge, the results of testicular biopsies performed in these men have not been reported. Therefore, the fact that the tubular segments furthest from the rete testis are located under the albuginea of the anterior face of the human testis (Nistal and Paniagua, 1984b), where biopsy specimens are obtained (Levin, 1979), should be taken into account because they were not distended in rat testes treated with the low dose here. Thus, the predictive value of the present study might be that not every biopsy from men exposed to DES in utero (Gill et al., 1977) which shows normal spermatogenesis must exclude the possibility of ectasia in tubular segments closest to the rete testis. This should be tested for using sonography (Tartar et al., 1993).

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