Reduced Testosterone During Puberty Results in a Midspermiogenic Lesion (43559)

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Abstract. **The aim of this study was to determine the role of testosterone, as reflected in the testicular interstitial fluid, in the completion of the first wave of spermatogenesis and to further elucidate its role in spermiogenesis. At weekly intervals beginning with 26-day-old rats, body and testis weights were obtained, testicular interstitial fluid testosterone (TIF-T) was assayed, daily sperm production (DSP) was determined, and testicular tissue was structurally analyzed by light and electron microscopy. At 40 days postpartum, half the rats were treated with ethane dimethanesulphonate (EDS) to temporarily reduce Leydig cells. The other half served as controls and were treated with the vehicle. The timing of EDS treatment was just prior to the elongation of spermatids. At Day 47 (1 week after EDS treatment), TIF-T, testis weight, DSP, and number of Leydig cells were significantly reduced. At Day 54 (2 weeks after treatment), TIF-T had returned to the normal adult level, Leydig cell repopulation was apparent, and testis weight was normal. The DSP returned to normal by Day 61 (3 weeks after treatment). At 1 and 2 weeks after treatment, Step 8–9 spermatids were partially or completely detached from Sertoli cells.**

Results indicate that a temporary reduction of testosterone during the peripubertal period leads to a temporary reduction of the DSP approximately 1 week later. It is suggested that reduced testosterone is associated with a mid-spermiogenic lesion interfering with stable attachment of Step 8–9 spermatids to Sertoli cells during Stage VIII-IX of the spermatogenic cycle. [P.S.E.B.M. 1993, Vol 202]

The precise roles of testosterone and follicle-stimulating hormone (FSH) in testicular maturation and the completion of the first wave of spermatogenesis are not yet clearly defined. Both hormones increase in the peripubertal period, FSH preceding testosterone (1–7). The rise of peripubertal testosterone begins around Day 40 in the rat and assumes the normal adult level by Day 60 (1, 6–9).

In the rat, Step 1 spermatids first appear at approximately Day 25 (10–16) and continue to differentiate as round cells up to the Step 8 stage of maturation. The Step 8 spermatid appears at around Day 40 (11–16). Pre-Step 9 spermatids are referred to as round spermatids. Post-Step 8 spermatids, referred to as elongated spermatids, continue the dynamic differentiational process of spermatid maturation (spermiogenesis), culminating in the formation of Step 19 spermatids (mature spermatids) by Day 45 (11–16). The Step 8 spermatid, which appears during midspermiogenesis at Stage VIII of the rat spermatogenic cycle (11–16), binds to the Sertoli cell at the well-described and unique Sertoli ectoplasmic specialization (17–19). The resulting stable Sertoli-spermatid junctional complex is extremely tight (9, 20, 21). It anchors the elongating post-Step 8 spermatid to the supportive epithelium, establishes orientation of the spermatids, and draws them into deep Sertoli cytoplasmic crypts (22) until their appropriate release (spermiation) at the completion of spermatid maturation. The control of these specific and pivotal events of the spermiogenic process has not been defined.

The peripubertal testosterone rise around Day 40 is coincident with the binding of round spermatids to Sertoli cells during Stage VIII of the spermatogenic cycle (11–16). Stable binding of Step 8 spermatids to Sertoli cells is presumed to be obligatory if complete differentiation of the spermatid is to be realized (22).

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In order to determine the influence of the peripubertal testosterone rise on the first wave of spermatogenesis and to better define its role in spermiogenesis, it would be desirable to delay the peripubertal testosterone rise at Day 40 without reducing the level of FSH. This was accomplished in the present study by whole animal treatment with ethane dimethanesulphonate (EDS). In this animal model, the reduction of both serum and testicular interstitial fluid (TIF) testosterone was focal, temporary, and moderate. It provided the opportunity to (i) examine the influence of the peripubertal testosterone rise on the completion of the first wave of spermatogenesis and (ii) determine the effects of testosterone deprivation on the specific spermiogenic event of spermatid binding to Sertoli cells and the subsequent completion of spermatid maturation. The effects of EDS treatment on the interactions between FSH and testosterone and systemic indications of puberty onset are addressed elsewhere (23).

Materials and Methods

Animals. A single group of weanling Sprague-Dawley male rats was obtained from the Harlan Corp. (Indianapolis, IN). At weekly intervals beginning with 26-day-old rats and continuing for 10 weeks (96-day-old rats), groups of animals ($n = 6$) were sacrificed by decapitation. From each animal in the study, body and testis weights were obtained and serum and testicular interstitial fluid were collected and frozen until assayed for testosterone by radioimmunoassay. One testis was processed for light and electron microscopy. The other testis was processed for the determination of the daily sperm production (DSP). At 40 days of age, half of the remaining animals were injected intraperitoneally with EDS at a dose of 100 mg/kg body wt (experimental), while the other half were injected with the dimethyl sulfoxide/water (1:3::vol:vol) vehicle (v-control). EDS was made immediately prior to its use following the technique described by Jackson and Jackson (24). EDS treatment was timed to coincide with the beginning of the peripubertal rise of testosterone and prior to spermatid elongation. The use of animals in this study received the prior approval of the University of South Florida College of Medicine Laboratory Animal Medicine and Ethics Committee (the local Institutional Animal Care and Use Committee).

Testicular Interstitial Fluid Testosterone. The right testis was suspended overnight at 5°C and testicular interstitial fluid was collected as described previously (25, 26). TIF was diluted 1/100 with assay buffer and frozen until assayed for testosterone by radioimmunoassay (27).

Testicular Morphology. The left testis was perfusion fixed via the testicular artery with 5% glutaraldehyde in 0.2 *M* *s*-collidine buffer as described previously (28, 29). Following secondary fixation with 1% osmium tetroxide, testicular tissue (five blocks/testis) was routinely dehydrated in alcohols, embedded in EM bed-812/araldite, and thick (0.5μm) and thin sectioned for light and electron microscopy, respectively. Thick sections (five slides/block) were stained with toluidine blue and thin sections were double stained with uranyl acetate and lead citrate.

Daily Sperm Production. Immediately after TIF collection, the left testis was processed for the determination of daily sperm production per gram of testicular parenchyma (DSP/g) by morphometric analysis of homogenized tissue as reported previously (28, 29) and modified from the technique described by Johnson *et al.* (30). Homogenization-resistant cells, which were counted to derive the DSP/g, were Step 15–19 spermatids. The total number of sperm produced daily by the testis (DSP/T) was estimated by multiplying the gram weight of the testicular parenchyma by the DSP/g.

Radioimmunoassays and Statistics. Testosterone concentrations were determined by use of a double-antibody radioimmunoassay as described previously (27). First, antibody and radioiodinated testosterone were obtained from Radioassay Systems Laboratories (Carson, CA). Mean organ weights, organ/body weights, DSP, and TIF testosterone were statistically analyzed to determine significance at the 95% confidence level. For radioimmunoassays, all samples were run in the same assay. Data were analyzed and significance was determined using Duncan's new multiple range test and Student's t test as appropriate. Each group contained six animals.

Results

Testis Weight. The absolute testis weight and the testis weight/100 g body wt were significantly ($P < 0.05$) reduced in 47-day-old experimental rats (1 week after EDS treatment) when compared with v-control rats of the same age (Fig. 1). With the exception of the absolute testis weight in the 75-day-old rat (5 weeks after treatment), all other testis weights (absolute and relative) were not different from controls (Fig. 1). The reduced absolute testis weight in the 75-day-old rat group was not accompanied by a significant reduction of the relative testis weight nor a reduction in the DSP (Figs. 1 and 2).

TIF Testosterone. The mean TIF testosterone concentration in testes from 47- and 54-day-old experimental rats (1 and 2 weeks after treatment) was significantly ($P < 0.05$) lower than in testes from v-controls of the same age (Fig. 2). The concentration of TIF testosterone in the 54-day-old experimental rat (2 weeks after treatment) was, however, not significantly different than TIF testosterone determined for all subsequent animals in the study (Fig. 2). At 3 weeks after EDS treatment and for the duration of the experiment, TIF

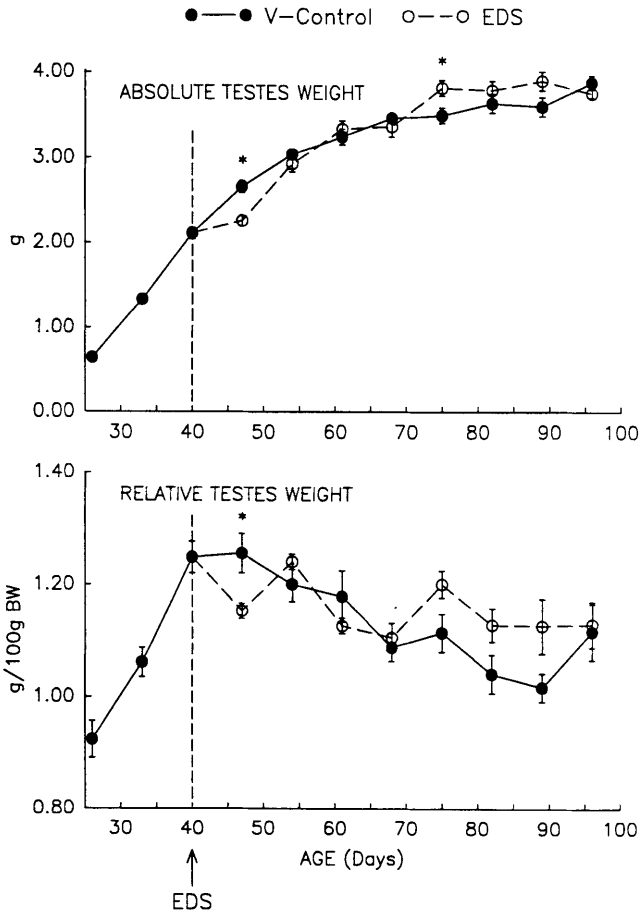

Figure 1. Graphs show the absolute testis weights (upper panel) and organ/body weights (lower panel) of EDS-treated rats (O) and vehicle-control rats collected at weekly intervals. Experimental animals were injected with EDS on Day 40. Vehicle-control rats were injected with dimethylsulfoxide/water also on Day 40. Mean ± SE. *$P < 0.05$, EDS-treated versus age-matched v-control.

testosterone in experimental rats was at the normal adult level and not significantly different than that determined in v-control rats of the same age (Fig. 2).

Daily Sperm Production. The daily sperm production was not detectable in rats younger than 40 days of age. In 47- and 54-day-old experimental rats (1 and 2 weeks after treatment), both the DSP/T and the DSP/g testicular parenchyma were significantly ($P < 0.05$) lower than those in v-control rats of the same age (Fig. 2). In 61-day-old experimental rats (4 weeks after treatment) and for the duration of the experiment, total and relative DSP were not significantly different from those of v-controls of the same age (Fig. 2).

Testicular Morphology. The reduction in number and repopulation of Leydig cells in the EDS-treated peripubertal rat was similar to that described for EDS treatment of the adult rat (9, 24, 31–33), except that Leydig cells were not completely eradicated. In the 47-day-old experimental rat (1 week after treatment), there was an obvious reduction of Leydig cells which ap-

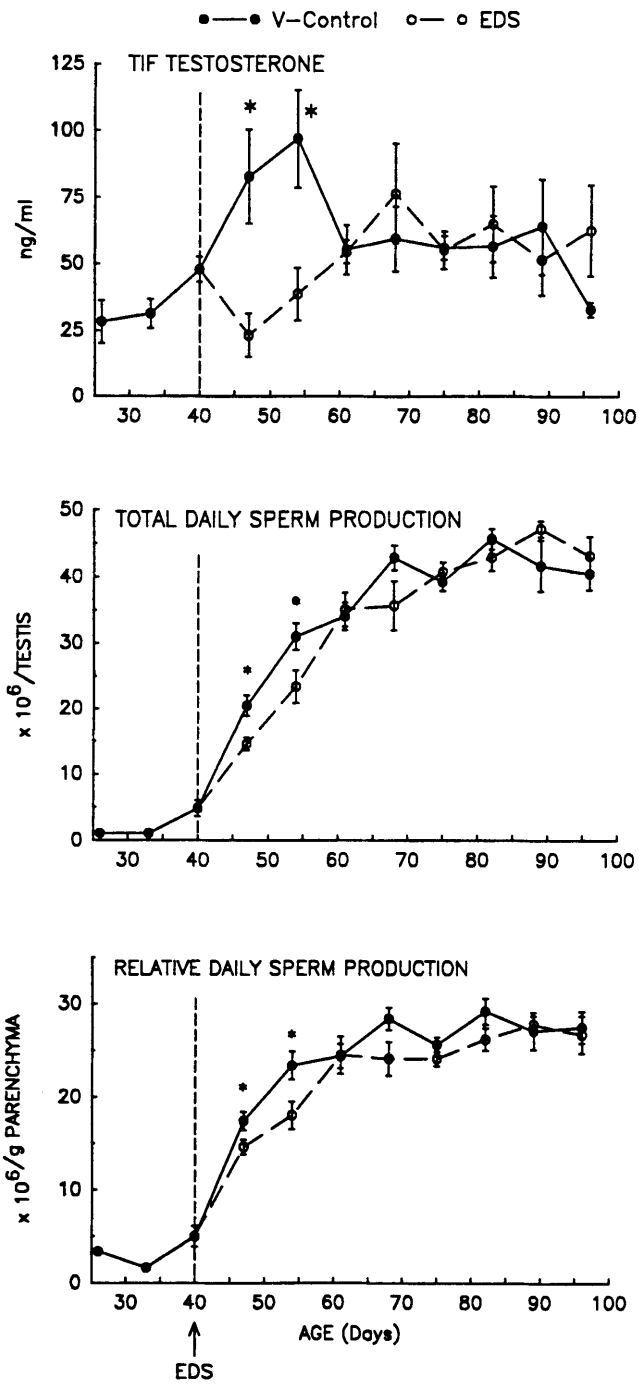

Figure 2. Graphs show the concentrations of TIF testosterone (upper panel), the total DSP/testis (middle panel), and the DSP/gram testicular parenchyma (lower panel) of EDS-treated rats (O) and v-control rats determined at weekly intervals. Experimental animals were injected with EDS on Day 40 and v-control animals were injected with the dimethylsulfoxide/water vehicle on Day 40. As illustrated in the upper panel, the mean TIF testosterone concentration in the 2-week posttreatment rats was significantly lower than its control. However, it was not significantly different than the mean TIF testosterone concentrations in the 3- to 8-week post-EDS-treated rats. Mean ± SE. *$P < 0.05$, EDS-treated versus age-matched v-control.

peared to have returned to normal in the 61-day-old rat (3 weeks after treatment).

The histology of seminiferous tubules from 47- and 54-day-old experimental rats (1 week and 2 weeks after treatment) appeared normal (Fig. 3), with one notable exception. In those seminiferous tubules displaying epithelia in Stages VIII-IX of the spermatogenic cycle, there was an overall loss of structural integrity of the epithelium, an increase of intercellular space, malorientation of spermatids, and partial or complete detachment of Step 8–9 spermatids (Fig. 3). This pattern of epithelial disaggregation was apparent in all Stage VIII-IX tubules observed in the 1- and 2-week posttreatment rats. In these rats, epithelia in earlier stages of the spermatogenic cycle appeared normal. Testicular morphology from all other experimental rats appeared normal and was not different from the testicular morphology of age-matched v-control rats.

At higher resolution, Sertoli ectoplasmic specializations observed in the 47- and 54-day-old experimental rats (1 and 2 weeks after treatment) were structurally intact, even in the morphologically aberrant Stage VIII-IX seminiferous epithelium (Fig. 4). However, some Step 8–9 spermatids were partially or completely detached from Sertoli cells (Fig. 4). Some Step 8–9 spermatids were without direct contact with any tissue structure and resident within the tubule lumen (Fig. 4). These "sloughed" cells were devoid of Sertoli cytoplasmic "tabs" adherent to their acrosomal poles (Fig. 4). Sertoli cytoplasmic tabs or remnants of ectoplasmic specializations are prevalent on Step 8–9 spermatids that have been mechanically detached from the epithelium (Fig. 4), as would be the case in rough handling of the tissue (17, 21).

Discussion

The effects of ethane dimethanesulphonate on the completion of the initial wave of spermatogenesis have not been reported previously. Results from this study showed that administration of EDS to 40-day-old rats led to a significant but temporary reduction of the testis weight occurring 1 week after EDS treatment, and a significant but temporary reduction of the daily sperm production occurring 1 and 2 weeks after treatment. EDS treatment also resulted in a significant reduction of testicular interstitial fluid testosterone concentrations 1 and 2 weeks after treatment when compared with vehicle-injected rats of the same age. The TIF testosterone at 2 weeks after treatment was not, however, significantly different than normal adult concentration of TIF testosterone as seen in both experimental and v-control rats 61 days old and older. EDS treatment delayed the normal peripubertal rise of both serum and TIF testosterone and, at this time, resulted in an elevation of the peripubertal concentration of FSH (23). A more detailed evaluation of the effects of EDS on the

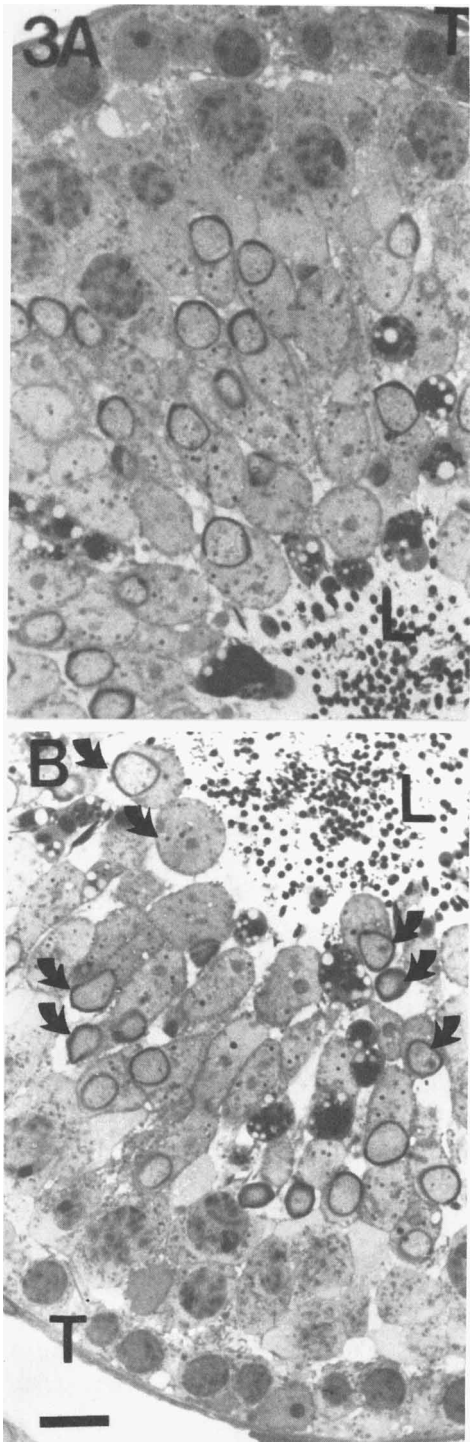

Figure 3. These two light micrographs illustrate a portion of the seminiferous epithelium from a (A) 47-day-old v-control rat testis and (B) a 47-day-old (1 week after EDS treatment) experimental rat testis. Epithelia in both micrographs are in Stage VIII of the spermatogenic cycle and contain Step 8 spermatids. In the (B) 1-week posttreatment rat, the tissue is disaggregated and some spermatids are partially or completely detached (arrows) from the epithelium. T, seminiferous tubule wall; L, tubule lumen; bar, 10 μm.

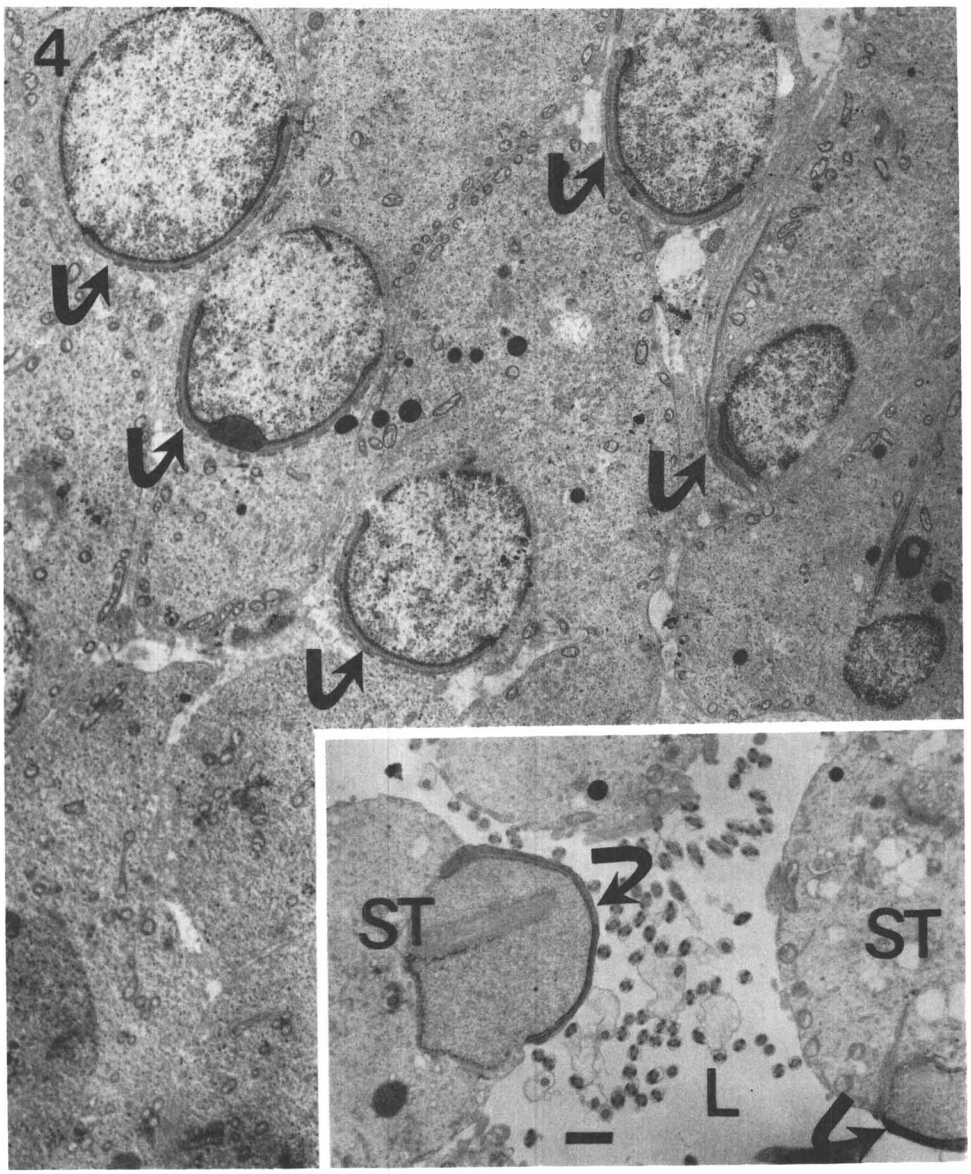

Figure 4. This electron micrograph shows Stage VIII seminiferous epithelium from the same tissue as illustrated in Figure 3B. Step 8 spermatids are associated with Sertoli ectoplasmic specializations (arrows). (Inset) Step 8 spermatids (ST) in the tubule lumen (L) are from the same seminiferous tubule shown in Figure 3B. Note that the acrosomal pole of the spermatids (arrows) are smooth and lacking Sertoli cell tabs or attached ectoplasmic specializations, as would be expected if the cell had been mechanically detached from the epithelium. Bar, 1 μm.

reproductive endocrinology of the peripubertal rat is addressed elsewhere (23). It was clear, in the present study, that EDS treatment did not completely eliminate testosterone and that the TIF testosterone returned to normal adult levels by the second week after treatment. The return to normal of the TIF testosterone preceded the return to normal of the DSP by at least 1 week.

Reduction of testis weight in the EDS-treated peripubertal rat was not as severe as reported in the EDS-treated adult rat. In the present study, this was most likely the result of a relatively moderate, but significant, reduction of testosterone in the peripubertal rat. It is not known why the effects of EDS on the peripubertal rat testis were less severe than that observed in the adult rat, but it may be related to the fact that peripubertal rat testes are in a growth phase rather than weight stabilized, as is the case in the adult rat. This model variation (peripubertal versus adult) is interesting and in need of further investigation.

Treatment with EDS in the adult rat results in the overt disruption of spermatogenesis, which has been attributed to the precipitous reduction of testosterone due to the eradication of Leydig cells (9, 24, 31, 32, 34). According to Verhoeven (35), EDS is selectively cytotoxic to Leydig cells even in the immature rat. It was suggested recently that EDS may have a cytotoxic effect directly on the seminiferous epithelium of intact animals (36). In this latter study, testosterone replacement in the EDS-treated rat failed to restore quantitatively normal spermatogenesis in experiments in which

rats were also exposed to exogenous estradiol. When testosterone replacement was not accompanied with the estradiol, there was a significant increase in the number of spermatids which, however, did not return to adult levels. In this latter experiment, the seminiferous tubule fluid concentration of testosterone was significantly lower than the control. An alternative explanation for these observations is that restoration of the adult number of mature spermatids, in the first experiment, was retarded by the presence of exogenous estradiol and, in the second experiment, was associated with the significantly reduced concentration of testosterone. In the experiments described in the present study, estradiol was not employed in the treatment protocol and restoration of testosterone, from endogenous sources, was to the normal adult level. For these reasons, we accept the more widely held opinion that EDS itself does not directly effect the spermatogenic process, but that the effects of EDS treatment are due to the reduction of testosterone.

Although, the process of spermatid maturation is known to be testosterone sensitive (34, 37–39), it is not known to what extent testosterone is involved and by what mechanisms, if any, this hormone promotes germ cell maturation. In the present study, the significant but temporary reduction of TIF testosterone was coincident with a significant but temporary reduction of the DSP. The return to normal adult levels of TIF testosterone 1 week later, or 2 weeks after EDS treatment, was not accompanied by a return to normal of the DSP (Step 15–19 spermatids). The DSP returned to normal the following week, or 3 weeks after treatment, and remained normal for the duration of the experiment. Because the return to normal of the DSP lagged behind the normalization of testosterone by approximately a week, the most likely stage for a spermatogenic lesion resulting in a reduction of Step 15–19 spermatids would be midspermiogenesis. If a disruption had occurred earlier in the spermatogenic cycle, then its expression as reduced DSP would have occurred later than 1 week from the perturbation, which was not the case in this study.

Results of the morphologic evaluation of testicular tissue support the hypothesis that a midspermiogenic lesion is associated with reduced testosterone. First, seminiferous epithelial pathology occurred only in the 1- and 2-week post-EDS-treated rats and second, the pathology was observed only in those tubules identified as being in Stage VIII-IX of the spermatogenic cycle. Although the morphologic change was subtle, when carefully compared with normal tissue, the epithelium appeared disaggregated, showed some Step 8–9 spermatids partially or completely detached from Sertoli cells, and showed some spermatids clearly resident within the lumen of the seminiferous tubule.

Although it could be argued that detachment of the cells was the result of rough handling and/or inadequate fixation, all tissues were handled and fixed in the same manner and, with this exception, disaggregated epithelia and sloughed germ cells were not observed. Additionally, sloughed Step 8–9 spermatids did not display attached Sertoli cytoplasmic tabs or remnants of Sertoli ectoplasmic specializations. Masri *et al.* (21) and others (17, 18) have shown that Step 8–19 spermatids are tightly anchored to Sertoli cells, and those which are mechanically detached from the epithelium can be identified by the presence of Sertoli cell fragments at their acrosomal poles. Results from the present study, therefore, indicate that the partial or complete detachment of Step 8–9 spermatids was not the result of inappropriate handling of the tissue, but rather the result of a physiologic or pathologic event.

It is possible that the reduced TIF testosterone adversely affected the structure and function of the Sertoli-spermatid junctional complex, resulting in spermatid detachment from the Sertoli cell. Uncoupling of the two cell types would eliminate, therefore, the mechanism by which post-Step 8 spermatids are pulled into the epithelium into deep Sertoli cell crypts (22, 40). These spermatids, then, would become "vulnerable" because of their persistent periluminal position in the pathway of the next generation of spermatids. As the new generation of spermatids would progressively occupy the periluminal area of the epithelium, they would displace the vulnerable spermatids into the lumen.

The loss of Step 8–9 spermatids from the epithelium at Stage VIII-IX of the spermatogenic cycle would lead to a subsequent reduction of mature spermatids by the end of that spermatogenic cycle. In the rat, sloughing of Step 8 spermatids would result in a reduction of the DSP (elongated spermatids at Steps 15–19) not sooner than 4–5 days later and not beyond 11–12 days after the resumption of normal binding. This is calculated based on the time it takes Step 8 spermatids to reach the Step 15 stage of spermatid differentiation (approximately 4 days in the rat) and the additional 7 days that it takes to reach Step 19 (11–14, 16). Results of this study closely accommodate these time limitations and support the hypothesis that reduced testosterone results in a reduction of the DSP mediated by sloughing of Step 8–9 spermatids.

Studies utilizing a Sertoli-spermatid coculture model support the proposal that testosterone regulates Sertoli-spermatid junctional interaction by demonstrating that maximal, stable binding of round spermatids to Sertoli cells requires testosterone (41) and that in the presence of FSH, spermatid binding appears to be dose responsive to testosterone (D. F. Cameron and K. E. Muffly, presented at Eleventh North American Testis Workshop, Montreal, Canada, 1991). These studies suggest that testosterone is directly involved with the actual cell-to-cell adhesion mechanism. Cell-adhesion

molecules including *N*-cadherins, have been identified in cells of the seminiferous epithelium (42–45) and, recently, Cyr *et al.* (46) showed that testosterone stimulates the gene expression of *N*-cadherins in epithelial cells of the epididymis. As appears to be the case with the epididymal epithelium, testosterone may affect Sertoli-spermatid cell-to-cell adhesion in the testis by stimulating the production of cell-adhesion molecules on that portion of the Sertoli cell membrane associated with the Sertoli-spermatid junction complex.

In the study reported here, there was no additional reduction of the DSP for up to 8 weeks after treatment, a time sufficient for the completion of several spermatogenic cycles (15, 16). The treatment clearly resulted in a lesion that was temporary and that affected the spermatogenic cycle in progress at the time of hormone reduction at a site not prior to midspermiogenesis. Our results do not rule out earlier sites of testosterone involvement in spermatogenesis as demonstrated by Sun *et al.* (34). However, because of the moderate and temporary nature of the testosterone reduction, we suggest that our results have identified the testosterone-dependent event that is most sensitive to hormone reduction.

Our results show that a temporary reduction of peripubertal testosterone in the rat was associated with a midspermiogenic lesion preceding initial spermatid elongation. It is suggested that the subsequent temporary reduction of the DSP was mediated by an interruption of the Sertoli-spermatid binding mechanism during Stages VIII-IX of the spermatogenic cycle.

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