Blackwell Publishing

Asian J Androl 2006; 8 (3): 289–299
DOI: 10.1111/j.1745-7262.2006.00140.x

AJA

·Original Article·

Histological changes of the testis and epididymis in adult rats as a result of Leydig cell destruction after ethane dimethane sulfonate treatment: a morphometric study

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Abstract

Aim: To quantitatively study the histological changes of the testis and epididymis as a result of a drastic reduction of testosterone secretion. **Methods:** Fourteen adult Sprague–Dawley rats were injected intraperitoneally with ethane dimethane sulfonate (EDS, 75 mg/kg) and the same number of animals were injected with normal saline as a control. At days 7 and 12 (after treatment), respectively, half of the animals from each group were killed. The testes and epididymides were removed and tissue blocks embedded in methacrylate resin. The cell number per testis was estimated using the stereological optical disector and some other parameters were obtained using other morphometric methods. **Results:** The EDS treatment resulted in an almost complete elimination of Leydig cells but had no effect on the numbers of Sertoli cells per testis. At day 7 after EDS treatment, many elongated spermatids were retained in the seminiferous epithelium and many round spermatids could be seen in the epididymal ducts. At day 12, a looser arrangement of spermatids and spermatocytes became evident, with apparent narrow empty spaces being formed between germ cells in an approximately radial direction towards the tubule lumen; the numbers (per testis) of non-type B spermatogonia and spermatocytes were similar to controls, whereas that of type B spermatogonia increased by 59%, and that of early round, elongating and late elongated spermatids decreased by 37%, 72% and 52%, respectively. **Conclusion:** The primary spermatogenic lesions following EDS administration were (i) spermiation failure and (ii) detachment of spermatids and spermatocytes associated with impairment in spermiogenesis and meiosis. *(Asian J Androl 2006 May; 8: 289–299)*

Keywords: epididymis; ethane dimethane sulfonate; Leydig cells; morphometry; spermatogenesis; stereology; testis; testosterone

1 Introduction

It was well established in the 1980s that ethane dimethane sulfonate (EDS), which is chemically related to alkylating cytotoxic agents, selectively kills Leydig cells in the mature rat testis [1]. A single dose of EDS would result in complete destruction of Leydig cells within 2 days, but a new population of Leydig cells would begin to regenerate at approximately day 14 after treatment and multiple doses of EDS would not prolong the effects of a single dose [1–3]. EDS treatment, alone or in combination with other treatments, is commonly used for

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Received 2005-04-27 Accepted 2006-01-13

the study of the hormonal control of spermatogenesis. EDS-induced intratesticular testosterone withdrawal is even more rapid and profound than that induced by hypophysectomy [4]. It produces degeneration or apoptosis of spermatogenic cells, especially spermatocytes and spermatids [5, 6]; the total daily sperm production, as obtained by counting sperm (elongated spermatids) in the homogenized testicular tissue, is reduced to 15% of the control at day 14 after EDS treatment [7]. However, there is still a paucity of data on the EDS-induced changes in, among other morphometric features of the testis and epididymis, the numbers of different categories of spermatogenic cells. The numerical changes must have been small, and unbiased stereological number estimation is therefore crucial in objectively evaluating the changes. The state-of-the-art optical disector, an unbiased and efficient stereological tool, was first used for the quantitative study of spermatogenesis in the 1990s [8], and has not been used in an EDS model. With careful morphometric analysis, the present study aimed to determine the histological changes of the testis and epididymis, especially the key spermatogenic changes, following a drastic testosterone withdrawal induced by EDS treatment.

2 Materials and methods

2.1 Animals and treatment

Animals were obtained from the Animal Center of North Sichuan Medical College. Experiment protocols were approved by the research section of the college and ethical guidelines were followed during the experiment.

Twenty-eight normal adult male Sprague–Dawley rats, aged approximately 90 days, were randomly divided into two groups. One was the control group (n = 14, weight 198 ± 7 g [160–230 g]), receiving a single intraperitoneal injection of normal saline; the other was the EDS group (n = 14, weight 196 ± 8 g [160–255 g]), receiving a single intraperitoneal injection of EDS (75 mg/kg bodyweight, dissolved in dimethyl sulfoxide). The same batch of EDS was used in a previous study [9]. The injection of normal saline instead of the EDS vehicle dimethyl sulfoxide in the control group was justified by a previous study in which no differences were found between the vehicle-treated and untreated rats [10].

Seven days after treatment, seven randomly-chosen animals in each group were anesthetized with ether to remove the ipsilateral testis and epididymis of one side (left or right, alternately chosen) and one random side of the seminal vesicles. After further 5 days, the organs were removed from the other animals in the same way. On removal, the organs were immersion-fixed in Bouin's fluid, and then, approximately 30 h later, stored in 70% ethanol for a few days. Then, the testis and epididymis were separated and the organs were weighed using an electronic balance (accuracy 0.1 mg). The organ volumes were calculated by dividing the weights by a density of 0.93 g/mL. The density measured after fixation in Bouin's fluid followed by dehydration or storage in 70% ethanol was consistently found to be approximately 0.93 for the testis [8, 11] or epididymis (unpublished data), and the testicular tissue volume obtained after fixation in the fixative and then storage in 70% ethanol had no significant shrinkage after further processing–dehydration, embedding in methacrylate and sectioning [8].

2.2 Sections

Two parallel slices perpendicular to the testicular long axis were cut from each testis, and a half or a quarter of each slice was cut for embedding. From each epididymis, two slices were cut around the middle of the caput and the cauda, respectively, along a random direction. An arbitrary segment was also cut from the thin and small intermediate part between the caput and cauda and embedded next to the caudal slice as one tissue block.

After dehydration in absolute ethanol and butanol, the tissue blocks (slices) were embedded in hydroxyethylmethacrylate resin (Historesin; Leica Microsystems Nusslock GmbH, Nussloch, Germany) according to the manufacturer's instructions. One 25-μm-thick section was cut from each block (average section area: ~24 ± 2 mm^2 for testicular sections and ~24 ± 3 mm^2 for epididymal sections) and stained with periodic acid-Schiff's reagent and hematoxylin (testis) or hematoxylin alone (epididymis).

The leaflike seminal vesicle was cut into four parallel pieces and two alternate pieces were sampled for further processing as described above. The sections were conventionally stained with hematoxylin-eosin.

2.3 Morphology and morphometry

The histology of the seminal vesicle was only qualitatively observed, and that of the testis and epididymis was studied both qualitatively and quantitatively. The quantitative methods were stereological or morphometric methods, which are mostly described in our previous studies [8, 11, 12] and only a brief account of which is given below.

2.3.1 Cell numbers

The numbers of all types of nuclei in the testis and the epididymal duct were estimated with the optical disector. Briefly, the section was observed using a × 100 oil lens on a computer monitor, and 4–6 computer-generated rectangular frames were superimposed on the image (final magnification × 2 677) for nuclear counting within 10 μm of the section in thickness. The numerical density (number per volume) of the nuclei in the organ was estimated by dividing the total number of nuclei counted by the total volume of "disectors" (3-D counting spaces inside the section), and then the total number per organ was calculated by multiplying the density by the volume of the organ. The areas of the counting frames used in the present study were 22 μm × 17 μm for nuclei in the testis and 8 μm × 6 μm for nuclei in the epididymal duct. The total numbers (per testis) of disectors counted for each type of nuclei were approximately 2 560 for spermatogonia and early spermatocytes, approximately 640 for other spermatocytes and nuclei in the interstitium, and approximately 320 for spermatids; the numbers of disectors counted per epididymis were approximately 360 for spermatozoa and approximately 2 106 for other nuclei within the epididymal duct.

2.3.2 Tubule volume, diameter and length

The volume fraction of the seminiferous tubules in the testis, or the epididymal duct in the epididymis, was conventionally estimated by point counting during the nuclear counting process (above), and the total tubule, or duct, volume per organ was calculated by multiplying the fraction by the volume of the organ.

All the testicular sections were observed again, using a × 10 objective lens on a computer monitor with one rectangular frame superimposed on the lower-left corner of the field (final magnification × 268). Forty (per testis) round or elliptical profiles of the seminiferous tubules with an apparent lumen were sampled with the frame, and then the diameter of round profiles or the short axis of elliptical profiles was measured as the tubule diameter. The tubule length per testis was then calculated by dividing the total tubule volume per testis by the cross-sectional area of the tubules, the cross-sectional area being calculated by multiplying the mean of the squared diameters by a constant ($\pi/4$).

2.3.3 Tubule morphology

After the diameter of each sampled seminiferous tubule profile was measured as described above, the profile was further observed at a higher magnification (using a × 20 objective lens) by focusing up and down in the section, and key morphological characteristics were recorded on, for example, whether there were elongated spermatids retained. (The retained spermatids were situated abnormally close to the basement membrane, or were present in the post-stages of the seminiferous epithelium in which elongated spermatids are normally absent – they have normally been released in the preceding stages of the seminiferous epithelium [12].) Thus the frequency of tubule profiles (corresponding to the relative length of the tubules) with abnormal features was obtained, as a complement to the unbiased numerical data obtained.

Epididymal sections were also examined, using × 10–40 objective lenses, for key morphological features.

2.4 Statistics

Data in the text and tables are shown as mean ± SEM. The effects of the two factors, EDS treatment and animal growth (duration of experiment), on morphometric data were detected with two-way ANOVA in conjunction with the Student-Newman-Keuls method for pairwise multiple comparisons. Other statistical tests used are indicated specifically in the text. The significance of difference was set at $P \leq 0.05$.

3 Results

In one of the seven animals receiving EDS treatment for 7 days, the number (37.8×10^6) of Leydig cells per testis remained within the normal range and the volume of the testis (1.60 cm^3) or seminal vesicle (0.261 cm^3) did not decrease, so no data obtained from this animal were included in the present study.

3.1 Bodyweights

In the vehicle (control)- and EDS-treated groups in which the animals were killed at either day 7 or 12 after treatment, neither EDS treatment nor animal growth had significant effects on the bodyweights measured before treatment and 7 days or 12 days after treatment (two-way repeated measures ANOVA).

3.2 Seminiferous tubules

Testicular volume was unaffected at day 7 after EDS treatment, which was, however, significantly decreased, by approximately 34%, at day 12. Similarly, the tubule

volume per testis was unchanged at day 7, but reduced significantly, by approximately 30%, at day 12 as a result of reduction in the tubule diameter (by ~14 %) rather than the tubule length (Table 1).

Seven days after EDS treatment, (i) abnormal (retained, malformed, disoriented or pyknotic) elongated spermatids, mostly retained ones (Figures 1, 2), were observed in some tubules (Table 2); (ii) fewer elongated spermatids (spermatozoa) were seen apparently released into the tubule lumen (Table 2); and (iii) more marked changes, similar to those seen in most animals treated with EDS for 12 days, were observed in one of the six animals.

At day 12 after EDS treatment, more obvious changes observed included the following: (i) a looser arrangement of spermatogenic cells, that is, abnormally large empty spaces (often radial cracks running towards the tubule lumen) were formed between lines, bundles or groups of spermatids and spermatocytes (Figure 1C, Figure 2B) in approximately 26% of the tubule profiles (Table 2); (ii) there were fewer elongated and elongating spermatids in the seminiferous epithelium; (iii) there were more detached (sloughed) round spermatids and spermatocytes in the tubule lumen; and (iv) severe spermatogenic damage was observed in one of the seven animals, in which few spermatids were left in the seminiferous epithelium and many pachytene spermatocytes were detached. An increase in the number of type B spermatogonia (Table 3) was confirmed on qualitative observation, with many type B spermatogonial nuclei being seen in a line along the basement membrane (Figure 2B).

EDS had no significant effects on the number (per testis) of Sertoli cells or non-type B (type A plus intermediate type) spermatogonia, but it significantly increased that of type B spermatogonia by approximately 50% at day 7 or 12. Spermatocyte numbers (per testis) were maintained after EDS treatment, and so were spermatid numbers at day 7; at day 12, however, early round, middle-stage elongating and late elongated spermatids were significantly decreased by 37%, 72% and 52%, respectively (Table 3). In the controls, few detached or pyknotic round spermatids or retained elongated spermatids were counted in the counting process. In the EDS-treated groups, few detached round spermatids were counted, but approximately 5% (counted in two of the six animals) and 7% (counted in four of the seven animals) of the round spermatids had pyknotic (abnormally dense) nuclei, and approximately 11% (counted in five animals) and 17% (counted in all seven animals) of the elongated spermatids were retained to some degree at days 7 and 12, respectively.

Table 1. Organ volumes and morphometric results on the seminiferous tubules and the epididymal duct (mean ± SEM). In general, [a]ethane dimethane sulfonate (EDS) treatment or [b]animal growth (duration of experiment) had a significant effect, [c]with a significant interaction between treatment and growth. For comparison between subgroups, [d]the control or EDS-treated subgroup at day 7 was significantly different from the corresponding subgroup at day 12, and [e]the EDS-treated subgroup was significantly different from the corresponding control (subgroup) at day 7 or day 12. [c']$P = 0.052$.

	Day 7		Day 12	
	Control ($n = 7$)	EDS-treated ($n = 6$)	Control ($n = 7$)	EDS-treated ($n = 7$)
Testis				
Volume (cm^3)[a,b,c]	1.27 ± 0.04	1.14 ± 0.06[d]	1.27 ± 0.06	0.85 ± 0.08[e]
Volume fraction of the tubules (%)[a]	81.6 ± 0.7	84.9 ± 0.7[e]	81.1 ± 0.7	84.5 ± 0.7[e]
Total tubule volume (cm^3)[a,b,c]	1.03 ± 0.03	0.97 ± 0.05[d]	1.03 ± 0.05	0.72 ± 0.07[e]
Tubule diameter (μm)[a,c']	303 ± 4	288 ± 6[d]	308 ± 5	264 ± 11[e]
Total tubule length (m)	14.2 ± 0.5	14.7 ± 0.8	13.8 ± 0.6	12.8 ± 1.0
Epididymis				
Volume (cm^3)[a]	0.339 ± 0.010	0.254 ± 0.035[e]	0.364 ± 0.025	0.186 ± 0.024[e]
Volume fraction of the duct (%)[a]	66.9 ± 1.6	43.6 ± 3.1[e]	68.6 ± 3.4	47.1 ± 5.5[e]
Total duct volume (cm^3)[a]	0.227 ± 0.008	0.109 ± 0.016[e]	0.252 ± 0.025	0.089 ± 0.017[e]
Seminal vesicle				
Volume (cm^3)[a,b,c]	0.138 ± 0.013[d]	0.065 ± 0.004[e]	0.266 ± 0.038	0.055 ± 0.008[e]

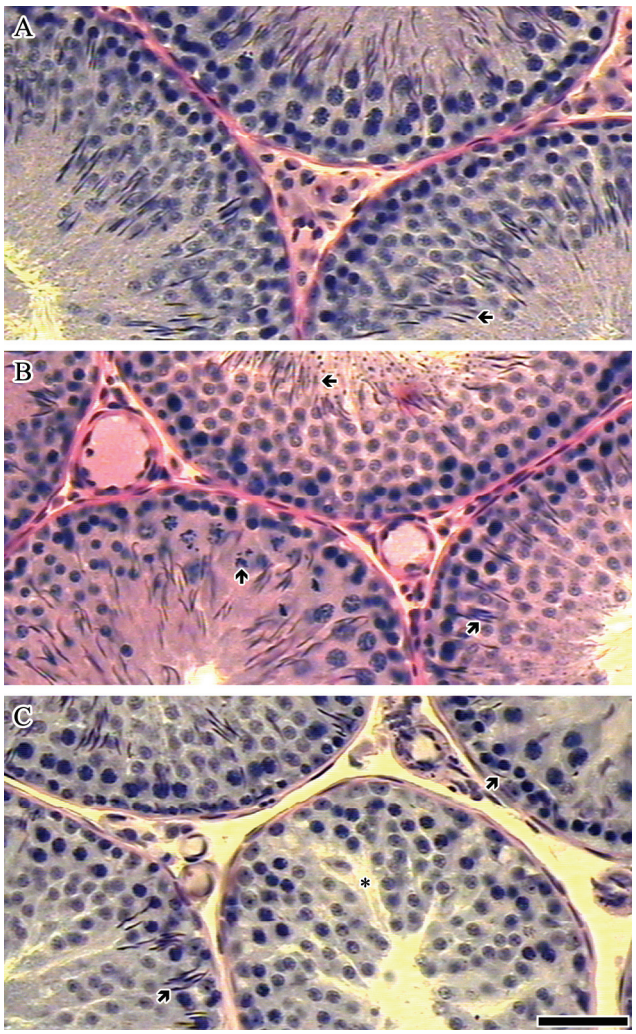

Figure 1. Typical testicular micrographs, taken on 25 μm thick methacrylate-embedded sections with a × 20 objective lens, from (A): vehicle-treated and (B, C): ethane dimethane sulfonate (EDS)-treated animals at days7, 7 and 12 after treatment, respectively. ←, late elongated spermatids (nuclei); ↗, apparently retained late elongated spermatids; ↑, secondary spermatocytes in division; *, empty space (crack) between spermatogenic cells. Scale bar, 50 μm.

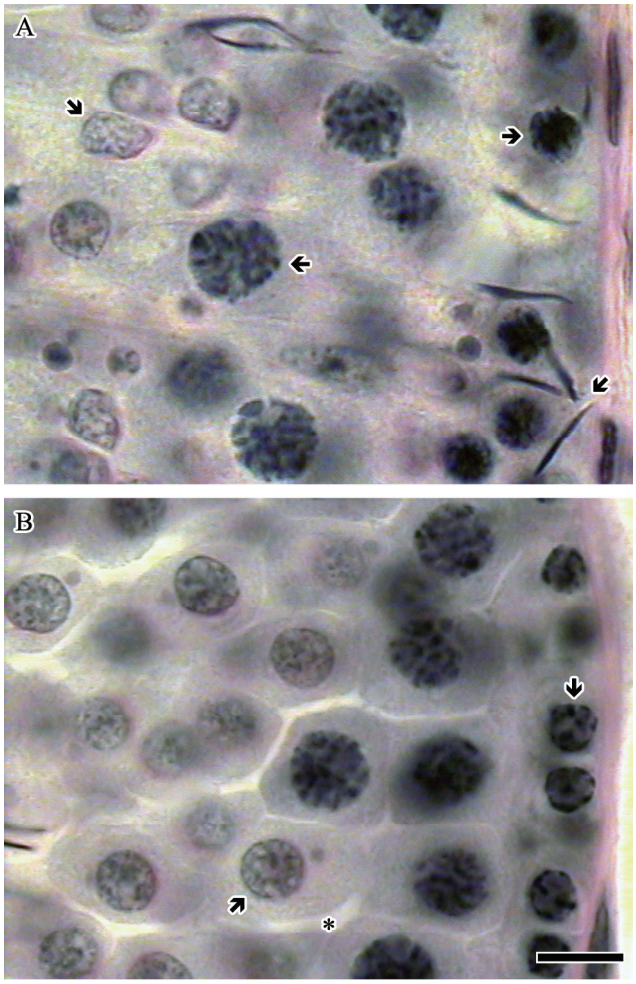

Figure 2. Typical testicular micrographs, taken on 25 μm thick methacrylate-embedded sections with a ×100 oil lens, from ethane dimethane sulfonate (EDS)-treated animals at (A): day 7 and (B): day 12 after treatment.↙, retained late elongated spermatids (nuclei); ↘, middle stage elongating spermatid; ↗, early round spermatid; ←, middle and late stage primary spermatocyte; →, early primary spermatocyte; ↓, type B spermatogonia; *, empty space (crack) between spermatocytes and spermatids. Scale bar, 10 μm.

3.3 Testicular interstitium

A consistent and dramatic change in the interstitial tissue was that typical Leydig cells [12] were rarely observed at either day 7 or 12 after EDS treatment. Suspected (probable) Leydig cells, which were slightly smaller in size and with less clear granules in the nucleus, were occasionally seen during the nuclear counting process.

The number (per testis) of probable Leydig cells at day 7 or 12 after EDS treatment was less than 8% of the Leydig cell number in the control, whereas those of other nuclear types in the interstitium were not significantly affected (Table 3).

3.4 Epididymis

At day 7 after EDS treatment, the epididymal volume and the volume of the epididymal duct per epididymis decreased significantly by 25% and 52%, respectively, and at day 12 the decreases were 49% and 64%, respec-

Table 2. Percentage (R.) of seminiferous tubule profiles with certain morphological features and number of nuclei observed per tubule profile (mean ± SEM). *Detached round spermatids and spermatocytes were counted in sections from only 1, 1, 2 and 4 of the animals in the subgroups (left to right), respectively, of all the counted sections obtained from all the animals. In general, [a]ethane dimethane sulfonate (EDS) treatment or [b]animal growth (duration of experiment) had a significant effect, [c]with a significant interaction between treatment and growth. For comparison between subgroups, [d]the control or EDS-treated subgroup at day 7 was significantly different from the corresponding subgroup at day 12, and [e]the EDS-treated subgroup was significantly different from the corresponding control (subgroup) at day 7 or 12. LSt, late elongated spermatids.

	Day 7		Day 12	
	Control (n = 7)	EDS-treated (n = 6)	Control (n = 7)	EDS-treated (n = 7)
R. with LSt released into the lumen[a]	15.2 ± 4.2	4.0 ± 1.8[e]	18.9 ± 1.3	3.2 ± 1.5[e]
No. LSt released into the lumen[a]	0.519 ± 0.175	0.111 ± 0.067	0.431 ± 0.081	0.125 ± 0.090
No. detached round spermatids and spermatocytes*	0.007 ± 0.007	0.046 ± 0.041	0.004 ± 0.004	0.093 ± 0.049
R. with abnormal LSt[a]	14.9 ± 1.6	60.7 ± 8.4[e]	12.3 ± 2.2	69.2 ± 12.7[e]
No. retained LSt[a]	0.06 ± 0.02	4.41 ± 0.74[e]	0.05 ± 0.02	5.47 ± 1.79[e]
No. other abnormal LSt[a]	0.29 ± 0.07	0.81 ± 0.20[e]	0.21 ± 0.07	0.74 ± 0.19[e]
R. with loose arrangement of spermatogenic cells[a,b,c]	0	4.6 ± 4.1[d]	0	26.4 ± 8.6[e]

tively (Table 1).

In the controls, round spermatids were occasionally scattered among densely packed spermatozoa. Seven days after EDS treatment, spermatozoal number (per area) appeared decreased and, more noticeably, groups of round spermatids, including a small number of pachytene spermatocytes, were seen in all six animals and on 10 of the 12 epididymal sections (Figures 3, 4). At day 12, the spermatozoal number appeared to be further decreased (Figure 3C) and more round spermatids and pachytene spermatocytes were present (Figure 4C) in six of the seven animals and on 11 of the 14 sections.

A considerable number of neutrophil nuclei, which were identified according to the morphology of neutrophils in the blood vessels and absent in the epididymal duct in control animals, were observed inside the epididymal duct on three sections from two animals at either day 7 or 12 after EDS treatment (Figure 4B). Furthermore, on three sections from three animals at day 7 and on two sections from one animal at day 12 after EDS treatment, sperm granuloma was observed in the epididymis, where the epithelial wall of the duct was invisible or ill-defined and sperm were mixed with more non-spermatogenic cells, probably inflammatory cells (Figure 4A).

The numbers (per epididymis) of spermatozoa were significantly decreased by 61% and 83% at days 7 and 12 after EDS treatment, respectively (Table 3). A considerable number of round spermatids were seen in the epididymal duct at day 7 (counted in five of the six animals) and the number increased approximately 5-fold at day 12 (counted in six of the seven animals); approximately 14% and 19% of the round spermatids had pyknotic nuclei at days 7 and 12 after EDS treatment, respectively. A considerable number of other nuclei were also counted in the epididymal duct; approximately 1% (counted in one animal) and 31% (counted in four animals) of them were pachytene spermatocytes at days 7 and 12, respectively, and most of the remaining cells were probably neutrophils.

3.5 Seminal vesicle

Compared to the control at days 7 and 12, the volume of the seminal vesicle after EDS treatment was significantly decreased by 53% and 79%, respectively (Table 1).

The lumen and mucosa of the tubular gland were markedly atrophied at day 7 after EDS treatment. At day 12, the gland was further atrophied and the lumen was almost obliterated. However, atrophy in the smooth muscle layer of the gland was less marked at either day 7 or 12.

4 Discussion

Observing morphology on methacrylate-embedded sections with good microscopic effects and using stereological methods to obtain morphometric data, the present study revealed that appreciable damage to sper-

Table 3. Numbers (10^6) of nuclei per testis or epididymis and numerical ratios (mean ± SEM). *The numbers of the cells (nuclei) counted in the control groups were zero, and no statistical tests were performed. [#]The number of probable (non-typical) Leydig cells. In general, [a]ethane dimethane sulfonate (EDS) treatment or [b]animal growth (duration of experiment) had a significant effect, [c]with a significant interaction between treatment and growth. For comparison between subgroups, [d]the control or EDS-treated subgroup at day 7 was significantly different from the corresponding subgroup at day 12, and [e]the EDS-treated subgroup was significantly different from the corresponding control (subgroup) at day 7 or 12. [b']$P = 0.050$. BSg, type B spermatogonia; ESc, early primary spermatocytes; ESt, early round spermatids; LSt, late elongated spermatids; MSt, middle stage elongating spermatids; Non-BSg, non-type B spermatogonia; Sc, middle and late stage primary spermatocytes; SSc, secondary spermatocytes.

	Day 7		Day 12	
	Control ($n = 7$)	EDS-treated ($n = 6$)	Control ($n = 7$)	EDS-treated ($n = 7$)
In the interstitial tissue				
Leydig cells[a]	28.2 ± 3.7	2.2 ± 0.8[e,#]	32.4 ± 2.5	1.5 ± 0.4[e,#]
Myoid cells	11.6 ± 1.1	12.6 ± 2.1	7.9 ± 0.7	11.6 ± 1.5
Other nuclei	29.3 ± 1.9	42.8 ± 3.4	38.2 ± 4.3	38.3 ± 5.5
In the seminiferous tubules				
Sertoli cells	36.7 ± 2.1	40.2 ± 3.6	33.8 ± 2.3	36.1 ± 3.8
Non-BSg[b]	6.95 ± 0.55	7.75 ± 0.44	5.09 ± 0.57	5.40 ± 0.89
BSg[a]	3.57 ± 0.22	5.38 ± 0.38[e]	3.04 ± 0.39	4.84 ± 0.59[e]
ESc	44.6 ± 3.0	50.7 ± 3.4	45.6 ± 3.8	52.1 ± 5.6
Sc	81.3 ± 2.1	78.1 ± 4.1	76.9 ± 4.2	70.9 ± 6.7
SSc	3.2 ± 0.8	4.0 ± 1.2	2.8 ± 0.8	2.2 ± 1.0
(ESt + MSt)[a,b,c]	296 ± 16	295 ± 25[d]	308 ± 20	168 ± 31[e]
ESt[b,c]	219 ± 13	244 ± 19[d]	231 ± 15	146 ± 25[e]
MSt[a]	77 ± 6	51 ± 11[d]	77 ± 8	22 ± 7[e]
LSt[a,c]	166 ± 13	163 ± 21[d]	195 ± 16	94 ± 24[e]
Ratio of numbers per (volume of) testis				
BSg : Non-BSg[a,b]	0.528 ± 0.047	0.699 ± 0.043[d]	0.623 ± 0.075	0.939 ± 0.065[e]
ESc : BSg[a]	12.9 ± 1.4	9.6 ± 0.9	16.4 ± 2.3	11.1 ± 1.0
Sc : ESc[a]	1.89 ± 0.16	1.58 ± 0.17	1.73 ± 0.13	1.39 ± 0.11
(ESt + MSt) : Sc[a,b',c]	3.67 ± 0.23	3.76 ± 0.22[d]	4.02 ± 0.18	2.29 ± 0.39[e]
ESt : Sc[c]	2.71 ± 0.17	3.12 ± 0.15[d]	3.01 ± 0.11	2.00 ± 0.32[e]
MSt : ESt[a]	0.353 ± 0.028	0.206 ± 0.051[e]	0.332 ± 0.028	0.135 ± 0.039[e]
LSt : MSt	2.23 ± 0.22	11.37 ± 8.33	2.63 ± 0.21	7.12 ± 2.13
LSt : (ESt + MSt)	0.559 ± 0.026	0.546 ± 0.042	0.635 ± 0.034	0.536 ± 0.057
In the epididymal duct				
Mature sperm[a]	271 ± 33	106 ± 51[e]	284 ± 40	48 ± 24 [e]
Round spermatids*	0	1.0 ± 0.4	0	4.7 ± 2.7
Other cells*	0	5.9 ± 3.8	0	4.3 ± 3.6

matogenesis occurred at day 7 after EDS treatment, which was primarily spermiation failure (i.e. retaining of late elongated spermatids in the seminiferous epithelium) and sloughing of spermatogenic cells into the epididymal duct. At day 12, detachment of spermatocytes and spermatids became evident, with apparent radial cracks being formed between the germ cells. It could also be concluded, if spermatocytes and spermatids were detached from the seminiferous epithelium in proportions, that key spermatogenic lesions also included impairment in meiosis (development of spermatids from spermatocytes) and spermiogenesis (transformation of elongated spermatids from round spermatids), as indicated by significantly smaller numerical ratios between round spermatids and

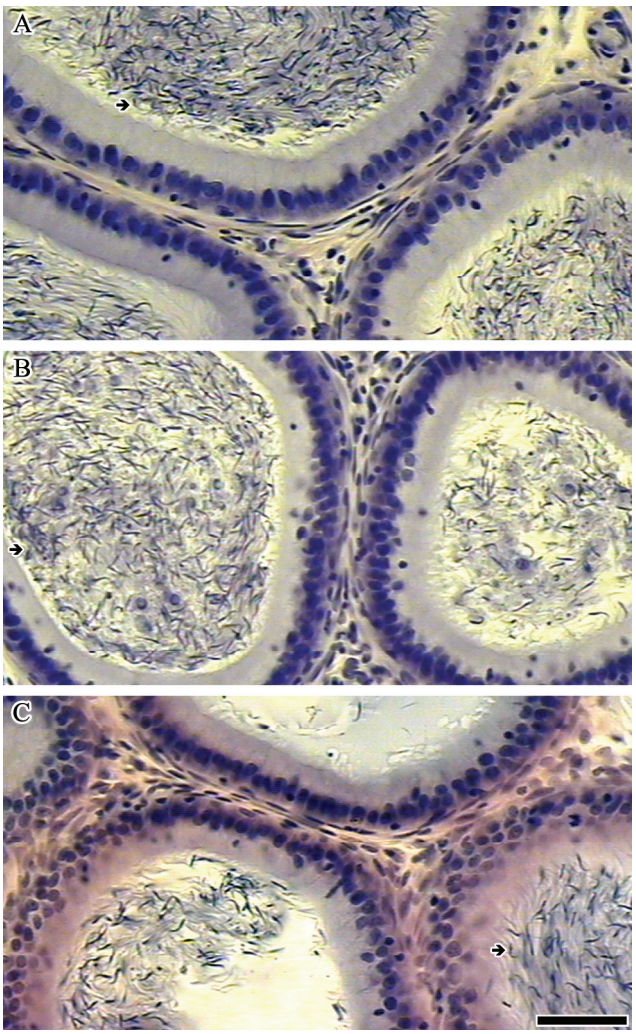

Figure 3. Typical epididymal micrographs, taken on 25 μm thick methacrylate-embedded sections with a × 20 objective lens, from (A): vehicle-treated and (B, C): ethane dimethane sulfonate (EDS)-treated animals at days7, 7 and 12 after treatment, respectively. ➔, densely packed spermatozoa inside the epididymal duct. Scale bar, 50 μm.

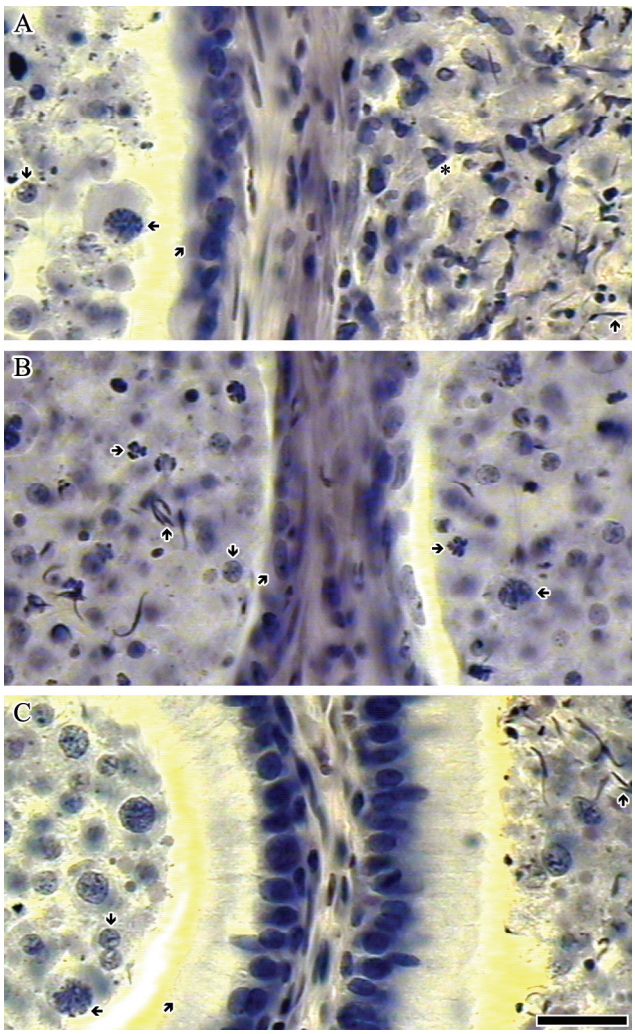

Figure 4. Typical epididymal micrographs, taken on 25 μm thick methacrylate-embedded sections with a × 40 objective lens, mainly showing the presence of round spermatids and spermatocytes inside the epididymal duct at (A): day 7 and (B, C): day 12 after ethane dimethane sulfonate (EDS) treatment. ➚, spermatozoon; ➘, round spermatid; ➔, primary spermatocyte; ➔, neutrophil (nucleus); ➚, epithelial wall of the epididymal duct; *, non-spermatogenic cells (around) mixed with a small number of spermatozoa, with ill-defined wall of the epididymal duct. Scale bar, 25 μm.

spermatocytes and between elongating spermatids and round spermatids (Table 3). Degeneration (pyknotic nuclei) or apoptosis of spermatocytes and spermatids as observed in the current study and previous studies [5, 6] also indicated impairment in meiosis and spermiogenesis. These changes should be testosterone-deficiency specific as gonadotrophin (luteinizing hormone [LH] and follicle stimulating hormone [FSH]) levels would be elevated rather than lowered following testosterone withdrawal. Although hormonal levels were not measured in the current study, we could assume that intratesticular testosterone levels were undetectable at day 7 or 12 after EDS treatment in the current study. This is supported by (i) the finding in the current study that almost all Leydig cells were destroyed and the testosterone-dependent seminal vesicle was severely atrophied; (ii) our previous study of an incomplete intratesticular testosterone withdrawal model induced by testosterone undecanoate injection, in which Leydig cells, although atrophied, had no significant change

in number and the testosterone levels in the testicular interstitial fluid fell to 7.2% of the control [12]; (iii) the previous study using the same EDS agent as used in the current study, which showed absence of Leydig cells with the 3β-hydroxysteroid dehydrogenase (Leydig cell marker) immunocytochemistry at days 2, 7 and 14 after treatment [9]; and (iv) the finding of previous studies that EDS treatment results in undetectable intratesticular testosterone levels [1]. As also shown previously, serum LH and FSH levels increased 4.2-fold and 1.3–1.8-fold (average 1.5) at day 3, 4.2–13.2-fold (average 7.4) and 1.5–2.7-fold (average 2.2) at day 7, and 4.4–9.0-fold (average 6.7) and 2.5–3.0-fold (average 2.8) at day 14 after EDS treatment, respectively [2, 7, 13–15].

Despite impairment in spermiation and spermiogenesis following EDS treatment, the number of type B spermatogonia was increased. This should be the effect of increased FSH levels since LH is known to act on Leydig cells and has no direct effects on spermatogenesis [16]. An increase in spermatogonial numbers was also observed in our previous study in monkeys with hemicastration, which increases FSH levels [17]. The finding was also notable that the number of spermatocytes was maintained in spite of their detachment. This should be partly because of the fact that their detachment still was not severe at day 12 after EDS treatment, and partly because of the increase of FSH levels after the treatment (above)–FSH alone could partially support the initial phase of spermatogenesis up to the level of round spermatids [16]. So although testosterone and FSH may act synergistically to support spermiation [18], testosterone is more important in this regard; while testosterone may initiate and maintain some degree of complete spermatogenesis in the apparent absence of FSH [16], FSH alone might be able to maintain normal spermatogenesis in the early stages if spermatocytes and spermatids should not be detached (also see below).

Using a rat model of testosterone plus estradiol treatment, which suppressed intratesticular testosterone (to ~3% of normal levels) but not serum FSH, previous studies showed that impairment of spermiation occurred at week 1 after treatment but spermatid sloughing appeared later, with round spermatids observed occasionally in the epididymal duct at week 3 [18, 19]. Using a presumably more profound intratesticular testosterone withdrawal model of EDS treatment, the current study demonstrates the concurrent presence of spermiation impairment and germ-cell sloughing 1 week after treatment, when the typical radial cracking (looser arrangement) of spermatogenic cells was not yet noticeable under light microscopy. In a chronic model of incomplete intratesticular testosterone withdrawal, we observed masses of the seminiferous epithelium appearing to be sloughing or bulging into the tubule lumen in 8% of the tubule profiles, while retention of elongated spermatids was apparent and there were not many detached round spermatids inside the tubule lumen [12]. Re-evaluating all the testicular sections used in this previous study [12] in the same way as was performed in the current study, we found that (i) a looser arrangement of spermatocytes and spermatids was seen in (11.5 ± 5.3)% of tubule profiles; and (ii) the looser arrangement was also characterized by radial cracks formed between spermatogenic cells, but the cracking was not as severe (i.e. with fewer and narrower cracks) compared to that observed in the current study (data not reported). These studies suggested that germ-cell sloughing, if any, following testosterone withdrawal involves, more likely, masses or pieces of the seminiferous epithelium next to the basal spermatogonial layer, rather than single round spermatids. The extent and duration of the intratesticular testosterone withdrawal determines the severity of spermatogenic lesions – germ-cell sloughing or detachment and impairment in spermiation and spermiogenesis. The lesions might be related [18], but it is yet to be demonstrated whether one lesion is the underlying or leading lesion of the other. Interestingly, testosterone replacement, if given immediately after hypophysectomy, maintains spermatogenesis, while delayed replacement (4-week replacement after 4 weeks of hypophysectomy) does not restore normal spermatogenesis [20]. Thus, we speculate that once spermatogenic cells are detached from Sertoli cells to some extent, degeneration and impaired movement of spermatogenic cells will follow and the effects of hormones or other factors on these cells will then be compromised.

We previously observed sperm granuloma in the epididymis after vasectomy [17] and around the vas deferens after intravasal laser irradiation [21], likely as a result of continual production of sperm from the testis, higher pressure inside the epididymal duct or leaking of sperm. But the presence of sperm granuloma or leukocyte infiltration in the epididymis after EDS treatment in the present study is hard to explain. It could possibly be a direct toxic effect of the chemical or an indirect result of the presence of immature spermatocytes and sperma-

tids in the epididymal duct. Furthermore, with the EDS-induced Leydig cell destruction model, the likelihood could not be completely excluded that EDS itself might have some direct effects on spermatogenesis [22], or on Sertoli cells and thus on spermatogenesis, and deficiency of factors other than testosterone secreted by Leydig cells might also have some effects on spermatogenesis, although these possible effects, which are not the result of testosterone depletion, have not been well recognized.

Some attention should also be given to the finding in the present study that one of the 14 EDS-treated rats was not responsive to the chemical (i.e. without destruction of Leydig cells). Not uniquely, EDS treatment in a previous study induced testosterone depletion in only six of 10 rats [15]. It was unclear whether this was a result of individual variation in response to the chemical or a result of misoperation of the injection (e.g. injection into the intestinal cavity or injection leakage out of the skin). Thus, it is important to confirm successful establishment of the animal model by observation of the Leydig cell morphology.

Acknowledgment

This study was supported by grants (Chuan-Ke-Ji [2001] 2 and 04ZQ026-025) from the Sichuan Youth Foundation of Science and Technology.

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