Journal of Andrology, Vol. 12, No. 1, January/February 1991

2,5-Hexanedione Exposure in the Rat Results in Long-Term Testicular Atrophy Despite the Presence of Residual Spermatogonia

KIM BOEKELHEIDE AND SUSAN J. HALL

From the Department of Pathology and Laboratory Medicine, Brown University, Providence, Rhode Island.

ABSTRACT: Charles River CD rats (approximate weight, 208 g) were exposed to 1.0% 2,5-hexanedione (2,5-HD) in drinking water for 5 weeks. Rats were killed 27, 60, and 75 weeks after exposure to evaluate the recovery potential following testicular injury. At 27 weeks, normal serum testosterone and significantly elevated serum luteinizing hormone and serum follicle-stimulating hormone levels were found in treated rats. The 2,5-HD–treated rats had low testicular and epididymal weights at all time points (28% and 72% of controls, respectively, at 75 weeks). Microscopically, there was a generalized loss of postspermatogonial germ cells at all time points, with no seminiferous tubules exhibiting normal spermatogenesis at 75 weeks. However, a relatively constant population of 3.1 to 3.7 spermatogonia/100 Sertoli cells was found in atrophic seminiferous tubules at all time points. The presence of a constant residual population of type A spermatogonia without a normal mass of more mature germ cells and the observed hormonal alterations suggest that 2,5-HD intoxication produced a lengthy disruption in local testicular homeostatic mechanisms that control spermatogenesis.

Key words: Germ cell repopulation, Sertoli cell toxicity, spermatogonia, stem cell mass.

J Androl 1991;12:18–26.

After exposure to germ cell toxicants, testes with a normal stem germ cell mass (approximately 2 spermatogonial stem cells [A_0] cells/100 Sertoli cells; Dym and Clermont, 1970; Erickson, 1976) rapidly repopulate with mature germ cells (Meistrich, 1986). However, the kinetics of germ cell recovery following exposure to Sertoli cell toxicants are unknown. This study examined the long-term effects on germ cell dynamics of exposure to 2,5-hexanedione (2,5-HD), a putative Sertoli cell toxicant (Chapin et al, 1982, 1983; Boekelheide, 1988a).

Distal peripheral polyneuropathy and testicular germ cell loss result from *n*-hexane exposure in the rat (Krasavage et al, 1980). Aliphatic precursors, such as *n*-hexane or methyl *n*-butyl ketone, are metabolically activated to the ultimate toxicant 2,5-HD by hepatic microsomal ω-1 hydroxylation and oxidation (DiVincenzo et al, 1977). 2,5-HD reacts with protein lysyl ε-amines to form pyrroles, intermediates in the development of toxicity (Graham et al, 1982; DeCaprio et al, 1982; Anthony et al, 1983a,b; Genter et al, 1987). A subsequent pyrrole-dependent crosslinking reaction appears necessary for the occurrence of both the neurotoxicity and the testicular injury (Boekelheide et al, 1988; St Clair et al, 1988).

The dynamics of 2,5-HD–induced testicular atrophy are complex; a time delay is evident between administration of the toxicant and the development of testicular injury, and the length and dose rate of exposure determine the magnitude of germ cell loss (Boekelheide, 1988a; Boekelheide and Eveleth, 1988). 2,5-HD exposure alters testicular microtubule assembly, and the extent of this assembly abnormality correlates with testicular injury as measured by testicular weight loss and histopathology (Boekelheide, 1987a,b, 1988a,b; Boekelheide and Eveleth, 1988). We have proposed that a failure of normal microtubule-dependent Sertoli cell functions results in germ cell loss, since the Sertoli cell cytoskeleton plays a critical role in the structural, nutritional, and hormonal support of germ cells (Russell et al, 1981; Vogl et al, 1983a,b; Neely and Boekelheide, 1988; Boekelheide et al, 1989).

In a previous study (Boekelheide, 1988a), rats were exposed to 1% 2,5-HD in drinking water for 5 weeks and examined periodically thereafter. Twelve weeks after the start of exposure, 99% of the seminiferous tubules in all rats studied had lost all their postspermatogonial germ cells. Twenty-two weeks after the start of exposure, a minority of the depleted seminiferous tubules had recovered and dem-

This work was supported by NIH grants K04 ES00193 and R01 ES05033 and the International Life Sciences Institute Research Foundation.

Correspondence to: Kim Boekelheide, MD, PhD, Department of Pathology and Laboratory Medicine, Division of Biology and Medicine, Brown University, Box G-B502, Providence, Rhode Island 02912.

Received for publication December 29, 1989; accepted for publication August 9, 1990.

onstrated normal spermatogenesis. At this last time point examined, a majority of the 2,5-HD–treated rats had no recovered seminiferous tubules and lacked postspermatogonial germ cells. Interestingly, these atrophic seminiferous tubules contained scattered, basally located spermatogonia in numbers indicative of type A_0 stem germ cells (approximately 2 germ cells/100 Sertoli cells).

In this study, we examined the possibility of germ cell recovery over a long period after acute 2,5-HD exposure. At 75 weeks, testes were uniformly and severely atrophic with few, if any, postspermatogonial germ cells, while containing an average of 2.37 type A spermatogonia/100 Sertoli cells. These results indicate that long-term disruption of normal testicular homeostatic mechanisms may result from exposure to Sertoli cell toxicants.

Materials and Methods

Virus and antibody-free Charles River CD rats (weight, approximately 208 g) were randomly assigned to treated and control groups after 6 days of acclimatization. Food (Pro-Lab Rat, Mouse and Hamster Chow No. 3000) was provided *ad libitum*. Rats were housed in autoclaved Thoren cages or hanging wire cages at 70 ± 2°F (21 ± 1°C) and 35% to 70% humidity with a 12-hour light:12-hour dark cycle. Body weight was determined at the times indicated in Fig 1. For statistical purposes, weights of paired organs were summed to give an aggregate organ weight for analysis. Data were analyzed for mean and SE and compared by the two-tailed Student's t test, analysis of variance, or the chi-squared test with significance at $P < 0.05$.

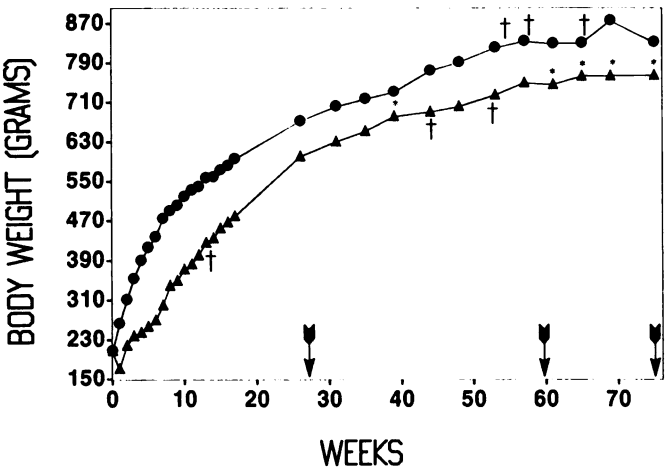

FIG. 1. Mean body weights of pooled treated (triangles) and control (circles) rats. In all cases, SE was <10% of the mean. Body weights of the treated rats differed significantly ($P < 0.05$) from control rats at all times except those indicated with an asterisk. Groups of treated and control rats were killed at 27, 60, and 75 weeks (arrows). Daggers indicate unscheduled deaths.

Experimental Design

Rats (n = 39) were treated with 1% 2,5-HD in drinking water for 5 weeks at an average dose rate of 3.1 mmol/kg per day. On day 10 of intoxication, 13 of the 39 treated rats were euthanized because they exceeded established humane criteria for maximum allowable weight loss (loss of > 33% of initial body weight). Groups of rats were randomly selected from treated survivors (n = 26) and controls (n = 18) to be killed at designated times after the beginning of 2,5-HD exposure: week 27 (treated rats, n = 7; control rats, n = 5), week 60 (treated rats, n = 7; control rats, n = 5), and week 75 (treated rats, n = 9; control rats, n = 5). Before the end of the experiment, three additional treated rats and three control rats were found dead in their cages or were killed due to progressive weight loss or clinical deterioration.

Rats were killed by intraperitoneal injection of pentobarbital. All control and treated rats sacrificed at the designated time points received complete necropsies, including determination of body weight, gross examination of organs, and determination of organ weights (testes, epididymides, seminal vesicles, prostate, kidneys, liver, spleen, lungs, heart, and brain). Seminal vesicle weights included associated fluids, and prostatic weights included membranous and prostatic urethra and bladder neck. For testes weight at week 75, only four control data points were available because of neoplastic replacement of a testis in one rat. Testes were fixed in 10% neutral buffered formalin, processed, and embedded in glycol methacrylate for cutting into 2.5-μm sections and staining with periodic acid Schiff and hematoxylin (PAS) as previously described (Chapin et al, 1984). Portions of other organs removed at necropsy, including pancreas and stomach, were fixed in 10% neutral buffered formalin, processed, and embedded in paraffin for cutting into 6-μm sections and staining with hematoxylin and eosin (H&E).

Endocrinology

At week 27 and before necropsy, cardiac blood was collected (between 9 AM and 1:30 PM) from pentobarbital-anesthetized rats; collections alternated between control and treated animals. Blood was allowed to clot at 37°C before separation in a clinical centrifuge for 10 minutes at 4°C. Sera were stored at −70°C until assayed. Hormonal determinations were performed by Hazleton Biotechnologies Co. (Vienna, VA). Samples were analyzed for: 1) follicle-stimulating hormone (FSH) using antibody S-11 from A. F. Parlow (coefficient of variation: within assay, 3.5%; between assay, 6.6%); 2) luteinizing hormone (LH) using antibody S-10 from A. F. Parlow and NIADDK-rLH RP-2 standard (coefficient of variation: within assay, 2.6%; between assay, 3.7%); and 3) total testosterone using an extraction/chromatography procedure (coefficient of variation: within assay, 7.6%; between assay, 9.2%).

Histopathology

Testicular cross sections, one from each rat, were examined for the presence of different types of germ cells. Testicular sections contained an average of 200 cross sections of seminiferous tubules/rat at 27 weeks (range, 53 to 379; n = 6 [one section was

Table 1. *Body and reproductive organ weights of 2,5-HD-treated and control rats after long-term recovery*

	Week 27		Week 60		Week 75	
	Control (n = 5)	Treated (n = 7)	Control (n = 5)	Treated (n = 7)	Control (n = 5)	Treated (n = 9)
Body (g)	676.4 ± 17.5	613.9 ± 16.8*	841.8 ± 39.6	716.3 ± 25.3*	831.2 ± 67.6	746.6 ± 34.8
Testes (g)	3.50 ± 0.12	1.59 ± 0.36*	3.83 ± 0.11	1.57 ± 0.35*	3.58 ± 0.11	0.99 ± 0.06*
Epididymides (g)	1.46 ± 0.05	0.95 ± 0.11*	1.67 ± 0.05	1.08 ± 0.08*	1.35 ± 0.04	0.97 ± 0.05*
Seminal vesicles (g)	1.83 ± 0.22	2.56 ± 0.16*	2.23 ± 0.18	2.09 ± 0.12	2.38 ± 0.22	2.07 ± 0.15
Prostate (g)	1.89 ± 0.15	2.13 ± 0.10	1.97 ± 0.28	2.13 ± 0.21	1.88 ± 0.17	2.05 ± 0.11

* $P < 0.05$ by analysis of variance.

inadequate for evaluation]), 375 tubular cross sections/rat at 60 weeks (range, 225 to 533; n = 7), and an average of 404 tubular cross sections/rat at 75 weeks (range, 254 to 487; n = 9).

Seminiferous tubules were considered to be "atrophic" if they lacked germ cells more mature than spermatogonia and "recovered" if they contained germ cells that had matured to at least the zygotene spermatocyte stage.* The number of spermatogonia/100 Sertoli cells was determined in testicular sections with atrophic seminiferous tubules by counting an average of 2015 total cells/rat at 27 weeks (range, 726 to 3070; n = 5 [one section was inadequate for evaluation and one section was totally recovered]), an average of 2688 total cells/rat at 60 weeks (range, 2572 to 2821; n = 6 [one section was totally recovered]), and an average of 1689 total cells/rat at 75 weeks (range, 1276 to 2011; n = 9). At week 75, the spermatogonia seen in atrophic seminiferous tubules were further categorized as either type A spermatogonia or intermediate plus type B spermatogonia. Type A spermatogonia were defined by their nuclear morphology, presence of a visible and limited cytoplasm, and their basal location in the seminiferous tubule (Clermont and Bustos-Obregon, 1968; Boekelheide, 1988a). Intermediate and type B spermatogonia were easily identified by their more condensed and basophilic nuclear morphology.

Distribution of type A spermatogonia and more mature spermatogonia among seminiferous tubules at week 75 was determined by counting these cell types in 75 randomly selected seminiferous tubules/treated rat (n = 9). A total of 352 type A spermatogonia and 132 intermediate plus type B spermatogonia were identified in 675 seminiferous tubules examined. To test if the distribution of these cell types among seminiferous tubules was random, the observed distribution of seminiferous tubules, with and without these germ cells, was compared with the expected Poisson distribution (Withers et al, 1974; Meistrich et al, 1978). By the Poisson distribution, occurrence of seminiferous tubules without a particular type of germ cell is predicted by the formula, $[e^{-\mu}]$, and occurrence of seminiferous tubules with a particular type of germ cell is predicted by the formula, $[1 - e^{-\mu}]$, where μ is the mean number of occurrences of that germ cell type per seminiferous tubule. For statistical purposes, observed and expected distribution of germ cells were compared by the chi-squared test.

*The morphological distinction between type B spermatogonia and spermatocytes of the preleptotene and leptotene stages can be difficult to determine by light microscopy. For the purposes of this study, cells with this morphology were considered to be type B spermatogonia.

Results

General Features of 2,5-HD Intoxication and Recovery

2,5-HD exposure induced a decrease in body weight throughout the postexposure period (Fig 1). Groups of control rats (n = 5) and treated rats (n = 7 to 9) were killed at 27, 60, and 75 weeks after the start of intoxication. At necropsy, grossly visible testicular atrophy was present in six of seven 2,5-HD–treated rats at 27 weeks, in six of seven treated rats at 60 weeks, and in all nine treated rats at 75 weeks. Control rats had uniformly normal appearing testes, with the exception of one rat at 75 weeks with an enlarged right testicle (testis weight, 2.89 g; microscopically, a Leydig cell tumor).

No consistent differences in the weights or microscopic appearance of nonreproductive organs (kidney, liver, spleen, lungs, heart, and brain) were observed between treated and control rats. Although the testicular and epididymal weights of treated rats were significantly decreased when compared with control rats at all time points, no differences were found in the weights of the seminal vesicles or prostate (Table 1). Microscopically, the prostate and seminal vesicles of both control and treated rats had a predominantly columnar epithelium that appeared actively secretory (data not shown).

At 27 weeks, both serum LH and serum FSH were significantly elevated in treated rats, while serum testosterone was normal (Table 2).

Histopathology of the Testes and the Epididymides

Testicular cross sections were scored for the presence or absence of recovered seminiferous tubules. At all time points, control rats had uniformly normal spermatogenesis in the majority of seminiferous tubules. Of the six 2,5-HD–treated rats examined histopathologically at 27 weeks, two rats had a partial recovery of spermatogenesis (one recovered seminiferous tubule of 379 examined and 38 recovered seminiferous tubules of 184 examined; Fig 2); one rat had a complete restoration of spermatogenesis in all of its seminiferous tubules (212 seminiferous tubular cross

Table 2. *Serum hormone levels in 2,5-HD-treated and control rats at 27 weeks*

	FSH (ng/ml)	LH (ng/ml)	Testosterone (ng/dl)
Control (n = 5)	167.0 ± 12.3	0.36 ± 0.02	304.0 ± 53.1
Treated (n = 7)	307.6 ± 30.3*	0.80 ± 0.10*	294.7 ± 14.3

* $P < 0.005$ by two-sided Student's t test.

sections examined); and three rats had complete atrophy of the seminiferous epithelium (an average of 200 seminiferous tubules/rat examined). At 60 weeks, one 2,5-HD–treated rat had a complete restoration of spermatogenesis (517 seminiferous tubular cross sections examined); however, the remaining six rats had only atrophic seminiferous tubules (an average of 351 seminiferous tubules/rat examined). At 75 weeks, no 2,5-HD–treated rats showed evidence of a recovery of spermatogenesis (an average of 404 seminiferous tubules/rat examined).

Atrophic seminiferous tubules identified in testicular sections from 2,5-HD–treated rats were also scored for the total number of spermatogonia/100 Sertoli cells. Spermatogonia were found in similar numbers at all time points: at 27 weeks, 3.67 ± 0.59 germ cells/100 Sertoli cells (n = 5); at 60 weeks, 3.10 ± 0.34 germ cells/100 Sertoli cells (n = 6); and at 75 weeks, 3.33 ± 0.76 germ cells/100 Sertoli cells (n = 9). The spermatogonia were further classified by their morphology: type A spermatogonia (Fig 3A) were distinguished from the more mature intermediate and type B spermatogonia (Fig 3B). Type A spermatogonia were the predominant cell type at all time points. This impression was measured at 75 weeks when 2.37 ± 0.49 type A spermatogonia/100 Sertoli cells and 0.96 ± 0.34 intermediate plus type B spermatogonia/100 Sertoli cells were found (n = 9).

To determine whether the germ cells of rats treated with 2,5-HD tend to cluster together, the distribution of spermatogonia among atrophic seminiferous tubules at week 75 was determined (Table 3). If random, the probability of finding germ cells in seminiferous tubules would be predicted by the Poisson distribution (Withers et al, 1974; Meistrich et al, 1978). This possibility was examined

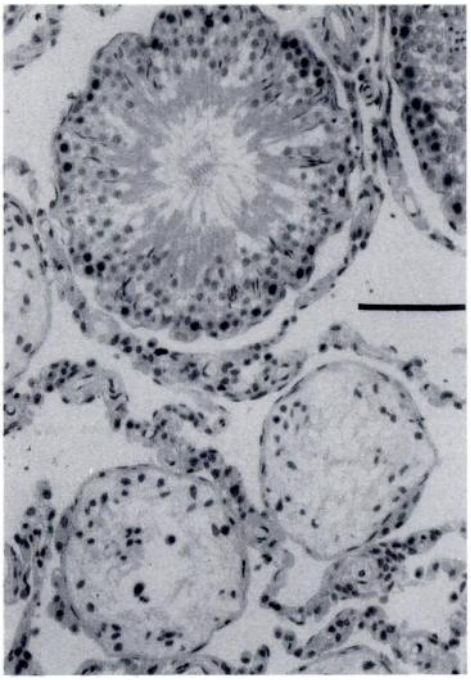

FIG 2. At 27 weeks, two of six testicular cross-sections showed partial recovery of spermatogenesis. A fully recovered seminiferous tubule (above) is adjacent to two atrophic seminiferous tubules (below). PAS, bar = 100 μm.

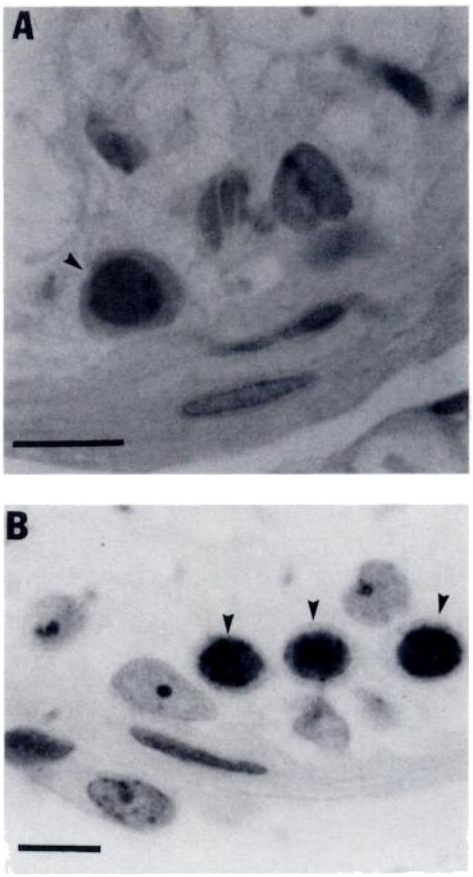

FIG 3. Cytological criteria were used to distinguish type A spermatogonia from intermediate plus type B spermatogonia in atrophic seminiferous tubules. (A) A Type A spermatogonium (arrowhead) can be distinguished from nearby Sertoli cell nuclei by its nuclear appearance, visible and limited cytoplasm, and basal location (75 weeks, PAS, bar = 10 μm). (B) A group of type B spermatogonia (arrowheads) are identified by their nuclear appearance. Note the presence of intercellular cytoplasmic bridges between these cells (75 weeks, PAS, bar = 10 μm).

Table 3. *Seminiferous tubules (%) with germ cells (number) at week 75*

	Germ cells per seminiferous tubule				
	0	1	2	3	>3
Type A spermatogonia	64.6 ± 7.8 (59.4)	23.3 ± 5.3 (31.0)	8.4 ± 2.1 (8.1)	3.0 ± 0.9 (1.4)	0.7 ± 0.4 (0.2)
More mature spermatogonia	91.9 ± 2.1 (82.2)	5.5 ± 1.3 (16.1)	0.9 ± 0.3 (1.6)	0.6 ± 0.5 (0.1)	1.2 ± 0.6 (0.005)

From each rat (n = 9), 75 seminiferous tubule cross-sections were randomly evaluated for their content of type A spermatogonia and more mature spermatogonia. The frequency of occurrence of seminiferous tubules with the indicated number of germ cells is shown as the mean ± SE. In parenthesis is shown the predicted frequency of seminiferous tubules with the noted number of germ cells if these germ cells were randomly distributed among seminiferous tubules according to the Poisson distribution.

by performing the chi-squared test on the observed and the Poisson-predicted distribution of germ cells in seminiferous tubules (Table 3). The distribution of type A spermatogonia and more mature spermatogonia was homogeneous among individual rats in these groups, allowing the statistical analysis to be performed on pooled data. A comparison of the observed and predicted incidence of seminiferous tubules, with and without germ cells (degree of freedom = 1), demonstrated a nonrandom distribution of both type A spermatogonia (chi-square = 7.65, $P < 0.01$) and more mature spermatogonia (chi-square = 42.72, $P < 1 \times 10^{-10}$). The extent of germ cell clustering was much greater for the intermediate plus type B spermatogonia, with occasional seminiferous tubule cross sections containing up to 16 spermatogonia.

Sections of the epididymides, including caput, corpus, and cauda, were stained with H&E and examined by light microscopy. All control rats had abundant spermatozoa present in all regions of the epididymis (Fig 4). In contrast, at all time points, treated rats (with the exception of those with spermatogenic recovery) lacked spermatozoa in the epididymal caput and corpus. At all time points, most of the treated rats contained spermatozoan nuclei embedded within a dense intraluminal eosinophilic matrix in the distal cauda epididymis (Fig 5). In addition, treated rats had numerous epididymal epithelial cells that were hugely distended by inspissated, slightly basophilic material (Fig 6). This cellular alteration was most frequent in the proximal cauda epididymis but was also present in the corpus and distal cauda epididymis.

Discussion

We have demonstrated that intense exposure to 2,5-HD, a putative Sertoli cell toxicant, produces an atrophic testis that is largely incapable of recovery. Histopathologic examination 75 weeks after beginning 2,5-HD exposure indicated the presence of a residual population of type A spermatogonia compatible with an intact stem cell population. Our experiments extend a study by Boekelheide (1988a) of the late complications of 2,5-HD exposure by quadrupling the postexposure observation period, determining the weights and histologic appearances of both testicular and nontesticular reproductive organs, and examining and quantifying the types of residual germ cells.

Previous experience with germ cell toxicants would predict a full and rapid recovery following an exposure that resulted in an intact residual stem cell mass (Meistrich, 1986); therefore, the clinical and toxicologic implications of exposure to germ cell and Sertoli cell toxicants may be quite different. Sertoli cell toxicants may produce a lengthy disruption in spermatogenesis by an as yet unidentified mech-

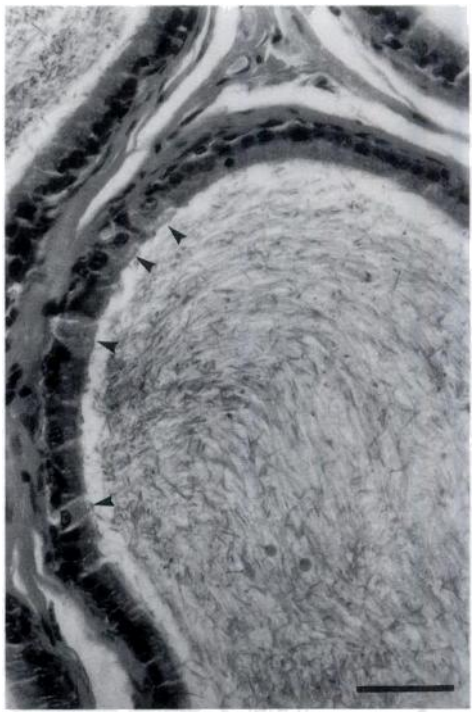

FIG. 4. Section of proximal cauda epididymis from a control rat showing abundant luminal spermatozoa and the normal appearance of the epithelial clear cells, indicated by arrowheads (75 weeks, H & E, bar = 50 μm).

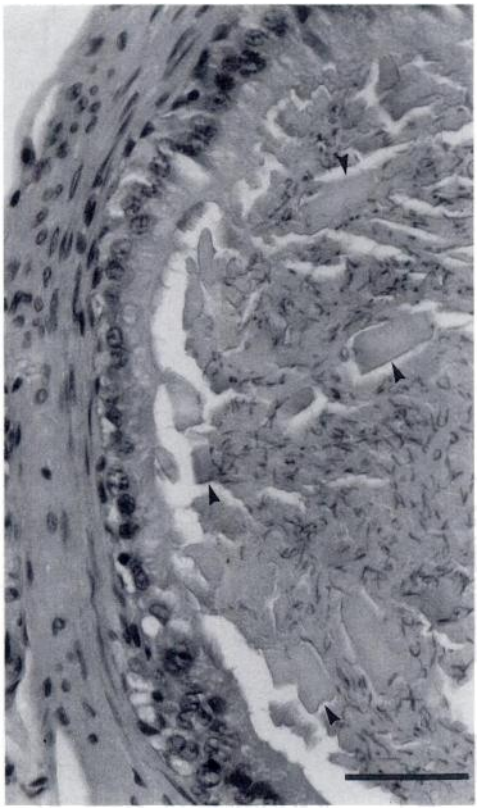

FIG. 5. All treated rats with atrophic seminiferous tubules lacked spermatozoa in the proximal epididymis. However, in the distal cauda epididymis, spermatozoa were retained within a dense, eosinophilic matrix that appeared focally crystalline, indicated by arrowheads (75 weeks, H&E, bar = 50 μm).

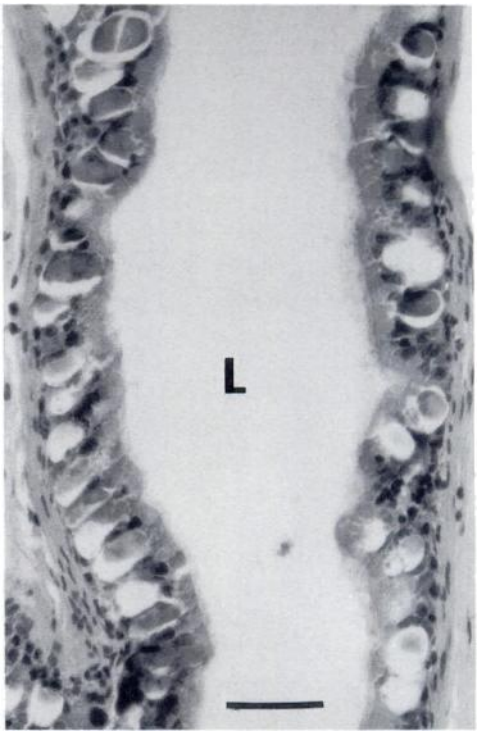

FIG. 6. A common alteration in the epididymis of treated rats, most prevalent in the proximal cauda epididymis as shown here, was the accumulation of dense, slightly basophilic concretions within numerous epithelial cells. These cells likely represent clear cells (see Fig 4) that may have an altered morphological appearance following toxicant exposure (Flickinger and Loving, 1976; Trasler et al, 1988). Note that the lumen (L) is devoid of spermatozoa (75 weeks, H&E, bar = 50 μm).

anism without depleting the spermatogonial stem cell mass. This conclusion should be viewed with two features of our model in mind: 1) the Sertoli cell specificity of 2,5-HD and 2) the identification of residual spermatogonia as type A_0 stem cells.

Both morphological and biochemical evidence indicate that the Sertoli cell is the initial target for 2,5-HD in the pathway leading to testicular injury. By light microscopy, vacuolization of the Sertoli cell cytoplasm (specific to stages XII, XIII, XIV, and I in the cycle of the seminiferous epithelium) is the first histopathologic sign of a testicular abnormality (Chapin et al, 1983; Boekelheide, 1988a). Biochemically, the activities of the Sertoli cell-specific enzymes β-glucuronidase and γ-glutamyl transpeptidase are decreased during 2,5-HD exposure at a time when the testis is morphologically normal (Chapin et al, 1982). However, no data are available that exclude germ cells as another site of action of 2,5-HD. Indeed, because of the extensive interactions among cells within the testis, demonstration of exclusive Sertoli cell specificity for any toxic agent would be quite difficult.

Type A spermatogonia were the predominant germ cell type present 75 weeks after beginning 2,5-HD treatment. Classification of spermatogonial cell types was accomplished using morphological criteria (nuclear appearance, a visible and limited cytoplasm, and a basal location). Type A spermatogonia represented 71% of all the germ cells present at 75 weeks (2.37 type A spermatogonia/100 Sertoli cells); the remaining germ cells were intermediate and type B spermatogonia. Spermatogenic stem cells (type A_0 spermatogonia) are known to occur with a frequency of approximately two per 100 Sertoli cells (Dym and Clermont, 1970; Erickson, 1976). Another characteristic of spermatogonial A_0 stem cells is their occurrence as isolated cells (Huckins, 1971). Statistically, the distribution of type A spermatogonia at 75 weeks was not random; however, they did not show a marked degree of germ cell clustering. This suggests that a sizable portion of these cells may well be "isolated" and fall into the category of presumptive A_0 cells. However, no specific marker or methodologic approach was used that would unequivocally identify the remaining spermatogonia as type A_0 stem cells. Indeed, no specific mark-

ers for type A_0 spermatogonia have been reported in the literature. An alternative methodologic approach (whole mounts of seminiferous tubules) failed to reliably distinguish isolated type A spermatogonia from Sertoli cells in injured seminiferous tubules.

Elevated levels of FSH and LH, along with normal testosterone levels, were found in treated sera 27 weeks after beginning the 5-week exposure to 1% 2,5-HD. This hormonal pattern may be explained by an intact hypothalamic-pituitary response to a dysfunctional gonad. Other examples of direct seminiferous tubule damage characterized by prolonged germ cell loss, such as that found after hydroxyurea exposure or vitamin A deficiency, elicit a similar hormonal response (Rich and de Kretser, 1977; Rich et al, 1979). The explanation for the elevation in LH in this setting of chronic seminiferous tubule damage is unclear. One possibility is that alterations in testicular hemodynamics, which are known to occur following germ cell loss, modulate the serum concentrations of LH and testosterone (Setchell and Galil, 1983). According to this hypothesis, the reduced blood flow in the germ cell depleted testis limits the amount of testosterone leaving the testis, which is compensated for by an elevation in LH. The normal microscopic appearance and weights of the seminal vesicles and prostates in the treated rats corroborate the normal serum testosterone and a compensated state of Leydig cell function. These results support previous hormonal and histopathologic studies (Chapin et al, 1982, 1983; Boekelheide, 1988a) that found that 2,5-HD–induced testicular injury was mediated by direct toxicity to the Sertoli cell: the hypothalamic-pituitary axis was spared despite the known neurotoxicity of this compound.

In light of this apparent sparing of the hypothalamic-pituitary axis, it is interesting to note the long-term deficit in body weight found in this study following 2,5-HD exposure during adolescent development in the rat. Decreased food intake during intoxication is an unlikely explanation for this long-term body weight deficit, as demonstrated by studies of the effect of short-term inanition in young rats on subsequent adult body weight (Stewart, 1916). Furthermore, in two studies (Gillies et al, 1980; Boekelheide, 1988a), treated rats weighed significantly less than pair-fed controls following 2,5-HD intoxication, indicating the presence of a weight-reducing, non-nutritional toxicant-related effect. The interpretation of this long-term weight deficit is complicated by the observation that organ weights of 2,5-HD–treated and control rats were similar.

2,5-HD–treated rats with atrophic testes lacked spermatozoa in the caput and corpus epididymis, but retained spermatozoa within a dense eosinophilic matrix in the distal cauda epididymis long after the cessation of spermatogenesis. This finding is consistent with a major alteration in the fluid dynamics of the ductal system, a potential consequence of exposure to a Sertoli cell toxicant (Gray and Gangolli, 1986). In addition, some epididymal epithelial cells, most prominently in the proximal cauda epididymis, were hugely distended with inspissated material. These cells may be epididymal clear cells that have been noted to enlarge and increase in number in response to toxic insults (Flickinger and Loving, 1976; Trasler et al, 1988).

Two other putative Sertoli cell toxicants, the phthalate esters and tri-*o*-cresyl phosphate, produce long-term alterations in testis weight or testis histopathology following exposure. A single injection of a phthalate ester produced a marked reduction in seminiferous tubule fluid secretion and androgen binding protein secretion, implicating the Sertoli cell as the target (Gray and Gangolli, 1986). Testis weight remained significantly depressed 70 days after completion of a 60-day dietary exposure to 20,000 ppm di(2-ethylhexyl)phthalate, suggesting long-term effects on germ cell production (Agarwal et al, 1986). Administration of tri-*o*-cresyl phosphate apparently impaired Sertoli cell phagocytosis of residual bodies (Somkuti et al, 1987a) and produced irreversible germ cell loss (Somkuti et al, 1987b). Both of these compounds induce alterations in Sertoli cell function and disrupt germ cell production for an extended period.

Sertoli cells are generally accepted as performing a central role in support of spermatogenesis. They provide the appropriate structural and nutritional environment for germ cell development, create the blood–testis barrier, and remodel the seminiferous epithelium as germ cells mature (Bardin et al, 1988). Recently, increased attention has focused on the importance of locally produced growth factors in spermatogenesis (Bellve and Zheng, 1989). Clearly, some of these growth factors are produced by Sertoli cells to modulate germ cell proliferation and function (Forti et al, 1989; Kancheva et al, 1990).

Given the hormonal evidence of prolonged seminiferous tubule damage and Sertoli cells as the target, 2,5-HD may be affecting local paracrine and autocrine regulation of spermatogenesis within seminiferous tubules. These morphological studies indicate that recovery from 2,5-HD exposure either occurs rapidly following intoxication or not at all. The quantitative analysis of cell populations at 75 weeks suggests that some A_0 cells can commit to germ cell maturation. The clustering of intermediate and type B spermatogonia indicates that clonal expansion of committed germ cells does occur. However, the rate of A_0 cell commitment to maturation appears to be low, and those germ cells committed to maturation fail to progress through the spermatocyte stage. A more detailed description of the mechanism of this failure in recovery and maturation requires ultrastructural studies and a better molecular understanding of paracrine and autocrine regulation of germ cell dynamics.

References

Agarwal DK, Eustis S, Lamb JC IV, Reel JR, Kluwe WM. Effects of di(2-ethylhexyl)phthalate on the general pathophysiology, sperm morphology, and reproductive performance of male rats. *Environ Health Perspect*. 1986;65:343–350.

Anthony DC, Boekelheide K, Graham DG. The effect of 3,4-dimethyl substitution on the neurotoxicity of 2,5-hexanedione. I. Accelerated clinical neuropathy is accompanied by more proximal axonal swellings. *Toxicol Appl Pharmacol*. 1983a;71:362–371.

Anthony DC, Boekelheide K, Anderson CW, Graham DG. The effect of 3,4-dimethyl substitution on the neurotoxicity of 2,5-hexanedione. II. Dimethyl substitution accelerates pyrrole formation and protein crosslinking. *Toxicol Appl Pharmacol*. 1983b;71:372–382.

Bardin CW, Cheng, CY, Musto NA, Gunsalus GL. The Sertoli cell. In: Knobil E, Neill JD, eds. *The Physiology of Reproduction*. New York: Raven Press; 1988:933–974.

Bellve AR, Zheng W. Growth factors as autocrine and paracrine modulators of male gondadal functions. *J Reprod Fertil*. 1989;85:771–793.

Boekelheide K. 2,5-Hexanedione alters microtubule assembly. I. Testicular atrophy, not nervous system toxicity, correlates with enhanced tubulin polymerization. *Toxicol Appl Pharmacol*. 1987a;88:370–382.

Boekelheide K. 2,5-Hexanedione alters microtubule assembly. II. Enhanced polymerization of crosslinked tubulin. *Toxicol Appl Pharmacol*. 1987b;88:383–396.

Boekelheide K. Rat testis during 2,5-hexanedione intoxication and recovery. I. Dose response and the reversibility of germ cell loss. *Toxicol Appl Pharmacol*. 1988a;92:18–27.

Boekelheide K. Rat testis during 2,5-hexanedione intoxication and recovery. II. Dynamics of pyrrole reactivity, tubulin content and microtubule assembly. *Toxicol Appl Pharmacol*. 1988b;92:28–33.

Boekelheide K, Eveleth J. The rate of 2,5-hexanedione intoxication, not total dose, determines the extent of testicular injury and altered microtubule assembly in the rat. *Toxicol Appl Pharmacol*. 1988;94:76–83.

Boekelheide K, Anthony DC, Giangaspero F, Gottfried MR, Graham DG. Aliphatic diketones: influence of dicarbonyl spacing on amine reactivity and toxicity. *Chem Res Toxicol*. 1988;1:200–203.

Boekelheide K, Neely MD, Sioussat TM. The Sertoli cell cytoskeleton: a target for toxicant-induced germ cell loss. *Toxicol Appl Pharmacol*. 1989;101:373–389.

Chapin RE, Norton RM, Popp JA, Bus JS. The effects of 2,5-hexanedione on reproductive hormones and testicular enzyme activities in the F-344 rat. *Toxicol Appl Pharmacol*. 1982;62:262–272.

Chapin RE, Morgan KT, Bus JS. The morphogenesis of testicular degeneration induced in rats by orally administered 2,5-hexanedione. *Exp Mol Pathol*. 1983;38:149–169.

Chapin RE, Ross MD, Lamb JC IV. Immersion fixation methods for glycol methacrylate-embedded testes. *Toxicol Pathol*. 1984;12:221–227.

Clermont Y, Bustos-Obregon E. Re-examination of spermatogonial renewal in the rat by means of seminiferous tubules mounted "in toto." *Am J Anat*. 1968;122:237–248.

DeCaprio AP, Olajos EJ, Weber P. Covalent binding of a neurotoxic *n*-hexane metabolite: conversion of primary amines to substituted pyrrole adducts by 2,5-hexanedione. *Toxicol Appl Pharmacol*. 1982;65:440–450.

DiVincenzo GD, Hamilton ML, Kaplan CJ, Dedinas J. Metabolic fate and disposition of ^{14}C-labeled methyl *n*-butyl ketone in the rat. *Toxicol Appl Pharmacol*. 1977;41:547–560.

Dym M, Clermont Y. Role of spermatogonia in the repair of the seminiferous epithelium following X-irradiation of the rat testis. *Am J Anat*. 1970;128:265–282.

Erickson BH. Effect of ^{60}Co γ radiation on the stem and differentiating spermatogonia of the post-pubertal rat. *Radiat Res*. 1976;68:433–448.

Flickinger CJ, Loving CK. Fine structure of the testis and epididymis of rats treated with cyproterone acetate. *Am J Anat*. 1976;146:359–384.

Forti G, Barni T, Vannelli BG, Balboni GC, Orlando C, Serio M. Sertoli cell proteins in the human seminiferous tubule. *J Steroid Biochem*. 1989;32:135–144.

Genter MB, Szakal-Quin Gy, Anderson CW, Anthony DC, Graham DG. Evidence that pyrrole formation is a pathogenetic step in γ-diketone neuropathy. *Toxicol Appl Pharmacol*. 1987;87:351–362.

Gillies PJ, Norton RM, Bus JS. Effect of 2,5-hexanedione on lipid biosynthesis in sciatic nerve and brain of the rat. *Toxicol Appl Pharmacol*. 1980;54:210–216.

Graham DG, Anthony DC, Boekelheide K, Maschmann NA, Richards RG, Wolfram JW, Shaw BR. Studies of the molecular pathogenesis of hexane neuropathy. II. Evidence that pyrrole derivatization of lysyl residues leads to protein crosslinking. *Toxicol Appl Pharmacol*. 1982;64:415–422.

Gray TJB, Gangolli SD. Aspects of the testicular toxicity of phthalate esters. *Environ Health Perspect*. 1986;65:229–235.

Huckins C. The spermatogonial stem cell population in adult rats. I. Their morphology, proliferation and maturation. *Anat Rec*. 1971;169:533–558.

Kancheva LS, Martinova YS, Georgiev VD. Prepubertal rat Sertoli cells secrete a mitogenic factor(s) that stimulates germ and somatic cell proliferation. *Mol Cell Endocrinol*. 1990;69:121–127.

Krasavage WJ, O'Donoghue JL, DiVincenzo G, Terhaar CJ. The relative neurotoxicity of methyl *n*-butyl ketone, *n*-hexane and their metabolites. *Toxicol Appl Pharmacol*. 1980;52:433–441.

Meistrich ML. Critical components of testicular function and sensitivity to disruption. *Biol Reprod*. 1986;34:17–28.

Meistrich ML, Hunter NR, Suzuki N, Trostle PK, Withers HR. Gradual regeneration of mouse testicular stem cells after exposure to ionizing radiation. *Radiat Res*. 1978;74:349–362.

Neely MD, Boekelheide K. Sertoli cell processes have axoplasmic features: an ordered microtubule distribution and an abundant high molecular weight microtubule associated protein (cytoplasmic dynein). *J Cell Biol*. 1988;107:1767–1776.

Rich KA, de Kretser DM. Effect of differing degrees of destruction of the rat seminiferous epithelium on levels of serum follicle stimulating hormone and androgen binding hormone. *Endocrinology*. 1977;101:959–968.

Rich KA, Kerr JB, de Kretser DM. Evidence for Leydig cell dysfunction in rats with seminiferous tubule damage. *Mol Cell Endocrinol*. 1979;13:123–135.

Russell LD, Malone JP, MacCurdy DS. Effect of the microtubule disrupting agents, colchicine, and vinblastine, on seminiferous tubule structure in the rat. *Tissue Cell*. 1981;13:349–367.

St Clair MBG, Amarnath V, Moody A, Anthony DC, Anderson CW, Graham DG. Pyrrole oxidation and protein cross-linking as necessary steps in the development of γ-diketone neuropathy. *Chem Res Toxicol*. 1988;1:179–185.

Setchell BP, Galil KAA. Limitations imposed by testicular blood flow on the function of Leydig cells in rats *in vivo*. *Aust J Biol Sci*. 1983;36:285–293.

Somkuti SG, Lapadula DM, Chapin RE, Lamb JC IV, Abou-Donia MB. Reproductive tract lesions resulting from subchronic administration (63 days) of tri-*o*-cresyl phosphate in male rats. *Toxicol Appl Pharmacol*. 1987a;89:49–63.

Somkuti SG, Lapadula DM, Chapin RE, Lamb JC IV, Abou-Donia MB. Time course of the tri-*o*-cresyl phosphate-induced testicular lesion in

F-344 rats: enzymatic, hormonal, and sperm parameter studies. *Toxicol Appl Pharmacol.* 1987b;89:64–72.

Stewart CA. Growth of the body and of the various organs of young albino rats after inanition for various periods. *Biol Bull.* 1916;31:16–51.

Trasler JM, Hermo L, Robaire B. Morphological changes in the testis and epididymis of rats treated with cyclophosphamide: a quantitative approach. *Biol Reprod.* 1988;38:463–479.

Vogl AW, Lin YC, Dym M, Fawcett DW. Sertoli cells of the golden-mantled ground squirrel (*Spermophilus lateralis*): a model system for the study of shape change. *Am J Anat.* 1983a;168:83–98.

Vogl AW, Linck RW, Dym M. Colchicine-induced changes in the cytoskeleton of the golden-mantled ground squirrel (*Spermophilus lateralis*) Sertoli cells. *Am J Anat.* 1983b;168:99–108.

Withers HR, Hunter N, Barkley HT Jr, Reid BO. Radiation survival and regeneration characteristics of spermatogenic stem cells of mouse testis. *Radiat Res.* 1974;57:88–103.