Journal of Andrology, Vol. 13, No. 6, November/December 1992

Acute and Long-term Effects of a Single Dose of the Fungicide Carbendazim (Methyl 2-Benzimidazole Carbamate) on the Male Reproductive System in the Rat

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ABSTRACT: The effects of carbendazim (methyl 2-benzimidazole carbamate) on the testis, efferent ductules, and sperm were determined in the adult rat after a single oral dose. Two experimental trials were performed: a time response between 2 hours and 32 days after exposure using 0 and 400 mg/kg, and a dose response at 2 and 70 days after exposure using 0 to 800 mg/kg doses. In experiment 1, effects were seen throughout the 32-day period, beginning 8 hours after exposure; the effects included first an increase in testis weight, then decreases in testicular spermatid numbers and in the percentage of morphologically normal cauda sperm. In experiment 2, significant testicular and efferent ductal alterations occurred in animals treated with doses of 100 mg/kg or greater. A dose-dependent increase in testicular weight 2 days after treatment was accompanied by increases in seminiferous tubular diameter and excessive loss of immature germ cells in a stage-dependent manner. There was also a dose-dependent increased incidence of occlusions in the efferent ductules. The occluded ductules were characterized by severe inflammation and exhibited disorganization of the epithelium. At 70 days, there were dose-dependent decreases in mean testis weight and mean seminiferous tubular diameter; however, only minimal long-term effects were seen at 50 mg/kg. In testes exhibiting seminiferous tubular atrophy of greater than 25% (100 mg/kg or greater doses), all of the testes were associated with efferent ductules containing occlusions. Caput sperm numbers were significantly reduced in these testes. Occlusions, abnormal ductules, fibrosis, spermatic granulomas, and mineralization were observed in the ductuli efferentes. Long-term effects of carbendazim on the testis were induced primarily by ductal occlusions. Results show that carbendazim produces more severe short- and long-term effects on the male reproductive system than the fungicide benomyl.

Key words: Fungicide, carbendazim, germ cell sloughing, microtubule poison, occlusion of ductuli efferentes, testicular atrophy.

J Androl 1992;13:507–518.

The benzimidazole carbendazim (U-32104; methyl 2-benzimidazole carbamate) has been shown to reduce fertility by 50% in male rats exposed to relatively high doses (Carter et al, 1987). This fungicide is a derivative of another male reproductive toxicant, benomyl (Douch, 1973; Teubert and Stringham, 1984), which induces long-term atrophy of seminiferous tubules at doses as low as 100 mg/kg (Hess et al, 1991). The fungicidal property of these chemicals resides in their ability to bind microtubules, thereby inhibiting mitosis (Davidse and Flach, 1977; Burland and Gull, 1984). In the mammalian system, this mechanism has been proposed to explain the long-term atrophy observed in the testis after exposure (Carter and Laskey, 1982; Carter et al, 1987; Gray et al, 1990). In a recent study from our laboratory (Hess et al, 1991), however, we found that efferent ductal occlusions induced by benomyl exposure contributed significantly to the inhibition of spermatogenesis at the higher doses.

When carbendazim is administered orally to mammals, it produces various adverse effects on male reproduction, such as sloughing of germ cells (Parvinen and Kormano, 1974; Gray et al, 1990), inhibition of germ cell division (Tyrkiel, 1984), seminiferous tubular atrophy (Gray et al, 1990), and alterations in hormone concentrations (Goldman et al, 1989; Rehnberg et al, 1989). These testicular effects resemble those induced by a single dose of benomyl (Hess et al, 1991); however, it is not known if these effects are related to the inhibition of microtubule formation or to the blockage of excurrent ducts of the testis. Therefore, this study was performed to determine the testicular and ductal effects of a single exposure to carbendazim.

Supported in part by National Institutes of Health Grant ES-05214, and Reproductive Hazards in the Workplace, Home, Community, and Environment Research Grant 15–140 from the March of Dimes Birth Defects Foundation.

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Received for publication March 16, 1992; accepted for publication April 22, 1992.

Materials and Methods

Experiment 1

Male, 86-day-old Sprague-Dawley rats (Charles River Breeding Laboratories, Inc., Kingston, NY) were ranked by body weight and randomly distributed into 16 groups of 8 rats. Animals were housed two per cage and were provided Purina Laboratory Chow and tap water *ad libitum*. Eight groups (controls) were gavaged with a single dose of corn oil (5 ml/kg) or an 8% suspension of carbendazim (U-32104) in corn oil (5 ml/kg) to give 400 mg carbendazim/kg body weight. Groups of control and carbendazim-treated animals were killed at 2, 4, 8, or 24 hours and 4, 8, 16, or 32 days after exposure. Under ether anesthesia, the testis and epididymis from one side of the reproductive tract were excised (alternating left and right sides). Animals were killed by exsanguination and organs were fixed by vascular perfusion. Wet weights of the testis (all time points) and epididymis (days 4 through 32) and fixed weights of the prostate and seminal vesicles (days 4 through 32) were determined.

Excised testes were frozen for determination of sonication-resistant sperm head counts (Cassidy et al, 1983). Sperm concentration in cauda luminal fluid, percentage of motile sperm, and percentage of morphologically normal sperm were determined as previously described (Linder et al, 1988). Mean values for carbendazim-treated and control animals were compared at each time point. Sperm counts, body weights, and organ weights were analyzed by ANOVA; group means were compared with Duncan's Multiple Range Test. Sperm motility was evaluated by analysis of covariance and least squares means. Wilcoxon scores and a Kruskal-Walis test were used to compare sperm morphology.

Experiment 2

This experiment was a dose–response study of a single exposure to carbendazim. To compare the results with a previous study of benomyl (Hess et al, 1991), testicular and epididymal effects of carbendazim were evaluated on days 2 and 70 postexposure. Treatment groups at the two periods included carbendazim doses of 0 (control), 50, 100, 200, 400, and 800 mg/kg. Male Sprague-Dawley rats between 97 and 105 days of age were housed two per cage with free access to food and water before treatment. Animals were ranked by body weight and randomly assigned to 12 groups, 6 groups with 8 animals each (killed on day 2 after exposure) and 6 groups with 12 animals each (killed on day 70 after exposure). Carbendazim, suspended in corn oil at appropriate concentrations, was given by gavage in a single oral dose of 1.5 to 2.0 ml volume.

Under sodium pentobarbital anesthesia, animals were fixed by vascular perfusion with a mixture of 2.0% glutaraldehyde/1% Acrolein in Ringer's phosphate buffered saline at pH 7.3 on either 2 or 70 days postexposure. After fixation, testes were excised and weighed; testes and excurrent ducts were processed for light microscopy in glycol methacrylate. Testicular sections were stained with periodic acid-Schiff (PAS) reaction and counterstained with hematoxylin (Hess, 1990). Longitudinal sections of efferent ductules and caput epididymides were stained with hematoxylin and eosin.

Parameters for quantitation of testicular alterations were obtained according to previous methods (Hess et al, 1991). Testicular weight and diameter of seminiferous tubules were measured for all animals. Two days after treatment, seminiferous tubular diameter and the percentage of tubules with sloughed epithelium were determined. For animals 70 days after treatment, tubules were classified as either normal, regressive, regenerative, or atrophic (Hess et al, 1988). Evaluations of seminiferous tubules were made randomly within all four quarters of each testicular cross-section. Ductuli efferentes were evaluated for the presence of occlusions, fibrosis, mineralization, granulomas, and abnormal growth. Caput epididymides were evaluated for sperm content and rated as 0 (azoospermia), 1 (small number of sperm), 2 (moderate number of sperm), or 3 (large number of sperm). Percentage data were transformed by arc sine and all data were evaluated first by ANOVA. If an overall difference was found ($P < 0.05$), then a multiple range analysis was used to determine significance among the means by dosage.

Results

Experiment 1

The effects of carbendazim were first apparent 8 hours after exposure when testicular weights were increased (Fig. 1).

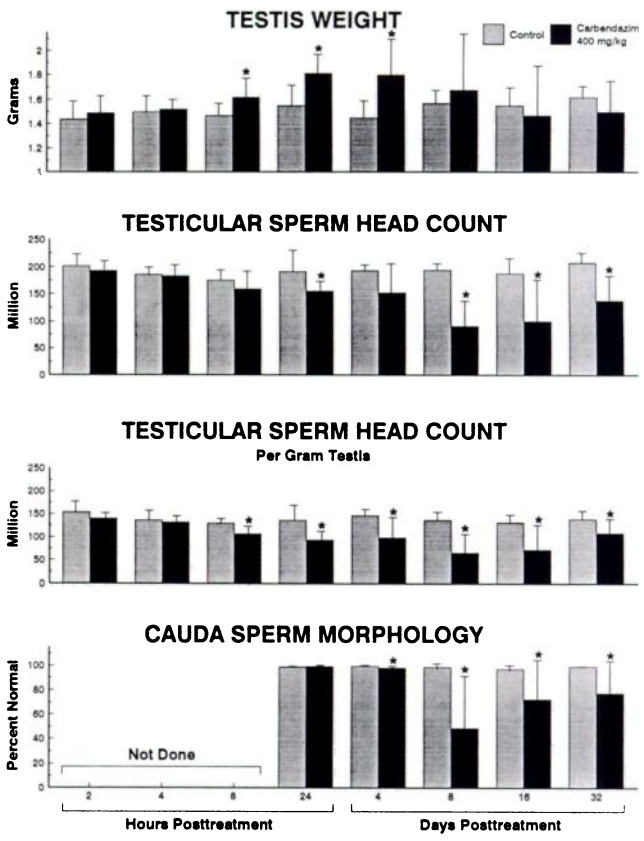

FIG. 1. Effects of carbendazim on testis and epididymal sperm. Responses are plotted as the mean N = 7 or 8 rats per group at each time period. *Significant differences between controls and treated animals at each timepoint ($P < 0.05$).

Testis weights continued to increase through day 4 and declined thereafter. On days 16 and 32 testis weights were substantially lower than controls in 5 of 16 animals, indicating the variable response of individual animals. A decrease in sonication-resistant sperm heads per testis was evident at 8 hours (four of eight rats appeared affected), but the decrease was not significant until 24 hours, when a mean decrease of 19% was observed. Maximum decreases in total sperm head counts per testis occurred on day 8, after which some recovery was apparent. Sperm head counts per gram testis, in general, parallelled the decline in total sperm head counts, but the decline was exaggerated from 8 hours through day 8 because of testicular edema, which increased testicular weight. The maximum effect on sperm production occurred between days 8 through 16, with some recovery apparent in most animals by day 32.

Epididymal weights were not determined until day 4, but were increased at this time ($P < 0.01$). The percentage of morphologically normal sperm in the cauda was minimally less than control on day 4 ($P < 0.05$), but we consider this an equivocal result. By day 8, however, many sperm heads were separated from flagella and 10% were misshapen; numerous sloughed round germ cells and/or cytoplasmic testicular debris also were evident. Many separated cells were also apparent on day 16, but only minimal numbers of round cells and debris were present. On day 32, an increased incidence of abnormal sperm was observed in three of eight animals. No effect on the percentage of motile sperm was seen at 2, 4, 8, 24 hours, or 4 days after treatment. Marked effects on the number of motile sperm were apparent on days 8 and 16; however, because of clumping and degeneration of spermatozoa, quantitative estimates of the percentage could not be obtained for these days. Similarly damaged sperm were seen in three of eight animals on day 32, whereas the percentage of motile sperm in the remaining animals was similar to controls. No effect on sperm concentration in the cauda luminal fluid was detected. Except for decreased prostate weight ($P < 0.01$) at day 16, no effect on body weight or accessory reproductive organ weights was observed.

Experiment 2

Day 2 After Treatment—Dose-dependent increases in testicular weights, seen with 100 mg/kg or greater doses (Fig. 2), were accompanied by significant increases in mean seminiferous tubular diameters at 400 and 800 mg/kg dosages (Fig. 3). Sloughing, the disappearance of immature germ cells from the seminiferous epithelium, had already started by day 2. At the 50 mg/kg dosage (Fig. 4), round spermatids were sloughed from stage (Hess, 1990) I and II epithelium (Fig. 5a) and elongated spermatids were sloughed from the stage VII epithelium (Fig. 5b). At 100 mg/kg, the disappearance of germ cells was more severe (Fig. 4) and extended into stages XII through XIV, where a greater number of elongated spermatids were sloughed (Fig. 5c). In some stage I and II tubules, the round spermatids were present, but the elongated spermatids were sloughed (Fig. 5d). An unusual observation in stages I and II was the presence of large secondary spermatocytes, some of which contained Golgi complexes and acrosomic granules.

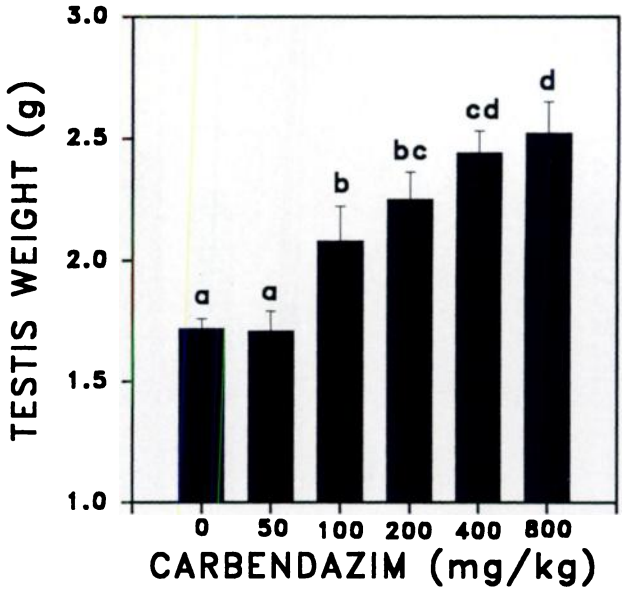

FIG. 2. The effect of carbendazim on testis weight 2 days after exposure (means ± SEM). Significant differences from control are indicated by different letters ($P < 0.05$).

At dosages higher than 100 mg/kg, missing germ cells extended into all stages (Fig. 5e–i), except for stages IX

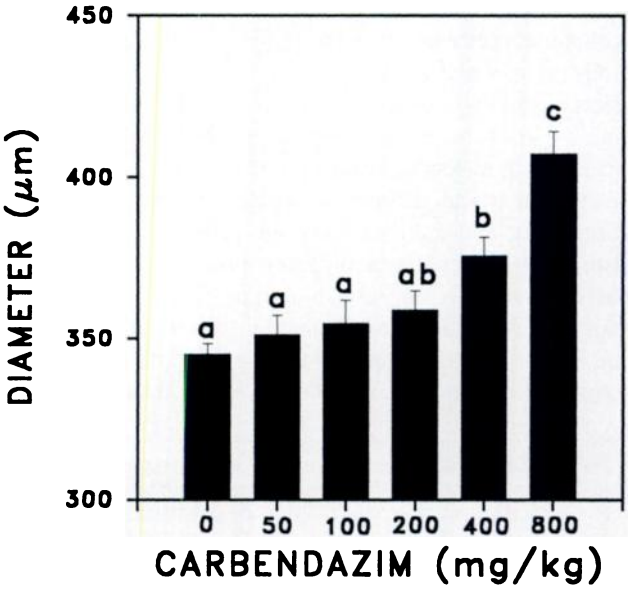

FIG. 3. The effect of carbendazim on the diameter of seminiferous tubules 2 days after exposure (means ± SEM). Significant differences from control are indicated by different letters ($P < 0.05$).

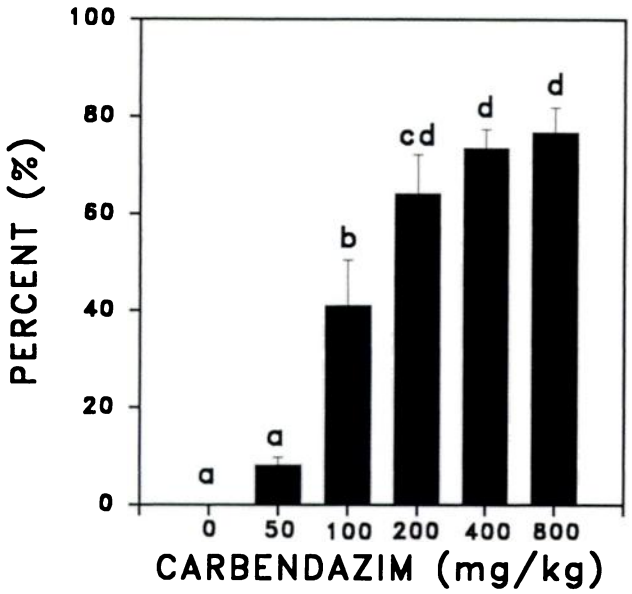

FIG. 4. The effect of carbendazim on the percentage of seminiferous tubules exhibiting epithelial sloughing 2 days after exposure (means ± SEM). Significant differences from control are indicated by different letters ($P < 0.05$).

through XI. Round and elongated spermatids and, to a lesser degree, spermatocytes were involved in sloughing or removal by Sertoli cell phagocytosis. Evidence of phagocytosis by the Sertoli cell was the presence of large PAS-positive bodies in the cytoplasm and occasional elongated spermatid heads, and was seen mainly in stages I and XIV. Secondary spermatocytes with prominent Golgi complexes continued to be seen in stages I and II (Fig. 5g), and rare giant cells of these spermatocytes were formed. Damaged pachytene spermatocytes (pyknotic) were observed only rarely at the higher dosages. Epithelia were not equally affected, and sometimes the degree of sloughing was not uniform in a tubular cross-section. At the 400 to 800 mg/kg dosages, some seminiferous epithelia were damaged so severely that it was difficult to identify the stage (Fig. 5i). Large clefts and multinuclear giant cells were found in the disorganized epithelia, and aggregations of sloughed material were seen in the tubular lumina (Fig. 5j). Also, the inhibition of mitosis was evident by the reduction of nearly one-half of the B type spermatogonia and preleptotene spermatocytes per tubular cross-section (data not shown).

In addition to the observed testicular effects of carbendazim, major pathogenic changes were seen in the excurrent ducts of the testis. The rete testis was swollen with sloughed germ cells, indicating that ductal blockages had occurred further down the tract. The occlusions were observed in efferent ductules of animals treated with 100 mg/kg or higher dosages (Table 1). The caput epididymidis exhibited no evidence of blockage. Occlusions of the ductuli efferentes appeared to be localized in the proximal regions (Guttroff et al, 1992) and the distended ductules were compacted with spermatozoa, sloughed immature germ cells, and cytoplasmic lobes (Fig. 6). The epithelium often was disorganized and contained fragments of spermatozoa and macrophages. Occluded ductules were surrounded by one or more layers of neutrophils and a lesser number of eosinophils and macrophages. Neutrophils often infiltrated the epithelium and the lumen (Fig. 6 inset). This inflammatory reaction occurred exclusively around occluded ductules, whereas the ductules that contained sparse luminal contents were not inflamed (Fig. 6). Neutrophils also were seen surrounding the distended rete testis, even within the tunica albuginea of the testis, but the inflammatory cells did not surround seminiferous tubules, regardless of their degree of swelling.

Day 70 After Treatment—Mean testis weight and mean seminiferous tubular diameter showed dose-dependent decreases at 70 days (Table 2). Histologically, these decreases were accounted for by various degrees of seminiferous tubular atrophy or regression (Fig. 7). The minimum effective dosage was 50 mg/kg, which produced atrophy of only a few seminiferous tubules in one testicle (Table 2). No atrophic tubules were seen in the control testes. In animals treated with higher doses (100 to 800 mg/kg), 37 of 96 testicles contained greater than 50% tubular atrophy (Table 2), and 100% atrophy (Fig. 7a) was observed in 14 testicles. Of the testicles containing between 26% to 100% tubular atrophy, all were associated with occluded efferent ductules (Table 3). The atrophied seminiferous tubules contained primarily Sertoli cells and occasional spermatogonia and were surrounded by a thickened basement membrane. A few tubules were similar in appearance to the prepubertal spermatogenic epithelium, evidence of an attempted regeneration. Mineralization of the luminal contents occurred in some seminiferous tubules.

Various pathologic alterations were observed in the ef-

FIG. 5. Seminiferous epithelium 2 days after exposure to carbendazim. Arabic numerals in each figure indicate steps of spermatids. A = type A spermatogonium; P = pachytene spermatocyte; S = Sertoli cell. Periodic acid-Schiff and hematoxylin; bars = 10 μm. (A) Round spermatids are missing in Stage I. Note the space (asterisks) in the epithelium. 50 mg/kg. (B) Elongated spermatids are completely missing in Stage VII (arrows). Step 7 round spermatids remain intact. 50 mg/kg. (C) Elongated spermatids are missing in Stage XIV (arrows). I = first miosis, II = second miosis. 100 mg/kg. (D) Most elongated spermatids are missing in Stage I (arrows). 100 mg/kg. (E) Elongated spermatids are missing in Stage IV (arrows). 200 mg/kg. (F) Elongated spermatids are missing in Stage VI (arrows). 200 mg/kg. (G) Secondary spermatocytes with Golgi complexes (arrowheads) are seen in Stage II. 200 mg/kg. (H) Residual bodies are missing in Stage VIII (arrows). Arrowheads indicates normally localized residual bodies in Stage VIII epithelium. 400 mg/kg. (I) Sloughing of all germ cells in the seminiferous epithelium (arrows). 400 mg/kg. (J) Sloughed materials in the lumen of the seminiferous tubules. Pachytene spermatocyte (P) and round (R) and elongated (E) spermatids are seen. 400 mg/kg.

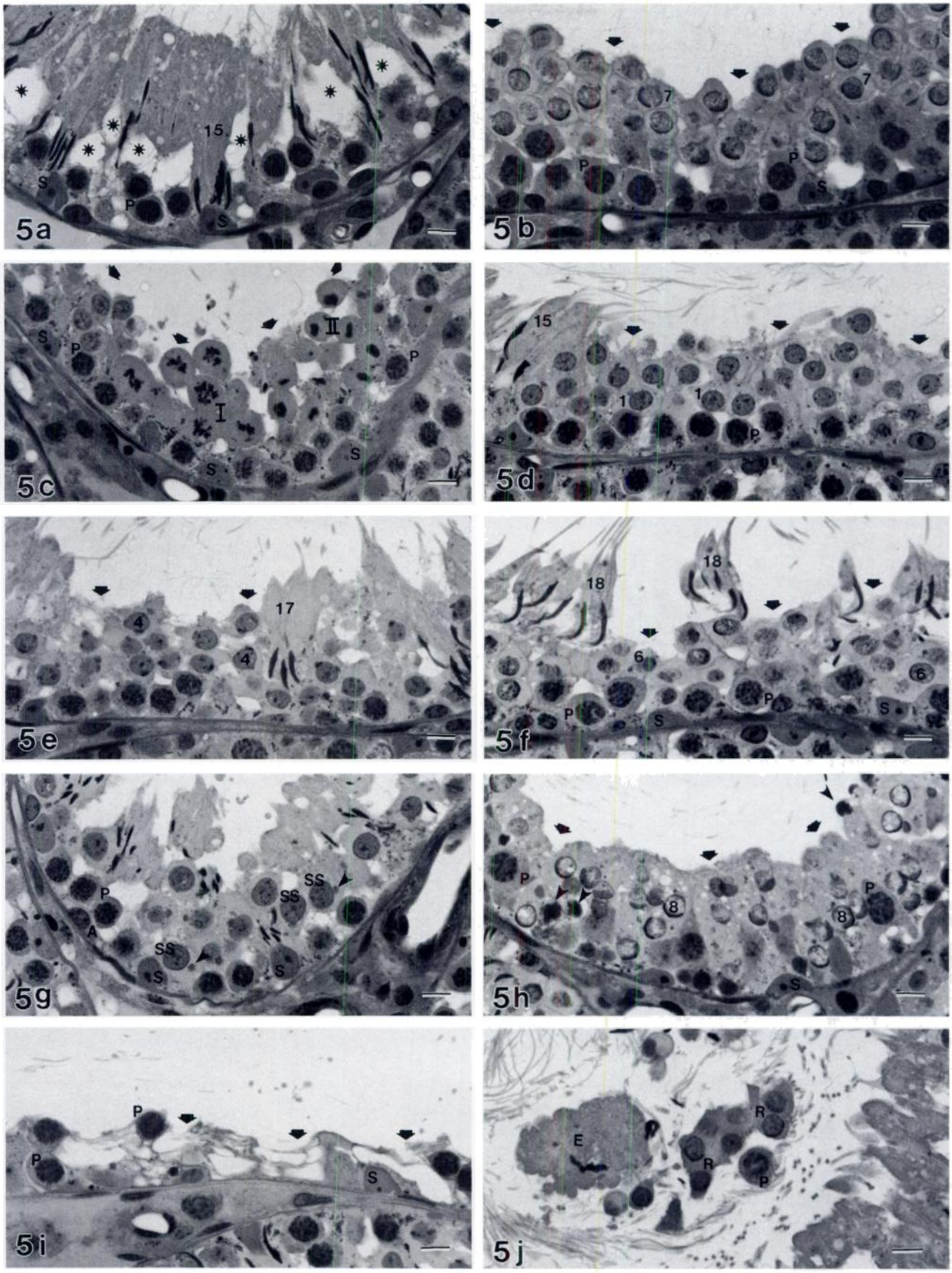
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Table 1. *Occlusions of the efferent ductules 2 days and 70 days after a single dose of carbendazim*

Dosage (mg/kg)	2 Days N*	2 Days Percent occluded	70 Days N*	70 Days Percent occluded
0	16	0	24	0
50	16	0	24	0
100	16	69	22	50
200	16	81	22	86
400	16	100	23	78
800	16	86	21	81

* Left and right excurrent ducts. Samples with incomplete sections of efferent ductules not included.

ferent ductules of the treated animals (Table 3). Minimal effects were seen at 50 mg/kg, where slight abnormal growth of the efferent ductules was seen in only one specimen. One or more alterations were observed in the animals treated with higher dosages. Occlusions of the efferent ductules were frequently observed (Table 1), and were most commonly characterized by compacted luminal contents, spermatic granulomas, mineralizations, and obliteration of the original lumen by fibrotic connective tissue. Abnormal growth of the efferent ductules and fibrosis also were common and usually occurred simultaneously. In these foci, a series of small, irregularly shaped ductules were seen along the perimeter of the fibrotic connective tissue (Fig. 8). The epithelium varied from cuboidal to columnar in shape and was composed of ciliated and nonciliated cells. Large fibrotic lesions without peripheral abnormal ductules were sometimes observed in the initial zone (Fig. 9). These lesions contained a large mass of macrophages and occasionally one or two thin ductules.

In spermatic granuloma, the lumina of efferent ductules were distended and occluded by numerous spermatozoa, macrophages, cell debris, and seminal plasma (Fig. 10). Numerous lymphocytes, and lower numbers of plasma cells, macrophages, and neutrophils were seen surrounding the ductules. Sometimes the epithelium of the efferent ductules near the spermatic granuloma showed hyperplastic growth over the luminal debris. Compacted luminal contents often showed various phases of mineralization. In

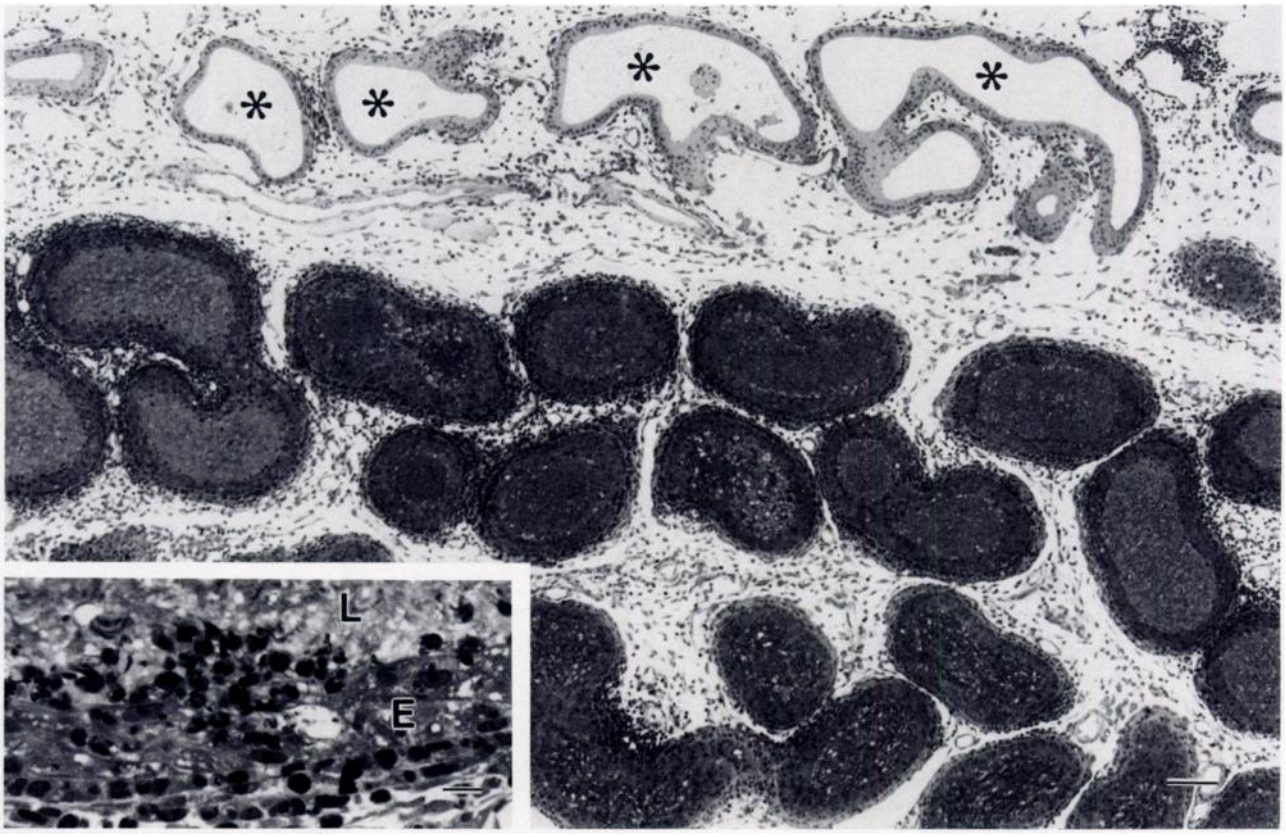

FIG. 6. Efferent ductules 2 days after exposure to carbendazim. Many ductules are occluded with numerous sloughed materials and are surrounded by inflammatory cells, but a few ductules with sparse luminal contents are not involved in the inflammation (asterisks). 800 mg/kg (hematoxylin and eosin; bar = 100 μm). (Inset) High magnification of disorganized efferent ductule. Numerous polymorphonuclear leukocytes are seen in the interstitium, the epithelium and the lumen. 400 mg/kg (hematoxylin and eosin; bar = 10 μm).

Table 2. *Testicular effects of carbendazim 70 days after exposure**

Dosage (mg/kg)	N	Testis weight (g)	Percent of testes†		Tubule diameter (μm)‡	Percent atrophied§	Number of testis >50% atrophy
			weight <1.60	weight >2.20			
0	24	1.95 ± 0.05	0	8.3	371.5 ± 2.6	0.0	0
50	24	1.88 ± 0.06	4.2	0.0	342.5 ± 4.8*	0.25 ± 0.24	0
100	24	1.76 ± 0.13*	26.9	7.7	321.2 ± 12.0*	24.0 ± 10.2*	8
200	24	1.56 ± 0.08*	45.8	0.0	314.0 ± 11.0*	30.2 ± 9.7*	6
400	24	1.46 ± 0.07*	66.7	4.2	291.6 ± 10.5*	48.3 ± 12.0*	13
800	24	1.54 ± 0.10*	45.8	0.0	293.0 ± 12.5*	42.9 ± 10.7*	10

* Values are presented as the mean ± SEM. Two testes per animal. Significant difference from control indicated by asterisks ($P \leq 0.05$).
† 1.60 g was the lowest and 2.26 g was the highest testicular weight in the control group.
‡ 100 tubules per testis.
§ Values presented as the mean percentage ± SEM.

specimens that contained more than 26% seminiferous tubular atrophy, the caput sperm showed a remarkable decrease in numbers (Table 3). Azoospermia (absence of spermatozoa in the caput epididymal cross-sections) was detected in 21 of 48 animals in the 100 to 800 mg/kg dose range.

Discussion

A single dose of carbendazim induced rapid testicular effects, detectible within hours as an increase in testis weight, followed by a decrease in the number of spermatids and

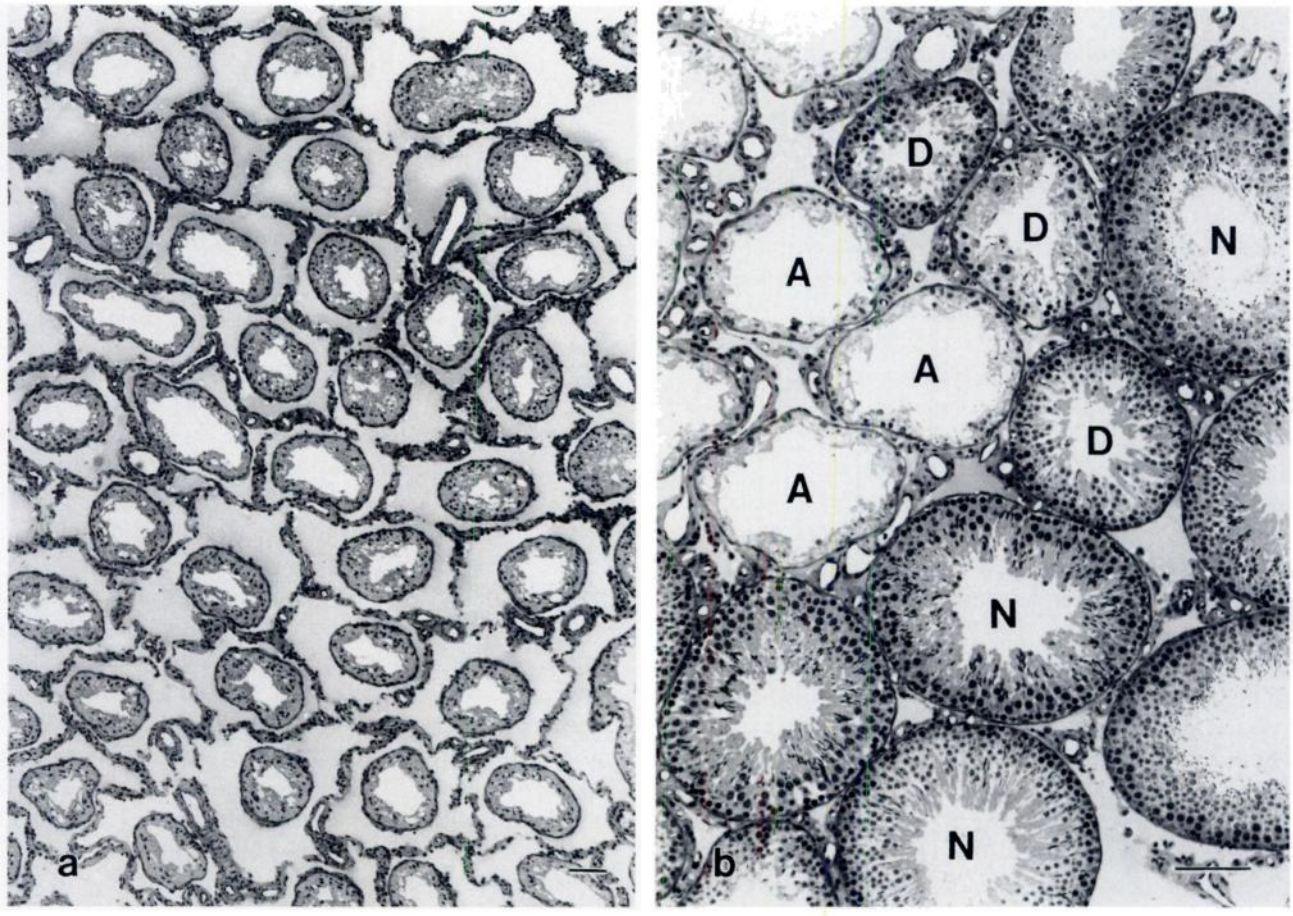

FIG. 7. Testis 70 days after exposure to carbendazim. (A) Total seminiferous tubular atrophy. 400 mg/kg (periodic acid-Schiff and hematoxylin; bar = 100 μm) (B) Normal (N), degenerative (D), and atrophied (A) seminiferous tubules. 200 mg/kg (periodic acid-Schiff and hematoxylin; staining bar = 100 μm).

Table 3. *Effect of carbendazim on excurrent ducts of the testis 70 days after exposure**

Seminiferous tubule atrophy (%)†	N‡	Histopathologic characteristics of efferent ductules§ Occlusion (%)	Fibrosis (%)	Granuloma (%)	Caput‖ sperm
0–25	52	56	54	17	2.89
26–100	36	100	94	25	0.49

* Data taken from treatment groups given 100, 200, 400, and 800 mg/kg of carbendazim.

† Percent of seminiferous tubules with Sertoli cell only or rare germ cells.

‡ Number of testes evaluated. Samples with incomplete sections of the efferent ductules not included.

§ Percent of reproductive tracts containing one or more occlusions, fibrotic lesions or granulomas in the efferent ductules.

‖ Sperm content was rated 0 (azoospermic), 1 (few number of sperm), 2 (moderate number of sperm), or 3 (large number of sperm).

finally the massive sloughing or phagocytosis of immature germ cells by day 2. Although testicular atrophy was an important end result of exposure to this chemical, the major inductive effect was found in the excurrent ducts of the testis, primarily in the ductuli efferentes, as seen with benomyl (Hess et al, 1991). These ductules consist of a series of tubules that conduct sperm from the rete testis to the caput epididymidis (Guttroff et al, 1991), and are responsible for sperm transport and the reabsorption of large quantities of water (Jones and Jurd, 1987) and protein (Veeramachaneni et al, 1990). As described in previous studies (Smith, 1962; Hess et al, 1991), occlusion of these ductules produces a fluid pressure that causes seminiferous tubular swelling, loss of germ cells, and eventual atrophy of the testis. Thus, the long-term testicular effects (seminiferous tubular atrophy and increases in seminiferous tubule concentrations of testosterone) reported in the current and previous studies (Carter et al, 1987; Rehnberg et al, 1989; Gray et al, 1990) can be explained by this pathophysiologic mechanism. The more subtle effects detected in epididymal sperm, at the lower dosages (50 and 100 mg/kg; Gray et al, 1990), also may be explained by the direct effects of carbendazim on the seminiferous epithelium demonstrated in the current study.

Sloughing of immature germ cells and the inhibition of cell division are the first testicular alterations observed after exposure to benzimidazoles (Parvinen and Kormano, 1974; Hess et al, 1991). Other microtubule-disrupting agents, colchicine and vinblastine, induce similar pathologic changes in the testis (Russell et al, 1981). Colchicine, however, causes the cleavage of apical Sertoli cell cytoplasm, which releases cohorts of immature elongated spermatids (Russell et al, 1981). In the current study of carbendazim, it was not possible to determine whether the sloughed materials consisted of germ cells and detached Sertoli cell cytoplasm. Within the epididymal lumen, however, the sloughed cells exhibited large cytoplasmic components, suggesting that

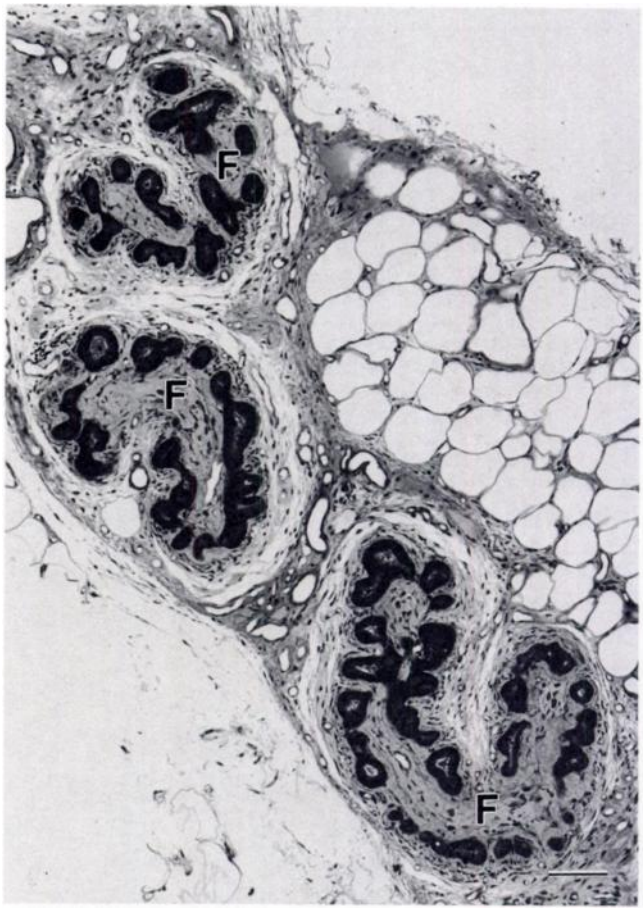

FIG. 8. Abnormal growth of the efferent ductules 70 days after-exposure to carbenzadim. Original lumen of the ductule is replaced with fibrotic connective tissue (F) and is surrounded by small abnormal ductules. 400 mg/kg (hematoxylin and eosin; bar = 100 μm).

portions of the Sertoli cell were released along with the spermatids. An increase in the cytoplasmic content within the epididymis, along with the possible reduction of sperm transport, could explain the increase found in epididymal weights. Because benzimidazole compounds have been shown to inhibit microtubule polymerization and the formation of mitotic spindles, by binding to tubulin (Burland and Gull, 1984), it is reasonable to postulate that the mechanism responsible for germ cell sloughing involves microtubule inhibition. This mechanism would account for the missing elongated spermatids that depend on the cytoskeletal support of the Sertoli cell cytoplasm (Russell, 1984) and the missing round spermatids in stages I and II. The missing steps 1 and 2 spermatids would have been spermatocytes in the second meiotic division on the day of exposure (Parvinen and Kormano, 1974; Hess and Chen, 1992). The inhibition of their cell division may have caused their degeneration and phagocytosis by the surrounding Sertoli cells, leaving large spaces in the epithelium.

It has been shown that microtubules are important in the

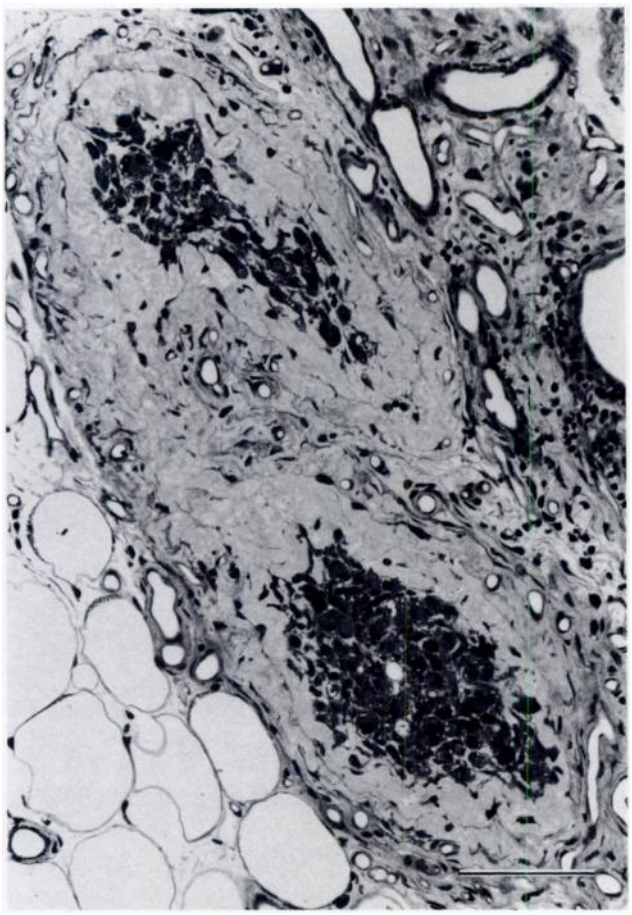

FIG. 9. Large fibrotic lesions without peripheral efferent ductules 70 days after treatment. Masses of macrophages are observed in the centers of the fibrotic lesions. 100 mg/kg (hematoxylin and eosin; bar = 100 μm).

cytoplasmic transport of secretory proteins (Achler et al, 1989), and other microtubule-disrupting agents, such as 2,5-hexanedione, inhibit the secretion of seminiferous tubular fluids (Johnson et al, 1991). Thus, the continued production of seminiferous tubular fluid and subsequent swelling of the testes after carbendazim-induced efferent ductal occlusions suggest that a nonmicrotubule target may also be involved in the carbendazim mechanism.

The current study demonstrates that carbendazim produces more severe long-term pathologic alterations of the testis and efferent ductules than does benomyl at all doses tested (Hess et al, 1991). At day 70, carbendazim resulted in decreased testicular weights beginning at the 100 mg/kg dosage, whereas benomyl had a significant effect only at 400 mg/kg. At the 100 mg/kg dosage, the number of atrophic seminiferous tubules after carbendazim treatment was more than double that obtained following benomyl exposure. Based on the effects, by day 70 it appears that carbendazim produces equivalent effects on male reproduction at half the dosage of benomyl. Although it is not known whether benomyl, or a metabolite such as carbendazim (Douch, 1973), have a direct effect on the testis, the current data showing that the metabolite carbendazim has double the long-term effects of an equivalent dosage of benomyl suggest that it is the metabolites of benomyl that inhibit male reproductive capacity.

At 2 days after exposure, the differences between carbendazim and benomyl treatments are not as distinct as with the long-term effects. Whereas carbendazim caused an increase in testis weight at a dose lower than benomyl (Hess et al, 1991), benomyl did cause a greater increase in seminiferous tubular diameters than carbendazim at the 100 to 400 mg/kg doses. The diameters showed steady increases with increases in carbendazim dosages, however, whereas with benomyl there was a slight decrease at the 50 mg/kg dosage followed by significant increases and then another decrease at the 800 mg/kg. The percentage of seminiferous tubular sloughing showed a similar comparative response, with carbendazim causing a steady increase in sloughing and reaching 78% with increasing dosage, whereas benomyl reached a plateau of approximately 50% at the 200 mg/kg dosage (Hess et al, 1991). From these data it appears that the carbendazim and benomyl exposures produce somewhat similar early effects, but long-term effects on the testis clearly are more severe with carbendazim. Because of the importance of efferent ductal involvement with the benzimidazole response, the difference seen in the long-term effects suggests that carbendazim has a greater effect on the efferent ductules, possibly occluding them for longer periods of time or completely inhibiting their repair.

Preliminary data (not shown) indicate that the maximum effect of carbendazim on testis weight is reached at 800 mg/kg, possibly due to the fact that testicular blood flow is stopped after maximum swelling of the testis (a conclusion derived from the fact that it was necessary to slit the tunica albuginea of swollen testes before fixative would perfuse the vasculature). A reduction in blood flow to the exposed testes would help explain the reported increases in testosterone and androgen binding protein concentrations within the interstitial spaces of the testis (Rehnberg et al, 1989).

It was surprising that some secondary spermatocytes, recognized by their large nuclei, did not degenerate and were observed in stages I and II. Haploid invertebrates have been induced to form diploid cells by the benzimidazole compound (Welker and Williams, 1980), but our study is the first evidence of diploid spermatid formation induced by the benzimidazoles in a mammalian species (Tates, 1979). These diploid cells appeared morphologically normal and developed with apparently normal function, since they formed a Golgi complex and acrosomal granules, typical of steps 1 through 3 spermatids (Hess, 1990). It is not known whether these cell developed into normal spermatozoa.

Carter et al (1987) reported a mean weight of 0.73 g for testes exhibiting 100% tubular atrophy at 245 days after

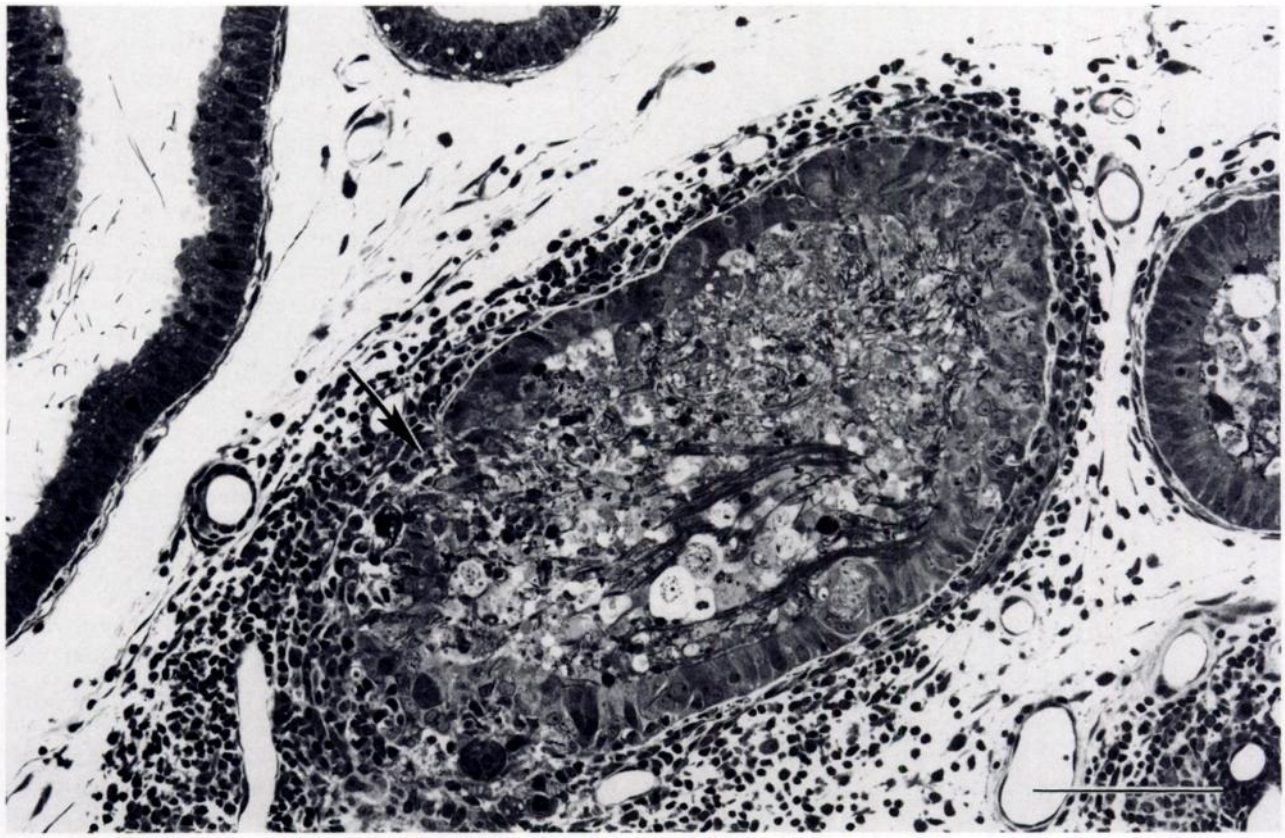

FIG. 10. Spermatic granuloma of the efferent ductules 70 days after treatment. The ductule is surrounded by numerous inflammatory cells. The epithelium shows degeneration and a rupture (arrow). 100 mg/kg (hematoxylin and eosin; bar = 100 μm).

treatment with carbendazim. In the current study at 70 days, we report a mean testicular weight of 1.46 g at the 400 mg/kg dosage, which consists of testes with varying degrees of seminiferous tubular atrophy. If only testes with 100% atrophy are included, however, the mean weight is 1.09 g, which is still larger than that obtained at 245 days. Therefore, the atrophic testes at 70 days after treatment continue to lose weight over time by an unknown mechanism, possibly through a further reduction in the secretion of seminiferous tubular fluid.

The reason that carbendazim causes more long-term testicular effects than benomyl appears to be related to the ability of carbendazim to induce more severe occlusions in the efferent ductules. Because other chemicals may cause sloughing of the seminiferous epithelium without ductal occlusions, the mechanism responsible for the blockage after carbendazim exposure will likely be a direct action of the chemical on the efferent ductules. The normal epithelium of the ductuli efferentes has been characterized as leaky (Nagano and Suzuki, 1980) and capable of reabsorbing much of the water in rete testis fluids (Jones and Jurd, 1987); therefore, damage to the epithelium could induce an acute inflammation and a rapid increase in the rate of water loss from the lumen. The inflammatory response may cause subsequent damage to the ductal epithelium and prevent the normal passage of the sloughed germinal cells. In addition, the sudden dehydration would then result in the compaction of an already crowded lumen of sloughed germ cells. This sequence of pathogenesis appears probable because a recent study by Ilio and Hess (unpublished data) has demonstrated that both benomyl and carbendazim treatments increase the activity of Na^+, K^+-ATPase, the ion pump thought to be ultimately responsible for water resorption in the efferent ductules.

Differences in the degree of inflammation could contribute to various alterations of the efferent ductules and further to testicular responses in individual animals at 70 and 245 days after exposure (Carter et al, 1987). It is likely that severe inflammations are followed by destruction of the efferent ductules and then by fibrosis. With a medium inflammatory response, surviving epithelial cells with intact basement membrane could regenerate (Cuppage and Tate, 1967; Vracko, 1974; Siegel and Bulger, 1975).

The reduction of the caput sperm numbers can be ascribed to either the regeneration of the seminiferous epithelium, or to occlusion of the efferent ductules or both. In the current study, testicles with many atrophied seminiferous tubules often exhibited epididymal azoospermia and ductal

occlusions. In testicles with total atrophy, early occlusions might occur in all efferent ductules and produce effects similar to experimental ligation (Harrison, 1953; Smith, 1962). In a few cases of azoospermia, however, the testicles contained up to 20% normal seminiferous tubules. These cases can be explained by the following. First, spermatic granulomas (present in some cases), as seen after vasectomy (Barratt and Cohen, 1987), could reabsorb the limited production of sperm, and thus provide a mechanism for sperm disposal that would reduce the fluid back-pressure induced by occlusion. Second, when the blockage is formed away from the rete testis, then the intact ductal epithelium between the rete and blockage could continue the reabsorption of fluid. Ligations in the distal efferent region have been shown to have less effect on the seminiferous tubules than the ligation on the proximal efferent region next to the rete testis (Smith, 1962; Soler et al, 1990a, 1990b). Therefore, if carbendazim induces some occlusions more distal to the rete testis, a limited amount of spermatogenesis would be possible. This may help explain why some carbendazim-treated animals were infertile only temporarily (Carter et al, 1987). Fluid pressure from the continued production of sperm could unblock some efferent ductules by pushing the spermatic plug into the epididymis before the ductules are permanently obstructed by fibrosis. It also is possible that a sustained pressure on the occluded ductules would induce new growth of the epithelium, similar to the attempted growth of the vas deferens after vasectomy (Cruickshank et al, 1989; Freund et al, 1989).

In conclusion, acute exposure to carbendazim, a microtubule poison and fungicide, produced more severe testicular effects than its parent compound, benomyl. Two major effects were observed: the disappearance of germ cells, either through degeneration or sloughing, and the occlusion of efferent ductules leading from the testis. Ductal occlusions appear to be the major cause of long-term testicular atrophy associated with higher dosages of carbendazim, whereas hypospermatogenesis results from exposure to lower dosages. Future studies should address the relationship between severity of ductal occlusions, their location, their potential for recanalization, and the onset of seminiferous tubular atrophy.

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