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Invited Review

Histopathology of the male reproductive system induced by the fungicide benomyl

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Summary. Benomyl is an effective fungicide that has been in use for many years. This chemical and its primary metabolite, carbendazim, are microtubule poisons that are relatively nontoxic to all mammalian organs, except for the male reproductive system. Its primary effects, at moderate to low dosages, are on the testis, where it causes sloughing of germ cells in a stage-dependent manner. Sloughing is caused by the effects of the chemical on microtubules and intermediate filaments of the Sertoli cell. These effects spread to dividing germ cells and also lead to abnormal development of the head of elongating spermatids. At higher dosages, it causes occlusion of the efferent ducts, blocking passage of sperm from the rete testis to epididymis. The mechanism of occlusion appears to be related to fluid reabsorption, sperm stasis, followed by leukocyte chemotaxis, sperm granulomas, fibrosis and often the formation of abnormal microcanals. The occlusion results in a rapid swelling of the testis and ultimately seminiferous tubular atrophy and infertility. In conclusion, studies that reveal long term testicular atrophy following chronic or subchronic exposure to a toxicant should be re-examined for histopathological lesions in the efferent ductules and head of the epididymis. Lesions in the male track that cause blockage may induce permanent testicular damage and a decrease in sperm production.

Key words: Testis, Epididymis, Toxicology, Fungicide, Microtubules

Introduction and early work

Benomyl [methyl 1-(butylcarbamoyl)-2-benzimidazole carbamate] is a classic benzimidazole carbamate fungicide and nematocide that has been used for many years on a variety of food crops, ornamental plants, trees and grasses. This well-known pesticide and its primary metabolite, carbendazim [methyl 2-benzimidazole carbamate], exhibit fungicidal action through their ability to disrupt microtubule formation. These chemicals bind B-tubulin subunits of microtubules, thereby inhibiting mitosis (Davidse and Flach, 1977; Burland and Gull, 1984). The U.S. Environmental Protection Agency regulated benomyl based partially on its reproductive toxicity, which was found to cause testicular atrophy and decreased fertility (Torchinskiy et al., 1976; Anonymous, 1979; Carter and Laskey, 1982; Barnes et al., 1983; Carter et al., 1984, 1987). A 70-day feeding study showed significant recovery 70 days after cessation of treatment, suggesting that testicular effects were reversible.

However, the early studies of benzimidazole compounds did not establish mechanisms of testicular atrophy; therefore, reversibility was not clearly established in relationship to dosage. Infertility occurred in 50% of the males after the first week of exposure to 10 daily doses of carbendazim (400 mg/kg), but histopathological examination of the testes revealed large variation in tubular atrophy (Carter et al., 1987). A few of the males showed only short-term infertility, but 50% of those infertile in the second week became irreversibly infertile. Histological examinations of testicular sections from males with irreversible infertility revealed greater than 85% of the seminiferous tubules as atrophic following treatment, and the tubules exhibited a "Sertoli cell-only" epithelium surrounded by a thickened basement membrane. In males with recovered fertility, there was a large variation in the number of atrophic seminiferous tubules, ranging from 13 to 85%.

At first, atrophy was assumed to be the result of carbendazim's action as a rnicrotubule poison, which has been shown subsequently to cause necrosis of mitotic and meiotic cells in the testis (Nakai and Hess, 1997) and the formation of abnormal spermatids (Nakai et al., 1997). Others found effects on hormones in serum and testicular fluids and attempted to relate the long-term effects on sperm production to an inhibition of the testicular-hypothalamus-pituitary feedback loop (Goldman et al., 1989; Rehnberg et al., 1989). However, upon further examination of the histopathology, it was found that two sequential events, first in the testis and

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then in the efferent ducts, could answer many of the questions regarding hormonal changes, long term atrophy of seminiferous tubules and why some males became irreversibly infertile.

Acute exposure - Time response

General response of the testis

An understanding of the mechanisms by which benzimidazole fungicides cause testicular atrophy did not become clear until studies were performed using single doses of the compounds followed by time response evaluations of testicular histopathology. In our first acute exposure study, it was found that after a single exposure, testis weight showed a significant increase, not a decrease, starting at 8 h and rising until 96 h post exposure, followed by a rapid decrease in testis weight (Fig. 2). This increase in testis weight occurred at the same time that germ cells were observed sloughing prematurely from the seminiferous epithelium and testicular sperm head counts were declining (Hess et al., 1991; Nakai et al., 1992). Sloughing of the epithelium was massive and rapid; therefore, it was hypothesized that the sloughed debris could fill the excurrent ducts of the testis and thus block the exit of sperm and fluids. An examination of the rete testis area of treated animals revealed massive swelling of the region. Thus, it seemed possible that the tubules transporting sperm from the testis to the epididymis could become blocked. Further examination uncovered an alternative explanation for ductal occlusions, as described below. At least two different mechanisms are involved in the male reproductive tract toxicity of the benzimidazole compounds: 1) a testicular response including germ cell degeneration and sloughing, and 2) efferent ductal occlusions that cause testicular swelling. An understanding of these separate mechanisms is just now beginning to surface and much has been gleaned from histopathological evaluations of testis and epididymis following sequential time responses.

Morphological response of the testis

Stage specificity

Benzimidazole carbamate compounds cause premature sloughing of germ cells along with cleaved processes of Sertoli cell cytoplasm (Hess et al., 1991; Nakai and Hess, 1994). This process of germ cell loss occurs within 2-4 hours after a single oral exposure and appears to be the first response of the male reproductive system (Hess et al., 1991; Nakai et al., 1992). Sloughing of immature germ cells was observed in all Stages of the cycle (Fig. 3) in the seminiferous epithelium, except for Stages III, IV and V, where elongating spermatids were embedded deep within the body of the Sertoli cell. A higher frequency of sloughing was observed in tubules of Stages VII, X through XIV and I (Nakai and Hess, 1994), although Lim and Miller (1997) reported no stage specificity of sloughing. Most of the sloughed germ cells were elongate spermatids, but occasional sloughing of

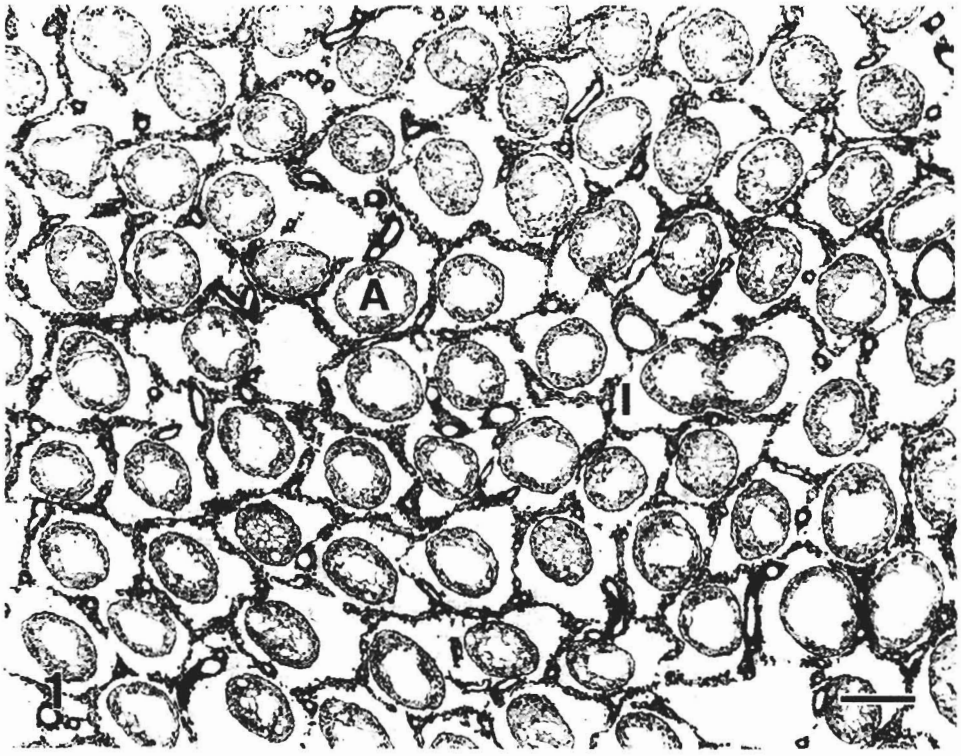


Fig. 1. Low magnification of the testis from a rat at 70 days post exposure to a single treatment of carbendazim (400 mg/kg). Note the complete atrophy of seminiferous tubules (A) and increased interstitial space (I). PAS-hematoxilin. Bar: 25 μm.

round spermatids and spermatocytes was seen. Sloughed spermatids in the tubular lumen were usually found in clusters and often in close apposition to one another as they normally would exist in the seminiferous epithelium, suggesting that the cells remained connected to each other by their intercellular bridges and/or cleaved cytoplasm of the Sertoli cell. Partially sloughed masses of immature germ cells were seen connected to the luminal border of the seminiferous epithelium by elongated apical cytoplasm of the Sertoli cells. The cells remaining within the epithelium often were loose in arrangement, exhibiting expanded intercellular spaces. Thus, the process of germ cell sloughing may have contributed to the long-term effects observed even 32 days post exposure (Nakai et al., 1992).

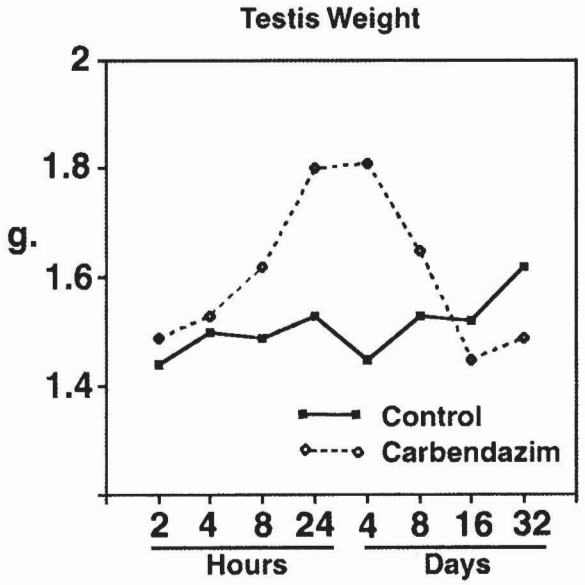


Fig. 2. Effects of carbendazim on testis weight over time. Significant differences between controls and treated animals are indicated by * ($p<0.05$). N=7 or 8 rats per group. Testis weight increases rapidly from 8 to 4 days, but then rapidly becomes atrophic. Modified and reprinted with permission from the American Society of Andrology (Nakai et al., 1992).

The reason for stage-specific effects of carbendazim remains unknown. In tubules where the germ cells were resistant, elongating spermatids are embedded deep within the body of the Sertoli cell and in such a configuration the spermatids may be more resistant to sloughing than in other tubules because of the greater structural integrity associated with increased Sertoli cell cytoplasm surrounding the heads and tails of the developing germ cells (Russell et al., 1990). It is also possible that Stage-specific resistance to sloughing is related to the presence of intermediate filaments within the Sertoli cell cytoplasm, which appear to help anchor germ cells in the seminiferous epithelium (Amlani and Vogl, 1988). Others have reported that disruption of vimentin (intermediate filaments), not tubulin, is correlated with sloughing induced by colchicine (Allard et al., 1993). Therefore, the distribution of cytoskeletal elements other than microtubules may be just as important in deriving an understanding of the effects of the benzimidazoles on Sertoli cell function.

Effects on Sertoli cells

In an effort to test the hypothesis that germ cell sloughing is caused by the disruption of Sertoli cell cytoskeletal elements, we have examined Sertoli cell morphology following carbendazim treatments (Nakai et al., 1992, 1995; Nakai and Hess, 1994). Like colchicine, carbendazim binds to tubulin subunits and inhibits the formation of microtubules (De Brabander et al., 1976; Ireland et al., 1979; Havercroft et al., 1981). Injection of

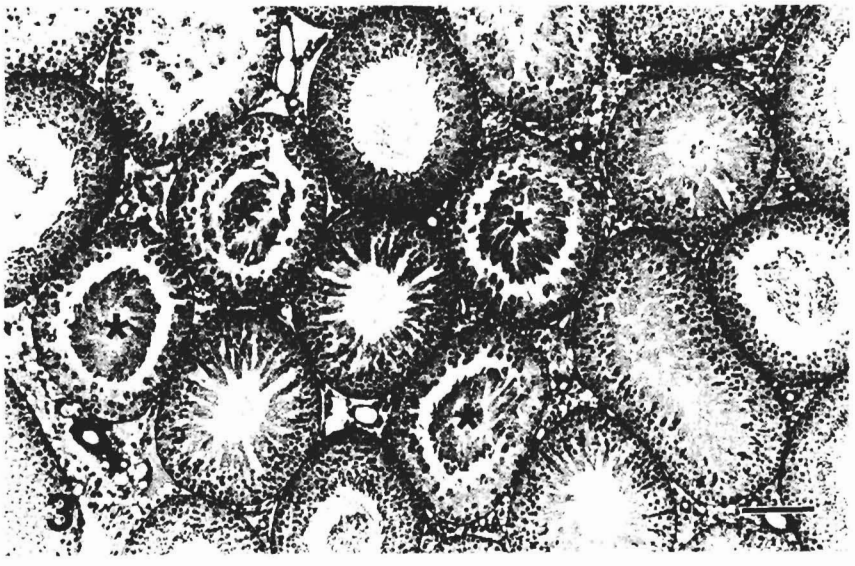


Fig. 3. Light micrograph of the testis at 3hr post treatment with carbendazim (400 mg/kg), showing stage dependent sloughing (*) of immature elongate

colchicine into the testis results in a dramatic decrease or disappearance of microtubules in the Sertoli cells (Russell et al., 1981a,b) and causes sloughing of immature germ cells along with the apical cytoplasmic processes of Sertoli cells in the rat. Russell et al. (1981a,b) described the rounding-up of the Sertoli cell and cleavage of its cytoplasm between the trunk and apical regions. Our studies also show a decrease in the number of microtubules in the body region of the Sertoli cells after treatment with carbendazim (Nakai et al., 1994). The loss of microtubules was associated with a failure of the Sertoli cell to maintain its proper shape. This was reflected in the shape of the Sertoli cell nucleus. In controls, most Sertoli cell nuclei were oval in shape and aligned with their longer axis either parallel (52%) or perpendicular (35%) to the basement membrane of the seminiferous epithelium. After treatment, however, nuclei with these configurations were significantly decreased in number, whereas round-shaped nuclei were the most numerous in tubules where germ cells were sloughed (Nakai et al., 1995).

In the normal Sertoli cell, mitochondria are elongated and stretched along the length of microtubule tracks (Figs. 4, 5) from basement membrane to the apical surface (Nakai and Hess, 1994). In contrast, carbendazim treatment causes mitochondria to form aggregates within the body region, where microtubules appear disrupted (Figs. 6, 7). This response is common to tissues and cells treated with other microtubule disrupting agents (Heggeness et al., 1978; Russell et al., 1981b; Vogl et al., 1983). However, one difference seen with carbendazim was the lack of round bodies in the lumen after sloughing. Instead, Sertoli cell cytoplasm attached to the sloughed germ cells retained their microtubules and original shape seen within the seminiferous epithelium. With colchicine, the sloughed cell aggregates formed rounded structures and ectoplasmic specialization and associated microtubules were disrupted (Russell et al., 1981b). Therefore, carbendazim does not exhibit as extensive an effect on microtubules as does colchicine treatment, which corresponds to *in vitro* binding studies indicating that colchicine binds tubulin with much greater affinity than does carbendazim (Friedman and Platzer, 1978).

The mechanism by which carbendazim causes the sloughing of germ cells appears to be associated with the disruption of microtubules and other cytoskeletal elements. Similar to the colchicine response, carbendazim disrupted the microtubules within the Sertoli cell body, but unlike colchicine it did not affect

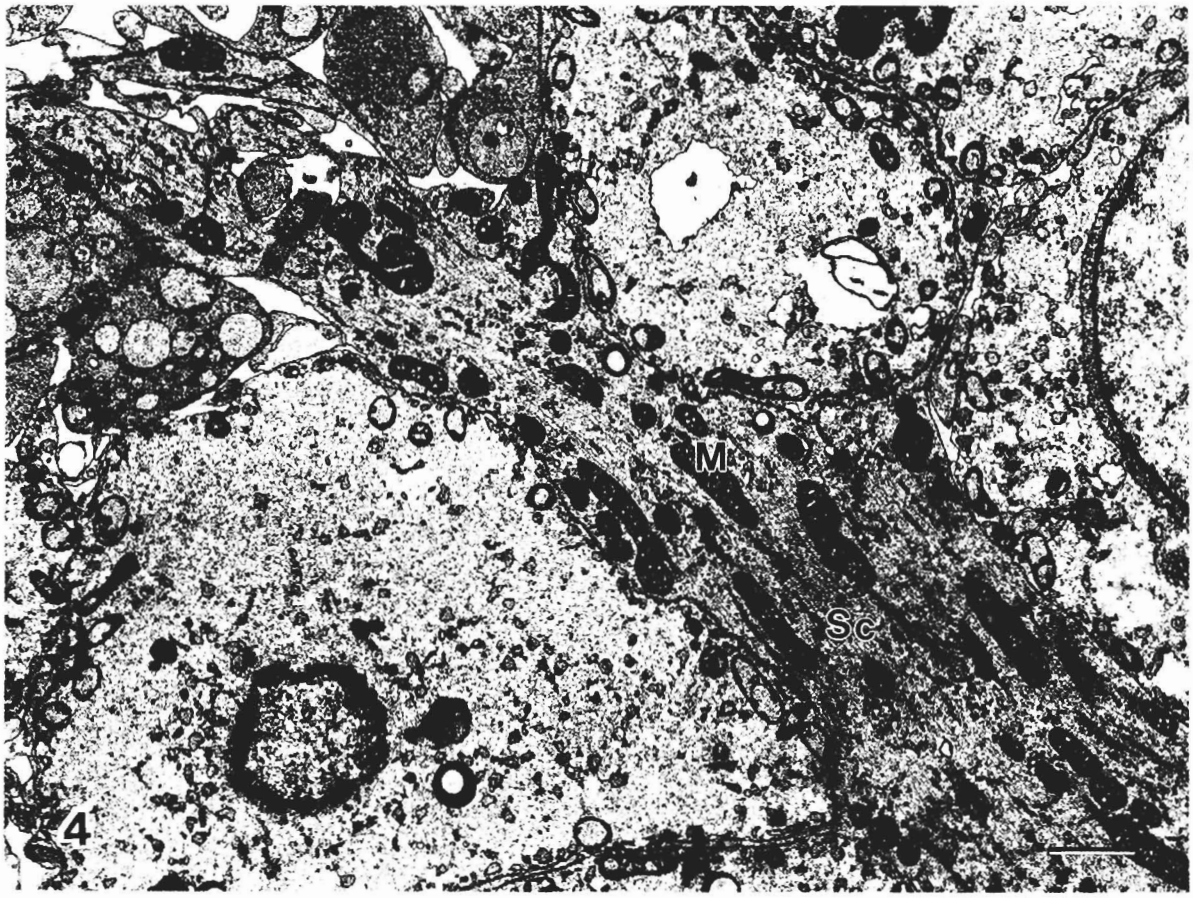


Fig. 4. Electron micrograph of control Sertoli cell (Sc). Mitochondria (M) are mostly elongated along the basal to apical axis. Bar: 2.5 μm.

the microtubules in the apical cytoplasm, even though the mature spermatids were sloughed along with the apical cytoplasm of the Sertoli cell. Thus, the apical processes maintained their original complex shape and were not withdrawn by the tension coming from the retraction of the body of the Sertoli cell following carbendazim treatment. The more complete destruction of Sertoli cell microtubules by colchicine is possibly one reason for the more wide-spread sloughing of the germinal epithelium, including the loss of round spermatids (Russell et al., 1981b) than was observed using carbendazim treatment (Nakai and Hess, 1994).

A decrease in the number of microtubules in the body region of the Sertoli cell, but not in the apical region following carbendazim treatment, might reflect a gradient in the concentration of carbendazim, which could have existed from the interstitium to the lumen of the seminiferous tubule. Alternatively, it is known that microtubules grow and shrink from their plus ends which are generally oriented toward the periphery of the cells (Darnell et al., 1990). Since microtubules of the Sertoli cells are oriented with their plus ends toward the base of the cell (Redenbach and Vogl, 1991), depolymerization of microtubules would most likely begin at their plus ends and progress toward their minus ends which are directed toward the seminiferous tubular lumen. Regardless of the mechanism, the disruption of microtubules can be attributed to the inhibitory action of carbendazim on microtubule formation (De Brabander et al., 1976). Another hypothesis is that carbendazim disrupts microtubule interactions with other cytoskeletal elements such as intermediate filaments (vimentin) or microtubule associated proteins, which play important roles in the stabilization of microtubules and in intracellular microtubule-based transport processes (Neely and Boekelheide, 1988; Achler et al., 1989; Darnell et al., 1990; Neely et al., 1990; Boekelheide et al., 1991; Hall et al., 1992).

Fig. 5. Higher magnification of control Sertoli cell cytoplasm showing the alignment of cytoplasmic organelles along tracks of microtubules (arrows), in a basal to apical orientation. Bar: 0.5 μm.

Recent efforts by our laboratory have shown that tyrosinated α-tubulin subunits and intermediate filaments are affected in the same Stages of spermatogenesis following carbendazim treatment. In seminiferous tubules immediately after sloughing of germ cells, there is a disappearance of α-tubulin staining (Figs. 8, 9) and a simultaneous collapse of the intermediate filaments (Figs. 10, 11). Thus, microtubule disruption may alter interactions with intermediate filaments, resulting in a loss of the germ cell anchor and consequently sloughing. The distribution of intermediate filaments has been shown to be related to the presence of microtubules, and the filaments exhibit perinuclear collapse in cells treated with microtubule disrupting agents (Klymkowsky, 1981; Darnell et al., 1990). Others have suggested that germ cells are held in place by a close association between the germ cell plasmalemma and specialized junctions of the Sertoli cell membrane, the ectoplasmic specializations (Vogl et al., 1991, 1993a,b; Vogl, 1996). It is interesting that intermediate filaments are extended toward the Sertoli cell crypts that hold these elongate spermatids at the specialized junctions (Amlani and Vogl, 1988; Vogl et al., 1991, 1993b). Therefore, we hypothesize that disappearance of vimentin filaments in the body region of the Sertoli cell may be a primary cause for sloughing of immature germ cells.

Effects on spermatids

In addition to inducing sloughing of elongate spermatids, the benzimidazole carbamate fungicides also produced effects on developing spermatids over a prolonged period of time after a single dose (Nakai and Hess, 1997; Nakai et al., 1998). Distortion of the nucleus was common in particular steps of spermatids 10 days post treatment (Fig. 12). The nucleus was irregular in shape and surrounded by dense aggregates of manchette microtubules. Three types of nuclear invagination were observed in spermatids: a) deep invaginations containing numerous manchette microtubules in the caudal aspects of nuclei of steps 9-11 spermatids (Fig. 13); b) a shallow pit or depression that occurred at multiple sites in non-acrosomal areas of the nucleus, mainly caudal; and c) irregular shaped invaginations of the nucleus that were larger in size. Distortion and invagination was also seen in binucleate cells. In binucleate cells, manchette microtubules extended along the lateral aspect of each nuclei. However, a single shared bundle of microtubules was located between the nuclei (Fig. 13).

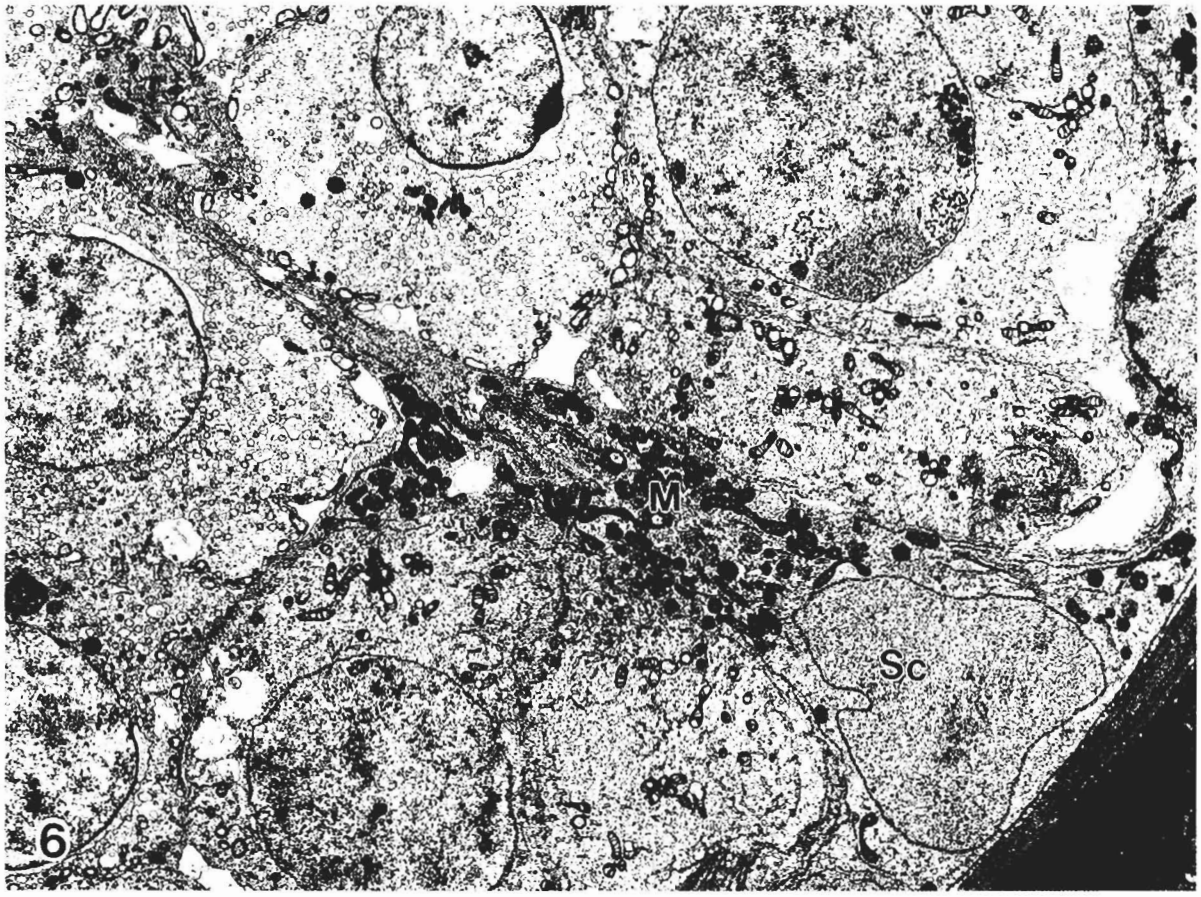


Fig. 6. Electron micrograph of Sertoli cell (Sc) from testis 3 h after carbendazim treatment (400 mg/kg). Note the aggregation of mitochondria (M) near the nucleus. Bar: 5 μm.

Abnormally formed acrosomes were also observed out to day 10.5 post treatment (Nakai et al., 1997). Acrosomes were spread over the nuclei in a normal manner as observed in controls, but were occasionally discontinuous at the periphery (Fig. 12). In other spermatids, fine PAS-positive granules adjacent to the nuclear surface were noted. Electron microscopy revealed that the granules were multiple vesicles containing circular cores of various electron densities. In addition to the abnormally formed acrosomes described above, spermatids showing complete lack of an acrosome were seen in Stages VII-X at days 7.5-10.0 (Figs. 14, 15). The nuclei of these acrosome-deficient spermatids were smaller in size than those of other spermatids found in the same tubules. They were round or oval in shape and showed eccentric position within the cell with their apex oriented toward the lumen. In the apical region of these cells, nuclear envelope was closely adjacent to the plasma membrane, where it showed the modification. However, acrosome was not present over

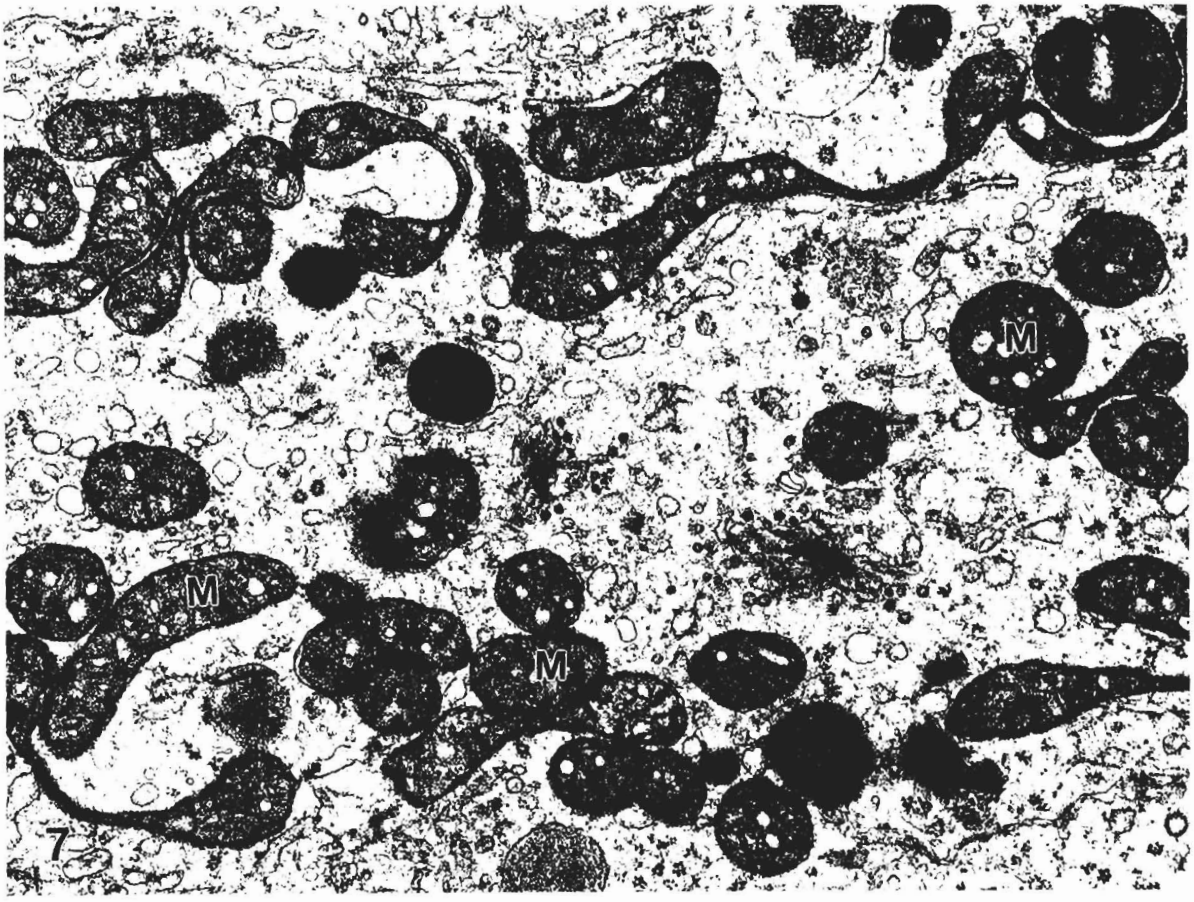


Fig. 7. Higher magnification of Sertoli cell cytoplasm after carbendazim treatment (400 mg/kg). Note the disappearance of microtubules and loss of elongation by the mitochondria (M) in the trunk region of the cytoplasm. Bar: 2 μm.

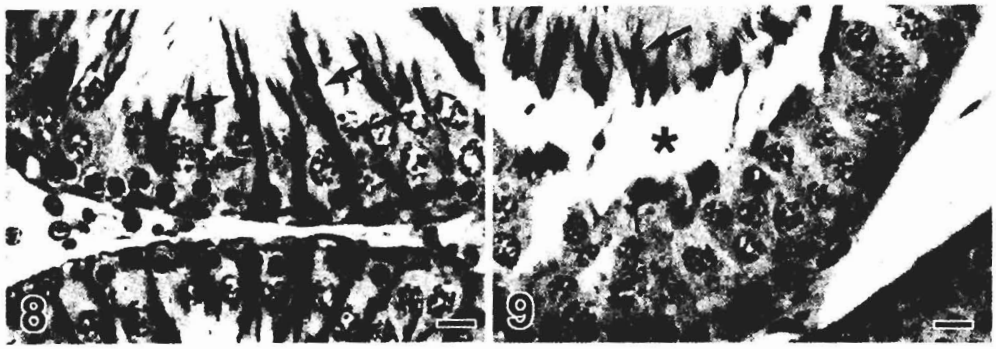


Fig. 8. Immunohistochemistry for tyrosinated α-tubulin in control testis. Microtubules (arrows) are abundant in Sertoli cell cytoplasm, extending from the base to the apex. Bar: 10 μm.

Fig. 9. Immunohistochemistry for tyrosinated α-tubulin in testis following carbendazim treatment (400 mg/kg). Elongated spermatids have sloughed, leaving a space (*) but retaining Sertoli cell microtubules (arrow). Microtubules in the remaining epithelium are missing, except in the very apical region. Bar: 10 μm.

this area. When the acrosome-deficient spermatids were located near the luminal border of the seminiferous epithelium the apical region was not covered by Sertoli cell cytoplasm, whereas when they were located within the seminiferous epithelium the apical region was covered by Sertoli cell cytoplasm. Poorly developed ectoplasmic specialization in Sertoli cells was often associated with the acrosome-deficient spermatids. Acrosomes of binucleate spermatids spread over the round and elongating nuclei, and fused to intervene between them. A single Golgi apparatus was shared by two nuclei. Because binucleate cells were observed as early as day 1.5 post treatment, it is likely that they were formed by the failure of cytokinesis of secondary spermatocytes following nuclear division, as explained for other treatments (Meistrich et al., 1982; Carter et al., 1987; Russell et al., 1987).

Effects on mitosis and meiosis

Benomyl and carbendazim impeded the spermatogenic process by causing necrosis of cells in division, both mitotic and meiotic, and by preventing pachytene spermatocytes from completing the second meiotic

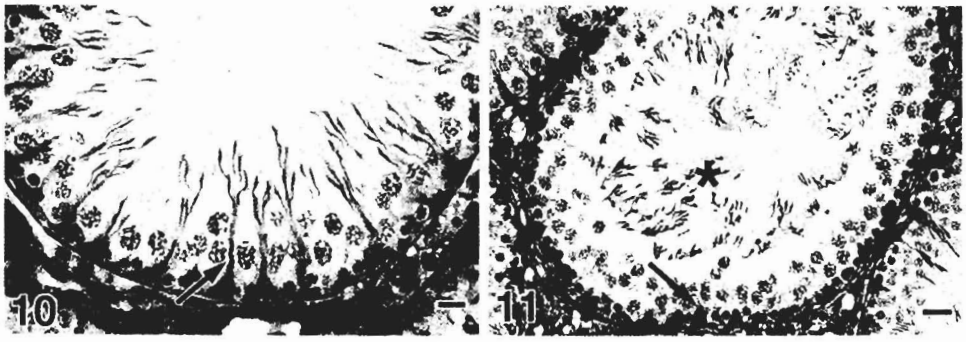


Fig. 10. Immunohistochemistry for vimentin intermediate filaments in control testis. Bar: 20 μm.

Fig. 11. Immunohistochemistry for vimentin intermediate filaments in testis following carbendazim treatment (400 mg/kg). After the spermatids slough (*), vimentin filaments are seen collapsed around the Sertoli cell nucleus (arrow). Bar: 10 μm.

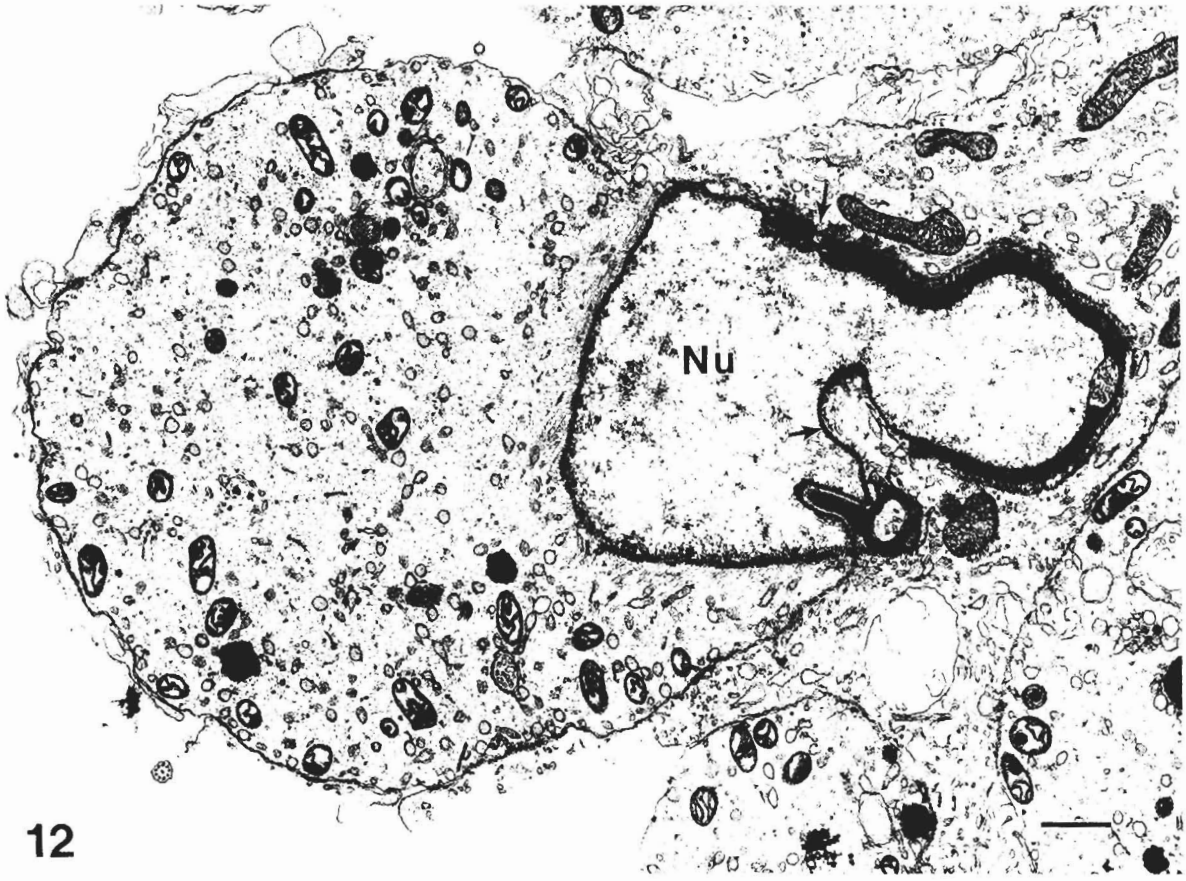


Fig. 12. Electron micrographs of abnormal spermatid induced by treatment with carbendazim (100 mg/kg). Spermatid with nuclear (Nu) distortion and discontinuous acrosome (arrows) seen at day 10.5 post treatment in stage X. Bar: 2 μm.

division (Fig. 16). There was a dramatic increase in the number of necrotic cells in mitosis between 3-6 hours post treatment with a single dose of benomyl (data unpublished). Effects seen on cells in meiosis peaked at 12 hours. Necrosis occurred most frequently in metaphase primary and secondary spermatocytes, but newly formed secondary spermatocytes occasionally showed necrosis.

When specific types of germ cells were destroyed, in time empty spaces appeared in the epithelium where cells were missing. Necrosis of meiotic spermatocytes resulted in windows of missing round spermatids in the expected Stages at individual post treatment intervals. For example, elongate spermatid steps 10-12 were missing in Stages X-XII at day 10.5 and and step 19 in Stage VII at day 20.0, which leads us to conclude that spermatogenesis was not arrested nor retarded, which is consistent with the claim that arrest and retardation of spermatogenesis does not generally occur (Russell et al., 1981a). However, there was evidence of the mixing of spermatid steps within a tubule cross section. Three or four different steps of spermatids were sometimes observed to coexist in Stages X-XI at day 10.5, but not in Stage VII at day 7.5, nor earlier. This suggested that carbendazim causes some spermatocytes that are in meiosis at the time of treatment to retard their development, and that the retardation likely becomes obvious in spermatids later than step 7 spermatids. However, the latest study from our laboratories (Nakai et al., 1998) further demonstrated that less developed spermatids than normal occurred in Stage III at day 3 post treatment. Therefore, it seems that carbendazim-induced asynchronization of spermatid development begins in the early phases of spermiogenesis. Retardation may be attributable to abnormalities in the spermatids and/or dysfunction of the associated Sertoli cells. However, if retardation is due to dysfunction of Sertoli cells, it should be observed in other stages and post treatment intervals besides Stage X-XI at day 10.5. Therefore, it is likely that the cause of retardation mainly resides in the spermatids. The fate of retarded spermatids is unknown, but they are probably removed from the seminiferous epithelium in the subsequent stages by phagocytosis or sloughing, as they are not observed in later stages.

Another major discovery following exposures to carbendazim was the presence of enlarged spermatids, or 'mega spermatids.' Mega spermatids were usually observed in tubules that also had missing spermatids. These abnormally large spermatids were proportionately

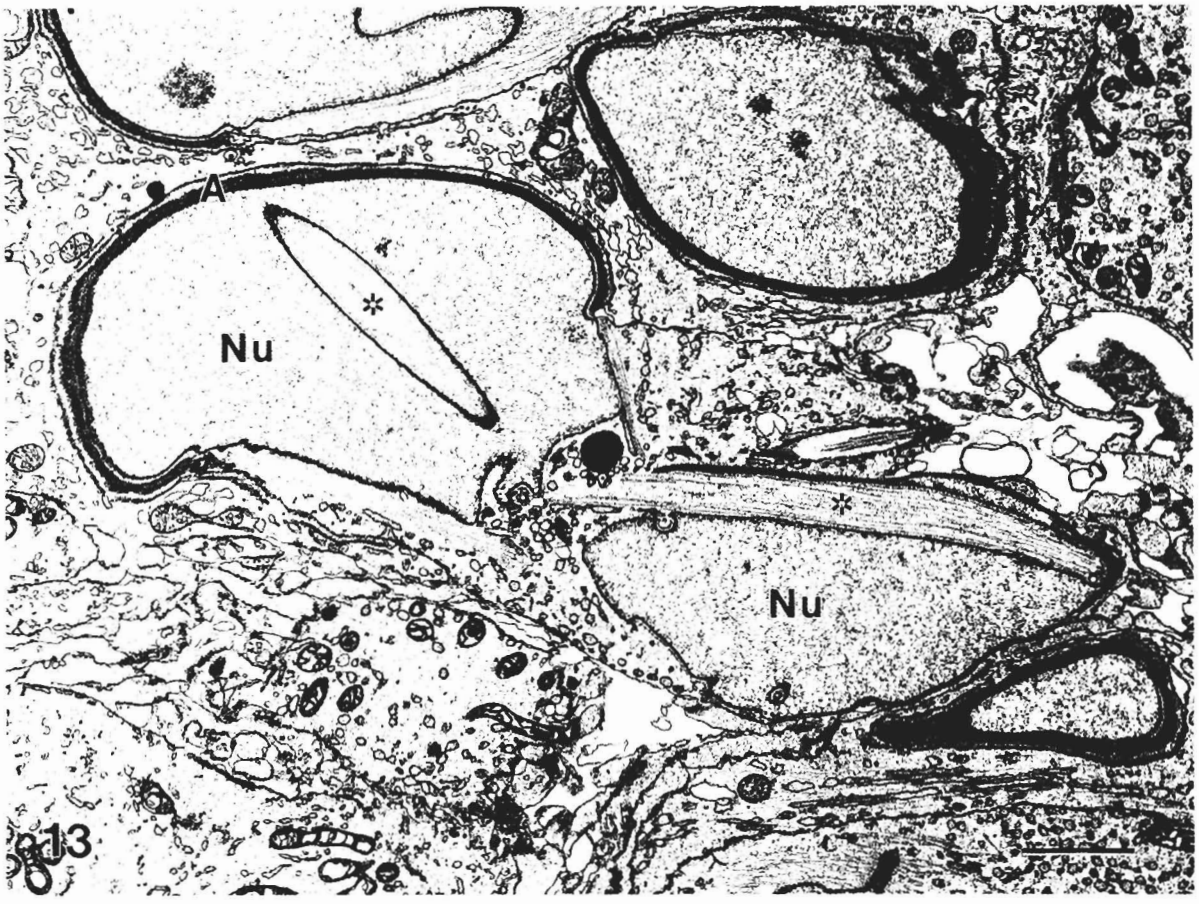


Fig. 13. Spermatid (binucleate) with deep nuclear invaginations containing manchette microtubules (*) seen at day 10.5 post treatment. Nu: nucleus; A: acrosome. Stage X. Bar: 2 μm.

normal in structure, but were approximately 30% larger than normal spermatids (Fig. 17). Mega spermatids were observed to develop up to step 12, but further study is required to determine whether they complete spermiation and have fertilizing capacity. It is reported that aneuploid spermatogenic precursor cells (secondary spermatocytes) develop to be mature spermatozoa despite the genetic defect (Stolla and Gropp, 1974). Large round spermatids have been observed in the mouse testis after treatment with various chemotherapeutic agents including microtubule disrupting agents (Lu and Meistrich, 1979; Meistrich et al., 1982). These cells are approximately the size of secondary spermatocytes and therefore are assumed to be diploid cells. In addition, it is reported that near-diploid spermatozoa, especially those with giant heads, contain near-diploid amount of DNA, and that they are originally due to an abnormal meiotic division (Stolla and Gropp, 1974). This is supported by recent studies indicating that colchicine and vinblastine induce aneuploidy in spermatocytes (Miller and Adler, 1992; Leopardi et al., 1993). An earlier study using the mouse showed that carbendazim causes an increase in the number of diploid cells of the testis at 7 days post treatment (Evenson et al., 1987). Presumably these cells could include a subpopulation of mega spermatids. The induction of aneuploidy by carbendazim or benomyl is also known to occur in other cell types (Beermann et al., 1988; Zelesco et al., 1990; Zuelke and Perreault, 1995).

Morphological response of the efferent ducts

Ductal occlusion and neutrophil chemotaxis

Occlusion of the efferent ducts is common following exposure to the fungicide benomyl or its metabolite carbendazim (Hess et al., 1991; Nakai et al., 1992). Sloughed germ cells are found in the lumen of the occluded efferent ducts and the epididymis (Figs. 18, 19). This response is rapid, as an increase in testis weight is detected as early as 8 hrs post exposure (Nakai et al., 1992). A single dose appears to be sufficient to induce total ductal occlusion (Fig. 20), but a comparison with a 10-day exposure period (Carter et al., 1987) is noteworthy because the percentage of testes with "total seminiferous tubular atrophy" was 21% on day 70 and 50% on day 245. This suggests that either seminiferous tubular regression continues long after treatment has stopped or that the additional doses were effective in spreading damage to a greater number of efferent ducts.

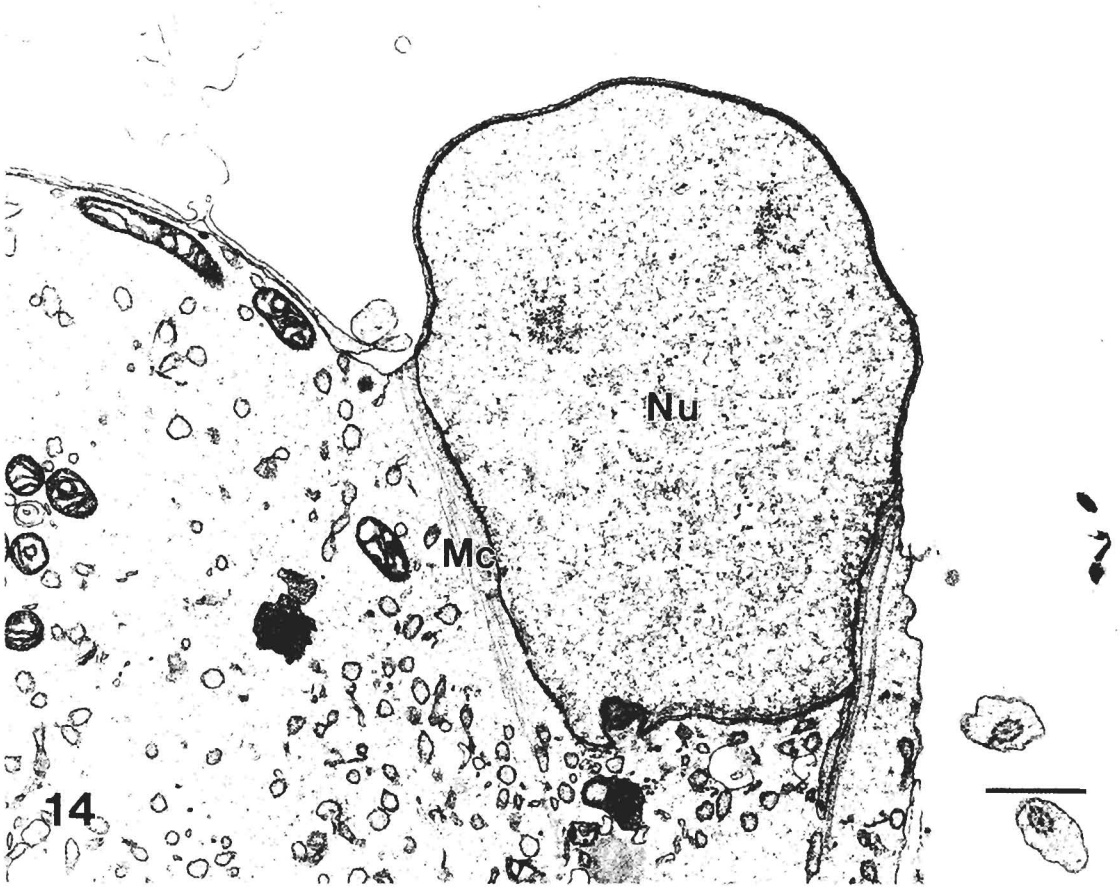


Fig. 14. Spermatid without acrosome in Stage X at day 10.0 post treatment. Nu: nucleus; Mc: manchette microtubules. Bar: 1 μm.

Microdissection of occluded efferent ducts was performed to determine specific locations of the benzimidazole lesions (Hess, 1998). At 12 h post treatment 75.8% of the ductules were occluded and by 24 h nearly 85% were occluded. Overall, 56% of the occlusions were located in the initial zone, 15% at junctions and 44% in the conus vasculosa. No occlusions were observed in the common efferent duct. Thus, the occlusions are primarily occurring in proximal efferent ducts, which causes us to reject our first hypothesis that occlusions were caused by an overwhelming presence of the sloughed germ cells in the common efferent duct, that is a single small diameter tubule, which could easily become blocked by large objects, similar to the clogging of a funnel (Guttroff et al., 1992). Other chemicals also cause massive sloughing of the seminiferous epithelium (Ericsson, 1971; Parvinen et al., 1978; Russell et al., 1981b; Chapin et al., 1983, 1984; Fukuoka et al., 1990; Chapin and Ku, 1994). However, it is not reported that these chemical cause occlusions in the efferent ducts; therefore, we must conclude that benzimidazole carbamates induce occlusions by direct action on the efferent ducts.

After occlusions were formed in the efferent ducts, an inflammatory response by neutrophilic leukocytes was initiated (Fig. 21), approximately 2-4 hrs later. Severity of response was dose-dependent, but the onset of occlusions and time to first appearance of neutrophils in the connective tissue remained the same regardless of dosage. In controls, occlusions were not present and neutrophilic leukocytes were absent. However, two hours after treatment, exfoliated spermatids were present in the lumen of the efferent ducts and most ductules were engorged by 4 h. Complete occlusion of the ductules occurred at 6-8 h (Fig. 20), before the infiltration of neutrophils, which corresponded to a buildup of fluid in the testis and increased testis weight (Nakai et al., 1992), therefore it does not appear that the resulting inflammation causes ductal occlusion, but rather that occlusion of the lumen and epithelial damage stimulates the inflammatory reaction.

The inflammatory reaction is quite extensive, with massive numbers of neutrophils surrounding the efferent ductules (Fig. 21). The reaction begins with neutrophils migrating between endothelial cells junctions lining small venules (Fig. 22), which produces limited hemorrhage into the connective tissue space (Hess, 1998). As the leukocytes migrate between the thin smooth muscle layers of the efferent ducts, some of the muscle cells are destroyed. By 48 h post treatment, neutrophils will have formed between 2-5 solid layers of cells surrounding the base of the ductules (Figs. 21, 22),

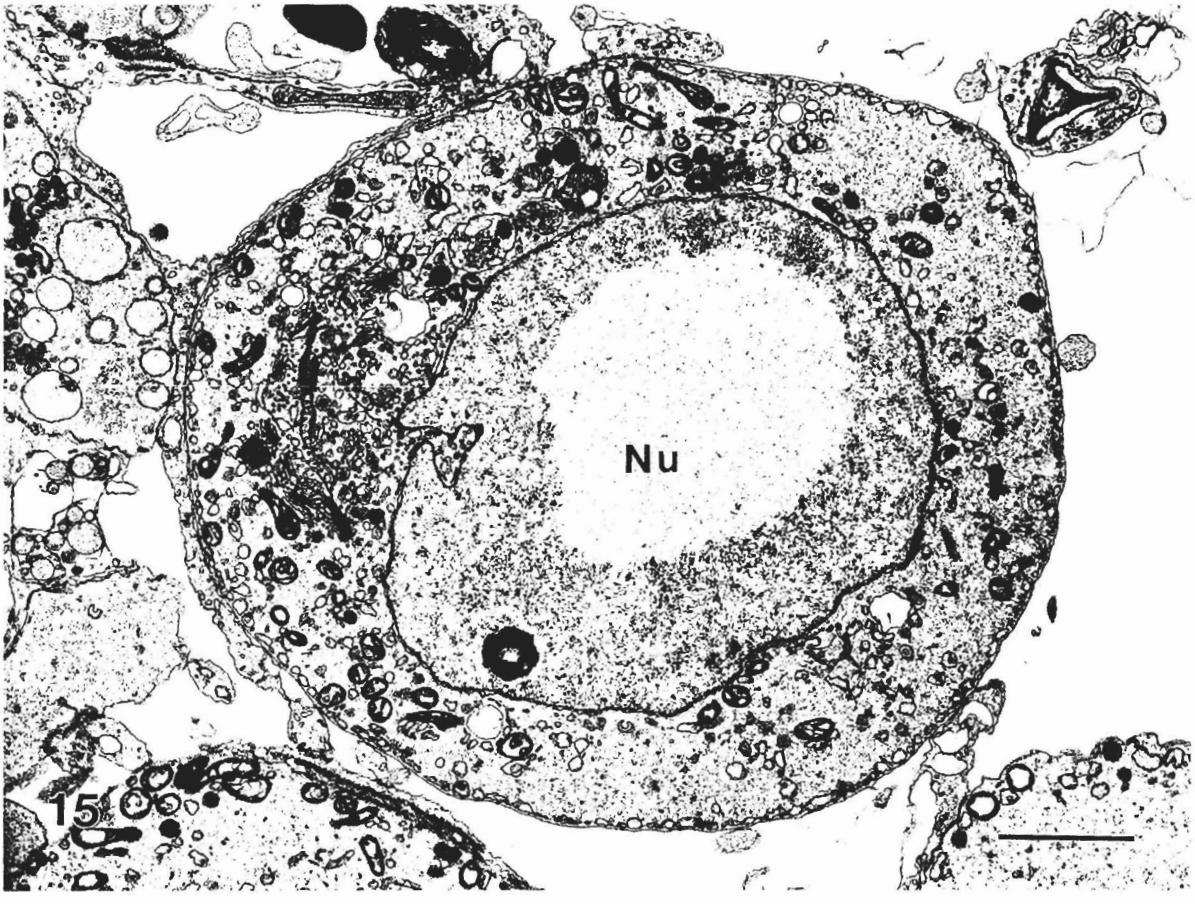
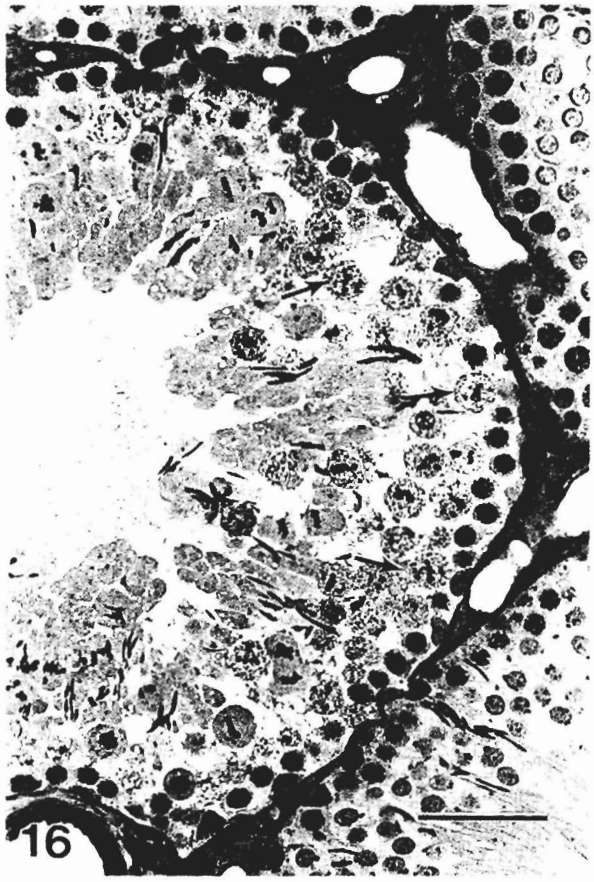


Fig. 15. Spermatid without acrosome in Stage VII at day 7.5 post treatment. Chromatin margination is noted in the nucleus (Nu). Bar: 2 μm.

where they erode basal lamina prior to penetrating between the epithelial cells (Fig. 22). Neutrophils then phagocytose luminal debris and cause luminal contents to erupt into the lamina propria. Thus, after carbendazim treatment, neutrophils exhibited specific chemotactic response toward efferent ducts containing stagnant sperm and exfoliated spermatids. The leukocytes attempted to seal off the luminal contents (Fig. 21) but



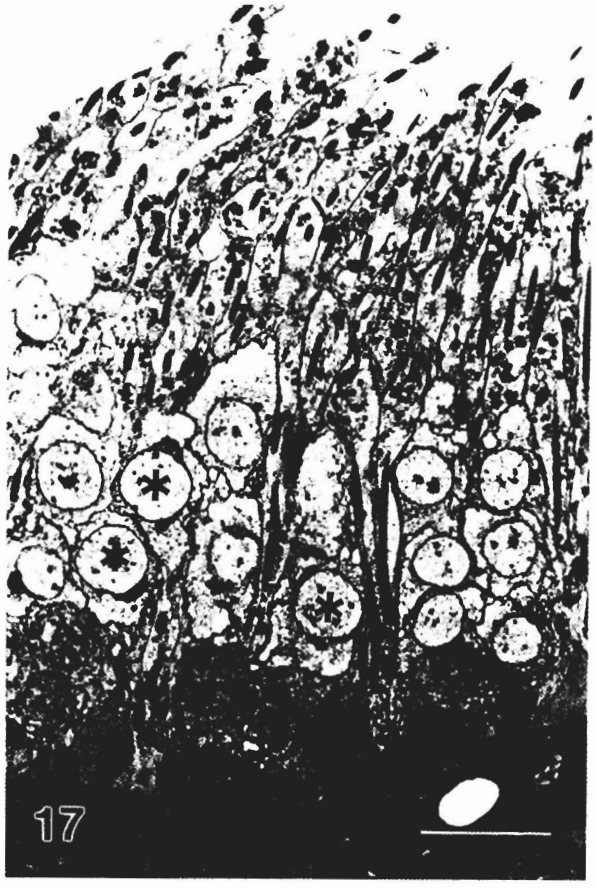


Fig. 16. Light micrograph of necrotic dividing spermatocytes (arrows) in Stage XIV at 8hr post treatment with carbendazim (100 mg/kg). PAS-hematoxylin. Bar: 50 μm.

Fig. 17. Mega round spermatids (*) in Stage V at day 4.5 post treatment with carbendazim (100 mg/kg). Mega spermatids are approximately 30% larger than the normal sized spermatids seen on the right side of the photograph. Toluidine blue. Bar: 20 μm.

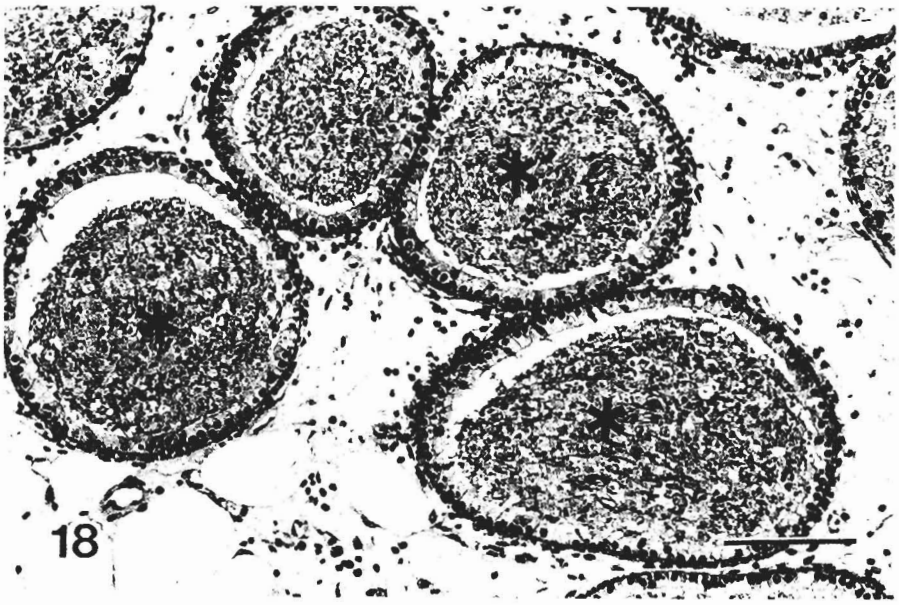


Fig. 18. Sloughed materials in the efferent ductules (*). Note the presence of neutrophils (arrrows) surrounding the efferent ductules. Hematoxylin and eosin. Bar: 100 μm.

appeared to damage the epithelium in the process (Fig. 22) which may have contributed to subsequent formation of fibrotic lesions (Fig. 23) and permanent infertility (Carter et al., 1987).

Micro-recanalization and fibrosis

The response of efferent ductal epithelium to injury induced by occlusions appears to be dependent upon the degree of inflammation caused by the trauma. An acute inflammatory reaction may be induced by the compacted luminal contents, causing the ductal lumen to dilate. It is likely that the ductal epithelium, stretched excessively by a large bolus of testicular debris, releases a chemotactic substance, possibly a cytokine of the interleukin superfamily, which then recruits massive numbers of neutrophils. Leakage of sperm antigens may draw the neutrophils toward the lumen and stimulate phagocytic activity. In other organ systems, indirect damage caused by neutrophil emigration into the interstitium and through the epithelium promotes granuloma formation and fibroblast activity (see review by Nakai et al., 1993). Thus, fibrotic lesions may be an indirect result of neutrophil damage rather than direct effects of epithelial injury (Fig. 23).

Epithelia with medium inflammatory responses often exhibited irregular epithelial growth along the edge of luminal contents and formed multiple abnormal ductules (Figs. 24). These abnormal ductules, formed by the migration of the original epithelia and growth at the

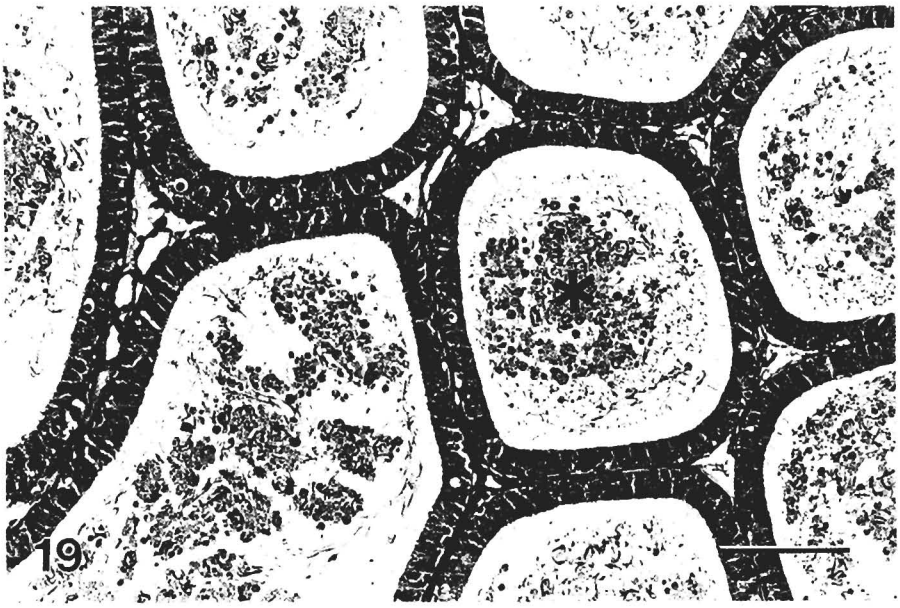


Fig. 19. The sloughed material is found in caput epididymis (*) at 24hr post treatment (carbendazim, 100 mg/kg). Hematoxylin and eosin. Bar: 100 μm.

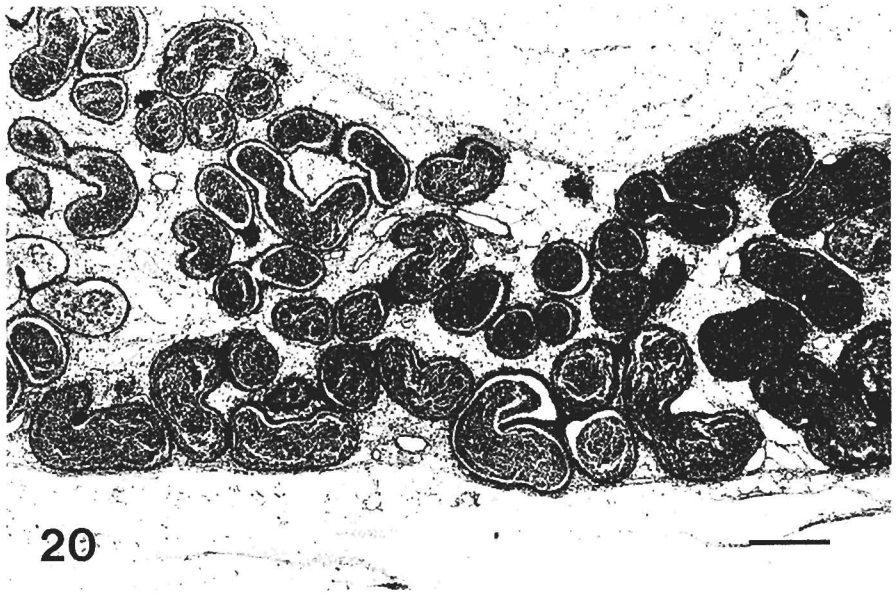


Fig. 20. Total occlusion of the efferent ductules at 24hr post treatment with carbendazim (400 mg/kg). PAS-hematoxylin. Bar: 300 μm.

periphery of the occluded lumen, indicated that recanalization was attempted by 16 days post treatment, (Nakai et al., 1993). Epithelial cells of the microcanals were similar in appearance to those of blind ending tubules (Guttroff et al., 1992). They contained few lysosomal granules and the epithelium was lower in height than normal. No evidence was found to indicate that microcanals formed patent connections between rete testis and epididymis.

Efferent ducts (ductuli efferentes) are the vital link between testis and epididymis, consisting of numerous tubules arising from rete testis chambers and coursing in a tortuous path within the epididymal fat pad before merging into a single terminus in the distal region (Guttroff et al., 1992; Ilio and Hess, 1994). The ductules are unique in the male reproductive tract, because they function similar to the proximal convoluted tubules of the kidney and reabsorb nearly 90% of the luminal fluids arriving from the testis (Jones and Clulow, 1987; Jones and Jurd, 1987; Clulow et al., 1994; Ilio and Hess, 1994). Thus, disruption of this vital function can easily upset the balance of physiological processes involved in the production of concentrated sperm in the epididymis. There is considerable literature defining the consequences of occlusion in the rete testis and efferent ducts of the testis. The result of occlusion is a rapid back up of fluid into the seminiferous tubules, swelling of the testis, and if prolonged, then subsequent degeneration of the seminiferous epithelium and atrophy of the testis (Harrison, 1953; MacMillan, 1953; Smith, 1962; Kuwahara, 1976; Jones, 1978; Anton, 1979).

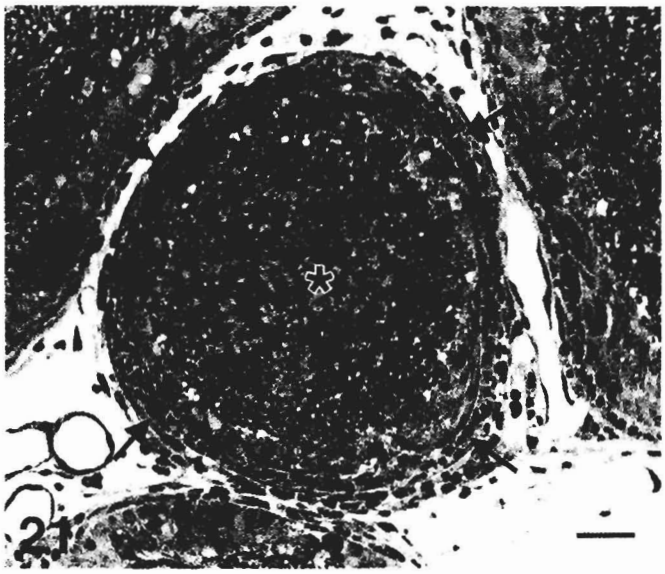


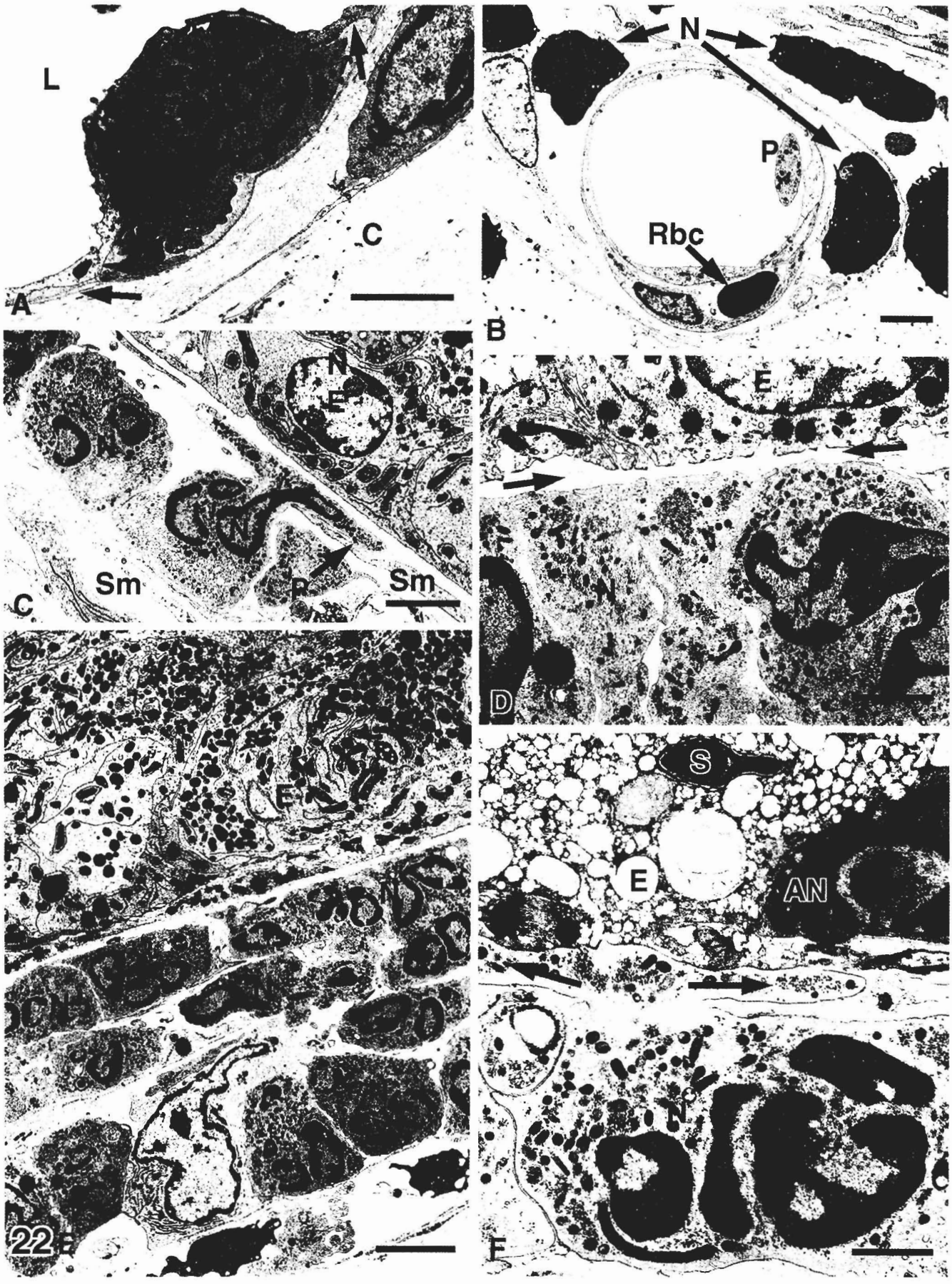
Fig. 21. A solid layer of neutrophils (arrows) surrounds an occluded ductule 24 h post treatment (carbendazim). Dehydrated luminal debris (*) is compacted in the lumen. Bar: 50 μm.

Mechanisms of toxicity and conclusions

Exposure to the benzimidazole carbamate fungicides in rodents produces two different pathological effects on the male reproductive system: 1) direct effects on the seminiferous epithelium that cause sloughing of germ cells, necrosis of dividing cells, and alterations in the formation of the nucleus of spermatids, and 2) occlusion of the efferent ductules. The first effects are primarily induced by low to moderate dosages, which are reversible. It is at the moderate to high dosages that occlusions of the efferent ductules occur. The data reviewed above suggests that the primary mechanism that leads to atrophy of the testis is the effects of these chemicals on efferent ducts of testis and not the effects observed on the seminiferous epithelium. Atrophy of the testis occurs after the efferent ducts become occluded. Efferent ducts respond to toxic insult by at least two different means: a) an increased rate of fluid reabsorption and/or decreased secretions (i.e., Cl^-); or b) a decreased rate of reabsorption and/or increased secretions. The first response leads to increased viscosity of luminal fluids, sperm stasis, ductal occlusions, granulomas and possibly fibrosis. The second response dilutes the luminal fluid, decreases sperm concentration, and leads to a decrease in sperm transit time through the epididymis.

The mechanisms by which benzimidazole chemicals disrupt fluid reabsorption are not known. However, we have limited data showing a dose dependent increase in the activity of Na^+,K^+-ATPase after treatment with carbendazim (Hess, 1998), which could result from the disruption of cytoskeletal elements (Jordan et al., 1995). The increase in activity was found to correspond to the proximal and mid-conus regions, which were also shown to be the major sites of ductal occlusions. Because Na^+,K^+ATPase is responsible for the lumen-to-interstitium movement of Na^+ with water following passively (Jones and Jurd, 1987; Clulow et al., 1994, 1996), it is probable that carbendazim-induced occlusions in the ductuli efferentes involves accelerated removal of water from the ductal lumen. However,

Fig. 22. This plate of electron micrographs shows the sequence of events that occur when the neutrophils migrate from the blood vascular system into the interstitial space surrounding the efferent ductules during the first 48 hours post treatment with carbendazim. **A.** A neutrophil (N) is migrating between the endothelial cells (arrows). L: lumen of the small venule; C: collagen. Bar: 5 μm. **B.** Small venule after neutrophils have migrated, leaving behind evidence of a slight hemmorhage. Note the platelet sticking to the endothelium (P) and the red blood cell (Rbc) outside of the lumen. Several neutrophils (N) are found in the interstitium. Bar: 5 μm. **C.** Neutrophils (N) migrate from the venules toward the efferent duct epithelium. Using pseudopodia, they migrate around the smooth muscle layer (Sm) and eventually destroy the muscle cells. E: epithelium. Bar: 5 μm. **D.** Neutrophils (N) line up beneath the basal lamina (arrows) of the epithelium (E). Bar: 2 μm. **E.** In some regions, the neutrophils (N) form 3-5 layers thick beneath the epithelium of the efferent ducts. Bar: 5 μm. **F.** Finally, the neutrophils (N) destroy the basal lamina and send pseudopodia (arrows) into the already damaged epithelium (E). S: sperm cell; An: apoptotic nucleus. Bar: 5 μm.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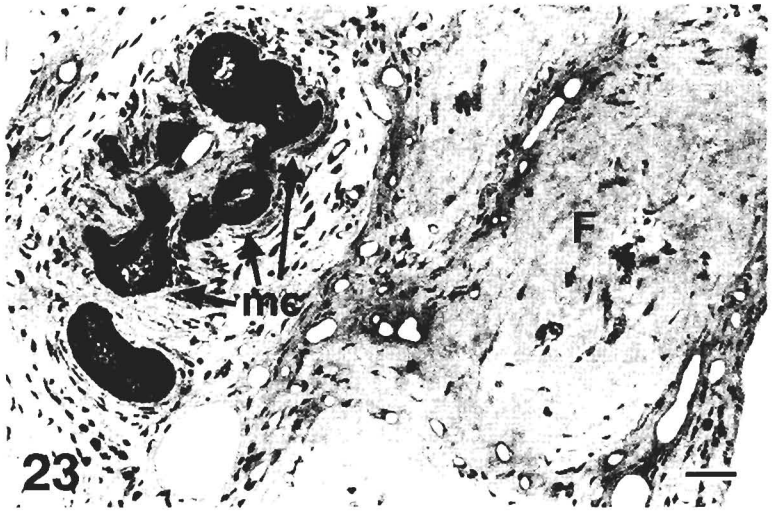

Fig. 23. Fibrotic lesion of the efferent ductules. The original lumen is replaced with the dense connective tissue (F), and surrounded by multiple microcanals (mc). Hematoxilin-eosin. Bar: 20 μm.

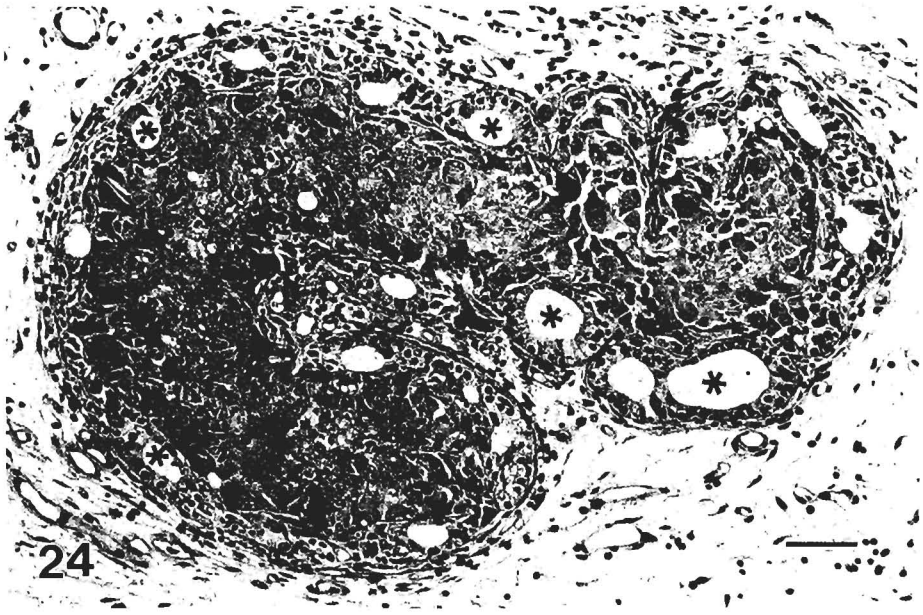

Fig. 24. Formation of microcanals (*) around the occluded efferent ductules at 16 days post treatment with carbendazim (400 mg/kg). Hematoxylin and eosin. Bar: 20 μm.

effects on other pathways, such as Cl^- secretion, should also be considered (Morris et al., 1998). It is possible that the disruption of microtubules prevents the recycling of membrane ion channels and thus causes an over resorption of sodium or a decrease in Cl^- secretion. In either case there would likely be an increased rate of reabsorption of lumina fluids, which would lead to compaction of luminal contents and subsequent occlusion of the ductules.

In conclusion, regardless of mechanism, once the efferent ductules become blocked, long term results are the same, as testicular atrophy and infertility are produced. Accordingly, long term testicular atrophy after subchronic and acute multiple exposures to any toxicant could be explained by potential efferent ductal dysfunction, a hypothesis synthesized from the results of studying the toxicity of benomyl and its metabolite carbendazim. Thus, the examination of efferent ductal histopathology should become routine procedure whenever it is suspected that a toxicant causes long term testicular atrophy.

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