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Masaaki Nakai - Kiyotaka Toshimori
Kazuya Yoshinaga - Tetsuo Nasu - Rex A. Hess

Carbendazim-induced abnormal development of the acrosome

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Abstract Effects of a single, high dose of orally administered carbendazim (100 mg/kg) on acrosome formation in the early phases of spermiogenesis were examined by electron microscopy and immunocytochemistry up to day 7.5 post-treatment. No obvious abnormality of acrosome development was noted in the Golgi phase spermatids on day 1.5 post-treatment. On day 3, step I spermatids were seen in stage III seminiferous tubules. In stage V tubules at this post-treatment interval, direct connections between the trans-side saccules of the Golgi stacks and the outer acrosomic membranes were observed in step 5 spermatids. Similar direct connections between these two organelles were also observed in the advanced round spermatids in later stages at days 4.5 and 7.5. On day 4.5, step I and 3 spermatids were seen in stage V tubules. On day 7.5, round spermatids with various abnormalities of acrosome development were observed in stage VII tubules, in addition to the discontinuous and granular acrosomes reported previously. These features were not observed in testes of control animals. In the imnumocytochemical analysis using an antibody mMN7 that recognizes a protein delivered from the Golgi apparatus to the acrosome, spermatids exposed to carbendazim showed various abnormal immunostaining patterns in the acrosomes. On the other hand, strong immunoreactivity was observed in the Golgi saccules connecting to the acrosomes. These results suggest that in testis treated with carbendazim acrosome development is impaired during the early phases of spermiogenesis, and material supply from the Golgi apparatus to the acrosome is perturbed, which is a possible cause of the abnormal development.

Key words Acrosome - Carbendazim mMN7 immunocytochemistry - Spermatids - Rat (Sprague Dawley)

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M. Nakai (r-1) - T. Nasu
Department of Veterinary Anatomy, Faculty of Agriculture, Miyazaki University, Miyazaki 889-2192, Japan
Tel.: +81 985 58 2811: Fax: +81 985 58 2884:
e-mail: aOdlOlu@cc.miyazaki-u.acjp
K. Toshimori - K. Yoshinaga
Department of Anatomy, Miyazaki Medical College, Kiyotake, Miyazaki 889-1692, Japan
R. A. Hess
Department of Veterinary Biosciences, University of Illinois, Urbana, IL 61801, USA

Introduction

There are growing concerns over the adverse effects of environmental chemicals on male reproductive function in many countries (Toppari et al. 1996). Benomyl, one these, is widely used as a fungicide on food crops and ornamental plants, and its metabolite, carbendazim, is known to exert benomyl's antifungal effect by binding to tubulin (Davidse and Flach 1977). However, benomyl and carbendazim are also known to be toxic to mammalian male reproductive organs (World Health Organization 1993). Testicular damage by these agents includes inhibition of testicular microtubule assembly (Lim and Miller 1997), sloughing of immature spermatids (Parvinen and Kormano 1974; Hess et al. 1991, Nakai and Hess 1994, Lim and Miller 1997), and seminiferous tubular atrophy (Carter and Laskey 1982; Carter et al. 1987; Hess et al. 1991; Nakai et al. 1992). In the most severely affected cases the animals show total testicular atrophy and infertility (Carter et al. 1987).

In addition to these adverse effects, we recently reported that carbendazim induced various morphological abnormalities in round and elogating spermatids including abnormally formed acrosomes at day 7.5 and longer posttreatment intervals even at the dose that did not cause testicular atrophy (Nakai and Hess 1997; Nakai et al. 1997). Backtracking in time indicated that suspected targets of carbendazim for acrosome abnormalities were germ cells somewhere between late spermatocytes (diplotene spermatocytes in stage XIII) and early round spermatids (step I spermatids in stage I) at the time of administration

(Nakai et al. 1997). Therefore, it is possible that carbendazim inhibits acrosome development in the early phases of spermiogenesis.

Acrosomal contents originating from the endoplasmic reticulurn and the Golgi apparatus are delivered via different pathways during spermiogenesis. Each pathway has been well characterized by routine light and electron microscopy (Leblond and Clermont 1952; Susi et al. 1971; Hess 1990), autoradiography (Clermont and Tang 1985), enzyme cytochemistry (Thorne-Tjomsland et al. 1988), and immunocytochemistry using different monoclonal antibodies (Tanii et al. 1992, 1994; Toshimori et al. 1992). Among these, a monoclonal antibody mMN7 specifically recognizes a 90-kDa protein (MN7) that is transported from the Golgi apparatus to the developing acrosome via Golgi-derived vesicles. Its immunoreactivity is first detectable as early as step 1-2 spermatids (Tanii et al. 1994). Therefore, MN7 seems to be a useful probe to examine the material transportation from the Golgi apparatus to the acrosome. The present study was conducted to test our hypothesis that carbendazim induces abnormalities of acrosome development in early spermiogenesis using electron microscopy and immunocytochemistry after a single oral dose of carbendazim.

Materials and methods

Male Sprague-Dawlev rats, 70-80 days old, were used in the present study. They were housed two or three per cage. and allowed free access to diet and water throughout the experiment. Carbendazim suspended in corn oil was administered by oral gavage at a dosage of 100 mg/kg. Post-treatment intervals selected in the present study were 1.5. 3.0, 4.5 and 7.5 days. Four or five animals were assigned to each interval. Animals given corn oil vehicle alone were used as controls. and their testes were collected al day 7.5 postadministration.

Routine electron microscopy

The testes were fixed with 4% glutaraldehyde in 0.1 M cacodylate buffer for 20-25 min using a vascular perfusion technique (Hess and Moore 1993) at the post-treatment intervals mentioned above. Tissue blocks were post-fixed in I% osmium tetroxide in 0. 1 M cacodylate buffer containing 1.0%, potassium ferrocyanide for 1.5 h (Russell and Burguet 1977), and embedded in Quetol 812. Thick sections were stained with toluidine blue for light microscopy. Ultrathin sections were stained with uranyl acetate and lead citrate, and observed by a Hitachi H-800MU electron microscope.

Immunocytochemistry

incubated without mMN7. Horseradish peroxidase-conjugated goat Fab' anti-mouse IgG (Protos Immunoresearch, San Francisco, Calif., USA) diluted at 1: 100 was used as the secondary antibody. The sections were fixed in 0.5% glutaraldehyde in 0. 1 M PBS for 5-10 ruin and reacted with diaminobenzidine and H_2O_2 in 0.05 M TRIS-HCI, followed by a post-fixation in 2% OsO_4 for I It. The sections were embedded in Epon 812. Ultrathin sections were observed without staining or following a brief staining with lead citrate.

Results

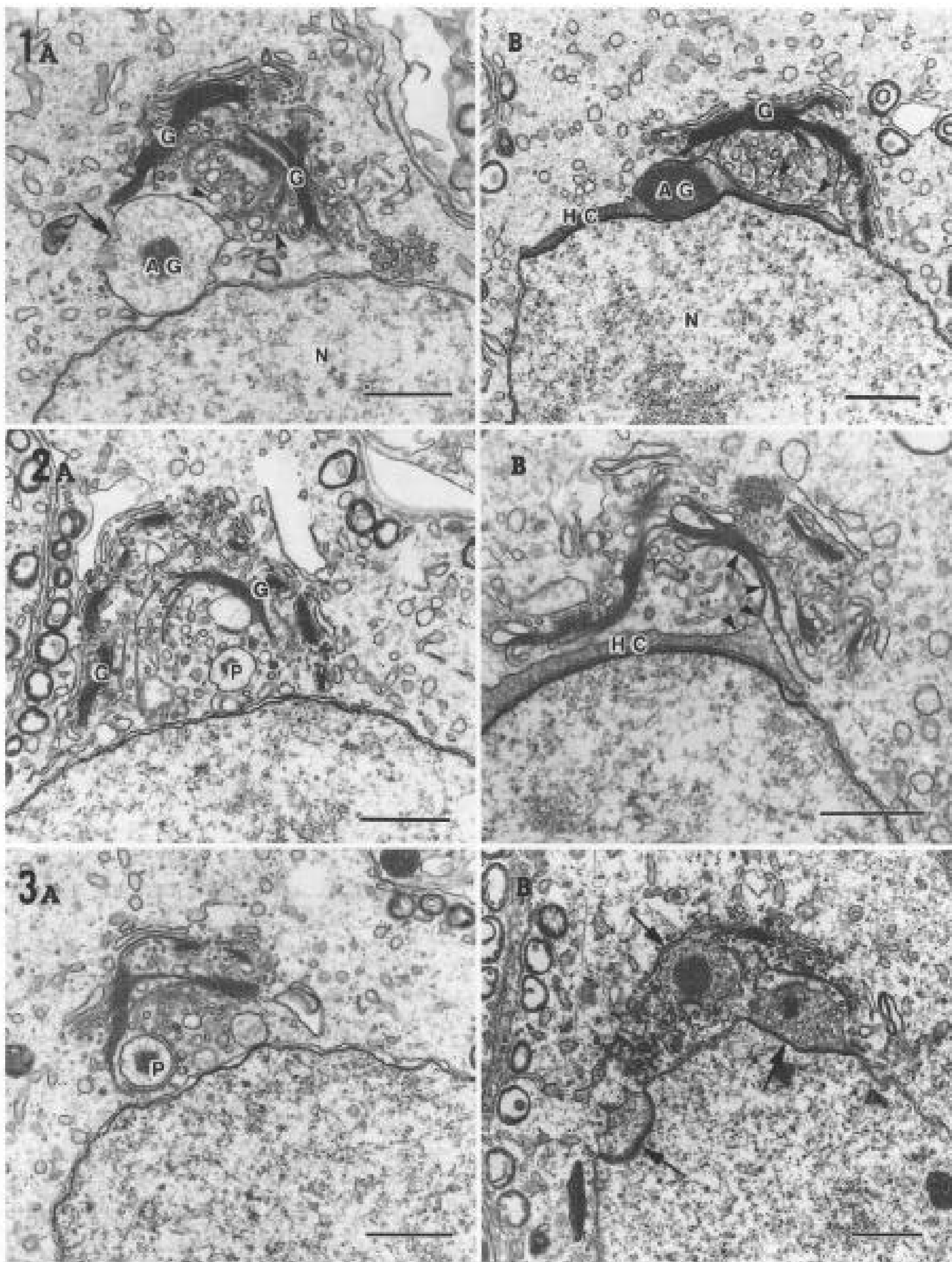
Routine electron microscopy

In testes of control animals (Fig. 1), the acrosome abnormalities described below were not observed except for a rare incidence of vacuoles in the acrosome. Abnormalities of acrosome development of spermatids were first noted in stage III and V tubules at day 3 post-treatment. In stage 111, step I spermatids, identified by the presence of proacrosomic vesicles containing granules and no obvious acrosomic vesicles, were observed among step 3 spermatids (Fig. 2A). Another abnormality in this stage was an indentation of acrosomic vesicles into the nuclei of step 3 spermatids, but this was also seen in later stages at longer post-treatment intervals as shown in Figs. 313 and 4A. In stage V, direct connections between the trans-side saccules of the Golgi stacks and the outer acrosomal membrane occurred in step 5 spermatids (Fig. 2B). Acrosome development of these spermatids appeared to be normal.

On day 4.5, acrosome abnormalities of spermatids were seen in stage IV-V and VII tubules. In stage IV, spermatids with less-developed acrosomes (approximately steps I and 3) were observed (Fig. 3A). Spermatids with multiple acrosomic vesicles also occurred in this stage (Fig. 3B). Again, direct connections between the trans-side saccules of the Golgi stacks and the outer acrosomal membrane were seen in step 5 and 7 spermatids in stages V and VII, respectively. Vacuoles of various sizes were observed in the head caps of step 5 and 7 spermatids (not shown).

Fig. I Conventional electron micrographs of normal step 2-3 (A) and step 5 (B) spermatids (AG acrosomic granule, G Golgi stacks, N nucleus, HC head cap, *arrow* acrosomic vesicle, *arrowheads* Golgiderived vesicles). *Bars I* μm

Fig. 2A, B Conventional electron micrographs of spermatids at day 3 post-treatment. A Spermatid with less-developed acrosome in stage 111. This spermatid has well-developed Golgi stacks (G) and proacrosmic granules (P), but does not form an acrosomic vesicle on the nuclear surface as shown in Fig. IA. B Spermatid wit" *a* direct connection between the trans-side Golgi saccule and the outer acrosomic membrane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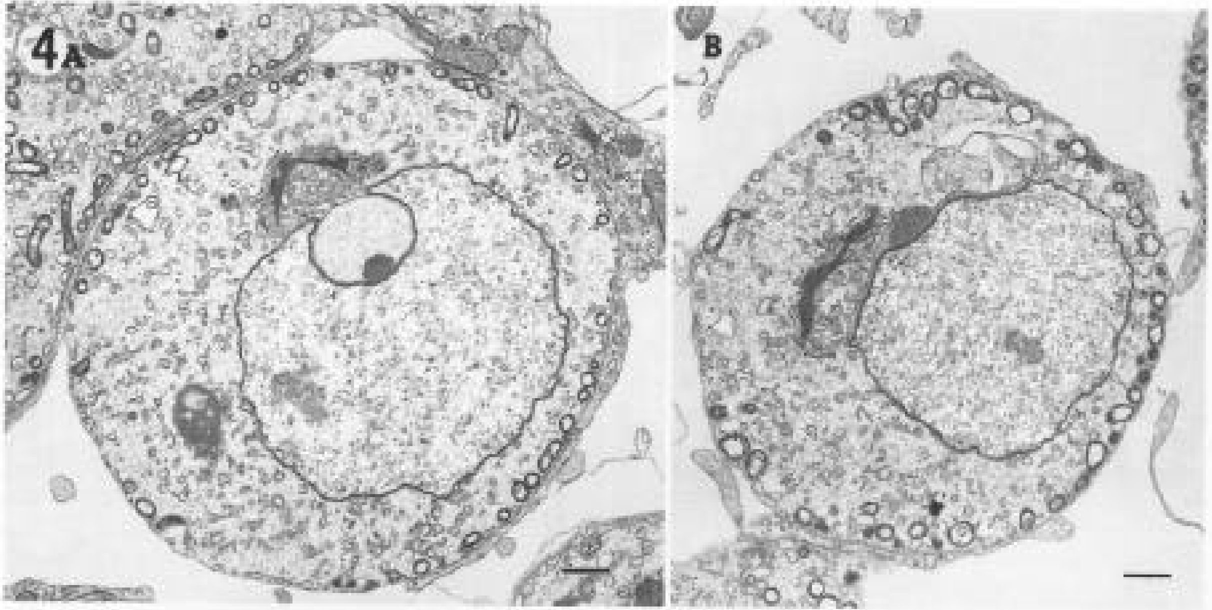


Fig. 4A, B Conventional electron micrographs of abnormal spermatids in stage VII at day 7.5 post-treatment. A The acrosome of this spermatid has developed only up to a level of approximately step 3, and deeply indents into the nucleus. B

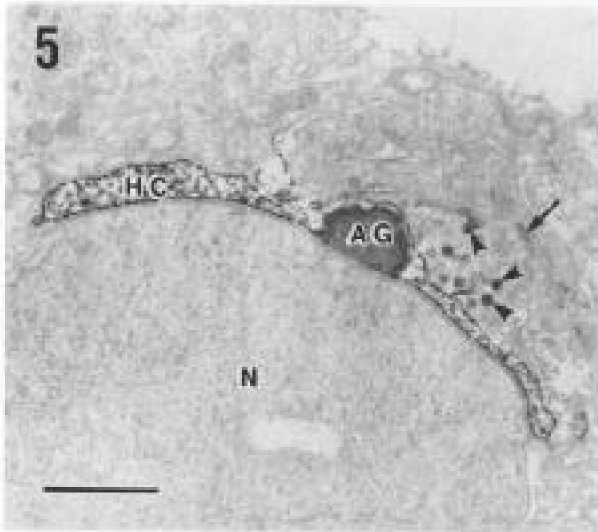


Fig.5 Immunocytochemistry with monoclonal antibody mMN7 showing mMN7 immunoreactivity in a normal step 5 spermatid. The reaction product is seen in the Golgi saccule *(arrow),* Golgi-derived vesicles *(arrowheads),* acrosomic granule (AG), and head cap region of the acrosome WC) *(N* nucleus). Unstained section. *Bal* pin

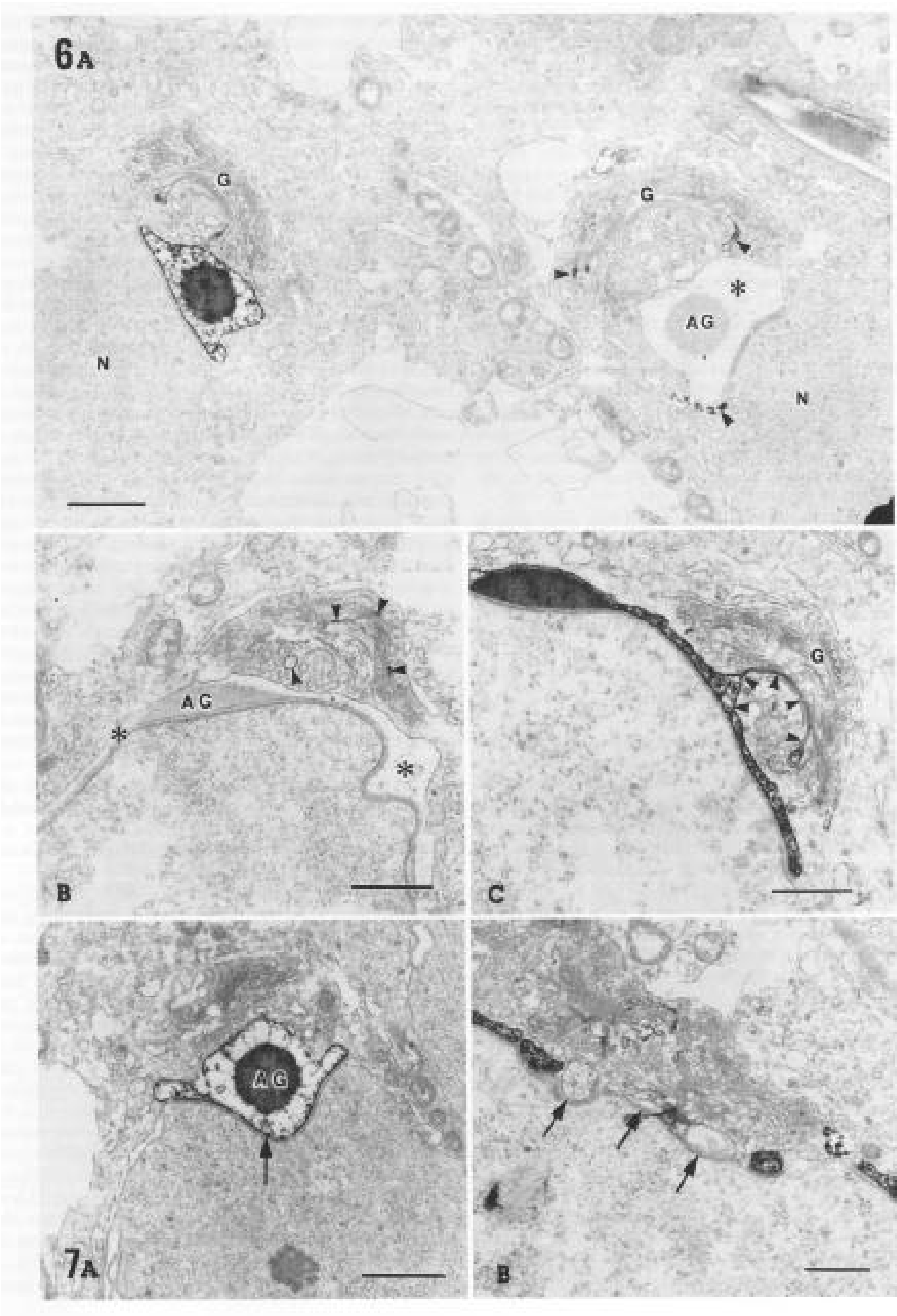
On day 7.5, acrosome abnormalities were observed in spermatids in stage VII tubules. In addition to the round spermatids with discontinuous acrosomes and multiple velopment of acrosomes were observed in the present study. These included spermatids that did not form acrosomes and spermatids in approximately steps 3-5 of development (Fig. 4). Nuclei *of* some spermatids with abnormally formed acrosomes showed chromatin margination. Furthermore, direct connections between the Golgi saccules and the head caps similar to those seen at shorter intervals occurred in

Immunoelectron microscopy

In testes of control animals, MN7 was immunolocalized in growing acrosomes of spermatids during Golgi and

Fig.6A-C Immunocytochemistry with monoclonal antibody mMN7 showing mMN7 immunoreacti v i ties in spermatids showing normal development of the acrosomes after treatment with carbendazim. Sections stained with lead citrate (G Golgi apparatus, *N* nucleus). A Step 3 spermatid on the left *half of* this figure shows a normal distribution *of* mMN7 immunoreaction. However, a step 3 spermatid on the *right* lacks the reaction product in the acrosomic vesicle *(asterisk)* and granule (AG), despite the presence of a positive reaction in the neighboring Golgi saccules and vesicles *(arrowheads).* Stage III at day 3. B Step 7 spermatid with little immunoreaction in the head cap *(asterisks)* and acrosomic granule (AG). Note the presence of weak immunoreaction in the neighboring Golgi saccules and vesicles *(arrowheads).* Stage VII at day 7.5. C Heavy reaction product is seen in the trans-side Golgi saccule connecting to the outer acrosomal membrane *(arrowheads).* Step 7 spermatid in stage VII at day 7.5. *Bars I µm*

Fig. 7A, B Immunocytochemistry with monoclonal antibody mMN7 showing mMN7 immunoreactivities in spermatids showing' abnormal development of the acrosomes. Sections stained with lead citrate. A This spermatid seen in stage V at day 4.5 has a less-developed acrosome (approximately two steps


6A
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cap phases of spermiogenesis (Fig. 5). In addition, one or more saccules of the Golgi stacks and neighboring Golgiderived vesicles often showed weak or medium intensity of mMN7 immunoreaction. No immunoreaction was observed in the control sections that had been incubated without the primary antibody.

In testes treated with carbendazim, the mMN7 immunostaining pattern in most spermatids that showed normal morphology of the acrosomes was the same as that of the control. However, step 3 spermatids at day 3 occasionally lacked the reaction products in the acrosomic vesicles, although immunoreaction was detected in the neighboring Golgi saccules and the Golgi-derived vesicles of the same cells (Fig. 6A). Likewise, it was observed on day 7.5 that step 7 spermatids occasionally lacked the reaction products in the head caps, despite the presence of an immunoreaction in the Golgi saccules and vesicles (Fig. 6B). The trans-side saccules of the Golgi stacks connecting directly to the outer acrosomal membrane of the head cap region contained heavy reaction products of mMN7 immunostaining (Fig. 6C). No reaction product was seen in the vacuoles formed in the head caps of any spermatids observed.

Acrosomes that were less developed than those seen in comparable stages of the control usually showed an mMN7 immunoreaction in the Golgi saccules, Golgi-derived vesicles and acrosomes (Fig. 7A). On the other hand, reaction products were not present in all fragments of discontinuous and multiple granular acrosomes as shown in Fig. 7B.

Discussion

Spermatogenesis of the rat testis has been classified into 14 stages based upon the germ cell associations within the seminiferous tubules, and cohorts of spermatids in each stage show a synchronized development of acrosome (Leblond and Clermont 1952; Hess 1991). However, we recently reported that step 7-10 spermatids coexisted in stage X-XI tubules at day 10.5 post-treatment with carbendazim, indicating that exposure of testis to this agent results in abnormal development of acrosome during spermiogenesis (Nakai and Hess 1997). The present study has further demonstrated that asynchronization of acrosome development in the carbendazim-treated testis occurs in the earlier phases of spermiogenesis. Spermatids with less-developed acrosomes than the controls were first noted in stage III at day 3.0 post-treatment, and those with various acrosome abnormalities were observed at the following post-treatment intervals, including spermatids having no acrosomes spreading over the nuclei in stage VII at day 7.5. These findings indicate that, in testis treated with carbendazim, spermatids arrest their acrosome development at a level of approximately step I or show various impairments of acrosome formation in later stages. Abnormal acrosomes observed in this study are not specialized effects appearing in damaged spermatids, as they have been reported in animals under different experimental conditions including chemical exposure (Russell et al. 1983a,b; Hodel et al. 1984; Tanii et al. 1998), ligation of the efferent ductules (Singh and Abe 1987), and mutations (Bryan 1977; Sotomayor and Handel 1986; Fouquet et al. 1992; Russell et al. 1994; Lalouette et al. 1996). The underlying mechanisms of these abnormalities are not known. However, various abnormal forms and MN7 distribution patterns would provide clues for further understanding of the acrosome abnormalities. For example, in acrosomes showing discontinuous and granular appearance, MN7-positive and negative fragments were seen in the same cells. It is possible in these cells that proacrosomic vesicles failed to fuse each other to form a single acrosomic vesicle, which resulted in an uneven distribution of MN7. On the other hand, spermatids occasionally lacked the reaction products of mMN7 immunostaining in the normally formed acrosomes, despite the presence of reaction products in the neighboring Golgi saccules and vesicles. This suggests that acrosomic contents including MN7 were synthesized and accumulated in the Golgi apparatus, but were prohibited from reaching the acrosomes, and, therefore, part of the normal acrosomal contents was more or less missing even though they appeared normal in morphology. The presence of MN7 in less-developed acrosomes (Fig. 7) could be accounted for in a similar manner; that is, it is possible in these cells that MN7 was normally transported to the acrosomes, but other acrosomal components including those that were essential for normal acrosome development were not delivered. Alternatively, every component was delivered, but it might be insufficient for acrosome formation. From these it can be speculated, at least in carbendazim-treated testis, that the degrees of acrosome abnormalities depend on how severely the material supply from the Golgi apparatus to acrosome is perturbed, al though it remains to be determined if this is true in abnormal acrosomes by other causes.

It is generally accepted that cytoplasmic vesicles are carried to their final destinations within cells along the tracks of microtubules (Lodish et al. 1995). However, as far as our knowledge goes, it has not been determined in spermatids whether the Golgi-acrosomic granule tract (see below) is a microtubule-dependent process. On the other hand, the bulk of orally administered carbendazim is rapidly excreted in urine and feces in the first 24 h (Gardiner et al. 1974), and the testicular level of carbendazim shows a rapid decline even after an intratesticular injection (Lim and Miller 1997). Assuming that the Golgi-acrosomic granule tract is a microtubule-dependent process, and that carbendazim temporally inhibits this process, the duration of carbendazim-induced impairment of acrosome development would be approximately 24 h or less. However, this duration is much shorter than that observed in the present study. For example, the duration of the

that temporal insults to the Golgi acrosomic granule tract during early spermiogenesis could result in a prolonged damage of acrosome formation. It is also possible that Golgi-derived vesicles are carried to the acrosome independent of microtubules, and that carbendazim causes damage of acrosome formation that becomes obvious as stages progress by directly and/or indirectly affecting another target beside microtubules.

In spermatids of Golgi and cap phases of spermiogenesis, Golgi-derived materials are mainly transported to the developing acrosome via Golgi-derived vesicles, and this pathway is called "Golgi-acrosomic granule tract" (Toshimori et al. 1992; Tanii et al. 1994; Toshimori 1998). In addition, direct connections between the Golgi saccules and acrosomes were reported in mouse spermatids (Sandoz 1970), although they were not seen in our control samples. This suggests that in normal spermatids part of the Golgi-derived materials may reach directly the developing acrosomes through the connections. In the carbendazim-treated testis, similar connections between these two organelles were observed, and strong mMN7 immunoreactivity was demonstrated in the Golgi saccules connecting to the acrosomes. Therefore, material transportation through the direct connections does not seem to be disturbed by carbendazim. This is supported by the fact that spermatids having direct connections between the Golgi saccules and acrosomes did not show marked abnormalities of acrosome.

In conclusion, the present observations suggest that a single, high dose of orally administered carbendazim results in a perturbation of the Golgi-acrosomic granule tract in early spermiogenesis, which causes prohibition of material

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