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Stage-specific effects of the fungicide carbendazim on Sertoli cell microtubules in rat testis

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Abstract. The aim of the present study is to provide a morphological explanation of carbendazim (CBZ)-induced sloughing of germ cells occurs in a stage-specific manner. Therefore, very early alterations in the seminiferous tubule epithelium were examined histologically in the rat testis after oral administration of CBZ (400 mg/kg). Gaps between the elongated and round spermatids, the first indication of germ cell sloughing (pre-sloughing), were observed in stage late VI–early VII seminiferous tubules at 90-min post-treatment. Tubulin immunoreaction in the Sertoli cells was reduced in intensity in tubules with pre-sloughing. However, electron microscopy demonstrated that there were some intact microtubules in these cells. At 120 min, sloughing was seen in stage late VI–early VII and XIII–XIV. Tubulin immunoreaction in the Sertoli cells was greatly decreased in intensity in tubules where cell sloughing was observed. Electron microscopy showed that there were few microtubules in the body region of these cells. Stages II–V and mid-VII–VIII were exempt from the sloughing effect at 180 min. These changes in microtubules were not observed in Sertoli cells that did not exhibit sloughing characteristics, regardless of the post-treatment intervals. The present results suggest that stage specificity of sloughing is due to the stage-specific susceptibility of Sertoli cell microtubules to CBZ. © 2002 Published by Elsevier Science Ltd.

Keywords: carbendazim, immunohistochemistry, microtubule, rat, Sertoli cell, vimentin

Introduction

Carbendazim (CBZ) is a metabolite of the benzimidazole fungicide, benomyl, and is responsible for BNL's fungicidal action by binding tubulin, thus disrupting microtubules and inhibiting mitosis (Davidse & Flach, 1977; Burland & Gull, 1984). Benomyl is well-recognized as being toxic to the male reproductive system (Hess et al., 1991), and CBZ mediates

the action of benomyl (Lim & Miller, 1997). Various alterations have been reported in the male reproductive organs after CBZ exposure (World Health Organization, 1993; Hess & Nakai, 2000). These include stage-specific sloughing of the spermatids (Parvinen & Korman, 1974; Nakai & Hess, 1994), occlusion of the efferent ductules (Nakai et al., 1992), testicular atrophy (Gotoh et al., 1999; Hess & Nakai, 2000), abnormal spermiogenesis (Nakai & Hess, 1997), chromosomal aberrations (Matsuo et al., 1999; de Stoppelaar et al., 1999) and infertility (Carter et al., 1987). Although these data have contributed to our understanding of CBZ's effects on the male reproductive system, the initial events by CBZ exposure are not thoroughly explained.

Microtubules are a prominent structure in normal Sertoli cells (Vogl, 1988), and are responsible for several functions including maintenance of cell shape and the positioning of germ cells within the seminiferous epithelium (Russell, 1997; Vogl et al., 1991, 1993). These functions were studied

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Received 20 August 2001
 Accepted 9 January 2002

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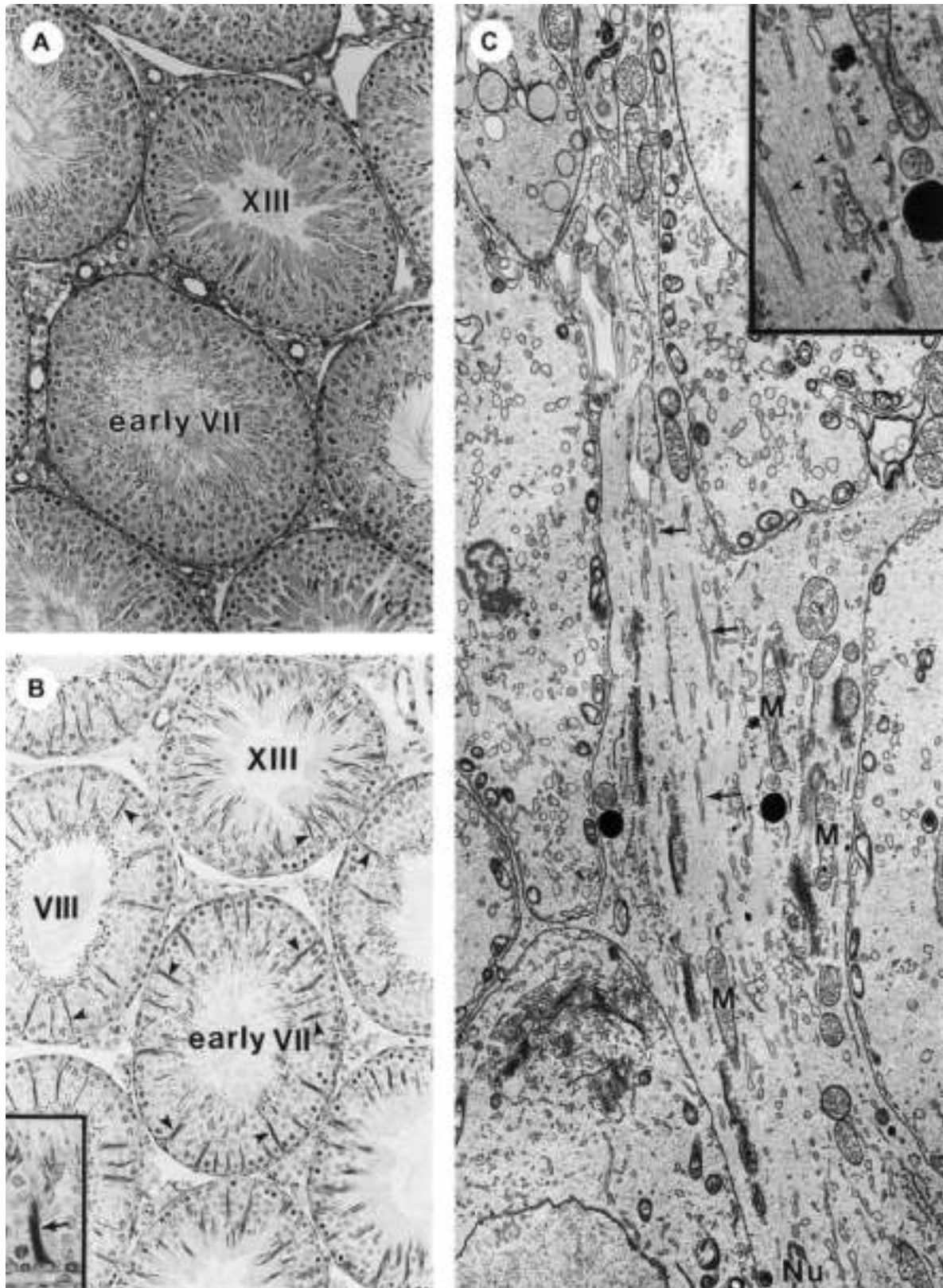


Fig. 1 Light and electron microscopic images of the control testis. Roman numerals represent stages of spermatogenesis. (A) Elongated spermatids attach to the luminal border of the seminiferous epithelium. Periodic acid–Schiff and hematoxylin. $\times 165$. (B) Immunostaining for tyrosinated α -tubulin. Sertoli cells show thick bundles of dense reaction products that extend from the perinuclear region to the luminal border of the seminiferous epithelium (arrowheads). $\times 165$. Inset: A high magnification of stage early VII showing a fine filamentous staining in the Sertoli cell (arrow). $\times 330$. (C) Sertoli cell in stage early VII. Mitochondria (M) and endoplasmic reticulum (arrows) are aligned parallel to the long axis of the cell. Nu: Sertoli cell nucleus. $\times 6000$. Inset: Numerous microtubules running in the body region of the cell (arrowheads). $\times 20\,000$.

using microtubule-disrupting agents such as colchicine and vinblastin and it was shown that Sertoli cells with depleted microtubules were deformed with sloughing of germ cells (Russell et al., 1981; Vogl et al., 1983). Similarly, it was demonstrated that Sertoli cells with complete sloughing by CBZ were associated with disruption of microtubules and cellular deformation (Nakai & Hess, 1994; Nakai et al., 1995). Therefore, we proposed previously a mechanism of CBZ-induced sloughing, in which Sertoli cells with disrupted microtubules fail to maintain their normal shape, retract their cytoplasm basally, and induce breakage of the cells at the apical cytoplasm (Nakai & Hess, 1994). This physical mechanism explains the CBZ-induced sloughing, but does not account for stage specificity of sloughing. In the present study, Sertoli cells were examined by immunohistochemistry for tyrosinated α -tubulin (Hermo et al., 1991; Wenz & Hess, 1998) and by electron microscopy during the initial 180-min post-exposure to CBZ.

Materials and methods

Animals and treatment

Male Sprague–Dawley rats, 10–13 weeks of age, were used in this study. They were given a single oral dose of CBZ suspended in corn oil (400 mg/kg), and deeply anesthetized with sodium pentobarbital 45 min ($n = 2$), 60 min ($n = 6$), 90 min ($n = 4$), 120 min ($n = 8$), 150 min ($n = 2$) and 180 min ($n = 3$) later. Control animals ($n = 4$) were given corn oil alone and killed after 180 min. While under deep anesthesia, the testes were fixed either with Bouin's solution for routine light microscopy and immunocytochemistry or with 4% glutaraldehyde in 0.1 M cacodylate buffer for electron microscopy using a vascular perfusion technique (Hess & Moore, 1993).

Light microscopy and immunohistochemistry

Tissue blocks fixed with Bouin's solution and those with glutaraldehyde were processed for paraffin and JB-4 plastic resin, respectively. Sections were stained with periodic acid–Schiff reaction and hematoxylin (PAS-H) for routine light microscopy. A total of 100 seminiferous tubules were randomly selected in individual testes and their stages were determined. They were examined for the presence of alter-

ations, and frequencies of sloughing of the elongated spermatids were obtained for individual stages.

Sections for tubulin immunostaining were treated in 0.3% hydrogen peroxide in methanol for 15 min to block the endogenous peroxidase. After incubation in 10% normal goat serum to prevent non-specific binding of the antibody, sections were incubated with a monoclonal antibody against tyrosine α -tubulin (clone TUB-1A2, Sigma, St Louis, MO, USA) diluted at 1:2000 for 2 h to overnight. Control sections were incubated without the primary antibody. Biotinylated goat anti-mouse immunoglobulin antibody was used as the secondary antibody at a dilution of 1:100 (DAKO, Denmark). The sections were then incubated in avidin biotin complex (ABC-kit, Vector Laboratories, California, USA). Positive reactions were visualized with DAB and H_2O_2 . Nuclei were lightly stained with hematoxylin.

Electron microscopy

Tissue blocks from 90- and 120-min exposure groups were processed for electron microscopy. They were rinsed in 0.1 M cacodylate buffer overnight and post-fixed in 1% OsO_4 in 0.1 M cacodylate buffer for 2 h. Potassium ferrocyanide (1% in final concentration) was added to OsO_4 for the last 30 min of post-fixation. The blocks were embedded in EMBED (Polysciences, Philadelphia, USA). Thick sections for light microscopy were stained with toluidine blue. Ultrathin sections were stained with uranyl acetate and lead citrate.

Results

Control

Few abnormalities were observed in the control testis (Fig. 1A). Immunohistochemical findings of tyrosinated α -tubulin of the Sertoli cell were the same as those previously reported from our laboratory (Wenz & Hess, 1998). Briefly, tubulin immunoreactions appeared as thick bundles of fine filaments extending from the supranuclear region to the luminal border of the cells in all stages (Fig. 1B). No immunoreaction was observed in sections incubated without the primary antibody.

Electron microscopy of the Sertoli cells revealed that there were numerous microtubules in the body region (cylindrical region between nucleus and luminal border) and further into the apical processes that hold the elongated spermatids

Table 1 Frequency of sloughing of elongated spermatids after carbendazim exposure (mean percent)^a

Stage	Control	45 min	60 min	90 min	120 min	150 min	180 min
I	0	0	0	0	0	0	19.1
II–early VI	0	0	0	0	0	0	0
Late VI–early VII	0	0	0	8.5 ^b	22.1	28.1	47.8
Mid-VII–VIII	0	0	0	0	0	0	0
IX–XI	0	0	0	0	0	0	7.3
XII–XIV	0	0	0	0	1.2 ^b	7.3	52.8

^a The numbers of seminiferous tubules examined are 400–900 per interval.

^b The frequency includes what appears to be pre-sloughing of the elongated spermatids.

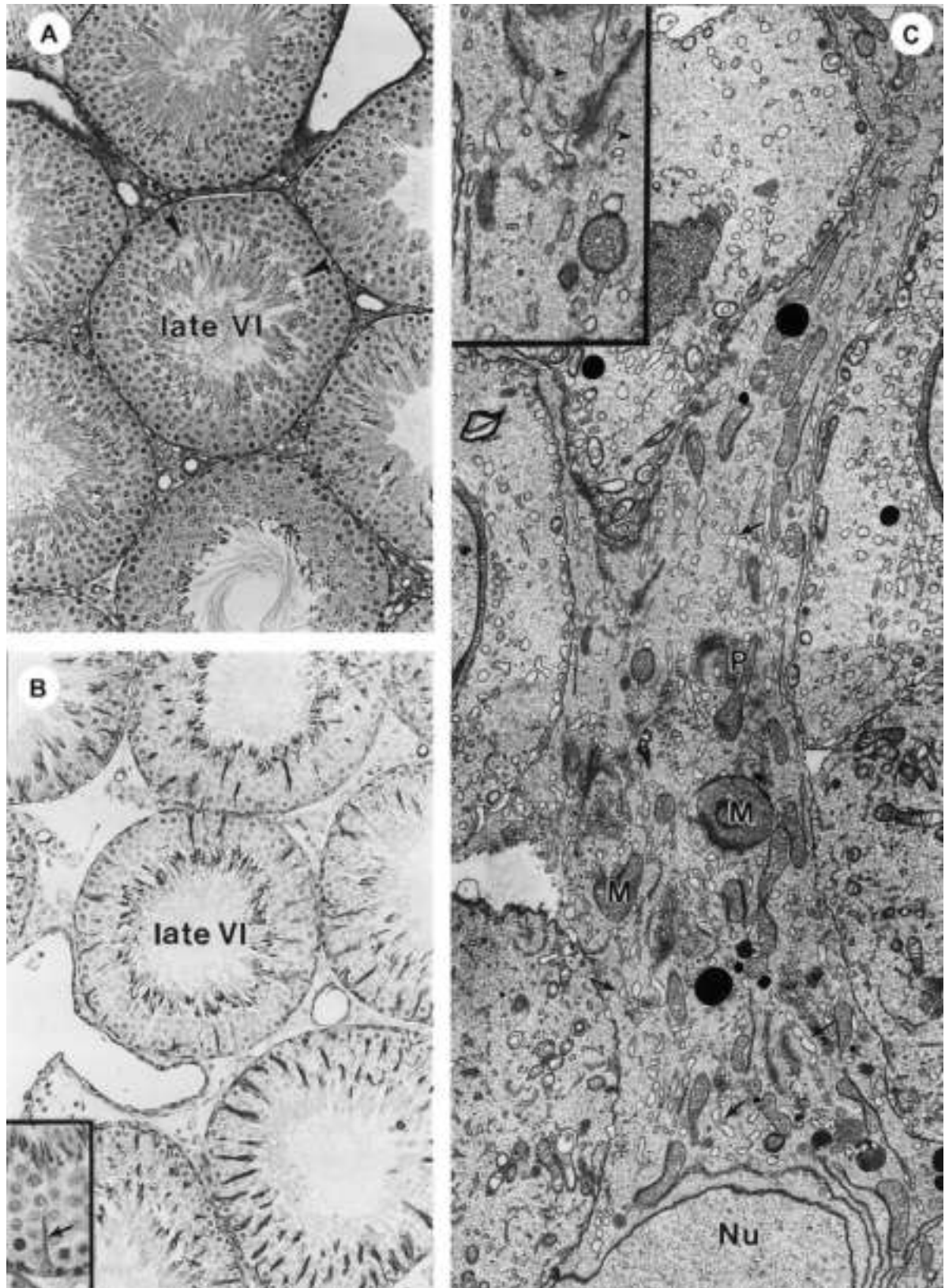


Fig. 2 Light and electron microscopic images of the testis 90 min after exposure to CBZ. Roman numerals represent the stages of spermatogenesis. (A) Pre-sloughing of the elongated spermatids in stage late VI. There are gaps between elongated and round spermatids (arrowheads). Periodic acid–Schiff and hematoxylin. $\times 165$. (B) Immunostaining for tyrosinated α -tubulin in stage late VI with pre-sloughing. Tubulin immunoreactivity in the Sertoli cells appear to be weaker than the control. $\times 165$. Inset: A high magnification of a Sertoli cell having a thinner and shorter bundle of tubulin immunoreaction (arrow). $\times 330$. (C) Sertoli cell with pre-sloughing in stage early VII. Rounded profiles of the endoplasmic reticulum (arrows) are more common than in the control, although there are intact microtubules in the cytoplasm (inset, arrowheads). Nu: nucleus of the Sertoli cell. $\times 6000$. Inset: $\times 20\,000$.

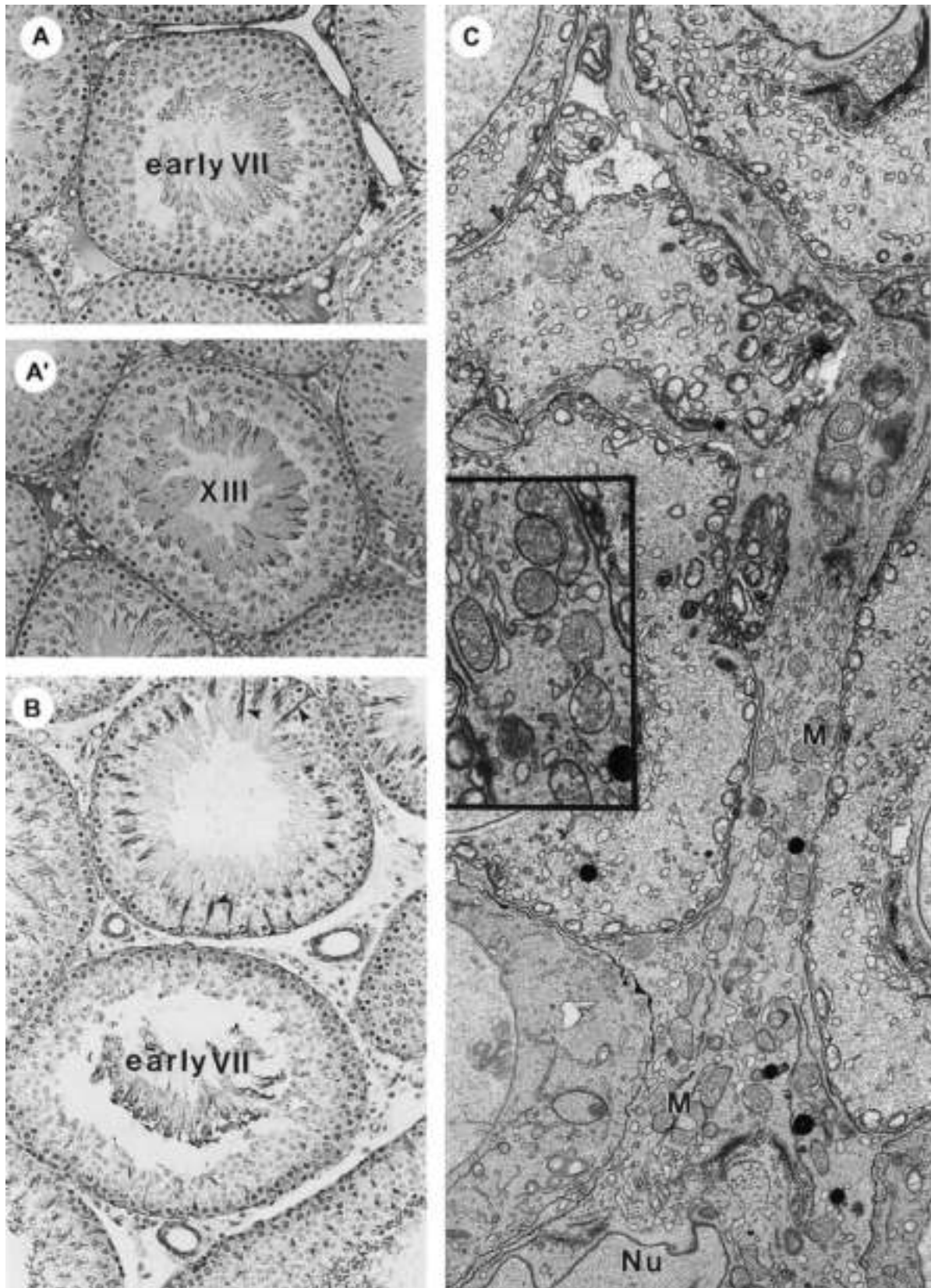


Fig. 3 Light and electron microscopic images of the testis 120 min after exposure to CBZ. Roman numerals represent the stages of spermatogenesis. (A) Sloughing in stage early VII (upper) and pre-sloughing in stage XIII (lower). Periodic acid–Schiff and hematoxylin $\times 165$. (B) Immunostaining for tyrosinated α -tubulin. Tubulin reaction in Sertoli cells has almost disappeared in stage early VII with sloughing. However, tubulin immunoreaction (arrowheads) in the neighboring tubule without sloughing remains unchanged. $\times 165$. (C) Sertoli cell with sloughing in early stage VII. Rounded mitochondria (M) are seen in the supranuclear region. Microtubules in the basal cytoplasm seem to be decreased (inset). Nu: nucleus of the Sertoli cell. $\times 6000$. Inset: $\times 20\,000$.

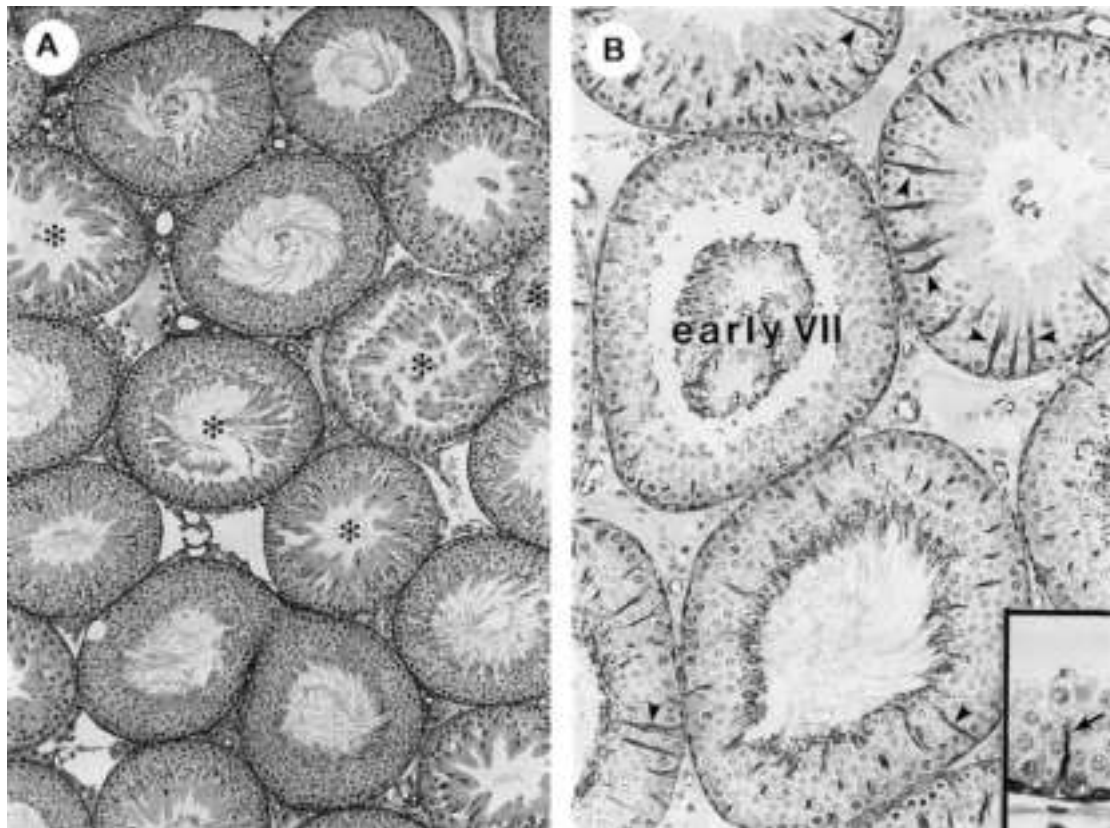


Fig. 4 Light microscopic images of the testis 180 min after exposure. Roman numerals represent the stages of spermatogenesis. (A) Pre-sloughing and sloughing of the elongated spermatids in multiple tubules (asterisks). Periodic acid–Schiff and hematoxylin. $\times 82.5$. (B) Immunostaining for tyrosinated α -tubulin. Sertoli cells showing greatly reduced or vanished reaction are predominant in this stage early VII tubule with sloughing. Note the normal tubulin immunoreaction of the Sertoli cells in tubules without sloughing (arrowheads). $\times 165$. Inset: A Sertoli cell that shows strong tubulin immunoreaction after sloughing (arrow). $\times 330$.

129 (Fig. 1C). The mitochondria and endoplasmic reticulum were
130 slender or elongated in shape and aligned with the long axes
131 parallel to microtubules.

132 The 45- and 60-min post-treatment

133 The testes exposed to CBZ for 45 and 60 min showed no
134 difference from the control.

135 The 90-min post-treatment

136 Although observed infrequently (Table 1), loose attachment
137 of the elongated spermatids to the seminiferous epithelium
138 in stages late VI–early VII was evident, indicating the begin-
139 ning of sloughing (pre-sloughing, Fig. 2A). In Sertoli cells
140 with pre-sloughing, tubulin immunoreaction remained fila-
141 mentous but staining intensity appeared to be reduced com-
142 pared to those in the control group (Fig. 2B). The bundles
143 of reaction product often tapered apically and did not reach
144 the luminal border. On the other hand, Sertoli cells without
145 pre-sloughing showed no change in tubulin immunoreaction
146 (Fig. 2B). Electron microscopy showed that Sertoli cells in
147 tubules with pre-sloughing had some intact microtubules in
148 the body region. Rounded profiles of the endoplasmic retic-
149 ulum were more common in these Sertoli cells than in the
150 control group (Fig. 2C).

The 120-min post-treatment

151 Sloughing of the elongated spermatids occurred in more
152 numerous stages late VI–early VII tubules than at 90-min
153 post-exposure (Fig. 3A, Table 1). Stages XIII and XIV also
154 showed pre-sloughing of the elongated spermatids (Fig. 3A).
155 In these tubules, tubulin immunoreaction of Sertoli cells was
156 significantly reduced in intensity and a filamentous staining
157 pattern was not obvious. Electron microscopy of the Sertoli
158 cells with sloughing indicated that microtubules in the basal
159 half of the body region were less numerous than in the con-
160 trol group (Fig. 3C). Mitochondria often lost the elongated
161 shape and were rounded in this region. In addition, the endo-
162 plasmic reticulum showed round or oval profiles, not slender
163 as seen in the control group. On the other hand, tubulin im-
164 munoreaction remained unchanged in seminiferous tubules
165 without sloughing.
166

The 150- and 180-min post-treatment

167 At 150-min post-treatment, sloughing was observed in more
168 numerous stages late VI–early VII and XII–XIV than at
169 120 min (Table 1). Stages II–V and mid-VII–VIII exhib-
170 ited no sloughing at 180 min, but all other stages displayed
171 sloughing of cells. Among these, stages late VI–early VII
172 and XII–XIV showed higher frequencies of sloughing than
173

174 did others (Table 1, Fig. 4A). Sertoli cells with sloughing
175 showed only weakly diffused immunoreaction of tubulin
176 in the supranuclear region. However, there were occasional
177 Sertoli cells that showed strong filamentous staining in the
178 body region even after complete sloughing of the elongated
179 spermatids (Fig. 4B, inset). Again tubulin immunoreaction
180 did not change in seminiferous tubules without sloughing.

181 Discussion

182 The present study showed that CBZ-induced sloughing
183 of the immature germ cells occurred first in stages late
184 VI–early VII between 90 and 120 min after treatment. No
185 sloughing was observed in stages II–V and mid-VII–VIII,
186 but all other stages exhibited sloughing by 180 min. Re-
187 duction of tubulin immunoreaction was observed in Sertoli
188 cells where sloughing had occurred. Electron microscopy
189 demonstrated that there were intact microtubules in Sertoli
190 cells with pre-sloughing. On the other hand, no change in
191 the tubulin immunoreaction was detected in Sertoli cells that
192 did not show sloughing. These results suggest that sloughing
193 is due to stage-specific susceptibility of Sertoli cell micro-
194 tubules to CBZ. We reported previously that stages III–V
195 showed no sloughing 3 h after a CBZ exposure and hypoth-
196 esized that spermatids were resistant to sloughing because
197 they were embedded deep in Sertoli cells in these stages.
198 However, this does not explain the absence of sloughing in
199 stages mid-VII–VIII where elongated spermatids are located
200 along the luminal border of the seminiferous epithelium.
201 Therefore, it seems that the susceptibility of Sertoli cell mi-
202 crotubules to CBZ, not the positioning of spermatids within
203 the seminiferous epithelium, is responsible for sloughing.

204 Although the factor(s) contributing to this stage-specific
205 susceptibility of microtubules is not known, a possible candi-
206 date is the microtubule-associated proteins (MAPs), as it is
207 well-recognized that MAPs participate in microtubule stabi-
208 lization (Alberts et al., 1994; Maccioni & Cambiazo, 1995).
209 Recently, it was reported that CBZ inhibited polymeriza-
210 tion of testicular microtubules by preventing the binding of
211 guanosine triphosphate to tubulin, and that this inhibition
212 was much greater in the absence of MAPs than in the pres-
213 ence of MAPs (Winder et al., 2001). These MAPs and CBZ
214 are thought to bind competitively to tubulin. Currently such
215 MAPs are not identified, but they should be characterized in
216 future studies, especially their expression in slough-sensitive
217 (late VI–early VII and XII–XIV) and -insensitive (II–early
218 VI and mid-VII–VIII) stages. Another possible factor is the
219 modification to tubulin, because it was shown in the isolated
220 rat seminiferous tubules that decreased tyrosination of tubu-
221 lin is associated with depolymerization of microtubules after
222 treatment with CBZ (Correa & Miller, 2001). Although the
223 relationship of detyrosination and the depolymerization of
224 microtubules has not been elucidated, this factor should also
225 be tested.

226 It was conceivable that the tubulin immunoreaction was
227 reduced wherever complete sloughing was seen at 120-min

and longer intervals, and that changes in shape and dis- 228
tribution of mitochondria and endoplasmic reticulum also 229
occurred (Nakai & Hess, 1994). In addition, we were hy- 230
pothesizing that microtubules would be severely disrupted or 231
totally absent in Sertoli cells by the beginning of sloughing. 232
Nevertheless, this was not observed, because intact micro- 233
tubules remained in the Sertoli cells that were associated with 234
pre-sloughing. This suggests that only a partial disruption of 235
Sertoli cell microtubules can induce germ cell sloughing. 236

In the present study, there were occasional Sertoli cells that 237
showed strong immunostaining of tubulin after sloughing. 238
However, this is not necessarily inconsistent with the mecha- 239
nism of sloughing, as the following explanation could account 240
for the loss of germ cells in Sertoli cells having CBZ insensi- 241
tivity. Multiple adjoining Sertoli cells normally support male 242
germ cells showing synchronous development, while the in- 243
tercellular bridges connect the germ cells to each other until 244
spermiation occurs (Weber & Russell, 1987). Therefore, it is 245
possible that when the elongated spermatids are sloughed, the 246
neighboring spermatids in the same cohort are also sloughed 247
by means of the intercellular bridges, even if the Sertoli cells 248
supporting them have intact microtubules. 249

In summary, the present study showed that sloughing of 250
immature germ cells occurred only in stages in which Sertoli 251
cells showed disruption of microtubules. It is suggested that 252
stage specificity of sloughing is due to stage-specific suscep- 253
tibility of Sertoli cell microtubules to CBZ. MAPs may be 254
a possible factor contributing to this stage-specific suscepti- 255
bility by protecting tubulin from CBZ binding (Winder et al., 256
2001). 257

Uncited references

Russell (1977), Wenz and Hess (1988).

ACKNOWLEDGEMENTS

The authors thank Dr Bruce Winder at the University of 261
California at Davis, for the collaboration and support. We 262
also thank the staff of the Center for Microscopic Imaging, 263
University of Illinois at Urbana-Champaign, for their excel- 264
lent technical assistance. This work is supported in part by 265
NIH Grant ES07832 (R.A.H.). 266

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