

Glycerol Disrupts Tight Junction–Associated Actin Microfilaments, Occludin, and Microtubules in Sertoli Cells

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ABSTRACT: Intratesticular injections of glycerol have been shown to result in a marked and prolonged reduction of spermatogenesis, accompanied by increased permeability of the blood-testis barrier. Because the permeability of the blood-testis barrier is regulated by Sertoli cell tight junctions, and tight junction organization is regulated by the cytoskeleton, we undertook to examine the effects of glycerol treatment on cytoskeletal actin microfilaments and microtubules, and on the tight junction protein, occludin, in Sertoli cells. Adult rats received a single intratesticular injection of either saline (controls) or a 10% glycerol solution. At 24 hours and 7, 15, and 21 days after injection, testes were collected and prepared for routine histology, cryosectioning, or whole seminiferous tubule immunohistochemical staining; and the preparations were viewed by light and confocal microscopy. In saline-injected testes, Sertoli cells had a cytoskeletal and junctional organization that resembled that of normal testes. F-actin microfilaments, located in the basal region, were arranged in regular bundles or chords that circumscribed the perimeter of each Sertoli cell at the level of the tight junction. Occludin colocalized with

tight junction–associated actin filament distribution and microtubules formed a geometric array associated with spermatogenic cells. In contrast, in glycerol-treated Sertoli cells, microfilament and microtubule organization and occludin distribution were partially or completely disrupted. From these results we conclude that glycerol treatment either directly or indirectly disrupts tight junction–associated F-actin and occludin and tubulin organization in rat Sertoli cells. Perturbation of the tight junction–associated proteins could explain the increase in permeability of the blood-testis barrier observed after glycerol treatment. Impaired spermatogenesis following glycerol treatment is likely a consequence of a leaky blood testis barrier and disrupted Sertoli cell cytoskeleton. Glycerol injections may serve as a useful tool in studying the relationship between cytoskeletal organization and the stabilization of Sertoli–Sertoli cell junctions.

Key words: Spermatogenesis, blood-testis barrier, seminiferous tubules, cytoskeleton, F-actin.

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Previous studies have shown that a single intratesticular injection of a glycerol solution results in long-term suppression of spermatogenesis in rats (Igdoura and Wiebe, 1994; Wiebe and Barr, 1984a,b), rabbits (Wiebe et al, 1986), and monkeys (Wiebe et al, 1989) without any apparent alterations in testicular steroidogenesis, serum levels of steroid hormones and gonadotropins, libido, and accessory sex structures. Studies with [³H]inulin and [¹²⁵I]albumin indicate that a single intratesticular glycerol injection results in significant increases in the permeability of the blood-testis barrier (Eng et al, 1994). The effects are long-term and remain in evidence for the duration (56 weeks) of the study (Eng et al, 1994), although metabolic studies using [¹⁴C]glycerol have demonstrated that 24 hours after injection, more than 99% of the glycerol has

disappeared from the testes (Wiebe et al, 1986). Because glycerol is present in and readily metabolized by all tissues and, in rats, may be present in serum at levels of 0.1 to 0.4 mM (Lin, 1977), it was of interest to examine the possible mechanism or mechanisms in which a single intratesticular injection of this ubiquitous substance exerts its long-term effect on the blood-testis barrier.

The blood-testis barrier is maintained by tight junctions between adjoining Sertoli cells (Dym and Fawcett, 1970; Setchell and Waites, 1975; Russell and Peterson, 1985; Byers and Pelletier, 1992). Sertoli-Sertoli cell tight junctions seal the cells together and divide the seminiferous epithelium into basal and adluminal compartments. The unique milieu in the adluminal compartment, preserved by these tight junctions, is required for successful meiosis and completion of spermatogenesis (Russell, 1978; Anderson et al, 1993). A normal organization of cytoskeletal components, specifically microfilaments and microtubules, is necessary for maintaining the integrity and proper functioning of these junctions (Hirsch and Noske, 1993) and for normal movement of spermatogenic cells (Russell and Peterson, 1985). Disruption of actin microfilaments in Sertoli cells by compounds such as cytochalasin D (Weber et al, 1988), dibutyryl cyclic adenosine

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monophosphate (Welsh et al, 1980), 2,5-hexanedione (Hall et al, 1991), *cis*-platinum (Pogach et al, 1989), and cadmium (Janecki et al, 1992; Hew et al, 1993a,b) has been shown to cause a breakdown in the Sertoli cell tight junction barrier and perturbation of spermatogenesis. Microtubules are involved in the transport of spermatogenic cells from the basal compartment to the adluminal compartment (Russell, 1977) and disruption of microtubules by various chemicals severely arrests germ cell mitosis and meiosis (Allard et al, 1993) and results in germ cell necrosis (Boekelheide et al, 1989). Several tight junction-associated peripheral membrane proteins have been identified, such as ZO-1 (Stevenson et al, 1986), ZO-2 (Gumbiner et al, 1991), ZO-3 (Haskins et al, 1998), cingulin (Citi et al, 1988), 7H6 antigen (Zhong et al, 1993), and symplekin (Keon et al, 1996). Until the recent demonstration that claudin-11/oligodendrocyte-specific protein (OSP) forms tight junction strands (Morita et al, 1999), occludin had been the only identified tight junction-specific integral membrane protein (ie, a component of the tight junction strand; Furuse et al, 1993; Fujimoto, 1995). Occludin has 4 transmembrane domains and 2 extracellular loops that form a tight seal between adjacent cells (Furuse et al, 1993; McCarthy et al, 1996; Mitic and Anderson, 1998; Moroi et al, 1998; Matter and Balda, 1999).

Because glycerol has been shown to alter microfilament and microtubule organization in cultured cells (Dinsdale et al, 1992), our objectives were to determine if intratesticular glycerol injections would result in a perturbation of Sertoli cell microfilaments, microtubules, occludin, or a combination of these at the tight junctions. The results indicate Sertoli cell microfilament and microtubule networks and occludin are all substantially disrupted by a glycerol injection. We conclude that the prolonged suppression of spermatogenesis and increased permeability resulting from a single glycerol pulse in rat testis may result from induced changes in perijunctional actin and tubulin and junctional occludin organization in Sertoli cells. Our studies did not ascertain whether the disruptions are a direct or indirect effect of glycerol. Intratesticular glycerol treatment could serve as a nonhormonal tool for studying the role of the Sertoli cell cytoskeleton in spermatogenesis.

Materials and Methods

Chemicals and Solutions

Glycerol, OCT compound, and paraffin (TissuePrep) were obtained from Fisher Scientific (Fair Lawn, NJ). Type I collagenase and pancreatin were obtained from Sigma Chemical Company (St Louis, Mo). Bovine serum albumin (BSA, fraction V) was purchased from Boehringer-Mannheim (Laval, Canada). Triton X-100, formaldehyde, glutaraldehyde, diethyl ether, Tween 20, and

salts used in saline solutions were obtained from BDH (Toronto, ON, Canada).

Animals and Treatments

Male Sprague-Dawley rats aged 55 to 60 days (each weighing 275–300 g) were obtained from Charles River Laboratories (St Constant, PQ, Canada). The animals were maintained according to approved laboratory conditions (2 rats per cage, a photoperiod of 14 h light/10 h dark, at 24°C). They were fed ProLab RMH3000 (Nutritional Inc, Brentwood, Mo) and water ad libitum. Prior to injection, each animal was lightly anaesthetized with ether and the scrotum was wiped with 70% ethanol. Each testis received 1 injection (300 μ L) of either 0.9% NaCl (control rats; n = 24) or 10% glycerol (treatment rats; n = 24) with a 27-gauge needle (Igdoura and Wiebe, 1994). Fresh solutions were prepared using double distilled deionized water and were sterilized through 0.2- μ m filters. Animals were killed by CO₂ asphyxiation at 24 hours or at 7, 15, or 21 days (n = 6 each time) after injection and the testes were removed through an abdominal incision. Generally, for each animal, one testis was rapidly detunicated and prepared for whole tubule staining while part of the other testis was prepared for cryosectioning and another part was prepared for routine histology.

Antibodies and Fluorescent Probes

Rabbit antioccludin polyclonal antibody was purchased from Zymed Laboratories (San Francisco, Calif). Mouse anti- β -tubulin monoclonal antibody (T-4026, clone TUB2.1), fluorescein isothiocyanate- (FITC) labeled goat anti-rabbit immunoglobulin G (IgG), and rhodamine-phalloidin were obtained from Sigma Chemical Company. FITC-conjugated goat anti-mouse IgG was purchased from Hyclone Laboratories, Inc (Logan, Utah), and propidium iodide was supplied by ICN (Costa Mesa, Calif).

Isolation of Seminiferous Tubule Fragments

Testes were placed onto a neoprene stopper, the seminiferous tubules were cut into fragments approximately 1 cm in length and placed into 1.5-mL microcentrifuge tubes containing ice cold phosphate buffered saline (PBS; pH 7.4). To remove the peritubular and connective tissue layers surrounding the seminiferous tubules, we employed the procedure described by Welsh and Wiebe (1975) with some modifications. The procedures were carried out in a calcium- and magnesium-free PBS (solution A), which consisted of 97.6 mM NaCl, 25 mM KCl, 3.7 mM Na₂HPO₄, 8.3 mM glucose, and 0.3 mM KH₂PO₄ at pH 7.3. After rinsing the tubules in solution A to remove germ cells and interstitial components they were treated with 20 mL of solution A containing 4 mg of Type I collagenase for 60 minutes at 28°C in a shaker bath. The collagenase solution was decanted and tubules were washed 3 times for 5 minutes in solution A. Tubules were resuspended for 20 minutes in 20 mL of solution A containing 4 mg of pancreatin and agitated in a shaker bath at 28°C. The pancreatin suspension was removed and tubules were rinsed 3 times for 5 minutes each in solution A. Removal of the peritubular layer by this procedure was histologically verified using Giemsa stain.

Immunohistochemistry

One testis from each animal was cut in half, embedded in OCT compound, snap-frozen in liquid nitrogen, and stored at -80°C until used. For cryosections, frozen testis portions were warmed to -15°C . Eight-micrometer sections were cut with a Leitz cryostat 1720 (Ernst Leitz, GMBH, Wetzlar, Germany), picked up on coverslips, and air-dried for at least 1 hour. The other testis was used to prepare seminiferous tubule fragments as outlined earlier (5–20 seminiferous tubule fragments per slide, 5–10 slides per testis).

Actin and Occludin—Both the cryosections and whole seminiferous tubule preparations were fixed in 2% formaldehyde in PBS for 10 minutes, permeabilized with 0.1% Triton X-100 for 10 minutes, and rinsed 3 times in PBS for a total of 15 minutes. Nonspecific protein binding was blocked for 1 hour with 5% BSA in a PBS-blocking solution containing 0.1% Tween 20. Actin microfilaments were stained with rhodamine-conjugated phalloidin (1 $\mu\text{g}/\text{mL}$ for cryosections and 2.5 $\mu\text{g}/\text{mL}$ for whole tubules), which was included in the secondary antibody solution. Cryosections to be used for occludin visualization were incubated overnight with rabbit antioccludin antibody (1:100) in blocking buffer, whereas whole tubules were stained for 1 hour (1:160). The tissues were then rinsed 3 times in the blocking solution (5% BSA + 0.1% Tween 20 in PBS) for a total of 15 minutes and incubated with FITC-conjugated goat-anti-rabbit IgG (1:40 in blocking buffer) for 1 hour. Controls for occludin antibody staining were treated with secondary antibody only.

Tubulin—Tubules were permeabilized for 45 minutes with 1% Triton X-100 in PEM buffer (80 mM PIPES, 5 mM EGTA, 1 mM MgCl_2 , pH 6.8). They were then rinsed in 3 5-minute changes of PEM buffer before fixation in 3.7% formaldehyde and 0.25% glutaraldehyde (5 min). To block nonspecific antibody reactions, tubules were incubated in 1% BSA. Microtubules were labeled by incubating fixed tissues in mouse anti- β -tubulin antibody (1:100) solution at 37°C . After 1 hour, the tubules were washed with PBS containing 0.005% Tween 20 and blocked again for 15 minutes in 1% BSA. The tubules were then incubated in FITC-conjugated goat-anti-mouse antibody (1:20) for 1 hour at 37°C . Controls for microtubule staining consisted of specimens that were incubated with the secondary antibody only.

Nucleic acids were stained by substituting 2 $\mu\text{g}/\text{mL}$ propidium iodide for rhodamine-phalloidin. Following incubations with the secondary antibodies, cryosections and whole tubules were washed in 3 changes of PBS (15 min), mounted in glycerol:PBS (9:1, pH 9), and stored at -20°C . Specimens were visualized using a BioRad MRC600 confocal laser scanning microscope equipped with an argon ion laser.

Histology

Sections of testes were fixed by immersion in Bouin's fixative and processed for embedding in paraffin wax. Sections (6 μm) were cut, mounted on glass slides, and stained with hematoxylin and eosin (BDH, Toronto, Canada) and viewed with a bright field microscope adjusted for Koehler illumination.

Statistical Analysis

Data were analyzed using Student's *t*-test. A probability of less than 5% ($P < .05$) was considered significant.

Results

Effect of Glycerol on Seminiferous Epithelium Organization

The appearance of the seminiferous epithelium was essentially the same in saline-injected (control) and untreated testes. The epithelium was composed of spermatogenic cells in various stages of differentiation and Sertoli cells. In cross sections, tubules had a characteristic stratified appearance and were composed of differentiating germ cells; spermatozoa were found in the lumens of most tubules (Figure 1A and B). A single intratesticular injection of 10% glycerol markedly disrupted the morphology and basic organization of the seminiferous epithelium. The disruption was evident within 24 hours of injection (not shown). By 2 weeks, large numbers of seminiferous tubule cross sections showed almost a complete absence of spermatogenic stages and consisted primarily of a basal layer of Sertoli cells (Figure 1C and D). In regions distal to the site of injection, some seminiferous tubule cross sections showed spermatogenic activity. At the immediate site of injection, some lumens were congested with amorphous aggregates of F-actin and nucleic acids, as demonstrated by rhodamine-phalloidin and propidium iodide staining (not shown). In contrast with the dramatic disruption of the seminiferous epithelium, the interstitial tissue appeared unchanged with apparently normal Leydig cells (Figure 1). Similar changes resulting from glycerol injections have been described in previous reports (Wiebe and Barr, 1984a; Igdoura and Wiebe, 1994).

Effect of Glycerol on Actin Microfilaments

The distribution of actin microfilaments in the seminiferous epithelium was examined in cryostat cross sections to determine whether perturbation of the cytoskeleton might account for the disruption of the seminiferous epithelium following glycerol treatment. In untreated and saline-injected testes, most seminiferous tubules showed apical and basal F-actin microfilament distributions (Figure 2A). In the apical cytoplasm, microfilaments formed tubulobulbar cups, which are associated with the heads of developing spermatids; in the basal region, microfilaments formed linear bundles on which the long axis was often parallel to the basal lamina. Glycerol treatment resulted in disruption of microfilament distribution, giving the appearance of a disorganized network of actin microfilaments between the apical and basal regions of the epithelium (Figure 2B). The disruption of F-actin organization was detectable in some tubules as soon as 24 hours postinjection. The frequency of tubules containing disorganized actin increased from near zero in the saline controls to more than 70% of tubules by 15 days after glycerol treatment (Figure 3), whereas the percentage of tubules with highly organized F-actin distribution decreased

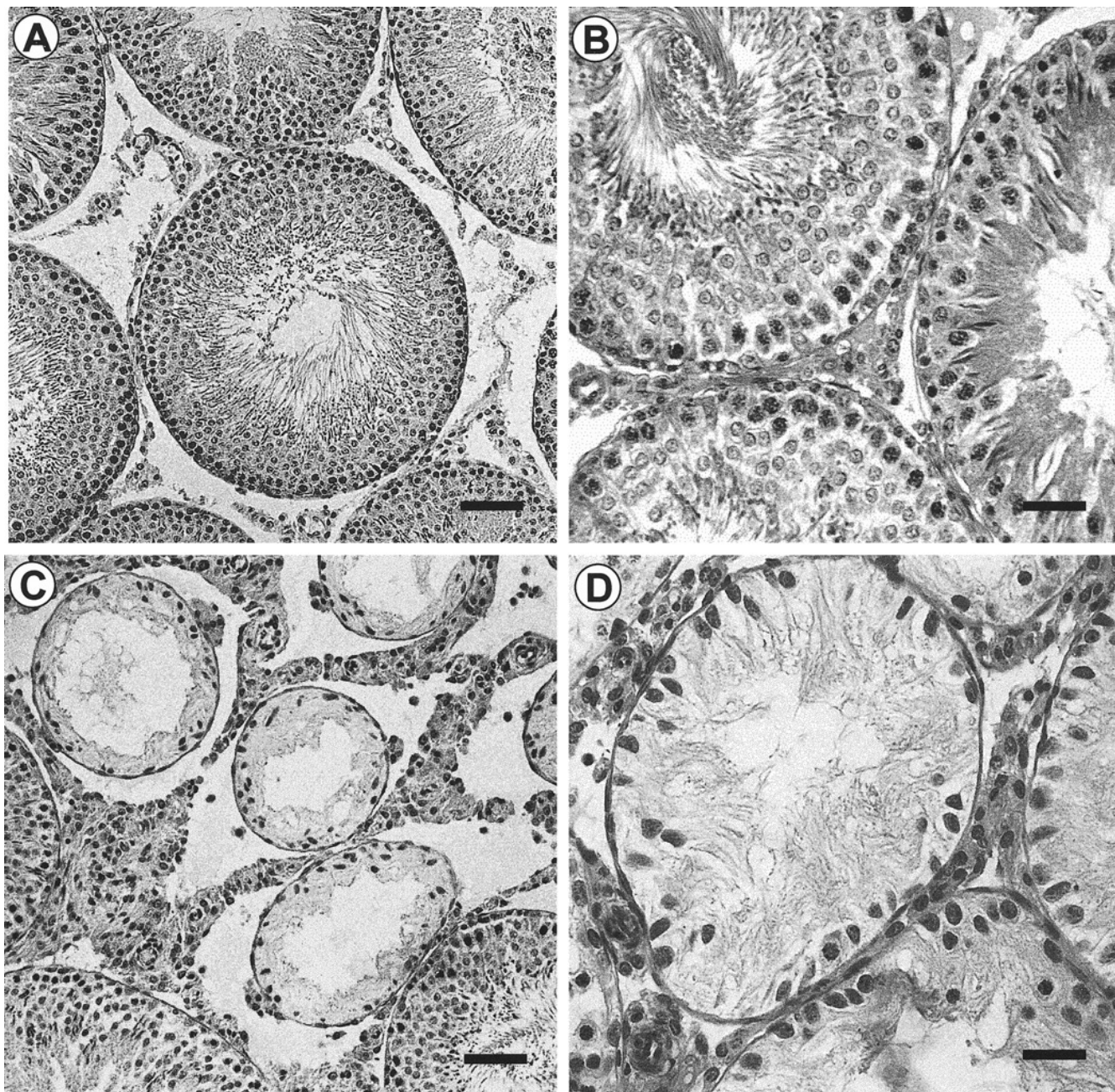


Figure 1. Histological cross sections of rat testes from saline- (A, B) and glycerol-injected (C, D) testes 21 days postinjection. In saline-injected testes (A, B) the seminiferous epithelium is composed of Sertoli cells and differentiating germ cells. In glycerol-treated testes (C, D) seminiferous tubules are lined with Sertoli cells and, in many tubules, differentiating germ cells are absent. Paraffin sections stained with hematoxylin and eosin. Scale bar = 80 μm for A and C; 40 μm for B and D.

from near 100% in the controls to just over 20% in the treated testes.

Whole mounts of seminiferous tubules stained for F-actin microfilaments were examined by confocal microscopy in longitudinal optical sections. Regular arrangements of F-actin networks were found approximately 2 to 5 μm from the basal surface of Sertoli cells in preparations of untreated and saline-treated testes (Figure 4A). Actin mi-

crofilaments, located in this basal perijunctional region, were arranged in bundles or chords circumscribing the perimeter of each Sertoli cell and exhibited a hexagonal, honeycomb-like pattern (Figure 4A). At a more distal plane (4–7 μm from the basal surface), individual filaments were visible as a fine meshwork extending toward the interior of each Sertoli cell and in circular arrays surrounding developing germ cells (Figure 4A, inset). The array of actin

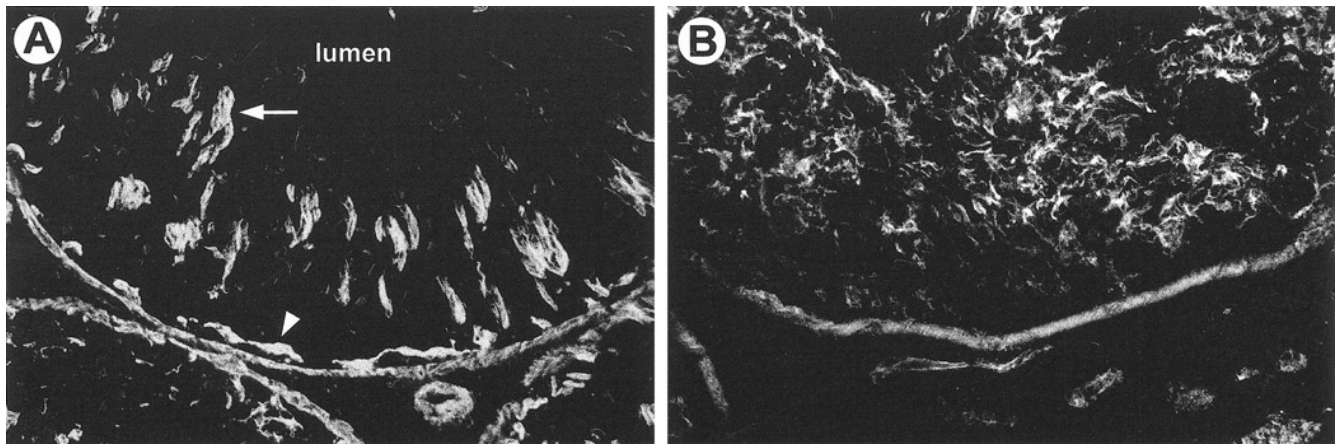


Figure 2. The effect of glycerol treatment on the polarized distribution of actin microfilaments in seminiferous epithelium. Cryosections stained with rhodamine-phalloidin of saline- (A) and glycerol-injected (B) testes show actin microfilaments in cross sections of seminiferous tubules viewed by confocal microscopy. In controls (A), distinct basal ectoplasmic specializations (arrowhead) composed of linear bundles of filaments adjacent to the basal lamina and apical (arrow) specializations line the lumen of each tubule. In glycerol-treated tubules (B) the distribution of microfilaments within Sertoli cells is disrupted, apical and basal ectoplasmic specializations are absent, and microfilaments in the seminiferous epithelium have a random distribution; however, actin in the tubule wall appears to remain organized. (Scale bar = 25 μ m).

filaments was still evident in the basal regions of most tubules 24 hours after treatment with glycerol, although they appeared to be somewhat thinner and deteriorated (Figure 4B). By 1 week after glycerol treatment, a loss of the regular arrangement of F-actin microfilaments had occurred and this condition remained unchanged at 3 weeks post-treatment. The treated testes displayed a range of effects. The filament bundles circumscribing the hexagonal array were greatly attenuated in some tubule segments (Figure 4B); in others, the hexagonal pattern was interrupted and discontinuous (Figure 4C); and in still others, the pattern was nearly (Figure 4D) to completely (Figure 4E) absent. In Figure 4E, the filament bundles appeared to be thin, irregular, and randomly distributed. Internal networks of filaments were dispersed and central regions of Sertoli cells were devoid of the microfilament pattern, which is associated with germ cells in control tubules (compare Figure 4A inset with Figure 4B, C, D, and E). Out of 1500 whole mounts of tubule segments from 12 rats examined by confocal microscopy, about 30% displayed partial disruption of the microfilaments and about 70% displayed a complete disruption of F-actin networks, in contrast to the normal networks that were observed in tubules from control testes. This confirms the observations made on cryosectioned testes (Figure 3). Overall, the glycerol treatment appeared to induce the dissolution of the F-actin filament array throughout the rat Sertoli cells and, in particular, in the basal regions, which are associated with tight junctions.

Whereas the F-actin microfilaments in Sertoli cells were markedly affected by glycerol treatment, there was no apparent effect in myoid peritubular cells. Preparations of glycerol-treated tubules (Figure 4G) exhibited F-actin microfilament staining and arrangements that appeared to

be no different from the saline controls (Figure 4F). These results suggest that the glycerol-induced disruptions are specific to Sertoli cells.

Effect of Glycerol on Occludin

To determine whether glycerol treatment affects occludin, the integral tight junction protein, we examined cross sections of cryopreserved pieces of testes and longitudinal optical sections of whole mount seminiferous tubules from rats 14 days after injection. Cryosections (Figure 5A and B) and seminiferous tubule mounts (Figure 5E and F) of saline-injected testes showed that occludin colocalized with actin microfilaments in the regions of the basal tight junction and ectoplasmic specializations. Occludin was not present in the apical regions of Sertoli cells or in peritubular cells. Cryosections of glycerol-treated testes showed that occludin was generally absent in the basal regions of severely disrupted epithelia (Figure 5D). Whole mounts of seminiferous tubules from control testes stained with antioccludin antibodies and propidium iodide showed an occludin network surrounding a Sertoli cell nucleus and several germ cell nuclei within each hexagonal array (Figure 5G). In glycerol-treated tubules the hexagonal occludin pattern was absent (Figure 5H) and only Sertoli cell (but not germ cell) nuclei were visible. These results indicate that, whereas Sertoli cells are present in disrupted regions of epithelia, glycerol has interfered with the maintenance, synthesis, or both of tight junction-associated occludin.

Effect of Glycerol on Microtubule Organization

Whole mounts of seminiferous tubules from saline-injected testes viewed by confocal microscopy in longitudinal optical sections showed microtubules in a regularly

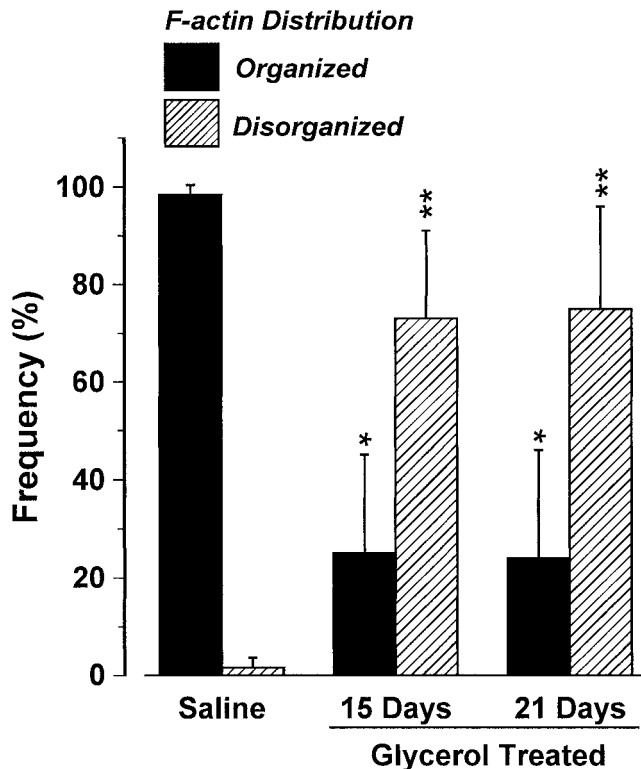


Figure 3. The effect of glycerol treatment on F-actin organization. In testes injected with glycerol the frequency of tubules containing epithelium with organized microfilament distribution was significantly less than in saline-injected animals, whereas the frequency of tubules containing disorganized actin distribution was greatly increased (saline-injected animals, $n = 4$; both glycerol injected groups, $n = 3$). Animals were killed 14 days postinjection. Significant differences between glycerol- and saline-injected testes are denoted by * ($P < .05$) or ** ($P < .01$), respectively.

spaced geometric array (Figure 6A). These arrays consisted of multiple connected rings of tubulin, presumably around individual dividing germ cells, and larger central areas that were devoid of microtubules. The microtubule organization was severely disrupted in seminiferous tubules from glycerol-treated testes (Figure 6B); only aggregates of tubulin, rather than a geometric array, were visible throughout the seminiferous epithelium. About 1200 sections of tubules from testes of 6 glycerol-treated rats were prepared and imaged and complete disruption of microtubule networks was consistently found in each.

Discussion

The present study confirms previous observations (Ig-doura and Wiebe, 1994) that a single intratesticular injection of a 10% glycerol solution induces long-term disruptions of spermatogenesis throughout a large part of the rat testis. Many of the affected seminiferous tubules are devoid of germinal cells beyond spermatogonia, showing

only an empty (cell-free) lumen and a lining of Sertoli cells. The mechanism through which glycerol exerts its antispermatogenic action is not understood. Previous *in vivo* and *in vitro* studies (Eng et al, 1994) had demonstrated that a single intratesticular injection of glycerol solution results in significant increases in permeability of the blood-testis barrier. Those studies showed that uptake of the macromolecules [^3H]inulin and [^{125}I]albumin by seminiferous tubules, rete testis fluid, and seminiferous tubule fluid was significantly greater in glycerol-treated testes than in control testes and that the effect was evident for up to 56 weeks after glycerol injection. The present study demonstrates that a single intratesticular injection of glycerol results in marked alterations in Sertoli cell tight junction-associated F-actin and occludin organization.

Glycerol treatment resulted in partial or complete disruption of actin microfilament arrays in Sertoli cells. Several lines of evidence suggest that actin microfilament bundles are intimately involved with tight junction complexes between neighboring Sertoli cells (Amlani and Vogl, 1988; Boekelheide, et al, 1989; Vogl, 1989). The assembly of microfilament networks closely parallels the formation of tight junction complexes of the blood-testis barrier (Redenbach et al, 1995). Actin filament bundles occur adjacent to junctional networks and are seen to form a belt that circumscribes the base of each Sertoli cell (Vogl and Soucy, 1985; Vogl, 1989) and to undergo organizational changes during spermatogenesis (Vogl and Soucy, 1985; Amlani and Vogl, 1988). Disruption of microfilaments in Sertoli cells by a number of compounds has been shown to result in failure of Sertoli cell-barrier function and to result in perturbation of spermatogenesis (Welsh et al, 1980; Weber et al, 1988; Pogach et al, 1989; Hall et al, 1991; Janecki et al, 1992; Hew et al, 1993a,b). The marked disruption of circumferential microfilament bundles at the level of tight junctions caused by glycerol treatment would result in major dissociations between F-actin and the tight junctions of Sertoli cells and, hence, could be expected to influence the integrity of the Sertoli cell (ie, blood-testis) barrier.

Actin microfilaments, localized in Sertoli cells at sites of intercellular contact, are linked to the transmembrane protein, occludin, via other tight junction-associated proteins (Stevenson et al, 1986; Madara, 1988; Pelletier et al, 1997; Balda and Matter, 1998; Denker and Nigam, 1998; Fanning et al, 1998). The action of glycerol on actin microfilaments in Sertoli cells was accompanied by marked effects on occludin. In control testes, occludin staining colocalized with actin filaments at the tight junctions. In seminiferous epithelium of glycerol-treated testes, the dissolution of the organized actin network was accompanied by a loss of occludin staining. The disruption by glycerol of both the junction-associated actin and

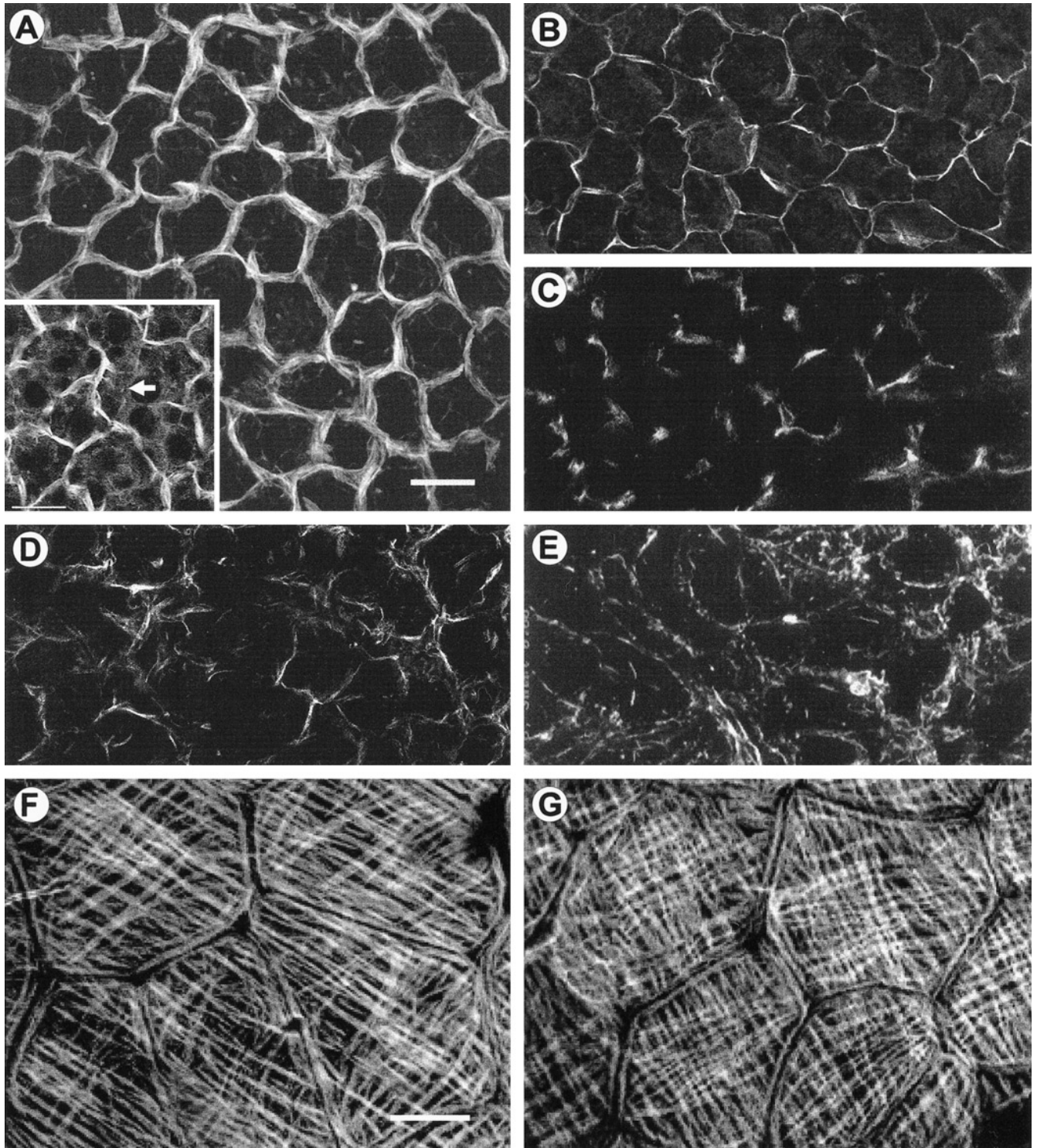


Figure 4. Distribution of actin microfilaments in longitudinal optical sections of seminiferous tubule (whole mount) segments at Sertoli-Sertoli tight junctions (2–5 μm from the basal surface of Sertoli cells) viewed by confocal microscopy (rhodamine-phalloidin stain). A seminiferous tubule from a saline-injected testis (**A**) showing microfilament networks arranged in typical circular arrays around the periphery of Sertoli cells; inset shows a fine internal meshwork of filaments (arrow) at about 4 to 7 μm surrounding germ cells (empty black areas). Twenty-four hours after glycerol treatment (**B**), actin bundles are somewhat thinner and exhibit deterioration. At 1 to 3 weeks postinjection, the filament bundles in some tubules were greatly attenuated (similar to **B**); in other tubule fragments the hexagonal pattern was interrupted (**C**); and in still others, the pattern was nearly (**D**) or completely (**E**) absent. In contrast, the microfilaments in peritubular cells do not appear to differ between saline- (**F**) and glycerol-treated (**G**) testes (14 d after injection). Scale bar = 20 μm .

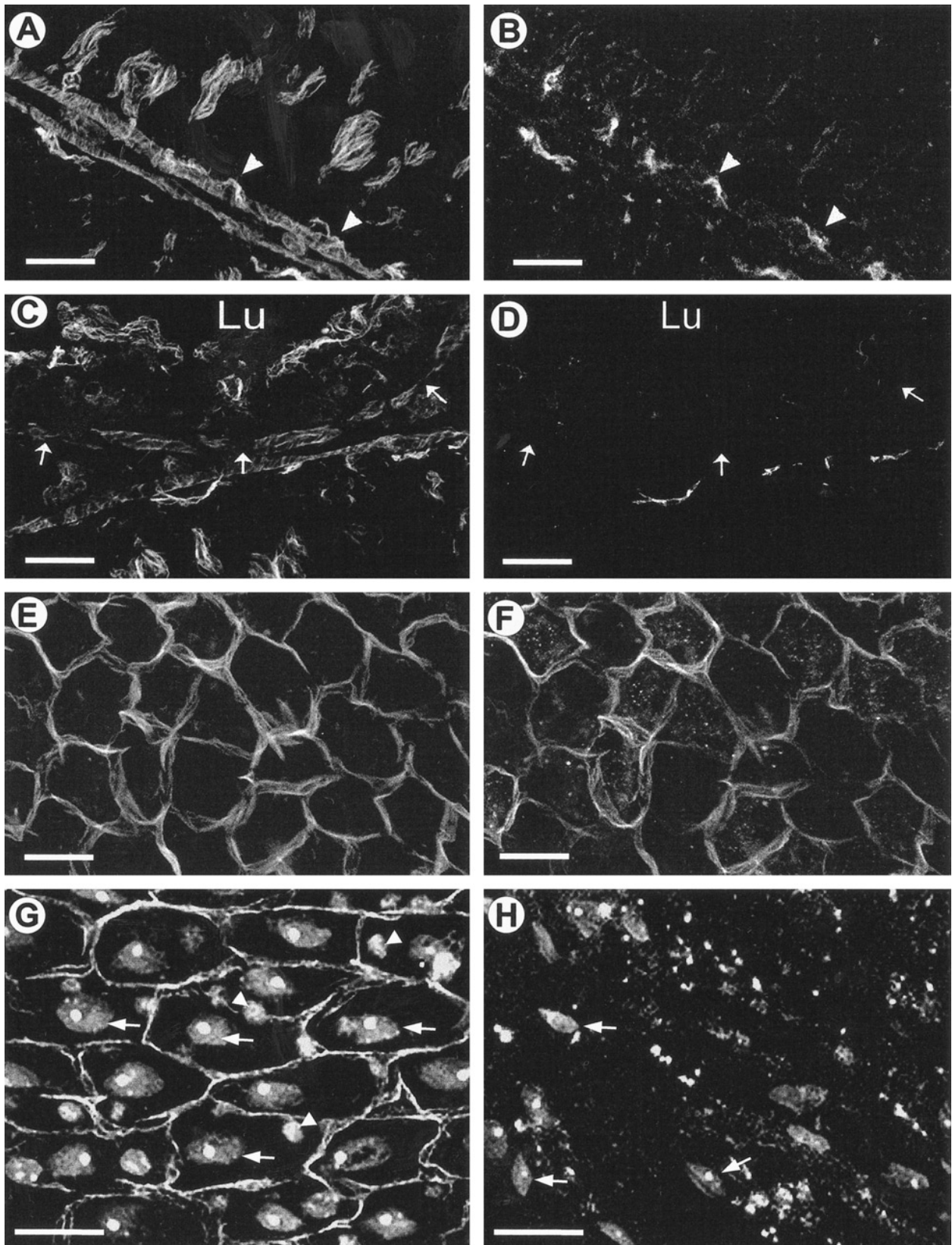


Figure 5. Effect of glycerol treatment on occludin distribution in cryosections of testes (A through D) and whole mounts of seminiferous tubules (E through H) 14 days after injection, viewed by confocal microscopy. Cross sections of saline- (A, B) and glycerol-treated (C, D) testes were double-labeled with rhodamine-phalloidin (A, C) and antioccludin antibodies (B, D). Sections of saline-injected testes show colocalization of actin and occludin

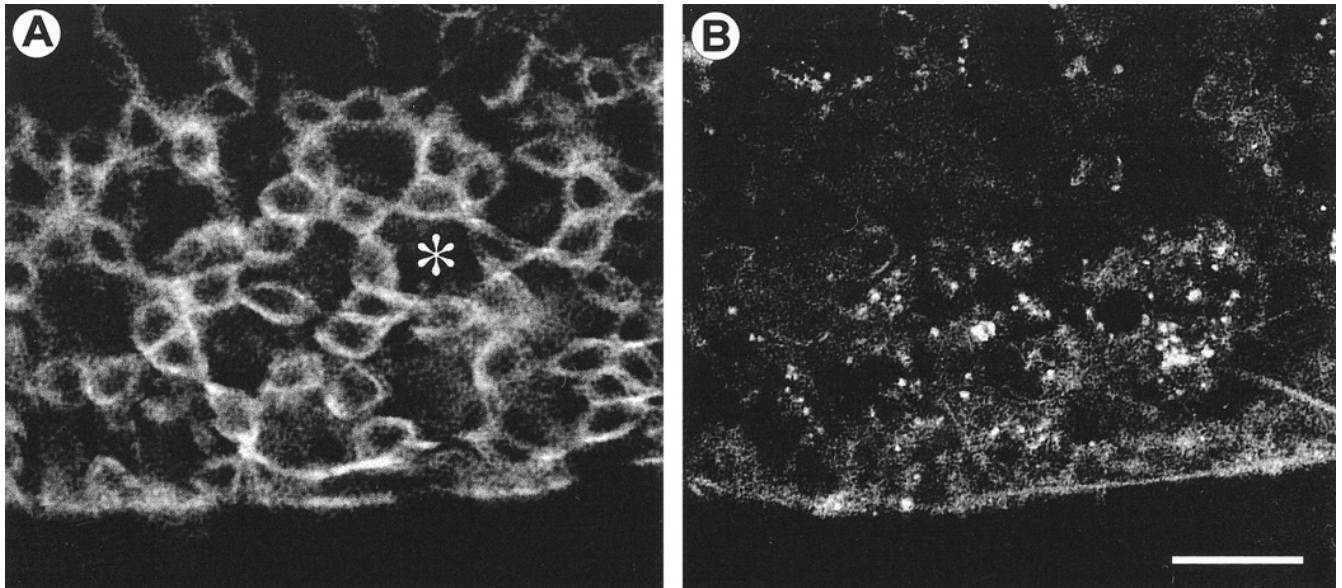


Figure 6. Effect of glycerol treatment on Sertoli cell microtubule organization. Whole mounts of seminiferous tubule segments were stained with anti- β -tubulin and viewed by confocal microscopy. In saline-injected testes (**A**), microtubule networks are arranged in circular arrays surrounding differentiating germ cells around the periphery of Sertoli cells (* indicates interior of 1 Sertoli cell). In glycerol-treated testes (**B**), the circular arrays of microtubules are disrupted. Scale bar = 20 μ m.

the transmembrane occludin barrier may be responsible for causing “leaks” in the tight junctions, which normally seal the intercellular space between adjacent Sertoli cells. This may explain the observed increases in permeability (Eng et al, 1994) of the blood-testis barrier following intratesticular glycerol injection.

In addition to the integrity of Sertoli cell tight junctions, the process of spermatogenesis requires an “unzipping” of junctions as well as movement of germ cells from the basal compartment to the tubule lumen (Russel, 1977). Normally, microtubules located in the peripheral areas of Sertoli cells undergo reorganization during spermatogenesis, which is associated with changes in Sertoli cell shape (Amlani and Vogl, 1988). These changes in cell shape and microtubule organization appear to support and position spermatogenic cells during their transit from the basal compartment to the apical compartment (Redenbach and Vogl, 1991). The importance of microtubule networks to spermatogenesis has been demonstrated using various agents that cause dispersion of microtubule organization (Russell et al, 1981; Boekelheide et al, 1989; Allard et al, 1993) and result in impaired spermatogenesis and germ cell necrosis. Sertoli cells from glycerol-treated

testes displayed a marked disruption or complete loss of microtubule networks. The circular arrays and individual microtubule networks that were common in the Sertoli cells of control testes were generally absent in glycerol-treated testes. The results lead us to suggest that the absence of normal microtubule organization could impair transport of spermatogenic cells toward the lumen of seminiferous tubules and that this, in turn, could lead to an arrest in their differentiation, development, and eventual loss.

The mechanisms by which glycerol disrupts the Sertoli cell cytoskeleton and junctional complexes are unknown. Previous studies in our laboratory have shown that treating baby hamster kidney (BHK) cell cultures with 6–10% glycerol leads to alterations in microtubule and stress fiber arrangements (Dinsdale et al, 1992). This observation suggests that glycerol may disrupt cell structure by acting directly on cytoskeletal filaments or associated proteins. However, if microfilament networks in different cells are structurally and biochemically similar, then one would expect glycerol to also disrupt the microfilament network in other testicular cells. Instead, glycerol specifically affected only Sertoli cells. Alternatively, glycerol may indi-

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(**A** and **B**, arrowheads) in the basal (tight junction) region of seminiferous tubules. In glycerol-injected testes, actin is disrupted (**C**) and occludin is absent (**D**) at the tight junctions. A longitudinal optical section of the basal region of a whole mount seminiferous tubule from a control testis stained for actin (**E**) and occludin (**F**) shows colocalization at Sertoli cell tight junctions forming hexagonal arrays. Whole mount seminiferous tubule preparations stained with propidium iodide for nucleic acids and with antioccludin antibodies show that, in controls (**G**), a single Sertoli cell nucleus (arrows) and occasional germ cell nuclei (arrowheads) are found within each hexagonal array; whereas glycerol-treated tubules (**H**) do not show the occludin pattern or germ cell nuclei but Sertoli cell nuclei only (arrows). Scale bar = 25 μ m.

rectly disrupt the Sertoli cell cytoskeleton. One possible explanation is that glycerol affords a sufficient osmotic stress to rupture the integrity of the tight junctions, and the resulting alteration in the adluminal compartment milieu may then further affect the Sertoli cell cytoskeleton. Our study did not address this potential route of glycerol action nor does it explain the apparent lack of repair, over time, of the cytoskeletal disarray caused by a single glycerol injection.

In summary, the present study demonstrates that glycerol injections result in the perturbation of Sertoli cell cytoskeletal and junctional protein organization. These effects were concurrent with changes in histology and spermatogenesis observed with glycerol treatment of rat testes as previously published (Wiebe and Barr, 1984a,b; Wiebe et al, 1986; Igdoura and Wiebe, 1994). The results suggest that these changes in the organization of actin, occludin, and tubulin may be responsible for the previously observed (Eng et al, 1994) weakening of the blood–testis barrier and cessation of normal spermatogenic cell development. These findings are significant because they demonstrate that glycerol, with no apparent untoward side effects, may be a valuable tool in determining the relationship between cytoskeletal organization and the stabilization of Sertoli–Sertoli cell junctions. Eventually, understanding the route of action of glycerol on spermatogenesis may be useful in the search for effective nonhormonal chemical male contraceptives that selectively act on Sertoli–Sertoli cell junctions.

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