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Toxicants target cell junctions in the testis: Insights from the indazole-carboxylic acid model

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Keywords: adjudin, cell junctions, germ cells, indazole-carboxylic acid, lonidamine, seminiferous epithelium, seminiferous epithelial cycle, Sertoli cells, spermatogenesis, testis

Abbreviations: AJ, adherens junction; BPA, bisphenol A; BTB, blood-testis barrier; CAR, coxsackievirus and adenovirus receptor; c-Yes, cellular Yamaguchi sarcoma viral oncogene homolog 1, a non-receptor protein tyrosine kinase; c-Src, cellular transforming gene of Rous sarcoma, a non-receptor protein tyrosine kinase; ES, ectoplasmic specialization; FAK, focal adhesion kinase; JAM, junctional adhesion molecule; MAPK, mitogen activated protein kinase; PFOS, perfluorooctanesulfonate; SHP2, SH2-domain-containing protein tyrosine phosphatase also known as PTPN11, tyrosine-protein phosphatase non-receptor type 11; SFK, Src family kinase; SSC, spermatogonial stem cell; TBC, tubulobulbar complex; TJ, tight junction; ZO-1, zonula occludens 1

There are numerous types of junctions in the seminiferous epithelium which are integrated with, and critically dependent on the Sertoli cell cytoskeleton. These include the basal tight junctions between Sertoli cells that form the main component of the blood–testis barrier, the basal ectoplasmic specializations (basal ES) and basal tubulobulbar complexes (basal TBC) between Sertoli cells; as well as apical ES and apical TBC between Sertoli cells and the developing spermatids that orchestrate spermiogenesis and spermiation. These junctions, namely TJ, ES, and TBC interact with actin microfilament-based cytoskeleton, which together with the desmosomal junctions that interact with the intermediate filament-based cytoskeleton plus the highly polarized microtubule-based cytoskeleton are working in concert to move spermatocytes and spermatids between the basal and luminal aspect of the seminiferous epithelium. In short, these various junctions are structurally complexed with the actin- and microtubule-based cytoskeleton or intermediate filaments of the Sertoli cell. Studies have shown toxicants (e.g., cadmium, bisphenol A (BPA), perfluorooctanesulfonate (PFOS), phthalates, and glycerol), and some male contraceptives under development (e.g., adjudin, gamendazole), exert their effects, at least in part, by targeting cell junctions in the testis. The disruption of Sertoli–Sertoli cell and Sertoli–germ cell junctions, results in the loss of germ cells from the seminiferous epithelium. Adjudin, a potential male contraceptive under investigation in our laboratory, produces loss of spermatids from the seminiferous tubules through disruption of the Sertoli cell spermatid junctions and disruption of the Sertoli cell cytoskeleton. The molecular and structural changes associated with adjudin administration are described, to provide an example of the profile of changes caused by disturbance of Sertoli-germ cell and also Sertoli cell-cell junctions.

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Introduction

Studies have shown many environmental toxicants, such as cadmium and bisphenol A, exert their effects by targeting cell junctions between testicular cells and also synapses between neurons in both rodents and humans.[1-11] Several toxicants also perturb gap junction-based cell–cell communication.[12-15] In the testis, Sertoli and germ cell junctional proteins, as well as the tight junction (TJ)-permeability barrier, are the early targets of toxicants based on studies in vitro[14,16-22] and/or in vivo.[22-28] Accumulating evidence indicates that toxicants activate and/or disrupt mitogen activated protein kinase (MAPK); focal adhesion kinase (FAK); Src family kinases (SFKs), such as c-Yes and c-Src; and phosphatases, such as tyrosine phosphatase SHP2, which are signaling proteins that function downstream of adhesion protein complexes at cell junctions[1,22,23,29-32] (also see Mathur this issue). These signaling molecules alter the phosphorylation states of proteins at the Sertoli–Sertoli cell and/or Sertoli–germ cell interface, which in turn disrupts protein localization (often, but not always, resulting in the re-localization of proteins from the cell membrane into the cytosol via endocytic vesicle-mediated protein trafficking), damages cell junctions and the blood–testis barrier, and results in germ cell loss from the seminiferous epithelium.[2,33–35] Furthermore, a disruption of kinase and/or phosphatase activity by environmental toxicants, such as cadmium, bisphenol A, phthalates, and other androgen disruptors can affect androgen action, including the transcriptional regulation of androgen-related genes necessary for the maintenance of spermatogenesis.[34,36-40] Because the disruption of cell junctions in the testis[2,4,6,33] and other organs[13,41] by environmental toxicants has been the subject of several reviews, we will use this short review to describe the main features of the different types of junctions in the testis as summarized in **Table 1**,

Table 1. Cell junctions in the seminiferous epithelium of mammalian testes that are targets of adjudin and other environmental toxicants*

Junction Type	Location	Adhesion protein complex
Anchoring Junction:		
Apical ES (testis-specific cell-cell actin-based)	Sertoli cell-spermatid (step 8–19) in the adluminal compartment	α6ß1-integrin-laminin α3ß3γ3; N-cadherin-ß-catenin; Nectin-2/3-afadin; JAM-C-ZO-1; CAR-ZO-1
Desmosome (cell-cell intermediate filament-based)	Sertoli cell-spermatocyte/spermatogonium in the basal compartment	
	Sertoli cell-spermatid (step 1–7) Desmoglein-desmocolin	Desmoglein-desmocollin
Hemidesmosome (cell-basement membrane intermediate filament-based)	Sertoli cell-basement membrane in the tunica propria	ß1-integrin/laminin α2
Communicating Junction		
Gap junction (a cell-cell actin-based)	Sertoli cell-spermatid (step 1–7) in the adluminal compartment**	Connexin 43-plakophilin-2
	Sertoli cell-spermatogonium in the basal compartment or stem cell niche	Connexins (e.g., connexin 43, connexin 33)
Blood-testis barrier:		
Tight junction (cell-cell actin-based)	Sertoli-Sertoli cell	Occludin-ZO-1; JAM-A-ZO-1; JAM-B-ZO-1; CAR-ZO-1
Basal ES (cell-cell actin-based)	Sertoli-Sertoli cell	N-cadherin-ß-catenin; Nectin-2-afadin
Desmosome (cell-cell Intermediate filament-based)	Sertoli-Sertoli cell	Desmoglein-2-desmocollin-2
Gap junction (cell-cell actin-based)	Sertoli-Sertoli cell	Connexin 43-plakophilin-2

*This Table was prepared based on studies in the rat testis, and updated from recent reviews from our laboratory.[85,86,108] Apical ES, basal ES, tight junction and gap junction are junctions at the cell-cell interface using actin for their attachment; desmosome and hemidesmosome, however, are junctions at the cell-cell and cell-matrix interface, respectively, using intermediate filament for their attachment. In the testis, basement membrane is a modified form of extracellular cell matrix (ECM).[54,55] While there is no specific adhesion protein complex known to use microtubule (MT) for their attachment in Sertoli and/or germ cells, the polarized MTs are found near the polarized actin microfilaments in Sertoli cells. Focal contact (also known as focal adhesion complex), a cell-matrix anchoring junction using actin for its attachment is *not* found in the testis. Abbreviations used: EC, ectoplasmic specialization; CAR, coxsackievirus and adenovirus receptor; JAM, junctional adhesion molecule; ZO-1, zonula occludens-1. **, it is noted that once apical ES is established at the Sertoli-spermatid (step 8) interface, it persists until step 19 spermatids when they are transformed to spermatozoa to prepare for spermiation, replacing desmosome and gap junction, becoming the only anchoring device that adhere developing spermatids onto the Sertoli cells during spermiogenesis. However, connexins, such as connexin 43 is found at the apical ES at the Sertoli-spermatid interface from step 8–19 spermatids, while no gap junction ultrastructures are detected, but connexin 43 alone can form hemichannels for the transport of signals as recently reviewed.[115]

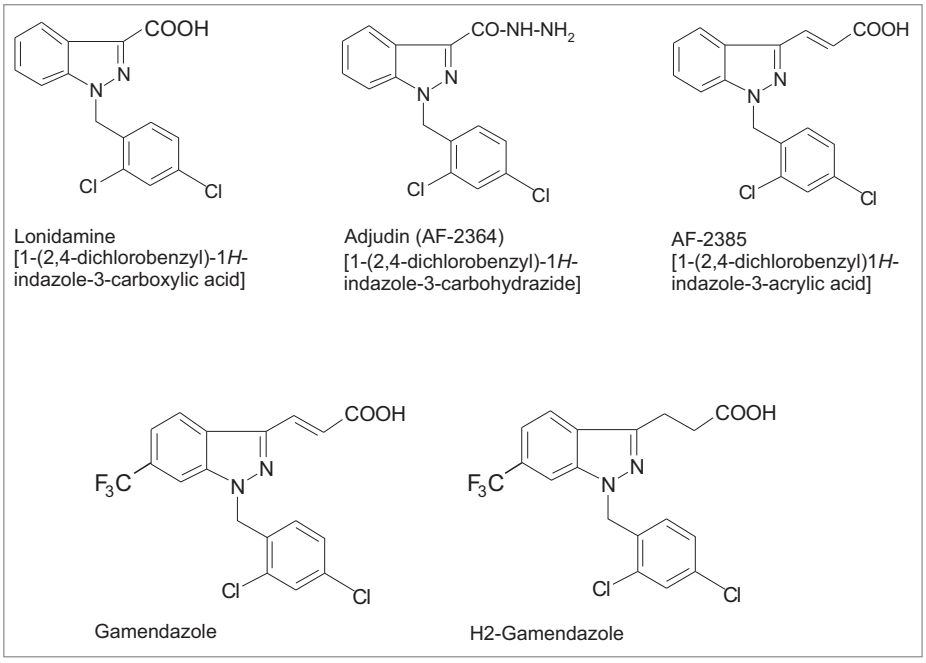

Figure 1. Structural formulae of adjudin and other indazole-based compounds that are being explored as potential male contraceptives. Detailed chemical synthesis of adjudin can be found in an earlier report.[134]

and discuss recent findings on adjudin, a potential male contraceptive that affects the testis similar to that of various toxicants.[42-45]

Signature Lesion

The most common manifestation of disrupted junctions between Sertoli and germ cells is sloughing of germ cells into the tubular lumen. The germ cells often retain relatively normal cytological features and may be present in considerable numbers. Specific germ cells may be shed, depending on which junctions have been damaged. It is noted that apical ectoplasmic specialization (apical ES, a testis-specific anchoring junction) is highly susceptible to the male contraceptive adjudin.[46,47] While apical ES is considered to be one of the strongest anchoring junctions, such as when compared to desmosome at the Sertoli-spermatid (pre-step 8) interface[48] that

utilizes intermediate filament for attachment (**Table 1**), it is most susceptible to adjudin treatment[49] because adjudin effectively perturbs the spatiotemporal expression of actin regulatory proteins that governs the organization of actin microfilaments at the apical ES based on recent studies in our laboratory reviewed in.[50] Thus, following adjudin, a male contraceptive (**Fig. 1**), treatment, such as a single oral dose at 50 mg/kg b.w., elongating/elongated spermatids are rapidly depleted from the seminiferous epithelium into the tubule lumen, and more than 50% of the tubules display signs of spermatid loss within ~6–9 hr following treatment. This is followed by round spermatid and spermatocyte depletion, which takes place by ~3- and ~6.5-day after adjudin treatment, respectively.[51] However, basal ES/BTB is not disrupted until after 2 wk of adjudin treatment,[52] likely due to the presence of 2 arrays of actin microfilament bundles on both sides of the Sertoli cells at the basal ES vs. a single array of actin microfilament bundles on the Sertoli cells at the apical ES.

The actin cytoskeleton and anchoring junctions

In mammalian epithelia/endothelia such as the seminiferous epithelium of the testis, cell junctions are broadly classified based on their function (such as occluding, anchoring or communicating function), relative location (such as at either the cell-cell or cell-matrix interface) and the cytoskeletal element that serves as attachment site for the constituting adhesion protein complex.[53] These include: (i) tight (or occluding) junction (TJ), (ii) anchoring junction, and (iii) communicating junction. TJ is constituted by adhesion protein complexes occludin-ZO-1, JAM-A-ZO-1, CAR-ZO-1 and others (see **Table 1**), utilizing actin microfilaments for attachment, in which each adhesion protein complex is composed of an integral membrane protein (e.g., occludin, JAM-A, CAR) and an adaptor protein that tethers the complex to the cytoskeleton. In the testis, TJ is restricted to the Sertoli cell-cell interface at the BTB.[47] While no TJ ultrastructures are visible at the Sertoli-germ cell interface, TJ proteins, however, are present at the Sertoli-spermatid (step 8–19) interface such as JAM-C and CAR. Anchoring junction is found either at the cell-cell interface or at the cell-matrix interface (basement membrane is a modified form of extracellular matrix in the testis[54,55]). For cell-cell anchoring junctions that use F-actin or intermediate filament for attachment, they are known as adherens junction (AJ) or desmosome, respectively. For cell-matrix anchoring junctions that use F-actin or intermediate filament for attachment, they are focal contact (or focal adhesion) or hemidesmosome, respectively. For communicating junction, gap junction is the best studied cell-cell communicating junction, and the other less studied communicating junction is intercellular bridge (also known as tunneling nanotube) which is used to transmit chemical/biological signaling molecules of larger molecular sizes.[56,57] In most epithelia, TJ usually lies at the apex between cells, underneath the TJ is the adhesion belt formed by aggregates of AJs, to be followed by the desmosome, and these junctional ultrastructures constitute the junctional complex.[53] Gap junction, and hemidesmosome or focal adhesion are then found behind the junctional complex.[53] In the testis, a testis-specific cell-cell adherens junction (AJ) called the ectoplasmic specialization (ES)[58] is limited to the Sertoli-spermatid (step 8–19 spermatids in the rat testis) and designated apical ES vs. the basal ES at the Sertoli cell-cell interface at the BTB.

In short, the anchoring junction at the Sertoli-spermatid (step 8–19 spermatids) interface in the rat testis is an F-actin-rich and a testis-specific adherens junction (AJ) known as apical ectoplasmic specialization (apical ES). The apical ES is limited to the apical (adluminal) compartment of the seminiferous epithelium.[58-60] Besides apical ES, ES is also found at the Sertoli cell-cell interface but restricted to the basal compartment known as the basal ES, and together with the actin-based tight junction (TJ) and gap junction, and the intermediate filament-based desmosome, they constitute the blood-tubule barrier (BTB),[47,60,61] which in turn physically divides the epithelium into the basal and the adluminal (apical) compartments. Basal ES shares similar ultrastructural features as of the apical ES.[47,62,63] For instance, both types of ES have bundles of actin microfilaments in the Sertoli cell that lie perpendicular to the Sertoli cell plasma membrane and are sandwiched either between cisternae of endoplasmic reticulum (ER) and the apposing plasma membranes of the Sertoli cell and the spermatid at the apical ES or between the ER and the apposing plasma membranes of the adjacent Sertoli cells at the basal ES.[47,58,60,64] Thus, basal ES has 2 arrays of actin filament bundles found on both sides of the adjacent Sertoli cells vs. just a single array of microfilament bundles at the apical ES, likely making basal ES structurally stronger. These actin filament bundles also confer unusual adhesive strength to the ES.[48] Interestingly, these actin microfilaments at the ES are rapidly reorganized from their "bundled" to "un-bundled/branched" configuration and *vice versa.* This is thought to give plasticity to the ES in order to accommodate the transport of either spermatids across the epithelium in the apical compartment during the epithelial cycle or transport of the preleptotene spermatocytes across the BTB at stage VIII of the cycle. Since adhesion protein complexes that confer adhesive function at the ES are using F-actin for their attachment, rapid re-organization of F-actin at the apical ES during the epithelial cycle also confers changes in spermatid adhesion and de-adhesion to facilitate spermatid transport during spermiogenesis. We have noted in earlier studies that adjudin is effective in disrupting actin microfilaments at the apical ES.[44,65,66] For instance, F-actin organization at the apical ES, but not basal ES/BTB, begins to show signs of disruption by ~12-hr after adjudin treatment; microfilaments are no longer well-organized surrounding the spermatid head,[67,68] and defragmentation of actin microfilaments is also detected.[65] Probably because of the 2 arrays of actin filament bundles that are found on both sides of the Sertoli cells at the basal ES, the BTB integrity remains undisturbed until after ~2-wk following adjudin treatment, and the disrupted BTB can be resealed thereafter and spermatogenesis rebounds[52,67] (**Fig. 2**). Based on these earlier studies using adjudin-treated rats as a study model, alongside with the use of RNAi to selectively disrupt the expression of target genes pertinent to the regulation of F-actin cytoskeleton at the ES to monitor the function of ES, it is becoming increasingly clear that apical ES restructuring is mediated by the spatiotemporal expression of actin bundling/barbed end capping protein

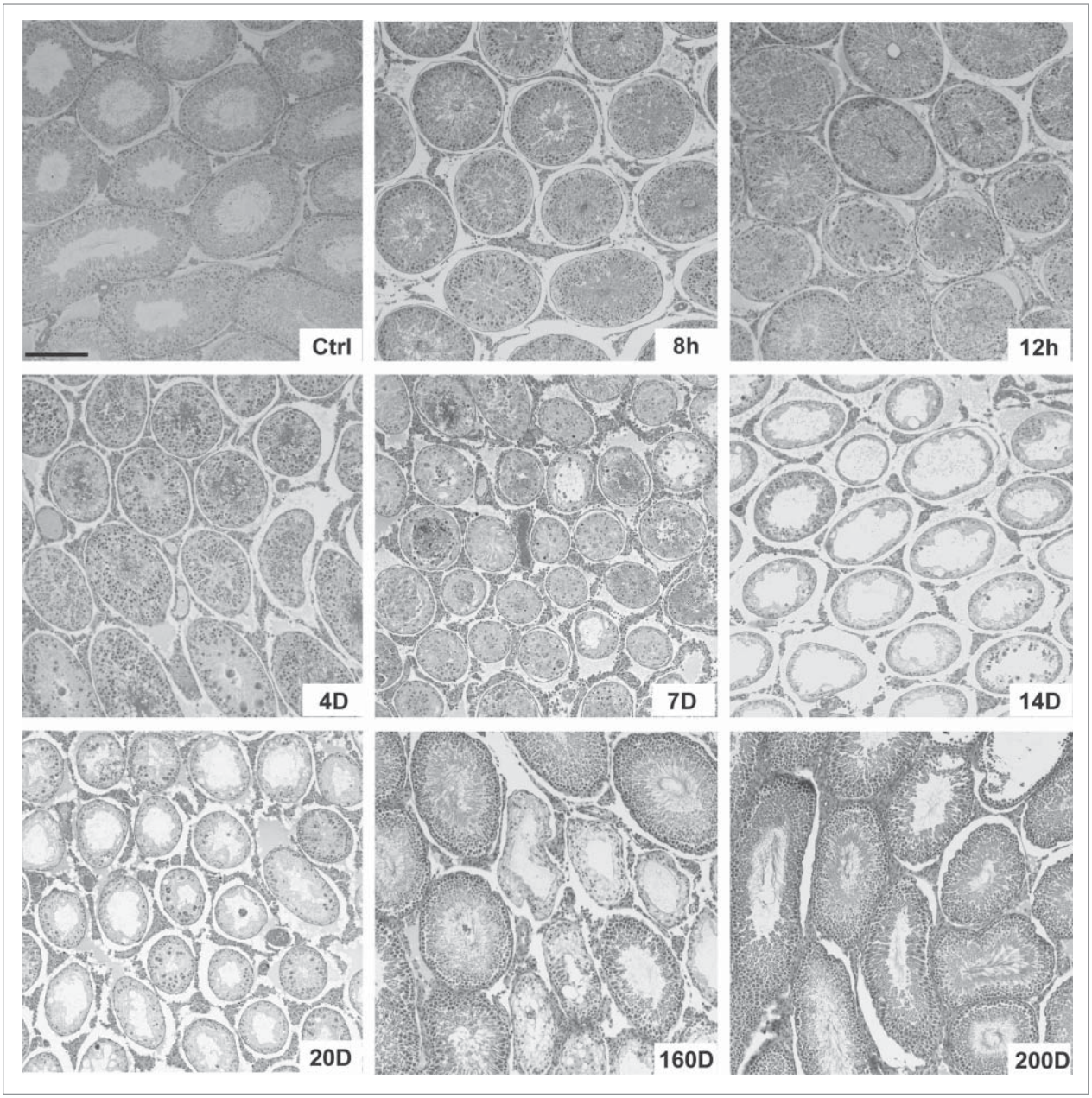

Figure 2. Changes in the seminiferous epithelium of testes following treatment of adult rats with adjudin (50 mg/kg b.w., by oral gavage). Adult Sprague-Dawley rats ($n = 4$ - 6 rats per time point) at 275–300 g b.w. received an oral dose of adjudin (50 mg/kg b.w., by oral gavage) suspended in 0.05% methylcellulose (0.05 g methylcellulose in 100 ml double distilled water, containing adjudin at 20 mg/ml) as earlier described.[42,52] At specified time points at 8 h (hour), 12 h, 4 D (day), 7D, 14D, 20D, 160D and 200D, rats were euthanized by CO_2 asphyxiation, testes removed, fixed in Bouin's fixative and embedded in paraffin for histological analysis following hematoxylin and eosin staining as described.[42,52] Scale bar, 150 μm which applies to other micrographs.

Eps8,[69] actin bundling/cross-linking protein palladin,[68] and barbed end nucleation protein Arp2/3 complex [70] at the apical ES junction between Sertoli cells and elongated spermatids. For instance, in stage VI-II tubules, Eps8 that confers actin bundling and prevents barbed end nucleation (i.e., it effectively prevents branching of an existing actin microfilament) is highly expressed at the apical and basal ES[50,69] to maintain the integrity of actin microfilament at both sites. In stage VIII tubules, the expression of Eps8 diminishes considerably to a level virtually undetectable at the apical and basal ES when these structures undergo degeneration and/or remodeling to facilitate the release of sperm at spermiation and the transport of preleptotene spermatocytes, respectively.[50,69] Treatment of rats with adjudin was found to down-regulate Eps8 expression at the apical ES in stage VI-VII tubules.[69,71] Additionally, branched actin-inducing protein Arp3, which effectively turns bundled microfilaments to an unbundled/branched configuration is highly expressed at the apical ES but confined to the concave (ventral) side of spermatid

head to facilitate endocytic vesicle-mediated protein trafficking, was found to become mis-localized, surrounding other parts of the spermatid heads. The combined down-regulation of Eps8 and the improper localization of Arp3 at the apical ES following adjudin treatment thus impedes spermatid adhesion to the Sertoli cell in the seminiferous epithelium. Studies have shown that the changes at the ES regarding the spatiotemporal expression of Eps8 and Arp3 during the epithelial cycle of spermatogenesis are mediated, at least in part, via the action of FAK[72] that serves as the molecular switch that regulates the organization of actin microfilaments at the Sertoli cell-spermatid interface.[50,71] In short, adjudin perturbs the spatiotemporal expression of these actin regulatory proteins, thereby disrupting the proper organization of F-actin by compromising the conversion of actin microfilaments between their "bundled" to "un-bundled/branched" configuration. This destabilizes the apical ES, leading to its disruption which is accompanied by the premature loss of spermatids from the seminiferous epithelium.[45,46,71] Based on histological analysis, many of these prematurely depleted germ cells following adjudin treatment were detected in the tubule lumen and the relative number of phagosomes in the tubules was not considerably induced (**Figs. 3–5**), so it is not likely that these germ cells undergo apoptosis and get phagocytosed by the Sertoli cell.[42,44,46] These findings are consistent with the underlying concept that adjudin exerts its effects at the Sertoli cell F-actin-based cytoskeleton via its effects on the spatiotemporal expression of actin regulatory proteins, which leads to apical ES disruption and germ cell loss.

Ectoplasmic specialization (ES), tubulobulbar complex (TBC), actin- and microtubule-based cytoskeletons

Once apical ES appears at the interface of step 8 spermatids and Sertoli cells at stage VIII of the epithelial cycle, it replaces the desmosome and gap junction, and it is the only anchoring device for spermatids until the step 19 spermatids are transformed to spermatozoa shortly before their release at spermiation at late stage VIII of the epithelial cycle.[58,60,64,73,74] However, at late stage VII, the concave (ventral) side, but not the convex (dorsal) side, of the step 19 spermatid head begins to undergo extensive endocytic vesicle-mediated trafficking, converting apical ES at this site to a transitional structure known as the apical tubulobulbar complex (apical TBC).[75-77] In short, apical TBC is an invagination of the plasma membranes of Sertoli cell and spermatid, which represents giant endocytic vesicles to facilitate the events of endocytosis, transcytosis and recycling so that "old" Sertoli apical ES proteins can be recycled to assemble "new" apical ES that will arise around the upcoming generation of elongating spermatids in stage VIII tubules.[58,76] This concept is supported by the abundant presence of proteins known to be involved in endocytic vesicle-mediated trafficking events, including clathrin, cortactin, N-WASP, vinculin, zyxin and others at the apical TBC.[70,78-81] Similar ultrastructure known as basal TBC derived from basal ES is also detected at the BTB, which is also used to facilitate endocytic vesicle-mediated trafficking to recycle adhesion proteins from the "old" to the "new" BTB during the transport of preleptotene spermatocytes across the immunologic blood-tubule barrier.[82,83] Thus, proteins can be rapidly "recycled" without requiring de novo synthesis of proteins to assemble new apical or basal ES during the epithelial cycle.

Besides serving as an anchoring device, ES is crucial to facilitate spermatid transport across the adluminal compartment during spermiogenesis and also preleptotene spermatocyte transport at the BTB.[64,84-86] Since late spermatocytes and post-meiotic spermatids are non-motile cells, they rely on the Sertoli cell for their transport to move from the base of the adluminal compartment to the edge of the tubule lumen while differentiating into more advanced germ cell types. It is now generally accepted that the cargoes (i.e., spermatids or preleptotene spermatocytes) are being transported by the vehicle (i.e., actin-based cytoskeleton with the motor proteins which likely serve as the engine) along the track (i.e., microtubules and the associated motor proteins) in Sertoli cells.[87-91] Thus, it is not surprising that microtubules are tightly associated with actin microfilaments in the Sertoli cell and they are 2 inseparable entities which support spermatid transport. It is noted that microtubules in the Sertoli cell are a highly polarized cytoskeleton in which their plus (+) and minus (−) ends are located to the basal and the apical region of the seminiferous epithelium, respectively, and they are stabilized by various microtubule-associated proteins (MAP) such as MARK4 (microtubule affinity regulating kinase 4).[87,92] Studies have shown that germ cell sloughing induced by some toxicants, such as colchicine and carbendazim, follows Sertoli cell microtubule disruption,[93-97] in which carbendazim exerts its effects by blocking tubulin polymerization[98] (also see Johnson, this issue). Furthermore, this effect is stage-specific, and spermatids that are embedded deep inside the seminiferous epithelium, such as stages I-V, are less susceptible to carbendazim-induced loss.[99] Treatment of rats with glycerol via intratesticular injection[100,101] is also known to disrupt microtubules and actin microfilaments.[26] In fact, studies have shown that microtubules are a primary target of numerous toxicants.[93,102,103] Adjudin also induces mis-localization of MARK4 (a microtubule stabilizing protein) in the rat testis, so that MARK4 at the apical ES in adjudin-treated rats no longer localizes to the concave (ventral) side of spermatid heads. Instead MARK4 diffuses away from the apical ES within 6–12 hr following adjudin exposure, and by 24 hr, the expression of MARK4 is considerably down-regulated.[104] Also, MARK4 is considerably diminished and virtually non-detectable in spermatid heads that have lost their polarity 12–24 hrs following adjudin exposure, and these spermatids also begin their sloughing from the epithelium.[104] Much work is needed to better understand the role of microtubule in anchoring junction integrity and spermatid transport during spermatogenesis and toxicant-induced aspermatogenesis. Also, the molecule(s) and the molecular mechanism(s) that provide the proper cross-talk between microtubules and microfilaments to elicit spermatid transport remain to be investigated.

Desmosome and intermediate-filament based cytoskeleton

The desmosome is considered to be one of the strongest adhesive junctions at the cell-cell interface in mammals,[105] most notably in the skin[106,107] when the force that required to disrupt ES

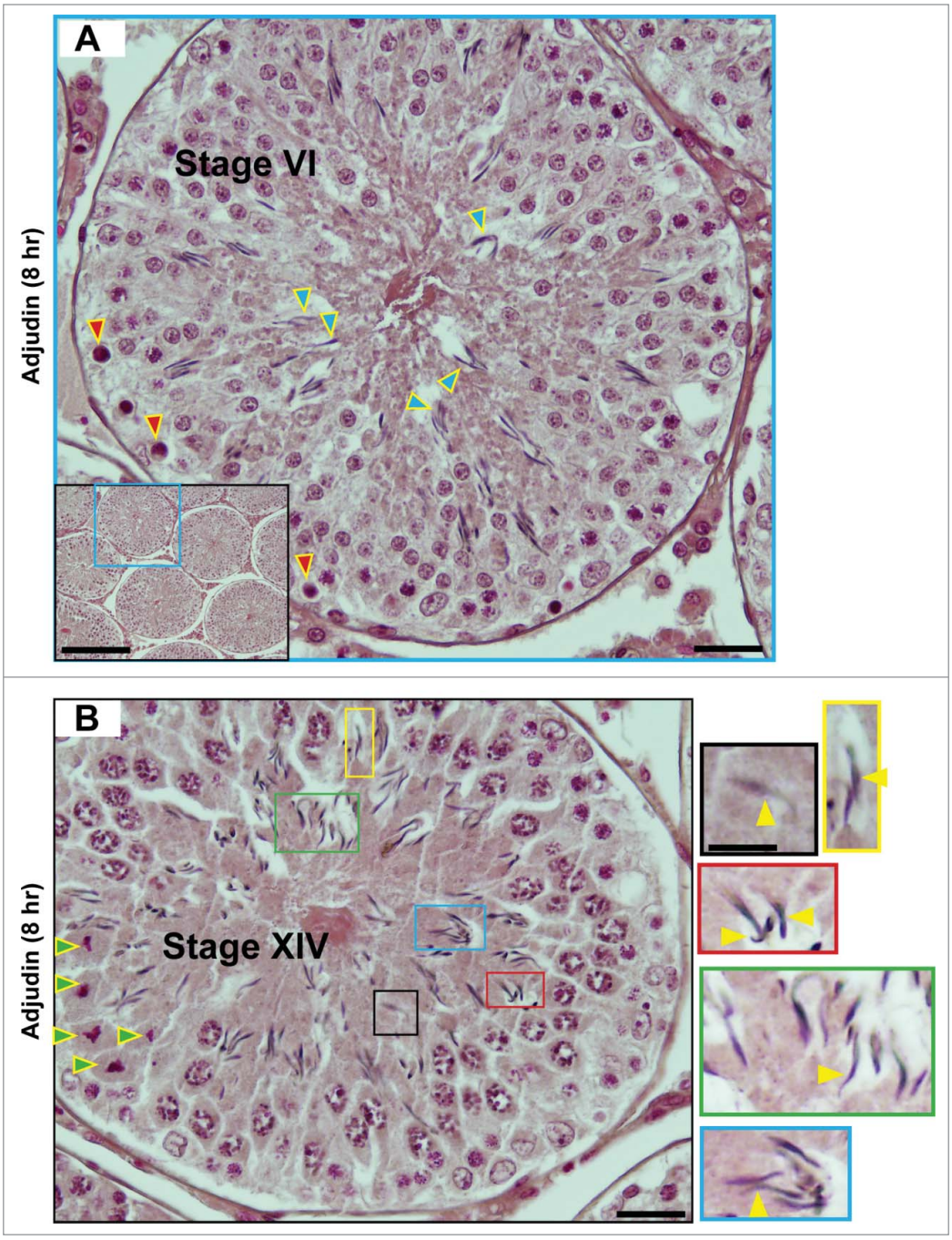

Figure 3. A, B. Sloughing of elongating/elongated spermatids from the seminiferous epithelium of rat testes by 8 hr after a single dose of adjudin (50 mg/kg b.w., by oral gavage). A stage VI (**A**) and a stage XIV (**B**) tubule are shown by H&E (hematoxylin and eosin) staining of paraffin-embedded testis sections. It is noted that elongating spermatids (step 18 spermatids in (**A**) and step 14 spermatids in (**B**)) are detected in the tubule lumen. These 2 micrographs illustrate as if the tubular lumen has closed, which may possibly be due to a shutdown of fluid secretion by Sertoli cells rather than physical shedding of spermatids, because the spermatid heads still look to be well embedded between the round spermatids, such as in (**A**) except for a few step 18 spermatids that are obviously found in the lumen, away from round spermatids as annotated by blue arrowheads. Also, there appears to be a layer of apoptotic pachytene spermatocytes around the basal layer of the tubule (annotated by red arrowheads). However, it is still likely that there is a disruption of spermatid adhesion to the Sertoli cell, at least an onset of apical ES disruption by 8 hr after adjudin treatment, so that spermatids are depleted at later time points. This possibility is supported by studies that have illustrated a disruption on the spatiotemporal expression of actin regulatory proteins Arp3, Eps8, and palladin in ~5- to 24-hr following adjudin treatment,[68-70] which subsequently perturbs F-actin organization, leading to eventual apical ES breakdown. In (**B**), this is a stage XIV tubule because meiosis is detected (meiotic germ cells are annotated by green arrowheads). Also, many spermatids have lost their polarity, recognized by heads, which are no longer pointing toward the basement membrane (annotated by yellow arrowheads in color-boxed areas which are the corresponding magnified images shown on the left panel). Scale bars: (**A**), 40 μm, and 150 μm in inset; (**B**), 40 μm, and 20 μm in inset, which applies to other insets in this panel.

vs. desmosome was quantified using a micropipette pressure transducing system.[48] In the testis, adhesion of spermatogonia, spermatocytes and pre-step 8 spermatids to the Sertoli cell in the seminiferous epithelium is largely dependent on desmosomes which utilize intermediate filaments for attachment.[108-111] The desmosome is also a structural component of the BTB.[47,85] In the Sertoli cell, intermediate filaments are constituted almost exclusively by vimentin[112] vs. keratins found in other cells and/or tissues such as the skin, neurons and intestines.[106] Desmosome is also an emerging platform for cell signaling functions.[106,113] Despite the presumed structural significance of desmosome, mice lacking vimentin are known to develop and reproduce normally without impaired spermatogenesis,[114] illustrating that its function can be superseded by other junctions in the testis, such as the gap junction which is an actin-based communicating junction.[115,116] These findings are not entirely unexpected since studies have shown that

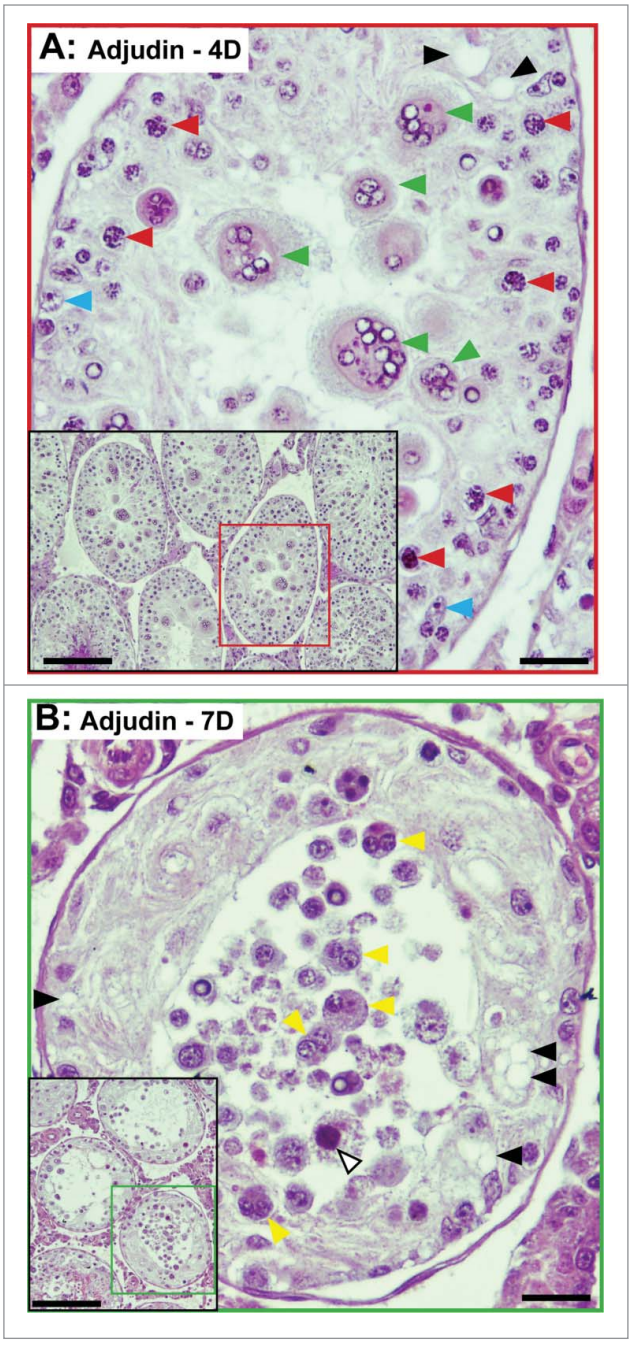

Figure 4. Sloughing of round spermatids (**A**) and spermatocytes (**B**) from the seminiferous epithelium of rat testes by 4- and 7-day after a single dose of adjudin (50 mg/kg b.w., by oral gavage). Following the loss of elongating/elongated spermatids which begins to take place in hours following exposure to adjudin, the sloughing of round spermatids (**A**) and spermatocytes is noted by (**B**) ~4D (day) and 7D, respectively. In (**A**), multinucleate round spermatid cells (green arrowheads), illustrating degenerating germ cells are shown. Spermatocytes (red arrowheads) and Sertoli cells (blue arrowheads) are also noted. In (**B**), multinucleate spermatocytes (yellow arrowheads), illustrating degenerating spermatocytes (annotated by white arrowhead) are shown. Sertoli cell vacuoles (annotated by black arrowheads) are also noted in both (**A**) and (**B**), illustrating Sertoli cell focal injury has occurred. These micrographs are magnified images of the corresponding tubules shown in insets. Scale bars, 40 μm; 150 μm in insets.

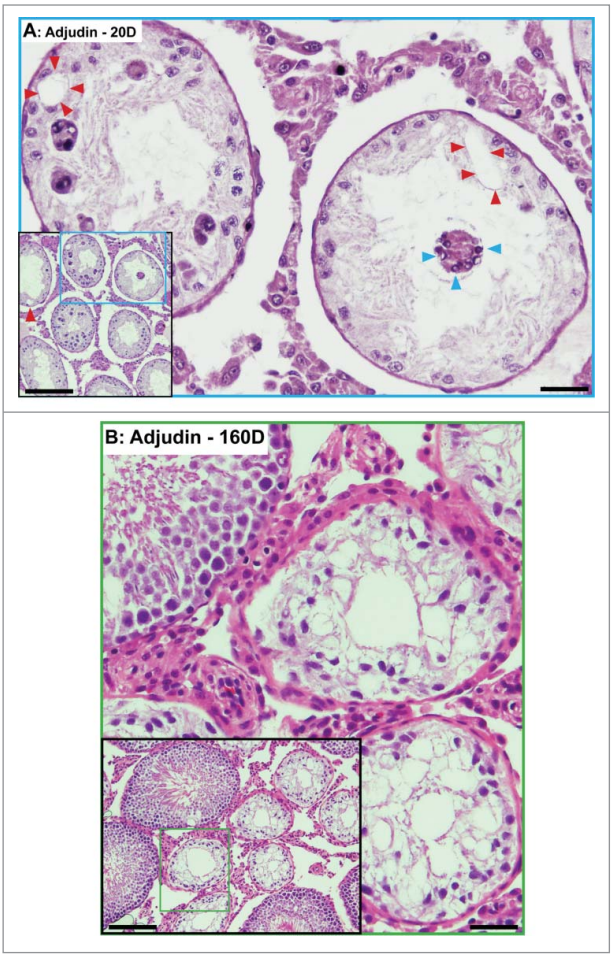

Figure 5. Phenotypes of the seminiferous epithelium in the testes of adult rats by day 20 (**A**) and day 160 (**B**) after treatment with a single dose of adjudin (50 mg/kg b.w., by oral gavage). (**A**) By day 20, tubules are virtually devoid of advanced germ cells. Only Sertoli cells and spermatogonia are found in the seminiferous epithelium. Occasionally, some multinucleate giant cells, such as multinucleate round spermatids are found (blue arrowheads). Vacuolization of seminiferous epithelium, illustrating Sertoli cell focal injury, is noted (red arrowheads). (**B**) By day 160, signs of rebounding of spermatogenesis is detected. As noted in the inset, at least 4 out of 10 tubules shown display signs of spermatogenesis with notable presence of elongating/elongated spermatids. Micrographs are magnified images from the corresponding boxed area shown in inset. Scale bars, 150 μm and 40 μm in insets.

gap junction in the testis also shares some of the common features of desmosomes,[111,117] suggesting that gap junction in the testis can be more than an intercellular communication junction. Furthermore, these findings suggest that the vimentin-based intermediate filaments that constitute desmosome in the testis are structurally and functionally engaged with the actin-based gap junction. In the adult rat testis, vimentin is localized intensely near the basement membrane, encircling the entire Sertoli cell nucleus, but relatively weak staining of vimentin is detected in the adluminal compartment.[89,118] In fact, desmosome is an integrated component of the BTB, and its function is likely to be tightly coupled with the actin-based cytoskeleton to

confer the immunological barrier function via signaling proteins that are recruited to the desmosome.[109] Interestingly, few studies are found in the literature, in particular the molecular mechanism(s), that specifically investigate changes in the intermediate filaments following exposure of animals to toxicants including adjudin except for studies based on the exposure of rodents to 2,5-hexanedione,[118] a known microtubule stabilizing toxicant.[119]

Lonidamine, a Derivative of Indazole-Carboxylic Acid and a Sibling of Adjudin

Adjudin, 1-(2,4-dichlorobenzyl)-1*H*-indazole-3-carbohydrazide (**Fig. 1**), is a derivative of lonidamine, an indazole-based compound first synthesized in the 1970s that was shown to have potent antispermatogenic activity.[120,121] Adjudin was selected from more than 2-dozen lonidamine analogs that were synthesized in the 1990s. The goal was to use an *in vivo* assay to identify a compound that could disrupt spermatid adhesion in the mammalian testis and result in infertility without the toxicity of lonidamine, serving as a potential non-hormonal male contraceptive. The assay was developed based on the observation that the level of testin, a signaling molecule highly expressed in the testis and ovary, was transiently up-regulated (usually within 4–6 h after treatment) whenever spermatid adhesion was disrupted; however, if the testin level remained elevated past ~6 hr, that chemical entity usually produced considerable toxicity.[42,121-123] Adjudin was selected based on this unique activity. Indeed, a subsequent study has shown that adjudin disrupts apical ES selectively since it preferentially perturbs apical ES adhesion and leaves the desmosomes unaffected, at least for a later time.[49]

Adjudin Disrupts the Cytoskeleton and Sertoli–Germ Cell Junctions

Adjudin initially affects the Sertoli cell actin cytoskeleton, which is followed by the disassembly of adhesion protein complexes at the Sertoli cell–spermatid interface and the sloughing of premature germ cells, most notably elongating/elongated spermatids, from the seminiferous epithelium.[44,46,66,68,71] With continued dosing, non-specific morphological changes such as germ cell degeneration and sloughing, seminiferous tubule atrophy, and focal Sertoli cell injury develop (**Figs. 2, 3, 4 and 5**). These non-specific changes are analogous to those seen after repeated exposure of rodents to other testicular toxicants, such as cadmium, phthalates, 2,5-hexandione, and glycerol, at both cellular and molecular levels.[2,24-26,28,93,102,103,124-126] In addition, the use of adjudin-treated rats has become a valuable *in vivo* model to study the regulation of spermatid adhesion to the Sertoli cell, and the transport of spermatids across the adluminal compartment during the epithelial cycle of spermatogenesis, as well as spermatid polarity.[58,73,127]

Studies have shown that adjudin effectively induces germ cell loss from the seminiferous epithelium[121,123] (**Fig. 2**). In fact, ~6 to 8 hr following exposure of rats to a single dose of adjudin at 50 mg/kg b.w. by oral gavage, over 50% of the tubules display signs of elongating/elongated spermatid loss from the seminiferous epithelium[46,51,85] (**Figs. 2, 3**). While adjudin preferentially and effectively disrupts the apical ES, non-apcial ES anchoring junctions at the Sertoli-spermatocyte/round spermatid interface are also found to be perturbed but at a later time. This notion is supported by the observation that depletion of round spermatids and spermatocytes occurs by day 3–4 and day 7, respectively,[51] instead of within ~6–8 hr; and by day 3–7, elongating/elongated spermatids are no longer seen in virtually any tubule examined (**Fig. 4**), consistent with the postulate that apical ES is an early target whereas desmosome/gap junction may be a later target. It is noted that the adhesion of spermatocytes and step 1–7 spermatids to the Sertoli cell in the seminiferous epithelium is supported by intermediate filament-based desmosome and actin-based gap junction.[85,108-110] The unusual adhesive strength of the apical ES vs. desmosome is supported by studies in which the force required to pull post step 8 spermatids from the Sertoli cell is at least twice as much as that required to pull pre-step 8 spermatids or spermatocytes away from the Sertoli cell.[48] Yet following exposure of Sertoli-germ cell cocultures to adjudin, less than half of the force is needed to disrupt the apical ES vs. desmosome,[49] illustrating apical ES is most sensitive to adjudin treatment. These findings thus support the notion that apical ES is more susceptible to adjudin vs. the desmosome and/or gap junction even though ES is considered to be one of the strongest anchoring junctions due to the presence of the array of actin filament bundles.[59,128]

Interestingly, spermatogonial adhesion is largely unaffected in rats treated with adjudin and the population of spermatogonia in the seminiferous tubule remains relatively unaltered following adjudin exposure.[52] A possible explanation for the lack of response of spermatogonia/spermatogonial stem cell (SSC) adhesion to adjudin is because these cells are located under the Sertoli cell BTB, or at the stem cell niche, namely at the juncture where three seminiferous tubules meet, adjacent to the microvessels in the interstitial space. But it is also possible that there are some yet-to-be defined adhesion protein complexes at the Sertoli-spermatogonia/SSC interface that are not susceptible to adjudin. For instance, studies by molecular modeling have identified α6β1-integrin, one of the best studied apical ES adhesion protein,[129-132] possesses a putative docking domain for adjudin,[133] suggesting that there is specific interaction of adjudin and adhesion proteins at the apical ES. If the predominant adhesion protein(s) at the Sertoli-spermatogonia/SSC interface is lacking a docking domain for adjudin, it becomes non-responsive to adjudin treatment. This postulate also explains the lack of cell depletion found in other organs, such as liver and kidney, in subchronic toxicity studies of adjudin in both male and female rats.[134]

As shown in **Figure 2**, within 8–12 hr following adjudin treatment, the tubule lumen in virtually all the seminiferous tubules is filled with elongating/elongated spermatids regardless of the stage of the epithelial cycle (see also **Fig. 3**), suggesting an onset of the apical ES disruption that eventually leads to spermatid depletion from the epithelium that is clearly visible by 4 d. This possibility is supported by findings in which the spatiotemporal expression

of branched actin inducing protein Arp, and actin microfilament bundling proteins Eps8, and palladin, are all perturbed at the apical ES within 5- to 24-hr following adjudin treatment,[68-70] which in turn facilitate the subsequent disorganization of actin microfilament bundles at the apical ES, leading to spermatid sloughing. In this context, it is of interest to note that the histological appearance at 8- to 12-h following adjudin treatment in which the tubular lumens have all closed down is plausible due to reduced fluid secretion by the Sertoli cell in the tubule, rather than the lumen being occluded by sloughed germ cells (**Fig. 3**). This possibility must be carefully evaluated in future studies to examine changes in fluid secretion by Sertoli cells following adjudin treatment. Also, there are no signs of an increase in phagocytic activity, such as an increase in the number of phagosomes in the epithelium, illustrating these depleting germ cells are not subjected to phagocytosis, at least not extensively. In this context, it is of interest to note that this phenotype of actin microfilament defragmentation at the ES also mimics the cadmium-induced defragmentation of actin filaments at the Sertoli cell-cell interface in the rat testis,[28] also known as basal ES that constitute the BTB.[47] This latter finding is also consistent with earlier reports demonstrating a disruption of occludin-based TJ-fibrils (note: TJ is also an actin-based occluding junction using F-actin for its attachment, see **Table 1**) at the Sertoli cell-cell interface at the basal ES/BTB in rats exposed to an acute dose of either $CdCl_2$ (via i.p.)[25] or glycerol (via intratesticular administration).[26] Due to the adjudin-induced defects at the apical ES, which is known to confer spermatid polarity, many spermatids also become misoriented and the heads of elongating/elongated spermatids are no longer pointing toward the basement membrane, but are either parallel to the basement membrane or point toward the tubule lumen (**Fig. 3B**). A recent report using human Sertoli cells cultured *in vitro* has also demonstrated toxicant-induced actin microfilament disruption in these cells, which is likely mediated by changes in the localization of actin bundling/barbed end capping protein Eps8 and branched actin polymerization protein Arp3 following exposure of human Sertoli cells to either cadmium or BPA,[11] consistent with a study using rat primary Sertoli cells following exposure to PFOS.[14] Collectively, these findings thus support the concept that at least some toxicants likely mediate germ cell sloughing via a disruption on the spatiotemporal and/or distribution of actin regulatory proteins (e.g.,, branched actin inducing protein Arp3, and actin bundling proteins Eps8, palladin and others) at the apical ES.

Disruption of the Sertoli cell blood-testis barrier (BTB)

When rats are exposed to acute doses of cadmium (administered via i.p.)[25,27,28] or glycerol[26] (via intratesticular injection), the BTB is irreversibly damaged via disruption of the actin microfilaments, microtubules and also TJ-fibrils at the site, causing irreversible infertility. However, a generally accepted view regarding cadmium toxicity in the testis is that cadmium mediates its effects through the vascular system by reducing blood flow, increasing microvessel permeability, causing interstitial edema and leading to ischemic damage to the Sertoli cells, thereby causing breakdown of the BTB,[135] and the toxicity to the cytoskeleton is likely to be an indirect effect due to shut down of blood flow. However, it was first reported that cadmium induced BTB disruption prior to microvessel damage in the rat.[27] A subsequent kinetics study in adult rats (cadmium chloride at 3 mg/kg b.w.; i.p.) based on histological analysis that monitored erythrocyte leakage into the interstitial space, coupled with electron microscopy to assess endothelial TJ-barrier disruption vs. the Sertoli cell BTB has shown that the BTB was damaged at least ~12–14 hr prior to endothelial TJ-barrier disruption in microvessels in the interstitium.[28] These findings thus suggest that the Sertoli cell is somehow highly susceptible to cadmium toxicity. Interestingly, in adjudin-treated rats, the BTB integrity remains robust until at least 2-wk after treatment, perhaps due to the presence of 2 arrays of actin microfilament bundles on both sides of the adjacent Sertoli cells at the basal ES that create the immunologic barrier.[52,85] Studies have shown that the BTB is disrupted after 6-wk following adjudin exposure, but the damage is transient because the disrupted BTB is resealed by 20-wk, unless a high dose of adjudin, such as 250 mg/kg b.w. is used vs. 50 mg/kg b.w., and this high acute dose of adjudin renders the BTB irreversibly disrupted, and BTB damage is detected as early as 2-wk.[52] Even though the population of spermatogonial stem cells/undifferentiated spermatogonia in the rats that are subjected to a high acute dose of adjudin remains comparable to control rats, spermatogenesis fails to resume possibly due to a permanently damaged BTB,[52] consistent with findings of rats exposed to acute doses of either cadmium or glycerol. It is of interest to note that even though the BTB was transiently compromised by adjudin, resident macrophages were not detected in the seminiferous epithelium (see **Figs. 3–5**),[52] unlike autoimmune orchitis that occurs spontaneously or induced by vasectomy or by immunization with testis antigens, in which macrophages are capable of entering the adluminal compartment following a disruption of the BTB.[136-139] These findings seemingly suggest that the nature of disruption at the BTB, such as a transient vs. a permanent disruption, determines if macrophages are freely permeable to the disrupted immunological barrier.

Concluding Remarks

As noted herein, many of the pathological findings in the testis induced by exposure of rodents to toxicants are also detected in the rat testis following exposure to adjudin – a male contraceptive actively under investigation in our laboratory.[42,44,46,85,108] These observations illustrate that these pathological changes are likely the physiological consequences in response to agents that exert their effects in the seminiferous epithelium behind the BTB. While many toxicants appear to exert their effects at the cell junction level, it is likely that these changes are secondary to the disruption of the actin-, intermediate filament- and/or microtubule-based cytoskeleton since adhesion protein complexes at the Sertoli cell-cell or Sertoli-germ cell interface use either actin or intermediate filament for their attachment, whereas microtubules are most notably used for spermatid transport and the transport of other essential organelles in the Sertoli cell cytosol during the epithelial cycle including endosome-based vesicles.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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