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Testicular Cell Junction: A Novel Target for Male Contraception

Nikki P.Y. Lee^{*}, Elissa W.P. Wong, Dolores D. Mruk, and C. Yan Cheng^{*}

Center for Biomedical Research, Population Council, 1230 York Avenue, New York, New York 10065, USA

Abstract

Even though various contraceptive methods are widely available, the number of unwanted pregnancies is still on the rise in developing countries, pressurizing the already resource limited nations. One of the major underlying reasons is the lack of effective, low cost, and safe contraceptives for couples. During the past decade, some studies were performed using animal models to decipher if the Sertoli-germ cell junction in the testis is a target for male fertility regulation. Some of these study models were based on the use of hormones and/or chemicals to disrupt the hypothalamic-pituitary-testicular axis (e.g., androgen-based implants or pills) and others utilized a panel of chemical entities or synthetic peptides to perturb spermatogenesis either reversibly or non-reversibly. Among them, adjudin, a potential male contraceptive, is one of the compounds exerting its action on the unique adherens junctions, known as ectoplasmic specializations, in the testis. Since the testis is equipped with inter-connected cell junctions, an initial targeting of one junction type may affect the others and these accumulative effects could lead to spermatogenic arrest. This review attempts to cover an innovative theme on how male infertility can be achieved by inducing junction instability and defects in the testis, opening a new window of research for male contraceptive development. While it will still take much time and effort of intensive investigation before a product can reach the consumable market, these findings have provided hope for better family planning involving men.

Keywords

Male contraception; spermatogenesis; Sertoli-germ cell interactions; cell junctions; blood-testis barrier; testis

INTRODUCTION

Testis is compartmentalized into two distinct, but functionally correlated segments, namely the interstitium and the seminiferous tubules. The sexual phenotypes of males are maintained by testosterone produced by Leydig cells in the interstitium, whereas the production of spermatozoa from spermatogonial stem cells occurs in the seminiferous epithelium of seminiferous tubules, which is supported by Sertoli cells. These two events, namely steroidogenesis and spermatogenesis, are regulated by luteinizing hormone (LH) and follicle stimulating hormone (FSH), respectively, which are released from the pituitary gland under the influence of gonadotropin releasing hormone (GnRH) from the hypothalamus [1]. Furthermore, androgen [2,3] and estrogen [4–8] that are mainly produced by Leydig cells also play a paramount role in germ cell development, apoptosis, and cell adhesion function,

^{*}Address correspondence to these authors at the Center for Biomedical Research, Population Council, 1230 York Avenue, New York, New York 10065, USA; Fax: 212 327 8733; Y-Cheng@popcbr.rockefeller.edu; or Department of Surgery, Faculty of Medicine, The University of Hong Kong, Hong Kong; nikkilee@hku.hk.
NPYL's present address: Nikki Lee, Ph.D., Research Assistant Professor, Department of Surgery, Faculty of Medicine, The University of Hong Kong, Hong Kong, China.

illustrating functional cross-talks between the seminiferous tubules and the inter-tubular interstitium.

In rodents, undifferentiated spermatogonia known as A single (A_s) (also referred as germline stem cells), A paired (A_{pr}), and A aligned (A_{al}), are found in the seminiferous epithelium adjacent to the basal lamina (or basement membrane, a modified form of extracellular matrix, ECM) [9]. During spermatogenesis, some of these cells enter *mitosis* and differentiate into type A spermatogonia (A_1 , A_2 , A_3 , and A_4), intermediate spermatogonia (A_{In}), and type B spermatogonia. Type B spermatogonium is the only cell type that enters *meiosis*, differentiating into primary and secondary spermatocytes, to be followed by cellular differentiation into round spermatid after undergoing two reduction divisions. These round spermatids will then transform into elongating and elongated spermatids, which eventually become spermatozoa *via spermiogenesis*. During spermiogenesis, each round spermatid undergoes extensive morphological changes, typified by the formation of acrosome, condensation of the chromatin in the nucleus behind the acrosome, changes in the shape of the spermatid head, elongation of the spermatid tail, and removal of the residual cytoplasm known as residual body. Eventually, spermatozoa are released to the tubule lumen at *spermiation* [10,11] at stage VIII of the seminiferous epithelial cycle in adult rat testes. These four phases of spermatogenesis: mitosis, meiosis, spermiogenesis and spermiation, take about 58 days to complete in rats [12–14]. In addition to the dividing germ cells, mammalian adult testes, including rodents, also harbor non-dividing cells, such as Sertoli cells in the seminiferous epithelium of the seminiferous tubules, and Leydig cells in the interstitial compartment [15]. Sertoli cells provide structural and nourishing supports to the developing germ cells in the seminiferous epithelium, and each Sertoli cell can support up to 30–50 germ cells at different stages of their development. Spermatocytes which include early preleptotene and late leptotene spermatocytes, will traverse the blood-testis barrier (BTB) at stages VIII-IX of the seminiferous epithelial cycle [16,17] while differentiating into zygote spermatocytes. Thus, spermatocytes migrate from the basal to the apical compartment of the seminiferous epithelium, where they differentiate gradually into round spermatids and undergo spermiogenesis.

During these developmental changes, it is obvious that extensive junction restructuring takes place at the Sertoli-Sertoli cell and Sertoli-germ cell interface to facilitate proper orientation and migration of germ cells in the seminiferous epithelium. In the testis, junctions are broadly classified into three major types: anchoring junction, tight junction (TJ), and gap junction (GJ) [18–21] (Fig. 1). Based on the associated underlying cytoskeleton, the anchoring junction can be further categorized into: (i) actin filament-based adherens junctions (AJs) and focal adhesions at the cell-cell and cell-matrix interface, respectively; and (ii) intermediate filament-based cell-cell desmosome-like junctions and cell-matrix hemidesmosomes (Fig. 1). Unlike other epithelia and/or endothelia where different junction types are present in separate discrete locations, testis has hybrid junctions scattered in the seminiferous epithelium. This results in the existence of various junctions in close proximity with each other in the seminiferous epithelium. The junction heterogeneity is best illustrated at the BTB, which is constituted by adjacent Sertoli cells, that segregates the epithelium into the basal and the apical compartment (Fig. 1). For instance, the BTB in rat testes is composed of TJ, AJ (i.e., basal ectoplasmic specialization, basal ES), GJ, and desmosome-like junctions [18,21–23]. The molecular architecture of some of the known integral membrane proteins and their peripheral proteins at the BTB is depicted in Fig. (2).

Sertoli cells regulate germ cell maturation, at least in part, by modulating the homeostasis of hormones, cytokines, proteases, and protease inhibitors in the microenvironment of the seminiferous epithelium [24,25]. Cytokines, such as transforming growth factor- β (TGF- β), are known to take an active role in germ cell development and maturation [24,26,27]. Proteases, such as the caspase family members, are shown to involve in germ cell apoptosis in the testis,

which is one of the crucial mechanisms to maintain the optimal germ cell number for development that can be functionally supported by the Sertoli cells, since the Sertoli cell number remains relatively constant after puberty by day 15–18 in rats and can only support a limited number of germ cells [28]. In fact, about 75% of the developing germ cells in the seminiferous epithelium undergo apoptosis and be phagocytosed by Sertoli cells. Other proteases and protease inhibitors, such as cathepsins and α 2-macroglobulin, are some other key players to sustain germ cell development and cellular reorganization in the seminiferous epithelium during the seminiferous epithelial cycle [29–31]. Thus, germ cell development and maturation are precisely coordinated at the Sertoli-germ cell interface. This prompted us and others to examine if a disruption of communication at the cell-cell interface would compromise spermatogenesis. To test this hypothesis, our laboratory performed some studies in the past to define the involvement of different target molecules during junction restructuring at the Sertoli-Sertoli and Sertoli-germ cell interface. Using an *in vitro* culture system of Sertoli cells isolated from 20-day-old rats, for which these cells are non-dividing and are capable of establishing a functional TJ-permeability barrier that mimics the BTB *in vivo*, it was shown that the assembly of the TJ-barrier coincided with a transient induction in the steady-state mRNA and/or protein levels of occludin and cadherins [4,20,32]. To further expand these earlier observations and to assess the significance of these results at the *in vivo* level, different animal models are developed and used as tools to examine the roles of junction dynamics on male fertility and these models are discussed in the following sections. These studies have illustrated conclusively that an abrogation of the junction integrity or functionality hampers spermatogenesis. Perhaps this is best summarized in the rat model that involves adjuvin treatment. Adjuvin [1-(2,4-dichlorobenzyl)-1H-indazole-3-carbohydrazide] (Fig. 3) is a potential male contraceptive that exerts its effects, at least in part, *via* a disruption of germ cell adhesion, leading to premature germ cell loss from the seminiferous epithelium [33]. During the course of this work, different junction proteins, including transmembrane proteins and peripheral proteins, were shown to be involved in the events of adjuvin-induced aspermatogenesis (Table 1). Furthermore, the related signaling pathways that regulate these changes were also identified [34]. Based on these findings, the interrelated signaling networks that connect different junction types in the seminiferous epithelium are unveiled.

ANIMAL MODELS

During the past two decades, investigators in the field have focused on studying the effects of different compounds in affecting spermatogenesis. A variety of compounds, such as adjuvin (an indazole-carboxylic acid derivative) (Fig. 3) or cadmium (a heavy metal), are administered to animals and their effects on spermatogenesis are investigated. On the other hand, attempts have also been made to disrupt spermatogenesis by interfering cell junction proteins, which is best illustrated in animal models involving the use of occludin and connexin peptides or by interfering androgen and vitamin A levels. Two *in vivo* approaches are described herein, in which animals become infertile reversibly or irreversibly following treatments with different compounds. It is apparent that findings derived from studies using the reversible models would have the potential of assisting the development of male contraceptives. However, studies from using the irreversible models not only can validate some critical findings derived from the reversible models, they also shed light on developing “*non-surgical*” sterilization approaches, products, or methods, possibly can replace surgical techniques such as vasectomy in men.

A. Reversible Models

i. Adjuvin—Adjuvin (Fig. 3) is a potential male contraceptive that induces the sloughing of germ cells from the seminiferous epithelium when administered to adult rats by gavage, *i.p.*, or *i.m.* at approximately 35–50 mg/kg b.w., 2 doses, *q*-1 wk [33,35]. Earlier studies have shown that one of the primary targets of adjuvin in the testis is the apical ES at the Sertoli cell-

elongating and/or elongated spermatid interface and desmosome-like junctions at the Sertoli-spermatocyte interface. This treatment causes premature release of germ cells, initially post-meiotic spermatids to be followed by spermatocytes, from the seminiferous epithelium, without apparent effects on the adhesion of spermatogonia to the basement membrane and/or to the Sertoli cells [35,36]. After the drug is metabolized and cleared, spermatogonia divide and subsequently differentiate and repopulate the epithelium within approximately 8–10 wk, which is about the time it takes for spermatogonia (diploid, 2n) to differentiate into spermatozoa (haploid, 1n) in rats [33]. Similar findings have recently been reported in rabbits. A pharmacokinetics study was performed in rabbits wherein adjuvin was administered either orally or *via* intravenous injection. In addition to the expected exfoliation of germ cells, this pharmacokinetics study also demonstrated a longer half-life of adjuvin in blood circulation in rabbits treated with adjuvin by gavage *versus* intravenous injection, whereas the oral route was less effective in triggering germ cell loss in comparison, possibly as a result of lower bioavailability [37]. Using the rat model, several anchoring junction proteins are known to have functional roles in sustaining Sertoli-germ cell adhesion during spermatogenesis. Among them, cadherins, catenins, and integrins, are the primary targets of adjuvin [17]. Besides, signaling molecules and their pathways associated with the adjuvin-mediated spermatogenic cell loss have been identified. It is known that (i) the integrin/Rho-associated protein kinase (ROCK)/LIM kinase (LIMK)/cofilin, (ii) the cadherin/c-Src (the cellular form of the *v-src* transforming gene of Rous Sarcoma virus, RSV)/carboxyl-terminal Src kinase (Csk), and (iii) the integrin β 1/phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB)/extracellular signal-regulated kinase (ERK) are three distinctive pathways that are activated during adjuvin-induced germ cell loss from the seminiferous epithelium. Studies conducted with the use of specific inhibitors against some of these signaling molecules have unequivocally demonstrated the involvement of these signaling proteins in anchoring junction restructuring pertinent to spermatogenesis [38–40].

In this context, it is noted that adjuvin is a potent compound to perturb germ cell adhesion in the seminiferous epithelium, in particular at those junctions between spermatids and Sertoli cells, causing premature release of germ cells from the seminiferous epithelium. Apart from cell junctions, adjuvin apparently affects spermatogenesis by altering the oxidative status of the mammalian testes. When rats were fed with adjuvin, a surge in oxidative stress was recorded which was associated with the event of AJ restructuring in the testes [41]. In another study, an activation of nitric oxide-associated pathway was detected in the testis when rats were treated with adjuvin by gavage [42]. These findings suggest that the adjuvin-induced germ cell loss from the epithelium is mediated *via* activation of several concurrent pathways involving signaling molecules and oxidative stresses. This transient infertility induced by adjuvin in adult animals implicates that adjuvin maybe developed as a male contraceptive. However, further investigation shows that its use in humans is limited due to the narrow margin between its efficacy and safety [33,35]. For instance, chronic administration of adjuvin to adult rats by gavage for 29 days at a dose that is effective to induce transient infertility leads to liver inflammation and skeletal muscle atrophy in 3 out of the 10 rats subjected to a chronic toxicity study [33]. An effort has been made in conjugating this drug to an FSH mutant, for which this FSH mutant-conjugated adjuvin is localized mainly to the testes by binding to the FSH receptors that are restricted to Sertoli cells. This FSH mutant-adjuvin conjugate causes germ cell loss and transient infertility in male rats and its side-effects in other organs are minimized due to its restricted localization in the testis [33]. However, the cost of preparing such a conjugate for contraceptive use is prohibitively expensive. Thus, much work is needed to address the issue of organ specificity and bioavailability. To this end, a formulation of adjuvin that can facilitate its absorption to the testes at the scrotum is of high priority to develop, since a first-pass of adjuvin in the testis instead of the liver can reduce hepatotoxicity and improve its bioavailability to the testes. This alternative approach takes advantage of the unique microvasculature of the testes that allows rapid heat exchange [43,44], since a 35°C optimal

temperature for spermatogenesis is maintained in the testis *versus* 37°C for the rest of the body in mammals (e.g., rats), such that a drug (e.g., adjuvin) can have its first-pass to the testis to exert its effects before it is metabolized and reaches the liver and kidney, thereby reducing its systemic toxicity. However, a more in-depth investigation is needed to verify the applicability of this method. Nonetheless, the use of adjuvin is a useful *in vivo* model to study Sertoli-germ cell adhesion and/or interactions pertinent to anchoring junction restructuring during spermatogenesis.

ii. Occludin Peptide—Occludin is one of the TJ integral membrane proteins at the Sertoli-Sertoli cell interface that confers TJ-permeability barrier function at the BTB in rat testes. It is noted that the use of a synthetic peptide against the second extracellular loop of rat occludin can cause a disruption of Sertoli cell TJ-permeability barrier both *in vitro* and *in vivo* [45]. This observation is consistent with an earlier study that used a chicken occludin peptide to disrupt TJ-permeability barrier function [46]. Furthermore, a direct administration of this synthetic peptide to testes can affect germ cell adhesion. A time-dependent loss of germ cells from the epithelium is observed until the time when the tubules are devoid of most of the germ cell populations except for spermatogonia, which will gradually repopulate the epithelium thereafter. Together, these studies have prompted us to speculate that a transient disruption of the occludin function at the BTB can lead to spermatogenic arrest. Indeed, when occludin peptide is administered directly to the testis *via* local injection [45] or *via* an FSH mutant as a carrier [47], its presence in the BTB microenvironment apparently disrupts the interactions between two interlocking occludin molecules of adjacent Sertoli cells. This causes a transient perturbation of the BTB and leads to a disruption of germ cell adhesion in the epithelium and germ cell exfoliation. After the occludin peptide is cleared metabolically, the BTB reseals and the disruptive effects of the occludin peptide will be reversed gradually by the time when spermatogonia enter the spermatogenic cycle. However, the precise mechanism(s) by which occludin peptide induces germ cell loss from the epithelium remains to be investigated. It is obvious that a cascade of signaling events is involved to cause germ cell exfoliation from the epithelium following a perturbation of the BTB. Since virtually all the post-meiotic germ cells are depleted from the epithelium when the BTB is compromised, it is unlikely that anti-sperm antibodies are produced so that normal spermatogenesis and fertility can be resumed in the treated rats.

iii. Intratesticular Androgen Suppression with Testosterone/Estrogen (T/E) Implants to Disrupt Spermatogenesis—The underlying mechanism of spermatogenic arrest mediated by the application of T/E implants in rats is analogous to the hormonal male “pills” that is currently under development for men [48–50]. In both systems, a transient surge in the systemic testosterone level leads to a shut-down of the hypothalamic GnRH production and reduces the release of pituitary LH, thus temporarily reducing the production of testosterone by Leydig cells [51,52]. The testosterone level within the testis under physiological conditions is approximately 100-fold higher than those found in the circulation and this high level is necessary for sustaining spermatogenesis [53]. In this model, a reduction in the intratesticular testosterone level can be achieved if testosterone is applied exogenously to the animals using T/E implants, thereby shutting down the release of GnRH from the hypothalamus as well as LH secretion from the pituitary gland, reducing the production of testosterone by Leydig cells in the testis, and this often leads to a failure in spermatogenesis with the occurrence of azoospermia and infertility. Recent studies have shown that a disruption of apical ES in the seminiferous epithelium is observed when the level of intratesticular testosterone is reduced [54,55]. In this approach, testosterone and estrogen are packaged separately into permeable ethylene and vinyl acetate tubing [54] and placed subcutaneously in adult rats, so that a steady release of both steroid hormones can be maintained over a period of time to suppress intratesticular androgen level [54,56]. It is noted that premature detachment of developing

spermatids from Sertoli cells and spermiation defect are observed in the testes of T/E-treated animals [2,57,58], in which these processes are resulted from the destruction of junction integrity at both apical ES and non-ES junctions [59]. Indeed, the disengagement of spermatids from Sertoli cells is shown to involve integrin-associated AJ complexes by stimulating the expression of $\alpha 6 \beta 1$ -integrin and the phosphorylation (i.e., activation) of focal adhesion kinase (FAK) [55], similar to those results obtained by using the adjuvin model wherein the integrin/FAK signaling pathway is activated during adjuvin-induced spermatid loss from the epithelium [60]. It is of interest to note that although the anchoring junction at the Sertoli-germ cell interface is compromised, possibly mediated by perturbing the cadherin/catenin-based AJ protein complexes *via* the action of kinases and phosphatases, such as myotubularin-related protein 2 and c-Src [61]; the BTB function, however, is strengthened plausibly *via* a surge in the production of integral membrane proteins (e.g., JAM-A, N-cadherin) at the BTB, perhaps being used to maintain the integrity of the immunological barrier [24,54]. This model is readily reversible, analogous to the adjuvin model, in which spermatogenesis resumes and germ cells repopulate the mostly spermatid-depleted epithelium following the removal of the implants.

iv. Vitamin A Depletion (VAD) and Post-Vitamin A Replenishment (PVA) Model

—Vitamin A is known to regulate male germ cell progression and maturation [62]. This model, perhaps, has some advantages over the other models. For instance, when vitamin A is depleted from diets over a period of time, the seminiferous epithelial stages in adult rat testes are synchronized, such that a homogenous stage is retained in the entire tubule at any given time [63,64]. After the replenishment of vitamin A, spermatogenesis resumes. Thus, each tubule possesses the same genomic and proteomic information and stage-specific molecules pertinent to spermatogenesis during the epithelial cycle can be tracked down [65,66]. Using this model coupled with differential display technology, a panel of cell type- and stage-specific genes was identified and separated from the bulk of gene pool. For instance, at least 24 differentially expressed genes were found using this approach [67]. Amongst these genes, AEP1 (acrosome expressed protein 1), previously known as VAD1.3, is found in developing testes from day 25 post-partum onwards, and this time period is associated specifically with the time of acrosome formation [68]. However, it remains to be determined how AEP1 is involved in acrosome formation. Also, much work is needed to assess the function of differentially regulated genes/proteins pertinent to their stage-specific expression/production during the epithelial cycle of spermatogenesis.

B. Irreversible Models

i. Cadmium Chloride—Cadmium chloride is a known environmental toxicant that affects male reproductive functions [69–71], in addition to its effects to induce cancers in the prostate, liver, and kidney [72–74]. The destructive and apparently nonreversible effects of cadmium chloride on spermatogenesis are mediated *via* its effects on different cell types in the testis. Among these, the Sertoli cell is the prime cellular target of cadmium toxicity [32,71,75]. It is known that mitochondrial damage in Sertoli cells is one of the ultrastructural phenotypes that can be detected readily after exposure of testes to cadmium chloride [76]. However, the mechanistic pathway(s) associated with these changes has not been fully deciphered. Recent studies investigating the mechanism(s) associated with cadmium toxicity in the testis, in which germ cell depletion from the seminiferous epithelium is observed, have shown that cadmium mediates its detrimental effects on spermatogenesis by impeding the junction integrity in the testes, and the BTB is one of the targets of cadmium toxicity. Besides TJ, both AJ and GJ are also vulnerable to cadmium toxicity [32,71,75,77] (Table 1). Thus, this model has been used to delineate the signaling pathways found in junction remodeling particularly at the BTB. For instance, transforming growth factor- $\beta 3$ (TGF- $\beta 3$)/p38 mitogen activated protein kinase (MAPK) is shown to be one of the signaling pathways that regulates BTB dynamics during the seminiferous epithelial cycle of spermatogenesis based on the studies using the cadmium

model [78]. These findings (see Table 1) provide a basis for identifying new means to protect testes against cadmium toxicity. The application of specific antagonist(s) or inhibitors of MAP kinases, for instance, can be used to examine whether it can block the destructive effects of cadmium toxicity on the BTB if it can be delivered specifically to the testis.

ii. Connexin Peptides—At least 11 connexins are found in the testes, while each connexin is unique but these proteins share some common consensus sequences [79,80]. Connexin peptides are antagonists, for which their sequences correspond to the extracellular loop of connexins, the connexin-binding domain. Recent studies have shown that their presence in the testis blocks the interactions between two connexin-binding domains of opposing connexins [81,82]. These properties of connexin have been used to design specific experimental tools to study the functions of connexins in rat testes. Connexin peptides were constructed and their sequences were based on those spanning either the conserved region of connexin family members (in the first extracellular loop) or the variable region of specific connexins (in the second extracellular loop). Each peptide was ~9 – 10 amino acid residues. After intratesticular injection of connexin peptides, it was reported that pan-connexin peptide targeting the conserved sequence among known testicular connexins induced germ cell apoptosis in the seminiferous epithelium, invoking the activation of a panel of apoptotic proteins, such as nuclear factor- κ B (NF- κ B) and caspase 3. This, in turn, led to germ cell dislodgment from the seminiferous epithelium. In the same study, connexin 31- and connexin 33-specific peptides had no apparent effects on the spermatogenesis status in the testis. Interestingly, using this model of functional knockout of connexins by peptide administration, it was shown that a disruption of the GJ function also led to a perturbation of the occludin-based TJ in the testis [29], illustrating the cross-talk and physiological links between different junction types in the seminiferous epithelium at the Sertoli-Sertoli and Sertoli-germ cell interface.

CROSS-TALKS BETWEEN JUNCTIONS IN THE SEMINIFEROUS EPITHELIUM: THEIR DISRUPTION CAN LEAD TO DYSFUNCTIONAL SPERMATOGENESIS

The testis is a unique organ with complex junction arrangements in the seminiferous epithelium (Fig. 1). The distribution of different junction types in the testis differs from those exhibited in other epithelia and endothelia wherein distinctive junction zones are found. Junctions in the seminiferous epithelium are scattered and co-exist at the same site throughout the Sertoli-Sertoli and Sertoli-germ cell interface [3,21] (Fig. 1). For instance, GJs are localized both at the basal and the apical compartments of the seminiferous epithelium, sometimes present side-by-side with anchoring junctions [21,83]. It is noted that the only exception to this atypical junction layout in the seminiferous epithelium is the apical ES wherein the appearance of apical ES surrounding the head of a step 8 spermatid becomes the “only” testis-specific AJ type at the Sertoli-spermatid interface until it is replaced by the apical tubulobulbar complex in late step 19 spermatid, just a few hours prior to spermiation. Regarding to the junction complexity, this is best exemplified by the BTB in the seminiferous epithelium, which lies adjacent to the basement membrane (Fig. 1).

At the BTB, it is composed of several coexisting junction types, such as TJ, anchoring junctions (e.g., basal ES), and GJ, interweaving with each others to form a complex junction network (Fig. 2). While the BTB is crucial to confer the immunological barrier, it must restructure (or “open”) to facilitate the transit of preleptotene and leptotene spermatocytes from the basal compartment to the adluminal compartment at stages VIII-IX of the epithelial cycle [16,84]. In order to accomplish these tasks, different junctions at this site are apparently under precise interactive coordination and regulation. It was proposed initially that these events occurred *via* changes in the structural interactions between the integral membrane proteins and their

peripheral adaptors [85]. These linkers and/or signaling proteins reside either in the cytosol, nucleus, or close to the plasma membrane, and they can shuttle between different protein complexes when required. This phenomenon was explored in several recent studies using different animal models. In a model to study TJ function following treatment of adult rats with cadmium chloride, the loss of occludin and its adaptor, ZO-1, from the BTB was shown to associate with a loss of adhesion of the cadherin/catenin and the nectin/afadin-based AJ complexes [86]. Also, a disruption of connexins and ZO-1 in GJ using pan-connexin peptide was demonstrated to trigger a mislocalization of N-cadherin (an AJ protein) and a reduction on the steady-state level of occludin (a TJ protein) [29]. However, these junction-to-junction interactions do not always lead to a down-regulation of adjacent adhesion protein complexes. In one study using the androgen suppression model, rats were treated with T/E implants to lower the intratesticular androgen level, thereby perturbing AJ adhesion between Sertoli and germ cells. This androgen suppression-induced loss on the AJ integrity, surprisingly, was shown to strengthen the BTB by inducing more occludin and ZO-1 production [54]. In light of these findings, we postulate that the junction-to-junction cross-talk is largely dependent on the reversibility of the study model. In the irreversible model, a primary destruction of one junction type (e.g., BTB damage induced by cadmium) usually leads to a secondary damage of the other junctions (e.g., anchoring junctions at the Sertoli-germ cell interface) that leads to germ cell loss. However, this phenomenon is not found in the reversible study models. For instance, in the androgen suppression model, the integrity of other junctions (e.g., BTB), however, is enhanced (as shown by a surge in occludin and ZO-1 production at the BTB) when a junction type is targeted (such as the apical ES). This also illustrates that in the reversible models involving the use of either adjuvin or T/E implants in adult rats, a physiologically 'protective' mechanism is 'switch-on' when one junction type is being destroyed (e.g., anchoring junction disruption in the adjuvin treated rats), this is being done to prevent any damage to the anchoring junctions at the BTB as manifested by the surge in occludin and ZO-1 production at the BTB [87] (see Fig. 1B). Apparently this maybe the same mechanism that the testis is used to protect BTB integrity during spermatid movement across the seminiferous epithelium involving extensive restructuring of anchoring junctions at the Sertoli-germ cell interface, such that the immunological barrier can be maintained (Fig. 1B). In short, studies using these *in vivo* models have shed lights in the regulation of different junction types during spermatogenesis.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

With the available information in the literature in recent years, it would be difficult to refute the concept that cell junction in the seminiferous epithelium is one of the prime targets for male fertility regulation, perhaps, its dysfunction is one of the many causes of male infertility. This notion is further supported by recent findings in different animal models. These animal models can now be used by investigators to decipher the molecular mechanisms associated with male infertility, such as those induced by environmental toxicants, this information should also be helpful to identify novel targets for developing male contraceptives. Some of these models are reversible and some are irreversible. For reversible models, several chemical entities, such as adjuvin and occludin peptide, were shown to cause spermatogenic arrest. Suppression of intratesticular androgen using T/E implants, for instance, also lead to reversible germ cell sloughing from the testes. In parallel to these models, animals treated with cadmium chloride or connexin peptide were shown to induce permanent infertility. Due to the irreversible nature of these models, they could be novel to develop new means of non-surgical sterilization for men.

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Fig. 1. A schematic drawing illustrating the different junction types found in the seminiferous epithelium, and the mechanism that induces germ cell depletion following treatment of adult rats with different chemicals/drugs being investigated for contraceptive development as illustrated in three study models

As discussed in the text, germ cell loss from the seminiferous epithelium occurs as a result of the disruption of adaptor dynamics and junction integrity in the seminiferous epithelium (A). Only three representative animal models for studying junction dynamics that can lead to germ cell loss from the seminiferous epithelium are shown in (B). When cell adhesion protein complexes fail to anchor onto the actin-based cytoskeletal network possibly *via* a disruption of the adaptor function, germ cells deplete from the epithelium, leading to a thinning of the seminiferous epithelium. During spermatogenesis, germ cells that migrate across the epithelium while differentiating into fully developed spermatids are released into the tubule lumen as spermatozoa at spermiation. During this process of germ cell movement, extensive restructuring of junctions occurs including tight junction (TJ), adherens junctions (AJ), and gap junction (GJ). These junctions share similar structural organization, such that each of them possesses a transmembrane protein and the associated adaptors as the basic architectural unit (see Fig. 2). For instance, connexin is a transmembrane protein that binds to ZO-1, a peripheral adaptor, at the site of GJ. These junctions are scattered throughout the seminiferous epithelium as shown in A. In order to decipher the correlation of junction dynamics and male fertility, different animal models are established, such as the adjudin model, the pan-connexin peptide model, and the occludin peptide model, for studying AJ, GJ, and TJ, respectively, as shown in B. Data derived from these *in vivo* models have shown that there are cross-talks among these junctions. Also, a dysfunction of a junction type can either reinforce (adjudin, Model I) or hamper (pan-connexin peptide, Model II) another junction type by stimulating or inhibiting the production of specific integral membrane proteins and their associated adaptors. For instance, a disruption of anchoring junctions in the adjudin model that causes germ cell loss can induce enhanced production of occludin and ZO-1 at the BTB to protect the immunological

barrier from being compromised (Model I). However, a disruption of GJ by the use of pan-connexin peptide (Model II) leads to a loss of anchoring junction function, inducing germ cell depletion. For the occludin peptide model (Model III), it is noted that the BTB disruption induced by the use of synthetic occludin peptide that leads to secondary germ cell loss is reversible. This is likely due to the fact that when the BTB is compromised, most of the post-meiotic germ cells have been depleted from the epithelium, thus, this fails to elicit anti-sperm antibody production. Spermatogonia can repopulate the epithelium with germ cells when the occludin peptides are metabolized and spermatogenesis resumes. Abbreviations used: Apical ES, apical ectoplasmic specializations; basal ES, basal ectoplasmic specializations; BTB, blood-testis barrier; desmosome, desmosome-like junctions; ES_p, elongated spermatid; N, Sertoli cell nucleus; P/L-SPC, preleptotene/leptotene spermatocyte; P-SPC, pachytene spermatocyte; RSp, round spermatid; SPG, spermatogonium.

Fig. 2. The molecular architecture of different protein complexes found at the blood-testis barrier (BTB) in rat testes

BTB in the testis is composed of co-existing tight junction (TJ), gap junction (GJ), and basal ectoplasmic specialization (basal ES, an atypical adherens junction, AJ, type). The components of the junction complexes are similar to those found in other epithelia and endothelia in different blood-tissue barriers, such as the blood-brain barrier or blood-retina barrier. GJ is composed of connexin-based complexes and at least 11 connexins are found in the testis. Connexin links to the underlying cytoskeletons *via* ZO-1 and ZO-2, these two adaptors have also been found to localize in TJ complexes. Occludin, junctional adhesion molecules (JAMs), and claudins are typical transmembrane proteins found at TJ in the BTB. ES is found in two locations in the

testis: one is present at the Sertoli cell-elongating or elongated spermatid interface at the apical ES, and the other is found at the BTB, known as basal ES, between two adjacent Sertoli cells in the seminiferous epithelium. Cadherin is the most studied ES protein and it is structurally associated with catenins, such as p120^{ctn}, β -catenin, and α -catenin. Fer, FAK, and c-Src, are several of the protein kinases known to be associated with the cadherin-based protein complex in the testis. Abbreviations used: FAK, focal adhesion kinase; Fer, Fer kinase; JAM, junctional adhesion molecule; ZO, zonula occludens.

Adjudin, 1-(2,4-dichlorobenzyl)-1H-indazole-3-carbohydrazide

Fig. 3.

The structural formula of adjudin, 1-(2,4-dichlorobenzyl)-1H-indazole-3-carbohydrazide.

Table 1

Studies using Different Animal Models to Assess the Effects of Different Compounds and/or Environmental Toxicants on Molecules at the Cell Junctions in the Testis

Animal models	Junctions	Target junction proteins	Selected references
Reversible study model:			
Adjudin	AJ	N-cadherin (↑), E-cadherin (↑), α -catenin (↑), β -catenin (↑), p120 ^{cas} (↑), zyxin (↓), axin (↓), WASP (↓), nectin-3 (↓), afadin (↓), laminin γ 3 (↓), integrin β 1 (↑), vinculin (↑)	Siu and Cheng, 2004 [88] Siu <i>et al.</i> , 2003 [60] Xia <i>et al.</i> , 2007 [89] Lee <i>et al.</i> , 2004 [90]
Occludin peptide	TJ	occludin (↓)	Chung <i>et al.</i> , 2001 [45] Wong <i>et al.</i> , 2007 [47] Wong and Gumbiner, 1997 [46]
T/E implant	AJ	α -actinin (↑), vinculin (↑), N-cadherin (↑), β -catenin (↑), β 1-integrin (↑), α -catenin (↑), nectin-3 (↑), I-afadin (↑), E-cadherin (↑)	Wong <i>et al.</i> , 2005 [31] Xia <i>et al.</i> , 2005 [54] Beardsley <i>et al.</i> , 2006 [55] Zhang <i>et al.</i> , 2005 [61]
	TJ	occludin (↑), JAM-1 (↑), ZO-1 (↑)	Xia <i>et al.</i> , 2005 [54]
Irreversible study model:			
Cadmium chloride	AJ	E-cadherin (↓), N-cadherin (↓), nectin-3 (↓), β -catenin (↓), γ -catenin (↓), I-afadin (↓), p120 ^{cas} (↑)	Wong <i>et al.</i> , 2004 [86]
	TJ	occludin (↓), ZO-1 (↓)	Wong <i>et al.</i> , 2004 [86]
	GJ	connexin 43 (↓)	Fiorini <i>et al.</i> , 2004 [77]
Pan-connexin peptide	GJ	connexin 31 (↓), connexin 32 (↓), connexin 33 (↑) connexin 43 (↓), ZO-1 (↓)	Lee <i>et al.</i> , 2006 [29]
	TJ	occludin (↓)	Lee <i>et al.</i> , 2006 [29]

Abbreviations used: JAM, junctional adhesion molecule; WASP, Wiskott-Aldrich syndrome protein; ZO, zonula occludens