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Sertoli cells are the target of environmental toxicants in the testis – a mechanistic and therapeutic insight

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Abstract

Introduction—Sertoli cells support germ cell development in the testis via an elaborate network of cell junctions that confers structural, communicating, and signaling support. However, Sertoli cell junctions and cytoskeletons are the target of environmental toxicants. Because germ cells rely on Sertoli cells for the provision of structural/functional/nutritional support, exposure of males to toxicants leads to germ cell exfoliation due to Sertoli cell injuries. Interestingly, the molecular mechanism(s) by which toxicants induce cytoskeletal disruption that leads to germ cell exfoliation is unclear, until recent years, which are discussed herein. This information can possibly be used to therapeutically manage toxicant-induced infertility/subfertility in human males.

Areas covered—In this review, we provide a brief update on the use of Sertoli cell system developed for rodents and humans *in vitro*, which can be deployed in any research laboratory with minimal upfront setup costs. These systems can be used to collect reliable data applicable to studies *in vivo*. We also discuss the latest findings on the mechanisms by which toxicants induce Sertoli cell injury, in particular cytoskeletal disruption. We also identify candidate molecules that are likely targets of toxicants.

Expert opinion—We provide two hypothetical models delineating the mechanism by which toxicants induce germ cell exfoliation and blood–testis barrier disruption. We also discuss molecules that are the targets of toxicants as therapeutic candidates.

Keywords

environmental toxicant; Sertoli cells; spermatogenesis; testis

1. Introduction

About 15% of couples are infertile, and almost 50% of these cases are contributed by men [1]. In fact, infertility is an emerging global public health issue after cancer and cardiovascular diseases, thus illustrating the necessity of identifying the causes of male infertility. Although there are reports demonstrating a trend of rising male infertility and subfertility in recent decades since the 1980s based on analysis of semen quality, such as

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reducing sperm counts in various countries [2,3], including a rise of testicular cancer [4], others failed to confirm these observations since there are regional variations [5,6]. Recent studies, however, have generally confirmed that the trend of reducing male fertility based on sperm counts and semen analyses is likely the result of an increase in exposure of men to environmental toxicants – an emerging determining key factor that links reduced sperm counts and infertility [7,8]. However, the precise molecular mechanisms by which environmental toxicants induce male reproductive dysfunction remain unknown. As such, therapeutic steps that can be taken to reverse the reduced semen quality such as declining sperm counts and/or sperm motility in men are not available. Recent advances in genomics, proteomics and metabolomics have identified a number of biomarkers that can be used to study the etiology of toxicant-mediated male infertility, which include cystic fibrosis transmembrane regulator, clusterin, protamine-2, androgen receptor (AR), A-kinase anchor protein 4, sperm-associated antigen 1B and others [9], thus illustrating that genetic defects can contribute to male infertility. Nonetheless, these defects could also be caused by environmental toxicants since many of these toxicants (e.g., cadmium, perfluorooctane sulfonate [PFOS]) are known carcinogens [10-12]. For instance, studies have shown that one of the effects by which cadmium induces carcinogenesis is mediated by mutagenesis [10]. Also, oxidative stress induced by toxicants is an emerging mechanism that disrupts male fertility [13,14]. Additionally, microtubule (MT)-based cytoskeleton in the testis is an emerging target of toxicants [15,16]. Collectively, these findings illustrate that environmental toxicant-induced male reproductive dysfunction can be better managed if the target(s) and/or candidate molecule(s) in the testis can be identified and better investigated. In this context, it is of interest to note that Sertoli cells, in particular Sertoli cell–cell and Sertoli–germ cell junctions and the associated underlying actin-based cytoskeleton, have recently been identified as one of the primarily targets of toxicants including human Sertoli cells [17]. Because the Sertoli cell is the only somatic cell type in the seminiferous epithelium that supports spermatogenesis, these findings coupled with earlier studies reporting that Sertoli cell MT-based cytoskeleton is the target of toxicants [15,16] thus provide a likely roadmap to tackle toxicant-induced male reproductive dysfunction.

Many environmental toxicants are natural or synthetic compounds that are extensively used in the industry, many of them have already been integrated into our food chain, yet they are known to disrupt normal function of the testis in particular following acute exposure or during fetal development in rodents and possibly humans [18,19]. These compounds include bisphenol A (BPA), cadmium, polychlorinated biphenyls, pesticides, phthalates, PFOS and others. Although we have reviewed the role of environmental toxicants on male reproductive dysfunction [18,20], the molecular mechanism(s) by which environmental toxicants perturb Sertoli cell function, in particular how toxicants perturb Sertoli cell–cell and Sertoli–spermatid cell junctions have not been critically evaluated based on recent findings in particular the use of human Sertoli cells. Moreover, studies have shown that the Sertoli cell culture system *in vitro*, in particular human Sertoli cells, can be a novel model to study the molecular mechanism by which toxicants induce male reproductive dysfunction. Thus, we critically evaluate these findings and propose two models which serve as the framework for future investigations.

2. *In vitro* models to study environmental toxicant-induced testicular damage

One of the major obstacles to identify the target(s) of environmental toxicants such as endocrine disrupting chemicals in the testis is the lack of a suitable *in vitro* model which can reliably translate findings from *in vitro* to *in vivo*. In the 1980s, several laboratories reported that Sertoli cells cultured *in vitro* in serum-free chemically defined medium can serve as a reliable model to study blood–testis barrier (BTB) function [21,22]. Subsequent studies have shown that this model mimics the Sertoli cell BTB *in vivo* both functionally and structurally since ultrastructures of tight junction (TJ), basal ectoplasmic specialization (basal ES), gap junction (GJ) and desmosome are found in these cultures besides the presence of a TJ-permeability barrier [23,24]. As such, multiple investigators have used this system for studies in their laboratories to better understand the biology of BTB, and many of these earlier findings have also been reproduced *in vivo*, illustrating the physiological relevancy of this *in vitro* model [23]. Because the BTB confers a major obstacle for the access of environmental toxicants to the testis, this model thus represents a major breakthrough to understand the biology of toxicant-induced testicular dysfunction, in particular how toxicants get access to the adluminal compartment to perturb germ cell function including meiosis and subsequent differentiation of haploid spermatids into spermatozoa. It is now established that Sertoli cells isolated from 20-day-old rat testes are capable of assembling a functional TJ-permeability barrier with ultrastructures of TJ, basal ES, GJ and desmosome in ~ 2 – 3 days in serum-free F12/DMEM with nutritional supplements, and Sertoli cell BTB function can be reliably monitored by assessing the transepithelial electrical resistance across the cell epithelium when Sertoli cells are cultured on Matrigel®-coated bicameral culture chambers/units [24]. Interestingly, these Sertoli cells can be obtained in high yield from 20-day-old male pups with a purity of ~ 98%; they are differentiated and cease to divide, mimicking adult Sertoli cells functionally, and contaminated with negligible Leydig, peritubular myoid and germ cells [24] versus Sertoli cells isolated from adult rodent testes with a maximal purity of ~ 85% [25]. Additionally, Sertoli cells can be cultured on Matrigel-coated coverslips, so that changes in localization and/or distribution of integral membrane proteins and associated peripheral adaptors at the Sertoli cell–cell interface as well as actin- and/or MT-based cytoskeletons can be assessed in parallel experiments. If needed, Sertoli cells can also be cultured in 12- or 24-well culture dishes, so that lysates can be obtained from these cells to assess changes in the steady-state levels of proteins and/or mRNAs by immunoblotting or reverse transcription polymerase chain reaction/quantitative polymerase chain reaction. Besides, additional biochemical assays can be performed to monitor changes in the bundling activity, as well as polymerization, and depolymerization kinetics of actin microfilaments and/or microtubules. These findings can then be used to validate and expand other morphological findings. If a target gene (or protein) or a set of relevant genes (or proteins) are known to be involved in mediating a toxicant-induced phenotype (e.g., a disruption or a tightening of the TJ barrier function), a downstream/common signaling molecule can be knocked down by RNA interference (RNAi) to confirm the finding before pertinent *in vivo* studies are conducted. Using such an approach, some advances are made in recent years, which are critically evaluated below. Furthermore, it is known that testes from rodents and humans can respond differently to the same EDC [26]; also, some TJ proteins,

such as occludin, are only found in rodent but not human testes [17,27], whereas others, such as claudin-3, are found in humans but not rat testes [28]. Thus, it is important to perform studies using human Sertoli cells instead of extrapolating data from studies in rodents to generalize the molecular mechanism(s) of a toxicant in the testis. An important development in recent years is the initial observation that Sertoli cells, both in rodents and humans, when cultured in serum-containing medium *in vitro* remain mitotically active [17,29,30]. Furthermore, these cells can be cryopreserved and stored in liquid nitrogen for years and remain viable for subcultures [17,30]. Typical ultrastructures of Sertoli cells (e.g., lipid droplets) and Sertoli cell-specific markers (e.g., GATA-4 and SOX9) are also detected in these cells [30]. When cultured in F12/DMEM containing fetal bovine serum (FBS, 5% vol/vol), penicillin (100 units/ml) and streptomycin (100 µg/ml) at 35°C in a humidified environment with 95% air/5% CO₂, the doubling time of human Sertoli cells is routinely ~ 4 days [17,30]. Furthermore, a functional TJ-permeability barrier is established when human Sertoli cells are cultured on human fibronectin-coated bicameral inserts [17,30], analogous to rat primary Sertoli cells. Thus, human Sertoli cells derived from a single individual can be used for multiple analyses without opting to obtain fresh human testes for their isolation, making it possible for a detailed and careful analysis of environmental toxicant-induced Sertoli cell injury using human Sertoli cells from a small group of men as illustrated in a recent report [17]. As such, this system provides a valuable tool in studies to investigate environmental toxicant-induced Sertoli cell injury such as the BTB function. However, it is noted that the metabolism of toxicants *in vitro* can be different from *in vivo*. Thus, other methods, such as *in vivo* animal models, and high-throughput genomics and proteomics, may be necessary to combine with this *in vitro* system to provide the mechanistic insights on toxicant-mediated male reproductive dysfunction.

3. Mechanisms of environmental toxicant-induced testicular damage

3.1 Bisphenol A

3.1.1 Introduction—BPA (2, 2-bis [4-hydroxyphenyl] propane) is an environmental toxicant widely used for manufacturing of polyesterstyrene, epoxy and polycarbonate resins, which are used for the production of household items such as water and baby bottles, food and beverage containers to confer their flexibility and durability. BPA is also present in dental fillings and sealants, thermal paper receipts and others. BPA is a reproductive toxicant, in particular, following *in utero* exposure and also in fetal/neonatal rodents and perhaps humans [31,32]. Human exposure to BPA is mainly through contaminated food and water, but BPA can also leach out of plastic or polycarbonate containers and bottles, especially under elevated temperature [33]. The estimated human daily intake of BPA is 34 ng/kg b.w. [19]. BPA is an EDC that exerts its effect by binding to estrogen receptors, ARs and/or thyroid hormone receptors [34]. Because these steroid receptors are found in Sertoli cells [35,36], BPA can exert its effects via these receptors to impair Sertoli cell function. Emerging epidemiological and experimental evidence indicates that BPA has adverse effects on male reproductive function following *in utero* and fetal/neonatal exposure because adults are more tolerant to BPA [37-39], perhaps due to its short half-life, < 2 h [39]. For instance, treatment of adult rats with BPA at 0.02 – 50 mg/kg b.w. by oral gavage does not impair spermatogenesis; however, it disrupts BTB integrity when neonatal rats at 20 dpp are

exposed to acute doses of BPA (50 mg/kg b.w./day for 6 doses) or when Sertoli cells isolated from rats are exposed to BPA at 40 – 200 μ M [37]. Administration of BPA to pubescent versus adult mice induces degenerative changes in the seminiferous epithelium, leading to reduced sperm counts (but not in adult mice) by maturation, and this disruptive effect of BPA in immature mice can be further potentiated by X-ray exposure [40].

3.1.2 BPA perturbs Sertoli cell function via MAPK signaling pathway—

In mammals, MAPK pathways are composed of three independent signaling cascades mediated by either extracellular-signal-regulated kinase (ERK), c-Jun N-terminal protein kinase (JNK) or p38. Fetal exposure of rats to BPA was shown to activate Raf1, p-ERK1 in the testis, and *in situ* hybridization analysis confirmed a BPA-induced upregulation on the expression of Raf1 and ERK1/2 mostly in Sertoli cells [41]. Consistent with this finding, BPA was also reported to upregulate p-ERK expression in Sertoli cells during BPA-mediated Sertoli cell TJ-permeability barrier disruption [37]. Subsequent study has shown that BPA-induced pERK1/2 activation quickly returned to its basal level following BPA removal in Sertoli cell cultures with an established functional TJ barrier, illustrating that BPA-induced Sertoli cell TJ barrier disruption is likely mediated via the ERK pathway [42]. Also, PD98059, a selective inhibitor of ERK, was shown to repress the BPA-induced ERK1/2 phosphorylation/activation in Sertoli cells [43]. BPA is also found to induce downregulation and redistribution of junction proteins, such as occludin, zonula occludens 1 (ZO-1), N-cadherin, β -catenin or connexin 43 (Cx43) at the cell–cell interface using Sertoli cells isolated from rat testes [37] or human testes [17] with an established TJ barrier that mimics the Sertoli cell BTB *in vivo*. It remains to be determined if these BPA-induced changes in cell junction protein expression and/or distribution/localization can be blocked or reversed by using specific inhibitors or antagonists of ERK1/2. Nonetheless, these data indicate that BPA exerts its disruptive effects on the Sertoli cell TJ barrier via ERK, at least in part, involving downregulation on the expression and/or localization of integral membrane proteins and their peripheral adaptors at the cell–cell interface.

3.1.3 BPA perturbs Sertoli cell GJ communication—

GJs are intercellular communication channels between adjacent cells in an epithelium, such as the seminiferous epithelium, so that chemical and/or biological signaling molecules (usually < 1 kDa) can be transported between adjacent cells (e.g., Sertoli–Sertoli, Sertoli–germ or germ–germ cells), which are necessary to coordinate cellular events of the epithelium in response to changes in the environment or intrinsic/extrinsic biological stimuli [44,45]. In the testis, Cx43 is the predominant GJ protein. BPA was found to downregulate the expression of Cx43 and its phosphorylation isoform p-Cx43-Ser³⁶⁸ in Sertoli cells, causing redistribution of Cx43 from Sertoli cell–cell interface by moving into cell cytosol via endocytosis (Table 1) [37,42]. Cx43 is known to have 19 phosphorylation sites, which can be phosphorylated by MAPK, c-Src, PKC or Akt (also known as PKB), and these kinases act on different phosphorylation sites to regulate GJ pore size, assembly and turnover, thereby modulating Cx43-based GJ function [46]. For instance, phosphorylation of Cx43 at Ser373 by Akt was found to eliminate the interactions of Cx43 with ZO-1, causing enlargement of GJ pore size [47,48]. PKC was shown to phosphorylate Cx43 at Ser368, contributing to a disruption of Cx43-based intercellular communications [49]. Using a functional GJ assay based on the

fluorescence recovery after photobleaching (FRAP), BPA was found to impede intercellular communications between Sertoli cells [42]. A study using molecular modeling analysis also revealed the presence of a putative docking domain in Cx26 that can structurally interact with BPA [18]. Collectively, these findings support the notion that GJ in Sertoli cells is likely one of the targets of BPA in the testis. Studies have shown that Cx43 interacts with desmosomal adaptor protein plakophilin-2 to create a functional protein complex, and this complex is necessary to coordinate other junction types at the BTB, such as TJ, basal ES, GJ and desmosome, to maintain the BTB integrity [50]. This notion is supported by findings that a knockdown of both Cx43 and plakophilin-2 by RNAi, but not either protein alone, induced Sertoli cell TJ-permeability barrier disruption, illustrating that a disruption of GJ function can perturb Sertoli cell TJ barrier integrity. Thus, when BPA disrupts Sertoli cell GJ communication at the BTB, this in turn perturbs coordination of various junction types at the BTB to maintain its integrity, effectively inducing BTB breakdown, making the testis more susceptible to toxicants.

3.1.4 BPA induces Sertoli and germ cell apoptosis—Studies have shown that BPA induces cytotoxicity in Sertoli cells causing apoptosis in Sertoli cells cultured *in vitro* [51]. The BPA-induced apoptosis in Sertoli cells is likely mediated via the phosphatase and tensin homolog (Pten)/Akt, JNK/p38 MAPK, NF- κ B and/or Fas/FasL signaling molecules downstream [52,53]. CaM-CaMKII-ERK [Calmodulin (CaM) and Ca²⁺/CaM-dependent protein kinase II-ERK] pathway was also shown to play a role in BPA-induced apoptosis in TM4 cells (a mouse Sertoli cell line) (Figure 1) [54]. BPA was also found to induce aggregation of actin filaments in Sertoli cells [51], suggesting that BPA may target cytoskeleton that leads to Sertoli cell apoptosis (Figure 1). In this context, it is of interest to note that BPA also induces germ cell apoptosis via p38 MAPK pathway and the transmembrane metalloprotease A Disintegrin and Metalloprotease-17 (Figure 1) [55]. It is likely that p38 MAPK is the common signaling molecule that mediates the BPA-induced Sertoli and germ cell apoptosis. It is also possible that there are crosstalks between MAPK and other signaling molecules shown in Figure 1 during BPA-induced Sertoli and germ cell apoptosis. Needless to say, further studies are needed to delineate the cascade of events by which these signaling molecules mediate BPA-induced Sertoli and/or germ cell apoptosis and if p38 MAPK is the key player in these events.

3.1.5 BPA perturbs human Sertoli cell actin cytoskeleton—When human Sertoli cells cultured *in vitro* are exposed to BPA, the organization of F-actin network is affected, and the extent of damage is dose-dependent [17]. For instance, at 0.4 μ M, BPA was found to cause truncation of actin microfilaments in Sertoli cells, but at higher concentrations, such as 40 and 200 μ M, BPA caused retraction and defragmentation of actin microfilaments, in which the truncated microfilaments retracted from cell cytosol and localized closely to the Sertoli cell nucleus [17]. These changes apparently are the result of mislocalization of two actin regulatory proteins, namely branched actin nucleation-inducing protein Arp3 and actin barbed end capping and bundling protein Eps8, so that actin microfilaments fail to properly organize into a bundled network to support Sertoli cell BTB function [17]. These findings should now be expanded to investigate if these BPA-induced cytoskeletal disruptions in

human Sertoli cells are mediated by p-focal adhesion kinase [FAK]-Tyr³⁹⁷ or p-FAK-Tyr⁴⁰⁷, as noted in the rat testis [56].

3.1.6 Summary—i) BPA disrupts Sertoli cell TJ barrier via the ERK pathway; ii) BPA also downregulates the expression and perturbs localization of GJ protein Cx43 and GJ communication at the BTB; iii) BPA induces Sertoli and germ cell apoptosis via different signaling pathways; and iv) BPA perturbs Sertoli cell actin cytoskeleton mediated by a disruption of spatiotemporal expression and/or localization of Arp3 and Eps8 in human Sertoli cells.

3.2 Cadmium

3.2.1 Introduction—Cadmium is a heavy metal and an EDC that is known to cause male reproductive toxicity in humans and rodents [19,20]. Cadmium-emitting industries including metal mining and refining, fertilizer production, waste incineration, fossil fuel combustion and battery manufacturing, which thus release cadmium to the atmosphere, water and soil, thereby entering the food chain [57]. Cigarette smoking is also another source of cadmium for humans [57]. The estimated human daily intake of cadmium is 1.06 µg/kg b.w. [19]. However, due to the long half-life of cadmium, ~ 20 – 40 years in humans, significant amount of cadmium can be accumulated in the body. The testis is highly sensitive to cadmium toxicity [58], and cadmium was found to induce severe testicular injury in adult rats in the 1950s [59]. The BTB is the primary target of cadmium and BTB disruption takes place sooner than micro-vessel disruption following an acute dose of cadmium in rodents [60]. CdCl₂ induces BTB disruption by causing defragmentation of actin filaments (and also TJ fibrils) in Sertoli cells [60,61] including human Sertoli cells [17], suggesting that cytoskeleton may also be a target of cadmium toxicity in the testis.

3.2.2 Cadmium perturbs Sertoli cell BTB function via MAPK signaling pathway—TGF-β3 is known to perturb the Sertoli cell TJ-permeability barrier *in vitro*, mediated by an activation of p38 MAPK downstream, leading to a downregulation of TJ integral membrane protein occludin, thereby destabilizing the TJ barrier [62]. The involvement of p38 MAPK in TGF-β3-induced Sertoli TJ barrier disruption has been confirmed in studies using specific inhibitors since SB202190 (a specific p38 MAPK inhibitor), but not U0126 (a specific MEK1/2 kinase inhibitor), effectively blocks TGF-β3 mediated TJ barrier disruption [62]. Subsequent studies have shown that cadmium also exerts its disruptive effects on the BTB in the rat testis *in vivo* by an initial upregulation of TGF-β3, to be followed by an activation of p38 MAPK, and this cadmium-mediated toxicity effect can be blocked by SB202190 [63,64]. Additionally, CdCl₂ also activates JNK pathway to induce an upregulation of α₂-macroglobulin, a nonspecific protease inhibitor produced by Sertoli cells, apparently being used to avoid unwanted proteolysis of the seminiferous epithelium [60], offering a protective function. This involvement of JNK pathway mediated by cadmium is confirmed in study using a JNK-specific inhibitor dimethylaminopurine (DMAP), because DMAP was found to worsen CdCl₂-induced testicular damage [60]. Collectively, these findings illustrate that cadmium mediates its disruptive effects at the BTB via the p38 MAPK signaling pathway; it also induces JNK signaling pathway which is likely used by the testis to serve as a protective mechanism to limit unwanted damage to the seminiferous

epithelium following cadmium exposure. The cadmium-induced activation of p38 and JNK pathways have also been confirmed in studies using three-dimensional Sertoli cell-gonocytes co-cultures [65]. Taken together, these findings provide insightful information regarding a therapeutic approach to modulate CdCl₂-induced male infertility, such as via the use of either specific p38 MAPK inhibitor or in combination with JNK activator.

3.2.3 Cadmium modulates Sertoli cell BTB function via FAK—FAK, a nonreceptor protein tyrosine kinase, is an emerging BTB regulator [66]. TJ proteins occludin and ZO-1 interact with FAK in the rat testis, and they are also putative substrates of FAK [67,68]. CdCl₂ is known to downregulate the expression of two activated/phosphorylated forms of FAK, p-FAK-Tyr³⁹⁷ and p-FAK-Tyr⁵⁷⁶, which are integrated component of the apical ES and they are highly expressed at the apical ES from stage VII through stage VIII until shortly before spermiation [68]. Furthermore, reducing the level of p-FAK-Tyr³⁹⁷, such as by overexpressing a non-phosphorylated FAK Y397F mutant in Sertoli cells or by increasing the level of p-FAK-Tyr⁴⁰⁷, such as by overexpressing a phosphomimetic FAK Y407E mutant in Sertoli cells, was shown to promote the Sertoli cell TJ barrier function (i.e., making the TJ barrier tighter) [56], illustrating that these activated/phosphorylated FAK are important regulators of BTB function. Collectively, these findings suggest that FAK may be a target of cadmium toxicity in the testis. Indeed, knockdown of FAK in Sertoli cells with a functional TJ barrier was found to desensitize these cells to CdCl₂-induced BTB disruption [67]. These findings also illustrate that FAK small interfering RNA duplexes, phosphomimetic FAK Y407E mutant or non-phosphorylated FAK Y397F mutant may be potential therapeutic drugs to mediate cadmium-induced toxicity in the testis.

3.2.4 Cadmium perturbs Sertoli cell actin cytoskeleton—Similar to BPA, cadmium is known to induce disruption of F-actin organization in human Sertoli cells dose-dependently (Table 1) [17]. Exposure of human Sertoli cells cultured *in vitro* to CdCl₂ at 0.5 μM caused only truncation of actin microfilaments in Sertoli cell cytosol; however, at 5 and 20 μM, although they are still not cytotoxic to Sertoli cells, CdCl₂ was shown to cause retraction of actin microfilaments from the cell cytosol to surrounding the cell nucleus, besides defragmentation, which is likely mediated by changes in the distribution and/or expression of actin regulatory proteins Arp3 and Eps8 [17]. Future studies need to focus on the role of p-FAK-Tyr³⁹⁷ and/or p-FAK-Tyr⁴⁰⁷ in the cadmium-induced cytoskeleton disruption in human Sertoli cells, since their overexpression may interfere with the cadmium-mediated testis toxicity.

3.2.5 Summary—i) Cadmium perturbs Sertoli cell BTB function by activating TGF-β3/p38 MAPK signaling pathway, and also JNK pathway which protects unwanted nonspecific proteolysis of the seminiferous epithelium; ii) cadmium downregulates the expression of both p-FAK-Tyr³⁹⁷ and p-FAK-Tyr⁵⁷⁶ during the cadmium-mediated BTB disruption; and iii) cadmium-induced actin cytoskeleton disruption at the human Sertoli cell BTB is mediated by mislocalization of Arp3 and Eps8.

3.3 Perfluorooctane sulfonate

3.3.1 Introduction—Perfluoroalkyl substances (PFAS) are a group of chemicals, most notably PFOS and perfluorooctanoic acid (PFOA), widely used in industries and are integrated in commercial products used for coatings of textiles, paper, upholstery and carpet, as well additives in fire-retardant products [69]. Human exposure to PFOS comes from contaminated food and drinking water [70]. The estimated human daily intake of PFOS and PFOA is 1.6 and 2.9 ng/kg b.w., respectively [19]. Due to the relatively long half-life of PFAS in humans, ~ 5.4 years for PFOS and ~ 3.8 years for PFOA [71], these toxicants can build up in tissues and/or organs overtime to cause toxicity [72]. High doses of PFOS and PFOA have shown to induce hepatotoxicity, developmental toxicity, immunotoxicity, endocrine disruption and neurotoxicity in humans and other mammals [73]. Human exposure to high levels of perfluoroalkyls is also associated with reduced number of normal spermatozoa in men [74], and infertility [75]. These findings illustrate that PFOS is a reproductive toxicant. In fact, PFOS has been banned in North America and Europe, but it remains widely used in China.

3.3.2 PFOS perturbs Sertoli cell actin cytoskeleton—In primary rat Sertoli cell cultures, PFOS was shown to induce defragmentation and truncation of actin microfilaments in Sertoli cells without downregulating the steady-state level of β -actin [76]. This PFOS-mediated disruption of actin filaments was also detected in rat Sertoli cell-gonocytes co-culture system [77]. It was shown that the PFOS-induced F-actin disruption is mediated via a downregulation of actin crosslinking and bundling proteins filamin A and palladin, leading to de-bundling of actin microfilaments necessary to maintain the integrity of the ES at the Sertoli cell–cell and/or Sertoli–spermatid interface [76]. This in turn causes redistribution of TJ (e.g., occludin/ZO-1) and basal ES (e.g., N-cadherin/ β -catenin) protein complexes, moving from cell–cell interface to cell cytosol, thereby destabilizing the Sertoli cell TJ-permeability barrier [76]. Consistent with these findings, PFOS was found to induce mouse Sertoli cell TJ barrier disruption, mediated by changes in the localization of adhesion proteins at the Sertoli cell–cell interface, and similar observations were also noted in the mouse testis *in vivo* [78]. Also, there was a loss of actin microfilaments at the basal ES/TJ at the BTB in the testis *in vivo* following treatment of mice with PFOS [78]. Interestingly, the PFOS-induced Sertoli cell TJ barrier disruption was also induced by p38 MAPK activation downstream, because the use of a selective p38 MAPK-specific inhibitor SB203580 could significantly block the PFOS-mediated TJ barrier disruption and the associated changes in adhesion protein distribution at the cell–cell interface [78]. In this context, it is of interest to note that the ERK1/2 was also activated by PFOS in the mouse testis but only when PFOS was used at high doses, such as 25 mg/kg/day [78]. These findings thus illustrate that the PFOS-induced Sertoli cell BTB disruption is also mediated via p38 MAPK, analogous to the cadmium-mediated Sertoli cell TJ disruption [63,64]. It remains to be determined if PFOS induces F-actin disruption in human Sertoli cells via changes in the expression and/or localization/distribution of actin regulatory proteins Eps8 and Arp3 similar to BPA and cadmium [17].

3.3.3 PFOS perturbs Sertoli cell GJ function—Treatment of rat or mouse Sertoli cell cultures with PFOS leads to a downregulation of Cx43, which is associated with a

redistribution of Cx43, moving away from the cell–cell interface [76,78] – apparently the result of enhanced endocytosis and endocytic vesicle-mediated intracellular degradation [76]. Based on the FRAP assay that functionally monitors GJ-based intercellular communication between Sertoli cells, PFOS perturbs GJ function in Sertoli cell cultures with an established TJ barrier [76]. In this context, it is of interest to note that PFOS also downregulates p-Cx43-Ser³⁶⁸ level in mouse Sertoli cells [78]. Nonylphenol, an environmental estrogenic toxicant, has been shown to downregulate p-Cx43 expression in TM4 cells (a mouse Sertoli cell line) via an inactivation of p38 MAPK but not PKC, thereby disrupting GJ communication [79]. However, PFOS was found to activate p38 and ERK pathways in primary mouse Sertoli cells during PFOS-mediated Cx43 reduction [78]. Although these differences could be the result of using primary Sertoli cell cultures versus cell line [80], they illustrate consistently that PFOS and other environmental toxicants (e.g., nonylphenol) can induce GJ communication failure, which is possibly mediated by MAPK downstream. This possibility must be carefully evaluated in future studies since these findings can lead to better therapeutic management of toxicant-induced male infertility.

3.3.4 PFOS modulates Sertoli cell BTB function via FAK—p-FAK-Tyr⁴⁰⁷ and p-FAK-Tyr³⁹⁷ are known to promote and perturb the Sertoli cell TJ-permeability barrier function, respectively, in the rat testis [76]. PFOS has shown to downregulate and upregulate p-FAK-Tyr⁴⁰⁷ and p-FAK-Tyr³⁹⁷ levels, respectively [76], suggesting that the PFOS-induced Sertoli cell BTB disruption is mediated by these phosphorylated forms of FAK downstream. In fact, overexpression of FAK Y407E phosphomimetic mutant in Sertoli cells alleviates PFOS-induced of TJ barrier disruption, and microRNA-mediated FAK knockdown worsens the disruptive effect of PFOS [76]. Collectively, these findings illustrate that p-FAK-Tyr⁴⁰⁷ is a likely therapeutic target to manage PFOS-induced infertility via the possible use of a FAK Y407E phosphomimetic mutant, such as an activator of p-FAK that mimics p-FAK-Tyr⁴⁰⁷.

3.3.5 Summary—i) PFOS disrupts Sertoli cell actin cytoskeleton in Sertoli cells by downregulating the expression of actin regulatory proteins filamin A and palladin; ii) PFOS exerts its disruptive effect via p38 and ERK pathways; iii) PFOS perturbs GJ communication by downregulating Cx43 and p-Cx43-Ser³⁶⁸ expression; and iv) PFOS perturbs Sertoli cell TJ barrier function by downregulating and upregulating p-FAK-Tyr⁴⁰⁷ and p-FAK-Tyr³⁹⁷ expression, respectively.

3.4 Crosstalk and interaction among environmental toxicants BPA, cadmium and PFOS

As briefly discussed herein, BPA, cadmium and PFOS share some common signaling pathways and/or target molecules to exert their disruptive effects in the testis. For instance, exposure of rat Sertoli cells to BPA and PFOS causes downregulation and redistribution of Cx43 at the Sertoli cell–cell interface, thereby disrupting GJ communication as both BPA and PFOS are known to perturb Sertoli cell GJ channel function [42,76]. These findings suggest that Cx43 is a likely therapeutic target to intervene the disruptive effects of BPA and PFOS at the Sertoli cell BTB. Furthermore, MAPK (e.g., ERK1/2, p38 MAPK) is the emerging downstream signaling pathway activated by BPA, cadmium and PFOS based on studies *in vitro* and *in vivo* (Table 1). Once the common signaling cascade(s) that involved

MAPK is better characterized, it will also provide insightful information on therapeutic intervention, such as the use of small-molecule inhibitors against MAPK. Studies have shown that there are crosstalks between FAK and MAPK. For instance, autophosphorylation of FAK at Tyr³⁹⁷ can unfold a binding site for c-Src, so that c-Src phosphorylates FAK at Tyr⁹²⁵, which in turn serves as a binding site for growth factor receptor bound protein-2 (Grb2)–son of sevenless (Sos) complex, and the Grb2-Sos complex acts as a mediator to activate Ras/MAPK pathway [81,82]. This, thus, leads to interaction between FAK and MAPK. As noted herein, both FAK and MAPK signal pathways are involved in environmental toxicant-induced testicular injury (Table 1), and research is needed to delineate the molecular mechanism(s) that regulates crosstalk between these signal pathways, which will be helpful in gaining insightful information to intervene toxicant-induced testicular injury.

4. Role of drug transporters in toxicant-mediated testis injury

Drug transporters are integral membrane proteins widely expressed by mammalian cells including Sertoli cells and cancer cells that prevent the entry and/or accumulation of toxicants and drugs, such as chemotherapeutic drugs and potential male contraceptives, inside eukaryotic cell [23,83]. Although drug transporters, including influx and efflux drug pumps, are crucial to maintain cell homeostasis and to protect cells from environmental toxicants, some toxicants appear to be able to disguise themselves, bypassing the network of drug transporters, such as in the testis [23,83]. Although efflux and influx drug transporters have been identified in the testis [23], few functional studies were done that probed the mechanism(s) by which environmental toxicants entered the testis. Since Sertoli cells near the basement membrane of the seminiferous tubule create the BTB, toxicants must be capable of passing the BTB to induce injury. Studies have shown that toxicants can traverse the Sertoli cell BTB by downregulating efflux drug transporters. For instance, cadmium at 15 μ M was shown to downregulate the expression of P-glycoprotein and multidrug resistance-related protein 1 in Sertoli cell cultures with an established functional TJ barrier [84]. Cadmium also downregulated the expression of influx drug transporters organic anion transporting polypeptide protein 3 (Oatp3) and solute carrier family 15 member 1 (also known as peptide transporter-1) [84]. This downregulation of multiple drug transporters can trigger the unwanted entry of xenobiotics and drugs (e.g., a potential male contraceptive adjuvin known to induce germ cell loss, most notably elongating/elongated spermatids from the testis [85]) into the testis behind the BTB as illustrated in studies by RNAi, in which the knockdown of drug transporters led to accumulation of [³H]-adjuvin behind the Sertoli cell BTB [86,87]. It is known that the entry of cadmium behind the BTB in the mouse testis is mediated by a cadmium-specific influx drug transporter known as Slc39a8 (also called Zip8) [88] since its mutation or deletion can insensitize the mutant mice to cadmium exposure/toxicity. However, it is not known if Slc39a8 is found in the rat or human testis. Furthermore, specific drug transporters for other environmental toxicants have now been identified. Also, PFOS, nonylphenol and other estrogenic (or androgenic)-based toxicants enter cells by polyspecific transporters such as influx drug transporter Oatp1d1 [89]. In short, much research is needed to better understand the interaction of environmental

toxicants and drug transporters in Sertoli cells and the testis so that activators of drug transporters can be used to protect humans from toxicant exposure.

5. Molecular models by which environmental toxicants perturb Sertoli and spermatid adhesion at the ES

Sertoli cell cytoskeleton appears to be one of the primary targets of environmental toxicants [16,85]. For instance, the MT-based cytoskeleton particularly in the Sertoli cell was shown to be disrupted during toxicant-induced testis injury following treatment of rodents with 2,5-hexanedione, carbendazim, phthalates and others [16]. Studies have shown that the polarized MT-based cytoskeleton in Sertoli cells is crucial for providing the 'track' for the transport of residual bodies as well as spermatids and also endocytic vesicles across the adluminal compartment throughout the epithelial cycle of spermatogenesis [90,91]. Thus, MT disruption induced by toxicants was found to cause: i) failure and defects in spermatid transport and spermiation in which spermatids remained embedded deep inside the epithelium in stage VIII or stage IX tubules; ii) failure in fluid secretion by Sertoli cells; and iii) germ cell apoptosis [16]. However, the underlying molecular mechanism(s) by which MT-based cytoskeleton was disrupted by toxicants remains to be elucidated. Recent studies have shown that toxicants may exert their effects in altering the spatiotemporal expression of MT regulatory proteins, such as MT-associated protein [MAP]/MT affinity-regulating kinase 4 (MARK4), which is a MT-stabilizing protein kinase [92]. In a study using adjuvin as model, considerable changes in the spatiotemporal expression of MARK4 as well as its downregulation at the apical ES were noted, which thus impeded spermatid adhesion, leading to defects in spermatid adhesion and transport [93]. Besides MT-based cytoskeleton, recent studies have also shown that environmental toxicants, such as PFOS, BPA and cadmium also perturb the organization of polarized actin microfilaments in Sertoli cells, most notably at the apical and basal ES, including human Sertoli cells (Table 1) [17,68,76]. This toxicant-induced disorganization of F-actin network at the ES, including truncation and/or defragmentation of actin microfilaments, thus perturbs spermatid adhesion and polarity, and also Sertoli cell-cell adhesion, leading to germ cell exfoliation and BTB disruption. Since cell junctions at the Sertoli cell-cell and Sertoli-spermatid interface are mostly using actin-based cytoskeleton as their site of attachment, defragmentation of F-actin thus destabilizes the TJ (i.e., occludin-ZO-1), basal ES (i.e., N-cadherin- β -catenin), and apical ES (i.e., β 1-integrin-laminin- α 3 β 3 γ 3), leading to a loss of cell adhesion in these anchoring junctions. However, the molecular mechanisms by which environment toxicants mediate these changes remain largely unknown until recent years. Whereas mechanistic study by which environmental toxicants induce cytoskeleton disruption remains scarce, studies in testicular toxicants such as potential male contraceptives (i.e., adjuvin) have provided some insightful information on this process [85]. For instance, many of the phenotypes detected in the rat or mouse testis following exposure of rodents to environmental toxicants such as cadmium, PFOS and others are also detected in the rat testis following treatment with adjuvin. These include the presence of multinucleated round spermatids and multinucleated spermatocytes in the seminiferous epithelium, degenerating spermatocytes, loss of spermatid polarity, presence of round spermatids/spermatocytes in tubule lumen, reduction of tubule diameter, closing up of tubule lumen due to defects in

tubule secretion and focal injury of Sertoli cells as manifested by the presence of large vacuoles in cell cytosol [85] and most notably defragmentation and truncation of actin microfilaments in Sertoli cells when the testis was examined by electron microscopy [94]. Studies performed in the past decade have shown that this adjuvin-induced testis injury regarding the actin-based cytoskeleton is mediated by changes in the spatiotemporal expression of two groups of actin-regulatory proteins at the ES [85]: i) branched actin-inducing proteins, such as the Arp2/3 complex, that effectively induce actin filaments from a bundled to an unbundled/branched configuration [95]; and ii) the actin bundling proteins, such as Eps8 (also an actin barbed end capping protein) [96], palladin [97] and fascin 1 [98] (both palladin and fascin 1 are actin crosslinking proteins), that confer actin filaments their bundled configuration. In normal testis, the expression of Arp3 at the apical ES is virtually limited to the concave (ventral) side of spermatid head at stage VII of the cycle, but none expressed at the basal ES/BTB at stage VII [95], which is being used to facilitate extensive endocytic vesicle-mediated protein trafficking events at the site so that 'old' apical ES proteins can be recycled to assemble 'new' apical ES that arises when step 8 spermatids first appear in stage VIII cycle [99]. At the basal ES/BTB, Arp3 is not expressed until in stage VIII tubules [95] when it is used to induce BTB restructuring to facilitate the transport of preleptotene spermatocytes across the immunological barrier [99]. On the other hand, Eps8 is also highly expressed at the apical ES in stage VII tubules, both at the concave and convex (dorsal) side of spermatid heads [96] to confer spermatid adhesion, whereas endocytic vesicle-mediated trafficking takes place at the concave side of spermatid heads to facilitate endocytosis, transcytosis and recycling of 'old' apical ES protein to assembly 'new' apical ES. However, Eps8 is prominently expressed at the basal ES/BTB in stage VII tubules (to confer actin filaments their bundled configuration to maintain basal ES function) but none at stage VIII [96]. This differential but spatiotemporal expression of these two groups of proteins thus provides a unique mechanism for the rapid reorganization of actin microfilaments at the ES in both sites by rapidly converting microfilaments from their bundled to unbundled/branched configuration and vice versa. However, following treatment of rats with adjuvin, this toxicant causes a disruption of the stage-specific spatiotemporal expression of Arp3, Eps8 and palladin via their mislocalization [95-97], as well as considerable downregulation and mislocalization of fascin 1 [98]. For instance, Eps8 and palladin no longer restrictively expressed at the apical ES at stage VII, instead, they are down-regulated and mislocalized, no longer limited to the apical and basal ES [96,97]. At the same time, Arp3 is no longer restricted to the concave side of spermatid heads, but spread across the entire apical ES and also expressed at the basal ES/BTB [95], thereby perturbing proper localization of cell adhesion proteins at both sites. The combined effect of these changes leads to spermatid loss and the eventual disruption of the BTB [99]. In short, toxicants induce restructuring of the apical ES in early and elongating spermatids by disrupting the actin microfilament organization, pushing immature spermatids to undergo 'spermiation'; toxicants also induce similar changes at the basal ES/BTB, forcing the BTB to enter a stage VIII-like restructuring, reversing the timely events of the epithelial cycle (Figure 2). These changes in the localization of actin regulatory proteins following toxicant-induced germ cell defoliation from the testis may be regulated, at least in part, by FAK, in particular p-FAK-Tyr⁴⁰⁷ and p-FAK-Tyr³⁹⁷, based on recent studies [56,100]. For instance, p-FAK-Tyr³⁹⁷ is known to confer apical ES integrity and its presence is detected at the

apical ES until spermiation at late stage VIII [56,101,102]. Overexpression of p-FAK-Tyr³⁹⁷ in the testis *in vivo* was found to disrupt the spatiotemporal expression of Eps8 and Arp3 at the apical ES in which some of these proteins persisted at the apical ES in stage VIII tubules, causing some spermatids to become embedded in the seminiferous epithelium, leading to defects in spermiation [100]. On the other hand, overexpression of p-FAK-Tyr⁴⁰⁷ in Sertoli cell epithelium with a functional TJ barrier via the use of a phosphomimetic mutant FAK Y407E was found to induce branched actin polymerization and the recruitment of N-WASP to Arp3 to activate the Arp2/3 complex [56]. Collectively, these findings illustrate the likelihood of involvement of p-FAK-Tyr³⁹⁷ and p-FAK-Tyr⁴⁰⁷ on the spatiotemporal localization and/or inducing intrinsic activity of these actin regulatory proteins. The concepts briefly outlined here help us to propose a model depicting toxicant-induced 'spermiation', as shown in Figure 2, and toxicant-induced BTB remodeling, as shown in Figure 3. Although the models depicted in Figure 2 are formulated based on studies using adjudin [85], recent studies as summarized and discussed in this review also support the hypothetical concepts that environmental toxicants exert their effects on cytoskeletons, mediated by signaling proteins, such as FAK and MAPK (e.g., ERK1/2, p38 MAPK). It is obvious that the models shown herein will be rapidly updated when more data are available. Nonetheless, these two models serve as the framework for future investigations.

6. Concluding remarks and future perspectives

As briefly discussed above, environmental toxicants appear to induce testis injury by activating some common signaling molecule(s) or event(s) in the Sertoli cell, which include: i) activation of MAPK pathway or FAK, in particular p-FAK-Tyr³⁹⁷ or p-FAK-Tyr⁴⁰⁷; and ii) disruption of GJ function. Furthermore, the MT- and actin-based cytoskeletons are tightly involved in toxicant-induced testis injury, and the role of the MT and actin regulatory proteins should be carefully elucidated in future studies since this information is exceedingly helpful in developing therapeutic approaches to interfere toxicant-induced male reproductive dysfunction. In this context, it is of interest to note that the component of MAPK pathway, especially p38, is also a drug target for inflammatory diseases and cancer by using small-molecular inhibitors of p38. Due to the ubiquitous function of MAPK in mammalian cells, a major obstacle of MAPK-directed therapeutic approach is tissue-specific delivery of the inhibitors. Considerable downregulation on the expression of Cx43 as noted in Sertoli cells following treatment with environment toxicants, such as BPA, CdCl₂ and PFOS, suggest that Cx43 may also be a molecular target of toxicants in the testis. Restoration of the lost Cx43-mediated GJ function may be a possible therapeutic strategy to reverse toxicant-induced testis injury. Furthermore, it must be noted that even though Sertoli cells, in particular human Sertoli cells, cultured *in vitro* may provide a novel model to study the molecular mechanism(s) by which environmental toxicants induce testis injury such as at the Sertoli cell BTB, cells cultured *in vitro* are different from cells in the *in vivo* microenvironment in many ways. For instance, cells *in vitro* can metabolize toxicants differently than cells *in vivo* in a multicellular environment. Thus, some of the observations seen *in vitro* may not necessarily be reproduced *in vivo*, and it is likely that a combination of both *in vitro* and *in vivo* approaches should be used. Nonetheless, the use of an *in vitro* system should reduce the burden of using a large number of life animals for tedious and also expensive *in vivo*

experiments until sufficient information is gathered before the launch of an expanded *in vivo* study.

7. Expert opinion

- 1) Research has shown that the Sertoli cell is a target of toxicant-mediated reproductive dysfunction in the male, both in rodents and in humans. However, much of the research that examined the effects of environmental toxicants on Sertoli cells are limited to changes in the expression of genes, sets of genes or a target protein relevant to Sertoli cell function. For instance, it is well established that the Sertoli cell microtubule-based cytoskeleton is a prime target of environmental toxicants, such as 2,5-hexanedione and phthalates; whereas the Sertoli cell actin-based cytoskeleton is a target of cadmium, PFOS and/or BPA. The underlying molecular mechanism(s), however, remain largely unexplored. Furthermore, most of the studies performed thus far were based on studies of Sertoli cells in rodents. However, recent advances in culture techniques allow the use of human Sertoli cells which remain mitotically active when cultured *in vitro* in the presence of fetal calf serum. These new findings are highlighted herein. But cautions are also noted that there are limitation of using Sertoli cells *in vitro*, and it is obvious that some limited studies *in vivo* are need to confirm and expand observations *in vitro*.
- 2) If the molecular targets and/or the underlying mechanism(s) are identified and known, toxicant-mediated Sertoli cell dysfunction that leads to male infertility can be therapeutically managed, and possibly treated. This thus provides hopes to many men suffering from unexplained infertility.
- 3) In recent years, the use of rat Sertoli cell culture system *in vitro* that mimics the Sertoli cell BTB *in vivo* has been exceedingly helpful in providing a better alternative to study the mechanism of toxicant-induced reproductive dysfunction. However, one of the limiting factors in the field is the lack of reliable human Sertoli cell culture system *in vitro* for studies since some of the phenotypes noted in rodents may not be physiologically relevant to humans. Recent advances in human Sertoli cell cultures have provide much excitement in the field; in particular, human Sertoli cells remain proliferative if cultured in the presence of fetal calf serum, so that freshly isolated human Sertoli cells are not needed for studies.
- 4) It is likely that the better use of human Sertoli cell cultures *in vitro* will energize the field of reproductive toxicology since human Sertoli cells can be maintained for long-term cultures with satisfactory proliferation rate. This also eliminates the reliance of Sertoli cells from rodents, speeding up the transition of research findings from laboratory bench to bed side.
- 5) Recent advances in the field, and areas of research that deserve more research and attentions are highlighted in this opinion article.

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Article highlights

- Sertoli cell junctions, and actin- and MT-based cytoskeletons are the target of environmental toxicants.
- Human and rodent Sertoli cells cultured *in vitro* provide the valuable tools to study blood-testis barrier function and toxicant-mediated testicular injury.

Figure 1. Illustration showing that BPA-induced apoptosis in Sertoli and germ cells are mediated by distinctive signaling molecules

ADAM17: A disintegrin and metalloprotease-17; BPA: Bisphenol A; ERK: Extracellular-signal-regulated kinase; JNK: c-Jun N-terminal protein kinase.

Figure 2. A schematic diagram that illustrates the likely mechanism by which environmental toxicants induce germ cell exfoliation from the mammalian testis

In the testis, such as in a stage VII to early VIII tubule, elongating and elongated spermatids remain adhered onto the Sertoli cell in the seminiferous epithelium by apical ES, conferred by actin microfilament bundles that are sandwiched between endoplasmic reticulum and the Sertoli cell plasma membrane (*left* panel). These actin microfilament bundles also serve as the attachment site for apical ES adhesion proteins (e.g., Sertoli cell-specific adhesion proteins nectin-2, $\alpha 6 \beta 1$ -integrin; and spermatid-specific adhesion proteins nectin-3, laminin- $\alpha 3 \beta 3 \gamma 3$). Actin microfilament bundles are maintained by the actin bundling proteins such as Eps8, palladin, fascin 1 and ezrin that are found in the testis. Furthermore, the proper expression and/or localization/distribution of these actin bundling proteins are likely maintained by several protein kinases including p-FAK-Tyr³⁹⁷, and also ERK and p38 MAPK. However, following exposure of the testis to the environmental toxicants, such as cadmium and PFOS, these toxicants induce unwanted activation of ERK1/2, p38 MAPK and/or downregulation of p-FAK-Tyr³⁹⁷, causing changes in the spatiotemporal expression of actin bundling proteins. Instead, branched actin-inducing proteins such as the Arp2/3 complex activated N-WASP, causing unwanted reorganization of F-actin network, rendering actin microfilaments to become a truncated and branched network, thereby destabilizing the adhesion proteins at the apical ES (*middle* panel). In short, the loss of actin microfilaments at the apical ES enhances endocytic vesicle-mediated protein trafficking with these adhesion proteins being internalized and transcytosed, to be followed by either recycling or endosome-mediated degradation. The net result is the unwanted depletion of germ cells from the seminiferous epithelium (*right* panel). This hypothetical model is prepared based on recent findings as briefly discussed in the text. This model, however, depicts several molecular targets that can be tackled to block toxicant-induced germ cell loss from the testis, such as the use of small-molecule inhibitors to block unwanted activation of MAPK and/or downregulation of p-FAK-Tyr³⁹⁷, as well as by blocking unwanted changes in the spatiotemporal expression of actin regulatory proteins.

ERK: Extracellular-signal-regulated kinase; ES: Ectoplasmic specialization; FAK: Focal adhesion kinase; N-WASP: Neuronal Wiskott-Aldrich Syndrome protein; PFOS: Perfluorooctane sulfonate.

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Figure 3. A schematic diagram that illustrates the likely mechanism by which environmental toxicants induce Sertoli cell BTB disruption in the mammalian testis

In the testis, such as in a stage VII tubule, the Sertoli cell BTB is maintained by properly organized F-actin network in which actin microfilaments are bundled and sandwiched between cisternae of endoplasmic reticulum and the Sertoli cell plasma membrane. These actin microfilament bundles also serve as the attachment site for adhesion protein complexes, such as TJ protein complexes: occludin/ZO-1, JAM-A/ZO-1; basal ES protein complex: N-cadherin/ β -catenin; and GJ protein complex: Cx43/plakophilin 2. Actin microfilaments at the basal ES/BTB are maintained by spatiotemporal expression of actin bundling proteins such as Eps8, palladin, fascin 1 and ezrin, which are conferred by proper expression of p-FAK-Tyr⁴⁰⁷, p38 MAPK and/or ERK1/2 (*left* panel). However, following exposure of the testis to environmental toxicants (e.g., cadmium, PFOS or BPA), either p38 MAPK/ERK1/2 is activated or p-FAK-Tyr⁴⁰⁷ is down-regulated (or both), this thus perturbs the spatiotemporal expression of actin bundling proteins vs. branched actin-inducing proteins (e.g., Arp2/3 complex and N-WASP). The predominant Arp2/3 complex/N-WASP thus converts F-actin from its bundled configuration to a branched/unbundled configuration, destabilizing adhesion protein complexes at the basal ES/BTB. This, in turn, induces endocytic vesicle-mediated protein trafficking, causing endocytosis of integral membrane proteins at the Sertoli cell BTB, either to be transcytosed and recycled or undergo endosome-mediated degradation (*middle* panel). The net result of these changes contributes to a disruption of the Sertoli cell BTB (*right* panel), so that environmental toxicants can get access to the adluminal compartment, causing extensive germ cell exfoliation as shown in Figure 2.

BPA: Bisphenol A; BTB: Blood–testis barrier; ERK: Extracellular-signal-regulated kinase; ES: Ectoplasmic specialization; FAK: Focal adhesion kinase; GJ: Gap junction; N-WASP: Neuronal Wiskott-Aldrich Syndrome protein; PFOS: Perfluorooctane sulfonate.

Effects of environmental toxicants on Sertoli cell functions and the involving signaling molecules *.

Table 1

Toxicant	<i>In vitro</i> / <i>in vivo</i> study/dosage	Junction protein expression	Junction protein localization/distribution	Modulation of pertinent signaling pathway(s)/ molecule(s)	F-actin organization	Cell apoptosis	Ref.
PFOS	<i>In vitro</i> study: 20 ~ 30 μ M	<i>In vitro</i> study: filamin A (-), palladin (-), Cx43 (-), p-Cx43-Ser ³⁶⁸ (-), ZO-1 (-), claudin-11 (-), occludin (-), N-cadherin (-), p-FAK-Tyr ⁴⁰⁷ (-), p-FAK-Tyr ³⁹⁷ (+)	<i>In vitro</i> study: filamin A (-), palladin (-), Cx43 (-), ZO-1 (-), occludin (-), N-cadherin (-), β -catenin (-)	FAK, p38, ERK	Truncation, defragmentation	No obvious apoptosis in Sertoli cell-gonocyte co-cultures	[76,78,103]
		<i>In vivo</i> study: ZO-1 (-), occludin (-), claudin-11 (-), Cx43 (-), p-Cx43-Ser ³⁶⁸ (-)	<i>In vivo</i> study: N.D.				
Cadmium	<i>In vitro</i> study: 3 μ M	<i>In vitro</i> study: occludin (-), ZO-1 (-), JAM-A (-), N-cadherin (-), β -catenin (-), FAK (-), p-FAK-Tyr ³⁹⁷ (-), c-Src (-), p-Src-Tyr ⁵²⁹ (-)	<i>In vitro</i> study: occludin (-), ZO-1 (-), FAK (-)	TGF- β 2, TGF- β 3, p38, JNK, FAK, c-Src	Truncation, defragmentation	Induction of apoptosis in Sertoli cell-gonocyte co-cultures	[17,60,63,64,67,68,104,105]
		<i>In vitro</i> study (human Sertoli cells): Arp3 (-)	<i>In vitro</i> study (human Sertoli cells): ZO-1 (-), N-cadherin (-), β -catenin (-), Eps8 (-), c-Src (-), annexin II (-)		Truncation, disorganization		
BPA	<i>In vitro</i> study: 3 mg/kg, b.w., i.p. 40, 200 μ M	<i>In vitro</i> study: α 2-MG (+), occludin (-), ZO-1 (-), N-cadherin (-), E-cadherin (-), β -catenin (-), nectin-3 (-), γ -catenin (-), I-afadin (-), Cx43 (-), β 1-integrin (-), actin (-), vimentin (-), α -tubulin (-), MMP-3 (-), uPA (-), cathepsin L (+), cystatin C (+), FAK (-), p-FAK-Tyr ³⁹⁷ (-), p-FAK-Tyr 576 (-), c-Src (-), p-c-Src-Tyr ⁴¹⁶ (-), p-c-Src-Tyr ⁵²⁹ (-)	<i>In vitro</i> study: α 2-MG (+), occludin (-), ZO-1 (-), N-cadherin (-), E-cadherin (-), β -catenin (-), nectin-3 (-), γ -catenin (-), I-afadin (-), Cx43 (-), β 1-integrin (-), actin (-), vimentin (-), α -tubulin (-), MMP-3 (-), uPA (-), cathepsin L (+), cystatin C (+), FAK (-), p-FAK-Tyr ³⁹⁷ (-), p-FAK-Tyr 576 (-), c-Src (-), p-c-Src-Tyr ⁴¹⁶ (-), p-c-Src-Tyr ⁵²⁹ (-)				
		<i>In vitro</i> study: Cx43 (-), p-Cx43-Ser ³⁶⁸ (-), occludin (-), JAM-A (-), ZO-1 (-), N-cadherin (-), α -catenin (-), β -catenin (-), β 1-integrin (-), c-Src (-), desmoglein-2 (-)	<i>In vitro</i> study: Cx43 (-), N-cadherin (-), occludin (-)	ERK1/2	Truncation, defragmentation	Induction of Sertoli cell apoptosis via CaM/CaMKII/ERK (in mouse TM4 cells), Pen/Akt, JNK/p38 MAPK, NF- κ B and/or Fas/	[17,20,37,38,52-54]

Toxicant	<i>In vitro</i> / <i>in vivo</i> study/dosage	Junction protein expression	Junction protein localization/distribution	Modulation of pertinent signaling pathway(s)/ molecule(s)	F-actin organization	Cell apoptosis	Ref.
	<i>In vitro</i> study (human Sertoli cells); 40, 200 μ M	<i>In vitro</i> study (human Sertoli cells): ZO-1 (–), N-cadherin (–), β -catenin (–), c-Src (–), Annexin II (–)	<i>In vitro</i> study (human Sertoli cells): ZO-1 (–), N-cadherin (–), β -catenin (–), Eps8 (–), c-Src (–), annexin II (–)		Truncation, retraction, disorganization	FasL signaling molecules downstream	
	<i>In vivo</i> study: 10, 50 mg/kg b.w., oral gavage	<i>In vivo</i> study: occludin (–), nectin-3 (–)	<i>In vivo</i> study: occludin (–)				

* Studies were performed using primary Sertoli cell cultures from rodents for *in vitro* studies and rodents for *in vivo* studies unless otherwise specified.

(–): Downregulation or mislocalization; (+): Upregulation; α 2-MG: α 2-macroglobulin; BPA: Bisphenol A; b.w.: Body weight; Cx43: Connexin 43; d: Day; ERK: Extracellular signal-regulated kinase; FAK: Focal adhesion kinase; i.p.: Intraperitoneal injection; JAM-A: Junctional adhesion molecule-A; JNK: c-Jun N-terminal protein kinase; N.D.: Not determined; PFOS: Perfluorooctane sulfonate; Pten: Phosphatase and tensin homolog; uPA: Urokinase plasminogen activator; ZO-1: Zonula occludens-1.