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Cadmium-induced Testicular Injury*

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Abstract

Cadmium (Cd) is an environmental toxicant and an endocrine disruptor in humans. Several organs (e.g., kidney, liver) are affected by Cd and recent studies have illustrated that the testis is exceedingly sensitive to Cd toxicity. More important, Cd and other toxicants, such as heavy metals (e.g., lead, mercury) and estrogenic-based compounds (e.g., bisphenols) may account for the recent declining fertility in men among developed countries by reducing sperm count and testis function. In this review, we critically discuss recent data in the field that have demonstrated the Cd-induced toxicity to the testis is probably the result of interactions of a complex network of causes. This is likely to involve the disruption of the blood-testis barrier (BTB) via specific signal transduction pathways and signaling molecules, such as p38 mitogen-activated protein kinase (MAPK). We also summarize current studies on factors that confer the testis sensitivity to Cd, such as Cd transporters and metallothioneins, and the impact of Cd on the testis as an endocrine disruptor, oxidative stress inducer and how it may disrupt the Zn⁺² and/or Ca⁺² mediated cellular events. While much work is needed before a unified mechanistic pathway of Cd-induced testicular toxicity is emerged, recent studies have helped to identify some of the likely mechanisms and/or events that take place during Cd-induced testis injury. Furthermore, some of the recent studies have shed lights on potential therapeutic or preventive approaches that can be developed in future studies by blocking or minimizing the destructive effects of Cd to testicular function in men.

Keywords

Environmental toxicant; cadmium; testis; spermatogenesis; seminiferous epithelial cycle; Sertoli cells; germ cells; blood-testis barrier; cell adhesion; infertility; cancer

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1. Introduction

Cadmium (Cd) is a heavy metal and a major environmental toxicant. The general population is exposed to Cd via contaminants found in drinking water and food (WHO, 2000; ATSDR, 2008), while occupational exposure to Cd usually takes place during mining or manufacturing of batteries and pigments that utilize Cd. Industrial activities, such as smelting and refining of metals, and municipal waste incineration also release Cd to the atmosphere as cadmium oxide, chloride or sulfide. The Cd levels in the air and drinking water are not alarming ($\sim 0.04 \mu\text{g}/\text{m}^3$ and less than $1 \mu\text{g}/\text{L}$, respectively) (ATSDR, 2008). However, an average person absorbs $\sim 1 \mu\text{g}$ Cd/day via food, while an additional 1-3 μg of Cd is absorbed by smoking one pack of cigarettes per day and, consequently, heavy smokers have more than double of the Cd body burden (Waalkes, 2003; ATSDR, 2008).

It has been suggested that Cd is involved in carcinogenesis in multiple organs including kidney, prostate, liver and pancreas [for reviews, see (Waalkes, 2003; Goyer *et al.*, 2004; Thompson and Bannigan, 2008)]. In fact, the International Agency for Research on Cancer classified Cd as a known human carcinogen in 1993 (IARC, 1993) and Cd is ranked the 7th toxicant in the Priority List of Hazardous Substances of the Agency for Toxic Substances and Disease Registry (ATSDR, 2007). International and governmental agencies have made efforts to control and lower the Cd exposure to the general public in recent years. Nevertheless, Cd has a long biological half-life, ~ 20 -40 years in humans, and it accumulates in the body over a considerable period of time – particularly in the kidneys and liver (WHO, 2000). It may also cause severe damage to embryos and the reproductive organs in adults including the ovary and testes, which are sensitive to Cd toxicity [for a review, see (Thompson and Bannigan, 2008)].

Although the testicular damage induced by Cd was recognized decades ago (Parizek, 1957), the precise mechanisms underlying its toxicity to the testes remained unclear. This review focuses on recent findings on Cd-induced testicular injury, highlighting the disruption of the blood-testis-barrier (BTB), which is a major target of Cd toxicity in the testis, and the underlying mechanisms of action. Also, we summarize current studies investigating the unusual sensitivity of the testis to Cd toxicity. Furthermore, we discuss the impact of Cd actions as an endocrine disruptor in males and the role of Cd-induced oxidative stress and the preventive effects of zinc (Zn) and antioxidants.

2. The blood-testis barrier (BTB) and cadmium toxicity

The testis is extremely sensitive to Cd toxicity. Since the 1950s, studies have shown that *in vivo* acute exposure to Cd caused BTB disruption, germ cell loss, testicular edema, hemorrhage, necrosis, and sterility in several mammalian species (e.g., rodents, rabbit, dog, calf, stallion) (Figure 1, Table 1), and *in vitro* studies have illustrated Cd-induced damage to testicular cells (Table 2) [for a review, see (Li and Heindel, 1998)]. Recent studies have also associated reduced male fertility, such as reduced sperm count and poor semen quality, in men exposed to Cd and/or other environmental toxicants [for a review, see (Benoff *et al.*, 2000)]. These correlation studies are significant since they illustrate the vulnerability of the testes to Cd toxicity, but the underlying mechanism(s) was not known at the time.

Initial studies that recognized Cd could induce profound and irreversible injury to mammalian testes described the disruption of endothelial cells of microvessels, edema and hemorrhage by morphological analysis (Parizek, 1960; Mason *et al.*, 1964), apparently as the result of a primary disruption in the vascular system (Parizek, 1965). This, in turn, would have affected the seminiferous epithelium, causing testicular ischemia and necrosis (Chiquoine, 1964). It was postulated that the mammalian testis was more sensitive than other organs because of its unique vasculature [for reviews, see (Mason *et al.*, 1964; Gunn and Gould, 1970)]. Although the vascular system is indeed a major target of Cd toxicity [for a review, see (Prozialeck *et*

et al., 2008)], Johnson suggested that the BTB could be the first target of Cd toxicity in guinea-pigs testes (Johnson, 1969). Afterwards, Setchell and Waites demonstrated that the BTB is more vulnerable to Cd toxicity than the tight junction (TJ) in the microvessels in adult rat testes, since the damage to the BTB occurred prior to the microvessels found in the interstitium (Setchell and Waites, 1970). In addition, a single low dose of Cd at 1 mg/kg b.w. disrupted TJ-associated microfilaments in rat Sertoli cells and also induced spermiation failure without visible vascular lesion in the testes (Hew *et al.*, 1993a; Hew *et al.*, 1993b). Taken together, these observations are rather unusual since the BTB is known to be one of the tightest blood-tissue barriers in mammals [for reviews, see (Wong and Cheng, 2005; Yan *et al.*, 2008a)]. In other words, although the BTB is better 'sealed' than the TJ-permeability barrier found in microvessels, it protects the testis from Cd toxicity less effectively.

While the barrier function of the blood-brain barrier, the blood-retina barrier or the microvessels, is attributed exclusively to the TJs of the endothelial cells in the blood vessels, the BTB is contributed mostly by adjacent Sertoli cells found near the basal compartment of the seminiferous (see Figure 2). More importantly, the BTB is created by coexisting tight junction, desmosome-like junction, gap junction, and basal ectoplasmic specialization (basal ES) [for reviews, see (Cheng and Mruk, 2002; Wong and Cheng, 2005; Mruk *et al.*, 2008)]. The basal ES is a testis-specific actin-based atypical adherens junction (AJ) type [for reviews, see (Wong and Cheng, 2005; Wong *et al.*, 2008)]. The physiological significance for the coexistence of these different junction types at the BTB remains unknown. However, recent studies have suggested that their coexistence is needed to permit the timely restructuring of the BTB to facilitate the transit of preleptotene spermatocytes at stage VIII of the epithelial cycle while maintaining the immunological barrier *simultaneously*. It was proposed that adaptors (e.g., α -catenin) are 'switching' between their association with the TJ- (e.g., occludin) or basal ES- (e.g., N-cadherin) based integral membrane proteins so that TJ can 'open'. Meanwhile the basal ES continues to maintain the barrier function transiently via an 'engagement' and 'disengagement' mechanism (Yan and Cheng, 2005; Yan *et al.*, 2008b). However, it is likely that because of this unique morphological layout of the BTB in the testis, which confers its unusual barrier function to facilitate the transit of preleptotene spermatocytes, this has also rendered this barrier vulnerable to environmental toxicants, such as Cd. For instance, other studies have shown that E-cadherin is one of the primary targets of Cd toxicity in epithelial cells (Prozialeck, 2000) since Cd interacts with the putative calcium-binding motif in E-cadherin, causing a disruption of the cadherin-based cell adhesion (Prozialeck and Lamar, 1999). Yet in all other epithelia and endothelia, the anchoring junctions are restricted behind the tight junctions forming the adherens plaque [for a review, see (Wong and Cheng, 2005)], as a result, E-cadherin, an integral membrane protein at the AJ, is being sealed from Cd. But since E-cadherin coexists with TJ-proteins (e.g., occludin, claudins, JAM-A) at the BTB, Cd would have immediate access to E-cadherin, making the testis more susceptible to Cd toxicity.

The Cd-induced BTB damage has also been studied *in vitro* by using a model in which primary Sertoli cells isolated from 20-day-old rat testes are plated at a cell density at $0.5\text{--}1.2 \times 10^6$ cells/cm² on a reconstituted basement membrane (e.g., Matrigel). These cells establish functional TJ, basal ES, and desmosome, which have been confirmed by both biochemical and morphological studies (Siu *et al.*, 2005). This system provides a good model to study BTB function *in vitro* that mimics the barrier *in vivo* (Janecki *et al.*, 1992; Chung and Cheng, 2001). By using this model, Cd has been shown to not only inhibit the assembly of Sertoli cells TJ-permeability barrier, but also to disrupt it dose-dependently (0.1-10 μM) without any apparent cytotoxicity (Janecki *et al.*, 1992; Chung and Cheng, 2001). Importantly, testosterone counteracted the Cd disruptive effects, possibly by inducing the expression of TJ integral membrane proteins such as occludin. Therefore, testosterone plays a crucial role in the regulation of Sertoli cells TJ-permeability barrier (Chung and Cheng, 2001), which is consistent with recent reports that androgen promotes the BTB integrity and cell adhesion

function in the testis (Meng *et al.*, 2005; Wang *et al.*, 2006). These observations also illustrate that androgen (or a manipulation of the androgen receptor in Sertoli cells) can be a potential target candidate to manage Cd-induced testicular toxicity, which should be explored in future studies. In this context, Cd-induced TJ disassembly in this *in vitro* model was further associated with a decline of occludin and a concomitant increase of uPA (a serine protease) mRNA steady-state levels (Chung and Cheng, 2001). No changes were observed in E-cadherin steady-state levels during Cd-induced Sertoli cell TJ-barrier disruption, despite the fact that E-cadherin was shown to be involved in the BTB assembly in postnatal development in rodents (Wu *et al.*, 1993) and it is a target of Cd toxicity in other epithelia (Prozialeck, 2000).

However, Cd and other environmental toxicants (e.g., DDT) may disrupt Sertoli cell TJ-barrier function and basal ES not only by decreasing the synthesis and/or expression of proteins, but also by promoting protein redistribution at the Sertoli-Sertoli cell interface (Chung and Cheng, 2001; Fiorini *et al.*, 2004; Wong *et al.*, 2004; Fiorini *et al.*, 2008), perhaps via an alteration of protein endocytosis and/or recycling. For instance, focal adhesion kinase (FAK), a non-receptor tyrosine kinase, has been shown to be localized to the basal ES in the testis at the BTB (Mulholland *et al.*, 2001; Siu *et al.*, 2003) whereas its activated phosphorylated form was restricted to the apical ES at the Sertoli cell-elongating spermatid interface (Siu *et al.*, 2003; Beardsley *et al.*, 2006). Recent studies from our laboratory have also localized FAK to the Sertoli-Sertoli cell interface *in vitro* after 4 days in culture (Figure 2), when the functional TJ-barrier is formed as assessed by transepithelial electrical resistance (TER) measurement across the cell epithelium. It was observed that FAK co-localized with occludin (a putative TJ protein in rodents) to the same site consistent with earlier reports for its localization at the BTB (Siu *et al.*, 2003). When these Sertoli cells were exposed to Cd, FAK and occludin redistribution became visible after 3 hours and it was more evident by 6 hours (see Figure 3), illustrating a time-dependent changes in redistribution at time points when differences in the steady-state levels of these proteins were not detectable by immunoblot analysis (Siu and Cheng, unpublished observations). These latest findings are also consistent with earlier studies which have shown E-cadherin redistribution after Cd treatment in Caco-2 and MDCK cells [for reviews, see (Prozialeck, 2000; Prozialeck *et al.*, 2003)]. Taken together, the studies using Sertoli cells *in vitro* have further complemented the previous *in vivo* investigations to better illustrate the BTB sensitivity to Cd. Nevertheless, the signaling pathways involved in the Cd-induced BTB disruption were not elucidated until recently, and these studies are described in the following section.

3. Signal transduction pathways activated by cadmium toxicity in the testis

The first study reporting the toxicity of Cd that affects the BTB is regulated by a signal transduction pathway (namely the stress activated p38 MAPK) appeared in 2003 (Lui *et al.*, 2003), when the use of a specific p38 MAPK inhibitor, SB202190, was shown to delay and partially block the damaging effects of Cd to the BTB in adult rats. This finding illustrates that the Cd-induced BTB damage is mediated via a defined signaling pathway instead of simple and/or general cell cytotoxicity. However, since the use of SB202190 failed to completely block the Cd-induced damage, this observation also indicates that other signaling events may operate concurrently to elicit the BTB disruption. This study was subsequently expanded and it was found that other non-receptor protein kinases, such as JNK, are indeed involved in this event (Wong *et al.*, 2004; Wong and Cheng, 2005; Wong *et al.*, 2005), possibly mediated via α_2 -macroglobulin, a non-specific protease inhibitor. Besides, an array of molecules including both proteases and protease inhibitors also take part in the Cd-induced BTB damage, the subsequent germ cell loss from the seminiferous epithelium and recovery (Wong *et al.*, 2004). These findings are significant, since they predict that a potential therapeutic approach can be developed to prevent, or at least to manage, Cd-induced infertility in men.

Based on these earlier reports (Wong *et al.*, 2004; Wong and Cheng, 2005), it is apparent that following exposure of the testis to Cd, the toxicant enhances the production of TGF- β 3 at the BTB microenvironment, which, in turn, activates the p38 MAPK signaling pathway downstream. The combination of these changes also leads to reducing the steady-state levels of integral membrane proteins (e.g., occludin, N-cadherin, E-cadherin) at the BTB site, compromising the BTB integrity. Besides, proteases (e.g., cathepsin L) are also activated, which further disrupt the BTB integrity, perhaps by disrupting cell adhesion molecules in the seminiferous epithelium, leading to germ cell loss from the epithelium. This apparently is followed by a subsequent activation of c-JNK signaling pathway and the production of α ₂-macroglobulin to 'contain' the proteolysis and/or other processes induced by Cd in the seminiferous epithelium, otherwise, general cellular necrosis will take hold in the epithelium. This hypothetical cascade of events is depicted in Figure 4. This hypothesis can now be tested in functional experiments in future studies. For instance, much work is needed to assess the precise role of MAPK in Cd-induced testicular injury. Perhaps this can be tested by Sertoli cell-specific knock-down of p38 MAPK by RNAi using specific siRNA duplexes in the *in vitro* system using Sertoli cells with established functional BTB that mimics the *in vivo* barrier. If the knock-down of p38 MAPK would render the loss of responsiveness of Sertoli cells to Cd, this would further support the physiological significance of p38 MAPK in Cd-induced injury. In this context, a therapeutic specific p38 MAPK inhibitor could be designed for its delivery to the Sertoli cell, eventually offering hopes to the Cd-induced male infertility and reduced sperm counts in industrialized nations.

4. Testicular sensitivity to cadmium exposure

As discussed before, the testis is extremely sensitive to Cd and one of the possible explanations for this sensitivity is the unique morphological layout of the BTB. However, studies on other important and exclusive features of the testis have also shed some lights on the intriguing aspect of the testicular sensitivity to Cd and they are summarized in this section.

4.1. The *cdm* gene and Cd-resistance

In the 1970's, Taylor and coworkers discovered genetic differences among some inbred mouse strains that conferred resistance to Cd-induced testicular damage. Indeed, mice lacking a single locus named *Cdm* displayed Cd-resistant testis, and this phenotype was transmitted as an autosomal recessive trait (Taylor *et al.*, 1973). In a recent study, the *cdm* gene was identified as the *solute-carrier (Slc)39a8* gene, encoding the Zrt-, Irt-like protein (ZIP)8, which is most likely responsible for conferring Cd testicular sensitivity in mice (Dalton *et al.*, 2005). ZIP8 has been characterized as a Cd²⁺, Zn²⁺ or Mn²⁺/HCO₃⁻ transporter (He *et al.*, 2006; Liu *et al.*, 2008) and it is expressed in Sertoli cells, and in vascular endothelial cells found in the interstitium of the testis in mouse strains sensitive to Cd toxicity. In contrast, almost no expression was detected in the microvascular endothelial cells in the Cd-resistant strains. Additionally, no changes were observed in ZIP8 expression in other Cd target tissues (e.g., lung, kidney and liver) when comparing the Cd-resistant and Cd-sensitive strains (Dalton *et al.*, 2005).

The generation of Slc39a8-transgenic mice (BTZIP8-3) has demonstrated that the gene-dose increase of *Slc39a8* in mice with a Cd-resistant genetic background could cause Cd-induced testicular injury similar to the Cd-sensitive strains (Wang *et al.*, 2007). In these animals, high levels of ZIP8 led to enhanced Cd uptake in the renal proximal tubules after Cd exposure, thus, ZIP8 is a potential key player in Cd-induced accumulation and toxicity in the testis and the proximal tubules in the kidney (Wang *et al.*, 2007) [for a review, see (Prozialeck *et al.*, 2008)]. The human ZIP8 mRNA is abundant in the pancreas and lung, and it is also expressed in the testis (Begum *et al.*, 2002). Although its role in Cd-induced testicular damage in humans

remains unclear, ZIP8 is a potential Cd transporter in testicular cells. Moreover, ZIP8 has been shown to transport Zn^{+2} in human lung epithelia (Besecker *et al.*, 2008).

Besides the *Slc39a8* gene, another potential gene product for the *cdm* locus has recently been identified as Ppp3ca, which is the catalytic subunit of calcineurin (CN, a calcium/calmodulin-dependent Ser/Thr phosphatase), also known as the α isoform of CN (Martin *et al.*, 2007). Ppp3ca was detected in testicular endothelial cells and at the Sertoli cell-round spermatid interface, and it has been suggested to be important for spermatogenesis, while CN could participate in the maintenance of the Sertoli cell actin cytoskeleton (Martin *et al.*, 2007). When FK506, a CN inhibitor, was administered to mice prior to Cd exposure, it could prevent testicular damage without decreasing the level of Cd in the testis (Martin *et al.*, 2007), illustrating the significance of CN in mediating Cd-induced testicular injury. Further studies are needed to delineate the intriguing involvement of ZIP8, CN and Ppp3ca on Cd-induced toxicity and Cd resistance. It remains unclear whether these gene products would affect Cd uptake and accumulation in Sertoli cells as well as in other cells and tissues in rats and humans.

4.2. Metallothioneins (MTs) in the testis

MTs are members of a family of low molecular weight proteins rich in cysteine. Four isoforms have been identified, namely MT-I to MT-IV. At present, several functions have been proposed for these interesting proteins, from chemotherapy resistance in tumor cells to neuroprotection [for reviews, see (Chung and West, 2004; Theocharis *et al.*, 2004)]. However, the initial studies on MTs were largely focused on their metal-binding properties to Cd, with the MT-I and MT-II isoforms alleviating cells from Cd toxicity. Cd and other physiologic and pathologic factors and conditions are known to induce MT-I and MT-II expression in various organs, such as the liver and kidney, to counteract heavy metal toxicity [for a review, see (Klaassen *et al.*, 1999)]. Since the testis is highly sensitive to Cd exposure, it raised the question whether this organ expressed sufficient MTs to protect it against heavy metals.

In rodents, MT-I/II have been localized to spermatogenic and Sertoli cells (Nishimura *et al.*, 1990; De *et al.*, 1991; Tohyama *et al.*, 1994; Suzuki *et al.*, 1998; Kusakabe *et al.*, 2008), as well as in interstitial cells, even though there are some discrepancies regarding their precise cellular distribution (Danielson *et al.*, 1982; McKenna *et al.*, 1996; Ren *et al.*, 2003a; Kusakabe *et al.*, 2008). Interestingly, under normal conditions the steady-state levels of MT-I/II in rodent testes are higher than those found in other organs (De *et al.*, 1991; Salehi-Ashtiani *et al.*, 1993; Suzuki *et al.*, 1998). It is well established that Cd treatment induces MT-I/II expression in several tissues (e.g., liver), yet the steady-state levels of MTI/II do not appear to be induced in sufficient levels in the testis, and there are some conflicting results in the literature regarding their responses to Cd treatment (McKenna *et al.*, 1996; Zhou *et al.*, 1999; Ren *et al.*, 2003a; Ren *et al.*, 2003b; Kusakabe *et al.*, 2008). Such discrepancies could be attributed to differences in experimental design and/or conditions in these studies, such as the Cd concentrations used were not identical, the time when samples were harvested post-treatment were different, antibodies used for different assays were obtained from different sources with varying specificities, and in some cases the levels of steady-state mRNA of MT-I/II were examined whereas their proteins were quantified in other studies. Nonetheless, it is important to note that the toxic effects of Cd in the testis could not be reduced in MT-I transgenic mice even though the expression of MT-I in these animals was 5.2-fold higher than the wild type (Dalton *et al.*, 1996). Therefore, the high susceptibility of the testis to Cd toxicity may not be entirely related to the levels of MT-I/II, but possibly by the genetic background as earlier suggested (Liu *et al.*, 2001). It remains unclear why the testis displays a relatively high basal level of MT-I/II *versus* other organs (e.g., liver), yet it fails to detoxify the heavy metal.

MT-III has been detected in the testis of humans and rodents (Moffatt and Seguin, 1998; Hozumi *et al.*, 2008). Its function in the testis has not been studied and, even though it binds

to Cd (Palumaa *et al.*, 2002), MT-III appears to be unrelated to Cd detoxification. Instead, it has been described to have neuroprotective effects and, interestingly, MT-III levels are reduced in Alzheimer's disease (Yu *et al.*, 2001; Chung and West, 2004; Meloni *et al.*, 2008). On the other hand, tesmin was firstly described in the testis as a metallothionein-like protein (Sugihara *et al.*, 1999). Although tesmin has been suggested to participate in spermatogenesis and, more recently, classified as a member of the CXC-hinge CXC family (Matsuura *et al.*, 2002; Sutou *et al.*, 2003; Olesen *et al.*, 2004), it may still display some characteristics related to MT-I/II, such as metal sensitivity, since the expression of tesmin appears to be responsive to Cd. It was shown that in normal mice, tesmin was detected in the cytoplasm in pachytene spermatocytes at stages I-VIII, and it became translocated to the nucleus in the late pachytene or diplotene spermatocytes at stages X-XII. After Cd treatment, this spatial translocation of tesmin in spermatocytes was affected (Matsuura *et al.*, 2002; Sutou *et al.*, 2003). Nonetheless, the role(s) of MTs and tesmin in the testis remains to be determined.

5. Cadmium as an endocrine disruptor

Cd is a known endocrine disruptor by affecting the synthesis and/or regulation of several hormones [for a review, see (Henson and Chedrese, 2004; Darbre, 2006)]. Indeed, Cd affected progesterone synthesis in JC-410 porcine granulosa cells (Smida *et al.*, 2004) and recent studies have shown that Cd may also activate the estrogen receptor (ER) α and/or mimic estrogen effects in different tissues (e.g., uterus and mammary gland) (Johnson *et al.*, 2003) and breast cancer cell lines (Brama *et al.*, 2007). Cd regulates androgen receptor (AR) gene expression and activity in LNCap cells, a hormone-dependent human prostate cancer cell line, and also mimics androgenic effects in orchidectomized rats and mice (Martin *et al.*, 2002).

In male rodents, it is well established that Cd significantly alters the circulating levels of several hormones (e.g., testosterone, LH, FSH) (Lafuente *et al.*, 2004). Previous studies have shown that Cd impairs the testosterone production in isolated Leydig cells without affecting their viability (Laskey and Phelps, 1991), demonstrating that steroidogenic disruption in Leydig cells is likely to be an initial target of Cd toxicity as an endocrine modulator. Cd also decreased steroidogenic acute regulatory protein (StAR), LH receptor and cAMP levels in the testis (Gunnarsson *et al.*, 2007). Cd can also modify hormone levels by affecting the hypothalamic-pituitary-testicular axis in different aspects, not only via its effects on Leydig cells. For instance, Cd affected the circadian pattern release of noradrenaline, a regulator of hypothalamus hormone secretion, which resulted in changes in the daily pattern of plasma testosterone and LH levels (Lafuente *et al.*, 2004). In addition, plasma levels of pituitary hormones (e.g., LH, FSH, prolactin, ACTH) were also modified after Cd exposure (Lafuente *et al.*, 2003). Nevertheless, it remains to be investigated if Cd acts as an endocrine modulator by interacting with ERs or ARs in the testis and/or Leydig cells.

In short, it is important to note that the endocrine disruption induced by Cd is likely to be multifactorial, mediated via its effects on Leydig cells and/or the hypothalamic-pituitary-testicular axis. Therefore, one should critically analyze the *in vivo* effects induced by Cd regarding hormonal disruption, especially considering that the effects of Cd on the hormonal production and/or secretion, and if these effects are dose- and time-dependent (Lafuente *et al.*, 2003). Moreover, the sequence of (or the concomitant) effects and the mechanisms involved remains to be explored and questions still remain unanswered. For example, it remains to be determined if Cd modulates AR and/or testosterone effects in the testis. Furthermore, the effects of Cd on the expression of androgen-regulated genes, both *in vivo* and *in vitro*, in the testis and/or Sertoli and germ cells will be helpful to address some of these questions.

6. Cadmium-induced cellular toxicity in the testis

Several mechanisms have been proposed to mediate Cd-induced cellular toxicity. It has been postulated that Cd exerts its effects via the physicochemical properties of the Cd^{+2} ion, namely its similarities to Ca^{+2} (e.g., ionic radii) and Zn^{+2} (e.g., electron configuration). As such, Cd^{+2} is likely to substitute Ca^{+2} or Zn^{+2} in crucial physiological processes that are mediated by these ions, resulting in the activation and/or inhibition of several signaling pathways. For instance, Cd may cause an increase in oxidative stress by binding to sulfhydryl groups of proteins and by depleting glutathione [for a review, see (Valko *et al.*, 2005)]. Thereafter, the oxidative stress may promote alteration of DNA repair mechanisms and induction of cell proliferation, which, in turn, may lead to tumorigenesis [for a review, see (Beyersmann and Hartwig, 2008)]. Overall, Cd-induced cellular toxicity is likely involved several signaling pathways, but few studies have been conducted using the testis as a model. Herein, we briefly discuss the current literature regarding the cellular toxicity and/or the likely cytotoxic mechanisms in the testis.

6.1. Cadmium-induced testicular oxidative stress: the preventive effects of zinc and antioxidants

At present, it is well established that testicular oxidative stress is commonly induced under different normal and/or pathophysiological conditions, leading to male infertility. In fact, oxidative stress is a common factor in about half of the infertile men examined to date, illustrating the importance of Cd as an inducer of oxidative stress [for reviews, see (Tremellen, 2008; Turner and Lysiak, 2008)]. Although the testis expresses several antioxidant enzymes, such as superoxide dismutase, catalase and glutathione peroxidase to counteract the oxidative stress, their levels are greatly diminished upon Cd exposure (Sen Gupta *et al.*, 2004). Therefore, it is reasonable to assume that antioxidant agents (enzymatic and non-enzymatic) may prevent or at least reduce the Cd toxicity to the testis. Indeed, Parizek was the first to report that Zn could prevent testicular damage induced by Cd (Parizek, 1957), while Gunn *et al.* observed the prevention of Cd-induced Leydig cell tumors by Zn (Gunn *et al.*, 1963). However, at that time the oxidative stress induced by Cd was not established, but the authors were correct when proposing that the prevention of the Cd-induced testicular injury by Zn could be due to the similarity between these ions and their ‘competition’ for the physiological binding sites of Zn. Subsequent studies with substances having antioxidant activities, such as vitamin C, vitamin E, Zn, selenium and melatonin, have also demonstrated that the oxidative stress was associated with the Cd-induced testicular damage, as these substances reduced and/or prevented both the oxidative stress and damage in the testis caused by Cd (Niewenhuis and Fende, 1978; Hu *et al.*, 2004; Kara *et al.*, 2007; Acharya *et al.*, 2008; Amara *et al.*, 2008; Burukoglu and Baycu, 2008).

6.2. Cd^{2+} mimics Ca^{+2} in physiological processes

Few studies have focused on the interaction between Cd and Ca-binding molecules, such as calmodulin, in Cd-induced testis damage. Indeed, Cd was shown to bind to calmodulin (Cheung, 1988) and, interestingly, calmodulin inhibitors (e.g., chlorpromazine, trifluoperazine and W-7) were shown to prevent the Cd-induced damage in the mouse and rat testis (Niewenhuis and Prozialeck, 1987; Shiraishi *et al.*, 1994). These findings were supported by a recent report in which an inhibitor of calcineurin (a Ca-calmodulin-dependent phosphatase) was shown to prevent Cd-induced testicular injury in the mouse (Martin *et al.*, 2007). These studies have shown that part of the Cd-induced damage to the testis is likely the result of the similarity between Cd^{2+} and Ca^{2+} , with the ‘improper’ and ‘unintentional’ induction of cellular events mediated by Ca-calmodulin. Further studies are needed to define which molecular effects are indeed elicited by Cd-calmodulin and/or whether Cd^{2+} binds to other regulators of the Ca^{2+} -induced signaling pathways.

7. Concluding remarks and future perspectives

As reviewed herein, recent findings in the field have shed light on the underlying mechanism(s), at least in part, that causes Cd-induced damage to the testis, such as the disruption of the BTB. These findings have shown that Cd likely exerts its effects, at least in part, via the stress-activated p38 MAPK pathway, suggesting that Cd-induced male infertility may possibly be managed by manipulating MAPK via the use of specific inhibitors. Another approach is to unravel the use of Zn, calmodulin inhibitors, or other antioxidants to prevent Cd-induced injury to the testis. While we cannot provide a unified mechanism of Cd-induced testicular injury due to limited number of studies in the field, the likely mechanism by which Cd disrupts the BTB mediated by MAPK as depicted in Figure 4 should be helpful to investigators in the field. Furthermore, as we discussed herein, Cd is a major environmental and occupational contaminant, while we can further reduce exposure of the general public to this environmental toxicant through tougher government sponsored restrictions and/or guidelines, it is unlikely we can eliminate Cd from our environment. As such, it is crucial to seek an expanded effort amongst investigators to further define its mechanism(s) of toxicity and exploit this new information to develop therapeutic approaches to manage Cd toxicity.

8. References

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Figure 1. Cd-induced testicular injury in the testis

A schematic drawing illustrating a normal testis and a damaged testis after Cd exposure, representing the most common gross morphological changes induced by Cd: hemorrhage and testis weight loss. The histological injury induced by Cd is noticeable when comparing micrographs of hematoxylin-eosin stained paraffin cross-sections of testes from rats terminated after 20 hr of treatment with vehicle (0.9% NaCl; upper panel) or CdCl₂ (3 mg/kg b.w., i.p.; lower panel). In normal testis (upper panel), the micrograph illustrates the well-organized distribution of cells in the seminiferous epithelium (SC, Sertoli cell; es, elongating spermatid; sp, spermatocyte); the interstitial Leydig cells (LC) and intact blood vessels in the interstitium. After Cd treatment, disruption of the tight junctions in the microvessels led to the leakage of blood cells (BC, lower panel), most notably erythrocytes, into the interstitial space, causing hemorrhage and edema. In addition, Cd affected Leydig cells (LC), thereby promoting a reduction in steroidogenesis, and Cd also induced cell necrosis, for example, by production of reactive oxygen species. Taken together, these effects induced by Cd exposure result in germ cell loss, causing irreversible sterility. Scale bar, 80 μ m.

Figure 2. A schematic drawing illustrating the relative location of the blood-testis barrier (BTB) and various junction types found in the seminiferous epithelium at the Sertoli-Sertoli and/or Sertoli-germ cell interface which are the targets of Cd toxicity in adult rat testes

The seminiferous epithelium, consisted of Sertoli cells and germ cells at different stages of their development, is overlying the tunica propria, which is composed of the non-cellular zone (basement membrane, type I collagen layer) and the cellular zone (peritubular myoid cell layer and the lymphatic vessel). The BTB, created by adjacent Sertoli cells near the basement membrane, divides the seminiferous epithelium into the basal and the adluminal compartments. The BTB is constituted by coexisting tight junction (TJ), desmosome-like junction, gap junction, and basal ectoplasmic specialization (basal ES, which is a testis-specific actin-based

atypical adherens junction type), which is one of the primary targets of Cd toxicity in the testis. It is noted that pre/leptotene spermatocytes traverse the BTB at stage VIII-IX of the seminiferous epithelial cycle of spermatogenesis, so that post-meiotic germ cell development takes place behind the BTB, wherein secondary spermatocytes develop into round, elongating and elongated spermatids.

Figure 3. Cellular localization of focal adhesion kinase (FAK) and occludin and their redistribution following CdCl₂ treatment in Sertoli cells cultured *in vitro*

Primary rat Sertoli cells (0.05×10^6 cells/cm²) were cultured on Matrigel-coated coverslips for 4 days, known to form functional tight junction, basal ES and desmosome-like junction, that mimics the BTB *in vivo* (Siu *et al.*, 2005), and stained for FAK by incubation with mouse monoclonal anti-FAK antibody (Upstate, Cat No.: 05-537, Lot: 0701049384) and goat anti-mouse IgG AlexaFluor 488-conjugated secondary antibody. FAK was localized to the Sertoli-Sertoli cells interface and some staining was found in the cytosol and nuclei (A, a). Treatment with 3 μ M CdCl₂ caused a redistribution of FAK from the cell-cell interface towards the cytoplasm that was visible after 3 hr (A, b) and more evident after 6 hr (A, c), illustrating a

time-dependent effect. The specificity of the FAK antibody used in this study was confirmed by immunoblot using Sertoli cell lysates (30 µg of protein), which shows a single band of ~125 kDa (B). FAK was shown to co-localize with occludin (C, a-c), which is an integral membrane tight junction protein at the BTB, illustrating it is a component of the BTB consistent with an earlier report (Siu *et al.*, 2003). Following CdCl₂ treatment (3 µM, 6 hr) both proteins were mis-localized (C, d-f), moving away from the Sertoli-Sertoli cell interface, suggesting that FAK may play a role in the damage of Sertoli-Sertoli cell junctions induced by Cd. Scale bars = 20 µM.

Figure 4. Cd-induced blood-testis barrier (BTB) and cell junction disruption in the seminiferous epithelium is mediated by the activation of specific signal transduction pathways

A schematic drawing illustrating the likely sequence of events in the seminiferous epithelium following Cd treatment (e.g., 3 mg/kg b.w. via i.p.) that leads to BTB disruption and germ cell loss in three phases. In Phase I, Cd ions likely enter Sertoli and/or germ cell via different mechanisms, such as diffusion, Ca^{2+} channels or using a $\text{Cd}^{2+}/\text{Zn}^{2+}$ transporter (Step 1). Although the Cd transporter, ZIP8, is present in Sertoli cells in the mouse, it is not known if it is present in all rodents and/or mammals, as well as in germ cells. Once inside the Sertoli and/or germ cell, Cd induces the synthesis and release of cytokines (e.g., TGF β -3), which, by interacting with their respective receptors, activates the stress activated p38 MAPK signaling pathway (Step 2) that can selectively disrupt the BTB. At the same time, possibly also mediated by the p38 MAPK, the production of proteases (e.g., cathepsin L) is also induced (Step 3). In Phase II, the BTB is disrupted via degradation of the integral membrane proteins (via the effects of proteases) and/or an acceleration of clathrin-mediated endocytosis of tight junction (e.g., occludin) and basal ES proteins (e.g., N-cadherin). The net results of all this contribute to the disruption of the BTB and Sertoli-germ cell junctions that lead to germ cell loss (Step 4). In Phase III, in order to limit unwanted proteolysis and avoid the dissolution of the entire seminiferous epithelium in the testis, there is an activation of c-JNK signaling pathway, which, in turn, stimulates the production of protease inhibitors (e.g., α_2 -macroglobulin) to initiate recovery of the seminiferous epithelium (Step 5). This cascade of events depicted herein is based on recent findings in the field as detailed in the text.

Table 1Cadmium-induced testicular injury and the target candidates in various mammalian species^a

Testicular injury (phenotype/disease)	Target candidates (structures, proteins or genes)	Animal species	References
Apoptosis	p53, c-JNK, Caspase-3, p53, c-JNK	Mouse	(Zhou <i>et al.</i> , 2004)
		Rat	(Xu <i>et al.</i> , 1999; Zhou <i>et al.</i> , 1999; Hu <i>et al.</i> , 2004)
BTB disruption ^b	n.d.	Guinea-pig	(Johnson, 1969)
	n.d.	Mouse	(Terada <i>et al.</i> , 2005)
	TGFβ-3, p38 MAPK, c-JNK, FAK ^b	Rat	(Setchell and Waites, 1970; Hew <i>et al.</i> , 1993b; Lui <i>et al.</i> , 2003; Wong <i>et al.</i> , 2004; Wong <i>et al.</i> , 2005) (Siu and Cheng, unpublished observations)
Edema, hemorrhage, necrosis and sterility ^c	BTB and vascular disruption	Calf	(Powell <i>et al.</i> , 1964)
		Dog	(Dixit <i>et al.</i> , 1975)
		Guinea-pig	(Parizek, 1960; Johnson, 1969)
		Hamster	(Parizek, 1960)
		Monkey	(Kar, 1961)
		Mouse	(Meek, 1959; Chiquoine, 1964)
		Rabbit	(Cameron and Foster, 1963)
Carcinogenesis	n.d. Testosterone, c-fos, c-JNK, antioxidant enzymes, DNA repair genes	Rat	(Roe <i>et al.</i> , 1964)
		Mouse	(Roe <i>et al.</i> , 1964; Koizumi and Li, 1992; Waalkes <i>et al.</i> , 1997; Hu <i>et al.</i> , 2004)
		Rat	(Roe <i>et al.</i> , 1964; Koizumi and Li, 1992; Waalkes <i>et al.</i> , 1997; Hu <i>et al.</i> , 2004)

^aThis table is not intended to be exhaustive, only selected examples are listed herein, illustrating different phenotypic changes/diseases as a result of cadmium-induced testicular damage;

^bSeveral cell junction proteins, proteases and protease inhibitors associated to the Cd-induced BTB disruption are described in Sections 2 and 3 in text;

^cTaken together, the disruption of the BTB and vascular system led to these pathological conditions; n.d., not described.

Table 2Cadmium effects on testicular cells *in vitro*

<i>In vitro</i> model	Observation(s) after Cd exposure	References
Primary rat Sertoli cell cultures	Low dose: impairment of development and maintenance of TJ-permeability barrier;	(Janecki <i>et al.</i> , 1992; Chung and Cheng, 2001)
	Decrease in uPA and occludin expression, testosterone can lessen Cd-induced TJ-barrier damage;	(Chung and Cheng, 2001)
	High dose: decreases in inhibin secretion and cell viability; cell death;	(Janecki <i>et al.</i> , 1992)
	Cell vacuolization and pyknosis, cells became round and then detached from the substratum;	(Syed <i>et al.</i> , 1997)
	Redistribution of FAK, occludin and ZO-1, decrease in TJ integral membrane proteins, activation of p38 MAPK	(Siu and Cheng, unpublished observations)
Rat Sertoli cell line (ASC-D19) cultures	TJs disruption, apoptosis	(Sorenson and Brabec, 2003)
Primary rat Sertoli cells and primary rat Leydig cells cultures	Sertoli cells are more sensitive to Cd exposure than Leydig cells	(Clough <i>et al.</i> , 1990)
Primary rat Sertoli cell-gonocyte cocultures	Morphological changes, decrease in cell viability, apoptosis associated with an increase in caspase-3/7-like activity, disruption of ubiquitin-dependent protein degradation, activation of SAPK, c-JNK and p38 MAPK	(Yu <i>et al.</i> , 2008)
Primary rat Leydig cell cultures	Decrease in testosterone synthesis, increase in oxidative stress and cellular DNA damage, decrease in cell viability	(Yang <i>et al.</i> , 2003)
Rat Leydig cell line (R2C) cultures	Cellular DNA damage	(Shiraishi <i>et al.</i> , 1995)
Isolated rat Leydig cells	Cd uptake via passive and non-passive (carrier mediated or active transport or both) diffusion, with the latter being inhibited by Zn treatment Decrease in testosterone synthesis	(Waalkes, 2003) (Laskey and Phelps, 1991)