

# Environmental Toxicants and Testicular Apoptosis

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**Abstract:** When apoptosis is improperly activated or regulated in the testis, infertility or even cancer can result. Studies have implicated elevated rates of apoptosis in infertile male patients. Pinpointing how environmental toxicity affects apoptosis is important for the advancement of preventative medicine and behavior, especially as potentially harmful compounds continue to proliferate in households and workplaces. Moreover, familiarity with testicular processes, particularly the induction of apoptosis, is essential for promoting male fertility. This review examines environmental toxicants that have been implicated in testicular apoptosis. We elucidate the mechanistic pathways through which specific xenobiotic compounds trigger cell death in the testis. This review highlights the role of oxidative stress in mediating these apoptotic actions.

**Keywords:** Oxidative stress, environmental toxicants, apoptosis, spermatogenesis, Leydig cell, Sertoli cell.

## INTRODUCTION

Apoptosis can be a two-faced companion reinforcing tissue homeostasis and physiological processes as a friend, instigating organ dysfunction and disease as a foe [1]. When apoptosis is improperly activated or regulated in the testis, infertility or even cancer can result. Studies have implicated elevated rates of apoptosis in infertile male patients [2]. Pinpointing how environmental toxicity affects apoptosis is important for the advancement of preventative medicine and behavior, especially as potentially harmful compounds continue to proliferate in households and workplaces. Moreover, familiarity with testicular processes, particularly the induction of apoptosis, is essential for promoting male fertility [3]. In male patients with idiopathic infertility, ROS-induced sperm damage is associated with increased apoptosis [4].

This review examines environmental toxicants that have been implicated in testicular apoptosis. We elucidate the mechanistic pathways through which specific xenobiotic compounds trigger cell death in the testis. This review highlights the role of oxidative stress in mediating these apoptotic actions.

## PHYSIOLOGY AND PATHOLOGY OF TESTICULAR APOPTOSIS

Apoptosis occurs under the direction of an active, intracellular death program that can be stimulated or inhibited by environmental agents [5]. Under developmental regulation, apoptosis proceeds in response to: a) deprivation of survival factors, such as testosterone, b) activation by ligated death

factors, or c) exposure to environmental stimuli, such as radiation, chemotherapeutic drugs, and ROS [6]. By activating caspases, ROS may initiate the propagation of a series of reactions that ultimately trigger apoptosis. Additionally, apoptosis may be induced by cell injury or stress. The principal function of testicular apoptosis is to help maintain tissue homeostasis during spermatogenesis [2].

The reproductive system involves dramatic cycles of tissue growth and degeneration, and spermatogenesis is no exception. Spermatogenesis is the process involving mitosis, meiosis, and cellular differentiation that transforms spermatogonia into mature spermatozoa [7]. During this process, the seminiferous epithelium proliferates rapidly in the testis.

The seminiferous epithelium consists of germ cells and Sertoli cells. Germ cells carry the genetic material and mature into the sperm cells that ultimately fertilize the egg. Helping to regulate the development of nascent sperm cells, Sertoli cells are the somatic cells that provide physical reinforcement to germ cells and mediate the movement of growth factors, hormones and signals into the seminiferous tubules where spermatogenesis commences. Additionally, Sertoli cells contribute to the consumption of excess byproducts after sperm cells have fully developed [2, 6, 8]. Any factor that impairs the viability and function of either germ cells or Sertoli cells may directly affect spermatogenesis.

Testicular apoptosis serves to deplete excess germ cells and remove abnormal spermatozoa during normal spermatogenesis. Indeed, apoptosis eliminates 75% of germ cells before they become fully mature [2, 3]. In this way, testicular apoptosis monitors germ cell population according to the support capacity of Sertoli cells [3].

While apoptosis is essential for maintaining testicular homeostasis during spermatogenesis, inappropriately occurring apoptosis has been linked to suboptimal male repro-

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ductive function [2]. Excessive apoptosis results from impaired regulation or improper activation, and can affect spermatogenesis and even lead to infertility [9, 10].

## PLAYERS AND PATHWAYS INVOLVED IN TESTICULAR APOPTOSIS

Executing apoptosis requires constitutively expressed proteins. Researchers have uncovered the genes for pro-apoptosis proteins, such as caspases and Apaf-1, and anti-apoptosis proteins, such as Bcl-2 [11-13]. These proteins, along with p53, NF- $\kappa$ B, and death receptors, play key roles in human apoptosis. Studying the nature of their responses to xenobiotic toxins may elucidate the mechanisms that oversee environmentally induced apoptosis.

### Caspases

The caspase family consists of different cysteine proteases that modulate apoptosis by cleaving specific intracellular proteins, which can then activate other destructive cell processes [5, 14]. Jacobson *et al.* have demonstrated that caspase inhibitors block apoptosis in animal cells [5]. Caspases are synthesized as zymogens, and undergo proteolytic activation at specific cleavage sites. Caspases may cleave other caspases [15].

Caspases act in a cascade, in which upstream initiator caspases amplify and integrate pro-apoptotic signals that then activate downstream effector caspases. Caspases-1, -2, -4, -5, -8, -9, -10, -11, and -12 comprise the upstream initiator caspases. They flock in inactive procaspase form to ligated death receptors, where they then undergo activation. The initiator caspases possess a long N-terminal prodomain, either a caspase recruitment domain (CARD) or a death effector domain (DED), which serves as scaffolding for the aggregation of caspase activating proteins. These protein complexes form as specialized responses to pro-apoptotic signals, and nudge their respective caspase cascades into motion [14]. For example, caspase-9 is activated in the mitochondrial pathway by the apoptosome, which engages with apoptotic protease-activating factor-1 (APAF-1) and cytochrome c. In the death signaling pathway, caspase-8 is triggered by the death inducing signaling complex (DISC), which forms at the Fas receptor. Proteolytic activation of caspases-8 and -10 ultimately triggers the caspase cascade [16].

The effector caspases consist of caspases-3, -6, -7, and -14. Activated initiator caspases send signals to the effector caspases, which then cleave their respective substrates [5, 14]. The target proteins include cytoplasmic structural proteins, such as actin, as well as nuclear proteins, such as PARP and lamins [17-19]. In addition to inducing protein degradation, activated caspase-3 can degrade DNA *via* proteolytic activation of DNases [20, 21]. These varieties of degradation trigger the execution of apoptosis.

### Bcl-2 Family Proteins

Bcl2 and its homologues regulate caspase activation. Among the more than 20 members of the mammalian Bcl2 family, five proteins are anti-apoptotic (Bcl-2, Bcl-xL, Bcl-

w, A1 and Mcl1) and two subgroups are pro-apoptotic (the BH3-only proteins and the effector proteins).

The antiapoptotic Bcl2-like proteins are embedded within the mitochondrial outer membrane, and regulate membrane integrity by directing blocking their proapoptotic cousins. Researchers have speculated that the Bcl2-like proteins could also regulate several initiator caspases that are upstream of or removed from mitochondrial disruption, although these caspases would be redundant triggers [22, 23].

The pro-apoptotic clan of the Bcl2 family is divided into the BH3-only proteins and the effector proteins (also called the Bax family). The BH1, BH2, BH3 and BH4 motifs from the Bcl-2 like proteins remain highly conserved in the effector proteins, whereas the BH3-only proteins contain only their namesake region. The BH3-only proteins (Bid, Bim, Bik, Bad, Bmf, Hrk, Noxa, Puma) promote apoptosis by inactivating their anti-apoptosis cousins, the Bcl2-like proteins, or by activating effector proteins, Bax and Bak. The balance between anti-apoptosis and BH3-only proteins determine tissue homeostasis; inactivation of Bcl2 boosts apoptosis. In addition to directly neutralizing anti-apoptotic proteins, the BH3-only family contributes to apoptosis by detecting intracellular damage and physiological death cues [22, 23].

The Bax family is involved further downstream in mitochondrial disruption. Involved in mediating mitochondrial membrane channels, Bax and Bak contribute to the release of apoptogenic proteins, such as cytochrome c. The mechanism of Bax and Bak activation remains unknown [23].

Non-Bcl-2 family proteins, such as p53, have also been implicated in the regulation of mitochondrial outer membrane permeability. Cytosolic p53 not only localizes to the mitochondria following stress induction, but also antagonizes Bcl2-like protein and directly activates effector proteins. By stimulating Bax expression, p53 skews the Bax to Bcl-2 ratio and promotes apoptosis [24].

### p53

p53 is the main human tumor suppressor protein, and mediates various cell cycle checkpoints to prevent replication of damaged DNA. To do so, p53 acts *via* gene regulation to induce cell cycle arrest or apoptosis [25]. p53-induced apoptosis occurs physiologically to remove excess or non-intact cells. p53-mediated apoptosis can occur by way of trans-activation, upregulating Bax activity or downregulating Bcl2 activity. When faced with DNA damage, p53 becomes active and can also activate transcription of p21. p21 gene overexpression stimulates the proapoptotic Bax gene, which warps the Bcl-2:Bax ratio. The transcription factor can also target the BH3-only class of proteins, such as Puma, Noxa and Bid, which act upstream of Bax [24].

The understanding of p53-induced apoptosis has long focused on p53's transcriptional activation of proapoptotic genes, such as BAX, PUMA, FAS/APO-1, NOXA, PIGs, and p53AIP1 [26]. However, p53 possesses a duality that alternates between trans-activation and trans-repression. Although its role as a gene repressor has been less re-

searched and less understood, a burgeoning wave of studies suggests that p53 can also trigger apoptosis by repressing transcription of antiapoptotic genes. Indeed, researchers showed that p53 acted as a transcriptional repressor of antiapoptotic genes PLK, PTTG1 and CHEK1, and that inhibiting repression of these specific genes significantly reduced the apoptotic response. Immunoprecipitation and reporter assays showed that p53 targets antiapoptotic signals both directly and indirectly. Speculated mechanisms of indirect gene repression by p53 include interfering with E2F-mediated transcription and recruiting a repressor complex *via* p21 [27].

If the Bax level is constitutively high, p53 may mediate apoptosis through a transcriptionally independent pathway. Additionally, p53 can induce apoptosis independent of transactivation. Cytoplasmic or mitochondrial p53 can assume the role of BH3-only proteins and dimerize with Bcl2 family proteins at the outer mitochondrial membrane. By antagonizing Bcl2 proteins, p53 can free Bax and Bak for apoptotic induction [24].

### NF- $\kappa$ B

NF- $\kappa$ B is a transcription factor associated with apoptosis. In its inactive state, NF- $\kappa$ B is bound to I $\kappa$ B in the cytoplasm. NF- $\kappa$ B inducers activate NF- $\kappa$ B by degrading I $\kappa$ B, thus freeing NF- $\kappa$ B for translocation to the nucleus [28]. NF- $\kappa$ B can act as both a proapoptotic and anti-apoptotic regulator within the same cell type, and so its specific function is determined by the environment.

NF- $\kappa$ B upregulates a number of proapoptotic genes, including Fas and death receptors 4, 5, and 6. In a rat study by Saradha *et al.*, NF- $\kappa$ B activation peaked within 24h of testicular germ cell exposure to lindane, whereas maximal Fas expression did not occur until 72h post-exposure. The staggered activity suggests that Fas expression increased in response to NF- $\kappa$ B up-regulation, indicating NF- $\kappa$ B's proapoptotic function [29].

Previous observations of glucocorticoid-induced upregulation of I $\kappa$ B, as well as mutual antagonism between NF- $\kappa$ B and glucocorticoid, suggest that NF- $\kappa$ B could act as an inhibiting agent in glucocorticoid-mediated apoptosis. In addition, NF- $\kappa$ B inhibited CORT-induced apoptosis in rat Leydig cells, as cells overexpressing NF- $\kappa$ B were less susceptible to CORT-induced apoptosis; cells receiving PDTC, a known NF- $\kappa$ B inhibitor, underwent higher levels of CORT-induced apoptosis [28]. This suggests that NF- $\kappa$ B acts as an anti-apoptotic agent in receptor-mediated apoptosis.

Testicular NF- $\kappa$ B responds to MEHP exposure, suggesting the regulatory role of NF- $\kappa$ B in germ cell apoptosis. NF- $\kappa$ B subunits showed a variety of localization patterns in rat testis following MEHP exposure. However, elevated activation in spermatocytes was particularly notable, since these germ cells are in the meiotic stage most sensitive to MEHP-mediated damage [8].

### Mitochondria-Mediated Pathway

Mitochondrial cell respiration is the main consumer of oxygen, as well as the primary location of endogenous ROS

production. Studies of different cell types show that several apoptotic pathways converge on the mitochondrion, which then becomes a key player in continuing the cell death sequence [30-33].

Intracellular stress triggers mitochondrial apoptosis, which is regulated by the Bcl2 family [23]. Cellular stress results in the release of cytochrome c from damaged mitochondria. Cellular stress or injury induces interaction between the Bax-like and BH3-only protein families, which permeabilizes the mitochondrial outer membrane and releases apoptotic proteins for diffusion into the cytosol. The release of cytochrome c and its subsequent interaction with cytosolic protein APAF-1 results in a protein complex called the apoptosome. This oligomer serves as scaffolding for caspase activation, which ultimately leads to apoptosis [22]. The activation of caspase-9 begins the caspase cascade towards apoptosis [23]. Furthermore, PT induces mitochondrial production of ROS [6].

Non-Bcl-2 family proteins, such as p53, have also been implicated in the regulation of mitochondrial outer membrane permeability. Cytosolic p53 not only localizes to the mitochondria following stress induction, but also antagonizes Bcl2-like protein and directly activates effector proteins.

It has been proposed that mitochondrial disruption is "neither necessary nor sufficient for apoptosis," as recent evidence shows that the caspase cascade can proceed sans cytochrome c release, and certain cells keep surviving for days after a mitochondrial breach [6].

### Receptor-Mediated Pathway

Spermatogenesis is a meticulously regulated process that incorporates autocrine, paracrine, and endocrine signaling. The Fas signal pathway is a critical paracrine regulator that comprises Fas ligand (FasL) expressed in Sertoli cells and the Fas receptor expressed in germ cells.

Caspase-8 and Fas-associated protein with Death Domain (FADD) are both components of DISC, and their interaction activates caspase-8 [34]. Similarly, when TNF $\alpha$  binds to TNF-R1 death receptor, TNF-R1 attracts the aggregation of various cytoplasmic signaling proteins, including TNF Receptor-Associated protein with Death Domain (TRADD). Upon interaction with TRADD, TNF-R1 is able to recruit and "unlock" Fas-associated protein with Death Domain (FADD), which in turn recruits procaspases-8 and -10 [6]. Fas signaling regulates NF- $\kappa$ B transcription, while activated NF- $\kappa$ B also regulates Fas transcription [8].

### OXIDATIVE DAMAGE IN THE TESTIS

Oxidative stress (OS) results from excessive biosynthesis or intake of pro-oxidants, impaired biosynthesis of antioxidants, or a combination of both. Balancing pro-oxidants and antioxidants is vital for normal testis function and sperm fertilization ability [7]. Consequently, much focus has been given to reactive oxygen species (ROS), a group of highly reactive oxidizing agents that are able to damage almost all molecular species in spermatozoa, including lipids, proteins and nucleic acids [7, 35]. Whereas low ROS levels are necessary for sperm maturation, high ROS levels wreak OS-

mediated pathological consequences on spermatozoa, including sperm DNA fragmentation [36] and abnormal sperm morphology [37]. Thus, excessive generation of ROS can cause cellular damage, and impair sperm structure and function.

### Spermatogenesis

Developing spermatozoa undergo a series of maturation processes involving ROS. This growth course involves membrane, nuclear and enzyme-related remodeling, the release, attachment and rearrangement of surface proteins, as well as the assembly of the signal transduction machinery. Aitken *et al.* demonstrated that ROS act as intracellular signaling molecules to aid proper chromatin packaging and stability [35].

The sperm cell is distinct from other germinal or somatic cells in its ability to generate ROS as well as its great vulnerability to such molecules. Not only do sperm cells contain high levels of polyunsaturated fatty acids and endogenous ROS, but they have a limited store of cytoplasmic defense enzymes that leaves them nearly incapable of membrane repair. Consequently, sperm cells are particularly susceptible to oxidative damage, especially lipid peroxidation. Due to evidence linking excessive ROS synthesis and failed spermoocyte fusion, oxidative stress has been proposed to damage the sperm plasma membrane [38].

Oxidative stress can inflict direct oxidative damage to genomic DNA or up-regulate apoptotic proteins. Either pathway leads to germ cell death and impaired spermatogenesis [39, 40]. Oxidative stress and the release of ROS during spermatogenesis have been linked by many studies to the role of germ cell apoptosis in the testis [41-46].

### Steroidogenesis

Steroidogenesis is the generation of steroid hormones by a variety of tissues in the endocrine system. Among the secreted hormones, testosterone and follicle stimulating hormone (FSH) act concomitantly as the two main regulators of spermatogenesis. Testosterone is an androgen synthesized in Leydig cells under the direction of luteinizing hormone (LH), and is essential for the initiation and progression of normal spermatogenesis. FSH is a glycoprotein synthesized and secreted by gonadotrophs of the anterior pituitary controlled by pulses of gonadotropin-releasing hormone (GnRH). It acts synergistically with LH in stimulating the maturation of germ cells as well as inducing the formation of Sertoli-Sertoli intracellular tight junctions. Both testosterone and FSH bind to target receptors on the Sertoli cells and inhibit death signals sent to germ cells [3, 47].

Serum corticosterones have been reported to induce Leydig cell apoptosis and impair Leydig cell steroidogenesis [48]. Additionally, elevated testosterone levels have been shown to induce germ cell death [49]. Kim *et al.* demonstrated that testosterone withdrawal induces spermatocyte apoptosis *via* caspase-3 activation and CAD expression [50]. Therefore, it is critical that normal hormone levels remain intact to ensure properly occurring steroidogenesis.

Studies have suggested various mechanistic pathways in which toxins may inhibit testosterone production [51, 52].

Ronco *et al.* demonstrated that lindane, an organochlorine pesticide, reduced the production of cAMP, a second messenger in the testicular steroidogenesis pathway [52].

The biosynthesis of steroid hormones from a cholesterol precursor requires oxidative enzymes located in the mitochondria and endoplasmic reticulum. As an oxygen-mediated process, steroidogenesis is particularly vulnerable to ROS-induced damage. Since physiological ROS activity and lipoprotein consumption in the plasma are both essential once steroidogenic cells are stimulated, excessive ROS levels may effect overproduction of steroid hormones and subsequently induce apoptosis [3, 53]. Diemer *et al.* report that ROS inhibit steroidogenesis in human Leydig cells by targeting hormone-sensitive mitochondrial cholesterol transfer [13].

### OS AND TESTICULAR APOPTOSIS: INIMICAL ROLE OF ENVIRONMENTAL TOXINS

Rising incidence of infertility over the past several decades has snared the attention of scientists and clinicians [54, 55]. Furthermore, environmental toxins have been well-documented in their deleterious effects on male reproductive function [35, 40, 56]. Numerous studies implicate such toxicants in inducing DNA fragmentation and chromatin damage and impairing testicular function [56-59]. Much research has focused on oxidative stress: its pathological implications with regards to reproductive health as well as its etiology in environmental toxicants [60].

Environmental contaminants are capable of elevating ROS levels and depleting ROS-scavenging antioxidants. By inducing oxidative imbalance, these compounds alter key processes, such as apoptosis, spermatogenesis and steroidogenesis. Research indicates that common mechanisms include up- or down-regulating the expression of apoptotic related proteins, in addition to directly triggering apoptosis in spermatocytes [7, 61]. Numerous toxins have been tested to elucidate the mechanistic pathways through which they induce OS-mediated testicular apoptosis. These findings are summarized in Table 1.

### Alkylphenol Polyethoxylates

During seminiferous tubule maturation, oestradiol acts synergistically with FSH to support germ cell survival. However, when FSH is deficient or absent, oestradiol has an inhibitory, pro-apoptotic effect [3]. Since estrogen receptors are present in the pituitary and spermatogenic cells, estrogen-like chemicals can act as agonists or antagonists for the hormone, thereby interfering with spermatogenesis [62].

Octylphenol (OP) is a metabolite of alkylphenol polyethoxylates, which are commonly found in industrial processing and in household and institutional cleaning products [63, 64]. OP is more persistent than its parent compounds, and can mimic naturally produced estrogen by interacting with estrogen receptors [65]. Bian *et al.* demonstrated that 150 mg/kg/d of OP significantly lowered sperm motility; raising the dose to 450 mg/kg/d also significantly decreased testicular sperm count and daily sperm production [66].

Blake *et al.* demonstrated that chronic administration of 4-tert-octylphenol (OP) to adult male rats resulted in lower

**Table 1. Summary of OS-Mediated Mechanisms of Environmentally Induced Apoptosis, by Toxin**

Compound	Study	Cells Observed	Genes/Proteins Involved	Proposed Mechanisms
Octylphenol [50, 63, 100]	Mishra <i>et al.</i>	Germ	↑FasL, ↑cyt c	Fas-signaling pathway, mitochondrial pathway
	Qian <i>et al.</i>	Sertoli	↑Bax, ↓Bcl-2, ↓downstream Fas/FasL effector	Altering Bcl-2/Bax ratio activates mitochondrial pathway
	Kim <i>et al.</i>	Germ	↓Bcl-xL, ↔Bcl-2, ↔Bax	Altering Bcl-2/Bax ratio activates mitochondrial pathway
Nonylphenol [72]	Han <i>et al.</i>	Sertoli	↑Fas, ↑FasL	Direct cell damage followed by compensatory activation of Fas-signaling pathway
Polycyclic aromatic hydrocarbons [74]	Coutts <i>et al.</i>	Germ	↑Bax, ↑PARP cleavage	Mitochondrial pathway
Bisphenol A [76; 78]	Li <i>et al.</i>	Germ, Leydig	↑Fas, ↑FasL, ↑caspase-3	Fas-signaling pathway triggers Leydig cell apoptosis that then induces germ cell apoptosis
	Takahashi <i>et al.</i>	Germ	↑Degradation of late spermatids and seminiferous tubules, ↔testosterone	Direct cell damage
Polychlorinated biphenyls [38]	Hsu <i>et al.</i>	Germ	↑caspase-3, ↑caspase-9, ↓Fas, ↓Bax, ↓Bcl-2, ↓p53	Activation of caspase cascade
Lindane [29]	Saradha <i>et al.</i> (2009)	Germ	↑cyt c, ↑caspase-9, ↑caspase-3, ↑Fas, ↑FasL	Mitochondrial pathway, Fas-signaling pathway
Methoxychlor [89]	Vaithinathan <i>et al.</i>	Germ	↑Fas, ↑FasL, ↑caspase-3, ↓NF-κB	Mitochondria and FasL-mediated signaling pathway
Toluene [91]	El-Nabi Kamel <i>et al.</i>	Germ	↔caspase-3	Inconclusive
	Yilmaz <i>et al.</i>	Leydig	↓testosterone	Inconclusive
<i>tert</i> -Butyl hydroperoxide [94]	Kalia <i>et al.</i>	Germ	↑p53, ↑p21	p53 and p21 upregulation trans-activates Bcl-2 family proteins
Mono-(2-ethylhexyl) phthalate [99]	Yao <i>et al.</i>	Germ	↑NF-κB, ↑sTNFα	Promotion of Fas-signaling pathway by direct and indirect up-regulation of Sertoli cell FasL expression
Di-(2-ethylhexyl) phthalate [97]	Kasahara <i>et al.</i>	Germ	↑cyt c, ↑MEHP	DEHP metabolism inflicts OS on mitochondria
2,5- Hexanedione (2,5-HD) [100]	Mishra <i>et al.</i>		↑Cleaved PARP, ↑Bcl-xS/Bcl-xL, ↔Bcl-2, ↔Bax	ROS-induced increase in intracellular Ca <sup>2+</sup> alters Bcl-2 family protein levels
1,3-Dinitrobenzene (1,3-DNB) [101]	Lee <i>et al.</i>	Germ	↑Bax, ↓Bcl-2	Alteration of Bcl-2/Bax ratio triggers mitochondrial pathway
Nitrobenzene [102]	Richburg <i>et al.</i>	Germ		
Ethanol [104-105]	Koh		↓pAkt, ↓pErk1/2, ↓Bad	Suppression of survival-signaling pathway
	Hu <i>et al.</i>	Germ	↑FasL	Fas-signaling pathway

levels of LH, FSH, prolactin and testosterone, and proposed that OP inhibited the secretion of reproductive hormones by mimicking estrogen [67].

An *in vitro* study by Mishra *et al.* indicates a link between increased estrogen exposure and sperm cell death [68]. The results suggest that FasL upregulation mediates estrogen-induced apoptosis in cells of spermatogenic lineage [68]. The study also suggests that estradiol exposure amplifies the death-inducing signal *via* mitochondrial release of cytochrome c [68]. This experiment was significant in establishing the importance of the independent capability of cells of the spermatogenic lineage to respond to estrogens, as

well as suggesting that low dose estrogen can potentially result in severe spermatogenic cellular dysfunction without interfering with the hypothalamo-hypophyseal axis [68].

Qian *et al.* demonstrated that apoptosis caused the reduction of rat Sertoli cell viability following OP treatment, as evidenced by cellular shrinkage, chromatin condensation, and DNA cleavage and fragmentation. Results showed that OP up-regulated Bax protein and down-regulated Bcl-2 protein, which together led to activation of the caspase-3 pathway. Furthermore, the study noted that OP down-regulated a downstream effector of the Fas/FasL pathway, suggesting the involvement of Fas/FasL [63]. Researchers

speculate that alkylphenols such as OP activate apoptosis by inhibiting ER  $\text{Ca}^{2+}$  pumps in the testis, activating Fas-signaling pathways, or mediating Bcl-2/Bax levels.

An *in vitro* study by Kim *et al.* looked at the effects of OP on the testicular development of prepubertal rats [69]. OP administration resulted in a marked increase in the occurrence of apoptosis in testicular germ cells. This was accompanied by a corresponding decrease in bcl-xL mRNA expression, although bcl-2 and bax mRNA expressions did not demonstrate any substantial change [69]. These results illustrate the ability of OP to severely reduce the size and/or impair the function of the male reproductive organs by increasing apoptosis of testicular germ cells [69].

In addition, Zhou *et al.* examined the role of apoptosis related Fas/FasL in azoospermia or oligozoospermia induced by testosterone undecanoate [70]. The researchers speculated that the Fas system may initiate and regulate the germ cell apoptosis induced by testosterone undecanoate [70].

Nonylphenols (NP) are used in industrial and household detergents, cosmetic products, and spermicides. Also found as water contaminants, they accumulate in fish and pose a hazard to successively higher vertebrates on the food chain. Low micromolar concentrations of NP have been shown to induce testicular oxidative stress and cytotoxicity *in vitro*. Exposing rat Sertoli cells to 40  $\mu\text{M}$  NP, Gong *et al.* observed significant increases in the levels of intracellular ROS and lipid peroxidation markers [71].

Han *et al.* showed that NP induced Sertoli cell apoptosis in rats. Observing increased Fas/FasL gene expression following direct Sertoli cell injury, the researchers proposed that the upregulation indicated a response by the Fas/FasL system to recover the Sertoli cells' support capacity [72].

### Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are environmental pollutants that are mainly produced during fuel combustion [73]. PAHs can ligate and activate aryl hydrocarbon receptors (AHRs), which are transcription factors involved in regulating xenobiotic metabolism, and are selectively expressed in solely the germ cells in the first- and second-trimester human fetus [74].

Coutts *et al.* demonstrated that PAHs induced germ cell apoptosis by interacting with the AHR receptor. PAH exposure stimulated Bax expression and PARP cleavage in human fetal testes, while an AHR antagonist neutralized these effects [74].

### Bisphenol A (BPA)

Bisphenol A (BPA) is a known environmental estrogen that is most commonly used to produce polycarbonate plastic. Found in the lining of food and beverage cans, as dental sealants, and as an additive to other consumer products, the resin can leach out if exposed to heat or acidic/basic compounds that hydrolyze its ester bonds [75].

High-dose BPA treatment was shown to upregulate FasL gene expression in rat Sertoli cells [76]. Another study showed that BPA altered the expression and localization of cell junctional proteins derived from a Sertoli cell line [77].

High doses of BPA led to concomitant Leydig and germ cell apoptosis in pubertal mice. Furthermore, Li *et al.* observed translocalized expression of Fas and active caspase-3 in these same cell types as apoptosis occurred, in addition to transactivation of Fas, FasL, and active caspase-3. The authors hypothesized that BPA exposure leads to germ cell apoptosis by inducing Leydig cell loss [76].

Another possible mechanism of germ cell apoptosis is direct cell damage, as Takahashi and Oishi found that BPA caused dose-related degeneration of late spermatids and seminiferous tubules without affecting serum testosterone levels [78].

### Polychlorinated Biphenyls (PCB)

Polychlorinated biphenyls (PCBs) lead to oxidative stress when PCB metabolites oxidize into quinines and semi-quinones, which act *via* free radicals to inflict oxidative damage on DNA [38]. Hsu *et al.* observed a bimodal effect on the expression of apoptotic genes and their protein products: low doses of PCB induced p53 gene expression and down-regulated caspase-3, whereas high doses down-regulated the gene expressions of Fas, Bax, bcl-2, and p53 and induced activation of caspase-3 and -9. Thus, researchers hypothesize that low-dose PCB treatment causes p53 to impair spermatogenesis by initiating cell cycle arrest, whereas high-dose PCB exposure is believed to lead to apoptosis by activating the caspase cascade [38].

### Lindane

In rats, lindane collects in the testis and deleteriously alters germinal epithelium and Sertoli cell fragmentation [29].

After treating adult rats with a single oral dose of lindane, Saradha *et al.* evaluated testicular levels of cytochrome c, a mitochondria-associated intermediate of apoptosis, and caspase-9, an upstream initiator of the mitochondrial signaling cascade for cell death. The study demonstrated a significant increase from control levels, even when amounts of cytochrome c and caspase-9 had dropped at the end of the 72h observation period [29].

Additionally, the lindane treatment produced significant, time-dependent increases in the testicular levels of caspase-3, Fas and FasL from 6h to 72h post-exposure. Immunofluorescence microscopy showed that caspase-3 and Fas colocalized in the peritubular germ cells of lindane-treated rat testis. Moreover, FasL was concentrated in the cytoplasmic extensions of Sertoli cells and the peritubular germ cells following lindane exposure [29].

Cytochrome c and caspase-9 decreased at 72h posttreatment, while caspase 3 showed continual increase. This is consistent with the mitochondrial apoptosis pathway, as cytochrome c and caspase-9 act upstream whereas caspase-3 is the main downstream effector in the caspase cascade. Similarly Fas and FasL levels rose significantly in parallel, and peaked at the 72h point [79].

Numerous studies have linked oxidative stress and ROS with lindane's harmful effects on testicular function [29, 46]. In the abovementioned study by Saradha *et al.*, maximal increases in cytochrome c and caspase-9 levels at 12h and

24h post-exposure corresponded to the time points at which transient elevation in H<sub>2</sub>O<sub>2</sub> levels had previously been observed [80]. Within 12h of exposing rat testis to a single dose of lindane, Saradha *et al.* observed the induction of oxidative stress [80, 29].

### Methoxychlor

Methoxychlor is a broad-spectrum chlorinated insecticide that is used extensively for controlling insects on agricultural crops, livestock, animal feeds, barns, pets, and in residential gardens. Methoxychlor is an atypical environmental estrogen. It binds to recombinant human ER $\alpha$  and ER $\beta$  with a relative binding affinity that is 10,000 times less than 17 $\beta$ -estradiol [81] and stimulates the transcriptional activity of both estrogen receptor subtypes, *in vitro*, at concentrations of approximately 1000 nM [81, 82]. More recently, it was reported that transient embryonic exposure to methoxychlor during a critical time of gestation reduced spermatogenic capacity in the testes of adult male rats of subsequent generations [83, 84]. Treatment of pregnant female rats with methoxychlor from embryonic day 8 to 15 at dosages of 100 and 200 mg/kg/day increased spermatogenic cell apoptosis and decreased sperm number and motility in adult animals of the F<sub>1</sub> and F<sub>2</sub> generations [83]. Studies from our laboratory has also demonstrated a state of oxidative stress induced in adult rat testis following short and long term exposure of methoxychlor shows that oxidative stress is induced by promoting lipid peroxidation, and a decrease in the activity of antioxidant enzymes in testis and epididymis [44, 85, 86]. Recently, we have demonstrated a transient inhibitory effect of methoxychlor (50 mg/kg body weight) on testicular steroidogenic enzymes,  $\Delta^5$  3 $\beta$ -hydroxysteroid dehydrogenase and  $\Delta^5$  17 $\beta$ -hydroxysteroid dehydrogenase within 6-12 h of exposure and a possible role of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) in mediating these effects was suggested [87]. Methoxychlor showed alteration in the levels of heat shock protein and clusterin accompanied by an induction of oxidative stress in the testis at a same dose [88]. In another study, exposure to a single dose of methoxychlor at 50 mg/kg body weight induced apoptosis in male rat germ cells in a time-dependent manner *via* mitochondrial mediated and FasL-dependent pathways [89].

### Toluene

Toluene is an organic solvent present in plastics, adhesives, paints, varnishes, and inks. When Nakai *et al.* treated rats with toluene, reduced epididymal sperm counts and serine testosterone concentration were associated with elevated testicular 8-oxodG levels; meanwhile, GnRH mRNA levels remained unchanged. As 8-oxodG indicates oxidative DNA damage, the authors concluded that toluene's toxicity is derived not from endocrine disruption, but direct oxidative damage of spermatozoa [90].

El-Nabi Kamel and Shehata implicated toluene in altering the redox state and triggering oxidative stress in the testis, but observed no postexposure change in testicular caspase-3 activity [91]. Furthermore, prolonged oral toluene exposure at the common abuse density in rats did not reduce testicular weight, nor did it significantly alter spermatogenesis [92]. However, Yilmaz *et al.* observed that toluene inhalation significantly lowered testicular weight and

seminiferous tubule diameters, and immunostaining showed that serine testosterone levels in Leydig cells had dropped significantly [93]. While toluene is an established generator of oxidative damage, it remains inconclusive as to whether toluene's toxicity extends to inducing apoptosis, and whether toluene targets spermatogenesis or steroidogenesis.

### *tert*-Butyl Hydroperoxide (tBHP)

*tert*-Butyl hydroperoxide (tBHP) decomposition involves the release of superoxide or superoxide-like radicals, which can then degrade the cellular membrane [94]. In a study that administered tBHP to mice, Kaur *et al.* linked a decrease in sperm parameters to increased lipid peroxidation. These changes indicate that tBHP-induced OS impairs spermatogenesis. tBHP treatment upregulated NF- $\kappa$ B transcripts p65 and p50, as well as NF- $\kappa$ B-regulated antioxidant enzymes. The study demonstrated that NF- $\kappa$ B regulates antioxidant enzyme GSH-Px through direct trans-activation. Together these findings suggest that the testicular response mechanism to tBHP-generated OS is to alter NF- $\kappa$ B's antioxidant enzyme gene regulation [95].

In a study by Kalia *et al.*, tBHP treatment led to significant elevation of ROS production, germ cell damage, and DNA degradation in rat testes. Furthermore, there were significant increases in mRNA and protein expression of p53 and p21, which were localized to different spermatogenesis stages [94]. While p21 is best known for regulating cell cycle arrest, it also displays proapoptotic function by upregulating Bax protein when overexpressed [96]. The observed association between p53 upregulation and ROS generation in tBHP-treated mice demonstrated that tBHP induces an apoptotic pathway, mediated by OS, that involves p53 [94].

### Phthalates

Phthalates are ubiquitous chemical compounds used as plasticizers for plastic devices, food packaging, blood storage bags, and medical tubing [97]. Recently conducted studies have verified that phthalates, are capable of generating severe germ cell apoptosis [98, 99]. Apoptosis occurred with significantly less frequency in FasL knockout mice than in wild-type mice, implicating the Fas system in phthalate-induced germ cell apoptosis [8].

In *in vitro* and *in vivo* experiments, Yao *et al.* demonstrated that mono-(2-ethylhexyl) phthalate (MEHP) can trigger germ cell apoptosis by increasing Sertoli cell FasL transcription both directly and indirectly. In the direct mechanism, Sertoli cells activate NF- $\kappa$ B following MEHP exposure. In the complementing pathway of action, MEHP injures Sertoli cells and consequently weakens the structural, hormonal, and nutritive support that germ cells normally receive. As a result, germ cells secrete soluble tumor necrosis factor alpha (sTNF $\alpha$ ) as a paracrine factor to activate the NF- $\kappa$ B signaling pathway in Sertoli cells. Triggering NF- $\kappa$ B upregulates Sertoli cell FasL expression, which promotes pro-apoptotic interaction with germ cell-expressed Fas receptor [99].

Kasahara *et al.* implicated oxidative stress in overseeing (DEHP)-induced testicular atrophy. In an *in vivo* rat study, 1 g/kg DEHP was the minimal dose needed to induce sperm-

atocyte apoptosis, elevate ROS generation and selectively reduce antioxidant levels in the testis. Notably, the authors discovered a significant increase in cytochrome *c* levels that coincided with the transformation of DEHP into MEHP. Since the observed MEHP concentrations sufficed for superoxide radical generation, the authors concluded that DEHP metabolism induced oxidative stress in and around the mitochondria. The ensuing release of cytochrome *c* began the series of events leading to apoptosis [97].

### 2,5-Hexanedione (2,5-HD)

2,5-Hexanedione (2,5-HD), a metabolite of the common industrial solvents *n*-hexane and methyl *n*-butyl ketone, is able to trigger apoptosis in male germ cells [100]. In an *in vitro* study by Mishra *et al.*, 2,5-HD treatment produced apoptosis in approximately 50 percent of the cells 8h post-exposure [100]. The study also identified evidence for the cleavage of poly (ADP-ribose) polymerase (PARP), which is a caspase-3 substrate, 6h following 2,5-HD administration [100].

Additionally, Mishra *et al.* demonstrated that 2,5-HD exposure *in vitro* caused a significant increase in testicular ROS with a subsequent elevation in intracellular calcium ( $Ca^{2+}$ ) levels. The rise in intracellular  $Ca^{2+}$  produced an elevation in Bcl-xS levels relative to Bcl-xL levels. However, since 2,5-HD did not alter levels of Bcl-2 and Bax, 2,5-HD's cytotoxic impact may be mediated by inverting the ratio between pro- and anti-apoptosis Bcl-2 family proteins. Furthermore, cytosolic accumulation of cytochrome *c* indicated cells had activated the mitochondrial pathway of cell death [100].

### 1,3-dinitrobenzene (1,3-DNB)

1,3-Dinitrobenzene (1,3-DNB) is a highly reactive nitro-aromatic compound that is incorporated in the manufacture of polymers, pesticides, and dyes [101]. This reactive nitrogen species (RNS) acts similar to ROS, damaging nearby cells if present in excess.

Previous rat research demonstrated that 1,3-DNB injured Sertoli cells and induced apoptosis in surrounding germinal cells [101]. Studying rat protein expression levels following 1,3-DNB treatment, Lee *et al.* (2009) observed down-regulation of the pro-survival protein Bcl-2, and up-regulation of the pro-apoptotic protein Bax. Although the ability of 1,3-DNB to inflict apoptosis has been well documented, there is limited information pertaining to the mechanistic pathways of cell death in Sertoli cells [101].

### Nitrobenzene

Nitrobenzene (NB) is a component of rubber, pesticides and pharmaceuticals, and acts as a toxicant by elevating the occurrence of testicular apoptosis [102]. Allenby *et al.* assessed the effect of NB on Sertoli cells *in vitro* and concluded that NB toxicity may disrupt Sertoli cell function [103].

Richburg *et al.* evaluated the sensitivity of testicular germ cells to NB [102]. Whereas activation of the Fas-signaling pathway is widely thought to be the mechanism through which toxicant-induced Sertoli cell injury results in

germ cell apoptosis, results indicated that Fas-mediated signaling is not required for NB-induced germ cell apoptosis. Rather, a dysfunctional Fas signaling system sensitized the mice to NB-induced germ cell death [102].

### Ethanol

Ethanol, a well-known environmental compound, affects the testis by disrupting the hypothalamic-pituitary-gonadal axis and hampering the secretory function of the Sertoli cells [27, 104]. In a rat study by Koh, ethanol suppressed testicular survival-signaling pathways by reducing levels of activated survival kinases, including pAkt and pErk1/2, and phosphorylated Bad at Ser112 and Ser136 [105]. Koh hypothesized that ethanol induced apoptotic cell death by suppressing the activation of survival kinases and the phosphorylation of their downstream targets [27].

Hu *et al.* further noted that chronic exposure to ethanol could result in testicular germ cell apoptosis [104]. In an *in vitro* transgenic mouse model, histopathological examination revealed that acute ethanol exposure produced epithelial degeneration of seminiferous tubules in FasL-overexpressing mice, whereas wild-type mice experienced no change in testicular morphology. These results suggested that FasL expression determines the sensitivity of testes to ethanol in mice, and that the Fas ligand mediates apoptosis of testicular germ cells [104].

### EXPERT COMMENTARY

The aim of this article is to elucidate the mechanism(s) of environmental toxicant induced apoptosis in male reproduction. Environmental contaminant exposure can cause oxidative stress in the testis, leading to apoptosis in germ, Sertoli, and Leydig cells. Numerous studies have identified the pathways through which specific toxicants trigger OS-induced testicular apoptosis. This information is relevant for preventive of environmental and occupational hazards, as it further evidences the need for reduced exposure to environmental contaminants.

### FIVE-YEAR REVIEW

In the existing scenario of massive industrialization and changing life styles, more focus should be on identification of populations at risk, evaluation of reproductive hazards, understanding the mechanism of action and to devise an appropriate preventive or intervention strategies to improve public health. Research on reproduction is complicated due to various constraints involved in evaluating and interpreting reproductive outcomes as the biology of reproduction is complex and the effect is not confined to the target alone.

### KEY POINTS

- Spermatogenesis generates ROS under physiological conditions that have regulatory influence on germ cell apoptosis.
- Toxicants induce massive germ cell death either by increasing the expression of apoptosis related proteins or by oxidative imbalance.

- Identifying the environmental contaminants that pose a major burden to the general population and prioritizing the agents that require intensive research could help in finding the hazards caused by environmental contaminants.

#### ABBREVIATIONS

1,3-DNB	=	1,3-Dinitrobenzene
2,5-HD	=	2,5-Hexanedione
AHR	=	Aryl hydrocarbon receptor
BPA	=	Bisphenol A
CARD	=	Caspase recruitment domain
DED	=	Death effector domain
DEHP	=	di-(2-ethylhexyl) phthalate
DISC	=	Death inducing signaling complex
FADD	=	Fas-associated protein with Death Domain
EDS	=	Ethylene dimethanesulfonate
FasL	=	Fas ligand
FSH	=	Follicle stimulating hormone
GnRH	=	Gonadotropin-releasing hormone
GST	=	Glutathione-S-transferase
hCG	=	Human chorionic gonadotropin
LH	=	Luteinizing hormone
MEHP	=	Mono-(2-ethylhexyl) phthalate
NB	=	Nitrobenzene
NP	=	Nonylphenol
OP	=	Octylphenol
OS	=	Oxidative stress
PCB	=	Polychlorinated biphenyl
ROS	=	Reactive oxygen species
sTNF $\alpha$	=	Soluble tumor necrosis factor alpha
tBHP	=	<i>tert</i> -butyl hydroperoxide
TRADD	=	TNF Receptor-Associated protein with Death Domain

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#### CONFLICT OF INTEREST

None Declared.

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