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# Disturbance in Testosterone Production in Leydig Cells by Polycyclic Aromatic Hydrocarbons

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**ABSTRACT :** Polycyclic aromatic hydrocarbons (PAHs), which are ubiquitous in the air, are present as volatile and particulate pollutants that result from incomplete combustion. Most PAHs have toxic, mutagenic, and/or carcinogenic properties. Among PAHs, benzo[a]pyrene (B[a]P) and dimethylbenz[a]anthracene (DMBA) are suspected endocrine disruptors. The testis is an important target for PAHs, yet effects on steroidogenesis in Leydig cells are yet to be ascertained. Particularly, disruption of testosterone production by these chemicals can result in serious defects in male reproduction. Exposure to B[a]P reduced serum and intratesticular fluid testosterone levels in rats. Of note, the testosterone level reductions were accompanied by decreased steroidogenic acute regulatory protein (StAR) and 3β-hydroxysteroid dehydrogenase isomerase (3β-HSD) expression in Leydig cells. B[a]P exposure can decrease epididymal sperm quality, possibly by disturbing the testosterone level. StAR may be a key steroidogenic protein that is targeted by B[a]P or other PAHs.

**Key words :** Polycyclic aromatic hydrocarbons, Endocrine disruptor, Steroidogenesis, Leydig cells

## Polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs), which are ubiquitous in the air, are present as volatile and particulate pollutants that result from incomplete combustion of fossil fuels, wood, and other organic matter (IARC, 1985; Menzie et al., 1992). PAHs are widely distributed in soils and sediments, groundwater, and the atmosphere. PAH molecules are composed of carbon and hydrogen atoms arranged in two or more fused benzene rings in linear, angular, or cluster arrangements (Sims & Overcash, 1983). PAHs are highly lipid-soluble, and therefore, they are readily absorbed in the gastrointestinal tract of mammals (Cerniglia, 1984). PAHs are rapidly distributed in a wide variety of tissues with a marked tendency for body fat localization. Many PAHs have toxic, mutagenic, and/or carcinogenic properties (Goldman et al., 2001). PAHs induce numerous enzymes that are involved in activation and PAH detoxification by acting on the aryl hydrocarbon receptor (AhR) (Nebert et al., 2004). The AhR is a transcription factor that, on binding of agonists, translocates from the cytoplasm to the nucleus, where it increases xenobiotic metabolizing enzyme expression. The U.S. Environmental Protection Agency (EPA) has promulgated 16 unsubstituted PAHs as priority pollutants (U.S. EPA. 1999). Of these 16 PAHs, 8 PAH compounds are considered to be possible carcinogens, namely benzo[a]anthracene (B[a]A), chrysene, benzo[b]fluoranthene (B[b]F), benzo[k]fluoranthene (B[k]F), benzo[a]pyrene (B[a]P),

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dibenzo[a,h]anthracene (DB[a,h]A), indeno[1,2,3-cd]-pyrene and benzo[g,h,i]perylene (Srogi et al., 2007). An important and very extensively studied prototype of this class of compounds is B[a]P (Knize et al., 1999). Two of the most potent PAH carcinogens include the environmentally relevant dibenzo[a,l]pyrene (DB[a,l]P) and 7-12-dimethyl-benz[a]anthracene (DMBA), both of which are more potent than B[a]P (Higginbotham et al., 1993).

## Benzo[a]pyrene and 7-12-dimethylbenz[a]anthracene

B[a]P is commonly found in tobacco smoke, broiled foods, and polluted environments and is widely regarded as a surrogate for PAH exposure. B[a]P is metabolically activated via a three-step process. First, cytochrome P450 (CYP) catalyzes the formation of (7R,8S)-epoxy-7,8-di-hydrobenzo[a]pyrene (B[a]P-7,8-oxide). This is converted to (7R,8S)-dihydroxy-7,8-dihydrobenzo[a]pyrene (B[a]P-7,8-diol), a reaction catalyzed by epoxide hydrolase. B[a]P-7,8-diol then is further oxidized, a process catalyzed by cytochrome P450 and other enzymes, producing mainly (7R, 8S)-dihydroxy-(9S,10R)-epoxy-7,8,9,10-tetrohydrobenzo-[a]pyrene (BPDE). Metabolic activation of B[a]P is highly selective. Initial conversion of B[a]P at positions 7 and 8 produces the R,R-dihydrodiol in high enantiomeric excess. Subsequent epoxidation at positions 9 and 10 then predominantly generates the diol-epoxide with R,S,S,R-(+)-anti-BP-7,8-diol-9,10-epoxide [(+)-anti-BPDE] (Yang et al., 1976). B[a]P-induced DNA damage predominantly results from covalent interaction between (R,S,S,R) diol-epoxide and 2'-deoxyguanosine (dG) residues through trans opening of the epoxide moiety (Cheng et al., 1989). The mutagenicity of BPDE- $N^2$-dG and its effects on DNA conformation have also been conclusively demonstrated (Kozack & Loechler, 1999).

7,12-Dimethylbenz[a]anthracene (DMBA) is a PAH that is a potent carcinogenic chemical with the ability to induce cancer in breast, ovary, skin, and other tissues in rodents (Cavalieri et al., 1991; Kanter et al., 2006). Humans are exposed to DMBA through burning of organic materials, as in cigarette smoke and car exhaust fumes, although there is little evidence that DMBA actually occurs in nature (Lawther & Waller, 1976). CYP1B1 metabolizes DMBA to DMBA-3,4-epoxide, which is hydrolyzed to DMBA-3,4-diol by microsomal epoxide hydrolase. DMBA-3,4-diol then undergoes epoxidation by CYP1A1 or CYP1B1 to form the ultimate cytotoxic and carcinogenic compound, DMBA-3,4-diol-1,2-epoxide (Miyata et al., 1999). In the nucleus, DMBA-DE covalently binds to DNA and causes the formation of a DNA-adduct, which can result in carcinogenicity, mutagenicity, and cytotoxicity (Buters et al., 2003).

## Leydig Cells

Leydig cells were discovered in 1859 by Franz von Leydig and are found in the testicles next to the seminiferous tubules. Leydig cells within the interstitial compartments produce testosterone, which is important to maintain spermatogenesis (Lipsett et al., 1966) and male secondary sex characteristics (Walsh et al., 1934). Pituitary gonadotropin luteinizing hormone (LH) stimulates testosterone production and subsequent downstream effects (Haider, 2004). Leydig cells first appear in the testis during day 15 of embryonic development in the rat (Siiteri & Wilson, 1974). The fetal Leydig cells present at birth are not progenitors of the adult Leydig cell population (Kerr & Knell, 1988). Leydig cells through pre- and postnatal development differ in their morphology as well as function (Hardy et al., 1991). In the adult, perhaps the most notable Leydig cell function is androgen production. Estrogen plays an inhibitory role in this process and therefore may be important in controlling the steroidogenic capacity of the adult testis. Leydig cells are responsible for testosterone production in the mammalian testis. Testosterone production depends upon stimulation of these cells with LH, which is secreted in pulses into the peripheral circulation by the pituitary gland in response to gonadotropin-releasing hormone (GnRH) from the hypothalamus. Testosterone and its aromatized product, estradiol, then feed back to the hypothalamus and pituitary to suppress transient LH and subsequent testosterone productions. In response to reduced testosterone, GnRH and LH are again produced. This negative feedback cycle results in pulsatile secretion of LH followed by pulsatile production of testosterone (Ellis et al., 1983). Normal Leydig cell function and development are important for male sexual development, testicular steroidogenesis during puberty and adulthood, and hence normal fertility.

## Steroidogenesis in Leydig cells

Testosterone biosynthesis is primarily controlled by pituitary gonadotropin LH. LH binds to specific receptors on the surface of Leydig cells and stimulates the production of cyclic AMP (camp), the intra-cellular second messenger for LH. cAMP has two principle activities in Leydig cell steroidogenesis control. The first action of cAMP is the acute testosterone biosynthesis stimulation via cholesterol mobilization and transport into the steroidogenic pathway, an action that takes place in less than a minute. The cAMP-dependent protein kinase PKA activates cholesterol mobilization from intracellular cholesterol pools and extracellular lipoprotein sources or *de novo* cholesterol synthesis from acetate. Regardless of origin, cholesterol transfer into the inner-mitochondrial membrane is a cAMP-dependent process, requiring the action of steroidogenic acute regulatory protein (StAR) (Stocco, 2000). StAR was initially identified as a 30/32-kDa phosphoprotein that accumulates in the mitochondria of Leydig cells in response to cAMP treatment and in a manner that parallels steroid formation (Epstein & Orme-Johnson, 1991). The StAR gene was cloned, and the 30 kDa phosphoprotein was shown to be processed from a 37 kDa cytosolic precursor protein containing a mitochondrial targeting sequence (Stocco, 2001). The second action of cAMP in Leydig cells is the chronic stimulation of steroidogenic enzyme gene expression and activity (Payne et al., 1996). Once cholesterol is transferred into the mitochondria, cholesterol side-chain cleavage cytochrome P450 (P450scc), which resides on the inner-face of the mitochondrial inner matrix membrane, converts it to pregnenolone. Pregnenolone diffuses to the smooth endoplasmic reticulum, where it is converted to progesterone by 3β-hydroxysteroid dehydrogenase isomerase (3β-HSD). 17α-hydroxylase/$C_{17\text{-}20}$ lyase (P450c17) in turn converts progesterone to 17α-hydroxy progesterone, then androstenedione, 17β-hydroxysteroid dehydrogenase (17β-HSD) then converts androstenedione to testosterone.

## Endocrine disruptor effects on male reproductive health

Endocrine disruptors (EDs) are exogenous substances that interfere with production or function of hormones that are responsible for the maintenance of homeostasis and the regulation of developmental processes in the body (US EPA, 1998). These substances have potential adverse effects on developmental, reproductive, immune, and cardiovascular systems in both humans and wildlife (Diamanti-Kandarkis et al., 2009). EDs are highly heterogeneous and include synthetic chemicals used as industrial solvents/lubricants and their by-products [e.g. PCBs, dioxins], plastics [bisphenol A (BPA)], plasticizers (phthalates), pesticides [dichlorodiphenyltrichloroethane (DDT), cypermethrin], fungicides (vinclozolin) and pharmaceutical agents [diethylstilbestrol (DES)]. Natural substances with hormonal activity have been found in human and animal food, including phytoestrogens and fungal estrogens (Diamanti-Kandarkis et al., 2009). EDs interfere with hormonal pathways through a multitude of mechanisms. They can compete for hormone receptor binding and activation, interfere with post-receptor signaling pathways, and modulate synthesis, bioactivity, or elimination of natural hormones, receptors, and cofactors. EDs were originally thought to function primarily through nuclear hormone receptors, including estrogen, androgen, progesterone, thyroid, and retinoid receptors (Diamanti-Kandarkis et al., 2009; Schug et al., 2011). However, recent evidence shows that the mechanisms are much broader than originally recognized. Thus, endocrine disruptors can act through nuclear hormone receptors, membrane receptors, non-steroid receptors, orphan receptors, transcript-tional coactivators, enzymatic pathways involved in steroid biosynthesis and/or metabolism, and numerous other mechanisms that converge upon the endocrine and reproductive systems (Diamanti-Kandarkis et al., 2009; Zoeller et al., 2012). AhR is the most studied protein with respect to ED interaction. This orphan receptor acts as a transcription factor for detoxifying enzymes (Yoshioka et al., 2011). Dioxins and some PCBs exert their endocrine-disruptive effects by binding AhR and impairing the usual gene transcription response (Beischlag et al., 2008). Moreover, AhR ligands enhance sex steroid receptor degradation (Ohtake et al., 2011).

Male reproductive health has been a major focus of research on endocrine disrupting substances since the early 1990s. There has also been an increase in the incidence of male reproductive disorders, including reduced semen quality and infertility, urogenital tract abnormalities, and testicular germ cell cancer (Skakkebaek et al., 2001; Sharpe and Skakkebeak, 2003). Male reproductive system development requires the activation of specific pathways by hormones, notably androgens and anti-Müllerian hormone. Although testis formation itself is not hormone-dependent, most other aspects of masculinization depend on normal testicular hormone production. Furthermore, testicular cell development is dependent on the local action of hormones. Therefore, disruption of testicular hormone production and action by EDs may lead to incomplete masculinization and malformations in the male reproductive tract of both humans and animals (Sharpe, 2006).

## Endocrine disrupting effects of non-PAHs on Leydig cells

Pesticides, such as vinclozolin or DDT and its derivatives, are all antagonists of AR and inhibit androgen-dependent tissue growth *in vivo* (Gray et al., 1999). Vinclozolin is a dicarboximide fungicide that has two active metabolites, M1 and M2, that both have anti-androgenic properties. These metabolites compete for androgen binding to AR and inhibit DHT-induced transcriptional activation by blocking AR binding to androgen response elements (AREs) in DNA (Wong et al., 1995). Oral vinclozolin administration delayed pubertal maturation, decreased sex accessory gland growth, and increased serum levels of LH and testosterone (Monosson et al., 1999). However, *in vitro* experiments revealed that vinclozolin did not affect basal or hCG-stimulated testosterone production of rat Leydig cells in primary culture (Murono and Derk, 2004). *p,p′*-DDE [1,1-Dichloro-2,2-bis(p-chlorophenyl) ethylene], a stable metabolite of persistent DDT, act as an antagonist of AR both *in vivo* and *in vitro* (Kelce et al., 1995). When *p,p′*-DDE was administrated to rats during gestation, anogenital distance was reduced, and hypospadias, nipple retention, and weight reduction of androgen-dependent tissues occurred (Gray et al., 1999). *In vitro*, *p,p′*-DDE binds to AR and prevents DHT-induced transcriptional activation in cells transfected with human AR (Kelce et al., 1995).

Phthalates are mainly used as plasticizers in the manufacturing of flexible vinyl plastic, which is used in consumer products, infant toys, food packaging, certain cosmetics, and medical devices (Thomas & Thomas, 1984). Although commonly used phthalates [e.g. diethylhexyl phthalate (DEHP) and dibutyl phthalate (DBP)] and their active metabolites [e.g. monoethylhexyl phthalate (MEHP) and monobutyl phthalate (MBP)] disrupt male reproductive development in an anti-androgenic manner, neither of these compounds binds AR (Park et al., 2000). Actually, phthalate-induced Leydig cells toxicity depends on the dosage and time of exposure during development. Prenatal exposure of rats to DEHP or MEHP during gestation significantly reduces fetal testosterone levels (Gray et al., 2000; Chauvigné et al., 2009), and DEHP reduces serum levels of both LH and testosterone in male offspring (Akingbemi et al., 2001). Paradoxically, chronic exposure of pubertal rats to low-dose DEHP significantly increases plasma levels of LH, testosterone, and E2 (Akingbemi et al., 2004a).

Bisphenol A (BPA) is an estrogenic compound that is widely used to manufacture polycarbonate plastics, which serve as containers for foods and beverages and are constituents of dental sealants (Akingbemi et al., 2004b). Although BPA structure resembles that of the natural estrogen E2, the affinity of BPA for binding ERs is at least a 10,000-fold lower than E2 (Welshons et al., 2003). BPA causes anti-androgenic effects on testicular function by interfering with androgen production and function (Akingbemi et al., 2004b). *In vivo*, exposure of prepubertal rats to environmentally-relevant BPA levels suppressed serum LH and testosterone levels (Akingbemi et al., 2004b). *In vitro*, BPA treatment of Leydig cells decreased testosterone biosynthesis as a result of decreased expression of the steroidogenic enzymes (Akingbemi et al., 2004b).

Dioxins are a class of highly toxic contaminants that are environmental pollutants and persistent organic pollutants, including polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and PCBs (Poland & Knutson 1982). Among these, 2,3,7,8-tetrachloro- dibenzo-p-dioxin (TCDD) is the most toxic contaminant in the environment. It is a by-product of industrial processes and is recognized as a potent developmental and reproductive toxicant (Gray et al., 1995). The most toxic actions of TCDD are mediated through the AhR, which is a ligand-activated transcription factor (Mimura & Fujii-Kuriyama, 2003). TCDD exerts its endocrine-disrupting effects through multitude mechanisms involving alteration of steroidogenesis (Mutoh et al., 2006), reduction of steroid hormone and LHRs (Fukuzawa et al., 2004), and induction of CYP1 family enzymes, resulting in inactivation of steroid hormones (Badawi et al., 2000). The effect of TCDD depends on the dosage during development. Low-dose exposure to TCDD to pregnant rats significantly reduced intratesticular testosterone levels of fetal males, while high doses decreased pituitary LH production of exposed male fetuses (Adamsson et al., 2009). In adult male rats, exposure to TCDD inhibits testicular steroidogenesis by inhibiting cholesterol mobilization to P450scc (Moore et al., 1991).

## Reduction of testosterone production in Leydig cells by PAHs

In contrast to non-PAHs, relatively few studies have investigated the effects of PAHs on Leydig cell steroidogenesis. One study showed that inhalation exposure to B[a]P in F-344 rats elevated serum LH levels (Archibong et al., 2008). Recently, however, a potent endocrine disrupting mechanism of testosterone production was proposed in Leydig cells after exposure to B[a]P (Chung et al., 2011). Long-term exposure to B[a]P significantly reduced both serum and intratesticular testosterone levels. The decrease was insufficient to cause testicular atrophy with massive germ cell apoptosis, but it was associated with a reduction in sperm quality in the epididymis. This study suggested that B[a]P exposure can decrease epididymal sperm quality by reducing the testosterone level and StAR could be an important steroidogenic protein that is targeted by B[a]P or other PAHs. In addition, DMBA, another representative PAH, also has a negative effect on testosterone production in Leydig cells (Personal communications; manuscript in preparation by Kim et al.). Kim et al. suggests that reduced testosterone production, caused by DMBA treatment, is associated with the direct effect of this chemical on steroidogenic machinery. Further studies are required to elucidate a precise mechanism(s) action of PAHs in steroid-ogenic Leydig cells.

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