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INVITED REVIEW

Reproductive Health

Similar causes of various reproductive disorders in early life

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During the past few decades, scientific evidence has been accumulated concerning the possible adverse effects of the exposure to environmental chemicals on the well-being of wildlife and human populations. One large and growing group of such compounds of anthropogenic or natural origin is referred to as endocrine-disrupting chemicals (EDCs), due to their deleterious action on the endocrine system. This concern was first focused on the control of reproductive function particularly in males, but has later been expanded to include all possible endocrine functions. The present review describes the underlying physiology behind the cascade of developmental events that occur during sexual differentiation of males and the specific role of androgen in the masculinization process and proper organogenesis of the external male genitalia. The impact of the genetic background, environmental exposures and lifestyle factors in the etiology of hypospadias, cryptorchidism and testicular cancer are reviewed and the possible role of EDCs in the development of these reproductive disorders is discussed critically. Finally, the possible direct and programming effects of exposures *in utero* to widely use therapeutic compounds, environmental estrogens and other chemicals on the incidence of reproductive abnormalities and poor semen quality in humans are also highlighted.

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INTRODUCTION

During recent years, there has been evidence that wildlife species and humans are exposed to ubiquitous endocrine-disrupting chemicals (EDCs) through environmental and lifestyle factors.[1,2] Despite having different chemical structures, these compounds share common features that interfere with the hormonal systems of living organisms. Such substances can act through many mechanisms, including agonistic or antagonistic action at the hormone receptor or post-receptor level or by interference with the synthesis, transport and metabolism of certain hormones. A recent general definition of an EDC is that it is a substance interfering with any aspect of hormone function.[3] There is a clear difference between EDCs and endocrine active substances (EAS). An EAS is a substance that has the inherent ability to interact or interfere with one or more components of the endocrine system, resulting in a biological effect without necessarily causing adverse effects. EDCs are compounds of natural or synthetic origin that can be identified according to three criteria: endocrine activity, adversity of effects and a plausible link between endocrine activity and the adverse effect.[4]

Because the reproductive function of humans, especially males, is regulated by a number of hormones and paracrine factors, it is not surprising that EDCs may contribute to adverse reproductive health outcomes in developing and adult humans. This idea is supported by reports of increased incidences of human reproductive disorders in recent decades, including cryptorchidism, hypospadias, testicular cancer and poor semen quality. This set of symptoms has been combined into one entity named testicular dysgenesis syndrome (TDS), which is caused by disturbed testicular development *in utero*.[1] Other male reproductive abnormalities in humans that have been associated with antiandrogenic action of certain EDCs are reduced anogenital distance (AGD) in newborn boys[5] and disorders of puberty.[6] The rapid increase in reproductive disorders suggests that environmental or lifestyle factors, rather than an accumulation of genetic defects, are the most likely causes. The presence of subpopulations of humans with genetic aberrations or polymorphisms that have a predisposition to deleterious impact (s) of environmental factors cannot be excluded.

Exposure to EDCs during development may result in different effects than exposure during adulthood. Adverse effects may be most pronounced in the developing organism and occur at concentrations of the chemical far lower than would be considered harmful in the adult.[7] Low-dose exposure during fetal development can result in long-standing adverse effects after the EDC is eliminated from the body. Such interactions between the developing organism *in utero* and the toxic components of the environment may determine the predisposition of that individual to develop disease later in life.[8] Environmental chemicals can cause dysregulation of the epigenetic control of gene expression in the fetus and alter the normal acetylation or methylation status of specific genes that can impact the clinical outcomes in postnatal life. One example, although recently challenged, is the observation that exposure to vinclozolin, a fungicide and antiandrogenic agent, during embryogenesis decreases the adult sperm motility and concentration, and this effect is transferred through several generations of male offspring and is associated with alterations in the sperm methylation profile.[9]

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It is well-documented that fetuses and children can be very sensitive to exogenous hormones[10,11] because the hormones interfere with the programming of normal hormone signaling and metabolic pathways.[12] In this review, we will describe the current evidence on the epidemiology, etiology and pathogenesis of male reproductive disorders in humans and link these disorders to possible prenatal exposure to environmental EDCs. We will also focus on the cascade of cellular events that occur during sex differentiation and how certain EDCs may disturb this process in animal models.

ROLE OF ANDROGENS IN MALE SEXUAL DIFFERENTIATION AND MASCULINIZATION

During male development, the human reproductive system undergoes several distinct cellular events, including sex determination, sexual differentiation and masculinization. The formation of a phenotypically normal male during sexual differentiation involves a cascade of changes initiated by activation of the *sex determining region Y* gene. This leads to testis formation, a process that is not dependent on androgen action. In the process of differentiation, the fetal testes develop the capacity to produce androgens, which trigger the organogenesis of the male reproductive organs. This process is named masculinization. Androgens (e.g., testosterone and dihydrotestosterone) play a pivotal role in the masculinization of the male fetus, governing the process of "making a male" during fetal development. Masculinization of the reproductive tract involves the differentiation of the internal and external genitalia. This period of development of the male fetus, in which the masculinization of the reproductive tract by androgens occurs, is named the "masculinization programming window".[12,13] This process involves differentiation of the internal (epididymis, vas deferens, seminal vesicles and prostate) and external (penis, scrotum and perineum) genitalia.[12,14]

In humans, testosterone reaches its maximal values between 11 and 18 weeks of gestation and stimulates differentiation of the Wolffian duct into the epididymis, vas deferens and seminal vesicles. The masculinization of the external genitalia and prostate is mediated primarily by dihydrotestosterone (DHT), a more potent metabolite of testosterone produced by the action of the enzyme 5α-reductase.[15] Simultaneously, the Sertoli cells of the fetal testis secrete anti-Müllerian hormone (AMH), which induces the regression of the Müllerian duct.[16] The Sertoli cells are the first cells that can be identified in the early fetal testis and are crucial for the seminiferous cord formation and Leydig cell functionality.[17] The Sertoli cells prevent the germ cells from entering meiosis and the further differentiation of the germ cells.[18] Failures during the maturation of the Sertoli cells caused by exposure to endogenous factors and xenobiotics are related to various spermatogenic failures and are among the crucial factors for TDS.[17,19]

The actions of testosterone and DHT are mediated by the androgen receptor (AR), which is expressed in the developing internal and external genitalia. In the human male fetus, the AR is expressed after 8 weeks of gestation prior to the onset of testicular androgen secretion; it is expressed at a higher level in the genitals than in the urogenital structures.[20] The absence of a functional AR in complete androgen insensitivity syndrome results in the development of the fetus into a phenotypic female irrespective of the otherwise normal formation and hormonal function of testes.[21] If the masculinization process is attenuated in more subtle ways, the individual appears phenotypically male, but may display disorder(s) such as hypospadias, cryptorchidism and micropenis because of insufficient androgenicity. Such disorders can be caused by deficient androgen production or action[12,22] and are among the most common congenital disorders in male children.

During the androgen-dependent phase of the reproductive organ development (8–13 gestational weeks), the male external genitalia becomes visible as a male structure because of the activation of AR-dependent genes by DHT. Under the influence of DHT, the genital tubercle (GT) differentiates into the glans, the genital folds become the shaft of the penis and the genital swellings become the scrotum.[23] Several androgen-dependent genes, such as *AR, steroid-5-alpha-reductase, alpha polypeptide 2* (*SRD5A2*), *hydroxysteroid (17-beta) dehydrogenase 3* (*HSD17B3*) and *FK506 binding protein 4* (*FKBP52*), are associated with the regulation of the external genitalia formation and defects in these genes significantly increase the risk of developing hypospadias.[13,23] A recent study showed that androgen-driven masculinization of various reproductive tract organs is programmed by androgen action earlier in fetal life, before the morphological differentiation of these organs occurs. Deficient androgen action within this early programming window in males can induce the common congenital disorders of cryptorchidism and hypospadias in rats.[13] Penis formation is dependent on androgen action before 19.5 day post coitum (dpc) in rats, and androgens play no role in normal penis formation after this age.[13]

The role of androgens in the development of the phallus has been demonstrated in humans with androgen insensitivity, in which the structures range from a normal male phallus to a hypospadic male phallus to an apparently female phallus depending on the degree of androgen action.[24] The transinguinal testis descent in humans and rodents is also androgen-dependent, but suppression of AR action by flutamide has been found to induce cryptorchidism in only 35%–60% of male rats, suggesting that there is not an absolute requirement for androgen action in the transinguinal testis descent or that other, yet unknown, factors may be able to compensate for deficient androgen action in the descending process.[13]

Poor masculinization of the reproductive tract by androgens can also cause shortening of the AGD, providing a noninvasive, lifelong "readout" of androgen action specifically within the programming window that predicts hypospadias and cryptorchidism.[13,25] Experiments in rodent models have shown that AGD can only be affected in rats by insufficient androgen action between 15.5 and 19.5 dpc, and no effect of androgen was observed after this period in the male fetal development.[13] AGD will be useful clinically as a noninvasive marker of androgen action during the critical masculinization programming window and will be predictive of adult-onset TDS.[13] A recent study showed that AGD is reduced in boys with hypospadias or cryptorchidism.[26]

One can conclude that the process of masculinization is an androgen-dependent process that is dependent on the proper function of fetal Leydig cells (FLCs), which are a major source of androgens in the prenatally developing male. Disruption of steroidogenesis in FLCs by environmental xenobiotics and/or metabolic endogenous factors during the masculinization programming window may cause undermasculinization and malformation of the male reproductive organs.

ROLE OF THE GENETIC BACKGROUND, ENVIRONMENT AND LIFESTYLE IN THE ETIOLOGY OF HYPOSPADIAS

The phenotypic features of abnormal development of the reproductive organs that are associated with insufficient androgen action during the sensitive masculinization window in male fetuses are hypospadias, micropenis and a shortened AGD. The typical anatomical anomalies in hypospadias are an abnormal ventral opening of the urethral meatus, an abnormal ventral curvature of the penis and an abnormal distribution of the foreskin around the glans with a ventrally deficient hooded foreskin.[27] Hypospadias shows familial clustering, with 7% of cases

having affected first, second or third degree relatives.[28] The chance that a brother of an affected boy will also have hypospadias is 9%–17%.[29,30] Segregation analysis suggests that the majority of cases of hypospadias have a multifactorial etiology involving genes and environmental factors.[31] Defects in several key genes that control phallus development, androgen biosynthesis and action can be enhanced by the environment; this promotes the development of hypospadias. Experimental animal models have demonstrated the importance of the homeobox genes A (HOXA) and D (HOXD) in the development of the phallus because deletion of these genes in mice induces malformation of the external genitalia consistent with hypospadias.[32] The FGF gene family, especially *FGF10*, also plays a role in the development of the external genitalia in mice.[33] In humans, polymorphisms of *FGF8*, *FGF10* and *FGFR2* may be associated with an increased risk of hypospadias.[34] Mutations in the genes controlling androgen biosynthesis by the Leydig cells (e.g., the luteinizing hormone (LH) receptor and 5α-reductase genes) also induce hypospadias, which can be associated with cryptorchidism and micropenis.[35,36] Extensive mutation screening in hypospadias patients has revealed disease-associated sequence alterations, especially in the AR gene. These mutations are typically found in AIS cases with severe hypospadias and they may not be common causes of isolated hypospadias.[37,38] There are some reports that an expanded CAG repeat length of the AR gene appears to be relevant in boys with isolated hypospadias.[39,40] A recent study reported that a higher number of the CAG repeat sequence in the AR gene may increase the risk of hypospadias in Caucasians.[41]

Another gene that is involved in the proper development of the phallus is *mastermind-like domain containing 1* (*MAMLD1*). This gene is expressed in the male gonad in mice and modulates SF-1-dependent synthesis of testosterone in the critical period of the masculinization of the reproductive tract.[42] In *Mamld1* knockout (KO) mice, downregulation of insulin-like factor 3 (Insl3) mRNA expression was reported, while mRNA levels of *Amh*, a marker of Sertoli cell function, remained unaffected.[43] In humans, mutations in these genes are associated with the development of disorders of sex development (DSD) syndrome, including micropenis, a bifid scrotum, penoscrotal hypospadias and cryptorchidism.[44]

Multiple studies show that environmental factors can contribute significantly to the occurrence of hypospadias. An increased incidence of this malformation was observed in wildlife species when their habitats were contaminated by pesticides.[45] Exposure of male fetuses *in utero* to different environmental chemicals such as vinclozolin, polychlorinated biphenyls (PCBs), phthalates and dioxins has been shown to induce hypospadias in boys of the resident families.[22,46,47] A significant number of studies have observed links between environmental chemicals and hypospadias. A residence location in the vicinity of hazardous waste disposal sites has been associated with a high incidence of hypospadias in boys of the resident families.[48,49] A high incidence of hypospadias was observed in male children of parents exposed to dioxin after the Seveso industrial accident.[50] Another study showed that fathers who worked in an agricultural region handling herbicides had more than one child with a birth defect.[51] Fathers who were vehicle mechanics[52] or firemen,[53] showed an increased risk of having a son with hypospadias. An increased incidence of hypospadias was reported in mothers exposed to farm chemicals.[54]

Hypospadias is thought to be associated with a transgenerational effect because treatment of pregnant mothers with diethylstilboestrol (DES) increased the incidence of hypospadias in the second generation.[55] Some studies have reported that paternal exposure to pesticides prior to pregnancy does not seem to be associated with hypospadias.[56,57] A large study in the United Kingdom based on the birth defects registry data reported that hypospadias was not more likely among infants born to women with any particular occupational exposures.[58] A study in South Africa observed no differences in the birth prevalence of hypospadias in villages that were or were not sprayed with dichlorodiphenyltrichloroethane (DDT) for malaria control.[59] These observations taken together suggest that the role of environmental chemicals in the etiology of hypospadias is debatable and might be associated with individual genotype-dependent sensitivities of specific subpopulation of humans to harmful effects of the environment.

The proposed role of the maternal lifestyle on the risk of developing hypospadias has not been elucidated. Alcohol consumption during pregnancy has not been associated with hypospadias.[60,61] Maternal smoking has not been linked with this disorder.[60,62] One study reported a relationship between maternal cocaine use during pregnancy and the development of hypospadias in their sons.[63] Some associations between dietary factors and the occurrence of hypospadias have been reported in several studies. Frequent consumption of fish was found to be associated with hypospadias; this was allegedly because of an accumulation of environmental chemicals in the fish.[64,65] A recent study showed that a decreased consumption of fruit, vegetables and protein-rich foods was associated with a higher risk of hypospadias compared to consuming healthy food.

ROLE OF THE GENETIC BACKGROUND, ENVIRONMENT AND LIFESTYLE IN THE ETIOLOGY OF CRYPTORCHIDISM

Cryptorchidism is often considered a mild malformation, but it represents the best characterized risk factor for infertility and testicular cancer in adulthood. Cryptorchidism is a heterogeneous disorder, and the testicular function in adulthood may be altered to different degrees with the sperm quality ranging from normozoospermia to complete azoospermia.[13,66] In a recent randomized study, we demonstrated that the position of the testes and the age related to the germ and Sertoli cell loss in boys suffering from cryptorchidism.[67] Between 9 months and 3 years of age, the number of germ and Sertoli cells and the diameter of the seminiferous cords decreased. This study demonstrated that the testicular volume in children can be used as a predictor of spermatogenesis, while the serum levels of inhibin B, follicle stimulating hormone (FSH), LH and testosterone are not useful in this respect.[67] The optimal age for orchidopexy to avoid severe alterations in spermatogenesis is suggested to be 9 months after birth.[67] The frequency of cryptorchidism of term boys at birth is approximately 2%–4% in many countries, but may vary among different countries.[68] Low birth weight, preterm delivery and small for gestational age weight are associated with a substantial increase in the incidence of cryptorchidism, which may reach 20%–25% in boys with birth weight less than 2.5 kg.[69] Over the last few decades, there has been clear evidence that the incidence of cryptorchidism has increased in Denmark[68,70] and the UK.[71] The etiology of cryptorchidism remains unknown for the most part, and cryptorchidism might be considered a complex disease. Currently, a large amount of data suggests that the interaction of environmental and genetic factors plays a critical role in the etiology of cryptorchidism. Testicular descent is regulated by two hormones, testosterone and Insl3, that are secreted by the FLCs in the testes.[72] Insl3 stimulates the development of the gubernaculum, which attaches the testis close to the inner opening of the inguinal canal. During late gestation, the testes migrate through the inguinal canals to the scrotum. This step is critically dependent on normal androgen action. Dysfunction of the FLCs leading to androgen and Insl3 deficiency retains the testis either in the abdomen or in

the inguinal canals. One can suggest that attenuation of Insl3 and testosterone production or action may contribute to the development of cryptorchidism. Lower Insl3 concentrations have been found in the cord blood of cryptorchid boys.[73] Cryptorchidism is a common feature of hypogonadotropic hypogonadism (HH), and treatment with human chorionic gonadotropin (hCG) and a gonadotropin releasing hormone (GnRH) agonist has been reported to induce the descent of the testes to a normal scrotal position of boys with cryptorchidism.[4] Patients with inactivating mutations in the LH receptor gene exhibit cryptorchidism and other phenotypic features.[75] All these pathological conditions suggest that insufficient stimulation of the FLCs to produce Insl3 and testosterone in the critical period of male fetal development increases the risk of developing cryptorchidism.

The genetic background plays an important role in the etiology of cryptorchidism. It was demonstrated that 22.7% of patients with undescended testes had a positive family history, compared to 7.5% of controls,[76] with a calculated increased risk factor of cryptorchidism in newborn males of 6.9 if a brother is affected and 4.6 if the father is affected. The genes that control the proper development of the gubernaculum and testicular descent are *INSL3*, the *INSL3* receptor *relaxin/insulin-like family peptide receptor 2* (*RXFP2*), luteinizing hormone *receptor* and other genes involved in the regulation of the biosynthesis and/or action of androgens. Mutations in the AR gene, steroidogenic enzymes and hypothalamic-pituitary regulators needed for testicular stimulation have been reported as rare causes of cryptorchidism.[77] One study reported an association between the GGN segment in the AR and the risk of cryptorchidism and hypospadias.[78] The CAG repeat length of the AR gene does not seem to play a major role in patients with unilateral cryptorchidism. In patients with bilateral undescended testes, a less functional AR because of a longer polyglutamine chain may play a role in the pathogenesis of cryptorchidism.[79]

The phenotypes of men with *INSL3 and RXFP2* mutations vary between unilateral to bilateral cryptorchidism, indicating differences of the penetrance of the genetic changes to the gubernaculum development.[80]

There have been considerable efforts to link maternal exposure to particular chemicals with the incidence and severity of cryptorchidism. Environmental compounds are thought to play a role in cryptorchidism by disturbing the action of hormones involved in the testicular descent. Endocrine disruptors with such possible effects are organohalogen pollutants, phthalates, antiandrogens and estrogens. Significantly higher levels of polybrominated diphenyl ethers (PBDEs) have been identified in the mother's milk of boys with undescended testicles compared to the mothers of boys with normal testicular development.[81] A significant association between congenital cryptorchidism and some persistent pesticides in breast milk has been found, suggesting that the testicular descent in the fetus may be adversely affected by EDCs.[82] The mechanism underlying this effect of EDCs is unknown, but can be proposed to be associated with their antiandrogenic effects.[83] The levels of phthalates in mother's breast milk were not linked with a risk of cryptorchidism in their sons,[84] even though EDCs have been found to cause an antiandrogenic effect on the anogenital index in boys.[5]

Estrogenic compounds also have the potential of disturbing cellular events that control the testicular descent in humans. Boys born to women treated with DES in early pregnancy had an increased incidence of cryptorchidism and other genital defects.[85] These congenital malformations can be associated with the suppression of production of androgen and Insl3 by FLCs.[86] The exact mechanism by which estrogens regulate Insl3 and androgen production by FLCs is unclear; a role of estrogen receptor (ER) α-dependent signaling has been suggested.[87] although direct action of estrogens on the gubernaculum, which expresses ER α (ERα)),[88]cannot be excluded. Several studies have reported that medicating pregnant mothers with mild analgesics is associated with an increased risk of giving birth to boys with cryptorchidism.[89,90] Such an association was observed in a Danish, but not a Finish, national birth cohort,[90] and it was very recently confirmed in a Dutch cohort.[66] In these three studies, drug use was established by a questionnaire.

Little is known about the role of the maternal lifestyle on the risk of developing cryptorchidism. Alcohol consumption by pregnant mothers was associated with an increased risk of cryptorchidism,[91] but other studies revealed a small increased risk of cryptorchidism only in the group of mothers with high consumption of alcohol during the early gestational period.[92,93]

ROLE OF THE GENETIC BACKGROUND, ENVIRONMENT AND LIFESTYLE IN THE ETIOLOGY OF TESTICULAR CANCER

Testicular cancer represents 1% of cancers in men. Germ cell tumors (GCTs) are the most common malignancy in men between 15 and 34 years of age[76] and account for 95% of testicular cancers. In 2011, Rosen *et al.*[94] described a study evaluating a global age-standardized incidence rate (ASIR) and age-standardized mortality rate (ASMR) for testicular cancer. In this study, the authors revealed a testicular cancer ASIR of 7.8% in Western Europe, 6.7% in North America, 6.5% in Australia, and an AISR below 1.0% in Asia and Africa.[94] The highest incidence rates occur in developed countries with mainly Caucasian populations.

Testicular germ cell tumors are known to be connected to mutations in several genes, including *kit-ligand (KITLG), sprouty homolog 4 (SPRY4), BCL2-antagonist/killer 1 (BAK1), telomerase reverse transcriptase (TERT), doublesex and mab-3 related transcription factor 1 (DMRT1)* and *activating transcription factor 7 interacting protein (ATF7IP).*[76] Detection of these malignant cells can be achieved by showing positive expression for placental alkaline phosphatase (PLAP); tyrosine-protein kinase Kit (C-KIT); POU domain, class 5, transcription factor 1 (POU5F1, also termed as OCT3/4); activating enhancer binding protein 2-gamma (AP-2gamma); and homeobox transcription factor NANOG (NANOG).[95] Recent and earlier studies demonstrate the role of *KITLG* and *DMRT1* gene alterations as the main factors involved in TGCT formation.[96,97] In addition to genetic factors, the relationship between abnormal functions of the Sertoli and Leydig cells has been discussed in several studies, and a delayed function has been associated with the formation of testicular cancer.[17,98] Reduced androgen production might act through the Sertoli cells on the germ cells and be the reason for the increased risk of developing TGCTs in patients with AISs.[95,98,99] In terms of tumor development, complete and partial AIS result in a different percentage of tumor development. Partial AIS patients have a 15% risk of developing TGCT, while the risk among complete AIS patients is approximately 0.8%.[95,100] The precursor cells for TGCT, the carcinoma *in situ* (CIS) cells, arise during fetal life and transform into TGCT cells later.[99] The effects of endogenous factors and xenobiotics on early fetal development might play an important role in TGCT formation by influencing the gonadal germ and somatic cells.

THE EFFECTS OF EDCS ON SEMEN QUALITY IN HUMANS

Because EDCs comprise a large number of different substances with dissimilar structures and diverse toxicities, some EDCs may directly affect spermatogenesis, for instance, by changing the structure or motility of spermatozoa, damaging the spermatogonia or destroying

Sertoli cells; while most other pesticides act indirectly through endocrine disruption. Several earlier studies showed that occupational exposure to specific pesticides such as dibromochloropropane, ethylene dibromide and chlordecone had detrimental effects on the semen quality, affecting the sperm count and the sperm motility and morphology.[101,102] Assessment of occupational or environmental exposure to EDCs in humans is very complicated, and the results from recent studies are inconsistent concerning the effects of pesticide exposure on semen quality. Earlier studies from the 1990s did not find an overall effect of pesticide exposure on semen characteristics.[103–105] One study reported some adverse effects of organophosphate pesticides on spermatogenesis, with significant reductions in the sperm concentration and the percentage of motile sperm and some reduction in the percentage of morphologically normal sperm observed among 32 workers in a Chinese pesticide production plant.[106] Exposure to pesticides has been shown to be associated with low sperm concentrations, low percentages of morphologically normal sperm, low percentages of motile sperm and increased serum estradiol concentrations. These associations were stronger in more frequently exposed men and in those with primary infertility, indicating an interaction between the pesticide exposure and predisposing genetic or medical risk factors.[107] A recent study reported that organophosphate pesticide sprayers had reduced percentages of morphologically normal sperm and motile sperm and decreased serum levels of LH and testosterone.[108]

Altogether, one can conclude that despite the heterogeneity in the study populations, the EDCs studied and the exposure assessment, environmental chemicals may affect spermatogenesis and lead to poor semen quality and reduced male fertility.

QUESTIONS FROM THE PANEL

Q1: Should we be concerned about medications used during pregnancy? Which drugs are candidates for activation of this type of fetal programming?

A1: There are a limited number of studies on the potential of certain therapeutic drugs to affect FLC steroidogenesis and impair masculinization of male fetuses. This chapter will describe the available data on the effects of some widely used therapeutic drugs on the FLC function and masculinization of the male fetus in humans and in animal models.

Glucocorticoids

The human fetus can be exposed to elevated physiologically relevant levels of glucocorticoids from maternal stress or after treatment with synthetic glucocorticoids. Dexamethasone administered to pregnant women to induce fetal lung maturation was reported to have no effect on the postnatal androgen production by the testes of preterm male infants.[109] Maternal use of corticosteroid medications was weakly associated with a risk of hypospadias, but the association was negligible after adjustment for potential confounders.[110] Little is known about the possible interactions between cortisol and testosterone in human fetuses. Two studies reported a positive correlation between cortisol and testosterone levels in the serum obtained from second and third trimester fetuses[111] and in amniotic fluid,[112] indicating that unlike the adult, where testosterone production is often inhibited by cortisol, there is a positive correlation between these steroid hormones in the fetus. In contrast to humans, the treatment of pregnant rats with dexamethasone and corticosterone reduced the AGD in newborn male offspring,[113] reflecting a disruption of the masculinization process because of FLC dysfunction induced by the glucocorticoids. Exposure to dexamethasone from e15 to e21 reduced the plasma testosterone and LH levels between e19 and e21.[114] These data strongly suggest that dexamethasone alters LH secretion, attenuating the production of testosterone in fetal rats; this finding contrasts with human fetal data.[12] Human fetal exposure to increased glucocorticoid concentrations appears to have no effect on the testosterone production or may even result in increased production; while in fetal rats, increased glucocorticoid concentrations suppress testosterone production, perhaps through effects on LH.[12]

Ketoconazole

Ketoconazole was originally developed as an antifungal agent for the treatment of fungal infections. This compound predominantly inhibits the 17,20-lyase reaction; it inhibits the 17α-hydroxylase and 11β-hydroxylase reactions to a lesser extent.[115] Ketoconazole has been used in the treatment of Cushing's syndrome, which is associated with increased blood levels of cortisol. One clinical study reported that ketoconazole administered during pregnancy did not affect the masculinization and development of the newborn male infant unless the drug was given between weeks 3 and 7,[116] suggesting that the dose of ketoconazole used was not high enough to inhibit the testosterone production enough to alter masculinization.[12] A population-based study of fetal exposure to ketoconazole during the second and third trimesters did not reveal a higher rate of congenital abnormalities after *in utero* exposure to ketoconazole.[117] In contrast, *in vitro* studies have shown that ketoconazole inhibits testicular steroidogenesis in human fetal testes.[118,119] These findings suggest that ketoconazole has the potential of decreasing androgen production by human FLCs and affecting proper masculinization and the development of male reproductive organs in humans. Experiments on pregnant rats showed that ketoconazole reduces the testosterone production and alters masculinization only after exposure of pregnant dams from e7 to e21,[120] with no demasculinization of males observed after treatment from e14 to lactational d3,[121] indicating that the androgen-dependent morphogenesis of the external genitalia take places specifically between e7 and e14 in rats.

Glitazones

Glitazones act through the activation of peroxisome proliferator-activated receptors to reduce insulin resistance and are widely used in treating type 2 diabetes.[122] This family of antidiabetic drugs can inhibit several steroidogenic enzymes, such as cytochrome P450, family 17 (CYP17) and hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1 (3βHSD) and can attenuate testosterone production.[123,124] Glitazones are often used to treat insulin resistance in women with polycystic ovarian syndrome,[125] and these drugs suppress androgen production by the ovary and reduce hyperandrogenism.[126] The fact that rosiglitazone can cross the human placenta in the first trimester[127] and the fact that it has a suppressive effect on steroidogenesis; suggests a potential for this compound to disturb the androgen-dependent process of reproductive organ development in human male fetuses. Several studies have been reported on human fetal exposure to rosiglitazone during pregnancy. One study observed that the treatment of women with polycystic ovarian syndrome with rosiglitazone before and during the first trimester did not induce any congenital abnormalities in the newborn infants.[128] Treatment with rosiglitazone to control type 2 diabetes during the first trimester has not been shown to have any harmful effects on the development of the newborn infants of either sex.[129] These clinical observations suggest that rosiglitazone used at therapeutic doses

has no negative effects on human FLC steroidogenesis because no androgen-dependent reproductive abnormalities were found.

Q2: Environmental estrogens seem to have made way for endocrine disruptors. Is the environmental estrogen hypothesis still tenable?

A2: The assumption that an increasing incidence of reproductive abnormalities and poor semen quality in the human male may be related to increased estrogen exposure *in utero* was first proposed in 1993 by Sharpe and Skakkebaek.[130] The so-called "estrogen" hypothesis was mainly based on accumulating evidence that the sons of women exposed to the potent estrogenic compound DES during pregnancy showed an increased incidence in reproductive abnormalities, suggesting that estrogens were antagonizing the androgen action in the male fetus. An important component of this hypothesis was based on the fact that Sertoli cell proliferation in humans and animals is controlled to a large extent by FSH,[131] and the inhibition of FSH secretion by environmental estrogens may attenuate Sertoli cell proliferation and suppress spermatogenesis.[130] Published studies on the adverse effects of environmental estrogens on male reproductive development have yielded conflicting results, in which either no effects[132,133] or adverse effects [134,135] on the reproductive organs were reported. One comprehensive study reported that neonatal exposure of rats to high doses of weak environmental estrogens, octylphenol (OP) and bisphenol A (BPA) slightly stimulated the first wave of spermatogenesis at puberty with no effect on the testes weight or fertility.[136] This finding allowed the authors to suggest that it is unlikely that human exposure to OP or BPA during neonatal life/puberty would result in any detectable adverse effect on spermatogenesis or fertility, because the level of human exposure is not as high as that used in the study. At present, there is a strong controversy regarding the adverse reproductive effects of xenoestrogen BPA in humans and animals. Recently, based on analysis of data obtained by Ryan *et al.*[137] and others[138,139] that focused on the investigation of estrogenic effects of BPA; Sharpe concluded that BPA, at doses considerably higher than human exposure levels, does not reliably affect the parameters of development and function in male or female rats/mice that are estrogen sensitive.[140] It has been further suggested that BPA could contribute to the additive effects of the mixture of estrogenic chemicals to which humans can be exposed,[141] but the low estrogenic potency of BPA *in vivo* through oral exposure and the low levels of exposure in the general population suggest that its contribution to mixed estrogenic effects is minimal.[140,142]

Although pregnancy is a period of high estrogen production,[143] a significant amount of this steroid is bound to sex hormone binding globulin (SHBG) in humans (but not in rodents), while synthetic estrogens, such as DES and ethinyl estradiol, do not bind to SHBG and have the potential to act directly on the developing reproductive organs in humans.[144] The negative effects of estrogens on male reproductive organ development are thought to be dose-dependent because low doses of ethinyl estradiol had no impact on human fetal testicular steroidogenesis.[145] Exposure to DES *in utero* was associated with a microphallus and a reduced testis size,[85] which are signs of disrupted androgen action on the male genitalia. Sixty percent of boys exposed to DES *in utero* showed various genital abnormalities including epididymal cysts, hypoplastic testes and undescended testes.[146] These studies, together with the finding that the expression of ERα is completely absent in the human fetal testis, including in the Leydig cells, suggest that the harmful effects of estrogenic agents on the development of the male reproductive organs in humans is not mediated by the suppressive action on testicular steroidogenesis as was observed in rodents,[87] but can be induced by other unknown mechanisms. This assumption was further supported by the observation that the reproductive tract abnormalities induced by DES in neonatal male rats are associated with reduced AR expression,[147] a finding that can provide a mechanism through which estrogens could alter the development of AR-dependent organs in fetal life, independently of any effect on testosterone production by the fetal testis. If this mechanism is operative during pregnancy in humans, it may explain the abnormalities in penis development (e.g., hypospadias, microphallus) by the direct action of estrogenic agents on penile formation. This assumption is consistent with the finding that the human fetal penis expresses both estrogen and ARs and estrogen-dependent signaling attenuates proliferation, while androgens stimulate this process in primary cultures of human fetal penile smooth muscle cells.[148]

The data presently available, allow us to conclude that the negative effects of estrogens on the development of the male genitalia in humans compared to rodents are not associated with attenuated testicular steroidogenesis. The negative effects seem to be induced by the direct suppressive effects on AR-mediated signaling in the target organs.

Q3: What type of factors influence the number of Sertoli cells and how does this occur? Specify the erroneous signals that are involved in the malignant transformation of immature germ cells.

A3: It is well-known that Sertoli cells play an important role in supporting normal spermatogenesis by functioning as "nurse" cells. They create niches for the differentiation of spermatogonial stem cells and supply the developing germ cells with structural support, nutrients and growth factors. The impairment of the Sertoli cell function by different metabolic perturbations or by external toxic environmental pollutants may significantly attenuate the process of spermatogenesis and may result in infertility.

The list of EDCs that affect the function of the testes, including the process of spermatogenesis, is growing and we will focus only on the effects of a few such compounds. It was shown that the treatment of pregnant rats with methoxychlor (MXC) from gestational day 7 to 15 reduced the number of germ cells at the age of 17 dpc because of increased apoptosis.[149] The treatment of perinatal and juvenile rats with MXC decreased the size of the Sertoli cell nuclei and the total Sertoli cell number, which was associated with an attenuated spermatogenic potential.[150] In addition to MXC, 1,1-dichloro-2,2-bis *(p*-chlorophenyl) ethylene (*p*, *p*'-DDE) and vinclozolin, a dicarboximide fungicide, were shown to suppress spermatogenesis by the activation of apoptosis.[151] Vinclozolin administered to pregnant mice during 15–22 dpc resulted in decreased sperm counts, lower prostate weight and a decreased AGD.[152]

Another compound that impairs rat spermatogenesis is the organochlorine pesticide lindane (gamma-hexachlorocyclohexane).[153] *In vitro* studies of Sertoli cells demonstrated an alteration of the gap junctions and impairment of the intercellular communications by affecting the distribution of connexion 43 (C×43) and zona occludens-1(ZO-1).[154] The xenoestrogen BPA was also reported to disrupt spermatogenesis in various species.[153] Maternal exposure to BPA was shown to decrease the efficiency of spermatogenesis and sperm production in male offspring.[155] BPA was reported to damage the Sertoli cell interactions and the blood-testis-barrier (BTB) in rats by affecting the distribution of C × 43 and ZO-1.[156,157] In addition to BPA, several other EDCs have the potential of suppressing spermatogenesis *in vivo*. An injection of 4-tert-octophenol was found to decrease the sperm counts and induce sperm head abnormalities in rats.[158] The exposure of male rats to Aroclor 1254, a PCB, was reported to cause decreased testicular weight, sperm count, sperm motility and daily sperm production and was associated with a depletion of mitochondrial antioxidant enzymes.[159] Lower sperm counts and an

increase of teratozoospermia and a depletion of the seminiferous tubules were also observed after treatment with the insecticide malathion in mice.[160] One can conclude that environmental toxicants may suppress spermatogenesis by a direct action on the developing germ cells and/or by alteration of the Sertoli cell function required to support spermatogenesis.

CONCLUSIONS AND UNRESOLVED QUESTIONS

The interaction between the environment and genetic factors is a fundamental aspect of development and some individuals may be more susceptible or resistant to EDCs than others. Little is known about the molecular mechanisms underlying such vulnerability of a person to certain environmental compounds.

Increasing evidence indicates that the risk of developing genital abnormalities (e.g., hypospadias, cryptorchidism, micropenis) in boys is tightly linked to the hormonal dysfunction of FLCs. Insl3 and androgens produced by these cells control the complex mechanisms of the formation and proper development of the male reproductive organs. A detailed knowledge of the cellular events that control a balanced production of these key factors by human FLCs is still limited. An important unresolved issue is whether estrogenic compounds can suppress androgen production by human FLCs. The observation that human FLCs do not express ERα strongly suggests that the susceptibility of masculinization to disruption by estrogen-like chemicals is questionable. Recent findings that human fetal testicular steroidogenesis is highly sensitive to low environmentally relevant doses of the xenoestrogen BPA give rise to the question of a possible role of ERβ and/or membrane-bound GPR30 in the regulation of the hormonal function of human FLCs.[161] Many environmental chemicals may have direct effects on the organogenesis of the male reproductive organs (e.g., the penis) by interfering with the AR and attenuating proper masculinization. This might lead to the development of hypospadias and micropenis in the male fetuses despite normal androgen production by the FLCs.

There is uncertainty whether phthalates or their metabolites can inhibit testicular steroidogenesis by the fetal human testis, as has been shown for rats. The present conclusions are based on the associations between maternal phthalate exposure and lower AGD in the sons of these women and suggest potential effects of environmentally relevant phthalate levels in humans. This result contrasts with the observation that monophthalates have no effects on steroidogenesis *in vitro* by human fetal testis explants.[12] These observed species differences of various EDCs' effects on androgen production by the FLCs of humans, rats and mice raised concerns about extrapolating the data from rodent studies to risk assessments in humans. Epidemiological data based on the increased incidence of hypospadias and cryptorchidism strongly support the hypothesis of a prevalent role of EDCs in the pathogenesis of these reproductive disorders. One can suggest that the disruptive effects of environmental EDCs on the developing male reproductive organs is a complex process that may include the attenuation of proper masculinization of the male genitalia because of androgen insufficiency resulting from FLCs targeted by EDCs and/or the attenuation of androgen action on the target reproductive organs by interference of certain EDCs with AR-dependent signaling that controls organogenesis (**Figure 1**).

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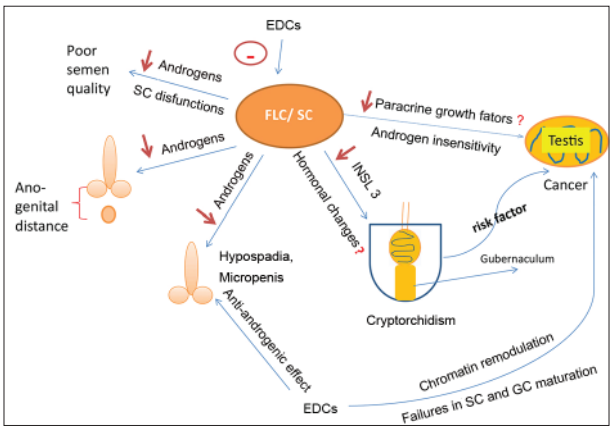

Figure 1: Summary diagram of the putative mechanisms underlying the development of reproductive disorders in human male fetuses. Different classes of endocrine-disrupting chemicals (EDCs) inhibit the production of androgens and insulin-like factor 3 (Insl3) by fetal Leydig cells (FLCs) in the sensitive androgen-dependent period of male reproductive organ development. Under-masculinization disturbs the proper formation and growth of the penis, increasing the risk of developing hypospadias and micropenis. A lack of Insl3 alters the normal testis descent, leading to cryptorchidism. EDCs can impair Sertoli cell (SC) differentiation from their progenitors and alter the proliferation of gonocytes that may lead to poor semen quality in postnatal life. EDCs may also directly impair the development of the reproductive organs by antiandrogenic or estrogen-mediated mechanisms and affect the methylation and/or acetylation status of the fetal testicular cells, which may create an abnormal genetic background and an increased risk of testicular cancer in postnatal life.

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