

Estrogen effects on fetal and neonatal testicular development

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Abstract

In recent years, evidences have accumulated that exposure to environmental components with estrogenic activity causes reproductive disorders in human populations. Studies conducted over the past 50 years have clearly shown a continual decline in semen quality accompanied by an increase in male reproductive disorders during this period in industrial countries. As healthy gametes are a prerequisite for healthy children, such disorders are a significant problem not only for the current society, but also for future generations. These male reproductive disorders have been attributed to xenobiotics, and particularly to xenoestrogens, which have steadily increased in diversity and concentration in the environment and food. Epidemiological, clinical, and experimental studies have suggested that excessive exposure to estrogens and xenoestrogens during fetal and neonatal development may induce testicular developmental disorders, leading to alterations in the adult male fertility. Recently, we have clearly demonstrated that fetal and neonatal testes are very sensitive to estrogens, as the inactivation of estrogen receptor α increases steroidogenesis and the inactivation of estrogen receptor β enhances development of the germ cell lineage in the male.

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Alterations in male reproductive function

Changes in the environment and their consequences for male reproductive function have been of major concern for the past 20 years.

Alterations in male reproduction were first observed in wild animals, in studies reporting the effects of accidental exposure of estrogenic chemicals on wildlife in the natural environment. These changes in male reproductive function vary from very subtle changes to permanent alterations, such as feminization or changes in reproductive behavior (Vos *et al.* 2000). Guillette *et al.* (1994) studied the male reproductive functions of alligators in two lakes in Florida. These two lakes are located very close to each other geographically, excluding the possibility of climate-based bias in these studies. They found that adult male alligators in Apopka Lake, which was polluted with agricultural waste and experienced a major chemical spill in 1980, had lower testosterone levels and presented micropenis and disorganized testes (Guillette *et al.* 1994, Guillette & Guillette 1996). A key part of this story is that no chemicals could be detected in the water of the apparently contaminated lake and thus the alligators were being

exposed simply by being at the top of the food chain. Other documented disruptions or alterations of reproductive activity and physiology have been correlated with exposure of contaminants in fish, amphibians, reptiles, birds, and mammals (examples in Table 1; for detailed review, see Vos *et al.* 2000, Edwards *et al.* 2006). Most of the reported effects on wildlife have been done on aquatic food chain organism making the causal link a direct or an indirect effect of pollutants hard to do.

In humans, there is increasing evidence that the birth sex ratio is altered in areas close to industry and exposed to environmental and industrial chemicals. The findings of the very recent report on the Aamjiwnaang First Nation community in Canada are striking (Mackenzie *et al.* 2005), i.e. the proportion of male live births in this community has been decreasing continually from 1990 to 2003, the sex ratio (number of male births/total number of births) reaching only 0.3. The epidemiologic data have also shown an increase in human male reproductive function disorder over the past 50 years, with the suggestion of a relation with the increase in the amounts of endocrine disruptors in the environment. Testicular cancer, which is the most prevalent cancer in young men, has steadily

Table 1 Effect of xenoestrogens on male reproduction in wildlife.

	Species	Country	Pollutant	Effects	References
Mammals	Panther	Florida, USA	Mercury, p,p'DDE, PCB	Cryptorchidism sperm count and motility	Facemire <i>et al.</i> (1995) Mansfield & Land (2002)
	Beluga whales	Quebec, Canada	PCB	Hermaphroditism	De Guise <i>et al.</i> (1994)
Birds	Polar bear	Svalbard, Russia	Organochlorine, PCB	Testosterone level	Oskam <i>et al.</i> (2003)
	Western gull	California, USA	DDT, methoxychlor	Feminization (ovotestis, persistent mullerian duct)	Fry & Toone (1981), Fry (1995)
Reptiles	Snapping turtles	Ontario, Canada	Organochlorine	Feminization	De Solla <i>et al.</i> (1998)
	Alligator	Florida, USA	P,p'DDE	Abnormal gonadal development and steroidogenesis penis size	Guillette <i>et al.</i> (1994), Guillette & Guillette (1996)
Amphibians Fish	Leopard frog	Different sites, USA	Atrazine	Gonadal dysgenesis hermaphroditism	Hayes <i>et al.</i> (2003)
	Roach fish	Leicestershire, UK	Endocrine disruptors	Intersex sperm count/motility	Jobling <i>et al.</i> (2002)
	Spottail shiner	Quebec, Canada	Estrogenic compounds	Intersex spermatogenesis impairment sperm count/motility	Aravindakshan <i>et al.</i> (2004)
	Catfish	Gauteng, South Africa	p-nonylphenol	Intersex	Barnhoorn <i>et al.</i> (2004)

increased in all countries studied, rising from 3.4% in 1973 to 5.5% in 1997 in North America (Toppari 2002). Hypospadias and cryptorchidism also increased from 0.2 and 2% respectively in 1970 to 0.38 and 3.5% respectively in 1991 (Toppari 2002). Finally, the sperm count decline have been controversial but large-scale prospective studies using standardized methodologies have shown a decline from 170 to 70 million spermatozoa per milliliter between 1940 and 1990 in Europe (Auger *et al.* 1995, Sharpe & Irvine 2004). There are grounds for linking these four disorders. For example, a comparative study in European countries showed that the incidence of each of these four abnormalities (sperm count decline, testicular cancer, hypospadias, and cryptorchidism) was maximal in Denmark and minimal in Finland (Virtanen *et al.* 2005). Moreover, having a history of cryptorchidism increases the risk of other three disorders (Kaleva *et al.* 2005) by a factor of 3–17 in the case of testicular cancer (Davenport 1997). Similarly, hypospadias increases the chances of developing testicular cancer (Sharpe & Irvine 2004) and oligospermia is frequently observed in men, who go on to develop testicular cancer (Moller & Skakkebaek 1999, Skakkebaek *et al.* 2001). Therefore, it has been suggested that these four alterations are symptoms of a single syndrome, the testicular dysgenesis syndrome (TDS; Skakkebaek *et al.* 2001, Skakkebaek & Jorgensen 2005).

These abnormalities first arise during fetal development

The testes begin to carry out their two major functions (gametogenesis and steroidogenesis) during fetal development. Sertoli cells are the first to differentiate. From 12 days postconception (dpc) in the mouse, 13.5 dpc in the rat, and 42–45 dpc in human, they surround the germ cells to form the seminiferous cords. Sertoli

cells divide actively until puberty, remaining quiescent thereafter. Primordial germ cells arise from the epiblast and migrate from the extra-embryonic mesoderm to colonize the genital ridge. The germ cells are named gonocytes once they reach the gonad, in which they proliferate until fetal day 15.5 in the mouse and fetal day 17.5 in the rat. The gonocytes (Fig. 1A) then enter a quiescent period during which their number does not change. This period extends to birth in the mouse and postnatal day 3 in the rat, when mitosis resumes and gonocytes differentiate into gonidia (Olaso & Habert 2000). Fetal Leydig cells (Fig. 1B) differentiate soon after Sertoli cells and produce the testosterone and insulin-like factor 3 (Insl3) necessary for masculinization of the fetus (Habert *et al.* 2001, Kubota *et al.* 2002).

It is currently thought that TDS is probably caused by changes in the development of the fetal testis (Skakkebaek *et al.* 2001) because the origins of all four characteristics of TDS can be traced to fetal development. Hypospadias results from a defect in the androgen production or action during fetal development. Cryptorchidism results from the abnormalities in the

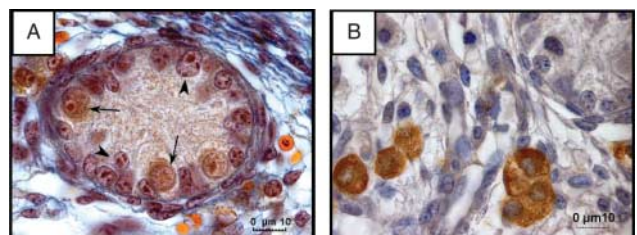


Figure 1 Histological aspect of the mouse neonatal testis observed 2 days after birth. (A) Seminiferous cord after coloration by Tushmann's blue. Arrows indicate the gonocytes, arrowhead indicates the Sertoli cells from Delbes *et al.* (2004). (B) Leydig cells, located in interstitial compartment, after immunohistochemical labeling of 3 β -hydroxysteroid dehydrogenase (3 β HSD); from Delbes *et al.* (2005).

production or activity of *Insl3* or the androgens regulating the transabdominal and transinguinal descent respectively of the testes (Kubota *et al.* 2002). The etiology of testicular cancer remains unclear, but there is considerable evidence to suggest that it originates early in development (Skakkebaek *et al.* 2001) when gonocytes would normally have differentiated into spermatogonia. Carcinoma *in situ* (CIS) is a local malignant lesion that precedes testicular cancer (seminomas and non-seminomas; Skakkebaek *et al.* 1981). CIS cells closely resemble fetal germ cells in terms of morphology and immunohistological markers (c-kit, alkaline phosphatase, etc; Rajpert-De Meyts *et al.* 1998). Moreover, CIS has been reported in only a few-months-old boys (Skakkebaek *et al.* 2001). Finally, sperm counts may have decreased for multiple reasons as the regulation of spermatogenesis remains poorly understood, but is known to involve complex endocrine, intratesticular, and intracellular regulation processes. The stock of gonocytes is determined during fetal development and takes part in determining the number of germ stem cells present in adulthood, since experimentally induced decreases in the number of gonocytes during fetal development lead to decreases in sperm count in adulthood (Moreno *et al.* 2001). Similar results are obtained if the number of Sertoli cells is reduced during perinatal life (Orth *et al.* 1988). Thus, adult sperm production depends partly on the fetal gametogenesis and partly on the capacity of gonocytes to differentiate into gonidia.

The hypothesis of fetal origin of TDS is also supported by clinical and experimental reports.

The major clinical data concern the boys born of women treated during their pregnancy with diethylstilbestrol (DES), a very potent estrogen agonist, from 1950 to 1970. Some studies have reported alterations in sperm quality and a higher incidence of genital malformations, cryptorchidism, and testicular cancer than for the control population (Glaze 1984, Strohsnitter *et al.* 2001), whereas others found no such change (Wilcox *et al.* 1995). These discrepancies may be due to differences in the period of treatment during pregnancy, suggesting that there may be a specific period of sensitivity to xenoestrogens in the testis. A recent analysis of these epidemiological studies by Storgaard *et al.* (2006) pointed out that DES seems to have a negative effect on sperm count only if administered at high dose during the first semester of pregnancy.

Another clinical argument concerns the recent demonstration that phthalates (found in cosmetics, paint, and PVC) have negative effects on human male reproductive tract development (Swan *et al.* 2005). Maternal urinary phthalate concentration during the pregnancy is inversely correlated with anogenital distance at birth. This finding is particularly important as it provides the first demonstration of negative effects of xenobiotics at environmental concentrations.

Experimentally, the following two approaches have been used in rodents to determine the effects of exposure to exogenous estrogens during fetal and neonatal development on male reproductive functions

In vitro studies in the rat are based on organ cultures and primary cell cultures (Table 2). These techniques are useful to detect short-term effects and have shown that estrogenic molecules (DES, 17 β -estradiol, etc.) may alter cord formation early in testis development (Cupp & Skinner 2001, Lassarguere *et al.* 2003) and disrupt the development of germ cells, Leydig cells, and Sertoli cells. Our recent unpublished results evidenced that rat testicular cells are more sensitive to estrogens during the early fetal period (Delbès G, Duquenne C, Habert R & Levacher C unpublished observation, Table 2).

In vivo, rodents can be treated with exogenous estrogens during fetal and neonatal development by injection, gavage, or via their drinking water. Numerous reports based on this experimental design have been published in recent years (Tables 3 and 4). The effects are observed in the short (before puberty; Table 3) or the long term (in adulthood; Table 4). Differences between treatment protocols (route of administration, age at treatment, duration, and time of treatment, etc.) and animal strains (rats: Wistar, Sprague–Dawley, etc. and mice: C57Bl/6, Swiss, NMRI, etc.) make it hard to draw a general process of effect. Indeed, sensitivity to estrogenic chemicals is known to depend on the species and the strain of animal (Spearow & Barkley 2001). However, all these studies suggest that exposure to high doses of exogenous estrogens during gestation can induce all the symptoms of TDS. These effects are dose-dependent and two studies (Atanassova *et al.* 2000, Fielden *et al.* 2003) in which low doses were administered reported no alterations. Furthermore, the alterations observed depend on the molecule tested, DES being the most potent, consistent with its three or four times higher affinity for the estrogen receptor than estradiol itself (Kuiper *et al.* 1997), and phytoestrogens the weakest (Fielden *et al.* 2003, Thuillier *et al.* 2003, Adachi *et al.* 2004). The timing of exposure is also an important criterion, with early exposure being the most deleterious, due to very rapid effects on testicular histology and functions and the probable induction of irreversible effects.

It is important to note that all these studies focused on a single estrogenic compound while in reality, individuals are exposed to a cocktail of chemicals that could lead to more potent effects.

Estrogen receptors in the fetal testis

To date, two estrogen receptors have been described, ER α (Green *et al.* 1986) and ER β (Kuiper *et al.* 1996). Both belong to the steroid nuclear receptor superfamily and regulate gene expression. ER α and ER β are encoded by two different genes located on different chromosomes (10 and 12 respectively in mouse, 1 and 6 in rat, and 6 and

Table 2 *In vitro* effects of estrogen treatment on fetal and neonatal rat testis or testicular cells.

Protocol	Age	Treatment	Effects	References
Organ culture	13.5 dpc 14.5 dpc	17 β -estradiol (4 μ M-72 h) DES (4 μ M-72 h)	Cord formation alteration ↓ Number of gonocytes ↓ Number of Sertoli cells ↓ Number of Leydig cells ↓ Testosterone secretion	Lassarguere <i>et al.</i> (2003) Cupp <i>et al.</i> (2001)
	20.5 dpc 3 dpp	17 β -estradiol (4 μ M-72 h) DES (4 μ M-72 h) 17 β -estradiol (4 μ M-72 h) DES (4 μ M-72 h)	No modification of testosterone secretion No modification of the number of gonocytes	Delbès <i>et al.</i> (unpublished data)
	Leydig cell culture	21.5 dpc	17 β -estradiol (2 μ M-72 h)	↓ Testosterone secretion
Leydig cell culture	16.5 dpc 20.5 dpc	17 β -estradiol (0.01 – 1 μ M-48 h) DES (0.01 – 1 μ M-48 h)	↓ Testosterone secretion	Delbès <i>et al.</i> (unpublished data)
	6–7 dpp	17 β -estradiol (1 μ M-24 h) Octylphenol (24 h)	No modification of testosterone secretion 1–10 nM: testosterone secretion 100–200 nM: ↓ testosterone secretion	Murono <i>et al.</i> (1999)
	Purified gonocyte culture	3 dpp	17 β -estradiol (1 μ M-20 h) 17 β -estradiol (0.1 and 10 μ M-20 h)	↑ Gonocyte proliferation No effect
Coculture sertoli cells/gonocytes	16.5 dpc 3 dpp	DES (1 μ M – 5 days) 17 β -estradiol (1 μ M– 5 days)	↓ Number of gonocytes only at 16.5 dpc	Delbès <i>et al.</i> (unpublished data)

dpc, day post-conception; dpp, day post-partum; DES, diethylstilbestrol.

14 in humans). Numerous human isoforms are generated due to alternative splicing of the C-terminal region (Hirata *et al.* 2003). Similar isoforms have also been described in rodents (Chu & Fuller 1997, Lu *et al.* 1998). No biological function has yet been reported for the truncated ER α protein, but many physiological implications of the ER β isoforms are currently being investigated.

The expression of these isoforms in the adult reproductive tract has recently been reviewed (Akingbemi 2005, Saunders 2005). ER α and ER β are present in the fetal testis very early in development and their distribution in various types of testicular cell has been extensively studied in mammals. Immunohistochemical data have shown that ER α protein is present in the undifferentiated gonad as early as 10.5 dpc in the mouse (Greco *et al.* 1992) and is localized in the fetal Leydig cells until birth in rodents review in O'Donnell *et al.* (2001). Only one study has shown some staining in the seminiferous cords (Greco *et al.* 1992). ER β mRNA is detected in the testis as early as 14 dpc in the mouse (Jefferson *et al.* 2000) and is present primarily in the gonocytes, and also in the Sertoli and Leydig cells, as early as 16 dpc in the rat (Van Pelt *et al.* 1999). ER β protein is present at 16 dpc in three main types of testicular cells in the rat but is found exclusively in the gonocytes in the mouse (Saunders *et al.* 1998, Jefferson *et al.* 2000). However, in a recent study, ER β was not detected in isolated Sertoli cells from 3-days-old rats (Wang *et al.* 2004). Immunohistochemical analysis has shown that in humans, ER β is not present in the testis but ER β is expressed in germ cells, Sertoli cells, and Leydig cells (Saunders *et al.* 2001).

Other rapid effects of estrogen have been described that cannot be accounted for by the 'classical' genomic action of estrogens. Some new 'non-classical' estrogen pathways involving a membrane receptor for estrogen capable of activating numerous intracellular pathways (G protein, kinases, etc.) have been proposed (Luconi *et al.* 2002). A G protein-coupled estrogen receptor was recently identified in a breast cancer cell line and shown to be present in the adult testis (Thomas *et al.* 2005). Studies of the effects of estrogen on the fetal and neonatal testis are still limited to the classical pathway, but further investigation of the involvement of other estrogen receptors is required.

The role of endogenous estrogens in fetal and neonatal testicular development

It is now widely accepted that estrogens play vital role in the control of reproductive function in the adult male reviewed in Carreau *et al.* (2003) and Akingbemi (2005). Few human cases of spontaneous mutation in the estrogen pathway have been described: one homozygous mutation of the ER α gene (Smith *et al.* 1994) and six cases of aromatase deficiency (Morishima *et al.* 1995, Carani *et al.* 1997, Deladoey *et al.* 1999, Herrmann *et al.* 2002, Maffei *et al.* 2004, Mitre Herve *et al.* 2004). These patients mostly display skeletal disorders, as they are very tall and continue to grow in adulthood. They display no masculinization disorders, but present high levels of testosterone, luteinizing

Table 3 Short-term effects of *in vivo* treatment with estrogenic compounds during fetal and neonatal life on testicular development in rodents.

Protocol	Treatment regimen	Treatment age	Observation age	Observations	References
Gavage (Ic1:MCR mice)	Ethinyl estradiol (0.02–2 mg/kg per day)	11–17 dpc	18 dpc	Ooestis, cryptorchidism ↑Number of gonocytes ↓Number of Sertoli cells Leydig cell hyperplasia	Yasuda <i>et al.</i> (1985a), Yasuda <i>et al.</i> (1985b)
Subcutaneous injection (NMRI mice)	α -Zeranol (150 μ g/kg per day) DES (150 μ g/kg per day) ^a	9–10 dpc	12–18 dpc	Changes to gonocyte differentiation Leydig cell hyperplasia Testicular descent delay	Perez-Martinez <i>et al.</i> (1996)
Subcutaneous injection (rat)	OP (600 mg/kg per day) DES (500 μ g/kg per day)	11 and 15 dpc	17 dpc	↓SF-1 mRNA in testis ↓SF-1 protein in Sertoli cells	Majdic <i>et al.</i> (1997)
Subcutaneous injection (Wistar rat)	OP (100–600 mg/kg per day) DES (100–500 μ g/kg per day) BPA (0.5 mg/animal per day)	11 and 15 dpc 2,4,6,8,10,12 dpp	17 dpc 18 dpp	No histological difference No modification of Leydig cell number ↓P450c17 and SF-1 protein levels ↓Testis weight ↓FSH β and inhibin α levels	Saunders <i>et al.</i> (1997)
Gavage (Sprague–Dawley rat)	DES (0.01–2 μ g/kg per day) BPA (0.1–200 mg/kg per day) Genistein (0.1–10 mg/kg per day) Coumestrol (1–100 mg/kg per day)	14–21 dpc	0–3 dpp	↑In hsp90 levels in gonocytes ↑PDGFR	Thuillier <i>et al.</i> (2003), Wang <i>et al.</i> (2004)
Subcutaneous injection (Sprague–Dawley rat)	DES (0.01–0.2 mg/kg per day) OP (0.1–100 mg/kg per day) BPA (1–100 mg/kg per day)	13,15,17 dpc	19 dpc	Alkylphenols have no effect Dose-dependent decrease in testosterone secretion in response to DES	Haavisto <i>et al.</i> (2003)
Subcutaneous injection (rat)	Estradiol benzoate (500 μ g)	1 dpp	10 dpp	↓Intratesticular testosterone content	Bellido <i>et al.</i> (1990)
Subcutaneous injection (Swiss mice)	17 β -estradiol (10 μ g/day)	1–9 dpp	10 dpp	↓Circulating testosterone level	Cooke and Eroschenko (1990)

DES, Diethylstilbestrol; OP, 4-octylphenol; BPA, Bisphenol A.
^a α -Zeranol is a mycoestrogen.

Table 4 Long-term effects of *in vivo* treatment with estrogenic compounds during fetal and neonatal life on testicular functions in rodents.

Protocol	Treatment	Treatment age	Observation age	Observations	References
Subcutaneous injection (Wistar rat)	Ethinyl-estradiol (10 µg/animal per day) DES (0.1–10 µg/animal per day)	2,4,6,8,10,12 dpp	18, 75, 90 dpp	Dose-dependent ↓ in testis weight and volume ↑ Seminiferous lumen size ↓ Sertoli cell number (at 18 dpp) ↓ Germ cell volume/testis ↑ Germ cell apoptosis Dose-dependent ↓ in testosterone secretion ↓ FSH and no effect on LH	Sharpe <i>et al.</i> (1998), Atanassova <i>et al.</i> (1999)
Subcutaneous injection (Wistar rat)	DES (0.1–10 µg/animal per day) OP (2 mg/kg per day) BPA (0.5 mg/animal per day)	2,4,6,8,10,12 dpp	18, 25, 35, 90 dpp	Transient dose-dependent ↓ in testosterone secretion Transient ↓ in Leydig cell volume/testicle (DES)	Sharpe <i>et al.</i> (2003)
Injection (ICR mice)	DES (50 µg/animal per day) Genistein (1 mg/animal per day)	1–5 dpp	84 dpp	No morphological alteration in response to genistein ↓ ERα and AR mRNA	Adachi <i>et al.</i> (2004)
Subcutaneous injection (Sprague–Dawley rat)	DES (0.001–10 µg/animal per day)	2–12 dpp	83–91 dpp	Dose-dependent ↓ of (from 1 µg/day): number of spermatozoa Morphology and motility of spermatozoa Daily sperm production number of pups per litter Testis and seminal vesicle weights Plasma testosterone level ↑ FSH and no effect on LH	Goyal <i>et al.</i> (2003)
Gavage (Long-evans rat)	BPA (2.4 µg/kg per day)	12–21 dpc 21–35 or 90 dpp	90 dpp 35 or 90 dpp	↓ Testis and seminal vesicle weights ↓ Testosterone production/Leydig cell ↓ Plasma LH and testosterone concentrations ↑ ERβ expression in the pituitary gland	Akingbemi <i>et al.</i> (2004)
Gavage (B6D2F1 mice)	Araclor 1242 (10–100 mg/kg per day) ^a DES (0.1–10 µg/kg per day) Genistein (0.1–10 µg/kg per day)	15 days before mating to 21 dpp 12 dpc–20 dpp 12 dpc–20 dpp	112 and 315 dpp 21, 105, and 315 dpp 21, 105, and 315 dpp	↓ <i>In vitro</i> fertilization ability ↓ Number of spermatozoa at 315 dpp ↓ <i>In vitro</i> fertilization ability from 105 dpp changes in expression of the <i>ERα</i> , <i>SF-1</i> , <i>P450c17</i> , <i>P450scc</i> , <i>StAR</i> genes ↓ Epididymis size	Fielden <i>et al.</i> (2001) Fielden <i>et al.</i> (2002) Fielden <i>et al.</i> (2003)
Subcutaneous injection (Wistar rat)	Estradiol benzoate (50 µg)	1 dpp	1–45 dpp	↓ FSH, LH and testosterone concentrations ↓ ERα and AR mRNA levels in the testis from 15 dpp ↑ ERβ mRNA in the testis from 5 dpp	Tena-Sempere <i>et al.</i> (2000)
Gavage (mice)	BPA (2–20 ng/g per day)	11 dpc–17 dpc		↓ Epididymis size ↓ Daily sperm production (–20%)	Vom Saal <i>et al.</i> (1998)

OP, 4-octylphenol; BPA, bisphenol A; ER, estrogen receptor; AR, androgen receptor.

^aAraclor 1242 belongs to the PCB family (polychlorinated biphenyl).

hormone (LH), and follicle-stimulating hormone (FSH). These men also have low sperm viability (Smith *et al.* 1994), number, and motility (Herrmann *et al.* 2002) with bilateral cryptorchidism in one case (Maffei *et al.* 2004).

The development of transgenic mouse models has improved our understanding of the role of estrogens in male reproduction. Male mice with inactivated estrogen receptor ($ER\alpha$ KO, $ER\beta$ KO and $ER\alpha\beta$ KO) or aromatase (ArKO) genes show reproductive disorders. $ER\alpha$ KO and ArKO mice are sterile due to epididymal reabsorption fluid deficiency in $ER\alpha$ KO mice and spermatogenesis disorders in ArKO mice reviewed in O'Donnell *et al.* (2001). Excess estrogens or xenoestrogens alter male reproductive function, but these data show that estrogen deficiency may also have negative effect, raising the possibility that endogenous estrogens are essential for the maintenance of male fertility.

We investigated the role of endogenous estrogens during fetal and neonatal testicular development by analyzing testis development in mice inactivated for $ER\alpha$ KO and $ER\beta$ KO (Delbes *et al.* 2004, 2005). Inactivation of the $ER\beta$ gene induced a 50% increase in the number of gonocytes observed 2 and 6 days after birth (Fig. 2) due to an increase in the proliferation and a decrease in the apoptosis of these cells, with no change in Sertoli cell number or Leydig cell number. $ER\alpha$ gene did not modify the number of gonocytes; it increased testosterone production from the earliest stage studied (13.5 dpc, i.e. just 2 days after the first fetal Leydig cells start to differentiate; Fig. 3). We also found that the negative effect of estrogens on fetal and neonatal steroidogenesis did not depend on the hypothalamo-pituitary axis (Delbes *et al.* 2005), in contrast to reports of adults (Akingbemi *et al.* 2003). The activity of each fetal Leydig cell is increased by inactivation of the $ER\alpha$ gene as shown by the hypertrophy of these cells and their higher levels of

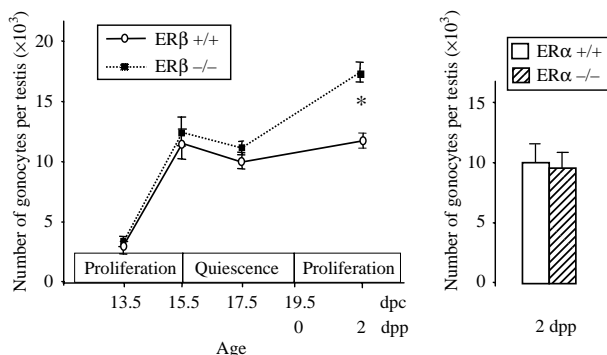


Figure 2 Number of gonocytes per testis during fetal and neonatal life after $ER\beta$ or $ER\alpha$ gene inactivation. Proliferation and quiescent periods of the gonocytes throughout the considered developmental period are indicated. Gonocytes were counted on histological testis sections from 13.5, 15.5, 17.5 dpc, and 2 dpp animals. Values are mean \pm S.E.M. of 4–13 animals. * P < 0.001 versus wild-type in Student's t -test. From Delbes *et al.* (2004).

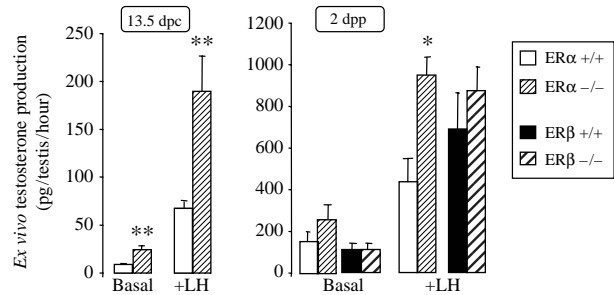


Figure 3 Production of testosterone by the fetal and neonatal mouse testis after $ER\beta$ or $ER\alpha$ gene inactivation. Testes were collected on day 13.5 (13.5 dpc) of gestation and on postnatal day 2 (2 dpp) and cultured on floating Millipore filters for 3 h in the presence (+LH) or the absence (basal) of 100 ng/ml ovine LH. The testosterone secreted in the medium was measured by radioimmunoassay. Values are mean \pm S.E.M. of 7–19 fetuses. * P < 0.01 and ** P < 0.001 versus wild-type in Student's t -test. From Delbes *et al.* (2005).

StAR, P450_{scc}, and P450 c17 mRNA. These data clearly show that endogenous estrogens inhibit testicular development and function during fetal and neonatal life. $ER\beta$ is involved in the control of gametogenesis, consistent with its location within the seminiferous cords, whereas $ER\alpha$ is present in the fetal Leydig cells and regulates steroidogenesis.

Origin and levels of estrogens acting on the developing testis

The question of the origin and level of endogenous estrogens acting on the developing testis during fetal and neonatal development is of prime importance, given the potential negative effect of exogenous estrogens and the need to improve our understanding of periods of sensitivity. Maternal plasma estrogen concentration peaks around gestational day 18. We showed that in C57Bl/6 mice, plasma 17 β -estradiol concentration is 0.19 nM at 13.5 dpc, 0.43 nM at 15.5 dpc, and 0.49 nM at 17.5 dpc (unpublished data), consistent with the previous reports (Barkley *et al.* 1979, Mahendroo *et al.* 1997). The only data dealing with intratesticular estradiol concentration in the fetal and neonatal mouse testis presently available is our measurement of a concentration equal to 4 nM at 2 dpp (Delbes *et al.* 2004). In the rat, intratesticular concentration of 17 β -estradiol is 0.55 and 0.34 nM at 18.5 and 20.5 dpc respectively and 0.36 nM in the fetal plasma at both ages (Habert & Picon 1984). Because of the presence of binding proteins in the plasma, these results suggest a production of estradiol by the testis.

Only one previous study reported the detection of aromatase mRNA in mouse fetal testis from 17.5 dpc onwards (Greco & Payne 1994). It had been suggested that expression of the aromatase gene in the fetal testis is

repressed by the product of *Sry* gene (Haqq *et al.* 1993), from embryonic days 12 to 16. We recently demonstrated the importance of aromatase activity by using organotypic culture. Addition of ICI 182,780, an antagonist of ER, to the culture medium increased the testosterone production by 20.5 dpc fetal rat testis, suggesting that estrogens produced by the cultured testis is sufficient to partially inhibit steroidogenesis (Delbès *et al.* unpublished data). On the contrary, ICI 182,780 did not change the testosterone production of 14.5 dpc testis when aromatase is probably not yet expressed (Lassurquere *et al.* 2003).

During late fetal life, aromatase is probably expressed by Sertoli cells at this stage in the rat as estrogens production is stimulated by FSH (Weniger & Zeis 1988, Rouiller-Fabre *et al.* 1998). Aromatase activity is weaker in the fetal Leydig cells than in adult Leydig cells (Saez 1994). Thus, the main source of estrogens is the Sertoli cells during fetal and neonatal life, whereas it is the Leydig cells in the adult. Furthermore, aromatase has been found in adult germ cells (Lambard *et al.* 2005), but no study has investigated whether aromatase is expressed in the gonocytes. This issue is of importance for the determination of the local concentration of estrogens in the target cell.

Remaining debates

Experimental data have shown that estrogens may have irreversible negative effects on testicular development and masculinization during fetal and neonatal life, consistent with the current hypothesis concerning the origin of TDS. However, controversy persists concerning the possible causal link between estrogen exposure during fetal and neonatal development and TDS (Storgaard *et al.* 2006). Recently, new data showed that polymorphism and lifestyle changes may also be responsible for male infertility (Aschim *et al.* 2005, Safe 2005).

We provided the first demonstration that endogenous estrogens physiologically regulate testicular development in a negative manner during fetal and neonatal life by controlling the two main functions of the testis, gametogenesis and steroidogenesis. As explained previously, changes in the establishment of the germ cell lineage may induce testicular cancer and abnormal sperm production. Furthermore, if testosterone secretion is regulated by estrogens, then estrogens control masculinization of the genital tract and testicular descent. This model is consistent with Sharpe & Skakkebaeck's (1993) hypothesis. Nevertheless, the link between abnormalities at birth and their real consequences for adult fertility is very weak, as the regulation of spermatogenesis changes considerably during puberty. Estrogen deficiency has positive effects during fetal development (increasing number of germ cells and steroidogenic activity) and negative effects in adulthood, with affected mice becoming infertile. Determination of the real impact of

estrogen deficiency during gestation on adult fertility would require studies of transiently transgenic mice, in which the expression of estrogen receptor genes was repressed only during this period of life.

Each estrogen receptor is involved in regulating one particular function, ER β being involved in gametogenesis and ER α in steroidogenesis processes. These precise mechanisms of action of estrogens in the fetal testis could help us to anticipate the effects of different estrogenic compounds depending on their affinity for each receptor. For example, genistein, which has a stronger affinity for ER β than ER α (Kuiper *et al.* 1997), would be expected to have a stronger effect on germ cells than that on steroidogenesis.

The existence of periods of development during which the testis is particularly sensitive to estrogens is strongly suggested by epidemiologic study of men exposed *in utero* to DES. These sensitive periods may vary depending on the susceptibility of the individual, as reported for the different strains of experimental animals (Spearow & Barkley 2001). We have shown in organ culture that exogenous estrogens inhibit gametogenesis only during fetal life (14.5 dpc) in the rat, whereas studies in ER β KO mice showed inhibition to occur later. It is unclear whether this difference is due to the difference in species (rat versus mice) or dose (addition of estrogens versus deficiency). Studies of the sensitivity of the human fetal testis would provide important new information, particularly as human testes differ from rodent testes in not expressing ER α and in containing numerous ER β mRNA variants (Moore *et al.* 1998).

Despite the accumulation of experimental data, the question of a possible direct effect of the increase in xenoestrogen levels in the environment on male fertility remains unanswered. With the exception of the report by Swan *et al.* (2005), studies using phytoestrogens or DES (Atanassova *et al.* 2000, Fielden *et al.* 2003) at low doses or at doses equivalent to human dietary exposure levels have reported no deleterious effect (Table 3). This has led some authors to conclude that endocrine disruptors do not reach sufficiently high concentrations in the body to have a deleterious effect on human health. However, individual can be exposed to a combination of chemicals with different activities (estrogenic, anti-androgonic, etc.). Moreover, the lipophilic characteristics of these molecules may result in their accumulation in fat tissues and these molecules may exert their effects by acting in combination. The developing fetus may be subjected to higher levels of xenoestrogens as maternal lipid stores are metabolized during pregnancy. Therefore, this raises the question of what is a physiologically relevant concentration of xenoestrogens.

Finally, a recent study highlighted the importance of this problem by demonstrating that rats descended from a great-grandfather exposed to high levels of endocrine disruptors during fetal development have low levels of sperm production (Anway *et al.* 2005, 2006). Thus,

changes in fetal testis development are observed not only in the contaminated individual during adulthood, but are also transmitted to subsequent generations by an undetermined epigenetic mechanism. This would have potentially major implications in terms of evolutionary biology and disease etiology and provides support for health care and environmental preventive action with respect to endocrine disruptors.

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