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 estrogentic 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.


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
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 gonad (Olaso & Habert 2000). Fetal Leydig cells (Fig. 1B) differentiate soon after Sertoli cells and produce the testosterone and insulin-like factor 3 (Ins3) 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

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**Table 1 Effect of xenoestrogens on male reproduction in wildlife.**

<table>
<thead>
<tr>
<th>Species</th>
<th>Country</th>
<th>Pollutant</th>
<th>Effects</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mammals</td>
<td>Panther</td>
<td>Florida, USA</td>
<td>Mercury, p,p’DDE, PCB</td>
<td>Cryptorchidism sperm count and motility</td>
</tr>
<tr>
<td></td>
<td>Beluga whales</td>
<td>Quebec, Canada</td>
<td>PCB</td>
<td>Hermaphroditism</td>
</tr>
<tr>
<td></td>
<td>Polar bear</td>
<td>Svalbard, Russia</td>
<td>Organochlorine, PCB DDT, methoxychlor</td>
<td>Testosterone level</td>
</tr>
<tr>
<td></td>
<td>Alligator</td>
<td>Florida, USA</td>
<td>P,p’DDE</td>
<td>Feminization (ovotestis, persistant mullerian duct)</td>
</tr>
<tr>
<td>Birds</td>
<td>Western gull</td>
<td>California, USA</td>
<td>Organochlorine</td>
<td>Feminization</td>
</tr>
<tr>
<td></td>
<td>Sanpping turtles</td>
<td>Ontario, Canada</td>
<td>Organochlorine</td>
<td>Abnormal gonadal development and steroidogenesis</td>
</tr>
<tr>
<td></td>
<td>Alligator</td>
<td>Florida, USA</td>
<td>P,p’DDE</td>
<td>Penis size</td>
</tr>
<tr>
<td>Amphibians</td>
<td>Leopard frog</td>
<td>Different sites, USA</td>
<td>Diiferent sites, UK</td>
<td>Gonadal dysgenesis hermaphroditism</td>
</tr>
<tr>
<td></td>
<td>Roach fish</td>
<td>Leicesterhire, UK</td>
<td>Atrazine</td>
<td>Intersex sperm count/motility</td>
</tr>
<tr>
<td></td>
<td>Spottail shiner</td>
<td>Quebec, Canada</td>
<td>Endocrine disruptors</td>
<td>Intersex spermatogenesis impairment</td>
</tr>
<tr>
<td></td>
<td>Catfish</td>
<td>Gauteng, South Africa</td>
<td>p-nonylphenol</td>
<td>Intersex</td>
</tr>
<tr>
<td>Fish</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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**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 3β-hydroxysteroid dehydrogenase (3βHSD); 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 nonseminomas; 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 gonia.

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, 17β-estradiol, etc.) may alter cord formation early in testis development (Cupp & Skinner 2001, Lassergue 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, ERα (Green et al. 1986) and ERβ (Kuiper et al. 1996). Both belong to the steroid nuclear receptor superfamily and regulate gene expression. ERα and ERβ are encoded by two different genes located on different chromosomes (10 and 12 respectively in mouse, 1 and 6 in rat, and 6 and
The expression of these isoforms in the adult reproductive tract has recently been reviewed (Akingbemi 2005, Saunders 2005). ERα and ERβ 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 ERα 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 (Chu & Fuller 1997, Lu et al. 1998). No biological function has yet been reported for the truncated ERβ protein, but many physiological implications of the ERβ isoforms are currently being investigated.

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 ERα 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, Mittre 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 2 In vitro effects of estrogen treatment on fetal and neonatal rat testis or testicular cells.

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Age</th>
<th>Treatment</th>
<th>Effects</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organ culture</td>
<td>13.5 dpc</td>
<td>17β-estradiol (4 μM-72 h)</td>
<td>Cord formation alteration</td>
<td>Lassargvuere et al. (2003)</td>
</tr>
<tr>
<td></td>
<td>14.5 dpc</td>
<td>DES (4 μM-72 h)</td>
<td>Number of gonocytes</td>
<td>Cupp et al. (2001)</td>
</tr>
<tr>
<td></td>
<td>20.5 dpc</td>
<td>17β-estradiol (4 μM-72 h)</td>
<td>No modification of testosterone secretion</td>
<td>Delbé's et al. (unpublished data)</td>
</tr>
<tr>
<td></td>
<td>3 dpp</td>
<td>DES (4 μM-72 h)</td>
<td>No modification of the number of gonocytes</td>
<td></td>
</tr>
<tr>
<td>Leydig cell culture</td>
<td>21.5 dpc</td>
<td>17β-estradiol (2 μM-72 h)</td>
<td>Testosterone secretion</td>
<td>Tsai-Morris et al. (1986)</td>
</tr>
<tr>
<td></td>
<td>16.5 dpc</td>
<td>17β-estradiol (0.01 – 1 μM-48 h)</td>
<td>Testosterone secretion</td>
<td>Delbé's et al. (unpublished data)</td>
</tr>
<tr>
<td></td>
<td>20.5 dpc</td>
<td>DES (0.01 – 1 μM-48 h)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6–7 dpp</td>
<td>17β-estradiol (1 μM-24 h)</td>
<td>10-10 nM: testosterone secretion</td>
<td>Murono et al. (1999)</td>
</tr>
<tr>
<td>Purified gonocyte culture</td>
<td>3 dpp</td>
<td>Octylphenol (24 h)</td>
<td>100-200 nM: testosterone secretion</td>
<td></td>
</tr>
<tr>
<td>Coculture sertoli cells</td>
<td>16.5 dpc</td>
<td>17β-estradiol (1 μM-20 h)</td>
<td>Gonocyte proliferation</td>
<td>Li et al. (1997)</td>
</tr>
<tr>
<td></td>
<td>3 dpp</td>
<td>17β-estradiol (0.1 and 10 μM-20 h)</td>
<td>No effect</td>
<td></td>
</tr>
<tr>
<td></td>
<td>17β-estradiol (1 μM-5 days)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

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

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Treatment regimen</th>
<th>Treatment age</th>
<th>Observation age</th>
<th>Observations</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gavage (Jc1:MCR mice)</td>
<td>Ethinyl estradiol (0.02–2 mg/kg per day)</td>
<td>11–17 dpc</td>
<td>18 dpc</td>
<td>Ootestis, cryptorchidism †Number of gonocytes ▼Number of Sertoli cells</td>
<td>Yasuda et al. (1985a), Yasuda et al. (1985b)</td>
</tr>
<tr>
<td>Subcutaneous injection (NMRI mice)</td>
<td>α-Zeranol (150 μg/kg per day) DES (150 μg/kg per day)a</td>
<td>9–10 dpc</td>
<td>12–18 dpc</td>
<td>Changes to gonocyte differentiation Leydig cell hyperplasia Testicular descent delay</td>
<td>Perez-Martinez et al. (1996)</td>
</tr>
<tr>
<td>Subcutaneous injection (rat)</td>
<td>OP (600 mg/kg per day) DES (500 μg/kg per day)</td>
<td>11 and 15 dpc</td>
<td>17 dpc</td>
<td>▼SF-1 mRNA in testis ▽SF-1 protein in Sertoli cells</td>
<td>Majdic et al. (1997)</td>
</tr>
<tr>
<td>Subcutaneous injection (Wistar rat)</td>
<td>OP (100–600 mg/kg per day) DES (100–300 μg/kg per day) BPA (0.5 mg/animal per day)</td>
<td>11 and 15 dpc</td>
<td>17 dpc</td>
<td>No histological difference No modification of Leydig cell number ▼P450c17 and SF-1 protein levels ▽Tests weight ▽FSHβ and inhibin α levels</td>
<td>Saunders et al. (1997)</td>
</tr>
<tr>
<td>Gavage (Sprague–Dawley rat)</td>
<td>DES (0.01–2 μg/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)</td>
<td>14–21 dpc</td>
<td>0–3 dpp</td>
<td>▼In hsp90 levels in gonocytes ▽PDGFR</td>
<td>Thuillier et al. (2003), Wang et al. (2004)</td>
</tr>
<tr>
<td>Subcutaneous injection (Sprague–Dawley rat)</td>
<td>DES (0.01–0.2 mg/kg per day) OP (0.1–100 mg/kg per day) BPA (1–100 mg/kg per day)</td>
<td>13,15,17 dpc</td>
<td>19 dpc</td>
<td>Alkylphenols have no effect Dose-dependent decrease in testosterone secretion in response to DES</td>
<td>Haavisto et al. (2003)</td>
</tr>
<tr>
<td>Subcutaneous injection (rat)</td>
<td>Estradiol benzoate (300 μg)</td>
<td>1 dpp</td>
<td>10 dpp</td>
<td>▼Intratesticular testosterone content</td>
<td>Bellido et al. (1990)</td>
</tr>
<tr>
<td>Subcutaneous injection (Swiss mice)</td>
<td>17β-estradiol (10 μg/day)</td>
<td>1–9 dpp</td>
<td>10 dpp</td>
<td>▼Circulating testosterone level</td>
<td>Cooke and Eroschenko (1990)</td>
</tr>
</tbody>
</table>

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.

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

OP, 4-octylphenol; BPA, bisphenol A; ER, estrogen receptor; AR, androgen receptor.<sup>a</sup>Araclor 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 (ERαKO, ERβKO and ERαβKO) or aromatase (ArKO) genes show reproductive disorders. ERαKO and ArKO mice are sterile due to epididymal reabsorption fluid deficiency in ERαKO mice and spermatogenesis disorders in ArKO mice reviewed in O’Donnell et al. (2001). Excess estrogens and 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 ERαKO and ERβKO (Delbes et al. 2004, 2005). Inactivation of the ERα 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 ERα 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 hypothalomo–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 ERα gene as shown by the hypertrophy of these cells and their higher levels of StAR, P450sc, and P450 c17 mRNA. These data clearly show that endogenous estrogens inhibit testicular development and function during fetal and neonatal life. ERβ is involved in the control of gametogenesis, consistent with its location within the seminiferous cords, whereas ERα 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 17β-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 17β-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 (Delbe`s 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 (Lassarguere 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 & Skakkebaek’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, ERβ being involved in gametogenesis and ERα 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 ERβ than ERα (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 ERβKO 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 ERα and in containing numerous ERβ 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 may be exposed to a combination of chemicals with different activities (estrogenic, anti-androgenic, etc.). Moreover, the lipophilic characteristics of these molecules may result in their accumulation in fat tissues and these molecules can 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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