

FETAL TESTIS AND ESTROGENIC ENDOCRINE DISRUPTERS

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RESUM

Des de fa ja molt temps, se sap que els estrògens tenen un paper molt important en la reproducció femenina, i hi ha ja també una forta evidència que poden també estar implicats en la regulació reproductiva masculina. En els humans, en diversos països i durant els darrers cinquanta anys, s'ha observat una disminució considerable en el recompte d'espermatozoides i un augment en la incidència de càncers testiculars, criptorquídies i hipospàdies. En les espècies salvatges, també s'han observat canvis semblants en la vida reproductiva dels mascles. Aquests desordres reproductius han estat atribuïts als xenobiòtics i, particularment, als xenoestrògens que, darrerament, han augmentat progressivament en diversitat i concentració en l'entorn i en el menjar. Estudis epidemiològics, clínics i experimentals han suggerit que l'exposició excessiva als estrògens i als xenoestrògens durant la vida fetal i neonatal podrien conduir a desordres reproductius en la vida adulta. En aquesta revisió presentem, en un model *in vitro*, que els estrògens afecten directament el desenvolupament del testicle fetal. També demostrem clarament que els testicles fetals i neonatals són molt sensibles als estrògens, ja que el silenciament del receptor d'estrògens alfa comporta un augment de l'esteroïdogènesi, i el silenciament del receptor d'estrògens beta facilita el desenvolupament de la línia cel·lular germinal en el mascle.

Paraules clau: estrògens, entorn, testicle, desenvolupament, fetus.

SUMMARY

Estrogens play a major role in female reproduction, but there is now compelling evidence that they may also be involved in the regulation of the male reproductive function. In humans, a decrease in sperm count and an increase in the incidences of testicular cancer, cryptorchidism and hypospadias, have been observed in many countries in the last fifty years. Changes in the male reproductive function have also been observed in wildlife. 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 in food. Epidemiological, clinical and experimental studies have suggested that excessive exposure to estrogens and xenoestrogen during fetal/neonatal life can lead to reproductive disorders in adulthood. We showed, in an *in vitro* model, that estrogens directly affected the development of the fetal testis. We also clearly demonstrated that fetal and neonatal testes were very sensitive to estrogens, as the invalidation of the estrogen receptor alpha led to an increase in steroidogenesis, and the invalidation of the estrogen receptor beta enhanced development of the germ cell lineage in the male.

Keywords: estrogens, environment, testis, development, fetus.

Concerns about environmental changes and their possible effects on reproduction in humans and animals have increased considerably over the last few decades.

CHANGES IN THE MALE REPRODUCTIVE FUNCTION

In the last few decades, we have observed an increase in the various abnormalities affecting the reproductive capacities of certain aquatic species and a qualitative and quantitative decrease in human sperm production. Furthermore, the incidence of testicular cancer (the most frequent cancer affecting young men) has increased steadily over the last twenty years, in all of the countries where studies have been made. The incidence of several other disorders affecting the male genital tract also seems to be increasing. This is the case with cryptorchidism (in which the testes do not descend into the scrotum), which is observed in 2 to 4% of neonates, and hypospadias (the ectopic opening of the urethra), which affects 0.3 to 0.7% of boys at birth (see figure 1).

Various lines of evidence suggest that these abnormalities could be linked. For example, a comparative study in various European countries showed that the incidence of each of these four types of abnormality (changes in sperm quality, testicular cancer, cryptorchidism and hypospadias) is highest in Denmark and lowest in Finland. It has

also been clearly demonstrated that cryptorchidism is a risk factor for the other three abnormalities and that hypospadias and oligospermia are risk factors for testicular cancer. Finally, men who go on to develop testicular cancer tend to be less fertile. Thus, these four abnormalities may be seen as different symptoms of the same syndrome: testicular dysgenesis syndrome (TDS).

ARE THESE CHANGES DUE TO THE DISRUPTION OF TESTICULAR DEVELOPMENT IN THE FETUS?

The testes begin to carry out their two major functions (gametogenesis and steroidogenesis) during fetal development. The Sertoli cells are the first cells to differentiate. They surround the germ cells, also known as gonocytes, to form the seminiferous tubules 12 days post-conception (dpc) in mice, 13.5 dpc in rats, and 42 to 45 dpc in humans. The Sertoli cells continue to actively multiply until puberty, but no further Sertoli cell proliferation is observed during the rest of a man's life. The germ cells are formed in the epiblast and migrate across the extra-embryonic and embryonic territories to the genital crests. The germ cells actively proliferate during this migration and after reaching the gonad. They then differentiate into A spermatogonia (also known as spermatogonial stem cells) after birth. The fetal Leydig cells differentiate shortly after the

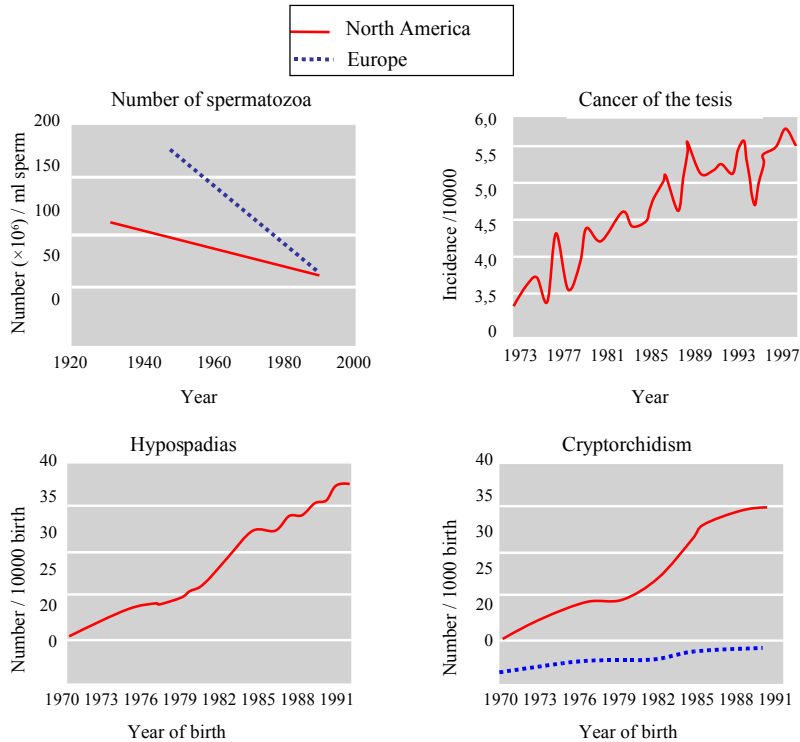


FIGURE 1. The evolution of the number of spermatozoa and of the abnormalities in the male reproductive system in recent years. (Toppari *et al.*, 2001; Sharpe and Irvine, 2004.)

Sertoli cells and secrete two hormones, essential for the masculinization of the fetus: testosterone and insulin-like factor 3 (Insl3).

It is currently believed that TDS is probably caused by changes in the development of the fetal testis (see figure 2) for the following reasons:

— Hypospadias results from a defect in the production or activity of androgens during fetal development.

— Cryptorchidism results from abnormalities in the production or activity of Insl3 or the androgens regulating the transabdominal and transinguinal descent, respectively, of the testes.

— Although the cellular origin of testicular cancer has not been demonstrated, there is evidence to suggest that the tumor cells originate from gonocytes that fail to differentiate nor-

mally into spermatogonia. Indeed, tumor cells in the early stages of this cancer (carcinoma *in situ*, CIS) have the same morphological characteristics and express the same immunohistochemical markers as gonocytes (e.g., alkaline phosphatase, c-kit, etc.). Furthermore, CIS has been reported in male infants, only a few months old, consistent with the idea of a fetal origin for the cancerous cells.

— Finally, decreases in sperm production in male adults may result from a wide variety of dysfunctions. Little is known about the control of spermatogenesis, but this control seems to involve extraordinarily complex intratesticular and intragerminal endocrine regulation. We know that the stock of gonocytes will go on to become spermatogonial stem cells in the adult forms during fetal testicular development. Experimentally decreasing the number

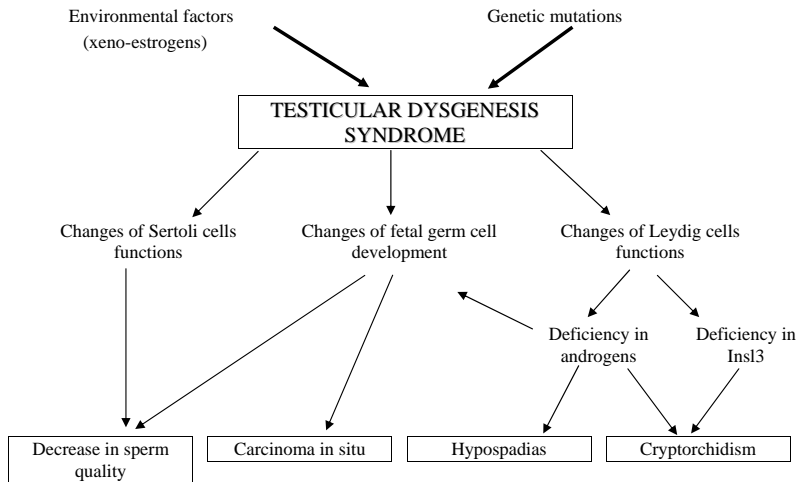


FIGURE 2. A schematic representation of the fetal origin of alterations in male reproductive functions. (Skakkebaek *et al.*, 2001.)

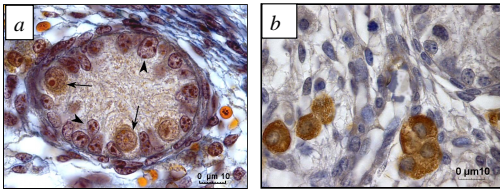


FIGURE 3. A histological aspect of the mouse neonatal testis observed 2 days after birth. *a*) A seminiferous cord after coloration using Tushmann's blue. Arrows indicate the gonocytes, which are identified by their large, spherical, lightly-stained nuclei, containing fine chromatin granules and two or more globular nucleoli and by a clearly visible cytoplasmic membrane. Arrowheads indicate the Sertoli cells, characterized by their irregular and densely-stained nucleus, located at the basal position in the seminiferous cord and by no clear cytoplasmic membrane. *b*) Leydig cells, located in the interstitial compartment, stained by immunohistochemical labeling of 3β -hydroxysteroid dehydrogenase (3β HSD), are often grouped in clusters at this developmental stage.

of gonocytes during fetal development leads to a decrease in sperm production in the adult. Similar decreases in adult sperm production result from the experimental reduction of the number of Sertoli cells during the perinatal period. Thus, adult sperm production depends partly on fetal gametogenesis and on

the capacity of gonocytes to differentiate into spermatogonial stem cells.

THE DELETERIOUS EFFECTS OF XENOESTROGENS

Sharpe and Skakkebaek suggested in 1993 that the reported problems in the domain of the male reproductive function could be due to the deleterious effects on the developing fetus of chemical pollutants, which have steadily increased in diversity and concentration in the environment. Many observations and experiments, over the course of the last twenty years, are consistent with this hypothesis. The principal chemical pollutants implicated in these effects are endocrine disrupters with estrogenic (xenoestrogens) or anti-androgenic activity. These compounds include pesticides, plastifying agents, surfactants and phytoestrogens, present in food and, to some extent, the atmosphere.

Studies of wildlife have provided evidence for the involvement of endocrine disrupters in the deterioration of the male reproductive function. A number of publications have re-

ported the effects of exposure to high concentrations of chemical pollutants with estrogenic properties on animals in their natural environment. The observed effects vary from subtle changes to permanent changes in the reproductive function, such as feminization or changes in sexual behavior.

A key, clinical argument concerns the abnormalities observed in the sons of women treated with diethylstilbestrol (DES), a strong estrogen agonist, during pregnancy in the 1950s, 60s and 70s. Some studies have shown that those men have a high incidence of genital malformations, cryptorchidism and testicular cancers, together with changes in sperm quality, whereas others have reported no change in fertility. These discrepancies in the results may depend on the phase of the pregnancy in which the treatment was given, suggesting that there could be particular periods of time, when the developing testis is more sensitive to xenoestrogens.

Another clinical argument concerns the recent demonstration of the negative effects of phthalates (plastifiers found in cosmetics, almost all PVC products, and many paints) on the development of the male reproductive apparatus. There is a correlation between the level of exposure (estimated by measuring the phthalates concentration in the urine of pregnant women) and the lower levels of masculinization in young children (a decrease in the distance between the anus and the base of the penis and an increase in the percentage of boys showing incomplete descent of the testes).

There is also a considerable body of experimental evidence. First, estrogen receptors ($ER\alpha$ and $ER\beta$) are present in the testis from the earliest stages of differentiation. $ER\alpha$ has been detected in the undifferentiated gonads in mice from 10 dpc onwards. This receptor is present in Leydig cells in rodent testes but is not present in human testes. $ER\beta$ has been detected in the testes of mice, from 14 dpc onwards. It is found primarily in the gono-

cytes, but also in the Leydig cells in mice and in the Sertoli cells of all of the species studied. Many studies have shown that, in rodents, males exposed to exogenous estrogens (e.g., DES, ethynylestradiol, bisphenol A), in utero or during neonatal development, develop hypospadias, display impaired testicular descent, or show a decrease in sperm production. Similarly, exposure to high doses of estrogens in utero has been shown to lead to testicular cancer in mice, rats, hamsters and dogs in adulthood.

ENDOGENOUS ESTROGENS AND FETAL/NEONATAL TESTIS DEVELOPMENT

Although exogenous estrogens were suspected of having a deleterious effect on testicular development, in the recent past little was known about the effects of endogenous estrogens.

Studies on male mice with invalidated receptors for estrogens ($ER\alpha$ KO or $ER\beta$ KO) or aromatase (ArKO), an analysis of patients lacking aromatase, and another study on an individual with an $ER\alpha$ mutation have shown that the absence of estrogens or of certain estrogen signaling pathways may also impair the reproductive capacity. Adult $ER\alpha$ KO and ArKO males are sterile, due to deficiencies in fluid reabsorption in the epididymis in $ER\alpha$ KO mice and abnormal spermatogenesis in ArKO mice. It has, therefore, been clearly shown that estrogens are required for male reproductive functions in the adult.

However, none of these studies provided information about the role of estrogens on fetal development. We recently studied the fetal and neonatal development of the testis (see figure 3) in $ER\alpha$ KO and $ER\beta$ KO mice. We showed that the invalidation of $ER\beta$ led to a 50% increase in the number of gonocytes two days post-partum (dpp) (see figure 4) and which was still observed at 6 dpp. This re-

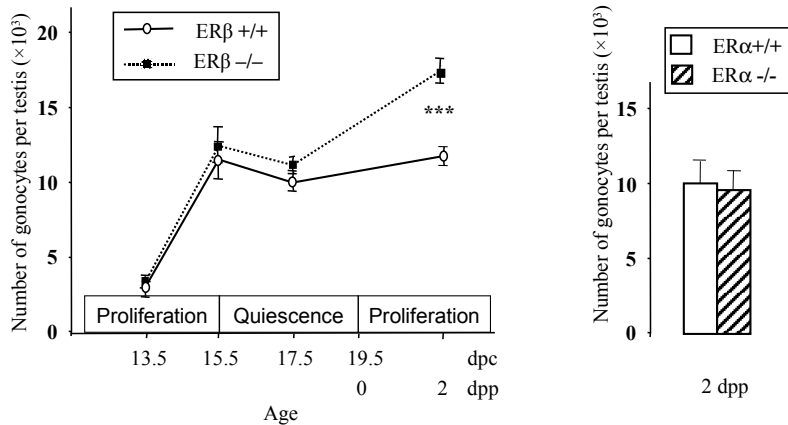


FIGURE 4. The evolution of the number of gonocytes in the mouse testis during fetal and neonatal stages after ER β or ER gene deactivation. Proliferation and quiescent periods of the gonocytes, throughout the considered developmental period, are indicated. Gonocytes on histological testis sections from 13.5 dpc, 15.5 dpc, 17.5 dpc and 2 dpp animals were counted. Values are means \pm SEM of 4 to 13 animals. ***: $P < 0.001$ versus wild-type in t test's Student. (Delbes *et al.*, 2004.)

sulted from an increase in the mitotic activity of these cells and a decrease in their apoptotic activity, with no change in the number of Sertoli cells. Conversely, the invalidation of ER α did not affect the number of germ or Sertoli cells, but it did increase testosterone production (see figure 5). This increase in testosterone production was observed from 13 dpc onwards. Thus, it began less than two days after the first Leydig cells differentiated and resulted from direct estrogenic action on the testis. There were no changes in the luteinizing hormone (LH) secretion, whereas changes in LH concentration seemed to play a key role in the case of the ER α KO adults. The activity of each fetal Leydig cell increased, as shown by the hypertrophy of these cells and the increase in the mRNA levels for three proteins involved in testosterone synthesis (StAR, P450scc and P450c17). In contrast, the invalidation of ER β had no effect on fetal testicular steroidogenesis (see figure 5).

We have thus shown that, in contrast to what is observed in adults, endogenous estrogens have a negative effect on the functions and development of the testis during

fetal and neonatal life. Each of the estrogen receptors plays a distinct role in testicular development, with ER β being involved in the regulation of germ cell development and ER α being involved in the regulation of Leydig cell development and function.

Over the last few years, we have developed an organotypic culture system for rat fetal and neonatal testes, which can reproduce the development *in vitro* of the various types of testis cells observed *in vivo*. Using this model in a collaborative study, we have shown that estrogens have a direct, negative effect on the development of the three principal cell types. Studies on the ontogenesis of estrogen effects *in vitro* during fetal and neonatal development are currently under investigation in our lab, and the first results have demonstrated that there are periods in which the testes are particularly sensitive to estrogens.

UNANSWERED QUESTIONS

The available experimental data suggest that the irreversible deleterious effects of es-

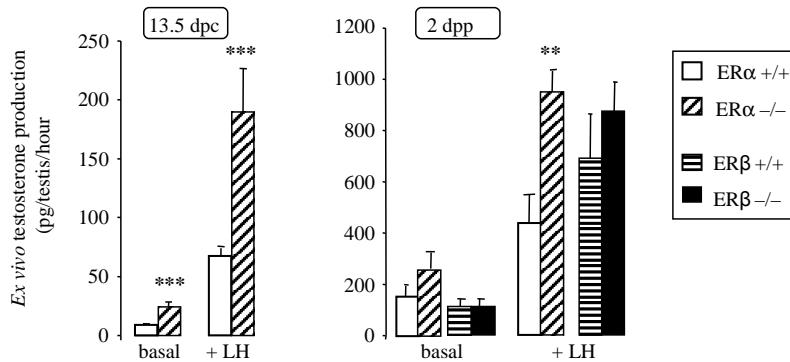


FIGURE 5. The production of testosterone by the fetal and neonatal mouse testis after $ER\beta$ or $ER\alpha$ gene deactivation. Testes were collected on day 13.5 (13.5 dpc) of gestation and on post-natal day 2 (2 dpp) and were cultured on floating Millipore filters for 3 h in the presence (+LH) or the absence (basal) of 100 ng/ml ovine LH. The testosterone secreted in the medium was measured by RIA. Values are means \pm SEM of 7 to 19 fetuses. **: $p < 0.01$ and ***: $p < 0.001$ versus wild-type in t test's Student. (Delbes *et al.*, 2005.)

trogens on testicular development and the masculinization of the genital tract occur during fetal and neonatal development. We have shown that endogenous estrogens regulate the physiological development of the testis during fetal and neonatal life, by controlling the two principal functions of the fetal and neonatal testis: gametogenesis and steroidogenesis. We have seen that dysfunctions in the establishment of the germ cell lineage are likely to induce testicular cancers and to lead to changes in sperm production. Estrogens also control the secretion of testosterone and, therefore, play an important role in the masculinization of the genital tract and the descent of the testicles.

However, the link between the effects observed at birth and their consequences for adult fertility remains unclear, particularly as many of the mechanisms regulating spermatogenesis do not become operational until puberty. Thus, deficiencies in estrogen pathways have positive effects on the fetus and neonate, but they have negative effects on adults. It would be advisable to develop mice strains with conditional gene knockouts, functioning only during fetal development; this would be helpful in demonstrating that the

abnormalities induced by the absence of estrogens or estrogens signaling, restricted to fetal and neonatal life, have consequences on the adult.

There seem to be periods during fetal and neonatal development, when the testes are particularly sensitive to estrogens, and this estrogen sensitivity depends on the species and the strain considered. It is, therefore, particularly important to study the sensitivity of the human fetal testes, as, unlike rodent testes, human testes do not contain $ER\alpha$ but do contain multiple variants of $ER\beta$.

Finally, all of the available experimental data demonstrate the deleterious effects of high doses of xenoestrogens, and some show that exposure to low doses has no irreversible deleterious effects. It, therefore, remains unclear whether increases in the xenoestrogen levels in the environment have a direct effect on the human male reproductive function, and some studies have suggested that the concentrations of endocrine disruptors reached in the body are too low for xenohormonal effects to be observed. However, the lipophilic nature of these compounds may lead to their accumulation, and their effects may be amplified by the combined consequences of diverse

xenoestrogens. The growing body of epidemiological and experimental evidence, linking the actions of estrogens with the male reproductive function, may justify medical and environmental preventive measures.

Lastly, a very recent study has 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. Thus, changes in fetal testis development are observed, not only in the contaminated individual during adulthood, but they are also transmitted to subsequent generations through an as yet undetermined mechanism.

FOOTNOTE BY RENÉ HABERT

This paper is dedicated to my mentor, José Maria Sáez, who became a very close friend. The members of my laboratory, who also collaborated with him and wanted to honor his memory, have signed their names to this work. The choice of a topic for this review, in relation to the work of José M. Sáez, was an

easy task, so intense were his scientific ideas and production. This paper, which deals with the actions of estrogens on the fetal testis, is in direct connection with the fact that José M. Sáez assumed very early on that estrogens played an important and direct role in testicular regulation. As early as 1978, he wrote, "it is apparent from the present study that oestradiol, at the doses used, produces a lowering effect on testosterone levels via a direct action at the testicular level, rather than an indirect action on the hypothalamic-pituitary axis"; *Acta endocrinologica*, 89: 379-392.

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