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Natural and anthropogenic environmental oestrogens: the scientific basis for risk assessment*

Environmental oestrogens and male infertility

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INTRODUCTION

The topics of adverse changes in human male reproductive health (fall in sperm counts, increase in the incidence of testicular cancer, cryptorchidism and hypospadias) and that of 'environmental oestrogens', have attracted enormous scientific, public and governmental attention over the past 6 or so years. Though the two topics have invariably been linked together in a postulated cause and effect manner, there is only circumstantial evidence to support such a relationship (35, 76). Whilst such 'absence of data' should not be interpreted as proof that such a causal relationship does not exist (see Table 1), it should also make us circumspect in dealing with this issue and cause us to use scientific rather than emotional criteria when judging or 'weighing' the relevant data. From these introductory remarks it should be clear already to the reader that I will be unable to offer any proof that environmental oestrogens either do or do not affect human male fertility. Whilst this may be less than ideal, it should not cause us to lose sight of three important facts. First, adverse trends in male reproductive health have occurred over the past 50 years, although the extent of this change and whether it is truly world-wide are more debatable (75). Second, a biologically plausible case can be made for environmental oestrogens inducing adverse changes in male reproductive health (Table 1; ref. 69), although plausibility does not tell us anything about likelihood (65). Third, there is no doubt that human exposure to environmental oestrogens has altered considerably in the past 50 years, although we do not have accurate measures of how great this change has been nor what relative impact it has had on 'total oestrogen exposure' of the individual (45, 76). This chapter will attempt to summarise our current understanding on each of these points and in doing so will hopefully highlight the strengths and weaknesses of the arguments which relate these three topics one to another.

ADVERSE TRENDS IN MALE REPRODUCTIVE HEALTH RELATING TO FERTILITY

As is outlined below, there are no simple straightforward means of assessing fertility in the male based on measurement of sperm numbers, motility, morphology etc. Insofar as we understand the causes of infertility in men, one thing is clear—there are multiple causes and the infertile male population are therefore a heterogeneous group. This means that assumptions about commonality of aetiology in individuals who present with the same semen analysis (e.g. oligozoospermia) cannot be made. Therefore, in considering male infertility in relation to exposure to environmental oestrogens, it is appropriate to take a very broad view rather than to simply focus on one aspect such as sperm counts, important though this may be. For this reason, I have chosen to consider male reproductive health and development in general.

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Table 1. Factors which provide a plausible theoretical basis for considering that abnormal oestrogen exposure could affect human male reproductive development and/or function

Factor	Site/mode/mechanism of action	Comments
Widespread distribution of oestrogen receptors (α and/or β) throughout the reproductive system of the male	Brain (sexual/other behaviors) Pituitary gland (FSH/LH secretion) Sertoli cells, certain germ cells, Leydig cells, efferent ducts, epididymis, vas deferens, prostate, seminal vesicles	Oestrogen receptors also expressed in most non- reproductive tissues Function of oestrogens at most of these sites is unknown
Oestrogen receptor-α knockout (ERKO) male mice are infertile	Infertility associated with abnormalities of fluid resorption from efferent ducts Possibly also abnormalities in sperm development and/or maturation	Unclear whether or not this data is relevant to man
Administration of oestrogens to adult males can reduce sperm counts and induce infertility	Oestrogens reduce testosterone secretion by direct effects on Leydig cells or indirectly by suppression of LH secretion from the pituitary gland Possible that oestrogens could directly affect spermatogenesis via effects on Sertoli or germ cells	These effects have only been documented after fairly prolonged exposure to potent oestrogens such as oestradiol or ethinyl oestradiol
Exposure of males to increased oestrogen levels perinatally can cause permanent disorders of the male reproductive tract and/ or reduce testis size and sperm production in adulthood	Mechanisms of action largely unknown but presumably occur via one or more of the sites listed above	High levels of exposure to potent oestrogens probably required to induce major adverse changes
	Suppression of FSH levels perinatally can reduce Sertoli cell numbers and hence reduce testis size and sperm production in adulthood	Human males exposed to DES *in utero* showed this range of abnormalities but these did not appear to affect their fertility

Decline in sperm counts

There had been numerous reports in the literature up to 1992 hinting that sperm counts in men might have fallen (reviewed in ref. 31) and these were finally crystallized in more definitive form in the paper by Carlsen *et al.* (15). Though this study, based on meta-analysis, was disputed with vigour and various reinterpretations offered (31), the most thorough re-analysis, which went back to the original data sources, reached the same and perhaps a slightly stronger conclusion, namely that sperm counts appeared to have fallen by approximately 1–3% per year over the past 30–50 years in both North America and Europe (73). Although a number of individual studies have also been published in the intervening period, some of which provide support for the fall in sperm counts (9, 20, 32, 77), others which do not (23, 53, 78), all of these retrospective studies are flawed in one or more ways and can therefore not be viewed as being definitive. Nevertheless, the study with the least flaws (9), in that over the years it used consistent recruiting methods, consistent semen analysis methods and was able to allow for confounding factors such as age at time of semen donation and period of abstinence, demonstrated in a large group of fertile French men that sperm counts had declined by an average of 1.6% for each later year of birth. This birth cohort effect has been confirmed in other studies (20, 32) and is of particular interest because (a) it implicates the perinatal period in the observed change, and (b) it ties in with the data for testicular cancer and for cryptorchidism and hypospadias (see below). Nevertheless, the issue of whether sperm counts have fallen and if so whether or not this is a general or region-specific phenomenon is likely to remain unresolved until properly designed, prospective studies are undertaken in which standardised semen analysis methodology is deployed. One such study has recently been completed (and is now at the analysis stage) in Europe involving centres in Denmark, Scotland, France and Finland and this will shed light on differences in sperm counts in recently fertile men in these different centres. Several other countries, including the USA and Japan as well as other European countries have recently aligned themselves with the European study and will use similar subject selection and semen analysis in

prospective studies. Over the next few years, important information on sperm counts in similarly recruited men in a range of countries should therefore become available and will undoubtedly be of considerable value. However, this data is unlikely to shed much light on the issue of the fall in sperm counts except for possibly providing some insight into the relationship between year of birth and sperm counts (though subject recruitment is limited to a relatively narrow age range).

Although it is scientifically correct to suspend judgement on whether sperm counts have fallen or not, the technical difficulties and time-issues involved in resolving this issue should not blind us to the clinical consequences and implications if sperm counts have fallen. One way of addressing this issue in terms of the possible birth cohort effect (see also below) is to undertake studies in young adult men (18–20 years of age) to seek reassurance that their sperm counts are not unusually low, as might be predicted if sperm counts were continuing to fall in relation to each later year of birth. Such studies are underway in two European countries based on military conscripts and results should be available in 1999. If sperm counts in these young men are found to be comparable to those found in older, fertile men (measured in the same Centres using the same methodology) then this can probably be viewed as evidence that sperm counts have not fallen in recent years, though it will still leave the argument open as to whether or not sperm counts were historically much higher. On the other hand, if sperm counts are found to be very low in the young men (taking account of abstinence etc.) then it will be difficult to escape from the conclusion that not only is the fall in sperm counts correct, but the decrease is ongoing. If this should prove to be the case it would have to be viewed with the utmost seriousness.

Increase in testicular cancer

Testicular germ cell cancers have increased progressively in incidence in Caucasian males throughout the last 20–50 years. This is a world-wide phenomenon and even though there are quite marked differences in incidence between different countries (2, 75), the types of cancers which occur and the age range of men who are affected is remarkably constant. In many countries, these cancers are now the commonest cancer in young men. Though increase in incidence of many cancers and other diseases is often attributable to improved diagnosis/reporting and/or to the increased longevity of the general population, it is clear that this is not the case for testicular cancer in which >95% of cases occur in males aged 15–45 years and diagnosis/reporting has always been extremely accurate (75).

Irrespective of whether a country has a high or a low incidence of testicular cancer the risk of a male developing the disease increases progressively with later year of birth i.e. it is a birth cohort effect (11, 86). Indeed, for a country such as Finland with a low incidence of testicular cancer, the risk of developing testicular cancer has increased by ≈11-fold for males born in the mid-1960's compared with those born in the early 1900's (11). As these cancers appear to develop from aberrant gonocytes which have persisted from foetal life (71), the change in incidence presumably implicates altered intrauterine exposure of the foetal testis to unknown factors. As disorders of hormone action, especially of androgen action but including increased exposure of the mother to oestrogens (62, 75), are known to be important risk factors for testicular cancer, the idea that environmental oestrogens might be implicated in this disease is a logical postulation. Of particular relevance to the associated change in sperm counts is the fact that poor semen quality is invariably found in individuals with testicular cancer (54). This is not due solely to the presence of the cancer as (a) when the contralateral testis is unaffected by cancer it frequently has poor spermatogenesis, and (b) similar changes in semen quality are not observed in men with non-testicular cancers at the time of diagnosis (54). A very recent study from the CECOS centre in Paris further ties the sperm count and testicular cancer data together. They showed that in 1356 men diagnosed as having testicular cancer, and who provided a semen specimen for frozen storage prior to commencing chemotherapy, sperm counts had fallen by 6.2% per year for each later year of birth (J Auger & P Jouannet, personal communication). It is difficult to think of a logical reason why this change should have occurred unless these men had been exposed **increasingly** to some factor responsible for causing both their cancer and for reducing their sperm counts. More than any other single piece of information, this finding implicates the same (or a related) cause for the increase in incidence of testicular cancer and a decline in sperm counts.

Incidence of cryptorchidism and hypospadias

In addition to testicular cancer there is evidence that the incidence of testicular maldescent (cryptorchidism) and hypospadias have increased in Western countries, though this evidence is nowhere near as certain as is the altered incidence of testicular cancer, primarily because of inconsistencies in diagnosis, ascertainment etc. (75). Several studies have reported an increase in incidence of cryptorchidism (75), though one large study of an ethnically mixed population from the USA reported no change (12). Results of carefully controlled, prospective studies, which are ongoing in several European countries, should help to resolve this uncertainty. The data for hypospadias is beginning to look a little more convincing as two recent studies in North America reported approximately a doubling in incidence from the 1970s to the 1980's (52), and most earlier data (of variable quality) reported similar trends in several countries (75). The relevance of these findings to the issue of human exposure to environmental oestrogens and fertility stems from (a) the increased incidence of both cryptorchidism and hypospadias in human males exposed in utero to DES (72, 75), (b) the fact that both of these are risk factors for the development of testicular cancer (46, 55), and (c) cryptorchidism is associated with poorer semen quality and higher risk of infertility in adulthood (30).

As a prelude to consideration of the pathways via which environmental oestrogens could impact on male reproductive function and fertility, it is first necessary to outline some important background information which summarises the important ingredients which together determine male fertility and which are susceptible to modulation by hormones such as oestrogens.

THE BASIS FOR MALE FERTILITY; SPERM COUNTS

Many factors contribute to the fertilising ability of an individual spermatozoon. These include factors such as an intact and fluid plasma membrane expressing a range of anchored proteins, the presence of an intact acrosome, full condensation of the DNA within the sperm head, the presence of a normal tail and its associated energy-generating mitochondria, the elimination of virtually all cytoplasm and the inherent ability of the spermatozoon to undergo capacitation and hyperactivation in the female reproductive tract as a prelude to binding to and fertilising the oocyte. The development and regulation of these multiple structural and functional properties are incompletely understood, though it is well established that the mechanisms which underlie these processes are laid down during the process of spermatogenesis in the testis and/or during sperm modification/maturation in the epididymis (57).

Fertility of the male does not depend just on the functional competence of individual spermatozoa, it also depends on the production of huge numbers of spermatozoa. A normal adult human male manufactures around 100–200 million new spermatozoa every day, each of which will have taken around 10 weeks to make (66). This high level of production ensures that the ejaculate contains on average 40–100 million spermatozoa/mL. Though it takes just one spermatozoon to fertilise the oocyte, only the production and ejaculation of astronomical numbers of spermatozoa will ensure a good chance of successful fertilisation. This is because the journey that the spermatozoa must make within the female reproductive tract is long (equivalent to 40 miles for a human) and arduous (the sperm are foreign invading cells which are subject to immune attack) and only tens or hundreds of cells are likely to make it to the site of fertilisation. To some extent, fertility is therefore dependent on sperm numbers in the ejaculate. However, the available scientific evidence (which is limited) suggests that the relationship between sperm number in the ejaculate and fertility is not linear. Instead, there appears to be a threshold concentration of spermatozoa below which qualitative (time to pregnancy) and quantitative (clinical pregnancy rate) fertility rates are related to sperm count whereas concentrations in excess of this threshold level do not appear to make any significant impact on fertility. Traditionally, this threshold has been set at $10–20 \times 10^6$ sperm/mL (83), but a recent prospective study (14) involving couples who had not previously put their fertility to the test suggests that sperm counts $<40 \times 10^6$ /mL are associated with qualitative changes in fertility i.e. longer time to achieve a pregnancy. Needless to say, these general principles only hold true for individual couples who have normal fertility and reproductive function. In instances where the male partner has one or more abnormalities in sperm quality (i.e. in sperm morphology, motility or functional ability to fertilise), these may result in a qualitative lowering of

fertility and in some instances in complete infertility, despite the presence of normal numbers of spermatozoa in the ejaculate. Based on these considerations, it is not surprising that the relationship between semen parameters and male fertility is anything but straightforward.

Infertility is an extremely common disorder in humans and is generally reckoned to affect about 1 in 6 couples at some stage of their reproductive life (49). Though the aetiology of infertility in couples is frequently idiopathic, there is general acceptance that male factors play a role in 50–60% of infertile couples (49). In affected men the cause of infertility can also be idiopathic and even when a disorder of sperm number, motility or morphology (or combinations of these factors) is clearly diagnosed, the aetiology of the observed changes is itself frequently unknown. It is therefore important to recognise that whilst male infertility/subfertility is extremely common, its causes and the mechanisms involved are, in general, poorly defined. This ignorance imposes important limitations when considering whether or not sperm counts in the human male have declined over recent decades and whether or not exposure to environmental oestrogens could have contributed to such a decline. A serious additional complication is that if exposures of the male in perinatal life are an important cause of lower sperm counts in men, then the >20 year time-lag between exposure and appearance/diagnosis of the effect greatly limits the chances of deducing the cause. Nevertheless, what is beyond dispute is that hormones, such as the gonadotrophins FSH and LH, androgens and even oestrogens, play a critical role in normal male reproductive function, both during development and throughout adulthood. Disorders of hormone production and/or action can clearly result in abnormalities of reproductive development (often permanent) and can affect fertility.

THE BASIS FOR MALE FERTILITY; STEROID NEGATIVE FEEDBACK LOOPS IN THE ADULT MALE

In the adult male the most important hormone, in terms of the maintenance of fertility, is testosterone (66). This hormone drives the process of sperm production via effects on the Sertoli cell, but also acts throughout the remainder of the reproductive tract (excurrent ducts, epididymis, vas deferens, prostate, seminal vesicles, genitalia) as well as throughout the body and the brain. The importance of testosterone in controlling sperm production and fertility is exemplified by the considerable database on normal men in whom intratesticular levels of testosterone have been lowered (by peripheral administration of steroids, including testosterone) as part of a programme to develop new male contraceptive methods (10, 84). Such individuals rapidly become azoospermic or severely oligozoospermic, but subsequently recover their fertility when treatment ceases. Infertility in these men results from inhibiting sperm production, and all other aspects of their reproductive function remain normal. The underlying principle that operates in these studies is the use of the negative feedback loop of testosterone from the testes to inhibit pituitary LH secretion; suppression of LH by peripherally administered steroids (a progestagen is frequently used in combination with an androgen) results in decreased drive to the Leydig cells within the testes and consequently to a lowering of intratesticular levels of testosterone. The levels of the latter within the testis need to be some 200-fold higher than the levels in peripheral blood to maintain normal sperm production, so any drastic lowering of testosterone production results in failure of sperm production (66). It is also recognised that partial suppression of intratesticular testosterone levels will lead to incomplete suppression of sperm production, though the spermatozoa produced under such suboptimal conditions may have an increased incidence of structural/functional abnormalities (66).

Environmental oestrogens and steroid negative feedback

Exposure of the male to any exogenous androgen or oestrogen has the potential to cause similar changes to those described above, provided that the level of exposure is sufficient. Therefore, increased exposure of the **adult** human male to environmental oestrogens could theoretically affect both the number and/or quality of sperm produced via such a mechanism. Such effects would be reversible as prolongation of any effects would require continued exposure to the causal agents. There is no direct evidence to indicate that exposure of human males in the general population to environmental oestrogens is sufficient to cause such changes, though it must also be admitted that no such studies have been undertaken and they would be practically difficult to perform. However, we know that in principle such effects are possible at least

as far as exposure to phyto-oestrogens is concerned. No such studies have yet been reported in normal adult men (though studies are in progress) but there has been a well-controlled study in Caucasian women with normal menstrual cycles who were fed phyto-oestrogen-containing soy products at a level comparable to that in the Japanese diet. This resulted in prolongation of the follicular phase of the menstrual cycle (compared with their own control cycle) as a consequence of lowering of FSH levels, presumably due to activation of the steroid negative feedback loop (16). There is every reason to expect that men exposed to a similar diet would exhibit a similar reduction in their FSH levels, though whether or not this would exert any effect on sperm production and sperm counts is far less certain in view of the current uncertainty as to how important a role FSH plays in the process of spermatogenesis in the adult male (66). However, suppression of FSH levels in the perinatal period via similar mechanisms could undoubtedly affect sperm production in adulthood as is discussed below.

Environmental anti-androgens

In the last 3 years it has become apparent that a number of environmental oestrogenic chemicals to which humans are exposed can exert anti-androgenic effects. Such chemicals include DDE (36), the main metabolite of DDT which accumulates in fat, dibutyl phthalate (47, 82), the fungicide vinclozolin (36) and dioxins (13, 56). Although the anti-androgenicity of some of these compounds is a straightforward case of the compound binding to and blocking the androgen receptor i.e. the compounds are androgen receptor antagonists (36), this may not account for the pronounced anti-androgenic effects of dibutyl phthalate or that of dioxin. Because of the central role of testosterone in regulating sperm production, exposure to such chemicals could theoretically reduce sperm counts in exposed men. In reality, this possibility may not be particularly relevant as administration of clinical anti-androgens to adult animals or man generally has little effect on sperm production because of the high testosterone levels within the testis and because of activation of increased LH secretion due to interference of the anti-androgen with steroid negative feedback on the hypothalamic-pituitary axis.

Steroid negative feedback loop in perinatal life

The steroid negative feedback loop from the testes to the hypothalamus and pituitary gland also operates in the foetus and neonate when the testis is developing. At this time in life, the process of sperm production is inactive and it is the development of Sertoli cells and gonocytes (the precursor germ cells) which is occurring as well as masculinization of the body via the secretion of testosterone by the foetal generation of Leydig cells. Exposure to exogenous steroids at this stage of life can have dramatic, and usually permanent, effects on the testis and reproductive system (4). It is uncertain to what extent these effects are due to activation of steroid negative feedback loops or to what extent they reflect more direct effects of the administered steroids (see below). With regard to the former there are two important considerations. First, suppression of pituitary FSH secretion leading to a reduced rate of Sertoli cell replication and thus to reduced capacity of the testis to produce spermatozoa in adulthood. Second, suppression of pituitary LH secretion leading to suppression of testosterone production by the foetal Leydig cells and thus to disorders of masculinization and/or gonocyte development. Whilst these are both largely theoretical possibilities as far as the human male is concerned, there is strong supporting data from experimental animals as well as data from humans which is less direct (65, 75). Finally, our emerging understanding of the sites and actions of oestrogens in the male reproductive system raises a third possibility, namely that oestrogens can directly act on various cell types in the testis and reproductive tract to induce changes which may adversely impact on male fertility (67). Each of these three areas is considered separately below.

SERTOLI CELL REPLICATION, FSH AND SPERM COUNTS; INHIBITION BY OESTROGENS

In all adult mammals which have been studied the number of Sertoli cells in the testes are crucial in determining both testicular size and daily sperm production (DSP). These relationships stem from the fundamental role of the Sertoli cell in supporting the germ cells during their development into

spermatozoa. Each Sertoli cell can only support a fixed number of germ cells (and this number varies from species to species) but as it is the germ cells which make up the bulk of the adult testis it is obvious why Sertoli cell number indirectly determines adult testis size (66). Similarly, the greater the number of germ cells per testis the greater will be the number of spermatozoa produced per day. In species such as man which do not store large numbers of spermatozoa, daily sperm production (which equates to Sertoli cell number; ref. 33) by the testes will therefore relate approximately to sperm count in the ejaculate if ejaculatory frequency is relatively constant. However, because of the lack of sperm storage in man, ejaculatory frequency (or period of abstinence) can exert an important effect on sperm counts.

The number of Sertoli cells formed is critical in determining the sperm count of an individual and factors which can alter Sertoli cell number can **permanently** alter DSP and thus the sperm count. The permanence of this effect stems from the fact that Sertoli cells only replicate when they are immature/undifferentiated and, in most species, once differentiation occurs the capacity to divide is permanently lost (66). In most species, including man, Sertoli cells multiply in foetal and neonatal life though in man there may also be further replication in early puberty (66). In some species of primates, such as the Rhesus monkey which has been used widely as a model for man, little or no Sertoli cell replication occurs neonatally and virtually all multiplication occurs during early puberty (42). The reason for this difference is unknown but may relate in some way to the delay of testicular descent in this species until puberty, in contrast to man in whom the testes are descended at birth. Recent unpublished studies of our own in Marmoset monkeys, a species which descends its testes at birth, as in man, shows that Sertoli cell proliferation in these animals occurs predominantly in the neonatal period.

Oestrogens and Sertoli cell numbers

Various hormones can influence both the rate and duration of Sertoli cell multiplication (66) and probably the most important is FSH which increases the rate at which the Sertoli cells divide (50). Suppression of FSH levels during the period of Sertoli cell replication leads, in the rat, to a permanent reduction in Sertoli cell numbers (66, 68) and similar changes can be inferred for human males with deficient FSH secretion (hypogonadotropic hypogonadism) (see ref. 66). Oestrogens are recognised as being powerful suppressors of FSH secretion in males, especially in the neonatal period (4, 68), and administration of high doses of the potent oestrogen diethylstilbestrol (DES) to neonatal rats can permanently lower Sertoli cell numbers (68). However, administration of moderately high levels of oestradiol to rats failed to alter Sertoli cell number (19) which may mean that only very high levels of oestrogen exposure will reduce Sertoli cell multiplication or that different oestrogens exert different effects. Based on the increased prevalence of small testes and low sperm counts in human males who were exposed in utero to high levels of DES (72), it is reasonable to conclude that similar effects can occur in man, though it is possible that effects of the DES directly on the testis rather than because of altered FSH secretion might have been involved (see below). Therefore, it can be logically concluded that inappropriate exposure of the human male to oestrogens during perinatal life has the theoretical potential to reduce Sertoli cell numbers, and hence to reduce sperm production and sperm counts in the ejaculate. However, there must be doubts as to whether human male infants are ever exposed to sufficient exogenous oestrogens to cause such effects if the data for the rat can be extrapolated to man and if the effects of candidate environmental oestrogens can be viewed as being predictable based on their weak oestrogenic potency.

One situation in which humans are exposed to exogenous oestrogens at a level sufficient to suppress FSH secretion is in infants who are fed on a soy formula milk diet for the first 6–9 months of life. In Western countries, 10–20% of infants are now reared on such a diet and it is clear that the blood levels of active phyto-oestrogens in such infants is 10-fold higher (63) than that required to suppress FSH secretion in adult women (16). Unfortunately, no studies have yet been conducted to investigate (a) whether this exposure does in fact affect FSH (or other hormone) levels in these infants, or (b) whether sperm counts or any other aspect of reproductive function in adulthood is affected. In this regard it is worth mentioning that the available evidence shows that men from Eastern Asian countries have smaller testes on average (5, 74, 80) than do comparably aged Caucasian men (e.g. 27). The reason for this difference is unknown. It may well reflect a genetic difference but it is at least theoretically possible that

it could be a consequence of higher exposure to phyto-oestrogens in the native diets in this region; note though that exposure of Oriental neonates to phyto-oestrogens from soy products (directly or via their mother's milk) is likely to be 2–3 orders of magnitude lower than that measured for Western infants consuming soy formula milk (63).

As well as ethnic differences in testis size, which are presumed to reflect underlying differences in Sertoli cell numbers, it is also well established that testicular size in Caucasian men is extremely variable (33). Analysis of Sertoli cell numbers and daily sperm production (DSP) in the testes of American men who had died suddenly revealed that Sertoli cell numbers varied over about a 100-fold range and showed a close correlation with DSP (33). The reasons for this enormous variation are unknown but once again emphasises the critical importance of Sertoli cell numbers in determining sperm output, and thus sperm counts.

LEYDIG CELL FUNCTION AND REPRODUCTIVE DEVELOPMENT; INHIBITION BY OESTROGENS

The process of masculinization of the sexually indifferent foetus requires the production of two hormones by the foetal testis once the latter organ has been induced to differentiate by expression of the testis-determining gene *Sry*. These two hormones are anti-Müllerian hormone (AMH), which is secreted by the Sertoli cells, and testosterone, which is secreted by the foetal Leydig cells. AMH causes regression of the Müllerian ducts which would otherwise give rise to the fallopian tubes, uterus and upper third of the vagina in the female. Testosterone ensures that the Wolffian ducts, which give rise to the efferent ducts, epididymis, vas deferens and seminal vesicles, are maintained. Furthermore, testosterone from the foetal Leydig cells is also secreted into the peripheral bloodstream and, following its conversion to the more potent androgen 5α-dihydrotesosterone (DHT) via the enzyme 5α-reductase, it acts on the external genitalia to induce its masculinization i.e. the formation of a scrotum and penis with the urethra opening at the tip of the penis. In addition to these changes, which occur in early to mid-pregnancy in the human male, testosterone and possibly AMH also play a role (together with other factors) in the descent of the testes from their point of origin alongside the foetal kidney to their final position in the scrotum, a process which extends into the third trimester of pregnancy. Inhibition of any of these various processes has the potential to interfere with the normal expression of male fertility, but the most important in the context of the present topic is the inhibition of testicular descent or cryptorchidism (30).

Maldescent of one or both testes is associated with poorer fertility in adulthood, especially if surgical correction of the disorder is delayed. Moreover, cryptorchidism is the most important established risk factor for the development of testicular cancer (46, 54, 75). Abnormalities in the site of the urethral opening on the penis (hypospadia), which reflects incomplete closure of the urethral folds during penis development, is of importance not because it drastically compromises fertility (though it can do so for mechanical or psychological reasons), but because of its frequent association with cryptorchidism, its apparently increasing incidence (52, 75), its clear reflection of inadequate androgen action and the observation that exposure of the human male foetus to DES in utero resulted in increased incidence of this disorder (72, 75). Similarly, DES exposure resulted in increased incidence of cryptorchidism (72, 75). In animal models, oestrogen exposure perinatally will induce similar changes, especially cryptorchidism, though as with the human case this followed exposure to relatively high levels of potent oestrogens such as DES or ethinyl oestradiol (4, 48). What is the mechanism of induction of these changes? It has generally been concluded that the pathway of oestrogen action is indirect and involves inhibition of testosterone production by the foetal Leydig cells. Though there is some supporting evidence from animal studies, which show inhibitory effects of DES administered to the mother on the expression and activity of one of the key steroidogenic enzymes in the testes of male foetuses (41), there are as yet no studies which have measured whether testosterone production has been inhibited, as would be predicted. In this regard there are two opposing arguments. On the one hand, it is established that foetal Leydig cells and their precursor cells are targets for oestrogens (24, 67) and data for the adult generation of Leydig cells clearly points to inhibition by oestrogens of both Leydig cell development (1) and steroidogenesis (17), which plausibly argues for a similar effect on the foetal Leydig cells. On the

other hand, normal foetal Leydig cells are extraordinarily active in terms of the amounts of testosterone which they synthesize, which might indicate that 'overproduction' of testosterone occurs normally and thus its partial inhibition would have little if any impact on development of the reproductive tract. At present, there is insufficient information to allow us to distinguish which, if either, of these interpretations is correct. An alternative explanation might be that oestrogen exposure of the foetus was able to inhibit androgen action, either by affecting the expression of androgen receptors or by inhibiting activity of 5α-reductase. These possibilities remain to be explored in detail, but there is reasonable data from animal studies which suggests that perinatal oestrogen exposure might permanently impair androgen levels or androgen action (4).

Androgen/oestrogen action and testicular cancer

Finally, it is well established that testicular germ cell cancers are found with very high incidence in individuals in whom androgen production or action is impaired in utero (62, 75). This would appear to indicate that a low androgen environment in the foetal testis is conducive to the abnormal arrest of development of gonocytes, as it is from these abnormal foetal germ cells that testicular cancers will arise in early adulthood. The mechanism for this effect is unknown as neither the gonocytes or the foetal Sertoli cells, with which the gonocytes are associated, express androgen receptors at this time of development. In contrast, it has been shown that both gonocytes and foetal Sertoli cells express oestrogen receptor-β (ERβ; see below and ref. 60), which means that androgens could act on gonocytes via conversion to an oestrogen. Based on a meta-analysis it appears that oestrogen administration to the mother does significantly increase the risk of subsequent testicular cancer in the male offspring (75), though the small increase in risk is unimpressive when considering the millions of women world-wide who were treated with DES during pregnancy (51).

Environmental anti-androgens and abnormal development of the male reproductive system

As has already been mentioned, some environmental chemicals (e.g. DDE, dibutyl phthalate, vinclozolin, dioxins) can also act as anti-androgens and, in view of the association of inhibition of androgen action with cryptorchidism, hypospadias and testicular cancer, the possibility is raised that anti-androgenic rather than oestrogenic chemicals could be involved in the aetiology of these disorders in man. There is no direct supporting evidence but studies in male rats exposed during pregnancy/lactation to dibutyl phthalate administered to their mothers showed an increased incidence of cryptorchidism and hypospadias as well as smaller testes etc. (47, 82). Although these abnormalities were only of high prevalence at rather high doses of dibutyl phthalate (>500 mg/kg/day), sporadic effects were still evident at 100-fold lower doses. There is also unpublished evidence from Earl Grey that other, even more ubiquitously used, phthalates may exert similar anti-androgenic effects in animals exposed developmentally. As human intake of phthalates is of the order of 10–30 μg/kg/day it would appear that more consideration may need to be given to the possible contribution of such effects to the reported changes in male reproductive health described above. Furthermore, human exposure to DDT/DDE was considerable 30–50 years ago in Western countries (36) whilst exposure to PCBs and dioxins, which can cause similar 'anti-androgenic' as well as 'oestrogenic-like effects (13, 56), has also been considerable. As other environmental anti-androgens such as vinclozolin are already known (36) and the likelihood that others will be identified, it may be that attention will switch from oestrogenic to anti-androgenic chemicals in the near future.

What is particularly remarkable is that exposure of developing males to either oestrogens or anti-androgens can result in more or less the same set of reproductive defects, as has been discussed above. This either means that both work via a common pathway (e.g.. interfering with androgen action) or that normal development of the male reproductive system requires a certain balance between androgen action and oestrogen action. There are several lines of evidence to support the latter possibility, not least being the fact that receptors for androgens and oestrogens are present throughout the reproductive system of the developing male together with local sources of aromatase to convert testosterone to oestradiol

(discussed in ref. 67). As is discussed below, this is now an area of active research, and important advances in our understanding are just beginning to appear.

DEVELOPMENT OF THE TESTIS AND REPRODUCTIVE TRACT; DIRECT INHIBITORY EFFECTS OF OESTROGENS

The original hypothesis suggesting a link between exposure to environmental oestrogens and reduced sperm counts centred on indirect suppression of FSH secretion leading to a reduction in Sertoli cell numbers as outlined above (64, 69). At the time, there was little in the way of evidence to implicate a more direct route of action of oestrogens. However, the discovery in 1996 of a second oestrogen receptor, ERβ (38), has dramatically changed this thinking. ERβ is far more widely distributed than is the 'original' oestrogen receptor, ERα, and this distribution includes Sertoli cells, gonocytes in the foetal testis and spermatogonia and spermatocytes in the pubertal and adult testis (22, 59, 60, 67). At present, the functions of oestrogens on these target cells are unknown, though there is recent indirect data of our own which suggests a role for oestrogens in maturational development of the Sertoli cells (68) whilst oestrogens have been shown to increase proliferation of gonocytes isolated from the foetal rat testis (89). The discovery of these new target cells for oestrogens means that there are potentially more direct pathways via which exogenous oestrogens could affect sperm production and/or the fertilizing ability of the spermatozoa. Although this possibility is entirely speculative, the fact that transgenic mice in which ERα is inactivated (ERKO mice) turn out to be infertile, because of poor fertilizing ability of their spermatozoa (21), suggests that oestrogen action on spermatogenesis and/or sperm maturation could be of fundamental importance (see below).

It has long been accepted that perinatal exposure to exogenous oestrogens can cause deleterious effects on development and/or function of the reproductive tract based both on studies in rodents (4) and the findings in human males who were exposed in utero to DES (72, 75). The range of abnormalities induced includes cryptorchidism, overgrowth of the rete testis, epididymal cysts, small penis, hypospadias and effects on the prostate gland. Although such effects have generally been interpreted as resulting from impairment of androgen production/action, as described above, the fact that ERα and/or ERβ are expressed throughout the length of the male reproductive tract (60, 67) also raises the possibility that oestrogens could directly affect the development and/or function of various parts of the tract. Unfortunately at present, so little is known about the functions of oestrogens at these various target sites that it is difficult to assess the likelihood of this possibility. However, there are two recent pieces of evidence which support this line of thinking. The first relates to the ERKO mouse in which abnormalities in function of the efferent ducts have been reported (29) resulting in an inability to reabsorb seminiferous tubule fluid. As a result this fluid accumulates in the rete testis and seminiferous tubule lumens and leads to progressive impairment of spermatogenesis. Whether or not this entirely accounts for the infertility of ERKO males is unclear. The second piece of evidence shows that exposure of rats neonatally to DES results also in permanent distension of the rete testis and efferent ducts (25). The fact that both over-exposure (DES expt) and under-exposure (ERKO mice) to oestrogen appears to result in similar permanent changes is paradoxical but may be explained by abnormal development of the efferent duct epithelium in both situations (25). This implies that the correct level of oestrogen exposure is required for normal development of these cells (67) and there may be similar roles for oestrogens at other target cells in the reproductive tract. If this is the case, then incorrect oestrogen exposure (either too much or too little) could cause permanent malfunction of a particular area of the reproductive tract and thus lead to impairment of fertility. As has been discussed above, it might also be important that either too little or too much oestrogen will equally distort the androgen:oestrogen balance. These possibilities cannot be considered further until a much clearer understanding of the roles of oestrogens and the importance of the androgen:oestrogen balance in the development of the reproductive tract of the male are established.

ROUTES AND LEVELS OF HUMAN EXPOSURE TO ENVIRONMENTAL OESTROGENS

There have been a number of recent reviews (35, 45, 58, 75) which have documented in some detail the changes in the routes and types of exposures of human males to environmental oestrogens over the past

half-century, and these are summarised in simple form in Table 2. The take-home message from these studies is that we are now exposed to a wider range of oestrogenic chemicals than was the case 50 years ago and that our level of exposure to 'environmental oestrogens' (or to hormonally active chemicals in general) is almost certain to have increased. Whether our level of exposure is at a peak now or whether it has peaked at some time in the intervening years is unknown as we do not have any reliable means of gauging what magnitude of increase in our oestrogen exposure has occurred. This uncertainty stems from a number of key, unanswered questions. First, how can the overall exposure or body-burden of oestrogens in humans be measured at different stages in life? Second, how complete or incomplete is the list of chemicals which have been identified as being oestrogenic? Third, what level of 'oestrogen' exposure is needed to induce an adverse or unacceptable effect and is this constant throughout life or does it vary with age or stage of development? It is dismaying to realise that we are scarcely even beginning to frame answers to these questions. To the uninformed reader this may seem surprising so it is worthwhile briefly expanding on why answers to these fundamentally important questions are a long way off.

Table 2. Routes of increased human exposure to environmental oestrogens over the past 20–50 years

Chemical type/usage	Routes of human exposure	Level of human exposure	Comments
Synthetic oestrogens (ethinyl oestradiol, DES) Oral contraception/HRT in women	Water recycling/contamination	Probably low in developed countries with advanced water treatment facilities	Historically, exposure may have been higher due to usage of high dose contraceptive pills + poorer water treatment
Growth promoters in animal husbandry	Residues in meat/contamination	Probably low	Illegal/improper usage may have led to significant exposure of small cohorts of people
Plant oestrogens (phyto-oestrogens)	Natural component of many plants. Added to >60% of processed foods in Western countries over past 10–15 years	Moderate to high (especially in vegetarians)	Differences in consumption of phyto-oestrogen-rich soy products is implicated in lower rates of breast and prostate cancers in Oriental compared with Western countries
	In Western countries, 10–15% of infants now reared on soy-formula milk	Very high. Probably sufficient to cause biological effects	Effects unknown but neonatal exposure to oestrogens is known to cause adverse effects in rodents
Industrial/agricultural chemicals, their metabolites or residues	Multiple. Food contamination, food component (fat-soluble compounds), leaching from plastics, PVC, food wraps Use in cosmetics (absorption via skin) Workplace/agricultural/ domestic exposures	Probably moderate but largely unknown for non-pesticide chemicals	Exposure to certain chemicals, especially DDT and its derivatives and PCBs, was high and widespread in the period 1940–1970 New oestrogenic chemicals are still being discovered

How can the overall exposure or body-burden of oestrogens in humans be measured at different stages in life?

There is no single factor of which we are aware that can indicate in a dose-response manner the level of exposure of an individual to oestrogens. Indeed, it looks likely that we will never have such an answer as different oestrogen target tissues clearly have different sensitivities to oestrogens, and chemicals which are oestrogenic in one tissue may be inactive in other oestrogen target tissues or may even be an

oestrogen antagonist! The best examples of this problem are the therapeutic 'anti-oestrogens' tamoxifen and raloxifene. Both are oestrogen antagonists in the breast but both are oestrogen agonists in bone (28, 85). Even more puzzlingly, tamoxifen is a weak oestrogen agonist on the endometrium of the uterus whereas raloxifene appears to be devoid of such activity. We still have little idea as to what makes a compound an oestrogen agonist in one tissue and an antagonist in another, and without such fundamentally important understanding then we cannot possibly make any realistic prediction of the effects on the body of exposure to individual or mixtures of 'oestrogenic' chemicals. Enlightenment is likely to come with improved understanding of how oestrogens interact with their receptors and then interact with the transcriptional machinery to effect a biological response (59). It also needs to be kept in mind that measurement of oestrogenic potency of individual chemicals in the various in vitro screening systems available may not be a reliable guide to the oestrogenic potency of the chemical in vivo at a specific oestrogen target site. Although for the moment it is logical to base risk assessment of individual chemicals on their known oestrogenicity in vitro, this should not blind us to the possibility that the chemical in question may behave very differently in vivo at a particular oestrogen target site. There is therefore no substitute for in vivo testing of the identified chemicals.

How complete or incomplete is the list of chemicals which have been identified as being oestrogenic?

New chemicals with oestrogenic activity, as determined mainly by in vitro screening systems, are being discovered regularly and there is no reason to suppose that the list is yet complete (8, 35, 45, 75). Until recently, the focus had been very much on pesticides and chlorinated compounds with oestrogenic activity, then attention switched to alkylphenolic compounds, bisphenolic compounds and phthalates. Now the search is spreading wider. As more screening is undertaken it has become clear that phenolic compounds in general have a more than reasonable chance of turning out to be oestrogenic, and as phenolic compounds are probably the most ubiquitous of industrial chemicals it seems reasonably likely that new oestrogenic chemicals will be discovered in the coming years. In addition, we must give careful thought to other new developments. The discovery of a second oestrogen receptor, ERβ, means that new in vitro screening systems which centre on the use of this receptor have to be deployed in order to test whether results obtained using current screens, based on ERα, are comparable. So far, only minor differences have been found in the affinity of the two receptors for a range of oestrogenic compounds based on studies in cells transfected with the relevant ER (37). However, we know already that ERα and ERβ can interact with the transcriptional machinery in very different ways in vivo and that heterodimers of the two receptors can be formed in tissues in which both receptors are expressed (59). What this will mean in terms of responsiveness to oestrogenic chemicals is unknown, but it is considered that such differences probably account in some way for the differential oestrogen agonistic and antagonistic activities of certain compounds, as described above for tamoxifen and raloxifene.

What level of 'oestrogen' exposure is needed to induce an adverse or unacceptable effect and is this constant throughout life or does it vary with age or stage of development?

Of the three questions posed this should be the most answerable if the full weight of toxicological experience can be focused on individual chemicals (8, 35). There are, however, new factors which have to be taken account of. First, we have only just become aware of how pervasive the effects of oestrogens are throughout the body of both the male and female, based on the sites at which ERα and/or ERβ are expressed (see above). Until we have developed a better understanding of the physiological functions of oestrogens at these various sites, we will remain unable to quell the concern that we may have been looking at the wrong endpoints or the wrong tissues when trying to assess whether or not a particular oestrogenic chemical is able to exert an adverse effect. Second, it is becoming ever clearer in the male that oestrogens exert fundamental 'programming' effects on the developing reproductive system, as was first shown by John McLachlan and his colleagues more than 20 years ago (reviewed in ref. 4). These irreversible changes can be quite devastating after exposure to high doses of potent oestrogens, which gives rise to the concern that other less obvious (but equally permanent) effects may be induced of which we are unaware. Improved understanding of the physiology of oestrogen action in the male during development is the only path forward which will help us to resolve this issue (67). Third, there is the

particularly thorny dilemma of the effects of very low doses of oestrogenic chemicals, their interactions and synergy etc. With regard to the latter, the prevailing view (43) is that this is unlikely to occur, at least in the way which was originally described (7). However, the evidence for effects of surprisingly low doses of oestrogenic chemicals on the prostate (79) or on the testis (88) is a little more difficult to dispel. With regard to the effects on the testis, some of this data has not proved repeatable in different laboratories (70) and the authors of the original paper have themselves expressed concern about the possible contributory role of other factors to such changes (70). With regard to the effects on the mouse prostate, such effects have been shown to be inducible in male offspring both by 2-fold elevations of circulating oestradiol levels in pregnant mice (within the physiological range) or by similar administration of remarkably low doses of bisphenol-A (87). There is as yet no published data from other laboratories to either confirm or not to confirm these findings, and without such data it is difficult to know what level of emphasis should be given to this data. Even though most experienced toxicologists consider such findings to be counter-intuitive, it is important in such situations to keep an open mind as science never fails to spring surprises. If the findings are correct and are repeatable in different laboratories, then the data would suggest that some oestrogenic chemicals may be surprisingly more potent in vivo than is indicated by in vitro measurement of their oestrogenic potency or indeed by measurement of the potency in vivo in the standard rat uterine weight assay. Even our present sketchy understanding of how different oestrogens interact with ERα and ERβ, and whether or not one or both receptors are present, points clearly to there being major differences in the relative ability of different oestrogens to activate gene transcription.

CONCLUSIONS AND FUTURE PROSPECTS

The possibility that exposure to environmental oestrogens has caused deleterious changes in male reproductive health remains a plausible hypothesis. However, we still lack any piece of direct evidence which would support this theoretical relationship. Nor do we have any evidence which shows directly that the overall level of human exposure to environmental oestrogens has ever been sufficient in the general population to cause biological or adverse changes in any aspect of human health. Reassuring though this may seem, this lack of certainty stems primarily from ignorance and lack of relevant data. More importantly, this deficiency has meant that all manner of claim and counter-claim can be made as to the threat that such exposures pose to man, backed up by 'lack of evidence' or presumption in the face of this lack. Faced with the evidence which we have regarding the effects of potent oestrogens, such as DES, on the male reproductive system, no-one will claim that we should be complacent in the face of so many types and routes of exposures to environmental oestrogens. On the other hand, we now live longer and generally we are healthier, so any risk from exposures to environmental oestrogens should be placed in this perspective. Indeed, the surprising finding (81) that men who had been exposed in utero to high levels of DES exhibited no significant qualitative change in their fertility in adulthood (based on time-to-pregnancy), despite the fact that we know that this exposure induced a range of abnormalities of reproductive development including lower sperm counts (72, 75), should serve as a reminder to us all that presumptions are no substitute for direct experimentation. If such a level of oestrogen exposure was unable to affect fertility then logic would argue that the less potent environmental oestrogens must surely pose a negligible threat to male fertility. Logical though this conclusion may be, it is nevertheless a presumption and we should wait for more direct evidence, lest we be lulled into drawing the wrong conclusion.

Risk assessment requires detailed information on the dose-response relationship between exposure to individual chemicals in animal studies (and human if these are available) and data on the levels of human exposure to such chemicals. At present, reasonable data on levels of human exposure to hormonally active chemicals is restricted to the persistent chlorinated compounds such as DDT and PCBs and to some extent also for dioxins (13, 36, 56). There is also data on human daily intake of various phthalates, though there is not good data on absorption and metabolism nor on whether such agents gain access to the foetus in man as they appear to do in the rat (47, 82); such information is critical if a realistic assessment is to be made of the potential involvement of phthalates in disorders of male reproductive health which are induced in utero. With regard to the more recently described oestrogenic chemicals,

such as bisphenolic and alkylphenolic compounds, there is no good data on the levels of human exposure. Obtaining such data is clearly essential for an informed risk assessment, as are detailed animal studies. However, both will require considerable resources. Pragmatism and the ever present shortage of funds for research might persuade people that less, rather than more, funds should be directed at this issue if logic suggests that the likely outcome is to show no risk to male fertility from environmental oestrogens. There are two powerful counter arguments to this line of thinking, namely (a) testicular cancer and male reproductive health in general, and (b) the likely benefits of understanding how oestrogens affect our health.

Testicular cancer: the visible manifestation of more widespread effects?

The phenomenal increase in testicular cancer remains unexplained, and what was once a very rare disease is now becoming quite common in Western countries. The fact that it has very low mortality (<5%) has perhaps blinded people to its impact on young men and to its undoubted morbidity. Our knowledge that this disease is hormonally modulable in foetal life and is increasing in men with each later year of birth should ring alarm bells about what environmental risk factors are at work to induce such effects and what else they might be inducing. The growing evidence for an association between low sperm counts, poor spermatogenesis and reduced fertility in men who go on to develop testicular germ cell cancers should surely make us continue to wonder whether the reported reduction in sperm counts with later age of birth in the normal population in some countries, is not a milder symptom of the same exposure(s)? Again, the fact that there appears to be an increasing prevalence of other, more common, reproductive developmental disorders which are related to hormonal problems (cryptorchidism and hypospadias) and which are risk factors for testicular cancer seems more than coincidental. As I have explained above, male fertility only declines once sperm counts are reduced drastically. Therefore, the fact that male fertility does not seem to have changed greatly in incidence in Western countries in recent decades (though this is not based on solid scientific documentation) and the fact that fertility of DES-exposed males is also unaffected should not be viewed as certain evidence that all is well with male reproductive health. Nor should we be complacent that matters will not get worse, to the point where male fertility is affected. If testicular cancer incidence is a beacon, then its light suggests that male reproductive health is going to get worse. However, even if we accept this view, we should not blind ourselves in the belief that environmental oestrogens or environmental chemicals in general are the cause of these changes. They remain a plausible, but theoretical, possibility but nothing more. We should remain open-minded and alert to alternative explanations or causes for the adverse changes in male reproductive health, though none are currently being advanced.

Health benefits of oestrogens

Environmental oestrogens are synonymous with 'doom and gloom', but there is another side to this issue. There are strong pointers to suggest that low levels of exposure to environmental oestrogens could be good news in turns of human health. The data from oriental countries which suggests a relationship between consumption of phyto-oestrogen-rich soy foods and the low incidence of cancers of the breast, prostate and testis could mean that such oestrogens are rather good for us (3, 6, 44). The similarly low incidence of cardiovascular diseases in such countries, compared with the West, is also arguably a consequence of increased intake of (weak) phyto-oestrogens, now that we know that oestrogens act throughout the cardiovascular system (18, 26) and that environmental oestrogens as well as oestradiol itself can induce vasodilatatory changes (57, 67). More speculatively, if tamoxifen and raloxifene are anything of a guide, we can expect that individual, environmental oestrogenic chemicals may act as selective oestrogen agonists and antagonists in different tissues and thus provide further possibilities for the development of tissue-specific therapy of oestrogen-dependent disease (45). Better understanding of how oestrogens regulate physiological processes throughout our bodies and how environmental oestrogens can impact on these processes therefore seems guaranteed to produce positive health benefits as well as enabling us to better estimate the risk, if any, that they pose to reproductive health and fertility in the human male.

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