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Neonatal exposure to potent and environmental oestrogens and abnormalities of the male reproductive system in the rat: evidence for importance of the androgen–oestrogen balance and assessment of the relevance to man

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The effects on reproductive tract development in male rats, of neonatal exposure to potent (reference) oestrogens, diethylstilboestrol (DES) and ethinyl oestradiol (EE), with those of two environmental oestrogens, octylphenol and bisphenol A were systematically compared. Other treatments, such as administration of a gonadotrophin-releasing hormone antagonist (GnRHa) or the anti-oestrogen tamoxifen or the anti-androgen flutamide, were used to aid interpretation of the pathways involved. All treatments were administered in the neonatal period before onset of puberty. The cellular sites of expression of androgen receptors (AR) and of oestrogen receptor-α (ERα) and ERβ were also established throughout development of the reproductive system. The main findings were as follows: (i) all cell types that express AR also express one or both ERs at all stages of development; (ii) Sertoli cell expression of ERβ occurs considerably earlier in development than does expression of AR; (iii) most germ cells, including fetal gonocytes, express ERβ but not AR; (iv) treatment with high, but not low, doses of potent oestrogens such as DES and EE, induces widespread structural and cellular abnormalities of the testis and reproductive tract before puberty; (v) the latter changes are associated with loss of immunoexpression of AR in all affected tissues and a reduction in Leydig cell volume per testis; (vi) none of the effects in (iv) and (v) can be duplicated by treating with high-dose octylphenol or bisphenol A; (vi) none of the reproductive tract changes in (iv) and (v) can be induced by simply suppressing androgen production (GnRHa treatment) or action (flutamide treatment); and (vii) the adverse changes induced by high-dose DES (iv and v) can be largely prevented by co-administration of testosterone. Thus, it is suggested that many of the adverse changes to the testis and reproductive tract induced by exposure to oestrogens result from a combination of high oestrogen and low androgen action. High oestrogen action or low androgen action on their own are unable to induce the same changes.

Key words: androgen:oestrogen balance/androgen receptors/environmental oestrogens/male reproductive tract/oestrogen receptors

TABLE OF CONTENTS

Introduction

In recent years concern has been raised about the possibility that reported increases in human male reproductive disorders (testicular cancer, cryptorchidism, hypospadias, low sperm counts) might stem from increased fetal (or neonatal) exposure of the developing male to oestrogens (Sharpe and Skakkebaek, 1993; Toppari *et al*., 1996). Though the original postulated link between increase in these disorders and increased oestrogen exposure offered a range of alternative pathways via which the increased hormone exposure could have occurred (Sharpe and Skakkebaek, 1993), most attention has been focused on the

possible involvement of exposure to environmental oestrogens (Toppari *et al*., 1996). The latter possibility has been fuelled by the demonstration that numerous, ubiquitous environmental chemicals possess weak oestrogenic activity when measured in a variety of in-vitro and in-vivo test systems (Toppari *et al*., 1996; Crisp *et al*., 1998). Though human exposure to these chemicals is widespread, all of the available data suggest that these chemicals are only very weakly oestrogenic, being 10 000- to >100 000-fold less potent than oestradiol itself (Toppari *et al*., 1996). Nevertheless, reports that exposure of mice *in utero* to extremely low concentrations of weak environmental oestrogens such as bisphenol A is able to increase significantly prostate size in adulthood (Nagel *et al*., 1997; vom Saal *et al*., 1998) has understandably raised concern about possible effects in humans. Although other, more detailed, studies have been unable to repeat the latter findings (Ashby *et al*., 1999; Cagen *et al*., 1999a,b), concern remains.

The primary reason why concern about environmental oestrogens persists is because there is good evidence that exposure of the human male fetus to the potent oestrogen diethylstilboestrol (DES), via administration to the mother, increased the incidence in the offspring of most of the disorders that are reportedly increasing in incidence in the general population (see Toppari *et al*., 1996; Sharpe, 1998a; Table I). Moreover, similar disorders can be induced in animal studies in which DES or other potent oestrogens (e.g. ethinyl oestradiol) are administered to the pregnant mother (Arai *et al*., 1983; Newbold and McLachlan, 1985; Toppari *et al*., 1996; Table I). However, an important fact that is often overlooked when considering these findings is that most, and possibly all, of the studies that have reported gross reproductive abnormalities in male offspring have involved the administration of very high doses of very potent oestrogens such as DES and ethinyl oestradiol. For example, most of the studies in pregnant or neonatal mice involve the administration of hundreds of micrograms per kg DES, and the human studies involved daily doses of 5 to 150 mg administered on a rising scale during pregnancy, commencing at 7 to 20 weeks of pregnancy (Stillman, 1982; Toppari *et al*., 1996). Compared to these huge doses, even vast amounts of weak environmental oestrogens would probably be insignificant in terms of their 'overall oestrogenicity'. Opinions remain firmly divided as to whether or not this massive difference in oestrogenic potency is reassuring or misleading. Another curious, and overlooked, fact is that the reproductive abnormalities induced by in-utero exposure of the male fetus to high concentrations of exogenous oestrogens bear remarkable similarities to those induced by interference with androgen action, whether as the result of administration of an anti-androgen or resulting from inactivating mutations in the androgen receptor (Table I).

Our approach to this issue has been systematic. First, to identify the cellular sites of action of oestrogens in the male reproductive system throughout development. Second, to establish via neonatal

Table I. Comparison of the effects on the male reproductive system of reduced androgen exposure of the fetus *in utero* with the effects of abnormally high oestrogen exposure due to administration of potent oestrogens such as diethylstilboestrol (DES) or ethinyl oestradiol. Information for the human derives from studies of the offspring of women treated with DES for threatened miscarriage (Stillman, 1982; Toppari *et al*., 1996), whereas data for animals (mainly the mouse) derives from numerous sources (Arai *et al*., 1983; Newbold and McLachlan, 1985; Imperato-McGinley *et al*., 1992; Silversides *et al*., 1995; Sharpe, 1998a,b; Mylchreest *et al*., 1999) including data from transgenic mice that over-express aromatase (Li *et al*., 1999)

Reduced androgen exposure (administration of an anti-androgen or partial androgen resistance syndromes)	Increased oestrogen exposure (administration of DES or ethinyl oestradiol at high doses or over-expression of aromatase)
Results in increased incidence of the abnormalities listed below	Results in increased incidence of the abnormalities listed below
At birth:	At birth:
Cryptorchidism[a]	Cryptorchidism[a]
Hypospadias[a]	Penile abnormalities[a]
Agenesis/abnormalities of development of the epididymis[a] and prostate	Epididymal abnormalities[a]
In adulthood:	*In adulthood:*
Small testes	Small testes
Low sperm counts	Low sperm counts
(Testicular germ cell cancer)[b]	Testicular germ cell cancer
Epididymal abnormalities	Epididymal cysts
Prostatic abnormalities[c]	Prostatic abnormalities[d]

[a]Note that the incidence of abnormalities of development of the reproductive system that are evident at birth in males exposed to insufficient androgens is notably higher than the corresponding incidence in males whose mothers were exposed to increased oestrogen concentrations.[b]Administration of anti-androgens to animals has not been shown to result in germ cell cancers, but in humans with disorders of androgen production or action (e.g. testicular feminization syndrome) there is an extremely high incidence of such tumours (Ottesen *et al*., 1999; Toppari *et al*., 1996).
[c]Small/under-developed.
[d]Small/under-developed; relative increase in stromal and decrease in epithelial volume; dysplasia.

treatment of the rat, what doses of DES will affect these target tissues. Third, to establish whether or not similar changes can be induced by administration of candidate environmental oestrogens. Fourth, where reproductive target tissues are affected grossly by oestrogen exposure, to establish the mechanism via which the changes are induced. This review summarizes the progress made toward these objectives and, using the example of the seminal vesicles, explores what appears to be a centrally important mechanism of action. Based on these findings, we provide our current view as to whether or not environmental oestrogens pose a significant risk to the developing human male.

Animals and treatments

The main approach used in our studies has been to administer the test compounds to Wistar rats during neonatal life, in most studies from day 2 to day 12 (day of birth = day 1). The rats were from our own colony, housed under standard conditions, and treated and managed according to Home Office-approved protocols. Compared with human babies, rodents such as the rat are born in a relatively non-advanced state, and in certain (but not all) respects the early part of the neonatal period (e.g. days 1–6) is akin to the last trimester of pregnancy in the human. Important developmental changes in the testis (Sertoli cell multiplication, emergence of the adult Leydig cell population) and reproductive tract (functional differentiation of the epididymis, prostate and seminal vesicles) occur or are initiated during the neonatal period in the laboratory rat, before the onset of puberty (about days 15–20). A summary of the treatment protocols used in these studies is provided in Table II. DES and ethinyl oestradiol were used as representative potent oestrogens, while octylphenol (an alkylphenolic surface-active agent/detergent) and bisphenol A (many uses, including in some plastics) were used as examples of environmental oestrogens to which there is significant human exposure (Toppari *et al*., 1996). It became apparent during our studies that suppression of androgen action, following DES treatment, was an important treatment-induced change. Therefore, the effect of neonatal administration of the androgen receptor antagonist flutamide (Sigma Chemical Co., Poole, Dorset, UK) was investigated to establish if this induced comparable effects to those of DES. Another possible explanation for the adverse effects of perinatal oestrogen exposure on male reproductive development is that it suppresses endogenous pituitary hormone production and thus retards normal testicular and reproductive tract development via this indirect route (Sharpe and Skakkebaek, 1993). To evaluate this possibility, some rats were treated with a gonadotrophin-releasing hormone (GnRH) antagonist (Antarelix; Europeptides, Argenteuil, France) to suppress endogenous gonadotrophin (and thus, androgen) concentrations and thus to induce simple retardation of development of the testis and male reproductive tract. Findings in these animals were then compared with those in the other treatment groups.

Sites of expression of oestrogen and androgen receptors

Though it is well established that androgen receptors (AR) are expressed widely throughout the developing male reproductive system (Bremner *et al*., 1994; Majdic *et al*., 1995), similar data for oestrogen receptors (ER) was generally lacking when our studies started and were compounded by the discovery of a second oestrogen receptor, ERβ (Kuiper *et al*., 1996, 1998). At this time the only information that was clear and unequivocal was that ERs were expressed in the epithelial cells of the efferent ducts and at lower levels in some Leydig cells. In order to focus our attention on cellular sites of oestrogen action, we therefore systematically evaluated where and when ERα and ERβ were expressed throughout development of the reproductive system of the male rat using either antibodies raised by ourselves (ERβ; Saunders *et al*., 1997, 1998) or which were available commercially (ERα; Nova-Castra or Dako; see Fisher *et al*., 1997). The pattern of ER immunolocalization was contrasted with that for AR, based on our previous (Bremner *et al*., 1994; Majdic *et al*.,

Table II. Summary of treatments administered to male rats during the neonatal period

Test compound per injection[a]	Dose administered	Dosing regimen[b]	Approximate equivalent in μg/kg/day[c]
Diethylstilboestrol	10 μg	Days 2, 4, 6, 8, 10, 12	370
(DES)	1 μg	Days 2, 4, 6, 8, 10, 12	37
	0.1 μg	Days 2, 4, 6, 8, 10, 12	3.7
	0.01 μg	Days 2, 4, 6, 8, 10, 12	0.37
Ethinyl oestradiol	10 μg	Days 2, 4, 6, 8, 10, 12	370
Flutamide	50 mg/kg/day	Days 2, 4, 6, 8, 10, 12	50 000
Octylphenol	2 mg	Days 2–12 inclusive	150 000
Bisphenol A	0.5 mg	Days 2–12 inclusive	37 000
GnRH antagonist	10 mg/kg	Days 2 and 5 only	NA

[a]All treatments were by subcutaneous injection in 0.02 ml corn oil.[b]DES, ethinyl oestradiol, and flutamide were administered only on alternate days.
[c]Calculation is based on average bodyweight for rats during the treatment period. Note that because most of the treatments were administered in oil to give a prolonged absorption pattern, this calculation may be somewhat misleading.
NA= not available.

1995) and more recent findings using a commercially available polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). All of these studies used optimised methods, including antigen retrieval and Bouin's fixation of tissue for 5–6 h, and the specificity of each of the antibodies used was confirmed by Western blots. Full details of the antibodies, immunolocalization procedures and specificity checks can be found elsewhere (Bremner *et al*., 1994; Fisher *et al*., 1997; Saunders *et al*., 1997, 1998, 2000, 2001; Williams *et al*., 2000; McKinnell *et al*., 2001). A summary of the findings is presented in Table III.

The main conclusions from these studies are as follows. First, ER (in particular ERβ) are expressed extremely widely in the male reproductive system stages (including fetal and neonatal stages) throughout life. Second, all cells and tissues that express

Table III. Summary of the pattern and intensity of sex steroid receptor expression in reproductive tract tissues of the male from fetal life through to adulthood. The details shown are based on our own findings for the rat (see text for references) but are broadly applicable to most species, including the human and non-human primate (Fisher *et al*., 1997; Saunders *et al*., 2001)

Reproductive tract tissue	Androgen receptor	Oestrogen receptor α	Oestrogen receptor β
Fetal			
Sertoli cells	–	–	++
Germ cells	–	–	+++
Leydig cells	–	+	++
Peritubular myoid cells	+++	–	+
Mesonephros/efferent ducts	++	++	++
Epididymis (W duct)	++	–	++
Vas deferens (W duct)	++	–	++
Seminal vesicles	++	?	?
Prostate	+	?	?
Neonatal			
Sertoli cells	+/–	–	++
Germ cells	–	–	++
Leydig cells	–	++	++
Peritubular myoid cells	+++	–	+
Efferent ducts	++	+++[a]	++
Epididymis	++	–	++
Vas deferens	++	–	++
Seminal vesicles	++	+[a]	++
Prostate	++	+[a]	++
Peripubertal			
Sertoli cells	++	–	++
Germ cells	–	–	++
Leydig cells	+	++	++
Peritubular myoid cells	+++	–	+
Efferent ducts	++	+++[a]	++
Epididymis	+++	+[a]	+++
Vas deferens	+++	+[a]	+++
Seminal vesicles	+++	+[a]	++
Prostate	+++	+[a]	+++
Adulthood			
Sertoli cells	+++	–	++
Germ cells	–	–	+++
Leydig cells	++	+/–	++
Peritubular myoid cells	+++	–	+
Efferent ducts	++	+++[a]	++
Epididymis	+++	–	+++
Vas deferens	+++	–	+++
Seminal vesicles	+++	+	+++
Prostate	+++	+[a]	+++

[a]Note that the cellular site of expression of ERα may vary depending on region and age and/or may be confined to selected epithelial or stromal cells. This is in contrast to the expression of AR and ERβ in the same tissues in which expression occurs widely in the majority of both epithelial and stromal cells

Intensity of immunoexpression is based on subjective, comparative evaluation, and ranges from weakly positive (+) to intensely positive (+++). Where not all cells of the same type are positive, a +/– score is shown. Cells/tissues which were immunonegative are indicated by –. ? indicates that this tissue has not yet been evaluated in detail.

AR also express one or both of the ER at all ages that have so far been investigated. Third, Sertoli cells, which are the primary androgen target cells in the testis, do not switch on immunoexpression of the AR until just before the onset of puberty but immunoexpress ERβ from very early in fetal life and then throughout life. Fourth, germ cells never express AR, but most germ cells (including fetal gonocytes) express ERβ at all ages. Fifth, all of the reported male reproductive tissues that are affected adversely by perinatal oestrogen treatment (Table I) clearly express ER during perinatal life. Although these findings are based on detailed evaluation of the rat, reasonably extensive studies of ours in other species, including the marmoset, macaque and human confirm that the main findings shown in Table III are broadly applicable to these other species as well as to other species of mammals (Fisher *et al*., 1997, 1999; Saunders *et al*., 2001; P.T.K.Saunders, R.M.Sharpe and C.McKinnell, unpublished data).

The findings in Table III raise several important questions to which we currently lack answers. For example, what role do oestrogens play in development of Sertoli cells and germ cells as well as in spermatogenesis in adulthood? More fundamentally, considering that all cell types that express AR also express one or both ER, what role do interactions between androgens and oestrogens play in these target cells? Finally, following on from the latter question what (if any) role do androgen–oestrogen interactions play in the aetiology of reproductive tract disorders after neonatal oestrogen exposure? The latter is a question that our studies have gone on to address and is outlined below.

Induction of reproductive tract abnormalities by neonatal oestrogen treatment

Administration of the highest of the doses of DES listed in Table II (10 μg/injection) to male rats consistently caused major developmental abnormalities of the testis and reproductive tract when evaluated at around the time of normal onset of puberty (days 18–25). The affected tissues and the types of changes induced are outlined in Table IV, and some are illustrated in Figure 1 (for full details, see Atanassova *et al*., 2001; McKinnell *et al*., 2001). The morphologically most dramatic effect of DES treatment was the induction of overgrowth and distension of the rete testis and distension of the efferent ducts, whereas the most pervasive effect was under-development of epithelial tissue, as this extended from the efferent ducts, through the epididymis and vas deferens to the seminal vesicles and prostate (Figure 1).

It is probably important to separate the effects of potent oestrogens on the testis from those on the reproductive tract as they show a somewhat different dose–response relationship and may thus reflect different mechanisms of induction. For example, the effects of DES/ethinyl oestradiol administration on Sertoli and germ cell numbers showed an extended dose–response curve, with even relatively low doses (e.g. 0.1 μg dose in Table II) still causing significant changes in cell numbers (Atanassova *et al*., 1999, 2000). In contrast, major abnormalities of the reproductive tract, characterized by under-development of epithelium and over-development of stromal tissue, were only grossly evident after treatment of rats with the highest dose of DES (10 μg dose in Table II), whereas lower doses exerted only minimal (if any)

Table IV. Summary of the reproductive tract tissues in which gross morphological abnormalities can be induced by oestrogen administration in neonatal life in the male rat (see also Table I). Results are based on our own findings (Sharpe *et al*., 1998; Atanassova *et al*., 1999, 2000, 2001; Fisher *et al*., 1998, 1999; Williams *et al*., 2000; McKinnell *et al*., 2001; R.M.Sharp, C.McKinnell and K.Williams, unpublished data) but are consistent with most data in the literature in which similar treatment regimens have been used. Note that any positive effects of treatment are shown in bold type

Reproductive tract tissue abnormality	Effect of GnRHa[a]	Effect of high dose DES[e]	Effect of low dose DES[e]	Effect of environmental oestrogens[b]
Sertoli cell number	Yes (–ve)	Yes (–ve) [c]	Yes (–ve) [c]	None
Germ cell number	Yes (–ve)	Yes (–ve) [c]	Yes (+ve) [c]	Yes (+ve) [c]
Leydig cell number	Yes (–ve)	Yes (–ve)	None	None
Rete/efferent ducts (overgrowth/distension)	None	Yes (–ve)	None	None
Epididymis (epithelial under-growth; stromal overgrowth)	None[d]	Yes (–ve)	None	None
Vas deferens (epithelial under-growth; stromal overgrowth)	None[d]	Yes (–ve)	None	None
Seminal vesicles (epithelial under-growth; stromal overgrowth)	None[d]	Yes (–ve)	None	None
Prostate (epithelial under-growth; stromal overgrowth	None[d]	Yes (–ve)	None	None

[a]Administered as a control for suppression of gonadotrophin secretion with consequent lowering of endogenous androgen concentrations.
[b]Bisphenol A and octylphenol both tested at a single high dose (see Table II).
[c]Effects may be secondary not primary (e.g. due to effects on pituitary hormone secretion).
[d]Note that these tissues exhibited some retardation of development due to suppression of gonadotrophin/androgen concentrations, but the morphological changes observed did not resemble those observed in oestrogen-treated animals.
[e]High and low doses of DES would be ~370 μg/kg/day and <4 μg/kg/day respectively (see Table II).

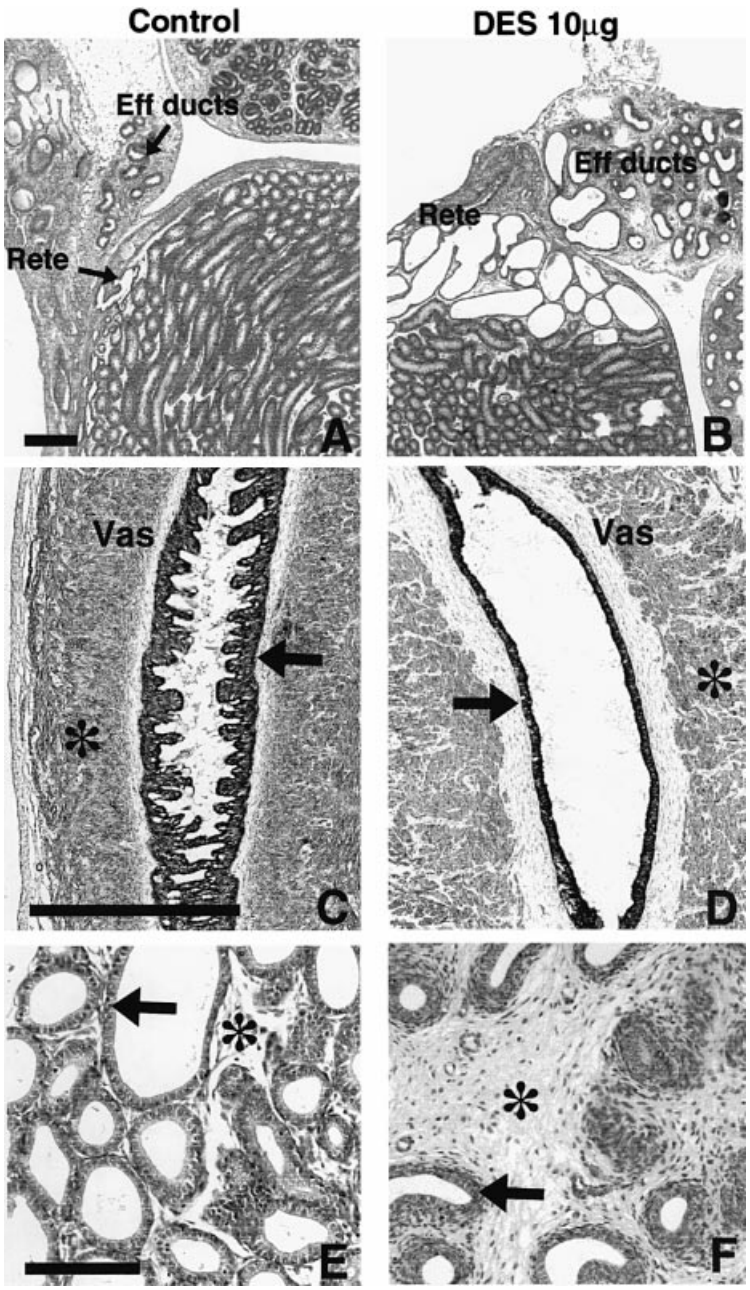

Figure 1. Induction of abnormalities of the testis and reproductive tract on day 18 by neonatal treatment of rats with a high dose (10 μg) of diethylstilboestrol (DES). (**A**, **B**) Midline sections of the upper pole of the testis at the junction between the rete and the efferent ducts in a control (A) and DES (10 μg)-treated (B) rat to illustrate the overgrowth and distension of the rete and distension of the efferent ducts (Eff ducts) that occurs in the latter group. (**C**, **D**) Cross-sections through the vas deferens of a control (C) and DES (10 μg)-treated (D) rat to illustrate the gross under-development of the epithelium (arrow = immunostained with a pan-cytokeratin antibody) of the vas in the latter group in comparison with the control. Asterisks show the muscle layer (immunostained for actin). (**E**, **F**) Sections through the ventral prostate of a control (E) and a DES (10 μg)-treated (F) rat to illustrate the relative under-development of the epithelium (arrows) and the relative overgrowth of stromal tissue (asterisk). Scale bars = 100 μm.

effect. In this regard, it is informative to consider the effects of administration of a GnRH antagonist. In terms of change in the numbers of Sertoli, germ and Leydig cells, GnRH antagonist treatment induced very similar effects to that of DES (Table IV; Figure 2), which may mean that the primary action of DES on the testis is indirect and involves suppression of FSH and LH secretion. Although this may be partly true, several other lines of evidence suggest that at least some of the changes in cell numbers induced in the testis by neonatal DES treatment might be the result of direct effects on this organ (Sharpe *et al*., 1998; Atanassova *et al*., 1999, 2000). Irrespective of the mechanism underlying these treatment-induced changes, it is clear that neither bisphenol A nor octylphenol was able to induce any of the negative effects on testis cell numbers following their neonatal administration to rats in a high dose (Table IV; Figure 2). However, these two compounds were able to mimic very low doses of DES in accelerating the normal onset of pubertal spermatogenesis as indicated by increase in germ cell volume/

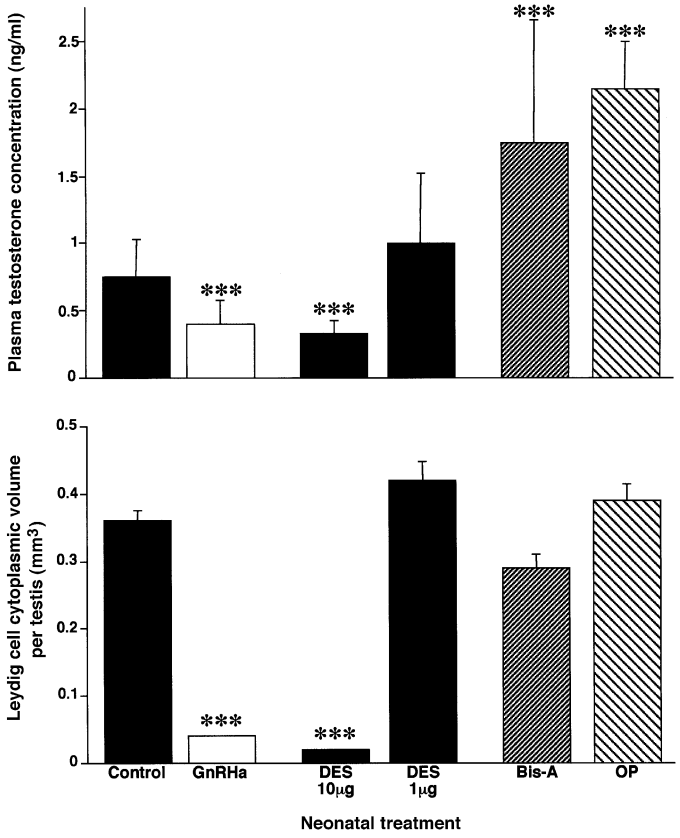

Figure 2. Effect of neonatal treatment of rats with a low (1 μg) or a high (10 μg) dose of diethylstilboestrol (DES), or with the environmental oestrogens, bisphenol A (Bis-A) or octylphenol (OP), or with a long-acting GnRH antagonist (GnRHa) on Leydig cell cytoplasmic volume per testis (bottom) and plasma testosterone concentrations (top) at day 18. Doses and treatment regimens are given in Table II. Leydig cells were identified by immunostaining of their cytoplasm for 3β-hydroxysteroid dehydrogenase and quantified by point-counting as detailed elsewhere (Atanassova *et al*., 2000). Values are mean ± SD for either 6–14 rats (top) or 5–6 animals per group (bottom). ***$P < 0.001$ versus control.

Sertoli cell (Table IV) and by parallel changes in other parameters (Atanassova *et al*., 2000). The latter findings indicate that, whereas high concentrations of oestrogens may have broadly negative effects on testicular development, low concentrations may have some stimulatory effects – in our view the latter effects are more likely to reflect the physiological effects of oestrogens on the testis, as there is emerging evidence for a role for oestrogens in the regulation of spermatogenesis (Atanassova *et al*., 2000; Ebling *et al*., 2000). Because of their weak oestrogenicity, environmental oestrogens may only be capable of mimicking the latter effects, and then only after exposure to very high concentrations (at least according to our findings). It should also be noted that these stimulatory effects of low oestrogen concentrations may be indirect and result from elevation of endogenous FSH concentrations (Atanassova *et al*., 2000; Ebling *et al*., 2000) and/or by precocious activation of Leydig cells and thus increase in testosterone concentrations (Figure 2).

In contrast to the effects of neonatal DES treatment on the testis, none of the effects of this compound on the rete testis, efferent ducts and rest of the reproductive tract (Figure 1) could be induced with either GnRHa or with bisphenol A or octylphenol (Table IV); the latter two compounds were administered at extremely high doses, and human exposure to such doses is highly unlikely. Additionally, neonatal treatment with the AR antagonist, flutamide, was also unable to induce any of the changes induced by DES (10 μg) as listed in Table IV (McKinnell *et al*., 2001; see example in Figure 3). Moreover, only the highest dose of DES (10 μg, Table II) was able to induce prominent changes to the reproductive tract, and after administration of a ten-fold lower dose (1 μg, Table II), the changes were either not evident or, in the case of the rete testis and efferent ducts, were quite mild compared with the 10 μg dose (Fisher *et al*., 1999; Williams *et al*., 2000; McKinnell *et al*., 2001). A further example of the effect of the 10 μg dose of DES on epithelial under-development is shown for the seminal vesicles in Figure 3, and similar or more pronounced changes are evident in other tissues of the male reproductive tract (Figure 1; Williams *et al*., 2000; Atanassova *et al*., 2001; McKinnell *et al*., 2001).

Oestrogen-induced reproductive abnormalities and the androgen:oestrogen balance

The clear differences between the effects of DES treatment on the testis versus its effects on downstream reproductive tract tissues raised the question of whether altered androgen action might be involved. This thinking stemmed from the fact that the testis is a relatively minor androgen target tissue in the neonatal period (pronounced androgen-responsiveness only develops during puberty), whereas it is accepted that development of the reproductive tract tissues is clearly androgen-driven and is reflected in the widespread and intense expression of AR in these downstream tissues (Table III). We therefore evaluated whether neonatal DES treatment had any effect on expression of AR in these tissues. The results showed unequivocally that treatment with the highest dose of DES (10 μg) virtually abolished immunoexpression of the AR in most reproductive tract tissues as well as in Sertoli cells in the testis (Table V; see also Figure 3 and Sharpe *et al*., 1998; McKinnell *et al*., 2001). Such changes were not induced by neonatal treatment with GnRHa (Sharpe *et al*., 1998), flutamide (Figure 3; McKinnell *et al*., 2001), lower doses of DES or by bisphenol A or octylphenol (Table V). Indeed, there was a strong and consistent relationship between loss of AR immunoexpression and the occurrence of gross morphological abnormalities in the same tissue (compare Tables IV and V; details in McKinnell *et al*., 2001). Although in most tissues there was loss of AR expression from both stromal and epithelial cells, in the seminal vesicles the loss of AR immunoexpression was complete for epithelial cells but was far less pronounced for stromal cells (Figure 3). The fact that neonatal GnRHa treatment was unable to induce loss of AR immunoexpression, especially in the reproductive tract tissues (Table V), yet was able to induce a similar reduction to DES (10 μg dose) in Leydig cell development and in testosterone concentrations (Figure 2) argues that the loss of AR in DES (10 μg)-treated animals is not simply a consequence of suppression of endogenous androgen concentrations/androgen action; results for flutamide treatment reinforce this view. It was therefore postulated that the changes induced by high-dose DES treatment might stem from disturbance of the androgen–oestrogen balance, as there are several pieces of evidence which suggest this is important in maintaining normal

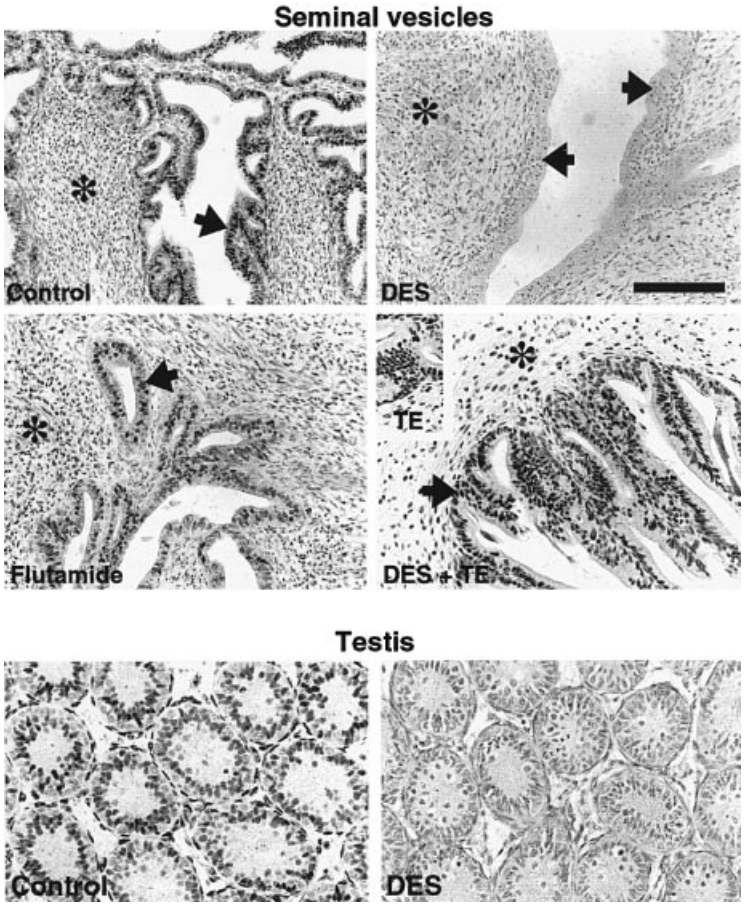

Figure 3. Effect of neonatal hormone treatment on immunoexpression of the androgen receptor (AR; black staining) in the seminal vesicles and testis (bottom row) of rats at day 18. Note that neonatal treatment with DES (10 µg) alone results in complete disappearance of AR immunoexpression from the testis (bottom right) and from epithelial cells of the seminal vesicles (top right) and that in the latter it is associated with under-development (less infolding) of the epithelium (see arrows). These changes are not reproduced by treatment with flutamide (middle left). AR immunoexpression and normal growth of the epithelial cells in the seminal vesicles is restored by testosterone esters (TE; 200 µg) co-administration to DES (10 µg)-treated rats (middle right). The inset in the middle right-hand panel shows a section from an animal treated with TE alone. Note that immunoexpression of AR in stromal cells (asterisks) of the seminal vesicles in the DES-treated animal (top right) is less affected than is epithelial expression. Doses and treatment regimens are given in Table II. Scale bar = 100 µm.

Table V. Summary of the effects of neonatal manipulation of androgen and/or oestrogen concentrations in rats on subsequent immunoexpression of the androgen receptor at day 18 (= early puberty). The treatments administered (see Table II) were: a high dose (10 µg) of diethylstilboestrol (DES), a long-acting GnRH antagonist or high doses of the environmental oestrogens bisphenol A (0.5 mg) or octylphenol (2.0 mg). The changes reported are by reference to vehicle-treated control animals and are based on published (McKinnell *et al*., 2001) and unpublished data

Reproductive tract tissue	Control	DES (10 µg)	GnRH antagonist	Environmental oestrogens[a]
Sertoli cells	++	–	++/+	++
Peritubular myoid cells	+++	+	+++	+++
Leydig cells	+	–	+	+
Rete/efferent ducts	++	–	++	++
Epididymis	+++	–	+++	+++
Vas deferens	+++	–/+	+++	+++
Seminal vesicles	+++	–/+	+++	+++
Prostate	+++	–/+	+++	+++

[a]Bisphenol A or octylphenol both tested at a single high dose (see Table II).
Intensity of immunoexpression is based on subjective, comparative evaluation, and ranges from weakly positive (+) to intensely positive (+++). Where some cells of the same type are negative but other different cell types in the same tissue are positive (see Figure 3), a –/+ score is shown. Cells/tissues which were immunonegative are indicated by –.

reproductive function in the male (Sharpe, 1998b). To test this possibility, animals were treated with DES (10 μg) alone or with DES (10 μg) + 200 μg testosterone esters (TE; Sustanon, Organon) or with the TE alone, using the same treatment protocol as shown for DES alone in Table II. Animals were then sampled at day 18 and the incidence of reproductive tract abnormalities was evaluated in conjunction with immunoexpression of AR.

The results obtained were clear-cut. Co-administration of TE with the DES prevented induction of virtually all of the reproductive tract abnormalities, such as epithelial underdevelopment, and this was accompanied by prevention of loss of AR immunoexpression (McKinnell *et al*., 2001). An example of this change is shown in Figure 3 for the seminal vesicles. Although the DES+TE treatment also prevented loss of DES-induced expression of AR in Sertoli cells (not shown; see McKinnell *et al*., 2001), it is not yet established whether any of the changes in testicular cell numbers induced by DES treatment alone are prevented by the TE co-treatment.

Discussion

As outlined in the Introduction, there is genuine concern as to whether or not the reported increase in human male reproductive disorders in recent decades might be a consequence of increased exposure of the fetus/neonate to environmental oestrogens or to oestrogens from other sources (e.g. from the mother during pregnancy). This possibility is hypothetical, but has led to great concern and scientific activity worldwide over the past decade, and in the USA has resulted in legislative changes that now demand testing and labelling of chemicals for their potential 'endocrine-disrupting' activity. Our approach to this issue has been to establish in the laboratory rat a biologically based understanding of how neonatal oestrogen exposure can affect development of the male reproductive system as well as establishing what chemicals and what doses can induce abnormalities of reproductive system development. The main reason for using the neonatal rat for these studies is that significant development and differentiation of the testis and reproductive tract occur during the neonatal period, and direct administration of the test compounds to the neonate is possible. In contrast, administration of high doses of potent oestrogens to pregnant rats (and thus to the male fetus) poses major practical problems, in particular the fact that even moderate doses of oestrogens can cause pregnancy failure or dystocia in the rat. Extrapolation of our findings to the fetus obviously needs to be considered cautiously, but in mice in which problems of dystocia are not encountered, major developmental abnormalities of the reproductive system (e.g. cryptorchidism, hypospadias, rete testis and epididymal abnormalities) are induced in the male offspring after administration of DES or other potent oestrogens during pregnancy (Arai *et al*., 1983; Newbold and McLachlan 1985; Newbold *et al*., 1986; Toppari *et al*., 1996). It is noteworthy that these studies also involved the administration of very high doses of DES (100 μg/kg or higher) to induce abnormalities in the male offspring, similar to our findings involving neonatal oestrogen treatment. Furthermore, as is discussed below, our evidence suggests that induction of reproductive tract abnormalities by DES administration neonatally is completely dependent on coincidental suppression of androgen action, a conclusion that again fits well with the type of reproductive tract abnormalities that are induced by in-utero exposure to potent oestrogens (see Table I).

The reproductive tract abnormalities that we have shown following neonatal treatment with a high dose of DES/other potent oestrogens are similar to changes reported by other workers in various species, but mainly in the rat and mouse. For example, distension/overgrowth of the rete testis (Newbold *et al*., 1986; Aceitero *et al*., 1998), relative overgrowth of stromal and undergrowth of epithelial tissue in the prostate (Prins, 1992; Pylkkänen *et al*., 1993), reduction in expression of AR in the prostate (Prins *et al*., 1993) and retarded development of Leydig cells (Abney, 1999). Our findings show that each of these oestrogen-induced changes is associated with—and is dependent on—coincident inhibition of androgen action via the suppression of expression of the AR. Though this finding might be interpreted at face value as evidence that DES is acting functionally as an 'anti-androgen', our findings are equally clear in demonstrating that none of the reproductive tract abnormalities that we have described, with the exception of retardation of testicular development/maturation, can be reproduced by simply suppressing androgen production (GnRH antagonist treatment) or by blocking androgen action via the neonatal administration of flutamide (McKinnell *et al*., 2001). It therefore appears that for DES and other potent oestrogens to induce major reproductive tract abnormalities there must not only be suppression of androgen action (due to reduced AR expression), there must also be coincident elevation of oestrogen action (McKinnell *et al*., 2001). Thus, administration of lower doses of DES did not induce major or widespread reproductive tract abnormalities, but it also did not induce major loss of AR expression either. Perhaps the most convincing piece of evidence to support the importance of collateral suppression of androgen action as a key factor in the DES-induced abnormalities come from our studies which show that co-administration of testosterone with a very high dose of DES is able to prevent most, if not all, of the abnormalities of the rete and reproductive tract that result from administration of the DES alone; thus in this situation, reproductive tract development is essentially normal despite the fact that there is massive exposure to oestrogens. The remarkable similarity in reproductive abnormalities induced by exposure *in utero* to anti-androgens/impaired androgen action on the one hand and those induced by high-dose oestrogen on the other hand (Table II), is consistent with a similar mechanism underlying the adverse reproductive effects induced in the male fetus, though this requires direct confirmation.

The fact that only very high doses of potent oestrogens are able to suppress AR expression explains why only such doses are able to induce reproductive tract abnormalities, but it does not explain the mechanism via which this suppression is induced. One recent study has produced evidence from cell transfection studies to indicate that ERα, but not ERβ, can interact directly with the AR to antagonise AR-mediated gene transactivation (Panet-Raymond *et al*., 2000) which raises one possible pathway. However, our studies show that, by both immunocytochemistry and Western analysis, the AR protein is not detectable or is grossly reduced in expression after high-dose DES treatment (McKinnell *et al*. 2001). This is perhaps more indicative of an effect of the DES on AR gene expression/protein production or

metabolism. Recent studies in ERα-knockout (ERKO) and ERβ-knockout (BERKO) mice administered DES, indicate that DES-induced reproductive tract abnormalities in both male and female animals occur in BERKO but not in ERKO mice, indicating that expression of ERα is an essential factor in mediating the adverse effects of DES (Couse *et al*., 2000). Some of our data would fit with this interpretation (Atanassova *et al*., 2001), and most sites in the male reproductive tract that are affected by DES treatment do express ERα in fetal/neonatal life (see Table III). However, it should also be noted that ERKO, but not BERKO, animals (both males and females) have supranormal androgen concentrations (Couse and Korach, 1999) and, in view of the present findings, it is possible that this might protect against the adverse reproductive effects of DES, at least in males.

Our limited studies with two candidate environmental oestrogens show that they are unable to induce loss of expression of AR or any of the gross reproductive tract abnormalities that are induced by administration of a high dose of DES. This negative result occurred despite the administration of extremely high doses (>30 mg/kg/day) over a 12-day period neonatally. In other studies we have shown that, in similarly treated animals, there is significant advancement of the onset of pubertal spermatogenesis, a change also induced by low (<4 μg/kg/day) doses of DES (Atanassova *et al*., 2000). The latter effects are consistent with the weak oestrogenicity of the environmental oestrogens that we have tested (bisphenol A, octylphenol), and the inability of these compounds to induce reproductive tract abnormalities is also consistent with their weak oestrogenicity. Our inability to detect any adverse reproductive effects after administration of these two compounds is consistent with some (Ashby *et al*., 1999; Cagen *et al*., 1999a,b; Chapin *et al*., 1999; Tyl *et al*., 1999) but not all (Lee, 1998; vom Saal *et al*., 1998; de Jager *et al*., 1999; Takao *et al*., 1999) reports in the literature, though it is emphasized that our studies have used different-aged animals and/or treatment regimens and have investigated different end-points compared with most of these published studies. It is emphasized also that our studies have used only a single high dose of either compound, and we are therefore unable to rule out the possibility that lower doses might, paradoxically, exert effects that these high doses do not. Even with this reservation in mind, we still feel that it is logical to predict that, based on our evidence that induction of major reproductive tract abnormalities requires both concurrent high oestrogen and low androgen action (McKinnell *et al*., 2001), most (and possibly all) of the environmental oestrogens so far identified will be incapable of inducing such effects, based on their oestrogenicity.

Somewhat surprisingly, our studies have also shown that low, as opposed to high, doses of DES can actually exert positive effects on the rat testis at around the time of puberty, and similar changes were induced by both weak environmental oestrogens, octylphenol and bisphenol A (Atanassova *et al*., 2000). In essence these effects represent a small advance in the onset of spermatogenesis. Such effects are consistent with the expression of ERβ in Sertoli cells and in most germ cells (Table III; Saunders *et al*., 2001), but it should be noted that in our studies FSH concentrations (Atanassova *et al*., 2000) were also elevated in the same treatment groups, and possibly testosterone concentrations (Figure 2), and both of these hormones play key roles in the activation of spermatogenesis. Therefore, some or all of the advancement of spermatogenesis could reflect indirect effects via elevation of FSH and/or testosterone concentrations. Nevertheless, a recent report (Ebling *et al*., 2000) showed that complete spermatogenesis can be induced by low concentrations of oestradiol in adult hypogonadal mice that lack GnRH without any significant change in blood concentrations of testosterone (though FSH was also increased). Similarly, in the human male there is evidence for premature activation of spermatogenesis by oestrogens emanating locally from an oestrogen-secreting tumour (Kula *et al*., 1996). Though these observations are lacking in detail they encourage the view that oestrogens might play a hitherto unknown role in spermatogenesis. The two environmental oestrogens that we investigated were both able also to advance the onset of pubertal spermatogenesis in rats, an effect that is consistent with their known level of oestrogenicity vis-à-vis DES (Atanassova *et al*., 2000), but both also elevated FSH and testosterone concentrations. Our studies have not established what is the lowest dose of 'oestrogen' that will exert stimulatory effects on spermatogenesis (via whatever route of action), which means that we are unable to dismiss the possibility that environmental oestrogens could induce 'positive' effects on the onset of spermatogenesis also in the human. However, it would be our expectation that extremely high levels of exposure to an oestrogenic compound would be required.

Based on our findings, we therefore conclude that weak environmental oestrogens are rather unlikely to pose a significant risk to the reproductive system of the developing human male unless the compound in question also possesses some other biological activity of relevance. In this regard, our findings would suggest that compounds identified as having anti-androgenic ± oestrogenic activity (e.g. phthalates; Mylchreest *et al*., 1999) would be the main concern. These conclusions are made tentatively but in due recognition of the following key points:

1. The processes and regulation of male reproductive development are highly conserved in mammals, so that extrapolation of findings from rodents to man is reasonable. A caveat is that rodents are born in a relatively under-developed state when compared with the human male so that some of the processes that occur neonatally in rodents occur *in utero* in the human. However, this does not mean that the processes are different, only that they occur with a different timing, and there is no reason to suppose that this difference renders the human male more susceptible to effects of environmental oestrogens. Indeed, the converse might be argued in view of the protected environment of the womb and the naturally high oestrogens of pregnancy in the human.

2. The adverse effects of in-utero exposure of the human fetus to high doses of DES administered to the mother are remarkably similar to those induced in comparable studies in pregnant mice, and this is equally true for both male and female offspring (see Newbold and McLachlan, 1985; Toppari *et al*., 1996).

3. Though human exposure data for most identified environmental oestrogens are lacking or are inadequate, there is general agreement that exposure to tens of milligrams per kg amounts of the compounds in question is unlikely in all but the most exceptional circumstances.

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