Institute of Experimental Morphology and Anthropology

Acta morphologica et anthropologica, 9
Sofia • 2004

Morphological, Quantitative and Functional Criteria for Evaluation of Estrogen Action and Disturbed Androgen-Estrogen Balance in the Male Rat Reproductive System

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In the present study we develop a complex system of morpho-functional criteria for identification and evaluation of the effect of estrogen action and disturbed androgen-estrogen balance in reproductive system of male developing rat. Severe retardation of testicular development is evident that involved inhibition of spermatogenesis, suppression of proliferation and functional maturation of Sertoli and Leydig cells. These changes could be result from direct effect DES on germ and Sertoli cells via ERβ and from indirect mechanism via gonadotropin suppression. Reproductive tract abnormalities include relative stromal overgrowth and epithelial underdevelopment and they are associated with loss of expression of AR and aberrant immunoexpression of ERα and induction of PR immunoexpression. These changes are result from disturbance of androgen-estrogen balance as they were prevented by co-administration with testosterone. In conclusion, our data provide a recent understanding for interaction between androgens and estrogens in normal male reproductive development.

Key words: estrogens, androgens, testis, epididymis, vas deferens.

Introduction

The incidence of disorders of human male reproductive health has more than doubled in the past 30 years while sperm counts have declined by about half. Similar abnormalities occur in sons of women treated with estrogenic hormones during pregnancy and they can be experimentally induced in animals by brief exposure to exogenous estrogens during perinatal life [14]. Hormones (mainly estrogens) determine subsequent risk of cancers of the male reproductive organs, e.g. testicular and prostate cancers. Environmental chemicals that can act as weak hormone agonists or antagonists cannot be ignored as a potential involvement in human reproductive disease [7].

There is growing evidence that estrogens play an important role in normal male reproductive development and function both are known to be androgen dependent. The physiological importance of estrogens is currently getting an increasing interest emerged from concerns that exposure to estrogens during perinatal life that disturb normal androgen/estrogen balance, can adversely affect male reproductive health [13]. A serious obstacle to address this concern is the absence of specific biological markers and crite-

ria for evaluation of estrogen action in the male. Our studies during the last few years found that disturbance of androgen/estrogen balance, in particular lowering the androgen side and at the same time elevating estrogen side, results in major reproductive system abnormalities in experimentally hormone manipulated animals. They involve suppression of germ cell development and Sertoli cell proliferation and maturation in the testis and abnormal structural and functional differentiation of male reproductive tract [1, 2, 3, 6, 8, 15, 17]. Based on our findings, the aim of the current work was to develop and to propose a complex system of morphological, quantitative and functional criteria for identification and evaluation of the effect of inappropriate hormone environment on male rat reproductive system resulting from neonatal estrogen exposure and disturbed androgen-estrogen balance. We also tried to distinguish estrogenic and anti-androgenic effect of diethylstilbestrol (DES, direct and indirect via gonadotropin suppression) from that resulting from disturbance of androgen/estrogen balance. The endpoints we used were: 1) quantitative parameters of spermatogenesis; 2) rete testis size; 3) epididymal epithelium cell high; 4) specific changes in expression of androgen (AR) and estrogen (ER) receptors.

Materials and Methods

The experimental manipulation of estrogen-androgen balance in male neonatal rats was performed as follow: beginning on postnatal day 2, rats were subjected to one of the following treatments administered by s.c. injection: a) DES at a dose of 10 or 0.1μg in 20μl corn oil on days 2,4,6,8,10 and 12; b) 10mg/kg of long acting GnRH-antagonist (GnRHa, Antarelix) in 20μl 5% mannitol on days 2 and 6; c) 10μg DES as in (a) + GnRHa as in (b); d) 0.1μg DES as in (a) + GnRHa as in (b); e) 50mg/kg of the AR antagonist flutamide in 20μl corn oil on days 2,4,6,8,10 and 12; f)) 0.1μg DES as in (a) + flutamide as in (e); g) 10μg DES as in (a) + 200μg testosterone at the same regimen; h) 20μl corn oil (vehicle) as control. Rats from all treatment groups were subsequently sampled on day 18, 25, 35, 75. Parraffin Bouin's fixed 5-μm testicular sections were used for cell quantification studies and visualization of apoptotic germ cells identified by TUNEL method as described previously [15]. Different testicular cell types were counted using 121-point eyepiece graticule and the data were used to determine the quantitative parameters of spermatogenesis [1, 2]. Leydig cell volume and number per testis was determined on sections immunostained for 3β-hydroxysteroid dexydrogenase (3β-HSD) [16]. The 3β-HSD positive nuclei and cytoplasm were scored separately by point counting and the data for absolute volumes (nuclear and cytoplasm) were collected. Measurement of efferent duct lumen area and epithelial cell height was performed using image analysis software "Image Pro Plus 4" [8]. Comparison of the different parameters for the various treatment groups were made using ANOVA. Immunohistochemical studies for inhibin-α, AR, ERα and progesterone receptor (PR) were performed on parraffin Bouin's fixed 5-μm sections from testis, epididymis and vas deferens of developing (d18, 25 and 35) and adult rats [3, 6, 15, 17].

Results

Our results clearly indicate that neonatal treatment of male rats with high doses of potent estrogens such as DES induced a range of developmental abnormalities in the testis and reproductive tract. Severe retardation of testicular development is evident, as it involved: 1) inhibition of spermatogenesis evaluated by the index of spermatogenic

Table 1. Morphological and quantitative criteria for identification and evaluation of the effect of neonatal estrogen action and disturbed androgen-estrogen balance in the testis and reproductive tract of male developing rat

CRITERIA	DES 10 μg	DES 10μg + TE	DES 0.1 μg	GnRHa	DES 0.1 μg + GnRHa	Flutamide	DES 0.1μg +Flutamide
TESTIS							
➢ Testis weight reduction	Major*	Moderate	Minor	Major*	Major	Moderate	Major
➢ Retardation of development	Major	Moderate	No	Major	Major	Moderate	Major
➢ Lumenal %volume reduction	Major	Major	Minor	Major	Major	Major	Major
➢ Sertoli cell number/volume reduction	Major*	Moderate	Minor	Major*	Major	Moderate	Moderate
➢ Total germ cell volume reduction	Major*	Moderate	No	Major ?*	Major	Moderate	Major
➢ Spermatogenic efficiency decrease	Major*	Minor	No	Major	Major	Moderate	Major
➢ Germ cell apoptotic index increase	Major*	Moderate	Minor	Major	Major	Minor	Moderate
➢ Leydig cell number/volume reduction	Major	No data	No	Major	Major	No data	No data
RETE TESTIS							
➢ Distension and overgrowth	Major*	No	No	No	Major	No	Moderate
EFFERENT DUCTS							
➢ Distension of lumenal area	Major	Moderate	Minor	Minor	Major	No	Major
➢ Epithelial cell height reduction	Major ?*	No	Minor	Moderate	Major	Minor	Major
EPIDIDYMIS							
➢ Relative stromal overgrowth + epithelial underdevelopment	Major*	No	No	Minor	Major	Minor	Moderate
VAS DEFERENS							
➢ Convoluted proximal VAS	Present*	No	Absent	Absent	Present	Absent	Not found
➢ Epithelial cell height reduction	Major	No	Minor	Moderate	Major	Minor	Minor

* - indicates changes that persist in adulthood

?* - indicates changes in adulthood that are not as pronounced as in developing animals

efficiency and total germ cell volume; 2) an increase in germ cell apoptosis; 3) suppression of proliferation (Sertoli cell volume and number per testis) and functional maturation of Sertoli cells (lumen formation, production of inhibin-α) and Leydig cells (Leydig cell volume and number per testis, testosterone production); 4) massive overgrowth/ distension of rete testis (Table 1, 3). Reproductive tract abnormalities include relative stromal overgrowth and epithelial underdevelopment and reduced epithelial cell height of the efferent duct, epididymis and vas deferens. All of these DES-induced changes are associated with a loss of expression of AR in Sertoli cells and rete testis as well as in the epithelial and stromal cells in efferent duct, epididymis and vas deferns (Table 2, 3). An aberrant immunoexpression of ERα was demonstrated, as normal switch from epithelial to stromal localization did not occur at the epididymal/vas boundary. Instead, an induction of ERα expression in vas epithelium caused uniform pattern of distribution in this region. Normally, the male reproductive tract is negative for PR but neonatal estrogenization induced pronounced immunoexpression of PR in stromal compartment of epididymis and vas deferens.

The neonatal administration of low dose DES (0.1 μg), which largely is ineffective on its own in inducing reproductive abnormalities, can induce a spectrum of adverse changes similar to high dose of DES (10 μg) if testosterone production (GnRHa administration) or action (flutamide administration) is suppressed at the same time (Table 1, 3). In contrast, the simple suppression of androgen production or action on its own is incapable of inducing the same reproductive abnormalities with an exception of the testis where GnRHa greatly suppressed its development and spermatogenesis as DES did whereas Flutamide exerted moderate effect.

Co-administration of testosterone (TE) with high dose of DES does prevent the induction of most of the abnormalities caused by DES. In contrast, suppression of testicular development and spermatogenic process as well as efferent duct lumenal distension was only partially prevented by co-administration of DES with TE. Measurements of TE and FSH levels showed elevated values of both hormones in DES 0.1 μg-treated rats and suppressed values due to single treatment with DES 10 μg or GnRHa and combined treatment of DES 0.1 μg + GnRHa (Table 1, 3).

Most of the testicular and reproductive tract abnormalities we found in the developing rats (day 18-35) still persist to adulthood that are testicular quantitative changes and an aberrant immunoexpression of AR and ERα in vas desferens.

Discussion

The mechanisms via which estrogens affect the male reproductive system are unclear. There are many similarities in the effects on the male reproductive tract produced by exposure to high levels of estrogens and those induced by interference with androgen production or action [12]. In terms of morphological and quantitative retardation of testis development our results revealed that the neonatal treatments with DES 10 μg or GnRHa are closely comparable indicating that gonadotropin suppression is a likely explanation for the observed adverse effects of estrogens. The strong evidence for this suggestion is our data on plasma FSH levels that paralleled the changes in pubertal spermatogenesis, i.e. lower FSH levels associated with retardation of spermatogenesis and higher FSH levels associated with advancement [2]. Another explanation for estrogen effect on spermatogenesis could involve direct action of estrogen on Sertoli and/or germ cells, as both cell types expressed ERβ [11] and direct adverse effect of high estrogen levels on Sertoli cells was reported [15]. This suggestion is reinforced by comparative analysis of DES- and GnRHa-induced changes in the testis by day 35 and

Table 2. Semi-quantitative assessment of the effect of neonatal estrogen treatment on the immunoexpression of AR, ERα and PR in the rat testis and male reproductive tract on day 18

TISSUE	ANDROGEN RECEPTOR		ESTROGEN RECEPTOR-α		PROGESTERONE RECEPTOR	
	CONTROL	DES-10 μg	CONTROL	DES-10μg	CONTROL	DES-10μg
TESTIS						
➢ Sertoli cells	+++	–/+	–	–	–	–
➢ Leydig cells	+++	–/+	++	++	–	–
➢ Peritubular cells	+++	+/++	++	++	–	–
RETE TESTIS	+++	–/+	++	–/+	–	–
EFFERENT DUCTS						
➢ Epithelial cells	+++	–/+	+++	+++	–	–
➢ Stromal cells	++	–/+	–	+	–	–
EPIDIDYMIS						
➢ Epithelial cells	+++	–/+	+	+	–	–
➢ Stromal cells	++	–/+	–	+	–	+
VAS DEFERNES						
➢ Epithelium	+++	–/+	–	++	–	–
➢ Periductal stroma	++	–/+	++	–/+	–	–/+
➢ Smooth muscle muscularis	++	–/+	–	++	–	+++

Table 3. Changes in immunoexpression of steroid receptors (AR, ERα, PR) as functional criteria for identification and evaluation of the effect of neonatal estrogen action and disturbed androgen/estrogen balance in testis and reproductive tract of male developing rat

CRITERIA	DES 10 μg	DES 10 μg + TE	DES 0.1 μg	GnRHa	DES 0.1 μg + GnRHa	Flutamide	DES 0.1 μg +Flutamide
TESTIS							
➢ AR	Abolished	Normal (N)	N	Almost N	Reduced	Slightly reduced	Slightly reduced
➢ ERα	N	N	N	N	N	N	N
➢ Inhibin-α	Reduced	N	N	Reduced	Reduced	No data	No data
RETE TESTIS							
➢ AR	Abolished	N	N	N	Reduced	N	Slightly reduced
➢ ERα	Abolished	N	N	N	Reduced	N	N
EFFERENT DUCTS							
➢ AR	Abolished	N	N	Almost N	Reduced	N	Reduced
➢ ERα	N**	N	N	N	N**	N	N**
EPIDIDYMIS AND VAS DEFERNES				N			
➢ AR	Abolished*	N	N	N	Reduced	N	Reduced
➢ ERα	N pattern changed*	N pattern	N	N	Almost N **	N	Almost N**
➢ PR	Induced	N(absent)	N		Induced	N	Slightly reduced

* - Indicates changes that persist in adulthood and are not as severe as that found on day 18.
** - Normal pattern of epithelial immunoexpression but intertubular stroma is positive compared to lack of immunostaining in controls.

adulthood, as we reported an improvement/recovering of spermatogenesis in GnRHa-treated rats compared to DES-animals [1, 15]. It is obviously difficult to disentangle direct from indirect effect of estrogens on the testis as the reduced Sertoli cell proliferation due to direct effect of DES on Sertoli cell via ERβ then resulted in inhibition of the FSH-stimulated intracellular signaling pathway that normally up-regulate Sertoli cell division.

The present study provides strong evidence that DES-induced disorders of reproductive tract development in the male result from disturbance of the balance between androgen and estrogen action rather than the absolute levels of either hormone alone. Morphological and functional abnormalities of the reproductive tract such as rete testis overgrowth, efferent duct lumenal distension reduced epithelial cell height of the reproductive tract as well as concomitant loss of AR protein expression [6] and aberrant immunoexpression of ERα in the epididymis and vas deferens [3] are changes that fall into this category. We demonstrated that none of the abnormalities induced by DES 10 μg can be induced by alteration of androgen production (GnRHa treatment) or action (Flutamide treatment) on their own. Combined treatment with low dose of DES (0.1 μg), that fail to produce adverse effect, plus either GnRHa or Flutamide is able to induce most of the abnormalities cause by DES 10 μg with the similar magnitude [8]. Interestingly, we found that such combined treatment did not exacerbate the effect on the epithelial cell height of vas deferens as did in the efferent duct and epididymis suggesting that there is different response of epithelial cell from anterior and posterior parts of reproductive tract to altered androgens and estrogens. Our data that treatment with DES 10 μg + GnRHa had no discernibly greater effect than either treatment alone are suggestive for the importance of androgen-estrogen balance. The role of that balance for normal male reproductive development is also demonstrated by our results that restoration of the balance by co-administration of DES 10 μg with testosterone prevents induction of most reproductive abnormalities cause by DES [9]. There were two exceptions – quantitative parameters of spermatogenesis and efferent duct lumenal area that were partially prevented by TE and they indicate the importance of estrogen action per se rather than androgen-estrogen balance. Efferent duct is known to be a major site of estrogen action with highest levels of expression of ERα within the male reproductive tract [4] a finding reinforced by studies on ERα-knockout mice [5].

In our studies we were unable to induce DES-like effect on ERα immunoexpression in the epididymis and vas deferens of rats treated with DES 0.1 μg + GnRHa or DES 0.1 + Flutamide. These results are in agreement with previous results suggested that estrogen-induced changes in ER expression were clearly demonstrable in this tissue [10]. Although our various findings emphasize that DES treatment has major effects on androgen production and action [6, 16] we have also shown that treatment with 10 μg of DES can alter estrogen action by affecting the distribution of ERα [3].

In conclusion, developing a complex system of morpho-functional criteria for evaluating the importance of estrogen action and androgen-estrogen balance we provide a recent understanding for interaction between androgens and estrogens in normal male reproductive development. We support the idea that induction of changes in the pattern of androgen and estrogen receptors in the developing male reproductive system is probably involved in the mechanisms of induction of morphological and quantitative abnormalities.

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