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Evaluation of Neonatal Estrogen Action on Rat Spermatogenesis in Adulthood: Comparative Relation to the Effect of DES in Puberty

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Several environmental contaminants are known to mimic natural hormones and thereby interfere in hormonal balance. After binding to hormonal receptors endocrine disrupters give rise to reproductive abnormalities resulting in male infertility. In this respect **the aim** of the study was to quantify the different stages of germ cell development in tandem with the changes in supporting function of the Sertoli cell toward different germ cell (GC) types in adult rat. We used experimental model for manipulation of neonatal hormonal environment by treatment with potent estrogen diethylstilbestrol (DES) in different doses or GnRH antagonist (GnRHa). Neonatal exposure to high dose of DES (10μg) greatly affected testis development and spermatogenesis in rat whereas GnRHa was quite less effective in producing negative impact in adulthood. Quantitative evaluation of spermatogenesis demonstrated that the most differentiated GC population - spermatids was the most sensitive to hormonal manipulation compared to spermatogonia and spermatocytes. Despite indirect gonadotrophin mediated effect we suggest direct estrogen action on GC differentiation.

Key words: estrogens, androgens, spermatogenesis

Introduction

The mammalian testis is a complex organ with two important functions: synthesis of male sex steroid hormones (testosterone) and production of male gametes (spermatozoa). The endocrine control of these functions involved gonadotrophins and locally synthesized steroids, among them androgens and estrogens [3]. Many reports in the literature demonstrate that the exposure of male rodent and/or human to exogenous estrogenic compounds during fetal/neonatal period suppress FSH (and probably LH) secretion at a time when this hormone is playing an important role in testicular development [9]. Besides this indirect pathway of action direct mechanism of estrogen action is suggested. The role of etrogens in the physiology of male reproductive tract has been

a subject of debate for a long time, even thought more and more evidence suggests that estrogens are involved via their specific receptors - α (ER-α) and β (ER-β) [4]. In prenatal Leydig cells ER-α is expressed before androgen receptor (AR) suggesting that estrogens may have a significant role very early in the testicular differentiation process in fetal life. Expression of ER-β in gonocytes, Sertoli and Leydig cells until birth was also reported. Around the time of birth the testis continues to express both ER subtypes and aromatase. In adult testis ER-α is restricted to Leydig cells whereas ER-β is widely distributed confined to the Leydig cells, peritubular cells, Sertoli cells and some populations of germ cells- spermatogonia (Sg), late primary spermatocytes (Sc) and round spermatids (Sd). This data support the hypothesis of direct estrogen action as on somatic cells (Sertoli cells, peritubular and Leydig cells) as well as on germ cells in the testis [1]. Several environmental contaminants are known to mimic natural hormones and thereby interfere in hormonal balance. By binding to hormonal receptors during fetal and postnatal life, the endocrine disrupters give rise to reproductive abnormalities (cryptorchidism, hypospadias, low sperm count, epididymal cysts) persisting to adulthood [12]. All studies in the literature concerning the importance of hormonal balance in regulation of spermatogenesis, have investigated Sertoli and total germ cell population. The mechanism of estrogen action on different stages of male germ cell development is poorly investigated. The absence of information about this problem requires implementation of profound study that would elucidate our understanding about the mechanisms via which estrogens regulate particular phases of spermatogenesis (mitotic, meiotic and postmeiotic stages). In this respect **the aim** of the present study was to quantify the different stages of germ cell development in tandem with the changes in supporting function of the Sertoli cell toward different germ cell types in mature rats. That would reveal their differential sensitivity to potent synthetic estrogen DES.

Materials and Methods

Wistar rats were maintained under standard conditions. Beginning on postnatal day 2, rats were subjected to one of the following treatments administered by s.c. injection: a) DES at doses of 10 μg or 1 μg or 0.1 μg in 20 μl corn oil on days 2, 4, 6, 8, 10 and 12; b) 10 mg/kg of long acting GnRH-antagonist (GnRHa, Antarelix) in 20 μl 5% mannitol on days 2 and 6; c) 20 μl corn oil (vehicle) as control. To evaluate the effects of phytoestrogens on male offspring there were used adult female Wistar rats fed on soy-free diet at least 3 weeks before copulation, during pregnancy and lactation. Their male pups continued independently with soy–free diet from 21 pnd to their sacrifice. Rats from all treatment groups were subsequently sampled on day 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 [11]. Different testicular, in particular germ cell types were counted using 121-point eyepiece graticule and the data were used to determine the quantitative parameters of spermatogenesis [2]. Using a systematic clock-face sampling pattern from random starting point, 32 fields were counted. Points falling over the nuclei of Sertoli cells, spermatogonia, spermatocytes and spermatids (apoptotic and viable) or over seminiferous tubule lumen and interstitium were scored and expressed as a percentage of the total points counted (3872). For each animal, the values for percent nuclear volume were converted to absolute nuclear volumes per testis by reference to testis volume (=weight), as shrinkage was minimal, i.e. testis weight before and after fixation were comparable in each treatment group. Comparison of the different parameters for the various treatment groups was made using Student's t- tests.

Results and Discussion

Histological observation of 75-day old rat testis showed presence of mature spermatozoa and their release into the tubular lumen in stage VIII of spermatogenic cycle, that is an evidence for completed spermatogenesis. The spermatogenesis is organized in 14 stages of classification of Clermont and Perey [5] and germ cells are arranged in 5-6 layers (Fig.1). There are many similarities in the effects on the male reproductive system produced by exposure to high levels of estrogens and those induced by gonadotrophin suppression which in turn inhibit testosterone production [10]. Some changes in testis weight (TW), germ cell number and luminal percent volume during pubertal development were reported in our previous papers, [6; 7] and in the current study we found them to persist in adulthood. They concerned dose-dependant effect of DES especially the negative alterations of high dose of 10 μg (Tabl.1). Neonatal treatment with any of the three doses of DES resulted in significant decrease in adult TW. Neonatal suppression of gonadotrophin levels via administration of GnRHa reduced TW to a similar extend as DES-10 (almost 50%) but caused weaker reduction in GC-volume per testis than did high dose of DES (45% reduction by DES-10 and 22% by GnRHa – Fig.2). In animals treated with GnRHa, DES-1, or 0.1 μg the gross morphology of the testis was comparable to that in controls. On day 75 in rats treated with DES-10 we observed thinner seminiferous epithelium with less germ cells in the tubules. At some places SCO-tubules (Sertoli-cell-only) were found and as well as tubules with increased numbers of degenerating/apoptotic germ cells. DES-10 exert adverse effects on the lumen manifested by 45% expansion of luminal volume resulted from disturbed absorbtion and accumulation of seminiferous fluid in the rete testis and efferent ducts. Accumulated fluid exerts pressure in seminiferous tubules, probably responsible for

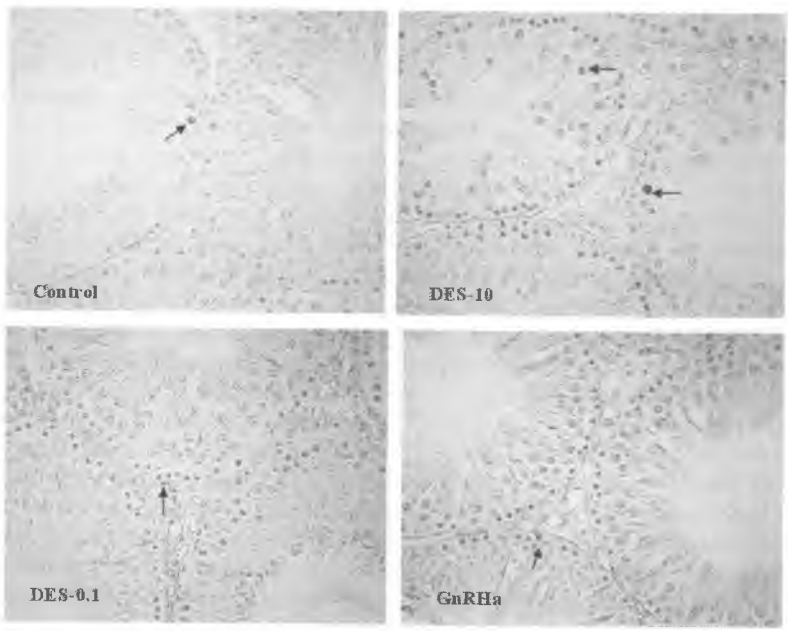

Fig. 1. Morphology of testis in rat (adulthood, day 75) in control and experimental animals, treated neonatally with different doses DES or GnRHa. TUNEL positive apoptotic cells (arrows) X 400.

Table. 1. Changes in testis weight and lumenal % volume of seminiferous tubules in adult rat testis (day 75).

	Testis weight (mg)	Lumen (% volume)
Control	1922.33 ± 30.18	18.53 ± 2.13
GnRHa	1133.25 ± 51.19	18.74 ± 1.52
DES-10	966.00 ± 31.24	26.70 ± 5.94
DES-1	1454.25 ± 141.44	21.00 ± 2.76
DES-0.1	1737 ± 79.61	16.40 ± 3.26

thinner seminiferous epithelium compared to control. Our previous data [6; 7] demonstrated that DES induced dose-dependent reduction in lumen formation, indicative for impaired Sertoli cell function. The effect of GnRHa is compared to that of DES-10 in early puberty (day 18) whereas GnRHa is less effective in late puberty (day 35).

In attempt to elucidate our understanding on the direct/indirect effects of oestrogens we focused our study on detailed characteristics of germ cell types as they are supposed to be a target for direct estrogen action. Quantitative study shows that after neonatal estrogen manipulation (reduced androgen and increased estrogens levels) the most sensitive germ cell populations in the testis were the most differentiated germ cells – spermatids (Sd) (Fig.2). After DES-10 their absolute nuclear volume (ANV) was reduced with 50% whereas GnRHa decreased Sd number to considerably lower extend, about 20% compared to control. Our data for differential effects of DES-10 and GnRHa on spermatid population are suggestive for direct estrogen action mediated by estrogen receptors (ER-β) [8]. In contrast to DES-10 after GnRHa treatment we found gradually restoration of spermatogenesis in adult rats. In adulthood we established that populations of spermatogonia and spermatocytes underwent similar reduction by DES-10 and GnRHa whereas during puberty spermatocytes were more affected by DES compared to GnRHa (Fig.2). Enumeration of Sg and Sc populations on day 75 after treatment with DES-10 showed reduction with respectively 43% and 40% than control. GnRHa induced respectively 37% and 20% decrease of absolute volume of both cell types. No significance was found in the effects of DES-10 and GnaRHa on Sg and Sc. Therefore the most differentiated germ cell type at each age/stage of postnatal development seems to be the most sensitive type to hormonal disbalance. Interestingly, in contrast to pubertal development a positive effect of lower doses of DES-0.1 emerged in adulthood. We established elevation in mean values of total GC-nuclear volume (20%), total Sc (50%) and total Sd (8%) compared to controls. The function of Sertoli cells to support GC, known as efficiency of spermatogenesis, was evaluated by estimation of ANV of germ cells per unit Sertoli cell ANV (data not shown). The ratios between GC polulations and Sertoli cells were considerably changed by total GC or any of the tree subtypes (Sg, Sc and Sd).

To identify the sensitivity of different subtypes GC to neonatal induced androgen/estrogen disbalance we applied detailed stereological analysis. In adulthood A-spermatogonia were more vulnerable compared to more differentiated Sg (In and B) (Fig.3). The number of A-Sg was similarly diminished after DES-10 and GnRHa treatment (about 40%). The lowest dose of estrogen shows values relatively similar to control. On the base of comparative analysis of our data we assume time dependent sensitivity of particular types of Sg, e. g. more advanced types are more vulnerable at puberty compared to adulthood when A-Sg are more sensitive to estrogens. Dose-dependent effect of DES on Sg population and its subtypes was emerged in adulthood – DES-1 was less

effective and DES-0.1 failed to produce effect. Similar tendency was observed in earlier ages (day 18 and 35).

In the populations of primary spermatocytes we established that the most differentiated subtype (pachytene and diplotene Sc) was the most affected by neonatal hormonal manipulations – their number were reduced two times (50%) after DES and 1.3 times after GnRHa (about 22%) (Fig.4). Significant difference was established between

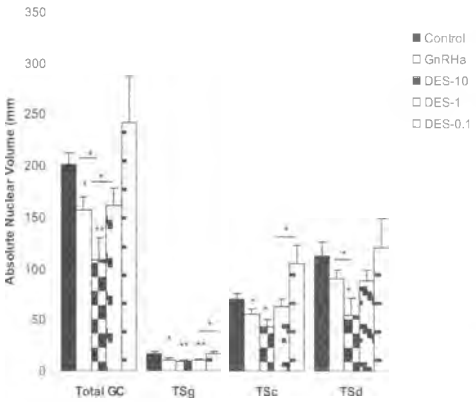

Fig. 2

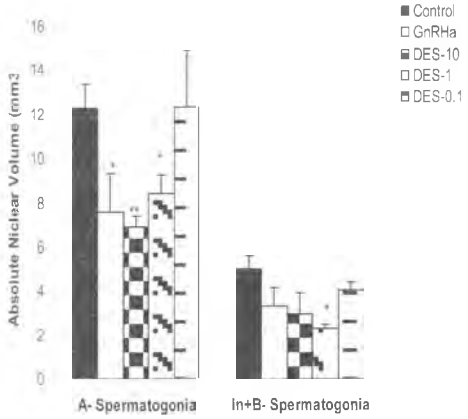

Fig. 3

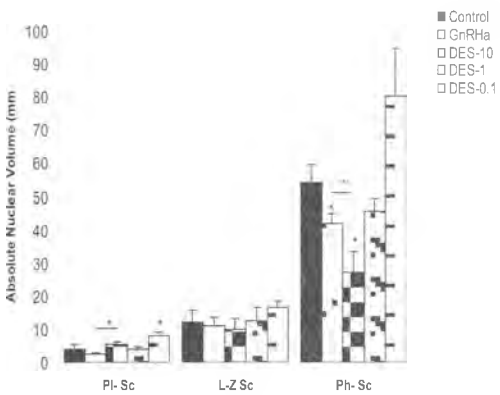

Fig. 4

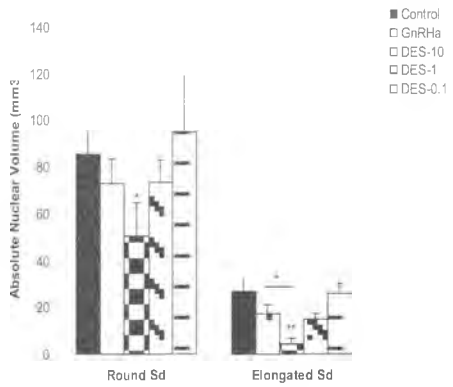

Fig. 5

Fig. 2. Quantification of spermatogenesis on day 75 of control and neonatally treated rats with GnRHa; DES-10; 1 or 0.1 μg. Absolute nuclear volume (mm^3) of different GC types; Data represent mean value ± SE (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). TGC- Total germ cells; TSg-Total spermatogonia; TSc-Total spermatocytes; TSd- Total spermatids;

Fig. 3-5. Quantification of absolute nuclear volume (mm^3) of subtypes of spermatogonia (Fig. 3), spermatocytes (Fig. 4) and spermatids (Fig. 5) on day 75 of control and neonatally treated rats with GnRHa; DES-10; 1 or 0.1 μg. Data represent mean value ± SE (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Pl Sc-preleptotene spermatocytes; L-Z Sc- Leptotene and zygotene Sc; Ph Sc- pachytene Sc.

DES and GnRHa data. Differential effect of DES-10 and GnRHa on advanced Sc types is suggestive for direct estrogen action in addition to indirect action via SC. Even meiosis of germ cells occurred beyond blood-testis barrier (established by SC) the Sc are target for direct estrogen action as they posses ER-β [12]. Similarly to Sg, dose-dependent effect of DES on Sc population and its subtypes was emerged in adulthood, suggestive for importance of estrogen action on all the stages of meiosis.

Quantitative analysis of spermatid subtypes (round and elongated Sd) demonstrated that DES-10 induced strong reduction of elongated Sd ANV (more than 6 times) compared to that of round Sd (40%). (Fig.5). Our results are in concert with general concept that more advanced GC types are more vulnerable to neonatal estrogen exposure. GnRHa treatment failed to produce any negative changes on Sd population and its subtypes suggestive for direct estrogen action on postmeiotic stages of spermatogenesis. Sertoli cell support toward elongated Sd was disturbed after estrogen administration and even being positive to round Sd after GnRHa treatment. Our data have yielded important information suggesting that relatively brief neonatal estrogen exposure can permanently and dose dependently alter the meiotic and postmeiotic germ cell development. It is well established in rats that androgen exerts its support on spermatogenesis at stages VII-VIII of the spermatogenic cycle and hence inadequate levels of testosterone within the testis result in appearance of more apoptotic GC compared to control. Certainly, that is one of the many causes but not the only one, responsible for impaired spermatogenesis. Despite it is difficult to distinguish the direct from indirect mechanism of estrogen action, our findings about differential sensitivity of particular steps of spermatogenesis to estrogen action and hormonal dis-balance would contribute to better understanding about the of importance of fine balance between estrogens and androgens for male reproduction.

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