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MORPHOLOGICAL AND QUANTITATIVE CHANGES IN RAT SEMINIFEROUS EPITHELIUM AT PUBERTY AFTER NEONATAL ESTROGEN EXPOSURE

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ABSTRACT. The present study aimed 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 and to evaluate the direct effect of estrogen on particular phases of spermatogenesis. Rats were neonatally treated with DES-10µg or GnRHa and testes were sampled on day 18 for quantitative analysis by point counting and image analysis. Neonatal treatment with DES and GnRHa caused similar adverse changes in the testis involving decrease in testis weight, reduction of seminiferous tubule area and mean diameter, suppression of lumen formation (% volume).DES but not GnRHa induced major overgrowth and lumen distension of rete testis. DES and GnRHa greatly suppressed germ cell development and Sertoli cell supporting function for spermatogenesis (Germ/Sertoli cell ratio). Meiotic germ cells (spermatocytes) were more affected than mitotic cells (spermatogonia) in both treatments. The effect of DES on germ cell differentiation (spermatocytes/ spermatogonia ratio) and Sertoli cell support for spermatocytes were more severe than those of GnRHa. In conclusion differences in effects of DES and GnRHa on spermatogonia and spermatocytes demonstrated differential sensitivity of mitotic and more advanced meiotic stages of spermatogenesis to neonatal hormonal dis-balance and different mechanisms are probably involved in mediation of effects of estrogens and gonadotropic antagonists.

KEY WORDS. estrogens, Sertoli cells, germ cells, testis, rat

INTRODUCTION

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 cause delayed development of the testis and permanent impairment of spermatogenesis adversely affecting male reproductive health (Arai et al., 1983, Atanassova et al., 1999). Neonatal administration of estrogens suppressed FSH production at the time when this hormone is essential for initiation of spermatogenesis at puberty (Bellido et al., 1990). For that reason in earlier studies the negative effect of estrogens were explained as a results of suppression of gonadotropin secretion during the treatment. Our comparative studies from the recent past on the effects of neonatal treatment with diethylstilboestrol (DES) or GnRH-antagonist (GnRHa) suggested direct and permanent effect of estrogens on the testis (Atanassova et al., 1999; Atanassova et al., 2003) mediated via estrogen receptor beta (ERβ) localized in germ and Sertoli cells (Saunders et al., 1998). Severe retardation of testicular development induced by DES involved elevation of germ cell apoptosis, inhibition of spermatogenesis evaluated by total germ cell nuclear volume and spermatogenic efficiency (germ/Sertoli cell ratio) (Atanassova et al., 2000). These abnormalities are associated with suppression of plasma testosterone levels and reduced Leydig cell number (Sharpe et al., 2003).

Quantification of spermatogenic development was performed on main testicular cell types, namely germ, Sertoli and Leydig cells and there were no data in the literature about DES effect on the kinetics of different germ cell types as spermatogonia and spermatocytes. 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. These data would elucidate our understanding about the mechanisms via which estrogens regulate particular phases of spermatogenic development.

MATERIAL 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 a dose of 10 μg 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) 20μl corn oil (vehicle) as control. Rats from all treatment groups were subsequently sampled on day 18. 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 (Sharpe et al., 1998). 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 (Atanassova et al., 1999). Using a systematic clock-face sampling pattern from random starting point, 25 fields were counted. Points falling over the nuclei of Sertoli cells, spermatogonia and spermatocytes (apoptotic and viable) or over seminiferous tubule lumen and interstitium were scored and expressed as a

percentage of the total points counted (3025). 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. Measurement of seminiferous tubule area and diameter was performed using image analysis software Axio Vision LE Rel. 4.1. Comparison of the different parameters for the various treatment groups was made using Student's t- tests.

RESULTS

Neonatal treatment with DES-10 μg or GnRHa caused more than 3-fold decrease in testis weight on day 18 compared to the control value (Tabl.1). The mean diameter and area of seminiferous tubules was reduced in both treatment groups. The formation of seminiferous tubule lumen as indicated by the luminal percent volume, was dramatically suppressed by both treatments and the effect of DES was more pronounced (7-fold decrease than control) compared to that of GnRHa (4.5-fold decrease). The luminal contour is easily distinguishable in the controls on day 18 whereas in DES and GnRHa testes the lumen area in most cases was filled with Sertoli cell cytoplasm. The percent volume of the testicular interstitium was slightly decreased in GnRHa whereas a slight increase was found in DES treated rats. The morphology and size of rete testis was greatly affected by DES but not GnRHa treatment (Fig. 1). Major overgrowth and luminal distension of the rete was observed in DES treated animals and as a result rete testis occupy a large portion of testis cross section.

Both the neonatal treatments, DES and GnRHa induced obvious morphological changes in the structure of the seminiferous epithelium and germ cell composition. In the controls on day 18 germ cell development proceeds to the late pachytene stage of meiotic prophase and numerous spermatocytes were present in the seminiferous tubules organized in 4 stages based on different germ cell associations. In treated animals with DES or GnRHa spermatogenesis was greatly affected and only few spermatocytes can be found in the tubules (Fig.2). Quantification of testicular cell types demonstrated more than 2-fold decrease in Sertoli cell absolute nuclear volume (ANV) in both treatment groups and total germ cell ANV was 4- and 5-time lower than control in GnRHa and DES rats, respectively (Fig.3). More general, DES and GnRHa caused more severe decrease in ANV of spermatocytes than that of spermatogonia, as compared to control value. Nuclear volume of spermatogonia was equally affected in both experimental groups (3-fold decrease), whereas spermatocyte volume decrease more in DES (10-fold) compared to GnRHa (6-fold). Hence, the ratio of spermatocytes/spermatogonia decreased in larger extent in DES (3.4-fold) than GnRHa (2-fold). The function of Sertoli cells to support germ cells, known as efficiency of spermatogenesis, was evaluated by estimation of ANV of germ cells per unit Sertoli cell ANV (Fig.4). Supporting function of Sertoli cells toward total germ cell population was significantly suppressed by both treatments and in DES animals this reduction was more pronounced (2 times lower than control) than GnRHa (1.5times). The ratio between spermatogonia and Sertoli cells were equally reduced

in both experimental groups whereas Sertoli cell support toward spermatocytes was more affected by DES (4-fold decrease that control) than GnRHa (2-fold). The differences between mean values of DES and GnRHa concerning spermatocytes were significant.

DISCUSSION

In the present study we performed a detailed comparative analysis of germ cell development on the rat testis in animals treated neonatally either with GnRHa or with a potent synthetic estrogenic compound (DES) in order to established if the adverse effect of DES could be attributed simply to the suppression of FSH that occurs during neonatal estrogen treatment (Arai et al., 1983, Bellido et al., 1990). We also considered that differences observed between these two treatments would provide a means of establishing whether the developing/differentiating germ cells are important target for estrogen action. Our data bring support for the role of gonadotropin suppression in many, but not all, of the adverse testicular changes induced by neonatal treatment with high dose of DES. We have not excluded the possibility that estrogen effects within the testis may negatively impact on the same signaling pathways as those activated by gonadotropins.

There are many similarities in the effects on the male reproductive system produced by exposure to high levels of estrogens and those induced by gonadotropin suppression which in turn inhibit testosterone production (Sharpe, 2003). In terms of morphological and quantitative retardation of the testis postnatal development, our data demonstrated that neonatal treatment with DES-10 μg or GnRHa are closely comparable, indicating that gonadotropin suppression is a likely explanation for the observed negative effects. Reduction of macro-parameters (testis weight, seminiferous tubule area and mean diameter, luminal percent volume) and cellular endpoints (Sertoli cell ANV, total germ cell ANV) are changes in support of this conclusion. Another strong evidence for this suggestion is our previous data (Atanassova et al., 2000) on plasma FSH levels that paralleled the changes in spermatogenesis (i.e low FSH levels associated with retardation of spermatogenesis and high FSH levels – with advancement).

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 (Saunders et al., 1998) and direct adverse effect of high estrogen levels on functional maturation of Sertoli cells was reported (Sharpe et al., 1998). Our present data for induction of major overgrowth of rete testis in DES but not in GnRHa could be interpreted as evidence for direct estrogenic effect. Moreover, loss of androgen receptor in the testis in DES treated animals but not in GnRHa is a finding that falls in the category of direct changes (McKinnel et al., 2001). This suggestion is reinforced by comparative analysis of DES- and GnRHa-induced changes in the testis by day 35 and adulthood, as we reported an improvement/recovering of spermatogenesis in GnRHa-treated rats compared to DES animals (Sharpe et al., 1998, Atanassova et al., 1999). 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.

In attempt to elucidate our understanding on the direct/indirect effect of estrogen we focused our present study on detailed characteristics of germ cell types as they are known to be a target for direct estrogen but not FSH action. Comparative analysis of quantitative changes in spermatogonia and spermatocytes in DES-10 μg and GnRHa testes provide new opportunity to reveal differential effects of both treatments and to access direct estrogen effect on spermatogenic development. Spermatogonial ANV is equally affected by both treatments whereas spermatocytes are more vulnerable to DES rather than GnRHa. Germ cell differentiation evaluated by spermatocyte/spermatogonia ratio was more retarded in DES than GnRHa. The function of Sertoli cells to support germ cells is more suppressed by DES than GnRHa and Sertoli cell support for spermatocytes is twice lower in DES than that in GnRHa. These data indicated that in contrast to spermatogonia, the more advanced germ cell types (spermatocytes) of developing testis are possible target for direct estrogen action in addition to FSH indirect effect mediated via Sertoli cells. Even germ cell meiosis occurred beyond blood-testis barrier (established by Sertoli cells) the spermatocytes are accessible for estrogens as they possess ERβ. Differential effect of DES and GnRHa on spermatogonia and spermatocytes demonstrated differential sensitivity of mitotic and meiotic stages of spermatogenesis to hormonal dis-balance and different mechanisms probably were involved in mediation the effect of estrogens and gonadotropic antagonists. In conclusion, data in the present study could improve our understanding for mechanisms of action of estrogens on germ cell development and differentiation and as well as the role of estrogens for particular steps of spermatogenesis.

Table. 1 .Measurements of testis weight, seminiferous tubule parameters (diameter, lumen) and interstitium in 18-day old control and treated rats with DES-10 μg and GnRHa. Data represent mean value ± SE. All the differences between control and treated rats are significant.

Parameters	Control	DES-10	GnRHa
Testis Weight (mg)	78.05 ± 4.22	24.24 ± 1.70	21.40 ± 1.53
ST Diameter (μm)	116.11± 1.70	89.30 ± 5.44	87.45 ± 1.04
ST Lumen (%Vol)	7.18 ± 0.77	1.03 ± 0.26	1.56 ± 0.15
Interstitium (%Vol)	19.82 ± 0.47	25.70 ± 1.13	16.82 ± 0.79

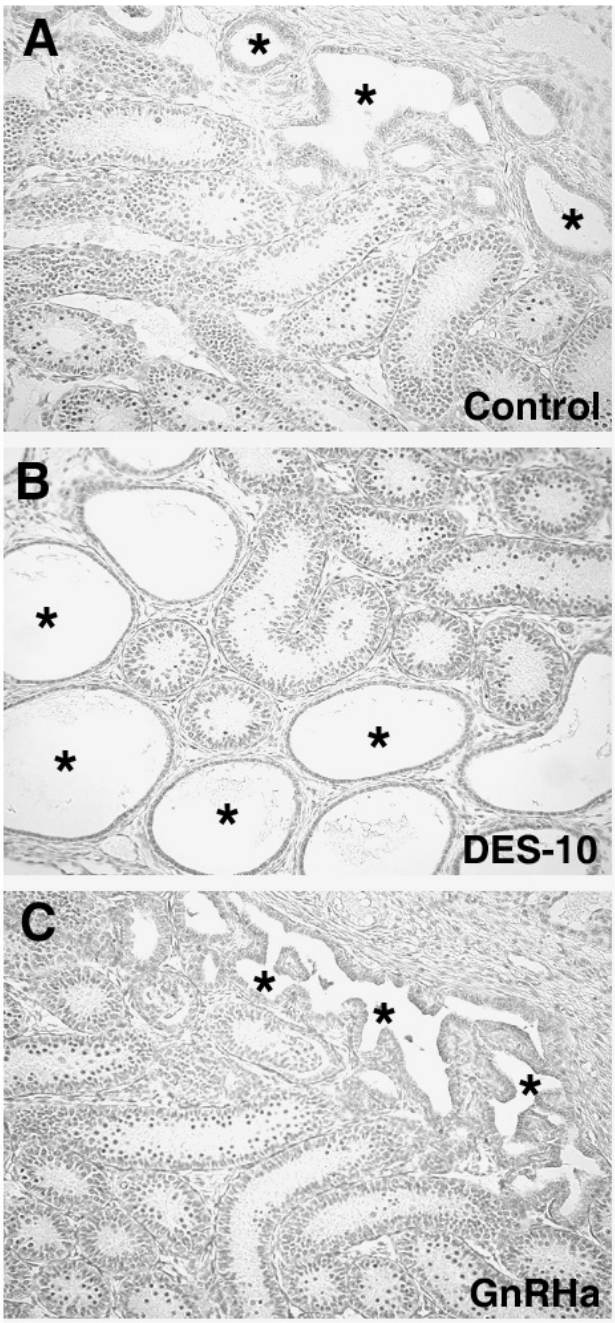

Fig.1. Morphology of the rete testis (asterisks) on testis cross section of 18 day-old rats x200. A – Control testis; B – Testis treated with DES-10 μg. Note overgrowth of rete testis area and luminal distension; C – Testis treated with GnRHa.

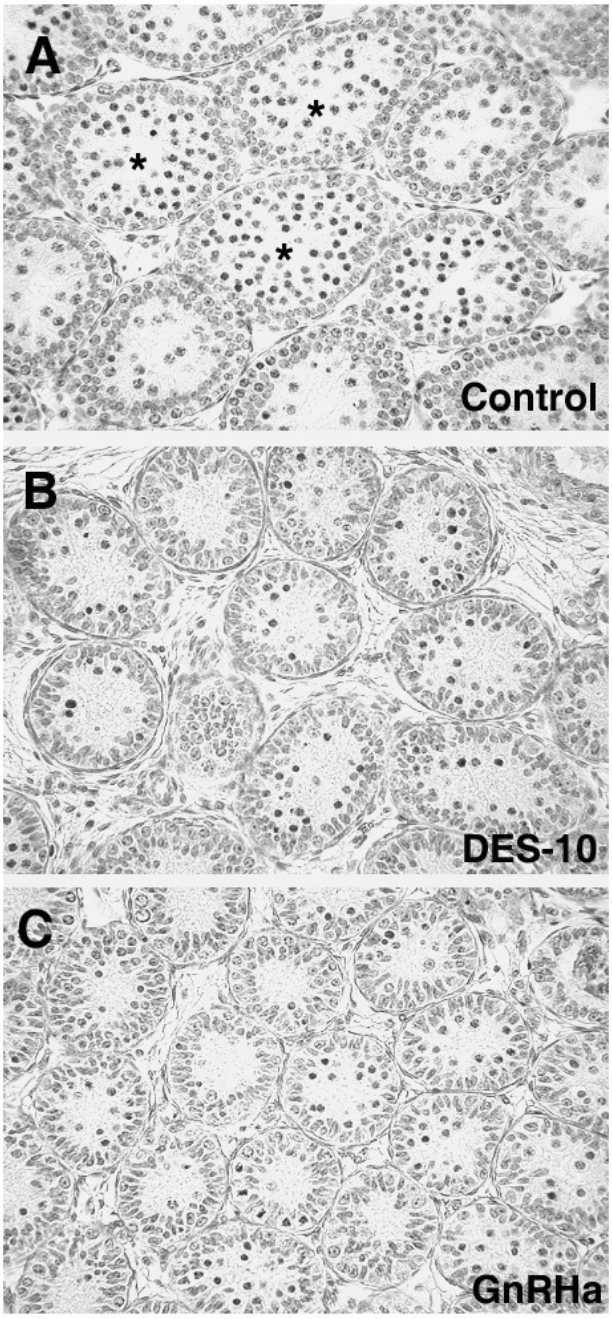

Fig.2. Morphology of the seminiferous tubules on testis cross section of 18 day-old rats x400. A - Control testis. Note numerous germ cells and lumen (asterisks) in the seminiferous tubules; B - Testis treated with DES-10 μg; C – Testis treated with GnRHa. Note suppression of spermatogenesis indicated by fewer germ cells and lack of lumen.

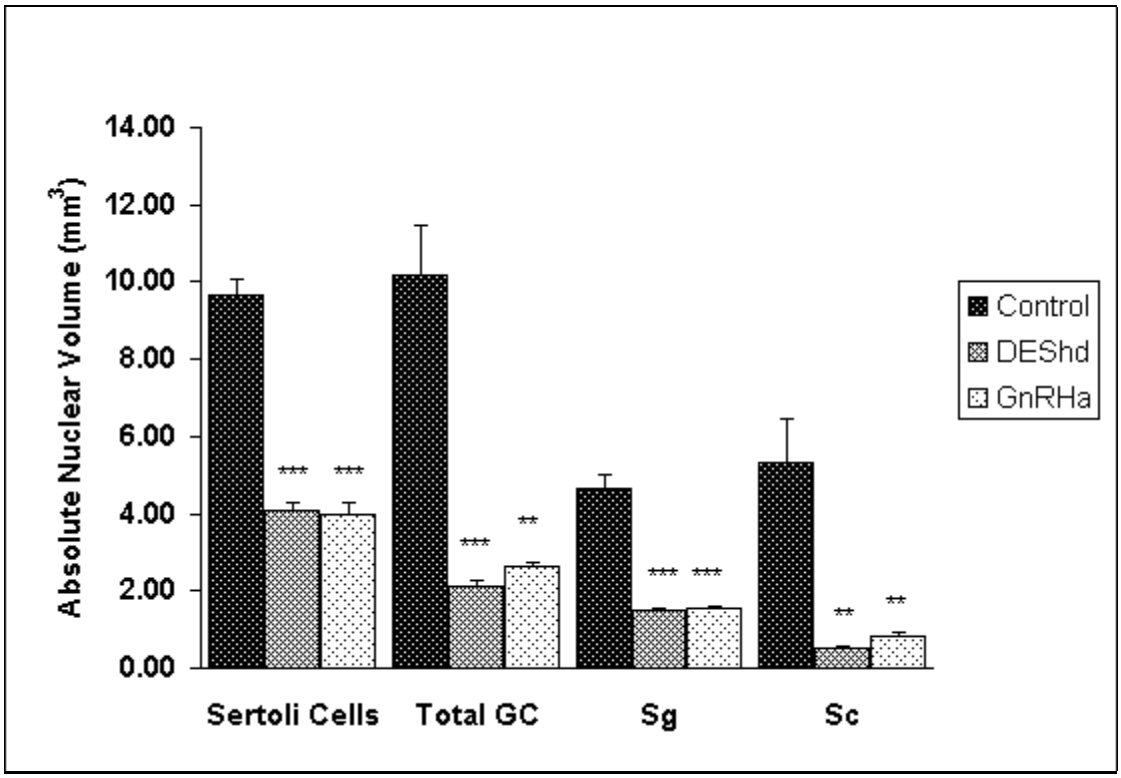

Fig.3. Absolute nuclear volume of testicular cell types on day 18 of control and neonatally treated rats with DES-10 μg and GnRHa. Data represent mean value ± SE (** p< 0.01; *** p< 0.001). TGC – total germ cells; Sg – spermatogonia; Sc- spermatocytes.

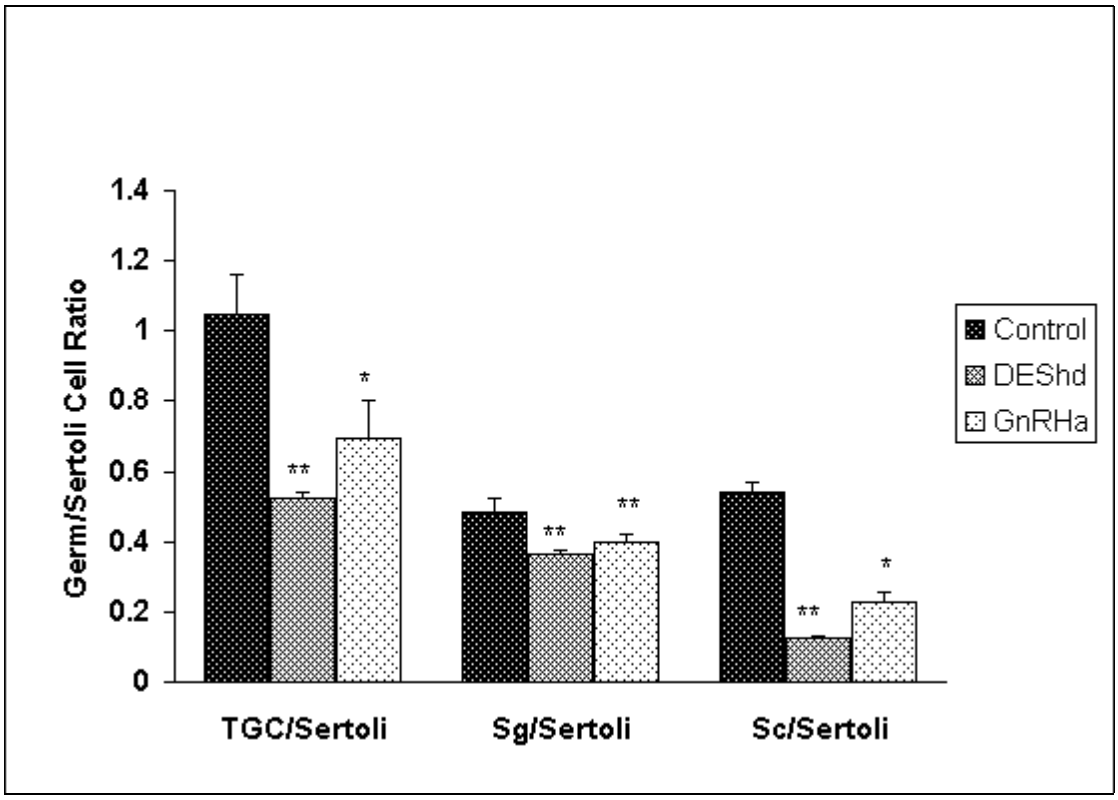

Fig.4. Absolute nuclear volume of germ cell types per unit Sertoli cell nuclear volume on day 18 of control and neonatally treated rats with DES-10 μg and GnRHa. Data represent mean value ± SE (* p< 0.05; ** p< 0.01). TGC – total germ cells; Sg – spermatogonia; Sc- spermatocytes.

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