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Direct and Indirect (Gonagotropin-mediated) Effects of Estrogens on Developing Rat Testis

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This study sought to establish the relative importance of direct and gonadotropin-mediated effects on the testis at age 18 days after neonatal treatment with a high (10 μg) or a low (0.1 μg) dose of diethylstilbestrol (DES). Rats were treated on alternative days from day 2 — 12 with DES or GnRH antagonist (GnRHa), alone or combined. Treatment with 10 μg DES or GnRHa, alone or combined, had similar negative effects on Sertoli cell nuclear volume/number per testis, germ cell apoptotic index, Leydig cell volume per testis and spermatocyte volume per unit Sertoli cell. Injection of DES 10 μg ± GnRHa, but not GnRHa or 0.1μg DES alone, induced major overgrowth/distension of rete testis. However, co-administration of 0.1μg DES + GnRHa caused a similar enlargement of the rete. Treatment with 0.1μg DES alone failed to produce adverse changes in spermatogenesis. However, when rats were treated with 0.1μg DES + GnRHA, the germ cell apoptotic index, spermatocyte volume/Sertoli cell and Leydig cell volume were all impaired to a lesser extent than in GnRHa treated rats. It is concluded that gonadotropin suppression is a likely explanation for the negative effects of neonatal treatment with 10 μg DES on Sertoli, Leydig and germ cells, but this plays no role in induction of rete testis overgrowth, which probably results form altered androgen:estrogen balance. A significant "stimulatory" effect of 0.1 μg DES, compared to GnRHa alone, on germ cell survival and Leydig cell volume, despite the co-administration of GnRHa, is suggestive of direct (estrogen receptor-mediated) effects of DES on one or more testis cell types.

Key words: estrogens, spermatogenesis, apoptosis, Leydig cells and testis.

Introduction

Numerous studies in rodents have demonstrated that neonatal exposure of the male to high doses of potent estrogens, such as DES, results in major adverse changes in the testis and reproductive tract [10, 3]. In earlier studies, the negative effects of estrogens were explained as a result of suppression of gonadotropin secretion during the treatment [5]. More recent studies of our own, involving comparison of the effects of neonatal treatment with DES or GnRHa, have raised the possibility of direct effects of the DES on testicular cells [13, 1]. This possibility has been reinforced by the demonstration that estrogen receptor-β (ERβ) is expressed in Sertoli and Leydig cells as well as in most germ cells [12] and Leydig cells also expressed

ERα [7]. However, because the Sertoli and Leydig cells are also target cells for gonadotropins, it is difficult to distinguish unequivocally between direct (ER-mediated) and indirect (gonadotropin-mediated) effects of DES on the testis.

Recently it was demonstrated a stimulatory effect of very low estrogen levels on spermatogenesis in adult hypogonadal mouse [6] and in the immature at [2] associated with elevated plasma levels of FSH. Therefore, for the effects of both low and high doses of estrogens on the rodent testis, it remains uncertain whether the major changes observed reflect modulation of gonadotropin secretion or direct activation of ERs in testis cell types or a combination of both effects. The present study was designed to address this issue by comparing the effects on the developing rat testis of treatment with a high or a low dose of DES alone or in combination with a GnRHA antagonist. We have evaluated endpoints that we have previously shown to reflect most clearly inappropriate estrogen exposure (germ cell volume per Sertoli cell, germ cell apoptotic index, Sertoli and Leydig cell volume/number per testis, rete testis size).

Materials and Methods

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 20 μl corn oil on days 2,4,6,8,10 and 12; b) 10 mg/kg of long acting GnRHa (Antarelix) in 20 μl 5% mannitol on days 2 and 6; c) 10 μg DES as in (a) + GnRHa as in (b); d) 0.1μg DES + GnRHa; e) 20 μl corn oil (vehicle) as control. Rats from all treatment groups were subsequently sampled on day 18. Paraffin 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 [13]. Different testicular cell types were counted using 121-point eyepiece graticule and the data were used to determine the following: a) nuclear volume of viable (non-apoptotic) spermatocytes per Sertoli cell nuclear volume as an index of spermatogenic efficiency; b) the germ cell apoptotic index as a ratio of the nuclear volume of apoptotic/normal germ cells per testis; c) seminiferous tubule lumen % volume as an indicator of the onset of spermatogenesis at puberty. Optical dissector method was applied for quantification of Sertoli cell number per testis [1] and to confirm data for Sertoli cell nuclear volume obtained by point counting. Leydig cell volume per testis was determined on sections immunostained for 3β-hydroxysteroid dexydrogenase (3β-HSD) [15]. The 3β-HSD positive nuclei and cytoplasm were scored separately by point counting and the data for absolute volumes (nuclear and cytoplasm) were collected. Comparison of the different parameters for the various treatment groups was made using ANOVA.

Results

Testicular weight and gross morphology, seminiferous tubule lumen formation
Testicular weight on day 18 was reduced to 70% by treatment with GnRHa, DES 10μg or DES 10μg + GnRHa, as compared to the control. Treatment with 0.1μg DES induced a small (18%) but not significant reduction in that parameter whereas combined treatment with DES 0.1μg + GnRHa caused a comparable reduction to that induced by treatment with GnRHa (Fig.1). Injection of DES 10μg ± GnRHa, but not GnRHa or 0.1μg DES alone induced major overgrowth/distension

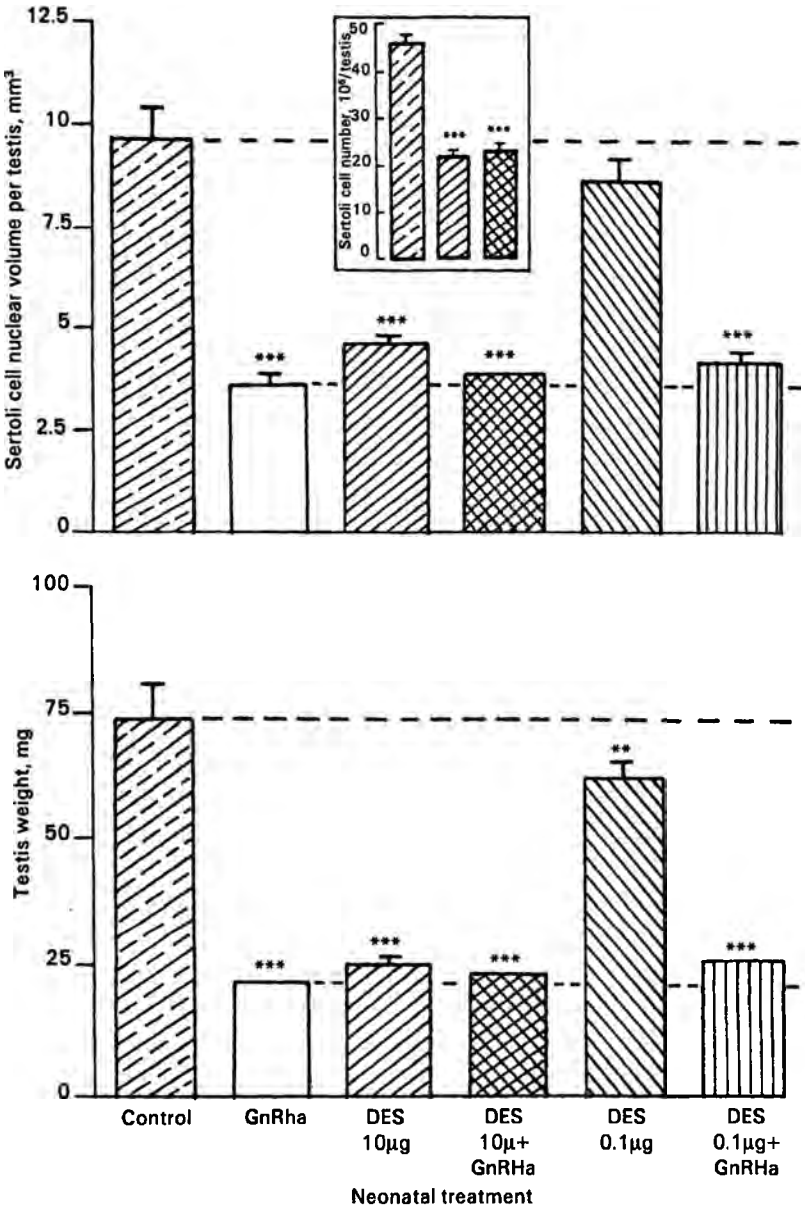

Fig. 1. Effect of neonatal treatment with DES (10 or 0.1 μg) or GnRHa, alone or in combination, on Sertoli cell nuclear volume per testis (top) and testis weight (bottom) on day 18th. Insert in the top panel shows the effect of treatment with DES 10μg alone or DES 10 μg + GnRHa on Sertoli cell number determined by optical disector method. Dashed lines show mean values for control and GnRHa-treated groups to aid comparison. Data represent the mean ± SEM (n=5 per group; ** p<0.01, *** p<0.001, in comparison with control)

of rete testis (data not shown). However co-administration of 0.1μg DES + GnRHa caused a similar enlargement of the rete. The formation of seminiferous tubule (% lumen volume per testis) was 7.0±0.4% in control and was reduced by 66, 80, 84, 6 and 61% respectively in groups treated with GnRHa, DES 10μg, DES 10μg + GnRHa, DES 0.1μg or DES 0.1μg + GnRHa. With an exception of the DES 0.1μg treatment group, all of these values were significantly reduced ($p<0.01$) compared with controls.

Changes in Sertoli and germ cell nuclear volume per testis

Based on point counting, Sertoli cell nuclear volume per testis showed changes after treatment that paralleled the reduction in testicular weight (Fig. 1). With an exception of the DES 0.1μg, all treatments resulted in a marked decrease in Sertoli cell nuclear volume. For three of the groups Sertoli cell number was determined by optical dissector method and the data confirmed those obtained for Sertoli cell nuclear volume using point counting (see insertion of the chart). Treatment with either GnRHa or DES 10 μg alone both significantly reduced spermatocyte nuclear volume per unit Sertoli cell volume, though the degree of suppression was slightly greater ($p<0.05$) with DES 10μg than with GnRHa (Fig. 2). Combined treatment with DES 10μg + GnRHa reduced germ cell volume to a comparable extent as did treatment with DES 10 μg alone. Treatment with DES 0.1μg alone had no effect on spermatocyte volume per unit Sertoli cell volume but combined treatment with DES 0.1 μg + GnRHa significantly suppressed this cellular ratio and the magnitude of this suppression was less than that observed in animals treated with GnRHa.

The germ cell apoptotic index was expressed as a ration of apoptotic/normal germ cells and showed the mirror image of changes to spermatocyte volume per Sertoli cell. Thus treatment with GnRHa or DES 10 μg alone, or these two treatments combined, increased the germ cell apoptotic index by comparable amounts (6-fold increase, Fig 2). In contrast, treatment with DES 0.1μg alone had no significant effect on the germ cell apoptosis. Combined treatment with DES 0.1 μg + GnRHa significantly increased ($p<0.01$) the germ cell apoptotic index when compared with control or DES 0.1μg alone. However, this increase was significantly less ($p<0.05$) than that induced by treatment with GnRHa alone.

Changes in Leydig cell volume per testis

Leydig cell volume per testis was reduced by 98% in rats treated with DES 10 μg or GnRHa alone or in combination (Fig. 3). Treatment with DES 0.1 μg alone reduced Leydig cell volume, compared with control, but this was not statistically significant. Combined treatment with DES 0.1μg + GnRHa reduced Leydig cell volume by 93%, but this reduction was significantly less ($p< 0.01$) than in rats treated with GnRHa alone.

Discussion

To assess the relative importance of direct and indirect effects of estrogens on the testis, we chose two doses of DES (10 μg and 0.1 μg) that our previous studies have shown to exert radically different effects on spermatogenesis and on FSH secretion [2]. Thus, the high dose (10 μg) of DES induced several major adverse effects on the testis and suppressed FSH secretion whereas the lower dose (0.1 μg) of DES

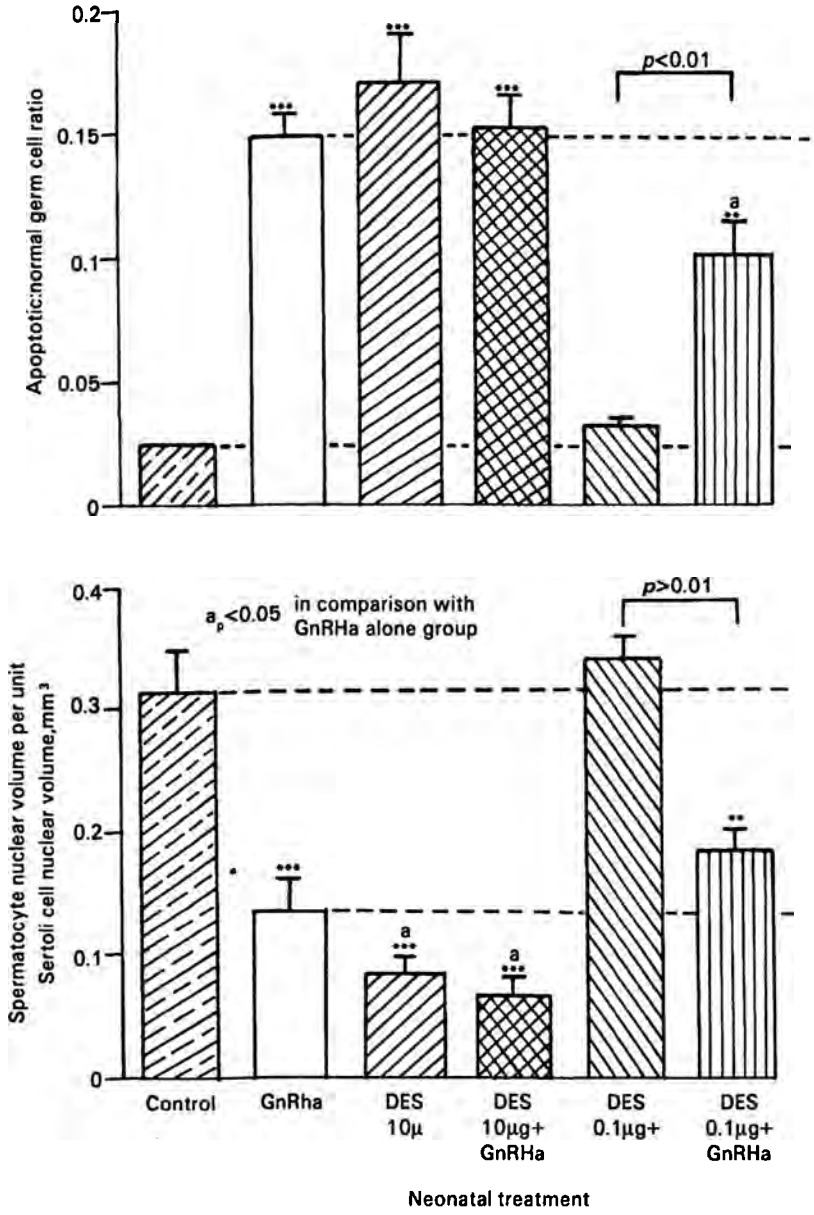

Fig. 2. Effect of neonatal treatment with DES (10 or 0.1 μg) or GnRHa, alone or in combination, on the germ cell apoptotic index expressed as a ratio of apoptotic/normal germ cells (top) and spermatocyte nuclear volume per unit Sertoli cell nuclear volume (bottom) on day 18th. Dashed lines show mean values for control and GnRHa-treated groups to aid comparison. Data represent the mean ± SEM (n=5 per group; ** p<0.01, *** p<0.001, in comparison with control)

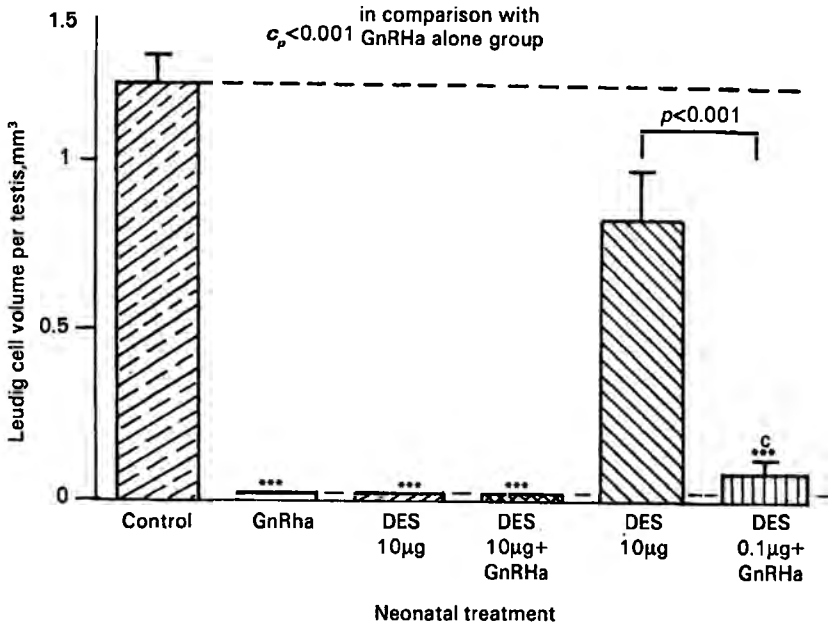

Fig. 3. Effect of neonatal treatment with DES (10 or 0.1 μg) or GnRHa, alone or in combination, on Leydig (3β-HSD positive) cell volume per testis on day 18th. Dashed lines show mean values for control and GnRHa-treated groups to aid comparison. Data represent the mean ± SEM (n=4 per group; *** p<0.001, in comparison with control)

failed to produce adverse effect on spermatogenic process. It was reasoned that demonstration of a testicular change in the DES + GnRHa-treated groups, but the absence of a comparable change in animals treated with GnRHa alone, would provide persuasive evidence for a direct effect of DES on the testis. Induction of rete testis overgrowth/distension is a change that fall into this category. We had earlier concluded that gross distortion of the normal androgen:estrogen balance (suppressed androgen production + supranormal estrogen action) underlay the gross morphological changes in the testis and reproductive tract of rats treated neonatally with high dose (eg 10 μg) of DES [3, 10] This interpretation is reinforced by some of the present findings. Thus, treatment with DES 0.1 μg alone will have raised estrogen levels but has no effect on testosterone levels [see 16] and did not affect the rete testis. Treatment with GnRHa alone will not have raised estrogen levels but does significantly reduce testosterone levels and also had no effect on the rete. However, when these two treatments were combined, presumably leading to raised estrogen and lowered testosterone levels concomitantly, it resulted in a marked distension of rete testis, comparable to that caused by high dose (10 μg) of DES. Similarly, the attenuation of germ cell apoptotic index in rats treated with DES 0.1μg + GnRHa when compared with that treated with GnRHa alone, is interpreted as possible evidence for a direct (stimulatory) effect of DES. The significantly lower suppression of Leydig cell volume in rats treated with DES 0.1 μg + GnRHa, compared with rats treated with GnRHa alone, may also fall into this category.

On the other hand, where similar testicular changes were induced by treatment with DES 10μg or GnRHa alone, and combined treatment had no additive effect,

we can conclude that gonadotropin suppression is a likely explanation for the observed adverse effects. Suppression of Sertoli cell nuclear volume/number and testis weight, increase in the germ cell apoptotic index and decrease in spermatocyte volume per unit Sertoli cell are endpoints which changes support this suggestion. We cannot exclude other possibilities, for example that treatment with DES 10 μg reduced Sertoli cell proliferation by a direct effect on Sertoli cells mediated via ERβ which then resulted in inhibition of the FSH-stimulated intracellular signaling pathway that normally up-regulated Sertoli cell proliferation. It is established that one of the major driving forces for Sertoli cell proliferation in the neonatal period of the rat is FSH [11, 14] and the present demonstration of an approximate halving of Sertoli cell number/volume after neonatal GnRHa treatments (alone or combined) reinforces this conclusion.

There is also a further, untested possibility, namely that the low dose of DES elevates prolactin secretion [5] which then positively acts on the function of Sertoli cell [8], germ [9] or Leydig [4, 9] cells. These various possibilities are difficult to dissect apart because of the complexity both of the endocrine system involved and of the testicular cell-cell interactions. It seems apparent from the points discussed above that future studies of direct, positive effect of estrogens on testicular germ cell development should use animals of a younger age or in vitro approaches such as germ cell-Sertoli cell co-cultures or short-term cultures of isolated seminiferous tubules from prepubertal rats.

In conclusion, the present study provides strong evidence in support of a role for 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 signalling pathways as those activated by gonadotropins. The present findings also demonstrate that DES-induced changes in the testis results also from a disturbance of the normal androgen:estrogen balance and therefore a close interrelationship between actions of androgens and estrogens is involved in regulating normal development of the testis.

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