Institute of Experimental Morphology and Anthropology

Acta morphologica et anthropologica, 6
Sofia • 2001

Dose-dependent and Differential Effects of Various Estrogenic Compounds on Spermatogenesis at Puberty and Relationship to Adulthood

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The current study aimed to investigate the effect of neonatal estrogen exposure on spermatogenesis during puberty (days 18 and 25) and in adulthood. Rats were treated neonatally with a range of doses (0.01-10μg) diethylstilbestrol (DES) and weak (environmental) estrogens, octilphenol (OP) and bisphenol A (Bis-A) or vehicle. DES administration caused dose dependent retardation of pubertal spermatogenesis on day 18 as evident by decreases in testis weight, lumen formation, efficiency of spermatogenesis and an elevation of germ cell apoptotic index. However, the two lowest doses of DES (0.1 and 0.01μg), and high dose of OP and Bis-A significantly advanced the same aspects of spermatogenesis. By day 25 all of stimulatory effects on spermatogenesis seen on day 18 were no longer evident. The dose-dependent negative changes in spermatogenesis still persist to adulthood that in higher doses of DES were associated with an increase in lumen size per testis. The lowest dose of DES as well as weak estrogens caused slight increase in testis weight but not in index of spermatogenic efficiency. It can be concluded that: 1. neonatal estrogen treatment dose dependently suppress spermatogenesis at puberty and in adulthood; 2. low levels of estrogens can advance the first wave of spermatogenesis at puberty but failed to maintain more efficient spermatogenesis in adulthood.

Key words: estrogens, spermatogenesis, apoptosis and testis.

Introduction

There is growing evidence that estrogens play a role in normal male reproductive development and function. This is based on information about an increasing concern whether weak environmental estrogens can induce impairment of spermatogenesis and whether human exposure to such compounds could have contributed to the reported fall in sperm counts or to other disorders of male reproductive health [13]. The latter concern has been supported by the demonstration of widespread expression of estrogen receptors in the testis and male reproductive tract in foetal and neonatal life [5, 11, 2] and by the numerous studies that have shown major adverse changes in the structure and function of these tissues after administration of potent estrogens in perinatal life [1, 14, 6].

In the past impairment of adult spermatogenesis in neonatally estrogenized rats has been attributed to reduced gonadotropin secretion induced during the treatment [4, 3], a conclusion that is difficult to reconcile with recent data providing evidence for direct modulation of Sertoli cell development by DES [14].

There are published studies that have shown effects (not necessarily adverse) of much lower levels of potent estrogens as well as of weak environmental estrogens [15]. Recently published studies that have addressed this issue have yielded conflicting results and this prompted us to investigate whether there were hidden effects of low estrogen levels that were not obvious, but were discernible by systematic analysis. In the context of evaluating human risk from exposure to environmental estrogens, an important question is what level of estrogen exposure will induce adverse changes to spermatogenesis and what endpoints most clearly reflect inappropriate estrogen exposure. The present study was undertaken to answer these questions and to establish which aspects of spermatogenic process are most sensitive to lifelong disruption by neonatal estrogen exposure.

Material and Methods

Beginning on postnatal day 2, rats were subjected to one of the following treatments: 1. sc. injection of DES at a dose of 10, 1, 0.1, or 0.01 μg in 20μl corn oil on days 2, 4, 6, 8, 10 and 12; 2. sc. injection of 2mg 4-tert-octilphenol in 20μl corn oil on each of days 2-12; 3. sc. injection of 0.5mg Bis-A in 20μl corn oil on each of days 2-12. Rats from the various treatment groups were subsequently sampled on day 18, 25, or 75-90 (=adults). Paraffin Bouin's fixed 5-μm testicular sections were used for cell quantification studies and visualisation of apoptotic germ cells identified applying TUNEL method as described in detail previously [14]. Cross-sections were counted using a 121-point eyepiece graticule. Standard point counting of cell nuclei was performed as described previously [14] to determine the nuclear volume per testis of Sertoli cell, germ cells (apoptotic and nonapoptotic), and seminiferous tubule lumen volume. These data then were used to determine the following: 1. the volume of total germ cells per unit Sertoli cell volume as an index of spermatogenic efficiency; 2. the germ cell apoptotic index based on the volume ratio of apoptotic/total germ cells per testis; 3. seminiferous tubule lumen per cent volume (=lumen formation) as an indicator of Sertoli cell functional development in immature rats. Plasma samples were stored at -20 °C until used for hormone analysis. Plasma levels of FSH were measured by RIA as described in detail previously [14]. Comparison of the different parameters for the various treatment groups were made using ANOVA.

Results

Effect of neonatal estrogen treatments on pubertal spermatogenesis

On day 18 neonatal treatment with DES resulted in dose-dependent, significant decrease in testis weight compared with controls (Fig.1 — *a*) with an exception of 0.1 μg dose of DES. In contrast neonatal treatment with Bis-A or OP significantly increased this parameter. The data for testis weight closely paralleled those for germ cell/Sertoli cell volume ratio (Fig. 2 — *b*), Sertoli or total germ cell volume per testis (not shown). All these parameters highly correlated with lumen formation, determined by per cent lumen volume per testis (Fig. 3 — *a*) that was inverse image of germ cell apoptotic index (Fig. 4 — *a*). Neonatal exposure to DES doses greater that 0.01 μg caused dose-related significant increase in germ cell apoptosis and a corresponding decrease in

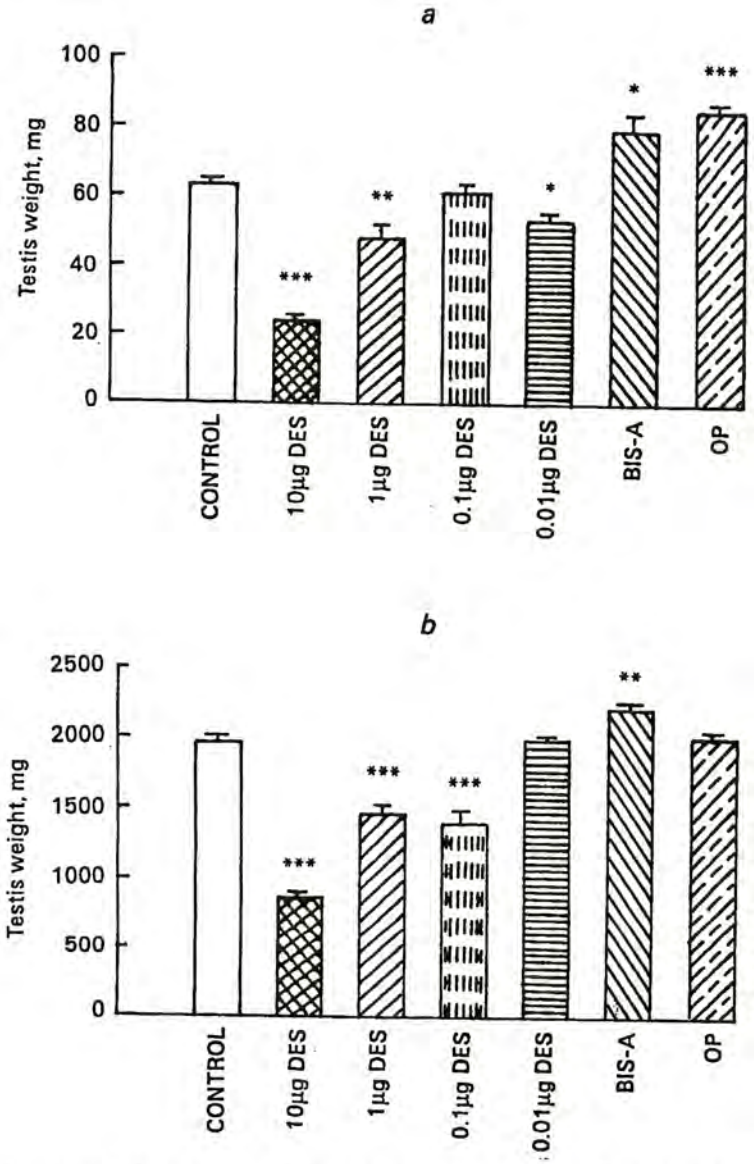

Fig. 1. Testis weight of 18-day-old (*a*), and adult (*b*) rats treated neonatally with various doses of DES or high dose of Bis-A or OP. Values shown are the mean ± SEM for 5-25 (d18), and 7-24 (adult) rats in each treatment group. Asterisks denote values significantly different from the controls (* $p< 0.05$; ** $p< 0.01$; *** $p< 0.001$)

lumen volume, although the latter reduction only achieved statistical significance with 10µg-dose of DES. Treatment with Bis-A or OP significantly advanced lumen formation and slightly decreased the germ cell apoptotic rate. Efficiency of Sertoli cells to support spermatogenesis, as indicated by the germ cell volume per unit Sertoli cell volume showed major changes according to treatment (Fig. 2 — *b*). Animals treated with 10µg DES showed a significant reduction in the index of spermatogenic effi-

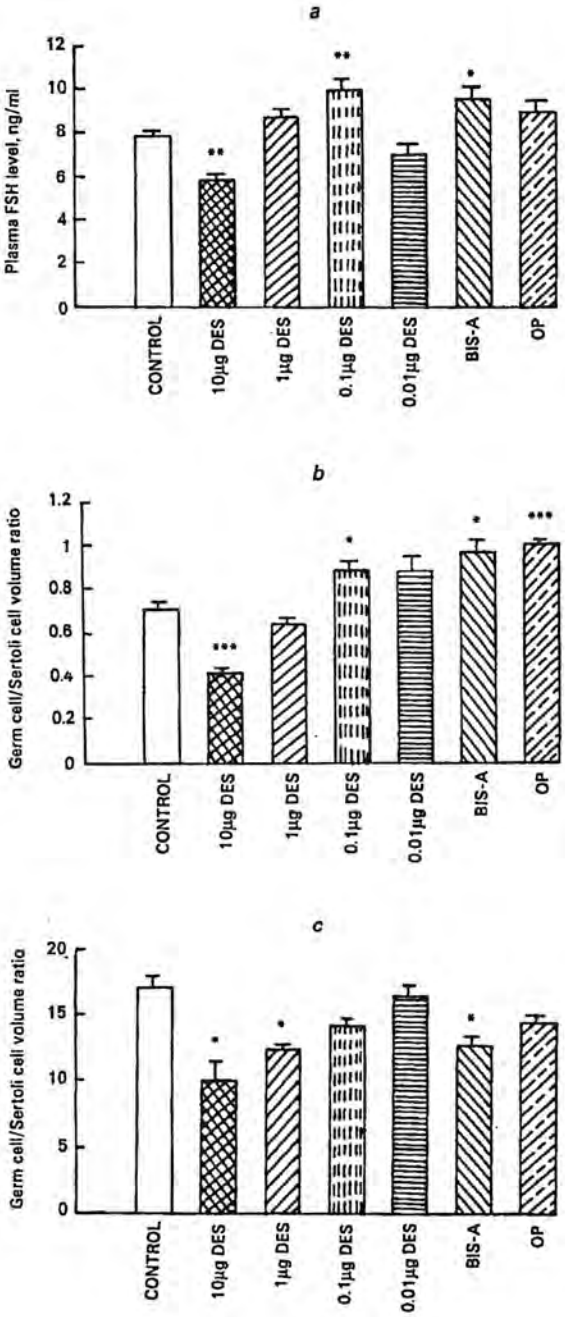

Fig. 2. Effect of neonatal treatment with DES, Bis-A or OP on plasma FSH levels on day 18 (*a*) and efficiency of spermatogenesis (total germ cell volume per unit Sertoli cell volume) on day 18 (*b*) and in adulthood (*c*). Values shown are the mean ± SEM for 5-14 (d18), and 3-4 (adult) rats per group. Asterisks denote values significantly different from the controls (* $p< 0.05$; ** $p< 0.01$; *** $p< 0.001$)

ciency. In contrast, animals exposed to either 0.1 or 0.01µg DES revealed a significant increase in this parameter as did animals treated with either Bis-A or OP. With one or two exceptions, the pronounced treatment changes, especially stimulatory effects, found on d18 were no longer evident on d25.

Effect of neonatal estrogen treatments on spermatogenesis in adulthood

Adult rats treated neonatally with DES showed a more or less dose-dependent reduction in testis weight whereas treatment with Bis-A or OP slightly increased this parameter (Fig. 1 — *b*). The germ cell volume per unit Sertoli cell volume (Fig. 2 — *c*) and total Sertoli or germ cell volume per testis (not shown) were dose-dependently reduced but statistically significant only at high doses DES (10- and 1μg). In contrast to day 18, Bis-A or OP did not increase both parameters. The proportion of germ cells that

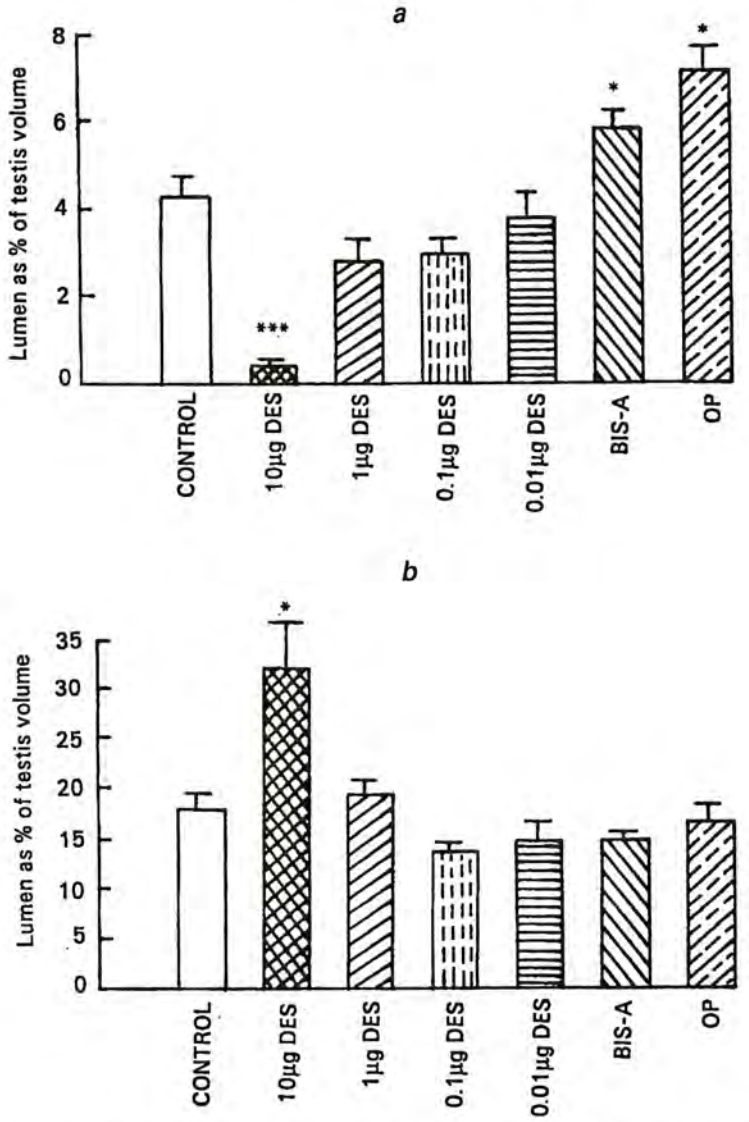

Fig. 3. Seminiferous tubule lumen volume as per cent of testis volume of 18-day-old (*a*) and adult (*b*) rats treated neonatally with various doses of DES or high dose of Bis-A or OP. Values shown are the mean ± SEM for 5-14 (d18) and 3-4 (adult) rats/group. Asterisks denote values significantly different from the controls (* $p< 0.05$; *** $p< 0.001$)

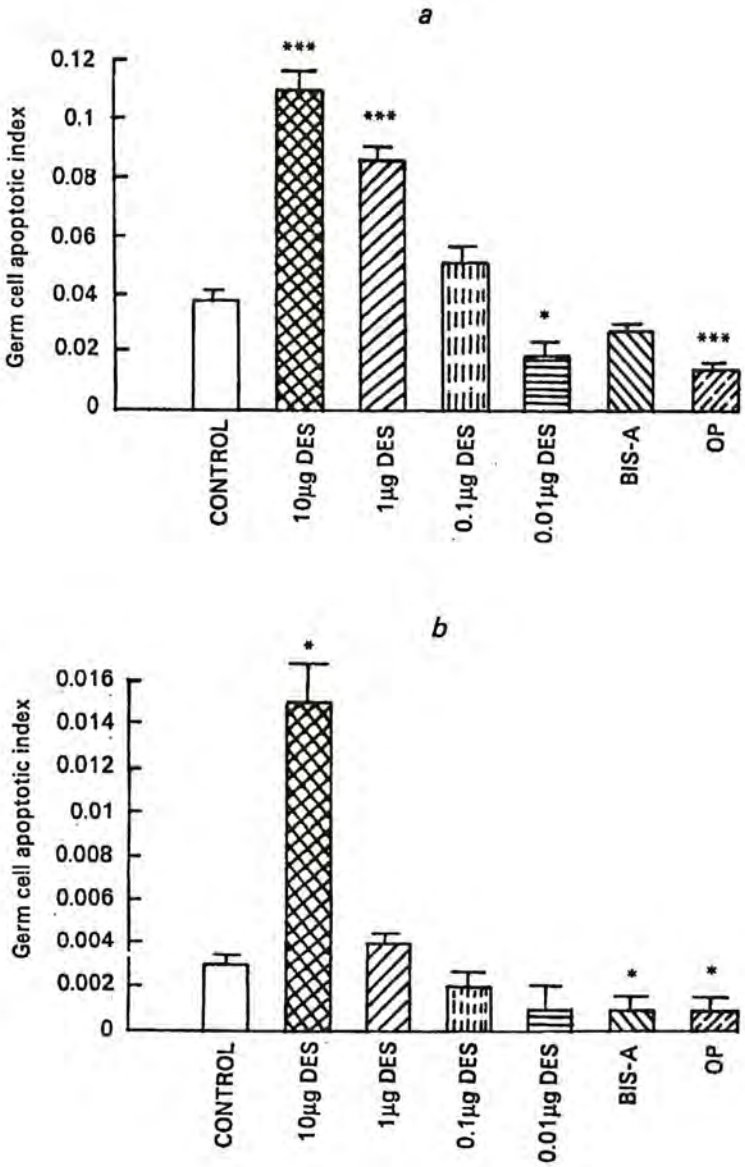

Fig. 4. Germ cell apoptotic index of rats treated neonatally with various doses of DES or high dose Bis-A or OP at puberty- day 18 (*a*) and in adulthood (*b*). Values shown are the mean ± SEM for 5-14 (d18), and 3-4 (adult) rats/group. Asterisks denote values significantly different from the controls (* $p< 0.05$; *** $p< 0.001$)

were apoptotic showed a stepwise diminishing pattern in DES group (Fig. 4 — *b*) with grossly elevated rates of apoptosis in animals treated with 10μg DES, and reduced rate of apoptosis in the 0.1μg-DES group. The reduction in this parameter was significant in Bis-A or OP treated animals. A possible cause for elevated apoptosis in DES-10μg group could result from accumulation of seminiferous tubule fluid (STF) in the tubule lumen. A dose-dependent increase in the percentage of testis volume occupied

by lumen (Fig. 3 — *b*) was evident in high doses DES a change that paralleled the increase in germ cell apoptopic index (Fig. 4 — *b*). The adult treatment groups that showed a more or less identical profile to that on day 18 were the DES-10μg and DES-1μg groups in which there were reduced testis weight, elevated germ cell apoptosis and less efficient spermatogenesis.

Plasma FSH levels on day 18 and in adulthood

The changes in FSH levels on day 18 broadly paralleled those for efficiency of spermatogenesis. In general, higher FSH levels were associated with advancement and lower FSH levels associated with retardation, of spermatogenesis (Fig. 2 — *a*). Surprisingly, in adulthood FSH levels in adulthood were elevated significantly, and to a similar level in all DES treatment groups (not sown).

Discussion

The first wave of spermatogenesis at puberty in the male is characterised by several interrelated temporal changes involving formation and progressive expansion of the seminiferous tubule lumen, a progressive increase in germ cell volume and number, and a progressive decrease in the number of degenerating germ cells [10, 12]. We have therefore used these three endpoints to characterise spermatogenic process in rats treated neonatally with DES, OP or Bis-A. Our data provide new information on the comparative effects of potent and weak estrogens on male reproductive development. They demonstrate that whereas high doses of potent estrogens, such as DES, are clearly inhibitory to testicular development in both the short and long term, as the dose is titrated downward a stimulatory (short-term) effect begin to emerge. Moreover, neonatal administration of high doses of weak estrogens (Bis-A, OP) was also able to advance pubertal spermatogenesis and their stimulatory effects were comparable to those of the lowest dose of DES. These data are somewhat at odds with other published studies on the action of Bis-A or OP, in which either no effect or adverse effects on reproductive development were reported [15]. However, these studies used different aged animals and/or treatment regimens and did not study the endpoints used in the present study.

There are several possible mechanisms that could explain differential effects of potent and weak estrogens. Most obviously, the changes in plasma FSH levels on day 18 broadly paralleled the changes in pubertal spermatogenesis, i.e. higher FSH levels associated with advancement, and lower FSH levels associated with retardation of spermatogenesis. Another explanation for estrogen effect on pubertal spermatogenesis could involve direct action of estrogens on the Sertoli and/or germ cells, as both cell types express ERβ during development [11] and direct adverse effect of high estrogen levels on Sertoli cell was reported [14]. There are also data for stimulatory effect of estrogens on gonocytes [8] and spermatogonia [7, 9]. It is obviously difficult in the present study to disentangle direct effects of estrogens on Sertoli/germ cells from effects due to altered FSH levels.

The present study revealed that exposure to a potent estrogen such as DES is able to induce long-term dose-dependent adverse changes in adult testis despite elevated FSH levels in all adult DES treatment groups. In the past impairment of adult spermatogenesis in neonatally estrogenized rats has been attributed to reduced gonadotropin levels during treatment [3, 4]. The studies on animals in which FSH levels are reduced by neonatal administration of a GnRH-antagonist do not support that conclusion [14]. Our data indicating reduced efficiency of spermatogenesis despite hyperse-

cretion of FSH also make it unlikely that gonadotropin suppression can account for most of the changes induced by DES. The most important factor that determines the ceiling of sperm production and output in mammals is the number of Sertoli cell per testis [12]. The present study confirms [14] and extends our previous observations demonstrating that neonatal DES treatment results in a permanent and dose-dependent reduction of Sertoli cell volume per testis. This effect may not be explicable only by a reduction in FSH levels during treatment (when Sertoli cell are replicating), but also reflects a direct effect of estrogens on the rate of division of developing Sertoil cells.

The reduced efficiency of spermatogenesis in DES treated rats stemmed in part from increased germ cell apoptosis and seemed to go hand in hand with increased seminiferous tubule lumen size. The latter changes is though to result from maldevelopment of rete testis and efferent ducts due to impaired fluid resorption by the epithelium of efferent ducts, previously showed in neonatally estrogenized rats [6]. It may therefore be that the positive stimulus of the Sertoli cell via increased FSH levels is counteracted by the negative influence on spermatogenesis of STF accumulation in the tubule lumen.

In conclusion, neonatal estrogen treatment dose dependently suppresses spermatogenesis at puberty and in adulthood. Low levels of estrogens can advance the first wave of spermatogenesis at puberty but failed to maintain more efficient spermatogenesis in adulthood. Aside from effects on spermatogenesis, the present study also provides evidence for permanent reprogramming of the hypothalamic-pituitary-testis axis at various levels by neonatal estrogen exposure.

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