

·Original Article·

Maturation, proliferation and apoptosis of seminal tubule cells at puberty after administration of estradiol, follicle stimulating hormone or both

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Abstract

Aim: To assess proliferative and apoptotic potential of the seminiferous epithelium cells in relation to Sertoli cell maturation in newborn rats under the influence of estradiol, follicle stimulating hormone (FSH) or both agents given together. **Methods:** From postnatal day (PND) 5 to 15 male rats were daily injected with 12.5 µg of 17β-estradiol benzoate (EB) or 7.5 IU of human purified FSH (hFSH) or EB + hFSH or solvents (control). On postnatal day 16, autopsy was performed. Sertoli cell maturation/function was assessed by morphometry. Proliferation of the seminiferous epithelium cells was quantitatively evaluated using immunohistochemical labeling against proliferating cell nuclear antigen and apoptosis using the TUNEL method. **Results:** Although EB inhibited Sertoli cell maturation and hFSH was not effective, a pronounced acceleration of Sertoli cell maturation occurred after EB + hFSH. Whereas hFSH stimulated Sertoli cell proliferation, EB or EB + hFSH inhibited Sertoli cell proliferation. All treatments significantly stimulated germ cell proliferation. Apoptosis of Sertoli cells increased 9-fold and germ cells 2-fold after EB, and was not affected by hFSH but was inhibited after EB + hFSH. **Conclusion:** At puberty, estradiol inhibits Sertoli cell maturation, increases Sertoli and germ cell apoptosis but stimulates germ cell proliferation. Estradiol in synergism with FSH, but neither of the hormones alone, accelerates Sertoli cell maturation associated with an increase in germ cell survival. Estradiol and FSH cooperate to induce seminal tubule maturation and trigger first spermatogenesis. (*Asian J Androl 2008 Jul; 10: 585–592*)

Keywords: estradiol; follicle stimulating hormone; germ cells; Sertoli cells; proliferation; apoptosis

1 Introduction

The precise orchestration of mammalian spermatogenesis

relies on functional interaction between germ and Sertoli cells. However, during early postnatal development the first series of spermatogonial divisions occur before the final size of mature, functional Sertoli cell population is established. In the rat, spermatogenesis commences shortly after birth. At about postnatal day (PND) 5, spermatogonia appear for the first time and between the PND 10 and 19 differentiate into the first meiotic spermatocytes [1]. For most of this time Sertoli cells are immature and still proliferate until PND 15 [2].

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Maturation of Sertoli cells involves cessation of proliferation and formation of the complex morphological and functional changes, terminating with, among others, the secretion of the seminal tubule fluid. The initiation of fluid secretion by maturing Sertoli cells begins around PND 15 with the formation of seminal tubule lumen [3].

Follicle stimulating hormone (FSH) and androgens are considered the principal regulators of Sertoli cell proliferation and function. The mechanism by which these hormones exert their effects on germ cells remains unknown, as germ cells do not express their receptors. It is becoming increasingly clear that estrogens play a role in testicular function. The testis expresses aromatase, an enzyme that converts androgens into estrogens and expresses α and β estrogen receptors (ER α and ER β) in Sertoli cells. Unlike androgen and FSH receptors, germ cells express ER β [4].

The stimulatory effect of estrogen on the number of the first A spermatogonia was reported as early as 1988 [5]. Precocious completion of spermatogenesis in young (4.5–8-year-old) boys with Leydig cell hyperplasia was associated with a prominent hypersecretion of estradiol [6]. In addition, endogenous estradiol was found to be a survival factor for spermatids in the adult human testis *in vitro* [7]. In 2000, Ebling *et al.* [8] induced qualitatively complete spermatogenesis by chronic administration of 17 β -estradiol to adult hypogonadal mice congenitally lacking gonadotropins and, consequently, sex steroid hormone production. In 2001, we demonstrated that the administration of 17 β -estradiol benzoate (EB) together with FSH in newborn rats greatly enhanced the stimulatory effect of FSH on the first spermatogenesis, resulting in quantitative precocious completion of premeiotic steps [9]. In this context, it seemed to be of interest to investigate some seminal tubule cellular kinetics parameters, such as Sertoli and germ cell proliferation, apoptosis and Sertoli cell maturation under similar experimental regime.

2 Materials and methods

2.1 Animals and hormone treatment

Five-day-old male Wistar rats, born on the same day, were randomly divided into experimental groups with five to six animals in each. Each group was kept in a separate cage together with a lactating female rat. Rats were daily injected s.c. from PND 5 to 15 with: (i) 12.5 μ g of EB (Oestradiolum Benzoicum, Jelfa, Jelenia Gora,

Poland); (ii) 7.5 IU of human FSH (hFSH) (Metrodin, Serono, Middlesex, UK); (iii) EB and hFSH together (EB + hFSH) or (iv) solvents for both hormones (control).

Animals were maintained at stable a temperature (22°C) and diurnal L:D cycles (12 h:12 h) with free access to food and water. Experiments were performed in accordance with Polish legal requirements, under the license given by the Commission of Animal Ethics at the Medical University of Lodz, Poland.

2.2 Processing of the tissue

Autopsy was performed on PND 16. Animals were anaesthetised with methohexital sodium (Brietal, Eli Lilly, Indianapolis, IN, USA) and fentanyl (Fentanyl, Polfa, Pabianice, Poland) and weighed. The testes were excised, weighed and fixed in Bouin's solution for up to 24 h. Subsequently, testes were processed through graded alcohol and embedded in paraffin.

2.3 Morphometric parameters of Sertoli cell maturation

5- μ m-thick sections of paraffin-embedded testes taken from equatorial cross-sections of the organ were routinely stained with haematoxylin and eosin. The percentage of seminal tubule cross-sections containing a clear lumen (larger than 100 μ m²) was determined by scoring 100 subsequent round-shaped cross-sections of the seminal tubules per animal. The surface area of seminal tubule lumen in 100 randomly selected round-shaped cross-sections were measured for each animal by planimetry. The nuclear area of Sertoli cells was measured by planimetry in 50 longitudinally sectioned Sertoli cells in each rat.

All measurements were performed using image analysis software LxAND version 3.60HM (Logitex, Lodz, Poland) able to carry out geometrical measurements of the marked objects.

2.4 Proliferating cell nuclear antigen labeling

Proliferative activity of the cells were studied by immunohistochemical labeling of proliferating cell nuclear antigen (PCNA). PCNA is a protein of 35 kDa that forms part of polymerase δ and which participates in the regulation of the cell cycle. 5- μ m-thick sections were placed onto slides coated with 0,1% poly-L-Lysine solution (Sigma-Aldrich, St. Louis, MO, USA). To optimize immunohistochemical staining, after deparaffinization and rehydration, the samples were exposed to antigen retrieval procedure by microwaving (800 W) the tissue

sections twice for 5 min in 10 mmol/L citrate buffer (pH 6.0). Sections were then washed in Tris-buffered saline (TBS, 0.05 mol/L Tris-HCL and 0.15 mol/L NaCl, pH 7.6) and the immunostaining procedure started. Sections were incubated in a humidified chamber in the presence of primary antibody: the monoclonal mouse anti-rat PCNA (ready to use, DAKO, Glostrup, Denmark) for 10 min. Next, the color reaction was developed using commercially available EnVision system-AP kit (DAKO, Glostrup, Denmark), which uses alkaline phosphatase labeled polymer and is conjugated to secondary antibodies. Briefly, the incubation with primary antibody was followed by incubation with labeled polymer for 20 min. After each step in this procedure, sections were rinsed and incubated twice for 5 min in TBS. The staining was completed with incubation using Fast Red Chromogen for 10 min, which resulted in red-coloured precipitate at the antigen site. Then sections were counterstained with Mayer's hematoxylin (5 min). Finally, the sections were rinsed in distilled water and mounted in Ultramount Medium (DAKO, Glostrup, Denmark) and coverslipped. As negative control, the sections were incubated with non-immune serum instead of the primary antibody.

The percentage of PCNA-positive (PCNA⁺) germ or Sertoli cells were examined in 1 000 subsequent cell nuclei at 1 000 × magnification in the light microscope (Nikon, Eclipse E600, Kanagawa, Japan).

2.5 TUNEL method

To detect nuclei with DNA fragmentation, representing a hallmark of apoptosis, the Terminal Deoxynucleotidyl Transferase (TdT) mediated dUTP Nick End Labeling (TUNEL) method was performed. 5- μ m-thick sections of the testis were mounted on silanized slides (SuperFrost Plus, Dako, CA, USA), deparaffinized, rehydrated and incubated with proteinase K (20 μ g/mL) (Sigma-Aldrich, St. Louis, MO, USA) for 15 min in room temperature. Endogenous peroxidase activity was blocked by immersion in 3% (v:v) H₂O₂ in methanol for 30 min. After two washes (5 min each) in phosphate buffered saline (PBS; 0.01 mol/L, pH 7.4) (Biomed, Krakow, Poland) the staining procedures were commenced. Apoptotic cells were visualized using the *in situ* Death Detection Kit POD (Roche Molecular Biochemicals, Mannheim, Germany). Briefly, the mixture of TdT and fluorescein-2'-deoxyuridine5'-triphosphate (dUTP) were added on the slides and immediately coverslipped using hybrid-slips (Sigma-Aldrich, St. Louis, MO, USA) and incubated at

37°C in a humidified chamber for 60 min (for negative control slides, only the enzyme buffer lacking TdT was added). The coverslips were then removed and the slides washed in PBS, followed by blocking for 20 min at room temperature with 5% normal lamb serum (Cytogen, Lodz, Poland).

Then anti-fluorescein-peroxidase antibody was applied, the slides were again coverslipped and incubated in 37°C for 30 min. After PBS washing (twice for 5 min), the reaction was visualized by 3,3'-diaminobenzidine (DAB) (DAKO, Glostrup, Denmark) by incubating the specimens for 5 min. Then sections were washed in distilled water (twice for 5 min) and counterstained with Mayer's hematoxylin (5 min). Finally, after gently rinsing in the distilled water the slides were mounted in Ultramount Medium (DAKO, Glostrup, Denmark) and coverslipped.

The number of TUNEL-positive (TUNEL⁺) germ or Sertoli cells were counted in 25 subsequent cross-sections of seminal tubules at 1 000 × magnification in the light microscope (Nikon, Eclipse E600, Kanagawa, Japan) and expressed as a percentage of the total number of the given cell type. Germ and Sertoli cells were identified based on their location within the tubule, their size and the shape of nucleus.

2.6 Statistics

Distribution of the data was analyzed using the Shapiro-Wilk test. For the data that were normally distributed, the parametric statistical analysis comparing two independent groups was conducted (unpaired *t*-test) and these data are presented as mean \pm SD. For the data where the normal distribution was not achieved ('Incidence of seminal tubules containing lumen' and 'Area of the seminal lumen'), the non-parametric statistical analysis comparing two independent groups was conducted (Mann-Whitney U test). The median (Md) and the range (Max–Min) were used to present these data. *P* < 0.05 was considered significant.

3 Results

3.1 Testicular weight and morphometry

Table 1 shows that all experimental treatments did not change mean body weights. EB reduced paired testicular weight by half (*P* < 0.001). In turn, after hFSH alone or hFSH in combination with EB, mean testicular weight was doubled (*P* < 0.001 compared with control).

After EB + hFSH, the incidence of tubules containing lumen and surface area of the seminal tubule lumen increased in comparison with the control ($P < 0.01$). These parameters were not significantly different from the control after hFSH alone. After EB alone none of the tubules presented lumen. The mean area of Sertoli cell nuclei was reduced after EB ($P < 0.001$), was unchanged after hFSH and increased after EB + hFSH ($P < 0.05$) in comparison with the control (Table 1).

3.2 Sertoli cell proliferation and apoptosis

Figure 1 (higher panel) shows that the mean number of Sertoli PCNA⁺ cells was increased 4-fold after hFSH ($32.4\% \pm 4.1\%$ vs. $7.7\% \pm 2.0\%$ in the control; $P < 0.001$) and was significantly reduced by EB and EB + hFSH ($1.9\% \pm 0.6\%$ and $2.1\% \pm 0.7\%$ vs. the control, respectively; $P < 0.01$). Incidence of Sertoli TUNEL⁺ cells was increased 9-fold after EB ($3.6\% \pm 2.1\%$ vs. $0.4\% \pm 0.2\%$ in the control; $P < 0.05$) and was eliminated after EB + hFSH ($0.7\% \pm 0.4\%$ vs. the control). As after EB + hFSH, after hFSH alone, Sertoli cell apoptosis was not affected ($0.5\% \pm 0.5\%$ vs. the control).

3.3 Germ cell proliferation and apoptosis

Figure 1 (lower panel) shows that the incidence of PCNA⁺ germ cells was significantly elevated to a similar extent in all experimental groups ($90.8\% \pm 3.7\%$ for EB, $92.5\% \pm 2.1\%$ for hFSH and $91.2\% \pm 1.6\%$ for EB + hFSH vs. $77.7\% \pm 2.2\%$ in the control, $P < 0.001$). The incidence of TUNEL⁺ germ cells was increased 2-fold after the administration of EB ($5.0\% \pm 1.2\%$ vs. $2.5\% \pm 1.3\%$ in the control, $P < 0.05$), unchanged by hFSH ($3.0\% \pm 1.8\%$ vs. the control) and reduced after EB + hFSH ($0.8\% \pm 0.4\%$ vs. the control; $P < 0.05$). Figure 2 presents the representative photomicrographs of seminal tubule cross-sections from the control and ex-

perimental groups labeled immunohistochemically for the presence of PCNA. Figure 3 presents photomicrographs of seminal tubules labeled *in situ* for DNA fragmentation (TUNEL method).

Graphic summary of different experimental regiments on seminal tubule cell maturation, proliferation and apoptosis in two seminal tubule compartments (Sertoli and germ cells) is presented in Figure 4.

4 Discussion

4.1 Doses of test substances

The dose of EB applied here was tested before and appeared to inhibit differentiation of spermatogonia in newborn rats, without affecting secretion of leuteinizing hormone (LH) or FSH ([9] and Kula [unpublished data]). It appears that this dose provides satisfactory filtration of estrogen to the testes. D'Souza *et al.* [10] showed that exogenously administered 17β -estradiol filters into the testis of mature rats with the administration of $100 \mu\text{g}/\text{kg}/\text{day}$ of 17β -estradiol. When increasing the dose to $200 \mu\text{g}/\text{kg}/\text{day}$ intratesticular concentration of estradiol is 10 times higher than normal, without influencing FSH and LH secretions and reduction of intratesticular testosterone concentration to 10% of normal value. In our experiment, the initial body weight of the rat was 10 g (data not shown), increasing to 30 g at the day of autopsy (Table 1). It gives the range from $1250 \mu\text{g}/\text{kg}/\text{day}$ of EB at the beginning to above $400 \mu\text{g}/\text{kg}/\text{day}$ of EB at the end of our experiment. Therefore, intratesticular estradiol concentration is expected to be more than 10 times higher than normal value.

The dose of hFSH was chosen based on the study in which comparable dose of recombinant FSH administered to prepubertal, hypophysectomized rats caused testicular maturation and spermatogenic progression [11].

Table 1. Body and paired testes weight and morphometric parameters of Sertoli cells maturation/function in rats receiving vehicles (control) or 17β -estradiol benzoate (EB) or human FSH (hFSH) or EB + hFSH. ^a $P < 0.001$, ^b $P < 0.05$, compared to the control (unpaired *t*-test); ^c $P < 0.01$, compared to the control (Mann-Whitney *U*-test).

Group (No. of animals)	Body weight (g) Mean \pm SD	Paired testes weight (mg) Mean \pm SD	Area of Sertoli cell nuclei (μm^2) Mean \pm SD	Incidence of seminal tubules containing lumen (%) Median (Min–Max)	Area of seminal tubule lumen (μm^2) Median (Min–Max)
Control (5)	30.8 ± 0.7	96.4 ± 3.4	29.6 ± 1.5	0.0 (0.0–9.0)	0.0 (0.0–27.8)
EB (6)	29.1 ± 2.3	47.0 ± 2.0^a	24.1 ± 0.6^a	Not present	Not present
hFSH (6)	30.9 ± 3.9	198.0 ± 4.7^a	31.6 ± 1.5	2.0 (0.0–44.0)	2.59 (0.0–175.3)
EB+hFSH (5)	29.5 ± 0.9	214.8 ± 10.6^a	33.1 ± 2.3^b	63.0 (58.0–68.0) ^c	264.9 (213.2–451.0) ^c

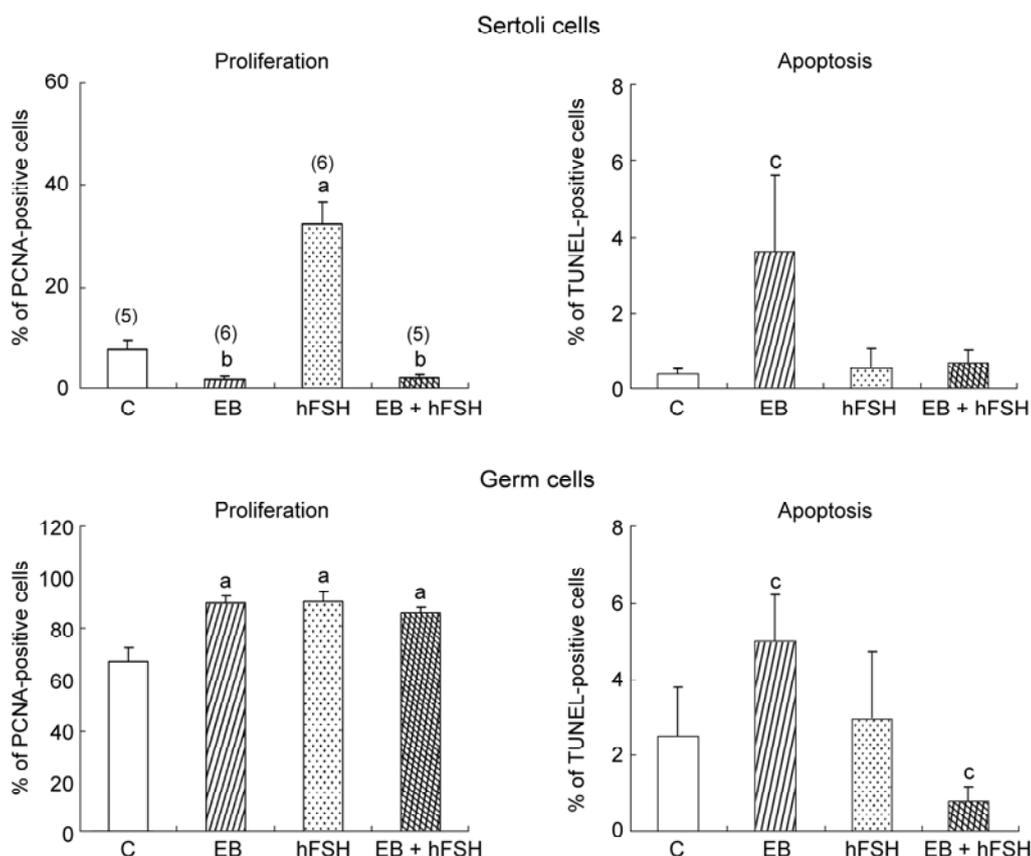


Figure 1. The influence of 17β -estradiol benzoate (EB), human follicle stimulating hormone (hFSH) and EB + hFSH on the incidence of proliferating seminiferous tubule cells (percent of PCNA-positive cells) and the incidence of cells in apoptosis (percent of TUNEL-positive cells) (mean \pm SD). C, control group. ^a $P < 0.001$, ^b $P < 0.01$, ^c $P < 0.05$, compared to C (unpaired *t*-test). Number of animals per group in parentheses. Data are mean \pm SD.

4.2 Sertoli cells

We have shown different effects of the examined substances on Sertoli cell maturation. The three main parameters were: mean area of Sertoli cell nucleus, incidence of the lumen in seminal tubule transsections and mean area of tubule lumen, the latter two being representative of intratubular fluid secretion. Sertoli cell maturation was inhibited by EB, remained unchanged by hFSH, but was unexpectedly stimulated after combined administration of EB and hFSH. Hormonal determinations in these animals are published in Kula *et al.* [9] and show that EB inhibits testosterone and stimulates the secretion of prolactin.

It is well known that FSH stimulates maturation of Sertoli cells, but our data demonstrates that the effect of FSH alone was not strong enough to produce changes in morphometry of Sertoli cells. Instead, estrogen facili-

tated the effect of FSH on Sertoli cell maturation. An enhancement of Sertoli cell maturation after EB + hFSH is intriguing. One possible explanation would be the synergistic action of estrogen and FSH on an arrangement of cell-to-cell contacts during seminal tubule development. MacCalman *et al.* [12] demonstrate that in immature mice, estradiol enhances the stimulatory effect of FSH on the production of mRNA transcript for N-kadherin biosynthesis in Sertoli cells, the protein necessary for intercellular adhesions within seminiferous epithelium. Cell-to-cell interactions (including acceleration of blood-testis barrier formation), might participate in acceleration of Sertoli cell maturation after EB + hFSH. Prevention by prolactin of the FSH-induced downregulation of testicular FSH receptor [13] would also be explanatory. Increased secretion of prolactin after estrogen administration [9] might enhance the stimulatory effect of exo-

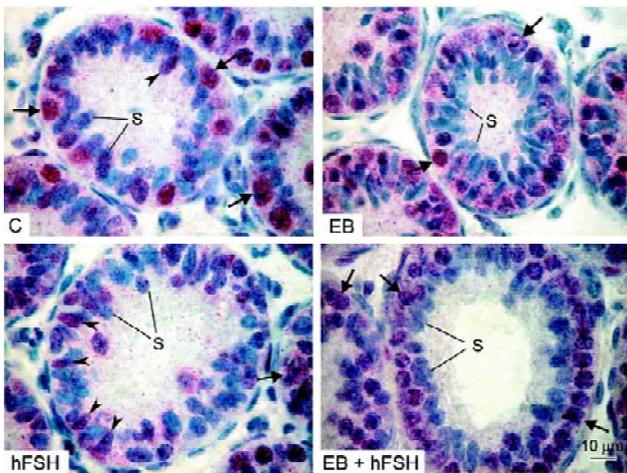


Figure 2. Representative photomicrographs of seminal tubules cross-sections labeled immunohistochemically for the presence of proliferating cell nuclear antigen (PCNA) in control animals (C) and after daily administration of 17 β -estradiol benzoate (EB), human follicle stimulating hormone (hFSH) and EB + hFSH. PCNA-positive germ cells (arrows) and PCNA-positive Sertoli cells (arrowheads) are stained in red. Note numerous PCNA-positive germ cells after all treatments. PCNA-positive Sertoli cells (S) after EB or EB + hFSH were less than after hFSH or in control group (C). Magnification 1 000 \times ; Bar = 10 μ m.

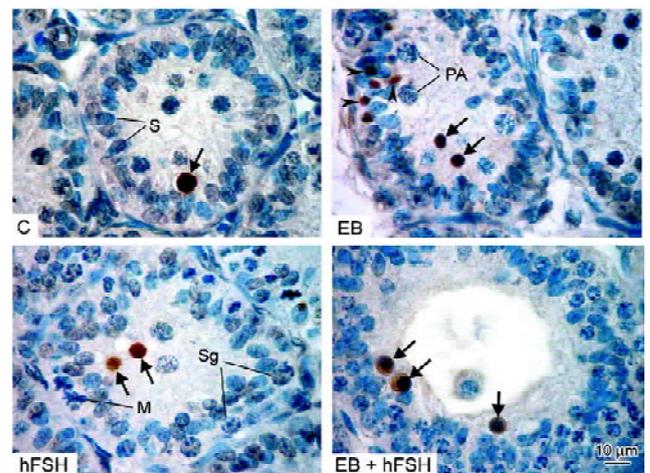


Figure 3. Representative photomicrographs of seminal tubules cross-sections of testes labeled *in situ* for DNA fragmentation (TUNEL method) in control animals (C) and after daily administration of 17 β -estradiol benzoate (EB), human follicle stimulating hormone (hFSH) and EB+hFSH. Brown-stained TUNEL-positive germ cells are marked with arrows and TUNEL-positive Sertoli cells with arrowheads. Note the prominent lumen in seminal tubule of rat treated with EB + hFSH. Sg, spermatogonia; PA, pachytene spermatocytes; M, mitosis of spermatogonia; S, Sertoli cells. Magnification 1 000 \times ; Bar = 10 μ m.

genous FSH on Sertoli cell maturation.

EB alone inhibited proliferation of Sertoli cells. To our knowledge, the inhibitory effect of estradiol on the Sertoli cell proliferation has not been described earlier but is in accordance with the data showing decreased Sertoli cell numbers after administration of a synthetic estrogen-like substance diethylstilbestrol (DES) to immature rats. Although initially related to feedback inhibition of FSH secretion by DES, more recent data shows that DES can decrease Sertoli cell numbers without altering FSH levels [14]. All these data might be in accordance with the recent results showing that reduction of estrogen synthesis in developing boars causes an increase in the final testis size, the number of Sertoli cells and total sperm production in adulthood [15]. Therefore, estrogen might be involved in shortening the proliferative period of Sertoli cells development and, therefore, might determine the final testis size.

To our knowledge, we present the second report describing Sertoli cell apoptosis during the early postnatal period [16]. We have shown that Sertoli cell apoptosis

is stimulated by estrogen. Except for the anti-apoptotic effect of estradiol on spermatids in adult male rats [10], the influence of estrogen on Sertoli or germ cell apoptosis and the first premeiotic steps of spermatogenesis has not been described until now. The cause of estradiol-induced apoptosis would be the decrease of testosterone production, as shown for hypogonadal immature rats by Hakamata *et al.* [17]. We showed here also that Sertoli cells are more sensitive to the pro-apoptotic influence of estradiol (9-fold increase in the incidence of apoptosis) than germ cell does (2-fold increase in the incidence of apoptosis), indicating that apoptotic changes in germ cells might follow those in Sertoli cells.

4.3 Germ cells

Our results show that although EB alone inhibits Sertoli cell function and the survival of Sertoli and germ cells, EB alone stimulates germ cell proliferation. This stimulating effect of EB seems to be a direct one, not mediated by still immature Sertoli cells. Assuming that blood-testis barrier was not yet completely formed [3], easier fil-

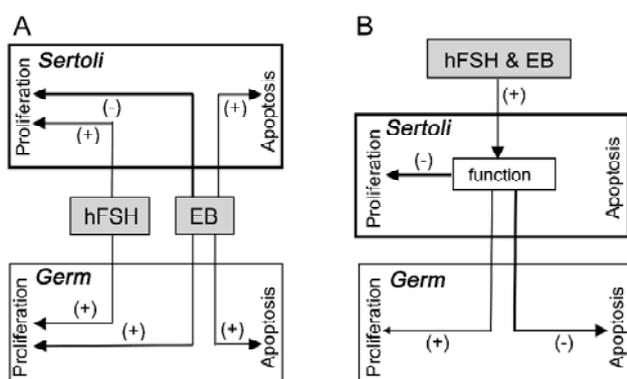


Figure 4. Schematic presentation of the stimulatory (+) and inhibitory (-) effects of 17 β -estradiol benzoate (EB) and human follicle stimulating hormone (hFSH) on cell proliferation, apoptosis and function in two compartments (Sertoli and germ cells) of maturing seminal tubules. (A): After separate administration EB stimulated cell apoptosis in both compartments while hFSH had no effect. Besides common stimulatory effect of both hFSH and EB on germ cell proliferation, the proliferation of Sertoli cells was stimulated by hFSH and inhibited by EB. (B): After combined administration (EB + hFSH), Sertoli cells acquired their mature function precociously. Then, the inhibitory effect of EB on Sertoli cell proliferation persisted, but stimulatory effect of hFSH on Sertoli cells proliferation was neutralized. Initiation of Sertoli cell maturation/function was associated also with the neutralization of the pro-apoptotic effect of EB on Sertoli cell and exerted an anti-apoptotic effect on germ cells.

tration of estrogen to spermatogonia at this age might facilitate the EB effect. Stimulation of the proliferation of spermatogonia by estrogen accords with earlier observations indicating that estradiol stimulates premeiotic germ cell numbers [5, 9]. Administration of 17- β estradiol in the lizard *Padarcis s. sicula* induced spermatogonial proliferation through non-genomic response, through activation of protein kinases 1 and 2. This effect required functional ER, as antiestrogen ICI 182-780 administration *in vivo* neutralized spermatogonial proliferation [18].

Estrogen-induced apoptosis of germ cells in animals was observed earlier and was attributed either to the suppression of FSH or testosterone secretion [19]. However, pro-apoptotic influence of estrogen on germ cells might be a direct one as well. Mishra and Shaha [20] demonstrate *in vitro* that estradiol-induced apoptosis in spermatogonia, spermatocytes and spermatids occurs in the absence of somatic cells, showing Sertoli cell-independent capability of germ cells to respond to estrogen.

Here, when Sertoli cell maturation was accelerated by the combined administration of EB and hFSH, germ cell survival was increased as well. Hence, the completion of Sertoli cell maturation might be responsible for the inhibition of germ cell apoptosis after EB + hFSH.

4.4 Conclusion

Estradiol might play a regulatory role in the seminal tubule maturation at the first spermatogenesis because it has both inhibitory and stimulatory effects. Inhibitory effects might include the inhibition of Sertoli cell proliferation, their maturation as well as Sertoli and germ cell survival. The stimulatory role of estradiol might involve the stimulation of germ cell proliferation and, when estradiol acts together with FSH, the acceleration of Sertoli cell maturation that protects germ cells from death.

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Theme: Environment, Life Style & Genetic/Epigenetic Factors and Men's Health

Date: October 10-13, 2009

Venue: International Conference Hotel, Nanjing, China

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Chairman: Professor Yi-Fei Wang, Acting President of Asian Society of Andrology, Editor-in-chief of AJA

Local Organizing Committee Chairman: Professor Jia-Hao Sha, Director of Laboratory of Reproductive Medicine, Nanjing Medical University, Nanjing, China

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