

# During seminiferous tubule maturation testosterone and synergistic action of FSH with estradiol support germ cell survival while estradiol alone has pro-apoptotic effect

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**Abstract:** During establishment of spermatogenesis at the prepubertal age, an early germ cells apoptotic wave occurs likely aimed to remove abnormal germ cells and to maintain a proper cell number ratio between maturing germ cells and Sertoli cells. Here we assessed Sertoli and germ cell apoptosis in relation to morphological parameters of Sertoli cell maturation in neonatal rats under the influence of testosterone, estradiol and FSH given alone or in combinations. From postnatal day (PND) 5th to 15th male rats were daily injected with: 1) 2.5 mg of testosterone propionate (TP), or 2) 12.5 µg of 17β-estradiol benzoate (EB), or 3) TP+EB, or 4) 7.5 IU of human purified FSH (hFSH), or 5) hFSH+EB or solvents (control-C). Autopsy was performed on PND 16th. Sertoli cell nuclei area and incidence of seminiferous tubule lumen formation (LF) were taken as markers of Sertoli cell maturation. Sertoli and germ cell apoptosis was assessed using TUNEL method. In comparison with C, the area of Sertoli cell nuclei was significantly reduced after EB (25.7±2.0 vs. 30.9±1.6 µm<sup>2</sup> for C, p<0.001) and increased after hFSH+EB (33.1±2.3 µm<sup>2</sup>, p<0.05). Incidence of LF was completely arrested by steroid hormone treatments given separately, significantly inhibited after TP+EB (median: 0.0%, vs. 2.0% for C p<0.05) and significantly enhanced after hFSH+EB (median: 51.0%, p<0.001). hFSH alone did not influence LF. Incidence of TUNEL positive Sertoli cells significantly increased after EB (median: 2.9% vs. 0.5% for C, p<0.05) or TP+EB (median: 2.2%, p<0.01) and was not affected by other treatments. Incidence of TUNEL positive germ cells increased significantly after EB alone (median: 4.4% vs. 2.5%, for C, p<0.01) and was significantly decreased by hFSH+EB (median: 0.5%, p<0.01). Conclusions: 1) Administration of testosterone or estradiol to immature rats inhibits Sertoli cell maturation. 2) Estradiol stimulates Sertoli and germ cell apoptosis while testosterone has no effect. 3) Testosterone eliminates estradiol-induced germ cell apoptosis when both hormones act in concert. 4) FSH in concert with estradiol, but neither one of the hormone alone, accelerate Sertoli cell differentiation and effectively inhibit germ cell apoptosis. 5) During seminiferous tubule maturation testosterone and the synergistic action of FSH with estradiol support germ cell survival while estradiol alone has an inhibitory, pro-apoptotic effect.

**Key words:** Estradiol - FSH - Testosterone - Germ cells - Sertoli cells - Apoptosis

## Introduction

In the rat several postnatal days (PND) overlap with initiation of sexual maturation. Sertoli cells proliferate during fetal and early postnatal period until around the 15th PND [1], setting the full complement of adult Sertoli cells that display a network of communication with germ cells. During establishment of spermatogenesis at the prepubertal age, an early germ cells apoptotic wave occurs likely aimed

to remove abnormal germ cells and to maintain a proper cell number ratio between maturing germ cells and Sertoli cells [2,3]. As a result, up to 75% spermatogenic cells are eliminated during seminiferous tubule maturation [4,5]. It has been shown that the intracellular balance of BclxL and Bax proteins is crucial for this early apoptotic wave [6]. The disturbances of the apoptosis during the first round of spermatogenesis appears to be critical for the normal development and function of the adult testis. However, a relative role of the hormones, that may control germ cell apoptosis, is not fully elucidated.

In addition to the established role of gonadotropins and androgens in spermatogenesis

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estrogens are now recognized as potential regulators of male reproduction [for review 7]. Testes produce estradiol [8] and express estrogen receptors (ER) [9]. Although findings from mice with transgenic inactivation of ER and aromatase have demonstrated no changes in the initiation of spermatogenesis [10], the other studies, analyzing foetal and neonatal testicular development, clearly demonstrated that endogenous estrogens regulate the establishment of the male germ cell lineage throughout these periods [11-13].

In this study we assessed the apoptotic potential of the seminiferous epithelium in relation to Sertoli cells maturation under the influence of testosterone, estradiol and follicle stimulating hormone (FSH). Experiments were performed in immature male rats in the period that covers multiplication of Sertoli cells and cessation of this process which co-exists with the beginning of Sertoli cell function including an establishment of the blood-testis barrier [14].

## Material and methods

**Animals and hormone treatment.** Five-day-old male Wistar rats, born on the same day, were randomly divided into experimental groups of 5-12 animals. Rats were daily subcutaneously injected from the 5th to 15th PND with following substances: 1) 2.5 mg of testosterone propionate (TP) (Testosteronum propionicum, Jelfa, Poland), 2) 12.5 µg of 17β-estradiol benzoate (EB) (Oestradiolum benzoicum, Jelfa, Poland), 3) TP and EB together (TP+EB), 4) 7.5 IU of human purified FSH (hFSH) (Metrodin, Serono, UK), 5) hFSH and EB together (hFSH+EB) and 6) solvents for tested hormones (control - C).

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

**Processing of the tissue.** Autopsy was performed on the 16th PND. Animals were anaesthetised with methohexital sodium (Brietal, Eli Lilly, USA) and fentanyl (Fentanyl, Polfa, Poland) and weighed. The testes were excised, weighed and fixed in Bouin's solution for up to 24 hours. Subsequently, testes were processed through graded alcohols and embedded in paraffin.

**Morphometric procedures.** Sections of paraffin-embedded testes, 5 µm thick, taken from equatorial cross-sections of the organ were routinely stained with haematoxylin and eosin. Using light microscope Nikon, Eclipse-E600 the percentage of seminiferous tubule cross-sections containing a clear lumen was determined. Scoring was performed in 100 subsequent round-shaped cross-sections of the seminiferous tubules per animal. The diameters of 30 randomly selected transverse sections of the round-shaped seminiferous tubules were measured for each animal across the minor axis of their cross-sectioned profiles. 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 v3.60HM (Logitex, Poland).

**Apoptosis of germ and Sertoli cells.** To detect nuclei with DNA fragmentation, representing a hallmark of apoptosis, Terminal Deoxynucleotidyl Transferase (TdT) mediated dUTP Nick End

Labelling (TUNEL) method was performed. Sections of the testis, 5 µm thick, were mounted on silanized slides (SuperFrost Plus, Dako, USA), deparaffinized, rehydrated and incubated with proteinase K (20 µg/ml) (Sigma, USA) for 15 min in room temperature. Endogenous peroxidase activity was blocked by immersion in 3% (v:v) H<sub>2</sub>O<sub>2</sub> in methanol for 30 min. After two washes (5 min each) in phosphate buffered saline (PBS, 0.01 M, pH 7.4) (Biomed, Poland) the staining procedures started. Apoptotic cells were visualised using In Situ Death Detection Kit POD (Roche Molecular Biochemicals, Germany) according the instruction provided. Briefly, the mixture of TdT and fluorescein-2'-deoxyuridine5'-triphosphate (dUTP) were added on the slides and incubated in 37°C in humidified chamber for 60 min (for negative control slides, only the enzyme buffer lacking TdT was added). Then the slides were washed in PBS, followed by blocking for 20 min at room temperature by 5% normal lamb serum (Cytogen, Poland). Anti-fluorescein-peroxidase antibody was applied and the slides were incubated in 37°C for 30 min. The reaction was visualized by 3,3'-diaminobenzidine (DAB) (DAKO, Denmark) by incubation the specimens for 5 min. The sections were counterstained with Mayer's hematoxylin (5 min) and mounted in Ultramount Medium (Dako, Denmark).

The number of TUNEL-positive (TUNEL+) germ or Sertoli cells were counted in 25 subsequent cross-sections of seminiferous tubules at 1000x magnification and expressed as a percent of the total number of the given cell type. Germ and Sertoli cells were identified basing on their location within the tubule, their size and the shape of nucleus.

**Statistical analysis.** Distribution of the data was analysed using Shapiro-Wilk's test. For the data that were normally distributed the parametric statistical analysis comparing two independent groups was conducted (t-test). These data were presented as mean SD. For the data where the normal distribution wasn't achieved the non-parametric statistical analysis comparing two independent groups was applied (Mann-Whitney's U test). These data were presented as median and the range. For all data  $p < 0.05$  was considered significant.

## Results

### *Testicular weight and morphometry*

Fig. 1 presents that in relation to C, mean paired testes weight (mg/100 g b.w.) was significantly reduced to 75% ( $p < 0.01$ ) after TP, to 50% after EB ( $p < 0.001$ ) and recovered to C values after TP+EB. After hFSH alone or hFSH + EB the mean relative paired testes weight doubled that of C ( $p < 0.001$ ). Seminiferous tubules diameter was significantly decreased after EB ( $75.6 \pm 7.6$  vs.  $82.7 \pm 3.3$  µm in C,  $p < 0.01$ ) and significantly increased after hFSH + EB ( $94.9 \pm 2.4$  µm;  $p < 0.001$ ). A significant increase in testes weight ( $p < 0.001$ ) and seminiferous tubules diameter ( $p < 0.05$ ) were present when compared EB alone treated animals with those treated with TP+EB or when compared seminiferous tubules diameter of hFSH alone treated with hFSH+EB ( $80.9 \pm 4.8$  for hFSH vs.  $94.9 \pm 2.4$  µm for hFSH+EB,  $p < 0.05$ ).

Fig. 1 presents also that mean area of Sertoli cell nuclei was reduced after EB ( $25.7 \pm 2.0$  vs.  $30.9 \pm 1.6$  µm<sup>2</sup> for C;  $p < 0.001$ ), did not change after hFSH or TP or TP+EB and increased after hFSH+EB ( $33.1 \pm 2.3$

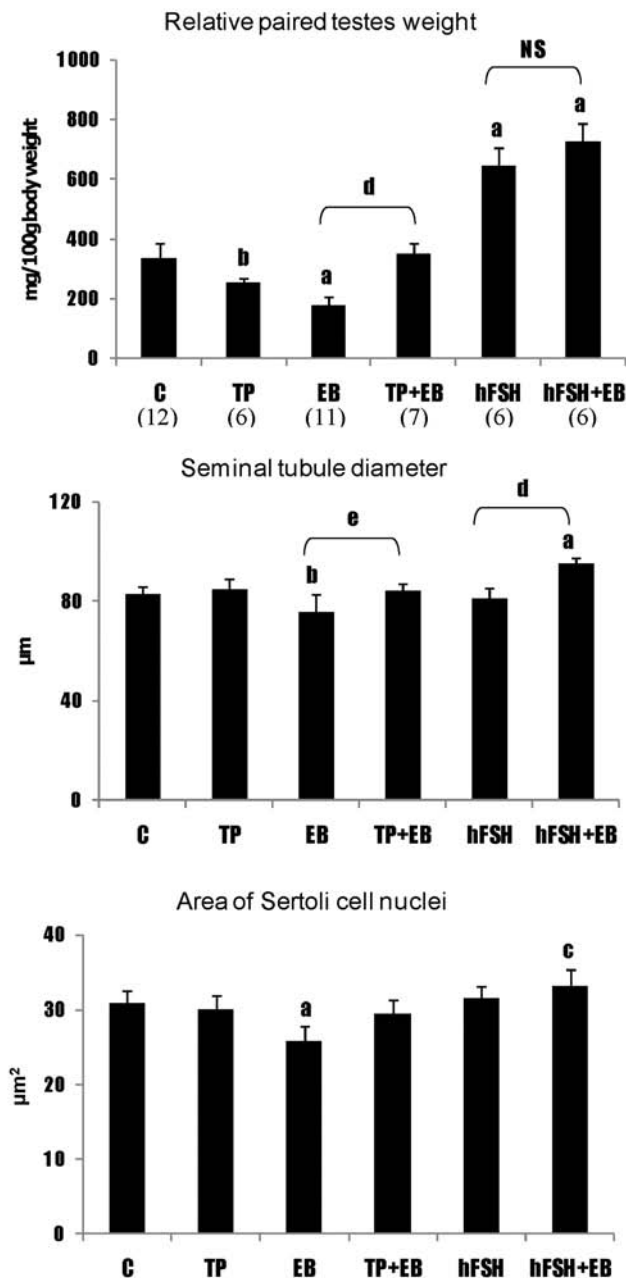


Fig. 1. Mean  $\pm$ SD of relative paired testes weight, seminiferous tubules diameter and the area of Sertoli cell nuclei in 16-day old rats receiving vehicles (C), testosterone propionate (TP) or 17 $\beta$ -estradiol benzoate (EB) or TP+EB or human FSH (hFSH) or hFSH+EB. <sup>a</sup> $p$ <0.001; <sup>b</sup> $p$ <0.01; <sup>c</sup> $p$ <0.05 vs. C; <sup>d</sup> $p$ <0.001; <sup>e</sup> $p$ <0.05 for EB vs. TP+EB or for hFSH vs. hFSH+EB. (*t*-test); NS-not significant. Number of animals in parentheses.

$\mu\text{m}^2$ ,  $p$ <0.05). The incidence of LF was arrested by TP or EB treatments, significantly inhibited by TP+EB (median: 0.0%; range 0.0%-2.0% vs. median: 2.0%; range: 0.0%-15.0% for C,  $p$ <0.05), was not influenced by hFSH given alone and greatly enhanced after hFSH+EB (median: 51.0%; range: 45.0%-63.0%,  $p$ <0.001) (Fig 2).

### Sertoli and germ cell apoptosis

Fig. 3 demonstrates that the incidence of TUNEL-positive Sertoli cells increased significantly after EB alone (median: 2.9%; range: 2.0%-7.2% vs. median: 0.5%; range: 0.2%-1.5% for C,  $p$ <0.05) or TP+EB (median: 2.2%; range: 1.7%-2.8%,  $p$ <0.01), but combined administration of hFSH and EB eliminated the effect of EB alone. hFSH alone or TP alone did not influence the incidence of apoptotic Sertoli cells.

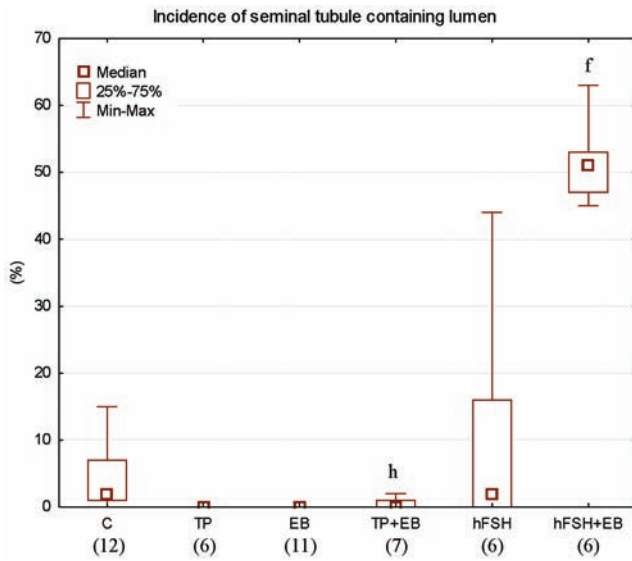
The incidence of TUNEL-positive germ cells increased after administration of EB (median: 4.4%; range: 2.9%-6.3% vs. median: 2.5%; range: 1.2%-3.9% for C,  $p$ <0.01) and was inhibited by hFSH+EB (median: 0.5%; range: 0.5%-1.4%,  $p$ <0.01). Neither TP nor hFSH alone did change the incidence of germ cells apoptosis but TP when given together with EB eliminated EB-induced increase in the incidence of TUNEL-positive germ cells.

### Discussion

The doses of TP and EB applied here were tested before and appeared to inhibit differentiation of spermatogonia in newborn rats, without affecting secretion of FSH [13,15, and our unpublished data]. Moreover, it appears that these doses provide satisfactory filtration of both steroids to the testes [16,17]. 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 [18].

### Testicular growth and Sertoli cell differentiation

Whether maturation of Sertoli cells involves a rapid switch from an immature to mature stage or a step-wise cascade of changes, that may occur over a period of time, is not yet entirely clear. Features of the progression in Sertoli cell maturation measured here were: 1) an increase in nucleus size and 2) formation of the tubule lumen, as a result of the beginning of intratubular fluid secretion that corresponds to the formation of the inter-Sertoli cell junctions (formation of blood-testis barrier) [14]. FSH stimulates both testicular growth and maturation of Sertoli cells [19,20], but our data demonstrated that the stimulatory effect of hFSH on Sertoli cell maturation was more pronounced, when hFSH was given together with EB than after hFSH alone. Namely, beside stimulation of testicular weight (seen after hFSH alone), after hFSH+EB seminiferous tubule diameter, the area of Sertoli cell nuclei and percentage of tubules containing lumen were also increased. All above parameters were indicative for greatly enhanced or precociously complete Sertoli cells maturation. The possible explanation for these would be the synergistic action of estradiol and



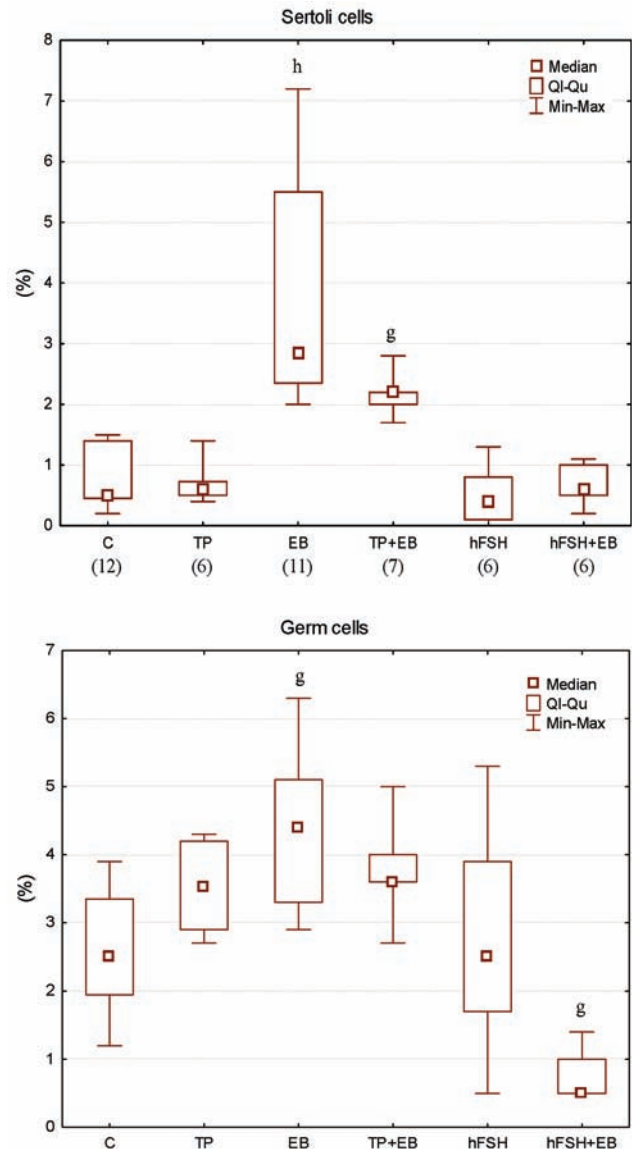
**Fig. 2.** The incidence (percent) of seminiferous tubule cross-sections containing lumen in 16-day old rats receiving vehicles (C), testosterone propionate (TP) or 17 $\beta$ -estradiol benzoate (EB) or TP+EB or human FSH (hFSH) or hFSH+EB. <sup>f</sup> $p < 0.001$ ; <sup>h</sup> $p < 0.05$  vs. C (Mann-Whitney U test). Data presented as median, Q1 - lower quartile, Qu - upper quartile and the range (min-max). Number of animals in parentheses.

FSH on an arrangement of cell-to-cell contacts during seminiferous tubule development. Mac Calman *et al.* [21] demonstrated that in immature mice estradiol enhanced the stimulatory effect of FSH on the production of mRNA transcript for N-cadherin biosynthesis in Sertoli cells, the protein necessary for intercellular adhesions within seminiferous epithelium. Inhibition of Sertoli cell maturation seen here after separate administration of TP or EB may be best explained by the feedback inhibition of FSH secretion exerted by sex steroids. However, since this feedback may not be effective in immature rats [13,15], discordant testosterone/estradiol availabilities within testes induced by either treatment with single hormone remains the other possibility.

### Cell apoptosis

Hormonal dependence of immature Sertoli cells apoptosis is unknown but increased apoptosis of Sertoli cells in congenitally hypogonadic (hypogonadotropic) rats during early postnatal period of life was presented [22], indicating that gonadotropins and/or sex steroids are protective for Sertoli cells survival. Our data suggest that estradiol is not protective as it stimulated Sertoli cell apoptosis and co-administration of testosterone could not eliminate this pro-apoptotic effect of estradiol.

What concerns apoptosis of germ cells, we demonstrated its significant increase after estradiol administration and inhibition after combined administration of



**Fig. 3.** The incidence (percent) of TUNEL-positive Sertoli and germ cells in 16-day old rats receiving vehicles (C), testosterone propionate (TP) or 17 $\beta$ -estradiol benzoate (EB) or TP+EB or human FSH (hFSH) or hFSH+EB. <sup>g</sup> $p < 0.01$ ; <sup>h</sup> $p < 0.05$  vs. C (Mann-Whitney U test). Data presented as median, Q1 - lower quartile, Qu - upper quartile and the range (min-max). Number of animals in parentheses.

FSH and estradiol. Estrogen-induced apoptosis of testicular germ cells in adult and immature animals is attributed either to the suppression of FSH or testosterone secretion [23,24]. However, pro-apoptotic influence of estrogens on germ cells may be direct one as well, mediated through hormone-ER interaction [25,26]. Mishra and Shaha [27] demonstrated that estradiol-induced apoptosis in different germ cells (spermatogonia, spermatocytes, spermatids) in vitro occurred in the absence of somatic cells, showing independent (even not mediated by Sertoli cells) capability of germ cells to respond to estrogen with apoptosis.

Antiapoptotic effect of estrogen on spermatogenesis has also been observed. Some studies *in vivo* and *in vitro* revealed that estrogen can act as germ cell survival factor and that this effect was dose-dependent [28,29]. For example, estradiol prevents apoptosis of germ cells within human seminiferous tubules *in vitro* even in the absence of gonadotropins [29]. Spermatogonial renewal in adult Japanese eel was stimulated by estrogens and was blocked by an estrogen antagonist administration [30]. In the view of our results it seems probable that proapoptotic effect of estradiol on germ cells may change into antiapoptotic one with dependence on the state of the progression of seminiferous tubule maturation and/or absence or presence of FSH.

In mature rats gonadotropins and testosterone have been shown to regulate testicular germ cell apoptosis in a stage-dependent manner [23]. We showed here that testosterone did not influence apoptosis of germ cells but that testosterone administration to estrogen-treated animals eliminated an estrogen-induced apoptosis of germ cells. These may indicate that testosterone protects germ cells from death. This finding is supported by other data demonstrating that in immature rat hypophysectomy or treatment with gonadotropin-releasing hormone (GnRH) antagonist results in the increased germ cell apoptosis, reversed by human chorionic gonadotropin (hCG) or testosterone administrations [5,24].

Apoptosis in immature rat testis can be induced by immunoneutralization of FSH [31]. Our data indicates, however, that not FSH alone but FSH in synergism with estradiol are effective to inhibit germ cell apoptosis. We have previously demonstrated that the administration of estradiol together with FSH in newborn rats greatly enhanced the stimulatory effect of FSH on the first spermatogenesis, resulting in the precocious quantitative completion of premeiotic steps [13]. In view of the presented here results this stimulatory synergistic effect on germ cell numbers might rely on the inhibition of germ cell apoptosis. It seems that the clue role might play precocious acquisition of Sertoli cell function. The most recent studies showed that the absence of blood-testis barrier can be one of the factors causing apoptosis of germ cells in immature rats [32].

Inhibition of germ cell apoptosis might engage the changes in the activity of the stem cell factor (SCF) c-kit system. Namely, in the *in vitro* studies [33] and in experiments on genetically modified animals [34] this system is involved in the protection of germ cells from apoptosis. This mechanism may be responsible for the changes in the Bcl-2 family members, since in cultured rat seminiferous tubules either FSH or Sertoli cell-derived SCF can up-regulate the anti-apoptotic Bcl-w [35,36]. The Bcl-2 family may also contribute to the anti-apoptotic effect of testosterone, as testosterone

withdrawal results in germ cell apoptosis together with down-regulation of Bcl-w and up-regulation of pro-apoptotic Bax and Bak [37,35]. FSH is able to stimulate SCF expression in the testis *in vitro* [38] and estradiol stimulates the production of SCF in the somatic cells of the fetal gonads [39], what may explain the presented here synergistic effect of FSH and estrogen on the inhibition of germ cell.

Neither hFSH alone nor TP alone did change the incidence of apoptotic germ cells. It may be because the level of apoptotic DNA fragmentation of the seminiferous epithelium in rats is age-dependent and naturally peaks between the 16th and 28th day of age, which is beyond a time window examined here. Furthermore the treatment with GnRH antagonist revealed the age-dependence of germ cell survival resulting from FSH action. Namely, in 16-32 day old animals treatment with GnRH antagonist (inhibiting FSH and testosterone secretion) increased apoptotic DNA fragmentation while it did not affect cell apoptosis in animals younger than 16 days [5]. Presumably, acceleration of Sertoli cell maturation would increase the response of germ cells to FSH and testosterone in immature rats.

It is concluded that: 1) Administration of testosterone or estradiol to immature rats inhibits Sertoli cell differentiation. 2) Estradiol stimulates Sertoli and germ cell apoptosis while testosterone has no effect. 3) Testosterone eliminates the estradiol - induced germ cell apoptosis when both hormones act in concert. 4) FSH in concert with estradiol, but neither one of the hormone alone, accelerate Sertoli cell differentiation and effectively inhibit germ cell apoptosis. 5) During seminiferous tubule maturation testosterone and the synergistic action of FSH with estradiol support germ cell survival while estradiol alone has a proapoptotic effect.

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