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Research Article

In Vitro and In Vivo Effects of Serine and Threonine on Follicle Growth, Differentiation and Atresia

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Abstract: This work was designed to test the effect of serine and threonine on follicle growth, differentiation and atresia under in vitro and in vivo conditions. Two experiments were conducted. In the first experiment, follicles 90-120 µm in diameter, from 21-day old mice, were dissected mechanically and cultured individually for 7 days. Follicles were subjected to 4 different treatments. Follicles in medium without amino acids and gonadotrophins were assigned as control groups. Follicles in medium with PMSG and without amino acids constituted a positive control whereas the other treatments were test groups. Every 24 h follicle diameters were measured and the media was changed. In the second experiment, the mice were injected daily with 0.2 ml of saline (control) or saline with a mixture of serine and threonine (test) for 5 days. After the last injection, the mice were cervically dislocated and the ovaries were removed and then stained with haematoxylin and eosin. The numbers of activated primordial follicles, primary follicles, preantral follicles, antral follicles and atretic follicles were blind counted in every 7 sections. In culture, neither of the treatments significantly increased the diameters of the follicles over those of the control group. According to the results of the second experiment, the injection of serine/threonine significantly reduced the number of activated primordial follicles entering the primary stage of growth, but after the primary stage the growth rate increased in the amino acid injected animals. Preantral follicle growth beyond the secondary stage was stimulated by amino acid injection and more follicles went through to the antral stage. As the growth rate increased, the number of atretic follicles was also increased by amino acid injection. In the early antral stage, the number of atretic follicles in the test animals was significantly lower, because atresia in the test group occurred mostly in the late antral stage.

Key Words: Mouse, amino acids, culture, follicle growth, atresia.

Serine ve Threonine Amino Asitlerinin İn Vitro ve İn Vivo Koşullarda Fare Foliküllerinin Büyümesine, Farklılaşmasına ve Dejenerasyonuna Etkisi

Özet: Bu çalışma, serine ve threonine amino asitlerinin foliküler büyümeye, farklılaşmaya ve dejenerasyona etkilerini in vivo ve in vitro koşullarda araştırmak için planlandı. Bu amaçla biri in vitro diğeri ise in vivo olmak üzere iki deney düzenlendi. Birinci deneyde, 21 günlük ev faresine ait foliküller kültürde dört değişik işleme (kontrol, pozitif kontrol (Gebe kısrak serum gonadotropini, PMSG), serine/threonine (AA) ve AA + PMSG) tabi tutularak her 24 saatte bir foliküllerin çapları ölçüldü. İkinci deneyde aynı farelere izotonik tuz çözeltisi (kontrol) ve 165 nmol serine ile 70 nmol threonine içeren bir karışım (test) günde bir kez beş gün boyunca enjekte edildi. Son enjeksiyondan sonra hayvanlara servikal dislokason yapıldı, sonra ovaryumlar histolojik analizler için alındı. Alınan organlar mikrotomla 10 µm kalınlıkta kesildikten sonra eosin ve hematoksilin ile boyandı. Kesitler üzerindeki foliküller mikroskop altında çaplarına göre, antrum oluşumuna göre ve dejenere olup olmadıklarına göre sınıflandırılarak sayıldı. Birinci deneyde amino asitlerin primer folikül gelişimine bir etkisinin olmadığı belirlendi. İkinci deneyde amino asit enjeksiyonu primer foliküller ile dejenerasyona uğrayan erken antral safhadaki folüküllerin sayısının azalmasına sebep olur iken antral safhadaki sağlıklı foliküllerin sayısını artırdı. Sonuç olarak in vitro koşullarda serine ve threonine amino asitlerinin yanlız veya PMSG ile birlikte kültüre ilavesinin küçük foliküllerin büyümesine etkisi olmadığı fakat bu amino asitlerin enjeksiyonu primer foliküllerin ve dejenere olmuş erken antral safhadaki foliküllerin sayısını azaltığı, antral safhadaki sağlıklı foliküllerin sayısını da artırdığı belirlenmiştir.

Anahtar Sözcükler: Fare, amino asit, kültür, foliküler büyüme, dejenerasyon.

Introduction

To understand the basic factors affecting follicle growth, differentiation and atresia, scientists have used in vivo and in vitro methods. Experiments on freshly dissected large follicles from various species showed that oocyte maturation and ovulation could be induced in vitro (1). Many studies on isolated and purified cell types have identified factors that might influence follicle

development. More recently, various methods of growing small intact follicles to maturity in vitro have been developed (2).

The amino acids serine and threonine are major substrates for phosphorylation by protein kinases (3). Some of the intra-cellular proteins that cause gene expression and hormone secretion are phosphorylated on serine and threonine residues, examples being the cAMP-responsive element binding proteins (CREB) and the cAMP-responsive enhancer element modulator (CREM) (4). This phosphorylation is important for the activation of other intra-cellular proteins involved in gene transcription and signal transduction resulting in the expression of TGF-β like proteins.

Serine and threonine amino acids may have an effect on follicle growth, differentiation and atresia due to the phosphorylation of these amino acids and consequent production of TGF-β like peptides. Therefore, the aim of this study was to determine the effects of a mixture of serine and threonine on mouse follicle growth, differentiation and atresia under in vivo and in vitro conditions.

Materials and Methods

Experiment (1)

Animals

Twenty-one-day old prepubertal mice were killed by cervical dislocation. The ovaries were removed and placed in a petri dish containing supplemented sterile dissection media (5).

Follicle dissection

Follicles were mechanically dissected in Leibovitz L15 dissection medium, using a 0.33 x 12 mm needle (Cat; AG D-34212, Omnicon, Belgium).

Follicle culture

Follicles 90-120 µm in diameter were cultured in α-MEM culture medium; (Cat; 11900-016, Life Technologies, Paisley, Scotland) (5).

Preparation of amino acid solution

The quantity of amino acids added to the culture medium was based on the amino acid concentrations in mouse plasma (6). The concentrations of L-serine, (cat; 21101-019, Life Technologies, Paisley, Scotland) and L-threonine (Cat; 103053, ICN, Ohio, USA) were adjusted to contain 165 nmol of L-serine and 70 nmol of L-threonine in 1 ml of culture medium.

Preparation of Pregnant Mare Serum Gonadotrophin Solution (PMSG)

In the study, 5000 IU of powdered PMSG (Cat; 2448; Intervet, Cambridge, England) was dissolved and 1 ml containing 200 IU PMSG was kept as a stock solution, and 5 µl (1 IU) of this was added to 1 ml of incubation medium.

Study groups

Control: Follicles were cultured individually for 7 days in supplemented α-MEM culture medium.

Positive control: Follicles were cultured in supplemented α-MEM medium for 2 days and PMSG was added for 5 days.

Test: Follicles were cultured in media containing a mixture of serine and threonine for 7 days (AA).

Test: Follicles were cultured in media containing a mixture of serine and threonine for 2 days and then PMSG was added for 5 days (AA + PMSG).

Experiment (2)

Treatments

Five to six mice from a litter were kept in the same box, and 2 to 3 of these were treated as controls and the other 2 to 3 were treated as the test group.

Experimental procedure

Mice were injected (intra-peritoneally) daily between 10:00 and 12:00 for 5 days. After 5 days, the mice were cervically dislocated 60 min after the last injection of saline or amino acids.

Histology

The ovaries were collected and dehydrated with increasing concentrations of alcohol and then embedded in wax. Each ovary was serially sectioned at 10 µm. Cut sections were mounted on glass slides and stained with 210 ml of Mayer's haematoxylin (containing 0.1% haematoxylin) and 1% eosin. Atretic and healthy follicles were identified under a light microscope at 400x magnification, by counting dark stained nuclei. All follicles were counted and the diameters were measured under the light microscope using an ocular micrometer at 100x magnification. Follicles with an oocyte and surrounded by a part layer of cubical granulosa cells were classified as

activated-primordial. Follicles with an oocyte completely surrounded by one layer of cubical granulosa cells were classified as primary follicles. Follicles with 2, 3 or 4 layers of granulosa cells were classified as pre-antral. Follicles at the beginning of antrum formation were classified as early antral (Figure 1). Follicles with a large, fully formed antral cavity were classified as late antral (Figure 2). All slides were bind counted (Figures 1 and 2).

Differentiation and classification of atretic and non-atretic follicles.

Cells with condensed chromosomes within the nuclei at x400 magnification were considered atretic due to their dark appearance. Depending on the number of atretic cells, follicles were classified as early atretic, late atretic or healthy. Follicles with 0-20 atretic cells were treated as healthy, follicles with 20-40 atretic cells were considered early atretic and follicles with more than 40 atretic cells were considered late atretic.

Statistical anlysis

Data were analysed by unpaired t-test

Results

In vitro follicle growth

Average follicle growth for the control group was 8 µm during the first 48 h of culture, after which follicles went to atresia (Figure 3). The addition of PMSG to serine and threonine supplemented follicles did not significantly increase the growth rate of cultured follicles above that of the follicles supplemented only with amino acids.

Addition of PMSG to serine and threonine supplemented media did not further increase the diameter of the follicles.

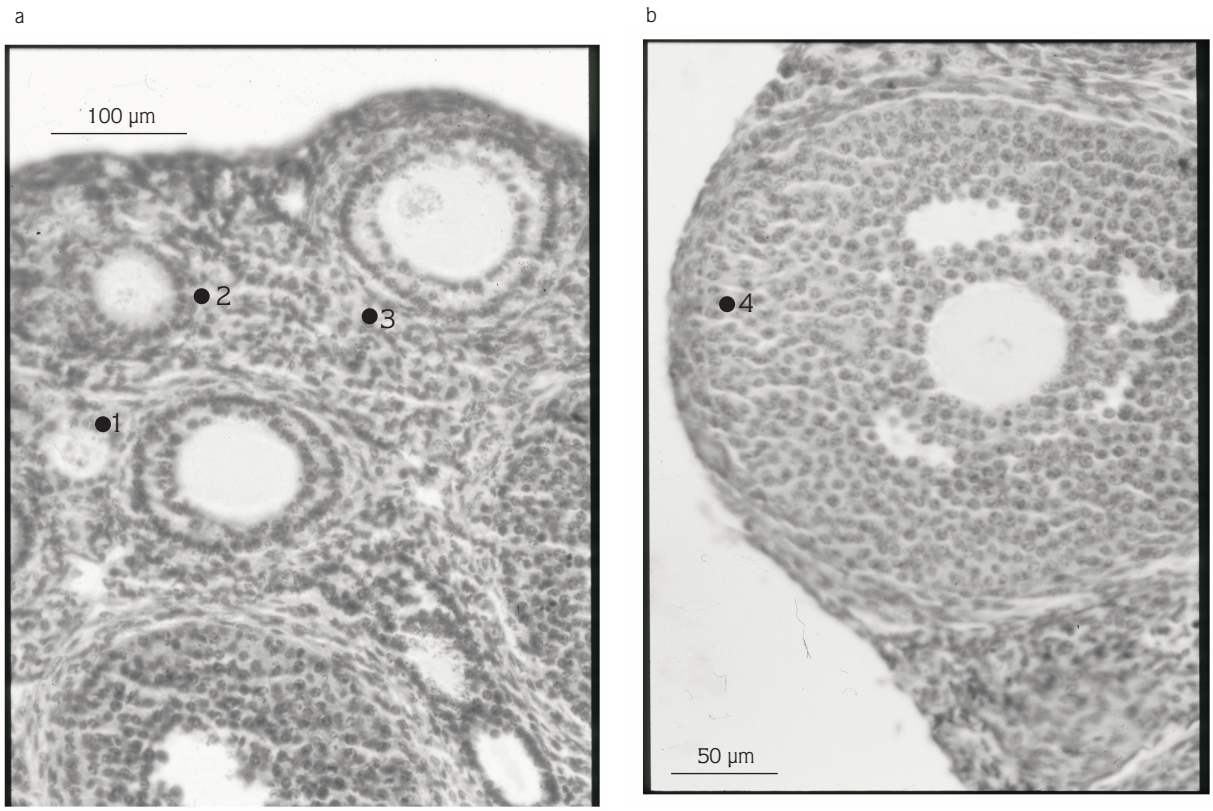

Figure 1. Photomicrographs of mice ovaries showing various classes of follicles at 200x magnification. a_1: Follicle in primordial-primary transitional stage surrounded by a few cubical granulosa cells around the oocyte. a_2: A primary follicle surrounded by one layer of granulosa cells. a_3: A secondary follicle containing 2 layers of granulosa cells. b_4: A healthy early antral follicle; the antrum is not completely formed.

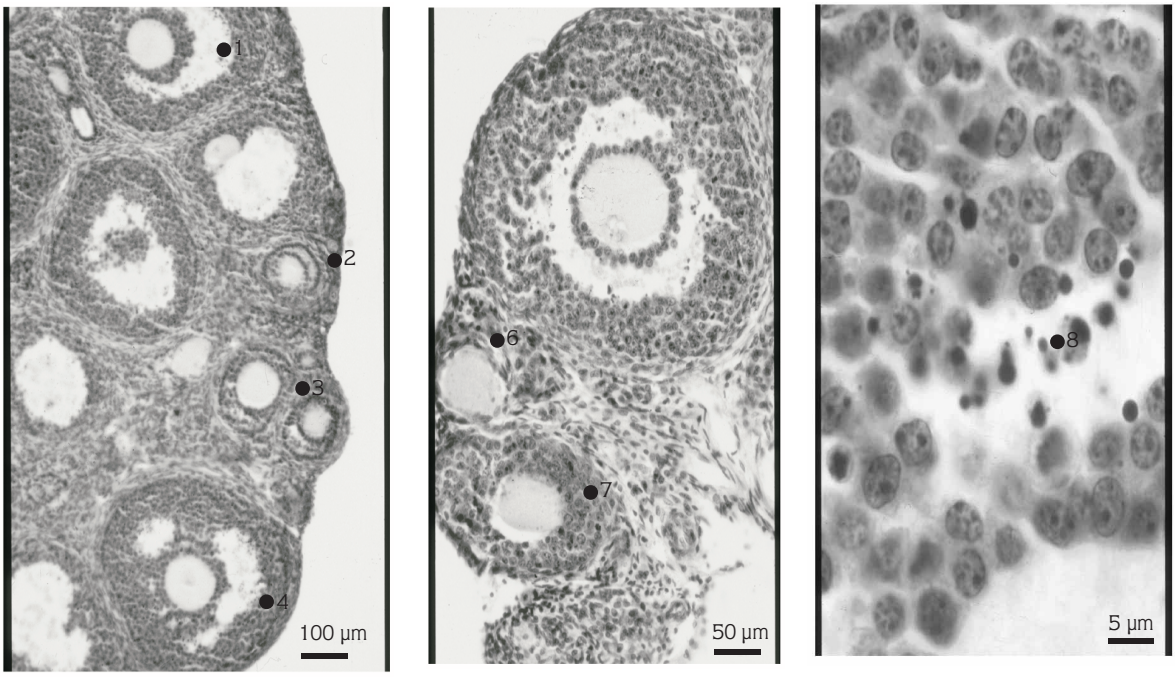

Figure 2. Three photomicrographs of mouse ovaries at 100x (a), 200x (b) and 400x (c) magnification showing: a_1: Healthy antral follicle. a_2: Secondary follicles with 2 layers of granulosa cells. a_3: Primary follicle with one layer of cubical granulosa cells. a_4: Healthy early antral follicle b_5: Atretic antral follicle. b_6: Atretic preantral follicle. b_7: Preantral follicle with more than 3 layers of granulosa cells. c_8: Dark stained nucleus indicating atresia.

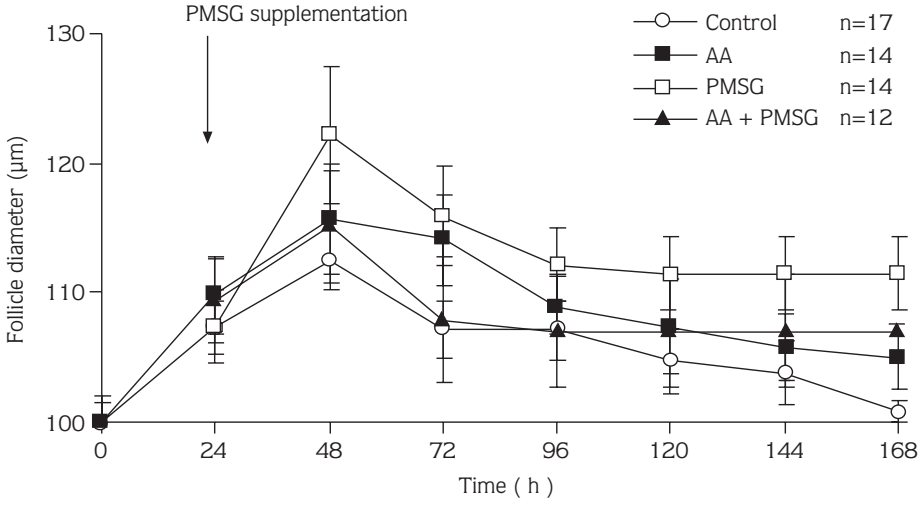

Figure 3. Changes in follicle diameters during 168 h in culture. Amino acids did not significantly increase follicle growth over that of the control group. Amino acids together with PMSG did not cause any significant change over the AA group. Pregnant mare serum gonadotrophin was added at 24 h and it caused a non-significant increase in follicle growth.

In vivo follicular growth, differentiation and atresia

Size distribution

The results show that there tended to be more follicles >200 µm in diameter in mice injected with serine and threonine (Table 1).

Class Distribution

Table 2 shows that amino acid injection reduced the number of primary follicles ($P < 0.05$) and there was no difference in the number of preantral and antral follicles ($P > 0.05$).

The injection of amino acids significantly reduced the number of primary follicles.

Table 3 also shows that ovaries of serine and threonine injected mice tended to contain more antral follicles than did the ovaries of the control mice.

Atresia

Atresia was not seen in activated primordial or primary follicles from each group. More preantral follicles in mice injected with amino acids were atretic ($P < 0.05$) (Table 4). The numbers of atretic late antral follicles were not different.

The numbers of healthy early antral follicles were significantly higher in mice injected with serine and threonine (Table 5).

Table 3. The number of antral follicles in mouse ovaries injected with saline or serine/threonine (mean ± sem).

Groups	Antral follicles		
	Early antral	Late antral	Total
Control	28 ± 4.0	24 ± 3.7	52 ± 7.7
Test	31 ± 3.2	26 ± 3.7	57 ± 6.9

Table 4. The number of atretic, preantral and antral follicles in mice ovaries injected with saline or serine/threonine for 5 days (mean ± sem).

Groups	Atretic preantral	Atretic early antral	Atretic late antral	Total
Control	45 ± 7.9	13 ± 2.5	20 ± 2.3	78 ± 12.7
Test	55 ± 11.5*	8 ± 2.1*	21 ± 2.1	84 ± 15.7

*P < 0.05 test compared to control.

Table 5. The number of healthy pre-antral and antral follicles in mouse ovaries injected with saline or serine/threonine (mean ± sem).

Groups	Healthy preantral	Healthy early antral	Healthy late antral	Total
Control	93 ± 15.2	15 ± 1.5	4 ± 1.4	112 ± 18.1
Test	100 ± 6.6	23 ± 1.1*	5 ± 1.6	128 ± 9.3

* P < 0.05 test compared to control.

Discussion

Follicle growth in the culture medium was slower than that expected from published papers. That is because the response of follicles to gonadotrophins and as well as to amino acids depends on their size at the beginning of culture (7,8). Small follicles require a longer time to enter a rapid growth phase but large follicles of about 280 µm in diameter grow quickly (7-10). Boland et al. (2) and Boland and Gosden (5) reported an average growth rate

Table 1. The number of follicles classified according to diameter in saline (control) or serine/threonine (test) animals (mean ± sem).

Groups	100-200 µm	200-400 µm	> 400 µm	Total
Control	84 ± 8.1	77 ± 11.0	30 ± 4.6	191 ± 23.7
Test	86 ± 6.8	84 ± 6.0	36 ± 2.6	206 ± 15.4

Table 2. The number of activated primordial, primary, preantral and antral follicles in saline (control) and serine/threonine (test) injected mice (mean ± sem).

Groups	Activ. Prmdl. follicles	Primary follicles	Preantral follicles	Antral follicles	Total
Control	205 ± 38.0	65 ± 6.4	138 ± 23.1	52 ± 7.7	460 ± 81.1
Test	278 ± 35.7	46 ± 5.0*	155 ± 18.1	57 ± 6.9	536 ± 65.7

*P < 0.05 test compared to control.

of 30 µm/day using similar culture systems to those in the present study, and they cultured follicles with initial diameters of 180 µm. In these studies, follicles with an average diameter of 100 µm were cultured and an average growth rate of 8 µm was reported. Smaller mouse follicles, 100-105 µm, were cultured by Liu et al. (11) for 4 days and they reported an average growth rate of 10 µm/day. The growth rate of follicles reported by Liu et al. (11) is quite close to that of follicles reported in this study.

In the second experiment, the number of follicles at the primary stage was significantly lower in the test group. This suggests that the progression from the primordial stage to the primary stage was significantly delayed by the injection of serine and threonine. The mechanisms that induce primordial follicles to enter the growth phase are not known, but one possibility might be reduced signalling activity or synthesis of transforming growth factor beta (TGF-ß). This growth factor is generally required for initiation of DNA synthesis (12).

Reduced atresia in amino acid injected mice can be a result of an increase in the LH response of granulosa cells due to the expression of LH receptors on the granulosa or increases in LH and FSH secretion because of expression of TGF-β. Late follicular differentiation is substantially affected by pituitary gonadotrophins. In culture, FSH did not affect the mean number of granulosa cells, but it increased follicular metabolism, in terms of lactate and oestradiol production (2).

The number of atretic pre-antral follicles increased in test mice. The increase in atresia might be caused by an increase in TGF-β like peptides. It was reported that treatment of immature rat thecal-interstitial cells with TGF-β decreased androgen production while it enhanced progesterone production (13). Increased progesterone production suggests an atretic effect of TGF-β because progesterone has a luteotrophic effect on follicular cells. Advanced studies are required to determine if there are any changes in the expression of TGF-β like proteins and steroidogenic enzymes within the ovary.

References

1. Hillensjo T.: Oocyte maturation and glycolysis in isolated preovulatory follicles of PMS-injected immature rats. Acta Endocrinol., 1976; 82: 352-359.
2. Boland N.I., Humpherson P.G., Leese H.J., Gosden R.G.: Pattern of lactate production and steroidogenesis during growth and maturation of mouse ovarian follicles in vitro. Biol. Reprod., 1993; 48: 798-806.
3. Alberts B., Bray D., Lewis J., Raff M., Roberts K., Watson J.D.: Cell signalling. In: Molecular Biology of the Cell. Third edition, Garland Publishing, New York & London, 1994: 732-734; 765-769.
4. Potchinsky M.B., Weston W.M., Lloyd M.R., Greene R.M.: TGF-β signalling in murine embryonic palate cells involves phosphorylation of CREB transcription factor. Exp. Cell Res., 1997; 231: 96-103.
5. Boland N.I., Gosden R.G.: Effects of epidermal growth factor on the growth and differentiation of cultured mouse ovarian follicles. J. Reprod. Fert. 1994; 101: 369-374.
6. Nagata Y., Konno R., Niwa A.: Amino acid levels in D-Alanine-administreted mutant mice lacking D-Amino acid oxidase. Metabolism, 1994; 43: 1153-1157
7. Hartshorne G.M., Clark C.: Growth and endocrine responses of mouse ovarian follicles in vitro. 48th Annual Meeting of the American Fertility Society, New Orleans, 31 October-5 November 1992 .
8. Mizunuma H., Liu X., Andoh K., Abe Y., Kobayashi J., Yamada K., Yokota H., Ibuki Y., Hasegawa Y.: Activin from secondary follicles causes small preantral follicles to remain dormant at the resting stage. Endocrinology, 1999; 140: 37-41.
9. Magoffin D.A., Gancedo B., Erickson G.F.: Transforming growth factor-beta promotes differentiation of ovarian thecal-interstitial cells but inhibits androgen production. Endocrinology, 1989; 125: 1951-1958.
10. Nayudu P.L., Osborn S.M.: Factors influencing the rate of preantral and antral growth of mouse ovarian follicles in vitro. J. Reprod. Fert.1992; 95: 349-362.
11. Liu X., Andoh K., Yokota H., Kobayashi J., Abe Y., Yamada K., Mizunuma H., Ibuki Y.: Effects of growth hormone, activin and follictatin on the development of preantral follicles from immature female mice. Endocrinology, 1998; 139: 2342-2347.
12. Dorrington J.H., Bendell J.J., Khan S.A.: Interactions between FSH, Estradiol-17β and Transforming Growth Factor-β regulate growth and differentiation of rat gonad. J. Steroid Biochem. Mol. Biol., 1993; 44: 441-447.
13. Fournet N., Weitsman S.R., Zachow R.J., Magoffin D.A.: Transforming growth factor-beta inhibits ovarian 17 alpha-hydroxylase activity by a direct noncompetitive mechanism. Endocrinology, 1996; 137: 166-174.