ISSN 0022-0930, Journal of Evolutionary Biochemistry and Physiology, 2023, Vol. 59, No. 1, pp. 57–68. © Pleiades Publishing, Ltd., 2023.
Russian Text © The Author(s), 2023, published in Zhurnal Evolyutsionnoi Biokhimii i Fiziologii, 2023, Vol. 59, No. 1, pp. 55–65.

EXPERIMENTAL PAPERS

Effect of Different Luteinizing Hormone Receptor Agonists on Ovarian Steroidogenesis in Mature Female Rats

A. A. Bakhtyukov[a], K. V. Derkach[a], E. A. Fokina[a], I. A. Lebedev[a], V. N. Sorokoumov[b], L. V. Bayunova[a], and A. O. Shpakov[a,*]

[a] *Sechenov Institute of Evolutionary Physiology and Biochemistry, Russian Academy of Sciences, St. Petersburg, Russia*

[b] *Institute of Chemistry, St. Petersburg State University, St. Petersburg, Russia*
**e-mail: alex_shpakov@list.ru*

Received November 2, 2022; revised November 29, 2022; accepted November 30, 2022

Abstract—In clinical practice, ovarian steroidogenesis is stimulated and ovulation is induced using such gonadotropin preparations as human chorionic gonadotropin (hCG) and luteinizing hormone (LH) which, however, have a number of side effects, including a reduction in ovarian sensitivity to endogenous LH and ovarian hyperstimulation syndrome. An alternative to hCG and LH could be allosteric LH/hCG receptor agonists, including the thieno[2,3-d]-pyrimidine derivative TP03 developed in our laboratory. This work was aimed to study the effect of TP03 (40 μg/kg, i.p.) versus hCG (30 IU/rat, s.c.) on ovarian steroidogenesis in mature female rats in the late proestrus phase, including those treated with the gonadotropin releasing hormone (GnRH) antagonist Orgalutran (100 μg/kg, s.c., 3 h before TP03 or hCG administration). Estradiol, progesterone and LH levels were measured in the blood, while expression levels of the steroidogenesis-related genes *Star*, *Cyp11a1*, *Hsd3b*, *Cyp17a1*, *Hsd17b*, *Cyp19a1* and LH/hCG receptor gene *Lhcgr* were assessed in the ovaries. Three hours after administration, TP03 and hCG increased blood progesterone levels and stimulated the expression of genes encoding the cholesterol-transporting protein StAR, cytochrome P450c17, and aromatase (cytochrome P450c19), with these effects detected both in control rats with normal LH levels and in Orgalutran-treated rats with reduced LH levels. The effects of TP03 were comparable to those of hCG, but in contrast to hCG, TP03 did not reduce the activity of the hypothalamic–pituitary–gonadal axis, as indicated by the lack of its influence on blood LH levels and ovarian expression of LH/hCG receptors. Our data suggest the ability of TP03 to effectively stimulate ovarian steroidogenesis, as well as good prospects for the development of TP03-based drugs for controlled ovulation induction.

DOI: 10.1134/S0022093023010052

Keywords: allosteric agonist, ovarian steroidogenesis, luteinizing hormone receptor, human chorionic gonadotropin, mature rats, progesterone, cytochrome P450c17

INTRODUCTION

The development of effective approaches to correcting ovarian steroidogenesis abnormalities in various reproductive disorders, as well as to implementing controlled ovulation induction in assisted reproductive technologies is one of the main challenges of modern reproductive endocrinology. The gonadotropins used for this purpose, luteinizing hormone (LH) and human chorionic

gonadotropin (hCG), have a number of significant drawbacks [1]. This is largely due to the use of hCG and LH in doses that entail a significant excess of physiological blood gonadotropin levels. In the case of ovulation induction in assisted reproductive technologies, this can lead to a severe complication, ovarian hyperstimulation syndrome [2]. The use of other pharmacological strategies, in which gonadotropins are replaced by gonadotropin releasing hormone (GnRH) antagonist or which use a GnRH/low-dose hCG coadministration protocol, reduces the risk of ovarian hyperstimulation syndrome, but in a significant number of cases reduces the occurrence of pregnancy as well [3]. Another reason may be significant differences in the N-glycosylation pattern of LH and hCG preparations, usually their recombinant forms, from that of endogenous gonadotropins, which influences their pharmacokinetics and activation specificity of intracellular effector proteins and transcription factors [4, 5]. LH/hCG receptor activation leads to a simultaneous stimulation of several types of heterotrimeric G proteins and β-arrestin adaptor proteins: cAMP-dependent signaling pathways are activated through G_s protein and adenylyl cyclase; calcium-dependent pathways are activated through $G_{q/11}$ protein and phospholipase Cβ; endocytosis of the ligand–receptor complex followed by receptor degradation or recyclization and the stimulation of the mitogen-activated protein kinase cascade occur through β-arrestins [5, 6]. A change of the gonadotropin N-glycosylation pattern can significantly alter the profile of gonadotropin-induced G protein and β-arrestin activation, one of the consequences of which can be a downregulation of LH/hCG receptors. For example, prolonged activation of follicular cells with LH or hCG causes a significant decrease in LH/hCG receptor gene expression and also reduces the number of active receptors on the cell surface, which results in the development of LH resistance [7–9]. Decreased sensitivity to endogenous LH has been demonstrated in males treated with hCG for a long time [10], as well as in male rats treated repeatedly with hCG at doses higher than the physiological, with a significant decrease in the number of LH/hCG receptors being in this case the key factor in the development of LH resistance [11, 12].

All of the above-said indicates the need of developing alternative pathways for specific LH/hCG receptor activation that would allow ovarian steroidogenesis stimulation and ovulation induction without causing ovarian hyperstimulation syndrome, the development of LH resistance and other undesirable sequelae of gonadotropin therapy. The main expectations in this area are associated with low-molecular-weight heterocyclic compounds sharing the properties of LH/hCG receptor agonists, primarily thieno[2,3-d]-pyrimidine derivatives [13–15]. We have previously developed such thieno[2,3-d]-pyrimidine derivatives as TP01, TP03 and TP04 having LH/hCG receptor agonist activity, whose intraperitoneal and oral administration to male rats increased testicular and blood testosterone levels, stimulated testicular steroidogenesis, restored testosterone levels and spermatogenesis in diabetic pathology and aging [11, 16, 17]. However, there are no data on the effect of low-molecular-weight allosteric LH/hCG receptor agonists on ovarian steroidogenesis in sexually mature female rodents.

The aim of the work was to study the effect of TP03, as well as hCG taken for comparison, on ovarian steroidogenesis in sexually mature female rats in the late proestrus phase, including those pretreated with Orgalutran, a GnRH antagonist with a prolonged action. Blood levels of estradiol, progesterone and LH, as well as the gene expression of steroidogenesis enzymes and LH/hCG receptor in the ovaries, were assessed.

MATERIALS AND METHODS

Four- to five-month-old female rats, kept in standard vivarium conditions by 5 animals per cage, with ad libitum access to water and dry food, were used in the experiments.

The estrous cycle phase was determined in sexually mature female rats before the onset of the experiment, and those animals that were in proestrus were selected. The estrous cycle phase was determined by microscopic examination of vaginal smears according to standard methods [18, 19]. Smears were taken daily at 11 a.m. according to the following scheme. The rat's forebody was immobilized, and the vaginal vestibule

was examined when lifting the animal by the tail. A cotton swab soaked in saline was inserted into the vagina and rotated several times, after which a smear was transferred from the swab to a dry slide. The preparation was air-dried and stained with methylene blue for microscopy. The stages of estrous cycle were determined based on the ratio of three cell types in the smear: nucleated epitheliocytes, keratinized epitheliocytes without nuclei, and leukocytes. In the proestrus phase, smears contained nucleated epitheliocytes only.

In animals with a verified proestrus phase, blood samples were withdrawn from the caudal vein to assess basal hormone levels (12:00), after which they were immediately treated with Orgalutran ("Organon N.V.", Netherlands), a GnRH antagonist, which was injected subcutaneously (s.c.) at a single dose of 100 μg/kg of body weight (b.w.). Orgalutran-untreated rats (control) were injected with physiological solution in the same volume and at the same time. Three hours after the treatment (15:00), blood samples were taken from the animals to assess the early effects of Orgalutran, after which the LH/hCG receptor agonists TP03 and hCG were injected once, using the doses selected in pilot experiments and evoking the maximum steroidogenic effect. In the case of TP03, the dose was 40 mg/kg (intraperitoneally, i.p.), and in the case of hCG, 30 IU/rat (s.c.). Control animals (Orgalutran-untreated) were also administered with TP03 or hCG in the same doses. Part of control and Orgalutran-treated animals, instead of LH/hCG receptor agonists, was injected with DMSO (i.p.) in the same volume (200 μL) as TP03. We have previously shown that DMSO administered i.p. in the above volume does not affect steroidogenesis and metabolic, hormonal and functional parameters [11]. Three hours after the administration of agonists (6 h after Orgalutran treatment), the animals were anesthetized (chloral hydrate, 400 mg/kg, i.p.), blood was re-sampled to assess hormone levels, and then the rats were decapitated. After decapitation, the ovaries were withdrawn, weighed, and the expression of target genes was measured in there using real-time PCR.

A total of the following 6 groups were formed and examined (by 5 rats from each group): control rats untreated with Orgalutran and LH/hCG receptor agonists (group C); rats treated with Orgalutran but untreated with LH/hCG receptor agonists (group Org); rats untreated with Orgalutran treatment but administered with TP03 (group C + TP) or hCG (group C + hCG); and rats treated with Orgalutran followed by TP03 (group Org + TP) or hCG (group Org + hCG) administration.

5-amino-N-*tert*-butyl-2-(methylsulfanyl)-4-(3(nicotinamido)phenyl)thieno[2,3-d]pyrimidin-6-carboxamide (TP03) was synthesized using the reaction between 1.0 equivalent of 5-amino-4-(3-aminophenyl)-N-*tert*-butyl-2-(methylsulfanyl) thieno[2,3-d]pyrimidin-6-carboxamide and 1.1 equivalent of nicotinic acid in the presence of 1.1 equivalent of 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU) and 1.2 equivalent of *N,N*-diisopropylethylamine in dry *N,N*-dimethylformamide, as described previously [17]. The reaction was carried out for 5 h at 22°C; the target product was purified by recrystallization from ethanol; column chromatography was used at the final stage. TP03 represented a yellow non-hygroscopic powder with a melting point of 157–159°C. TP03 structure was verified by NMR spectroscopy and high-resolution mass spectrometry. The results of ^{1}H-NMR spectroscopy (DMSO-d_6), δ, ppm (*J*, Hz): 1.37 (9H, s, $C(CH_3)_3$); 2.62 (3H, s, SCH_3); 6.14 (2H, s, NH_2); 6.98 (1H, s, NH-*t*-Bu); 7.42 (1H, d, J = 7.8, 4′-H); 7.57-7.64 (2H, m, 5′-H and 5″-H); 8.04 (1H, d, J = 8.2, 2′-H); 8.08 (1H, s, 6′-H); 8.33 (1H, d, J = 7.7, 4″-H); 8.78 (1H, d, J = 4.8, 6″-H); 9.14 (1H, s, 2″-H), 10.72 (1H, s, 3′-NH). The results of $_{13}$C-NMR spectroscopy (DMSO-d_6), δ, ppm: (14.3 (SCH_3); 29.2 ($C(CH_3)_3$); 51.9 ($C(CH_3)_3$); 97. 7; 117.8; 120.9; 122.5; 124.0; 124.9; 129.8; 130.8; 136.0; 137.1; 139.6; 144.7; 149.2; 152.8; 162.5; 164.9; 165.2; 167.8; 168.8). By the data of high-resolution electrospray ionization time-of-flight (ESI-TOF) mass spectrometry, the mass of the TP03 molecular ion ($M+Na^+$) was 515.1304, which coincided with the calculated molecular ion mass of this compound (515.1294). Mass spectra were recorded using a Bruker micrOTOF spectrometer ("Bruker", Germany); NMR spectra were recorded using a Bruker Avance III 400 spectrometer (400.13 MHz for ^{1}H-NMR and

Table 1. Primer sequences used to assess gene expression in the rat ovaries

Gene	Forward and reverse primers	GenBank
Lhcgr	(For) CTGCGCTGTCCTGGCC; (Rev) CGACCTCATTAAGTCCCCTGAA	NM_012978.1
Star	(For) AAGGCTGGAAGAAGGAAAGC; (Rev) CACCTGGCACCACCTTACTT	NM_031558.3
Cyp11a1	(For) TATTCCGCTTTGCCTTTGAG; (Rev) CACGATCTCCTCCAACATCC	NM_017286.3
Hsd3b	(For) AGGCCTGTGTCCAAGCTAGTGT; (Rev) CTCGGCCATCTTTTTGCTGTAT	XM_017591325.1
Cyp17a1	(For) CATCCCCCACAAGGCTAAC; (Rev) TGTGTCCTTGGGGACAGTAAA	XM_006231435.3
Hsd17b	(For) CCTTTGGCTTTGCCATGAGA; (Rev) CAATCCATCCTGCTCCAACCT	NM_024392.2
Cyp19a1	(For) GGTATCAGCCTGTCGTGGAC; (Rev) AGCCTGTGCATTCTTCCGAT	NM_017085.2
Actb	(For) CTGGCACCACACCTTCTACA; (Rev) AGGTCTCAAACATGATCTGGGT	NM_031144.3

100.61 MHz for ^{13}C-NMR) ("Bruker", Germany).

Estradiol and progesterone blood levels were measured by enzyme immunoassay using the "Estradiol ELISA" and "Progesterone ELISA" kits ("HEMA", Russia). The LH levels were determined using the ELISA for LH, Rat ("Cloud-Clone Corp.", USA).

Total RNA from the ovaries was isolated using the ExtractRNA reagent ("Eurogen", Russia), then reverse transcription was performed to obtain cDNA using the MMLV RT Kit ("Eurogen", Russia). Real-time PCR was carried out using an Applied Biosystems® 7500 Real-Time PCR System amplifier ("Life Technologies, Thermo Fisher Scientific Inc.", USA) in a mixture containing 0.4 μM of forward and reverse primers, using the qPCR-HS SYBR+Low ROX reagent ("Eurogen", Russia). The primer sequences are shown in Table 1. For gene expression quantitative data analysis, the delta–delta C_t method was used [20]. The 7500 Software v2.0.6 and Expression Suite Software v1.0.3 were used for calculations. RQ values for gene expression were calculated versus control, which was taken as one. The expression of the *Actb* gene encoding actin B was used as an endogenous control.

Microsoft Office Excel 2007 software was used for statistical data analysis. Normality of distribution was assessed by the Shapiro–Wilk test. To compare two samples with normal distribution, the Student's *t*-test was applied, while to compare three groups, the analysis of variance with Bonferroni correction was used. Differences were considered statistically significant at $p < 0.05$. Data were presented as $M \pm SEM$.

RESULTS

The ovarian weight (0.114 ± 0.010 g in the control group of female rats) did not change significantly after TP03 and hCG treatment and was 0.132 ± 0.007 and 0.138 ± 0.007 g, respectively ($p > 0.05$ vs. group C). Orgalutran treatment also did not change the ovarian weight (0.104 ± 0.011 g, $p > 0.05$ vs. group C). There was an upward trend for the ovarian weight in groups Org + TP (0.136 ± 0.010 g) and Org + hCG (0.140 ± 0.011 g), although the differences with group Org were non-significant ($p > 0.05$).

Blood LH levels in control rats varied during the experiment, significantly increasing by 15:00 vs. the starting point (12:00 noon) and remained

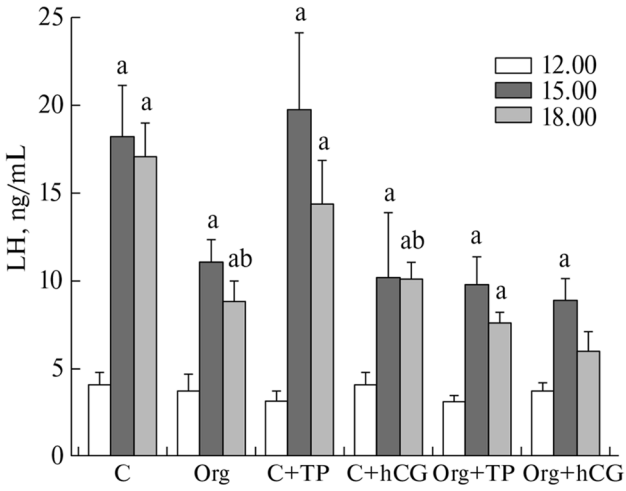

Fig. 1. Luteinizing hormone levels in the blood of female rats in the proestrus phase in control and after the treatment with the GnRH antagonist Orgalutran (100 μg/kg, single, s.c.), as well as LH/hCG receptor agonists TP03 (40 mg/kg, single, i.p.) and hCG (30 IU/rat, single, s.c.). [a]—Significant ($p < 0.05$) intragroup differences at time points 15.00 and 18.00 vs. starting point (12.00), [b]—significant ($p < 0.05$) differences between groups Org, C + TP and C + hCG vs. control group C at the same time points. In all groups, $n = 5$; the values are presented as $M \pm SEM$.

Table 2. AUC (integrated area under the hormone concentration–time curve) values for progesterone, estradiol and luteinizing hormone in control rats and animals treated with Orgalutran and LH/hCG receptor

Group	LH	Progesterone	Estradiol
C	85.9 ± 7.8	271 ± 38	3.09 ± 0.22
Org	52.6 ± 6.1[a]	125 ± 14[a]	2.27 ± 0.20[a]
C + TP	85.9 ± 16.0	407 ± 29[a]	3.80 ± 0.32
C + hCG	79.2 ± 11.3	381 ± 52	3.47 ± 0.44
Org + TP	46.0 ± 4.7	249 ± 32[b]	2.66 ± 0.33
Org + hCG	41.7 ± 4.4	262 ± 24[b]	2.84 ± 0.26

AUC values include a 6-h interval (12.00–18.00) and are expressed in relative units. [a]—Significant ($p < 0.05$) differences between groups Org, C + TP and C + hCG vs. control group C; [b]—significant ($p < 0.05$) differences between group Org vs. groups Org + TP and Org + hCG. In all groups, $n = 5$; $M \pm SEM$.

at a high level until 18:00 (Fig. 1). Orgalutran, a GnRH antagonist, suppressed LH production, as reflected in a significant decrease in hormone levels, which were almost twice as low as in group C 6 h after Orgalutran administration, as indicated by the values of $AUC_{12:00–18:00}$, the area under the LH concentration (ng/mL)–time (h) curve (Table 2). TP03 treatment, 3 h after its administration (18:00), had a little effect on blood LH levels in groups with and without Orgalutran treatment, whereas hCG treatment after 3 h significantly reduced LH levels in control rats (C + hCG vs. group C, $p = 0.016$), but had no effect on LH levels in group Org + hCG (Fig. 1). However, the $AUC_{12:00–18:00}$ values in groups C and C + hCG were statistically indistinguishable (Table 2).

Blood progesterone levels in control rats in the proestrus phase did not change significantly throughout the experiment, whereas 3 h after the treatment with TP03 and hCG, it significantly increased, with the effectiveness of both drugs at the given doses being similar (Fig. 2, Table 2). After Orgalutran treatment, there was a significant decrease in progesterone levels: 6 h after treatment, its blood concentration in Org rats was 4.3 times lower vs. control animals (Fig. 2). The attenuation of progesterone production was indicated by the $AUC_{12:00–18:00}$ values for the progesterone concentration (nM)–time (h) curve, which were 54% lower in group Org vs. control group (Table 2). Both LH/hCG receptor agonists increased progesterone levels in Orgalutran-treated rats, but their effects were less pronounced vs. control groups (Fig. 2). Blood estradiol levels changed little throughout the experiment in control group, and the treatment with Orgalutran and LH/hCG receptor agonists had only a minor effect on it (Fig. 2).

A study of the ovarian expression of the steroidogenesis genes and the gene encoding the LH/hCG receptor yielded the following results. The expression of the *Star* gene encoding the steroidogenic acute regulatory (StAR) protein responsible for cholesterol transport decreased in Orgalutran-treated group and increased when control and Orgalutran-treated animals were treated with both LH/hCG receptor agonists (Table 3). The expression of the *Cyp11a1* gene encoding cytochrome P450scc (cholesterol side chain cleavage enzyme, CYP11A1), which con-

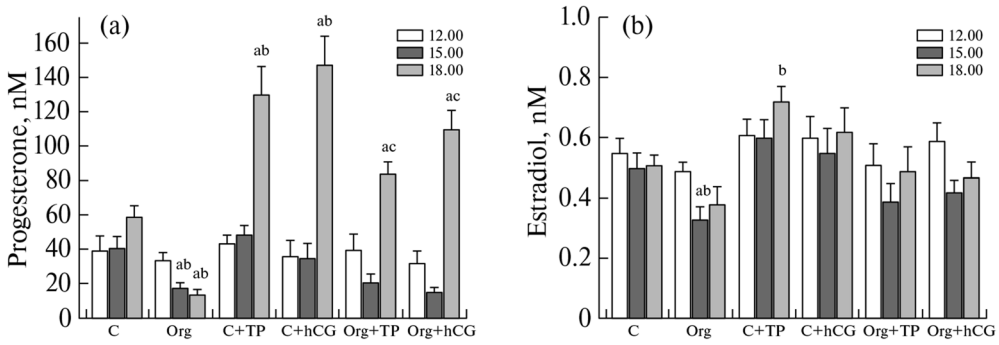

Fig. 2. Progesterone (a) and estradiol (b) levels in the blood of female rats in the proestrus phase in control and after the treatment with Orgalutran and the LH/hCG receptor agonists TP03 and hCG. [a]—Significant ($p < 0.05$) intragroup differences at time points 15.00 and 18.00 vs. starting point (12.00); [b]—significant ($p < 0.05$) differences between groups Org, C + TP and C + hCG vs. control; [c]—significant ($p < 0.05$) differences between group Org vs. groups Org + TP and Org + hCG at the same time points. In all groups, $n = 5$; $M \pm SEM$.

Table 3. Expression of steroidogenic genes in the rat ovaries in control and after the treatment with Orgalutran (100 μg/kg) and LH/hCG receptor agonists TP03 (40 mg/kg) and hCG (30 IU/rat)

Group	*Star*	*Cyp11a1*	*Hsd3b*	*Cyp17a1*	*Hsd17b*
	RQ, rel. u.				
C	1.03 ± 0.13	1.03 ± 0.16	1.01 ± 0.13	1.05 ± 0.13	1.01 ± 0.09
Org	0.57 ± 0.11[a]	0.65 ± 0.08	0.80 ± 0.10	0.15 ± 0.03[a]	1.11 ± 0.04
C + TP	1.51 ± 0.10[a,b]	1.37 ± 0.03[b]	0.96 ± 0.06	2.44 ± 0.33[a,b]	1.29 ± 0.15
C + hCG	1.62 ± 0.13[a,b]	1.24 ± 0.18[b]	0.76 ± 0.08	3.42 ± 0.55[a,b]	0.72 ± 0.06[a,b]
Org + TP	1.52 ± 0.19[b]	1.41 ± 0.16[b]	1.08 ± 0.16	1.75 ± 0.20[a,b]	1.52 ± 0.19
Org + hCG	1.56 ± 0.15[b]	1.25 ± 0.12[b]	1.07 ± 0.09	2.98 ± 0.53[a,b]	1.15 ± 0.10

[a]—Significant ($p < 0.05$) differences vs. control; [b]—significant ($p < 0.05$) differences vs. group Org. In all groups, $n = 5$; $M \pm SEM$.

verts cholesterol into pregnenolone, showed a downward trend in group Org, although the differences vs. controls were non-significant ($p = 0.069$). At the same time, TP03 and hCG had little effect on *Cyp11a1* gene expression in control groups, but significantly increased it in Orgalutran-treated groups (Table 3).

The treatment with the GnRH antagonist led to a 7-fold decrease in the expression of the *Cyp17a1* gene encoding cytochrome P450c17 (CYP17A1) which catalyzes a two-stage conversion of pregnenolone into dehydroepiandrosterone (DHEA), or progesterone into androstenedione. Meanwhile, TP03 and hCG treatment effectively stimulated the expression of this gene in both control and Orgalutran-treated animals (Table 3). It should be noted, however, that the stimulating effect of hCG in both cases exceeded that of TP03. The expression of *Hsd3b* and *Hsd17b* genes encoding 3β- and 17β-hydroxysteroid dehydrogenases (3β-HSD and 17β-HSD), respectively, did not change significantly under the treatment with both Orgalutran and hCG receptor agonists, except for group C + hCG, in which *Hsd17b* gene expression decreased vs. both control and Org animals (Table 3). The enzyme 3β-HSD converts pregnenolone into progesterone, whereas 17β-HSD, depending on the enzyme subtype, catalyzes a wide range of reactions at the final stages of ovarian steroidogenesis, including estrogen synthesis.

The expression of the *Cyp19a1* gene encoding

aromatase that converts androgens into estrogens changed little when female rats were treated with Orgalutran, but significantly increased when treated with TP03 and hCG in both control and GnRH antagonist-treated animal groups (Fig. 3). The expression of the *Lhcgr* gene encoding the LH/hCG receptor tended to increase ($p = 0.05$) after Orgalutran treatment, which was associated with a decrease in blood levels of endogenous LH (Fig. 3). The treatment with hCG significantly decreased *Cyp19a1* gene expression vs. group Org, but not vs. control animals. In all TP03-treated groups, *Lhcgr* gene expression was similar to its control values (Fig. 3).

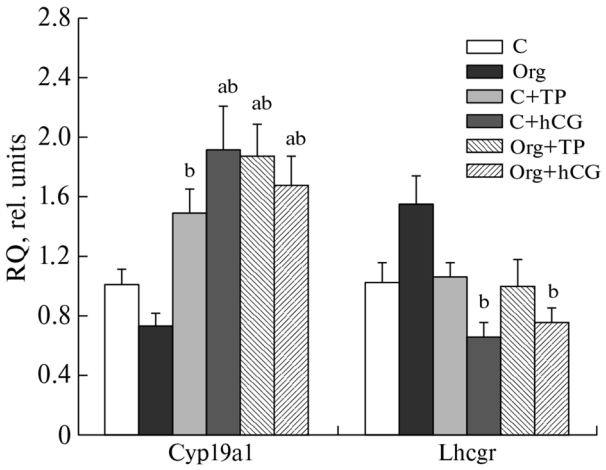

Fig. 3. Expression of genes encoding aromatase (*Cyp19a1*, (a)) and LH/hCG receptor (b) in the rat ovaries in control and after the treatment with Orgalutran (100 μg/kg), TP03 (40 mg/kg) and hCG (30 IU/rat). [a]—Significant ($p < 0.05$) differences vs. control group; [b]—significant ($p < 0.05$) differences vs. group Org. In all groups, $n = 5$; $M \pm SEM$.

DISCUSSION

Urinary hCG and recombinant LH preparations, widely used for the treatment of reproductive dysfunctions in women and in assisted reproductive technologies, have significant drawbacks due to hyperactivation of ovarian steroidogenesis, decreased ovarian sensitivity to endogenous gonadotropins and the development of ovarian hyperstimulation syndrome. Low-molecular-weight agonists of the LH/hCG receptor that interact with its allosteric site located inside the transmembrane domain are considered as a possible alternative. Previously, we have obtained thieno[2,3-d]-pyrimidine derivatives, compounds TP01, TP03 and TP04, capable of activating the LH/hCG receptor via an allosteric mechanism, which stimulated testicular steroidogenesis in male rats when administered intraperitoneally and orally [11, 16, 17], and restored androgen deficiency and spermatogenesis in diabetes and physiological aging [11, 17]. Other authors used thieno[2,3-d]-pyrimidine derivatives, including the Org43553 compound, for in vitro steroidogenesis stimulation in ovarian cells and in vivo ovulation induction in female rats [14, 21, 22], as well as for controlled ovulation induction in female volunteers [23]. It is noteworthy that, along with agonists, we and other authors have now developed low-molecular-weight LH/hCG receptor antagonists that interact with the same transmembrane allosteric site, but at the same time stabilize an inactive conformation of the receptor [24, 25].

In the present study, the effect of TP03, the most active of the thieno[2,3-d]-pyrimidine derivatives we developed in our laboratory [17], on blood steroid hormone levels and the expression of key genes of ovarian steroidogenesis in mature female rats, including those treated with Orgalutran. When administered intraperitoneally, TP03 evoked a significant increase in progesterone levels both in control rats and in animals treated with Orgalutran (Ganirelix as a reactant). By the magnitude of the stimulating effect, TP03 was comparable with hCG in both groups of animals (Fig. 2). The revealed ability of TP03 to effectively stimulate progesterone production is consistent with the results obtained in in vitro experiments by other authors when treating follicular cells with another thieno[2,3-d]-pyrimidine derivative, Org43553 [14, 21]. It should be noted that GnRH antagonists with prolonged action, including Ganirelix, under conditions rapidly and effectively reduce LH secretion both in vitro and in vivo and thus inhibit progesterone production in the ovaries [26, 27]. In our case, Orgalutran significantly reduced the circulating LH level in the blood 6 h after treatment, although a downward tendency for the hormone level was already observed after 3 h (Fig. 1). As a consequence, the increase in progesterone levels, induced by TP03 and hCG in Orgalutran-treated rats, was due

almost exclusively to the stimulating effect of the injected drugs on ovarian steroidogenesis.

The progesterone production-stimulating effects of TP03 and hCG, as assessed by the increment in hormone concentration over its level in the corresponding rat group untreated with LH/hCG receptor agonists, were similar, suggesting no significant changes in the sensitivity of LH/hCG receptors and the ovarian steroidogenesis system under conditions of Orgalutran-induced suppression of LH secretion. In this regard, it should be noted that 6 h after the treatment with a GnRH antagonist, there was detected an upward tendency for LH/hCG receptor gene expression in the ovaries as a compensatory reaction to a decreased blood LH level and thus insufficient activation of the LH-dependent signaling pathways in the follicular cells. This is supported by the fact that hCG administration evoked a twofold decrease in *Lhcgr* gene expression (Fig. 3). Importantly, a hCG-induced decrease in *Lhcgr* gene expression has been shown by other authors in ovarian follicular cells [7–9], as well as in rat testicular Leydig cells in our previous experiments [11, 25]. Along with this, the treatment of control rats with hCG resulted in a significant decrease in blood LH levels, indicative of hCG-induced suppression of endogenous gonadotropin production.

We found that TP03 and hCG had virtually no effect on estradiol production. This is because, during the estrous cycle, estradiol peaks at the final stage of the diestrus and the first half of the proestrus phase, while the study included the second half of the proestrus phase with its characteristic increase in progesterone production induced by LH/hCG receptor agonists, including endogenous LH [28, 29].

A study of steroidogenic gene expression showed that TP03 and hCG administration to control and Orgalutran-treated rats significantly increased the ovarian expression of the *Star* and *Cyp17a1* genes encoding the key proteins of ovarian steroidogenesis, cholesterol-transporting protein StAR and cytochrome P450c17 (CYP17A1). StAR is one of the main targets during LH- and hCG-induced stimulation of ovarian theca and granulosa cells, since the *Star* gene promoter contains binding sites for cAMP-dependent transcription factors, primarily CREB, which are activated through the LH/hCG receptor–G_s protein–adenylyl cyclase–protein kinase A signaling pathway [30]. According to our previous in vitro data, TP03 and its structural analogs selectively stimulate adenylyl cyclase and increase the intracellular cAMP level, acting similarly to hCG, albeit more selectively, on the G_s protein-dependent pathway through the LH/hCG receptor [31, 32]. As a result, the capability of TP03 to activate cAMP-dependent signaling cascades is a cause of an increase in *Star* gene expression, comparable with that when using hCG. The fact that the stimulating effect in the ovaries is not as significant as in the case of the TP03 effect on *Star* gene expression in testicular cells [17] may be due to a relatively high basal level of this gene expression at the late proestrus stage [33].

Cytochrome P450c17 combines the activities of 17-hydroxylase and 17,20-lyase and catalyzes various steroidogenic reactions, depending on the phase of the estrous cycle, cell type, and animal species. The main reactions catalyzed by cytochrome P450c17 are pregnenolone conversion into 17OH-pregnenolone and then into dehydroepiandrosterone (DHEA), as well as progesterone conversion into 17OH-progesterone and then into androstenedione [34]. It should be noted that in the proestrus phase, cytochrome P450c17 expression and activity significantly increase due to increased androgen synthesis in theca cells [34]. The regulation of *Cyp17a1* gene expression in theca cells is mainly implemented through the cAMP-dependent signaling pathways due to gonadotropin-induced LH/hCG receptor activation, but other signaling pathways may also be involved in this process, including those realized through the stimulation of phorbol-sensitive protein kinase C isoforms. At the same time, the activation of cAMP-dependent pathways and protein kinase A leads to increased *Cyp17a1* gene expression, whereas the activation of protein kinase C causes a decrease in cAMP-stimulated expression of this gene [34, 35]. It should be noted that in granulosa cells, *Cyp17a1* gene expression is appreciably lower and less influenced by cAMP-dependent signaling [34]. In our experiments, stimulating effects of TP03 and hCG on *Cyp17a1* gene expression were clearly expressed not only in control animals, but also in Orgalutran-pretreated

female rats which, in the absence of stimulation with LH/hCG receptor agonists, had an extremely low gene expression level of cytochrome P450c17 (Table 3). With consideration to the TP03-induced selective stimulation of cAMP signaling, it can be concluded that it is the activation of cAMP-dependent pathways in the ovaries of rats with endogenous LH deficiency that is the main cause of the increased *Cyp17a1* gene expression. However, the temporal dynamics of this effect should be taken into account, because Western blotting showed that 7 h after hCG administration, cytochrome P450c17 content in rat follicular cells significantly decreased, which can be considered as a compensatory reaction following a burst of steroidogenic activity [36].

The fact that in the control group there was no significant stimulating effect of TP03 and hCG on the expression of the *Cyp11a1* gene encoding cytochrome P450scc (CYP11A1), which catalyzes cholesterol conversion into pregnenolone, was quite unexpected. On the other hand, in Orgalutran-treated rats, in which there was an insignificant decrease in *Cyp11a1* gene expression, the stimulating effects of both LH/hCG receptor agonists were statistically significant (Table 3). Based on the literature data, there are grounds to believe that this is due to relatively high level of *Cyp11a1* gene expression and the amount of cytochrome P450scc in the late proestrus phase [37, 38], as well as a slower (vs. the *Star* and *Cyp17a1* genes) stimulating effect of LH/hCG receptor agonists on *Cyp11a1* gene expression [39]. For example, in control rats, the level of *Cyp11a1* gene expression, increased in the proestrus, masks the stimulating effects of TP03 and hCG, but with a small decrease in this level under conditions of endogenous LH deficiency, these effects begin to show up.

Aromatase (cytochrome P450c19) is one of the key enzymes of ovarian steroidogenesis, which catalyzes the conversion of androgens into estrogens. During the estrous cycle, aromatase expression and activity are highly variable, with a change in aromatase activity "lagging behind" *Cyp19a1* gene expression. For example, *Cyp19a1* gene expression is maximal in the diestrus, slightly decreases in the first half of the proestrus, and sharply decreases, becoming almost undetectable, in the estrus phase, whereas the peak of the enzyme activity falls on the proestrus and then gradually declines until the onset of the diestrus phase of the next cycle [40], positively correlating with aromatase and estradiol blood levels during these periods of follicular development [41]. These changes in activity are also associated with a rise in blood LH levels and the activation of LH-dependent signaling pathways in granulosa and, to a lesser extent, theca cells [41]. We demonstrated a comparable (in its magnitude) stimulating effect of hCG and TP03 on *Cyp19a1* gene expression in both control female rats and Orgalutran-treated animals, and this increase positively correlated with the steroidogenic activity of both LH/hCG receptor agonists (Fig. 3). Interestingly, the stimulating effect of hCG on aromatase gene expression, in contrast to that on *Cyp17a1* gene expression, was poorly different from that of TP03. For example, the ratio of *Cyp17a1* gene expressions in groups Org + hCG and Org + TP was 1.70, while that of aromatase gene expression was 0.89.

Thus, we demonstrate here for the first time that the administration of TP03, a low-molecular-weight allosteric LH/hCG receptor agonist, to sexually mature female rats evokes an increase in blood progesterone levels and, to varying degrees, enhances the expression of a number of steroidogenic genes, such as the cholesterol-transporting protein StAR, cytochrome P450c17 and aromatase, and this effect is evident in both control rats in the presence of normal endogenous LH levels, as well as in animals treated with a GnRH antagonist, with reduced endogenous LH levels. The effects of TP03 are comparable to those of hCG, which is widely used in clinical practice as an ovarian steroidogenesis activator and ovulation inducer. At the same time, in contrast to hCG, TP03 evokes no attenuation of gonadal axis components, as indicated by the lack of its effect on blood LH levels and ovarian LH/hCG receptor expression. There is reason to believe that the milder TP03-induced stimulation of steroidogenesis may reduce the risk of ovarian hyperstimulation, which requires further investigation. Our data suggest the ability of TP03 to stimulate ovarian steroidogenesis in mature rats and promise good prospects for developing TP03-based dosage forms for controlled ovulation induction.

ACKNOWLEDGMENTS

NMR studies were carried out using the equipment of the St. Petersburg State University Resource Center "Magnetic Resonance Research Methods"; high-resolution mass spectra were obtained on the equipment of the St. Petersburg State University Resource Center "Methods for Compositional Analysis of Matter".

AUTHORS' CONTRIBUTION

Conceptualization and experimental design (A.A.B., K.V.D., A.O.Sh.); data collection (A.A.B., E.A.F., I.A.L., L.V.B.), organic synthesis (V.N.S.); data processing (A.A.B., K.V.D., E.A.F.); writing and editing the manuscript (A.O.Sh., K.V.D.).

FUNDING

This work was supported by the Russian Science Foundation, project no. 19-75-20122.

COMPLIANCE WITH ETHICAL STANDARDS

All experimental procedures with animals were carried out in compliance with the requirements of the Ethics Committee at the Sechenov Institute of Evolutionary Physiology and Biochemistry of the Russian Academy of Sciences (protocol no. 2-5/2021, 20/04/2021), as well as the Guidelines for the Care and Use of Laboratory Animals, and the Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest associated with the publication of this article.

REFERENCES

1. Martinez F, Racca A, Rodríguez I, Polyzos NP (2021) Ovarian stimulation for oocyte donation: a systematic review and meta-analysis. Hum Reprod Update 27(4): 673–696. https://doi.org/10.1093/humupd/dmab008
2. Namavar Jahromi B, Parsanezhad ME, Shomali Z, Bakhshai P, Alborzi M, Moin Vaziri N, Anvar Z (2018) Ovarian Hyperstimulation Syndrome: A Narrative Review of Its Pathophysiology, Risk Factors, Prevention, Classification, and Management. Iran J Med Sci 43(3): 248–260. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5993897
3. Engmann LL, Maslow BS, Kaye LA, Griffin DW, DiLuigi AJ, Schmidt DW, Grow DR, Nulsen JC, Benadiva CA (2019) Low dose human chorionic gonadotropin administration at the time of gonadotropin releasing-hormone agonist trigger versus 35h later in women at high risk of developing ovarian hyperstimulation syndrome—a prospective randomized double-blind clinical trial. J Ovarian Res 12(1): 8. https://doi.org/10.1186/s13048-019-0483-7
4. Jiang X, Dias JA, He X (2014) Structural biology of glycoprotein hormones and their receptors: insights to signaling. Mol Cell Endocrinol 382(1): 424–451. https://doi.org/10.1016/j.mce.2013.08.021
5. Casarini L, Simoni M (2021) Recent advances in understanding gonadotropin signaling. Fac Rev 10: 41. https://doi.org/10.12703/r/10-41
6. Riccetti L, Yvinec R, Klett D, Gallay N, Combarnous Y, Reiter E, Simoni M, Casarini L, Ayoub MA (2017) Human Luteinizing Hormone and Chorionic Gonadotropin Display Biased Agonism at the LH and LH/CG Receptors. Sci Rep 7(1): 940. https://doi.org/10.1038/s41598-017-01078-8
7. Segaloff DL, Wang HY, Richards JS (1990) Hormonal regulation of luteinizing hormone/chorionic gonadotropin receptor mRNA in rat ovarian cells during follicular development and luteinization. Mol Endocrinol 4(12): 1856–1865. https://doi.org/10.1210/mend-4-12-1856
8. Menon B, Sinden J, Franzo-Romain M, Botta RB, Menon KM (2013) Regulation of LH receptor mRNA binding protein by miR-122 in rat ovaries. Endocrinology 154(12): 4826–4834. https://doi.org/10.1210/en.2013-1619
9. Menon B, Gulappa T, Menon KM (2015) miR-122 Regulates LH Receptor Expression by Activating Sterol Response Element Binding Protein in Rat Ovaries. Endocrinology 156(9): 3370–3380. https://doi.org/10.1210/en.2015-1121

10. Veldhuis JD, Liu PY, Takahashi PY, Keenan DM (2012) Dynamic testosterone responses to near-physiological LH pulses are determined by the time pattern of prior intravenous LH infusion. Am J Physiol Endocrinol Metab 303(6): E720–728. https://doi.org/10.1152/ajpendo.00200.2012
11. Bakhtyukov AA, Derkach KV, Gureev MA, Dar'in DV, Sorokoumov VN, Romanova IV, Morina IY, Stepochkina AM, Shpakov AO (2020) Comparative Study of the Steroidogenic Effects of Human Chorionic Gonadotropin and Thieno[2,3-D]pyrimidine-Based Allosteric Agonist of Luteinizing Hormone Receptor in Young Adult, Aging and Diabetic Male Rats. Int J Mol Sci 21(20): 7493. https://doi.org/10.3390/ijms21207493
12. Bakhtyukov AA, Derkach KV, Romanova IV, Sorokoumov VN, Sokolova TV, Govdi AI, Morina IYu, Perminova AA, Shpakov AO (2021) Effect of low-molecular-weight allosteric agonists of the luteinizing hormone receptor on its expression and distribution in rat testes. J Evol Biochem Physiol 57(2): 208–220. https://doi.org/10.1134/S0022093021020034
13. Heitman LH, Oosterom J, Bonger KM, Timmers CM, Wiegerinck PH, Ijzerman AP (2008) [^{3}H]Org 43553, the first low-molecular-weight agonistic and allosteric radioligand for the human luteinizing hormone receptor. Mol Pharmacol 73(2): 518–524. https://doi.org/10.1124/mol.107.039875
14. van Koppen CJ, Zaman GJ, Timmers CM, Kelder J, Mosselman S, van de Lagemaat R, Smit MJ, Hanssen RG (2008) A signaling-selective, nanomolar potent allosteric low molecular weight agonist for the human luteinizing hormone receptor. Naunyn Schmiedebergs Arch Pharmacol 378(5): 503–514. https://doi.org/10.1007/s00210-008-0318-3
15. Nataraja SG, Yu HN, Palmer SS (2015) Discovery and Development of Small Molecule Allosteric Modulators of Glycoprotein Hormone Receptors. Front Endocrinol (Lausanne) 6: 142. https://doi.org/10.3389/fendo.2015.00142
16. Derkach KV, Dar'in DV, Lobanov PS, Shpakov AO (2014) Intratesticular, intraperitoneal, and oral administration of thienopyrimidine derivatives increases the testosterone level in male rats. Dokl Biol Sci 459: 326–329. https://doi.org/10.1134/S0012496614060040
17. Bakhtyukov AA, Derkach KV, Sorokoumov VN, Stepochkina AM, Romanova IV, Morina IY, Zakharova IO, Bayunova LV, Shpakov AO (2021) The Effects of Separate and Combined Treatment of Male Rats with Type 2 Diabetes with Metformin and Orthosteric and Allosteric Agonists of Luteinizing Hormone Receptor on Steroidogenesis and Spermatogenesis. Int J Mol Sci 23(1): 198. https://doi.org/10.3390/ijms23010198
18. Cora MC, Kooistra L, Travlos G (2015) Vaginal Cytology of the Laboratory Rat and Mouse: Review and Criteria for the Staging of the Estrous Cycle Using Stained Vaginal Smears. Toxicol Pathol 43(6): 776–793. https://doi.org/10.1177/0192623315570339
19. Ajayi AF, Akhigbe RE (2020) Staging of the estrous cycle and induction of estrus in experimental rodents: an update. Fertil Res Pract 6: 5. https://doi.org/10.1186/s40738-020-00074-3
20. Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 3(6): 1101–1108. https://doi.org/10.1038/nprot.2008.73
21. van de Lagemaat R, Timmers CM, Kelder J, van Koppen C, Mosselman S, Hanssen RG (2009) Induction of ovulation by a potent, orally active, low molecular weight agonist (Org 43553) of the luteinizing hormone receptor. Hum Reprod 24(3): 640–648. https://doi.org/10.1093/humrep/den412
22. van de Lagemaat R, Raafs BC, van Koppen C, Timmers CM, Mulders SM, Hanssen RG (2011) Prevention of the onset of ovarian hyperstimulation syndrome (OHSS) in the rat after ovulation induction with a low molecular weight agonist of the LH receptor compared with hCG and rec-LH. Endocrinology 152(11): 4350–4357. https://doi.org/10.1210/en.2011-1077
23. Gerrits M, Mannaerts B, Kramer H, Addo S, Hanssen R (2013) First evidence of ovulation induced by oral LH agonists in healthy female volunteers of reproductive age. J Clin Endocrinol Metab 98(4): 1558–1566. https://doi.org/10.1210/jc.2012-3404
24. Wortmann L, Lindenthal B, Muhn P, Walter A, Nubbemeyer R, Heldmann D, Sobek L, Morandi F, Schrey AK, Moosmayer D, Günther J, Kuhnke J, Koppitz M, Lücking U, Röhn U, Schäfer M, Nowak-Reppel K, Kühne R, Weinmann H, Langer G (2019) Discovery of BAY-298 and BAY-899: Tetrahydro-1,6-naphthyridine-Based, Potent, and Selective Antagonists of the Luteinizing Hormone Receptor Which Reduce Sex Hormone Levels in Vivo. J Med Chem 62(22): 10321–10341. https://doi.org/10.1021/acs.jmedchem.9b01382
25. Derkach KV, Dar'in DV, Shpakov AO (2020) Low-Molecular-Weight Ligands of Luteinizing

Hormone with the Activity of Antagonists. Biochemistry (Moscow) Suppl Ser A: Membrane and Cell Biology 14(3): 223–231. https://doi.org/10.1134/S1990747820030034
26. Broqua P, Riviere PJ, Conn PM, Rivier JE, Aubert ML, Junien JL (2002) Pharmacological profile of a new, potent, and long-acting gonadotropin-releasing hormone antagonist: degarelix. J Pharmacol Exp Ther 301(1): 95–102. https://doi.org/10.1124/jpet.301.1.95
27. Weiss JM, König SJ, Polack S, Emons G, Schulz KD, Diedrich K, Ortmann O (2006) Actions of gonadotropin-releasing hormone analogues in pituitary gonadotrophs and their modulation by ovarian steroids. J Steroid Biochem Mol Biol 101(2-3): 118–126. https://doi.org/10.1016/j.jsbmb.2006.06.009
28. Smith MS, Freeman ME, Neill JD (1975) The control of progesterone secretion during the estrous cycle and early pseudopregnancy in the rat: prolactin, gonadotropin and steroid levels associated with rescue of the corpus luteum of pseudopregnancy. Endocrinology 96(1): 219–226. https://doi.org/10.1210/endo-96-1-219
29. Donner NC, Lowry CA (2013) Sex differences in anxiety and emotional behavior. Pflugers Arch 465(5): 601–626. https://doi.org/10.1007/s00424-013-1271-7
30. Manna PR, Dyson MT, Stocco DM (2009) Regulation of the steroidogenic acute regulatory protein gene expression: present and future perspectives. Mol Hum Reprod 15(6): 321–333. https://doi.org/10.1093/molehr/gap025
31. Shpakov AO, Derkach KV, Dar'in DV, Lobanov PS (2014) Activation of adenylyl cyclase by thienopyrimidine derivatives in rat testes and ovaries. Cell Tissue Biol 8(5): 400–406. https://doi.org/10.1134/S1990519X14050071
32. Derkach KV, Dar'in DV, Bakhtyukov AA, Lobanov PS, Shpakov AO (2016) In vitro and in vivo studies of functional activity of new low molecular weight agonists of the luteinizing hormone receptor. Biochemistry (Moscow) Suppl Ser A: Membrane and Cell Biology 10(4): 294–300. https://doi.org/10.1134/S1990747816030132
33. Manna PR, Stetson CL, Slominski AT, Pruitt K (2016) Role of the steroidogenic acute regulatory protein in health and disease. Endocrine 51(1): 7–21. https://doi.org/10.1007/s12020-015-0715-6
34. Patel SS, Beshay VE, Escobar JC, Carr BR (2010) 17α-Hydroxylase (CYP17) expression and subsequent androstenedione production in the human ovary. Reprod Sci 17(11): 978–986. https://doi.org/10.1177/1933719110379055
35. Beshay VE, Havelock JC, Sirianni R, Ye P, Suzuki T, Rainey WE, Carr BR (2007) The mechanism for protein kinase C inhibition of androgen production and 17alpha-hydroxylase expression in a theca cell tumor model. J Clin Endocrinol Metab 92(12): 4802–4809. https://doi.org/10.1210/jc.2007-1394
36. Hedin L, Rodgers RJ, Simpson ER, Richards JS (1987) Changes in content of cytochrome P450(17)alpha, cytochrome P450scc, and 3-hydroxy-3-methylglutaryl CoA reductase in developing rat ovarian follicles and corpora lutea: correlation with theca cell steroidogenesis. Biol Reprod 37(1): 211–223. https://doi.org/10.1095/biolreprod37.1.211
37. Conley AJ, Howard HJ, Slanger WD, Ford JJ (1994) Steroidogenesis in the preovulatory porcine follicle. Biol Reprod 51(4): 655–661. https://doi.org/10.1095/biolreprod51.4.655
38. Peca-Rico M, Guadalupe Ortiz-López M, Camacho-Castillo L, Cȯrdenas M, Pedraza-Chaverri J, Menjívar M (2006) Steroidogenic impairment due to reduced ovarian transcription of cytochrome P450 side-chain-cleavage (P450scc) and steroidogenic acute regulatory protein (StAR) during experimental nephrotic syndrome. Life Sci 79(7): 702–708. https://doi.org/10.1016/j.lfs.2006.02.023
39. Lavoie HA, King SR (2009) Transcriptional regulation of steroidogenic genes: STARD1, CYP11A1 and HSD3B. Exp Biol Med (Maywood) 234(8): 880–907. https://doi.org/10.3181/0903-MR-97
40. Lephart ED, Doody KJ, McPhaul MJ, Simpson ER (1992) Inverse relationship between ovarian aromatase cytochrome P450 and 5 alpha-reductase enzyme activities and mRNA levels during the estrous cycle in the rat. J Steroid Biochem Mol Biol 42(5): 439–447. https://doi.org/10.1016/0960-0760(92)90255-h
41. Zurvarra FM, Salvetti NR, Mason JI, Velazquez MM, Alfaro NS, Ortega HH (2009) Disruption in the expression and immunolocalisation of steroid receptors and steroidogenic enzymes in letrozole-induced polycystic ovaries in rat. Reprod Fertil Dev 21(7): 827–839. https://doi.org/10.1071/RD09026

Translated by A. Polyanovsky