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BIOCHEMISTRY, BIOPHYSICS, AND MOLECULAR BIOLOGY

Stimulation of Ovulation in Immature Female Rats Using Orthosteric and Allosteric Luteinizing Hormone Receptor Agonists

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Abstract—Human chorionic gonadotropin (hCG) and luteinizing hormone (LH) are widely used for the treatment of reproductive disorders and for controlled ovulation induction, but their use is limited by side effects. Allosteric agonists of the LH/hCG receptor, including thieno[2,3-d]thienopyrimidine TP03 developed by us, can become an alternative. TP03 (50 mg/rat, i.p.) when administered to immature female rats treated 48 h before with Follimag has been shown to increase progesterone levels (maximum 8 h post-treatment) and induce ovulation, as indicated by the appearance at 24 h corpus luteum (8.6 ± 0.5 per ovary). In terms of its activity, TP03 is comparable to hCG, although it acts more moderately. In the ovaries, unlike hCG, TP03 does not lead to an increase in the expression of vascular endothelial growth factor, which can cause ovarian hyperstimulation syndrome. Thus, TP03 is a promising drug as an ovulation inducer and ovarian steroidogenesis stimulator.

Keywords: ovulation induction, ovarian steroidogenesis, immature females, thienopyrimidine, allosteric agonist, chorionic gonadotropin, luteinizing hormone

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The development of effective strategies for controlled ovulation induction, including those in assisted reproductive technologies, is a relevant task of modern endocrinology and reproductive medicine. Currently, gonadotropins (urinary and recombinant human chorionic gonadotropin (hCG)) and recombinant luteinizing hormone (LH) are used for this purpose; however, they are used at relatively high doses, which leads to hyperactivation of the ovarian LH/hCG receptor and enhances the expression of vascular endothelial growth factor (VEGF), which can cause ovarian hyperstimulation syndrome (OHSS) [1]. In addition, gonadotropins exhibit low specificity with respect to intracellular cascades in target cells, which is due to the interaction of the hormone-activated LH/hCG receptor with various types of heterotrimeric G proteins (G_s, $G_{q/11}$) and β-arrestins [2, 3]. Many side effects of LH and hCG pharmaceuticals are determined by the fact that their N-glycosylation pattern differs from that of natural hormones [4]. It is important to note that N-glycosylation not only affects the affinity of gonadotropins to the receptor but also plays the key role in their effector specificity [3, 4].

All of the above requires the development of alternative approaches for ovulation induction, and great expectations are associated with allosteric regulators of the LH/hCG receptor, including thieno[2,3-d]-pyrimidine derivatives [5, 6]. We have previously developed and studied two thieno[2,3-d]-pyrimidine derivatives TP03 and TP04, which exhibit the properties of full allosteric agonists of the LH/hCG receptor. When administered to male rats with androgen deficiency caused by aging or diabetes mellitus, they restored testicular steroidogenesis, normalized testosterone levels, and improved spermatogenesis [7, 8]. We assumed that these compounds, when administered to female rats, can function as ovulation inducers and stimulate ovarian steroidogenesis. This assumption is supported by the data that the compound Org43553, which exhibits the activity of an LH/hCG receptor agonist and also belongs to the class of thieno[2,3-d]-pyrimidines, can induce ovulation in animals and in female volunteers under clinical conditions [9, 10]. In accordance with this, the aim of this study was to investigate in a comparative aspect the ability of hCG and TP03 to induce ovulation in imma-

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Table 1. The specific mass of the ovaries, the number of preovulatory follicles and corpus luteum in immature female rats after ovulation induction with TP03 and hCG

Group	Specifi covarian mass, %*	Number of preovulatory follicles, units/ovary	Number of corpus luteum (ovulatory follicles), units/ovary
Control	0.020 ± 0.002	ND	ND
F	0.046 ± 0.004 [a]	1.0 ± 0.5	ND
FT-4	0.042 ± 0.002 [a]	1.8 ± 0.4	ND
FT-8	0.037 ± 0.002 [a]	2.8 ± 0.6	ND
FT-16	0.065 ± 0.006 [a,b]	5.6 ± 0.9	ND
FT-24	0.054 ± 0.003 [a]	1.4 ± 0.7	8.6 ± 0.5
FG-4	0.066 ± 0.005 [a,b]	2.4 ± 0.5	ND
FG-8	0.059 ± 0.010 [a]	3.2 ± 0.4	ND
FG-16	0.078 ± 0.005 [a,b]	7.0 ± 0.8	0.8 ± 0.5**
FG-24	0.060 ± 0.004 [a,b]	ND	16.6 ± 1.2

* The specific ovarian mass was calculated as the ratio of the sum of the masses of the left and right ovaries to the total body weight (%).
** Two out of five rats had two ovulatory follicles, and the remainder had no ovulatory follicles.
The differences are significant at $p < 0.05$: [a] with Control group, [b] with group F. Data are presented as $M \pm SEM$, $n = 5$ in each group.

ture female rats, regulate ovarian steroidogenesis, and influence the expression of the *VegfA* and *VegfB* genes encoding various isoforms of vascular endothelial growth factor (VEGF-A, VEGF-B), involved in the development of OHSS [11]. The temporal dynamics of the studied effects of TP03 and hCG was evaluated.

Experiments were performed using immature female Wistar rats aged 22–24 days. Folliculogenesis was stimulated by the gonadotropin from the serum of pregnant mares Follimag (1000 IU/vial, Mosagrogen, Russia) (subcutaneously (s.c.), 15 IU/rat). Forty-eight hours after the Follimag administration, the rats were injected with ovulation inducers TP03 (intraperitoneally (i.p.), 50 mg/kg) or hCG (s.c., 15 IU/rat). Then, 4, 8, 16, or 24 h after the administration of TP03 or hCG, vaginal swabs were taken from the animals for microscopy. Before and at appropriate time intervals after the administration of ovulation inducers, rats were anesthetized (chloral hydrate, 400 mg/kg, i.p.) and decapitated. Their ovaries were taken and weighed, the number of mature follicles was morphologically assessed, and the expression of ovarian genes was measured. Blood was taken from the neck veins to determine the steroid hormones. Ten groups were formed ($n = 5$ in all groups): the control rats (C) that who received solvents instead of drugs; the rats that were injected with Follimag alone (48 h after injection) (F); and the rats that were injected with TP03 (i.p., 50 mg/kg) or with hCG (s.c., 15 IU/rat) 48 h after Follimag injection and decapitated 4, 8, 16, and 24 h after the injection of TP03 (FT-4, FT-8, FT-16, FT-24) or hCG (FG-4, FG-8, FG-16, and FG-24).

For morphological analysis, the ovaries were fixed for 48 h (4°C) in a 4% paraformaldehyde solution, which was prepared in phosphate buffer (0.1 M, pH 7.2) containing 0.9% NaCl (PBS). After washing in PBS, the samples were placed in a 30% sucrose solution in PBS (4°C) and, after cryoprotection, were frozen on dry ice in Tissue-Tek® medium (Sacura, Finetek Europe, Netherlands). Longitudinal sections of the ovary (10 μm thick) were obtained using a Leica CM-1520 cryostat (Leica Microsystems, Germany); every tenth section was mounted on Super-Frost/plus glasses (Menzel, Germany) and dried. After treatment with 50% ethanol solution (15 min), the slides were stained with Sudan-3 (15 min), counterstained with hematoxylin, and placed under a coverslip with glycerol. The images were analyzed with a Carl Zeiss Imager A1 microscope (Carl Zeiss, Germany) equipped with Axiocam 712 video camera and Zen 3.4 software. According to the histological atlas [12], the number of preovulatory follicles and corpora lutea on sections was counted to estimate the number of ovulated follicles. The levels of estradiol and progesterone in the blood of rats were determined by ELISA analysis using Estradiol-ELISA and Progesterone-ELISA kits (CHEMA, Russia). Total ovarian RNA was isolated using the ExtractRNA reagent (Evrogen, Russia), and reverse transcription was performed using the MMLV RT Kit (Evrogen, Russia). PCR was performed in an Applied Biosystems® 7500 Real-Time PCR System amplifier (Life Technologies, Thermo Fisher Scientific Inc., United States) in a mixture containing 0.4 μM forward and reverse primers using the qPCR-HS SYBR+Low ROX reagent (Evrogen, Russia). Expression of the *VegfA* and *VegfB* genes was assessed by the $2^{-\Delta\Delta C_T}$ method using the actin B (*Actb*) gene as a reference gene as described previously [7]. Statistical analysis was performed using Microsoft Office Excel 2007 (United States). The results were presented as $M \pm SEM$. Distribution normality was assessed using the Shapiro–Wilk test. Differences were considered significant at $p < 0.05$.

The study of the specific weight of the ovaries showed its significant increase in all groups administered with Follimag, including the administration with TP03 and hCG (Table 1). Morphological analysis of the ovaries showed that the number of preovulatory follicles after the administration of rats with both ovulation inducers increased during 16 h after their administration. After 24 h, the number of preovulatory follicles sharply decreased (TP03) or they were not detected at all (hCG) (Table 1). Corpora luteus corre-

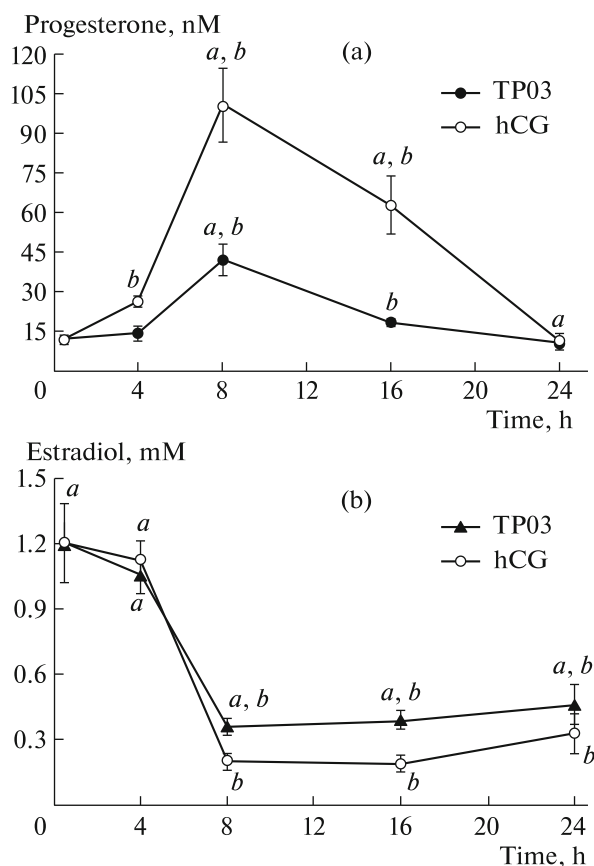

Fig. 1. Changes in the content of progesterone (a) and estradiol (b) in the blood of immature female rats during 24 h after the administration of ovulation inducers TP03 and hCG. In the control group, the concentration of progesterone in blood was 20.9 ± 3.7 nM, and the concentration of estradiol was 0.19 ± 0.03 nM. In the experimental groups, blood serum samples were obtained 48 h after folliculogenesis stimulation (group F, point 0) and then 4, 8, 16, and 24 h after ovulation induction with TP03 (groups FT-4, FT-8, FT-16, and FT-24) or with hCG (groups FG-4, FG-8, FG-16, and FG-24). The differences are significant at $p < 0.05$: [a] with Control group, [b] with group F. Data are presented as $M \pm SEM$, $n = 5$ in each group.

sponding to ovulatory follicles were found in significant amounts in animals in the FT-24 and FG-24 groups, which indicates the induction of ovulation in them (Table 1).

The level of progesterone in the blood increased significantly 8 h after administration with TP03 and hCG, to a greater extent in the case of hCG (Fig. 1a). The increase in progesterone levels persisted 16 h but disappeared 24 h after hCG administration, which is consistent with the data of other authors on an acute peak in the progesterone level in the blood of immature rats after hCG administration and its subsequent decrease [13, 14]. The calculated AUC_{4-24} value for the progesterone concentration (nM)–time (h) curve in the case of hCG significantly exceeded the corresponding value for TP03 (1208 ± 156 vs 475 ± 42, $p <$ 0.002). The estradiol level in the TP03 and hCG administration groups, on the contrary, significantly decreased in comparison with the F group (Fig. 1b). The decrease was more singificant in the case of gonadotropin, judging by the lower AUC_{4-24} value for the estradiol concentration (nM)–time (h) curve in the case of hCG in comparison with TP03 (6.30 ± 0.74 vs. 9.29 ± 0.72, $p < 0.05$). A decrease in estradiol levels after ovulation induction with hCG in immature rats was also reported by other authors [13, 15]. One of the caused for this is the change in the availability of testosterone for aromatase, which converts it into estradiol [15]. Thus, the dynamics of steroidogenic effects of TP03 and hCG was similar, but hCG was more effective.

The data obtained indicate that, as in the case of stimulation of testicular steroidogenesis [7, 8], the effect of the allosteric agonist TP03 on ovarian steroidogenesis is more moderate compared to hCG, and the activity of TP03 as an ovulation inducer is less pronounced. However, this has certain advantages because it makes it possible to prevent OHSS, ovarian reserve depletion, and decreased egg quality, which are often observed in the case of hCG [1]. For a comparative study of the possible risks of OHSS development in the ovaries when TP03 and hCG are used, the expression of the *VegfA* and *VegfB* genes, encoding VEGF-A and VEGF-B, which can be OHSS inducers, was evaluated [11]. It was shown that the expression of *VegfA* and *VegfB* in group F (administration with Follimag alone) decreased (Fig. 2). TP03 and hCG had little effect on the reduced expression of the *VegfA* gene in group F, except for the time interval of 24 h, when both agonists suppressed it to an even greater extent (Fig. 2a). In the case of *VegfB* gene expression, the situation was different. hCG significantly stimulated it in comparison with group F, whereas TP03 had little effect on the *VegfB* expression, significantly increasing it only in the rats of group FT-16 (Fig. 2b). These data indicate that hCG, despite the relatively low dose of the drug used in our study (close to the physiological range of endogenous LH concentrations), maintains a high level of VEGF-B expression during 1 day after administration. This, together with the higher specific ovarian mass throughout the experiment, indicates significant risks of development of polycystic ovary syndrome (PCOS), which is often observed with the use of gonadotropins for controlled ovulation induction [1]. In turn, TP03 had no significant effect on the VEGF-B expression, which may indicate a low probability of OHSS development.

Thus, we have shown for the first time that TP03, an LH/hCG receptor agonist interacting with its transmembrane allosteric site, similarly to the commercial drug hCG, interacting with the extracellular orthosteric site of the receptor, can stimulate progesterone production and induce ovulation in immature

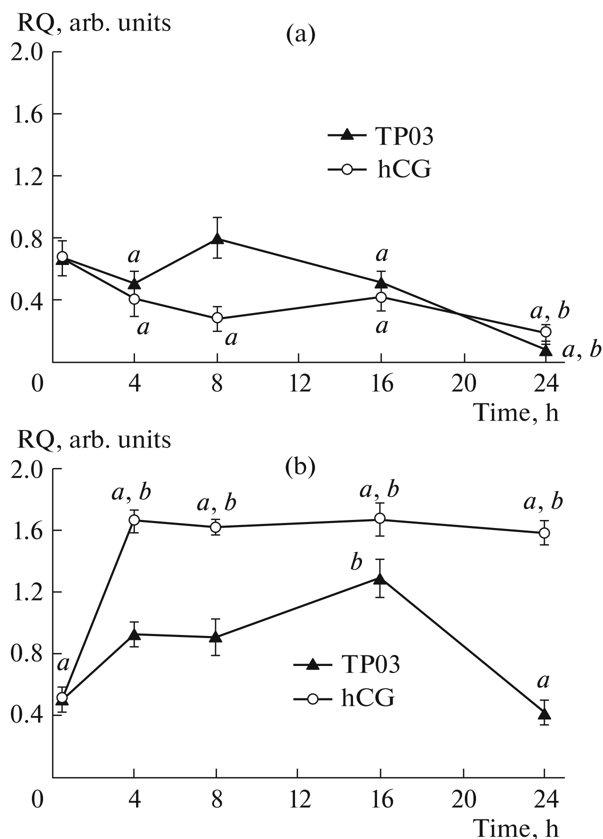

Fig. 2. Expression of the *VegfA* (a) and *VegfB* (b) genes encoding isoforms A and B of vascular endothelial growth factor in the ovaries of immature rats and the effect of ovulation inducers TP03 and hCG on them. In the control group, the expression of the *VegfA* and *VegfB* genes in the ovaries was 1.03 ± 0.09 and 1.04 ± 0.08 relative units, respectively. In the experimental groups, ovarian tissues were obtained 48 h after folliculogenesis stimulation (group F, point 0) and then 4, 8, 16, and 24 h after ovulation induction with TP03 (groups FT-4, FT-8, FT-16, and FT-24) or with hCG (groups FG-4, FG-8, FG-16, and FG-24). The differences are significant at $p < 0.05$: [a] with control group, [b] with group F. Data are presented as $M \pm SEM$, $n = 5$ in each group.

female rats. TP03, in comparison with hCG, had a milder effect on the ovaries, increasing the progesterone level in blood to a lesser extent and leading to a twofold decrease in the formation of corpora lutea. At the same time, TP03, unlike hCG, caused no increase in the *VEGF-B* gene expression in the ovaries, which minimizes the probability of developing OHSS, which is associated with the activation of VEGF-dependent signaling pathways and is one of the severe complications in using gonadotropins in controlled ovulation induction in assisted reproductive technologies.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest. The authors declare that they have no conflicts of interest.

Statement on the welfare of animals. All procedures for the care of animals and their use in the study were carried out in strict accordance with the requirements of the Ethics Committee of the Sechenov Institute of Evolutionary Physiology and Biochemistry of the Russian Academy of Sciences (minutes no. 2/4-2021), European Communities Council Directive 1986 (86/609/EEC), and "Guide for the Care and Use of Laboratory Animals."

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