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EXPERIMENTAL PAPERS

The Effect of Metformin Therapy on Luteinizing Hormone Receptor Agonist-Mediated Stimulation of Testosterone Production and Spermatogenesis in Diabetic Rats

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Abstract—The functional restoration of the male reproductive system in type 2 diabetes mellitus (DM2) is one of the urgent global challenges in modern endocrinology. This problem can be solved through the application of both the drugs that improve glucose homeostasis and insulin sensitivity, primarily metformin, and the activators of luteinizing hormone receptor (LHR), such as human chorionic gonadotropin (hCG) and low-molecular-weight allosteric LHR agonists. The aim of the work was to study the effect of metformin therapy (4 weeks, 120 mg/kg/day) on steroidogenesis- and spermatogenesis-stimulating effects induced by the 5-day administration of 5-amino-*N*-tert-butyl-2-(methylsulfanyl)-4-(3-(nicotinamido)phenyl)thieno[2,3-d]pyrimidine-6-carboxamide (TP03), an allosteric LHR agonist (15 mg/kg/day), and hCG (20 IU/rat/day) to male Wistar rats with DM2. The DM2 was induced by a high-fat diet and a low-dose streptozotocin (25 mg/kg). Metformin therapy partially restored testosterone levels and normalized spermatogenesis in DM2 rats. On the first day, metformin treatment enhanced the steroidogenic effects of TP03 and hCG, however, in the following days its potentiating effect was not detected. After five days of treatment of diabetic rats with TP03 and hCG, epididymal sperm count was restored, including the spermatozoa with progressive motility, and the percentage of abnormal sperm was decreased. Spermatogenesis indices when treating with metformin or LHR agonists separately were comparable to those resulted from their co-administration. Thus, metformin therapy enhances TP03- and hCG-induced testosterone production on the first day of treatment with these drugs, however, later on, steroidogenic and spermatogenic effects of LHR agonists in the groups of diabetic rats treated and untreated with metformin did not differ significantly.

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Keywords: steroidogenesis, spermatogenesis, type 2 diabetes mellitus, metformin, chorionic gonadotropin, thienopyrimidine, testosterone

INTRODUCTION

One of the complications of type 2 diabetes mellitus (DM2), which is characterized by insulin resistance, impaired glucose homeostasis and lipid metabolism, are dysfunctions of the reproductive system and impaired functional activity of the hypothalamic–pituitary–gonadal (HPG) axis. Male patients with DM2 develop androgen deficiency and abnormal spermatogenesis, which leads to reduced fertility and can even cause male infertility [1–4]. The search for effective approaches to restore the functions of the male reproductive system under DM2 conditions represents one of the urgent global challenges of modern endocrinology, which is explained by both high growth rates of DM2 incidence worldwide and the general trend toward a decline in fertility, including due to the prevalence of male infertility.

To normalize the male reproductive potential, gonadotropins are used most often, including human chorionic gonadotropin (hCG) targeting the luteinizing hormone receptor (LHR) in testicular cells [5]. Like luteinizing hormone (LH), hCG is characterized by a high-affinity interaction with the extracellular domain of LHR, which refers to G protein-coupled receptors. A resultant conformational change in the transmembrane LHR domain triggers the activation of gonadotropin-dependent intracellular signaling pathways [6]. The cAMP-dependent pathway plays a pivotal role in this process. This pathway includes the G_s protein, adenylate cyclase and protein kinase A and leads to the activation of the cholesterol-transporting steroidogenic acute regulatory (StAR) protein. In turn, StAR catalyzes the first, rate-limiting, stage of steroidogenesis, as well as steroidogenic enzymes responsible for the synthesis of testosterone and its precursors in Leydig cells. The phosphoinositide pathway, which is triggered via LHR-mediated activation of $G_{q/11}$ proteins and phospholipase Cβ, also plays a modulatory role in the regulation of steroidogenesis [7]. Testosterone synthesized in Leydig cells affects Sertoli cells and thereby controls sperm maturation and functional activity. However, long-term LHR stimulation by gonadotropins and the use of their high doses needed to attain a significant steroidogenic effect cause the activation of β-arrestins responsible for the internalization and downregulation of LHR and trigger the other negative feedback mechanisms. All this leads to the development of gonadotropin resistance of Leydig cells, due to which their elimination can lead to androgenic insufficiency and abnormal spermatogenesis [5]. Under conditions of diabetic pathology caused by hyperglycemia, inflammation and increased oxidative stress, testicular LHR expression declines and the structure of its binding domains is disrupted, leading to a decrease in the receptor affinity for gonadotropins and the attenuation of their effects on steroidogenesis and spermatogenesis [8, 9].

All of the above-said indicates the need to design novel LHR agonists that would be more specific toward intracellular signaling cascades and able to stimulate steroidogenic pathways more mildly compared to hCG and LH, causing no resistance of testicular cells to endogenous gonadotropins. In this regard, of greatest interest are thienopyrimidine derivatives (TPs) that have been developing over the past years in our laboratory [10–14] and by other authors [15–17]. Unlike gonadotropins, they interact with an allosteric site located in the transmembrane domain of LHR and do not influence the accessibility of the extracellular site for gonadotropins. Their advantages include the selectivity toward the cAMP-dependent pathway responsible for the activation of steroidogenesis, the absence of immunogenicity, and the ability to retain activity when administered orally, since TPs are resistant to degradation in the gastrointestinal tract and are well absorbed by enterocytes [10, 11, 18]. The steroidogenic effects of TPs are being intensively studied now [12, 18], but their impact on spermatogenesis, both in normal conditions and in DM, remains poorly understood.

The first-line drug in the treatment of DM2 is metformin, which activates AMP-activated protein kinase (AMPK), a crucial cellular energy sensor, and thereby not only restores metabolism and insulin sensitivity, but also improves the functioning of the male reproductive system [19, 20]. We have previously shown that metformin therapy of male rats and mice with various DM forms restores steroidogenesis, improves the morphol-

ogy of the seminiferous tubules, and increases sperm motility [9, 21, 22]. Based on the data that metformin administration increases testicular sensitivity of diabetic rats to a single hCG exposure [9], we hypothesized that metformin therapy can potentiate the restoration of the testicular function in rats with DM2 when treated with orthosteric (hCG) and allosteric (TP) LHR agonists.

The aim of this work was to study the effect of 4-week metformin therapy on the stimulation of steroidogenesis and spermatogenesis in male rats with DM2, induced by the treatment with 5-amino-*N*-tert-butyl-2-(methylsulfanyl)-4-(3-(nicotinamido)phenyl)thieno[2,3-d]pyrimidine-6-carboxamide (TP03), an allosteric LHR agonist developed in our laboratory, and gonadotropin (hCG).

MATERIALS AND METHODS

The experiments were carried out on 2-month-old male Wistar rats (body weight 170–200 g) that were kept under standard conditions with ad libitum access to food and water. All experimental protocols complied with the requirements of the Bioethics Committee at the Sechenov Institute of Evolutionary Physiology and Biochemistry and European Communities Council Directive 1986 (86/609/EEC) and the Guide for the Care and Use of Laboratory Animals.

DM2 was induced using a 9-week high-fat diet (1 kg of the food mixture contained 524 g of lard, 417 g of cottage cheese, 50 g of liver, 5.3 g of L-methionine, 1.85 g of yeast, 1.85 g of NaCl) [23] and a subsequent one-time administration of a low dose (25 mg/kg) of streptozotocin (Sigma, USA) with a diet continuation for another 6 weeks. Control animals (C, $n = 18$) were receiving standard pelleted food instead of a high-fat diet, and, instead of streptozotocin, were injected with its solvent, 0.1 M sodium citrate buffer (pH 4.5). Throughout the experiment, the body weight was monitored and the blood glucose level was measured (2 h after meal) using One Touch Ultra test strips (USA) and a Life Scan glucometer (Johnson & Johnson, Denmark). The degree of hyperglycemia was assessed by the level of glycated hemoglobin (HbA1c) using a Multi Test HbA1c System kit (Polymer Technology Systems, Inc., USA).

Two weeks after streptozotocin administration, before the onset of metformin therapy, diabetic groups were composed from rats with glucose levels above 7 mM (120 min after glucose load) and HbA1c levels above 5.5%, as followed from the results of the glucose tolerance test (GTT). During GTT, the animals (after 12-h fasting) were injected intraperitoneally (i.p.) with a glucose solution at a rate of 2 g of glucose per kg of body weight, and the blood glucose level was measured afterwards for 120 min. Diabetic rats were randomly assigned to groups with no treatment (D, $n = 18$) and with metformin treatment (DM, $n = 18$). Metformin treatment (orally, daily dose 120 mg/kg; Sigma-Aldrich, USA) of DM rats was carried out for 4 weeks, whereas D and C rats, instead of metformin, received its solvent (distilled water) at the same time and in the same volume. Meanwhile, the diabetic rats continued to be kept on a high-fat diet. Three weeks after the onset of treatment, in order to assess the therapeutic effect of metformin in C, D and DM groups, the GTT was repeated to measure the glucose level for 120 min, and after that, to assess the insulin level using a Rat Insulin ELISA kit (Mercodia AB, Sweden).

Five days before the end of the experiment, each of the groups of control, untreated diabetic and metformin-treated diabetic rats was further divided into three subgroups (C0/CT/CG, D0/DT/DG and DM0/DMT/DMG), forming a total of 9 groups ($n = 6$ in each). C0, D0, and DM0 rats ($n = 6$), instead of the drugs, received dimethyl sulfoxide (DMSO), a TP03 solvent. CT, DT, and DMT rats were i.p. injected at a daily basis with TP03 compound dissolved in DMSO at a daily dose of 15 mg/kg. CG, DG and DMG rats ($n = 6$) were injected subcutaneously (s.c.) with hCG (Moscow Endocrine Factory, Russia) for 5 days at a daily dose of 20 IU/rat. According to previous studies, DMSO has no significant effect on the parameters being evaluated, as well as on the functional state of animals [14]. TP03 compound was synthesized as described elsewhere [11] and characterized using a high-performance liquid chromatography and mass spectrometry. On days 1 and 5 of the treatment with LHR ago-

Table 1. Body weight, glycemic indices, insulin and testosterone levels in control male rats (C) and diabetic animals, untreated (D) and metformin-treated (DM)

Index	C	D	DM
Body weight, g	337 ± 29	376 ± 38*	354 ± 34
Fasting glucose, mM (before GTT)	4.3 ± 0.5	6.8 ± 0.9*	5.4 ± 0.7*#
Postprandial glucose, mM (120 min after GTT)	5.2 ± 0.7	10.5 ± 2.0*	6.9 ± 1.3*#
HbA1c, %	4.5 ± 0.4	8.1 ± 1.5*	5.7 ± 1.1*#
AUC_{0-120} (GTT), arb. u.*	985 ± 123	1448 ± 258*	1209 ± 185*#
Postprandial insulin, ng/mL (120 min after GTT)	0.93 ± 0.23	1.46 ± 0.37*	1.05 ± 0.31*#
Testosterone, nM	13.0 ± 4.3	7.1 ± 2.8*	10.6 ± 3.0#

*—Differences vs. control are statistically significant at $p < 0.05$; #—differences between D vs. DM groups are statistically significant at $p < 0.05$. Data are presented as $M \pm SD$, $n = 18$.

nists, the testosterone level was evaluated before the administration of the agonists (10 am) and 60, 180 and 300 min (11 am, 1 pm, and 3 pm, respectively) after the administration of TP03 and hCG. On days 2 to 4, the testosterone level was evaluated 180 min after drug administration (1 pm), when, according to our pilot results, the steroidogenic effect of gonadotropin peaked, while the corresponding effect of TP03 began to plateau [12]. To measure testosterone concentration, a commercial Testosterone-ELISA kit (Alkor-Bio, Russia) was used.

At the end of the experiment, the animals were anesthetized (4–5% isoflurane inhalation) and decapitated, after which their spermogram indices were assessed as described previously [24]. To do this, 5 mg of sperm was collected from the caudal part of the epididymis, placed in 195 μL of the Quinn's Advantage^{TM} Medium With HEPES fertilization medium (In Vitro Fertilization Inc., Cooper Surgical Company, USA), and incubated for 30 min at 37°C therein. Then, 10 μL of diluted semen was added to the Makler sperm counting chamber (depth 10 μm, Sefi Medical Instruments, Israel), where cell count was performed using a MICMED-5 microscope (magnification ×400; LOMO, Russia), and the results were presented as the number of cells per g of the epididymis. The number of motile spermatozoa and functionally active spermatozoa with progressive motility was calculated as a percentage of the total sperm count, which was taken as 100%. Sperm morphology was examined after azure-eosin staining a smear placed on a glass slide using a Spermo-Diff-200 reagent kit (Syntacon, Russia). The number of morphologically abnormal forms, including the sperm with a coiled tail or head defects [25], was calculated per 100 cells in each preparation. Sperm digital images were obtained using an Axio Lab.A1 MAT microscope (Carl Zeiss, Germany) with a built-in TV camera (magnification ×1000) and Axio-Vision 4.8.

Statistical data analysis was carried out using Microsoft Office Excel 2007. The data distribution normality was checked using the Shapiro–Wilk test. To compare samples with normal distribution, the Student's *t*-test was used. Differences were considered statistically significant at $p < 0.05$. Data were presented as $M \pm SD$.

RESULTS

Long-term high-fat diet and the treatment of rats with a low dose of streptozotocin led to the development of DM2 with its intrinsic hyperglycemia and impaired glucose tolerance, as indicated by elevated levels of postprandial glucose and HbA1c, as well as increased values of the integrated area under the "glucose concentration (mM) vs. time (min)" curves (AUC_{0-120}) for glucose in GTT, and hyperinsulinemia, as evidenced by an increased insulin level 120 min after glucose loading (Table 1). Metformin treatment significantly normalized glucose and insulin levels and restored glucose tolerance (Table 1). In rats with DM2, baseline testosterone levels were reduced, and metformin treatment partially restored them (Table 1).

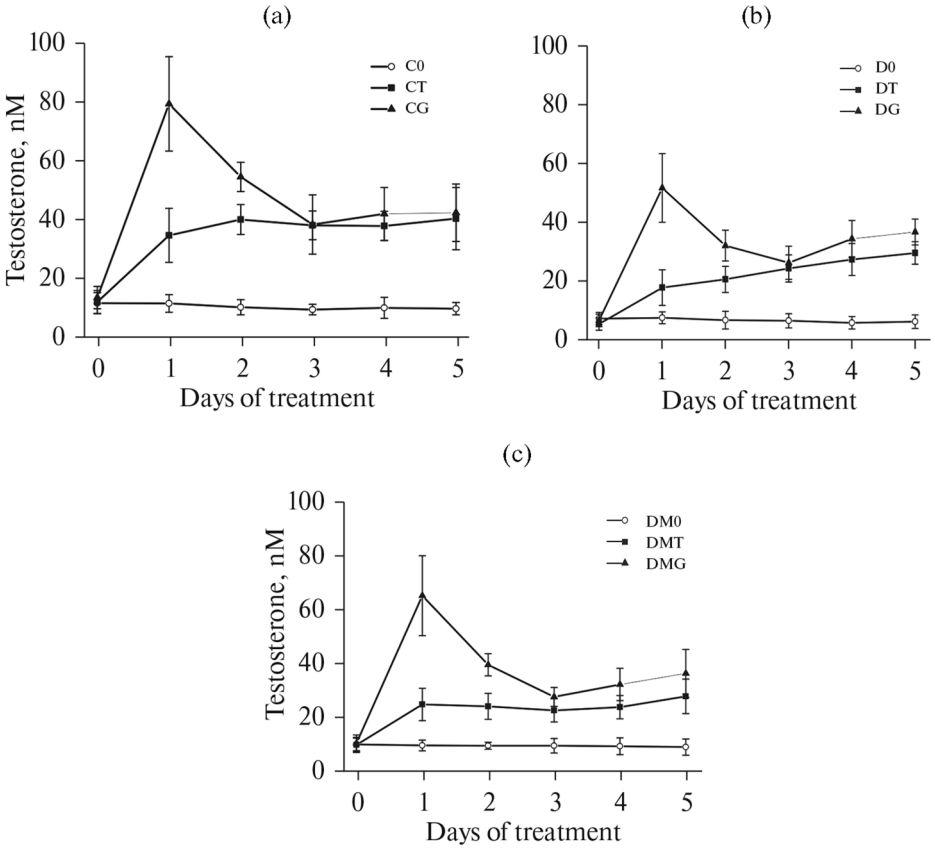

Fig. 1. Testosterone level dynamics throughout 5-day treatment of male control (a), diabetic untreated (b) and diabetic metformin-treated (c) rats induced by TP03 (15 mg/kg) and hCG (20 IU/rat). C0—Control, CT—control + TP03, CG—control + hCG, D0—diabetes, DT—diabetes + TP03, DG—diabetes + hCG, DM0—diabetes + metformin, DMT—diabetes + metformin + TP03, DMG—diabetes + metformin + hCG. The testosterone level was measured daily 3 h after drug administration. Data are presented as $M \pm SD$. $n = 6$.

Five-day TP03 and hCG administration to control rats increased the testosterone level, with hCG being more effective in the first two days, while on days 3 to 5, steroidogenic effects of TP03 and hCG were indistinguishable (Fig. 1, Table 2), which is consistent with the data we obtained previously in the study of TP03 [11] and its structural analog TP4/2 [14]. In rats with DM2, both baseline and LHR agonist-stimulated testosterone levels were reduced, illustrating the lower (compared to the corresponding control groups) AUC_{0-300} and AUC_{0-5} values representing the integrated area under the "testosterone concentration (nM) vs. treatment time (min or days)" curve (Table 2). As in the control, the steroidogenic effect of hCG, as evaluated by the AUC_{0-300} value in the DG group, significantly exceeded that of TP03 on day 1 of treatment (Table 2). At the same time, on day 5, the effects of hCG and TP03 were comparable, and the AUC_{0-300} value in the DG group exceeded that in the DT group only by 40% (Fig. 1, Table 2).

Metformin treatment of diabetic rats led to an increase in baseline testosterone levels and enhanced the steroidogenic effects of TP03 and hCG on day 1 of treatment (Fig. 1, Table 2). The differences between AUC_{0-300} in DT vs. DMT and DG vs. DMG groups were statistically significant ($p < 0.05$) (Table 2). From days 2 to 5, the effects of TP03 and hCG in diabetic rats treated and untreated with metformin were comparable (Fig. 1). For example, the differences in AUC_{0-300} values on day 5 of treatment between the DT vs. DMT and DG vs. DMG groups were not statistically significant, while in the case of AUC_{0-5}, significant differences persisted only between DG vs.

Table 2. Values of AUC_{0-300}, an integrated area under the "testosterone concentration (nM) vs. treatment time (min)" curve, for the testosterone level on days 1 and 5 of treatment, and values of AUC_{0-5}, an integrated area under the "testosterone concentration (nM) vs. treatment time (days)" curve, for the testosterone level during 5-day treatment with TP03 (15 mg/kg) and hCG (20 IU/rat) of control rats and diabetic untreated and metformin-treated animals

Group	Day 1, AUC_{0-300}, arb.u.	Day 5, AUC_{0-300}, arb.u.	5-day treatment, AUC_{0-5}, arb.u.
C0	58.0 ± 5.9	50.9 ± 5.7	52.2 ± 5.1
CT	142.5 ± 12.4^{c}	155.8 ± 14.4^{c}	177.4 ± 8.4^{c}
CG	$325.8 \pm 23.2^{c,d}$	178.5 ± 14.7^{c}	$242.9 \pm 15.7^{c,d}$
D0	35.9 ± 3.3^{a}	30.5 ± 4.5^{a}	33.6 ± 4.1^{a}
DT	73.4 ± 8.5^{c}	107.8 ± 4.9^{c}	107.6 ± 7.7^{c}
DG	$210.1 \pm 13.7^{c,d}$	$151.4 \pm 4.6^{c,d}$	$165.6 \pm 8.2^{c,d}$
DM0	50.6 ± 4.3^{b}	48.4 ± 5.7^{b}	47.8 ± 4.0^{b}
DMT	$113.0 \pm 7.0^{c,e}$	112.6 ± 8.6^{c}	117.6 ± 8.5^{c}
DMG	$269.2 \pm 19.1^{c,d,e}$	149.8 ± 14.9^{c}	$188.3 \pm 5.1^{c,d,e}$

Differences are statistically significant ($p < 0.05$) between: [a]—diabetic groups (D0, DM0) vs. control group (C0); [b]—D0 vs. DM0 groups; [c]—untreated groups (C0, D0, DM0) vs. corresponding groups treated with TP03 (CT, DT, DMT) or hCG (CG, DG, DMG); [d]—CT vs. CG, DT vs. DG or DMT vs. DMG groups; [e]—DT vs. DMT and DG vs. DMG groups. Data presented as $M \pm SD$, $n = 6$.

DMG groups (Table 2).

Next, the effects of DM2, metformin treatment, and treatment with TP03 and hCG on spermatogenesis indices were studied. In DM2, the percentage of motile sperm, including their functionally active forms with progressive rectilinear motility, was lower vs. control, while the percentage of abnormal sperm was higher vs. control (Fig. 2). There was a downward trend in the total epididymal sperm count, although the differences between C0 and D0 groups were not statistically significant. Metformin treatment increased the total number and motility of sperm, while the percentage of the sperm with progressive motility increased in comparison not only with the D0 group, but also with the control (Fig. 2). The treatment of control rats with TP03 and hCG increased the percentage of sperm with progressive motility, and in the case of hCG, also the total sperm count. The percentage of sperm with progressive motility, as calculated with consideration to the total sperm count, in CT and CG groups increased compared to the control untreated with the drugs by 43 and 85%, respectively. In diabetic rats, TP03 and hCG also increased the percentage of motile sperm, including those with progressive motility (Fig. 2). There was an upward trend in the total sperm count in DG group, but the differences with D0 group were not statistically significant. In DMT and DMG groups, the total number and percentage of motile sperm in comparison with DM0 group did not change (Fig. 2). The data obtained indicate an increase in sperm fertility when treating control and diabetic rats with TP03 and hCG and diabetic rats with metformin, however, they also attest to the absence of an enhancement of the stimulatory effect of LHR agonists on spermatogenesis in rats that received metformin therapy.

DISCUSSION

Under conditions of prolonged DM2, both steroidogenesis and spermatogenesis are impaired, leading to reproductive dysfunctions and infertility, as demonstrated both in rodents with DM2 [8, 9, 26–28] and in patients with the same pathology [2, 29]. The data of our previous study on male rats with DM2 induced by a high-fat diet and low-dose of streptozotocin also indicate a negative

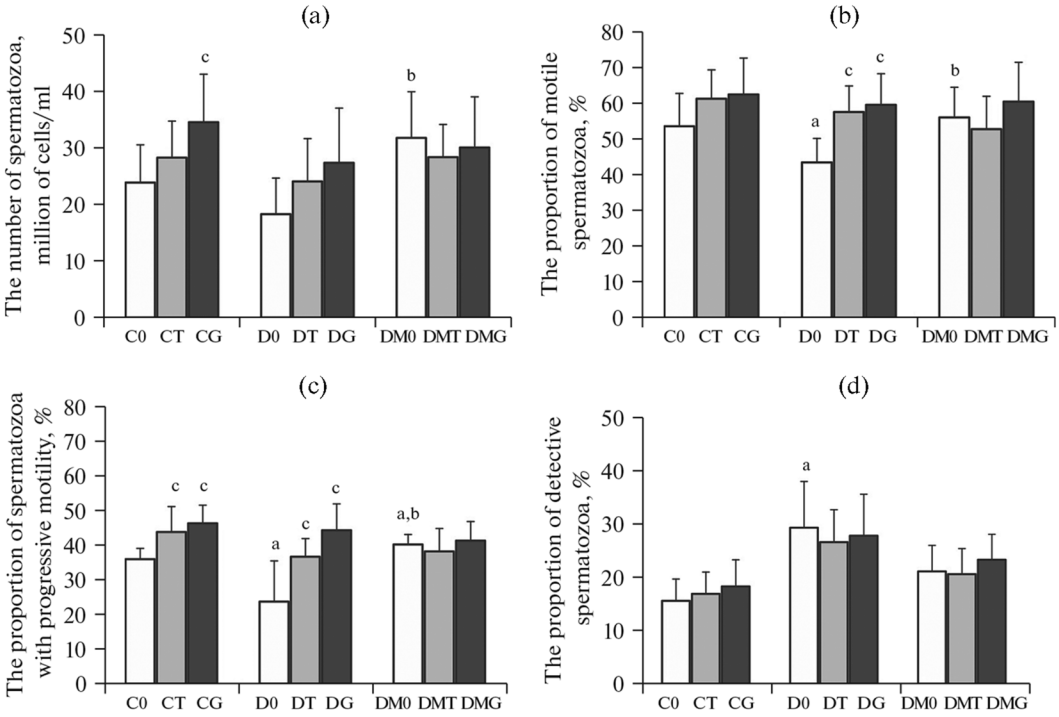

Fig. 2. Effects of 5-day treatment of diabetic male rats with TP03 (15 mg/kg) and hCG (20 IU/rat) on the sperm count (a), percentage of motile sperm (b), percentage of sperm with progressive rectilinear motility (c), and percentage of sperm with a coiled tail or head defects (d), and the impact of metformin therapy on these effects. C0—control, CT—control + TP03, CG—control + hCG, D0—diabetes, DT—diabetes + TP03, DG—diabetes + hCG, DM0—diabetes + metformin, DMT—diabetes + metformin + TP03, DMG—diabetes + metformin + hCG. Differences are statistically significant ($p < 0.05$) between: [a]—diabetic groups (D0, DM0) vs. control group (C0); [b]—D0 vs. DM0 groups; [c]—untreated groups (C, D, DM) vs. corresponding groups treated with TP03 (CT, DT, DMT) or hCG (CG, DG, DMG). Data are presented as $M \pm SD$, $n = 6$.

effect of hyperglycemia, impaired glucose tolerance and insulin resistance on the androgenic status, sperm count and motility, morphology of the seminiferous tubules [9].

A study of the steroidogenic effect of hCG, a structural and functional homolog of LH, which is a potent stimulator of testosterone production by Leydig cells [6], showed that in diabetic rats, as compared to control animals, this effect declined (Fig. 1, Table 2), whereas the stimulatory effect of the gonadotropin on spermatogenesis in DM2 underwent no significant changes (Fig. 2). At the same time, on days 3 to 5, the steroidogenic effect of gonadotropin attenuated in both control and diabetic rats and became comparable to that of TP03, which, unlike hCG, stimulates LHR activity by binding to an allosteric site located in the transmembrane domain of the receptor [14, 16–18]. Importantly, the steroidogenic effect of TP03, in contrast to hCG, did not attenuate during the 5-day administration, which may be due to both the accumulation of this hydrophobic compound in testicular tissue and the peculiarities of its action on LHR. For example, gonadotropins bind to the extracellular domain of LHR, which undergoes excessive glycation under conditions of prolonged hyperglycemia and damage induced by reactive oxygen species, while the allosteric site, the TP03 target, is less accessible for such exposures. In addition, as a hydrophobic compound, TP03 is able not only to bind to LHR located in the plasma membrane, but also penetrate into the cell and interact with yet "immature" forms of the receptor [18, 30]. In the case of DM2, this seems to be especially important, since oxidative stress, inflammation and endoplasmic reticulum stress, which are strongly pronounced in diabetic pathology, disrupt the post-translational processing of LHR and their translocation into the membrane, reducing thereby the number of receptors on the surface of target cells [9, 27].

One of the most widely and successfully used

drugs for the treatment of DM2 is metformin, which not only improves metabolic and hormonal indices, but also partially restores reproductive functions [8, 19, 27, 31]. In the present work, metformin treatment of diabetic male rats (4 weeks, 120 mg/kg/day) not only normalized glucose homeostasis, glucose tolerance and insulin levels, but partially restored the indices of steroidogenesis and spermatogenesis. For example, in the DM0 group, there was an increase in the basal testosterone level reduced in DM2 (Table 1), a restoration of the epididymal sperm count to control values and the proportion of their motile forms, including those with progressive rectilinear motility (Fig. 2). These data are in good agreement with those we and other authors obtained previously on the restorative effect of metformin therapy toward the testicular function in rodents with different models of diabetes [8, 9, 19, 21, 22, 27, 31].

Since in DM2 metformin therapy restores metabolic and hormonal indices and improves the functioning of the HPG axis, we hypothesized that such a treatment would enhance the stimulatory effects of LHR agonists on testicular steroidogenesis and spermatogenesis. This hypothesis was based on the enhancing effect of metformin therapy on steroidogenic effects of hCG that we revealed after its one-time administration to diabetic rats [9].

We demonstrated that metformin treatment of diabetic rats boosts the steroidogenic effect of TP03 and hCG on treatment day 1 (Fig. 1). On day 2, in DMT and DMG groups, there was an upward tendency in the effects of TP03 and hCG, but the differences with DT and DG groups were no longer statistically significant. However, later on, the potentiating effect of metformin therapy on the effects of LHR agonists disappeared (Figs. 1, 2). While enhancing the testosterone production-stimulating effects of LHR agonists with different mechanisms of action upon their single administration, metformin therapy does not affect these effects during long-term drug administration. The unexpected decay of the potentiating effect of metformin therapy on the steroidogenic effects of TP03 and hCG may owe the following reasons.

Metformin is a stimulant of AMPK, a key energy sensor of the cell, the functional activity of which declines in most tissues, including the testes, under diabetic pathology [32, 33]. Metformin therapy normalizes AMPK activity, which is one of the reasons behind the restoration of steroidogenesis in DM0 group and potentiation of the response of testicular cells to a single administration of TP03 and hCG in DMT and DMG groups (Fig. 1, Table 2). At the same time, with the long-term effect of LHR agonists on steroidogenesis, permanent activation of AMPK by metformin may trigger the mechanisms that prevent the potentiation of the steroidogenic effect of TP03 and hCG. This may be due to a negative effect of metformin-activated AMPK on the expression and activity of steroidogenic proteins [34–37]. For example, in experiments with ovarian granulosa cells of broiler breeder chickens, it was shown that an increase in AMPK activity is associated with a decrease in the expression of genes encoding the transport protein StAR, cytochrome CYP11A1, and dehydrogenase HSD3B, which results in a decrease in progesterone synthesis [38]. In human luteinized granulosa cells, AMPK stimulation by an adiponectin agonist inhibits cAMP-dependent cascades, decreasing aromatase expression and estrogen production [39]. In luteal cells, AMPK hyperactivation also suppresses steroidogenesis, but not via suppression of cAMP signaling and reduced gene expression of steroidogenic proteins, but through inhibition of hormone-sensitive lipase (HSL) [40]. This enzyme hydrolyzes cholesterol esters that accumulate in lipid droplets inside the cell, and thus makes cholesterol available for the transport into mitochondria, where the initial stages of steroidogenesis take place. It should be noted that LH suppresses AMPK activity in luteal cells, preventing its negative effect on steroidogenesis [41]. Permanent AMPK stimulation by metformin during 4-week therapy can thus be a limiting factor that prevents the potentiation of the steroidogenic effect of hCG and TP03.

As shown in the present study, metformin therapy, along with improving testicular steroidogenesis, normalizes spermatogenesis, bringing the evaluated parameters closer to their control values, and in the case of spermatozoa with progressive rectilinear motility, their count even exceeds that in the control (Fig. 2). These data are consistent with

the results obtained previously in our laboratory and by other authors on the restoration of the spermatogenic function and morphology of the seminiferous tubules in metformin-treated rodents with different models of diabetes [8, 9, 21, 27]. The key role in these processes belongs to the restoration of the polarity of Sertoli cell and their tight junctions (impaired in diabetes mellitus) that determine the integrity of the blood-testis barrier and normal spermatogenesis [36, 42]. At the same time, against the background of metformin-induced normalization of spermatogenesis, additional stimulation of spermatogenesis during the treatment of diabetic rats with TP03 and hCG in DMT and DMG groups was not detected (Fig. 2). This is probably due to the fact that the resource for spermatogenesis restoration within metformin therapy is completely exhausted and its indices do not differ from or even exceed those in the control (Fig. 2).

Thus, metformin treatment of male rats with DM2 induced by a high-fat diet and low-dose streptozotocin improves their testicular steroidogenesis and normalizes spermatogenesis impaired under diabetic pathology. We established for the first time that metformin treatment enhances the steroidogenic effects of LHR agonists interacting with both allosteric (TP03) and orthosteric (hCG) sites of the receptor, but only on the first day of drug administration. Later on, the potentiating effect of metformin therapy is not detected, probably due to the metformin-activated negative effect of AMPK-dependent pathways in Leydig cells on steroidogenic cascades. Both metformin and LHR agonists restore spermatogenesis, with spermatogenesis indices in the groups treated with metformin, TP03 and hCG separately being comparable to those recorded after co-administration of metformin and LHR agonists. Based on the data obtained, it was concluded that long-term co-administration of metformin and LHR agonists in DM2 does not enhance their restorative effect on the androgenic status and fertility, but is able to increase the steroidogenic effect of gonadotropin and TP03 after a single administration.

AUTHORS' CONTRIBUTION

Conceptualization and experimental design (A.O.Sh., A.A.B., K.V.D.); data collection (A.A.B., A.M.S., V.N.S., I.A.L., L.V.B., K.V.D.); data processing (A.A.B., V.N.S., K.V.D., A.O.Sh.); writing and editing a manuscript (A.O.Sh., A.A.B., K.V.D.).

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CONFLICT OF INTEREST

The authors declare that they have neither evident nor potential conflict of interest related to the publication of this article.

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