BASIC SCIENCE

Ipidacrine (Axamon), A Reversible Cholinesterase Inhibitor, Improves Erectile Function in Male Rats With Diabetes Mellitus-Induced Erectile Dysfunction

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ABSTRACTS

Background: Management of diabetes mellitus-induced erectile dysfunction (DMED) is challenging because of its insufficient responses to phosphodiesterase type 5 inhibitors.

Aim: To compare the effects of ipidacrine, a reversible cholinesterase inhibitor, and sildenafil on DMED in a rat model of streptozotocin (STZ)-induced diabetes.

Methods: Erectile dysfunction (ED) caused by STZ-induced diabetes mellitus was modeled in adult male Wistar rats, which were randomized to 4 groups: untreated diabetic rats, sildenafil (5 mg/kg), ipidacrine (3.6 mg/kg) and ipidacrine (6.7 mg/kg). The test drug (ipidacrine), comparator (sildenafil) or control substance (1% starch solution) were administered orally for 5 days or 14 days. Erectile function was assessed by the change in the maximum intracavernous pressure (ICPmax) following cavernous nerve electrical stimulation. The mean arterial pressure (MAP) was recorded, and the ICPmax/MAP ratio was calculated. Sexual behavior, cholinesterase activity and blood testosterone level tests assessed.

Main Outcome Measure: The quantitative value of ICPmax/MAP 14 days after the start of administration of the test drug and the comparison drug.

Results: Animals with STZ-induced diabetes mellitus showed a significant decrease in ICPmax and ICPmax/MAP ratio compared to the intact control group. When ipidacrine was administered to rats with DMED for 14 days, an increase in these indicators was noted. It was proved that ipidacrine at a dose of 6.7 mg/kg has noninferiority compared to sildenafil on the DMED model. Significant increase in ICPmax compared to STZ-control after electrostimulation of the cavernous nerve was recorded following administration of ipidacrine at a dose of 6.7 mg/kg ($P < .05$) and sildenafil at a dose 5 mg/kg ($P < .05$). Neither the test drug, nor the comparator were associated with increase in testosterone levels in blood; as well both drugs did not promote activation of sexual behavior.

Clinical Implications: Ipidacrine may be considered as an effective therapy for DMED but needs to be verified in human investigations.

Strengths & Limitations: The role of ipidacrine, was firstly demonstrated in rats with DMED. However, the results were obtained in animal experiments, and will be further tested in the study of receptor interactions and the determination of cellular targets.

Conclusion: This is the first study to show that administration of ipidacrine, the reversible cholinesterase inhibitor, improved erectile function in diabetic rats and these results may be beneficial in further studies using ipidacrine for treatment of DMED, particularly in non-responders to PDE5 inhibitors. **Bykov V, Gushchina E, Morozov S, et al. Ipidacrine (Axamon), A Reversible Cholinesterase Inhibitor, Improves Erectile Function in Male Rats With Diabetes Mellitus-Induced Erectile Dysfunction. Sex Med 2022;10:100477.**

KEY WORDS: Erectile Dysfunction; Ipidacrine; Maximum Intracavernous Pressure

INTRODUCTION

According to various reports, 9−54% of men aged 40 to 70 years have erectile dysfunction (ED).[1,2] The mean prevalence of this disorder is 20−40%.[3] The risk of ED is increased in subjects with diabetes mellitus; thus, the detection rate of ED is 35−90% in men on anti-diabetic therapy and over 65% in those older than 40 years of age.[4,5]

At the same time, the effectiveness of drugs traditionally used to manage ED (PDE-5 inhibitors) decreases in patients with long-term diabetes mellitus. However, if in the general population the effectiveness of sildenafil for the treatment of erectile dysfunction is 69−88%, for patients with diabetes this frequency is on average 58%,[6] and in some categories of patients it decreases to 40% and below.[7] This is due to a decrease in the production of NO and cGMP in the cavernous bodies, a decrease in blood flow in the arteries of the penis, as well as a violation of neurogenic and endothelium-dependent relaxation of the arteries of the penis.[6] The effectiveness of reversible cholinesterase inhibitors, as ipidacrine, has not been studied in ED previously. It is assumed that the use of ipidacrine, characterized by a different mechanism of action than PDE-5 inhibitors, will provide an effective treatment for DMED resistant to other drugs.Erection is an end result of a complex neurovascular event mediated by stimulation of parasympathetic preganglionic neurons in S2-S4 sacral spinal cord segments. A nerve impulse is transmitted via cholinergic fibers which are the part of mixed nervi splanchnici pelvici and nervi hypogastrici forming nervi cavernosi penis. Stimulation of cholinergic and non-cholinergic postganglionic neurons promotes the release of NO and vasoactive intestinal polypeptide in nerve terminals and endothelial cells of penile vessels and cavernous bodies.[8,9] In addition to the above-stated, main neurotransmitters involved in transmission of the nerve impulse in the parasympathetic system and penile erection include acetylcholine which however has no direct relaxing effect on penile smooth muscle fibers.[10,11]

Acetylcholine exerts its effects mediated by increased NO release in penile cavernous bodies through M2-cholinergic receptors of smooth muscle fibers and M3-cholinergic receptors of endothelial cells as well as through nicotinic receptors located on NO-ergic nerve fibers. Moreover, acetylcholine prevents a vasoconstricting effect of noradrenaline which facilitates erection subsidence through muscarinic receptors on adrenergic nerve endings.[8] Histological examinations in monkeys revealed a high acetylcholinesterase activity in the vicinity of a cavernous artery, in the nerves surrounding this artery and the tissue of a cavernous body.[12]

Experiments in animals of different species demonstrated that intracavernous administration of acetylcholine induces a dose-related erectile response associated with increase in arterial blood flow, relaxation of cavernous smooth muscles and prevention of cavernous outflow and promotes increase in intracavernous pressure (ICP) following cavernous nerve electrical stimulation.[13,14] M-anticholinergic agents, atropine or scopolamine, decrease cavernous blood flow, however the complete suppression of erectile response induced by acetylcholine is observed only following combination of M- and H-anticholinergics.[12] Cholinesterase inhibitors (neostigmine) which increase acetylcholine concentrations cancel the effects of anticholinergic agents.[15] However, these effects of cholinesterase inhibitors and anticholinergics could not be confirmed following systemic and intracavernous administration in healthy subjects.[16] This may be due to a limited number of these studies.

Thus, the effects of the reversible cholinesterase inhibitor ipidacrine in erectile dysfunction may be associated with the activation of muscarinic cholinergic receptors in the nerves innervating the penis, due to increased concentration of acetylcholine. This leads to direct stimulation of erection, as well as indirect action due to relaxation of the smooth muscles of the cavernous bodies and occlusion of the veins of the penis against the background of stimulation of NO-ergic synapses of cholinergic nerve fibers, as well as due to the vasodilating action of acetylcholine itself.[12,17,18]

The effects of cholinesterase inhibitors on ED in various chronic conditions have not been intentionally assessed in clinical studies. Meanwhile, series of our experiments showed that ipidacrine may be successively used for recovery of sexual function in rats with spontaneously decreased one and for treatment of sexual activity disturbances associated with decreased secretion of sexual hormones and chronic stress.[19] This agent belongs to non-selective reversible cholinesterase inhibitors from a class of 4-aminopyridine derivatives which penetrate the blood- brain barrier and has a good safety profile.[20] In addition, ipidacrine inhibits K^+ and Na^+-channels in the neuronal membrane,[21] promotes prolongation of the repolarization phase of action potential and acts as an antagonist of M1, M3-cholinergic receptors and a partial agonist of M2-cholinergic receptors.[22] Ipidacrine is used in different conditions characterized by deficits in central or peripheral cholinergic regulation, such as vascular encephalopathy and ischemic stroke,[23,24] peripheral neuropathies of various origin, focal neuropathies of the upper extremities[25] and alcoholic neuropathies.[26]

Impairment of the peripheral nervous system is characteristic of diabetes mellitus as well.[27,28]

The prevalence of neuropathies in subjects with diabetes mellitus is rather high, that is, about 8% in those with newly diagnosed condition and more than 50% in subjects with confirmed diagnosis.[29] Predominant types include sensomotor and vegetative neuropathies, for which ED is one of manifestations.[29,30] However, efficacy of ipidacrine in diabetic neuropathies and mellitus-induced erectile dysfunction (DMED) has not been studied.

The hypothesis to study[31]: does ipidacrine have a non-inferiority than sildenafil on DMED in a rat model of streptozotocin (STZ)-induced diabetes?

The aim of this study is to compare the effects of ipidacrine (Axamon), a reversible cholinesterase inhibitor, and sildenafil on DMED in a rat model of STZ-induced diabetes.

MATERIALS AND METODS

Animals

The study protocol was approved by the Bioethics Commission of RMC "Home of Pharmacy" and was in agreement with the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes and Guide for the care and use of laboratory animals. National Academy −Washington, D.C. 1996. All animal procedures were carried out in accordance with the Directive 2010/63/EU of the European Parliament and of the council of 22 September 2010 on the protection of animals used for scientific purposes. Two hundred and sixty-six male Wistar rats aged 10 −12 weeks weighing 280−320 g (RMC "Home of Pharmacy," Leningrad region, Russia) were kept under temperature-controlled and humidity-controlled conditions with a 12-hours light−dark cycle and fed a standard rat diet with water ad libitum. Twenty-four animals were included in the intact control group. Experimental diabetes was modeled in 242 animals. The death rate during induction of experimental diabetes mellitus was 40.4% and in 29.5% of animals the experimental pathology was not induced successfully. Thus, 73 STZ-diabetic rats were included in the experiment to assess the effect of ipidacrine on ED in experimental diabetes mellitus.

Induction of Diabetes Mellitus

A single intraperitoneal injection of STZ at a dose of 65 mg/kg diluted in sterile citrate buffer solution (0.1 M, pH 4.5) was used to induce diabetes. Nondiabetic control animals were injected with an equivalent volume (average 0.5−0.7 mL) of citrate buffer solution. The period of formation of experimental diabetes mellitus after administration of STZ was 12 weeks. Preliminary studies showed that this period is sufficient for formation of ED in animals with diabetes mellitus.

The following indicators were used as criteria for formation of experimental diabetes mellitus:

- the blood glucose level 72 hours after administration of STZ was more than 16.7 mol/L. Blood glucose levels in serum samples obtained from the tail vein in all rats were measured using a blood glucose meter (OneTouch Select® Plus, manufacture No: EBLPZPRS, LifeScan, USA);
- the amount of glycosylated hemoglobin as % of total hemoglobin indicating the severity of hyperglycemia (%HbA1) 9 weeks after administration of STZ was statistically significantly higher than in intact animals. %HbA1 indicated the severity of hyperglycemia[32] and can serve as a key marker of a vascular pathology associated with hyperglycemia.[33]

Changes in body weight, blood glucose level and %HbA1 during formation of STZ-induced diabetes mellitus in rats are presented in Table 1.

Test Drug, Comparator and Control Substance

The test drug, ipidacrine (trade name − Axamon, manufactured by PIQ-PHARMA LEC LLC, Russia), was administered in courses of 5 days and 14 days. The comparator, sildenafil (trade name − Viagra, manufactured by Pfizer Inc.), was administered in courses of 14 days. Drugs were crushed and suspended in 1% starch solution and administered intragastrically. Animals in intact control and STZ-control groups were administered 1% starch intragastrically (control substance) 3 times a day as a control substance.

Dosing of the test drug, comparator or control substance was initiated 12 weeks after diabetes induction. Ipidacrine is used to be as polyneuropathy treatment due to its reversible cholinesterase inhibition properties more than 40 years in some countries.

Table 1. Changes in body weight, glucose level and %HbA1 in male rats during formation of STZ-induced diabetes mellitus, Me (Q1;Q3)

Parameter, experiment day		Normal control	STZ-diabetic rats
Body weight, g	Before STZ	302 (288; 310)	286 (272; 301)
	in 9 wk	404 (390; 425)	202 (184; 224)[a]
Glucose level, mmol/L	Before STZ	4.5 (4.3; 4.8)	4.8 (4.4; 5.1)
	in 72 h	4.5 (4.3; 4.8)	22.6 (20.4; 24.6) *
	in 9 wk	5.2 (4.9; 5.8)	22.1 (13.7; 25.3) *
HbA1,%	in 9 wk	3.39 (3.13; 3.58)	4.97 (4.56; 5.38) *

*Statistically significant difference compared to intact control, $P < .05$.
HbA1 = glycosylated hemoglobin; STZ = streptozotocin.

Dosage was calculated in accordance with dosage recommendation for authorized indications using common interspecies scaling equation.[34]

Study Plan

The main hypothesis was to evaluate whether non-inferiority of 6.7 мg/kg/day ipidacrine over 5 мg/kg/day sildenafil in 14 days on STZ-induced DMED in rats using ICPmax/MAP rising effect. Sildenafil was chosen as comparator for its proved effectiveness in DMED.[35,36]

It was decided to use quantitative value of ICPmax/MAP in 14 days of first administration of test drug and comparator as a primary endpoint.

As secondary endpoints were chosen next indicators:

- quantitative values of ICPmax and MAP in 14 days of first administration of 6.7 mg/kg test drug and 5 mg/kg comparator;
- quantitative value ICPmax/MAP in 5 and in 14 days of first administration of 3.6 mg/kg and 6.7 mg/kg test drug;
- measurements, describing experimental pathology development and test drug vs comparator possible effects: body weight, glucose blood concentration, testosterone blood concentration, the results of Mating behavior tests;
- measurements, describing test drug possible adverse effects: Red Cell Cholinesterase and Plasma Cholinesterase Activities.
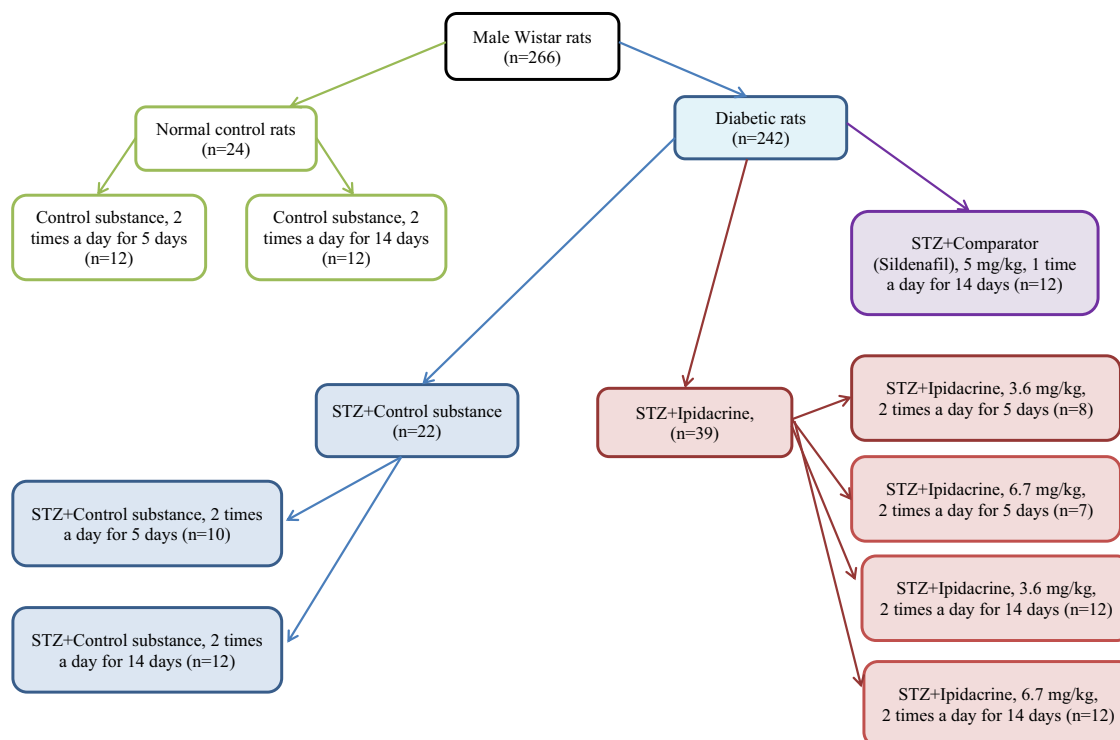
Animals were divided into 7 group (Figure 1):

In Group 1 (normal control, n = 24) intact animals divided into subgroups (n = 12; 12) were given the control substance 2 times a day for 5 days and 14 days, respectively.

In Group 2 (STZ control, n = 22) animals divided into subgroups (n = 10; 12) were given the control substance 2 times a day for 5 days and 14 days, respectively.

In Group 3 (Comparator, n = 12) STZ-induced diabetic rats were given the comparator, sildenafil, at a dose of 5 mg/kg once a day for 14 days. The dose and duration of sildenafil administration were selected based on previous studies.[37]

In Group 4 (test drug 3.6; 5 days, n = 8) STZ-induced diabetic rats were given the test drug, ipidacrine, twice a day at a dose of 3.6 mg/kg/day for 5 days.

In Group 5 (test drug 6.7; 5 days, n = 7) STZ-induced diabetic rats were given the test drug, ipidacrine, twice a day at a dose of 6.7 mg/kg/day for 5 days.

In Group 6 (test drug 3.6; 14 days, n = 12) STZ-induced diabetic rats were given the test drug, ipidacrine, twice a day at a dose of 3.6 mg/kg/day for 14 days.

In Group 7 (test drug 6.7; 14 days, n = 12) STZ-induced diabetic rats were given the test drug, ipidacrine, twice a day at a dose of 6.7 mg/kg/day for 14 days.

Measurements of Mean Arterial Pressure (MAP) and Intracavernous Pressure (ICPmax)

Numbers of different in vivo approaches have been used to investigate the erectile function in experimental animal models. These include but are not limited to penile plethysmography, screening tests of sexual behavior, apomorphine-induced erectile response, and dynamic cavernosography. However, none of these

Figure 1. Animals groups.

assays is ideal for the neurophysiological evaluation of erection because of lack of accuracy and reproducibility. Intracavernosal pressure (ICP) measurement after electrical stimulation of the major pelvic ganglion or cavernous nerve elicited penile erection in anesthetized animals is known as an objective and accurate biometric on the evaluation of erectile function. It provides the most reliable and quantitative response of the penis to peripheral and central nervous system neural activation. Accordingly, it contributes greatly to the assessment of a normal or ED experimental animal model as well as the development of potential therapeutic strategies.[38,39] MAP and ICPmax in response to electrical stimulation of the cavernous nerve were measured one day after the last dose in all groups. Animals were anesthetized with 1.75-3.0 % Isoflurane (Laboratorios Karizoo, S.A., Spain)[40] using Zoomed Minor Vet (Manufactory No 2014239, Zoomed, Russia). After a carotid artery was catheterized and a pressure sensor attached to the artery (SPR-407 Micro-Tip catheter pressure transducer for rats, size 2F, REF No 840-4079, Rev. E 320-7051, Millar, Inc., USA), incision of the skin of the anterior abdominal wall was performed. Once a cavernous body was isolated, the pelvic ganglion and cavernous nerve were determined on the surface of the dorsal lobe of the prostate. Stainless steel hook-type electrodes d = 0.4 mm were inserted under the cavernous nerve. The cavernous body was catheterized by inserting a needle with a diameter of 25G and a length of 18 mm, connected to a cannula Tubing− PE Catheter SP0109 (ADInstruments Ltd., Australia), filled with heparin solution at a concentration of 500 IU/mL. The cannula was connected to MLT844 Physiological Pressure Transducer (Part. No 32030, Series No AC0172, ADInstruments Ltd, Australia). Both pressure sensor and pressure transducer were connected to multi-channel recorder PowerLab 8/30 ML 870/P (manufacture No 830-1679, ADInstruments Ltd., USA) via Bridges Amp FE221 (ADInstruments Ltd, Australia). MAP and ICP were continuously measured using LabChart 6 software (v. 6.1.3, ADInstruments Ltd., USA) under electrical stimulation of the cavernous nerve with the following electric current parameters: output pulse voltage 19V and 10V, frequency 30 Hz, 0.5-millisecond (ms) pulse width, duration of stimuli − 30 seconds. Each period of stimulation was separated by a resting period of 5 minutes. In addition, ICPmax/MAP ratios were calculated using LabChart 8 Reader software (v. 8.0.30.09.2013, ADInstruments Ltd., USA).

Table 2. ChE and AChE activities in male rats in the normal control group compared to STZ- diabetic rats, Me (Q1; Q3)

Group	ChE (U/L)	AChE (mM $\times$ min^{-1} $\times$ L^{-1})
Normal control	578 (499; 691)	0.704 (0.584; 0.777)
STZ-diabetic rats	556 (370; 716)	0.528 (0.388; 0.703)*

*$P < .05$ compared to normal control, using the Mann-Whitney test.
AChE = acetylcholinesterase; ChE = cholinesterase; STZ = streptozotocin.

Blood Testosterone Level Assessments

The testosterone concentrations in blood plasma were measured in STZ-diabetic rats before the dosing period and 1 hour after the last dose. In the normal control group, the assessments were carried out in a similar time frame. Assays were carried out using blood samples collected from the tail vein between 12:00 and 13:00.

Red Cell Cholinesterase and Plasma Cholinesterase Activities Assessments

The cholinesterase activity in blood plasma (ChE) and acetylcholinesterase activity in red blood cells (AChE) were assessed in the tail vein blood samples in STZ-diabetic rats before the dosing period and after the last dose. The ChE activity was assessed using ELISA and the activity of AChE was assessed using the modified Ellman method.[41,42] The results are presented in Table 2.

The activity of ChE in STZ-induced diabetes mellitus remained unchanged, while the AChE activity was significantly reduced ($P < .05$).

Mating Behavior Tests

The sexual behavior of male rats was evaluated on the last day of dosing. Sexual behavior was assessed using the following parameters: mount latency (ML), intromission latency (IL), ejaculation latency (EL), postejaculation interval (PEI), mount frequency (MF), and intromission frequency (IF).[42]

Chemicals

Streptozocin (STZ) and all analytical reagents were purchased from Sigma-Aldrich (USA). %HbA1 was determined by ion-exchange columnless method. ChE activities in blood plasma were assessed using a Cholinesterase-Novo kit (Series No 8, expiry date 02. 2022, Vector- Best, Novosibirsk, Russia). The measurement range was 123−25000 units/L. Testosterone concentrations in blood plasma were assessed using a Testosterone-EIA-BEST kit (No X3972, lot 129, expiry date 02.25.2021, Vector-Best, Novosibirsk, Russia). The lower limit of quantitative determination for the method was 0.17 nmol/L. %HbA1, ChE activities and Testosterone concentrations was determined using microplate analyzer CLARIOstar (manufactory No 430-0796, BMG LABTECH, Germany).

Statistical Analysis

The analysis was conducted using Statistica version 10.0 software (StatSoft, USA). To analyze the normality of the distribution, the Shapiro-Wilk's W test and graphical methods was applied. In case of normal distribution, we used the Student's criterion for related and unrelated data, univariate analysis (ANOVA) using the Tukey criterion. Nonparametric analysis was used where at least one of the experimental groups had data inconsistent with the law of normal

Table 3. Changes in body weight and blood glucose levels following administration of the test drug and comparator for 5 days (Me (Q1; Q3))

Group	Body weight, g			Glucose level, mmol/L		
		After 5 days of administration of the drug			After 5 days of administration of the drug	
	Baseline value	value	% relative to the baseline value	Baseline value[‡]	value	% relative to the baseline value
Normal control	439 (417; 453)	405 (391; 429)	94.0 (92.4; 94.6)	4.70 (4.55; 4.95)	6.60 (5.75; 6.85)	142.7 (117.0; 145.7)
STZ-control	213 (192; 227)	169 (157; 187)	82.7 (81.8; 84.4)*	24.9 (17.5; 28.5)	11.4 (4.7; 15.5)	49.1 (20.4; 65.3)*
Test drug, 3.6 mg/kg	210 (199; 224)	208 (189; 218)	96.5 (92.3; 100.2)[†]	22.5 (21.2; 26.0)	20.0 (14.6; 23.7)	84.4 (64.4; 102.0)
Test drug, 6.7 mg/kg	234 (214; 257)	211 (195; 235)	90.7 (85.8; 93.8)	22.5 (17.9; 25.6)	16.4 (10.0; 21.8)	66.8 (55.6; 84.2)*

*P < .05 compared to normal control.
[†]P < .05 compared to STZ-control.
[‡]The baseline glucose level before dosing was assessed 9 weeks after STZ injection. The dosing was started after 12 weeks.
STZ = streptozotocin

distribution. In this case, we used the Mann-Whitney test, the Wilcoxon test for related samples, and the Kruskal-Wallis test with multiple comparisons of average ranks. *P* value < .05 was considered statistically significant. The 95% reference interval for glycosylated hemoglobin was calculated using the formula $\bar{X} \pm 1.96S$, where X is the arithmetic mean of the amount of glycosylated hemoglobin in intact animals and S is the standard deviation.

To test the non-inferiority hypothesis, the difference between ICPmax/MAP was determined in animals that were administered the test drug at a dose of 6.7 mg/kg (T) and or a comparator at a dose of 5 mg/kg (C) for 14 days. Then, a 95% 2-way confidence interval of this indicator was calculated. If the lower limit of the interval was less than or equal to -δ, then the null-hypothesis (H0) was accepted, and it was concluded that the comparison drug was superior to the tested drug. If the lower limit of the

interval was greater than -δ, then an alternative hypothesis (H1) was accepted that the tested drug is not worse than the comparator.[43]

$$H0 : T - C \leq -\delta; \quad H1 : T - C > -\delta \quad (1)$$

where δ — minimal measure of differences, allowing to reject null-hypothesis («noninferiority margin»). Variable δ, which value was set as 0.1, was determined on a basis of similar trials results.[44]

Minimum sample size was calculated in accordance with recommendations,[45] used following equation:

$$N = (Z\alpha - Z\beta)^2 * (s_1{}^2 + s_2{}^2)/\delta \quad (2)$$

where $s_1{}^2$ and $s_2{}^2$ — condition dispersion in both groups, $Z\alpha$ and $Z\beta$ — rejection limits of normal distribution, corresponding to the given levels of errors of the 1st kind and the chosen level of

Table 4. Changes in body weight and blood glucose levels following administration of the test drug and comparator for 14 days (Me (Q1; Q3))

Group	Body weight, g			Glucose level, mmol/L		
		After 14 days of administration of the drug			After 14 days of administration of the drug	
	Baseline value	value	% relative to the baseline value	Baseline value[§]	value	% relative to the baseline value
Normal control	446 (424; 459)	429 (391; 448)	94.8 (92.9; 97.0)	4.6 (4.3; 4.7)	6.6 (5.6; 7.0)	142.5 (122.5; 149.5)
STZ-control	219 (208; 243)	190 (169; 210)	83.8 (81.1; 89.4)	24.0 (17.7; 27.4)[§]	9.2 (4.5; 12.2)	40.2 (24.4; 54.0)*
Comparator, 5.0 mg/kg	246 (229; 280)	238 (213; 253)	91.3 (86.7; 98.5)	22.1 (15.4; 26.4)[§]	21.3 (14.4; 24.1)	94.2 (52.2; 109.3)
Test drug, 3.6 mg/kg	185 (169; 212)	221 (185; 234)	111.5 (106.9; 118.1)[†,*,‡]	21.6 (15.6; 24.3)[§]	26.1 (15.3; 31.0)	122.8 (62.0; 181.9)*
Test drug, 6.7 mg/kg	247 (232; 258)	232 (221; 247)	93.9 (90.6; 97.9)	20.1 (15.4; 22.5)[§]	14.9 (8.0; 24.1)	81.2 (70.7; 118.9)

*P < .05 compared to normal control.
[†]P < .05 compared to STZ-control.
[‡]P < .05 compared to comparator.
[§]The baseline glucose level before dosing was assessed 9 weeks after STZ injection. The dosing was started after 12 weeks.
STZ = streptozotocin.

significance α. The calculation was carried out for a significance level of 0.05 with a test power of 0.75, taking into account that the standard deviation for the ICPmax / MAP index is approximately 0.05 (according to the results of preliminary experiments).

The sufficiency of the sample size was checked in the groups to which the test drug was administered at a dose of 6.7 mg / kg and the comparator drug at a dose of 5 mg / kg for 14 days. The calculated value was in the range of 12.1-12.3 / in group excluding retired animals.

RESULTS

Body Weight and Blood Glucose

In all animals following administration of STZ a decrease in body weight and hypoglycemia were noted (Tables 3-4). Following administration of the comparator, the change in body weight of the animals corresponded to the STZ-diabetic rats' group without treatment. After administration of the test drug at a dose of 3.6 mg/kg, there was a more pronounced increase in body weight compared to all other groups. Following administration of the test drug at a dose of 6.7 mg/kg, the weight gain was less pronounced.
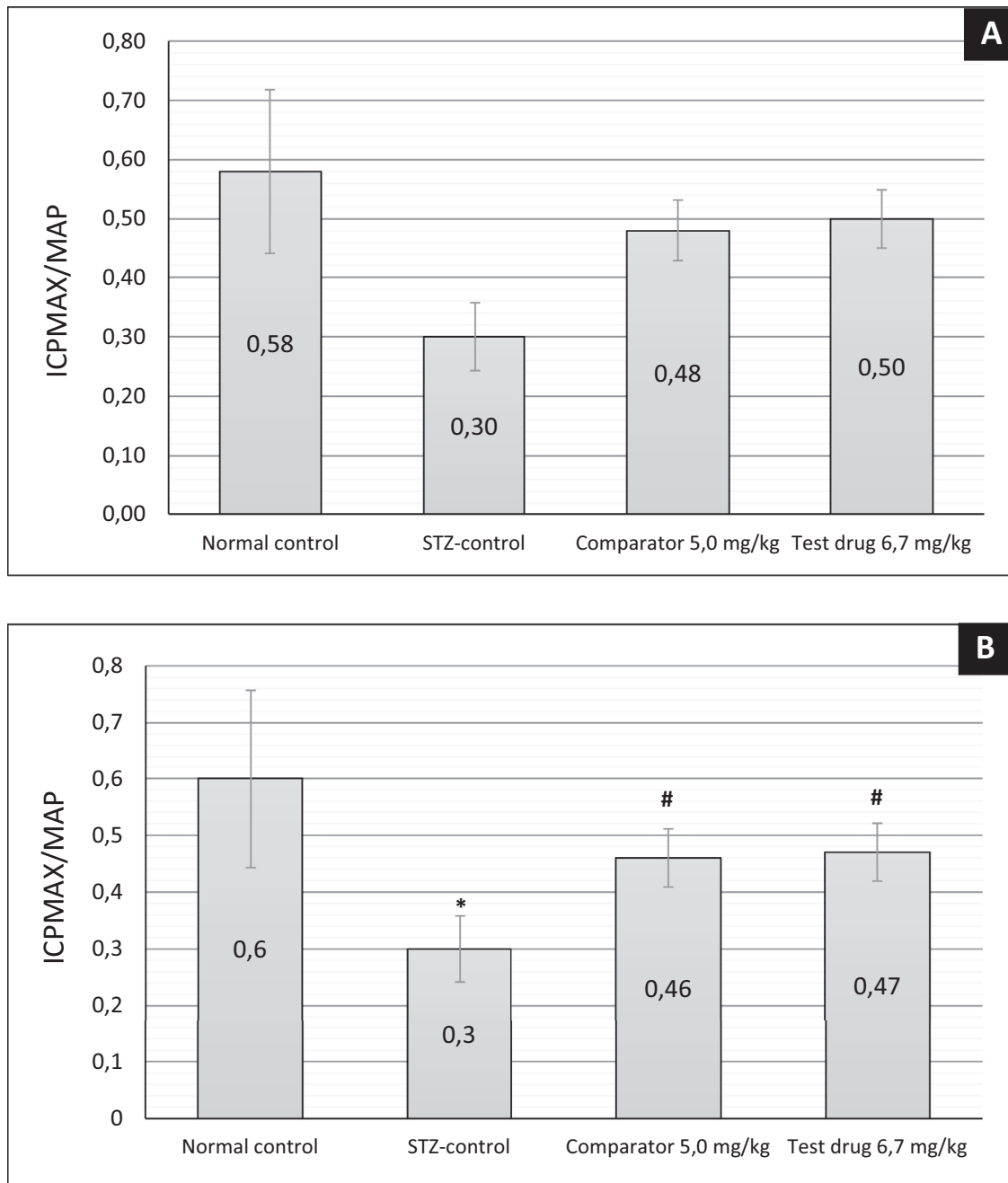
Figure 2. Changes in ICP max/MAP after 14-day administration following electrical stimulation of 10 V (A) and 19 V (B), respectively. Mean and standard deviation, n = 12. * $P < .05$ compared to normal control. # $P < .05$ compared to STZ-control. STZ = streptozotocin.

Table 5. ICPmax, MAP and ICPmax/MAP in the normal and STZ-diabetic rats after 14-day administration following 10 V and 19 V electrical stimulation, Me (Q1; Q3)

Group	10 V			19 V		
	ICPmax/MAP	ICPmax	MAP	ICPmax/MAP	ICPmax	MAP
Normal control (n = 12)	0..59 (0.44; 0.17)	75.1 (49.8; 79.1)	120.5 (108.5; 128.2)	0.64 (0.47; 0.70)	68.2 (49.4; 87.9)	116.4 (108.7; 122.6)
STZ-control (n = 12)	0.32 (0.26; 0.34)*	34.0 (28.6; 35.9)*	103.6 (96.2; 111.8)	0.32 (0.26; 0.34)*	34.2 (31.9; 35.0)*	105.0 (102.9; 112.6)
Comparator 5.0 mg/kg (n = 12)	0.47 (0.46; 0.50)†	52.2 (48.2; 56.7)†	109.1 (102.5; 125.0)	0.46 (0.43; 0.48)†	49.7 (47.8; 55.1)†	113.5 (105.3; 124.5)
Test drug 6.7 mg/kg (n = 12)	0.51 (0.47; 0.55)†	58.1 (48.1; 63.2)†	110.7 (101.4; 120.1)	0.47 (0.44; 0.51)†	50.8 (47.1; 54.7)†	106.9 (105.7; 111.3)

*$P < .05$ compared to normal control.
†$P < .05$ compared to STZ-control.

Despite the confirmed diabetes mellitus, a significant decrease in hyperglycemia was observed in the STZ-control group following 5-day administration of the control substance (1% starch solution) ($P < .05$). Similar changes were noted after administration of ipidacrine at a dose of 6.7 mg/kg. At a lower dose of the drug, the decrease was less pronounced ($P > .05$). In contrast, a slight increase in blood glucose levels ($P > .05$) was recorded in the intact control group.

ICP and MAP

The results of comparing ICPmax / MAP after administration of ipidacrine at a dose of 6.7 mg/kg or sildenafil at a dose of 5 mg/kg for 14 days are shown in Figure 2, and the values of ICPmax, MAP and ICP / MAP ratio are shown in Table 5.

STZ-induced diabetes mellitus was accompanied by the development of erectile dysfunction, manifested by a decrease in intracavernous pressure on the background of electrical stimulation. The decrease in ICPmax and ICPmax/MAP was observed in the STZ-group after 14-day administration of the control substance (1% starch). A statistically significant increase in ICPmax following the cavernous nerve stimulation of 10V and 19V was recorded both after administration of ipidacrine at a dose of 6.7 mg/kg and after administration of the comparator. The increase in the ICP max/MAP ratio is mainly attributed to the stimulating effect of drugs on intracavernous pressure since no increase in MAP was observed.

The results of testing the hypothesis of noninferiority are presented in Table 6.

The obtained results allowed to accept the alternative hypothesis H1 about the non-inferiority of ipidacrine (14 days of administration of 6.7 mg / kg / day) compared with sildenafil (14 days of administration of 5 mg / kg / day) in rats with STZ-induced DMED.

Along with the proof of the main hypothesis, it was studied the effect of ipidacrine on ED when administered in doses 3.6 mg/kg и 6.7 mg/kg for 5 and 14 days. Results of ICPmax/MAP during electrostimulation of the cavernous nerve with 10 V and 19 V voltage comparison are shown on Figure 3.

It was estimated that 5-days administration of ipidacrine does not induce statistically significant evolve of ICPmax/MAP in comparison with STZ-control group in none of the studied dosages. Fourteen-days administration of ipidacrine leads to statistically significant evolving of ICPmax/MAP in comparison with STZ-control group only in high dose − 6.7 mg/kg/day.

Testosterone Levels

The testosterone levels in blood of experimental animals are shown in Figure 4.

A sharp increase in the blood testosterone level in the normal control group was observed after 5 days and 14 days. This could be related to the assessment of sexual behavior performed before blood sampling. Following development of experimental diabetes mellitus, there was a statistically significant decrease in blood testosterone levels in male rats, which is in line with literature data.[35,46] The administration of either test drug or the comparator did not have a pronounced effect on the blood level of testosterone.

Table 6. Testing the hypothesis of noninferiority: comparison of the 95% confidence interval of the difference between the sample mean values of ICPmax/MAP with the noninferiority margin (δ)

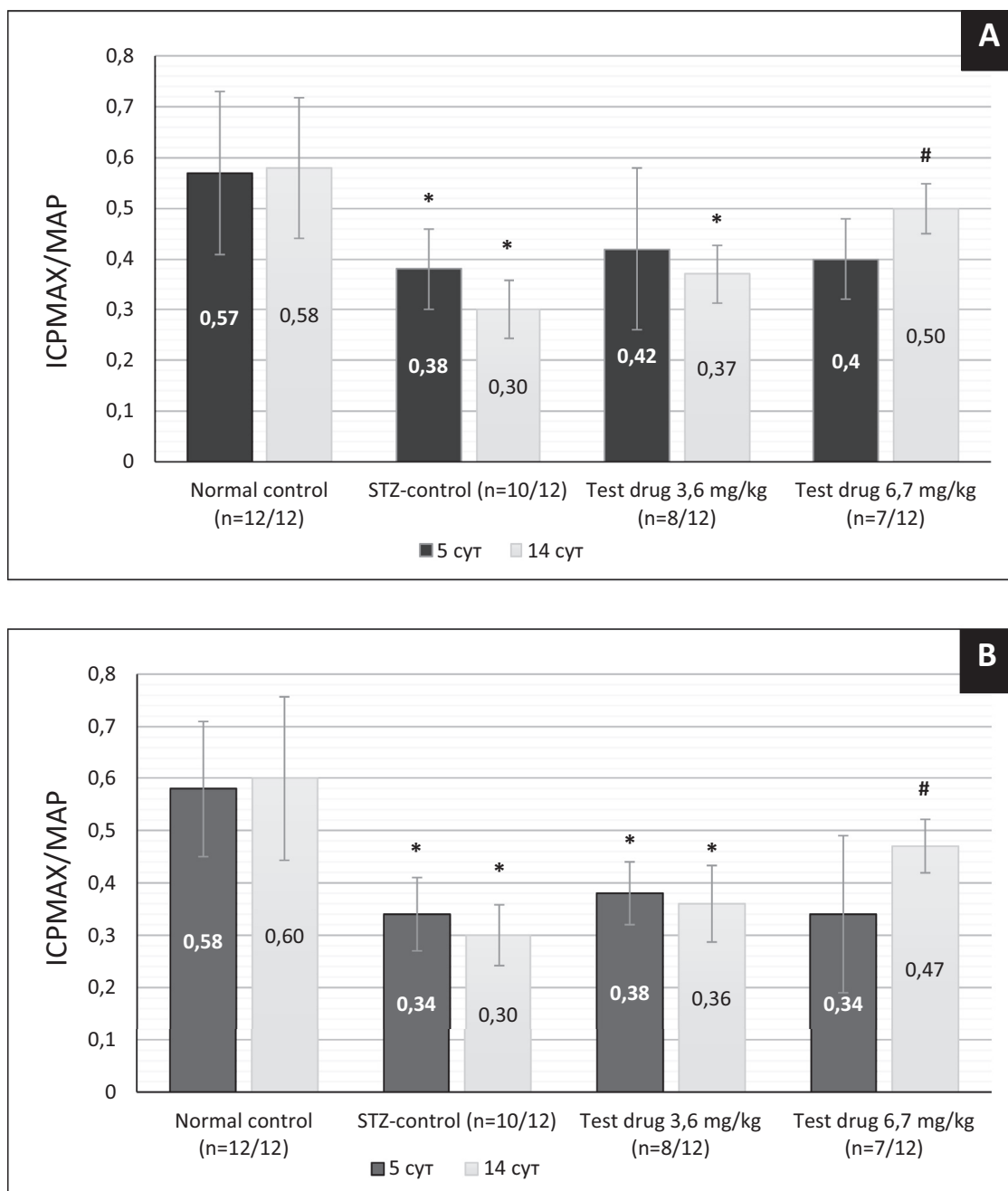| Group	Mean sample value difference ICPmax/MAP ($\tilde{x}_T - \tilde{x}_C$)	95% 2-way confidence interval of ICPmax/MAP difference	δ	Result
10 V	0.021	-0.022; 0.063	0.1	-0.022>-0.1 => H1
19 V	0.002	-0.041; 0.045		-0.022>-0.1 => H1

Figure 3. Changes in ICP max/MAP after 5 and 14-day administration following electrical stimulation of 10 V (A) and 19 V (B), respectively. Mean and standard deviation. The number of animals in groups after 5 days /14 days is indicated in parentheses * $P < .05$ compared to normal control. #$P < .05$ compared to STZ-control. STZ = streptozotocin.

Red Cell Cholinesterase and Plasma Cholinesterase Activities

The measured ChE activities in blood plasma of male rats are presented in Table 7.

Following 5-day administration of ipidacrine at doses of 3.6 mg/kg and 6.7 mg/kg, there was a marked decrease in ChE activity ($P < .05$). Following 14-day administration, Ipidacrine at doses of 3.6 and 6.7 mg/kg and the comparator showed a marked increase in ChE activity compared to baseline values ($P < .05$). ChE activities in the intact control and STZ-control groups did not significantly change after administration of the control substance (1% starch solution) for 14 days ($P > .05$).

AChE activities in red blood cells of male rats are presented in Table 8.

A significant decrease in AChE activity was observed following 5-day administration of Ipidacrine at a dose of 3.6 mg/kg ($P < .05$). In contrast, following the prolonged course of Ipidacrine
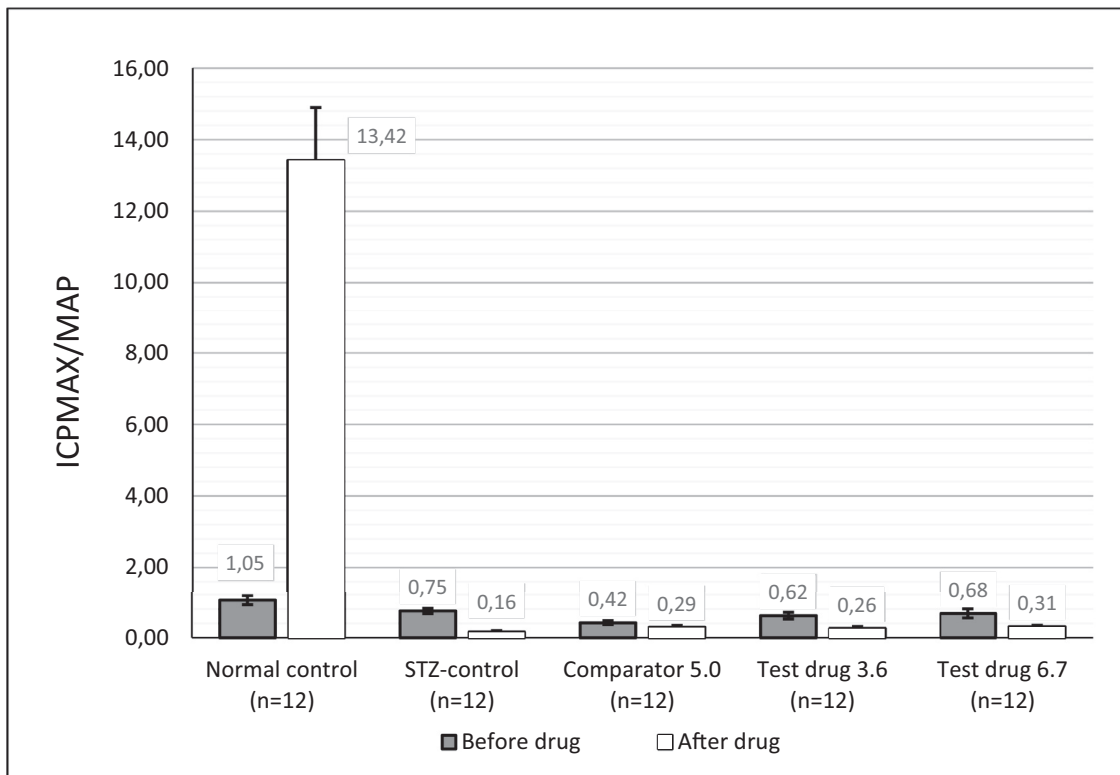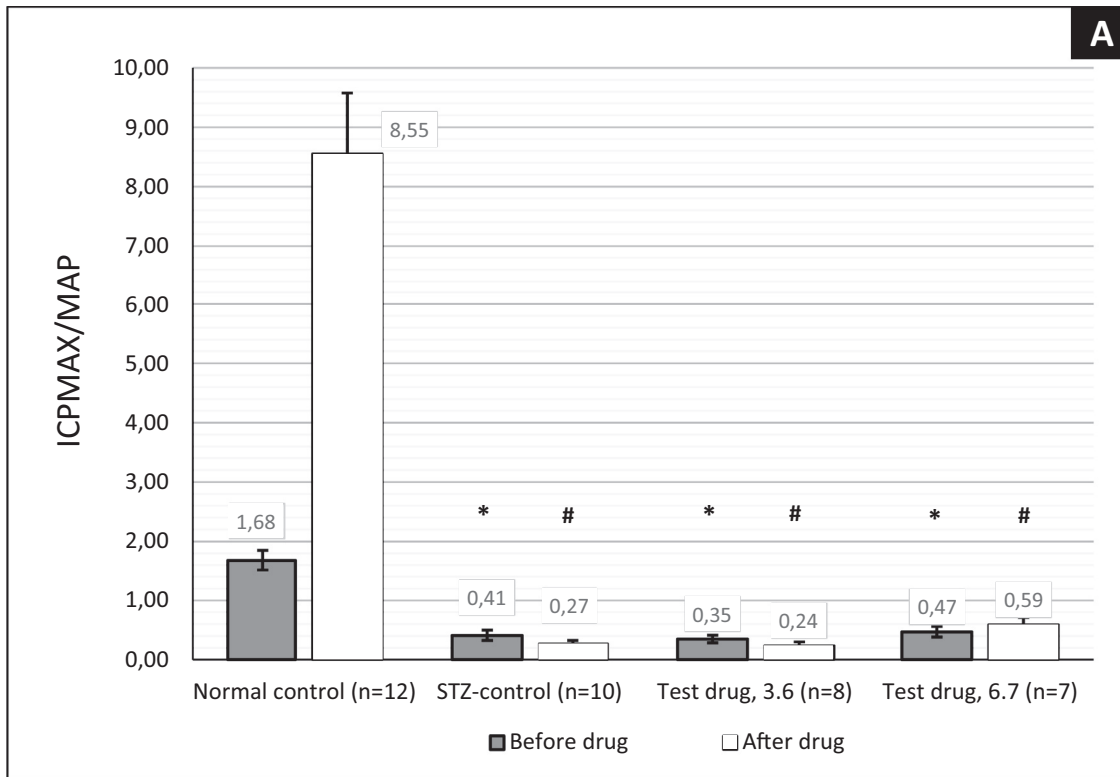
Figure 4. Testosterone levels in blood of animals before, 5 days (*A*) and 14 days (B) and 14 days after the start of the dosing. * *P* < .05 compared to normal control before dosing. #*P* < .05 compared to normal control after dosing.

Table 7. AChE activities in red blood cells of male rats, M ± SD

	ChE activity, U/L	
Group	Before dosing	After dosing
5-d administration of the drug or control substance		
Normal control	634.0 (537.5;714.0)	516.5 (168.0;701.0)
STZ-control	647.0 (572.0;766.0)	714.0 (533.0;789.0)
Test drug (3.6 mg/kg)	657.0 (526.0;766.5)	149.5 (71.0;456.5)* †
Test drug (6.7 mg/kg)	556.0 (148.0;937.0)	161.0 (111.0;538.0)*
14-d administration of the drug or control substance		
Normal control	442.5 (327.5;499.5)	651.0 (456.0;734.0)
STZ-control	831.0 (650.0;923.0)	559.0 (131.0;675.0)
Comparator (5.0 mg/kg)	507.0 (430.0;626.0)	778.0 (575.0;813.0)*
Test drug (3.6 mg/kg)	168.0 (128.5;586.5)	713.0 (444.0;920.0)*
Test drug (6.7 mg/kg)	387.0 (117.0;451.5)	641.5 (621.0;823.0)*

*$P < .05$ compared to baseline.
†$P < .05$ compared to STZ-control.
ChE = cholinesterase; STZ = streptozotocin.

Table 8. AChE activities in red blood cells of male rats, M ± SD

	AChE activity, mM × min⁻¹ × L⁻¹	
Group	Before dosing	After dosing
5-d administration of the drug or control substance		
Normal control	0.64 ± 0.102	0.67 ± 0.155
STZ-control	0.46 ± 0.093	0.56 ± 0.106
Test drug (3.6 mg/kg)	0.82 ± 0.062	0.60 ± 0.065*
Test drug (6.7 mg/kg)	0.55 ± 0.158	0.57 ± 0.061
14-d administration of the drug or control substance		
Normal control	0.62 ± 0.147	0.66 ± 0.206
STZ-control	0.62 ± 0.210	0.60 ± 0.214
Comparator (5.0 mg/kg)	0.52 ± 0.037	0.51 ± 0.026
Test drug (3.6 mg/kg)	0.41 ± 0.129	0.69 ± 0.156*
Test drug (6.7 mg/kg)	0.55 ± 0.173	0.56 ± 0.245

*$P < .05$ compared to baseline, using the paired t-test.
STZ = streptozotocin; AChE = acetylcholinesterase.

administration for 14 days, the activity of AChE significantly increased. In other groups, no significant changes in AChE activity were recorded.

Mating Behavior Tests

The results of Mating behavior tests in male rats are presented in Table 9.

Thus, a significant decrease in sexual behavior of male rats with erectile dysfunction was revealed. Ipidacrine had no pronounced effect on sexual behavior of male rats with erectile dysfunction associated with STZ-induced diabetes mellitus. Following 14-day administration of the comparator, a statistically significant decrease in the latent period of intromission and number thereof was observed in male rats compared to the STZ-control group.

DISCUSSION

At present PDE5 inhibitors are considered to be a main class of agents for management of ED of various origins.[47] Their efficacy is 74−89% on average but does not exceed 56−62% in diabetic subjects.[37,48] Therefore, the search for treatment options and agents for management of diabetic ED with other mechanisms of action is ongoing. However, no reports on use of agents activating the cholinergic system (cholinergic agonists,

Table 9. Mating behavior tests results (Me (Q1; Q3))

Group	Mount latency, sec	Intromission latency, sec	Ejaculation latency, sec	Mount frequency	Intromission frequency	Post ejaculation interval, sec
Normal control 5 d	80 (43; 98)	63 (0; 124)	0 (0; 285)	10 (5; 17)	5 (0; 9)	0 (0; 0)
Normal control 14 d	93 (29; 410)	95 (0; 288)	0 (0; 340)	7 (2; 15)	3 (0; 8)	0 (0; 0)
STZ-control 5 d	0 (0; 286)	0 (0; 0)*	0 (0; 0)*	0 (0; 1)*	0 (0; 0)*	0 (0; 0)
STZ-control 14 d	0 (0; 0)*	0 (0; 0)*	0 (0; 0)*	0 (0; 0)*	0 (0; 0)*	0 (0; 0)
Test drug, 3.6 mg/kg 5 d	40 (0; 150)	0 (0; 104)	0 (0; 21)	3 (0; 4)*	0 (0; 2)	0 (0; 0)
Test drug, 3.6 mg/kg 14 d	0 (0; 145)	0 (0; 0)	0 (0; 0)	0 (0; 1)*	0 (0; 0)	0 (0; 0)
Test drug, 6.7 mg/kg 5 d	0 (0; 88)	0 (0; 0)	0 (0; 0)	0 (0; 0)*	0 (0; 0)*	0 (0; 0)
Test drug, 6.7 mg/kg 14 d	0 (0; 94)*	0 (0; 0)*	0 (0; 0)	0 (0; 2)*	0 (0; 0)*	0 (0; 0)

*$P < .05$ compared to normal control.
STZ = streptozotocin.

cholinesterase inhibitors) in treatment of diabetes mellitus-associated ED could be found. This may be due to negative outcomes obtained following intracavernous administration of acetylcholine and neostigmine in healthy subjects[16] or potential side effects associated with inhibition of cholinesterase.

The mechanism of action of typical cholinesterase inhibitors (tacrine, neostigmine) comprises accumulation of acetylcholine in the synaptic cleft and enhancement of its stimulating action on a postsynaptic cell. In contrast to these agents, ipidacrine affects all components involved in a nerve impulse conduction. It stimulates a presynaptic nerve fiber, increases a release of the neurotransmitter in the synaptic cleft and decreases a breakdown of the transmitter.[49] This agent has a satisfactory safety profile since adverse effects related to anticholinesterase action were observed in the experiment at high doses exceeding 10 mg/kg.[22]

This is the first study to show that administration of ipidacrine, the reversible cholinesterase inhibitor, improved erectile function in diabetic rats and these results may be beneficial in further studies using ipidacrine for treatment of DMED, particularly in non-responders to PDE5 inhibitors. The study demonstrated that a 14-day administration of ipidacrine (Axamon) has a stimulating effect in ED associated with experimental diabetes mellitus induced by STZ. In particular, it selectively increased ICPmax with minimal impact on MAP resulting in increased ICPmax/MAP ratio. Lack of increase in MAP suggests a low risk of systemic side effects, such as arterial hypertension, following a course treatment with ipidacrine in diabetes mellitus.

Effect of ipidacrine, administered for 14 days in dose of 6.7 mg/kg/day had non-inferiority than effect of reference drug sildenafil in dose of 5 mg/kg/day for 14 days. For both agents, the effect on DMED is not related to stimulation of testosterone secretion since no changes in testosterone blood levels were observed. Both drugs do not stimulate sexual behavior in male rats, the patterns of which are significantly decreased in STZ-induced diabetes mellitus. This may be due to the peculiarities in development of the pharmacological effects of sildenafil and ipidacrine, which are ultimately associated with an increase in blood flow in the vessels of the penis. The point of application of the pharmacological effects of sildenafil is the endothelium of the vessels and cavernous bodies of the penis, and the point of application of the effects of ipidacrine is the peripheral nerves, ganglia and cholinergic synapses. At the same time, both drugs do not have a stimulating effect on the formation of testosterone, and also do not have pronounced central effects associated with the influence on sexual behavior. Perhaps this is due to the relative severity of DMED in the experimental model used.

Results concerning the effects of ipidacrine on ChE and AChE activities are of a certain interest. Although this is a reversible cholinesterase inhibitor, no dose-related inhibition of the enzyme was noted in the experimental diabetes mellitus. At lower dose, 3.6 mg/kg, following a 5-day administration this agent exerts cholinesterase inhibitory activity for both ChE and AChE. However,

with the prolonged course of treatment the effect reverses and administration of the agent is associated with significant increase in both ChE and AChE activities. At higher dose, 6.7 mg/kg, ipidacrine had a similar effect on ChE. Meanwhile, AChE activity virtually remained unchanged and was not related to the treatment course duration. It can be assumed that the effect of ipidacrine on the activity of various forms of cholinesterase in the blood is not related to its effects on cholinesterase of peripheral nerve fibers and ganglia, and does not determine its effects on blood flow in the penis and indicators of intracavernous pressure. A similar relationship may be revealed during the determination of the activity of cholinesterase in the nerve fibers innervating the penis. Similar studies are planned, and their results will be presented in the future.

The conducted studies suggest that ipidacrine may be considered as an effective drug in ED associated with diabetes mellitus. The obtained results may be confirmed in further studies with ipidacrine being used to treat diabetic ED, particularly in non-responders to PDE5 inhibitors.

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Conflicts of Interest: None.

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