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Effect of inhibiting carnitine biosynthesis on male rat sexual performance

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ABSTRACT

L-carnitine has a documented role as a cofactor in cellular energy metabolism and fatty acid β-oxidation pathways and it has also been considered to function in reproductive biology. We investigated whether decreasing concentrations of L-carnitine using an inhibitor of its biosynthesis, mildronate (3-(2,2,2-trimethylhydrazinium)-propionate), would influence the sexual behavior or sperm quality in male rats. Mildronate treatment induced a significant decrease in carnitine concentration and an increase in γ-butyrobetaine (GBB) concentration in both plasma and testes extracts. However, the expression of carnitine palmitoyltransferase I in testes and testosterone concentration in plasma was not changed in mildronate treated rat. Behavioral experiments demonstrated that mildronate treatment did not decrease the sexual motivation in both sexually *naive* and sexually experienced rats. The densities of spermatozoa in the *cauda epididymis*, as well as motility, were unchanged after mildronate treatment at a dose of 100 mg/kg. In conclusion, our study provides experimental evidence that mildronate induces decrease in the free carnitine concentration in rat testes, but does not decrease the sexual activity or sperm quality of male rats.
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L-carnitine (3-hydroxy-4-*N*-trimethylaminobutyrate) is a conditionally essential amino acid which plays an important role as a cofactor in cellular energy production in the mitochondrial matrix. L-carnitine aids in the transport of activated acyl-groups over the mitochondrial inner membrane, and it is needed for oxidation of long-chain fatty acids in mitochondria of all cells [1]. The rate of fatty acid oxidation in mitochondria is further regulated by availability of fatty acids, oxygen, and the activity of carnitine palmitoyltransferase I (CPT I), which is regulated by a variety of factors [1].

In mammals, complex homeostatic mechanisms maintain the circulating L-carnitine concentration in a physiological range. L-carnitine is absorbed from dietary products and biosynthesized from lysine and methionine; its excretion is efficiently regulated by renal reabsorbtion [2]. In addition to the recognized role of L-carnitine in the functioning of muscle cells and in energy metabolism, L-carnitine has been implicated in reproductive biology. For example, Marson et al. [3] have shown that the total amount of L-carnitine in seminal fluid increases during maturation of the male chimpanzee genital tract. Among the enzymes that catalyze the subsequent steps in carnitine biosynthesis, γ-butyrobetaine (GBB) hydroxylase (E.C. 2.1.2.1.) is expressed not only in liver and kidney, but also in the testis [4,5]. The activity of rat testicular GBB hydroxylase increases with sexual maturity, and it was found to be present in the seminiferous tubules [6]. Therefore, it was suggested that carnitine production could be associated with the maturation process of spermatogenesis [6]. Interestingly, L-carnitine is highly concentrated in the epididymal lumen where it is taken up by spermatozoa [7]. Therefore, the role of L-carnitine-dependent pathways in the functional capacity and motility of spermatozoa has been of particular interest [8,9].

Because of some anti-apoptotic and antioxidant-like effects in addition to promising effects in sperm motility studies, L-carnitine is used as a nutritional supplement in dietary therapy for male infertility [10,11]. It was suggested recently that the concentration of free L-carnitine in human seminal plasma correlated with semen quality [12]. In a clinical study, propionyl-L-carnitine was shown to be effective for treatment of symptoms associated with male aging [13]. The oral administration of L-carnitine might improve sperm quality at least in patients with idiopathic asthenozoospermia [14]. Although several experimental and clinical studies have been undertaken to determine the effects of L-carnitine and a related compound administered for the treatment of erectile dysfunction and infertility, results have not been conclusive and effects on motility did not correlate with clinically relevant endpoints, such as actual pregnancies [9,15].

Abbreviations: GBB, γ-butyrobetaine; CPT I, carnitine palmitoyltransferase I; OCTN, organic cation-carnitine transporter.

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The effects of L-carnitine administration on male sexual performance might be masked by its highly regulated uptake mechanisms and homeostasis, which preserves the carnitine concentration *in vivo*. Thus, in several studies, it was found that L-carnitine levels in semen did not change despite the administration of a 2 g daily dose [16,17]. The current experimental study was designed with these results in mind; we investigated whether the reduction of L-carnitine concentration by mildronate (3-(2,2,2-trimethylhydrazinium)-propionate dihydrate) influenced the sperm quality of adult male rats. The testing of sexual activity was performed because in addition to carnitine lowering effect mildronate treatment is known to induce an increase in GBB concentration [18]. This effect itself might be important for sexual performance as it was previously reported that the pharmaceutical composition comprising GBB can be used for stimulating sexual activity and potency [19]. Since it was shown earlier that the accumulation of carnitine in epididymis might be androgen-dependent [20], but mildronate was used in ageing men with clinical features of androgen deficiency to retard prostate growth by androgen administration [21], the concentration of testosterone was also tested.

It has been shown in several experimental studies that the administration of mildronate significantly reduces the carnitine contents in body tissues because mildronate is both an inhibitor of GBB hydroxylase [22] and an inhibitor of carnitine re-absorption in kidneys [23]. The carnitine lowering effects of mildronate administration have been detected in rat blood plasma, heart, brain, and liver tissues [18,24]. Carnitine concentration within the male reproductive organs is regulated by organic cation-carnitine transporters such as OCTN1 and OCTN2 [25]. OCTN2 is also expressed in cardiac cells, and mildronate is known to inhibit OCTN2 in a competitive manner [26]. To date, no studies have investigated the influence of mildronate on carnitine transport proteins and carnitine and GBB contents within the male genital tract.

In this study, we used mildronate as a carnitine lowering and GBB increasing agent and subsequently tested both sexually unexperienced and experienced male rat sexual performance, sperm quality measures and testosterone concentration. In addition, the concentration of carnitine and GBB was measured in male rat plasma and testis homogenates. To investigate possible effects of mildronate administration on testicular cell energy metabolism pathways, we examined the expression of CPT 1, which is the rate limiting enzyme in fatty acid oxidation [1].

1. Methods

1.1. Subjects

The subjects were experimentally naive male and female Wistar rats weighing 230–250 g at the beginning of the experiments. Animals for *Experiment 1* were obtained from Riga Stradins University (Riga, Latvia); animals for *Experiment 2* were obtained from the breeding centre "Rappolovo" (St. Petersburg, Russia). Animals were housed in groups of five, with males and females housed separately (in different rooms), in standard T4 Plexiglas cages, maintained under standard laboratory conditions (21 ± 1 °C; relative humidity 60–70%). All rats had *ad libitum* access to standard rat lab chow LABFOR (Lactamin AB, Sweden) or recipe ПК 120-1 ("Laboratorsnab", Moscow, Russia) and filtered tap water throughout the experiments. Rats were kept under a 12/12 h reversed light/dark cycle.

All experimental procedures were carried out in accordance with guidelines of the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by Ethics Council of Animal Protection at the Veterinary and Food Service, Riga, Latvia and Ethics Committee of Pavlov Medical University, St. Petersburg, Russia, respectively.

1.2. Sexual activity of inexperienced male rats (Experiment 1)

Before the start of vehicle/mildronate administration, the male rats were randomly assigned to one of the following groups: vehicle treated control group (VEH, $n = 10$) and three groups of rats treated with mildronate (daily for 14 days) in doses of 30 mg/kg (M30, $n = 10$), 100 mg/kg (M100, $n = 10$) and 300 mg/kg (M300, $n = 10$) perorally (p.o.). A sexual behavior test was performed 24 h after the last administration of vehicle/mildronate (Fig. 1). After the test session, the rats were decapitated. Blood was collected into EDTA-containing vacutainers, centrifuged to obtain just plasma, and then frozen (−80 °C) until analysed. Organs of rats were removed and used for evaluation of sex organs and sperm quality. One testis of each rat was used for tissue extract preparation.

1.3. Recovery of sexual activity in sexually experienced male rats (Experiment 2)

After gathering sexual experience data during the first test session (Fig. 1), male rats were divided into three experimental groups based on the results of the first "sexual satiation" test to balance the baseline level of sexual activity (number of copulatory series in a completed cycle of sexual behavior) between treatment groups. Immediately after the completion of the first test procedure, rats received either the vehicle or mildronate (Fig. 1). The second and third sexual satiation tests were performed 24 h after the 10th and 14th vehicle/mildronate administration, respectively. It was observed earlier that after the copulation test employing a criterion of satiety, male rats of the same strain and from the same breeder need at least 9 days for their sexual activity to be fully restored (Belozertseva, unpublished observations). Therefore, only 4 days long interval between the second and third tests (Fig. 1) was used to test the effect of treatment on recovery of sexual activity. The following experimental groups were used: vehicle (VEH, $n = 12$), mildronate 30 mg/kg (M30, $n = 11$), and mildronate 300 mg/kg (M300, $n = 10$), p.o.

1.4. Sexual satiation test

Behavioral tests were conducted during the dark phase of the day/night cycle under dim, red illumination. Single male rats were placed in a standard rectangular Plexiglas observation cage and allowed to acclimate to the test chamber for 30 min. The test was initiated with the introduction of a sexually receptive stimulus female. Females were brought into sexual receptivity by a sequential treatment with estradiol benzoate (12 μg/rat; 48 h before mating) and progesterone (500 μg/rat; 4–5 h before mating). Hormones were dissolved in olive oil and injected subcutaneously (s.c.) in a volume of 0.1 ml/rat.

DAY	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Experiment 1															♂+♀ (1)
Experiment 2	♂+♀ (1)										♂+♀ (2)				♂+♀ (3)
TREATMENT	Vehicle/Mildronate daily oral administration														

Fig. 1. Design of sexual behavior experiments.

The following parameters were recorded or calculated according to the standard procedure as described by Agmo [27]:

(a) Mount latency (ML): time from the introduction of the female until the first mount.
(b) Intromission latency (IL): time from introduction of the female until the first intromission (vaginal penetration).
(c) Ejaculatory latency (EL): time from the first mount or intromission to ejaculation.
(d) Refractory period (RP) or Post-ejaculatory interval (PEI): time from ejaculation until the next intromission.
(e) Mount frequency (MF): the number of mounts preceding ejaculation.
(f) Intromission frequency (IF): the number of intromissions preceding ejaculation.
(g) Interintromission interval (III): average interval between successive intromissions (calculated as ejaculation latency divided by intromission frequency).
(h) Copulatory efficiency (CE): a measure of intromissive success (calculated as intromission frequency divided by mount frequency plus intromission frequency).

The tests ended when the male rats met one of the following criteria: (1) no intromissions for 30 min after the receptive female was introduced, (2) no sexual activity for 30 min after the last ejaculation; or (3) no ejaculation for 60 min after the copulatory series had begun.

1.5. Body and organ weights

Body weight was monitored throughout the experiment. After scarifying the testicles and accessory sex organs, the viz. *cauda epididymis*, seminal vesicles and prostate were dissected out and the attached tissues were trimmed off. The organs were then washed in saline (0.9% NaCl; w/v) and weighed. The relative weight of organs (average weight for paired organs) was expressed as g/100 g of body weight. One testis of each rat was used for preparation of tissue extract for the determination of mildronate, carnitine and GBB concentrations.

1.6. Epididymal sperm and motility analysis

A sample of epididymal fluid was collected using a pipette tip, diluted with isotonic saline, and immediately examined [28]. The sample was placed on a pre-warmed hemocytometer. The density and agglutination rates were determined by random counting of spermatozoa in 25 fields on each side of the hemocytometer by light microscopy, with a magnification of 10×16. The number of i) mobile spermatozoa; ii) vibrant spermatozoa, moving without migration; and iii) immobile spermatozoa were counted and the results were expressed as a percentage of the total count.

1.7. Determination of carnitine, GBB and mildronate by UPLC/MS/MS

Blood was collected into ethylenediaminetetraacetic acid (EDTA) containing vacutainers, centrifuged and the obtained plasma was stored frozen (−80 °C) until analysed. Testes were excised and homogenized at 1500 rpm for 1 min with a glass-Teflon homogenizer in ice-cold distilled water at w/v ratio 1:5. The obtained homogenate was spun at 14,000 g for 10 min at 4 °C. The supernatant was then decanted, but the pellet was homogenized in the same volume of distilled water as before. The obtained homogenate was spun at 14,000 g for 10 min at 4 °C. The supernatants were combined and stored frozen (−80 °C) until analysed.

Determination of carnitine, gamma-butyrobetaine and mildronate concentration in testis tissue samples was performed by ultra performance liquid chromatography–tandem mass spectrometry (UPLC/MS/MS) method in positive ion electrospray mode. Sample preparation was performed by deproteinization with a methanol/acetonitrile mixture. Internal standard — 3-(2,2-dimethyl-2-prop-1-yl-hydrazinium)propionate was applied for calibration. Thus, 50–200 μl of plasma sample was mixed with 1.0 ml of internal standard solution in acetonitril-methanol (3:1, v/v), vortexed and centrifuged at 10,000 ×g for 20 min. The supernatant was transferred into UPLC vial and used for UPLC/MS/MS analysis. UPLC was carried out using Waters Acquity UPLC system. Waters Acquity HILIC BEH 1.7 μm 2.1×100 mm column was used and volume of injection was 5 μl. Chromatographic separation was performed in 10 mM ammonium acetate (pH4) and acetonitrile gradient at a flow rate of 0.2 ml/min. MS/MS analysis was performed on a Micromass Quatro MicroTM tandem mass spectrometer in positive ion electrospray mode using multiple reaction monitoring mode with the precursor-to-product ion transition m/z 162.4→m/z 103.0 for carnitine, m/z 146.3→m/z 87.9 for GBB; m/z 147.3→m/z 58.4 for mildronate and m/z 175.4→m/z 86.0 for internal standard. Cone voltage and collision energy values were optimized for each compound. MassLynx 4.1. software with QuanLynx 4.1. module (Waters) was used for data acquisition and processing.

1.8. Determination of testosterone concentration

Blood was collected into EDTA-containing vacutainers, centrifuged and the obtained plasma was stored frozen (−80 °C) until analysed. The plasma concentration of testosterone was measured by Testosterone EIA Kit (Cayman Chemical) following producers test protocol.

1.9. Western blot analysis

Testes were homogenized 1:5 (w/v) at +4 °C temperature in a buffer containing 10 mM Tris–HCl, pH 7.4, 1 mM EDTA, 0.25 M sucrose, 25 mM NaF and protease inhibitors, 10 μM leupeptin, 1 μM pepstatin, 1 μM aprotinin, and 50 μM phenylmethylsulfonyl fluoride by using a motor-driven Teflon/glass homogenizer. Homogenates were centrifuged at 1000 ×g for 10 min at +4 °C. Supernatant was decanted and used for SDS-PAGE separation. Proteins (50 μg for total extracts) were heated to 100 °C for 3 min in the 2× loading buffer (EC-886, National Diagnostics, Atlanta, GA) and separated on 10% SDS-PAGE gels for 1 h at 150 V. The electrophoresis running buffer contained 25 mM Tris base, 192 mM glycine, and 0.1% SDS. Proteins were transferred onto a PVDF membrane (Immobilon, Millipore) in blotting buffer (25 mM Tris base, 192 mM glycine, and 10% methanol (v/v)) for 20 min at 180 mA. Membranes were stained with Ponceau S to verify equal protein loading and transfer. Membranes were blocked with 5% BSA (Sigma) in PBS (137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, pH 7.4) for 1 h at room temperature and then incubated 1 h at room temperature with one of the following antibodies diluted in the blocking solution: rabbit monoclonal antibodies for mCPT I and α-tubulin (1 μg/ml, Millipore, Chemicon). After washing with PBS (3 times, 10 min each), the blots were incubated for 30 min at room temperature with peroxidase-coupled goat anti-rabbit IgG (1:15,000; Chemicon/Millipore) diluted in blocking solution and then washed again in PBS. The blots were developed using chemiluminescence reagents (Millipore). The same blot was stripped (30 min at 50 °C in 62.5 mM Tris base, 2% SDS, 100 mM β-mercaptoethanol) and re-stained with a monoclonal β-actin antibody (ABCAM) as an internal control. Quantification was performed by densitometric scanning.

1.10. Chemicals

Mildronate (3-(2,2,2-trimethylhydrazinium)propionate) was provided from JSC Grindeks, Latvia, and dissolved in distilled water. Estradiole benzoate, γ-butyrobetaine (GBB) and progesterone were from Sigma Chemical, St Louis, MO, USA. Methanol was purchased from Merck (Darmstadt, Germany). Acetone, acetic acid, chloroform, diethyl ether, potassium dihydrogen phosphate, potassium hydrogen orthophosphate and ammonium acetate were purchased from Acros Organics (Geel, Belgium) and used without further purification.

Table 1
Influence of mildronate treatment on naive male rat sexual behavior during the first copulatory series

Behavioral measures	Treatment (mg/kg)			
	Vehicle	Mildronate, 30	Mildronate, 100	Mildronate, 300
ML (s)	251 ± 173	392 ± 198	710 ± 259	273 ± 171
IL (s)	251 ± 173	397 ± 197	711 ± 259	281 ± 170
EL (s)	961 ± 125	993 ± 222	949 ± 248	1277 ± 299
PEI (s)	517 ± 51	445 ± 41	443 ± 20	459 ± 26
MF	1.8 ± 0.4	1.4 ± 0.5	1.9 ± 0.6	2.2 ± 0.7
IF	15.3 ± 1.8	16.1 ± 0.7	15.6 ± 2.4	17.9 ± 3.0
III (s)	70.2 ± 12.8	59.8 ± 11.1	57.1 ± 6.3	71.4 ± 15.1
CE	89.6 ± 2.4	92.1 ± 2.7	90.3 ± 1.9	89.8 ± 1.4

Specific sexual behavior measures of sexually active male rats treated with vehicle/mildronate (30, 100, 300 mg/kg). ML = mount latency, IL = intromission latency, EL = ejaculation latency, PEI = post-ejaculatory interval, MF = number of mounts, IF = number of intromissions, III = interintromission interval, CE = copulatory efficiency. Values are expressed as mean ± S.E.M.

1.11. Statistical analysis

Data are presented as the mean ± SEM. Between-group differences in sperm quality, sex organ weight and levels of hormones and L-carnitine were analysed with a two-tailed *t* test for independent samples. Behavioral data were analysed by a one-way analysis of variance (ANOVA) with a *post-hoc* Mann–Witney test in Experiment 1. Analysis of variance for repeated measures (ANOVAR) was used for estimation of interactions between the treatment group and test day on behavioral measurements in Experiment 2. The difference in individual time-points (test day) was analysed with a two-tailed *t* test for independent samples with a Bonferroni correction. To analyse mount and intromission latencies in Experiment 2, we employed a Kaplan–Meier analysis using Log-Rank statistics. P value <0.05 was considered significant.

2. Results

2.1. Effects of mildronate on the sexual behavior of inexperienced male rats

The number of ejaculations in series was 4.6 ± 0.7 for control group and mildronate administration did not induce significant changes. As can be seen in Table 1, mildronate treatment did not induce any

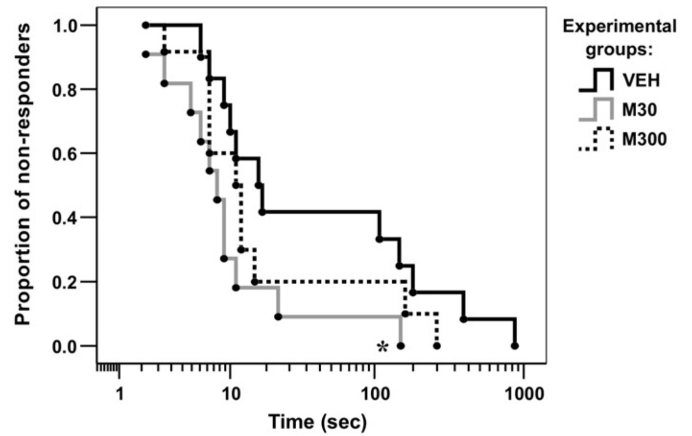

Fig. 3. Effect of mildronate administration for 14 days on the initiation of copulation (latency of intromission). The Kaplan–Meier analysis: Log-Rank = 5.99, $df=2$, $P=0.05$. * — significantly vs. VEH group by pairwise comparisons with Log-Rank test (Log-Rank = 4.81, $df=1$, $P=0.03$).

statistically significant and dose dependent effects also on other behavioral measures of sexual activity.

2.2. Effect of mildronate on the sexual activity in experienced male rats

The first measurement of sexual behavior was performed immediately before vehicle/mildronate treatment. There were no differences between experimental groups in the baseline sexual activity of male rats. The second measurement of copulation was performed after ten days of vehicle/mildronate treatment. As expected, based on the preliminary observations (data not published), an interval of 10 days between two consecutive sexual interaction tests was sufficient for the animals' sexual activity to be completely restored in VEH group. Fig. 2 (data points above '0') reveals no difference in the number of ejaculations in the first and second sexual satiation tests in the VEH group only. In mildronate treated male rats, the number of ejaculations during the second sexual interaction test (10 days of treatment) was reduced when compared to the first (baseline) sexual interaction test (significantly for M30 group). During

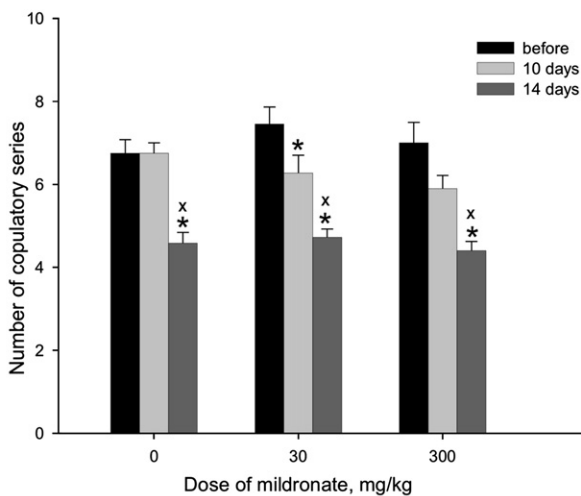

Fig. 2. Effect of mildronate on the number of ejaculations until sexual satiation. Significance was assessed using a Tukey–Kramer multiple comparisons test following separate repeated measures ANOVAs: * — vs. first test (before treatment); x — vs. second test (24 h after 10-th infusion). ANOVAs: VEH — $F(2,35)=16.9$, $P<0.01$; M30 — $F(2,32)=19.1$, $P<0.01$; M300 — $F(2,29)=15.5$, $P<0.01$.

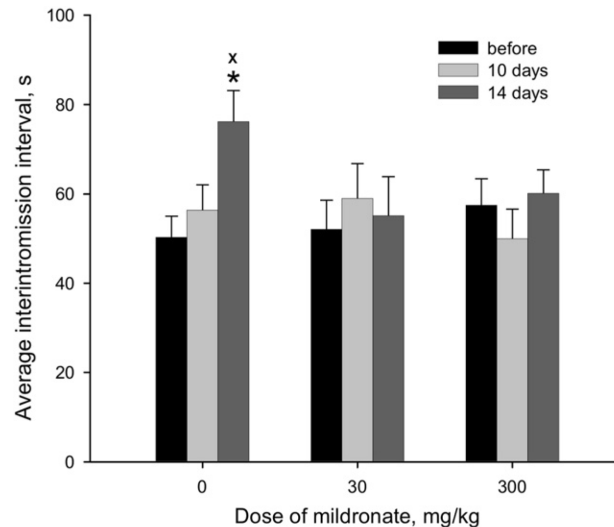

Fig. 4. Effect of mildronate on the average interintromission interval per copulatory cycle. Significance was assessed using a Tukey–Kramer test ($P<0.05$): * — compared to the baseline; x — compared to the second test. Separate repeated measures: VEH — $F(2,35)=6.439$, $P<0.01$; M30 — $F(2,32)=0.22$, $P=0.80$; M300 — $F(2,29)=0.09$, $P=0.36$.

Table 2
Influence of mildronate on experienced male rat sexual behavior during the first copulatory series in the third sexual satiation test

Behavioral measures	Treatment (mg/kg)		
	Vehicle	Mildronate, 30	Mildronate, 300
ML (s)	16.0±8.4	6.9±1.78	33.1±25.4
IL (s)	147.9±73.8	21.1±12.9	50.0±27.9
EL (s)	686±125	508±61	503±71
PEI (s)	480±25	503±46	511±27
MF	3.3±1.1	2.5±0.5	2.7±0.6
IF	9.0±0.82	9.0±1.1	7.7±0.42
CE	76.6±4.4	77.0±3.6	77.9±5.9

Specific sexual behavior measures of sexually active male rats treated with vehicle/mildronate (30, 100, 300 mg/kg). ML = mount latency, IL = intromission latency, EL = ejaculation latency, PEI = post-ejaculatory interval, MF = number of mounts, IF = number of intromissions, CE = copulatory efficiency. Values are expressed as mean±S.E.M.

the third sexual satiation test (after 14 days of treatment), in all experimental groups, the incidence of ejaculation was reduced in comparison to the previous (second) test and there were no differences between treatment groups. The Kaplan–Meier analysis of IL showed a significant difference between treatment groups after 14 days (Test 3) of vehicle/mildronate administration and males from the M30 group beginning intromission significantly faster than animals from VEH group (Fig. 3). The average number of intromissions per copulatory series (threshold of ejaculation) was not different in treatment groups of any test. The length of the average III per copulatory cycle, which reflects intensity of genital stimulation, was significantly different in treatment groups only during the last test ($F(2,30)=2.416$, $P=0.106$). The significant increase of this measure from test to test was indicated in the VEH group only (Fig. 4). Although additional sexual performance measures (PEI, ML, MF, EL and CE) were recorded, there were no significant treatment differences found in our statistical analysis (Table 2).

2.3. Effect of mildronate on reproductive organ weight and sperm quality

The average body weight of animals was not different between experimental groups. The effect of mildronate administration on sperm quality was tested using a dose of 100 mg/kg. As can be seen in Table 3, mildronate did not bring about any significant influence on male rat reproductive organ weight, with the exception of vesicular gland weight which was found to be slightly increased in mildronate treated male rats (i.e., 0.3±0.02 in control rats and 0.4±0.03 in M100 group animals).

The densities of spermatozoa in the *cauda epididymis*, as well as motility, also remained unchanged in the M100 treatment group (Table 4). As can be seen in Table 4, agglutination was observed in fewer samples of the mildronate treated group, but the effect was not statistically significant.

2.4. Effect of mildronate on carnitine, mildronate and GBB concentrations

Mildronate was administered (p.o., 30, 100 and 300 mg/kg/daily) to rats for 14 days and blood plasma and testes tissue homogenate samples were analysed by UPLC/MS/MS. Analysis of control and mildronate treated rat plasma samples is shown in Fig. 5. The average concentrations of free carnitine and GBB in saline treated animal plasma were 28±2 and 3±0.3 nmol/ml, respectively. As can be seen in Fig. 5B, the administration of mildronate resulted in a significant and dose dependent change in both carnitine and GBB concentrations. Thus, the lowest dose of 30 mg/kg of mildronate induced a statistically significant decrease in the plasma carnitine concentration. In contrast, the concentration of GBB was increased in the plasma of the mildronate treated group, and the effect was statistically significant at doses of 100 and 300 mg/kg (Fig. 5). The rat plasma mildronate concentration in the mildronate treated group was about 20 nmol/ml in all mildronate treatment groups, regardless of the dose used (Fig. 5B).

The analysis of rat testis tissue extracts revealed that, consistent with the concentration changes in plasma samples, the free carnitine and GBB concentrations in testis tissues also changed with mildronate treatment (Fig. 5A). As can be seen in Fig. 5A, the concentrations of free carnitine and GBB in the control group rat testes were 221±10 and 15±0.6 nmol/g, respectively. In the M100 group, the carnitine concentration was reduced by 36% and it reached almost a 3-fold decrease (to 76±2 nmol/g) in 300 mg/kg mildronate treatment group rat testes extracts (Fig. 5A). Alternatively, the GBB concentration increased in a dose dependent manner, up to 49±1 nmol/g (3-fold increase in comparison to control samples) in the M300 group rat testes (Fig. 5A). As presented in Fig. 5A, the mildronate concentration in rat testes extracts increased dose dependently and was 7.4±1; 58.6±7 and 142±10 nmol/g in M30, M100 and M300 groups, respectively.

Table 3
The effect of mildronate on genital gland weight (g)

Treatment group	Testes	Vesicular gland	Prostate	*Cauda epididymis*
Control	1.1±2.7	0.3±0.02	0.2±0.01	0.03±0.004
M100	1.1±3.2	0.4±0.03*	0.2±0.01	0.04±0.004

*$P<0.02$, statistically significant in relation to the control ($N=10$).

Table 4
The effect of mildronate on rat male spermatozoa (in the *cauda epididymis*)

Treatment group	Density of spermatozoa, %	Mobile spermatozoa, %	Immobile spermatozoa, %	Vibrant spermatozoa, %	Agglutination, incidence
Control	67.0±5.9	28.0±5.9	47.0±6.8	25.0±1.8	9/10
M(100)	54.8±7.2	24.0±5.0	53.0±6.3	23.0±2.0	5/10

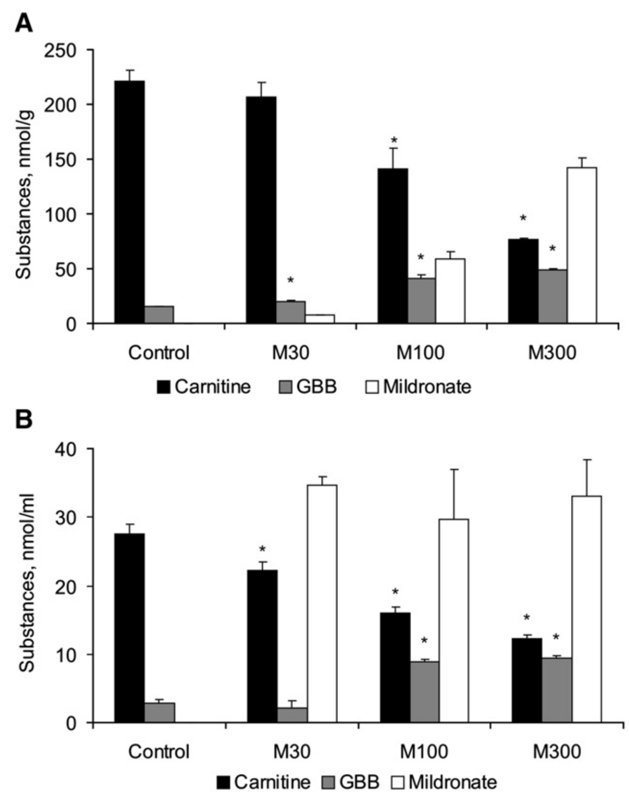

Fig. 5. Effect of mildronate administration on carnitine, GBB and mildronate contents in male rat testis extracts (A) and blood plasma (B). Vehicle or mildronate (30, 100, 300 mg/kg) were orally administered for 14 days. Values are expressed as mean±S.E.M. ($N=10$). *t* test, *$P<0.005$, statistically significant relative to control.

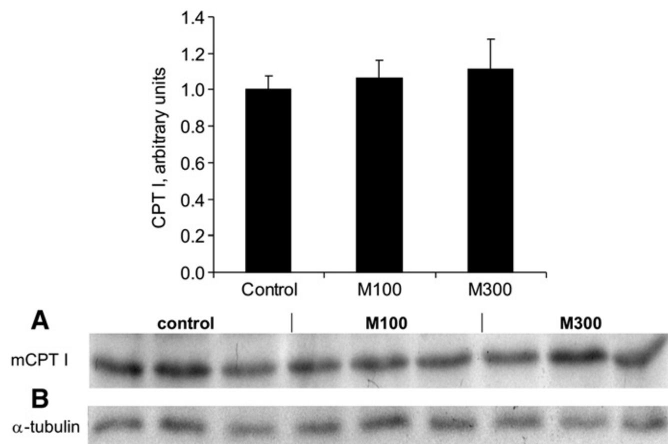

Fig. 6. Western blot analysis of mCPT I protein content (A) and α-tubulin (B) in the rat testis with or without administration of 100 and 300 mg/kg of mildronate. Data are the means ± S.E.M. and expressed as arbitrary units. Results are expressed relative to α-tubulin content under basal conditions (given an arbitrary value of 1). The bar graph is calculated from results of three control and six mildronate treated rats.

2.5. Effect of mildronate on plasma testosterone concentration

The testosterone concentration in control rat plasma collected 1 h after finishing behavioral tests was 1.9 ± 0.3 ng/ml. Administration of mildronate at all doses tested (p.o., 30, 100 and 300 mg/kg/daily) did not induce any significant changes in testosterone concentration (data not shown).

2.6. Effect of mildronate on CPT1 expression

The influence of lowered carnitine concentration on CPT1 protein expression in male rat testes was investigated after a 14 day administration of mildronate using daily doses of 100 and 300 mg/kg using Western blotting. As can be seen in Fig. 6, mildronate treatment did not influence the expression of CPT-1 in male rat testes tissues.

3. Discussion

In the present study, we applied mildronate as an inhibitor of carnitine biosynthesis and transport systems to determine the effects on sexual behavior and sperm quality in rats. Our study demonstrates that mildronate treatment significantly decreased carnitine contents in male rat plasma and testis, but it did not bring about changes in sexual performance in an applied experimental model of male rat sexual behavior. Only slight activation of a sexual response after mildronate treatment was observed in both sexually naive (Experiment 1) and experienced animals after a short period (4 days) of rest between successive second and third sexual satiation tests (Experiment 2), but the effects were not dose dependent. Thus, mildronate treatment at a dose of 30 mg/kg induced a reduction of the first refractory period in naive animals (Table 1). Faster initiation of copulation (decreased IL) and increase of copulatory speed (diminished III) were revealed in sexually experienced male rats (Figs. 3 and 4). In addition, measures of sperm quality were similar to that of control rats, as determined by examination of sperm samples from the *cauda epididymis* after mildronate treatment at the dose of 100 mg/kg (Table 4).

Similar to previously reported effects of mildronate administration on blood plasma carnitine concentrations, in this study we found that 30 mg/kg mildronate treatment significantly lowered the carnitine concentration in a statistically significant manner (Fig. 5B). To date, the effect of mildronate on carnitine concentrations had been measured in such tissues as heart and liver [29,18]. Previous studies showed that a significant drop in carnitine concentration in heart and liver tissues can be obtained after only a 10 day treatment with a dose of 50 mg/kg mildronate [29]. The results of this study provide evidence that mildronate treatment affects carnitine concentration in male rat testes, but the effect is significant only at a dose of 100 mg/kg (Fig. 5A). This effect might be due to the highly regulated carnitine uptake and transport systems, which counteract the mildronate effects in testes tissues. Interestingly, it has been previously reported that even though mildronate treatment at a high dose of 800 mg/kg decreased carnitine concentration in rat heart tissues, the total carnitine content of isolated mitochondria was higher in mildronate treated rat hearts than in control rat heart mitochondria [30]. Thus, in addition to the functional location of carnitine for fatty acid oxidation in cardiac mitochondria, the presence of carnitine in male rat testes might also be preserved by adaptive transport systems.

In the present study, we also measured an increased concentration of GBB both in mildronate treated rat plasma and testes (Fig. 5). This is in agreement with previously reported findings for the rat heart and plasma [31,18]. The concentration of GBB in mildronate treated rat testes, however, was just three-fold higher than that in control animal tissues. In comparison, in mildronate treated rat heart tissues, GBB was found to be up to 20-fold higher after a 10 day treatment of 100 mg/kg [31]. The lower expression of mildronate in the testes was unexpected because mildronate is a known inhibitor of GBB hydroxylase, which is present in the testes, but not in heart tissues [6]. Therefore in testes, mildronate would block the transformation of GBB and maintain its concentration at a higher level. The K_i of mildronate for GBB hydroxylase is in the micromolar range [22], suggesting that high concentrations of mildronate are needed to inhibit carnitine biosynthesis by GBB hydroxylase. Our study demonstrated that the concentration of mildronate in testes increased in a dose dependent manner and reached about 140 nmol/g in tissues with an administration dose of 300 mg/kg. It might be that such a concentration of mildronate is not sufficient to block the activity of GBB hydroxylase and thus, GBB is not accumulated, but is instead converted to carnitine under conditions of carnitine shortage.

The carnitine concentration in male reproductive organs is regulated by a set of carnitine transport proteins [25]. This is the first study to show that mildronate affects the carnitine concentration in rat testes. The inhibitory constants of mildronate for carnitine transporters in different tissues are not precisely determined, but our data suggest that mildronate might inhibit carnitine transport through the blood–testis barrier. The transporter involved could be OCTN2, since Grube and colleagues showed that mildronate inhibits OCTN2 in the heart [26]. However, a recent study [32] concluded that mildronate fully inhibits L-carnitine uptake by muscle cells, but remains inefficient in inhibiting L-carnitine uptake by heart cells. In our study, the administration of mildronate at a dose of 100 mg/kg induced a decrease of carnitine concentration by 36% (Fig. 5A), suggesting that mildronate affects carnitine transporters responsible for carnitine supply to the testes. Since 100 mg/kg of mildronate corresponds to the cardioprotective dose used in clinics, the sperm quality measures of male rats treated with M100 were also evaluated. Our data show that in M100 group rats, the weight of the vesicular gland was slightly increased and agglutination was observed in fewer samples, but the densities of spermatozoa in the *cauda epididymis*, as well as motility, were comparable to those of control samples (Tables 3 and 4). The observed effects might not be attributed to changes in testosterone, since mildronate treatment did not change blood plasma concentration of testosterone. Thus, we conclude that the decreased carnitine concentration in the testes did not bring about any significant changes in reproductive organ weight or sperm quality.

Western blot analysis of tissues unexpectedly showed that, in mildronate treated male rat testes, the expression of CPT1 remained unchanged. Increased CPT1 activity and gene expression was reported previously for rat heart and liver tissues after mildronate treatment at a dose of 800 mg/kg [30,33]. In our study, we did not use as high a dose of mildronate, and our findings provide evidence that mildronate used

at lower doses might affect energy metabolism-related gene and protein expression differently.

The mean value of plasma carnitine found in men is significantly higher than that in women [34]. Our data show that mildronate treatment decreased the concentration of carnitine in both the plasma and testes without disturbing the sexual performance of male rats. The uptake of L-carnitine from plasma in mature spermatozoa is considered to be a protective form of mitochondrial metabolism, useful for the survival of the cell [7]. Mildronate is structurally related to carnitine. It has been shown in isolated rat heart experiments that oral pretreatment with mildronate protects energy metabolism against hydrogen peroxide-induced derangement and it was concluded that this effect was mediated by GBB that accumulated in the myocardium because of the inhibition of GBB hydroxylase [31]. In our study of mildronate treated animal testes, we also found an increased concentration of GBB, which might have some protective effect under conditions of decreased carnitine. Carefully designed experimental and clinical studies are necessary for further evaluation of the importance of carnitine and related molecules in reproductive biology and pathology.

In clinics, mildronate is used as a cellular energy metabolism regulating drug for cardiac and CNS applications [35,36]. In addition to carnitine reducing effects, it was shown that mildronate treatment leads to an increase of nitric oxide concentrations in rat tissues [37]. Thus, an increase in the nitric oxide levels in various rat tissues was detected after administration of mildronate along with GBB, as measured by electron paramagnetic resonance [37]. Interestingly, the simultaneous administration of mildronate and GBB resulted in increased nitric oxide concentrations in testes tissues as well [38]. To some extent, the mildronate treatment-induced increase in sexual motivation in the present study might be associated with an increased concentration of GBB and mildronate in rat testes and an influence of both compounds on nitric oxide concentration. Similar changes in rat sexual behavior were previously obtained after intraperitoneal administration of L-arginine, the natural substrate for nitric oxide synthase [39]. In addition, it has been hypothesized that the anti-ischemic mechanism of action of mildronate could result from the mildronate-induced accumulation of GBB and related molecules [36]. Therefore, we cannot exclude the possibility that the results of the present study might be related to both lowered carnitine concentrations and direct pharmacological activity of mildronate itself.

In conclusion, our study provides experimental evidence that administration of mildronate decreases the free carnitine concentration in rat testes, but this effect does not decrease the sexual activity or sperm quality of male rats.

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