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Research article

Short- and long-term cognitive and metabolic effects of medium-chain triglyceride supplementation in rats

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ABSTRACT

Medium-chain triglycerides (MCT) possess neuroprotective properties. However, the long-term metabolic consequences of supplementing a regular diet with cognition-enhancing doses of MCT are largely unknown. We studied the effects of chronic (28 days) supplementation of regular diet with different doses of MCT oil (1, 3, or 6 g/kg/day) or water (control) on working memory (Y-maze), behavior in the Open Field, spatial learning (Morris water maze), and weight of internal organs in male Wistar 2.5-m.o. Rats. In a separate experiment, we evaluated acute (single gavage) and chronic (28 days) effects of MCT or lard supplementation (3 g/kg) on blood biochemical parameters. MCT-1 and MCT-3 doses improved working memory in YM. In MWM, MCT-6 treatment improved spatial memory. Chronic MCT-1 or MCT-3 treatment did not affect internal organ weight, while MCT-6 dose increased liver weight and the brown/white adipose tissue ratio. Acutely, MCT administration elevated blood β-hydroxybutyrate and malondialdehyde levels. Chronic MCT administration (3 g/kg) did not affect the blood levels of glucose, lactate, pyruvate, acetoacetate, β-hydroxybutyrate, total and HDL cholesterol, triglycerides, malondialdehyde, and aspartate transaminase and alanine transaminase activities. Therefore, daily supplementation of standard feed with MCT resulted in mild intermittent ketosis. It improved working memory at lower concentrations without significant adverse side effects. At higher concentrations, it improved long-term spatial memory but also resulted in organ weight changes and is likely unsafe. These results highlight the importance of monitoring the metabolic effects of MCT supplementation alongside cognitive assessment in future studies of MCT's neuroprotective properties.

1. Introduction

It is well established that the ketogenic diet (KD), fasting, and their mediators ketone bodies (KB) demonstrate certain

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neuroprotective properties [1–4]. The brain's ability to metabolize glucose is often reduced in natural aging and neurodegenerative conditions such as Alzheimer's disease (AD), while the brain's capacity to utilize KB as an energy source remains more intact [5,6]. Therefore, various approaches have been developed to elevate the blood KB levels without the limitations of a strict KD, including the use of β-HB salts and esters and medium-chain triglycerides (MCTs) [2].

MCTs are triglycerides (TG) of saturated medium-chain fatty acids (MCFAs, C6–C10). Unlike long-chain fatty acids (LCFAs), MCFAs do not trigger chylomicron formation in enterocytes and instead reach the liver via the portal vein. In the liver cells, MCFAs largely avoid activation in the cytosol and therefore do not require the carnitine transport system to enter mitochondria, whereas the LCFA's entry to mitochondria is limited in the presence of glucose [7]. Therefore, MCFAs in hepatocytes are primarily oxidized in the mitochondria, while LCFAs are primarily esterified and directed towards TG storage, phospholipid synthesis, or excretion in very-low-density lipoprotein (VLDL) particles [8]. Unregulated MCFAs oxidation in the mitochondria can generate amounts of acetyl-CoA exceeding the capacity of the tricarboxylic acid (TCA) cycle. This acetyl-CoA can feed various metabolic pathways, including the ketogenesis in the mitochondria, as well as cholesterol synthesis and *de novo* lipogenesis in the cytosol. The KB, acetoacetate (AcAc) and β-hydroxybutyrate (β-HB), produced in the liver can travel in the bloodstream to other organs, including the brain, where they can be converted to acetyl-CoA and used in the TCA cycle to generate ATP [7]. While LCFAs are only significantly ketogenic under conditions such as starvation, ketogenic diet (KD), diabetes, or strenuous exercise, carbohydrates do not affect the ketogenesis from MCFAs to the same extent [9,10]. Consistently, both acute and chronic consumption of MCT increased KB uptake and utilization [11,12]. Both acute and chronic MCT supplementation improved cognitive function in various tests in elderly with normal cognition [13], patients suffering from neurodegenerative diseases [14–16], and healthy adults [17]. Although most human and animal MCT supplementation studies are designed in line with the hypothesis that its neuroprotective effects are mediated by KB, MCFAs may also exert neuroprotective and procognitive effects via mechanisms independent of KB metabolism [18–20]. Some studies report elevated brain levels of MCFAs but not β-HB [19,21]. Despite the number of studies demonstrating the efficacy of MCT supplementation in supporting cognitive function, the mechanisms remain poorly understood.

Additionally, depending on the physiological conditions, the acetyl-CoA generated during MCFA oxidation in the liver can stimulate cholesterol synthesis and lipogenesis and may further limit LCFA oxidation, making more LCFAs available for TG storage and VLDL excretion. Both protective and toxic effects of MCT consumption on liver and blood lipids have been reported [22–29]. Although MCT consumption is considered safe in doses not exceeding 1 g/kg [30], and most human clinical trials used concentrations within this range [20], in some cases, the dose may be increased until the desired neuroprotective effect has been achieved [31]. The long-term effects of MCT supplementation without any dietary restrictions on cardiovascular and metabolic health are unknown.

This study aimed to assess the metabolic effects of acute and chronic MCT administration in neuroprotective concentrations in adult male rats. We tested three doses corresponding to low, typical, and high MCT doses used in human clinical trials, adjusted for metabolic rate [32].

2. Materials and methods

2.1. Animals

The study was performed on male 2.5–3.5 month-old Wistar rats. At the beginning of the testing, their weight averaged 316 ± 28.0 g (M ± SD) and reached 372 ± 38.7 g at the end. 4–5 weeks-old animals (weighing 160–180 g on average) were obtained from Rappolovo breeding center (Leningradskaya region, Russia). They were kept in standard cages (2 animals per cage) under standard conditions with *ad libitum* access to tap water and standard chow (Table 1) under 12 h light/dark cycle. The room temperature was maintained at 22 ± 2 °C. The animals were given 1 month to get used to the environment before the beginning of the testing. All animal experiments were approved by the ethics authorities of the Institute for Experimental Medicine (St. Petersburg, Russia) and were designed to comply with the Directive 2010/63/EU regulations.

Table 1
Diet composition.[a].

Nutrient[b]	Amount, % by weight
Protein	19.38
Fat	5.00
Fiber	4.00
Ash	7.00
Lysine	1.2
Methionine + Cysteine	0.7
Ca	1.01
P	0.66
NaCl	0.18

[a] Laboratokorm "PK-120-2_1211", Moscow.
[b] Ingredients: corn, wheat, sunflower grist, soy grist, meat meal, sunflower oil, premix "Pushnovit" 0.5%, L-lysine monochloride, DL-methionine, defluorinated feed phosphate, mineral powder.

2.2. Study design

The study design is shown in Fig. 1.

2.2.1. Experiment I

Experiment I aimed to assess behavioral effects of MCT supplementation in different doses and to select the administration dose for further study of the acute and chronic effects of MCT supplementation on biochemical markers in the blood serum. Young adult animals (2.5 m. o.) were tested in the Y-maze (YM with 3 equal arms; tested once for 8 min) to evaluate the baseline values for the number of arm entries and spontaneous alternations (visits to all three different arms consecutively) and then in the Open Field test (OF: 3 consecutive days, 3 min) to determine the baseline of the extinction of locomotor exploratory activity. Upon completion of these baseline tests, the rats started receiving 1, 3, or 6 g/kg/day MCT (Jarrow Formulas® MCT Oil; C8 and C10 mixture; 951,7kCal/g) or water (n = 8–10 in each group) through orogastric gavage, daily for 28 days. From the 17th day of the MCT administration, the animals were once again tested in the YM and OF under the same protocols as used for the pre-supplementation assessment. At the end of the MCT gavage period, animals were tested in the Morris water maze (4 consecutive days of 4 training trials per day and a 90-s probe trial on the 5th day). 20 h after the final MCT administration, the animals were decapitated. The internal organs (heart, lungs, thymus, liver, spleen, kidneys, adrenal glands, retroperitoneal white adipose (rWAT), and brown adipose tissues (inter-scapular (iBAT) and sub-scapular (sBAT)) were weighed. Body weight, chow consumption, and calorie consumption were assessed daily. Chow consumption and calorie intake were assessed per cage (with 2 animals housed per cage).

2.2.2. Experiment II

Based on the results of Experiment I, a dose of 3 g/kg was selected for the investigation of the acute and chronic metabolic effects of MCT supplementation in Experiment II. Control animals in this experiment were receiving either water or lard (3 g/kg; 897 kCal/g), orogastrically. The animals were decapitated 30 or 120 min after the administration or after 28 days of daily gavage. Only in the MCT group, animals were also sacrificed at 60 or 180 min after MCT administration for further assessment of the blood ketone body level dynamics. The blood was collected during the decapitation, stored at +4 °C overnight, centrifuged at 2000 g (20 min), and the serum was stored at −70 °C until biochemical analyses.

2.3. Behavioral testing

2.3.1. Y-maze

Working memory was assessed in the Y-maze test (YM) as a rate of spontaneous alternations (SA), i.e., consecutive entries to 3 different arms of the Y-maze, during an 8-min session. The Y-maze consisted of 3 equal arms (width 10 cm, length 45 cm, wall height 30 cm) attached at 120° to each other. The test was performed at dim lighting conditions (5 lux). The behavior of the animals during the test was filmed with a video camera located above the maze, and the sequence of arm entries (AE) was recorded manually. The percentage of spontaneous alternations was calculated as follows: SA% = [SA/(number of AE - 2)] * 100. Only trials with 8 arm arteries or more were included in the statistical analysis.

Two different Y-maze apparatuses with identical dimensions were used for pre-treatment and post-treatment assessment in order to

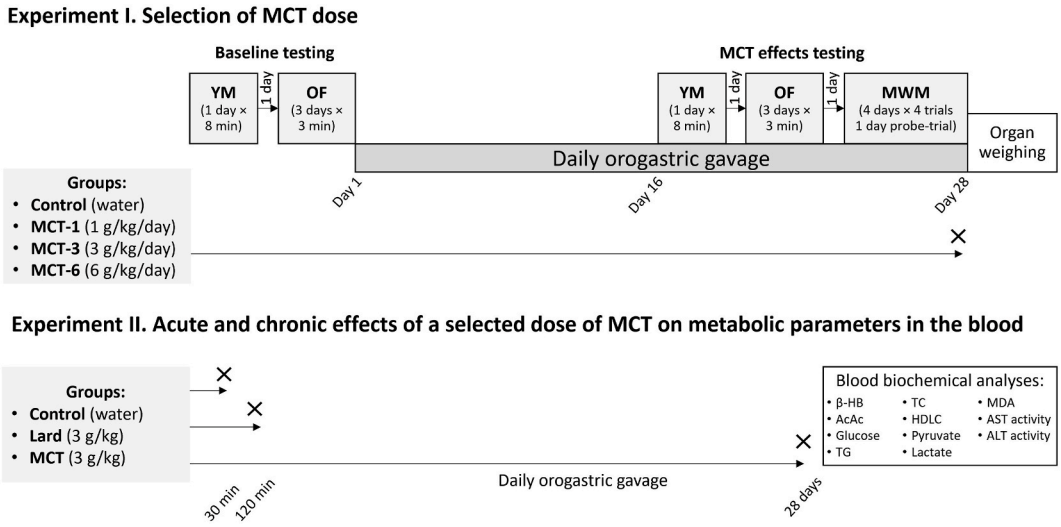

Fig. 1. Study design. See description in the text. YM – Y-maze, OF – Open field test, MWM – Morris water maze, β-HB – beta-hydroxybutyrate, AcAc – acetoacetate, TG – triglycerides, TC – total cholesterol, HDLC – high-density lipoprotein cholesterol, MDA – malondialdehyde, AST – aspartate transaminase, ALT – alanine transaminase.

avoid the extinction of exploratory behavior.

2.3.2. Open Field

The locomotor exploratory activity of animals and its extinction were assessed in 6 trials of the Open Field test (OF), carried out 3 times before the treatment to evaluate the baseline performance and 3 times from 18 to 20 days of MCT-treatment in the same arena in order to study not only the dynamics of extinction in the pre- and post-treatment sessions, but also to evaluate memory in a familiar environment. Each set of testing was carried out on three consecutive days, 3 min per day, from 18:00 to 23:00.

The testing was performed in a round arena (diameter 1 m, wall height of 30 cm, illumination 10 lux). The rat was placed in the center of the platform and a video of the animal's behavior was recorded from above for 3 min.

A tracking program 'Pavlovian tracking' (developed at the I.P. Pavlov Department of Physiology (FSBSI "Institute of Experimental Medicine", St. Petersburg, Russia) and previously validated [33]) was used for tracking and analysis of the following parameters: total distance traveled (a measure of locomotor and exploratory activity), distance covered in the first minute (a measure of orientation and exploratory behavior), and time spent in the periphery (the outer ring, 10 cm wide) of the arena (a measure of anxiety). Extinction index within each trial was calculated as a ratio of distance covered during the 3rd min to 1st min, expressed in %.

2.3.3. Morris Water Maze

Spatial learning and memory were assessed in the Morris Water Maze (MWM) [34], a tank 150 cm in diameter with a wall height of 70 cm. Four different visual cues printed on A4 paper were placed equidistantly on the inner walls in the northeast (NE), southeast (SE), southwest (SW), and northwest (NW) quadrants. Four times a day for four consecutive days, animals were placed for 90 s in the tank filled with water mixed with milk, with 90 s intervals between trials. The platform (10 × 10 cm) was submerged 1 cm below the water in the center of the NW quadrant (target quadrant) [33]. SE, S, E, and NE quadrants were used as starting locations. After the rat reached the platform or was placed on it at the end of an unsuccessful 90-s trial, it was left there for 30 s. On the 5th day of the testing, a 90-s probe-trial was carried out starting from the SE location.

The behavior of the animals in each trial was filmed from above. The video files were further processed with the Pavlovian Tracking software, measuring the path length and the percentage of time spent in the target quadrant [35]. For statistical analysis, the path length was averaged over each training day.

2.4. Biochemical analyses

The levels of acetoacetate (AcAc), β-hydroxybutyrate (β-HB), glucose, lactate, pyruvate, total cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides (TG), malondialdehyde (MDA), and aspartate transaminase (AST) and alanine transaminase (ALT) activities were measured in serum samples without deproteinization by enzymatic colorimetric assays using commercial kits according to the manufacturers' instructions (Sigma-Aldrich, MO, USA (β-HB, AcAc); Olvex Diagnosticum (AST, ALT), Vital Development Corp., St. Petersburg, Russia). ImmunoChem-2100 Microplate Reader (High Technology, Inc., MA, USA) was used to measure absorbance.

2.4.1. Ketone bodies

β-Hydroxybutyrate (β-HB) and acetoacetate (AcAc) concentrations were measured using Sigma-Aldrich ketone body assay kit (MAK134) according to the manufacturer's instructions, using an enzymatic assay based on *3-hydroxybutyrate dehydrogenase* catalyzed reactions, in which the change in NADH absorbance is directly related to either the AcAc or β-HB concentrations. The absorbance of the samples was measured at 340 nm.

2.4.2. Glucose

Glucose levels were measured using a kit B 05.01 (Vital Development Corp., St. Petersburg, Russia) according to the manufacturer's instructions. Briefly, β-D-glucose is oxidized by atmospheric oxygen under the action of *glucose oxidase* with a formation of an equimolar amount of hydrogen peroxide. Under the action of peroxidase, *hydrogen peroxide* oxidizes chromogenic substrates in the presence of phenol with the formation of a colored product, the color intensity of which is proportional to the glucose concentration in the sample and is measured photometrically at 510 nm.

2.4.3. Pyruvate and lactate

Pyruvic acid levels were measured by reaction with 2,4-dinitrophenylhydrazine (2,4-DNPH) in alkaline medium to form 2,4-dinitrophenylhydrazone of pyruvic acid, which has a yellow-orange color. The color intensity is proportional to the pyruvate content. Proteins in the sample were precipitated with 10% Trichloroacetic acid. The mixture was incubated for 20 min with a 5 mM solution of 2,4-DNPH in the dark. Then, 12% NaOH was added and the optical density was measured at 405 nm.

Lactic acid levels were measured using a kit B 19.01 (Vital Development Corp., St. Petersburg, Russia) according to the manufacturer's instructions. Briefly, lactate, under the influence of lactate oxidase in the presence of oxygen, is split into pyruvic acid and hydrogen peroxide. The latter, in the presence of 4-aminoantipyrine and *p*-chlorophenol under the influence of peroxidase, forms a quinone imine compound, the color of which is proportional to the lactate content in the analyzed sample and is measured photometrically at 505 nm.

2.4.4. Total and HDL cholesterol

The concentration of total cholesterol was measured using a kit B 13.11 (Vital Development Corp., St. Petersburg, Russia) according

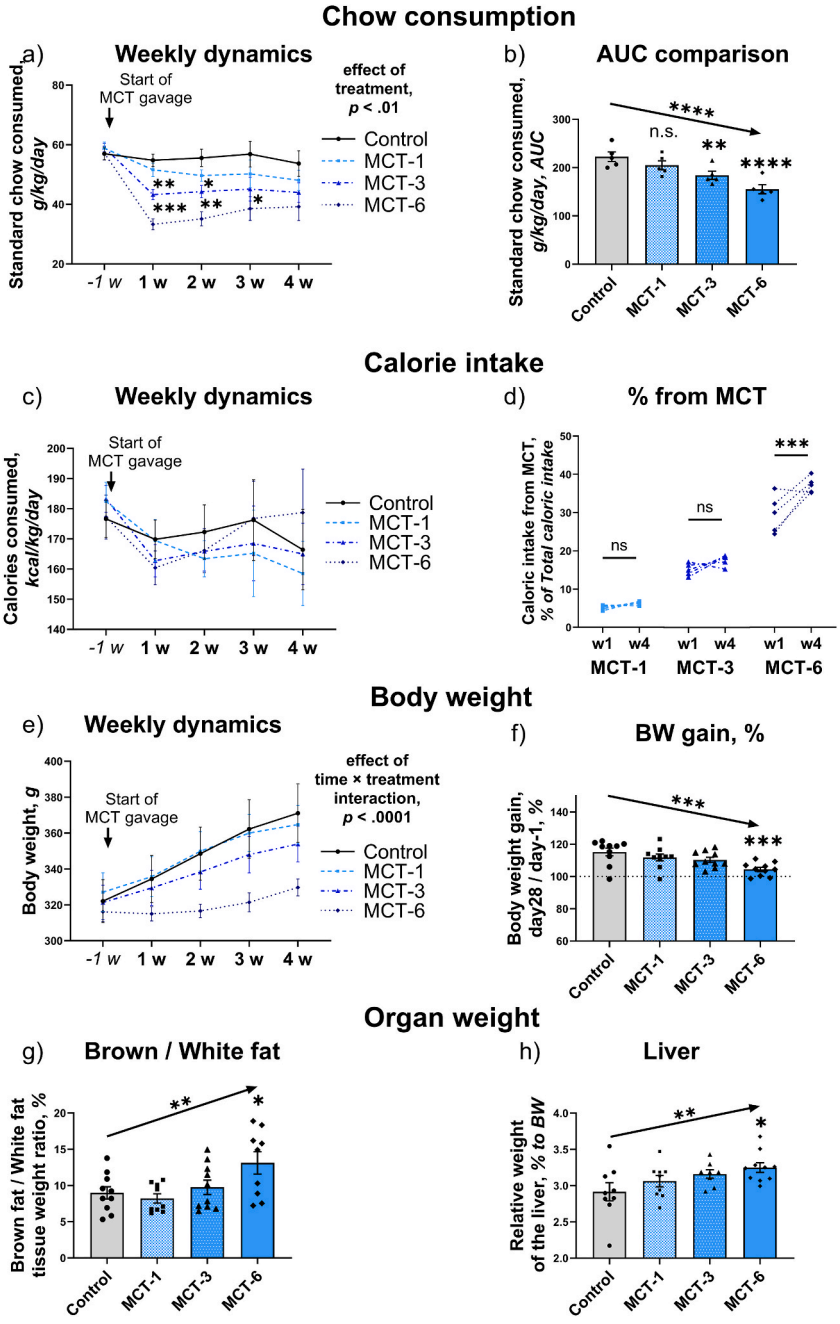

Fig. 2. Effects of daily MCT administration on chow consumption, caloric intake, body and organ weight. Dynamics **(a)** and AUC comparison **(b)** of standard chow consumption (g/kg/day; MCT-3 and MCT-6, but not MCT-1 groups were characterized by lower chow consumption compared to control starting from week 1 of MCT gavage). **(c)** Dynamics of caloric intake (no differences among groups observed, $P > .05$). **(d)** The proportion of caloric intake from MCT significantly increased over the 4-week gavage period in MCT-6, but not in MCT-1 and MCT-3 groups. **(e)** Dynamics of body weight gain: although 2-way ANOVA detected a significant effect of week × dose interaction, pairwise *post hoc* comparisons by BKY procedure showed no differences between groups at either time point. **(f)** Body weight (BW) gain measured as a ratio of BW at the end of experiment to BW at 1 day prior to the start of MCT gavage: MCT-6, but not MCT-1 and MCT-3, group had significantly lower BW gain compared to control with a significant inverse linear trend of dose-dependency. **(g)** The ratio of brown to retroperitoneal white adipose tissue weight was higher in MCT-6, but not MCT-1 and MCT-3 groups, compared to the control group. **(h)** MCT-6 animals had higher relative liver weight compared to the control group. M ± SEM; **(a), (c), (d), (e)** — 2-way rm-ANOVA and *post hoc* BKY procedure (*$q \leq 0.05$, **$q \leq 0.01$, ***$q \leq 0.001$); **(b), (f), (g), (h)** —ANOVA, *post hoc* BKY procedure and test for linear trend (*$q \leq 0.05$, **$q \leq 0.01$, ***$q \leq 0.001$, ****$q \leq 0.0001$ vs. Control); $n = 8$–10 rats per group; for chow consumption and calorie intake, $n = 5$ (2 animals per cage). BW – body weight, AUC – the Area Under the Curve. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

to the manufacturer's instructions. The analysis is based on conducting coupled reactions catalyzed by *cholesterol esterase* (cholesterol esters —> cholesterol + fatty acids), *cholesterol oxidase* (cholesterol + O_2 —> cholestenone + H_2O_2), and *peroxidase* (H_2O_2 + chromogen —> H_2O + colored product). Hydrogen peroxide oxidizes *peroxidase* substrates with a formation of a colored product, the concentration of which is directly proportional to the cholesterol content in the analyzed sample and is determined photometrically at 500 nm.

The level of HDL-cholesterol was determined similarly to the determination of the level of total cholesterol with preliminary binding of all lipoproteins, except for HDL, with antibodies against β-lipoproteins, which prevents their participation in reactions. A kit B 13.85 (Vital Development Corp., St. Petersburg, Russia) was used for this purpose according to the manufacturer's instructions.

2.4.5. Triglycerides

The levels of triglycerides (TG) were measured using a kit B 17.01 (Vital Development Corp., St. Petersburg, Russia) according to the manufacturer's instructions. The free glycerol formed during the hydrolysis of TGs by *lipase* as a result of successive enzymatic reactions is oxidized by atmospheric oxygen to form hydrogen peroxide (*lipase*: TG —> glycerol + FA; *glycerol kinase*: glycerol + ATP —> glycerol-3-phosphate + ADP; *glycerol-3-phosphate oxidase*: glycerol-3-phosphate + O_2 —> dihydroxyacetone phosphate + H_2O_2). Under the action of *peroxidase*, hydrogen peroxide oxidizes chromogens, forming colored quinone imine, the concentration of which is directly proportional to the content of triglycerides in the sample and is determined photometrically at 505 nm.

2.4.6. Malondialdehyde

The detection of malondialdehyde (MDA) was based on its reaction in a serum sample (100 μL) with 0.5% 2-thiobarbituric acid (TBA, 50 μL) in acidic medium (pH = 4, established by 40% Trichloroacetic acid, 100 μL) at high temperatures (~100 °C, in a boiling water bath) for 45 min to form a colored Thiobarbituric acid reactive substances (TBARS), which has an absorbance maximum at 532 nm. After incubation, the mixture was cooled down to room temperature, and the solution was purified from insoluble products by their precipitation in 96% ethanol (100 μL) and centrifugation for 20 min at 6000 g.

2.4.7. Aspartate transaminase and alanine transaminase

The kinetic spectrophotometric determination of the activity of *aspartate transaminase (AST)* and *alanine aminotransferase (ALT)* was performed using kits 002.024 and 001.024, respectively (Olvex Diagnosticum, St. Petersburg, Russia), according to the manufacturer's instructions. The analysis is based on measuring a decrease in the optical density of the analyzed sample at 340 nm as a result of the following reactions.

ALT: L-alanine + α-ketoglutarate —> pyruvate + L-glutamate. The pyruvate formed in this reaction further oxidizes NADH in the presence of *lactate dehydrogenase* (*LDH*: pyruvate + NADH + H^+ —> lactate + NAD^+), which is accompanied by a decrease in optical density at 340 nm, and this decrease is proportional to the activity of *ALT* in the sample.

AST: L-aspartate + α-ketoglutarate —> oxaloacetate + L-glutamate. Oxaloacetate formed in this reaction further oxidizes NADH in the presence of *malate dehydrogenase* (*MDH*: oxaloacetate + NADH + H^+ —> L-malate + NAD^+), which is accompanied by a decrease in optical density at 340 nm, and this decrease is proportional to the activity of *AST* in the sample.

2.5. Statistical analysis

GraphPad Prism v.8 (GraphPad Software, Inc., CA, USA) software was used for statistical analyses and graph plotting. The normality of the data distribution was assessed with D'Agostino–Pearson and Shapiro–Wilk tests. Between-group differences were assessed using one-way ANOVA analysis with *post hoc* two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli (BKY procedure) and two-way repeated measures (rm)-ANOVA with factors for trial/time and treatment with *post hoc* BKY procedure. Non-parametric Kruskal–Wallis H-test was applied to the biochemical data obtained after chronic lard or MCT administration. The effects of MCT and lard treatments on the dynamics of blood biochemical parameter changes following a single administration were assessed by linear regression analysis and comparison of slopes. The graphs were plotted as M ± SEM. Differences were considered significant at $P \leq .05$ (or $q \leq 0.05$ in the case of BKY procedure).

3. Results

3.1. Chow consumption, caloric intake, body weight and organ weight in MCT-fed animals

Daily MCT administration significantly decreased standard chow consumption in MCT-3 and MCT-6, but not MCT-1 groups compared to control animals during the first 2 or 3 weeks of treatment. However, by the 4th experimental week, the difference from control in neither of the MCT-fed groups was statistically significant (Fig. 2a; 2-way rm-ANOVA: $F_{(3, 16)} = 8.83$, $P < .01$ (effect of treatment), $F_{(1.273, 20.36)} = 18.36$, $P < .001$ (effect of time), $F_{(12, 64)} = 2.59$, $P < .01$ (effect of time × treatment interaction); *post hoc* BKY procedure: **week 1**, Control vs. MCT-3: t = 4.28, $q = 0.0015$, Control vs. MCT-6: t = 7.85, $q < 0.0001$, **week 2**, Control vs. MCT-3: t = 3.16, $q = 0.015$, Control vs. MCT-6: t = 5.46, $q < 0.001$, **week 3**, Control vs. MCT-3: t = 1.99, $q = 0.08$, Control vs. MCT-6: t = 3.11, $q = 0.014$, **week 4**, Control vs. MCT-3: t = 1.79, $q = 0.11$, Control vs. MCT-6: t = 2.28, $q = 0.052$).

Cumulative chow consumption was assessed by AUC analysis: compared to control, this measure significantly decreased in MCT-3 and MCT-6, but not MCT-1, groups (Fig. 2b; ANOVA: $F_{(3, 16)} = 9.028$, $P < .001$; *post hoc* BKY procedure: Control vs. MCT-1: t = 1.29, $q = 0.074$, Control vs. MCT-3: t = 2.81, $q = 0.0065$, Control vs. MCT-6: t = 4.94, $q = 0.0002$) with a significant dose-dependent inverse

linear trend (test for linear trend: slope = −22.29, SE of slope = −4.31, $F_{(1, 16)} = 26.73$, $P < .0001$). Considering the amounts of calories obtained from the standard chow and the MCT supplement (9.517 kcal/ml of MCT oil), the caloric intake did not differ among groups (Fig. 2c; 2-way rm-ANOVA: $P > .05$). Notably, the proportion of calories consumed in the form of MCT increased significantly over the 4 weeks of treatment in the MCT-6 group. No changes were registered in the MCT-1 and MCT-3 groups (Fig. 2d; 2-way rm-ANOVA: $F_{(1, 12)} = 17.03$, $P < .01$ (effect of time), $F_{(2, 12)} = 4.54$, $P < .05$ (effect of time × dose interaction); *post hoc* BKY procedure w1 vs. w4: **MCT-1**, t = 0.77, $q = 0.31$; **MCT-3**, t = 1.57, $q = 0.14$; **MCT-6**, t = 4.8, $q = 0.0009$).

Although a significant effect of time × treatment interaction on body weight (BW) gain was observed (Fig. 2e; 2-way rm-ANOVA: $F_{(12, 144)} = 5.31$, $P < .0001$), *post hoc* analysis did not reveal any significant differences among groups at any time point (BKY procedure: $q > 0.05$). The ratio of BW at the end of the experiment (Day 28 of gavage) to that at the beginning of the experiment (Day −1, the last before starting the gavage) was significantly lower in MCT-6, but not MCT-3 or MCT-1 animals in comparison with the control group (Fig. 2f; ANOVA: $F_{(3, 36)} = 5.74$, $P < .01$; *post hoc* BKY procedure: Control vs. MCT-1: t = 1.27, $q = 0.14$, Control vs. MCT-3: t = 1.84, $q = 0.077$, Control vs. MCT-6: t = 4.05, $q = 0.0005$) with a significant dose-dependent inverse linear trend (test for linear trend: slope = −3.38, SE of slope = −0.84, $F_{(1, 36)} = 16.24$, $P < .001$).

At the end of the 28-day MCT gavage period, MCT-6, but not MCT-1 or MCT-3 animals had a significantly higher ratio of brown/retroperitoneal white adipose tissue (B/W ratio) than control animals (Fig. 2g; ANOVA: $F_{(3, 34)} = 4.06$, $P = .014$, *post hoc* BKY procedure: Control vs. MCT-1: t = 0.51, $q = 0.42$, Control vs. MCT-3: t = 0.53, $q = 0.42$, Control vs. MCT-6: t = 2.78, $q = 0.018$) with a significant dose-dependent direct linear trend (test for linear trend: slope = 1.35, SE of slope = .46, $F_{(1, 34)} = 8.3$, $P < .01$). The animals fed with 6 g/kg/day MCT had significantly enlarged livers at Day 28 compared to control (Fig. 2h; ANOVA: $F_{(3, 35)} = 3.34$, $P = .03$, *post hoc* BKY procedure: Control vs. MCT-6: t = 3.01, $q = 0.02$), while the liver weight of the MCT-1 and MCT-3 animals did not differ from that in the control animals (*post hoc* BKY procedure: $q > 0.05$). A significant direct linear dose-dependent trend was also observed for the relative weight of the liver (test for linear trend: slope = .11, SE of slope = .035, $F_{(1, 35)} = 9.8$, $P < .01$). The relative weight of other measured organs (thymus, heart, lungs, spleen, kidneys, adrenal glands, testicles, retroperitoneal white adipose tissue, inter-scapular and subscapular brown adipose tissue) was not affected by any dosage of MCT treatment compared to control (Fig. 1S; $P > .05$).

3.2. Results of the behavioral testing

The number of arm entries in the Y-maze test during the second trial was lower in all groups (Fig. 3a; 2-way rm-ANOVA: $F_{(1, 35)} = 103.5$, $P < .0001$ (effect of trial), $F_{(3, 35)} = 0.84$, $P > .05$ (no effect of treatment); *post hoc* BKY procedure Before vs. After: **Control** t = 4.54, $P < .0001$, **MCT-1** t = 6.32, $P < .0001$; **MCT-3** t = 3.67, $P = .0008$; **MCT-6** t = 5.73, $P < .0001$) with no statistical difference among groups (Fig. 3b; ANOVA: $F_{(3, 28)} = 2.03$, $P = .13$), indicating that the MCT supplement in neither dose had any significant effect

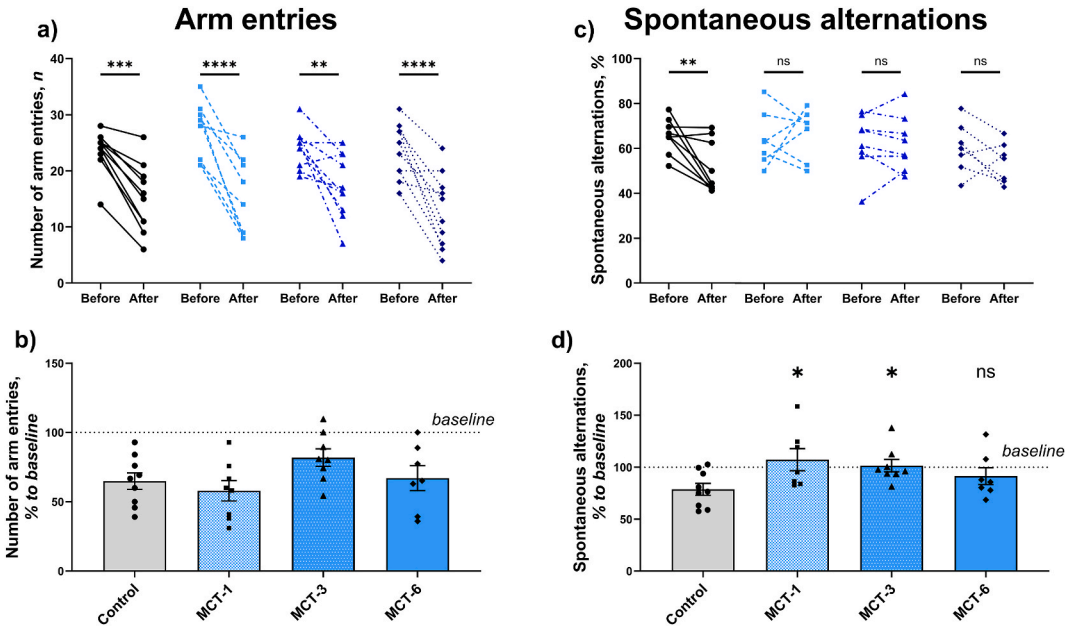

Fig. 3. Effects of chronic MCT administration (1, 3, or 6 g/kg/day) on working memory assessed in the Y-maze test. (a) The number of arm entries (AE) in the Y-maze test before and after chronic treatment decreased in all groups. **(b)** The average change in the number of AE compared to the baseline value in each group did not differ among groups. **(c)** The frequency of spontaneous alternations (SA) before and after chronic treatment significantly decreased only in the control group, but not in either of the MCT-fed groups, while **(d)** the ratio of SA during the second session vs. baseline was higher in MCT-1 and MCT-3 groups (but not MCT-6) compared to control. M ± SEM; **(a), (c)** — 2-way rm-ANOVA and *post hoc* BKY procedure (**$q \leq 0.01$, ***$q \leq 0.001$, ****$q \leq 0.0001$); **(b)**, **(d)** —ANOVA and *post hoc* BKY procedure (*$q \leq 0.05$ vs. Control); n = 7–9 rats per group.

on locomotor activity. MCT administration attenuated the decrease in spontaneous alternations (SA) in the Y-maze compared to control (Fig. 3c; 2-way rm-ANOVA: $F_{(3, 27)} = 3.13$, $P = .042$ (effect of trial × treatment interaction); $F_{(1, 27)} = 4.4$, $P = .046$ (trial); $F_{(3, 27)} = 1.3$, $P = .3$ (no effect of dose); *post hoc* BKY procedure Before vs. After: **Control** t = 3.52, $P = .004$, **MCT-1** t = 0.57, $P = .59$; **MCT-3** t = 0.044, $P = .75$; **MCT-6** t = 1.42, $P = .26$). The degree of the treatment-induced SA frequency change compared to baseline was greater in rats fed with 1 or 3, but not 6, g/kg/day (compared to control), indicating improved working memory in MCT-1 and MCT-3 groups (Fig. 3d; ANOVA: $F_{(3.27)} = 4.9$, $P = .05$; *post hoc* BKY procedure: Control vs. MCT-1 t = 2.7, $q = 0.025$, Control vs. MCT-3 t = 2.23, $q = 0.035$, Control vs. MCT-6 t = 1.2, $q = 0.16$).

In the Open field test, no differences were observed among groups in pre- and post-treatment sessions (Fig. 4; 2-way rm-ANOVA: $P > .05$).

Spatial learning acquisition, assessed in the Morris water maze as average distance traveled to the hidden underwater platform at each testing day, did not differ among groups (Fig. 5a; 2-way rm-ANOVA: $P > .05$). At probe trial, animals fed with 6, but not 1 or 3, g/kg/day MCT, spent significantly more time in the target quadrant compared to control group, indicating improved spatial memory in MCT-6 animals (Fig. 5b and c; ANOVA: $F_{(3, 30)} = 3.6$, $P = .02$, *post hoc* BKY procedure: Control vs. MCT-1 t = 0.2, $q = 0.83$, Control vs. MCT-3 t = 0.97, $q = 0.35$, Control vs. MCT-6 t = 2.9, $q = 0.016$).

3.3. Metabolic effects of MCT supplementation

Based on the results of Experiment I, the 3 g/kg/day MCT dose was selected for further assessment of its acute and chronic effects on the metabolic health parameters, as it was high enough to elicit procognitive effects in the Y-maze test, but did not affect the liver weight and the brown/white adipose tissue ratio. Lard (3 g/kg/day), rich in long-chain fatty acids, and water were used as control treatments in Experiment II.

Acutely, MCT administration (3 g/kg) elevated blood β-HB level peaking at 2 h and decreasing by 3 h after gavage (Fig. 6a; ANOVA: $F_{(4, 22)} = 10.29$, $P < .0001$; *post hoc* BKY procedure: 0 vs. 30 t = 4.31, $q = 0.0009$; 0 vs. 60 t = 3.6, $q = 0.0016$; 0 vs. 120 t = 5.73, $q <$

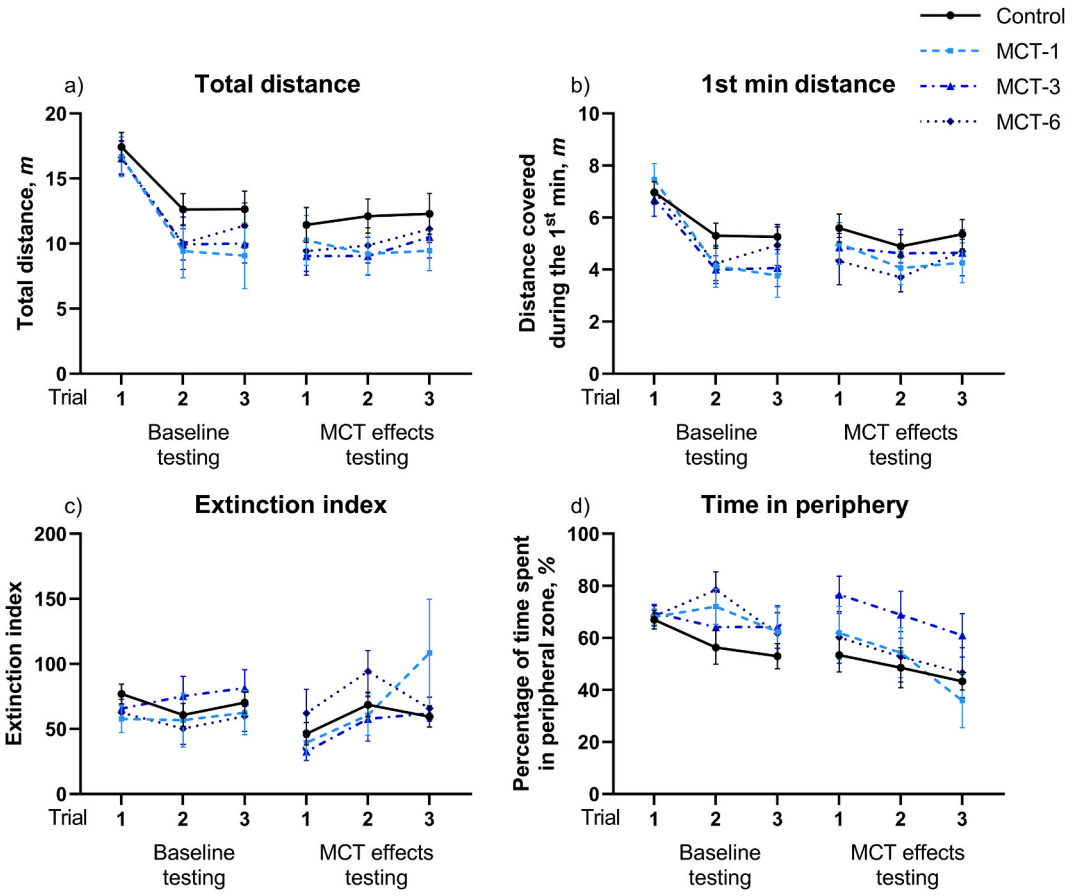

Fig. 4. No effect of MCT administration in the Open Field. Dynamics of the following parameters for 3 days prior to the MCT administration and for 3 days from days 19–21 of the MCT administration: **(a)** Total distance traveled; **(b)** Distance covered during the 1st min of the test; **(c)** Extinction index (calculated as a ratio of distance covered during the 3rd min to that covered during the 1st min, expressed in %; **(d)** Time spent in the peripheral zone. M ± SD. 2-Way rm-ANOVA ($P > .05$); $n = 8–9$ rats per group.

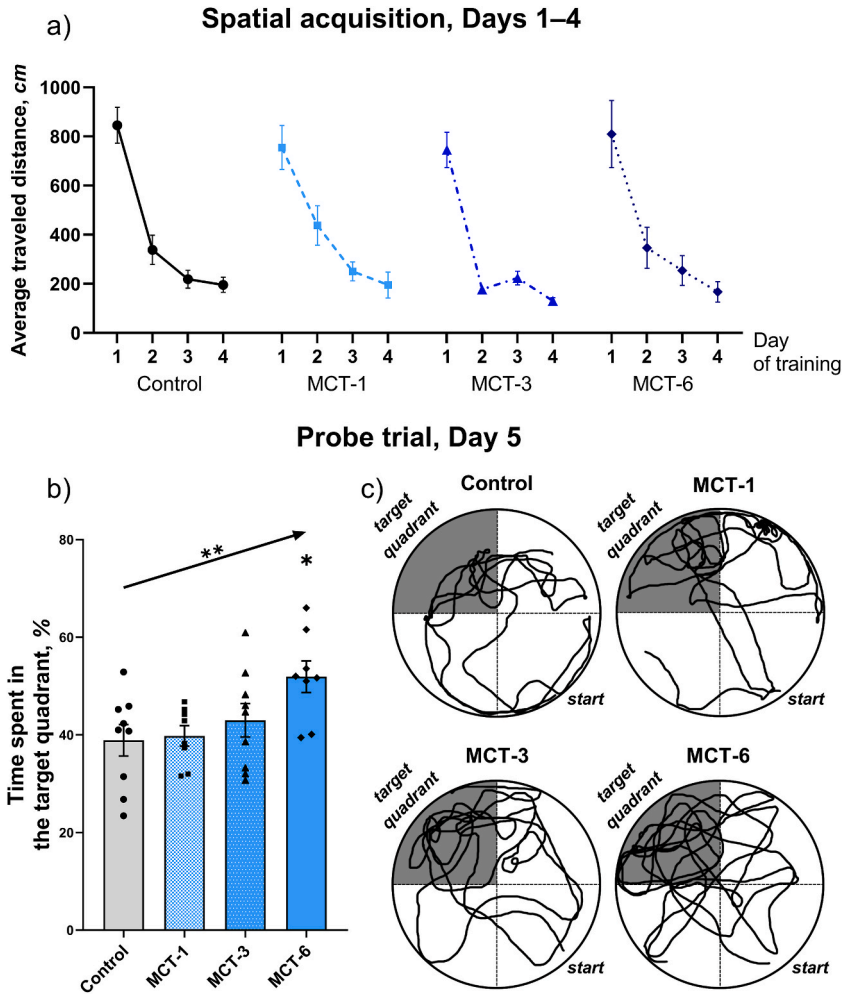

Fig. 5. Effects of chronic MCT administration (1, 3, or 6 g/kg/day) on spatial memory assessed in the Morris water maze. (a) No differences in the learning dynamics among groups. **(b)** Compared to control, MCT-6 animals spent significantly more time in the target quadrant during the 90-s probe trial with a significant dose-dependent trend. **(c)** Most representative tracks in probe-trial. M ± SEM; (a) — 2-way rm-ANOVA ($P > .05$); (b) —ANOVA and *post hoc* BKY procedure (*$q \leq 0.05$ vs. Control) and linear trend analysis (**$P \leq .01$); $n = 8$–9 rats per group.

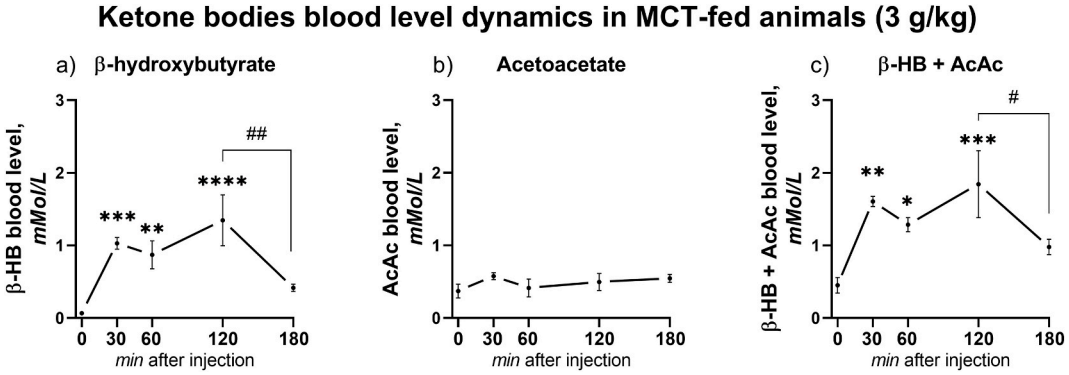

Fig. 6. Dynamics of blood ketone body levels after a single oral dose of MCT (3 g/kg). (a) The level of β-HB increased over 2 h post-administration with a peak concentration of 1.347 ± 0.3497 mMol/L and decreased by 3 h post-administration. **(b)** The level of AcAc did not change over 3 h after MCT gavage. **(c)** Cumulative level of both ketone bodies elevated over 2 h after MCT injection peaked at 1.844 ± 0.4604 mMol/L and decreased by 3 h after administration. M ± SEM. ANOVA and *post hoc* BKY procedure (*$q \leq 0.05$, **$q \leq 0.01$, ***$q \leq 0.001$ – vs. Control; #$q \leq 0.05$, ##$q \leq 0.01$–120 vs. 180 min). $N = 4$–8 rats per group.

0.0001; 0 vs. 180 t = 1.45, $q = 0.12$; 120 vs. 180 t = 3.54, $q = 0.0029$), while lard administration (3 g/kg) did not affect the blood ketone body levels (Fig. 7a; linear regression comparison: $F_{(1, 28)} = 34.32$, $P < .0001$; ANOVA 30 min: $F_{(2, 14)} = 140.4$, $P < .0001$; ANOVA 120 min: $F_{(2, 11)} = 63.56$, $P < .0001$). Administration of neither fat significantly affected the AcAc level (Figs. 6b and 7b; $P > .05$). The level of AcAc and β-HB combined was elevated after the administration of MCT but not lard (Fig. 6c: ANOVA: $F_{(4, 21)} = 7.17$, $P = .0008$; *post hoc* BKY procedure: 0 vs. 30 t = 3.98, $q = 0.002$; 0 vs. 60 t = 2.87, $q = 0.019$; 0 vs. 120 t = 4.8, $q = 0.0006$; 0 vs. 180 t = 1.69, $q = 0.09$; 120 vs. 180 t = 2.6, $q = 0.026$; Fig. 7c: linear regression comparison: $F_{(1, 26)} = 19.5$, $P = .0002$; ANOVA 30 min: $F_{(2, 13)} = 43.1$, $P < .0001$; ANOVA 120 min: $F_{(2, 11)} = 30.01$, $P < .0001$). The β-HB/AcAc ratio was elevated after MCT administration, peaking at 2 h, closely following β-HB (Fig. 7d; linear regression comparison: $F_{(1, 26)} = 6.92$, $P = .0139$; ANOVA 30 min: $F_{(2, 13)} = 39.53$, $P < .0001$; ANOVA 120 min: $F_{(2, 11)} = 17.2$, $P < .0004$).

The glucose level decreased 30 min after MCT administration compared to control (Fig. 7e; ANOVA: $F_{(2, 18)} = 3.67$, $P = .046$, *post hoc* BKY procedure Control vs. MCT: t = 2.67, $q = 0.0325$), while no changes in glucose level were observed in the lard-fed group ($q > 0.05$).

The pyruvate level was elevated 120 min after the administration of MCT but not lard compared to control (Fig. 7f; ANOVA: $F_{(2, 17)} = 3.81$, $P = .043$, *post hoc* BKY procedure Control vs. MCT: t = 3.86, $q = 0.035$), while neither fat impacted the lactate level (Fig. 7g; $P > .05$). As a result, the lactate/pyruvate ratio was lower in the MCT-fed animals compared to control 120 min after the treatment (Fig. 7h; ANOVA: $F_{(2, 17)} = 3.63$, $P = .048$, *post hoc* BKY procedure Control vs. MCT: t = 2.61, $q = 0.019$).

Lard but not MCT administration acutely increased the blood level of triglycerides (Fig. 7i; linear regression comparison: $F_{(1, 35)} = 10.18$, $P = .003$) and cholesterol (Fig. 7j; linear regression comparison: $F_{(1, 37)} = 15.00$, $P = .0004$), and ALT activity (Fig. 7n; linear

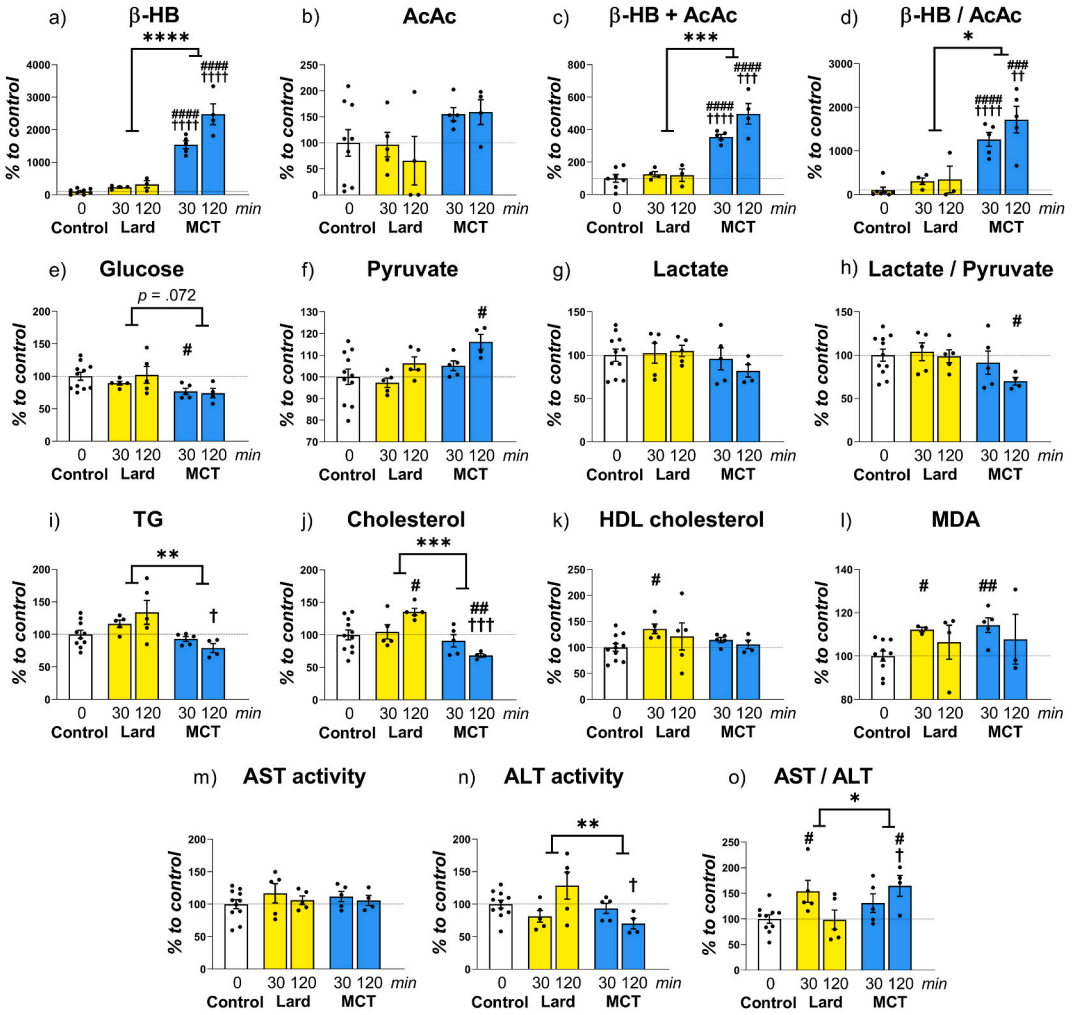

Fig. 7. Acute effects of MCT and lard administration (3 g/kg) on the biochemical and metabolic parameters in blood. See description in the text. MCT vs. Lard difference: linear regression analysis (*$P \leq .05$, **$P \leq .01$, ***$P \leq .001$, ****$P \leq .0001$); ANOVA and *post hoc* BKY procedure: # — fat vs. control difference, † — MCT vs. the respective time-point Lard difference (#, †$q \leq 0.05$, ##, ††$q \leq 0.01$, ###, †††$q \leq 0.001$, ####, ††††$q \leq 0.0001$). $N = 5$–11 rats per group. β-HB –, AcAc – acetoacetate, TG – triglycerides, HDL cholesterol – high-density lipoprotein cholesterol, MDA – malondialdehyde, AST – aspartate transaminase, ALT – alanine transaminase.

regression comparison: $F_{(1, 37)} = 9.34$, $P = .004$. The HDL cholesterol level was elevated 30 min after lard but not MCT treatment compared to control (Fig. 7k; ANOVA: $F_{(2, 18)} = 4.95$, $P = .019$, *post hoc* BKY procedure Control vs. Lard: $t = 3.13$, $q = 0.012$).

The level of malondialdehyde was elevated at 30 min after gavage in both lard and MCT groups but decreased to almost the control level at 120 min (Fig. 7l; ANOVA: $F_{(2, 15)} = 8.46$, $P = .0035$, *post hoc* BKY procedure: Control vs. Lard $t = 2.68$, $q = 0.017$, Control vs. MCT $t = 3.74$, $q = 0.002$).

When administered chronically, neither MCT nor lard (3 g/kg/day) impacted the blood levels of glucose, triglycerides, total and HDL cholesterol, MDA, AST and ALT activities and their ratio (de Ritis ratio) (Fig. 8; $P > .05$).

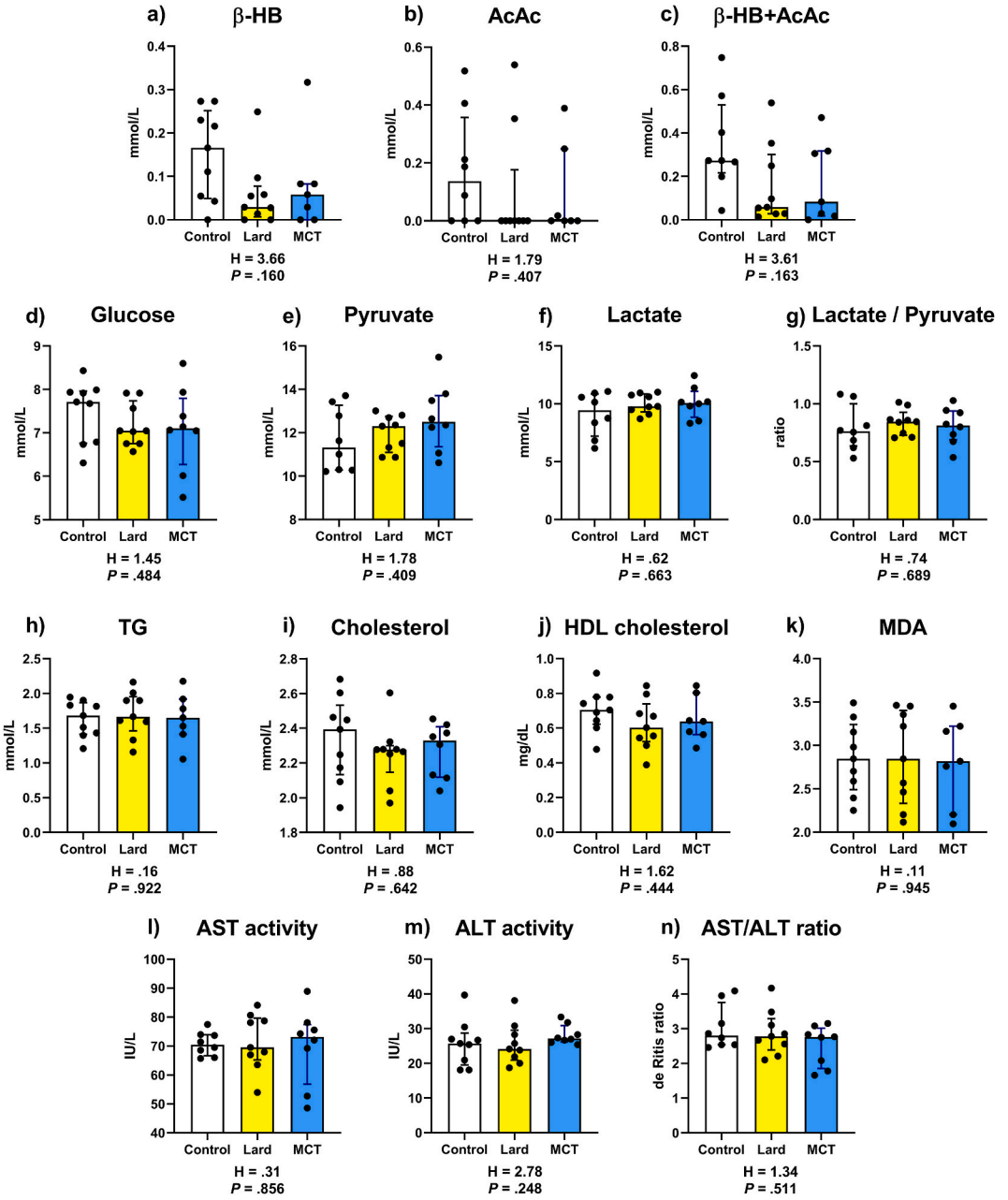

Fig. 8. Chronic effects of MCT and lard administration (3 g/kg) on the biochemical and metabolic parameters in blood. MCT or lard oral gavage for 28 consecutive days (3 g/kg/day) had no effect on blood ketone bodies, glucose, pyruvate, lactate and lactate/pyruvate ratio, triglycerides, total and HDL cholesterol, MDA level, AST and ALT activities, measured 20 h after the last orogastric administration. Median and interquartile range. Kruskal–Wallis H-test, $P > .05$. $N = 7–9$ rats per group. β-HB – beta-hydroxybutyrate, AcAc – acetoacetate, TG – triglycerides, HDL cholesterol – high-density lipoprotein cholesterol, MDA – malondialdehyde, AST – aspartate transaminase, ALT – alanine transaminase.

4. Discussion

In clinical trials of cognition-enhancing effects of MCTs, the doses given to adults most typically range from 20 to 30 g [13,15,16,35–37]. However, with the growing public awareness of the beneficial effects of MCTs, it is reasonable to expect that individuals may decide to consume larger amounts to achieve cognition-enhancing effects. For example, one case study reported a 43-year-old man supplementing his regular diet with 4 tablespoons of MCT twice daily (up to about 112 g/day) to control seizures [31]. Most clinical trials of the cognition-enhancing effects of MCT consumption do not monitor the metabolic effects of MCT supplementation, even when administered in higher doses (e.g., 56 g/day [14]). Therefore, in the present study, we sought to examine the behavioral effects of various MCT doses and, at the same time, assess the metabolic effects of MCT supplementation of a regular diet (standard rat chow). We tested 3 doses: 1, 3, and 6 g/kg/day. Adjusted for metabolic rate [32], they correspond to approximately 10, 30, and 60 g (low, typical, and high) of MCT for an adult human subject.

In agreement with previous studies [38,39], MCT-fed animals gained less body weight (Fig. 2e and f). The animals were weighed daily, and the daily dose of the MCT supplement was calculated based on their weight on that day. Interestingly, with this study design, we observed that in the animals fed 6 g/kg/day of MCT, the proportion of MCT calories to total calories consumed increased significantly from 34.0 ± 3.1% during the 1st week to 37.2 ± 2.1% over the 4th week of the experiment (Fig. 2d), while no significant change in the proportion of caloric intake from MCT was observed at lower doses. In addition, MCT administration at the highest tested dose increased the relative liver mass (Fig. 2g) and the weight ratio of brown to white adipose tissue (Fig. 2h). Both these findings are consistent with previous reports. For example, it has been shown that MCT consumption in mice increased liver mass when fed in large quantities [40] or moderate quantities when added to a high-fructose diet [41]. MCFAs enhance thermogenesis in the brown adipose tissue [42], and promote lipolysis in the white adipose tissue activating the hormone-sensitive lipase [43]. Therefore, increasing the MCT concentration from 3 to 6 g/kg/day in rats resulted in significant metabolic effects in multiple organs and tissues, suggesting that at the highest dose, the amount of MCFAs exceeded the capacity of the ketogenic pathway.

In the Y-maze test, the MCT-1 and MCT-3 animals demonstrated an attenuated decrease in the frequency of spontaneous alterations during the second trial, which can be interpreted as an improvement in working memory [44] (Fig. 3d). Interestingly, the MCT-6 animals showed no improved performance in this test. We observed a significant dose-dependent trend in the Morris water maze test; however, only the MCT-6 animals spent significantly more time in the target quadrant during the probe trial, indicating improved spatial memory (Fig. 5b). These findings are in line with some previous studies showing that LCFA-based KD improved performance in the Y-maze [45], and MCT supplementation had a positive effect on spatial memory [46] in rats. On the other hand, MCT-enriched KD demonstrated no positive effect on cognitive performance in 2 murine transgenic AD models [47]. In another study, MCT supplementation attenuated anxiety in highly anxious animals but offered no benefits to animals with normal baseline anxiety levels [48]. Furthermore, we found no effect of MCT supplementation on locomotor/exploratory activity and anxiety, assessed in the Open field test (Fig. 4). Thus, in our study, different concentrations of MCT were required to achieve measurable effects depending on the selected test. In the Y-maze test, 1 g/kg/day and 3 g/kg/day led to similar improvements; however, the effect disappeared at the higher concentration. All these findings highlight the necessity for further detailed studies of different concentrations and administration protocols on various cognitive metrics to define the scope of cognitive functions and impairments, which can be targeted with MCT supplementation without metabolic side effects.

Since the 3 g/kg/day MCT dose exhibited certain procognitive benefits in the Y-maze task and did not result in measurable changes in any tissues or organs, we selected this dose to assess the effects of acute and chronic MCT supplementation on common markers of metabolic health. This 3 g/kg dose, given by orogastric gavage to non-fasted young adult rats, was sufficient to achieve mild intermittent ketosis, elevating the blood KB levels (AcAc + β-HB) 3-4-fold with the peak concentration at around 2 h (Fig. 6).

The acute effects of MCT ingestion on serum glucose, lactate, and pyruvate observed in our study (Fig. 7) are mostly in line with the classical studies in non-fasted rats [49]. However, in our study, the pyruvate level increased while the lactate/pyruvate ratio decreased at 120 min post-administration, which is the opposite of the results reported by Bach's team [49]), who observed a decrease in pyruvate level. This discrepancy may potentially be explained by the differences in dosage and the timing of measurements. A dose-dependent decrease in pyruvate level in Ref. [49] has been observed at 35 min. However, this decrease in male rats was not significant compared to control. The authors did not take a measurement at 120 min. They administered MCT at approximately 1.2 g/kg concentration to assess the dynamics of pyruvate level fluctuations [49]. We used a higher dose, and the elevation of pyruvate in our study coincided with the peak of KB. Therefore, the observed elevation of pyruvate may be explained by the KB's inhibitory effect on pyruvate dehydrogenase [50]. Notably, the fluctuations of pyruvate level reported in Ref. [49] were much more pronounced in female rats than in males. Therefore, the effect of MCT on pyruvate and the lactate/pyruvate ratio may be different depending on the MCT dose and other factors.

Activities of ALT and AST are common biomarkers of liver injury. ALT activity was lower after MCT administration and higher after lard, consistent with some previous reports [51,52]. MCT lowered TG and cholesterol levels at 120 min, consistent with studies in healthy human subjects, and can be explained by reduced chylomicron formation after MCT administration [53,54]. Both fats slightly increased the level of malondialdehyde (MDA), the main end product of lipid peroxidation often used as a biomarker of oxidative stress. Fats are known to affect postprandial MDA levels stronger than other macronutrients [55], and elevated MDA levels are associated with heart disease [56]. Although 4 weeks of our supplementation regime did not elevate the baseline blood MDA level, it is unknown what the result might be after longer interventions or in the case of pre-existing pathological conditions.

Chronic supplementation with MCT (3 g/kg) for 28 days had no significant effect on any measured markers of metabolic health (Fig. 8). When a similar dose of 4 g/kg MCT was used to supplement a fructose-rich diet, 12 weeks of such feeding has been reported to exacerbate the liver damage associated with a high-fructose diet in mice [41]. An even larger dose of 10 g/kg/day of MCT added to

standard feed for 28 days has been reported to increase liver size and reduce HDL cholesterol in rats [57]. These values agree well with our findings in the present study, where 28 days of 6 g/kg/day MCT supplementation protocol resulted in increased liver weight. Therefore, 3 g/kg/day MCT dose used in our study was high enough to improve cognition in young adult rats but low enough to avoid hepatotoxic effects. It must also be highlighted that all the behavioral tests in our study were conducted prior to MCT administration on that day. Therefore, the improved performance in behavioral tests cannot be attributed to ketone bodies acting as an alternative energy source during the test. Instead, it appears to be a cumulative effect of continuous MCT supplementation or regular intermittent ketosis. More detailed studies are needed to better understand how intermittent ketosis may affect cognitive functions between the peaks of elevated KB levels. More studies are needed to determine optimal MCT dosage for procognitive benefits under varying conditions and confirm whether long-term MCT supplementation of a regular diet without carbohydrate or LCFA limitation is safe for cardiovascular and metabolic health.

5. Conclusions

Daily supplementation of regular feed with 6 g/kg/day MCT improved spatial memory in rats (corresponding to 60 g/day dose in human adults) but resulted in significant metabolic changes and cannot be considered safe. These results suggest that it may be necessary to monitor the metabolic effects of MCT supplementation alongside cognitive assessment, especially in human studies, in order to determine the long-term consequences of MCT supplementation of regular diet on cardiovascular health and metabolic health.

Daily supplementation of regular feed with 1–3 g/kg/day MCT improved working memory in young adult rats (corresponding to 10–30 g/day dose in humans) without adverse effects on common markers of metabolic health. Furthermore, the working memory assessment test was conducted when the serum KB concentration had already returned to baseline. Therefore, MCT supplementation resulted in a long-lasting effect in the brain outside the KB peaks of the intermittent ketosis established by the MCT supplementation. Further studies are needed to clarify the mechanisms of these effects.

Author contribution statement

Ksenia Shcherbakova: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Alexander Schwarz, Marina Karpenko: Analyzed and interpreted the data; Wrote the paper.

Irina Ivleva: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Veronika Nikitina: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Darya Krytskaya: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Sergey Apryatin: Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Alexander Trofimov: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data included in article/supp. Material/referenced in article.

Declaration of interest's statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e13446.

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