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Administration of L-carnitine and mildronate improves endothelial function and decreases mortality in hypertensive Dahl rats

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Abstract:
Hypertension is a well established risk factor for the development of cardiovascular diseases and increased mortality. This study was performed to investigate the effects of the administration of L-carnitine or mildronate, an inhibitor of L-carnitine biosynthesis, or their combination on the development of hypertension-related complications in Dahl salt-sensitive (DS) rats fed with a high salt diet. Male DS rats were fed laboratory chow containing 8% NaCl from 7 weeks of age. Experimental animals were divided into five groups and treated for 8 weeks with vehicle (water; n = 10), L-carnitine (100 mg/kg, n = 10), mildronate (100 mg/kg, n = 10) or a combination of L-carnitine and mildronate at the doses above (n = 10). During the experiment, control group animals continued to consume a diet with normal salt content. Administration of the combination significantly improved the survival rate for 50% of the population. None of the tested compounds or their combination influenced high salt intake-induced hypertension, while treatment with mildronate and the combination for 8 weeks significantly decreased resting heart rate by 12% and 10%, respectively. Feeding with high salt diet had no influence on systolic function of the heart, but it induced thickening of the ventricular walls and development of heart hypertrophy that was not improved by the administration of tested compounds. In addition, administration of the combination attenuated the development of endothelial dysfunction in isolated aortic rings. In conclusion, our results demonstrate that treatment with a combination of L-carnitine and mildronate is protective against hypertension-induced complications in an experimental model of salt-induced hypertension.

Key words:
mildronate; L-carnitine; Dahl salt-sensitive rats; hypertension; endothelial dysfunction

Abbreviations: BW – body weight, DS rats – Dahl salt-sensitive rats, GBB – γ-butyrobetaine, IVSd – interventricular septal thickness at end-diastole, IVSs – interventricular septal thickness at end-systole, LVEF – left ventricular ejection fraction; LVFS – left ventricular fractional shortening, LVIDd – left ventricular internal dimension at end-diastole, LVIDs – left ventricular internal dimension at end-systole, LVPWd – left ventricular posterior wall thickness at end-diastole, LVPWs – left ventricular posterior wall thickness at end-systole, LVW – left ventricular weight, UPLC/MS/MS – ultra performance liquid chromatography-tandem mass spectrometry

Introduction

Hypertension is a well established risk factor for myocardial infarction and stroke [13], the development of endothelial dysfunction [17] and atherosclerosis [4]. Large clinical trials have shown that a reduction of arterial blood pressure attenuates the development of complications of hypertension [23, 45]. Nevertheless, it has recently been demonstrated that drugs that do

not have a direct impact on blood pressure were also able to attenuate the development of complications of hypertension [21, 44]; thus, a reduction of blood pressure is not the only therapeutic strategy to prevent complications of hypertension. Some experimental data have indicated that treatment with L-carnitine has a certain impact on the development of hypertension-related complications [5, 9].

L-Carnitine is an amino acid derivative that is essential for the transport of long-chain fatty acids from the cytoplasm into sites of β-oxidation in the mitochondria [12]. Additional supplementation of L-carnitine has been shown to be beneficial in different pathologies that could be induced by hypertension. Results from several studies have proven the beneficial effects of the administration of L-carnitine in the case of cardiovascular pathologies [10], and treatment with L-carnitine has attenuated the development of atherosclerosis [39] and reduced endothelial dysfunction [42]. Recent experimental data have also shown that in some experimental models, the administration of L-carnitine was able to normalize elevated systolic blood pressure [29, 36].

Mildronate [3-(2,2,2-trimethylhydrazinium) propionate] is an inhibitor of biosynthesis of L-carnitine [43] and its reabsorption in kidneys [22]; thus, the administration of mildronate decreases the L-carnitine content in plasma and different tissues [16, 24]. The pharmacological effects of mildronate are based on its regulatory effect on L-carnitine concentration, with subsequent changes to downstream pathways of energy metabolism [8, 25]. Similar to L-carnitine, mildronate treatment has shown an impact on complications and diseases that could be induced by hypertension. The anti-atherosclerotic and angioprotective effects of mildronate have been proven in experimental models of atherosclerosis and diabetes [26, 48]. Moreover, mildronate treatment protected myocardium against ischemia-reperfusion-induced damage [24, 41] and improved cardiac function in experimental models of heart failure [2, 15].

The aim of the present study was to investigate whether the administration of L-carnitine, mildronate or their combination is protective against hypertension-induced complications. Effects on the development of hypertension-induced complications were tested in Dahl salt-sensitive (DS) rats fed with a high salt diet, because during a high salt load, DS rats develop marked hypertension with cardiovascular complications [27, 51].

Our results provide the first experimental evidence that treatment with a combination of mildronate and L-carnitine is protective against hypertension-induced endothelial dysfunction, and at the same time decreases overall morality.

Materials and Methods

Chemicals

Mildronate dihydrate was obtained from JSC Grindeks (Latvia). Ketamine hydrochloride solution (Bioketan) was from Vetoquinol Biowet (Poland) and xylazine hydrochloride solution (Seton 2%) was from Laboratorios Calier (Spain). Acetonitrile and methanol were obtained from Merck (Germany). (2,2-Dimethyl-2-prop-1-yl-hydrazinium)propionate was prepared in-house. L-Carnitine, sodium chloride, potassium chloride, calcium chloride dihydrate, magnesium chloride hexahydrate, sodium hydrogencarbonate, potassium dihydrogenphosphate, glucose, ethylenediaminetetraacetic acid (EDTA), L-phenylephrine hydrochloride, acetylcholine chloride, ammonium acetate and sodium pyruvate were purchased from Sigma (USA).

Experimental animals and protocol

The experimental procedures were carried out in accordance with the guidelines of the European Community and local laws and policies and were approved by the Latvian Animal Protection Ethical Committee of the Food and Veterinary Service, Riga, Latvia.

Six week old male DS rats were purchased from Charles River (Germany) and maintained under controlled conditions of light, temperature and humidity. After a week of adaptation to a new environment, the rats were divided into 5 groups according to treatment and diet: rats of the first group received a normal salt (0.3% NaCl) diet and drinking water (NS group); rats of the second group received a high salt (8% NaCl) diet (Special Diets Services, Great Britain) and drinking water (HS group); rats of the third group received a high salt (8% NaCl) diet and L-carnitine at a dose of 100 mg/kg together with drinking water (HS + C100 group); rats of the fourth group received a high salt diet and mildronate at a dose of 100 mg/kg together with drinking water (HS + M100 group); rats of the

fifth group received a high salt diet and mildronate and L-carnitine, both at doses of 100 mg/kg together with drinking water (HS + CM group). Experimental animals received the experimental diets and test compounds for 8 weeks. The dosing of mildronate, L-carnitine or their combination was confirmed by measuring the consumption of drinking water every 2 days and adjusting the concentration of supplemented substances.

At the beginning of the experiment, when DS rats started to receive diet with high salt load, the concentrations of tested compounds in the drinking water were as follows: L-carnitine 1.004 g/l; mildronate dihydrate 1.406 g/l and in the drinking water of the combination group: L-carnitine 0.876 g/l and mildronate dihydrate 1.086 g/l. At the end of the study when experimental animals consumed larger amounts of drinking water because of high salt diet, solutions of the tested compound had to be more diluted. Thus, at the end of the experiment concentrations of tested drugs in the drinking water were as follows: L-carnitine 0.202 g/l; mildronate dihydrate 0.218 g/l and in the drinking water of the combination group: L-carnitine 0.199 g/l and mildronate dihydrate 0.246 g/l.

Physiological and echocardiographic measurements in DS rats were performed at the beginning of the experiment and 4 and 8 weeks after the treatment. At 15 weeks of age, DS rats were sacrificed under anesthesia. Plasma was taken for the quantitative determination of L-carnitine, γ-butyrobetaine (GBB) and mildronate. The thoracic aorta was dissected for organ chamber experiments as described below. All analyses were performed by an observer blinded to the treatment group.

Physiological and echocardiographic measurements

Systolic blood pressure and heart rate were measured in conscious DS rats at the beginning of the experiment and after 4 and 8 weeks of administration of the test compounds using a Non-Invasive Blood Pressure Controller ML125 connected to PowerLab8/30 system (ADInstruments) and a PC.

Transthoracic echocardiographic studies were performed on DS rats with iE33 ultrasonograph using linear L15-7io transducer (Philips Ultrasound Inc., USA). Rats were lightly anesthetized with an intraperitoneal injection of ketamine and xylazine (50 and 10 mg/kg) and were held in the decubitus position. The rats were allowed to breathe spontaneously during the procedure. M-mode tracings of the left ventricle were recorded at the papillary muscle level to measure left ventricular weight (LVW), interventricular septal thickness at end-diastole (IVSd), interventricular septal thickness at end-systole (IVSs), left ventricular posterior wall thickness at end-diastole (LVPWd), left ventricular posterior wall thickness at end-systole (LVPWs), left ventricular internal dimension at end-diastole (LVIDd), left ventricular internal dimension at end-systole (LVIDs), left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS). The average of three values for each rat was calculated.

Organ chamber experiment

Endothelial function was examined in aortic rings using an organ chamber bath as described previously [3]. In brief, the thoracic aorta was excised, immersed in ice-cold Krebs-Henseleit (K-H) buffer (content in mmol/l: NaCl 118, CaCl2 2.5, $MgCl_2$ 1.64, $NaHCO_3$ 24.88, KH_2PO_4 1.18, glucose 10.0, EDTA 0.05) pH 7.4 and cleaned of fatty and connective tissue. The aorta was cut into 3 mm long aortic rings that were mounted between two stainless steel hooks in oxygenated K-H buffer (pH 7.4, 37°C). The passive tension was fixed at 40 mN. After a period of equilibration (1 h), the maximal contractile function was assessed by application of 60 mM KCl. The aortic rings were then precontracted to 70% of maximal contraction with phenylephrine and the cumulative response to acetylcholine (10^{-10} – 10^{-5} M) was assessed. Relaxation of aortic rings to acetylcholine was expressed as a percent of phenylephrine-induced constriction.

Determination of L-carnitine, GBB and mildronate

The determination of L-carnitine, GBB and mildronate concentrations in the blood plasma samples was performed by ultra performance liquid chromatography-tandem mass spectrometry (UPLC/MS/MS) in positive ion electrospray mode, as described previously [7].

Data analysis

Data were expressed as the mean ± SEM. Statistical calculations were performed using the GraphPad Prism 3.0 software package (USA). Survival curves

were constructed for each group using the Kaplan-Meier method, and comparison of survival distributions among the groups was performed using the log-rank test. For the comparison of the response of aortic rings to acetylcholine, two-way ANOVA with the Bonferroni *post-hoc* test was performed. In case of other parameters differences between means were evaluated by one way ANOVA followed by a Mann-Whitney U test. The differences were considered significant when $p < 0.05$.

Results

Survival rate

Kaplan-Meier analysis revealed that the survival rate of rats receiving a diet with high salt content (HS group) was markedly reduced compared with that of animals from the NS group (Fig. 1). In the HS group after 8 weeks of treatment, the survival rate was 30% (3 of 10 animals), while there were no lethal cases in the NS group. Administration of a combination of L-carnitine and mildronate significantly increased the survival rate compared with the HS group, and after 8 weeks of treatment, the HS + CM group survival rate was 80% (8 of 10 animals). The obtained data showed that there was no statistically significant increase in survival rate in the HS + C100 (survived 8 of 10 animals) and HS + M100 (survived 8 of 10 animals) groups compared with the HS group although the number of survived animals were similar as in HS + CM group.

Systolic blood pressure and heart rate

At the beginning of the experiment, there were no significant differences among the mean systolic blood pressure of animals in all of the experimental groups (Fig. 2A), and the average systolic blood pressure was about 125 mmHg. After 4 and 8 weeks of treatment, the systolic blood pressure of DS rats receiving a high salt diet had increased to above 170 mmHg. As can be seen in Figure 2A, none of the test compounds or their combination influenced systolic blood pressure during the experiment. The analysis of heart rate showed that there were no differences among the heart rate of animals of all of the experimental groups before switching to the high salt diet or on the fourth week of the treatment. However, on the eighth week of the experiment, the mean heart rate in the HS group was 510 ± 10 bpm, but administration of mildronate and a combination of mildronate and L-carnitine decreased it by 12 and 10%, respectively (Fig. 2B).

Organ weight and echocardiographic analysis

High salt loading in DS rats induced hypertension with the subsequent development of left ventricular hypertrophy. As can be seen in Table 1, significant hypertrophy of the left ventricle of the heart was found after 4 weeks of feeding with the high salt diet. In NS group animals, the left ventricle/body weight index (LVW/BW) on the fourth and eighth week of the experiment was 2.8 ± 0.1 g/kg, while in HS group animals, LVW/BW on the fourth and eighth week of the experiment were 3.7 ± 0.1 g/kg and 4.4 ± 0.3 g/kg, respec-

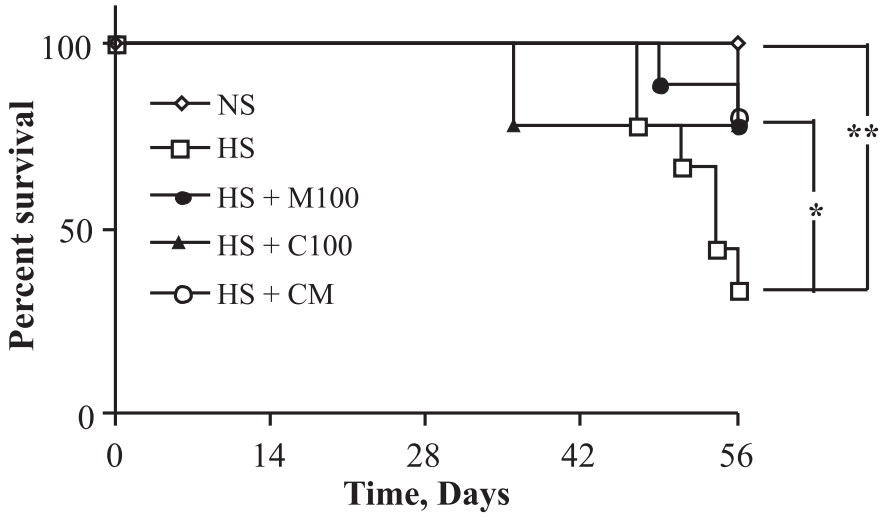

Fig. 1. Kaplan-Meier plots of the survival rate of DS rats fed a normal-salt diet or a high salt diet and treated either with vehicle, L-carnitine, mildronate or their combination. * $p < 0.05$ *vs.* HS group; ** $p < 0.01$ *vs.* HS group

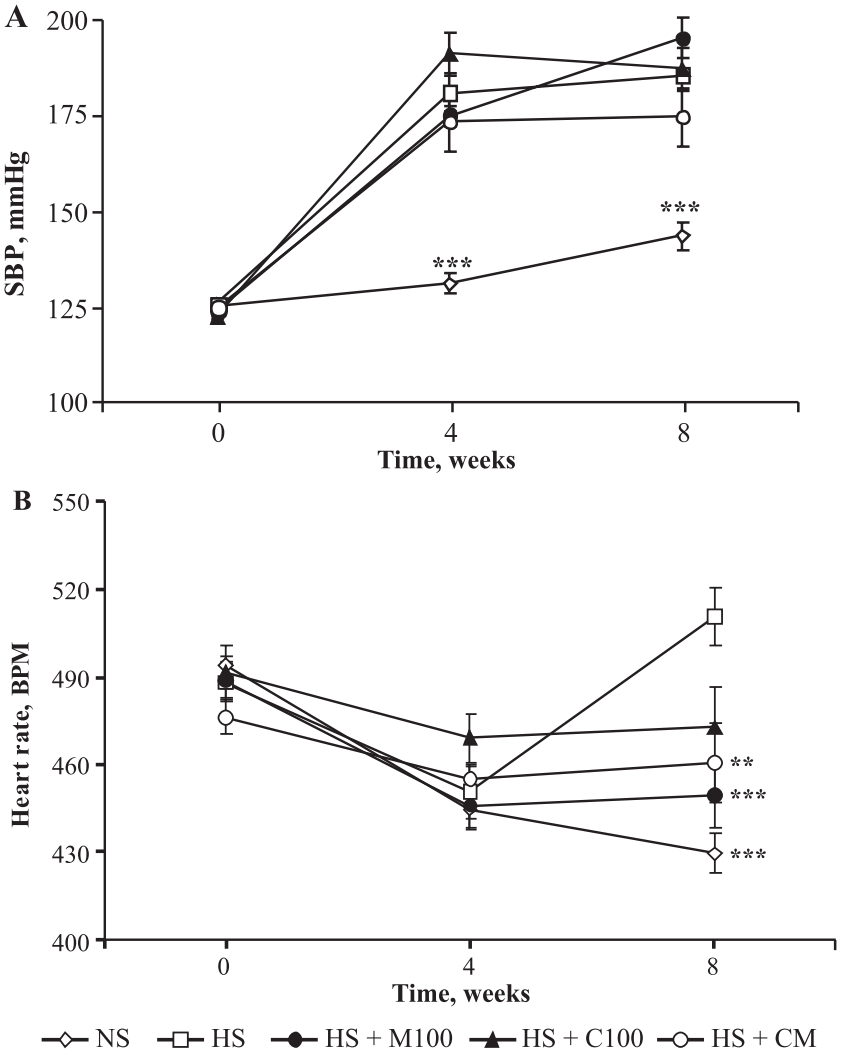

Fig. 2. Time course of systolic blood pressure (**A**) and heart rate (**B**) of DS rats fed a normal-salt diet (NS) or a high salt diet (HS) and treated either with vehicle, L-carnitine (HS + C100), mildronate (HS + M100) or their combination (HS + CM). Results are presented as the mean ± SEM of at least three animals. ** $p < 0.01$ *vs.* HS group; *** $p < 0.001$ *vs.* HS group

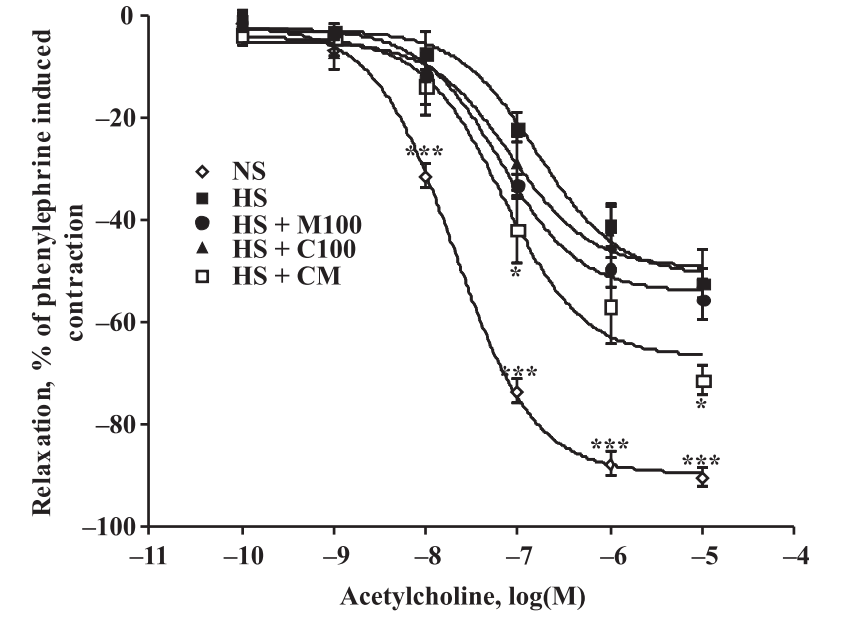

Fig. 3. Endothelium-dependent relaxation to acetylcholine in aortic rings of DS rats fed a normal-salt diet (NS) or a high salt diet (HS) and treated either with vehicle, L-carnitine (HS + C100), mildronate (HS + M100) or their combination (HS + CM). Results are presented as the mean ± SEM of at least three animals. * $p < 0.05$ *vs.* HS group; *** $p < 0.001$ *vs.* HS group

Tab. 1. Body weights and organ to body weight indexes

	BW (g)	LVW/BW (g/kg)	Lung/BW (g/kg)	Kidney/BW (g/kg)
NS group				
0. week	229 ± 6	3.1 ± 0.2	–	–
4. week	362 ± 7**	2.8 ± 0.1**	–	–
8. week	408 ± 9**	2.8 ± 0.1**	4.7 ± 0.7	8.9 ± 0.2**
HS group				
0. week	223 ± 5	3.0 ± 0.1	–	–
4. week	329 ± 7	3.7 ± 0.1	–	–
8. week	334 ± 19	4.4 ± 0.3	5.5 ± 0.9	12.9 ± 1.0
HS + M100 group				
0. week	224 ± 4	2.9 ± 0.1	–	–
4. week	340 ± 6	3.7 ± 0.1	–	–
8. week	342 ± 14	4.6 ± 0.6	5.9 ± 1.0	12.7 ± 0.7
HS + C100 group				
0. week	224 ± 5	3.2 ± 0.1	–	–
4. week	333 ± 4	3.6 ± 0.1	–	–
8. week	300 ± 14	5.3 ± 0.3	5.0 ± 0.5	12.2 ± 0.8
HS + CM group				
0. week	224 ± 5	3.3 ± 0.2	–	–
4. week	337 ± 9	3.7 ± 0.1	–	–
8. week	324 ± 15	4.1 ± 0.3	5.9 ± 1.2	13.4 ± 0.7

The body weight (BW), left ventricle to body weight index (LVB/BW), lung to body weight index (lung/BW) and kidney to body weight index (kidney/BW) of the DS rats before the study and on the fourth and eighth weeks of the experiment. Results are presented as the mean ± SEM of at least three animals. ** $p < 0.01$ *vs.* HS group

tively. In addition, renal hypertrophy was observed in experimental animals receiving diet with high salt load. None of the tested compounds or their combination influenced the development of heart or renal hypertrophy. Moreover, data from our study showed that in DS rats receiving a high salt diet for 8 weeks, significant pulmonary congestion was still absent (Tab. 1).

Results from the echocardiographic analysis are summarized in Table 2. Feeding experimental animals with diet with high salt load induced thickening of the walls of the left ventricle during diastole as well as systole. None of the tested compounds or their combination influenced thickening of the walls of the left ventricle. Analysis of the systolic parameters of the left ventricle (LVEF and LVFS) revealed that there were no significant differences between mean values of the experimental groups (Tab. 2).

Endothelium-dependent relaxation to acetylcholine in aortic rings

Endothelium-dependent relaxation to acetylcholine in the aortic rings of HS group animals was significantly impaired compared with the aortic rings of NS group animals (Fig. 3). Treatment with a combination of mildronate and L-carnitine improved endothelium-dependent relaxation, but the administration of mildronate or L-carnitine alone for 8 weeks had no impact on acetylcholine-induced relaxation (Fig. 3).

L-carnitine, GBB and mildronate content in rat plasma

The UPLC/MS/MS analysis of plasma samples revealed that the consumption of diet with high salt load decreased concentration of L-carnitine nearly 2-fold compared with that of rats consuming diet with normal salt load (Tab. 3). Administration of L-carnitine or combination significantly increased the concentration of L-carnitine in the plasma, but the treatment with mildronate at the dose of 100 mg/kg decreased it. The obtained data showed that the concentration of mildronate in plasma samples of HS + CM group animals was three times lower than in HS + M100 group animal plasma samples (Tab. 3). Moreover, the administration of mildronate, carnitine or their combination significantly increased the concentration of GBB in plasma samples.

Discussion

In the present experimental study, we investigated whether the administration of L-carnitine, mildronate or their combination could attenuate the progression of hypertension-related complications in DS hypertensive rats. The obtained results showed that only administration of a combination consisting of mildronate and L-carnitine had a significant effect on mortality and the development of endothelial dysfunction compared to a vehicle group with no pharmacotherapy that was consuming the same salt diet.

The UPLC/MS/MS analysis of plasma samples revealed that experimental animals from HS group had decreased plasma concentration of carnitine compared with NS group animals (Tab. 3). Differences between both groups could be due to increased excretion of urine in HS group animals (data not shown) as

Tab. 2. Changes in echocardiographic parameters

	NS group	HS group	HS + M100 group	HS + C100 group	HS + CM group
IVSd (mm)					
0. week	1.39 ± 0.01	1.43 ± 0.04	1.40 ± 0.08	1.39 ± 0.03	1.41 ± 0.03
4. week	1.69 ± 0.03*	1.90 ± 0.04	1.88 ± 0.05	1.90 ± 0.04	1.92 ± 0.09
8. week	1.76 ± 0.04*	2.20 ± 0.04	2.32 ± 0.05	2.26 ± 0.06	2.23 ± 0.09
IVSs (mm)					
0. week	2.62 ± 0.02	2.66 ± 0.13	2.42 ± 0.17	2.71 ± 0.16	2.72 ± 0.11
4. week	2.76 ± 0.05*	3.20 ± 0.08	3.26 ± 0.11	3.27 ± 0.11	3.11 ± 0.06
8. week	2.92 ± 0.05*	3.68 ± 0.16	3.76 ± 0.16	3.73 ± 0.12	3.45 ± 0.10
LVPWd (mm)					
0. week	1.48 ± 0.04	1.67 ± 0.11	1.54 ± 0.12	1.71 ± 0.16	1.73 ± 0.12
4. week	1.79 ± 0.06*	2.07 ± 0.06	2.11 ± 0.04	2.05 ± 0.05	2.08 ± 0.04
8. week	1.87 ± 0.05*	2.29 ± 0.04	2.56 ± 0.13	2.73 ± 0.21	2.39 ± 0.17
LVPWs (mm)					
0. week	2.71 ± 0.27	2.86 ± 0.05	2.74 ± 0.18	3.18 ± 0.20	3.10 ± 0.05
4. week	2.81 ± 0.11*	3.22 ± 0.11	3.35 ± 0.05	3.29 ± 0.10	3.36 ± 0.12
8. week	2.94 ± 0.08*	3.73 ± 0.16	3.87 ± 0.16	3.96 ± 0.12	3.72 ± 0.21
LVIDd (mm)					
0. week	7.21 ± 0.12	6.84 ± 0.18	6.59 ± 0.14	6.81 ± 0.45	6.77 ± 0.20
4. week	7.85 ± 0.08	7.86 ± 0.15	8.02 ± 0.11	7.85 ± 0.16	7.90 ± 0.19
8. week	8.05 ± 0.12	7.88 ± 0.19	7.42 ± 0.26	7.01 ± 0.24*	7.73 ± 0.17
LVIDs (mm)					
0. week	3.67 ± 0.01	3.61 ± 0.27	3.44 ± 0.01	3.12 ± 0.33	3.37 ± 0.22
4. week	4.85 ± 0.15	4.72 ± 0.16	4.69 ± 0.15	4.57 ± 0.25	4.68 ± 0.25
8. week	5.01 ± 0.15	4.30 ± 0.33	4.05 ± 0.31	3.66 ± 0.11	4.39 ± 0.25
LVFS (%)					
0. week	50 ± 3	47 ± 3	48 ± 1	54 ± 2	48 ± 1
4. week	41 ± 2	40 ± 1	42 ± 1	42 ± 2	42 ± 1
8. week	41 ± 2	45 ± 4	41 ± 2	47 ± 3	41 ± 2
LVEF (%)					
0. week	84 ± 1	83 ± 3	83 ± 1	86 ± 2	85 ± 3
4. week	75 ± 2	76 ± 2	77 ± 1	77 ± 2	76 ± 2
8. week	77 ± 2	80 ± 4	77 ± 2	82 ± 2	77 ± 3

Interventricular septal thickness at end-diastole (IVSd), interventricular septal thickness at end-systole (IVSs), left ventricular posterior wall thickness at end-diastole (LVPWd), left ventricular posterior wall thickness at end-systole (LVPWS), left ventricular internal dimension at end-diastole (LVIDd), left ventricular internal dimension at end-systole (LVIDs), left ventricular fractional shortening (LVFS) and left ventricular ejection fraction (LVEF) of the DS rats before the study and on the fourth and eighth weeks of the experiment. Results are presented as the mean ± SEM of at least three animals. * $p < 0.05$ *vs.* HS group

Tab. 3. Concentrations of L-carnitine, γ-butyrobetaine and mildronate in the plasma

	NS group	HS group	HS + M100 group	HS + C100 group	HS + CM group
Carnitine (μM)	55 ± 6$^{*,\#}$	23 ± 3$^{\#}$	5 ± 1*	56 ± 4$^{*,\#}$	36 ± 2$^{*,\#}$
GBB (μM)	1.5 ± 0.1$^{\#}$	1.5 ± 0.1$^{\#}$	8.6 ± 1.0*	2.2 ± 0.4$^{*,\#}$	18.1 ± 3.4$^{*,\#}$
Mildronate (μM)	–	–	98 ± 12	–	29 ± 3$^{\#}$

Concentrations of L-carnitine, γ-butyrobetaine and mildronate in plasma were measured by UPLC/MS/MS on the eighth week of the experiment. Values are expressed as the mean ± SEM of at least three animals. * $p < 0.05$ *vs.* HS group; $^{\#}$ $p < 0.05$ *vs.* HS + M100 group

well as developing renal injuries evidenced in other studies [11, 52] that could result in an increased renal loss of L-carnitine. Previous studies that have investigated effects of L-carnitine administration on cardiovascular diseases, including hypertension-related complications, used at least two times higher dose of L-carnitine [5, 9, 32]. In our study we chose the dose of administered L-carnitine that restored plasma concentration of carnitine of HS group animals to the level of NS group animals to study the effects of normal carnitine concentration on the development of hypertension-related complications. Administration of mildronate at the dose of 100 mg/kg induced significant decrease in plasma L-carnitine concentration that was in accordance with previous studies [24, 26]. In addition, simultaneous administration of L-carnitine and mildronate did not restore the plasma concentration of L-carnitine to the level of NS group and plasma concentration of mildronate was lower than found in HS + M100 group animals (Tab. 3). Lower plasma concentrations of both compounds could be due to similar reabsorbtion pathways [14] and influence of mildronate on the synthesis and reabsorbtion of L-carnitine [22, 43]. Despite to lower plasma concentrations of mildronate and L-carnitine in plasma of HS + CM group animals, we found pronounced increase in plasma GBB concentration (Tab. 3). We suppose that the increase in GBB concentration is firstly due to action of mildronate on the concentration of GBB [24] and secondly due to reabsorbed GBB, that could occur as a result of metabolism of L-carnitine [37]. Moreover, we observed elevated GBB concentration in the plasma of HS + C100 group animals, but the increase was not so pronounced probably because of conversion of GBB to L-carnitine [38].

Significant differences in the rate of mortality were noted between HS group and NS group animals, and the survival rate was increased by administration of a combination of L-carnitine and mildronate (Fig. 1). Results from previous studies have shown that there can be several causes of death of DS rats fed on high salt diet at the age of fifteen weeks [35]. Although we did not observe differences between the functional parameters of the heart and lung to body weight indexes of NS group animals and rats receiving a high salt diet (Tabs. 1 and 2), it has been shown that DS rats of that age can die of heart failure [35], while other studies have shown that heart failure develops with further feeding with high salt diet [18, 20]. In addition, data from previous studies have shown that DS rats receiving a high salt diet have a high incidence of lethal strokes [27, 35]. Moreover, DS rats fed with a high salt diet develop marked left ventricular hypertrophy [50] that could also be a cause of myocardial infarction and lethal ventricular arrhythmias [28]. Kamei et al. [19] showed that DS rats have increased susceptibility to ventricular tachycardia that supports the theory about lethal ventricular arrhythmias in DS rats. Although we do not have experimental data in this experimental model on the effects of the tested substances on cardiac arrhythmias and the development of stroke, there is experimental evidence that mildronate and L-carnitine can exert some anti-arrhythmic [31, 47] and cardioprotective [2, 10, 41] properties. It is therefore tempting to speculate that the beneficial effect of the combination of L-carnitine and mildronate on mortality in DS rats could be linked to their cardioprotective and anti-arrhythmic activities.

Analysis of heart rates among the experimental groups revealed that HS group rats on the eighth week of treatment had developed an elevated heart rate compared with NS group rats (Fig. 2B). Previously, it has been shown that resting heart rate is an independent predictor of cardiovascular morbidity and mortality, irrespective of the presence of co-morbidities [33]. This finding was in compliance with our results,

because the highest heart rate was found in HS group animals, as was the highest mortality (Fig. 1). Moreover, the administration of a combination of mildronate and L-carnitine decreased heart rate and significantly reduced mortality. Although mildronate treatment for 8 weeks also decreased heart rate, the protective effect against mortality was not so pronounced ($p < 0.06$ *vs.* HS group) as in the combination group. These findings could also indicate that a reduction of mortality in the HS + CM group is related to the effects of the tested compounds on the heart.

Similar to resting heart rate, endothelial dysfunction has been shown to be associated with the occurrence of cardiovascular events [34] and hypertension-induced organ damage [49]. In our study, the effects of treatment with L-carnitine, mildronate or their combination on the development of endothelial dysfunction were assessed in isolated aortic rings. The angioprotective effects of L-carnitine in hypertension-induced endothelial dysfunction have been already documented [5, 9]. In addition, previous studies have also shown the beneficial effects of mildronate treatment in diseases involving endothelial dysfunction like atherosclerosis [48]. Surprisingly, we did not observe any improvement of endothelial function in aortic rings of HS + C100 or HS + M100 group animals compared to aortic rings of HS group animals (Fig. 3). This could be explained by the two times lower dose of L-carnitine used in our experiment compared to other studies that have shown protective effects of L-carnitine on the development of endothelial dysfunction [5, 9]. While mildronate or L-carnitine treatment had no impact on endothelial dysfunction, administration of their combination attenuated the development of endothelial dysfunction (Fig. 3), but similar to the administration of mildronate or L-carnitine alone, did not reduced blood pressure (Fig. 2A). Although results from a recent study demonstrated a positive relationship between a reduction in blood pressure and an improvement in arterial endothelium-dependent relaxation [6], it has been suggested that the mechanism of attenuation of the development of endothelial dysfunction was based on direct influence on endothelium and vascular tissues, and not on the hypotensive activity [40, 46]. Our results support this theory, as endothelial function was improved without significant effect on arterial blood pressure.

Data from previous studies have shown beneficial effects of the separate administration of L-carnitine or mildronate in the case of different diseases and pathologies, e.g., type 2 diabetes mellitus [26, 30] and cardiovascular diseases [2, 10, 24]. For the first time, our study demonstrates that the simultaneous administration of L-carnitine and mildronate, an inhibitor of carnitine biosynthesis and reabsorption, shows pharmacological effects better than when the two agents are administered separately. Thus, our study showed that L-carnitine and mildronate could share some common properties that are beneficial in the prevention of hypertension-induced complications. Although there is not much experimental evidence about protective effects of GBB, our previous study showed that an increase of GBB concentration induced by administration of mildronate inversely correlated with the size of infarction in experimental heart infarction model in rats *in vitro* [24]. Moreover, it has been shown that GBB protects isolated heart from hydrogen peroxide induced damage [1]. Thus, we can hypothesize that beneficial effects of administration of combination of mildronate and L-carnitine could be due to increased GBB concentration, but further experiments are required to confirm this hypothesis.

In summary, our study demonstrated that treatment with a combination of L-carnitine and mildronate is protective in hypertension-related complications.

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