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Mitochondria-targeting compounds for management of metabolic and hemostatic abnormalities associated with heart dysfunctions in experimental type 2 diabetes

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Objective. Cardiovascular complications are highly prevalent in type 2 diabetes mellitus (T2DM) driven by obesity, dyslipidemia, hypertension, and hypercoagulability associated with insulin resistance. The purpose of this study was to elucidate the effects of combined treatment with acetyl-L-carnitine (ALC), alpha-lipoic acid (ALA), and nicotinamide (NAm) on diabetes-induced metabolic, hemostatic, and heart abnormalities.

Methods. Male non-linear Wistar rats were fed with a high-calorie diet for 2 months followed by a single low-dose streptozotocin injection to induce T2DM. Two weeks later, the diabetic rats received ALC (100 mg/kg), ALA (50 mg/kg), and NAm (100 mg/kg) for 2 weeks in separate daily injections. Fasting blood glucose, glycated hemoglobin (HbA1c), and hemostatic parameters: fibrinogen, protein C, factor X, plasminogen activator inhibitor-1 (PAI-1), were measured. The NAD+ content and NAD^+/NADH ratio were assessed in the heart tissue.

Results. After 12 weeks, blood glucose and HbA1c levels in diabetic rats were 1.8-fold and 2-fold higher, respectively. Diabetes increased fibrinogen (1.5-fold) and PAI-1 (1.7-fold) levels, caused the appearance of soluble fibrin monomers complexes, while protein C and factor X levels were decreased by 18% and 19%, respectively, indicating hypercoagulability and impaired fibrinolysis. In diabetic rats, the cardiac NAD+ level was reduced by 48%. The NAD+/NADH ratio decreased by 2-fold. Combined treatment lowered the glucose levels by 1.3-fold and HbA1c by 1.7-fold and improved the NAD+ metabolism and partially corrected the hemostatic abnormalities.

Conclusion. Co-treatment with ALC, ALA, and NAm improved the glycemic control, partially restored the cardiac NAD^+ metabolism and reduced the hemostatic abnormalities in T2DM suggesting their potential as a safe adjunct therapy for diabetes-associated cardiovascular complications.

Keywords: type 2 diabetes mellitus, coagulation, heart, NAD+, NAD+/NADH ratio, mitochondria-targeted compounds

The type 2 diabetes mellitus (T2DM) is one of the prior health problems affecting millions of people worldwide. The prevalence of diabetes increases rapidly due to the reduced physical activity, population aging, and obesity. The main mechanisms of insulin resistance and islet β-cell dysfunctions

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in T2DM are the oxidative stress, the endoplasmic reticulum stress (ER stress), amyloid deposition in the pancreas, ectopic lipid deposition in the muscles, liver and pancreas, and increased levels of free fatty acids in blood leading to lipotoxicity associated with glucotoxicity (Marushchak et al. 2022; Lu et al. 2024).

The cardiovascular diseases (CVDs) including myocardial infarction, heart failure, and ischemic heart disease, are the major causes for the early death for at least 50% of patients with T2DM (Einarson et al. 2018). The high prevalence of CVDs, as the widespread complication in T2DM patients is related to the common risk factors such as obesity, dyslipidemia, hypertension, and insulin resistance, which lead to the development of chronic inflammation (De Rosa et al. 2018). Moreover, development of cardiovascular abnormalities in T2DM are also related to the hyperglycemia-induced oxidative stress, activation of protein kinase C pathway, and elevation of advanced glycation end-products, which promote the impaired vascular cell functioning (Henning 2018).

The pathogenesis of cardiovascular disorders in large extent is associated with impaired hemostasis. Under physiological conditions, the system of hemostasis promotes the maintenance of blood in a liquid state within the vascular bed. When activated after injury of the vessel, the hemostatic system prevents the blood loss through the formation of platelet plug and fibrin clot. After repair of the damaged vessel, the clot is dissolved to restore the blood flow. However, the persistent hyperglycemia and dyslipidemia in T2DM lead to the development of endothelial dysfunction, which disrupts the balance of coagulation and fibrinolysis. Moreover, the impaired hemostasis can be a risk factor for development of thrombosis, which results from elevated activity of platelets, activation of blood coagulation factors, reduced activity of antithrombin, and decreased levels of thrombomodulin, protein C, and factor X (Addai-Mensah et al. 2019; Sobczak and Stewart 2019; Li et al. 2021).

Impairments of the primary and secondary hemostasis in T2DM are associated with elevated fibrinogen levels due to the activation of its synthesis in hepatocytes under hyperglycemia and insulin resistance. The synthesis of fibrinogen is also stimulated under chronic inflammation caused by diabetes (Li et al. 2021). Moreover, fibrinogen glycation induced by persistent hyperglycemia in T2DM alters its structure that causes formation of the denser clots, which are more resistant to fibrinolysis (Pieters et al. 2007; Luzak et al. 2020).

The insulin resistance also contributes to impaired fibrinolysis through the reduced level of tissue plasminogen activator (tPA) and elevated level of plasminogen activator inhibitor-1 (PAI-1), which is the main physiological regulator of fibrinolysis preventing the excessive lysis of the clots (Sillen and Declerck 2020). Thus, the modulation of PAI-1 activity can be considered as the potent strategy to control fibrinolysis and reduce the risk of thrombosis in T2DM patients.

Nowadays, there is a great interest to study the use of natural biologically active compounds, in particular vitamins, antioxidants, and microelements, as supplements for the additional therapy of diabetic complications including CVDs (Jimenez-Cortegana et al. 2021; Senesi et al. 2021; Barrea et al. 2023). Regarding the role of oxidative stress and ER stress in pathogenesis of CVDs, it was important to investigate whether the supplementation with naturally occurring compounds possessing the antioxidant properties can be a strategy to improve the indexes of hemostasis and to prevent development of cardiovascular complications in T2DM.

From the biologically active compounds with antioxidant properties, we concentrated our attention on alpha-lipoic acid (ALA), acetyl-L-carnitine (ALC), and nicotinamide (NAm). Alpha-lipoic acid is a mitochondrial cofactor of pyruvate dehydrogenase and alpha-ketoglutarate dehydrogenase complexes that improves the mitochondrial bioenergetics and has an antioxidant activity (Rochette et al. 2013). Acetyl-L-carnitine is a compound produced at acetylation of carnitine in the mitochondria, which is involved in lipid oxidation and glucose metabolism. ALC supplementation may have beneficial effects in individuals with the high risk of CVDs (Ruggenenti et al. 2010). Nicotinamide, as a precursor in $NAD(P)^+$ biosynthesis, plays a critical role in fundamental cellular processes including energy metabolism and ATP generation. It is also involved in the development of inflammation, vascular dysfunction, and oxidative stress (Fan et al. 2020). We believe that the combination of these compounds could be more efficient against the negative effects of oxidative stress and inflammation induced by T2DM. Therefore, the aim of this study was to estimate the effects of combined treatment with mitochondria-specific antioxidants (ALC and ALA) with NAm to prevent the metabolic and hemostatic abnormalities induced by T2DM.

Material and Methods

Animals and diabetes induction. Twenty-four two-month-old nonlinear male Wistar rats (180±17 g)

were used in *in vivo* experiments. Animals were housed in Plexiglas cages with a 12-h light/dark cycle and free access to standard diet and water *ad libitum*. After one week of adaptation, the animals were randomly divided into three groups: 1 – control group (control); 2 – diabetic group (diabetes); 3 – diabetic animals treated with the combination of ALC, ALA, and NAm (diabetes+CTr). The animals of the control group were fed with a standard diet of vivarium all along. In the experimental model of T2DM, the animals of groups diabetes and diabetes+CTr were fed with a high-calorie diet containing 54% of the standard diet of vivarium, 25% of fat, 20% of fructose, and 1% of bile for 2 months. After this period of high-calorie diet, a freshly prepared streptozotocin (STZ) in 0.1 M cold sodium citrate buffer (pH 4.5) at a dose of 25 mg/kg was administered intraperitoneally (i.p.) after overnight fasting. Then the animals were housed with a standard diet of vivarium. Two weeks after the injection of STZ, the group of animals (diabetes+CTr) was injected i.p. with the combination of ALC, ALA, and NAm as separate daily injections at a dose of 100, 50, and 100 mg/kg of body weight (b.w.), respectively, for two weeks. Administration of these compounds separately provides the stability, bioavailability, and reproducibility of their individual pharmacological effects. It also promotes their solubility with proper dilution to obtain a small volume of injected solution, as recommended for i.p. injections (Shoyaib 2019). The blood glucose levels were measured by glucometer "Accu-chek" active (Roshe diagnostics, USA).

The oral glucose tolerance test was used to confirm the development of T2DM. After the overnight fasting, the initial level of blood glucose (baseline) was measured. Then, the rats were administered with glucose (2.5 g/kg b.w.) using an oral gavage. Further, the blood glucose levels were measured in 15, 30, 60, and 90 min after glucose administration (Bowe et al. 2014). The data of blood glucose levels versus time were plotted as graphs. The areas under curves (AUC) were calculated by trapezoidal rule.

The blood samples were taken from the retrobulbar sinus and were centrifuged at 4000 g for 10 min to obtain the blood plasma. The levels of glycated hemoglobin (HbA1c) were measured with the Lachema (Czech Republic) kit of reagents by spectrophotometer according to the kit instruction.

After twelve weeks of diabetes duration, the experimental rats were sacrificed via cervical dislocation under mild diethyl ether narcosis. The blood plasma and hearts were used for analysis.

The indexes of hemostasis. Fibrinogen levels in the blood plasma were measured by total clottable fibrinogen assay (Lowe et al. 2004) with some modifications. Fibrinogen was precipitated from citrated blood plasma by thrombin reagent (thrombin from bovine plasma, Sigma-Aldrich) at 37 °C during 30 min. The fibrin clot was removed and dissolved in 1.5% acetic acid. The content of protein in solution was determined by spectrophotometer by measuring the absorption at 280 nm.

The method of qualitative determination of appearance of soluble fibrin-monomer complexes (SFMCs) is based on estimation whether the visible fibrin flakes appear in blood plasma during 120 s after adding the 0.78% solution of o-phenanthroline (SCMC test U/400, Ukraine) according to instruction of the manufacturer.

The protein C level was estimated by chromogenic method based on ability of activated protein C to hydrolyze the specific chromogenic substrate (S-2366 Chromogenix, Sweden) generating the colored p-nitroaniline, which concentration is directly proportional to protein C in the blood plasma (Roshan et al. 2019). The activation of protein C in the samples was performed by protein C activator ("Protac", Pentapharm AG, Switzerland). Factor X level was measured by chromogenic method with chromogenic substrate S-2765 (Chromogenix, Sweden), primarily activating factor X in the samples by RVV factor X activator (RVV-X, Pentapharm AG, Switzerland). The concentration of p-nitroaniline was measured spectrophotometrically at 405 nm. The results were represented as a percent from the control values, which were taken as 100%.

The level of PAI-1 was determined using the commercial ELISA kit (Elabscience, USA) according to instruction of the manufacturer.

Preparation of protein-free perchloric acid extracts of the heart. After sacrification, the hearts of experimental animals were quickly removed and frozen in a liquid nitrogen and grounded to a powdery state. One g of grounded samples was mixed with 7 ml of cold 0.6 N $HClO_4$. The samples were kept on ice 30 min for more complete protein sedimentation. The protein precipitates were separated by centrifugation at 4000 g for 15 min. Then, the supernatants were neutralized by half-saturated solution of KOH to pH 6 and kept on ice for 30 min. The formed potassium perchlorate precipitates were removed by centrifugation at 4000 g for 15 min (Bergmeyer 1974). The neutralized protein-free perchloric acid extracts were used for the further analysis.

Determination of metabolite levels in perchloric extracts of the heart. Lactate, pyruvate, and NAD^+ levels were measured in protein-free perchloric acid

extracts of the heart tissue by enzymatic assays (Bergmeyer 1974). The levels of NAD^+ in aliquots of perchloric acid extracts of the heart tissue were measured spectrophotometrically by their specific reduction to NADH in reaction of ethanol oxidation to acetaldehyde catalyzed by alcohol dehydrogenase (Cornell and Veech 1983).

The cytosolic NAD^+/NADH ratio was determined by the method of steady-state metabolite concentrations and equilibrium constant of lactate dehydrogenase (Williamson et al. 1967).

Chemicals. Streptozotocin (STZ), ALC hydrochloride, ALA, and NAm were of analytical reagent grade quality and purchased from Sigma-Aldrich (St. Louis, Missouri, USA). The other reagents are specified in the text.

Statistical analysis. The statistical processing of experimental data was performed by generally accepted methods of variation statistics. The results of all experiments were expressed by mean±SD (standard deviation). The reliability of differences between groups was determined by the method of one-way analysis of variance (one-way ANOVA) using the computer program Origin v.9.0. The differences were considered as reliable at $p<0.05$.

Ethical statement. All experimental procedures were performed in accordance with the Directive 2010/63/ EU and approved by the Animal Care and Use Committee of Palladin Institute of Biochemistry of NAS of Ukraine Protocol for the Animal Studies and international principles proclaimed by European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, Strasbourg, 1986. All efforts were made to minimize the number of used animals and their sufferings.

Results

At the beginning of the experiments, the average body weight and blood glucose levels in all groups of animals were 180±17 g and 4.7±0.6 mmol/L, respectively. In twelve weeks of duration of experiment, the body mass of animals of the control group was 210±19 g with blood glucose within the same range. The levels of the HbA1c in the control group were 4.2±0.5%. After twelve weeks of diabetes duration, the group of diabetic animals gained weight (225±27 g) compared to the control group. Treatment with the combination of ALC, ALA, and NAm did not influence the body weight of animals. Development of T2DM was proved by elevation of blood glucose and HbA1c levels by 1.8-fold and 2-fold, respectively. Combined treatment caused the reduction of glucose and HbA1c levels by 1.3- and 1.7- folds, respectively.

To prove the development of T2DM, glucose tolerance test was performed (Figure 1A). The glycemic reaction to glucose load for the control group and for the diabetes and diabetes+CTr groups was estimated as AUC. Diabetes caused 36% elevation of AUC index for the group of diabetic animals. Combined treatment caused the partial improvement of this index indicating better glycemic control (Figure 1B).

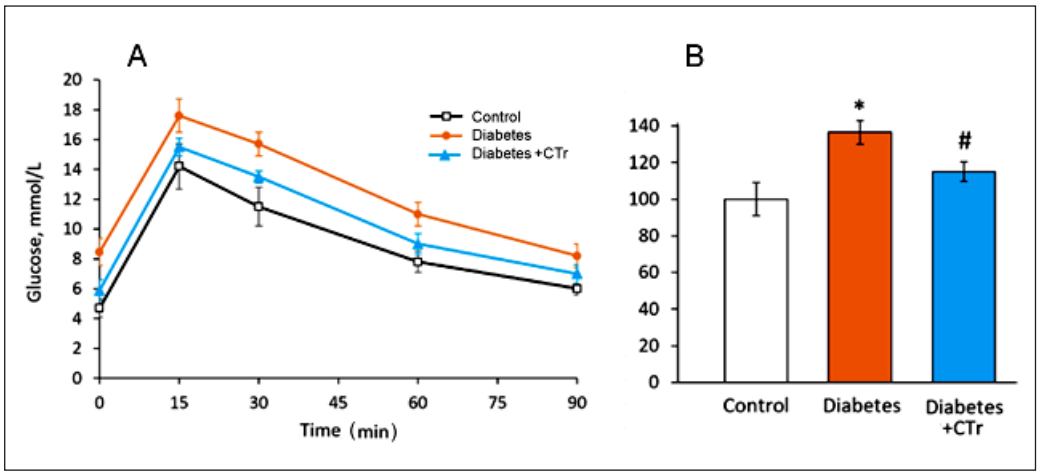

Figure 1. The oral glucose tolerance test: the plot of the blood glucose levels versus time (**A**) and the area under the curves (**B**) in the control group (control), animals with type 2 diabetes mellitus (T2DM) (diabetes), and diabetic rats with combined treatment (alpha-lipoic acid, acetyl-L-carnitine, and nicotinamide) (diabetes+CTr) (n=8). Data are presented as mean±SD; *$p<0.05$ compared to the control group; #$p<0.05$ compared to the diabetic group of rats.

The hemostatic system was altered in T2DM animals. Despite the plasma levels of fibrinogen in the control group (3.4±0.4 mg/ml), in diabetic group, the fibrinogen level was 1.5-fold higher compared to the controls. Combined treatment of diabetic animals led to the reduction of fibrinogen levels by 32% compared to diabetic group (Figure 2).

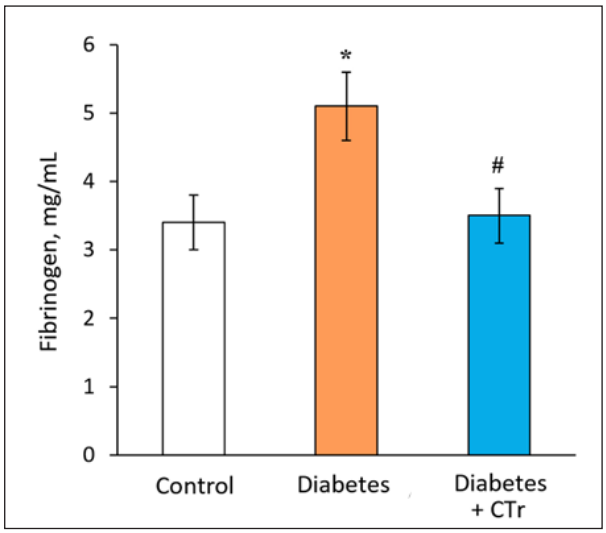

Figure 2. The level of fibrinogen in the plasma of the control group of rats (control), animals with type 2 diabetes mellitus (T2DM) (diabetes), and diabetic rats with combined treatment (alpha-lipoic acid, acetyl-L-carnitine, and nicotinamide) (diabetes+CTr) (n=8). Data are presented as mean±SD; *p<0.05 compared to the control group; #p<0.05 compared to the diabetic group of rats.

In the blood plasma of the diabetic animals, the appearance of SFMCs, which are the markers of hypercoagulability, was established. The levels of protein C (Figure 3A) and factor X (Figure 3B) in the blood plasma of diabetic rats decreased by 18% and 19%, respectively compared to the control group. Supplementation with ALC, ALA, and NAm slightly influenced the diabetes-induced alterations in protein C and factor X levels; however, the observed elevations were within the confidence interval when compared to the group of diabetic animals.

The estimation of indexes of fibrinolysis has shown 1.7-fold elevation of PAI-1 compared to the control group (6.8±0.5 ng/mL). Combined treatment of diabetic rats reduced the levels of PAI-1 by 18% compared to the diabetic group (Figure 4).

Impairment of the hemostatic system is one of the key mechanisms associated with the development of CVDs in T2DM. It often leads to changes of energy homeostasis in the heart tissue, which also makes an important contribution to pathobiochemistry of CVDs. It was established that the level of NAD^+ as well as the ratio of free NAD^+/NADH couples were altered in diabetes. The level of NAD^+ in the heart tissue of the control group was 0.27±0.04 μmol/g. In diabetic rats, the level of NAD^+ in the heart tissue was lower by 48% (0.14±0.02 μmol/g) than those in the control group (Figure 5A). The ratio of NAD^+/NADH in the control group of animals was 274±26. In the group of diabetic animals, the ratio of NAD^+/NADH in the heart tissue was almost 2 times lower (139±17) than those in the control group (Figure 5B).

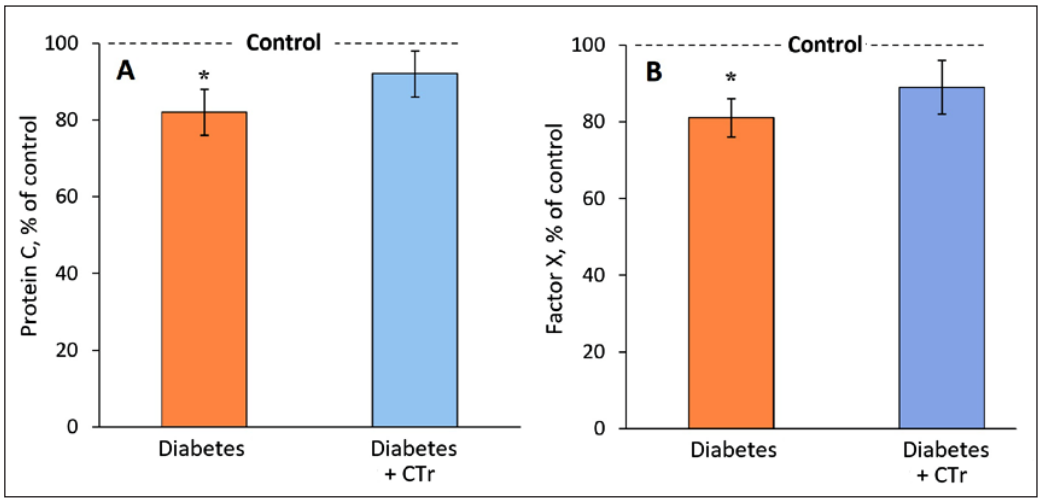

Figure 3. The levels of protein C (**A**) and factor X (**B**) in the plasma of the control group of rats (control), animals with type 2 diabetes mellitus (T2DM) (diabetes), and diabetic rats with combined treatment (alpha-lipoic acid, acetyl-L-carnitine, and nicotinamide) (diabetes+CTr) (n=8). Data are presented as mean±SD; *p<0.05 compared to the control group.

Combined treatment caused the elevation of NAD^+ levels (0.19±0.02 μmol/g) and NAD^+/NADH ratio in the heart tissue (257±24).

Discussion

This study was carried out to assess whether the diabetes-induced abnormalities in the hemostatic system can be attenuated by combined treatment with vitamin B3 (NAm), ALC, and ALA.

For this purpose, we used the high-fat diet (HFD)/STZ model of T2DM modified by fructose load, which nearly imitates the human pathophysiology of the disease. The model of HFD/STZ promotes the development of obesity and insulin resistance followed by injection of a low dose of STZ to reduce the pancreatic β-cell mass causing their partial destroying, thereby, causing faster and stable induction of T2DM (Wickramasinghe et al. 2022). This model reproduces the key characteristics of T2DM such as persistent hyperglycemia, elevated HbA1c, hypoinsulinemia, dyslipidemia, and causes the development of late-stage complications, in particular cardiovascular disease, nephropathy, and hepatic dysfunctions (Sharma et al. 2019). Nevertheless, the combination of HFD and fructose load more effectively induces obesity, dyslipidemia, and hyperinsulinemia compared to the HFD alone creating metabolic conditions that closely mimic the prediabetic stage in humans before the low-dose STZ injection (Barriere et al. 2018; Lozano et al. 2016).

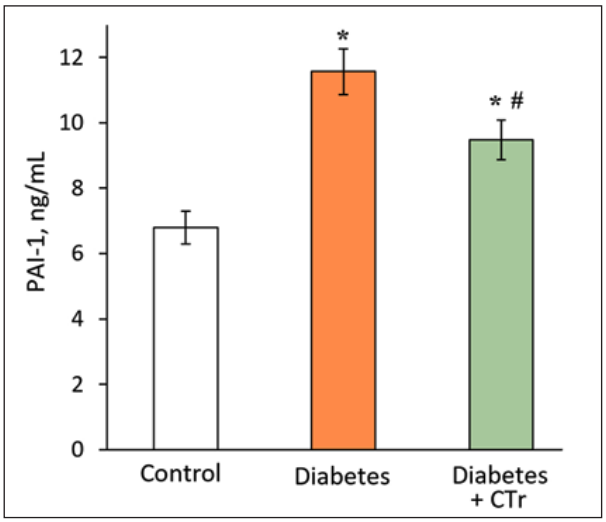

Figure 4. The level of plasminogen activator inhibitor-1 (PAI-1) in the plasma of the control group of rats (control), animals with type 2 diabetes mellitus (T2DM) (diabetes), and diabetic rats with combined treatment (alpha-lipoic acid, acetyl-L-carnitine, and nicotinamide) (diabetes+CTr) (n=8). Data are presented as mean±SD; *p<0.05 compared to the control group; #p<0.05 compared to the diabetic group of rats.

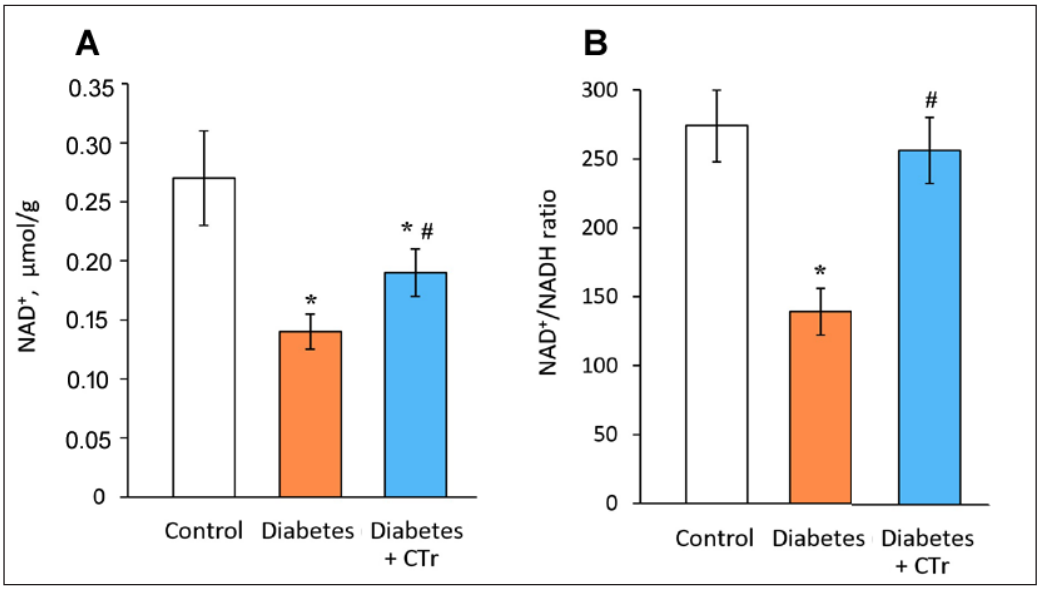

Figure 5. Effects of combined treatment with acetyl-L-carnitine (ALC), alpha-lipoic acid (ALA), and nicotinamide (Nam) on the levels of NAD+ (**A**) and NAD+/NADH ratio (**B**) in the heart tissue of the control group of rats (control), animals with type 2 diabetes mellitus (T2DM) (diabetes), and diabetic rats with combined treatment (ALC, ALA, and NAm) (diabetes+CTr (n=8).Data are presented as mean±SD; *p<0.05 compared to the control group; #p<0.05 compared to the diabetic group of rats.

Our earlier studies have demonstrated that supplementation with NAm or its derivatives such as nicotinoyl-GABA (N-GABA) and N-methylnicotinamide (MNA) as well as the combinations of NAm with other non-toxic naturally occurring biologically active compounds to diabetic animals exerted the neuroprotective effects and improved metabolic alterations induced by diabetes (Kuchmerovska et al. 2010; Kuchmerovska et al. 2019).

Specifically, N-GABA was found to modulate apoptosis-related proteins and upregulate the angiogenesis-related markers, thereby preserving the blood-brain barrier (BBB) integrity and enhancing the metabolic capacity of diabetic brain (Tykhonenko el al. 2022). The NAm and N-GABA may exert the neuroprotective effects against diabetes-induced impairments of the nervous system by improving myelination processes in the brain and partially restoring the functioning of sciatic nerves (Kuchmerovska et al. 2025). Similarly, administration of MNA, a methylated metabolite of NAm produced by nicotinamide N-methyltransferase (NNMT), attenuated the impairments in CNS of diabetic rats. These effects were probably mediated through the modulation of serotonergic system, stabilization of ion gradients, and restoration of NAD^+ levels, potentially via NNMT inhibition (Kuchmerovska et al. 2010).

Our prior work has also suggested that co-administration of NAm with mitochondria-targeted antioxidants such as ALC and ALA offers greater efficacy against the long-term complications of diabetes than their use all alone. Indeed, in type 1 diabetes mellitus (T1DM) model, this combination reduced the oxidative stress, enhanced the energy metabolism, and normalized the activity of Na^+/K^+-ATPase in synaptic membranes isolated from brain (Kuchmerovska et al. 2019). These findings collectively support the hypothesis that targeting mitochondrial dysfunction and oxidative stress through the multicomponent therapy may provide the synergistic neuroprotective effects in diabetes.

Development of diabetes in our study was confirmed by elevation of blood glucose and HbA1c levels by 1.8- and 2-fold, respectively. Treatment with the combination of ALC, ALA, and NAm resulted in the reduction of both blood glucose and HbA1c levels with a more pronounced effect on HbA1c indicating the improved glycemic control. This can be preferably associated with the biological effects of ALC as the component of combined treatment. It is known that carnitine and its derivatives are essential for mitochondrial fatty acid transport and β-oxidation. Disruptions in lipid metabolism are the key contributors to insulin resistance in pathogenesis of T2DM (Bene et al. 2018). Additionally, NAm, a precursor of NAD^+, may influence the level of glycemia because of its critical role in cellular metabolism and energy generation (Fan et al. 2020).

The oral glucose tolerance test was used as an additional tool to confirm development of T2DM and to check the influence of co-treatment on overall glycemic control (Figure 1A). It was established 36% elevation of AUC parameter (group diabetes), while in the group diabetes+CTr, the value of AUC was only 15% higher than those in the control group indicating the improved glycemic control (Figure 1B).

In conditions of persistent hyperglycemia, the elevated level of HbA1c and insulin resistance result in impaired hemostasis (Li et al. 2021). Indeed, in our study, it was evidenced by the appearance of soluble fibrin monomer complexes and by alterations in plasma levels of fibrinogen, PAI-1, protein C and factor X, which are the key components in regulation of coagulation and fibrinolysis. It was demonstrated 1.5-fold increase of plasma fibrinogen level in response to elevation of HbA1c and fasting blood glucose concentration (Figure 2). Considering the central role of fibrinogen in atherogenesis and thrombosis, its elevation may indicate the higher risks of CVDs development. Noteworthy, the clinical studies have shown that fibrinogen levels in T2DM patients correlate with the levels of HbA1c, age, BMI, and hypertension (Bembde 2012, Razak and Sultan 2019). Combined treatment with ALC, ALA, and NAm effectively reduced the level of fibrinogen almost to those in the control group (Figure 2) suggesting their indirect positive effect on indexes of hemostatic system, which may result in reduced risks of thrombosis, cerebrovascular, and cardiovascular complications. Our findings generally coincide with the data of other researchers, who have shown that vitamin C supplementation alone or in combination with vitamin E, but not vitamin E alone, reduced the level of plasma fibrinogen and LDL cholesterol in STZ-induced T2DM Sprague Dawley albino rats (Talat et al. 2011). We hypothesize that the observed improvements in glycemic control and reduction of fibrinogen levels may derive from the anti-inflammatory and antioxidant properties of the components of co-treatment. Fibrinogen also belongs to acute-phase proteins and its elevation can be associated with development of systemic inflammation, which is typical for T2DM. Moreover, fibrinogen glycation in diabetes can lead to formation of more stable fibrin clots. The long-term glycemic control may decrease

fibrinogen glycation, improving clot degradation, and reducing thrombotic risk. The lowering of fasting blood glucose and HbA1c, observed in our study under combined treatment in long-term experiments potentially may also reduce fibrinogen glycation and prevent the formation of more stable clots.

Moreover, we observed that elevation of fibrinogen was accompanied by the appearance of soluble-fibrin-monomers complexes, which are the intermediates of fibrin clot formation and markers of thrombin generation and activation of coagulation. In normoglycemic conditions and balanced hemostasis, the concentration of SFMCs is typically low (Potaskalova et al. 2019). The appearance of SFMCs in the blood plasma of diabetic rats may be a signal of predisposition to thrombosis.

To evaluate the anticoagulant balance, it was important to assess the level of protein C, which is a vitamin K-dependent anticoagulant proenzyme that once activated degrades factors Va and VIIIa inhibiting coagulation. In blood plasma of diabetic rats, the protein C level was 18% decreased compared to those in the control group (Figure 3A) contributing to the hypercoagulability. Noteworthy, it has been demonstrated that in T2DM patients the alterations in levels of both pro-coagulant and anticoagulant proteins are associated with uncontrolled glycemia and can be reversed by better glycemic control (Sobczak and Stewart 2019). Despite the improved glycemic parameters in the group of diabetic animals treated with ALC, ALA, and NAm, the level of protein C was practically not changed (Figure 3A) most likely due to the relatively short time of co-treatment.

We also observed a 19% decrease in factor X level in the group diabetes compared to control (Figure 3B), suggesting the impaired mechanisms of coagulation. In T2DM patients, the altered hemostasis is often accompanied by elevation of factor X levels (Sobczak and Stewart 2019). However, in another report concerning patients with T2DM (Ceriello et al. 1990), it was also found the lowering of factor X levels. We suppose, it may be a result of more complex regulation of pro- and anti-coagulant proteins, such as factor X, thrombin, antithrombin III, fibrinopeptide A, etc. Combined treatment slightly elevated the factor X levels in the blood plasma (Figure 3B).

Development of inflammation, which is typical for both T1DM and T2DM, is associated with metabolic disorders also including the imbalance of coagulation and fibrinolysis, which is manifested by elevation of blood plasma PAI-1 level. Indeed, it was found 1.7-fold increase of PAI-1 level in diabetic rats compared to control (Figure 4). It should be noted that the PAI-1 gene is stress-regulated, since mental and physical stress-factors may negatively influence the fibrinolytic activity, potentially increasing the risk of thrombotic complications (Sandrini et al. 2020). In patients with T2DM, the elevation of PAI-1 is mediated by vascular inflammation, decreased NO synthesis, increased formation of reactive oxygen species (ROS), elevated production of endothelin-1, and tissue factor (TF) and contributes to vasoconstriction, impaired fibrinolysis, and increased risk of thrombosis (Pechlivani and Ajjan 2018; Speelman et al. 2022). Combined treatment with ALC, ALA and NAm led to the partial decrease in PAI-1; however, its level still remained 1.4 times higher than in the control group of animals (Figure 4). We suppose that the effects of combined treatment on PAI-1 levels can be mediated through the reduction of systemic inflammation by the compounds, which possess the antioxidant properties.

The results of our study showed that the hyperglycemia-induced alterations in key hemostatic factors, in particular, fibrinogen, PAI-1, protein C and factor X levels, as well as the appearance of SFMCs, reflect the T2DM severity and duration. It is not excluded that combined treatment with mitochondria-targeted antioxidants partially corrected the alterations of hemostatic parameters likely through the reduction of oxidative stress and inflammation, thereby reducing hypercoagulation.

In turn, hypercoagulation is the risk factor of cerebrovascular and cardiovascular complications in T2DM. Consequently, it was important to assess whether these complications in T2DM are associated with impaired myocardial energy metabolism, NAD^+ depletion, and reduced NAD^+/NADH ratio as a marker of the cellular redox status. The impaired NAD^+/NADH ratio contributes to oxidative stress, mitochondrial dysfunctions, and progression of metabolic diseases, including T2DM. The proper maintenance of this ratio is essential for energy production, redox homeostasis, and activity of NAD-dependent signaling pathways important for cell survival and functioning (Fan et al. 2020).

In the current study, persistent hyperglycemia led to 1.9-fold lowering of NAD^+ (Figure 5A) and almost 2-fold decrease of NAD^+/NADH ratio (Figure 5B) in heart tissue of diabetic rats compared to the control group. Notably, the processes of energy generation in myocardium depend on glucose and fatty acid oxidation as well as lactate utilization, all of which require the sufficient NAD^+ availability. Thus, the impaired NAD^+ metabolism and NAD^+/NADH ratio may result in improper course of aerobic

energy-generating mechanisms in the heart tissue of diabetic rats. Altered NAD^+ metabolism may also decline cardiac functions through the multiple pathogenic mechanisms including hyperacetylation of proteins, reduced enzymatic antioxidant activity, enhanced ROS generation, and suppression of AMPK signaling (Chiao et al. 2021). Previously we have shown that NAm, as the precursor of NAD^+ synthesis, also counteracts to development of diabetes-induced alterations in the rat liver improving its detoxifying functions, reducing the oxidative stress, and normalizing the redox state of free cytosolic NAD^+/NADH couples (Kuchmerovska et al. 2023). Moreover, the metabolic and hemostatic abnormalities in T2DM are closely related to hepatic dysfunction, oxidative-nitrosative stress, and disordered protein synthesis and degradation. Since the liver is the principal source of the most circulating plasma proteins, including fibrinogen, albumin, and apolipoproteins, the changes in their content serve as sensitive indicators of systemic metabolic and functional recovery (Liu et al. 2025).

Importantly, the combined treatment with ALC, ALA, and NAm resulted in 36% increase of NAD^+ level (Figure 5A) in the heart tissue in group (Diabetes + CTr) compared to diabetic animals. The NAD^+/NADH ratio in heart tissue of diabetic rats after the co-treatment was practically restored. These improvements in treatment with ALC, ALA, and NAm can be mediated through the synergistic mechanisms including enhanced mitochondrial fatty acid transport (via ALC), supply of exogenous NAD^+ precursor (via NAm), and reduced oxidative stress and inflammation (via ALA). Collectively, these effects suggest a promising therapeutic potential of this combination to prevent/reduce metabolic and cardiovascular dysfunctions in T2DM.

Conclusion

Our findings suggest the benefits of combined treatment of T2DM with ALC, ALA, and NAm by chosen doses of each of the components for prevention of diabetic complications development and thus providing a rationale for the further clinical studies. However, it cannot be excluded that the other doses of the components and other terms of co-treatment could be more efficient for treatment of T2DM and its complications at least as adjunct therapy.

Conflict of interest: *The authors declare no conflict of interest.*

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