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Nicotinamide prevention in diabetes-induced alterations in the rat liver

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Objective. The study was performed to elucidate whether nicotinamide (NAm) can attenuate the diabetes-induced liver damage by correction of ammonia detoxifying function and disbalance of NAD-dependent processes in diabetic rats.

Methods. After four weeks of streptozotocin-induced diabetes, Wistar male rats were treated for two weeks with or without NAm. Urea concentration, arginase, and glutamine synthetase activities, NAD+ levels, and NAD+/NADH ratio were measured in cytosolic liver extracts. Expression of *parp-1* gene in the liver was estimated by quantitative polymerase chain reaction and PARP-1 cleavage evaluated by Western blotting.

Results. Despite the blood plasma lipid peroxidation products in diabetic rats were increased by 60%, the activity of superoxide dismutase (SOD) was reduced. NAm attenuated the oxidative stress, but did not affect the enzyme activity in diabetic rats. In liver of the diabetic rats, urea concentration and arginase activity were significantly higher than in the controls. The glutamine synthetase activity was decreased. Decline in NAD+ level and cytosolic NAD+/NADH ratio in the liver of diabetic rats was observed. Western blot analysis demonstrated a significant up-regulation of PARP-1 expression accompanied by the enzyme cleavage in the diabetic rat liver. However, no correlation was seen between mRNA expression of *parp-1* gene and PARP-1 protein in the liver of diabetic rats. NAm markedly attenuated PARP-1 cleavage induced by diabetes, but did not affect the *parp-1* gene expression.

Conclusions. NAm counteracts diabetes-induced impairments in the rat liver through improvement of its detoxifying function, partial restoration of oxidative stress, NAD+ level, normalization of redox state of free cytosolic NAD+/NADH-couples, and prevention of PARP-1 cleavage.

Key words: type 1 diabetes mellitus, nicotinamide, urea cycle, NAD+, NAD+/NADH ratio, *parp-1* gene, PARP-1

Diabetes is a worldwide chronic epidemic accompanied by functional, metabolic, and morphological alterations (Lin et al. 2020). Type 1 diabetes mellitus (T1DM) is predominantly an autoimmune disease characterized by a near or a total lack of insulin secretion due to the destruction or even demise of β-cells leading to dependence of diabetic patients on exogenous insulin for survival (DiMeglio et al. 2018). If not treated well, it leads to development of numerous complications including cardiovascular pathologies, retinopathy, nephropathy, liver pathologies, neuropathy, etc. (Powers 2021). However,

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the mechanisms of their development at both types of diabetes are not completely understood (Mertens et al. 2021).

To study the development of diabetes-induced complications, animal models of streptozotocin-induced (STZ) diabetes are widely used. They are considered as most relevant, especially for T1DM. STZ destroys β-cells in experimental animals and induces structural, functional, and biochemical changes similar to those occurring in the human diabetes (Furman 2021). We have previously shown *in vitro* that nicotinamide (NAm) can protect the pancreatic cells of the rats Langerhans islets against the cytotoxic effects of STZ. However, the protective effects of NAm vary depending on its concentration (Kuchmerovska et al. 2012).

In both types of diabetes, the development of complications is largely associated with oxidative stress caused by chronic hyperglycemia (Ighodaro 2018; Aranda-Rivera et al. 2022). Overproduction of reactive oxygen species (ROS) and the oppressed activity of the enzymes of antioxidant defence system result in an activation of lipid peroxidation and damage of macromolecules such as proteins and nucleic acids. These alterations can also cause the impairment of cellular signaling pathways. One of the key enzymes of antioxidant defence system is superoxide dismutase (SOD, EC 1.15.1.1), which activation leads to reduction of superoxide radical level. It means that assessment of SOD activity in the blood plasma and tissues at diabetes allows to characterize the efficiency of antioxidant defence system in dependence on the severity of oxidative stress development.

Under T1DM, the deficiency of insulin stimulates the processes of protein and amino acid degradation. It results in an elevated formation of ammonia in the tissues and organs and an enhanced flux of its transport forms to the liver as the major cite of detoxication. In the liver, the urea cycle is functioning as a nitrogen clearance system. It converts ammonia produced at breakdown of proteins and other nitrogen-containing molecules into urea that plays a key role especially under pathological conditions. It should be emphasized that even partial deficiency of urea cycle enzymes may lead to accumulation of ammonia, which is harmful for brain. That is why the activity of urea cycle enzymes can be used to estimate the efficiency of urea cycle functioning in the liver. From all the enzymes of urea cycle, arginase (EC 3.5.3.1) deserves special attention. Changes of its activity can be associated with the development of diabetes mellitus complications due to the important role of this enzyme in the regulation of arginine metabolism, nitric oxide production, and inflammatory response (Ren et al. 2022). In addition, urea cycle and glutamine synthetase (EC 6.3.1.2) are the two main pathways for waste nitrogen removal. Glutamine plays an important role in intermediary metabolism as a substrate for protein, nucleotide, and amino sugar synthesis. It is important for transport of ammonia between tissues; it maintains energy production, and metabolism (Cruzat et al. 2018). Glutamine is also involved in multiple metabolic pathways such as gluconeogenesis, glycemic control, synthesis of nitric oxide, and γ-aminobutyric acid. It maintains L-glutamine metabolism and ammonia homeostasis (Darmaun et al. 2019). The endogenous glutamine synthesis, which is provided by glutamine synthetase, makes important the enzyme activity determination in the liver to estimate the response of glutamine exchange to development of diabetes.

Nowadays, there are many approaches for treatment of both types of diabetes, which use a wide range of synthetic medical drugs. However, the great attention is also devoted to the natural biologically active compounds as additional therapy. In our investigations we used NAm, which preferably realizes its function through the nicotinamide adenine dinucleotide (NAD+) playing a critical role in cellular metabolism. NAD+ is essential for energy exchange and ATP production and it is involved in cellular pathways that are impaired under pathological processes such as inflammation, vascular dysfunction, and oxidative stress. Moreover, the changes in NAD+ metabolism are accompanied by alterations of free NAD+/NADH couple ratio in the kidneys, neurons, heart, and retina leading to disorder of regulatory processes in different organs and tissues (Fan et al. 2020).

It is important to emphasize that NAD+ is not only an electron carrier, but it is also utilized in metabolic pathways as a substrate. The pathway of poly(ADP)-ribosylation of proteins is catalyzed by poly(ADP-ribose) polymerase (PARP, EC 2.4.2.30). One of the PARP family enzymes is poly(ADP-ribose) polymerase-1 (PARP-1), which deserves a considerable attention due to its localization in the nucleus. PARP-1 activation is involved in DNA repair, chromatin remodeling, transcriptional regulation, etc. (Kraus and Hottiger 2013; Dillmann 2019). At the same time, overactivation of PARP-1 under pathological condition can lead to impairments of metabolic and/or signaling pathways in different cells and tissues. Indeed, the activation and overexpression of PARP-1 is seen at many pathological

conditions associated with oxidative stress, such as diabetes, arthritis, and neurodegenerative disorders (Pazzaglia and Pioli 2020). Therefore, the overactivation of PARP-1 can be a reliable marker of oxidative-nitrosative and genotoxic stress in the tissues.

As reported previously, we have shown that PARP-1 is overactivated in diabetes causing a depletion of NAD+ and impaired NAD+/NADH balance in the brain cells nuclei (Kuchmerovska et al. 2004). NAD+-dependent silent information regulator proteins (sirtuins, SIRT, EC 2.3.1.286) pathways use NAD+ for protein deacetylation. During this process, NAD+ is degraded to form NAm and 2'-O-acetyl-ADP ribose. We established that decreased ratio of free NAD+/NADH couples is accompanied by alterations of SIRT1 and SIRT2 expression in brain cell nuclei of diabetic rats that can lead to development of brain dysfunctions (Guzyk et al. 2019). Noteworthy, under the condition of increased NAD+ consumption by sirtuins, NADH is more effectively used to maintain the redox balance (Connell et al. 2019).

There exist some studies directed to investigations of protective effect of NAm and other NAD+ precursors on prevention of diabetes-induced complications in the brain and kidneys and their treatment (Lee and Yang 2019; Chandrasekaran et al. 2020; Yasuda et al. 2021). The results of our previous studies on the animal models have demonstrated that NAm administration improved, at least partially, the metabolic processes in retina (Guzyk et al. 2016) and brain also in combination with acetyl-L-carnitine and α-lipoic acid (Guzyk et al. 2017; Kuchmerovska et al. 2019) impaired by diabetes. However, the mechanisms concerning the putative protective NAm action on liver disorders induced by T1DM are not still completely elucidated.

The aim of the present study was to find out whether NAm administration is able to exert hepatoprotective effects attenuating diabetes-induced damage in the liver by correction of its ammonia detoxifying function, alterations of NAD-dependent processes, and some biochemical and metabolic parameters in diabetic rats.

Materials and Methods

Ethics 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 (Protocol #1, 26/01/2023) 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.

Chemicals. All chemicals were of analytical reagent grade quality and purchased from Sigma-Aldrich (St. Louis, Missouri, USA), except of those listed below or otherwise specified in the text. Streptozotocin (STZ, S0130), nicotinamide (NAm, N0636), alcohol dehydrogenase (74931), and lactate dehydrogenase (427211) were purchased from Sigma-Aldrich.

Animals and diabetes induction. Twenty-five male Wistar rats (150–170 g) were used for *in vivo* experiments. Animals were randomly divided onto three groups: 1) control group (Control); 2) diabetic group (Diabetes, D); 3) diabetic group treated with NAm (D+NAm). The rats were housed under standard chewing diet and water *ad libitum*. After one week of acclimation period, the T1DM was induced in the rats by a single intraperitoneal (i.p.) injection of a freshly prepared solution of STZ in 0.1 M cold sodium citrate buffer (pH 4.5) at a dose of 65 mg/kg after overnight fasting. During one week after STZ injection, four animals have died spontaneously. Four weeks after the induction of diabetes, the group of diabetic animals (D+NAm) was treated with NAm at a dose of 100 mg/kg of body weight during two weeks. After six weeks of diabetes duration, the fasting blood glucose levels were measured with Accu-Chek (Roshe Diagnostics, Switzerland). The rats with a blood glucose level 21.2±2.9 mmol/L were considered as diabetic. Moreover, three rats were excluded from the experiment because they did not match the trend. After six weeks of the diabetes duration, the experimental rats were sacrificed via cervical dislocation under mild diethyl ether narcosis. The liver and blood plasma were used for analysis.

Preparation of protein-free perchloric acid and cytosolic extracts of the liver. After sacrification followed by decapitation, the portion of livers of experimental animals was quickly removed and frozen in liquid nitrogen and treated with $HClO_4$ (1:7 w/v). Then liver samples were centrifuged at 4000 g for 10 min. After centrifugation, they were neutralized with 49% KOH (some drops) to pH 7.0 and then centrifuged at 4000 g for 5 min to remove the insoluble $KClO_4$ (Bermeyer and Bernt 1974). Another part of liver tissue was homogenized in 0.25 M sucrose at 4°C and centrifuged at 12 000 g for 15 min. Then Ca^{2+} and Mg^{2+} ions in 10 mM Tris-HCl buffer were added to supernatants, the pH was 7.4. Received samples were centrifuged at 10 000 g for 10 min. The precipitates of microsomal fraction were

removed and the received samples of liver supernatants used in experiments as cytosolic fractions.

Protein samples preparations. Freshly isolated portion of rat livers were snap-frozen in liquid nitrogen and finely ground. Then, the liver tissues from all investigated animals were lyzed and proteins extracted in RIPA buffer (20 mM tris-HCl, pH 7.4) containing 0.15 M NaCl, 1% sodium dodecylsulphate, 1 mM ethyleneglycoltetraacetic acid, 2.5 mM ethylenediaminetetraacetic acid, 6.5 μM aprotinin, 1.5 μM pepstatin A, 23 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 5 μg/mL soybean trypsin inhibitor, 1 μM sodium orthovanadate (tissue: buffer 1:9, w/v). All lysates were sonicated three times for 30 s using an ultrasonic disintegrator "Sartorius" (Labsonic® M) and centrifuged at 16 000g for 30 min at 4 °C, as previously described (Tykhonenko et al. 2022). The protein concentration in supernatant samples was determined spectrophotometrically as described elsewhere (Stoscheck 1990). The supernatants were diluted in 5 × Laemmli sample buffer supplemented with 0.1 M dithiothreitol, and boiled for 5 min. Protein samples were frozen and stored at –20 °C before analysis.

Western blot analysis. The blotting of liver proteins was performed on a Mini Trans-Blot Cell device (Bio-Rad, USA) at a voltage of 100 V for 90 min. Protein samples (75 μg/track) were run in 7% or 10% denaturing gels and transferred on nitrocellulose membrane (RPN 203D, GE Healthcare, Amersham Bioscience) with 0.45 μm pore diameter. After blotting, the membranes were blocked for 90 min at 37 °C with 5% (w/v) non-fat dried milk and diluted in phosphate buffer saline containing 0.05% Triton X-100 (v/v) (PBST) according to manufacturer's recommendations and incubated overnight at +4 °C with primary PARP (Cell Signaling Technology, #9542, 1:1000), or beta actin (Abcam, ab20272, 1:5000) antibodies. Then membranes were washed 5 times with PBST and incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies diluted in PBST for 60 min. The unbound antibodies were removed by 7-times washes in PBST for 5 min each. Immunoreactive bands were visualized using the enhanced chemiluminescence method (ECL). Densitometric analysis of the autoradiographs was performed using densitometry software TotalLab TL120 (Nonlinear Inc., USA) and normalized to the intensity of the respective bands obtained for β-actin.

Quantitative reverse transcription-polymerase chain reaction (RT-PCR). RT-PCR is an experimental method for amplification, isolation or identification of a known mRNA sequence. Isolation of RNA from the liver tissue was performed as it was previously described (Kuchmerovska et al. 2021). Brifly, the amount of total RNA isolated by extraction in the presence of impurities of proteins and carbohydrates from the liver tissue specimens was determined by the optical density of solutions at 260 nm and by the ratio A260/A280 and A260/230. The total RNA was reverse transcribed into the end product, which is known as complementary DNA (cDNA) in PCR with the detection of products in real time according to method of Heid et al. (1996). As it is known, DNA is more stable than RNA that is the reason why the total liver tissue RNA was used as a template for the synthesis of cDNA using the kit "QuaniTect Reverse Transcription" (QIAGEN, Germany). The obtained cDNA was used to perform a quantitative PCR. The following primers were used to amplify of *parp-1* mRNA: forward 5'-AAGGTCAAGAAGACCGCAGA-3' and reverse 5'-AGAGGAGGCTAAAGCCCTTG-3'. The expression of *β-actin* mRNA served as an additional control for the amount of RNA taken for analysis. The following primers were used to amplify of *β-actin* mRNA: forward 5'-CGTACCACTGGCATCGTGAT-3' and reverse 5'-GTGTTGGCGTACAGGTCTTT-3'. RT-PCR was performed on a CFX96 apparatus (Bio-Rad, USA). Four independently selected RNA preparations were used to perform the quantitative PCR. The results were calculated using the program CFX95 (Bio-Rad) and the graphs constructed in Microsoft Excel.

The biochemical assays. Urea concentration in the liver cytosolic extracts was determined by diagnostic kit of "Felicit diagnostics" for determination of urea by colorimetric method at 470 nm according to the kit instruction.

The activity of arginase was determined in cytosolic liver extracts by measuring the amount of urea produced in reaction samples using the method Paik et al. (1984) with some modifications. The samples were prepared in 1.5 mL of 0.2 M glycine buffer (pH 9.5) containing 0.5 mL liver cytosolic extract, 0.2 mL 5 μM $MnCl_2$, and 0.4 mL of 50 μM L-arginine. 1 mL of each sample was incubated for 10 min at 37°C, and then the reaction was stopped by adding 0.25 mL of 3% (w/v) diacetyl monoxime. After centrifugation, the samples were kept in an ice bath for 5 min, and then the content of urea in the samples was measured spectrophotometrically at 480 nm. The activity of arginase was expressed in μmol of urea/mg protein/min.

The activity of glutamine synthetase in reaction mixture containing ammonia (Tate et al. 1972) was

determined by measuring the inorganic phosphate by Fiske and Subbarow (1925) method. The enzyme activity was expressed in μmol of Pi/mg protein/min.

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 lipid peroxidation products in the blood plasma were measured using thiobarbituric acid (TBA) according to the method of Ohkawa et al. (1979) with some modifications. Briefly, the mixture containing 100 μL of blood plasma and 200 μL of trichloroacetic acid was kept 15 min at cooling and then centrifuged at 2200 g at 4 °C for 15 min. 200 μL of 0.67% solution of TBA was added to 200 μL of supernatant and kept on the boiling water bath for 20 min. The level of thiobarbituric acid reactive species (TBARS) was determined by measuring the absorbance of the samples at 532 nm and was expressed in nmol/mL.

The activity of the SOD in the blood plasma of rats was measured by the method based on ability of SOD to inhibit the reduction of nitro blue tetrazolium (NBT) to yellow colored formazan (Sun and Oberley 1988). The unit of SOD was estimated as the amount of enzyme, which inhibits the rate of NBT reduction by 50%. The enzyme activity was further represented in percentage compared to the control group, which was taken as 100%.

Lactate, pyruvate, and NAD^+ levels were measured in protein-free and ion-free perchloric acid extracts of liver by enzymatic assays (Bermeyer and Bernt 1974). The cytosolic NAD^+/NADH ratio in cytoplasm was calculated by the method of steady-state metabolite concentrations and equilibrium constant for lactate dehydrogenase following Williamson et al. (1967).

Statistical analysis. The results of all experiments were expressed by mean±SD and considered as statistically meaningful at $p<0.05$. For comparison of differences between groups in metabolite parameters and enzyme activities, the statistical significance was determined using Student's t-test by the program STATISTICA 10.0 with non-parametric Mann-Whitney U-test. Differences between means were also estimated using the ANOVA followed by Tukey *post hoc* test.

Results

Mortality. Four animals died during the 1st week after STZ injection and three animals did not match the trend of glucose level, therefore, were not included into the final groups of animals used for investigations.

Blood glucose levels and body weights in experimental rats. Increased blood glucose concentration and body weight loss are the main characteristics of development of experimental T1DM. The initial (prior to STZ administration) body weight of rats was similar in all experimental groups. The final fasting blood glucose concentration in diabetic rats was measured after six weeks of diabetes duration. Hyperglycemia was observed starting from one week after the induction of T1DM. At the end of experiments, the blood glucose concentration in diabetic rats was elevated (21.2±2.9 vs. 4.8±0.5 mmol/L), while the average body weight was decreased (158.3±9.8 vs. 205.0±15.3 g) compared to the control group. These data confirmed the development of T1DM in experimental rats. NAm administration did not influence neither the body weight nor the blood glucose level in diabetic rats compared to the controls.

Indexes of ammonia detoxication in the liver. According to our data, the urea concentration in the liver of diabetic animals was increased by 38% compared to controls. Administration of NAm to diabetic rats reduced the urea concentration (Table 1). Elevation of urea in the liver of diabetic rats correlated with increased arginase activity by 43%. NAm supplementation to diabetic rats has partially reduced the arginase activity by 18% (Table 1).

Glutamine synthetase activity in the liver was reduced by 15% indicating the switching of glutamine metabolism towards its uptake by the liver rather than its synthesis. We did not observe the effect of NAm on the activity of glutamine synthetase in the liver of diabetic rats (Table 1).

Table 1
The urea concentration, the activities of arginase and glutamine synthetase in the rat liver

Parameter	Group		
	Control	Diabetes (D)	D+NAm
Urea (mmol/L)	18.2±1.6	25.1±2.0*	19.5±1.8#
Arginase (μmol of urea/mg protein/min)	15.9±1.3	22.7±1.9*	18.6±1.7*#
Glutamine synthetase (μmol of Pi/mg protein/min)	0.73±0.07	0.62±0.04*	0.65±0.05

Data are presented as mean ± SD (n=6). *$p<0.05$ compared to the control group; #$p<0.05$ compared to the group of diabetic rats (D).

The indexes of oxidative stress. The level of lipid peroxidation products, TBARS, as markers of oxidative stress development in the blood plasma of diabetic rats, was increased by 60%. NAm treatment of diabetic rats led to reduction of oxidative stress that was proved by 25% drop of TBARS level (Figure 1). In response to oxidative stress development, it was reasonable to evaluate the activity of SOD, one of the key enzymes of antioxidant defence system. However, we did not observe the elevation of SOD activity in the blood plasma, but its activity was reduced (Figure 2). Positive effects of NAm on SOD activity was negligible.

Assessment of NAD+ content and NAD+/NADH ratio in rat liver. The assessment of NAD+ levels in the liver of experimental rats under diabetes and NAm treatment allows to estimate the diabetes-induced alterations and protective NAm action. Diabetes led to 1.37-fold decline in NAD^+ content in the rat livers compared to the controls. NAm administration normalized the NAD^+ levels in the liver (Table 2).

Lactate concentration in the liver was 1.44-fold increased in diabetic rats compared to the control group. The diabetes-associated alterations were partially countered by NAm treatment (Table 2). It was not observed a statistically significant difference in pyruvate levels in the liver between the control and diabetic groups. However, the pyruvate level in rat liver was increased at NAm-treated diabetes. Free cytosolic NAD^+/NADH ratio in liver was 1.53-fold reduced in diabetic rats compared to the control group (Table 2). The NAm-treated animals exhibited a normalizing effect of NAm on the redox state of free cytosolic NAD^+/NADH-couples in the liver.

The mRNA expression level of *parp-1*. To determine the effect of NAm supplementation and to understand better the possible mechanism of its

Table 2
The levels of lactate, pyruvate and NAD+ in the rat liver and redox-state of free cytosolic NAD^+/NADH couples

Parameter	Group		
	Control	Diabetes (D)	D+NAm
Lactate (μmol/g)	1.90±0.16	2.74±0.23*	1.57±0.12*#
Pyruvate (μmol/g)	0.173±0.015	0.163±0.013	0.141±0.011*#
NAD+ (μmol/g)	0.761±0.068	0.554±0.040*	0.695±0.057#
NAD+/NADH	820±63	536±40*	809±74#

Data are presented as mean ± SD (n=6). *p<0.05 compared to the control group; #p<0.05 compared to the group of diabetic rats (D).

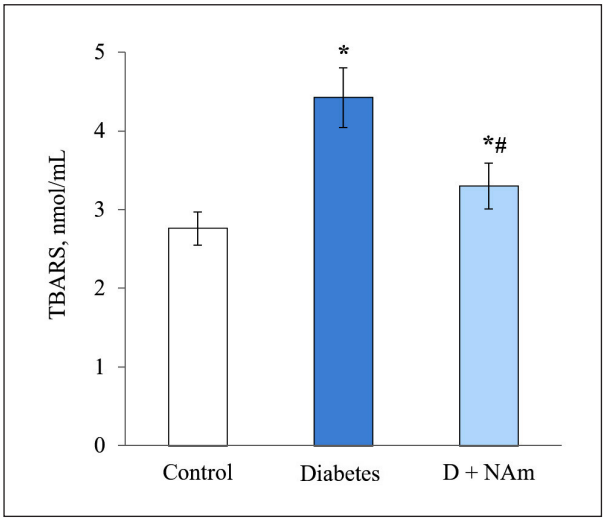

Figure 1. The levels of TBARS in the blood plasma of control group of rats (Control), rats with type 1 diabetes mellitus (Diabetes) and diabetic rats treated with nicotinamide (D+NAm). Data are presented as mean±SD (n=6). *p<0.05 compared to the control group; #p<0.05 compared to the diabetic rats.

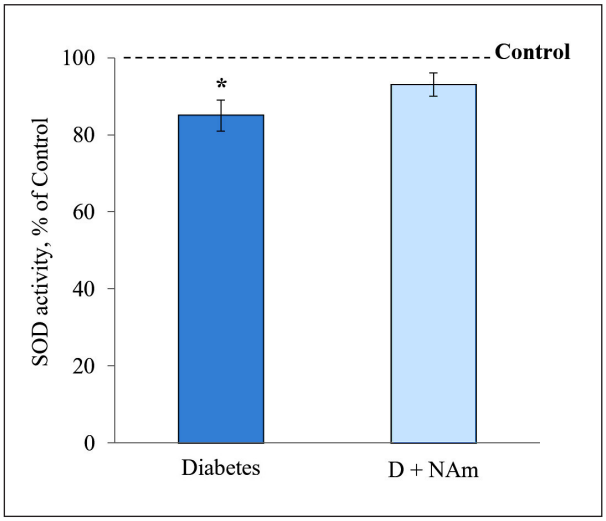

Figure 2. Superoxide dismutase (SOD) activity in the blood plasma of control group of rats (Control), rats with type 1 diabetes mellitus (Diabetes) and diabetic rats treated with nicotinamide (D+NAm). Data are presented as mean±SD (n=6). *p<0.05 compared to the controls.

involvement in hepatoprotective action, the *parp-1* expression at post-transcriptional level in the liver of diabetic rats was analyzed. As presented in Figure 3, it was established that diabetes led to a decrease of mRNA expression of *parp-1* in liver tissue by 30% compared with the control animals. The mRNA expression of *parp-1* gene did not change after NAm administration to diabetic rats.

PARP-1 fragmentation in rat livers. In the liver of diabetic rats, poly(ADP)ribosylation was activated confirmed by PARP-1 cleavage resulted in appearance of 89 kDa PARP-1 fragment. According to the Western blot analysis, a significant up-regulation of PARP-1 expression (Figure 4A) and increased ratio of cleaved PARP-1 fragment (89 kDa) to full-length PARP-1 (116 kDa) in diabetic liver was demonstrated (Figure 4B). The normalizing effect of NAm on poly(ADP)-ribosylation in the liver tissue of diabetic rats was shown for the first time under this T1DM duration (Figure 4).

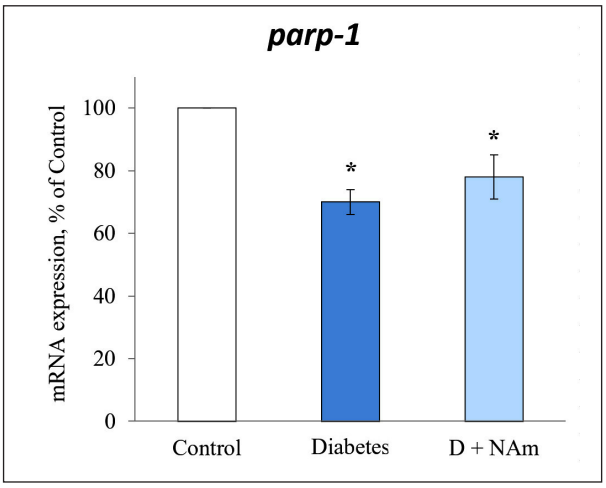

Figure 3. The mRNA level of *parp-1* in total RNA isolated from the liver tissue of control group of rats (Control), rats with type 1 diabetes mellitus (Diabetes) and diabetic rats treated with nicotinamide (D+NAm). Data are presented as mean±SD (n=5). *p<0.05 compared to the controls.

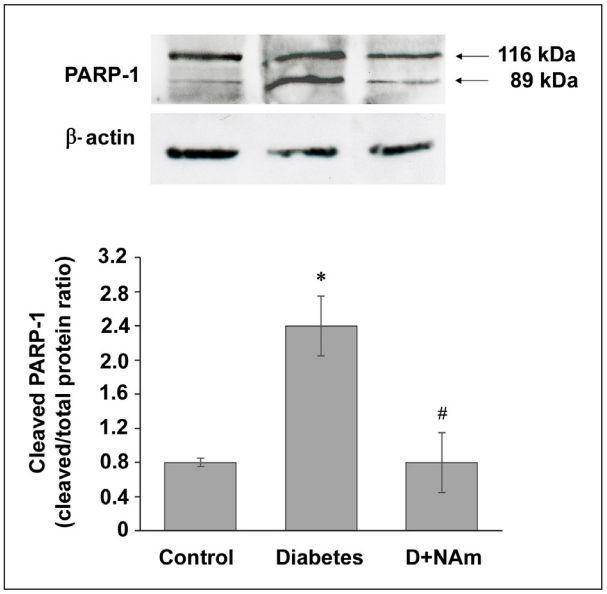

Figure 4. Effects of NAm on PARP-1 expression and fragmentation in the liver of rats with streptozotocin-induced diabetes in the control group of rats (Control), rats with type 1 diabetes mellitus (Diabetes) and diabetic rats treated with nicotinamide (D+NAm). **A)** Representative Western blot of PARP. **B)** Results of the densitometric analysis of immunoblots, in which bar plot shows the ratio of cleaved PARP-1 (89 kDa) fragment to full-length PARP-1 (116 kDa). Equal loading was confirmed by reprobing for β-actin. Data are expressed as mean±SD (n=5). *p<0.05 vs. control group; #p<0.05 vs. diabetic group.

Discussion

It is well known that the liver plays a key role in metabolic processes in the body that makes expediently important to elucidate the mechanisms of its diabetes-induced dysfunctions. The consequences of diabetes mellitus in the liver involve not only biochemical and metabolic alterations, as it was demonstrated herein, but also a spectrum of structural and functional impairments, which are characteristic for this endocrine disease. Impaired urea cycle functioning may have a detrimental role in liver disorders, which can also be associated with diabetes (Nagamani et al. 2021). Indeed, urea, as a non-toxic excretable form of ammonia, reflects protein and amino acid turnover in the body that makes important to investigate how hyperglycemia can influence the detoxication ability of the liver. The better understanding of the mechanisms resulting in liver impairments may promote a development of more effective treatments. We believe that NAm and its biologically active derivatives can be the promising candidates for the protection and/or recovery of liver functioning without undesirable side effects. During a long time, NAm has been considered as main player in energy metabolism, as precursor of NAD+ synthesis related to energy production in functioning of organism (Canto et al. 2015). Additionally, NAD+ is the precursor for phosphorylated dinucleotide NADP+ and both reduced forms, NADH and NADPH that are the cofactors of numerous dehydrogenases involved in maintaining of cellular redox homeostasis and cellular biosynthetic pathways. It should be emphasized that the ratio of free NAD+/NADH couples is crucial for driving a

wide range of oxidation-reduction (redox) reactions in cellular bioenergetics and especially in regulatory processes. Moreover, NAD+ and its metabolites play a significant role in cell signaling pathways since they are substrates for PARP, apoptosis-inducing factors, cyclic ADP-ribose, which induces Ca^{2+} release from endoplasmic reticulum via the ryanodine receptor intracellular Ca^{2+} channel complex, etc. (Takasawa 2022).

Our investigations were performed to assess the diabetes-induced biochemical and metabolic abnormalities in the liver to elucidate possible protective potential of NAm. To be sure that T1DM was developed in animals, we compared glucose level and body weight in rats at the beginning of experiments and at the end before the start of investigations. Results suggested the development of T1DM, a proof of which was a 23% weight loss in diabetic animals and a 4.4-fold increase in glucose concentration, as a result of poor glycemic control.

Hyperglycemia is accompanied by an elevated production of ROS promoting oxidative damage of the biomolecules in the tissues and membranous structures of the cells. In our experiments, we have observed 60% increase in the plasma TBARS levels as markers of lipid peroxidation and oxidative stress development in diabetic animals. Our data are consistent with the data of other authors, who have shown elevation of TBARS in the liver and kidneys associated with development of oxidative stress under STZ-induced diabetes (Mushtaq et al. 2015). After treatment of diabetic rats with NAm, the level of TBARS in the blood plasma was decreased by 25% as shown in Figure 1. It is important to note that other naturally occurring biologically active compounds such as flavonoids (Aloud et al. 2018), curcuminoids (Roxo et al. 2019), and L-carnitine (ElGendy and Abbas 2014; Rezaei et al. 2018) reduced the level of TBARS in the blood plasma and tissues and protected the organs against the oxidative stress.

Moreover, hyperglycemia leads to an imbalanced functioning of pro-oxidant and antioxidant systems. It was unexpected that estimated activity of SOD was not elevated in response to overproduction of ROS and was even lower than in the blood plasma of the control group of rats (Figure 2). We assumed that the activity of SOD in the blood plasma characterizes the level of the tissue damage caused by oxidative stress. The activity of SOD in the blood plasma of diabetic rats was lower by 15% compared to the control group. These results of our experiments are consistent with the data showing the reduced activity of SOD in the liver and heart in animal models of diabetes (Tabei et al. 2015; Chis et al. 2016). The activity of other enzymes of antioxidant defense system, in particularly catalase and glutathione peroxidase, were also affected by diabetes (Aloud et al. 2018). The activity of SOD, the key enzyme of antioxidant defense system in diabetic rats, was practically not changed by NAm administration.

Development of an oxidative stress was accompanied by impaired functioning of the liver, in particularly, by altered urea production. It was proved by the results showing 38% increase of urea concentration in the liver of diabetic animals (Table 1). Our results correlate with the data of the study, where elevation of plasma urea in diabetic rats has been shown (Ahmadvand 2012). Administration of NAm reduced urea concentration (Table 1), which can be explained not by its direct effect on urea cycle, but through its involvement in other NAD-dependent processes in the liver including energy production (Mitchell et al. 2018).

According to the obtained results, the activity of arginase, an enzyme of the last reaction of urea cycle, was elevated by 43% in the liver that can indicate higher flux of ammonia through the urea cycle and elevation of amino acid breakdown induced by T1DM (Table 1). Furthermore, the increased arginase activity and/or expression due to impaired arginine metabolism can also be an additional cause for the development of kidneys, central nervous system, cardiovascular system pathologies, and vascular impairments (Caldwell et al. 2018).

It should be noted that arginine serves as a substrate for the synthesis of nitric oxide, i.e., an important signaling molecule (Gawrys et al. 2020), which is also involved in the development of oxidative stress. The elevation of nitric oxide and arginase activity and decreased SOD activity in the liver of diabetic rats possibly can be explained by an increased oxidative damage of tissue (Kisacam et al. 2022). We suppose that arginase, rather than nitric oxide synthase, appears to be dominant for arginine metabolism in the liver under investigated model of T1DM.

Moreover, the increased L-ornithine level can lead to structural impairments and neuronal toxicity in vascular system in hepatocytes. In the previous study, we have demonstrated increased ROS production in the blood leukocytes and significant changes in the redistribution between their two main types accompanied by the increased number of granulocytes in the blood of diabetic rats (Guzyk et al. 2013). The chronic NAm administration to the diabetic rats led to a partial decrease in the level of ROS in leukocytes and partial restoration of the balance

between the number of granulocytes and agranulocytes. Therefore, the elevation of arginase activity in these experiments can be explained by intensification of inflammatory processes in the liver of diabetic rats. Furthermore, it can be supposed that chronic NAm administration to the diabetic rats influenced not only inflammation, but also arginase activity. Indeed, as we showed in this study, NAm reduced the activity of arginase by 18% compared to the diabetic animals (Table 1), which can be also explained by stimulation of NAD+ synthesis and improvement of hepatic metabolism (Mitchell et al. 2018).

In our experiments, we found a decrease in glutamine synthetase activity by 15% in the liver of diabetic rats (Table 1). This may contribute to both activation of urea cycle functioning and alteration of glutamine metabolism in the liver from synthesis to its uptake. Reduced glutamine synthetase activity in the liver of diabetic rats (Table 1) can be caused not only by activation of urea cycle, but also by elevated glutamine uptake. In addition, in response to oxidative nitrosative stress induced by diabetes, the activity of glutamine synthetase can be impaired due to tyrosine nitration at modification of this enzyme (Frieg et al. 2021). It is known that under pathological conditions including diabetes, the alterations of glutamine synthetase functioning occur not only in the liver but also in the other tissues, espessially in brain, where this enzyme prevents possible accumulation of ammonia maintaining it on low and non-toxic levels. Indeed, the reduced activity of glutamine synthetase has been observed in retina of diabetic rats that was accompanied by disordered glutamate metabolism resulting in an excitotoxicity and even neurodegeneration (Fernandez et al. 2012; Ola and Alhomida 2014). Moreover, regulation of intercellular glutamine cycle in the liver depends on ammonia content and acid-base balance; it may be switched from urea to glutamine synthesis (Frieg et al. 2021).

We also concentrated our attention on NAm as a supplement for treatment of diabetes. It was important to analyze how it can mediate its action through the intracellular NAD+ depended processes. It is known that maintaining of NAD+ level in the cells of different organs is considered as one of the regulating factors of energy generation (Canto et al. 2015). In response to the development of oxidative stress at T1DM, 1.37-fold depletion of NAD+ content in the liver of diabetic rats was established (Table 2). Such depletion can be associated with the use of NAD+ by non-redox NAD-dependent enzymes such as sirtuins and poly(ADP-ribose) polymerases, as we have shown for the brain of diabetic rats (Kuchmerovska et al. 2010; Guzyk et al. 2019). It can be also due to the shift of NAD+/NADH ratio towards NADH that results in downregulation of energy-producing pathways including glycolysis. It may lead to increased demands for ATP since NAD+ resynthesis in mammals requires several moles of ATP per one mole of NAD+ (Chandrasekaran et al. 2022).

The reduced NAD+/NADH ratio results in downregulation of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) that contributes glyceraldehyde-3-phosphate and the preceding intermediates to pass through the metabolic pathways which normally function as minor (Paul et al. 2020). We suppose that reduced glucose flux through G3PDH and oxidative phase of glycolysis can also be associated with intensification of oxidative stress in leukocytes induced by diabetes (Guzyk et al. 2013). Moreover, the increased flow of glucose through the polyol (sorbitol) pathway contributes to hyperglycemia-induced oxidative stress via several mechanisms. In polyol pathway, aldose reductase competes with glutathione reductase for the use of NADPH. It may cause the depletion of NADPH required by glutathione reductase to maintain the pool of GSH (Yan 2018). The elevated sorbitol dehydrogenase activity also increases the NADH level, which serves as a substrate for NADH-dependent oxidase leading to production of superoxide anion. Fructose through the formation of fructose-3-phosphate and 3-deoxyglucosone promotes formation of advanced glycation end products (Russell et al. 2007) and activation of protein kinase C pathway also contribute to formation of ROS and development of oxidative stress (Lien et al. 2021).

The more reduced state of cytosolic NAD+/NADH-couple can be associated with diabetes-mediated changes in the adenine nucleotide system and implied in impairment of energy metabolism. Our results demonstrated that diabetes led to 44% increase of lactate level in the liver without significant changes in pyruvate (Table 2). The measurement of lactate and pyruvate levels in the liver allowed to estimate the cytosolic NAD+/NADH ratio. The free NAD+/NADH ratio was decreased by 35% in the liver of diabetic animals at the end of the diabetes duration compared to the control group (Table 2), indicating the impaired regulation of energy metabolism in hepatocytes. Improvement of redox balance regulation in the liver under T1DM by NAm was established.

Reduction of NAD+ level in the liver at T1DM (Table 2) can be explained by its use in other metabolic processes. Among the other causes of NAD+ depletion in the liver of diabetic rats can be:

the deficiency or low activity of enzymes, which take part in NAD+ biosynthesis or high content and activity of enzymes that break down the nucleotide. Moreover, cADP-ribose synthases, which are ubiquitous and can use nucleotide as a substrate to generation of secondary messengers such as cADP-ribose that are involved in the calcium mobilization thereby regulating calcium homeostasis (Takasawa 2022). NAD+ as a substrate is also used by NAD+–dependent deacetylases (sirtuins) that play an important role in transcription regulation, DNA repair, energy metabolism modulation, inflammation, etc. (Fan et al. 2020). NAD+ is also used for biosynthesis of several molecules as well as NADP+ and NAADP+ (nicotinic acid adenine dinucleotide phosphate) that play key role in energy transduction and cell signaling (Chini et al. 2021). Indeed, in liver of diabetic rats, we observed the activation of poly(ADP)ribosylation that was confirmed by PARP-1 cleavage resulted in appearance of the 89 kDa PARP-1 fragment (Figure 4).

Diabetes-induced decrease in *parp-1* mRNA expression in liver tissue by 30% (Figure 3) may reflect a response to PARP-1 activation. Indeed, using NAD+ as a substrate, PARP-1 in the liver was activated that was proved by this enzyme cleavage with formation of fragments (Figure 4). PARP-1 activation may be associated with the requirement to repair DNA strand breaks caused by T1DM to maintain the integrity of the genome. PARP-1 is preferably localized in the nuclei of liver cells; thereby, this enzyme can be closely associated with DNA repair, as we have previously shown for the brain of diabetic rats (Kuchmerovska et al. 2004; Guzyk et al. 2017). However, we did not observe the NAm influence on *parp-1* gene expression (Figure 3). The obtained data of this part of the study can mean that in the liver tissue of diabetic rats the apoptosis of endothelial cells can develop leading to alterations of permeability and dysfunctions of hepatic cells. In general, the protective action of NAm in the liver can be realized through the suppression of PARP-1 cleavage, due to inhibition of PARP-1 activation (Figure 4) and promotion of intracellular NAD+ pool recovering (Table 2).

All established positive effects of NAm treatment on the liver dysfunction induced by T1DM can be realized through the described mechanisms. It does not exclude that many other factors rather interact than exist separately. As we have recently shown, NAm, as a charged organic cation, can penetrate the blood-brain-barrier and through the regulation of investigated brain protein levels, can improve cell survival, vascular functions, enhancing metabolic processes, and cellular signaling impaired by T1DM (Tykhonenko et al. 2022).

The improved diabetic brain functioning after NAm treatment is another convincing explanation for indirect positive NAm effect on the function of other organs including liver, as we established. Thus, the effects of NAm on the processes other than those investigated in this study on liver functioning at diabetes require further investigation.

Conclusions

Our results provide a rationale for further clinical studies of NAm to determine its usefulness in improving the management of liver disorders induced by diabetes. Although the experimental studies of NAm were conducted on the experimental T1DM only, they can also be relevant for T2DM, because the latter is also associated with the oxidative stress, urea cycle disorders, and impairments of NAD-dependent processes, especially poly(ADP)ribosylation that uses NAD+ as a substrate. The present findings identify the NAm as a multitarget compound alleviating multiple manifestations not only liver disorders, but also potentially the other organs diabetic complications. The key finding of this study demonstrates that mechanism of NAm action against the diabetes-induced liver impairments is realized through improvement of ammonia detoxified functions, reduction of oxidative stress, partial restoration of NAD+ content, normalization of free cytosolic NAD+/NADH-couple ratio, and preventing overactivation of PARP-1 in the liver of diabetic rats.

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

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