THE EFFECT OF PHYTOPROTECTORS ON THE FUNCTIONING OF LIVER NAD$^+$- AND NADP$^+$-MALIC ENZYMES IN RATS WITH ALLOXAN DIABETES

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The development of experimental alloxan diabetes in rats was accompanied by the increase the activity of liver NAD$^+$- and NADP$^+$-dependent malic enzymes (ME; NAD$^+$-ME, EC 1.1.1.39 and NADP$^+$-ME, 1.1.1.40) associated with an increase in the rate of transcription of genes encoding these enzymes. Oral administration of aqueous extracts of Jerusalem artichoke and olive to diabetic rats caused a noticeable decrease in blood glucose, a decrease in the rate of transcription of the studied genes; and a decrease in ME activity towards normal values. Thus, extracts of Jerusalem artichoke and olive can be used as additives to the standard therapy of diabetes mellitus.

Key words: malic enzyme; decarboxylating malate dehydrogenase; diabetes; alloxan; activity; expression; gene; isoenzyme; phytoprotector

DOI: 10.18097/PBMC20236902104

INTRODUCTION

Chronic metabolic disorder in diabetes mellitus (DM) is a global problem with huge social, medical and economic consequences [1]. Like a number of other diseases, such as cancer and neurodegenerative disorders, the development of DM is associated with oxidative stress [2]. NADPH is known to serve as a substrate for NADPH oxidases, which produce reactive oxygen species. In addition, NADPH is also involved in cellular antioxidant defense (NADPH-dependent conversion of oxidized glutathione to a reduced form catalyzed by glutathione reductase) [3].

Malic enzymes, or decarboxylating malate dehydrogenases (ME; NAD$^+$-ME, EC 1.1.1.39 and NADP$^+$-ME, EC 1.1.1.40), located in the cytoplasmic (ME1) and mitochondrial (ME2, ME3) compartments, catalyze oxidative decarboxylation of malate to pyruvate with simultaneous accumulation of reducing equivalents in the form of NADPH (ME1, ME2, ME3) or NADH (ME2) [4]. Therefore, MEs are believed to play an important role in the substrate supply of gluconeogenesis, glycolysis, and fatty acid synthesis, and in the antioxidant protection of cells as well.

There is a growing interest in the use of plant extracts for the prevention and treatment of patients with diabetes and related complications [5]. Jerusalem artichoke (*Helianthus tuberosus* L.) is cultivated in many countries, including Russia. Oral administration of Jerusalem artichoke preparations had a positive effect on the glycemic control in humans and animals; therefore, it is considered as a medicinal plant [6]. The main component of fructans and dietary fiber present in Jerusalem artichoke is inulin. Two-month administration of inulin isolated from Jerusalem artichoke tubers improved the glycemic control and reduced obesity in diabetic patients [7]. Oleuropein, the main phenolic component of the olive (*Olea europaea* L.), attracts the attention of scientists for its antioxidant, anti-inflammatory, cardio- and neuroprotective, and anticancer actions. The beneficial effect of oleuropein on diabetes mellitus, chronic kidney disease and cancer has been also demonstrated [8].

Thus, the aim of this study was to investigate functioning of the liver ME isoenzymes in rats with alloxan diabetes and to elucidate the effect of aqueous extracts of Jerusalem artichoke (JAAE) and olive (OAE) on the activity and the rate of transcription of decarboxylating ME in the liver of diabetic rats.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 150-200 g were obtained from the bionursery Stezar (Vladimir, Russia). The animals were kept under standard vivarium conditions and a natural light regime with free access to water and food during the day.

Experimental Setup

Diabetes mellitus was induced by a single intraperitoneal injection of 5% (w/v) alloxan monohydrate in 0.9% (w/v) sodium citrate solution at a dose of 150 mg/kg body weight [9]. Control animals were injected with 0.9% (w/v) sodium citrate solution. Blood sampling from the tail vein was performed in the morning on an empty stomach under sterile conditions in accordance with sanitary rules. The glucose level was measured with a glucometer Satellite plus (ELTA, Russia). After 14 days,

the anesthetized animals were decapitated. Analysis of the biomaterial (liver) was performed 14 days after the modeling of alloxan diabetes. After appearance of signs of diabetes, thirtysix rats were randomly divided into three groups of twelve rats each: "Diabetes" — diabetic rats, "Diabetes + JAAE" and "Diabetes + OAE" — animals with alloxan diabetes treated with Jerusalem artichoke extract (JAAE) and olive extract (OAE) respectively. The fourth group of rats ("Norm", n=12), which were not treated with alloxan, was used as a control. Rats from the "Norm" and "Diabetes" groups received distilled water, and "Diabetes + JAAE" and "Diabetes + OAE" — aqueous extracts of Jerusalem artichoke and olive, respectively, daily for 14 days.

Preparation of Plant Extracts

To prepare an aqueous extract of Jerusalem artichoke (*H. tuberosus*) tubers, dry plant material was powdered, poured with hot water (5 g per 100 ml of water), infused for 24 h, filtered, and added to drinkers at a dose of 60 mg/kg body weight once a day. To prepare an aqueous extract of the olive, dry leaves were powdered and 10 g of the powder was mixed with 100 ml of boiling distilled water, infused for 24 h, filtered, and the resultant supernatant was placed in an oven for 30 min at a temperature of 100°C to obtain a dry extract powder. The powder was dissolved in distilled water and added to drinkers at a dose of 100 mg/kg body weight [10].

Isolation of MEs and Measurement of Enzymatic Activity

To obtain a homogenate, rat liver was ground in a mortar in the cold (1 g of the liver per 5 ml of isolation medium) using the isolation medium, containing 50 mM Tris-HCl buffer, pH 6.5; 1 mM EDTA, 2 mM KCl, 3 mM DTT. After centrifugation for 5 min at 3000 *g* and 4°C the resulting supernatant was used in subsequent experiments.

ME Activity Assay

ME activity was determined spectrophotometrically at 340 nm by a decrease in the optical density due to NAD(P)H oxidation. To determine the rate of pyruvate reduction, the reaction medium contained 100 mM Tris-HCl, pH 6.5 (for NAD^+-ME) or 50 mM Tris-HCl, pH 6.5 (for $NADP^+$-ME) as well as 1.5 mM pyruvate, 0.15 mM NADH or NADPH. Manganese chloride (2 mM $MnCl_2$) was used as the ME cofactor and an inhibitor of oxidoreducing malate dehydrogenases. The enzyme activity was expressed as units (U) of ME; 1U corresponded to the amount of the enzyme catalyzing the conversion of 1 μmole of the substrate per 1 min at 25°C.

The protein content was determined by the Lowry method. Enzyme activity was expressed as U/g of wet wt (total activity) and U/mg protein (specific activity).

Study of the isoenzyme composition of ME

The isoenzyme composition of MEs was analyzed by polyacrylamide gel electrophoresis, which was performed according to the modified Davis method in two polyacrylamide gels of different concentrations [11]. The bottom (running) gel (8%) contained: 8% acrylamide/methylenebisacrylamide solution (A/B; 74:1; w/v); 1.5 M Tris-HCl, pH 8.8; 3 mM freshly prepared ammonium persulfate solution; 3 mM TEMED. The stacking (upper gel) (4%) contained: 4% A/B solution; 0.5 M Tris-HCl, pH 6.8; 3 mM ammonium persulfate; 3 mM TEMED. Tris-glycine buffer (0.025 M Tris – 0.192 M glycine), pH 8.3, was used as the electrode buffer. Bromophenol blue (0.01% (w/v) was used to control for the movement of the front line. Electrophoresis was run with a constant electric current of 1.5-2.5 mA [12]. All procedures were carried out at 4°C. ME detection in the gel was carried out by the tetrazolium method in a medium containing 100 mM Tris-HCl buffer, pH 6.5 (for NAD^+-ME) or 50 mM Tris-HCl buffer, pH 6.5 (for $NADP^+$-ME) as well as 2 mM sodium malate; 3 mM $NAD(P)^+$; 0.01 M nitroblue tetrazolium (NBT) (pre-dissolved in 0.5 ml ethylene glycol); 0.01 M phenazine methosulfate (PMS). 5 μg of protein of the test solution was added to the gel well. The electrophoretic mobility (R_f) was determined as the ratio of the migration distance of the protein through the gel (L_1) to the migration distance of the dye front (L_2): $R_f = L_1 / L_2$.

Isolation of RNA

Total cellular RNA was isolated using the guanidine isothiocyanate-phenol-chloroform extraction method and LiCl as the precipitating agent [13]. Electrophoresis in 1% agarose gel (w/v) was used to visualize and analyze the quality of the isolated RNA. The gel was stained with 0.1% alcohol solution of ethidium bromide. Reverse transcription of mRNA was performed using the M-MuLV reverse transcriptase enzyme and oligo(dT) primers (Sibenzyme, Russia) for synthesis of the first cDNA strand according to the manufacturer's instructions.

Primer Selection

Specific primers were selected using the nucleotide sequences of the *me-1*, *me-2*, and *me-3* rat genes taken from the NCBI database [14] and the Primer-BLAST program [15]. The obtained oligonucleotide sequences were tested for specificity to the studied genes by the BLAST program [16] and for the formation of hairpins and other secondary structures by the Clustal Omega program [17]. Based on the analyzed sequences, specific primers for the *me-1*, *me-2*, and *me-3* genes, encoding rat MEs were selected (Table 1).

Table 1. Specific primers for the *me-1*, *me-2*, and *me-3* genes, encoding rat MEs

Gene	Primer	Sequence	Annealing temperature	Theoretical product size, b.p.
me-1	Forward	GAGAAAGAGGTGTTTGCCCA	58.0°C	249
	Reverse	GGAAACAGAGTCCGTCCATC		
me-2	Forward	TCCGTACTACTTTGCTTGGC	58.0°C	328
	Reverse	GTGCTAGAGGACTGAGTGGA		
me-3	Forward	CAATGCCTATGTGTTTCCCG	58.0°C	253
	Reverse	GTTTGTACGCATAGTCGAGGA		

Real-Time PCR

Polymerase chain reaction was carried using a Bio-Rad DNA Engine Thermal Cycler Chromo 4 PCR analyzer (Bio-Rad, USA), HS-Taq PCR Extra-Mix kits (Diam, Russia) as reagents and the dye SYBR Green I (Evrogen, Russia). The following amplification parameters were used: initial denaturation at 95°C for 5 min, then the cycle: 95°C for 20 s, 58°C for 30 s, 72°C for 40 s (detection), final elongation at 72°C for 10 min. The amount of cDNA was controlled using parallel amplification of the *ef-1α* elongation factor and gene-specific primers developed by us. The total RNA that did not undergo reverse transcription served as a negative control. The relative expression level of the studied genes was determined using the $2^{-\Delta\Delta Ct}$ method [18] and Opticon Monitor™ Software (Bio-Rad).

Statistical Data Processing

The experiments were carried out in three analytical and five biological repeats. Stattech v.1.2.0 commercial software was used for statistical analysis. Since the number of studied parameters did not exceed 50, the correspondence to the normal distribution was calculated using the Shapiro-Wilk test. To describe quantitative indicators, the normal distribution of which was confirmed, they used the calculation of the arithmetic mean (M) and standard deviation (SD), and also determined the boundaries of the 95 percent confidence interval (95% CI). Comparison of three or more groups in terms of a quantitative indicator having a normal distribution was performed using one-way analysis of variance, post hoc comparisons were performed using Tukey's test (equal variances between the groups).

RESULTS AND DISCUSSION

Induction of Experimental Diabetes

Initial concentration of blood glucose in rats used in the experiments was within the normal range (4.9±0.12 mmol/l). Alloxan introduction caused a significant increase of blood glucose to 14.8±0.30 mmol/l on the second day after the injection; in the "Diabetes" group, this parameter demonstrated a further increase during the observation period (Fig. 1). In control animals, which were not treated with alloxan, the blood glucose remained within the normal range (5.0±0.15 mmol/l) during the whole experiment. The impact of alloxan causes destruction of β-cells of the islets in the pancreas, resulting in a lack of insulin and the development of type I diabetes mellitus (Fig. 1) [9]. The cycle of redox transformations leads to the formation of free radicals, which play a key role in the degeneration of β-cells [19].

Rats from the groups "Norm" and "Diabetes" received distilled water. For animals of the group "Diabetes + JAAE", water was replaced with the aqueous extract of Jerusalem artichoke (60 mg/kg of body weight) [20]; for rats of the group "Diabetes + OAE" group, water was replaced for the olive aqueous extract (100 mg/kg of body weight) [10] once a day for 14 days. By the end of the experiment, the concentration of blood glucose in rats treated with the plant extracts was higher than in the control animals, but significantly lower than in the rats of the group "Diabetes" ("Diabetes" — 17.2 [17.1–17.5] mmol/l; "Diabetes + JAAE" — 10.2 [9.9–10.4] mmol/l; "Diabetes + OAE" — 12.6 [12.2–13.0] mmol/l). The most probable reason for the decrease of hyperglycemia in diabetic rats treated with the aqueous extract of the leaves of *H. tuberosus* (JAAE) may be attributed to absorbance of dietary glucose uncleaved inulin molecules promoted by hydrochloric acid in the stomach. In addition, it was found that fructose, penetrating into the cells of all organs without the participation of insulin, could fully replace glucose in metabolic processes [21]. Oleuropein is known to be the most common phenolic component in olive leaves [22]. Experimental studies have demonstrated the valuable effects of oleuropein and its products, such as hydroxytyrosol, on various biological processes, including free radical scavenging, as well as antihyperglycemic effects [23]. Treatment of diabetic rabbits with oleuropein, caused a decrease in blood glucose [24]. These studies support our findings that Jerusalem artichoke and olive extracts can be used as additives conventional therapy of diabetes.

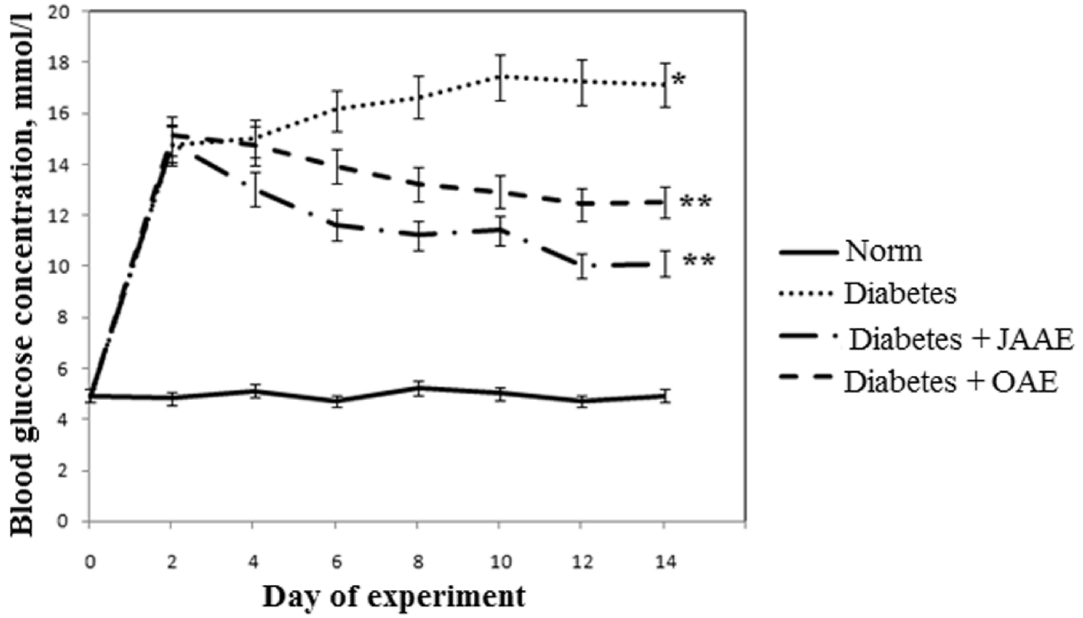

Figure 1. Time-course of blood glucose in healthy rats ("Norm"), rats with alloxan diabetes ("Diabetes") and rats with alloxan diabetes, treated with aqueous extracts of Jerusalem artichoke ("Diabetes + JAAE") and olive ("Diabetes + OAE"). * – differences in parameters between the groups "Norm" and "Diabetes" are statistically significant (p=0.001); ** – differences in parameters between the groups "Diabetes", "Diabetes + JAAE" and "Diabetes + OAE" are statistically significant ($p \leq 0.05$).

Table 2. Total and specific activity of NAD^+- и $NADP^+$-MEs in norm and alloxan diabetes, $p \leq 0.05$

Group of rats	$NADP^+$-ME, U/g wet wt	$NADP^+$-ME, U/mg protein	NAD^+-ME, U/g wet wt	NAD^+-ME, E/mg protein
Norm	12.4±0.62	1.3±0.25	11.3±0.56	1.5±0.21
Diabetes	16.7±0.84	2.1±0.13	24.9±0.24	2.9±0.15
Diabetes + JAAE	14.2±0.74	1.5±0.44	18.4±0.92	1.8±0.37
Diabetes + OAE	14.9±0.75	1.6±0.36	20.8±0.31	2.0±0.46

Rat Liver ME Activity

It is known that MEs localized in the pancreas play an important role in insulin secretion [25, 26]. However, we did not find any information on the activity of these enzymes in the liver in normal conditions and during the development of DM. Previously, we found a significant increase in the activity of NAD-dependent oxidoreducing malate dehydrogenase [20] and lactate dehydrogenase [27] in the rat liver. In this work, we detected an increase in the total activity of $NADP^+$-ME in diabetes mellitus by 1.3 times, and NAD^+-ME by 2.2 times, while the specific activity increased by 1.6 and 1.9 times, respectively (Table 2). Our data on the increase in $NADP^+$-ME activity under conditions of alloxan diabetes are consistent with the literature data. For example, in 2000, Savchenko et al. showed an increase in the activity of $NADP^+$-ME in lymphocytes of children with insulin-dependent DM compared with the norm [28]. However, at the end of the last century, there were reports on the insulin regulation of $NADP^+$-ME gene expression, which demonstrated a decrease in the activity of the studied enzymes in diabetes, and insulin administration restored the rate of its functioning [29, 30]. Probably, such discrepancies in the results can be associated with different duration and conditions of the experiment. In the study by Drake et al. rats were kept on a special carbohydrate-rich diet [30]. Therefore, this issue needs further research.

The use of phytoprotectors led to a decrease in ME activity as compared with the enzyme activity in the group "Diabetes". However, it should be noted that this was not sufficient to normalize metabolic processes.

The Study of the Isoenzyme Composition of MEs

Changes in the rate of enzyme activity may be associated with variations in their isoenzyme composition. Electrophoretic studies, performed in polyacrylamide gel, revealed the presence in the liver

homogenate of healthy rats of two isoenzymes of $NADP^+$-MEs, differing in molecular weights with R_f = 0.18 and R_f = 0.21, and one form of NAD^+-ME with R_f = 0.34. The development of DM did not lead to appearance of new additional isoforms (Fig. 2). This suggests that the increase in ME activity is not associated with the appearance of an additional isoform, in contrast to NAD^+-dependent malate dehydrogenase, which has oxidoreductase activity (EC 1.1.1.37); for this enzyme we previously revealed the appearance of an additional isoform in DM [19]. Probably, an increase in the activity of NAD^+- and $NADP^+$-ME during the development of DM is associated with the increase in the functioning of already existing forms of the enzyme, and not with synthesis of additional proteins.

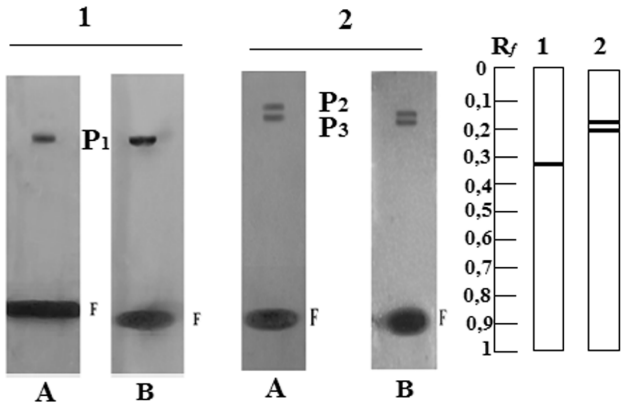

Figure 2. Distribution of NAD^+- (1) and $NADP^+$-MEs (2) in the livers of: **A** – healthy rats; **B** – rats with alloxan diabetes ("Diabetes"); P1, P2, and P3 – proteins bands exhibiting enzymatic activity (R_f of 0.34; 0.18, and 0.21, respectively); F – dye front.

The use of JAAE and OAE did not influence the isozyme composition of the studied enzymes (data not shown).

Analysis of Expression of ME Coding Genes

Analysis of the GeneBank database [31] has shown that in most mammals, including rats, $NADP^+$-ME is encoded by two genes: *me-1* (cytoplasmic isoenzyme) and *me-3* (mitochondrial isoenzyme); the NAD^+-dependent form of the enzyme is encoded by one gene (*me-2*). To analyze the expression rate of these genes, we selected specific primers (Table 1).

The expression rate of genes encoding NAD^+- and $NADP^+$-dependent MEs, was evaluated using a real-time polymerase chain reaction. The results obtained indicate that the development of alloxan diabetes is accompanied by a significant increase in expression of the genes of interest: *me-1* by 9 times; *me-2* by 5.7 times, and *me-3* by 2.6 times relative to the control group (Fig. 3). The data on the levels

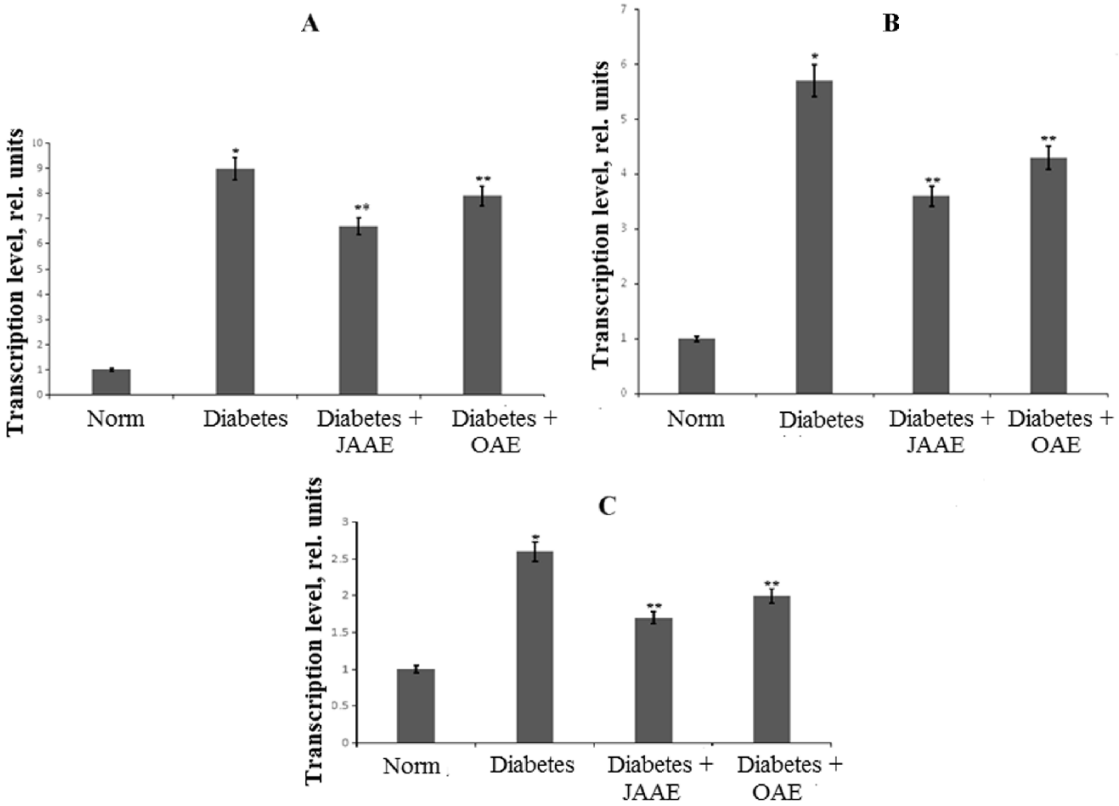

Figure 3. The relative level of transcription of the liver genes *me-1* (**A**), *me-2* (**B**), and *me-3* (**C**) in healthy rats ("Norm"), animals with alloxan diabetes ("Diabetes"), animals with alloxan diabetes treated with aqueous extracts of Jerusalem artichoke ("Diabetes + JAAE") or olive ("Diabetes + VEO"). * – differences in parameters between the groups "Norm" and "Diabetes" are statistically significant ($p \leq 0.05$); ** – differences in parameters between the groups "Diabetes", "Diabetes + JAAE" and "Diabetes + OAE" are statistically significant ($p \leq 0.05$).

of expression of the genes encoding NAD^+- and $NADP^+$-ME in normal conditions and in alloxan diabetes correlate with the values of the studied enzyme activities in the experimental and control groups. The data obtained suggest that in the liver of rats with alloxan diabetes there is an active synthesis of additional ME proteins in order to activate their work. It is known that under conditions of glucose deficiency in liver cells, the process of gluconeogenesis is triggered [32]. Probably, an increase in the activity and rate of functioning of decarboxylating malate dehydrogenases is necessary for the formation of pyruvate, which is used in gluconeogenetic processes. In addition, NADPH molecules can be used in the NADPH-dependent reduction of glutathione, which, in turn, is used by the body to maintain the balance of redox processes that are disturbed in alloxan diabetes [3].

Administration of plant extracts significantly decreased the relative level of transcripts of the studied genes; however, it did not reach the control level. It can be assumed that plant extracts are involved in the regulation of transcription factors that control the expression of various genes. For example, Li et al. showed that black raspberry fractions inhibited activation of AP-1, NF-κB, and nuclear factor of activated T cells (NFAT) by BaPDE, as well as their upstream PI-3K/Akt-p70 (S6K) and mitogen-activated protein kinase pathways [33].

The data obtained suggest that the increase in ME activity is associated with the increase in the transcription rate of their genes. At the same time, oral administration of plant extracts attenuates changes in the transcriptional activity of the *me-1*, *me-2*, and *me-3* genes thus causing a decrease in the rate of ME functioning, which still does not reach the control values.

CONCLUSIONS

An increase in the activity of liver NAD^+- and $NADP^+$-MEs in rats with alloxan diabetes is associated with the increase in the rate of transcription of the *me-1*, *me-2*, and *me-3* and may reflect the need in additional resources of pyruvate and NADPH molecules under stress conditions.

The studied plant extracts exhibit a hypoglycemic effect, which manifests itself in a marked decrease in the blood glucose in rats with alloxan diabetes. These extracts attenuate diabetes-induced changes in the transcriptional activity of the studied genes and thus promote normalization of biochemical processes.

ACKNOWLEDGMENTS

The authors thank Evrogen for the synthesis of the oligonucleotides used in the study.

FUNDING

The study was financially supported by the Russian Foundation for Basic Research (project No. 20-04-00296).

COMPLIANCE WITH ETHICAL STANDARDS

All experimental procedures complied with the requirements of international rules for the humane treatment of animals, reflected in the sanitary rules for the selection and maintenance of experimental biological clinics (vivariums).

CONFLICT OF INTEREST

The authors declare no conflict of interests.

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Received: 10. 10. 2022.
Revised: 09. 02. 2023.
Accepted: 28. 02. 2023.

ВЛИЯНИЕ ФИТОПРОТЕКТОРОВ НА ФУНКЦИОНИРОВАНИЕ NAD$^+$- И NADP$^+$-МАЛИК-ЭНЗИМОВ В ПЕЧЕНИ КРЫС ПРИ АЛЛОКСАНОВОМ ДИАБЕТЕ

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Показано увеличение активности NAD$^+$- и NADP$^+$-зависимых малик-энзимов (МЭ; NAD$^+$-МЭ, КФ 1.1.1.39 и NADP$^+$-МЭ, КФ 1.1.1.40) в печени крыс при развитии аллоксанового диабета, связанное с усилением скорости транскрипции кодирующих данные ферменты генов. Пероральное введение водных экстрактов топинамбура и оливы крысам с диабетом способствовало заметному снижению концентрации глюкозы в крови крыс при развитии патологии, приводило к уменьшению скорости транскрипции исследуемых генов; активность МЭ принимала значения, близкие к норме. Предлагается использовать экстракты топинамбура и оливы в качестве дополнительных средств терапии сахарного диабета.

Полный текст статьи на русском языке доступен на сайте журнала (http://pbmc.ibmc.msk.ru).

Ключевые слова: малик-энзим; декарбоксилирующая малатдегидрогеназа; диабет; аллоксан; активность; экспрессия; ген; изофермент; фитопротектор

Финансирование. Исследование выполнено при финансовой поддержке РФФИ проекта № 20-04-00296.

Поступила в редакцию: 10.10.2022; после доработки: 09.02.2023; принята к печати: 28.02.2023