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ANALYSIS OF THE EFFECTIVENESS OF INULIN IN ALLOXAN DIABETES TREATMENT

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ABSTRACT

An increase in the activity and the appearance of a new isoform of NAD-dependent malate dehydrogenase (MDH) in the liver of rats with alloxan diabetes was revealed. This fact confirms the possibility of the participation of the malate dehydrogenase enzyme system in the adaptive reaction of the body under oxidative stress caused by biochemical changes in diabetic cells. An increase in the activity of the enzyme under study in experimental type I diabetes is associated with the formation of an additional MDH isoform in peroxisomes. Data on the expression of mdh1 and mdh2 MDH genes confirm that in diabetes, activation of enzymes occurs at the transcription level of their genes. The use of inulin contributed to a noticeable decrease in the concentration of glucose in the blood of rats with alloxan diabetes, leveled the change in the transcriptional activity of the studied genes, and, as a consequence, blocked the formation of a new isoform of MDH in rats with experimental alloxan diabetes. As a result, this extract may be of considerable interest from the point of view of pharmacological correction of metabolic changes in the development of pathologies of this kind.

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Introduction

Diabetes mellitus (DM) is one of the most common diseases in the world. The number of DM patients is constantly growing [1, 2]. According to the IDF (International Diabetes Federation, International Diabetes Federation), by 2040, the number of DM patients will exceed 600 million [3].

Among the ways to protect against cell damage caused by DM, natural substances contained in plant extracts play an important role [4, 5]. For example, extracts of Jerusalem artichoke leaves and tubers (Helianthus tuberosus), which are used for the treatment of diabetes as a source of inulin, contain a significant amount of fructans, food-soluble fibers, sesquiterpenes, diterpenes, and chlorogenic acid analogs [6, 7]. Inulin and a complex of other biologically active substances in the composition of Jerusalem artichoke dry concentrate affect the activity of the formation of islands in the pancreas, especially during the first three weeks, thereby alleviating the severity of the course of alloxan diabetes in rats [8, 9]. In addition, inulin adsorbs glucose and affects insulin-dependent cells, increasing their affinity for insulin and thereby reducing blood glucose levels [10].

The adaptation of the animal organism to alloxan experimental diabetes is a complex multi-stage process, the main link of which is the transformation of cellular metabolism [11, 12]. The induction of enzymes of the glyoxylate cycle and the

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tricarboxylic acid cycle in animal tissues changes the main metabolic pathways caused by glycogen resynthesis in the liver of rats with pathologies associated with food deprivation and experimental diabetes [13, 14]. In addition to the fact that gluconeogenesis acts as the most important process in the adaptive reaction of the body to extreme conditions, an important role is played by energy metabolism, mainly associated with the functioning of the Krebs cycle [15, 16]. Consequently, the adaptation of cellular metabolism is ensured by the ratio of the intensity of catabolism and anabolism of glucose in the cells of the liver and other organs of the animal body. An important role in the implementation of adaptive reactions in the body belongs to the malate dehydrogenase enzyme system [17]. Malate dehydrogenase (MDH), being a multifunctional enzyme complex, ensures the flow of constructive and energy exchanges [18].

In connection with the above, the purpose of this work was to study the functioning of the MDH system in rat hepatocytes with alloxan diabetes and to clarify the effect of inulin of Jerusalem artichoke extract on the activity and rate of transcription of MDH in the liver of rats with pathology.

Materials and Methods

The study objective was male white laboratory rats of the Wistar line weighing 150-200 grams. The animals were kept in a vivarium at a standard temperature with natural light and free access to water and feed.

The induction of diabetes mellitus was caused by a single intraperitoneal injection of 5% (weight/volume) alloxane monohydrate in 0.9% (mass/volume) NaCl solution. Control animals were injected with 0.9% (weight/volume) NaCl solution. After showing signs of diabetes, thirty—six rats were randomly divided into three groups of twelve rats each; "Norm" — rats of the control group, "Diabetes" — diabetic rats, and "Diabetes + INULIN" - animals with alloxan diabetes that received inulin. Rats from the "Norm" and "Diabetes" groups received distilled water and "Diabetes+INULIN" — inulin for 14 days.

To prepare an aqueous extract of jerusalem artichoke leaves (Helianthus tuberosus), dry vegetable raw materials were used, which were ground into powder, poured with hot water in a ratio of 5 g of raw materials per 100 ml of water, infused for 24 hours, filtered and added to drinkers at a dose of 60 mg/kg of body weight once a day [19].

To obtain liver tissue, experimental animals were decapitated under ether anesthesia [20]. Glucose determination was performed using a glucose meter, "Satellite Plus" (Russia). Blood sampling was carried out from the tip of the tail on an empty stomach in the morning.

To obtain rat liver homogenate, the tissue sample was rubbed in three times the volume of a cooled isolation medium (50 mm tris-HCl buffer (pH 7.8) containing 1 mM EDTA, 2 mM MgCl2, 2 mM DTT) and centrifuged at 5000 g for 10 min. The resulting supernatant was used for further studies.

MDH activity was measured spectrophotometrically on an SF-2000 spectrophotometer (Russia) at 340 nm by changing the optical density of reaction mixtures determined by the rate of NADH consumption. The medium contained 50 mm tris-HCl buffer, pH 7.5; 1.5 mM oxaloacetate and 0.15 mM NADH, 5 mM MgCl2, and 4 mM DTT. For the unit of activity (E) MDH, the amount of the enzyme needed to convert 1 micromole of the substrate into 1 min at 25oC was taken. The Lowry method was used to determine the protein content [21]. Enzyme activity was expressed as specific activity (E/mg protein).

Electrophoresis was performed in polyacrylamide gel according to the modified Davis method [22], using 7.5% separating and 2% concentrating gels in order to analyze the isoenzyme composition of MDH from rat liver hepatocytes. The electrode buffer was a mixture of 0.05 M tris-glycine buffer, pH 8.6, with 0.01% bromophenol blue [23]. The specific manifestation of the gel on MDH activity was carried out by tetrazolium method in the medium of the following composition: 0.05 M tris-HCI buffer, pH 7.5; 0.2 M sodium malate, 3 mM NAD+; 1 mM MgCl2; 0.01 M phenazine metasulfate and 0.01 M nitrosine tetrazolium. 5 micrograms of the protein of the test solution were inserted into the gel pocket. To prevent partial degradation of proteins, protease inhibitors were added to the medium: 0.1 mM n-chloromercury benzoate and 0.1 mM phenylmethylsulfonyl fluoride.

Subcellular localization was determined by isodensity centrifugation in a sucrose gradient on a Beckman centrifuge (USA) at 100,000 g 90 min at 0oC in a stepwise gradient of the following composition: 50 mM tris-HCl, pH 7.5; 2 mM EDTA; 3 mM DTT; sucrose in concentrations of 2.5 M; 2.3 M; 1.8 M; 1.5 M; 1.3 M. The obtained fractions were carefully collected with a Pasteur pipette, diluted with a buffer to a sucrose concentration of 0.4-0.5 M, and then centrifuged for 30 min at 12000 g to precipitate organelles. The resulting sediments were destroyed by osmotic shock (in a 50 mM Tris-HCl buffer, pH 7.5). Cross-contamination was determined by the activity of marker enzymes of cytoplasm (alcohol dehydrogenase), mitochondria (succinate dehydrogenase), and peroxisomes (catalase). The total cellular RNA was isolated using the phenol-chloroform extraction method using LiCl as a precipitator [24].

Electrophoresis in 1 agarose gel mass volume was utilized to visualize and analyze the quality of the isolated RNA. Gel staining was carried out with a 0.1 alcohol solution of ethidium bromide. Reverse mRNA translation was performed utilizing the M MuLV reverse transcriptase protein and oligo dT primers SibEnzyme, Russia, to synthesize the first cDNA chain concurring with the manufacturer's instructions.

Specific primers were selected using rat mdh1 and mdh2 nucleotide sequences taken from the NCBI international database (https://www.ncbi.nlm.nih.gov/genes /), using the Primer-BLAST program (https://www.ncbi.nlm.nih.gov/tools/primer-blast /). Primers to the mdh1 gene: direct — 5-gctctactcgttccctgtcg-3; reverse — 5-acgactgtgtagtcatgcgg-3; to the mdh2 gene: direct — 5-acccccaaggttgactttcc-3; reverse — 5-ttccttcccattcatggcgt-3.

Polymerase chain reaction with gene-specific primers was carried out using a set of AmpliSence reagents (Helicon, Russia) on a LightCycler 96 device (Roche, Switzerland), using SYBR Green as a dye. Amplification parameters — preliminary denaturation: 95℃ — 5 min, then cycle: 95℃ — 20 s, 60℃ — 30 s, 72℃ — 40 s (detection), final elongation: 72℃ —10 min.

Statistical analysis was performed using the StatTech v. 1.2.0 program (Stattech, Russia). Quantitative indicators were evaluated for compliance with the normal distribution using the Shapiro-Wilk criterion (with the number of subjects less than 50) or the Kolmogorov-Smirnov criterion (with the number of subjects more than 50) [25]. Quantitative indicators having a normal distribution were described using arithmetic averages (M) and standard deviations (SD), the boundaries of the 95% confidence interval (95% CI). Comparison of three or more groups by a quantitative indicator having a normal distribution was performed using a one-factor analysis of variance, and posterior comparisons were carried out using the Tukey criterion (provided that the variances are equal). Statistically significant differences at $p<0.05$ are discussed.

Results and Discussion

Before the introduction of alloxan, the blood glucose levels in the animals of the studied groups were almost the same ($p=0.006$). 72 hours after the administration of alloxan, the animals were found to have elevated glucose levels (Diabetes = 19.3 mmol/L and Diabetes+INULIN = 19.72 mmol/l) **(Figure 1)**. Rats from the Norm and Diabetes groups received distilled water. In the Diabetes+INULIN group, water was replaced with an aqueous solution of inulin at a dose of 60 mg/kg of body weight once a day (based on preliminary data obtained in our laboratory) for 14 days. At the end of the experimental period, the glucose level in the Diabetes+INULIN group was still elevated compared to the control group but significantly lower than in the Diabetes group (Diabetes = 19.95 mmol/L and Diabetes+INULIN = 8.95 mmol/l). In the control group of healthy animals, the blood glucose level fluctuated within the normal range throughout the experiment and averaged 4.8-5.4 mmol/l. The reason for the decrease in hyperglycemia when using an aqueous extract of the leaves of H. tuberosus before normoglycemia may be associated with its ability to inhibit the absorption of glucose from the intestine, which promotes the release of glucose from the liver [26, 27]. At the same time, the inulin contained in the extract stimulates insulin synthesis by the pancreas [16].

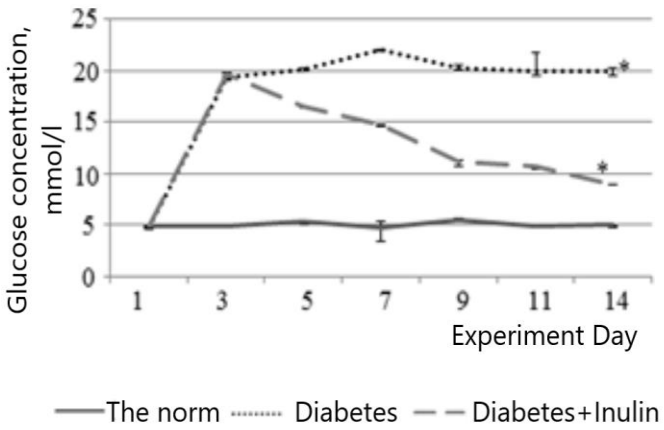

Figure 1. Dynamics of changes in blood glucose levels in rats. Norm — rats of the control group (n=12); Diabetes — rats exposed to alloxan diabetes (n=12); Diabetes+INULIN — animals with pathology who took inulin solution (n=12).

According to the data obtained when comparing the dependence of glucose concentration for the group's Diabetes and Diabetes+INULIN from the day of the experiment, we found statistically significant differences ($p=0.007$ ($p_{11day–1day}=0.009$), $p=0.006$ ($p_{3day–1day}=0.009$), respectively). No statistically significant differences could be detected for the Norm group ($p=0.207$). Method used: The Kraskel-Wallis criterion.

A comparison of the specific activity of MDH from rat liver hepatocytes shows the existence of two pools of MDH activity in the liver cell compartments of healthy rats: cytoplasmic and mitochondrial, whereas, in rats with diabetes, the activity of this enzyme was also found in the peroxisomal fraction **(Table 1)**. The ratio of cytoplasmic and mitochondrial forms of MDH was 46.0% and 54.0% under normal conditions, in experimental diabetes — 25.0% and 58.0% in cytoplasm and mitochondria, respectively, and in peroxisomes — 16.0%. It should be noted that the level of cross-contamination was 4-6%, which is considered acceptable for data interpretation.

Table 1. The specific activity of malate dehydrogenase in subcellular compartments of liver hepatocytes of control group rats (Norm) exposed to alloxan diabetes (diabetes) and animals with pathology taking inulin (diabetes+INULIN)

Indicator	The norm	Diabetes	Diabetes + INULIN
MDH activity in the cytoplasm, Units/mg of protein; M±SD (95% CI)	1,4±0,27*(1,13-1,67)	2,12±0,15**(1,95-2,29)	1,57±0,27**(1,26-1,87)
MDH activity in mitochondria, Units/mg of protein; M±SD (95% CI)	1,5±0,19***(1,3-1,69)	4,62±0,1*(4,51-4,74)	1,85±0,1***(1,73-1,96)
MDH activity in peroxisomes, Units/mg of protein; M±SD (95% CI)	—	0,81±0,13(0,66-0,96)	—

Note: Based on the data obtained, when comparing the activity of MDH in the cytoplasm and mitochondria, statistically significant differences were revealed (*$p<0.001$; **$p_{Diabetes–Diabetes+INULIN} = 0.003$; ***$p_{NORM–Diabetes+INULIN} = 0.027$*). Method used: Variance analysis.

When determining MDH activity in animals with DM treated with INULIN, activity in the peroxisomal fraction was not detected, and the value of MDH activity practically corresponds to normal indicators (the ratio of cytoplasmic and mitochondrial MDH activity is 44% and 56%, respectively). A similar pattern was observed when studying the effect of an aqueous extract of the European Olive (Olea europaea) on the functioning of the enzymes of the glyoxylate cycle and the tricarboxylic acid cycle in rats under DM conditions. Olive extract injected into rats has been shown to have a leveling effect on the change in MDH activity [28].

The increased MDH activity indicates accelerating metabolic processes in which this enzyme participates. However, the exact reasons for its growth are not fully understood and require more detailed study. Interestingly, previous studies have shown a significant redistribution of malate dehydrogenase activity towards the peroxisomal fraction of hepatocytes during starvation of rats [29].

To solve the issue of activation of certain metabolic processes associated with malate dehydrogenase activity in alloxan diabetes, its isoenzyme composition was studied. Electrophoretic studies carried out in polyacrylamide gel showed the following: compared with control animals and rats that took plant extracts, in whose hepatocytes there are two isoforms — MDH1 (cytoplasmic) and MDH2 (mitochondrial) with Rf 0.29 and 0.36, respectively, an additional isoform MDH3 appears in animals with experimental diabetes. The induced form was characterized by a slower movement velocity through the gel (its Rf was 0.20) and was detected in the peroxisomal fraction **(Figure 2)**.

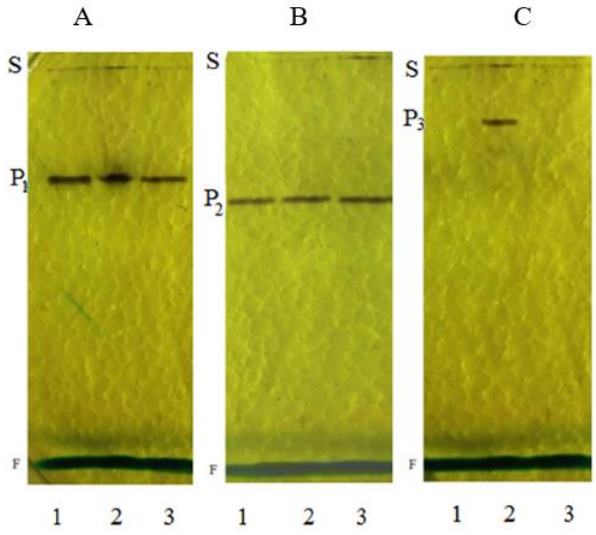

Figure 2. Isoenzyme composition of malate dehydrogenase in cytoplasmic (A), mitochondrial (B), and peroxisomal (C) liver fractions of control group rats (1) exposed to alloxan diabetes (2) and animals with pathology taking inulin (3). S is the start line, P1, P2, and P3 are isoforms of malate dehydrogenase, and F is the front of the marker-dye bromophenol blue.

Analysis of the data obtained suggests that an increase in MDH activity is associated with the appearance of an additional isoform localized in the peroxisomal fraction. It was previously shown that in conditions of experimental diabetes and food deprivation, the activity of marker enzymes of the glyoxylate cycle, isocitrate lyase, and malate synthase occurs in the liver and kidneys of rats [30]. Therefore, we assume that the inducible form of MDH can participate in the processes of gluconeogenesis. Using inulin blocks the formation of peroxisomal MDH in rats with experimental diabetes.

The change in the isoenzyme composition in rats with alloxan diabetes may be due to the differential expression of genes encoding MDH. The results of the PCR-RV study are shown in **Figure 3**, from which it can be seen that in the liver of diabetic rats, the concentration of transcripts of the studied genes increased compared to this indicator in the control group of rats. An increase in the transcription level of the mdh2 gene in rats exposed to alloxan diabetes by more than 2 times compared to the

control group correlates with an increase in the activity of the mitochondrial form of MDH. An increase in the rate of the mdh1 gene by 1.8 times in diabetic conditions may be associated with the appearance of an additional peroxisomal form of MDH, probably due to alternative splicing. According to Stibler *et al*. the c-terminally expanded isoform containing the peroxisomal target signal 1 (PTS1) is formed using an alternative stop codon, and this isoform is localized in peroxisomes.

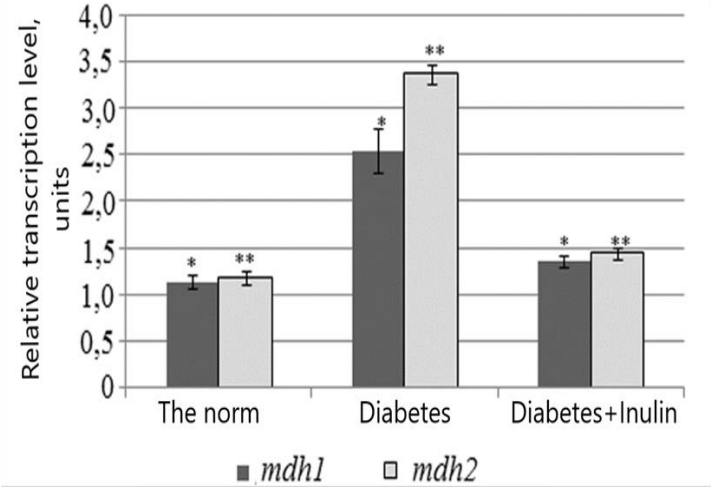

Figure 3. Relative level of transcripts of mdh1 (cytoplasmic) and mdh2 (mitochondrial) malate dehydrogenase genes from the liver of control group rats (Norm) exposed to alloxan diabetes (Diabetes) and animals with pathology taking inulin (Diabetes+INULIN). When assessing the relative level of transcription of the mlh1 and msh2 genes in all groups of the studied animals, statistically significant differences were revealed (*p=0.039; **p<0.001). Method used: Variance analysis.

Conclusion

Thus, we revealed an increase in the activity and appearance of the peroxisomal isoform of MDH in the liver of rats in experimental diabetes. Data on the expression of the mlh1 and msh2 genes indicate that in diabetes, an increase in the activity of isoenzymes occurs at the transcription level of the corresponding genes.
In addition, the results obtained indicate that the studied plant extract has a hypoglycemic effect, manifested in a noticeable decrease in the concentration of glucose in the blood of rats with alloxan diabetes; it levels the change in the transcriptional activity of the studied genes and, as a consequence, blocks the formation of a new isoform of MDH in rats with pathology.

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