UDC 616.12:616-018:616.379-008.64-039:599.323.4

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Morphofunctional features of tissues and blood in rats against the background of experimental diabetes mellitus

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Modern Pediatrics. Ukraine. (2023). 1(129): 99-104. doi 10.15574/SP.2023.129.99

For citation: Havrylenko YuV, Laiko AA, Syniachenko VV, Kyianytsia MA, Osadcha TM. (2023). Morphofunctional features of tissues and blood in rats against the background of experimental diabetes mellitus. Modern Pediatrics. Ukraine. 1(129): 99-104. doi 10.15574/SP.2023.129.99.

Introduction. Increased susceptibility to infections in patients with type 1 diabetes mellitus is associated with impaired body protective functions as a result of suppressed immunity, increased cellular adhesion of microorganisms, susceptibility to catabolic processes.
Purpose — to determine the morphofunctional features of tissues and blood in rats against the background of experimental diabetes mellitus (EDM); to investigate the effect of the extract of seven medicinal plants BNO 1030 in them.
Materials and methods. Experimental studies were conducted on 20 male Wistar rats in order to determine their level of nicotineamideadeninedinucleotide (NAD), reactive oxygen species in white blood cells and superoxide dismutase. All animals were separated: the Group 1 was the control group of healthy rats, the Group 2 — the control group receiving BNO 1030 extract, the Group 3 included animals with the EDM without treatment, the Group 4 — rats with the EDM, which received the BNO 1030 extract.
Results. After 6 weeks of development of the EDM, the NAD blood level in diabetic rats was reduced and reached (0.270±0.017) μmol/L, which is lower than that in the control group ((0.357±0.021) μmol/L).
The group of diabetic rats treated with BNO 1030 extract showed its increase to (0.341±0.012) μmol/L compared to the group of diabetic animals, which did not receive the drug. While assessing the viability of white blood cells, it has been established that the death of most of these cells occurs by apoptosis against the background of the EDM. When BNO 1030 was used in the group of diabetic rats, the level of apoptotic cells decreased and amounted to 13.7% compared to the group, which was not administered with the drug (20.8%). In addition, the level of apoptosis in control rats, which also received BNO 1030, decreased by 3% versus the control group.
Conclusions. The reduction of NAD content in tissues and blood in experimental animals with the EDM was determined, and its increase by 24% following the administration of BNO 1030 was established, which proves positive effect of the drug on the course of energy processes and glycolysis — the main route of carbohydrate metabolism.
When carrying out experiments with laboratory animals, all bioethical norms and recommendations were observed.
No conflict of interests was declared by the authors.
Keywords: experimental diabetes mellitus, BNO 1030, white blood cells, antioxidant system.

Морфофункціональні особливості тканин та крові у щурів на тлі експериментального цукрового діабету

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Вступ. Підвищена сприйнятливість до інфекцій у пацієнтів із цукровим діабетом 1-го типу пов'язана з порушенням захисних функцій організму внаслідок пригніченого імунітету, підвищеної клітинної адгезії мікроорганізмів та схильності до катаболічних процесів.
Мета — визначити морфофункціональні особливості тканин та крові щурів на тлі експериментального цукрового діабету (ЕЦД); вивчити вплив на них екстракту семи лікарських рослин BNO 1030.
Матеріали та методи. Експериментальні дослідження проведено на 20 щурах-самцях лінії Вістар з метою визначення в них рівня нікотинамідаденіндинуклеотиду (НАД), активних форм кисню в лейкоцитах та супероксиддисмутази. Усі тварин поділено: група 1 — контрольна група здорових щурів, група 2 — контрольна група, яка отримувала екстракт BNO 1030, група 3 — тварини з ЕЦД без лікування, група 4 — щури з ЕЦД, які отримували екстракт BNO 1030.
Результати. Через 6 тижнів розвитку ЕЦД рівень НАД у крові діабетичних щурів знизився і досяг (0,270±0,017) мкмоль/л, що нижче, ніж у контрольній групі ((0,357±0,021) мкмоль/л).
Група діабетичних щурів, які отримували екстракт BNO 1030, показала його збільшення до (0,341±0,012) мкмоль/л порівняно з групою діабетичних тварин, які не отримували препарат. Оцінюючи життєздатності лейкоцитів, встановлено, що загибель більшості цих клітин відбувається шляхом апоптозу і натомість ЕЦД. У разі застосуванні BNO 1030 у групі діабетичних щурів рівень апоптотичних клітин знизився та становив 13,7% порівняно з групою, у якій препарат не вводили (20,8%). Крім того, рівень апоптозу в контрольних щурів, що також отримували BNO 1030, знизився на 3% порівняно з контрольною групою.
Висновки. Встановлено зниження вмісту НАД у тканинах та крові в експериментальних тварин з ЕЦД, а також його підвищення на 24% після введення BNO 1030, що доводить позитивний вплив препарату на перебіг енергетичних процесів та гліколізу — основного шляху вуглеводного обміну.
Під час експериментів із лабораторними тваринами всі біоетичні норми та рекомендації дотримано.
Автори заявляють про відсутність конфлікту інтересів.
Ключові слова: експериментальний цукровий діабет, BNO 1030, лейкоцити, антиоксидантна система.

Introduction

Disorders of the major metabolic pathways in the body that occur in type 1 diabetes mellitus (T1DM) are closely linked to the compromise of the immune system [8]. Increased susceptibility to infections in patients with T1DM is associated with impaired protective functions of the body as a result of suppressed immunity, increased cellular adhesion of microorganisms, and susceptibility to catabolic processes. The oxidative stress is one of the main pathogenetic mechanisms lying at the core of development of many late complications of T1DM, in particular micro- and macroangiopathies, and nephropathy [11]. Under these conditions, there is an excessive production of reactive oxygen species (ROS) and boosted processes of glucose autooxidation. At the same time, there is an imbalance between prooxidant processes and the antioxidant protection system in the body [1]. The ROS, which are produced under physiological conditions, are involved in regulatory processes, are signaling molecules, have an effect on the activity of some proteinases and phosphatases, as well as gene expression. However, due to their extremely high activity and excessive concentrations of the ROS, they are capable of causing cell metabolism disturbance as a result of oxidative stress, which lseads to disruption of membrane transport processes, changes in enzyme activity, gene mutations, damage to protein structure, lipids, etc. [6]. Moreover, the oxidative stress induced by hyperglycemia triggers the β-cell damaging mechanisms, and thus accelerates the development and progression of T1DM.

Therefore, constant regulation of the production of ROS in order to maintain their level within the physiological limits is essential for the functioning of cells in human and animal bodies. The antioxidant system (AOS) performs such regulation against the harmful effects of the overproduction of the ROS. Its main links are enzymatic and non-enzymatic. The superoxide dismutase (SOD) is one of the key components of the AOS enzymatic link. It catalyzes the conversion of the superoxide anion radical with the formation of hydrogen peroxide and, subsequently, oxygen and water. High activity of the SOD was detected in the liver, heart, and red blood cells [12].

Treatment of T1DM patients with other chronic comorbidities, such as chronic tonsillitis (CT), is a particularly complex clinical issue in pediatrics. Under these conditions, CT exacerbates the metabolic disorders induced by T1DM, leads to decompensation of carbohydrate metabolism and ketoacidosis, which, in turn, exacerbates the course of the pathological process in the tonsils [7]. Therefore, complex therapy with modern immune rehabilitation medicinal products is of great importance in treatment of T1DM in the presence of CT.

Among the means of non-specific immunopharmacotherapy, BNO 1030 (an active ingredient in the composition of Imupret, manufactured by Bionorica SE, Germany), is of particular interest in terms of clinical efficacy. Its ingredients are the active components of marshmallow root, chamomile flowers, horsetail herbs, walnut leaves, yarrow herb, oak bark, and medicinal dandelion herb. Due to these components, the drug has a clear immunomodulatory effect, which is aimed at activating the non-specific immune protection (increase in the number and activity of phagocytes, killer protection, as well as the restoration of antibody formation in the setting of immunosuppression). Yet, no studies on the use of this drug in children with CT and T1DM have been found in the literature.

Purpose of the study — is to determine the morphofunctional features of tissues and blood in rats against the background of the experimental diabetes mellitus (EDM) and to investigate the effect of the BNO 1030 extract in them.

Materials and methods

Experimental studies were carried out at Shupyk National Healthcare University of Ukraine and Palladin Institute of Biochemistry of the NAS of Ukraine on 20 male Wistar rats, of which the Group 1 (C) was the control group of healthy rats, the Group 2 (C + BNO 1030) — the control group receiving BNO 1030 extract, the Group 3 (EDM) included animals with the EDM without treatment, the Group 4 (EDM + BNO 1030) — rats with the EDM, which received the BNO 1030 extract.

The EDM was induced in animals by a single intraperitoneal administration of Streptozotocin (S0130, Sigma-Aldrich Co. LLC, USA) at a dose of 55.0 mg/kg diluted in 0.1 M citrate buffer, pH 4.5. Rats in the control group of the same age were intraperitoneally injected with 0.5 mL of 0.1 M citrate buffer, pH 4.5. After four weeks of EDM development, rats were administered with the BNO 1030 extract for 14 days *per os* at a dose of 0.05 mL/animal 3 times a day, the cho-

sen concentration meets the instructions for use of a daily dose for children over 12 years old and adults, taking into account the species-specific resistance factor for humans and rats (0.45 and 1.89, respectively)

Animal blood from the retrobulbar venous sinus of the eye was used as a study material to identify the key enzymes of antioxidant blood protection: the ROS level in white blood cells (WBCs) serum SOD activity level, living, apoptotic, and necrotic count in WBCs by flow cytofluorometry, state of tissue energy processes based on the nicotineamide adenine dinucleotide (NAD) content in peripheral blood using a spectrophotometer.

Determination of ROS production in WBCs was measured using 2',7'-dichlorofluorescin diacetate (Sigma-Aldrich) at a final concentration of 25 μmol/L.

The contents of living, apoptotic, and necrotic WBCs were detected using GFP-conjugated Annexin V (Green fluorescent Protein) and PI (Propidium iodide, Sigma-Aldrich, USA). To achieve that, the cells were resuspended in 1 mL of annexin-binding buffer (10 mol/L Hepes, 140 mol/L NaCl, 2.5 mol/L $CaCl_2$) and precipitated at 400g for 7 min. The supernatant was removed, the cells were resuspended in 500 μL of annexin-binding buffer with the addition of annexin-GFP at a final concentration of 0.6 μg/mL, PI — 5.0 μg/mL. The samples were vortexed and incubated at room temperature for 15 min.

The samples were then analyzed using a COULTER EPICS XL flow cytometer. The fluorescence signals of the test samples were recorded on the FL1 channel (515–535 nm) for GFP, and FL3 (620–630 nm) for PI. More than 20,000 events from each sample were analyzed. The results were processed using FCS Express V3.

The SOD activity was determined using the microplate technique, the total volume of the sample was 0.25 mL. 25 μL of the sample was added to 200 μL of freshly prepared mixture (0.1 mmol/L EDTA, 62 mmol/L NBT (nitro blue tetrazolium), 294 mmol/L NADH in 50 mmol/L of phosphate buffer pH 7.4). The reaction was triggered by adding 25 μL of freshly prepared 33 mmol/L PMS (phenazine methosulfate) in 50 mmol/L of phosphate buffer pH 7.4 containing 0.1 mmol/L EDTA. Extinction was measured at 560 nm. Comparison of the SOD activity in the EDM setting and exposure to the drug was performed versus control, which were taken as 100%.

Statistical processing of the obtained data samples was performed using Statistica 6.0 software. The sets were compared using Student's t-test. The results are presented as a mean (M) and a standard error of the mean (±m). The difference was considered statistically significant at $P<0.05$.

The meeting of the Ethics Committee of Shupyk National Healthcare University of Ukraine approved and granted permission to conduct this clinical study in accordance with the current Ukrainian legislation in force, current ethical standards and principles for experimental studies. When carrying out experiments with laboratory animals, all bioethical norms and recommendations were observed.

Results

At the beginning of the experimental studies, the body weight and blood glucose were measured in animals, which were virtually the same in all study groups. However, after 6 weeks of EDM development, the body weight in diabetic rats decreased by 19% (Fig. 1), while blood glucose levels increased 2.3 times compared to control animals (Fig. 2). The data obtained confirm the development of uncompensated hyperglycemia in diabetic animals.

According to the findings, no effect of BNO 1030 on body weight was detected in control rats. At the same time, it has been established that the administration of the herbal medicinal product resulted in a slight decrease in blood glucose in the group of diabetic animals, while the product had no effect on the body weight of the animals.

Based on the literature data, it is known that depletion of energy resources in human and animal bodies leads to disruption of many metabolic processes. There is a possibility that significant changes in the metabolism of carbohydrates, in particular, glucose, by glycolysis, the final metabolite of which — pyruvate — enters the cycle of tricarbo-

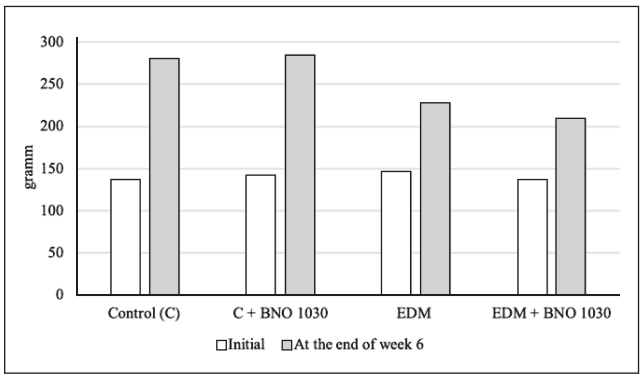


Fig. 1. Body weight in the study rats by observation groups (n=5)

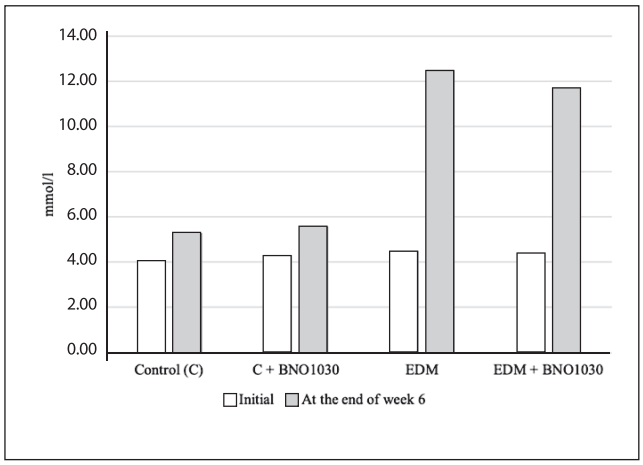


Fig. 2. Blood glucose levels in the study rats by observation groups (n=5)

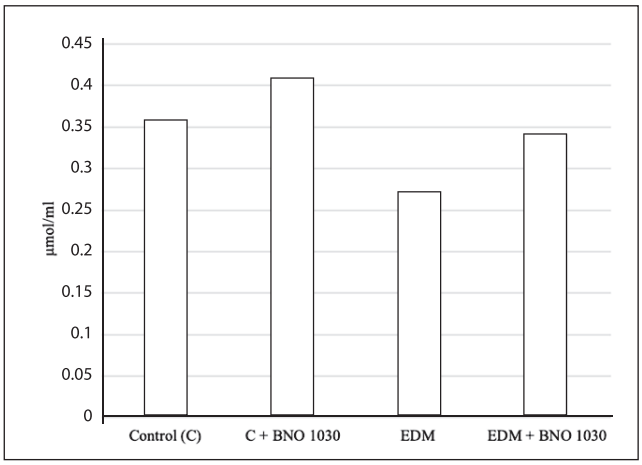
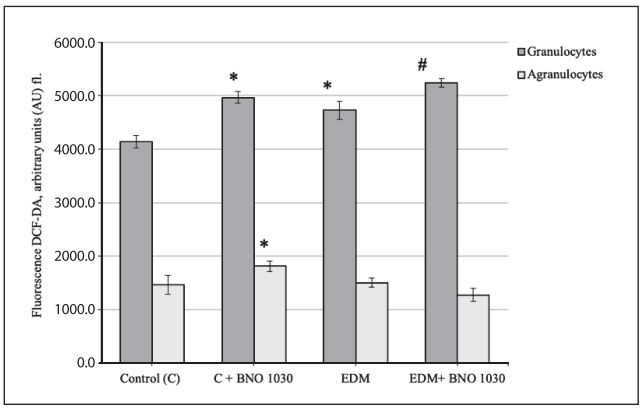
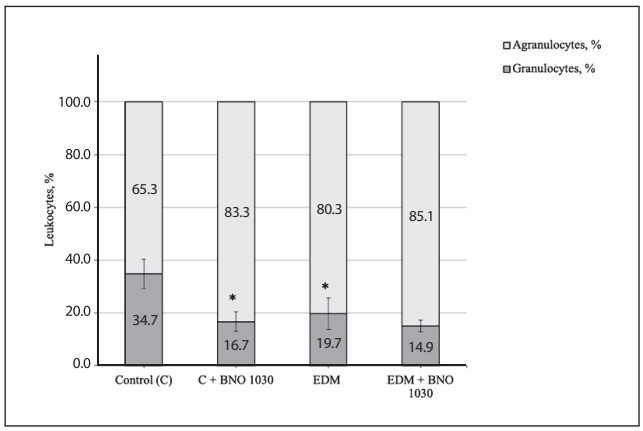


Fig. 3. NAD blood level in the study rats by observation groups (n=5)



Notes: * — p<0.05 versus the control group; # — p<0.05 versus the EDM group.

Fig. 4. Relative fluorescence of Dichlorodihydrofluorescein in agranulocytes and blood agranulocytes in diabetic rats (n=5)



Notes: * — p<0.05 versus the control group; # — p<0.05 versus the EDM group.

Fig. 5. Viability of WBCs by observation groups (n=5)

xylic acids, providing synthesis of ATP, will occur against the background of hyperglycemia.

However, as the content of NAD and its reduced form NADH decrease, these pathways will undergo significant changes, so it was important to evaluate the NAD concentration in protein-free blood extract in diabetic rats. As the data presented in Fig. 3 shows, after 6 weeks of development of the EDM, the NAD blood level in diabetic rats was reduced and reached (0.270±0.017) µmol/L, which is 24% lower than that in the control group ((0.357±0.021) µmol/L).

When the NAD level was analyzed in the group of diabetic rats treated with BNO 1030 extract, its increase to (0.341±0.012) µmol/L (by 26%) was observed compared to the group of diabetic animals, which did not receive the drug.

When analyzing the ROS level in WBCs, it has been established that it increased by 17% in agranulocytes of diabetic rats compared to the group of control animals. However, the level of ROS in blood agranulocytes in diabetic rats did not change and was the same as in the control group.

The level of ROS in blood agranulocytes in diabetic rats administered with BNO 1030 increased by 8%, whereas in agranulocytes it decreased by 5% compared to the group of animals, which did not receive the drug (Fig. 4).

When assessing the viability of WBCs, it has been established that the death of most of these cells occurs by apoptosis against the background of the EDM. When BNO 1030 was used in the group of diabetic rats, the level of apoptotic cells decreased and amounted to 13.7% compared to the group, which was not administered with the drug (20.8%). In addition, the level of apoptosis in control rats, which also received BNO 1030, decreased by 3% versus the control group (Fig. 5).

Against the background of intensifying oxidative stress, it was advisable to determine the state of the AOS. In determination of the activity of the key AOS enzyme (SOD), it has been established

that it decreased by 17.6% in the serum of diabetic rats. This is an indication that the production of superoxide radicals (O_2) significantly exceeds the functional capacity of the enzyme (Fig. 6). Administration of BNO 1030 in diabetic rats for two weeks led to a partial increase in SOD activity, indicating its protective effect on AOS.

Discussion

In patients suffering from T1DM, the cardiovascular system undergoes the greatest changes, its disorders leading to involvement of the vascular, nervous and other systems, in particular the immune system, as well as to disability and mortality, one of the reasons for which is the accumulation of the end products of glycosylation [5].

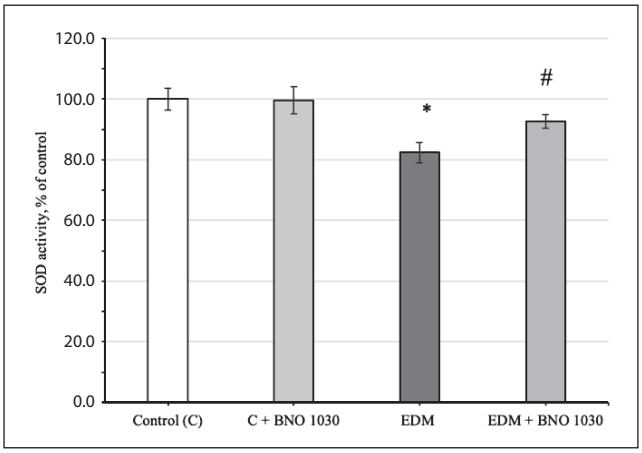
With the development of pathological conditions, in particular T1DM, and especially with prolonged decompensation, significant changes occur in the flow of energy processes, which in turn leads to disruption of physiological oxygen consumption, as well as the regulation of tissue metabolism, one of the main roles in which is played by NAD. Since the NAD plays an important role as a coenzyme of numerous dehydrogenases, alterations in its content will lead to significant impaired cellular metabolism, as well as suppression of humoral immunity, which may be accompanied by the risk of development of susceptibility to infections in patients with T1DM [3,4].

The decrease in NAD content under the conditions of the EDM may be the result of a decrease in the content of precursors of its biosynthesis, namely nicotinic acid, nicotinamide, and tryptophan, as well as the use of the NAD as a substrate in poly-ADP-ribosylation processes as a result of breakages in DNA molecules induced by the EDM [10].

That is, NAD is not only an important coenzyme of numerous dehydrogenases, but also a vital compound for the regulation of cellular metabolism, on the level of which energy processes in the body depend.

We have obtained some data indicative of the fact, that there is an increased production of ROS, which triggers the development of oxidative stress and increases the rate of glucose oxidation against the background of the EDM-induced hyperglycemia.

These findings are consistent with the findings of other researchers, who have shown that there is a relationship between the development of oxidative stress in WBCs, blood glucose, blood pressure, and C-reactive protein [2].



Notes: * — p<0.05 versus the control group; # — p<0.05 versus the diabetes mellitus group.

Fig. 6. SOD activity in serum by observation groups (n=5)

Our data on the ability of BNO 1030 to prevent the death of WBCs may indicate that the drug components, when combined, are potentially capable of exhibiting mediated antibacterial action. This may be confirmed by data from other researchers, who have shown that drug made of a combination of medicinal plants effectively prevent the development of recurrent infections of the upper respiratory tract [9].

Conclusions

Thus, the development of the EDM positively leads to a decrease in body weight and an increase in blood glucose levels in diabetic rats. Against the background of hyperglycemia, the NAD level in the blood of diabetic animals was significantly reduced by 24%. The use of the BNO 1030 extract increased the NAD blood values, which indicates its positive effect on the flow of energy processes, especially on the main pathway for carbohydrate metabolism — glycolysis.

The results of experimental studies showed that the administration of the BNO 1030 extract led to a slight intensification of ROS production in control and diabetic groups, but its effect positively increased the viability of immune cells, preventing their death.

The use of BNO 1030 increased the SOD activity by 10% compared to the group of diabetic animals, which suggests that among the components of this medicinal product there are compounds that have antioxidant activity and inhibit the enhanced formation of superoxide radicals induced by the presence of the EDM.

No conflict of interests was declared by the authors.

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Стаття надійшла до редакції 16.09.2022 р., прийнята до друку 07.02.2023 р.