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DIABETES-CORRECTING AND ANTIOXIDANT EFFECTS OF GRAPE POMACE EXTRACT RICH IN NATURAL COMPLEX OF POLYPHENOLS

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Background. The positive health effects of polyphenols have led to an increased scientific interest in these natural compounds over the past decade. Many studies confirm the effectiveness of polyphenols as additional therapy in diabetes, especially due to the sugar-lowering effect of polyphenols. The aim of the research was to investigate the morphological and functional state of peripheral blood erythrocytes and the indices of oxidative stress in the liver of rats with experimental diabetes and after the administration of grape pomace extract rich in natural complex of polyphenols.

Materials and Methods. We obtained grape pomace extract, which contains a variety of polyphenolic compounds. Rats of the following groups were used in the experiments: control animals, animals treated with grape pomace extract rich in natural complex of polyphenols for 14 days, animals with streptozotocin-induced diabetes mellitus, and animals with streptozotocin-induced diabetes mellitus treated with grape pomace extract rich in natural complex of polyphenols for 14 days. The number of erythrocytes and reticulocytes, the concentration of hemoglobin and glycated hemoglobin were determined in the peripheral blood of rats. The activities of catalase, superoxide dismutase and the content of thiobarbituric acid-reactive substance and carbonyl groups of proteins were determined in the liver tissues of rats.

Results. The study has shown an increase in the number of erythrocytes and the level of hemoglobin, a decrease in the level of glycated hemoglobin and the number of reticulocytes in the peripheral blood of rats after administration of grape pomace extract rich in natural complex of polyphenols to rats with experimental diabetes. A decrease in the content of thiobarbituric acid-reactive substance and the content of carbonyl groups

of proteins of neutral and basic character and an increase in the activity of catalase and superoxide dismutase in liver tissues were found under the same conditions.

Conclusions. The results indicate that the extract of the natural complex of polyphenols is capable of correcting the morphological and functional state of erythrocytes, as well as improving the activity of antioxidant enzymes and the content of marker molecules of oxidative stress in hepatocytes of rats under experimental diabetes mellitus.

Keywords: diabetes mellitus, oxidative stress, polyphenolic compounds, grape pomace, erythrocytes, hepatocytes

INTRODUCTION

Diabetes mellitus (DM) is a chronic disease of the endocrine system caused by a violation of insulin synthesis and/or its action and is accompanied by an increase in the level of glucose in the blood. According to statistics, the number of people suffering from DM has increased from 108 million in 1980 to 422 million in 2016 (American Diabetes Association, 2021).

DM is known to affect blood cells. Erythrocytes are the first cells that react to changes in plasma composition. Erythrocytes in patients with DM face hyperglycemia, oxidative stress, inflammation, and impaired lipid metabolism, leading to increased aggregation and/or decreased cell deformation (Wang *et al.*, 2021).

The impact of reactive oxygen species (ROS) (Volpe *et al.*, 2018) leads to increased lipid peroxidation (Ayala *et al.*, 2014) and protein post-translational modification (Zheng *et al.*, 2016; Sabadashka *et al.*, 2021b). Such modifications usually cause the loss of protein function and can distort the signaling pathways involved in the antioxidant defense and cellular homeostasis (Hecker & Wagner, 2018).

A large number of common drugs are used to treat hyperglycemia; however, most of them do not provide long-term control (Pandey & Rizvi, 2014). Nevertheless, when choosing antidiabetic drugs, not only their sugar-lowering effectiveness, but also the effect on the functional state of blood cells as well as oxidative stress in organs that are affected in the pathogenesis of diabetes should be taken into account.

Polyphenols that exhibit a hypoglycemic effect are increasingly attracting the attention of scientists. Plants synthesize polyphenols that are secondary metabolites, the main functions of which are to protect them in adverse conditions and to provide resistance against microbial infections. Catechins, proanthocyanidins, and anthocyanins and their oxidation derivates are natural antioxidants present in the highest concentration in red grapes and red wine (Pandey & Rizvi, 2014). Grape pomace of *Vitis vinifera* L. is one of the main waste products of the wine industry and can be regarded as an excellent and affordable source of polyphenols.

Considering this, the study of the biochemical mechanisms of the influence of polyphenols from grape pomace and their effect on the state of the blood system and hepatocytes, which determines the state of the body in diabetes and the likelihood of developing acute or chronic diabetic complications, deserves considerable attention. The aim of the research was to investigate the morphological and functional state of peripheral blood erythrocytes and the indicators characterizing oxidative stress in the liver of rats with streptozotocin-induced diabetes under the administration of grape pomace extract rich in a natural complex of polyphenols (NCP).

MATERIALS AND METHODS

The research was performed on male white outbred rats weighing 120–180 g. The experiments were conducted according to „General Principles of Experiments on Animals", approved by the First National Congress on Bioethics (Kyiv, Ukraine, 2001) and agreed with the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and the Law of Ukraine „On Protection of Animals from Cruelty" of February 26, 2006, and also approved by the Ethics Committee of Ivan Franko National University of Lviv, Ukraine (protocol No 29-01-2023 from January 29, 2023).

Preparation of grape pomace extract rich in natural complex of polyphenols. The used grape pomace was provided by Odesa National University of Technology (Ukraine) and its characteristics were described previously (Sabadashka *et al.*, 2021a). The extract was prepared according to the modified method described earlier (Ohai *et al.*, 2001). The total content of polyphenols in the obtained extract was measured using Folin-Ciocalteu reagent according to gallic acid equivalent (Singleton *et al.*, 1999) simple, and require only common equipment and have produced a large body of comparable data. Under proper conditions, the assay is inclusive of monophenols and gives predictable reactions with the types of phenols found in nature. Because different phenols react to different degrees, expression of the results as a single number – such as milligrams per liter gallic acid equivalence – is necessarily arbitrary. Because the reaction is independent, quantitative, and predictable, analysis of a mixture of phenols can be recalculated in terms of any other standard. The assay measures all compounds readily oxidizable under the reaction conditions and its very inclusiveness allows certain substances to also react that are either not phenols or seldom thought of as phenols (e.g., proteins). The concentration of polyphenols in the obtained extract was 80 mg/mL.

Experimental DM was induced by intraperitoneal injection of streptozotocin (Sigma, USA), dissolved in 10 mM citrate buffer (pH 5.5), at a dose of 60 mg/kg of body weight. The development of DM was controlled using fasting blood glucose, which was determined 72 hours after streptozotocin injection. In the research, animals with glucose concentrations of 12 mmol/L and higher were used. Glucose concentration was determined with a Contour plus glucometer (Bayer, Switzerland).

All experimental rats were divided into four groups: 1st – control animals (C); 2nd – healthy animals that were treated with grape pomace extract rich in NCP (C + NCP); 3rd – animals with experimental DM (DM); 4th – animals with experimental DM that were treated with grape pomace extract rich in NCP (DM + NCP).

The extract was administered for 14 days per os with water at a dose of 45 mg of polyphenols per 1 kg of body weight, which corresponds to the theoretical average concentration of polyphenols contained in 300 ml of wine (which, according to the literature, corresponds to the optimal daily norm for a person weighing 70 kg (Hrelia *et al.*, 2022)).

Rats from all experimental groups were decapitated under ether anesthesia on the 29th day of the experiment. Samples collection was carried out after the decapitation of the animals. The blood was collected into a porcelain cup. Heparin was used as an anticoagulant (final dilution of heparin to whole blood = 1:100).

Extirpation of the liver was also performed. For the research, the 10% W/V homogenate in hypotonic 50 mM Na-K phosphate buffer (pH 7.4) was prepared. The studied parameters were determined in the supernatant obtained after centrifugation of the homogenate for 5 min at 10,000 rpm.

The concentration of proteins in the supernatant samples was measured according to the conventional Lowri method (Lowry *et al.*, 1951). The number of erythrocytes was determined by the method of counting in the Goryaev chamber (Sabadashka *et al.*, 2021a). The concentration of hemoglobin was determined by the photometric cyanmethemoglobin method (Sabadashka *et al.*, 2021a). The number of reticulocytes was determined by the method of Heilmeyer L. (Vitak *et al.*, 2015). The activity of superoxide dismutase (SOD, EC 1.15.1.1) and catalase (CAT, EC 1.11.1.6) were determined according to (Sabadashka *et al.*, 2021a). The content of carbonyl groups of proteins and thiobarbituric acid-reactive substance (TBARS) were determined according to (Sabadashka *et al.*, 2021a).

Statistical analysis was carried out using Microsoft Excel. The calculation of basic statistical parameters was performed by direct quantitative data obtained from the study (arithmetic mean – AM, the standard deviation of the arithmetic mean – SD). The probability of statistical significance of differences between group means was assessed by one-way analysis of variance (ANOVA) with post hoc analysis. The difference was considered significant under the indications of reliability $p \geq 0.95$ (significance level $P < 0.05$).

RESULTS AND DISCUSSION

High blood glucose (hyperglycemia) is a key indicator of DM. During long-term hyperglycemia, the morphology and functions of erythrocytes inevitably undergo a series of changes that further affect microcirculation (Wang *et al.*, 2021). Therefore, it is worth investigating changes in the number of erythrocytes and reticulocytes, concentration of hemoglobin in peripheral blood of rats with DM without and with the extract administration.

According to the obtained data, there was a significant decrease in the number of erythrocytes (by 1.2 times) in the peripheral blood of rats under experimental DM compared to control animals (see **Table**). When studying the number of reticulocytes, which are the precursors of erythrocytes that enter the bloodstream, we found a significant increase (by 1.9 times) in the peripheral blood of rats with experimental DM compared to control animals (see Table). These data are consistent with the literature data (Riley *et al.*, 2001). Under the administration of grape pomace extract rich in NCP to non-diabetic animals, we detected a tendency for the number of erythrocytes to increase and the number of reticulocites to decrease, compared to the control animals (see Table). Under the administration of grape pomace extract rich in NCP to animals with DM, the number of erythrocytes increased by 1.2 times, while the number of reticulocytes decreased by 2.2 times compared to animals with DM (see Table). The anti-inflammatory and antioxidant properties of polyphenols allow them to improve erythropoiesis, especially under the stress conditions. For example, resveratrol accelerates erythroid maturation by decreasing red cell membrane oxidative damage (Xu *et al.*, 2021). Therefore, the normalization of the number of reticulocytes in animals with DM testifies to the positive effect of the grape pomace extract rich in NCP.

The most important function of the erythrocyte is the transportation of molecules, especially oxygen, which is due to the content of hemoglobin in the erythrocyte (Wang *et al.*, 2021). In rats with DM, there was a decrease in hemoglobin concentration by 1.1 times in the peripheral blood when compared to the control animals (see Table). Under the administration of grape pomace extract rich in NCP to animals with DM, there was an increase in hemoglobin concentration by 1.2 times compared to the data obtained in animals with DM (see Table). Polyphenols can absorb ROS, lipoperoxide

radicals, as well as chelate metal ions, such as Fe^{2+} and Cu^{2+}, which play an important role in initiating of free radical reactions. Therefore, due to the effect of polyphenols, hemoglobin biosynthesis is activated (Hussain *et al.*, 2016).

The main hematological parameters of the peripheral blood of rats in normal conditions, under diabetes and after the introduction of grape pomace extract rich in natural complex of polyphenols (M±SD, n = 6–8)

Hematological parameters	Groups			
	Conrol	Control + NCP	Diabetes mellitus	Diabetes mellitus + NCP
Number of erythrocytes, mln of cells/μL	9.91±0.47	10.42±0.87	8.32±0.24*	10.16±0.30#
Hemoglobin concentration, g%	15.50±0.50	14.65±0.58	13.42±0.34*	15.68±0.84#
Content of glycated hemoglobin, %	6.57±0.25	6.13±0.24	13.70±1.7***	6.83±0.38###
Number of reticulocytes, %	3.17±0.19	2.57±0.36	5.98±0.53***	2.66±0.34###

Comments: *, *** – the difference is significant compared to control (p ≥0.95, p ≥0.999); #, ### – the difference is significant compared to DM (p ≥0.95, p ≥0.999)
Herein after, control animals (C), control animals treated with an extract from grape pomace rich in natural complex of polyphenols (C + NCP), animals with experimental diabetes mellitus (DM), and animals with experimental diabetes mellitus treated with an extract from grape pomace rich in natural complex of polyphenols (DM + NCP)

One of the complex cascades of events that lead to cellular failure in response to high glucose is the formation of glycated hemoglobin. Glycated hemoglobin forms a strong bond with oxygen and contributes to the development of tissue hypoxia in DM (Welsh *et al.*, 2016). A two-fold increase in the content of glycated hemoglobin in the peripheral blood of rats with experimental DM compared to control animals was observed (see Table). Under the administration of grape pomace extract rich in NCP to animals with DM, the level of glycated hemoglobin significantly decreased (by 2 times) compared to the data obtained in DM (see Table). A decrease in the level of glycated hemoglobin under the administration of the grape pomace extract rich in NCP in diabetes indicates a hypoglycemic effect of the extract.

Similar results were obtained when studying the influence of polyphenolic compounds from red wine on the number of erythrocytes, reticulocytes, the total hemoglobin concent and its glycated form (Sabadashka *et al.*, 2021a). The normalization of the studied parameters in animals with DM testifies to the positive effect of the grape pomace extract rich in NCP.

The antioxidant system plays an important role in maintaining redox balance. CAT and SOD are the most important enzymes among endogenous antioxidants involved in various physiological and pathological processes (Nocella *et al.*, 2019).

We studied activities of SOD and CAT in the liver of rats with and without grape pomace extract rich in NCP under DM because it is the central organ responsible for the metabolism of a large number of exogenous and endogenous substances. Liver is the main target organ for various xenobiotics and a number of polyphenols have shown promising therapeutic effects on liver damage in animal models (Li *et al.*, 2018).

SOD catalyzes the dismutation of superoxide anion into hydrogen peroxide and molecular oxygen, whilst CAT enzymatically converts hydrogen peroxide into oxygen and water and thus neutralizes it. The activities of CAT and SOD decreased by 1.3 and 1.6 times in the liver tissues of animals with DM compared to control (**Fig. 1**). Under the administration of grape pomace extract rich in NCP to animals with DM there was an increase in CAT and SOD activities by 1.4 and 1.8 times compared to diabetes (Fig. 1). Similar results were obtained in different cells and tissues (plasma, kidneys, heart, and brain) of rats with experimental diabetes after the administration of polyphenols from red wine and are consistent with literature data. The decreased activity of CAT and SOD under DM may be caused by inactivation of these enzymes due to the glycation of enzymes active sites and/or oxidation and nitration by ROS (Hertsyk *et al.*, 2021). Antioxidant properties of polyphenols are mainly explained by their capability of absorbing free radicals, in particular ROS and peroxynitrite, and chelating metals (Yang *et al.*, 2020). Polyphenols from grape pomace extract can reduce the level of ROS and peroxinitrite thus increasing activities of SOD and CAT under DM (Hertsyk *et al.*, 2021). Also, the normalization of the studied enzymes activities under DM after treatment with extract rich in NCP can be caused by inhibition of glycation of active sites of CAT and SOD due to sugar-lowering activity of polyphenolic compounds (Sabadashka *et al.*, 2021a).

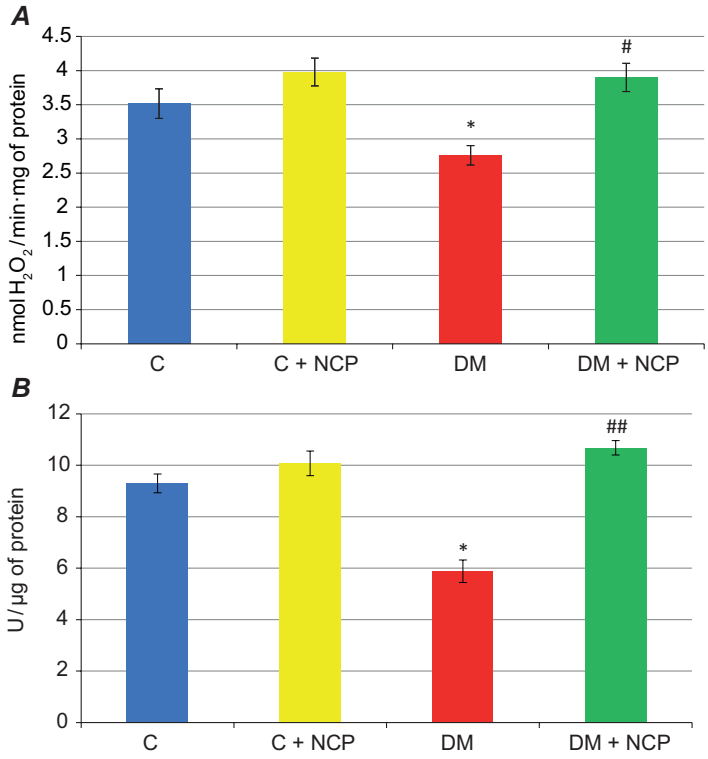

Fig. 1. The activity of catalase (***A***) and superoxide dismutase (***B***) in the liver tissues in control animals, animals with experimental diabetes mellitus with and without treatment with grape pomace extract rich in natural complex of polyphenols.
* – the difference is significant compared to control (p ≥0.95); #, ## – the difference is significant compared to DM (p ≥0.95; p ≥0.99)

Protein carbonyls are formed during protein oxidation and are the biomarkes of oxidative stress. Damage of proteins with a subsequent accumulation of their oxidation products negatively affects cell physiology. Carbonyl groups (aldehydes and ketones) are produced on protein side chains (especially of Pro, Arg, Lys, and Thr) when they are oxidised. Protein carbonyls can be generated directly (by amino acids oxidation and the α-amidation pathway), or indirectly, by forming adducts with lipid peroxidation products or glycation and advanced glycation end-products (Dalle-Donne *et al.*, 2003).

An increase in the content of protein carbonyls of neutral and basic characters by 120.3 % and 142.1 %, respectively, was revealed in the liver tissues of animals with experimental DM compared to control animals (**Fig. 2**). Under the administration of grape pomace extract rich in NCP to animals with DM, there was a decrease in the content of protein carbonyls of neutral and basic nature by 79.2 % and 119.4 %, respectively, compared to DM (Fig. 2). Polyphenols can inhibit protein carbonyls formation due to the action of ROS. Polyphenolic compounds are known as scavengers of ROS, therefore they can protect proteins from oxidation processes (Pandey & Rizvi, 2014).

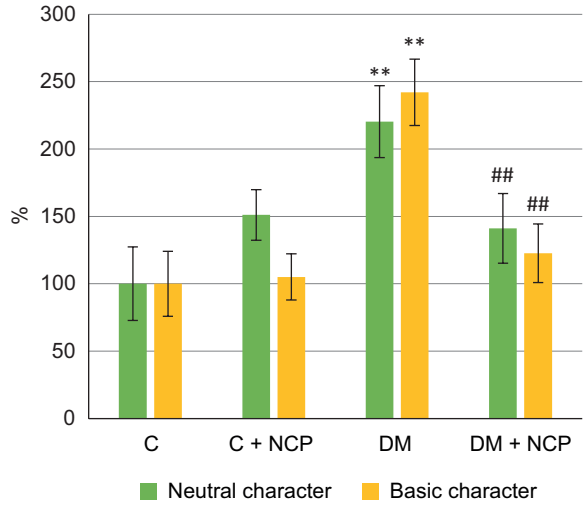

Fig. 2. The content of protein carbonyls of a neutral and basic character in the liver tissue in control animals, animals with experimental diabetes mellitus with and without treatment with grape pomace extract rich in natural complex of polyphenols. The content of protein carbonyls of a neutral and basic character in control was taken as 100 %.
** – the difference is significant compared to control (p ≥0.99); ## – the difference is significant compared to DM (p ≥0.99)

ROS initiate lipid peroxidation (LPO) or oxidation of unsaturated lipids, which leads to the formation of various oxidation products. The main primary products of lipid peroxidation are lipid hydroperoxides. Among the many different aldehydes that can be formed as secondary products during lipid peroxidation are malondialdehyde (MDA), propanal, hexanal, and 4-hydroxynonenal. MDA has been widely used for many years as a convenient biomarker for lipid peroxidation due to its easy reaction with thiobarbituric acid (Ayala *et al.*, 2014). A significant increase in the content of TBARS by 59.2 % in the liver tissues of animals with experimental DM compared to control animals was observed (**Fig. 3**).

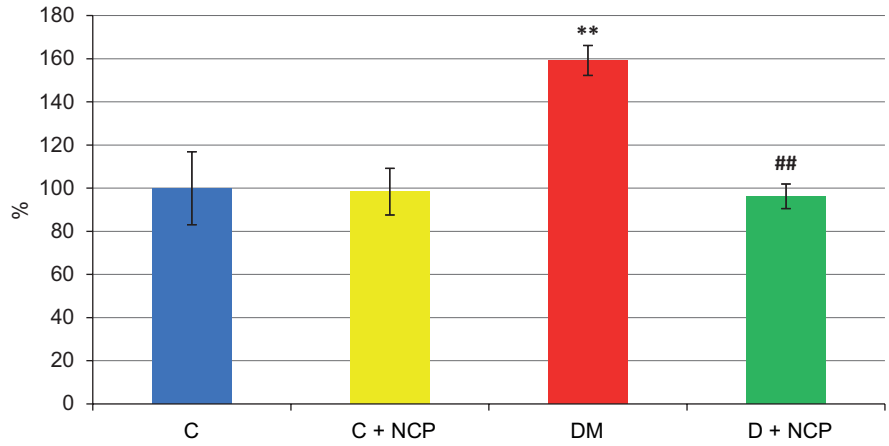

Fig. 3. The content of TBARS in the liver tissues of control animals, animals with experimental diabetes mellitus with and without treatment with grape pomace extract rich in natural complex of polyphenols. Values of control rats were taken as 100 %.
** – the difference is significant compared to control (p ≥0.99); ## – the difference is significant compared to DM (p ≥0.99)

Under the administration of grape pomace extract rich in NCP to animals with DM, there was an increase in the content of TBARS by 63 % compared to the data obtained for animals with experimental DM (Fig. 3). The high content of TBARS in liver tissues indicates oxidative stress. It is known that polyphenolic compounds have a positive effect on lipid peroxidation as they can act as hydrogen donors or chelate metal ions such as iron and copper (Lupoli *et al.*, 2020). In addition, polyphenolic compounds prevent the development of oxidative stress in DM due to their ability to scavenge free radicals (Pandey & Rizvi, 2014).

CONCLUSIONS

The obtained results enable the evaluation of the state of peripheral blood erythrocytes of rats, changes in distinct molecular markers of oxidative stress in the liver of rats with streptozotocin-induced DM, and the effect of grape pomace extract rich in natural complex of polyphenols on these indices.

All studied parameters indicate that grape pomace extract rich in NCP does not significantly affect the state of the organism without DM.

The parameters related to peripheral blood erythrocytes and oxidative injury of the liver of rats with DM after treatment with grape pomace extract rich in NCP were returned to control values, which confirm the diabetes-correcting activity of the studied extract, in particular sugar-lowering and antioxidant activities.

Thus, it is worth considering the extract rich in NCP as a basis for new diabetes-correcting medicinal product. However, the molecular mechanisms of action of these compounds require further research.

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COMPLIANCE WHITH ETHICAL STANDARDS

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Human Rights: This article does not contain any studies with human subjects performed by any of the authors.

Animal studies: All international, national and institutional guidelines for the care and use of laboratory animals were followed.

AUTHOR CONTRIBUTIONS

Conceptualization, [S.M.; S.N.]; methodology, [S.V.; Ch.D.; S.M.]; validation, [S.V.; Ch.D.; S.M.]; formal analysis, [S.V.; S.M.;].; investigation, [S.V.; Ch.D.]; resources, [S.V.; Ch.D.; S.M.]; data curation, [S.V.; Ch.D.; S.M.]; writing original draft preparation, [S.V.; S.M.]; writing – review and editing, [S.V.; S.M.; S.N.]; visualization, [S.V.]; supervision, [S.M.; S.N.]; project administration, [S.M.; S.N.]; funding acquisition, [S.M.].

All authors have read and agreed to the published version of the manuscript.

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ДІАБЕТ-КОРИГУЮЧИЙ І АНТИОКСИДАНТНИЙ ЕФЕКТИ ЕКСТРАКТУ З ВИНОГРАДНИХ ВИЧАВОК, ЗБАГАЧЕНОГО ПРИРОДНИМ КОМПЛЕКСОМ ПОЛІФЕНОЛІВ

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Вступ. Позитивний вплив поліфенолів на здоров'я зумовив зростання наукового інтересу до цих природних сполук упродовж останнього десятиліття. Багато наукових досліджень підтверджують ефективність використання поліфенолів як додаткових засобів у лікуванні діабету, зокрема, завдяки їхній цукрознижуючій дії. Мета дослідження – вивчити морфофункціональний стан еритроцитів периферичної крові та показники, що характеризують оксидативний стрес у печінці щурів зі стрептозотоцин-індукованим діабетом і під час застосування екстракту виноградних вичавок, збагаченого природним комплексом поліфенолів.

Матеріали та методи. Отримано екстракт виноградних вичавок, до складу якого входять різноманітні поліфенольні сполуки.

У дослідженні використовували такі групи щурів: контрольні тварини, контрольні тварини, яким вводили екстракт із виноградних вичавок, збагачений природним комплексом поліфенолів, упродовж 14 днів, тварини зі стрептозотоцин-індукованим цукровим діабетом і тварини з експериментальним цукровим діабетом, яким вводили екстракт із виноградних вичавок, збагачений природним комплексом поліфенолів, упродовж 14 днів. У периферичній крові щурів визначали кількість еритроцитів і ретикулоцитів, концентрацію гемоглобіну та глікованого гемоглобіну. У тканинах печінки щурів визначали активність каталази, супероксиддисмутази та вміст ТБК-позитивних продуктів і карбонільних груп білків.

Результати. Встановлено підвищення кількості еритроцитів, рівня гемоглобіну, зниження рівня глікованого гемоглобіну та кількості ретикулоцитів у пери-

феричній крові щурів після введення щурам з експериментальним цукровим діабетом екстракту з виноградних вичавок, збагаченого природним комплексом поліфенолів. За цих же умов виявлено зниження вмісту ТБК-позитивних продуктів і вмісту карбонільних груп білків нейтрального й основного характеру та підвищення активності каталази і супероксиддисмутази у тканинах печінки.

Висновки. Отримані результати доводять, що екстракт природного комплексу поліфенолів коригує морфологічний і функціональний стан еритроцитів, а також впливає на активність антиоксидантних ферментів і на вміст маркерних молекул оксидативного стресу – продуктів окиснення білків та ліпідів у гепатоцитах щурів за умов цукрового діабету.

Ключові слова: цукровий діабет, оксидативний стрес, поліфенольні сполуки, виноградні вичавки, еритроцити, гепатоцити

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