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СЕРЫЯ БІЯЛАГІЧНЫХ НАВУК

ТОМ 68

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PHYSIOLOGICAL CHANGES OF MITOCHONDRIA UNDER THE DIABETES CONDITION AND PHARMACOLOGICAL APPROACHES TO ELIMINATE THEM

Abstract. The state of lipid peroxidation (LPO), respiration and oxidative phosphorylation, mitochondrial permeability transition pore (mPTP), and the antiradical activity of rat liver mitochondria in the streptozotocin (STZ)-induced diabetes condition were studied with the consideration of the ways of correcting detected membrane damages using gossitan isolated from the cotton plant *Gossypium hirsutum* L. It was shown that the rate of respiration of liver mitochondria in states V3 and V4 increases during STZ-induced diabetes, which significantly reduces the respiratory control (RC) and ADP/O coefficients in comparison with the control. The findings suggest that the uncoupling of respiration and oxidative phosphorylation takes place during STZ-induced diabetes. It was shown that in the STZ-induced diabetes condition, the rate of swelling of rat liver mitochondria is higher than that of the healthy ones; this means that mPTP of rat liver mitochondria is in the open state. Gossitan recovers mPTP to the normal condition, thereby removing the STZ effect on mitochondria. Gossitan (a personal dose is 10 mg/kg of body weight, during 8 days) eliminates the detected functional disorders of rat liver mitochondria, probably due to its antioxidant properties.

Keywords: polyphenol compounds, mitochondria, antiradical activity, antioxidant activity, diabetes

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ФИЗИОЛОГИЧЕСКИЕ ИЗМЕНЕНИЯ МИТОХОНДРИЙ ПРИ САХАРНОМ ДИАБЕТЕ И ФАРМАКОЛОГИЧЕСКИЕ ПОДХОДЫ ПО ИХ УСТРАНЕНИЮ

Аннотация. Изучено состояние перекисного окисления липидов (ПОЛ), дыхания и окислительного фосфорилирования, митохондриальной Ca^{2+}-зависимой поры (mPTP) и антирадикальной активности митохондрий печени крыс при диабете, индуцированном стрептозотоцином (STZ). Рассмотрены пути коррекции выявленных повреждений митохондриальных мембран с использованием полифенола госситана, выделенного из хлопчатника *Gossypium hirsutum* L. Показано, что скорость дыхания митохондрий печени в состояниях V3 и V4 при STZ-индуцированном диабете увеличивается, что выражается в значительном снижении коэффициентов дыхательного контроля (V3/V4) и фосфорилирования (ADP/O). Полученные данные свидетельствуют о том, что во время STZ-индуцированного диа-

бета происходит разобщение дыхания и окислительного фосфорилирования. Показано, что в условиях STZ-индуцированного диабета скорость набухания митохондрий печени выше, чем у интактных крыс, что предполагает открытое состояние mPTP. Госситан нормализует динамику mPTP, снижая эффекты STZ на митохондрии печени. Позитивное влияние госситана (пероральная доза составляет 10 мг/кг массы тела в течение 8 дней) может быть опосредовано также антиоксидантными свойствами в отношении ПОЛ.

Ключевые слова: полифенолы, митохондрии, антирадикальная активность, антиоксидантная активность, сахарный диабет

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Introduction. At the development of various pathologies, along with alterations in the physiological processes of the cell, the functional state of the mitochondria is disturbed [1]. The function of mitochondria is important in the vital activity of each cell. In addition to disturbances in GLUT (the major membrane protein) signaling pathways of the cell membrane and insulin in diabetes mellitus, changes in the mitochondrial energetics and the ion transport system are observed [2, 3]. In experimental diabetes, the formation of free radicals in the mitochondrial respiratory chain increases. In conditions of diabetes as a result of violations of the transfer of electrons along the respiratory pathway, a decrease in the synthesis of ATP and the release of cytochrome C from the matrix, there is an energy deficit [4]. As a result of this, disturbances in the mitochondrial function can lead to a disruption in the synthesis of nucleic acids and protein in cells, as well as to enhance LPO. In diabetes, the number of free fatty acids and various phospholipids increases in mitochondria [5]. In diabetes, along with the study of degenerative alterations in pancreatic β-cells, the study of mitochondrial liver disorders and their correction by plant substances is an important urgent problem [6]. Since the study of ATP synthesis and ion transport systems in mitochondria as a source of cellular energy is considered extremely important in illuminating the mechanisms of diabetes and their treatment. However, in diabetes, natural compounds that have hypoglycemic activity and effectively affect cell organelles are very rare. For the first time, it was revealed that one of such compounds – polyphenol gossitan in conditions of STZ-diabetes shows hypoglycemic activity and effectively affects the bioenergetic system of liver mitochondria. The aim of the study was the investigation of the corrective effect of polyphenol gossitan [7] (Fig. 1) isolated from the *Gossypium hirsutum* L. plant on the dysfunction of LPO processes, respiration and oxidative phosphorylation of rat liver mitochondria under conditions of STZ-diabetes.

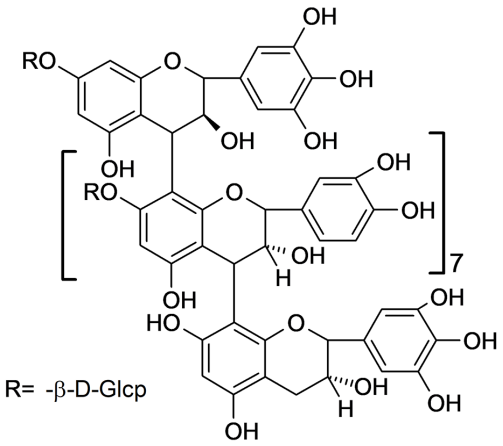

Fig. 1. Chemical structure of gossitan

Materials and research methods. ***STZ-induced diabetes model.*** Experiments were performed on 30 white male rats weighing 180–200 g. Laboratory animals were divided into three groups: I group – control (n = 10), II group – STZ-induced diabetes (n = 10), III group – STZ-induced diabetes + gossitan (n = 10). Control animals after daily starvation were entered intraperitoneally 0.2 ml of 0.9 % of the physiological solution. STZ-induced diabetes in rats by a single intraperitoneal injection of STZ (Sigma)

in 0.2 ml of citrate buffer at a dose of 50 mg/kg of body weight after a 24-hour fast. After the injection of STZ to rats, in 12 days when the level of the glucose in blood reached 11 mmol/l, during 8 days animals were entered intraperitoneally: 0.2 ml of 0.9 % of the physiological solution – II group and 10 mg/kg gossitan – III group (per os dose of 10 mg/kg body of weight). Previously, it was shown that these doses are STZ diabetogenic [8].

Blood glucose was determined using the glucose oxidase method set "Glucose – enzymatic-colorimetric test" (Cypress diagnostic, Belgium).

Mitochondrial isolation method. Mitochondria isolated from rat liver by differential centrifugation according to Schneider [9]. Nuclei and cellular fragments were removed by centrifugation at 600 g for 7 minutes in a centrifuge. The mitochondria are pelleted at 10 000 g for 15 minutes at the same temperature. The mitochondrial pellet was washed twice in the isolation EDTA-free medium. The content of mitochondrial protein was determined by the Lowry method in the modification of the Peterson [10].

mPTP condition measurement. mPTP condition assessed by the speed of Ca^{2+}-dependent swelling of mitochondria, the mitochondrial suspension recording light scattering at 540 nm. Experiments at 26 °C in a swelling medium of 200 mM sucrose, 20 μM EGTA, 5 mM succinate, 2 μM rotenone, 1 μg/ml oligomycin, 20 mM Tris, 20 mM HEPES, and 1 mM KH_2PO_4, pH 7.2 [11].

The concentration of mitochondria in the swelling experiments was 0.5 mg protein/ml.

Mitochondrial respiration and oxidative phosphorylation measurement. Mitochondrial respiration and oxidative phosphorylation were measured by polarography method (polarograph OH-105, Hungary) at 25 ºC. The assay medium contained 100 mM sucrose, 75 mM KC1, 10 mM Tris-HCl, 2.5 mM K_2HPO_4, pH 7.4 and 10 mM succinate as respiratory substrates. The protein concentration of mitochondria corresponded to 3 mg/ml of the reaction medium ADP (200 μM) was added as a respiratory stimulant. It was calculated the rate of mitochondrial respiration in different metabolic states: V_3 – respiration rate after making ADP, V_4 – respiration rate after spending ADP. The indices characterizing pair of oxidation and phosphorylation in mitochondria: respiratory control (RC) ratio ($RC = V_3/V_4$) and the coefficient of phosphorylation of ADP/O. Mitochondrial respiration rate in different metabolic states is expressed in nanograms of consumed oxygen atoms per 1 minute per 1 mg of mitochondrial protein. The respiratory control and ADP/O ratio was calculated according to the method of Chance [12].

Lipid peroxidation measurement. LPO experiments were carried out using thiobarbituric acid (TBA). The reaction was stopped by adding 0.220 ml of 70 % trichloroacetic acid in the incubation medium. Thereafter mitochondrial suspensions were centrifuged for 15 minutes at 4000 r/min. Then 1 ml of 75 % solution of TBA was added to 2 ml of the supernatant. The control tube was poured 2 ml of distilled water and 1 ml of TBA. The mixtures were incubated at 37 ºC water bath for 30 min. After cooling the absorbance was measured at 540 nm. The quantity of malondialdehyde (MDA) was calculated using a molar extinction coefficient equal to 1.56/10 sm. The rate of LPO reaction expressed in nM of MDA/mg of protein in hour [13].

DPPH kinetic analysis. The antiradical activity of polyphenols was determined by the standard method of measuring the kinetics of the optical density of an alcohol solution of the free radical (1.1-diphenyl-2-picrylhydrazyl) DPPH. The concentration of free radicals was 0.1 mM. The ratio of DPPH/polyphenol was 1:10. The change in the optical density of the alcohol solution of a pair of planetary plasmatic hormones was carried out in cuvettes with a long optical path of 1 cm, in a volume of 3 ml, on an SF-26 spectrophotometer [14].

Drugs and chemicals. These given chemical reagents were used: EGTA, EDTA, cyclosporine A (Sandoz, Switzerland), rotenone, tris-HCl (Serva, Germany), Sucrose (Russia), DPPH (Sigma, USA), $CaCl_2$ (Sigma, USA). Other reagents were chemically pure and received from local companies. Gossitan was kindly provided by Experimental technology laboratory of the Institute of bioorganic chemistry Academy of Sciences of Uzbekistan.

Data analysis. Statistical analyses were performed using the statistical package Origin 6 (OriginLab Corporation, USA). The data were evaluated using parametric Student's *t*-test, we expressed as M ± m. Deemed authentic results are expressed at $p < 0.05$, $p < 0.01$.

Results and its discussion. ***Antioxidant activity of gossitan*.** Our previous results indicate the uncoupling of oxidative phosphorylation in the mitochondria of the liver in STZ-induced diabetes, with the development of ATP deficiency in the rat tissues and the transition of mPTP to the open state, i. e. permeabilization of mitochondrial membranes is observed [15]. Gossitan reduces the effect of STZ diabetes on the function of mitochondria.

It was shown that STZ intoxication increases the rate of LPO in liver mitochondria, causes a hyper compensated low-energy shift with increasing respiratory rates in all metabolic states and dissociation of oxidative phosphorylation. Amber antitoxic therapy normalized the processes of succinate and NAD-dependent energy production in mitochondria, with the restoration of the conjugation of oxidation and phosphorylation, and LPO in the liver decreased [16].

The high antioxidant activity of gossitan has been shown recently on the model of alloxan diabetes in rats [17].

LPO is a radical molecular process in biological membranes and this way oxidizes free fatty acids that are part of unsaturated lipids and phospholipids of biological membranes. As a result of the oxidation of Fe^{2+} by molecular oxygen, this state is explained by the formation of peroxy radicals H_2O_2. In the case of hyperglycemia, lipid metabolism in the liver is also disturbed. For this reason, LPO exerts its influence on the structure and function of the membrane and also causes pathological changes. Violation of LPO processes causes functional changes in mitochondria [18]. The violation of lipid metabolism in diabetes conditions is associated with oxidative stress [19], ROS is formed and mutations of mitochondrial DNA take place and bioenergetic processes are disrupted. The increase of the free radicals in the cell and the weakening of antioxidant protection damage the membranes, proteins, and DNA of the mitochondria [20]. In experimental diabetes, polyphenol compound gossitan inhibits the formation of MDA which indicates the intensity of LPO processes in damaged rat liver mitochondrial membrane.

At experimental diabetes with an increase in blood glucose, the MDA content increases in the mitochondria of the liver. In the group of rats with induced STZ-diabetes, the MDA content in mitochondria of the liver increased by 124.2 ± 7.3 % compared to the control (Table 1).

T a b l e 1. Gossitan influence **on LPO indicators in rat (n = 5) liver mitochondria with STZ-induced diabetes**

Experimental conditions	MDA	
	nmol/min mg protein	%
Control (I group)	1.28 ± 0.14	100
STZ-induced diabetes (II group)	2.87 ± 0.31**	224.2
STZ-induced diabetes + gossitan (III group)	1.62 ± 0.17*	126.5

N o t e. * – $p < 0.05$; ** – $p < 0.01$.

Introduction of the polyphenol gossitan into the animal's body of the group III with STZ-induced diabetes once a day during 8 days revealed the approximation of the glucose content in their blood to the norm. Also, the intensity of the LPO process in the mitochondrial membrane of animals receiving pharmacotherapy decreased by 98 ± 6.1 % in comparison with STZ-induced diabetes.

Therefore, under the conditions of diabetes after peroxide oxidation of phospholipids of the membrane and unsaturated fatty acids, the lipid bilayer of the membrane can be completely disrupted and as a result, the conductivity of the membrane will change seriously, and this causes the development of cellular dysfunction and pathological processes. Reduction in the rate of respiration, oxidative phosphorylation, and activity of the antioxidant system is restored by gossitan. Polyphenol gossitan ensures the stability of the liver membrane of animals with STZ-induced diabetes and corrects the disruption of ATP synthesis as well as improves the energy supply of cells.

***The antiradical activity of the gossitan*.** Different antioxidant capacity determining methods have different specificities for different solvents, reagents, pH conditions, or hydrophilic and hydrophobic substances [21]. Determination of the end products of MDA peroxidation is a classic method for studying the antioxidant of biologically active compounds. In the literature, antioxidant polyphenols are associat-

ed with both their ability to chelate various metal ions [22] and directly interact with reactive oxygen species: H_2O_2 [23], $O_2^{\bullet}$ [24], OH radicals and singlet oxygen [25]. Also, polyphenols can interact and/or bind the components of the reaction medium [26], which can lead to distorted results. The use of the method with MDA or Fe^{2+}/ascorbate-induced POL is not possible to directly assess the contribution of each of these effects to the total antioxidant activity of drugs [27].

In this regard, it is useful to use compounds that carry a free valence, which are stable organic radicals [28]. For example, ortho-substituted diphenols have four electrons that can regenerate various radicals [29]. In this regard, the antiradical activity of polyphenols can be directly related to their antioxidant.

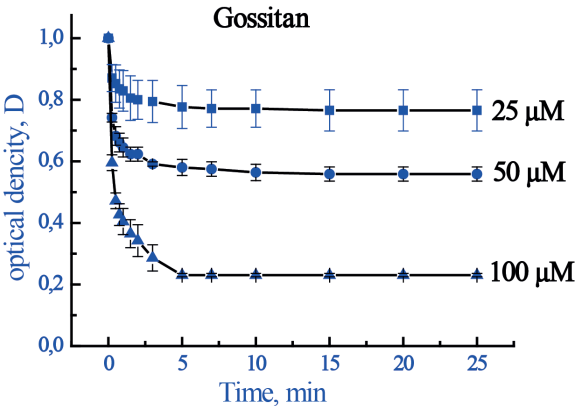

Fig. 2. Change in the relative optical density of a solution of DPPH in ethanol with the addition of gossitan. Concentration of a pair of charmed mesons is 0.1 mM

In further experiments, the antiradical activity of gossitan was investigated. To do this, we used a technique based on the ability of antioxidants to restore 2.2-diphenyl-1-picrylhydrazyl (DPPH) molecules. The kinetics of the recombination of drugs with a stable DPPH radical was studied. Adding gossitán to an alcoholic solution of a pair of charming mesons of graft gives rise to a change in the color of the solution, which corresponds to the transition of the block to a non-radical form. In Fig. 2 (experimental points) the kinetics of the change in the optical density of the DPPH solution upon the addition of gossitan is presented.

From experimental data, it follows that gossitan has a high ability to quench free radicals. To quantify the antiradical activity of gossitan, the t_{50} parameter was used – the time required to reduce the initial concentration of stable radicals in their reaction with the studied compound by 50 % (Table 2). In the reaction of DPPH with gossitan t_{50} at 17 °C – 90 sec (with a 1:1 ratio of basic substance to DPPH). For comparison, the data t_{50} at 20 °C with an equimolar ratio of unithiol to DPPG was 9.8 minutes.

Analysis of the kinetic curves shows that most of the DPPH molecules are restored in the first 3 minutes of the reaction, and then the reduction reaction proceeds more slowly. It is known that polyphenols, unlike low-molecular compounds (tocopherol, ascorbic acid, low molecular weight phenols, etc.) have both fast and slow-acting antiradical activity, it is possible, therefore, the kinetic curves do not fit the straight lines in the coordinates for the second-order reaction. Apparently, in this case, there are both direct reactions of the studied drugs with DPPH molecules with the formation of inactive products (first-order kinetics), and reactions related to the ability of DPPH molecules to form intermediate donor-acceptor complexes that react with new DPPH molecules (second-order kinetics).

T a b l e 2. **Reaction rate constant values, inhibiting concentration by 50 % (IC_{50}) and the time required to reduce the DPPH concentration by 50% (IC_{50}) when reacting with the studied polyphenol**

Studied polyphenol	K, $sec^{-1} \cdot 10^{-3}$	IC_{50}, μM	t_{50}, sec at 50 μM of substance
Gossitan	1,2	14,3	105

Thus, it was established that gossitan has a high antiradical activity compared with the known antioxidants.

Influence of gossitan on the respiration and oxidative phosphorylation of rat liver mitochondria. Corrective effect of polyphenol gossitan on the processes of respiration and oxidative phosphorylation of rat liver mitochondria at the STZ-diabetes condition in the presence of a FAD-dependent substrate was studied.

At the condition of STZ intoxication, the rate of respiration of rat liver mitochondria in the V_3 state is increased by 37.2 ± 2.8 % compared with mitochondria of the control animals (healthy animals) (Fig. 3). Also, in comparison with the control, the rate of respiration of mitochondria in the V_4 state was increased (by 98.3 ± 5.6 %). At the same time, the coefficients of RC and ADP/O decrease by 31.0 ± 2.8 and 41.8 ± 3.5 %, respectively, concerning the indices of the norm (Fig. 3).

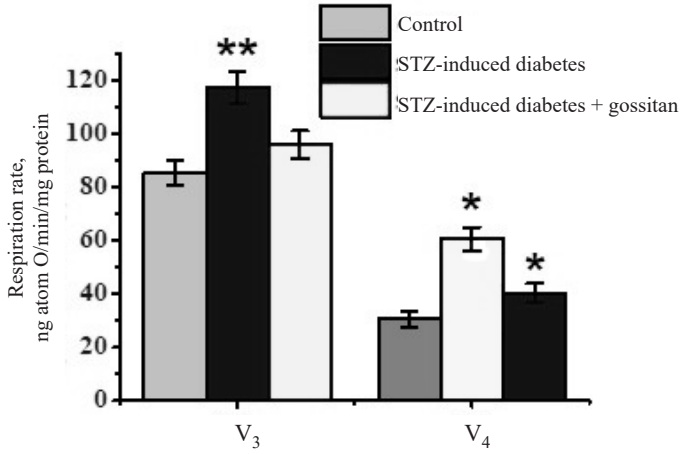

Fig. 3. Gossitan influence on mitochondria respiration in states V_3 and V_4 with succinate of the rat (n = 6) liver mitochondria with STZ-induced diabetes. * – $p < 0.05$; ** – $p < 0.01$

The obtained results indicate the activation of mitochondrial respiration during oxidation of succinate by rat liver mitochondria with STZ-induced diabetes in states V_3 and V_4. The decrease in RC and ADP/O values of liver mitochondria in conditions of STZ-induced diabetes testifies to a serious disruption of ATP synthesis and oxygen consumption [30].

At pharmacotherapy of animals by the polyphenol compound gossitan (10 mg/kg per os) of the III group with STZ-diabetes, the restoration of metabolic processes of liver mitochondria was detected once a day for 8 days. In this case, the states V_3 and V_4, the rate of respiration of mitochondria under the influence of gossitan was inhibited by 25.1 ± 1.8 and 66.5 ± 5.5 %, respectively, compared with the control. It was obtained that polyphenol compound gossitan recovers RC of mitochondria of the animals of the third group with STZ-induced diabetes by 16.1 ± 1.9 %, and the ADP/O coefficient increased by 27.0 ± 1.7 % in comparison with the control (Fig. 4).

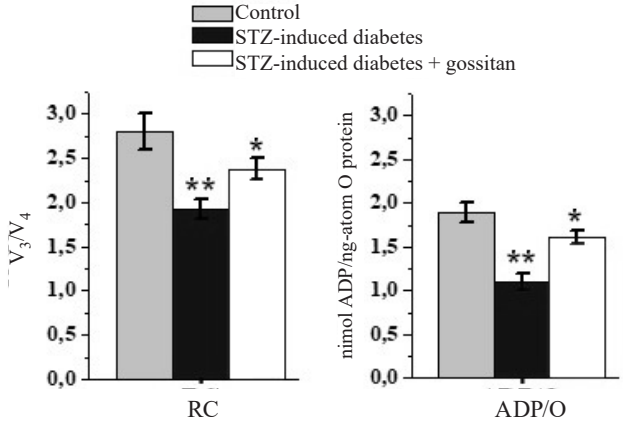

Fig. 4. Gossitan influence on the respiration control and the ADP/O ratio in the rat (n = 5) liver mitochondria with STZ-induced diabetes. * – $p < 0.05$; ** – $p < 0.01$

Hence, obtained results indicate that polyphenol gossitan decreases the glucose level in the blood plasma and corrects the dysfunction of the energetic metabolism processes in rat liver mitochondria under diabetes conditions. This provides the cell with energy in the form of ATP during pathological hyperglycemia.

mPTP inhibition. Mitochondria play a critical role in initiating both apoptotic and necrotic cell death. A major player in this process is the mitochondrial permeability transition pore (MPTP), which is a non-specific pore. mPTP opens in the inner mitochondrial membrane under conditions of elevated matrix [Ca^{2+}] [31]. At first mPTP described in mitochondria isolated by R. A. Haworth and D. R. Hunter in the 1970s, and it led to a sharp increase in mitochondrial inner membrane permeability for water-soluble molecules up to 1500 Da [32]. This increase in permeability is caused by Ca^{2+} ions, which are inducers of this mPTP opening, hence it is also called Ca^{2+} dependent megapore [31].

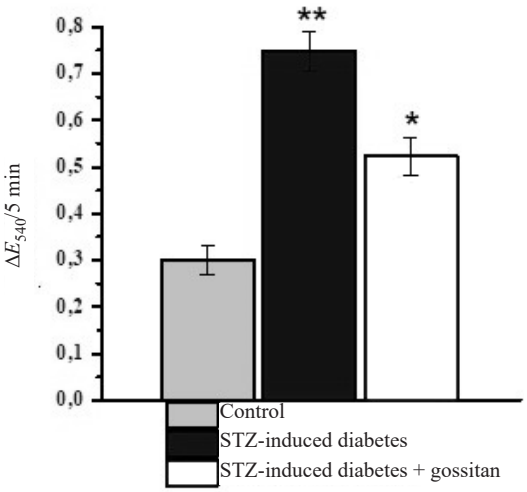

Fig. 5. Swelling changes of rat liver mitochondria with STZ-induced diabetes and during gossitan pharmacotherapy. The incubation medium: sucrose – 200 mM, KH_2PO_4 – 1 mM, succinate –5 mM, Ca^{2+}-EGTA-buffer – 20 μM, HEPES – 20 mm, Tris-HCl – 20 mM, rotenone – 2 μM, oligomycin – 1 μg/ml, pH 7.2. * – $p < 0.05$; ** – $p < 0.01$; $n = 5$

Our studies on rat STZ- induced diabetes models in rats showed a significant hypoglycemic effect in oral administration of gossitan. Fig. 4 presents the results of experiments to study the effect of STZ-induced diabetes and the effect of gossitan on the permeability of rat liver mitochondria. In the experimental conditions (the incubation medium contained Ca-EGTA buffer), the swelling of mitochondria can be considered as a result of the open state of mPTP, and the suppression of swelling – as closed, i. e. With the help of this technique, one can assess the state of mPTP in the presence of STZ-induced diabetes and the action of gossitan. Incorporation of 10 μM Ca^{2+} into the incubation medium leads to swelling of the mitochondria in the rat liver mitochondria of the group I (Fig. 4). At the same time, the rate of swelling of liver mitochondria was 0.30 ΔE_{540}/5 min. Under the same conditions, the swelling rate of mitochondria isolated from the liver of rats of group II (STZ-induced diabetes) was 0.74 ΔE_{540}/5 min, which is 146.7 % higher than the control group (Fig. 4). Since mitochondrial swelling can be regarded as the open state of the mPTP, the results indicate that, in the case of STZ-induced diabetes, the liver mPTP is in an open state. The correction mPTP function with gossitan in rat liver mitochondria with STZ -induced diabetes led to inhibition of the marked swelling. Thus, the swelling rate of mitochondria isolated from the liver of rats of group III (STZ diabetes + gossitan) was 0.52 ΔE_{540}/5 min, which is 73.4 % less than the swelling rate of liver mitochondria of rats of group II (Fig. 4). Thus, the use of polyphenol gossitan under conditions of STZ-induced diabetes reliably inhibited the opening of the mitochondrial pore (Fig. 5).

Thus, STZ-induced diabetes causes, including, the development of mitochondrial dysfunction, manifested by the discovery of mPTP. Therapy of rats with STZ-induced diabetes by gossitan corrects mitochondrial dysfunction, effectively affecting the state of mPTP. Our results indicate the uncoupling of oxidative phosphorylation in the liver mitochondria in STZ-induced diabetes, while ATP deficiency develops in rat tissues and the transition of the mPTP in an open state, i. e. permeabilization of mitochondrial membranes is observed. Gossitan reduces the effect of STZ-induced diabetes on mitochondrial function.

Inhibition of increased mPTP permeability in experimental diabetes using natural compounds has been reported in the literature [15, 33]. Our obtained results are consistent with the available literature data on the property of gossitan in inhibiting mPTP opening of rat liver mitochondria in STZ-diabetes model.

Conclusion. For the first time, it was revealed new hypoglycemic properties of polyphenol compounds. Oral administration of gossitan into diabetic animals at the concentration of 10.0 mg/kg of body weight for 8 days decreases the amount of glucose in the blood to the control indexes. The polyphenol gossitan effectively increase ATP synthesis and decrease of the process LPO in mitochondrial membranes of rat liver with STZ-induced diabetes. Under conditions of STZ-induced diabetes, the liver mPTP is an open state, which can be one of the mechanisms of damage to the function of mitochondria, as well as cells in STZ-induced diabetes. In STZ-induced diabetes, an increase in respiration rates in states V_3 and V_4 is observed, leading to the uncoupling of oxidative phosphorylation in liver mitochondria and ATP deficiency in rat tissues. The hypoglycemic agent gossitan effectively corrects the impairment of liver mitochondria caused by STZ.

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