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THE EFFECT OF QUERCETIN ON OXIDATIVE STRESS MARKERS AND MITOCHONDRIAL PERMEABILITY TRANSITION IN THE HEART OF RATS WITH TYPE 2 DIABETES

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Increasing evidence suggests that oxidative stress and induction of mitochondrial permeability transition in cardiomyocytes are linked to tissue damage and the development of diabetic cardiovascular complications. The aim of this study was to assess the effects of quercetin (Q) on oxidative stress and mitochondrial permeability transition in the heart of rats with type 2 diabetes mellitus (DM). Type 2 DM was induced in 12-week-old male Wistar rats by intraperitoneal injections of 25 mg/kg streptozotocin twice per week followed by a high-fat diet during four weeks. The rats were divided into three groups: control intact group (C, n = 8), untreated diabetic group (Diabetes, n = 8) and diabetic rats treated with Q (50 mg/kg/day per os for 8 weeks) after diabetes induction (Diabetes+Q, n = 8). Administration of Q increased insulin sensitivity and normalized the functional state of cardiac mitochondria due to increased aconitase and succinate dehydrogenase activities in rats with type 2 DM. Q also ameliorated oxidative stress, decreasing the level of advanced oxidation protein products and increasing the activity of thioredoxin-reductase in heart mitochondria of diabetic rats. In addition, Ca^{2+}-induced opening of the mitochondrial permeability transition pore was significantly inhibited in diabetic rats treated with Q in comparison with the untreated diabetic group. These data demonstrate that Q can protect against oxidative stress, mitochondrial permeability transition induction and mitochondrial dysfunction in cardiomyocytes of diabetic rats. We suggest that the use of Q may contribute to the amelioration of cardiovascular risk in type 2 DM.

K e y w o r d s: quercetin, type 2 diabetes mellitus, cardiomyocytes, oxidative stress, non-specific mitochondrial pore.

Diabetes mellitus (DM) is one of the most prevalent non-infectious diseases associated with increased health expenditure, and low quality of life. According to the International Diabetes Federation, 425 million people were living with type 2 DM in 2017, and by 2045 the number will be almost 629 million [1].

It is believed that DM causes not only microangiopathy (diabetic retinopathy, nephropathy and neuropathy) but also constitutes a major risk factor for diabetic cardiomyopathy (DCM). Patients with DM are two to four times more likely to develop cardiovascular diseases and have a three times higher overall mortality rate compared to those without DM [2].

Mitochondrial dysfunction is recognized as a reduction of energy production, oxidative stress, and disruption of redox- and Ca^{2+}-dependent intracellular signalling and is considered to be a driving force for the pathogenesis of DCM [3]. One of the earliest manifestations of mitochondrial dysfunction is an increase in the production of reactive oxygen species (ROS) in the respiratory chain, which leads to a dissipation of the transmembrane potential, and

decreasing of oxidative phosphorylation and production of ATP [4]. The mitochondrial permeability transition, in this case, is, to a considerable extent, the downstream effector. The opening of an unspecific permeation pathway in the inner mitochondrial membrane causes depolarization, uncoupling, loss of metabolites and respiration factors such as NADH from the mitochondrial matrix, ATP depletion and leads to necrotic cell death [5].

There is much evidence indicating that oxidative stress and induction of mitochondrial permeability transition in cardiomyocytes are linked with tissue damage and development of diabetic cardiovascular complications. The excessive ROS production by mitochondria can directly affect the contractile function of the myocardium by oxidative modification of enzymes and ion channels that participate in the regulation of the excitation-relaxation cycle [6, 7]. In addition, ROS stimulate proliferation of cardiomyocytes, and activate fibroblasts and matrix metalloproteinases, which lead to hypertrophy and remodelling of the myocardium and, as a result, the formation of heart failure [8].

It can be assumed that a pharmacological intervention aimed to enhance the oxidative capacity of mitochondria and reduce the hyperproduction of ROS in the respiratory chain may be effective in preventing and correcting disorders associated with type 2 DM. Plant polyphenols are generally considered as antioxidants and candidates for the role of mitochondria-protecting agents. Quercetin (Q; 3,5,7,3′4′-pentahydroxy flavon) is the most abundant flavonoid in the human diet. There is a wide range of biological functions of Q that has been reported including effects on biological pathways involved in inflammation, diabetes and cardiovascular disease [9].

The aim of this study was to assess the effects of Q on oxidative stress and mitochondrial permeability transition in the heart of rats with type 2 DM.

Materials and Methods

Chemicals and experimental design. All chemicals used were of analytical reagent grade quality and purchased from Sigma Chemical Co. (St. Louis, MO, USA). Q was provided by the PJSC SIC Borshchahivskiy Chemical-Pharmaceutical Plant (Kyiv, Ukraine), which contained a minimum 96.5% of active substance.

The present study was approved by the bioethics committee of the V. Danilevsky Institute of Endocrine Pathology Problems, National Academy of Medical Sciences of Ukraine (Kharkiv, Ukraine) and performed in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 1986).

The experiments were performed on 24 male Wistar rats (12-week-old, 200–230 g body weight), which were housed in Plexiglas cages (3 animals per cage) at a temperature of (22 ± 1) °C, in a constant 12-hour light/dark cycle.

We used the animal model of type 2 DM induced by a high-caloric diet combined with multiple low-dose streptozotocin (STZ) injections. This model provides the development of two main features of type 2 DM: the high-caloric diet initiates insulin resistance and the low-dose STZ induces a mild impairment of insulin secretion. Thus, this model mimics the natural history of the disease events (from insulin resistance to β cell dysfunction) as well as the metabolic characteristics of human type 2 DM.

The control intact group (C; n = 8) was fed a standard diet *ad libitum* during 14 weeks. The experimental group (n = 16) was fed the high-caloric diet, containing 16% fat, 28% carbohydrates, 6% proteins for 14 weeks. Both groups had free access to water. In four weeks, rats in the experimental group were injected intraperitoneally with small doses of STZ (25 mg/kg) twice per week [10]. The experimental group was further divided into two groups: untreated diabetic rats (Diabetes, n = 8) and diabetic rats treated with Q for 8 weeks (50 mg/kg/day per os by gavage) after diabetes induction (Diabetes+Q, n = 8).

Measurement of glucose homeostasis. The intraperitoneal insulin tolerance test (IPITT) was performed on overnight fasted rats. Blood glucose concentrations were initially measured at the basal condition (0 min), then the animals were administered an intraperitoneal injection of insulin 0.25 U/kg (Actrapid, Novo Nordisk, Agsvaerd, Denmark) followed by an intraperitoneal injection of glucose (2 g/kg) [11]. Subsequently, tail blood glucose levels were measured using a glucose analyser (Exan-G, Analita, Vilnius, Republic of Lithuania) at 15, 30, 60, 90 and 120 min after the glucose load.

Mitochondria isolation. Mitochondria were isolated by conventional procedures of differential centrifugation. Freshly excised rat hearts were homogenized in a medium containing 0.18 M KCl, 10 mM EDTA, 0.5% bovine serum albumin (BSA),

10 mM HEPES, pH 7.4. To remove EDTA and albumin, mitochondrial pellets were washed twice with 0.18 M KCl, 10 mM HEPES, pH 7.4 and resuspended in wash buffer [12].

Spectrophotometry. All spectrophotometry was performed using a Shimadzu UV-1800 spectrophotometer (Kyoto, Japan).

Measurement of enzyme activities. Mitochondrial aconitase activity was measured by tracking the appearance of NADPH at 340 nm and an open stirred glass cuvette [13]. Mitochondria from heart (0.1 mg protein) were incubated for 20 min at 37 °C in 3.5 ml of assay buffer: 120 mM KCl, 5 mM KH_2PO_4, 3 mM HEPES, 1 mM EGTA, 1 mM $MgCl_2$, 0.4 mM NADP, 0.1% Triton-X100, 2 U/ml isocitrate dehydrogenase and 5 mM citrate, pH 7.2.

Succinate dehydrogenase activity was measured by analysis of 2,6-dichlorophenolindophenol reduction within 20 min at a 600 nm wavelength [14].

Thioredoxin reductase activity was measured using the DTNB (5,5′-dithiobis-(2-nitrobenzoic acid))–assay method [15]. The assay mixture contained: 0.1 M potassium phosphate buffer pH 7.0, 10 mM EDTA, 0.2 mM NADPH, 0.2 mg/ml BSA, 5 mM DTNB and 0.5 mg/ml mitochondrial protein with or without 100 μM 1-chloro-2,4-dinitrochlorobenzene as a thioredoxin reductase inhibitor. Normally, the increase in absorption at 412 nm between the first and second minutes of the measurement was used as an indicator and measure of the enzymatic reaction.

Measurement of advanced oxidation protein products. Determination of the levels of advanced oxidation protein products (AOPP) was performed by the modified method of Witko-Sarsat et al. [16]. 0.1 ml of 1.16 M KI was added to 2.0 ml of mitochondrial suspension (0.1 mg protein/ml) in a phosphate buffer solution containing 0.1% Triton-X100. After 2 min, 0.2 ml of acetic acid was also added. The absorbance of the reaction mixture was immediately read at 340 nm against the blank. APOP concentrations were expressed as nmol-equivalent chloramine T/mg protein.

Measurement of protein content in the samples. Mitochondrial protein was determined by the Lowry protein assay method with BSA as the standard [17].

Mitochondrial permeability transition pore (mPTP) opening. Mitochondrial swelling was monitored throughout changes in absorbance at 540 nm [18]. Incubations were carried out at 25 °C. The concentration of mitochondrial protein in the reaction buffer medium (0.25 M sucrose, 10 mM Tris-Mops pH 7.4, 0.05 mM EGTA, 5 mM sodium pyruvate, and 1 mM KH_2PO_4) was 0.25 mg of protein per ml. Opening of the mPTP was induced by the addition of 0.25 mM $CaCl_2$ to mitochondria treated with 1 mM cyclosporin A (non-specific swelling) or to untreated mitochondria.

Statistical analysis. Data normality were rated using the Shapiro-Wilk test, and all normally distributed data are expressed as the means ± standard error of the mean (SEM). Group comparisons of quantitative variables were performed by one-factor analysis of variance (ANOVA). The Newman-Keuls test was used for multiple comparisons of the groups. Values were considered significant at $P < 0.05$.

Results and Discussion

As shown in Table, the basal glucose level in diabetic rats was significantly higher in comparison with control rats (Diabetes group versus Control group, $P < 0.05$). In addition, the areas under the glycemic curves (AUC) during the IPITT were larger for diabetic rats than for control rats. This difference confirms the development of relative insulin deficiency and insulin resistance in rats of the experimental group.

There was no significant difference in basal hyperglycemia between diabetic rats and those treated with Q. However, Q significantly increased insulin sensitivity compared with diabetic rats (Table).

Previous studies have demonstrated the ability of Q to reduce insulin resistance [19]. Q increases glucose uptake in insulin-responsive tissues and reduces the production of glucose by the liver through numerous cell-signalling pathways. Q activates the

Indexes of glucose homeostasis in control and diabetic rats

Group	Basal glucose level, mmol/l	IPITT, AUC (mmol/l)×min
Control	3.75 ± 0.08	568.20 ± 37.54
Diabetes	9.00 ± 1.19[a]	1691.06 ± 147.74[a]
Diabetes+Q	8.30 ± 0.61[a]	999.32 ± 152.56[a,b]

Data are shown as mean ± standard error of the mean (SEM), $n = 8$. AUC, area under the curve; IPITT, intraperitoneal insulin tolerance test; Q, quercetin. [a]Significant versus Control group ($P < 0.05$), [b]significant versus Diabetes group ($P < 0.05$).

insulin-independent adenosine monophosphate-activated protein kinase (AMPK) signalling pathway which results in the increase of the translocation of GLUT4 to the plasma membrane in muscle cells and also in the inhibition of the key enzymes of gluconeogenesis in the liver [20].

AOPP accumulation in the heart of diabetic rats was significantly increased confirming the development of oxidative stress (Diabetes group versus Control group, $P < 0.05$, Fig. 1). AOPP are recognized as a relatively novel marker of oxidative damage of proteins and the intensity of oxidant-antioxidant imbalance and inflammation. The AOPP level is elevated in type 2 DM and the concentration of AOPP correlates with insulin resistance and the presence of severe diabetic complications [21, 22]. The oxidized protein modifications play a crucial role in the development of diabetic myocardial dysfunction. Intensified oxidation of amino acid residues, aggregation, cross-linking, fragmentation, and loss of enzymatic or other functional properties of cardiac proteins may affect the cardiac muscle contraction process. In addition, AOPP can induce proapoptotic signalling in cardiomyocytes, leading to development of DCM [23].

In our study Q administration resulted in significant lowering of the AOPP level in the heart of diabetic rats in comparison with the untreated diabetic group, suggesting its antioxidant properties (Diabetes+Q group versus Diabetes group, $P < 0.05$, Fig. 1).

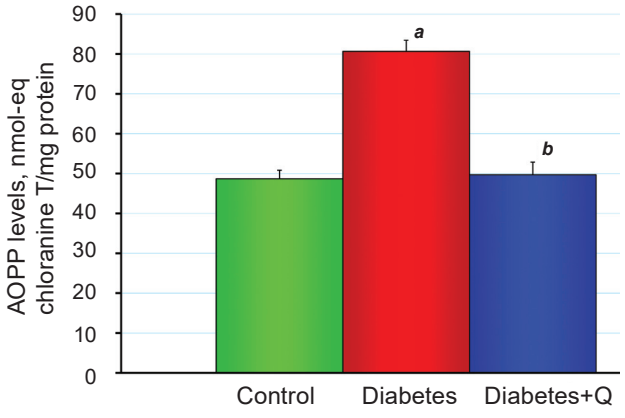

Fig. 1. Levels of advanced oxidation protein products (AOPP) in the isolated heart mitochondria of rats. Data are shown as mean ± standard error of the mean (SEM), n = 8. [a]Significant versus Control group (P < 0.05), [b]significant versus Diabetes group (P < 0.05)

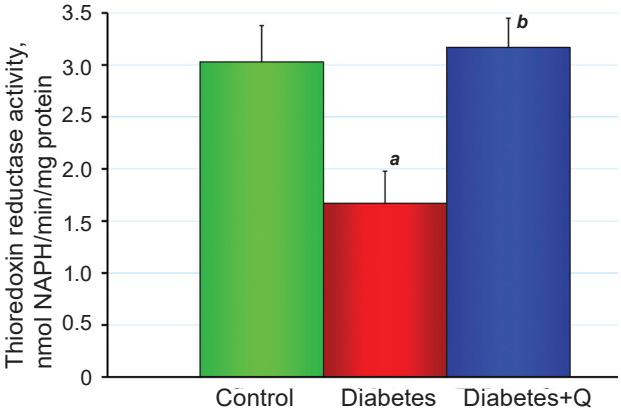

Fig. 2. Thioredoxin reductase activity in the isolated heart mitochondria of rats. Data are shown as mean ± standard error of the mean (SEM), n = 8. [a]Significant versus Control group (P < 0.05), [b]significant versus Diabetes group (P < 0.05)

The increase in oxidative stress was accompanied by a significant 2-fold reduction of thioredoxin reductase activity (Diabetes group versus Control group, $P < 0.05$, Fig. 2).

It is known that the mitochondrial isoform of this enzyme and its substrate thioredoxin do not play a substantial role in ROS detoxication but indirectly support an active (reduced) peroxiredoxins 3 and 5 pool. The basic function of the thioredoxin/thioreductase system involves participation in redox signalling performed throughout reversible reductive-oxidative protein modification [24].

It is supposed that thioredoxin reductase plays an important role in proliferation regulation, apoptosis of cardiomyocytes and contractile activity of myocardium. Earlier it was shown that reduction of thioredoxin reductase activity in type 2 DM can cause the intensification of cardiomyocyte apoptosis and development of DCM [25].

Q increased the thioredoxin reductase activity in diabetic rats in comparison with the untreated diabetic group (Diabetes+Q group versus Diabetes group, $P < 0.05$, Fig. 2). This finding indicates the ability of Q to decrease the intensity of oxidative stress and to prevent the induction of apoptotic processes in cardiomyocytes.

Aconitase is one of the most sensitive enzymes of energy metabolism in mitochondria to the damaging effect of ROS. As aconitase catalyzes the conversion of citrate to isocitrate, high ROS production in the respiratory chain leads to the accumulation of citrate in the mitochondrial matrix by inactiva-

tion of aconitase. Citrate is an intermediate product of acetyl-CoA oxidation and can be exported to the cytoplasm and be involved in the de novo synthesis of fatty acids. Chronic hyperproduction of ROS is associated with an excessive accumulation of fatty acids and their derivatives, such as diacylglycerol. Fatty acid accumulation in peripheral tissues is an important factor in insulin resistance development and metabolic disorders associated with lipotoxicity in diabetes [26, 27].

Aconitase activity in the heart mitochondria was significantly decreased by 50% in diabetic rats compared with control rats (Diabetes group versus Control group, $P < 0.05$, Fig. 3).

Aconitase activity was increased in diabetic rats treated with Q compared to untreated diabetic rats (Diabetes+Q group versus Diabetes group, $P < 0.05$, Fig. 3). The increase of aconitase activity is associated with the ability of Q to reduce ROS production in the mitochondrial respiratory chain; superoxide anion interacts with the iron-sulfur clusters of the aconitase active centre and inhibits the activity of this enzyme.

Q has a positive effect on the functional state of mitochondria by increasing NAD^+-dependent deacetylase Sirt1 expression. This enzyme deacetylates and activates peroxisomal proliferator activator receptor-gamma coactivator-1 alpha (PGC-1α), which is the key regulator of mitochondrial function and biogenesis [28]. PGC-1α induces gene expression of the tricarboxylic acid cycle and electron transport chain components, antioxidant defence and the mitochondrial transcription factor Tfam, which controls the transcription, translation and repair of mitochondrial DNA [29].

Activity of succinate dehydrogenase which is one of the enzymes of the tricarboxylic acid cycle and the component of the mitochondrial respiratory chain (complex II) was significantly decreased in diabetic rats compared to control rats (Diabetes group versus Control group, $P < 0.05$, Fig. 4). Activity of this enzyme was increased in heart mitochondria of diabetic rats treated with Q compared to untreated diabetic rats (Diabetes+Q group versus Diabetes group, $P < 0.05$, Fig. 4).

The oxidative inactivation of redox-sensitive aconitase is associated with oxidative stress and can decrease the activity of succinate dehydrogenase. The inactivation of succinate dehydrogenase inhibits the tricarboxylic acid cycle and also stimulates the accumulation of acetyl-CoA which enhances the enzymatic and non-enzymatic acetylation of proteins [30].

The acetylation and deacetylation processes are an important signalling system of post-translational protein modifications and play significant roles in the regulation of various cell functions. It is known that signalling system disbalance is one of the pathogenetic mechanisms of type 2 DM. It has been shown that acetylation of the catalytic subunit of succinate dehydrogenase is associated with a decrease in its activity by 30%. Moreover, deacetylation of this

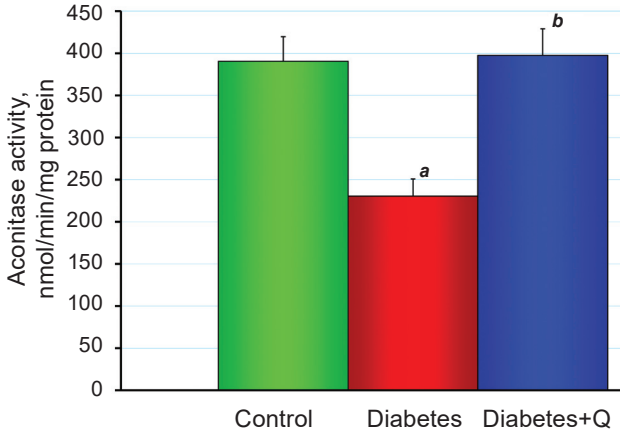

Fig. 3. Aconitase activity in the isolated heart mitochondria of rats. Data are shown as mean ± standard error of the mean (SEM), n = 8. [a]Significant versus Control group (P < 0.05), [b]significant versus Diabetes group (P < 0.05)

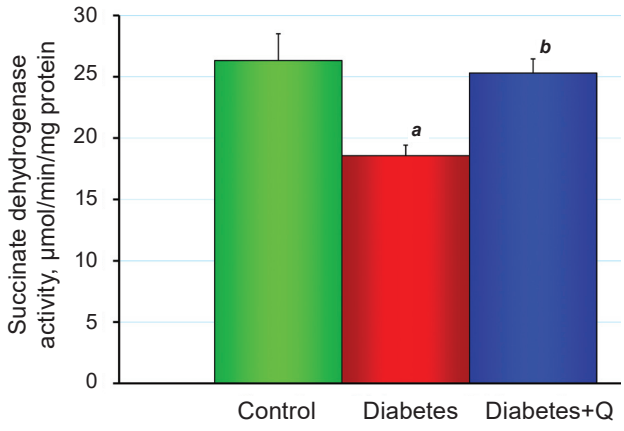

Fig. 4. Succinate dehydrogenase activity in the isolated heart mitochondria of rats. Data are shown as mean ± standard error of the mean (SEM), n = 8. [a]Significant versus Control group (P < 0.05), [b]significant versus Diabetes group (P < 0.05)

subunit with mitochondrial NAD^+-dependent Sirt 3 deacetylase restores its activity [31].

We suggest that Q prevents succinate dehydrogenase deactivation in several ways. Firstly, Q reduces oxidative stress and prevents tricarboxylic acid cycle inhibition and the accumulation of acetyl-CoA. Secondly, flavonoids can be activators of deacetylases, in particular, Sirt 3 [32].

The mPTP plays a crucial role in the initiation of apoptotic and necrotic cardiomyocyte death. The mPTP is a non-specific pore that opens in the inner mitochondrial membrane under conditions of elevated matrix [Ca^{2+}], especially when it is accompanied by oxidative stress, for examples in diabetes. Opening of the mPTP causes massive swelling of mitochondria, rupture of the outer membrane and release of intermembrane components that induce apoptosis. In addition, mitochondria became depolarized causing inhibition of oxidative phosphorylation and stimulation of ATP hydrolysis [33]. Activation of the mPTP promotes the release of mitochondrial death factors, such as cytochrome *c* and apoptosis-inducing factor (AIF). Cytochrome *c* is involved in caspase-dependent cell death mechanisms, while AIF induces caspase-independent cell death. Factors inducing mPTP opening, such as oxidative stress and mitochondrial membrane potential decrease, depleted adenine nucleotide ratio (ATP/ADP) and mitochondrial matrix pH elevation are specific for mitochondrial dysfunction that is closely related to DCM development [34].

Several *in vitro* studies have shown that Q can have protective or cytotoxic effects, by acting as either an agonist or antagonist of the mPTP depending on its concentration and the type of cells [35, 36].

In our study, the rate of Ca^{2+} dependent mitochondrial swelling was two times faster in diabetic rats compared to control rats (Diabetes group versus Control group, $P < 0.05$, Fig. 5). Q administration significantly increased the mitochondrial resistance to Ca^{2+} dependent swelling in rats with type 2 DM (Diabetes+Q group versus Diabetes group, $P < 0.05$).

Our data correspond to those of Shahbaz et al. (2011), who reported that Q attenuated mPTP opening in heart mitochondria in rats with aldosteronism [37].

The data of the present study confirmed the positive effect of Q on redox homeostasis and metabolic activity of heart mitochondria in rats with type 2 DM.

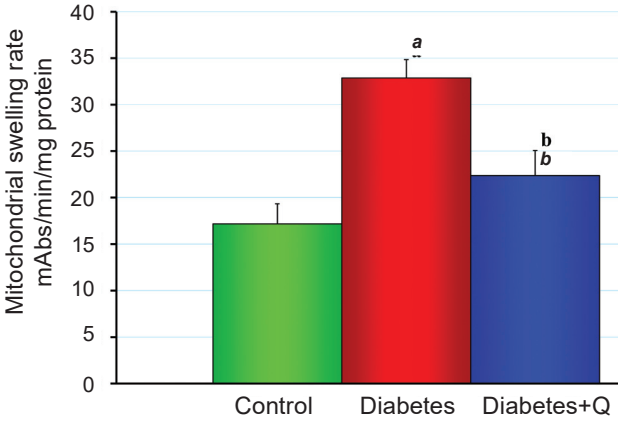

Fig. 5. Ca^{2+}-induced opening of mitochondrial permeability transition pore in the isolated heart mitochondria of rats. Data are shown as mean ± standard error of the mean (SEM), n = 8. [a]Significant versus Control group ($P < 0.05$), [b]significant versus Diabetes group ($P < 0.05$)

Due to its chemical structure, Q may interact directly with mitochondrial membranes and components of the mitochondrial electron transport chain, affecting the production of ATP and the dynamics of the mPTP [36]. Furthermore, Q may elicit inhibitory effects on mPTP formation due to interactions with molecular targets in mitochondria, for example decreasing the capacity of Ca^{2+} ions to induce mPTP opening [37]. Q has been shown to counteract a classical mPTP inducer - oxidative stress - by activating signaling pathways related to mitochondrial antioxidant defence in cultured cells and animal tissues. Q is able to activate the master regulator nuclear factor erythroid 2-related factor 2 (Nrf2) which may occur through phosphorylation mediated by different protein kinases [38]. Additionally, orally-administered Q in relatively low doses may modulate antioxidant enzymes expression through a pro-oxidant manner as a hormetic agent causing ameliorations in the cellular redox environment [39].

Our results suggest that Q normalizes metabolic activity of heart mitochondria activating aconitase and succinate dehydrogenase, and increases the resistance of myocardium cells to pro-apoptotic stimuli decreasing the production of AOPP, increasing thioredoxin reductase activity and reducing sensitivity of the mPTP to calcium ions.

We suggest that the use of Q may contribute to the amelioration of cardiovascular risk in type 2 DM.

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ВПЛИВ КВЕРЦЕТИНУ НА МАРКЕРИ ОКСИДАТИВНОГО СТРЕСУ ТА ПРОНИКНІСТЬ МІТОХОНДРІАЛЬНОЇ МЕМБРАНИ В СЕРЦІ ЩУРІВ ІЗ ЦУКРОВИМ ДІАБЕТОМ 2 ТИПУ

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Відомо, що окисидативний стрес і активація мітохондріальної пори в кардіоміоцитах спричинюють пошкодження тканин і розвиток діабетичних серцево-судинних ускладнень. Метою дослідження було визначення впливу кверцетину на показники оксидативного стресу та проникність мітохондріальної мембрани в серце щурів із цукровим діабетом (ЦД) 2 типу. ЦД 2 типу було індуковано у 12-місячних самців щурів Wistar за допомогою високожирової дієти протягом чотирьох тижнів та ін'єкцій стрептозотоцину (25 мг/кг маси тіла внутрішньочеревно двічі на тиждень). Всіх тварин було розподілено на три групи: інтактна група (С, $n = 8$), контрольна діабетична група (Діабет, $n = 8$) і діабетичні щури, що отримували кверцетин (50 мг/кг/добу внутрішньошлунково) протягом 8 тижнів після індукції діабету (Діабет+Кверцетин, $n = 8$). Встановлено, що введення кверцетину щурам із ЦД 2 типу супроводжується поліпшенням чутливості до інсуліну та нормалізацією функціонального стану мітохондрій серця за рахунок підвищення активності аконітази та сукцинатдегідрогенази. Кверцетин також зменшував інтенсивність оксидативного стресу, знижуючи рівень продуктів посиленого окислення протеїнів і збільшуючи активність тіоредоксинредуктази в мітохондріях серця щурів у порівнянні з діабетичним контролем. Крім того, кверцетин пригнічував Ca^{2+}-індуковану активацію мітохондріальної пори в серці діабетичних тварин. Одержані дані свідчать про наявність протективного ефекту у кверцетину відносно оксидативного стресу, активації мітохондріальної проникності і дисфункції мітохондрій в кардіоміоцитах діабетичних щурів, що обґрунтовує перспективність його використання з метою ослаблення кардіо-васкулярного ризику за умов цукрового діабету 2 типу.

Ключові слова: кверцетин, цукровий діабет 2 типу, кардіоміоцити, оксидативний стрес, неспецифічна мітохондріальна пора.

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