INFLUENCE OF QUERCETIN AND DIHYDROQUERCETIN ON SOME FUNCTIONAL PARAMETERS OF RAT LIVER MITOCHONDRIA

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ABSTRACT

Flavonoids are proved to be prominent plant-derived compounds generally found in fruit- and vegetable- rich diets with free radicals quenching ability. Quercetin, a member of flavonoids subclass – flavonols, has gained major attention due to its overwhelming potential. Quercetin was demonstrated to preserve diverse degenerative diseases by inhibiting lipid peroxidation. In the present paper, we deal with two polyphenolic compounds – quercetin and dihydroquercetin – influence on functional parameters of mitochondria. Thus, the fore mentioned flavonols showed the mitochondrial permeability transition pore (mPTP) inhibition and ATP-sensitive potassium channel activation capacities as well as strong antioxidant/antiradical activity.

Improving functional parameters of mitochondria by both quercetin and dihydroquercetin formed a basic idea of the hypothesis that the studied flavonoids may provide antihypoxic defense.

Keywords: flavonoids, antioxidant activity, antihypoxic agents, mPTP, mitoKATP

INTRODUCTION

Mitochondria play a major role in the apoptosis and necrosis regulation (Dave, Byfield, et al., 2008; Vladimirov, 2002; Shimizu, Tsujimoto, 2007). The parameters like mPTP, oxidation phosphorylation, antioxidant defense systems, and others regulate the functionality of mitochondria (Pozhilova, Levchenkova, et al., 2014). The swollen mitochondria initiates opening of mPTP that leads to the rupture of the outer mitochondria membrane as well as intermembrane components leakage into the cytosol provoking the process of cell apoptosis. The Ca2+ ions accumulation in the matrix of the mitochondria is also a direct regulatory mechanism for opening the mitochondrial pore, a key link in the launch of the cell apoptosis program (Dave, Byfield et al., 2008; Tsujimoto, Shimizu, 2007). The formation and opening of mPTP is not the only mechanism for the leakage of mitochondria intermembrane components into the cytosol. However, the search for medicinal substances, the target of which will be the mitochondrial mega pore, can become a promising direction in the correction of many pathological conditions (Novikov, Levchenkova, 2013). Also, recently, interest in the study of the mitochondrial ATP-sensitive potassium channel (mitoKATP channel) has increased, since it has been established that its activation plays a key role in protecting organs and tissues from the effects of ischemia/reperfusion (Akao, Ohter et al., 2001) and in addition to its important role, the mitoKATP channel plays a huge role in adapting the body to various stress factors (Danilenko, Lokrovsky et al., 2010; Hund, Mohler, 2011).

It is known that the opening of the mitoKATP channel leads to depolarization of the membrane potential of mitochondria and causes the reversible oxidation of flavoproteins of the respiratory chain, thereby increasing the production of reactive oxygen species (ROS) in mitochondria (Samaratil, Monick et al., 2002). But in a review, Pozhilova et al. show that the natural metabolic activators of the mitoKATP channel, uridine and its nucleotides in micromolar concentrations, significantly reduce the area of myocardial infarction, normalize the level of ATP, creatine phosphate and antioxidant defense systems, reducing the formation of ROS, and normalize the heart rate by rat myocardial infarction models (Pozhilova, Levchenkova et al., 2014). It is also mentioned that the opening of the mitoKATP channel leads to a decrease in ROS in tissue cells, thereby regulating it (Ferranti, da Silva et al., 2003; Facundo, de Paula et al., 2007; Fornazari, de Paula et al., 2008) Thus, the activation of the potassium cycle upon adaptation to the stress factor and the subsequent decrease in ROS formation can explain the known protective role of the mitoKATP channel in ischemia/reperfusion (Garlid and Halestrap, 2012; Meng, Ma et al., 2016).

Activation of lipid peroxidation (LPO) is often one of the triggers of many diseases and is also an aggravating factor in many pathological conditions (Mylonas, Kouretas, 1999; Lobo, Patil et al., 2010; Chesnokova, Morrison et al., 2009). Moreover, the use of antioxidants in the treatment of various pathological conditions of the body slows down the course of pathological conditions caused by an excess of free radicals, since antioxidants can prevent the activation of induced free radical reactions (Breuer, 2011). Thus, it can be concluded from the above material that compounds with antioxidant activity can be considered as promising universal remedies promoting the prevention and treatment of oxidative stress caused disorders.

Given the above, studies regarding the molecular and cellular mechanisms of plant compounds actions are currently considered relevant to detect potential pharmacological agents with effect on the organism. One of the potential pharmacological agents is polyphenol compounds of plants. Polyphenolic compounds have a very wide spectrum of action on biological objects, exhibiting antioxidant, hepatoprotective, antihypoxic, membrane-tropic and many other effects (Asrarov, Komilov et al., 2015; Rustamova, Irgasheva et al., 2008; Tsibulsky, Popov et al., 2011). The manifested physiological responds of cells exposed to polyphenols are observed due to polyphenols’ ability to alter the mitochondrial membrane permeability with respect to particular ions that is crucial in preventing of different pathological conditions in an organism. It’s been widely accepted that quercetin, typical component of onions, buckwheat and citrus fruit and utilized in traditional medicine as preventive agent against variety of diseases, such as cancer (Zhang et al. 2011; Jovanovic, 1999; Mullen, 2008), cardiovascular and nervous disorders (Shankar, 2007; Labinsky, 2006), obesity (Yang et al. 2008), and chronic inflammation (Teixeira S., 2002; García-Mediavilla V., 2007) possesses its therapeutic effect through immense antioxidant activity. Although the underlying mechanisms of biological action are largely under debate, the quercetin structure suggests multi-functional and mitochondria-mediated mechanism (Karbarz B, 2008).

Being a significant member of the flavonol subclass, quercetin, has attracted considerable attention. Moreover, quercetin and its glucosylated forms contribute to 60-75% of flavonoid intake (Boktaib et al., 2002).
Oxidized low-density lipoproteins (LDL), that are hallmarks of certain diseases, such as cancer, atherosclerosis, and chronic inflammation (Hollman and Katan 1997; Murota and Terao 2003), were shown to be strongly inhibited by quercetin both by free radicals scavenging mechanism and chelating transition metal ions. Thus, in this work, we studied the comparative mechanism of effect of two flavonoids (the quercetin and its derivative dihydroquercetin) on some functional parameters of mitochondria, such as mPTP, mitoKATP channel, and LPO of rat liver mitochondria.

![Chemical structures of quercetin (A) and dihydroquercetin (B)](image)

**Figure 1** Chemical structures of quercetin (A) and dihydroquercetin (B)

### MATERIALS AND METHODS

#### Mitochondria isolation

Mitochondria isolation protocol was followed as described by (Schneider, Hageboom, 1951) from rat liver tissue homogenate (white outbred rats, both sexes, 180-200 g). In brief, rat liver tissue was homogenized on ice, medium A contained sucrose (250 mM), EDTA (0.5 mM), Tris-HCl buffer (10 mM) adjusted to pH 7.4, centrifuged at 1500 g for 7 min (-2 to -4 °C). After centrifugation supernatant was collected and mitochondria was precipitated at 6000 g for 15 min (-2 to -4 °C), resuspended in medium A lacking EDTA, and stored on ice until needed. The mitochondrial protein amount was measured by the Lowry protein assay with minor modifications (Peterson, 1977). All experiments were performed at room temperature, on colorimeter Granat, Russia, 1991.

#### Mitochondria swelling assay

The opening of mPTP was observed through mitochondrial swelling by recording the spatial light scatter intensity at 540 nm in the presence of 10 mM Ca²⁺ as a control. 1 ml of mitochondrial solution contained 0.3-0.4 mg protein. The buffer contained sucrose (200 mM), EGTA (20 μM), succinate (5 mM), rotenone (2 μM), oligomycin (1 μg/ml), Tris (20 mM), HEPES (20 mM), KH₂PO₄ (1 mM), adjusted to pH 7.2 (He, Lemasters, 2003). Polyphenols were added at the 1-100 μM concentration range.

**mitoK⁺/ATP current recording**

mitoKATP channel current recording was monitored at 540 nm in external solution contained KCl (125 mM), Heps (10 mM), succinate (5 mM), MgCl₂ (1 mM), K₂HPO₄ (2.5 mM), KH₂PO₄ (2.5 mM), rotenone (0.005 mM), oligomycin (0.001 mM) (Vadzyuk, Kosterin, 2008). Polyphenols were added at the 1-50 μM concentration range. 10 mM of ATP was accepted as control.

#### Lipid peroxidation assay

Lipid peroxidation (LPO) was initiated by Fe²⁺/ascorbate system in mitochondria suspension, external medium contained: KCl (125 mM), Tris-HCl (10 mM), pH adjusted to 7.4, according to (Schneider et al., 1949). 1 ml of mitochondrial solution contained 0.5 mg protein. 10 μM FeSO₄, together with 600 μM ascorbate were added to medium to generate process of mitochondrial swelling. The experiments were carried out 24-26°C.

**DPPH assay**

DPPH kinetics was carried out as described elsewhere (Gayibova, 2019). Briefly, the kinetic results regarding DPPH (0.1 mM) decolorization in the presence of polyphenols (up to 10 μM) was recorded on SF-46 (LOMO, Russia, 1996). The spectroscopic data was fixed every 15 sec until the reaction reached steady state.

#### Conductometry

The conductivity of the solutions was measured using a Sartorius Professional meter PP-20 conductometer (USA) at a temperature of 25 °C, at a constant salt concentration and a variable concentration of polyphenols. During the experiment, a weighed portion of polyphenols was dissolved in 20 ml of water, and then the conductivity was measured by successively diluting a solution of divalent metal salt (initial concentration of 10⁻⁸ M) with certain volumes of the initial solution of the substance (0.01 M) (Andreev, 1971).

#### Chemical reagent

EDTA, EGTA and cyclosporin A were purchased from “Sandoz”, Switzerland, rotenone and tris-HCl were from “Serva”, Germany, DPPH and CaCl₂ were from “Sigma”, USA. Other reagents were purchased from the local companies with grade marked as “chemically pure”.

#### Data analysis

A statistical analysis was based on three replicates computed on the statistical package Origin 6.1 (Origin Lab Corporation, USA). Statistical significance was performed as Student’s t-test. Mean values and standard deviations were calculated. Statistically significant results were expressed at * - P<0.05; ** - P<0.01; ***- P<0.001.

### RESULTS AND DISCUSSION

#### Antioxidant activity

Plant-derived compounds are an enduring source of phytochemicals possessing a list of therapeutic implementations. In the molecular pathogenesis of plants’, animals’, and humans’ disorders, oxidative stress contributed with free radicals overproduction is suspected to play significant role. Therefore, the screening for the free radicals scavenging regulators of natural and synthetic nature and studying their antiradical processes remain relevant.

All plant compounds concerning animal organisms have biological activity of an extremely wide spectrum, due to the diversity of their chemical structure, and are currently in the center of scientific attention. In connection with the foregoing, the search for antioxidants and the study of their inhibitory effect on the processes of free radical oxidation. In this regard, the effect of various concentrations of quercetin and dihydroquercetin on preventing Fe²⁺/ascorbate-induced mitochondrial lipid peroxidation was studied in vitro. The introduction into the external medium the system, including Fe²⁺/ascorbate, activates lipid oxidation, that in turn destroys mitochondria membrane barrier function and leads to mitochondria swelling (Fig. 2). When quercetin and dihydroquercetin at a minimal dose of 2 μM are added into external solution, containing Fe²⁺/ascorbate, the antioxidant properties of polyphenols are observed that is reflected in mitochondria swelling inhibition. The effect of quercetin and dihydroquercetin on the process of mitochondrial membranes peroxidation revealed dose-dependent manner. Also, at the concentration of 10 μM both compounds totally suppressed the mitochondria swelling proving the inhibition of lipid oxidation with IC₅₀ values as follow 6.08 ± 0.06 μM (quercetin) and IC₅₀=8.1 ± 0.09 μM (dihydroquercetin).
Thus, it can be concluded that AOA of polyphenols is not associated with the chelation of divalent cations by the studied polyphenols.

**Antiradical activity**

Determination of mitochondrial swelling induced by the Fe$^{2+}$/ascorbate system is one classic method for studying the antioxidant properties of biologically active compounds.

In the literature, AOA of polyphenol compounds is associated both with their ability to chelate various metal ions (Tripathi, Rastogi, 1981), and directly interact with reactive oxygen species: OH radicals (Takahama, 1984), O$_2^.$ (Takahama, 1985), and singlet oxygen (Youngman, Takahama et al., 1984). Besides, polyphenols can interact and/or bind the components of the reaction medium (Riedl, Carando et al., 2002), which can lead to distortion of the results. The use of the Fe$^{2+}$/ascorbate-induced system does not directly assess the contribution of every single effect to the total antioxidant activity of the polyphenols.

Thus screening of compounds, that having free valence electrons are still stable organic radicals, seems reasonable (Gayibov, Komilovi, et al., 2017). For example, ortho-substituted diphenols have four electrons that can reduce various radicals (Pochinok, Tarakhovsky, et al., 1985). In this regard, the antiradical activity of polyphenols can be directly associated with their AOA.

In this regard, the antiradical activity of quercetin and dihydroquercetin against DPPH free radical was studied. To do this, we used a technique based on antioxidant's ability to reduce the molecules of 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Gayibov et al., 2012). We studied the kinetics of drug recombination with the stable radical DPPH. When the studied compounds are added to the alcohol solution of DPPH, the color of the solution changes, which corresponds to the transition of DPPH to a nonradical form. In fig. 4 (experimental points) presents the kinetics of changes in the DPPH optical density with the addition of the studied compounds.

**Conductivity**

It is known that alkaline earth metal cations are capable, under certain conditions, of forming coordination bonds with oxygen-containing molecules. To obtain more direct experimental evidence on the complexing (chelating) properties of the studied polyphenols with cations of iron, the method of conductivity was used. As iron-binding property of polyphenols has been also proposed as their mechanism of cell protection against oxidative stress, the equilibrium constant between polyphenol and iron ions may provide insight into the mechanism of antioxidant effect.

The study of the electrical conductivity of divalent metal salts (Fe$^{2+}$) in an aqueous solution in the presence of studied polyphenols did not confirm the assumption that these compounds are capable of forming coordination type compounds. An analysis of experimental data shows that with an increase in the concentration of the studied polyphenol compound, as well as an increase in the concentration of divalent metal ions, the electrical conductivity of the solution containing Fe$^{2+}$ cations does not change and does not reach a plateau (Fig. 3). However, presence EDTA in solution the electrical conductivity changes and reach plateau which indicates about chelating of metal ions with EDTA.

An analysis of experimental data shows that an increase in the concentration of polyphenols and divalent metal ions in a solution does not change the electrical conductivity.

**Figure 3** Typical conductometric titration curve of quercetin and dihydroquercetin with a Fe$^{2+}$ solution (where S is the salt concentration, L is the polyphenol concentration, µM).

To compare the ARA of the studied polyphenols, we selected a concentration of 50 µl for each compound from the prepared alcohol solution of the substance at a concentration of 10$^{-5}$ M. Analyzing the results, we can conclude that the optical density of the ethanol solution of DPPH decreases which indicates their antiradical ability.
From experimental data, it follows that the studied compounds have a high ability to quench free radicals. To quantify the antiradical activity, we used the parameter $t_{50} - e$ — the time required for the studied drugs to decrease the initial concentration of the radical to 50%, chemical reaction constant (k) and half inhibition concentration (IC$_{50}$).

| $K$ 10$^{-2}$, e$^{-1}$ | IC$_{50}$, mc | $t_{50}$, sec |
|--------------|-----------|-------------|
| DQ           | Q         | DQ          | Q         | DQ          | Q         |
| 1.2          | 5.3       | 14.5        | 7.2       | 105         | 9.6       |

Thus, the studied compounds are characterized by a strong ability of components to react with free radicals.

The correlation coefficient between ARA and AOA of polyphenol compounds is $r=0.89$.

Our main interest in the investigation of AOAA/ARA activity was to clarify the mechanism of AOAA of two studied polyphenols depending on their chemical structure. It is well known that the antiradical/antioxidant of polyphenolic compounds is up to the number and location of hydroxyl groups in the benzene ring of polyphenols (Yashin, 2014). In the molecules of quercetin and dihydroquercetin there is the same number of hydroxyl groups. However, the antiradical activity of dihydroquercetin is significantly lower than the ARA of quercetin. Higher ARA of quercetin, might possibly due to the numerous existence of double bonds in comparison to the molecule of dihydroquercetin.

Thus, we can assume that the high AOA/ARA of quercetin compared with its derivative is associated with the most energetically favorable state due to the distribution of electron density in the benzene ring.

When the quercetin donates an electron to free radical, initiating quercetin radical formation, due to resonance this newly formed radical lacks sufficient energy to approach reactive nature (Mariani, et al., 2008). The structural groups that stabilize quercetin and specify its antioxidant features are considered to be the 3- and 5-hydroxyl groups together with 4-oxo group and B ring $\sigma$-dihydroxyl groups (Hollman and Katan, 1997). This electron donating groups transfer electrons to the functional rings thus increasing the amount of resonance forms (Mariani, et al., 2008).

On the base of the conductometric titration, we considered that there are no complexes of polyphenols with Fe$^{2+}$ ions which could decrease the LPO process in the membrane due to chelate of metal ions by polyphenols. It is reasonable to suppose that AOA of polyphenols related to the non-polar part of quercetin and dihydroquercetin molecules extend into the acyl chain region of mitochondrial membrane and quench free radicals.

**Inhibition of mPTP**

Mitochondrial permeability transition pore (mPTP) approved to manifest the key role in the functional parameters of the cell. Thus, mPTP plays crucial part as trigger of apoptosis and necrosis under oxidative stress, ischemia/reperfusion as well as Ca$^{2+}$ toxicity in liver cells (Kim et al., 2013). In this case the mechanism of cardioprotection of the most cardiovascular drugs is based on direct mPTP inhibition, as well as decreasing level of mPTP opening triggers, calcium overload and/or scavenging reactive oxygen species. According to the Akopova’s data (Akopova, Kolchinskaya et al., 2011), mPTP goes into the open state as a result of the Ca$^{2+}$ overload, induction of lipid peroxidation, the formation of ROS and closes in the presence of antioxidants or cation chelators (Asrarov, Komilov, et al., 2015).

Since Ca$^{2+}$-dependent mPTP is a key factor in ensuring the permeability of mitochondrial membranes (Akopova, Nosar et al., 2013), in the following experiments the effect of quercetin and its derivative dihydroquercetin on mPTP activity (Fig. 5) was investigated. The introduction of 10 μM Ca$^{2+}$ ions into the external solution induces mitochondrial swelling (Fig. 5, cont.), which indicates that mPTP is in the open state. Cyclosporin A (CsA), a specific mPTP blocker, avoids mitochondria from being swollen under the above-mentioned conditions (Fig. 5, A), i.e. mPTP remains in closed state even when Ca$^{2+}$ ions are in mitochondria.

According to experimental data, in Ca$^{2+}$ containing medium, quercetin at 50 μM inhibits opening of mPTP up to 18.6%, at 200 μM inhibits opening of mPTP up to 89.7% in comparison to control experiment. The data suggests that quercetin, depending on the concentration, inhibits the opening of rat liver mPTP. This is probably due to the antioxidant properties of quercetin (Gayibov, Komilov et al., 2017), that is universal mechanism of stabilizing membranes. Our further investigations were devoted to studying the influence of dihydroquercetin on mPTP activity on the mitochondrial membrane. We were interested in the comparison interaction of quercetin and its derivative dihydroquercetin with mitochondria. The concentration of dihydroquercetin was the same (10-100 μM).

From the data in fig. 5 (B) inhibition of mPTP opening by dihydroquercetin is not much different from quercetin activity.

mPTP opening leads to mitochondrial dysfunction through ATP hydrolysis and oxidative phosphorylation uncoupling, ultimately resulting in cell death. The opening state of mPTP has been examined explored broadly in pathology of cardiovascular disorders such as ischemia/reperfusion and heart failure. Hence, mPTP is recognized as a therapeutic target for pharmacological and non-pharmacological strategies by blocking pore activation over direct blockade of mPTP components and/or indirect mechanism of blockage of mPTP inducers (Javidov et al., 2009). The list of indirect mechanisms of mPTP blockade includes $\Delta$Pm modulation, redox state, mitochondrial mass regulation, calcium retention capacity and fusion/fission processes. Closer approach reveals alterations in protein expression, post-translational modifications and interactome disturbance, thus affecting signaling pathways.

**mitoKATP activation**

Potassium ions are the main ionic component of not only the cytoplasm but also the mitochondrial matrix. The direction of the electric field on the inner membrane of mitochondria favors the transport of potassium ions from the cytoplasm to the matrix. Thus, the presence of any K$^+$-selective channels, including the mitoKATP channel, in the inner mitochondrial membrane will be accompanied by changes in the mitochondrial volume and regulation of the state of the Ca$^{2+}$-dependent mPTP. Modulation of the conductivity of the mitoKATP channel underlies changes in mitochondrial functions under different pathologic conditions, however, the physiological role of this phenomenon is not entirely clear (Akopova, Kolchinskaya et al., 2011). Fig. 6 shows experimental data using quercetin as mitoKATP channel activator. The data shows that quercetin, starting from a concentration of 10 and 20 μM, stimulates the mitoKATP channel opening (Fig. 6), and at concentrations of 80 μM or more, an exit to the board is observed. Regarding the action of quercetin on the activity of the mitoKATP channel, the experimental line observed is saturation curve line representing dose-dependent effect.
**Mitochondrial swelling, %**

**Concentration of compound, µM**

**Figure 6** Influence of quercetin (A) and dihydroquercetin (B) on the mitoKATP channel activation. External medium was as follow (mM): KCl (125), Hepes (10), succinate (5), MgCl₂ (1), K₃HPO₄ (2.50), KH₂PO₄ (2.5), rotenone (0.005) and oligomycin (0.001), pH 7.4. (** - P<0.01, *** - P<0.001; n=4)**

**Mitochondrial swelling, %**

**Concentration of quercetin, µM**

**Figure 7** The effect of dihydroquercetin on the state of the mitoKATP channel of rat liver (** - P<0.01; n = 3).**

Open: open conformation, control without ATP (activity of the mito-CATF channel).

ATP: MitoCATF channel state in the presence of 200 µM ATP.

DZX: mitoKATP channel activity in the presence of 30 µM diazoxide + 200 µM ATP.

DHQ: MitoATF channel activity with 80 µM DHQ + 200 µM ATP.

DZX + DHQ: mitoCATF channel activity with 30 µM diazoxide + 80 µM DHQ + 200 µM ATP.

The dual role of reactive oxygen species was widely accepted. On one hand in high concentrations ROS can damages cells; on the other hand they display messenger’s activity in cellular signaling thus inducing cells survival in stress conditions. Interestingly ROS also can stimulate mitoKATP activation under oxidation stress in the heart and brain, conferring the cells protective effect (Badziak et al., 1999). The functioning of mitoKATP depends on the redox state of the active groups of the channel protein. It has been found that redox agents modulate the work of mitoKATP. For example, the electron donor n-dimethylaminomethyl benzoate activates mitoKATP, and the electron acceptors — pelargonidine inhibits the channel (Grigoriev et al., 1999), which is probably due to their effect on the SH groups of the channel. Perhaps the activation of this channel by quercetin and dihydroquercetin is related to the fact that they are electron donors (antioxidants).

**CONCLUSION**

It is known that polyphenolic compounds exhibit antioxidant, anti-inflammatory, immunomodulatory and many other actions, which creates the prerequisites for creating drugs based on them. In this case polyphenols are advantageous compounds due to their low toxicity and sufficient aqueous solubility. Summarizing above mentioned data of polyphenol compounds activity on the mitochondrial level we can conclude that compounds are determined to some
extent by the influence on the mitochondrial membrane structure, free radical oxidation and oxidative phosphorylation processes.

As it was shown earlier by M.I. Asrarov et al. (2015), a trend to improve some functional characteristics of mitochondria by the policosanol-Ruspolia induces the background to assume possible antioxidant, cardio protective and antihypertensive effects of quercetin and dihydroquercetin. Further studies are required to validate the role of quercetin and dihydroquercetin as antioxidant, cardio protective and antihypertensive agent on organismal level.

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