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The effect of Crataegus fruit extract and some of its flavonoids on mitochondrial oxidative phosphorylation in the heart

The effect of Crataegus extract on the heart mitochondrial function

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SUMMARY

*Crataegus* (Hawthorn) fruit extracts (CE) are widely used for the treatment of various cardiovascular diseases (arrhythmias, heart failure, myocardial weakness, etc.). Despite the fact that many of these diseases are associated with disturbances of mitochondria, no data have been found on the effect of CE on their function. The aim of this study was to perform an oxygraphic investigation of the effect of CE (in concentration range from 70 ng/ml to 13.9 µg/ml of *Crataegus* phenolic compounds (PC)) and its several pure flavonoids on isolated rat heart mitochondria respiring on pyruvate+malate, succinate and palmitoyl-L-carnitine+malate. CE at doses under 278 ng/ml of PC had no effect on mitochondrial functions. At concentrations from 278 ng/ml to 13.9 µg/ml of PC, CE stimulated State 2 respiration by 11% - 34% with all used substrates, and decreased the mitochondrial membrane potential by 1.2-4.4 mV measured with a tetraphenylphosphonium-selective electrode and \( \text{H}_2\text{O}_2 \) production measured fluorimetrically. Similar uncoupling effects on mitochondrial respiration were observed with several pure CE flavonoids. The highest CE concentration also slightly reduced the maximal ADP-stimulated and uncoupled respiration, which might be due to inhibition of mitochondrial respiratory chain between flavoprotein and cytochrome c. Whether or not the uncoupling and other effects of CE on mitochondria may be realized *in vivo* remains to be determined.

**Keywords:** Crataegus fruit extract, heart mitochondria, oxidative phosphorylation, flavonoids
**Abbreviations:** CE - *Crataegus* fruit extract; PC - *Crataegus* phenolic compounds; ROS – reactive oxygen species; BSA – bovine serum albumin; TMPD - N,N,N',N' -tetramethyl-p-phenylenediamine; CCCP – carbonyl cyanide 3-chlorophenylhydrazone; FCCP - carbonyl cyanide-4- (trifluromethoxy)phenylhydrazone.
INTRODUCTION

Cardiovascular diseases remain the principal cause of mortality in both developed and developing countries. Many of them are associated with the structural and functional disturbances in heart mitochondria. Since mitochondria make up 35% of the cell volume in the cardiac muscle cells and produce 95% of energy necessary for the heart functioning, their role in the cell is obvious. Therefore, the therapeutic agents that could influence mitochondrial dysfunction are of special importance, and this leads to an increased attention paid to the effect of various medicinal preparations on the mitochondrial structure and function.

The range of effective plant products for the target phytotherapy of cardiovascular system diseases is not wide. *Crataegus monogyna* Jacq. (Hawthorn) extract is among the most popular herbal medicinal products and has been used around the world since ancient times (Fong and Bauman, 2002). Nowadays extracts of *Crataegus* fruit, leaves and flowers are used for the prevention and treatment of various cardiovascular diseases (hypertension, arrhythmias, congestive heart failure, coronary artery disease, myocardial weakness etc.) due to their positive inotropic and negative chronotropic effects, reduction in coronary spasm, blood pressure and total plasma cholesterol, and for their antioxidant and antiinflammatory properties (Chang et al., 2005; Rigelsky and Sweet, 2002). Such a wide pharmacological effect is due to polyphenol substances in the extract, consisting of flavonoids, triterpenes, phenolic acids, etc.

Despite a number of preclinical and clinical studies, the mechanisms of the protective effects of *Crataegus* extract remain poorly understood. Moreover, there are no
findings on its action on energetic metabolism of the cardiac muscle cells, which is essentially determined by the activity of mitochondria, ensuring the main physiological function of cardiac muscles, i.e. continuous mechanical contraction. Much more is known on the effects of polyphenols on mitochondrial function. Although their ability to protect cells from the “oxidative stress” (antioxidant activity) has been demonstrated, there is increasing evidence that the same polyphenol compounds could behave as both antioxidants and prooxidants, depending on the concentration and the source of free radicals (Nemeikaite-Ceniene et al., 2005; Laughton et al., 1989). Because some of Crataegus flavonoids are the same as in Ginkgo biloba, one can assume that CE, like Ginkgo biloba extract (Trumbeckaite et al., 2007), could possess the uncoupling activity on heart mitochondria, which was also demonstrated in our study (Trumbeckaite et al., 2006) for some pure flavonoids. However, it is difficult to predict the behavior of the same flavonoids in the natural extracts containing many different components, including those of unknown nature.

In this regard, it is interesting to note that moderate mitochondrial depolarization (uncoupling of oxidative phosphorylation) is known to attenuate the generation of mitochondrial reactive oxygen species (ROS) and could be beneficial in protection against cardiac ischemia-reperfusion injury (Skulachev, 1998; Sack, 2006; Halestrap et al., 2007) and, possibly, against other pathological situations. ROS are formed within mitochondria under physiological and pathological conditions, particularly under ischemia/reperfusion.

Therefore, the aim of this study was to investigate in vitro the direct influence of Crataegus fruit extract and some of its flavonoids on the respiration of isolated rat heart
mitochondria in order to test for their uncoupling potential and get more insight into the mechanism of their action.

MATERIAL AND METHODS

Preparation of CE and quantitative analysis. The dry fruits of *Crataegus monogyna* Jacq. were extracted (1:10) with 70% ethanol, the particle size was 2-3 mm, the production method was percolation, and the flow speed of the extract was 0.5 ml/min (Bernatoniene et al., 2003).

The content of total phenolic compounds in CE was determined spectrophotometrically (Hitachi 557) using the Folin-Ciocalteu method. 10 ml of appropriate dilutions of CE were added to a 50 ml volumetric flask containing 10 ml of distilled water, 4 ml of Folin-Ciocalteu reagent, and 6 ml of a 20% sodium carbonate. Distilled water was added to increase the volume up to 50 ml. The color was developed for 2 hours at room temperature, the suspension was centrifuged at 5000 rpm, and the absorption of the obtained solution was measured at 760 nm wavelength. The measurement was compared to a standard curve of prepared gallic acid solution. Total phenolic contents are expressed as Gallic Acid Equivalent.

HPLC analysis was carried out using the Waters 2690 Alliance HPLC system (Waters Corporation, Milford, MA, USA), equipped with a Waters 2487 UV/Vis detector, an on-line degasser and an auto sampler, and a Waters X Terra RP18 150×3.9 mm column. UV detection was achieved at 360 nm on the detector. The chromatographic elution was accomplished by a gradient solvent system consisting of water containing 0.1% TFA (A) and acetonitrile containing 0.1% TFA (B). The gradient conditions were
the following: 0 min, 95% A, 5% B; 45 min, 55% A, 55% B, kept to 50 min; 55 min, 95% A, 5% B at the flow rate of 400 µl/min. The injection volume was 10 µl. Buffer solutions were filtered through 0.2 µm disposable membrane filter (Roth, Karlsruhe, Germany) and degassed prior to use. Data were collected and analyzed using the Waters Millennium 2000® chromatographic manager system. The eluted constituents were identified by comparison of the retention time. Regression of the calibration curves of the reference standards was linear, correlation coefficient ($R^2$) of all curves was $>0.9999$, and the resolution ($R_s$) of standards peaks was $>1.5$.

**Isolation of rat heart mitochondria.** Male Wistar rats weighing 250-300 g were used for our study. The animals were killed according to the rules defined by the European Convention for the protection of Vertebrate Animals Used for Experimental and Other Purposes (License No.0006). Hearts of rats were excised and rinsed in ice-cold 0.9% KCl solution. Heart mitochondria were isolated in the medium containing 220 mM mannitol, 70 mM sucrose, 5 mM N-tris[Hydroxymethyl]methyl-2-aminoethane-sulfonic acid and 0.5 mM EGTA (pH 7.4, adjusted with Trizma base; 2 °C) and 2 mg/ml bovine serum albumin (BSA; fraction V, A4503, Sigma). The homogenate was centrifuged at 750 x g for 5 min, then the supernatant was re-centrifuged at 10,000 x g for 10 min, and the pellet was washed once (10 min 10,000 x g) in the isolation medium without BSA, suspended in it and kept on ice. The mitochondrial protein concentration was determined by applying the biuret method with BSA used as standard.

**Measurement of mitochondrial respiration, membrane potential and $\text{H}_2\text{O}_2$ generation.** Oxygen uptake rates were recorded at 37°C by means of the Clark-type
electrode system in a solution containing 20 mM imidazole, 20 mM taurine, 0.5 mM dithiothreitol, 1.6 mM MgCl₂, 100 mM MES, 3 mM KH₂PO₄, 3.0 mM CaK₂EGTA, and 7.1 mM K₂EGTA (free Ca²⁺ concentration - 0.1 µM) (pH 7.1 adjusted with KOH at 37°C) separately (1) with 6 mM pyruvate + 6 mM malate, (2) 12 mM succinate (+2 mM amytal), and (3) 9 µM palmitoyl-L-carnitine + 0.24 mM malate as substrates. The solubility of oxygen was estimated to be 422 nmol O₂/ml. The activity of cytochrome c oxidase was measured with 10 mM ascorbate + 0.5 mM N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) as electron donors. Mitochondrial respiration rates were expressed as nmol O₂/min/mg protein. The final mitochondrial protein concentration in all experiments was 0.5 mg/ml. All stock solutions of pure flavonoids (Carl Roth GmbH, Karlsruhe, Germany) that were used in our study were prepared in 70% ethanol. Control measurements showed that the maximal used ethanol concentration (10 µl of 70% ethanol per 1 ml of the mitochondrial incubation medium) did not affect any functional parameters of mitochondria.

Mitochondrial membrane potential was measured in a closed, stirred and thermostatically controlled 1.5 ml vessel fitted with a tetraphenylphosphonium (TPP⁺)-selective electrode using a TPP⁺-binding correction factor of 0.162 (µl/mg) (Brown and Brand, 1988). The binding correction factor was determined from the ratio of Rb⁺ to TPP⁺ accumulation as a function of mitochondrial volume. The experiments were performed at 37 °C using 6 mM pyruvate + 6 mM malate as oxidizable substrate, and 0.5 mg/ml mitochondria in the same medium as mitochondrial respiration.

ROS generation was estimated as the release of H₂O₂ from isolated rat heart mitochondria (0.25 mg/ml) fluorimetrically (Thermo Scientific). Mitochondria were
incubated for 7 min at 37°C in the same medium as for mitochondrial respiration supplemented with pyruvate (5 mM), malate (5 mM) and antimycin A (0.016 µg/ml). After incubation, Amplex Red (5 µM) and horseradish peroxidase (2U/ml) were added, and fluorescence (excitation at 544 nm, emission at 590 nm) was measured. Amplex Red fluorescence response was calibrated by adding known amounts of H$_2$O$_2$.

**Statistical analysis.** Data are presented as means ± S.E.M. Nonparametric methods were applied for making inferences about the data. Differences between mean values in dependent groups were tested using Wilcoxon matched pairs test. Differences between mean values in independent groups were tested using nonparametric Kruskal-Wallis test with Dunn's post-hoc evaluation. $P < 0.05$ was taken as the level of significance. Statistical analysis was performed using the software package Statistica 1999, 5.5, StatSoft Inc., USA.

**RESULTS**

In this study we used *Crataegus* fruit extract that contained 139 ± 8 mg/100 ml of total phenolic compounds. Using the HPLC analysis method, we identified several classes of flavonoids in our investigated CE: flavonols (quercetin – 1.26 ± 0.1 µg/ml, hyperoside – 29.32 ± 0.5 µg/ml, rutin – 2.64 ± 0.2 µg/ml, quercitrin – 0.49 ± 0.01 µg/ml), flavanols (epicatechin – 44.92 ± 0.9 µg/ml, procyanidin B$_2$ – 0.71 ± 0.06 µg/ml), and phenolic acid (chlorogenic acid – 3.46±0.03 µg/ml).

The effect of CE on the integrity and function of mitochondrial membranes was determined oxygraphically using isolated rat heart mitochondria. The State 2 respiration
rate ($V_0$) with different substrates was monitored for 1 min and then various amounts of CE were added in order to assess their effect on the State 2 respiration rate. Subsequently, ADP was added, and the maximal respiration in State 3 ($V_{ADP}$) was recorded. It reflects the maximal capacity of respiratory chain and ATP production rate. In order to check the intactness of the outer mitochondrial membrane, we used cytochrome c test and for this we added exogenous cytochrome c in State 3. The degree of respiration stimulation by cytochrome c ($V_{ADP+C}/V_{ADP}$) allows for assessing the integrity of the outer mitochondrial membrane. Changes in $V_0$ show changes in the permeability of the inner mitochondrial membrane to protons or ions, and, together with calculated respiratory control index (RCI = $V_{ADP}/V_0$), allows for determining the effectiveness of ADP phosphorylation in mitochondria.

In the first series of experiments, we investigated the effect of various concentrations of CE (70 ng/ml – 13.9 µg/ml of PC) on the mitochondrial respiration with complex–I dependent substrates pyruvate+malate. CE was added into incubation medium after 1 min registration of State 2 or State 3 respiration. The percentage change in the mitochondrial respiration parameters is presented in Fig. 1. Our findings showed that CE at the lowest investigated concentration (70 ng/ml of PC) had no effect on any mitochondrial respiratory parameters. Higher concentrations of CE (278 ng/ml – 6.95 µg/ml of PC) increased $V_0$ (11-24%) but did not affect any other mitochondrial respiration rates ($V_{ADP}, V_{ADP+C}$). The highest CE concentration used in our experiments (13.9 µg/ml of PC) induced a 34% increase in State 2 respiration rate and a decrease in State 3 respiration rate by 18%, leading to the most pronounced decrease in RCI. The decrease in State 3 was not restored after the addition of exogenous cytochrome c. Thus,
these results showed that the decrease in State 3 respiration rate at highest used CE concentration was not due to the loss of cytochrome c. Further data demonstrated that CE at the concentration 13.9 µg/ml of PC also suppressed the CCCP-uncoupled respiration rate with pyruvate+malate as substrates by 25-53% (data not shown). Thus, this demonstrates that the respiration rate at the highest CE concentration was decreased due to the inhibition of mitochondrial respiratory chain. Our experiments also showed that the activity of the terminal respiratory chain complex IV (cytochrome c oxidase) measured by recording ascorbate + TMPD oxidation was not affected by the highest CE concentration (control, 586 ± 41 nmol O₂/min/mg protein; in the presence of CE, 627 ± 42 nmol O₂/min/mg protein, n=4 measurements).

Mitochondrial membrane potential in State 2 was the same (163.2 ± 1.3 mV) in both control mitochondria oxidizing pyruvate + malate and at the lowest used CE concentration (70 ng/ml). At higher CE concentrations in the medium (278 ng/ml, 6.95 µg/ml and 13.9 µg/ml of PC), the mitochondrial membrane potential decreased by 1.2 mV, 2.5 mV and 4.4 mV (P<0.05, all versus control, respectively; n = 3).

Our fluorimetric measurements demonstrated that CE at the maximal (13.9 µg/ml of PC) and at the half-maximal (6.95 µg/ml of PC) concentrations decreased H₂O₂ production in mitochondria by 62.8±12% and 35.3±7%, respectively (n=5, p<0.05; control rate of H₂O₂ production was 0.31 ± 0.03 nmol/min/mg protein).

In the next series of studies, we investigated the effect of CE on the oxidation of FAD-specific substrate succinate and palmitoyl-L-carnitine, the main respiratory substrate of the heart (Table 1). CE extract at the concentration of 70 ng/ml of PC had no effect on V₀. At higher CE concentrations (6.95 and 13.9 µg/ml of PC), V₀ increased by
27% and 30% with succinate, and by 14% and 27% - with palmitoyl-L-carnitine. Only at the highest used concentration of CE (13.9 µg/ml of PC), the maximal respiration rate ($V_{ADP}$) decreased by 12% and 19% with substrate succinate and palmitoyl-L-carnitine, respectively. Lower concentrations of CE had no effect on the State 3 respiration rate.

In further experiments we investigated the effect of some pure active substances present in the composition of CE on mitochondrial respiration with pyruvate+malate. Their concentrations were identical to those found in CE at the maximal used amounts (13.9 µg/ml of PC). Rutin had the greatest effect (Fig. 2) - mitochondrial State 2 respiration rate was stimulated by 70%; epicatechin, procyanidin, hyperoside, quercetin, and quercitrin increased it by 17-30%, whereas the chlorogenic acid had no effect on State 2. Rutin and quercitrin had no effect on State 3 respiration rate whereas it was slightly inhibited (-11%) by hyperoside and quercetin (data not shown).

**DISCUSSION**

One of the main functions of mitochondria is to supply a cell with energy, which is required for maintaining the regular functioning of heart. Therefore damage to these organelles may be the key factor for myocardial dysfunction. For this reason, the role of mitochondria in physiological and pathological conditions and the effect of various medical preparations on the mitochondrial processes are intensively investigated. In this study we prepared the ethanolic extract from *Crataegus monogyna* fruit and determined its effect *in vitro* on the function of mitochondria isolated from rat hearts.

Our investigation showed that at concentrations of 278 ng/ml – 13.9 µg/ml of PC, CE stimulated State 2 respiration by 11% - 34%, similarly to all used Complex I and
Complex II substrates, and statistically significantly (by 1.2-4.4 mV) decreased mitochondrial membrane potential. Thus, the decrease in mitochondrial membrane potential and the increase in $V_0$ indicates that CE in vitro possesses a slight direct uncoupling effect in isolated cardiac mitochondria. This effect was dose-dependent, yet it was practically independent of the respiratory substrate. It is interesting to note that the CE-induced uncoupling was clearly lower than that induced by Ginkgo biloba extract even at much lower concentrations of flavonoids, as it had been shown in our previous study (Trumbeckaite et al., 2007). It could be that certain unknown components affected (potentiated or inhibited) the flavonoid action.

It is known that "mild" uncoupling of isolated mitochondria, which leads to a slight decrease in mitochondrial membrane potential, declines ROS production (Skulachev, 1998; Sack, 2006; Halestrap et al., 2007). Therefore, it was proposed that such uncoupling is involved in the cellular defense system against oxidative stress. In good agreement with the data obtained using classical uncouplers of oxidative phosphorylation (Skulachev, 1998), our fluorimetical measurements demonstrated that CE at concentrations causing mild uncoupling of oxidative phosphorylation also significantly decreased H$_2$O$_2$ production in mitochondria. However, Brennan et al. (2006) showed that adding low doses of an uncoupler (100 nM FCCP) prior to ischemia of an isolated perfused rat heart, increasing oxygen consumption (mild uncoupling) and ROS production but having no effect on the mitochondrial membrane potential, can also protect the heart against subsequent ischemia-reperfusion injury.

Several other studies showed that CE can be beneficial due to its antioxidant effects. It was shown that 15-day oral administration of Crataegus flavonoids extracted from
leaves decreased ROS production and protected the brain against delayed cell death caused by ischemia/reperfusion injury (Zhang et al., 2004). Oral pretreatment of rats for 7 days with Crataegus extract from leaves with flowers (10 mg/kg or 100 mg/kg) improved cardiac function and reduced infarct size in a rat model of prolonged coronary ischemia and reperfusion (Veveris et al., 2004). Jayalakshmi et al. (2006) showed that oral pretreatment of rats for 30 days with the ethanolic extract of Crataegus oxycantha fruit at a dosage of 0.5 ml/100 g body weight/day maintained mitochondrial antioxidant status, and prevented mitochondrial lipid peroxidative damage and decrease in Krebs cycle enzymes induced by isoproterenol in rat heart. Considerable antioxidant potential of water extracts of Crataegus aronia leaves and fruit (1 µg/mL –1 mg/mL) was also demonstrated on cultured Hep G2 cells (Ljubuncic et al., 2005). Noteworthy, during Crataegus extract administration by above mentioned research groups, the concentrations of total flavonoids varied widely, approximately between 20-170 µg/g body weight/day, thus it is difficult to compare these data to our in vitro study.

The much more drastic differences between doses were noted for pure flavonoids. For example, the amount of quercetin administered to rats per os differed by about 24,000 times, i.e. from 0.033 mg/kg (Brookes et al., 2002) to 100 mg/kg (Guzy et al., 2003) and even 800 mg/kg per day (de Boer et al., 2005). Surprisingly, despite a 3,000-fold difference of the amount of quercetin administered (i.e. at concentrations ranging from antioxidant to prooxidant) in the first two studies, cardioprotective effects in ischemia-reperfusion and daunorubicin-induced injury, respectively, were noted.

It should be also noted that the concentrations of CE used in our in vitro study were higher than therapeutic doses of ethanolic CE in human studies. However, they were
comparable to those used with rats in vivo (Jayalakshmi et al., 2006). The highest CE concentration used in our investigations (13.9 µg/ml of PC) not only increased State 2 respiration rate (similarly to all respiratory substrates), but also reduced the maximal ADP-stimulated mitochondrial respiration rate by 12-19% and the CCCP-uncoupled respiration rate with pyruvate+malate as substrates. Thus, this clearly demonstrates that higher concentrations of CE suppress some mitochondrial respiratory chain complexes. Since the cytochrome c oxidase (complex IV) activity was not affected by this CE concentration, these data also suggest that the inhibition of electron transport in mitochondria is localized between flavoprotein and cytochrome c, i.e. between Complex I and III. However, we cannot exclude that ADP/ATP translocase might be inhibited to some extent as well. Concerning ATP synthase (complex V), it was revealed that some polyphenolic phytochemicals bind to and inhibit mitochondrial ATP synthase (Gledhill et al., 2007; Zheng and Ramirez, 2000); however its inhibition in our experiments seems unlikely due to much lower flavonoid concentrations used.

Since some of CE polyphenols may damage membranes and cause cytochrome c release from mitochondria, we used cytochrome c test in order to check this possibility and elucidate the reason for the inhibition of mitochondrial respiration. Our findings showing that the degree of external cytochrome c-induced stimulation of mitochondrial respiration (\(V_{ADP+c}/V_{ADP}\)) was similar in the control group and after the addition of CE, denied such possibility.

It is known that inhibition of mitochondrial respiration by various respiratory chain inhibitors augments ROS production and oxidative stress that occurs under the conditions of inhibitor-diminished membrane potential (Byun et al., 2008), and could also protect
the cells at ischemia and reperfusion (Halestrap et al., 2007; Kabir et al., 2006). In contrast, the treatment of isolated, perfused rabbit heart with rotenone (mitochondrial complex I inhibitor) decreased the production of ROS in mitochondria and preserved the contents of cardiolipin and cytochrome c measured after 45 min of ischemia (Chen et al., 2007). Interestingly, in isolated rat cardiocytes, antimycin A-caused ROS production was abolished by the mitochondrial uncoupler (Kabir et al., 2006). Thus, some inhibition of mitochondrial respiration observed at the highest CE concentration can be beneficial in some circumstances and may complement the effect of uncoupling. The blockade of electron transport and the partial uncoupling of respiration were regarded as two mechanisms whereby manipulation of mitochondrial metabolism during ischemia decreases cardiac injury (Chen et al., 2007).

Investigations concerning the action of individual classes of flavonoids, namely chalcones, flavones, and flavonols, showed that at higher concentrations (100 µM) they inhibit beef heart mitochondrial succinatoxidase, NADH-oxidase, ATPases, and can have a pro-oxidant effect (Hodnick et al., 1994). Flavonoids at lower concentrations (IC$_{50}$ ≤1.5 µM) possess antioxidant activity and do not inhibit cellular enzymes (Laughton et al., 1989).

In our study, we also tested the effect of some pure flavonoids present in the CE at the maximal concentration (13.9 µg/ml of PC) on the heart mitochondrial respiration with pyruvate+malate. We found (Fig.2) that epicatechin (1.5 µM), procyanidin (0.012 nM), hyperoside (0.96 µM), quercetin (45 nM), and quercitrin (5.3 nM) stimulated State 2 respiration by 18-30%, i.e. similarly to the CE. Rutin (47 nM) increased it more effectively - by 70%. Thus, it seems likely that the effect of CE is mainly determined by
these compounds. Noteworthy, these concentrations were far lesser than those mentioned above (Hodnick et al., 1994), and not optimal to achieve the maximal increase in State 2 respiration rate (Trumbeckaite et al., 2006).

It still remains to be determined whether or not the effects of CE may be realized \textit{in vivo} - through uncoupling of oxidative phosphorylation in mitochondria observed in our \textit{in vitro} studies or via other mechanisms. This issue is complicated due to several circumstances. It is known (Brookes et al., 2002) that polyphenols, after intake, undergo various conversions, but the effects of most metabolites on mitochondria are not known. Moreover, because of the long half-lives of elimination and the hydrophobic nature of polyphenols, repeated consumption of polyphenol-containing products (food or CE) should cause their accumulation in blood and membranes, including those of mitochondria. Since the daily dietary flavonoid intake (1) varies considerably in different populations (65-250 mg/day – for Western populations) (Erdman et al., 2007) and, in addition, (2) there exist very large blood, tissue and species differences with regard to the distribution and concentrations of flavonoids and its metabolites (de Boer et al., 2005), including differences in plasma half-life for these compounds in various species, differences in the background concentrations of flavonoids in the cells should be expected. Accordingly, it should entail the differences in the effect of CE on various subjects and tissues. Thus, to facilitate the solution of a problem/interpretation of the data, it seems reasonable that the concentrations of flavonoids in the plasma, cells, and mitochondria should be determined before and after treatment with CE. Based on the effects of chronic respiratory uncoupling of Hep G2 cells by classical uncoupler dinitrophenol (10 and 50 µM, 3 days) - an increase in cellular oxygen consumption,
oxidative capacity and cytochrome c oxidase activity -which was associated with an upregulation of its subunit IV and adenine nucleotide translocase 3 gene expression (Desquiret et al., 2006) - one may assume that long-term treatment with CE may induce mitochondrial biogenesis (for rev. see Tapia, 2006), which would be beneficial in heart failure treatment. On the other hand, large numbers of protein kinases (that have been reported as being potential targets for flavonoids due to their ability to bind to ATP sites on enzymes) as well as other accumulating evidence suggest that the cellular effects of flavonoids may be mediated by their interactions with specific proteins central to intracellular signaling cascades (Williams et al., 2004).

In sum, our in vitro findings clearly demonstrated that CE and its main biologically active components cause partial uncoupling of oxidative phosphorylation and suppression of H₂O₂ production in the isolated rat heart mitochondria. It remains to be determined whether these effects may be realized in vivo and thus could reduce the generation of free radicals within mitochondria, as well as whether slight mitochondrial respiratory chain inhibition plays any significant role in cardioprotection.

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Table 1. The effect of *Crataegus* fruit extract on mitochondrial respiration parameters

|                  | **Succinate** | | **Palmitoyl–L–carnitine** | | | |
|------------------|---------------|--|---------------------------|--|----------------|--|----------------|--|----------------|--|
|                  | Control CE 70 ng/ml | CE 6.95 µg/ml | CE 13.9 µg/ml | Control CE 70 ng/ml | CE 6.95 µg/ml | CE 13.9 µg/ml |
| **V₀** (before adding CE) | 158±6 | 154±4 | 157±6 | 162±3 | 83±2 | 82±1 | 74±1 | 70±4 |
| **V₀** (after adding CE) | - | 154±4 | 200±7* | 217±7* | - | 82±1 | 84±2 | 89±3* |
| **V<sub>ADP</sub>** | 315±7 | 290±12 | 312±8 | 277±4* | 187±13 | 201±6 | 165±8 | 153±5* |
| **V<sub>ADP+C</sub>** | 490±11 | 451±13 | 482±10 | 495±13 | 226±11 | 236±10 | 188±12 | 184±11* |
| **RCI** | 2.0±0.1 | 1.8±0.03 | 1.9±0.09 | 1.3±0.03* | 2.3±0.1 | 2.5±0.07 | 1.9±0.1 | 1.7±0.09* |

Measurements were performed in the presence of 12 mM succinate (+2 mM amytal) or 9 µM palmitoyl-L-carnitine + 0.24 mM malate as substrates (V₀); Further additions: 1 mM ADP (V<sub>ADP</sub>); 32 µM cytochrome c (V<sub>ADP+C</sub>); RCI – respiratory control index; number of experiments – 3; * - P<0.05 vs control.
FIGURE LEGENDS

Fig. 1. The effect of different concentrations of Crataegus fruit extract on the mitochondrial respiratory parameters (substrate - pyruvate+malate)

The reagents were added in the following order: \(V_0\) – 6 mM pyruvate + 6 mM malate; \(V_{ADP}\) - 1 mM ADP; \(V_{ADP+C}\) - respiration rate in the presence of 32 µM cytochrome c; RCI – respiratory control index; number of experiments – 3; *p<0.05 vs control.

Fig. 2. The effect of the main active compounds of Crataegus fruit extract on mitochondrial respiration in State 2.

Concentrations were identical to those in the extract CE 13.9 µg/ml of PC

Substrates were 6 mM pyruvate + 6 mM malate; number of experiments – 3-5; *p<0.05, vs control
Fig. 1
Fig. 2

- Epicatechin 449 ng/ml
- Hyperoside 293 ng/ml
- Chlorogenic acid 30 ng/ml
- Rutin 26 ng/ml
- Quercetin 12.6 ng/ml
- Quercitrin 4.9 ng/ml
- Procyanidin B2 7 ng/ml

Changes, %

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