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Effect of *Ginkgo biloba* extract on the rat heart mitochondrial function

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Abstract

Ginkgo biloba L. (Ginkgoaceae) originated from China, first introduced to Europe in the 18th century, it is now distributed all over the world. The leaves of *Ginkgo biloba* include a rich complex of active compounds responsible for various pharmacological properties. *Ginkgo biloba* extract improves blood circulation, protects against oxidative cell damage, blocks platelet aggregation that could be important for prevention of cardiovascular diseases. Therefore the fluid extract from *Ginkgo biloba* leaves was prepared and tested for it is effect on rat mitochondrial function. Our data showed that 0.5 μl/ml of GE (containing 0.57 ng/ml of rutin, 0.23 ng/ml of quercitrin, 0.105 ng/ml of hyperosid and 0.02 ng/ml of quercetin) had no effect on the State 2 respiration rate of mitochondria with all used substrates: pyruvate + malate, succinate and palmitoyl-L-carnitine. Further increase in GE concentration (2 and 4 μl/ml), increased the State 2 respiration rate with all respiratory substrates in a dose-dependent manner (by 35–116%). The State 3 respiration rate was not affected by GE. In order to identify which compounds of GE could be responsible for the observed effects, we measured the effect of pure flavonoids: rutin, quercetin, hyperosid and quercitrin on mitochondrial respiration. All flavonoids (except of hyperosid) at maximal used concentration, comparable/identical to that in GE, stimulated the State 2 respiration rate only by 8–20%, i.e. less effectively as compared to GE. Therefore, for the explanation of the GE-induced uncoupling of oxidative phosphorylation, other biologically active compounds of GE have to be taken into account in future studies.

Keywords: Mitochondria; Oxidative phosphorylation; *Ginkgo biloba* extract

1. Introduction

Ginkgo biloba L. (Ginkgoaceae) in traditional Chinese medicine has been used for a very long time. Originated from China, first introduced to Europe in the 18th century (Stromgaard and Nakanishi, 2004), it is now distributed all over the world. Over the past 20 years *Ginkgo biloba*-derived preparations have became widely known and belong to the one of best selling phytomedicine in Europe. Wide pharmacological application of *Ginkgo biloba* extracts (GE) is determined by the main active substances: flavonoids (flavone glycosides, primarily composed of quercetin) and terpenoids (ginkolides and bilobalides). To this day, due to their vasorelaxing, anticoagulant, antioxidative, and anti-inflammatory properties GE have most frequently been prescribed as preparations that improve cerebral blood circulation, and especially for memory improvement (Christen, 2004; Luo, 2001). Evidence from different studies on the antioxidant properties of GE supports the protective effect of this extract against ischemia-reperfusion injury and oxidative stress conditions (Pietri et al., 1997). It is postulated that both flavonoid and terpenoid constituents are involved in the antioxidant effects of GE due to decreasing of tissue level of reactive oxygen species (ROS) and inhibiting membrane lipid peroxidation (DeFeudis and Drieu, 2000). It is well known that mitochondria may generate free radicals on the level of the respiratory chain. As ROS produced by mitochondria are increased in a variety of pathological conditions including hypoxia, ischemia-reperfusion injury, thus GE may be effective against cardiovascular disorders. Clinical trials and studies on experimental animals showed that EGb 761, a standardized dry extract of *Ginkgo biloba* leaves, consisting of 24% ginkgo flavone glycosides and 6% terpenoids, improve myocardial functional recovery, reduce the number of

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ventricular extrasystoles and the duration of ventricular tachycardia induced by reperfusion (Clostre, 2001; Varga et al., 1999; Varga, 2002). Other authors reported that EGb 761 inhibit NO production and improve the recovery of post-ischemic cardiac function in the ischemic/reperfused myocardium (Varga et al., 1999). Rioufol proposed that EGb 761 can attenuate myocardial stunning following a brief ischemic stroke in pig hearts, and the effect of functional recovery involves both its non-ginkgolide and ginkgolide constituents (Rioufol et al., 2003). We presume that *Ginkgo biloba* might have a cardioprotective effect and that mitochondria could be involved into this mechanism, as they are the main cell organells that produce ATP requiring for the functioning of the heart. Thus, the aim of this work was to investigate the effect of the GE, produced in Lithuania, on the oxidative phosphorylation in rat heart mitochondria.

2. Materials and methods

2.1. Preparation of GE and HPLC conditions

After the selection of the optimal production technology, the extract was produced from leaves of *Ginkgo biloba* tree growing in Lithuania. Dried *Ginkgo biloba* leaves (*Ginkgo biloba* L.) were extracted using 70% ethanol (1:5); the particles size 2–3 mm, the production method percolation, and the flow speed of extract 0.5 ml/min.

HPLC analysis was carried out using a Waters 2690 Alliance HPLC system (Waters Corporation, Milford, MA, USA), equipped with a Waters 2487 UV/vis detector, an online degasser and an auto sampler, and a Waters XTerra RP18 150 mm × 3.9 mm column. UV detection was achieved in the 360 nm on detector. The chromatographic elution was accomplished by a gradient solvent system consisting of water containing 0.1% TFA (A), acetonitrile containing 0.1% TFA (B). The gradient conditions were: 0 min, 95% A, 5% B; 45 min, 55% A, 55% B, kept to 50 min; 55 min, 95% A, 5% B at a flow rate 400 μl/min. 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 calibration curves of reference standards is linear, correlation coefficient (R^2) of all curves >0.9999, resolution (R_s) of standards peaks >1.5.

The majority of flavonoids identified using the HPLC method and its amounts were quercetin derivates (Table 1). For investigation of mitochondrial function we added different amounts (0.5, 1, 2 and 4 μl) of diluted GE (1:200) into 1 ml of incubation medium. 1 μl of this extract contained: 1.14 ng of rutin, 0.46 ng of quercitrin, 0.21 ng of hyperosid and 0.04 ng of quercetin.

Table 1
The amounts of quercetin and its glycosides in the extract of *Ginkgo biloba* leaves

Retention time (min)	Constituent	Detected amount (μg/ml), $n = 3$
26.6	Rutin	227.5 ± 8.0
30.9	Quercitrin	93.1 ± 6.9
28.2	Hyperosid	41.2 ± 4.2
42.1	Quercetin	8.8 ± 1.9

2.2. Preparation of rat heart mitochondria

Approval of the Lithuanian Ethic Committee for Laboratory Animal Use was obtained before commencement of the experiments. Hearts of male Wistar rats weighing 250–300 g were excised and rinsed in ice-cold 0.9% KCl solution. Subsequently the tissue was cut into small pieces and homogenized in a buffer containing 160 mM KCl, 10 mM NaCl, 20 mM Tris/HCl, 5 mM EGTA, and 1 mg/ml BSA (pH 7.7). The homogenate was centrifuged at 450 × *g*, and the supernatant obtained was centrifuged at 6300 × *g*. The mitochondrial pellet was resuspended in buffer containing 180 mM KCl, 20 mM Tris/HCl, and 3 mM EGTA (pH 7.35) to approximately 50 mg/ml protein, and stored on ice. The whole procedure was performed at 4 °C. The mitochondrial protein concentration was determined by the biuret method using bovine serum albumin as a standard.

2.3. Measurements of mitochondrial respiratory rates

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 $MgCl_2$, 100 mM MES, 3 mM KH_2PO_4, 3.0 mM CaK_2EGTA, and 7.1 mM K_2EGTA (free Ca^{2+} concentration – 0.1 μM) (pH 7.1 adjusted with KOH at 37 °C) with 6 mM pyruvate + 6 mM malate, 12 mM succinate (+2 mM amytal), and 9 μM palmitoyl-L-carnitine + 0.24 mM malate as substrates. The solubility of oxygen was estimated to be 422 nmol O/ml. Mitochondrial respiration rates were expressed as nmol O/min mg protein. The final mitochondrial protein concentration in all experiments was 0.5 mg/ml.

2.4. Statistical analyses

Data are presented as mean ± S.E.M. nonparametric methods were applied for making inferences about data. Differences between means in dependent groups were tested using Wilcoxon matched pairs test. Differences among means in independent groups were tested using nonparametric Kruskal–Wallis test with Dunns post-hoc evaluation. $p < 0.05$ was taken as the level of significance. Statistical analysis was performed by using programm Statistica 1999, 5.5 StatSoft Inc., USA.

3. Results

In order to investigate the effect of GE on the heart mitochondrial parameters, their respiration rate in various metabolic states was measured in the absence and in the presence of GE. According to our scheme of measurements, at the beginning we measured the basal (State 2) respiration rate (V_o) with complex I dependent substrates pyruvate + malate (Fig. 1). Then different amounts of GE were added into the incubation medium dur-