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The Effect of Ginkgo biloba extract on mitochondrial oxidative phosphorylation in the normal and ischemic rat heart

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5 **The Effect of *Ginkgo biloba* extract on mitochondrial oxidative phosphorylation**
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7 **in the normal and ischemic rat heart**
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14 Jurga Bernatoniene^{1*}, Daiva Majiene^{1,2}, Rimantas Peciura¹, Ale Laukeviciene³, Ruta Bernatoniene⁴,
15 Tauras Mekas¹, Arturas Kasauskas⁵, Dalia Kopustinskiene^{2,5}
16
17
18

19
20 ¹Department of Drug Technology and Social Pharmacy, Kaunas University of Medicine, A.
21 Mickeviciaus str. 9, LT-44307 Kaunas, Lithuania
22

23 ²Institute for Biomedical Research, Kaunas University of Medicine, Eiveniu str. 4, LT-50009
24 Kaunas, Lithuania
25

26 ³Department of Physiology, Kaunas University of Medicine, Kaunas, A. Mickeviciaus str. 9, LT-
27 44307 Kaunas, Lithuania
28

29 ⁴Department of Pharmaceutical Chemistry, Kaunas University of Medicine, A. Mickeviciaus str. 9,
30 LT-44307 Kaunas, Lithuania
31

32 ⁵Department of Biochemistry, Kaunas University of Medicine, Eiveniu str. 4, LT-50009, Kaunas,
33 Lithuania
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59 *Corresponding author: Jurga Bernatoniene, Department of Drug Technology and Social
60 Pharmacy, Kaunas University of Medicine, Lithuania, Tel.: +37060063349
E-mail: jurgabernatoniene@yahoo.com

ABSTRACT

Free radicals-induced myocardial damage and impairment of vascular endothelium-dependent relaxation are amongst the most important mechanisms responsible for the ischemic heart injury. *Ginkgo biloba* leaf extract (GE) has been reported to improve blood circulation in the brain and have beneficial impact on the cardiovascular system but its cardioprotective effects have not been elucidated yet. Therefore, in this study we investigated the influence of GE in 70% ethanol (1:5) administered orally to rats on the functions of isolated heart mitochondria under normal and ischemic conditions. *Wistar* rats were given GE or ethanol (solvent control) at a dosage of 0.32 mL/kg in drinking water for 10 and 18 days, while control animals received untreated drinking water. Mitochondrial respiration rates were determined oxygraphically. Pyruvate and malate, succinate or palmitoyl-L-carnitine and malate were used as substrates.

GE treatment partially uncoupled mitochondrial oxidation from phosphorylation, reduced generation of free radicals in the mitochondria, diminished ischemia-induced V_3 decrease and the degree of respiration stimulation by exogenous cytochrome c. Thus, these results indicate that GE exerts cardioprotective effects reducing ischemia-caused impairment of the functions of heart mitochondria.

Keywords: *Ginkgo biloba*; heart mitochondrial respiration; ischemic injury; cardioprotection.

Abbreviations: ADP – adenosine 5-diphosphate; BSA – bovine serum albumin; GE – *Ginkgo biloba* leaf extract; ROS – reactive oxygen species.

INTRODUCTION

Ischemic heart injury is the leading cause of morbidity and mortality although the molecular mechanisms responsible for it, as well as for its prevention, have not been fully clarified yet (for recent review see Ostadal, 2009). Free-radical induced myocardial damage is one of the main causes of the cardiovascular pathology. (Di Lisa *et al.*, 2009). The major targets of free radical

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4 attacks are supposed to be heart mitochondria which are the main sites of cellular energy supply
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6 (Korshunov *et al.*, 1997; Skulachev, 2009). Therefore, during investigation of myocardial damage it
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8 is very important to evaluate mitochondrial function changes responsible for 1) impaired cardiac
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10 muscle contractions and 2) cell death.
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14 Recent studies demonstrate that herbal preparations could decrease the effects of pathogenic
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16 processes such as oxidative stress, improving rheological properties of the blood and increasing
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18 blood flow to the various organs of the body (Addabbo *et al.*, 2009; Yapar *et al.*, 2010). *Ginkgo*
19
20 *biloba* preparations are most widely used for this purpose; however, their action was investigated
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22 mainly on brain cells and subcellular organelles (Gertz and Kiefer, 2004). It was shown that *Ginkgo*
23
24 *biloba* preparations, especially EGb 761 (1), improved blood flow to the brain, particularly, in the
25
26 region of microcirculation, (2) inactivated toxic oxygen radicals and prevented cell damage, (3)
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28 inhibited platelet-activating factor responsible for a number of CNS pathologies (Abdel-Kader *et*
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30 *al.*, 2007; Mahadevan and Park, 2008). Furthermore, in a randomized, double-blind, placebo-
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32 controlled, parallel design trial *Ginkgo biloba* (EGb 761, 300 mg a day for 4 weeks) was shown to
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34 have no impact on indices on blood coagulation and no adverse bleeding effects (Gardner *et al.*,
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36 2007). These broad pharmacological activities of *Ginkgo biloba* are predetermined by two active
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38 ingredient groups that have different functions: flavonoid fraction (quercetin, kaempferol,
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40 isorhamnetin glycosides) and terpenoid fraction (ginkgolides; bilobalide). Flavonoid fraction was
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42 demonstrated to neutralize effectively free radicals – superoxide anions, hydroxyl radicals and NO
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44 (Oyama *et al.*, 1996). Also, flavonoids influenced hemodynamic disorders, vasomotoric functions
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46 of ischemia zones, permeability of capillaries, ion regulation of cellular homeostasis, and some
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48 intracellular enzymes – phospholipases, phosphodiesterases and protein kinases (DeFeudis *et al.*,
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50 2003). Terpenoid fraction controlled platelet-activating factor, possessed vaso-relaxant properties,
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52 improved cerebral blood flow (Kleijnen and Knipschild, 1992). In most investigations, GE rich in
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54 both flavonoid and terpenoid mixtures was used. The obtained results showed that active
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4 ingredients of GE effectively improved brain functions, therefore, *Ginkgo biloba* preparations are
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6 widely used for prevention of numerous neurological disorders (dementia, macula degeneration,
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8 tinnitus, winter depression, difficulties in concentration and learning capacity, headache, *etc*)
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12 (Leistner and Drewke, 2010).

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14 EGb 761 exerted beneficial effects in endothelial dysfunction increasing production of
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16 endothelial nitric oxide. The putative mechanism for this effect was suggested to be increased
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18 endothelial nitric oxide synthase (eNOS) promoter activity and eNOS expression *in vitro*
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20 (Koltemann *et al.*, 2007). Also, *Ginkgo biloba* EGb 761 attenuated arteriosclerotic nanoplaque
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22 formation, reduced lipoprotein a concentration, increased concentration of vasoactive substances –
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24 cAMP and cGMP (Siegel *et al.*, 2009). Recently the effect of different active ingredients of *Ginkgo*
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27 *biloba* and the biological effect of its tinctures and extracts has been intensively studied not only on
28
29 the brain, but also on the heart function. Trumbeckaite with co-workers showed that GE (1:5)
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31 exerted uncoupling activity in heart mitochondria *in vitro* in normal conditions (Trumbeckaite *et al.*,
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33 2007; Baliutyte *et al.*, 2010). It has been demonstrated that EGb 761, rich in bilobalides, decreased
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35 ischemic damage in mitochondria and protected the components of mitochondrial respiratory chain
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37 (Shen and Zhou, 1995; Janssens *et al.*, 2000). The terpenoid constituents of EGb 761 and the
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39 flavonoid metabolites, that were formed after *in vivo* administration of the extract, acted in a
40
41 complementary manner to protect against myocardial ischemia-reperfusion injury (Liebgott *et al.*,
42
43 2000). These findings have led us to consider the possibility that GE could protect heart from
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45 injuries. However, there is very few data about the influence of GE on the energy metabolism in
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47 heart during ischemia. Therefore, the aim of this study was to investigate the effects of GE
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49 administrated *per os* for 10 and 18 days on rat heart mitochondrial functions under normal and
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60 ischemic conditions.

MATERIAL AND METHODS

Chemicals

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4 All chemicals were from Sigma-Aldrich (St. Louis, MO), except Amplex Red which was from
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6 Invitrogen (Carlsbad, CA).
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8 9 **Preparation of Ginkgo extract**

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11 Dried *Ginkgo biloba* L. leaves (Poland, Acorus Calamus) were extracted using 70% ethanol
12 (1:5); particle size in the extract ranged from 2 to 3 mm, the production method was percolation,
13 and the flow speed of extract - 0.5 mL/min (Bernatoniene *et al.*, 2002). GE contained 3.4 ± 0.08
14 mg/ mL phenolic compounds. Several classes of flavonoids: quercetin - 8.8 ± 2 µg/mL, hyperoside -
15 41.2 ± 4 µg/mL, rutin - 227.5 ± 8 µg/mL, and quercetrin - 93.1 ± 7 µg/mL were identified in this
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17 extract by HPLC analysis (Trumbeckaite *et al.*, 2007).
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26 **GE administration per os to rats**

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28 Male *Wistar* rats weighing 250-300 g were used for our study. GE or ethanol (solvent
29 control) were administered to rats at a dosage of 0.32 mL/kg in drinking water for 10 and 18 days,
30 while control animals received untreated drinking water. The animals were handled according to the
31 rules defined by the European Convention for the protection of Vertebrate Animals Used for
32 Experimental and Other Purposes, the experiments were approved by Lithuanian State Food and
33 Veterinary service (License No.0006).
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43 **Isolation of rat heart mitochondria**

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45 Mitochondria were isolated by differential centrifugation procedure (Scholte *et al.*, 1973).
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47 Hearts were excised and divided into two halves. Control heart halves were rinsed in ice-cold 0.9%
48 KCl solution. Heart halves subjected to total ischemia, were rinsed in warm (37°C) KCl solution,
49 placed in a humidified thermostated chamber and allowed to autolyse at 37°C for 45 min. After
50 these procedures, the heart tissues were cut into small pieces and homogenized in a glass-Teflon
51 homogenizer in the medium containing 160 mmol/L KCl, 10 mmol/L NaCl, 20 mmol/L Tris-HCl, 5
52 mmol/L EGTA and 1 mg/mL bovine serum albumin (BSA; fraction V, A4503, Sigma) (pH 7.4,
53 2°C). The homogenate was centrifuged at 750×g for 5 min, then the supernatant was re-centrifuged
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4 at 10 000×g for 10 min and the pellet was washed once (10 min 10 000×g) in the isolation medium
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6 without BSA, suspended in it and kept on ice. The mitochondrial protein concentration was
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8 determined by the biuret method (Gornall *et al.*, 1949) using BSA as standard. The final
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10 mitochondrial protein concentration in all experiments was 0.5 mg/mL.
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13 14 **Measurement of mitochondrial respiration rate**

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16 Oxygen uptake rates were recorded at 37°C by means of the Clark-type electrode system in a
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18 solution containing 20 mmol/L imidazole, 20 mmol/L taurine, 0.5 mmol/L dithiothreitol, 1.6
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20 mmol/L MgCl₂, 100 mmol/L MES, 3 mmol/L KH₂PO₄, 3.0 mmol/L CaK₂EGTA, and 7.1 mmol/L
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22 K₂EGTA (free Ca²⁺ concentration - 0.1 μmol/L) (pH 7.1 adjusted with KOH at 37°C) with 6
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24 mmol/L pyruvate + 6 mmol/L malate, 12 mmol/L succinate (+2 mmol/L amytal), and 9 μmol/L
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26 palmitoyl-L-carnitine + 0.24 mmol/L malate as substrates. Mitochondrial respiration rates were
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28 expressed as nmol O₂/min/mg protein. The solubility of oxygen was estimated to be 211 nmol
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30 O₂/ml (Holtzman, 1976).
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34 35 **Measurement of mitochondrial ROS generation**

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37 The generation of ROS in mitochondria was monitored using the Amplex Red assay for
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39 H₂O₂. The evaluation of ROS levels was performed at 37 °C in the same medium as for
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41 mitochondrial respiration, which was supplemented with mitochondria (0.25 mg/mL), 6 mmol/L
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43 pyruvate + 6 mmol/L malate, 10 μmol/L Amplex Red, 1 U/mL horseradish peroxidase, and 1
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45 μmol/L antimycin A and continuously stirred. Fluorescence of the oxidized probe was measured
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47 with a Thermo Scientific fluorometer using the excitation at 544 nm and emission at 590 nm.
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51 52 **Statistics**

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54 Statistical analysis was performed using Prism 3.0 program (GraphPad Software Inc., La
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56 Jolla, CA, USA). The results are presented as means ± S.E. of 5 independent experiments. The data
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58 were analysed with repeated measures analysis of variance (ANOVA) followed by Tukey's
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60 multiple comparison test. p < 0.05 was taken as the level of significance.

RESULTS

In our study, mitochondrial functions were evaluated by recording mitochondrial respiration rate in the State 2 (substrate oxidation, V_2), State 3 (substrates + 1 mmol/L ADP, V_3) and State 3 in the presence of exogenous cytochrome c (32 μ mol/L, $V_{3+Cyt\ c}$). Pyruvate and malate, succinate or palmitoyl-L-carnitine and malate were used as substrates. Changes in V_2 reflected alterations of the permeability of the inner mitochondrial membrane to protons or ions, and, together with calculated respiratory control index ($RCI = V_3/V_2$), allowed to determine the effectiveness of ADP phosphorylation in mitochondria. V_3 reflected the capacity of mitochondria to synthesize ATP. The degree of respiration stimulation by exogenous cytochrome c ($V_{3+Cyt\ c}/V_3$) allowed for assessing the integrity of the outer mitochondrial membrane.

First, we performed solvent control test and found that rat treatment *per os* with ethanol for 10 or 18 days using the same concentration as in studied GE, 0.32 mL/kg, did not influence respiration rates of isolated heart mitochondrial oxidizing all substrates used in our study (see Table).

Fig. 1 shows that under normal non-ischemic conditions, 10 days and 18 days administration of GE did not significantly affected State 2 respiration of mitochondria oxidizing complex-I dependent substrates pyruvate and malate, but induced a 14% ($p < 0.01$) and 26% ($p < 0.001$) increase in State 3 respiration rate, respectively, compared to control. 45 min ischemia had a tendency to increase State 2 respiration rate and caused a significant decrease in V_3 by 60% ($p < 0.001$), as well as in RCI by 64% ($p < 0.001$). The intactness of outer mitochondrial membrane was assessed by cytochrome c test. Cytochrome c effect was 54% higher ($p < 0.001$) in mitochondria isolated from ischemic hearts. When GE was administered for 10 days and 18 days, 45 min of total ischemia caused less damage to mitochondrial functions: State 3 respiration rate of isolated heart mitochondria was decreased only by 35% ($p < 0.001$) and 30% ($p < 0.001$), respectively. Treatment with GE for 10 and 18 days also decreased the damage of outer mitochondrial membrane under ischemic conditions by 17% ($p < 0.001$) and 16% ($p < 0.001$), respectively.

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Further, we have tested the influence of GE treatment to rats on the generation of ROS by mitochondria (Fig. 2). The obtained results showed that both under normal conditions and after 45 min total ischemia 10-day treatment with GE significantly by 28% ($p < 0.05$) and 21% ($p < 0.05$) decreased H_2O_2 production in isolated rat heart mitochondria.

In the next series of studies, the effect of GE treatment on the oxidation of FAD-specific substrate succinate was investigated under normal and ischemic conditions (Fig. 3). The results showed that after 10-day administration of GE under normal conditions mitochondrial respiration rate in State 2 and in State 3 increased by 32% ($p < 0.001$) and 9% ($p < 0.001$), respectively. After 18 days, the increase was, accordingly, 22% ($p < 0.001$) and 8% ($p < 0.001$). After 45 min of total ischemia, mitochondrial respiration rate in control rat mitochondria in State 2 decreased by 14% ($p < 0.001$), while in mitochondria isolated from those rats that did receive GE, respiration rate in State 2 was diminished less – by 5% ($p < 0.001$) and 9% ($p < 0.001$), respectively, after 10 and 18 days. Respiratory rate in State 3 following total ischemia in control rat heart mitochondria decreased by 40% ($p < 0.001$), while in mitochondria isolated from rats that received GE for 10 and 18 days, it decreased by, accordingly, 34% ($p < 0.001$) and 32% ($p < 0.001$). GE treatment for 10 and 18 days decreased cytochrome c effect in control mitochondria from 1.5 to 1.3 ($p < 0.01$) and 1.2 ($p < 0.001$) respectively, whereas in ischemic mitochondria the effect was decreased from 1.73 to 1.53 ($p < 0.001$) and 1.43 ($p < 0.001$), indicating that GE treatment protects mitochondrial outer membrane integrity both under normal and ischemic conditions.

In further experiments the effect of GE treatment on State 2 respiration rate of mitochondria, oxidizing the main respiratory substrate of heart mitochondria - palmitoyl-L-carnitine was investigated under normal and ischemic conditions (Fig. 4). It is interesting to note that 10-days and 18-days of treatment with GE had no effect on the State 2 respiration rate under normal conditions, whereas V_3 increased by 16-25% ($p < 0.001$). Furthermore, 45 min ischemia had a tendency to increase V_2 in control group and significantly decreased V_3 by 52% ($p < 0.001$). In groups which

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4 received GE, mitochondrial respiration rate in State 2 remained unchanged, whereas V_3 decreased
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6 only by 40-38% ($p < 0.001$) in the groups treated with GE for 10 and 18 days, respectively, under
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8 ischemic conditions. Also, addition of exogenous cytochrome c increased the State 3 respiration rate
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10 by 24-28% ($p < 0.001$) less in mitochondria isolated from rats treated with GE under ischemic
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12 conditions.
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18 DISCUSSION

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20 Cardiovascular diseases are associated with the alteration of mitochondria – main producers
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22 of ATP in the cardiac muscle cells. Numerous investigations have shown that ischemia in heart
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24 impairs mitochondrial oxidative phosphorylation (for review, see Piper *et al.*, 1994; Solaini and
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26 Harris, 2005). Ischemia decreases State 3 mitochondrial respiration rate (Jennings *et al.*, 1969;
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28 Schwartz *et al.*, 1973; Toleikis *et al.*, 1989), increases permeability of the inner mitochondrial
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30 membrane to protons or ions in State 2 (Toleikis *et al.*, 1989; Piper *et al.*, 1994), inhibits the activity
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32 of complexes I, II, III of mitochondrial respiratory chain (Rouslin, 1983; Piper *et al.*, 1985),
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34 ADP/ATP carrier (Shug *et al.*, 1975; Regitz *et al.*, 1984; Borutaite *et al.*, 1996; Halestrap, 2009),
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36 ATP-synthase (Rouslin, 1983), reduces amounts of membrane proteins and proteins located in the
37
38 intermembrane space, especially cytochrome c (Balasiavichius *et al.*, 1984; Piper *et al.*, 1985).
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45 Since it has been demonstrated that EGb 761, rich in bilobalides, decreases ischemic damage
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47 in heart and protects the components of mitochondrial respiratory chain (Shen and Zhou, 1995;
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49 Janssens *et al.*, 2000), we investigated the effect of GE treatment on heart mitochondrial function
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51 under normal and ischemic conditions. The amount of GE (0.32 mL/kg) administered to the studied
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53 animals for 10 or 18 days was selected based on the recalculation from the standard therapeutical
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55 dosages used for human treatment (mL for a person with an average weight of 70 kg). Our
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57 investigations showed that in rats which received GE, the State 2 respiration in normal conditions
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59 had a tendency to increase, the most pronounced effect was observed in the case of succinate
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oxidation (23%) (Fig. 1, Fig. 3 and Fig. 4). In the State 2 mitochondrial respiration rate to the great extent depends on the passive proton flux through the mitochondrial inner membrane (Groen *et al.*, 1982). Thus, this indicates that GE *in vivo* – **similarly** as previously detected *in vitro* (Trumbeckaite *et al.*, 2007) - **causes** a slight uncoupling **of** cardiac mitochondria.

Korshunov with co-workers and Starkov reported that "mild" uncoupling in mitochondria is involved in the cellular defence system against free radicals (Korshunov *et al.*, 1997; Starkov, 1997), therefore in the next series we investigated the effect of ROS generation in mitochondria. Our results showed that both under normal conditions and after 45 min total ischemia 10-day treatment with GE significantly by 28% and 21% decreased H₂O₂ production. GE (1) directly scavenges free radicals and (2) reduces their production via mitochondria, therefore these results imply that GE may reduce mitochondrial damage by ischemia in cardiac muscle cells acting as a very strong antioxidant.

With all the studied substrates, mitochondrial respiration rate in State 3 was increased in mitochondria isolated from rats treated with GE by up to 8-25% under normal conditions, with the most pronounced effect (25%) in the case of complex I dependent substrate oxidation (pyruvate and malate as well as palmitoyl-L-carnitine and malate, Fig. 1, Fig. 4) and ~8% in the case of succinate oxidation (Fig. 3). 45 min ischemia decreased V₃ by 40-60%, also the lowest effect was observed in the case of succinate oxidation. GE treatment reduced V₃ decrease during ischemia, the most effectively in the case of complex I dependent substrate oxidation (up to 50% of decrease without GE treatment). In the State 3 complex I-dependent substrates-supported mitochondrial respiration rate depends both on the activity of mitochondrial respiratory chain and phosphorylation (Hafner *et al.*, 1990; Brown, 1992), whereas in the case of succinate oxidation, ATP synthesis, transport and hydrolysis is responsible only for ~10% of respiration rate (Borutaite *et al.*, 1995). Thus, our results indicate that GE treatment improves ATP synthesis in mitochondria and protects phosphorylation system from ischemic damage.

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5 In our study, the degree of respiration stimulation by exogenous cytochrome c ($V_{3+Cyt\ c}/V_3$)
6 was used to assess the integrity of the outer mitochondrial membrane. When mitochondria were
7 oxidizing complex I-dependent substrates under ischemic conditions, exogenous cytochrome c
8 stimulated respiration by 60%, this stimulation was reduced by 35-50% in mitochondria isolated
9 from GE-treated rat hearts. In the case of succinate oxidation, cytochrome c effect was more
10 pronounced even under normal conditions, and GE also significantly reduced it implicating that GE
11 treatment protects integrity of the outer mitochondrial membrane.
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21 The ability of GE to reduce risk of cardiovascular events was questioned in the large recent
22 clinical trial – the Ginkgo Evaluation of Memory (GEM) Study conducted in 2000 – 2008 where
23 GE therapy was beneficial only in the case of peripheral vascular disease but not in the case of
24 cardiovascular or coronary heart diseases (Kuller *et al.*, 2010). However, literature sources also list
25 several studies demonstrating the protective effect on heart mitochondrial function of *Ginkgo biloba*
26 preparations. It was reported that after 30 min of regional cardiac ischemia and 120 min of
27 reperfusion, the control ischemic rabbit hearts demonstrated severe histological damage such as
28 swelling and vacuolization of the mitochondria, whereas in animals after treatment with EGb 761
29 (10 mg/kg injected into the coronary artery), cardiac ischemia/reperfusion caused only slightly
30 damage (Shen and Zhou, 1995). Janssens with co-workers also demonstrated that EGb 761 and one
31 of its constituents – bilobalide - inhibited hypoxia-induced decrease in ATP content in endothelial
32 cells *in vitro* (Janssens *et al.*, 1999) and treatment of rats with 8 mg/kg of bilobalide protected
33 complex I and III activities and prevented ischemia-induced decrease in State 3 respiration rate
34 (Janssens *et al.*, 1999; 2000). The data of our experiments show that the administration of GE *per os*
35 may lessen the ischemic damage of the heart muscle. It is likely that the protective effect of GE may
36 be associated with the antioxidant activity of the extract.
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59 It is noteworthy that the cardiovascular effect of EGb 761 was dose-dependent. In rats that
60 received EGb for prolonged periods, the indexes of peripheral blood circulation were worse than in

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4 control rats (Tada *et al.*, 2008). Our results have shown that effects of both 10 days and 18 days rat
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6 treatment with GE were similar, indicating that prolonged treatment might not be useful for
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8 cardioprotection by GE.
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11 In summary, GE preparation partially uncouples mitochondrial oxidation from
12 phosphorylation and reduces generation of free radicals in the mitochondria, diminishes ischemia-
13 induced V_3 decrease and the degree of respiration stimulation by exogenous cytochrome c. It is
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15 probable that by inhibiting free radical generation, as well as by improving ATP synthesis and
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17 preserving mitochondrial outer membrane integrity, GE protects cardiac muscle from damages
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19 caused by oxidative stress.
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Table. Influence of rat treatment *per os* with ethanol (0.32 mL/kg) on the respiration parameters of isolated heart mitochondria.

Mitochondrial respiration rates were expressed as nmol O₂/min/mg protein. V₂ – State 2 (substrates only), V₃ – State 3 (substrates + 1 mmol/L ADP), V_{3+Cyt c} – State 3 in the presence of exogenous cytochrome c (32 μmol/L) respiration rate, n = 5.

	Pyruvate and malate (6+6 mmol/L)			Succinate (12 mmol/L + 2 mmol/L amytal)			Palmitoyl-L-carnitine and malate (9 μmol/L + 0.24 mmol/L)		
	Control	Ethanol _{10 days}	Ethanol _{18 days}	Control	Ethanol _{10 days}	Ethanol _{18 days}	Control	Ethanol _{10 days}	Ethanol _{18 days}
V ₂	50 ± 1	51 ± 1	51 ± 1	78 ± 2	76 ± 1	73 ± 1	40 ± 1	40 ± 1	40 ± 1
V ₃	185 ± 3	187 ± 2	186 ± 1	167 ± 2	163 ± 1	159 ± 1	130 ± 2	125 ± 3	126 ± 2
V _{3+Cyt c}	196 ± 4	195 ± 2	195 ± 1	247 ± 2	245 ± 1	241 ± 4	150 ± 4	148 ± 3	148 ± 3

Figure legends:

Figure 1. Influence of rat treatment *per os* with *Ginkgo biloba* extract (GE, 0.32 mL/kg) on the respiration (A) and functional parameters (B) of isolated heart mitochondria oxidizing pyruvate and malate (6 + 6 mmol/L) under normal and ischemic conditions.

RCI – mitochondrial respiratory control index (V_3/V_2); Cyt c effect - the degree of respiration stimulation by exogenous cytochrome c ($V_{3+Cyt\ c}/V_3$). * $p < 0.05$ statistically significant effect of GE treatment compared to control under normal conditions, # $p < 0.05$ statistically significant effect of ischemia compared to control under normal conditions, ## $p < 0.05$ statistically significant effect of GE treatment under ischemic conditions compared to control under ischemic conditions, $n = 5$.

Figure 2. Effect of rat treatment *per os* for 10 days with *Ginkgo biloba* extract (GE, 0.32 mL/kg) on H_2O_2 generation in isolated heart mitochondria under normal and ischemic conditions.

For experimental details, see Material and Methods. Similar curves were obtained in 5 independent experiments.

Figure 3. Influence of rat treatment *per os* with *Ginkgo biloba* extract (GE, 0.32 mL/kg) on the respiration (A) and functional parameters (B) of isolated heart mitochondria oxidizing succinate (12 mmol/L + 2 mmol/L amytal) under normal and ischemic conditions.

V_2 – State 2 (substrates only), V_3 – State 3 (substrates + 1 mmol/L ADP), $V_{3+Cyt\ c}$ – State 3 in the presence of exogenous cytochrome c (32 μ mol/L) respiration rate; RCI – mitochondrial respiratory control index (V_3/V_2); Cyt c effect - the degree of respiration stimulation by exogenous cytochrome c ($V_{3+Cyt\ c}/V_3$). * $p < 0.05$ statistically significant effect of GE treatment compared to control under normal conditions, # $p < 0.05$ statistically significant effect of ischemia compared to control under normal conditions, ## $p < 0.05$ statistically significant effect of GE treatment under ischemic conditions compared to control under ischemic conditions, $n = 5$.

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5 **Figure 4. Influence of rat treatment *per os* with *Ginkgo biloba* extract (GE, 0.32 mL/kg) on the**
6 **respiration (A) and functional parameters (B) of isolated heart mitochondria oxidizing**
7 **palmitoyl-L-carnitine and malate (9 $\mu\text{mol/L}$ + 0.24 mmol/L) under normal and ischemic**
8 **conditions.**
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14 V_2 – State 2 (substrates only), V_3 – State 3 (substrates + 1 mmol/L ADP), $V_{3+\text{Cyt c}}$ – State 3 in the
15 presence of exogenous cytochrome c (32 $\mu\text{mol/L}$) respiration rate; RCI – mitochondrial respiratory
16 control index (V_3/V_2); Cyt c effect - the degree of respiration stimulation by exogenous cytochrome
17 c ($V_{3+\text{Cyt c}}/V_3$). * $p < 0.05$ statistically significant effect of GE treatment compared to control under
18 normal conditions, # $p < 0.05$ statistically significant effect of ischemia compared to control under
19 normal conditions, ## $p < 0.05$ statistically significant effect of GE treatment under ischemic
20 conditions compared to control under ischemic conditions, $n = 5$.
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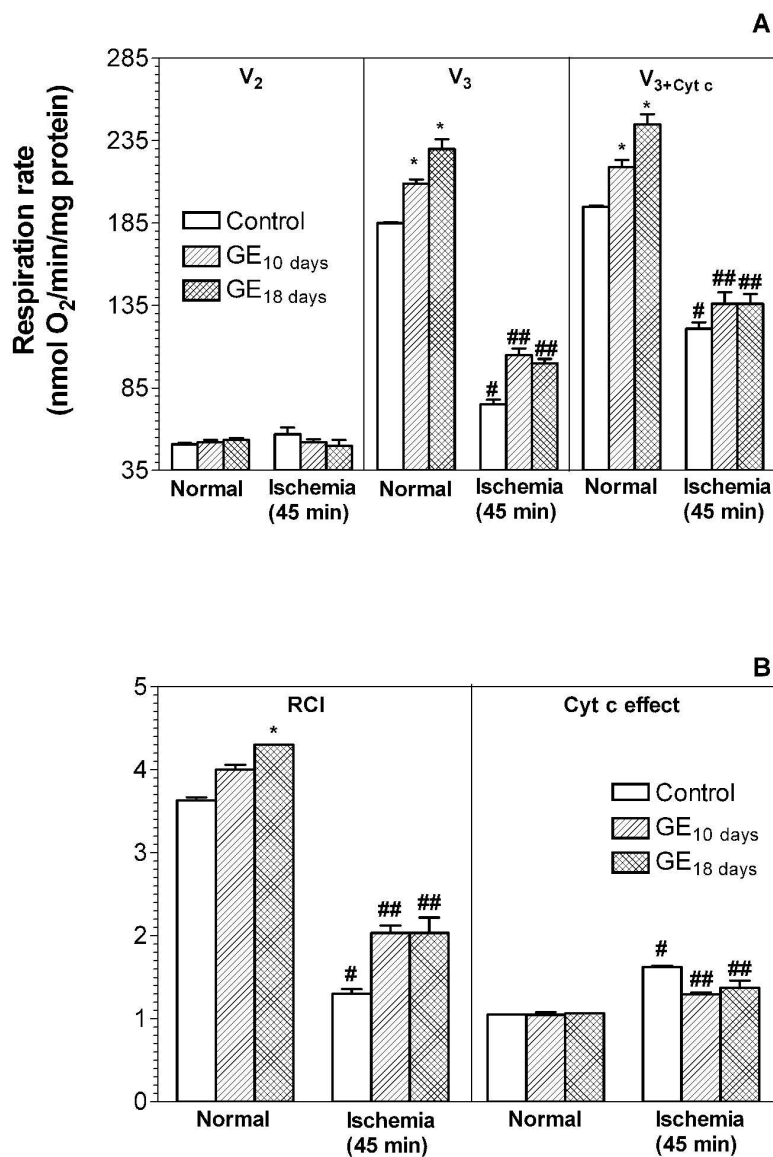


Figure 1. Influence of rat treatment per os with Ginkgo biloba extract (GE, 0.32 mL/kg) on the respiration (A) and functional parameters (B) of isolated heart mitochondria oxidizing pyruvate and malate (6 + 6 mmol/L) under normal and ischemic conditions.
179x257mm (600 x 600 DPI)

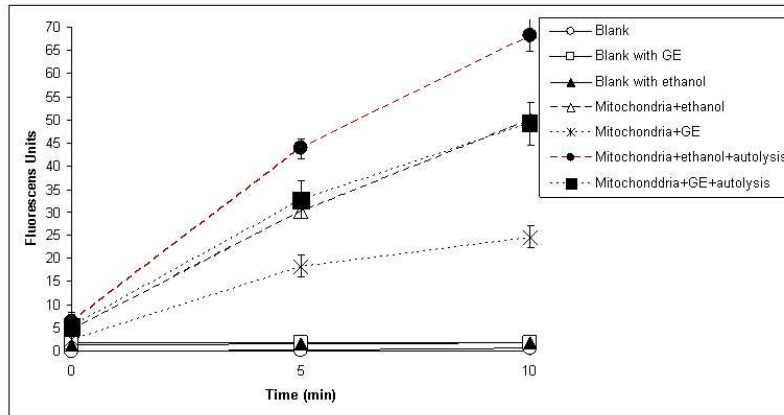


Figure 2. Effect of rat treatment per os for 10 days with Ginkgo biloba extract (GE, 0.32 mL/kg) on H₂O₂ generation in isolated heart mitochondria under normal and ischemic conditions.
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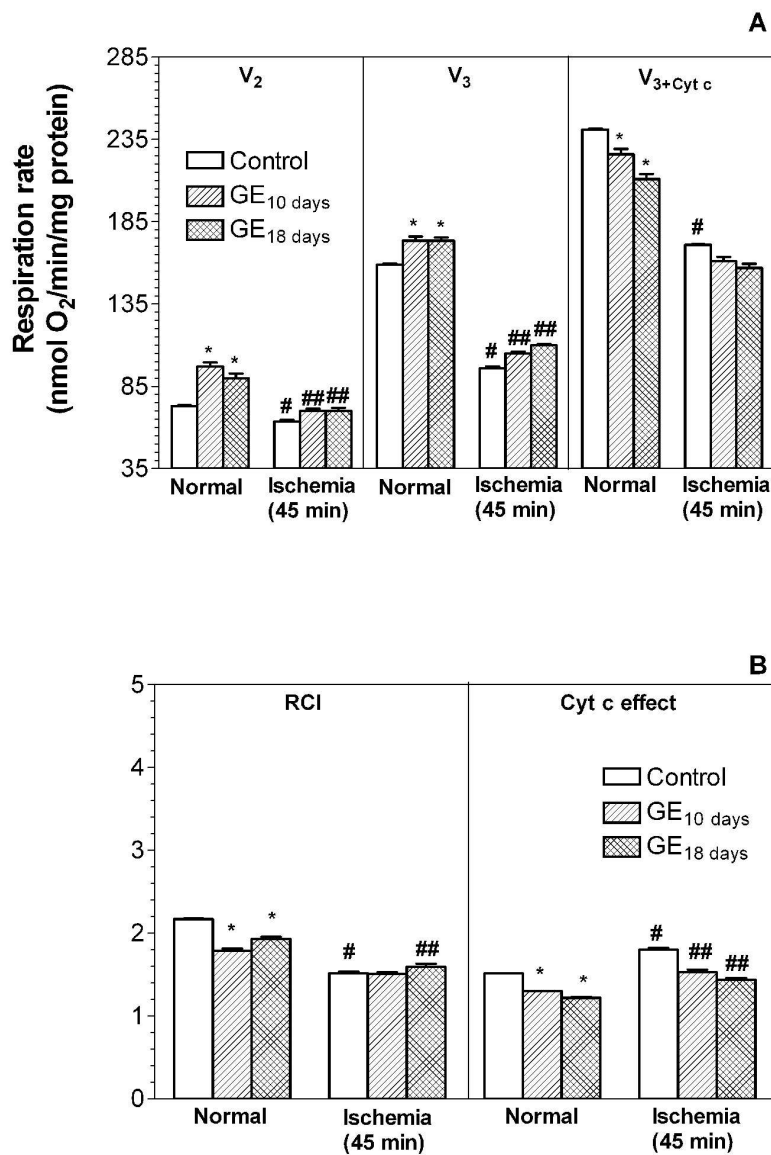


Figure 3. Influence of rat treatment per os with Ginkgo biloba extract (GE, 0.32 mL/kg) on the respiration (A) and functional parameters (B) of isolated heart mitochondria oxidizing succinate (12 mmol/L + 2 mmol/L amytal) under normal and ischemic conditions.
179x257mm (600 x 600 DPI)

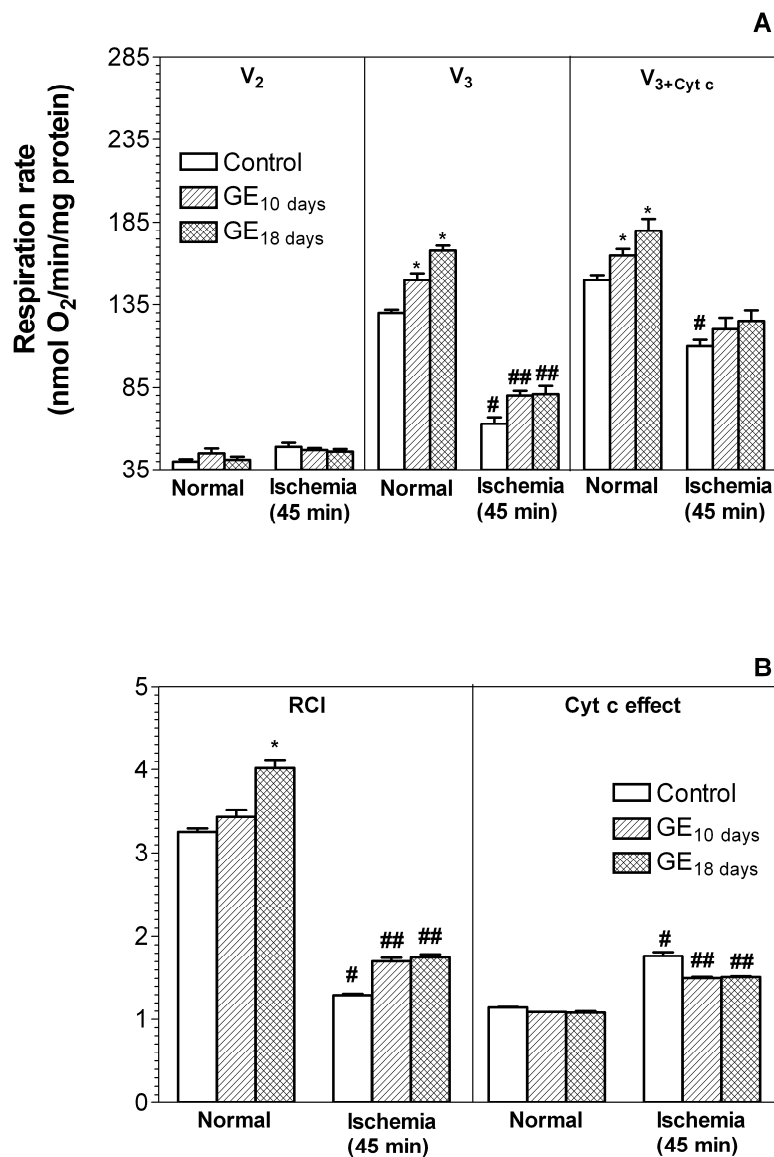


Figure 4. Influence of rat treatment per os with Ginkgo biloba extract (GE, 0.32 mL/kg) on the respiration (A) and functional parameters (B) of isolated heart mitochondria oxidizing palmitoyl-L-carnitine and malate (9 μ mol/L + 0.24 mmol/L) under normal and ischemic conditions.
179x257mm (600 x 600 DPI)