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Full Length Research Paper

Vaccinium myrtillus improves liver mitochondrial oxidative phosphorylation of diabetic Goto-Kakizaki rats

Fernanda M. Ferreira[1]*, Francisco P. Peixoto[2], Elsa Nunes[3], Cristina Sena[3], Raquel Seiça[3] and Maria Sancha Santos[4]

[1]Department of Environmental Sciences (CERNAS), Agricultural College of Coimbra, Coimbra, Portugal.
[2]Chemistry Department (CECAV), University of Trás-os-Montes and Alto Douro, Vila Real, Portugal.
[3]Department of Physiology and Institute of Biomedical Research in Light and Image, Faculty of Medicine, University of Coimbra, Portugal.
[4]Department of Zoology, Center for Neurosciences and Cell Biology of Coimbra, University of Coimbra, Portugal.

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Despite medicinal plants increasing use in diabetes mellitus therapy, recurrently, however, beneficial effects are mainly empirical. In this study, the effects of decoctions of *Vaccinium myrtillus* L. (bilberry) leaves were investigated on GK rats, a type 2 diabetes mellitus animal model. The possible toxic effects of *V. myrtillus* over mitochondrial respiratory activity indexes were evaluated. Our results show that *V. myrtillus* leaf decoctions presented significant benefits on glycaemic control. Moreover, GK rats treated during four weeks with *V. myrtillus* decoction presented an improvement of mitochondrial respiratory parameters evaluated (RCR and FCCP stimulated respiration) which could be explained due to mitochondrial biogenesis improvement by quercetins present in *V. myrtillus* leaves.

Key words: Goto-Kakizaki (GK) rats, type 2 diabetes mellitus, *Vaccinium myrtillus* L., phytotherapy, oxidative phosphorylation, toxicology.

INTRODUCTION

Type 2 diabetes is the usual designation for a group of pathologies that have a common feature: hyperglycaemia. This hyperglycaemia, leading to subsequent pancreatic beta-cell failure, as a result of hepatic and peripheral tissue insulin resistance, causes severe injuries (William and Pickup 2004). Therefore, the major purpose of diabetes mellitus therapy is both to reach normal glycaemic levels and to neutralize free radicals, preventing oxidative stress (Rolo and Palmeira, 2006).

Medicinal plants stand for a useful alternative or complementation of synthetic drugs used in type 2 diabetes therapies. Some of these synthetic drugs (as metformin or guanidine) are based in active compounds previously extracted from medicinal plants (Mueller and Jungbauer, 2009). *Vaccinium myrtillus* L. (bilberry or European blueberry) an Ericaceae, is a shrubby perennial plant used since ancient times both due to its high nutritive value and to its therapeutic properties (Canter and Ernst 2004; Valentová et al., 2007; Bao et al., 2008). V. myrtillus leaves tea is used as a folk medicine treatment of type 2 diabetes (Cignarella et al., 1996). Nevertheless, several advices are found in order to avoid V. myrtillus leaf decoctions for long-lasting treatments, since the high tannins content can cause liver injury. However, the possible toxicological effects have not been studied until now. Therefore, the current study was undertaken to investigate the outcome of *V. myrtillus* leaves tea over the glycaemic levels of a diabetic animal model, the Goto-Kakizaki (GK) rats (Goto et al., 1975), one of the best - characterised animal model of

*Corresponding author. E-mail: fmlferreira@gmail.com. Tel: 351-239-802940. Fax: 351-239-802979

Abbreviations: **FCCP,** carbonyl cyanide p-trifluoromethoxyphenylhydrazone; **GK,** Goto-Kakizaki; **IPGTT,** intraperitoneal glucose tolerance test; **RCR,** respiratory control ratio; **ROS,** reactive oxygen species.

spontaneous non-obese type 2 diabetes mellitus (McIntosh and Pederson, 1999; Portha et al., 2009). To access possible toxicological effects, liver mitochondria from these rats were isolated and mitochondrial parameters (RCR and FCCP stimulated respiration) were evaluated. Mitochondria have proven to be a good model to study the action of many xenobiotics on cell toxicity and data obtained from the studies are usually in good agreement with citotoxicity parameters reported in cell cultures and whole organisms (Haubenstricker et al., 1990; Knobeloch et al., 1990), since constitute the major energy-producing organelles of the cell. Any interference with mitochondrial bioenergetics is known to be a part of cell injury process, by a multiplicity of mechanisms and assorted agents (Wallace, 2008).

MATERIALS AND METHODS

Materials

All reagents and chemicals used were of the highest grade of purity commercially available. Inhibitors and drugs were dissolved in water or ethanol. In control experiments, ethanol was added to isolated mitochondria at concentrations not exceeding 0.2%.

Preparation of the extracts

The leaves of *V. myrtillus* L. were dried in the dark and obtained from "11 anos - Segredo da planta - Produtos naturais e biológicos, Lda" (Seixal, Portugal), and were imported from Romenia. An V. myrtillus voucher (number 2422) has been identified by specialists and deposited at the Herbarium of the University of Trás-os-Montes and Alto Douro, Vila Real, Portugal.

Plant aerial parts, as used in the decoctions, were ground to a powder. The decoction were prepared by boiling 125 g of the dried material in 1000 ml of deionised water for 15 min., filtered and centrifuged at 7000 x g for 5 min and kept at -20°C.

Animals

Male spontaneously diabetic GK rats were obtained from a local breeding colony (Animal Research Center Laboratory, University Hospitals, Coimbra), established in 1995 with breeding couples from the colony at the Tohoku University School of Medicine (Sendai, Japan; courtesy of Dr. K. Suzuki). Animals were kept under controlled light and humidity conditions and with free access to powdered rodent chow (diet C.R.F. 20, Charles Rivers, France) and water (or plant decoction) in accordance to European Community guidelines. GK rats were randomly divided and housed in 2 separated groups, one of them drinking *V. myrtillus* decoction and the other, used as control, drinking distilled water. The experiments lasted for an interval of 4 weeks.

Occasional glycaemia

Glycaemia in a non fasting condition was determined twice a week through the glucose oxidase reaction by using a glucometer (Glucometer Elite - Bayer SA, Portugal) and compatible reactive test strips. Blood samples were collected from the vein tail.

Intraperitoneal glucose tolerance test

Intraperitoneal glucose tolerance test (IPGTT - 1.8 g glucose/ kg body weight, i.p.) was carried out after fasting glycaemia determination (fasting period 16 - 18 h). Blood glucose concentration was measured 30, 60, 90 and 120 min after glucose load, using the above described method.

Preparation of mitochondria

GK rats were maintained ad libitum, for at least 12 h, before being sacrificed for cervical displacement, according to a pre-established method (Gazotti et al., 1979), with slight modifications (Ferreira et al., 1997). Protein concentration was determined by the Biuret method, using BSA (bovine serum albumin) as a standard (Gornall et al., 1949).

Mitochondrial respiration

Oxygen consumption of isolated mitochondria was determined polarographically at 25°C with a Clark oxygen electrode, connected to a suitable recorder in a closed chamber with magnetic stirring. Mitochondria (1 mg), 2 µM of rotenone and succinate (5 mM), as respiratory substrate, were added to 1 ml of reaction medium (130 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, 5 mM KH_2PO_4, 5 mM HEPES, pH 7.2). To induce state 3 respirations (V3), 300 nmol of ADP (magnesium salt) were used. The respiratory control ratio (RCR) was calculated according to Chance and Williams (1956). FCCP-uncoupled respiration (VFCCP) was performed by adding 1.5 µM of FCCP (carbonyl cyanide p-trifluoromethoxyphenylhydrazone) to mitochondria energized with succinate (Ferreira et al., 1999), after a phosphorylative cycle. In order to validate respiratory activity assays, 1 mM KCN was added and the slope due to the possible O_2 diffusion was discounted in all assays.

Statistics

The results are presented as mean ± SEM of the number of experiments shown on the legends of the figures and tables. Statistical significance was determined using paired Student's t-test and $p < 0.05$ values were considered significant.

RESULTS

Our results showed that *V. myrtillus* leaf decoctions lead to a slight decrease of occasional glycaemia (Table 1) and improved IPGTT response, mainly during initial 60 min (Figure 1). These results corroborate the anti-diabetic properties attributed to this plant. The amount of liquid and food ingested were significantly lower, probably related to lowest *V. myrtillus* treated rats glycaemia. However, no significant weight changes were observed between *V. myrtillus* and control groups during the experiment.

Furthermore, our results revealed that GK rats treated with *V. myrtillus* decoctions presented a stimulated respiration, using succinate as respiratory substrate (Table 2). In fact, oxidative activity seems to be largely increased, since FCCP-uncoupled respiratory activity is much higher in GK treated rats (around 180% of control).

Table 1. Glycaemic levels, food and liquid intake, and weight gain of GK rats.

Condition (➔) Extract (🡻)	Food ingested (g/ day/ rat)	Liquid ingested (ml/day/rat)	Final weight (%)	Occasional glycaemia (mM)
Control	23.7 ± 0.44	60.0 ± 1.41	106.2 ± 0.44	10.89 ± 0.70
V. myrtillus	20.2 ± 0.54*	33.1 ± 1.04**	108.0 ± 1.08	6.91 ± 0.21**

Occasional glycaemia was evaluated as described in Material and Methods section. The amount of food and liquid ingested were recorded 3 times a week. Final weight of each rat was expressed as the percentage of its initial weight, to decrease inaccuracies. Data were present as means ± SEM. Values statistically different from control (distilled water): ** $p < 0.01$, * $p < 0.05$.

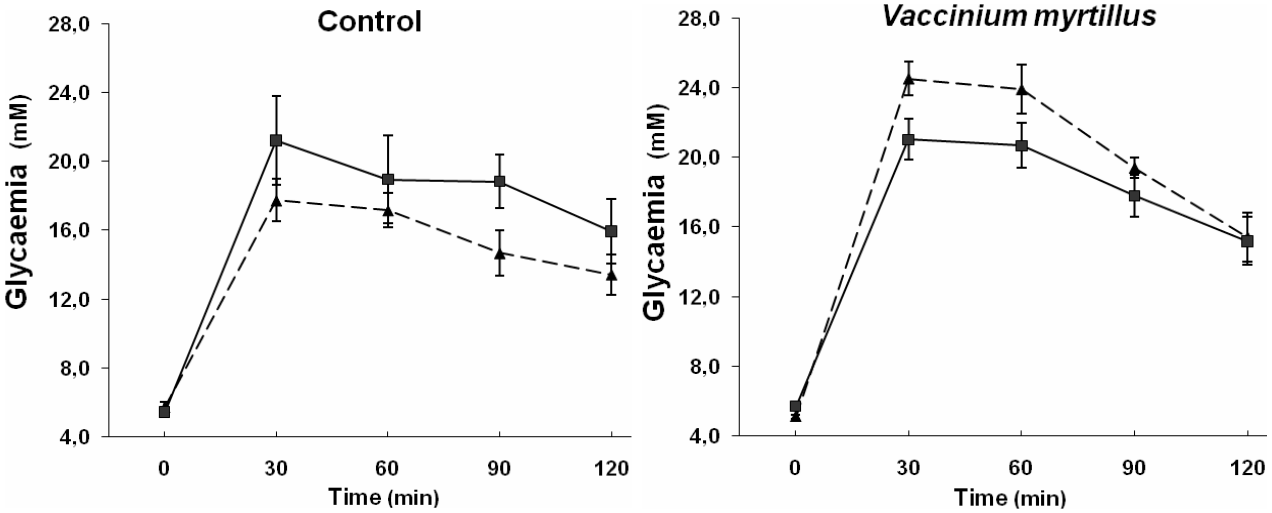

Figure 1. Determination of intraperitoneal glucose tolerance test (IPGTT). Intraperitoneal glucose tolerance test (IPGTT - 1.8g glucose/ kg body weight, i.p.) was carried out after fasting glycaemia determination. Symbols: ▲correspond to glycaemias before treatment with plant extract (or distilled water) and ■ stand for glycaemias after treatment, evaluated 4 weeks later. Data were present as means ± SEM of 4 different rats.

Table 2. Effect of *V. myrtillus* on GK rat liver mitochondrial parameters.

Condition (➔) Extract (🡻)	V_2 nmol $O_2.mg^{-1}.\ min^{-1}$ (%)	V_3 nmol $O_2.mg^{-1}.\ min^{-1}$ (%)	FCCP nmol $O_2.mg^{-1}.\ min^{-1}$ (%)	RCR (%)
Control	8.96 ± 0.78 (100.0 ± 6.50)	36.36 ± 4.14 (100.0 ± 12.40)	42.30 ± 5.69 (100.0 ± 13.42)	4.21± 0.59 (100.0 ± 14.00)
V. myrtillus	14.16 ± 0.91 (158.0 ± 10.21)**	78.78 ± 2.99 (216.7 ± 9.11)***	79.93 ± 10.76 (189.0 ± 25.39)***	5.73 ± 0.46 (136.1 ±10.92)*

Mitochondria (1 mg protein) were incubated in 1 ml of the respiratory standard medium supplemented with rotenone (2 µg) and succinate (5 mM). In order to achieve state 3 respirations 300 nmol ADP were added. All values are also expressed as percentage of control (GK rats drinking distilled water). Results are presented as mean ± SEM of triplicates of experiments performed with 3 different mitochondrial preparations of GK rats. Statistics: ***$p < 0.0001$; ** $p < 0.01$; * $p < 0.05$, as compared to controls.

Moreover, respiratory rate in the presence of ADP (V3) was increased in GK treated rats, showing a good coupling between oxidative and phosphorylative activities. This is reflected in respiratory control ratio (RCR) activity, which is significantly higher in *V. myrtillus* GK rats (around 135% of control).

DISCUSSION

V. myrtillus is commonly used in the production of tea beverages with attributed therapeutic properties, namely, protective properties for (micro and macro) vascular system (Valentová et al., 2007; Bao et al., 2008).

However, almost all these studies point towards the use of *V. myrtillus* fruit's extracts or teas. *V. myrtillus* leaf use in therapeutic treatments is less known, despite being used due to its anti-diabetic properties. Though, in vivo studies to support this empirical data are missing (Cignarella et al., 1996). Our results show that, IPGTT test performed after GK-rats 4 week-lasting treatment with *V. myrtillus* leaf decoction show a better response. This could be due to the presence of neomyrtillin, a glucoside compound found in V. myrtillus leaves, also known as "plant insulin" (Edgars, 1936; Duke, 1992).

Because of its high content in tannins (Duke 1992), it is believed that long-term therapy with *V. myrtillus* leaf tea could induce toxic effects. In order to clarify this, toxicological effects of *V. myrtillus* leaf decoctions were evaluated. For that we used liver mitochondria isolated from GK rats treated with leaf decoctions during 4 weeks. Liver is the organ responsible for detoxification processes and therefore liver mitochondria are one of the main targets for ingested xenobiotic compounds. Our results showed that mitochondrial oxidative and phosphorylative activities were largely increased in *V. myrtillus* treated rats. These results could probably be explained by an increase of respiratory complexes per mitochondria. Despite a higher phosphorylative efficiency could raised ROS production, this undesirable effect could be counteracted due to the antioxidant activity present in *V. myrtillus* extract.

Quercetin is part of a broad group of natural polyphenolic flavonoids also present in *V. myrtillus* leaves (Riihinen et al., 2008). These flavonoid compounds are reported to exhibit a wide variety of biological effects, including antioxidant and free radical-scavenging activities (Ichiyanagi et al., 2004; Rohn et al., 2004; Davis et al., 2009). Moreover, recent evidence suggests that quercetins can improve mitochondrial biogenesis, being observed an increased mRNA expression of mitochondrial genes and a higher cytochrome c concentration (Davis et al., 2009), which, therefore, corroborates the respiratory stimulation observed in our study.

Anthocyanosides and quercetins found in *V. myrtillus* constitute important therapeutic agents in diabetes, delaying the onset of diabetic complications, generally related to vascular damages produced by oxidative stress. Proanthocyanosides found in *V. myrtillus* leaves (Duke 1992; Riihinen et al., 2008), as well as anthocyanosides, are high antioxidant compounds that prevent angiogenesis and are also responsible for collagen stabilization (Roy et al., 2002; Matsunaga et al., 2009), being worthy for diabetic patients. Furthermore, the increased respiratory chain activity of GK treated rats can be explained due to quercetins effect on mitochondrial genes expression.

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