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Full Length Research Paper

Inhibitory effect of *Arctium minus* on mitochondrial bioenergetics in diabetic Goto-Kakizaki rats

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Despite an increasing use of medicinal plants in diabetes mellitus therapy, their use is usually based on empirical knowledge. This work aims to evaluate the effects of decoctions prepared from *Arctium minus* Bernh. on glycaemic control of goto-kakizaki (GK) rats, a type 2 diabetes mellitus animal model, and access the potential toxicity of *A. minus* decoctions, using liver mitochondrial preparations. GK rats were drinking *ad libitum A. minus* root decoction for an interval of 4 weeks. During this time, food and beverage ingested and weight increase were controlled. Occasional glycaemias were also evaluated twice a week. Intraperitoneal glucose tolerance was determined at the beginning and at the end of the experiment. Four weeks later, GK rats liver mitochondria were isolated and respiratory parameters evaluated. *A. minus* root decoctions did not significantly affect the glycaemic control and long-lasting treatments induced noxious effects, considering that the liver mitochondria isolated from *A. minus* treated GK rats presented a significant decrease in mitochondrial respiratory parameters (RCR and FCCP stimulated respiration). This study showed that long lasting treatments with *A. minus* tea induced deleterious effects on cellular metabolism and, thus, should be avoided in type 2 diabetes long-term therapy.

Key words: Goto-Kakizaki (GK) rats, type 2 diabetes mellitus, *Arctium minus* Bernh., phytotherapy, oxidative phosphorylation, toxicology.

INTRODUCTION

Type 2 diabetes is caused by hepatic and peripheral tissue insulin resistance and pancreatic beta-cell dysfunction (William and Pickup, 2004). Due to generalization of developing countries western lifestyles and to the consequent increasing rates of childhood and adult obesity, type 2 diabetes has become an epidemiologic problem, attaining pandemic dimensions (WHO, 2006). Around 85 - 90% of diabetic people suffer from type 2 diabetes, a common designation for a group of pathologies with a shared feature: hyperglycaemia. This hyperglycaemia, as a result of uncontrolled glucose regulation, causes severe diabetic complications, such as nephropathy, retinopathy, neuropathy and cardiovascular disease, which severely influence the quality of diabetic patients life (Engelgau and Gueiss, 2000; Nuorooz-Zadeh, 2000; Yorek, 2003). Therefore, the major aim of diabetes therapy is to reach normal glycaemic levels (Agius, 2007; Yu et al., 2010). Nevertheless, this target usually remains unattainable using regular therapies and chemical anti-hyperglycaemic agents.

Medicinal plants seem to be a useful alternative or complementation to the synthetic drugs used in type 2 diabetes' therapy. In fact, several of these synthetic drugs (such as metformin or guanidine) are based on active

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compounds previously extracted from medicinal plants (Mueller and Jungbauer, 2009; Petlevski et al., 2001). As a result, there is an increasing use of medicinal plants in the treatment of diabetes mellitus (Halat and Dennerhy, 2003; Ryan et al., 2001). However, there is little knowledge about the mechanism of action and the therapeutic effects of several of these plants with attributed anti-diabetic action by folk medicine. Moreover, toxicological effects of most medicinal plants only in recent years are being investigated (Aguilar-Santamaría et al., 2007; Déciga-Campos et al., 2007), but for most plants they remain poorly characterized (Street et al., 2008; Jordan et al., 2010).

In Portugal, there is also an increasing demand of phytotherapy as a complement for traditional therapy (oral anti-diabetic medication or insulin), in order to reach normal glycemic levels and prevent later complications (Barata, 2008). *Arctium minus* (Hill) Bernh (lesser burdock), a widespread spontaneous Asteraceae, commonly known as "pegamasso-menor", is traditionally used to treat skin problems and type 2 diabetes mellitus (Cunha et al., 2009). Indeed, *A. minus* phytotherapy applications in Portugal are similar to those of *A. lappa* and roots decoctions are frequently used for diabetes treatment and skin problems (although, sometimes leaves decoctions are also used for dermatological problems), the later with external applications (Carvalho, 2005; Cunha et al., 2009). However, in Portuguese territory, *A. lappa* is rare, in that *A. minus* is more common, it is usually found in uncultivated lands and rural roads (Carvalho, 2005; Cunha et al., 2009). Also, in some Asian countries, burdock (*A. lappa*) root salad is a common dish. In fact, Arctium roots contain inulin, a fructan compound, indigestible in the upper gastrointestinal tract with inhibitory action over the absorption of lipids and cholesterol in small intestine (Causey et al., 2000; Duke et al., 2002; Kardosová et al., 2003; Li et al., 2008). Some other studies with *A. minus* leaves water extracts reported a high antioxidant potential, due to the presence of flavonoids (quercetin and rutin) (Erdemoglu et al., 2009), being also beneficial to reduce oxidative stress associated with hyperglycaemia.

Nevertheless, the therapeutic effect of *A. minus* is still unclear and until now the possible toxicological effects have not been studied. The goal of this study was to investigate the influence of *A. minus* root decoction on the glycaemic levels in a diabetic animal model, the Goto-Kakizaki (GK) rats (Goto et al., 1975), one of the best-characterised animal model of spontaneous non-obese type 2 diabetes mellitus (McIntosh and Pederson, 1999; Portha et al., 2009). To evaluate the possible toxicological effects, liver mitochondria from these rats were isolated and mitochondrial parameters (RCR and FCCP stimulated respiration) evaluated. Liver is majorly responsible for detoxification processes and mitochondria constitute the major energy-producing organelles of the cell and 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; Wallace et al., 1997). In spite of this, mitochondria has proved to be a good model to study the action of many xenobiotics on cell toxicity and data obtained are usually in good agreement with citotoxicity parameters reported in cell cultures and whole organisms (Haubenstricker et al., 1990; Knobeloch et al., 1990).

MATERIALS AND METHODS

Materials

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

Plant material

The roots of *A. minus* Bernh. originally from Central Asia, were dried in the dark and obtained from "11 anos - Segredo da planta - Produtos naturais e biológicos, Lda" (Seixal, Portugal). An *A. minus* voucher (number 3198) has been identified and deposited at the Herbarium of the University of Trás-os-Montes and Alto Douro, Vila Real, Portugal.

Preparation of the extracts

The roots of the plant, used in the decoction, were ground to a powder. The decoction was prepared by boiling 125 g of the dried material in 1000 mL of deionised water for 15 min, filtered and centrifuged at 7000 × g for 5 min and kept at -20°C.

Metals quantification

The plant extract was digested with nitric acid (supra pure grade) at 60°C overnight. The digested samples were analysed in a graphite furnace atomic absorption spectrophotometer (UNICAMP 939 AA - GF90). For each set of triplicates, blank assays with Milli-Q50 water were performed, using the same procedure. The results were expressed in mg. g^{-1} (or µg. g^{-1}) of dry weight.

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 *ad libitum A. minus* decoction and the other, used as control, drinking distilled water. The experiments lasted for an interval of 4 weeks and were carried out in accordance with the National requirements for vertebrate animal research and European convention for the protection of animals used for experimental and other scientific purposes.

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
A. minus	21.3 ± 0.51*	42.3 ± 1.03***	109.1 ± 1.19	9.06 ± 0.69*

The amount of food and liquid ingested and occasional glycaemia were recorded twice a week. The final weight of each rat was expressed as the percentage of its initial weight. Data are presented as means ± SEM. Values statistically different from control (distilled water): *** $p < 0.001$, * $p < 0.05$.

Table 2. Glycaemia, fasting and two hours after glucose loading.

Condition (➔)	Glycaemia (mM)			
	Initial		Final	
Extract (🡻)	Fasting	120 min	Fasting	120 min
Control	5.7 ± 0.2	16.0 ± 2.2	5.4 ± 0.3	15.9 ± 1.2
A. minus	4.7 ± 0.1	16.6 ± 2.2	5.9 ± 0.4	18.5 ± 0.6

Blood glucose determination was carried out as described in materials and methods section. Initial glycaemias were evaluated before the ingestion of *A. minus* (or distilled water) and final glycaemias were evaluated 4 weeks later. Data are presented as means ± SEM of 3 different rats.

Glycaemia

Glycaemia in a non-fasting condition (occasional) was determined twice a week. Fasting glycaemia (fasting period 16-18 hours) and glycaemia 2 h after glucose load (1.8 g glucose/ kg body weight, *i.p.*) were carried out at the beginning of the experiment and at the end. Blood glucose levels were determined 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 tail vein.

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 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 (Estabrook, 1967). 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 respiration (*V3*), 300 nmol of ADP (magnesium salt) were used. The respiratory control ratio (RCR) was calculated according to Chance and Williams (Chance and Williams, 1956). FCCP-uncoupled respiration (V*FCCP*) 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 (standard error mean) of the number of experiments shown on the legends of the tables and figures. Statistical significance was determined using unpaired Student's t-test and p values < 0.05 were considered significant.

RESULTS

In the present study, a group of 6 diabetic GK rats with 10 weeks of age, showing an initial occasional glycaemia of 8.8 ± 1.50 mM were randomly divided and housed for 4 weeks in 2 separated groups, one drinking *A. minus* root decoctions *ad libitum* and control group drinking distilled water.

Our results show that *A. minus* decoctions lead to a slight but significant ($p < 0.05$) decrease of occasional glycaemia (Table 1) of GK rats; however, the study did not observe any difference on GK rats glycaemia, both under fasting and 2 h after intraperitoneal glucose loading, as compared to control group (Table 2). The amount of liquid and food ingested was lower in *A. minus* drinking group. Nevertheless, the increase in weight was not significantly different between both groups (Table 1).

Due to the importance of metal ions in the mechanisms of insulin release and action (Cunningham, 1998; McCarty, 1996), this study analysed the ionic contents in plant extracts. The results showed that the plant extracts used in this study contained a significant

Table 3. Metal content in water plant extract of *A. minus*.

Al	Ca	Cu	Fe	K	Mg	Mn	Na	P	Sr	Zn	Cd	Co	Cr	Ni
Milligrams												Micrograms		
0.314	4.000	0.022	0.163	22.800	2.200	0.035	0.180	5.300	0.025	0.036	0.146	nd	nd	1.927

Water plant extract was performed as described in Materials and Methods section. Metal contents were evaluated by atomic absorption spectroscopy. Results are expressed as milligrams or micrograms of metal per gram of plant. nd – not determined.

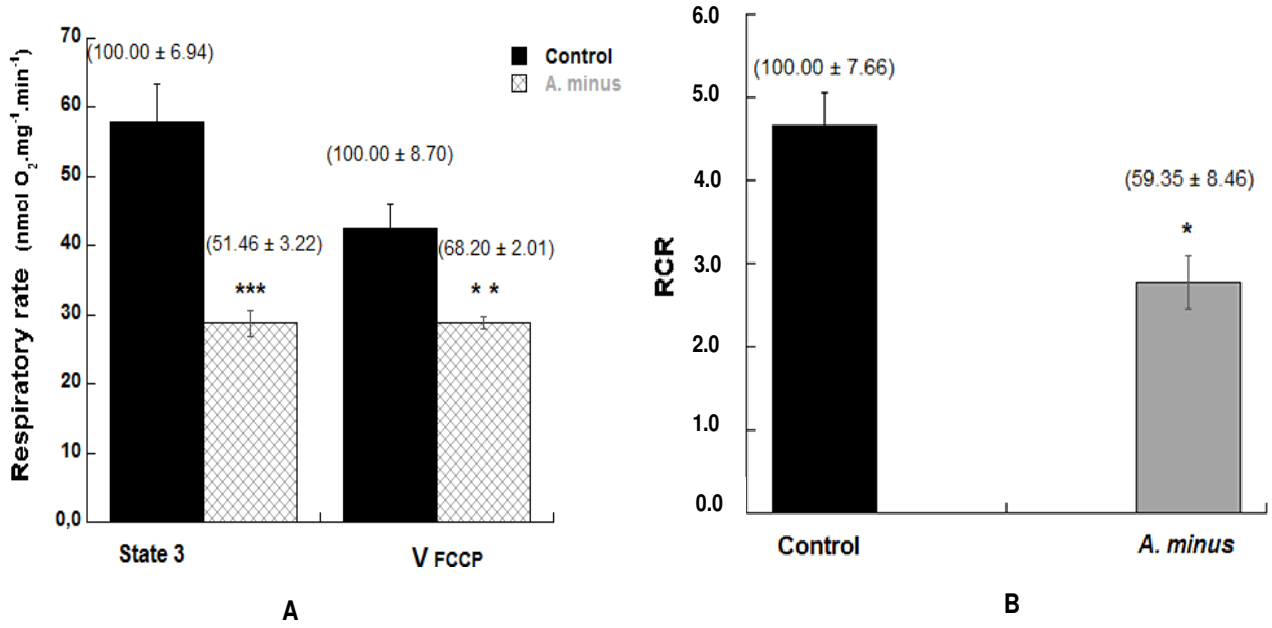

Figure 1. Effect of *A. minus* on GK rat liver mitochondrial parameters. Mitochondria (1 mg protein) were incubated in 1 ml of the respiratory standard medium supplemented with rotenone (2 mg) and succinate (5 mM). In order to achieve state 3 respiration 300 nmol ADP were added. Values of respiratory rates state 3 and FCCP stimulated respiration are expressed as nmol O2.mg protein-1.min-1 (A). RCR was determined accordingly to Chance and Williams (1956) (B). 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: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ as compared to control.

amount of metal ions important in insulin release, such as magnesium or zinc; however, the extracts contained about 4 µM of nickel and cadmium (Table 3); metal elements responsible for the inhibition of insulin secretion.

To evaluate the possible toxic effects over hepatic mitochondrial bioenergetics, this study isolated liver mitochondria of GK rats after 4 weeks of treatment with *A. minus* decoction. *A. minus* induced a decrease in several parameters of GK liver mitochondria respiratory activity (Figure 1). In fact, in the presence of succinate as respiratory substrate, we observed a decrease in state 3 (V_3; 50% inhibition) and FCCP-stimulated respirations (VFCCP; about 30% inhibition), as compared to controls. The respiratory control ratio (RCR), therefore, is substantially decreased in *A. minus* drinking rats. These results point towards a lower activity in several complexes of mitochondrial respiratory chain.

DISCUSSION

Diabetes mellitus nowadays has become a severe threat to public health, affecting about 6% of human population (WHO, 2006), with a negative economy impact to the countries all over the world (ADA, 2003). The major purpose of diabetes therapy is to maintain normal glycaemic levels (Agius, 2007). Thus, in order to achieve this, several liver crucial enzymes in glucose metabolism, such as glucokinase, which catalyses the first step in glycolysis, and enzymes of gluconeogenesis and/or glycogenolysis, such as glucose 6-phosphatase, fructose 1,6-bisphosphatase and glycogen phosphorylase and the glucagon receptor have received special attention (Agius, 2007). Also, new α-glucosidase inhibitors, preventing the digestion of carbohydrates and reducing the amount of absorbed glucose are still under research (Xu et al.,

2007). Furthermore, dipeptidyl peptidase IV (DPP IV) seems to be a novel target for the treatment of type 2 diabetes (Nielsen, 2005, Benbaw et al., 2009). In fact, DPP IV inhibitors improve the impaired insulin secretion and decrease postprandial concentrations of glucagon by enhancing the incretin hormone levels glucagon-like peptide-1(GLP-1) and glucose-dependent insulinotropic polypeptide/gastric inhibitory polypeptide (GIP) (Green et al., 2006).

Nonetheless, this goal frequently remains unattainable using chemical hypoglycaemic agents. In recent years, in Portugal, like in many other countries, there is also an increasing use of medicinal plants with attributed antidiabetic effects, often simultaneously with antidiabetic oral drugs. Indeed, ethnomedical studies reveal that the use of phytotherapy in some countries is sometimes in concerted use with antidiabetic drugs, in order to achieve a better glycaemic control (Baldé et al., 2006). Among these plants, *A. minus* is commonly used in the production of tea beverages with attributed antidiabetic properties (Cunha et al., 2009). Despite its antibacterial, antioxidant and immunomodulatory activities (Abraham et al., 1946; Basaran et al., 1997), little is known about the effect of *A. minus in vivo* studies on diabetic patients or about its possible toxic effects. Owing to simple use of plants, users do not generally recognize the potential risks of phytotherapy, because plants are “natural” products. Nevertheless, ingestion of plant extracts can have adverse effects or potential toxicity (Pepato et al., 2004). In this study, a common *A. minus* root preparation was used, usually found in Portuguese supermarkets and herb stores, being easily available to common people.

Our results showed that *A. minus* decoction lead to a GK rats occasional glycaemia decrease. However, the study did not observe any significant difference on GK rats fasting glycaemia and glucose tolerance, as compared to control group, which is contradictory to the anti-diabetic properties attributed to this plant roots. GK rats drinking *A. minus* decoction ingested less food and probably this is the main reason for the lower occasional glycaemias that were evaluated in normal eating conditions. Nevertheless, inulin reduced the absorption of lipids in small intestine (Davidson et al., 1998; Causey et al., 2000), but inulin does not seem to reduce glucose absorption (Alles et al., 1999) and, thus, the improved occasional glycaemia may be due to the reduced amount of ingested food. Furthermore, as glucose loading was intraperiteonal, differences in intestinal absorption do not contribute to the discrepancies observed between occasional glycaemia and glucose load results in *A. minus* treated-rats.

It is well known that certain inorganic mineral elements (potassium, zinc, calcium, magnesium, etc.) play an important role either in normal glucose-tolerance maintenance and release of insulin from β-cells of islets of Langerhans (Kar et al., 1999). Metal ions analysis showed that our plant extracts contained a great content of potassium (K) and magnesium (Mg) ions. It was described that a poor intracellular Mg^{2+} concentration found in type 2 diabetes accounts for the impairment in insulin action and worsening of insulin resistance, and oral magnesium supplementation reduced type 2 diabetes development in predisposed rats (Barbagallo and Dominguez, 2007; Barbagallo et al., 2003; de Valk et al., 1995). However, fasting glycaemia and glucose tolerance were not improved, possibly due to antagonistic effects of other metal ions, such as nickel (Ni) and cadmium (Cd). A long time ago, it is recognized that nickel is a potent pancreatic β-cells insulin release inhibitor (Dormer et al., 1974; Gupta et al., 2000). Cadmium, a toxic metal, responsible for decreasing insulin release, is present in *A. minus* extract (Lei et al., 2007). Indeed, most aromatic and medicinal plants have the ability to bioaccumulate several heavy metals (Broadley et al., 2001). The ingestion of metals ions like cadmium will lead to an accumulation in liver and kidneys (Lind et al., 1997). The extent of metal ions accumulation is dependent both on the time and concentration, but also on the form. In fact, when metals are chelated with proteins or other biomolecules, the absorption rate could be increased in that it is more close to the natural contamination source (Haouem et al., 2007). Furthermore, cadmium is slowly eliminated from the organism (Zabulyte et al., 2007), which also contribute to the organ-accumulation.

Hence, since Cd possesses a cumulative effect and a long half-life, despite being present in the plant extract in a low amount, the noxious effects will be permanent (Lei et al., 2007; WHO, 1992). Nevertheless, further studies are needed to confirm pancreatic insulin release and the levels of cadmium in blood (or urine) and tissues, including pancreas.

In this experimental work, a decrease in succinate supported oxygen consumption was observed either in the presence of ADP, corresponding respectively to respiratory state 3 and (Chance and Williams, 1956) also a decrease in FCCP stimulated respiration in the presence of succinate in GK rats drinking *A. minus* decoction. This large decrease in respiratory activity is reflected by the respiratory control ratio (RCR; around 60% of control), and by the decrease in the oxygen consumption observed in the presence of FCCP, a classic uncoupler, that dissociates the oxidative and phosphorylative activities. This point towards an inhibitory effect on the respiratory complexes (succinate dehydrogenase, coenzyme Q: cytohrome-*c*-oxidoreductase and/or cytochrome-*c*-oxidase). Indeed, it was previously described that Cd inhibits succinate dehydrogenase and coenzyme Q: cytohrome-*c*-oxidoreductase activities of mitochondrial electron transfer chains from liver, brain and heart (Kondoh et al., 2002), and this seems to be a plausible explanation to the study’s results. The observed decrease in V_3 rate could also be explained by the increased permeabilization of inner mitochondrial membrane, induced by reactive oxygen species (ROS)

produced in the presence of Cadmium (Kondoh et al., 2002; Lasfer et al., 2008; Pham et al., 2006). Furthermore, the decreased respiratory efficiency can be counterweighed by glycolysis stimulation (Holloszy and Booth, 1976) and also account for lower (occasional) glycaemia.

Conclusion

Despite the fact that the genus *Arctium* (family Asteraceae) comprises of some other different species such as *A. lappa* L. (greater burdock), a plant morphologically similar to *A. minus* (which is widely used due to its anti-diabetic properties) (Li et al., 2004), this study allow us to believe that long-term treatments with at least some chemiotypes of *A. minus* may induce deleterious effects on cellular metabolism and therefore should be avoided in type 2 diabetes long-term therapy. Moreover, this study remarks the major importance of chemical characterization and standardization of plant extracts used in phytotherapy treatments (Ong, 2004), in this study, the presence of some heavy metals such as nickel and cadmium, can overlap the beneficial effects of phytochemicals present in the plant. However, further studies are recommended to clarify this point.

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