Phytomedicine 21 (2014) 595–601

Contents lists available at ScienceDirect

Phytomedicine

journal homepage: www.elsevier.de/phymed

The cardioprotective effect of an aqueous extract of fermented rooibos (*Aspalathus linearis*) on cultured cardiomyocytes derived from diabetic rats

P.V. Dludla[a,b], C.J.F. Muller[a], J. Louw[a], E. Joubert[c,d], R. Salie[a,e], A.R. Opoku[b], R. Johnson[a,*]

[a] *Diabetes Discovery Platform, South African Medical Research Council, Tygerberg 7505, South Africa*
[b] *Department of Biochemistry and Microbiology, University of Zululand, KwaDlangezwa 3886, South Africa*
[c] *Post-Harvest and Wine Technology Division, Agricultural Research Council (ARC) Infruitec-Nietvoorbij, Stellenbosch 7599, South Africa*
[d] *Department of Food Science, Stellenbosch University, Stellenbosch 7602, South Africa*
[e] *Division of Medical Physiology, Faculty of Health Sciences, Stellenbosch University, Tygerberg 7505, South Africa*

ARTICLE INFO

Article history:
Received 2 September 2013
Received in revised form
26 September 2013
Accepted 27 October 2013

Keywords:
Rooibos
Aspalathus linearis
Diabetic cardiomyopathy
Hyperglycemia
Oxidative stress

ABSTRACT

Diabetic cardiomyopathy (DCM) is a disorder of the heart muscle that contributes to cardiovascular deaths in the diabetic population. Excessive generation of free radicals has been directly implicated in the pathogenesis of DCM. The use of antioxidants, through dietary supplementation, to combat increased cellular oxidative stress has gained popularity worldwide. *Aspalathus linearis* (rooibos) is a popular herbal tea that contains a novel antioxidant, aspalathin. Literature has reported on the antidiabetic, anti-inflammatory and free radical scavenging effects of rooibos. However, its protective effect against DCM has not been established. Therefore, this study investigated whether chronic exposure to an aqueous extract of fermented rooibos (FRE) has an *ex vivo* cardioprotective effect on hearts obtained from streptozotocin (STZ) induced diabetic rats. Adult Wistar rats were injected with 40 mg/kg of STZ. Two weeks after STZ injection, cardiomyocytes were isolated and cultured. Cultured cardiomyocytes were treated with FRE (1 and 10 μg/ml), vitamin E (50 μg/ml), and n-acetyl cysteine (1 mM) for 6 h, before exposure to either hydrogen peroxide (H_2O_2) or an ischemic solution. Cardiomyocytes exposed to H_2O_2 or an ischemic solution showed a decrease in metabolic activity and glutathione content with a concomitant increase in apoptosis and intracellular reactive oxygen species. Pretreatment with FRE was able to combat these effects and the observed amelioration was better than the known antioxidant vitamin E. This study provides evidence that an aqueous extract of fermented rooibos protects cardiomyocytes, derived from diabetic rats, against experimentally induced oxidative stress and ischemia.

Introduction

The International Diabetes Federation estimates that the prevalence of diabetes mellitus (DM) will increase to 552 million by the year 2030, with type 2 diabetes (T2D) contributing 95% to the epidemic in industrialized countries (Garg et al., 2012). In diabetics, the risk of developing cardiovascular disease (CVD), especially diabetic cardiomyopathy (DCM), increases up to 4-fold compared to nondiabetic individuals (Voulgari et al., 2010).

The first association between DM and CVDs was made in the Framingham heart study, where chronic hyperglycemia was identified as an independent risk factor for the development of DCM (Rubler et al., 1972). In uncontrolled diabetes, chronic hyperglycemia is implicated in the augmented production of free radicals (King and Loeken, 2004). Increase in free radical production is associated with altered cardiac energy substrate metabolism resulting in myocardial damage. The exacerbation of myocardial damage may eventually lead to cardiomyocyte apoptosis and impaired

Abbreviations: ATP, adenosine triphosphate; $CaCl_2$, calcium chloride; CVD, cardiovascular disease; DCFH-DA, 2′,7′-dichlorfluorescein-diacetate; DCM, diabetic cardiomyopathy; DM, diabetes mellitus; DMEM, Dulbecco's modified Eagle's medium; FBS, foetal bovine serum; FFAs, free fatty acids; FRE, fermented rooibos extract; GSH, glutathione; HBSS, Hank's balanced salt solution; HFABP, heart fatty acid binding protein; HPLC-DAD, high performance liquid chromatography with diode-array detection; H_2O_2, hydrogen peroxide; KCl, potassium chloride; $MgCl_2{\cdot}6H_2O$, magnesium chloride hexahydrate; NAC, n-acetyl cysteine; NaCl, sodium chloride; $NaHCO_3$, sodium hydrogen carbonate; $NaH_2PO_4{\cdot}2H_2O$, sodium dihydrogen phosphate; PBS, phosphate buffered saline; PI, propidium iodide; PPAG, phenylpyruvic acid-2-*O*-β-D-glucoside; ROS, reactive oxygen species; SGLT, sodium-dependent glucose transporters; STZ, streptozotocin; T2D, type 2 diabetes mellitus.

* Corresponding author at: South African Medical Research Council, Diabetes Discovery Platform, P.O. Box 19070, Tygerberg 7505, South Africa. Tel.: +27 219380866; fax: +27 219380456.
E-mail address: rabia.johnson@mrc.ac.za (R. Johnson).

0944-7113/$ – see front matter © 2013 Elsevier GmbH. All rights reserved.
http://dx.doi.org/10.1016/j.phymed.2013.10.029

myocardial function. Thus, investigation into new research areas focusing on suppression of oxidative stress, during chronic hyperglycemia, may be a reasonable therapeutic strategy to protect the diabetic heart at risk from developing DCM.

Improving the quality of life for individuals with diabetes through optimal glycemic control is important. However, optimal control of blood glucose levels still remains a big challenge. Current therapies used to control blood glucose levels do not offer much protection against DCM. The use of plant derived antioxidants as an adjunct to current therapies in ameliorating metabolic disturbances continues to show promise. In recent years, research has highlighted the strong antioxidant properties of polyphenols and their potential to ameliorate diabetic complications (Kumarappan and Mandal, 2008; Zang et al., 2006). Tea consumption is one of the major sources of polyphenolic dietary intake and the relationship between drinking tea and a reduced risk of CVD has been reported (Bohn et al., 2012). The herbal tea, rooibos, produced from the leaves and stems of *Aspalathus linearis*, is a well known beverage with a high phenolic content. It is mostly consumed as an infusion prepared from the "fermented" (oxidised) plant material. Studies investigating its efficacy have reported on its antioxidant, anti-inflammatory and antidiabetic properties (Joubert et al., 2008; Joubert and De Beer, 2011). Furthermore, rooibos has been shown to prevent oxidative stress in rats and in humans (Ulicna et al., 2006; Marnewick et al., 2011).

To date, little is known about the beneficial potential that rooibos could have on DCM. Therefore, the purpose of the present study was to elucidate whether an aqueous extract of fermented rooibos could protect cardiomyocytes isolated from diabetic rats against experimentally induced oxidative stress and ischemia.

Materials and methods

Chemicals and reagents

CellTracker Blue CMAC dye was purchased from Invitrogen (Carlsbad, CA, USA) and taurine from Acros Organics (Geel, Belgium). Fetal bovine serum (FBS), Hank's balanced salt solution (HBSS), Dulbecco's modified Eagle's medium (DMEM), phosphate buffered saline (PBS), Media 199 and penicillin/streptomycin amphotericin B were from Lonza BioWhittaker (Verviers, Belgium) and hydrogen peroxide (H_2O_2) was from Merck (Whitehouse Station, NJ, USA). All other consumables as well as reagents were purchased from Sigma–Aldrich Corp. (St. Louis, MO, USA).

Preparation and analysis of rooibos extract

Fermented rooibos was batch extracted on industrial scale (600 kg) using a percolator type extraction vessel as described for unfermented *C. subternata* extract (Dudhia et al., 2013). High performance liquid chromatography with diode - array detection (HPLC-DAD) analysis was used to determine the major phenolic/phenyl compounds present in the fermented rooibos extract (FRE) (Beelders et al., 2012).

Induction of diabetes

Six-month-old adult male Wistar rats (350–450 g) were used for the study. The animals were housed at the Primate Unit and Delft Animal Centre of the South African Medical Research Council (MRC) in a controlled environment with a 12 h light/dark cycle in a temperature range of 23–25 °C (relative humidity: ~50%). The rats received standard laboratory chow pellets (Afresh Vention, Cape Town, South Africa) *ad libitum* and had free access to drinking water. Ethical approval for this study was granted by the MRC Ethics Committee for Research on Animals (ECRA no. 11/03/G). Diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ) (40 mg/kg body weight). Five days later (following a 4 h fast), tail pricks were performed to measure blood glucose. Rats with a fasting blood glucose concentration of ≥14 mmol/l were classified as diabetic and included in the study. Rat hearts were harvested 2 weeks after induction of diabetes.

Radioimmunoassay for insulin determination

Rat insulin was determined using a radioimmunoassay kit as per manufacturer's instruction (Linco Research, Inc., St. Charles, MO, USA).

Ex vivo cardiomyocyte culture

Rat hearts were removed and cardiomyocytes isolated by using a previously described method (Fischer et al., 1991). Cardiomyocytes isolated from the perfused rat heart were resuspended in 10 ml of supplemented Media 199 (5 mM carnitine, 5 mM taurine, 0.1 mM bromodeoxyuridine, 5 mM creatine monohydrate, 5% FBS and 0.5% penicillin/streptomycin amphoterin B) and incubated in 100 mm tissue culture dishes for a period of 1 h under standard tissue culture conditions to eliminate nonmyocytic cells. The nonadherent cardiomyocytes were harvested and seeded onto laminin coated 6 well tissue culture plates at a density of 5.94×10^5 cells/well. Cell viability count was determined by trypan blue assay (Invitrogen, Carlsbad, CA, USA) and cell viability counts of >70% were used for subsequent experiments.

Rooibos treatment

The study consisted of 2 experimental conditions (H_2O_2 and ischemic solution exposure) and each experimental condition had 6 experimental groups pretreated with either: (i) negative control (experimental control), (ii) 1 μg/ml FRE, (iii) 10 μg/ml FRE, (iv) 1 mM n-acetyl cysteine (NAC), (v) 50 μg/ml vitamin E and (vi) untreated controls. Cardiomyocytes were pretreated for 6 h before exposure to either 32 μM H_2O_2 or an ischemic solution (116 mM NaCl, 50 mM KCl, 1.8 mM $CaCl_2$, 2 mM $MgCl_2 \cdot 6H_2O$, 26 mM $NaHCO_3$, 1 mM $NaH_2PO_4 \cdot 2H_2O$) for 24 h and 2 h, respectively. Cells that served as negative controls were treated with media only.

Measurement of metabolic activity

The adenosine triphosphate (ATP) assay was done as a measurement of metabolic activity using a ViaLight Plus kit (Lonza, Basel, Switzerland), following manufacturer's instructions.

Annexin V and propidium iodide (PI)

Apoptosis was evaluated by labelling cells with a combination of annexin V and PI. The annexin V conjugate was prepared according to manufacturer's instructions (Invitrogen, Carlsbad, CA, USA) and PI was prepared in PBS (20 μg/ml). The cells were incubated at room temperature for 15 min with annexin V/PI before the solution was removed and cells resuspended in PBS for microscopic analysis.

Determination of membrane leakage

The myocardial membrane leakage was detected using a rat heart fatty acid binding protein (HFABP) ELISA kit according to manufacturer's instructions (HycultBiotech, Uden, Netherlands).