Cardiovascular Topics

Rooibos (*Aspalathus linearis*) protects against nicotine-induced vascular injury and oxidative stress in Wistar rats

Michelle Smit-van Schalkwyk, Shantal Windvogel, Hans Strijdom

Abstract

Background: Rooibos (*Aspalathus linearis*) is an indigenous South African plant, traditionally used by the local population as a remedy against several ailments. More recently, rooibos was shown to exhibit potent antioxidant properties, attributed to its polyphenols. We assessed whether treatment with fermented rooibos (RF), unfermented rooibos (RUF) and melatonin (Mel), a well-documented antioxidant included for comparison, could counter the harmful vascular and pro-oxidant effects of nicotine.

Methods: Vascular function, antioxidant enzyme activity and lipid peroxidation were assessed in male adult rats treated with nicotine (5 mg/kg body weight/day) and 2% RF, 2% RUF or 4% Mel co-administration. Nitric oxide (NO) production and cell viability were measured in nicotine-exposed rat aortic endothelial cells (AECs) pre-treated with RF (0.015 mg/ml).

Results: Vascular studies showed that co-administration with RF or Mel exerted anti-contractile and pro-relaxation responses in aortic rings, and increased hepatic superoxide dismutase and catalase activity in nicotine-exposed animals. Co-treatment with Mel additionally decreased lipid peroxidation in nicotine-exposed rats. RUF exerted anti-contractile responses in aortic rings of nicotine-treated animals, while in nicotine-exposed AECs, RF pre-treatment increased intracellular NO levels.

Conclusion: For the first time, we have shown that rooibos co-treatment exerted beneficial vascular effects in nicotine-exposed rats, and that this was associated with increased antioxidant enzyme activity.

Keywords: nicotine, *Aspalathus linearis*, rooibos, melatonin, endothelial dysfunction

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Centre for Cardio-metabolic Research in Africa, Division of Medical Physiology, Biomedical Sciences, Faculty of Medicine and Health Sciences, Stellenbosch University, Tygerberg, South Africa

Michelle Smit-van Schalkwyk, PhD, michelle.smitvanschalkwyk@gmail.com

Shantal Windvogel, PhD

Hans Strijdom, PhD

Tobacco smoking is one of the most important risk factors for the development of cardiovascular disease and is responsible for approximately 12% (6.2 million) of all deaths globally.[1] It is estimated that over five million people are current or ex tobacco users and that over 600 000 non-smokers die from exposure to second-hand smoke.[2] Nicotine, the addictive substance in tobacco, is associated with the development of endothelial dysfunction (ED) through oxidative stress. ED is an early, reversible precursor of atherosclerosis.[3] In turn, atherosclerosis is the underlying pathology for many cardiovascular diseases, often resulting in myocardial infarction and stroke.[4]

Nitric oxide (NO) plays an important role in protection against the onset and progression of cardiovascular disease. The ability of the endothelium to synthesise and release NO is essential in regulating haemostasis, vessel tone, blood pressure and vascular remodelling.[5] Furthermore, reactive oxygen species (ROS) and the resultant oxidative stress are important mediators of the pathological manifestations of ED. ROS reduce or eliminate the protective abilities of NO, which in turn could lead to ED.[6]

Experimental and clinical data indicate that exposure to nicotine increases oxidative stress and has the potential to induce ED.[7] While endogenous mechanisms such as antioxidant enzymes as well as non-enzymatic defences exist to combat the deleterious effects of oxidative stress, they might not offer sufficient protection against the ROS produced during nicotine exposure.[8,9]

Early endothelial changes such as ED are reversible,[10] rendering it clinically relevant to identify possible treatment modalities such as anti-oxidant therapy, which could counter the harmful effects of increased ROS production, and hence restore the release of endothelioprotective NO. Protecting the endothelium will result in reduced or delayed atherogenesis, which lowers the risk of cardiovascular mortality. Such therapies may include dietary supplementation with natural plant products or chemically synthesised versions of endogenous molecules with known antioxidant capacity.

Rooibos (*Aspalathus linearis*) is an indigenous South African plant that is popularly consumed as a beverage and is known to possess bio-active properties.[11] Rooibos boasts a unique flavonoid content and contains various dihydrochalcones, including aspalathin, a C-linked dihydrochalcone glucoside,[12,13] and aspalanin, a cyclic dihydrochalcone,[14] which are both unique to *Aspalathus linearis*.

Both unfermented (green) and fermented (red) forms of rooibos are commercially available. Green rooibos is immediately dried after the cutting phase, whereas non-enzymatic oxidative degradation of aspalathin results in the characteristic red-brown fermented form.[15] Rooibos has been shown to exert potent antioxidant, immune-modulating and chemo-protective actions, with the additional benefit of having minimal adverse effects.[16] In addition, rooibos possesses cardioprotective properties, including the improvement of dyslipidaemia and redox status in human study participants,[17] as well as being able to exert protective effects on cultured cardiomyocytes from diabetic rats.[18]

A seven-week rooibos treatment protocol was shown to protect against ischaemia/reperfusion injury in isolated perfused rat hearts.[19] Furthermore, in a rat model of chronic rooibos consumption, no adverse effects were found.[20] However, despite these promising results, studies into the cardioprotective effects of rooibos, both fermented and unfermented, remain limited, with investigations into the effects of rooibos on the vascular endothelium, in particular, lacking.

In addition to controlling circadian rhythms, the hormone melatonin has been shown to be a versatile biological signalling molecule,[21] involved in many physiological processes in humans and animals, including blood pressure control and the scavenging of free radicals.[22,23] In addition to the pineal gland, melatonin is secreted from a variety of organs (regarded as non-endocrine organs) and tissues, including the retina, Harder's glands, gastroenteric mucous membrane, megakaryocytes, platelets, lymphocytes, bone marrow and the skin, but at lower and varying rates.[24,25] Under experimental conditions, chronic melatonin administration was demonstrated to be cardioprotective, which can be attributed to its free-radical scavenging and antioxidant properties.[26,27] Melatonin has also been suggested to be atheroprotective and may slow the progression of atherosclerotic development.[28] Melatonin has been shown to act as a vasoconstrictor in the caudal artery and a vasorelaxant in the mesenteric artery and aorta.[25] In addition, melatonin treatment has not been associated with any toxic effects.[29]

In view of the above, this study aimed to address a considerable knowledge gap related to the putative beneficial effects of rooibos on nicotine-induced vascular injury and oxidative stress. It is of particular interest and importance to investigate whether medicinal plants such as rooibos may protect the vascular endothelium by countering the harmful effects of increased ROS production associated with nicotine exposure and restoring the release of NO. Melatonin was included in the study as it is known to be a potent antioxidant and cardioprotective molecule, hence, it served as a control for rooibos.

Methods

Ethics approval was received from Stellenbosch University; project number SU-ACUM12-00041. Experiments were conducted according to the Revised South African National Standard for the Care and Use of Animals for Scientific Purposes (South African Bureau of Standards, SANS 10386, 2008).

A total of 90 adult male Wistar rats, weighing between 220 and 310 g at the start of the study, were housed in the central animal facility of the Faculty of Medicine and Health Sciences, Stellenbosch University. Animals were housed at room temperature (23°C ± 2°C) under normal 12-hour light and 12-hour dark cycles with free access to rat chow and fluids, and allowed to adapt to laboratory conditions for seven days prior to the start of treatment. Animals were randomly assigned to treatment groups of 10 rats per group in order to prevent bias, and individually caged in order to monitor fluid intake. The experimental rats were weighed daily.

Nicotine [(-)-nicotine, Sigma-Aldrich, St. Louis, MO, USA] was dissolved in sterile 0.9% physiological saline and injected subcutaneously. Physiological saline (0.9%) served as the vehicle control for nicotine and was also injected subcutaneously. Rooibos (2% fermented and unfermented) was a gift from the Promec Unit of the South African Medical Research Council and was prepared according to a standard laboratory protocol.[30] Rooibos solution served as the drinking fluid in the cages housing the rats assigned to the rooibos experimental groups. Melatonin (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 1 ml absolute ethanol and then added to the drinking water at a final concentration of 0.05% (v/v) ethanol with melatonin, as previously described.[27] Fresh melatonin preparations were supplied on a daily basis and rat fluid intake was monitored daily to ensure that the correct concentration of melatonin was received. The melatonin solution served as the drinking fluid in the cages housing the rats assigned to the melatonin experimental groups. See Table 1 for the treatment groups, as well as their abbreviations, used in the remainder of the text.

At the end of the six-week treatment period, the rats were fasted overnight and euthanised with an overdose of sodium pentobarbital (160 mg/kg) by means of intra-peritoneal injection. Blood was collected and allowed to clot on ice for 30 minutes, after which it was centrifuged at 1 200 g for 10 minutes at 4°C and the serum was aspirated. Liver tissue was excised, rinsed in saline solution, blotted dry and snap frozen in liquid nitrogen. Serum and snap-frozen liver tissue were then stored at –80°C for subsequent analysis. The aorta was excised and immediately used for vascular contraction/relaxation studies.

Biochemical analysis of rooibos

The soluble solid content of the rooibos preparation was determined gravimetrically (six repetitions each) after drying 1-ml aliquots at 70°C for 24 hours, and these were subsequently placed in a desiccator for 24 hours. Total polyphenol content and analysis for known flavonoid compounds were determined by the ARC Infruitec-Nietvoorbij, Post-Harvest Wine Technology Division, Stellenbosch, South Africa. The total polyphenol content was determined using the Folin-Ciocalteu's phenol reagent, as described by Arthur *et al*.[31] The absorbance was read at 765 nm and expressed as mg gallic equivalents per mg soluble solids.

Table 1. Treatment groups and abbreviations

Group	*Abbreviation*
Control groups (*n* = 10/group)	
Saline vehicle control	Veh control
Drinking control (tap water)	Water control
Melatonin 4 mg/kg bw/day	Mel
2% fermented rooibos	RF
2% unfermented rooibos	RUF
Treatment groups (*n* = 10/group)	
Nicotine 5 mg/kg bw/day	Nicotine
Nicotine 5 mg/kg bw/day + melatonin 4 mg/kg bw/day	NMel
Nicotine 5 mg/kg bw/day + 2% fermented rooibos	NRF
Nicotine 5 mg/kg bw/day + 2% unfermented rooibos	NRUF

bw: body weight.

Analysis for known flavonoid compounds was determined according to an established HPLC method.[32] Flavonol content was spectrophotometrically determined using a Spectronic® 20 GenesysTM photospectrometer (Spectronic Instruments, Leeds, UK) at 360 nm, according to a standard protocol,[33] utilising quercetin as standard. Both quercetin and rooibos were diluted in 95% ethanol.

Flavanol content was determined at 640 nm using a Spectronic® 20 GenesysTM photospectrometer (Spectronic Instruments, Leeds, UK) according to a standard protocol,[33] using the 4-(dimethylamino)-cinnamaldehyde (DAC) reaction. DAC and rooibos were dissolved in HCl-MeOH (1:3). Catechin was dissolved in methanol to prepare a 0.05% solution and this served as standard for the flavanol determinations. For both flavonol and flavanol determinations, the optimal dilution factor of rooibos was determined and subsequent analysis was performed in triplicate.[33]

Ex vivo investigations: aortic ring isometric tension studies

The thoracic aorta was excised and immediately placed in ice-cold Krebs Henseleit buffer (KHB, composition in mM: NaCl 119, $NaHCO_3$ 25, KCl 4.75, KH_2PO_4 1.2, $MgSO_4.7H_2O$ 0.6, Na_2SO_4 0.6, $CaCl_2.H_2O$ 1.25 and glucose 10). All connective tissue and perivascular fat were removed and the aorta was cut into 3–4-mm segments and mounted in a 25-ml organ bath containing oxygenated (95% O_2 and 5% CO_2) KHB and maintained at 37°C. The rings were equilibrated for 30 minutes under a resting tension of 1.5 g. The tension (in grams of tension) of the aortic ring was recorded with an isometric force transducer (TRI202PAD, Panlab, ICornellà, BCN, Spain) and the data were analysed with LabChart 7 software (Dunedin, New Zealand).

Following the initial equilibration period, aortic rings were exposed to a first round of contraction (100 nM phenylephrine; Phe) (Sigma-Aldrich, St Louis, MO, USA) and relaxation (10 µM acetylcholine; ACh) (Sigma-Aldrich, St Louis, MO, USA) in order to establish the functionality of the endothelium. Following wash-out of the Phe and ACh, the aortic rings were equilibrated for a further 30 minutes.

The contractile response of the aortic rings was determined at cumulative concentrations of Phe (100 nM – 1 µM). After each addition of Phe, a plateau response was reached before the addition of the next dose. At the end of the plateau phase of the final Phe concentration (1 µM), the rings were subjected to cumulative concentrations of ACh (30 nM – 10 µM) to induce relaxation. The final concentration of ACh resulted in maximum percentage relaxation of contraction and was the endpoint of the experiment. The relaxation responses to ACh were expressed as a percentage of the contraction caused by the final Phe concentration (1 µM).

Antioxidant enzyme activity

The activities of superoxide dismutase (SOD) and catalase (CAT) were determined in liver tissue. Liver tissue homogenates were prepared in phosphate buffer-containing microcentrifuge tubes using the Bullet Blender 24 and 0.5-mm zirconium oxide beads (Next Advance, NY, USA). The supernatant was collected after centrifugation at 12 000 rpm for 20 minutes and aliquots were stored at –80°C until the day of analysis.

SOD activity was determined using a commercially available superoxide dismutase assay kit (Cayman Chemical Company, Ann Arbor, MI, USA), which measured total SOD (Cu/Zn and Mn) of mammalian tissue. One unit (U) of SOD was defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide free radical. Tetrazolium salt was used for the detection of superoxide radicals, and bovine erythrocyte SOD (Cu/Zn) served as standard.

The protocol by Ellerby and Bredesen was adapted for use in a 96-well plate to determine CAT activity.[34] In a 96-well clear UV plate, 5 µl diluted sample and 170 µl buffer (50 mM potassium phosphate, pH 7.0) were added, where after 0.1% H_2O_2 was added to initiate the reaction. The linear decline in absorbance was monitored every 30 seconds at 240 nm for five minutes in a FLUOstar Omega Microplate Reader (BMG Labtech, Offenburg, Germany). CAT activity (µmole/min/µg protein) was determined using the molar extinction coefficient of 43.6/M/cm.

Lipid peroxidation

Thiobarbituric acid reactive substances (TBARS) were measured by spectrophotometric methods using a Labsystems multiscan MS analyser (AEC Amersham Co, South Africa) according to a method described previously.[34] Serum samples (200 µl) were mixed with 10 µl butylated hydroxytoluene (BHT) (Fluka Chemie, Switzerland) in ethanol (85%) (Merck Chemicals, South Africa) and orthophosphoric acid (15 mol/l) (Sigma-Aldrich, St Louis, MO, USA) buffer at pH 3.6 and vortexed. Thiobarbituric acid (TBA) (Sigma-Aldrich, St Louis, MO, USA) reagent (25 µl) was added and vortexed again. After incubation at 90°C for 45 minutes in a water bath, the reaction was terminated by placing the tubes on ice.

TBARS were extracted with n-butanol, saturated NaCl (50 µl) was added and the mixture was centrifuged at 12 000 rpm for one minute. Absorbance was read at 532 nm and values were expressed in µmol/l of serum.

Supplementary *in vitro* investigations

Adult rat aortic endothelial cell (AEC) cultures were purchased from VEC Technologies (Rensselear, New York, USA) and received in culture. Cell cultures were maintained in a standard tissue culture incubator (Forma Series II, Thermo Electron Corporation, Waltham, MA, USA) at an atmospheric composition of 21% O_2, 5% CO_2, 40–60% humidity, and temperature was maintained at 37°C. The endothelial cell growth medium (EGM-2, Clonetics, Cambrex Bio Science, Walkersville, USA) was supplemented with 10% FBS (Highveld Biological, Lyndhurst, South Africa) and standard endothelial growth factors [vascular endothelial growth factor (VEGF), human epidermal growth factor (hEGF), long-chain human insulin-like growth factor (R3-IGF-1), human fibroblastic growth factor (hFGF), hydrocortisone, antibiotics (gentamicin and amphotericin B) and ascorbic acid] according to the manufacturer's instructions. Cells were grown to confluency, as determined by microscopic evaluation and passaging to the next generation was performed in a 1:2 ratio.

Cells grown to confluency were exposed to 100 µM for 24

Table 2. Soluble solid, total polyphenolic, flavonol and flavanol content of 2% fermented and 2% unfermented rooibos

Variables	*2% RF*	*2% RUF*	*NRF*	*NRUF*
Soluble solids (mg/ml)	3.50 ± 0.22	4.60 ± 0.40#		
Total phenolic content (mg gallic acid/mg soluble solids)	0.16 ± 0.01	0.23 ± 0.03#		
Daily total phenolic intake (mg gallic acid equivalents/day/100 g bw)	5.17 ± 0.28	9.43 ± 0.46#	4.86 ± 0.31	8.07 ± 0.26#
Flavonol content (mg quercetin equivalents/mg soluble solids)	0.36 ± 0.02@	0.18 ± 0.02		
Daily flavonol intake (mg quercetin equivalents/ day/100 g bw)	1.11 ± 0.06@	0.74 ± 0.04	1.04 ± 0.07@	0.63 ± 0.02
Flavanol content (mg catechin equivalents/mg soluble solids)	0.05 ± 0.00	0.10 ± 0.01#		
Daily flavanol intake (mg catechin equivalents/ day/100 g bw)	0.10 ± 0.01	0.37 ± 0.02#	0.09 ± 0.01	0.32 ± 0.01#

bw: body weight; NRF: nicotine 5 mg/kg bw/day + 2% RF co-treatment; NRUF: nicotine 5 mg/kg bw/day + 2% RUF co-treatment.
#$p < 0.05$ vs 2% RF treatment groups; @$p < 0.05$ vs 2% RUF treatment groups; n = 5–6.

hours. Nicotine was diluted with phosphate-buffered saline (PBS). RF was freeze dried in a FreeZone6 (Labconco, Kansas City, MO, USA) freeze drier to remove the aqueous fraction. Freeze-dried RF was made up to a 20 mg/ml stock solution in cell culture medium and further diluted in cell culture medium to a concentration of 0.015 mg/ml. Cells were co-treated with nicotine and RF. In all cases, cells were examined for NO production and necrosis.

Flow cytometric analysis: NO production was measured by 4,5-diaminofluorescin-2 diacetate (DAF-2/DA) fluorescence (Calbiochem, San Diego, CA, USA) according to a previously established protocol.[36,37] Diethylamine NONOate diethylammonium salt (DEA/NO) served as positive control. Propidium iodide (PI, Sigma-Aldrich, St Louis, MO, USA) was used to determine necrosis,[38] and osmotic stress-induced cell injury served as a positive control.

Statistical analysis

All data are expressed as mean ± standard error of the mean (SEM). When comparisons between two groups were made, an unpaired *t*-test was performed. For multiple comparisons, the ANOVA (two-way where appropriate), followed by the Bonferroni correction, was applied. A *p*-value < 0.05 was considered significant. All data were analysed using GraphPad Prism® 5 software (GraphPad Software, San Diego, CA, USA). All aortic ring isometric tension data are expressed as the percentage contraction from a resting tension of 1.5 g or percentage relaxation of maximum contraction, respectively. For *in vitro* investigations, controls were adjusted to 100% and values are expressed as a percentage of the controls.

Results

Biochemical analysis of rooibos

RUF had a significantly higher soluble solid content and total polyphenolic content compared to RF, while the daily total phenolic intake of the RUF treatment groups (2% RUF, and 2% RUF and 5 mg/kg bw/day nicotine co-treatment) was also significantly higher than that of the RF treatment groups (2% RF, and 2% RF and 5 mg/kg bw/day nicotine co-treatment) (Table 2).

RF had a significantly higher flavonol content than RUF. The daily flavonol intake of the RF treatment groups was also significantly higher than that of the RUF treatment groups (Table 2), while RUF had a significantly higher flavanol content than RF. The daily flavanol intake of the RUF treatment groups was significantly higher than that of the RF treatment groups (Table 2). Values of known flavonoid compounds, as determined by HPLC analysis, are given in Table 3.

Ex vivo investigations: aortic ring isometric tension studies

The vascular function of all treatment groups was assessed by means of aortic ring isometric tension studies. The experimental protocol consisted of cumulative additions of Phe and ACh to test the functionality of the endothelium. Aortic rings from the nicotine-treated rats showed a significant pro-contractile response to Phe administration when compared to the saline vehicle control (Fig. 1A), with E_{max} values of 131.3 ± 17.33% (nicotine) vs 102.9 ± 4.99% (vehicle control), but Phe had no significant effect on relaxation (Fig. 1B). Aortic rings from Mel-treated rats (E_{max} value of 78.06 ± 7.39%) showed a significant anti-contractile response to Phe administration when compared to the water control, RF and RUF treatment groups (Fig. 2A) (E_{max} values of 110.9 ± 10.64, 112.9 ± 9.67 and 108.3 ± 8.11%, respectively). Aortic rings from Mel, RF and RUF treatment rats (E_{max} values of 86.62 ± 4.5, 70.84 ± 6.91 and 79.94 ± 7.01%, respectively) showed a significant pro-relaxation response to ACh administration when compared to the water control group (E_{max} value of 63.28 ± 4.03%) (Fig. 2B).

Aortic rings from NMel, NRF and NRUF treatment rats (E_{max} values of 84.64 ± 6.67, 109.2 ± 9.87 and 110.2 ± 6.29%,

Table 3. HPLC quantification of flavonoids in 2% fermented and 2% unfermented rooibos consumed by rats

	2% fermented rooibos		*2% unfermented rooibos*	
Flavonoid compounds	*% of soluble solids*	*Daily intake (mg/100 g bw)*	*% of soluble solids*	*Daily intake (mg/100 g bw)*
Phenylpyruvic acid-2-O-glucoside (PPAG)	0.391 ± 0.03	0.124 ± 0.01	0.361 ± 0.04	0.148 ± 0.01
Aspalathin	0.221 ± 0.01	0.070 ± 0.01	8.907 ± 1.05	3.645 ± 0.18
Nothofagin	0.051 ± 0.01	0.016 ± 0.00	1.311 ± 0.15	0.537 ± 0.03
Isoorientin	0.933 ± 0.06	0.295 ± 0.02	1.478 ± 0.17	0.605 ± 0.03
Orientin	0.842 ± 0.05	0.266 ± 0.01	1.132 ± 0.13	0.463 ± 0.02
Ferulic acid	0.055 ± 0.01	0.017 ± 0.00	not detected	
Quercetin-3-robinobioside	0.562 ± 0.04	0.178 ± 0.01	0.395 ± 0.05	0.162 ± 0.01
Vitexin	0.165 ± 0.01	0.052 ± 0.01	0.168 ± 0.02	0.069 ± 0.01
Hyperoside	0.156 ± 0.01	0.050 ± 0.01	0.066 ± 0.01	0.027 ± 0.01
Rutin	0.047 ± 0.01	0.015 ± 0.00	0.313 ± 0.04	0.128 ± 0.01
Isovitexin	0.168 ± 0.01	0.053 ± 0.01	0.224 ± 0.03	0.091 ± 0.01
Isoquercitrin	0.106 ± 0.01	0.034 ± 0.01	0.099 ± 0.01	0.041 ± 0.01
Luteolin-7-glucoside	0.024 ± 0.01	0.008 ± 0.00	0.031 ± 0.01	0.013 ± 0.00

bw: body weight; n = 5–6.

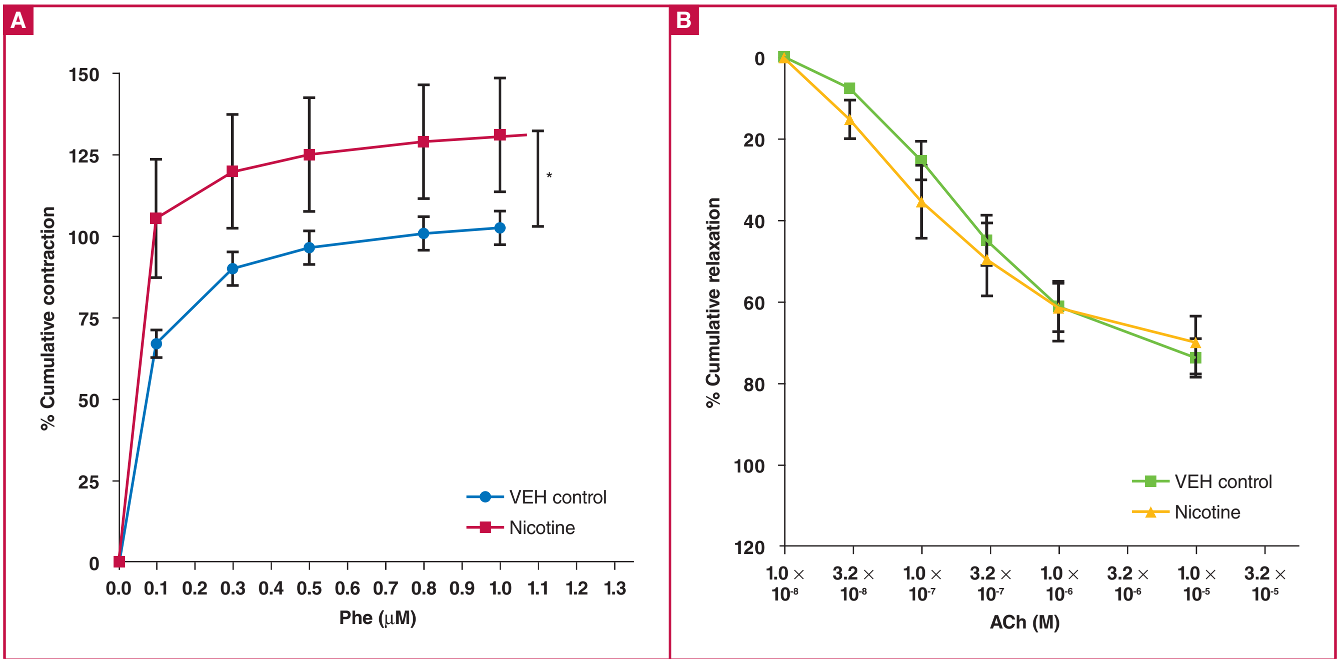

Fig. 1. (A) Contractile responses of aortic rings harvested from the veh control and nicotine-treated rats following cumulative Phe administration (*$p < 0.05$ nicotine vs veh control). (B) relaxation response of aortic rings harvested from veh control and nicotine treated rats following cumulative ACh administration.

respectively) showed a significant anti-contractile response to Phe administration when compared to the nicotine-treated group (E_{max} value of 131.3 ± 17.33%). Additionally, aortic rings from NMel-treated rats also showed a significant anti-contractile response to Phe administration when compared to the NRF- and NRUF-treated groups (Fig. 3A). Aortic rings from NMel- and NRF-treated rats (E_{max} values of 93.11 ± 3.28 and 89.60 ± 5.96%, respectively) showed a significant pro-relaxation response to ACh administration when compared to the nicotine- and NRUF-treated groups (E_{max} values of 69.8 ± 6.02 and 70.55 ± 6.49%, respectively) (Fig. 3B).

Antioxidant enzyme activity

Nicotine has a high affinity for the liver[39] and is also metabolised by the liver.[40] It has previously been demonstrated that nicotine

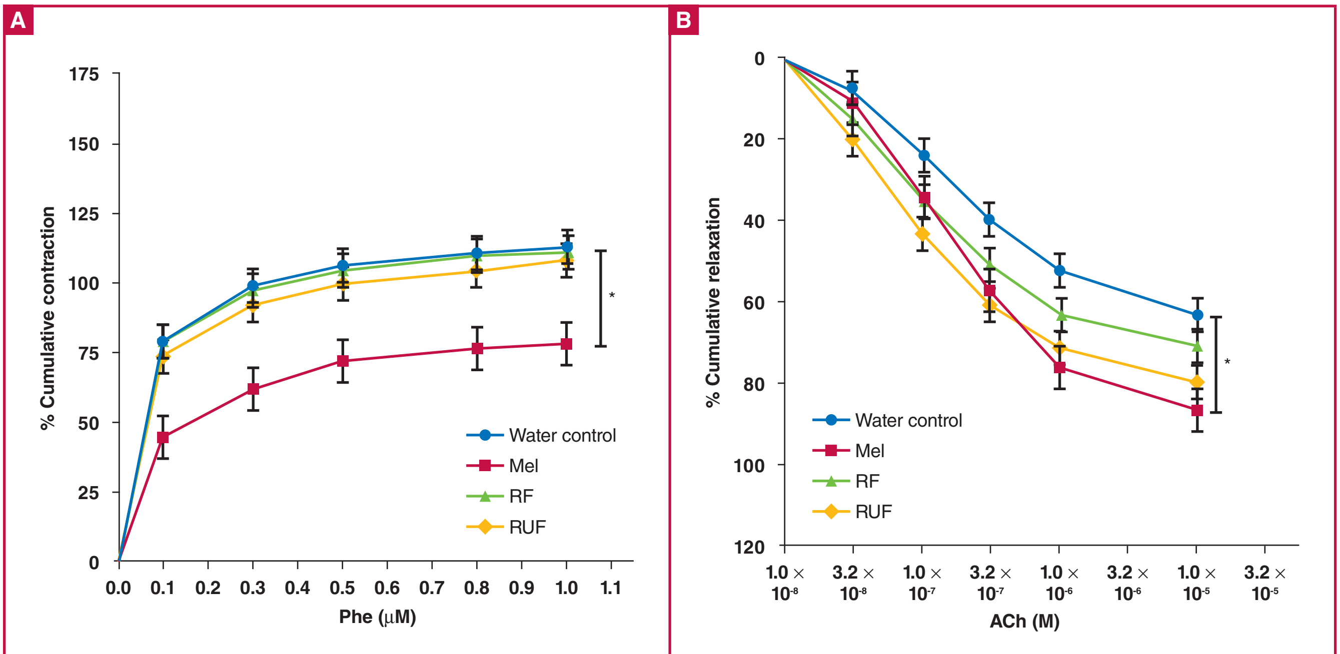

Fig. 2. (A) Contractile responses of aortic rings from Mel, RF, RUF and water control animals following cumulative Phe administration (*$p < 0.05$ Mel vs RF, RUF and water control). (B) Relaxation response of aortic rings harvested from Mel, RF, RUF and water control-treated rats following cumulative ACh administration. (*$p < 0.05$ water control vs Mel, RF and RUF).

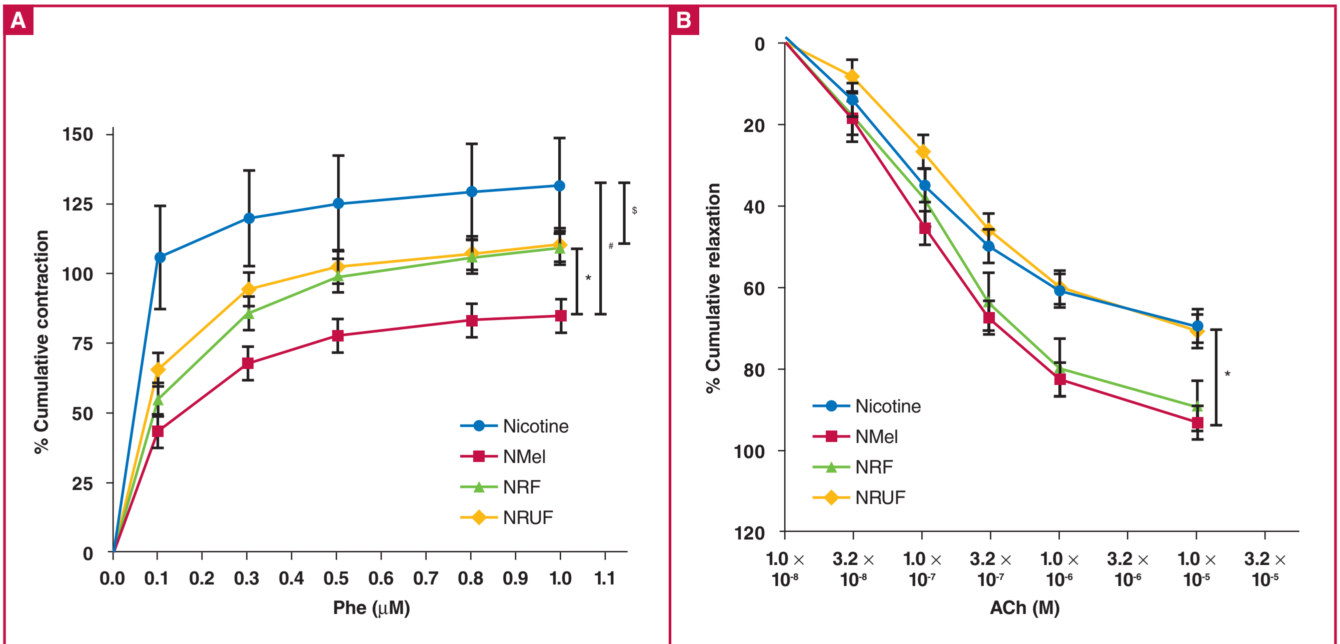

Fig. 3. (A) Contractile responses of aortic rings harvested from nicotine, NMel, NRF and NRUF-treated rats following cumulative Phe administration (*$p < 0.05$ NMel vs NRF and NRUF; #$p < 0.05$ NMel vs nicotine; $$p < 0.05$ NRF, NRUF vs nicotine). (B) Relaxation response of aortic rings harvested from nicotine, NMel, NRF and NRUF-treated rats following cumulative ACh administration (*$p < 0.05$ NMel and NRF vs nicotine and NRUF).

treatment resulted in a decrease in SOD[41,42] and CAT[42] activity in the liver, compared to untreated controls. Our results indicate that SOD activity in liver tissue homogenates was significantly increased in the veh control, RF, NMel and NRF groups compared to the nicotine-treated group. SOD activity was also increased in the RF and RUF groups compared to the water control. Additionally, SOD activity in the RF group was increased when compared to the Mel group (Table 4). CAT activity in liver tissue homogenates was significantly increased in the veh control, Mel, NMel and NRF groups compared to the nicotine-treated group. CAT activity was also increased in the veh control group compared to the water control (Table 4).

Lipid peroxidation

TBARS levels in serum of the nicotine-treated group were significantly increased when compared to the RF, veh control, water control, RUF, Mel and NMel treatment groups. TBARS levels were also significantly increased in the NRF- and NRUF-treated groups compared to the RF treatment group (Table 5).

Supplementary *in vitro* investigations

Based on the effects of RF on nicotine-induced vascular changes in the *in vivo* investigations, RF was selected for performing additional *in vitro* investigations. According to separate dose–response experiments for the NO production and necrosis investigations (data not shown), nicotine was used at a concentration of 100 µM and RF at a concentration of 0.015 mg/ml. Nicotine at a concentration of 100 µM over a treatment period of 24 hours resulted in significant reduction in NO

Table 4. Effects of melatonin and rooibos (fermented and unfermented) treatment on SOD activity (U/mg protein) and CAT activity (µmole/min/µg) in liver tissue homogenates of all treatment groups

Treatment group	*SOD activity (U/mg protein)*	*CAT activity (µmole/min/µg)*
Veh control	155.3 ± 6.7*	868.3 ± 138.4*#
Water control	135.5 ± 5.0	427.2 ± 51.4
Nicotine	121.4 ± 14.7	295.8 ± 76.7
Mel	133.4 ± 10.4	597.7 ± 98.2*
RF	192 ± 21.1*#$	597.5 ± 111.7
RUF	182.7 ± 20.4#	515.6 ± 111.9
NMel	180.8 ± 9.0*	565.8 ± 87*
NRF	160.7 ± 6.4*	727.3 ± 158.6*
NRUF	164.7 ± 16.6	191.9 ± 27.6

Values are mean ± SEM of 9–10 rats per group.
*Significantly different compared to nicotine treatment group ($p < 0.05$);
#significantly different compared to water control group ($p < 0.05$);
$significantly different compared to Mel treatment group ($p < 0.05$).

Table 5. Effects of melatonin and rooibos treatment on lipid peroxidation in serum of all treatment groups

Treatment groups	*TBARS (µmol MDA equivalents/l)*
Veh control	2.907 ± 0.2
Water control	2.997 ± 0.3
Nicotine	4.615 ± 0.3#$&@!*
Mel	2.829 ± 0.9
RF	2.472 ± 0.3
RUF	3.350 ± 0.3
NMel	3.411 ± 0.3
NRF	3.772 ± 0.3#
NRUF	3.707 ± 0.1#

Values are mean ± SEM of 10 rats per group.
#Significantly different compared to RF ($p < 0.05$); $significantly different compared to veh control ($p < 0.05$); &significantly different compared to water control ($p < 0.05$); @significantly different compared to RUF ($p < 0.05$); !significantly different compared to Mel ($p < 0.05$); *significantly different compared to NMel ($p < 0.05$).

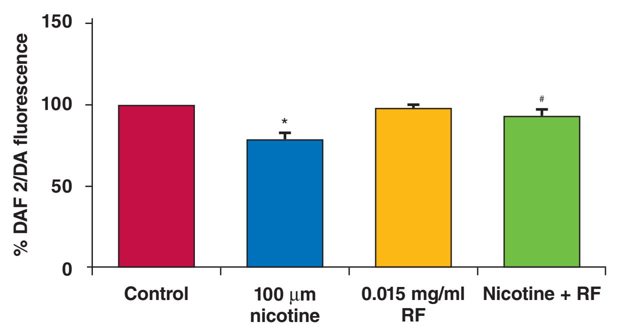

Fig. 4. The effects of RF pre-treatment on NO-production as measured by DAF-2/DA fluorescence. $^{*}p < 0.05$ vs control; $^{\$}p < 0.05$ vs 100 µM nicotine (n = 6– 8 per group).

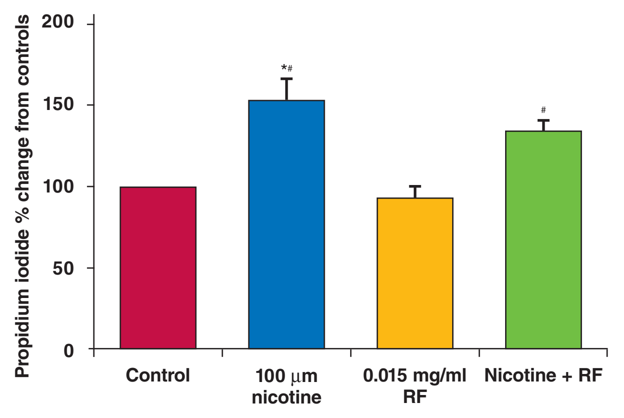

Fig. 5. The effects of RF pre-treatment on cell viability. Percentage change in necrosis indicated by PI fluorescence. $^{*}p < 0.05$ compared to control. $^{\#}p < 0.05$ compared to 0.015 mg/ml RF; (n = 6–8 per group).

production, as indicated by DAF-2/DA fluorescence (Fig. 4), and an increase in necrosis, as indicated by PI fluorescence (Fig. 5), when compared to controls.

AECs were pre-treated for one hour with 0.015 mg/ml RF, followed by the addition of 100 µM nicotine for a further 24 hours. Pre-treatment with 0.015 mg/ml RF was associated with a modest but significant increase in NO production in nicotine-injured cells compared to cells treated with nicotine only, as indicated by DAF-2/DA fluorescence (Fig. 4). However, pre-treatment with 0.015 mg/ml RF was not able to significantly reduce necrosis in nicotine-injured cells, as indicated by PI fluorescence (Fig. 5).

Discussion

To the best of our knowledge, this is the first study to investigate the effects of rooibos, both fermented and unfermented, in a rat model of nicotine-induced vascular changes and oxidative stress. The protective effects of RF and RUF were compared to the known beneficial effects of the potent antioxidant and free-radical scavenger, melatonin.

Exposure to 5 mg/kg bw/day nicotine over a six-week treatment period resulted in increased vascular contractility in aortic rings and a reduction in antioxidant enzyme activity (SOD and CAT) in liver tissue. Lipid peroxidation, as indicated by TBARS levels, was increased in serum samples of nicotine-exposed rats, therefore indicating that nicotine increases oxidative stress. The harmful vascular endothelial effects of nicotine were further characterised in a model of cultured rat AECs, where nicotine treatment (100 nM; 24 hours) was associated with reduced NO production and reduced cell viability.

In vascular studies, when RF (2%) and melatonin (4 mg/kg bw/day) were co-administered with nicotine, the harmful pro-contractile effects observed in aortic rings from rats treated with nicotine only were attenuated. Additionally, endothelium-dependent vasorelaxation was significantly enhanced in groups co-treated with RF and melatonin. The effects of RUF were limited to reducing contractility in aortic rings of nicotine-treated animals. Furthermore, co-administration of RF and melatonin with nicotine resulted in increased SOD and CAT activity in liver tissue of rats compared to those treated with nicotine only, whereas co-administration of RUF with nicotine did not result in any significant increase in SOD or CAT activity. Co-treatment with melatonin additionally decreased lipid peroxidation. In the nicotine-injured AECs, pre-treatment with RF (0.015 mg/ml) significantly increased NO production.

Nicotine-induced vascular changes and oxidative stress have previously been demonstrated by others. Nicotine exposure resulted in pro-contractile responses in the aortic rings of rats, where aortic rings were challenged with Phe[43] or KCl[44,45] to elicit contractile responses. Furthermore, exposure to nicotine resulted in decreased SOD activity in the liver and increased lipid peroxidation in Sprague-Dawley rats,[41] as well as decreased CAT activity, when compared to untreated controls in Wistar rats.[42] In these studies, oxidative damage, resulting in impaired integrity of the vascular endothelium, was suggested as a possible mechanism of action.[43-45]

It is, to the best of our knowledge, the first time that oral ingestion of RF over a period of six weeks has been demonstrated to improve vascular endothelial function, associated with increased activity of important antioxidant enzymes, in a rat model of nicotine-induced injury. The potential of rooibos to enhance antioxidant defences, including SOD and CAT activity, has previously been demonstrated in rat brain extracts in an immobilisation stress model,[46] while SOD levels were significantly higher in RUF-treated animals in a rat colitis model.[47]

These actions have been attributed to the flavonoid content in rooibos[46] and the potential ability of rooibos to reduce DNA damage caused by oxidative reactions.[47] Epidemiological evidence suggests that dietary-derived antioxidants have the potential for disease prevention,[48] and it has been shown that dietary polyphenols can increase endothelium-dependent NO generation by modulating cellular sensors for oxidative stress. NO is capable of reacting with $O_2^{\cdot-}$ to form peroxynitrite, which can lead to the nuclear accumulation of nuclear factor erythroid 2-related factor (Nrf2).[49] Nrf2 is a redox-sensitive transcription factor, involved in antioxidant response element (*ARE*)-dependent gene expression,[50] and under conditions of oxidative stress, Nrf2 is capable of activating *ARE*-dependent transcription of phase II and antioxidant defence enzymes, such as glutathione-*S*-transferase, GPx and heme-oxygenase-1.[51]

Although the beneficial effects of RF on vascular endothelial function and oxidative stress were comparable to those observed with melatonin, the effects of RUF treatment were more modest and limited to vascular contractility only. The difference in the effects of RF and RUF is particularly interesting, since the phytochemical content of rooibos changes considerably during the fermentation process. The main difference was in the aspalathin and nothofagin contents, which were considerably higher in RUF. This is consistent with previous findings showing that the amount of aspalathin can decrease by 98% during fermentation.[52]

However, in the present study, the antioxidant and free-radical scavenger ferulic acid was found to be present in RF, but not RUF. Ferulic acid is a potent antioxidant and free-radical scavenger,[53] which also possesses blood pressure-lowering effects.[54] It has also been suggested that ferulic acid has multifactorial vasodilating effects, involving reduction of angiotensin II and activation of eNOS, leading to an increase in NO levels.[55] The presence of ferulic acid could therefore help to explain the modulatory capacity of RF in this experimental setting of nicotine-induced vascular injury.

The modulatory capabilities of melatonin were expected, since melatonin is a known antioxidant and free-radical scavenger and the effects of melatonin to reduce or abolish vascular injury have previously been demonstrated. Our findings support previous data by showing that melatonin was capable of decreasing contraction and enhancing relaxation in the aortas of nicotine-treated animals. The pro-relaxation action of melatonin in aortic ring studies was first demonstrated in the rabbit aorta,[56] and it has been suggested that melatonin could enhance endothelium-dependent vasodilation, which could be explained by the enhancement of the vascular NOS pathway.[57]

A four-week melatonin treatment period has previously been shown to increase SOD activity in liver tissue of nicotine-treated rats,[58] while an eight-week treatment period increased SOD activity in liver tissue in a fructose-induced model of the metabolic syndrome.[59] In a rat model of renovascular hypertension, a nine-week treatment period with melatonin led to an increase in SOD and CAT activity in kidney and heart tissue.[60]

Even though both melatonin and rooibos exerted beneficial effects on the vascular system and increased antioxidant activity in nicotine-exposed rats, it is possible that melatonin and rooibos exert their effects through different mechanisms. It is, however, possible that these mechanisms result in a restoration of vascular homeostasis and, in particular, the function of NO.

The addition of Western blotting analysis of aortic rings could provide more information on the underlying cellular mechanisms of the different treatment groups. Proteins of interest that would add value to our understanding of the underlying mechanisms include eNOS, the main enzyme responsible for vascular production of NO, and protein kinase B (PKB)/AKT, a cell growth and survival protein and upstream activator of eNOS and an important anti-apoptosis protein. Furthermore, investigating the role of p22phox, a marker of NADPH-oxidase activity, which is an important vascular source of ROS and oxidative stress, may also further elucidate the cellular mechanism involved. Proteomic analysis of aortic rings to explore large-scale protein expression patterns and differential protein regulation could greatly contribute to a better understanding and identification of novel cellular pathways and mechanisms involved in vascular injury and protection.

Limitations of the study include the absence of blood pressure measurements in the rodent model, which would have provided clinically relevant data relating to vascular function, and should be considered in future studies. In addition, *in vitro* investigations into the effect of melatonin on nicotine-injured rat AECs would have supplied valuable insights into cellular mechanisms and are worth exploring.

Conclusions

Nicotine administration resulted in significant vascular and endothelial injury, associated with increased oxidative stress and reduced antioxidant activity. In a novel finding, our data showed that rooibos, specifically RF, exerted beneficial effects on the vascular and endothelial system of nicotine-exposed rats, and increased liver antioxidant enzyme activity. The results shown with RF are similar to those observed with melatonin, whose protective actions in the cardiovascular system are well established. However, RUF did not exert beneficial effects to the same extent as RF and melatonin, and was capable of reducing contractility in aortic rings of nicotine-treated animals only.

It is plausible that both RF and Mel exerted their beneficial vascular effects through their antioxidant properties, although other mechanisms cannot be ruled out. Restoration of vascular homeostasis, underscored by eNOS activation and subsequent increased release of NO, as shown in the cultured cell experiments, may also underlie the protective actions of both rooibos and melatonin. Based on the data presented in this study, fermented rooibos may show promise as a future cost-effective therapeutic option on its own or as adjuvant therapy in combatting the harmful effects of nicotine exposure on the vasculature system, endothelium and redox status.

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