Cardiovascular Topics

The cardiovascular effects of *Aspalathus linearis* supplementation in male Wistar rats receiving fixed-dose combination first-line antiretroviral therapy

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Abstract

HIV-infected populations receiving antiretroviral therapy (ART) have an increased risk of cardiovascular disease. The beneficial cardiovascular effects of rooibos are well described; however, it is unknown whether rooibos ameliorates harmful ART-induced cardiovascular side effects. We investigated the cardiometabolic effects of rooibos co-treatment in rats receiving ART (efavirenz, emtricitabine, tenofovir) for nine weeks. Rooibos treatment reduced total cholesterol levels; however, triglyceride, phospholipid and thiobarbituric acid-reactive substance levels were unaffected by ART, rooibos or combination treatment. In isolated hearts exposed to ischaemia–reperfusion injury, ART resulted in increased infarct sizes compared to controls, which was not observed when co-treated with rooibos. Vascular studies showed reduced aortic relaxation with ART, and improved relaxation when co-treated with rooibos. In conclusion, we show that rooibos treatment reduced total cholesterol levels in control rats, and that rooibos co-treatment ameliorated the harmful ART-induced cardiovascular effects. These findings are novel and warrant further studies into underlying mechanisms and clinical relevance.

Keywords: HIV, antiretrovirals, cardiovascular, *Aspalathus linearis,* vascular reactivity

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Highly active antiretroviral therapy (HAART) was introduced in 1996 to target the replication and spread of the human immunodeficiency virus (HIV).[1] These drug combinations have become very effective and now comprise three or more drugs in a single tablet.[2,3] The effectiveness of HAART is evidenced in the decreased rate of HIV-related mortalities since its introduction.[1] In South Africa, particularly, an increased roll-out of antiretroviral drugs in 2005 was associated with a significant decline in AIDS-related deaths in the short term. ART, especially the protease inhibitor (PI) class, has been associated with increased cardiovascular complications; however, findings remain contradictory and more studies are needed for it to be conclusive.[4-6]

In 2012 the South African Department of Health approved the use of a new first-line ART consisting of the nucleoside reverse transcriptase inhibitors (NRTIs): tenofovir diphosphate (TDF) and emtricitabine 5′-triphosphate (FTC), and the non-nucleoside reverse transcriptase inhibitor (NNRTI), efavirenz (EFV), in a single-tablet fixed-dose combination (FDC).[7] Studies investigating the long-term effects of this specific FDC are sparse. South Africa (SA) currently has the largest ART roll-out programme in the world and in 2016 became the first African country to implement pre-exposure prophylaxis (PrEP), specifically in the form of TDF or the combination of TDF/FTC, as a preventative treatment given to HIV-negative people.[8] The importance of extensive research into the long-term cardiovascular effects of the current first-line FDC ART regimen is, therefore, undeniably high. Not only is this research fundamental, but further extrapolations into the exact mechanisms and possible co-treatments will aid in the on-going battle against HIV/AIDS.

Prolonged use of HAART has been associated with toxicity and a number of detrimental effects on the body, including nephrotoxicity and lipodystrophy.[9,10] Furthermore, previous studies have linked the long-term use of ART to myocardial infarctions (MI) and increased risk of developing cardiovascular disease (CVD).[1,11,12] This may be caused by the HIV infection itself,[12] the immunological responses to the virus, or by the effects of HAART through its effects on both lipid and glucose metabolism.[13]

Reactive oxygen species (ROS) and oxidative stress have also been identified as key role players in the pathogenesis of CVD and an important precursor of CVD, endothelial dysfunction (ED). ED encompasses diminished production/availability of nitric oxide and/or a disparity in the endothelium-derived relaxing and contracting factors, which can lead to impaired endothelium-

dependent vasodilatation.[14,15] Viral infections, energy deprivation, oxidative stress or calcium depletion in the endoplasmic reticulum are known to induce an inflammatory response, which can result in ED.[14,15] The development of ED has been associated with protease inhibitor-induced toxicity in isolated vasculature models in particular; furthermore, subsequent CVD and long-term (18 months) FDC ART treatment have also been associated with decreased endothelial function, as measured by percentage flow-mediated dilatation (FMD%).[16-18]

Antioxidants counter excessive ROS generation and the development of oxidative stress.[19] *Aspalathus linearis*, commonly known as rooibos, is a well-established source of antioxidants.[20] It is a plant that is indigenous to SA and its stems and leaves are used in a popular beverage, commonly known as 'rooibos tea'.[21] Rooibos does not contain caffeine or other stimulants, and has a high concentration of potent antioxidants, vitamins and minerals.[22] Its antioxidant-driven cardio-protective properties are well established and substantiated by scientific research.[20,23,24] These have, however, been poorly investigated in the context of ART-induced oxidative stress, even though a couple of studies have shown favourable effects of rooibos on suppressing HIV-binding to MT-4 cells.[25,26] The aims of this study, therefore, were to examine the effects of treatment with the first-line FDC drug on blood lipid levels and oxidative stress markers, myocardial ischaemic tolerance and vascular endothelial function in male Wistar rats, and furthermore, to determine whether co-treatment with an aqueous rooibos extract exerts protective effects.

Methods

Approval for the use of male Wistar rats for this study was obtained from the Research Ethics Committee for Animal Care and Use, Stellenbosch University (Protocol #: SU-ACUD14-00021). Rats were housed in the animal facility at Stellenbosch University. They were handled according to the institutional ethical guidelines and the Revised South African National Standard for the Care and Use of Animals for Scientific Purposes (South African Bureau of Standards, SANS 10386, 2008).

The animals had *ad libitum* access to standard rat chow and fluids (water or rooibos). They were housed in groups of three rats per cage under 12 hours light and 12 hours dark. The rats were weighed and assessed for hair loss and other signs of stress on a weekly basis.

The study made use of a total of 100 rats. They were randomly assigned into four treatment groups: (1) control (25 rats): gavaged with tap water and received tap water to drink; (2) rooibos (25 rats): gavaged with tap water and received rooibos to drink; (3) antiretroviral therapy (ART) control (22 rats): gavaged with ART and received tap water to drink; ART + rooibos (24 rats): gavaged with ART and received rooibos to drink.

A total of 65 rats were used for isolated heart perfusion studies and aortic ring isometric tension studies. Of these, 34 hearts were used to determine functional recovery by inducing global ischaemia and 31 hearts to determine infarct size through the induction of regional ischaemia. Twenty-two rats were fasted overnight and blood serum was collected from the thoracic cavity after sacrifice. A total of four rats were lost to gavage during the treatment period. The total treatment period was nine weeks of ART, rooibos or combination treatment.

ART drug preparation and treatment

Each FDC tablet [Odimune, Cipla MedPro (Pty) Ltd, Bellville, Western Cape, SA], containing the daily dose of active ingredients for an average human weighing 70 kg, was crushed and the human:rat dosage conversion was calculated at six-fold the human dosage, according to previously published guidelines.[27] The dose per rat was calculated weekly according to the average total body mass of the rats per cage (human daily dose: 600 mg EFV, 200 mg FTC and 300 mg TDF). The calculated dose of crushed powder was suspended in 1 ml tap water/rat/day and vortexed thoroughly. It was administered to the rats daily via oral gavage by a qualified laboratory animal husbandry professional. Vehicle control rats were gavaged with an equal volume of ART-free tap water.

Rooibos preparation and treatment

The rooibos leaves were mixed with freshly boiled tap water, left to steep for half an hour and then filtered to yield an aqueous extract of a final concentration of 2% (w/v), according to a previously published method.[28] The rooibos mixture was placed in light-resistant drinking bottles in the animal cages and substituted for the drinking water. The volume of the rooibos solution remaining in the bottles was measured weekly to calculate the amount of fluid consumed, and replenished with a fresh batch weekly (800–1 000 ml/week). The same was done with the normal drinking water of the control and ART groups.

Liquid chromatography mass spectrometry analysis was conducted on the rooibos infusions after applying different storage methods (fresh, fridge for one week or frozen at –20°C for more than one week). It showed no differences in polyphenol levels and the composition for all was comparable to that of rooibos infusions used in previously published data.[29] The rats were treated from four weeks of age for nine weeks, according to previously published rooibos treatment protocols in rats.[20]

Rat euthanasia, organ harvesting and blood collection

Rats were euthanised via an intraperitoneal injection of sodium pentobarbitone (160 mg/kg), conducted by a researcher authorised by the South African Veterinary Council (SAVC). Upon disappearance of the pedal reflex, a thoracotomy was performed to quickly remove the heart. The heart and thoracic aorta were carefully excised and placed in ice cold Krebs-Henseleit buffer (KHB) containing, in mM: NaCl 119; $NaHCO_3$ 24.9; KCl 4.74; KH_2PO_4 1.19; $MgSO_4$ 0.6; $NaSO_4$ 0.59; $CaCl_2$ 1.25; glucose 10. After excision of the heart, blood was collected from the chest cavity and placed in blood collection tubes on ice.

Blood samples were centrifuged at 2 000 × *g* for 15 minutes at 4°C, after which the serum and/ or plasma was removed and stored at –80°C. Serum and plasma were subsequently analysed at the Division of Chemical Pathology, University of Cape Town, for triglyceride (TG), phospholipid (PL) and thiobarbituric acid-reactive substance (TBARS) levels.

Lipid and TBARS analyses

Serum TG and PL levels were determined using commercially available enzymatic colorimetric kits [Wako LabAssay™ TG (290-63701) and PL (296-63801), Wako Chemicals, GmbH,

Germany] and read on a microplate reader (Spectra-Max Plus 384 with SoftMax Pro 4.8 data-acquisition and analysis software: Separations, Cape Town, SA). In principle, performing the assay involved pipetting the triglyceride standard and samples into a microtitre plate (Greiner: Merck, South Africa), adding an enzymatic colour reagent to each well and incubating at room temperature for 30 minutes before reading the absorbance at 600 nm.

The concentrations of TBARS were determined spectrophotometrically according to the method of Jentzsch *et al.*,[30] and calculated using the appropriate molar extinction coefficient. Briefly, 25 µl of 4-mM butylated hydroxytoluene and 200 µl of ortho-phosphoric acid was added to 200 µl of each plasma sample in 5-ml macrotube 5 tubes (MTCBio: Lasec, SA) and vortexed for 10 seconds. Twenty-five µl of thiobarbituric acid reagent was added, the tubes were vortexed as before and incubated at 90°C for 45 minutes, prior to cooling on ice. TBARS were extracted into 500 µl of butanol per tube, using saturated NaCl to facilitate phase separation. Each tube was centrifuged at 2 000 × *g* for one minute and the absorbance was read at 532 nm. The concentrations of TBARS were normalised to TG + PL concentrations.

Directly after sacrifice of each rat, the total cholesterol (TC) level was measured with the Cardiocheck PA® system (Polymer Technology Systems Inc, Indianapolis, USA), as previously described. This entailed a capillary being filled with blood and introduced into the analyser.[30]

Isolated rat heart perfusion technique and protocols

Myocardial ischaemic tolerance was determined by means of a regional ischaemia protocol to assess infarct development, and global ischaemia to assess functional recovery. In both protocols, hearts were mounted on the perfusion system and subjected to 10 minutes of retrograde perfusion in Langendorff mode at a constant pressure, followed by 20 minutes of working-heart mode at a preload of 15 cm H_2O and an afterload of 100 cm H_2O. Perfusions were performed with KHB. During mounting, the aortic cannula was inserted into the aorta and the second cannula into the left pulmonary vein to perfuse the heart via the left atrium during the 10 minutes of working-heart perfusion. The pressure transducer was inserted into the aortic outflow tract.

Regional ischaemia was induced for 35 minutes by ligation of the proximal left anterior descending coronary artery with a silk surgical suture. After 35 minutes, the ligature was loosened and reperfusion followed for 60 minutes (Fig. 1A). To measure risk zone and infarct size, the heart was stained with 0.5% Evan's Blue dye solution (Sigma, St Louis, MO, USA), frozen overnight and then cut into 2-mm slices. Slices were incubated with 1% w/v triphenyltetrazoliumchloride (TTC) [Merck (Pty) Ltd (Darmstadt, Germany)] in phosphate buffer solution for 15 minutes (pH 7.4) before being placed in 10% formalin.

The slices were analysed with computerised planimetry (UTHSCSA Image Tool program, University of Texas Health Science Center, San Antonio, Texas). Infarct size (IS) was expressed as a percentage of the area at risk (%AAR).

To induce global ischaemia, perfusion to the heart was completely shut off (coronary flow rate = 0 ml/min) for 20 minutes at a constant temperature of 36.5°C. This was followed by 30 minutes of reperfusion and freeze clamping (Fig. 1B). The

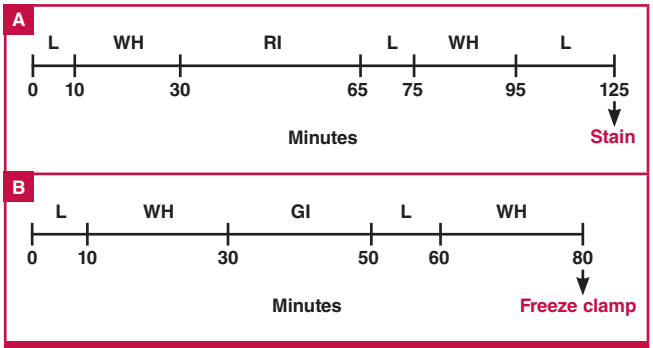

Fig. 1. Isolated rat heart ischaemia–reperfusion protocols. (A) Experimental protocol of regional ischaemia. (B) Experimental protocol of global ischaemia. L: Langendorff; WH: working heart; RI: regional ischaemia; GI: global ischaemia.

parameters recorded and calculated to determine mechanical function were: coronary flow rate (CF; ml/min), aortic output (AO; ml/min), peak systolic and diastolic pressures (PSP and PDP; mmHg), and heart rate (HR; bpm) recorded with a Viggo-Spectramed pressure transducer coupled to a computer system. The parameters were measured at the 20-minute baseline, and again at the end of the 30-minute recovery in working-heart mode. Cardiac output (CO) was calculated as follows:

CO (ml/min) = CF + AO.

Total work (TW) was calculated as a function of the CO and PSP:

TW = CO × PSP × 0.0022.[31]

Experimental protocol for vascular reactivity studies

After excision, the aorta was cleaned by removing all surrounding connective tissue and perivascular adipose tissue (PVAT). Great care was taken not to damage or stretch the aorta while cleaning. Next, a 3–4-mm ring segment was cut out and mounted between two steel hooks, one of which was connected to an isometric force transducer (TRI202PAD, Panlab, ICornellà, BCN, Spain). The ring was lowered into the organ bath (AD Instruments, Bella Vista, New South Wales, Australia) filled with 25 ml KHB and gassed with 95% O_2 and 5% CO_2 at 36.5–37°C. Tension in the aortic ring was recorded throughout the experiment using LabChart 7 software (Dunedin, New Zealand), as previously described in our laboratory.[32] Stock solutions of phenylephrine (Phe) and acetylcholine (Ach) were prepared daily by dissolving each drug in a 0.9% saline solution.

The protocol was initiated by a 30-minute stabilising period at a resting tension of 1.5 g with a KHB change every 10 minutes. This was followed by a test for contraction and relaxation with the addition of 100 nM Phe until the Phe-induced contraction curve reached a plateau, followed by 10 µM Ach administration to induce relaxation. Only rings displaying at least 70% relaxation of the maximum contraction were used for further experimentation. At this point, the organ bath was rinsed three times with fresh KHB, followed by another 30-minute stabilisation period. KHB was changed with pre-warmed buffer every 10 minutes.

Following this, aortic ring contraction was evaluated by adding cumulative concentrations of Phe to the waterbath (final Phe concentration after each of the five consecutive additions:

100, 300, 500, 800 nM and 1 µM). Once the contraction curve of the final Phe administration reached a plateau, relaxation was induced by the administration of cumulative Ach concentrations (final Ach concentration after each addition: 30, 100, 300 nM, 1 and 10 µM). The same protocol was followed for control, ART, rooibos and combination treatment aortas.

Statistical analyses

All data were analysed on GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA) using either the one-way or two-way analysis of variance (ANOVA), followed by Bonferroni's method for *post hoc* testing between selected groups. Relaxation of rings is expressed as percentage relaxation of the contraction reached with the final Phe concentration. Differences were regarded as statistically significant at a p-value of < 0.05; n values are displayed beneath the figures.

Results

Fluid intake was measured weekly per cage and calculated weekly per group. Although there were no significant differences between the groups in the weekly fluid intake over the experimental period, there was a trend for the ART + rooibos group to have all-round higher fluid consumption.

The rats were weighed at the start of the project, weekly and at the end of the nine-week protocol. No differences were seen in absolute body weight gain (weight gain = final weight minus starting weight) (control: 118.9 ± 7.09 g; rooibos: 124.1 ± 7.11 g; ART: 108.4 ± 7.72 g; ART + rooibos: 119 ± 8.77 g) or percentage weight gain (control: 67.63 ± 2.65%; rooibos: 65.45 ± 1.55%; ART: 70.58 ± 1.73% and ART + rooibos: 66.92 ± 1.95%).

There were no differences in the levels of TG (control: 0.8 ± 0.09 mmol/l); rooibos: 0.99 ± 0.26 mmol/l; ART: 0.61 ± 0.08 mmol/l; ART + rooibos: 0.83 ± 0.14 mmol/l), PL (control: 1.57 ± 0.07 mmol/l; rooibos: 1.44 ± 0.08 mmol/l; ART: 1.54 ± 0.10 mmol/l; ART + rooibos: 1.56 ± 0.14 mmol/l) and TBARS (control: 7.69 ± 0.55 µmol/l; rooibos: 7.49 ± 0.39 µmol/l; ART: 7.54 ± 0.74 µmol/l; ART + rooibos: 5.39 ± 0.53 µmol/l). TC levels were, however, significantly decreased in the rooibos group compared to the controls (control: 3.03 ± 0.16 mmol/l; rooibos: 2.48 ± 0.05 mmol/l; ART: 2.67 ± 0.13 mmol/l; ART + rooibos: 2.61 ± 0.16 mmol/l; $p = 0.05$; $n = 6$ per group) (Fig. 2).

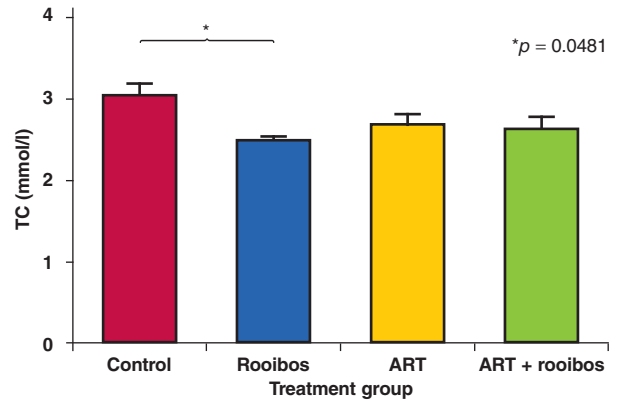

Fig. 2. Serum total cholesterol (TC) levels at the end of the nine-week treatment period. One-way ANOVA, $p < 0.05$; Bonferroni *post hoc* test, *$p = 0.04$; n = six per group.

Isolated heart perfusions: myocardial function and infarct size

The pre-ischaemic hearts from ART + rooibos showed significantly ($p = 0.007$) increased coronary flow rates compared to the ART group (Table 1). No inter-group differences were seen in any of the other pre- and post-global ischaemic functional parameters. There were also no differences observed in the percentage recovery in any of the parameters (Table 1). Furthermore, following regional ischaemia–reperfusion, infarct sizes were significantly larger in the ART-treated group compared to the control (control: 28.17 ± 5.10%; rooibos: 39.37 ± 5.83%; ART: 50.56 ± 4.08%; ART + rooibos: 46.75 ± 4.72%; $p = 0.03$: control vs ART) (Fig. 3). No significant inter-group differences were observed in the percentage area at risk (control: 49.47 ± 7.56%; rooibos: 44.29 ± 2.00%; ART: 46.29 ± 3.71%; control + ART: 47.94 ± 4.45%; $p > 0.05$).

Vascular reactivity studies

In response to Ach, the rings from the ART-treated group relaxed significantly less compared to both the control aortas ($p = 0.03$) as well as the ART + rooibos group ($p = 0.003$) (Fig. 4). There were no differences in relaxation between the other groups.

Table 1. Functional parameters of hearts pre-ischaemia and post 20-minute global ischaemia. *$p < 0.05$ vs rooibos + ART; one-way ANOVA with Bonferonni *post hoc* test, or Student's *t*-test where relevant

Mean ± SEM	*Control* (n = 9)	*Rooibos* (n = 9)	*ART* (n = 9)	*Rooibos + ART* (n = 9)
Aortic output (ml/min)				
Pre	37.78 ± 1.87	38 ± 1.7	33 ± 5.85	40.5 ± 1.80
Post	10.89 ± 3.06	18.67 ± 2.92	12.44 ± 3.80	13.75 ± 3.88
% Aortic output recovery	28.30 ± 5.35	47.36 ± 4.03	31.08 ± 5.89	32.63 ± 6.02
Coronary flow (ml/min)				
Pre	13.94 ± 0.77	12.83 ± 0.75	11.63 ± 0.90*	15.06 ± 0.72
Post	12.06 ± 0.52	11.44 ± 1.01	9.00 ± 2.03	11.75 ± 1.33
% Coronary flow recovery	87.87 ± 5.00	89.56 ± 6.27	76.80 ± 17.29	78.51 ± 7.95
Cardiac output (ml/min)				
Pre	51.72 ± 2.37	50.83 ± 2.26	44.63 ± 6.62	55.56 ± 2.10
Post	22.94 ± 3.22	30.11 ± 3.78	21.44 ± 5.51	25.50 ± 4.45
% Cardiac output recovery	44.41 ± 6.09	57.87 ± 5.43	44.80 ± 10.3	45.35 ± 7.48
Peak systolic pressure (mmHg)				
Pre	88.44 ± 0.58	89.22 ± 1.11	88.13 ± 2.11	90.25 ± 0.97
Post	80.89 ± 2.32	84.78 ± 1.33	62.75 ± 13.73	75.75 ± 9.54
% Peak systolic pressure recovery	91.53 ± 2.8	95.01 ± 0.59	69.99 ± 15.32	83.74 ± 10.43
Heart rate (bpm)				
Pre	280.20 ± 13.76	268.20 ± 15.71	237.90 ± 5.81	263.50 ± 6.30
Post	260.80 ± 8.67	229.30 ± 11.57	187.80 ± 41.69	232.60 ± 34.34
% Heart rate recovery	94.26 ± 4.3	87.02 ± 4.89	81.25 ± 18.05	88.43 ± 12.93
Total work (TW) (mW)				
Pre	10.08 ± 0.52	10.01 ± 0.54	8.85±1.44	11.05 ± 0.47
Post	4.17 ± 0.66	5.68 ± 0.77	3.98 ± 1.01	4.73 ± 0.97
% TW recovery	41.54 ± 6.47	55.17 ± 5.36	41.96 ± 9.77	41.99 ± 7.91

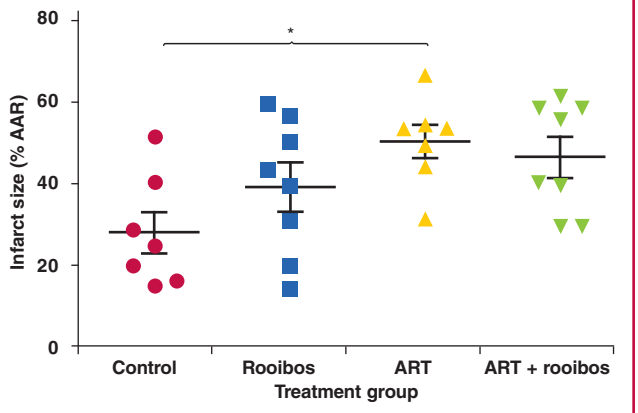

Fig. 3. Infarct size expressed as a percentage of the area at risk (%AAR) at the end of the nine-week treatment period. One-way ANOVA with Bonferonni *post hoc* test, $*p < 0.05$; n = seven to eight per group.

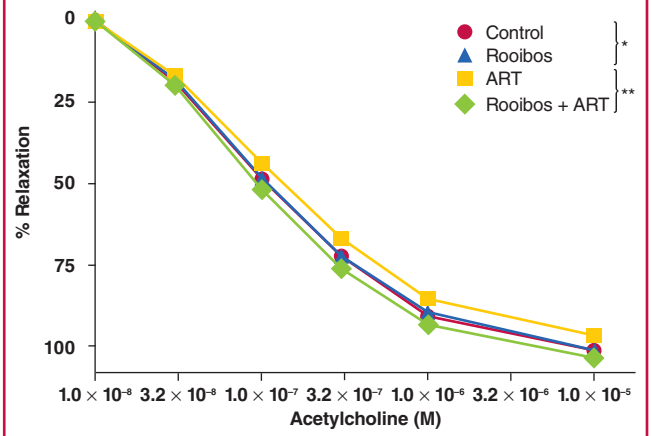

Fig. 4. Ach-induced relaxation of aortic rings at the end of the nine-week treatment period; n = 20 to 25 per group; $*p < 0.05$; two-way ANOVA, $**p < 0.01$.

Discussion

Similar to the findings of previous studies, our results showed that rooibos did not alter body weight or fluid consumption.[33-36] Furthermore, ART alone had no effect on body weight, also similar to previous findings in humans.[37] Consequently, it was not unexpected, that the combination of ART and rooibos also had no effect on body weight.

Previous clinical studies have reported elevated blood lipid levels in ART-exposed compared to ART-naïve individuals, depending on the duration and class of ART.[38-40] In a South African study, patients receiving ART for less than six months were found to have significantly increased TG, TC, low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) levels when compared to the ART-naïve patients.[41] However in another study, patients on ART for less than two years had relatively less risk of dyslipidaemia than those on it for longer.[42] In the present study, ART demonstrated no significant effect on lipid profiles. This lack of effect may be due to the fact that changes in lipid profiles have been noted mostly in ART treatment other than the FDC used in this study,[43] or the relatively short treatment period.[44]

Rooibos consumption exerted TC-lowering effects in this study; however, the other blood lipid parameters were unaffected. Lipid lowering with rooibos intake has been seen in both humans and rats.[45,46]

No significant differences were found in the TBARS levels between the groups. This was partially unexpected as ART (particularly PI and EFV) has previously been found to increase TBARS in both cell-based and epidemiological studies.[45,47-49] On the other hand, our findings do not contradict those of an epidemiological study by Masia *et al.*,[46] who showed increased oxidative stress by lipid peroxidation in subjects receiving PI-containing ART, while ART containing NNRTIs (including EFV), such as in our study, induced oxidative stress levels similar to those observed in ART-naïve patients.[46] A study in rat testes showed no increase in TBARS in testicular tissue after eight weeks of therapy with the same FDC as was used in the present study.[50]

The novelty of our findings provides insight on the effects of the EFV/FTC/TDF combination drug. From the results, it appears that this specific FDC is possibly not associated with increased lipid perdoxidation, as seen in other previously investigated combinations, or that it would need a longer treatment period to exert observable changes in lipid peroxidation.

Improved antioxidant capacity due to rooibos and the ability of rooibos to inhibit lipid peroxidation has been demonstrated in several studies.[28,51-54] However, it has also been shown to have no effect in other studies.[34,55] Conducting one or two complementary techniques, such as determination of the total antioxidant capacity together with the TBARS analysis may have provided a more cohesive insight on the antioxidant capacity of the rats. However, our results seem to be similar to those of other studies in that rooibos treatment did not adversely affect oxidative stress.

Coronary flow in ART-exposed animals was lower than in the ART animals supplemented with rooibos. A decrease in myocardial perfusion was observed during ART in a longitudinal study in humans with positron emission tomography and flow-mediated dilation, where myocardial perfusion was seen to decrease.[56] Although a significant decrease in coronary flow from control values in either the ART or the ART + rooibos groups was not seen in this study, the ability of rooibos co-treatment with ART to significantly improve coronary flow compared to ART only is, as far as we are aware, a novel finding.

Certain flavonoids, such as quercetin, which are found in rooibos, have been shown to increase endothelial nitric oxide synthase 3 (eNOS) activity and endothelium-dependent vasorelaxation in aortic rings from spontaneously hypertensive rats.[57] However, this is the first time rooibos has been directly linked to increased coronary flow in the baseline (pre-ischaemic) setting, specifically in the context of ART, and further investigations are warranted to explore underlying mechanisms.

Interestingly, no other functional parameters (pre- and post-ischaemia) or percentage recovery values were affected by either rooibos or ART, or in combination. Although difficult to explain, it may be due to a too-short treatment period that was not sufficient to elicit functional effects in the hearts, despite showing demonstrable effects in terms of infarct size and vascular function, as discussed below. It is also possible that the FDC takes longer to provoke functional changes in the heart. Furthermore,

the majority of previous studies investigated the effects of PIs *in vivo* or *in vitro*, or the effect of NRTIs and NNRTIs in the *ex vivo* setting or on organs other than the heart.[16,57,58]

The infarct size of the ART treated group was significantly greater than that of the control group, which suggests that, in our hands, the TDF/FTC/EFV combination treatment was associated with decreased myocardial ischaemic tolerance. This may be explained by an ART-induced reduction in cell viability as a result of toxicity. EFV, particularly, has previously been demonstrated to result in endoplasmic reticulum stress-induced apoptosis and therefore decreased cell viability in human brain endothelial cells and human umbilical vein endothelial cells.[57,59]

When ART-treated rats were co-treated with rooibos, the significance compared to the control group was lost. However, similarly, the infarct size of the ART + rooibos group also remained unchanged compared to ART only, which excludes pronounced infarct sparing. Although the small reduction in infarct size when rooibos was added is unlikely to be biologically significant, this finding, as well as the improved baseline coronary flow observed in the ART + rooibos group compared to ART only, warrants further investigation to explore any possible cardio-protective properties.

ART was associated with a significant reduction in Ach-induced aortic ring relaxation compared to the control and co-treatment groups. The anti-relaxation effects may be an indicator of impaired endothelial function as a result of ART, as previously shown in humans.[13,60] Although ART has been shown to decrease markers of ED (sVCAM-1, sICAM-1, von Willebrand factor) and increase flow-mediated dilation (FMD) in HIV-infected individuals, the markers did not completely return to values during no HIV infection.[61,62] ART has previously been demonstrated to induce ED in clinical and experimental models by decreasing NO production or release through mechanistically inhibiting eNOS expression and increasing reactive oxygen species (ROS) production.[63,64] This has mostly been observed with PIs, but there is some evidence to suggest that some NRTIs (AZT and abacavir) also induce ED through these mechanisms.[65,66]

EFV, but not TNF or FTC, has also recently been shown to impair Ach-induced relaxation in rat thoracic aortic rings and cause apoptosis and necrosis in EA.hy926 cells.[16] However, in contrast to our study, the drugs were administered directly to the aortic rings in an *ex vivo* fashion.

Co-treatment with rooibos resulted in relaxation that was similar to that observed in the control rings, and significantly increased compared to the ART-treated group. This result demonstrates that the anti-relaxation effect observed in the ART group was attenuated when co-treated with rooibos, suggesting that rooibos may have beneficial effects on vascular function, possibly via antioxidant effects (not shown in the present study). Rooibos has previously been shown to directly scavenge ROS and inhibit inflammation.[67] *Aspalathus linearis* has been shown to have strong anti-HIV activity; however, scant research has been published on its effects when co-administered with ART.[25]

While two-week rooibos consumption in rats was shown to increase CYP3A4 inhibitory activity, any influence on ART effectivity has not been shown.[68,69] Therefore, to our knowledge, the present study is the first to show that rooibos in conjunction with ART decreased ART-induced ED.

In studies by Nakano *et al.*,[25,26] acid polysaccharides extracted from the leaves of *A linearis* suppressed the cytopathicity of HIV (HTLV-III)-infected MT-4 cells, while polysaccharides from Japanese green tea leaves and a hot water extract of *A linearis* did not. The polysaccharide, composed of reducing sugars (27%), neutral sugars (46%) and uronic acid (22%), also almost completely inhibited the binding of HIV-1 to MT-4 cells.

Conclusion

The significant increase in infarct size and decrease in baseline coronary flow observed in the hearts from the ART group was mirrored by a decreased response to Ach-induced relaxation in the aortic rings of the same animals. Although further investigations are needed, our findings suggest that the specific FDC used may have caused impaired endothelial function.

Rooibos co-treatment proved effective to reduce the detrimental ART-associated effects on infarct size and vasorelaxation. These findings are novel and should be further investigated to explore the possible future therapeutic potential of rooibos in high-risk cardiovascular patients receiving ART.

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