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Long-term consumption of fermented rooibos herbal tea offers neuroprotection against ischemic brain injury in rats

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Stroke is the second leading cause of death worldwide, affecting about 240 people a day in South Africa and leaving survivors with residual disabilities. At the moment, there is no clinically approved neuroprotective product for stroke but the consumption of plant polyphenols has been suggested to offer some protection against stroke. In this study, we investigated the effects of long-term consumption of fermented rooibos herbal tea (FRHT) on ischemia/reperfusion (I/R)-induced brain injury in adult male Wistar rats. FRHT was administered to the animals *ad libitum* for 7 weeks prior to the induction of ischemic injury via a 20-minute bilateral occlusion of the common carotid arteries (BCCAO) followed by reperfusion for 24, 96 and 168 hours respectively. Neurobehavioural deficits, brain oedema, blood-brain barrier (BBB) damage, apoptosis, lipid peroxidation and total antioxidant capacity were subsequently evaluated using standard methods. Our results showed that long-term consumption of FRHT by Wistar rats significantly reduced brain oedema and neuronal apoptosis, but did not attenuate BBB damage following cerebral ischemia. Analysis of whole-brain homogenates showed significantly reduced lipid peroxidation levels, increased total antioxidant capacity and resulted in improved neurobehavioural outcomes in FRHT-treated rats when compared with untreated animals. Taken together, our results tend to suggest that continuous consumption of FRHT could confer some protection against ischemic brain injury (IBI) and is therefore highly recommended for patients with stroke-predisposing conditions.

Key words: apoptosis, brain oedema, cerebral ischemia, fermented rooibos herbal tea, lipid peroxidation, total antioxidant capacity

INTRODUCTION

Recent statistics from the Heart and Stroke Foundation, South Africa (HSFSA) show that about 130 heart attacks and 240 stroke episodes occur daily in South Africa, which implies that about 10 people will suffer a stroke and 5 people will suffer a heart attack every passing hour (HSFSA 2013). The burden of stroke does not only lie in its high mortality but also in its high morbidity rate as up to 50% of stroke survivors often become chronically disabled (Wilkinson et al. 1997).

Although most of the medications approved for the treatment of stroke worldwide (e.g. alteplase (rt-PA), reteplase, tenecteplase, anistreplase, streptokinase and urokinase) are known to restore blood flow (Duggal and Harger 2011), some have been found to be neurotoxic, to disrupt neurovascular matrix and to increase the risk of intracerebral hemorrhage (ICH) (Wang et al. 2003). Even compounds that showed promising neuroprotective activity in experimental brain ischemia models have failed to successfully translate in clinical human trials (Ziemka-Nalecz and Zalewska 2014), with the result that there is hitherto no clinically effective neuroprotective drug licensed for stroke (Macrae 2011).

Oxidative stress is considered a major contributing factor in cerebral I/R-induced injury (Liu 2015). A stroke usually produces oxidative stress leading to production of reactive oxygen species (ROS) and other free radicals, mainly due to low-oxygen inhibition of cellular respiration, inflammation and excitotoxicity (Pradeep et al. 2012). Free radicals are known to attack important intracellular and extracellular macromolecules in the body such as DNA, RNA, carbohydrate moieties, unsaturated lipids, proteins and micronutrients, leading to cell damage and homeostatic disruption (Lobo et al. 2010). To protect against free radical damage, the body has a defense system of

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endogenous antioxidants which can be boosted with intake of antioxidant-rich foods and supplements.

There is increasing scientific interest in the potential health benefits and possible neuroprotective effects of antioxidant-rich food substances and beverages in literature. Some studies have focused on the effects of these substances when consumed prior to the onset of an IBI. Rooibos tea, a very popular beverage in South Africa, is known to contain such polyphenol antioxidants as the monomeric flavonoids aspalathin, chrysoeriol, isoorientin, isoquercitrin, isovitexin, luteolin, nothofagin, orientin, quercetin, rutin and vitexin (Erickson 2003). It has since gained increasing popularity among international consumers due to its many acclaimed health benefits (Mahomoodally 2013). Another study has shown that rooibos tea could prevent DNA damage and inflammation via its anti-oxidative activity (Baba et al. 2009). A study by Inanami and others (1995) found that rooibos tea could protect against age-related changes in the brains of rats compared to the controls, mainly due to its ability to prevent the age-related accumulation of lipid peroxides in the brain.

Recent animal studies have shown that rooibos tea has potent antioxidant, antimutagenic, immune-modulating and chemopreventive effects (Van der Merwe et al. 2006, Ichiyama et al. 2007, Marnewick et al. 2011). Another study showed that high intake of rooibos tea resulted in significant reductions in lipid peroxidation, low density lipoprotein (LDL) cholesterol, triglycerides and an increase in high density lipoprotein (HDL) cholesterol levels, suggesting that rooibos tea could lower the risks of cardiovascular and degenerative diseases (Marnewick et al. 2011).

Previous studies have reported the cardioprotective (Dludla et al. 2014) as well as hepatoprotective (Ulicná et al. 2003) properties of FRHT following experimental injury and ischemia. However, information on the neuroprotective effects of FRHT is scanty in literature, but maternal consumption of pomegranate juice was reported to be neuroprotective for the neonatal brain (Loren et al. 2005), probably through the effects of its bioactive compounds. Rooibos tea has a rare source of the dietary dihydrochalcones, aspalathin and nothofagin (McKay and Blumberg 2007) which could cross the BBB like other flavonoid compounds (Youdim et al. 2003, Rashid et al. 2014) to possibly exert neuroprotective effects against IBI.

The present study therefore aims to determine the neuroprotective effects of FRHT on brain oedema, apoptosis, neurologic deficits, BBB damage, lipid peroxidation, and total antioxidant capacity following IBI induced by bilateral common carotid artery occlusion (BCCAO).

EXPERIMENTAL PROCEDURE

Ethical considerations

Ethical guidelines as specified by the Animal Research Ethics Committee (AREC) of the University of the Western Cape, Bellville, South Africa were followed, with assigned ethics and project registration numbers: 13/10/94 and ScR1Rc2013/07/18 respectively.

Animals

Fifty (50) healthy male Wistar rats with an average weight of 250 g were procured from the University of Stellenbosch animal facility in Cape Town, South Africa and maintained at the Animal House of the University of the Western Cape, Bellville, South Africa, under standard laboratory conditions of temperature (25±2°C), humidity (50±15%) and 12 hours light-dark cycle. Animals were acclimatized for two weeks and fed on standard rat chow and tap water *ad libitum* and received humane care in accordance with National Institutes of Health (NIH) Guide for Care and Use of Laboratory Animals.

Experimental design and grouping

Animals were randomly separated into four groups – Control-sham (C-SHAM) group (15 rats), Rooibos-sham (R-SHAM) group (5 rats), Rooibos+ischemia (R+I) group (15 rats) and the ISCHEMIA group (15 rats). All animals in the respective groups were used for the assessment of brain oedema, BBB integrity, histological and immunohistochemistry (IHC) assessment of apoptosis and neurochemical studies (NS) of lipid peroxidation, oxygen radical antioxidant capacity assay (ORAC) and ferric reducing antioxidant power (FRAP). All rats in the C-SHAM and ISCHEMIA groups received food and tap water *ad libitum* throughout the study while rats in the R-SHAM and R+I groups had daily access to food and FRHT *ad libitum* for 7 weeks prior to BCCAO or sham surgery and were sacrificed at the end of 24-hour, 96-hour and 168-hourpost-BCCAO periods respectively, depending on the experimental protocol.

Rooibos tea preparation

Aqueous extracts of FRHT (generous gift from Rooibos Ltd; Clanwilliam, South Africa) at a concentration of 2 g/100 ml FRHT was used throughout this study as this concentration has been reported to be routine for tea-making purposes

(Marnewick et al. 2003, Pantsi et al. 2011). Fresh preparations were made every day and administered to the experimental rats *ad libitum* (Opuwari and Monsees 2014).

Bilateral common carotid artery occlusion (BCCAO) surgery

Animal weights were recorded prior to a 20-minute BCCAO with a 4-0 silk suture as previously reported (Speetzen et al. 2013). Isoflurane was used for anesthesia and surgery occurred in an aseptic environment. The animals were maintained under Isoflurane anesthesia throughout the occlusion period until reperfusion. The analgesic – Meloxicam was administered after surgery to prevent post-surgical pain and distress. Rectal temperature of the animals was monitored during and after surgery and maintained at about 37°C by a heating pad and Infrared Lamp in the recovery cage. In the sham groups (C-SHAM and R-SHAM), the same surgical procedures were followed but BCCAO was not done.

Physiologic parameters

As previously described by Ord and others (2012) and while under anesthesia, physiological parameters were measured prior to, during and after the occlusion of the right and left common carotid arteries. Systolic and diastolic blood pressure and heart rate were measured by the CODA non-invasive tail cuff blood pressure monitor (Kent Scientific, USA), which uses the volume pressure technology. Values from each animal were determined from the means of a minimum of 3 separate blood pressure and heart rate measurements. Temperature was monitored using a rectal thermometer and was maintained at about 37°C using a heating pad and infrared heating lamp.

Brain oedema assessment

Twenty four hours after reperfusion, rats were sacrificed under deep anesthesia (Sodium pentobarbital, 150 mg/bw i.p) and decapitated. The brains were harvested and the cerebellum, pons, and olfactory bulbs removed and weighed immediately (wet weight (WW)). Brain sections were then placed in an oven (Memmert, Germany), dehydrated at 105°C for 48 hours and reweighed (dry weight (DW)). Brain oedema was estimated as the difference in percentage of brain water and calculated with the formula below (Bigdeli et al. 2007).

Brain water content (BWC)=[(WW–DW)/WW]×100.

Blood-brain barrier assessment

Extravasation of plasma contents into the brain tissue occurs when there is disruption of BBB integrity (Klohs et al. 2009). In this study, BBB integrity was evaluated as Evans Blue (EB) (Sigma Aldrich, USA) extravasation into the brain parenchyma as previously described (Bigdeli et al. 2007). Briefly, 4 ml/kg of 2% EB solution in PBS was injected into tail vein of each rat an hour before sacrifice. The brains were weighed, homogenized in 1:10 w/v PBS and an equal volume of 60% trichloroacetic acid (TCA) (Sigma Aldrich, USA) before centrifugation at 1000×g for 30 minutes at 4°C. Readings were then taken at 610 nm using a POLAR star omega spectrophotometer (BMG Labtech, Ortenberg, Germany) and a standard curve was used to determine EB levels in each brain sample.

Histological and immunohistological studies

Rats were sacrificed 24 hours, 4 days and 7 days after BCCAO with an overdose of Sodium pentobarbital injection (150 mg/bw i.p). The thoracic cavity was opened and the rat perfused transcardially with a 300 ml of cold Phosphate buffered saline (PBS). A pale color of the liver was indicative of a successful perfusion after which, the rats were decapitated and the brains removed, weighed and bisected along the mid-sagittal plane. The right hemisphere was fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 24 hours for histological and immunohistochemical analysis. The left hemisphere was also quickly immersed in 1:10 w/v 0.1 M cold PBS solution and stored at –80°C to be used for biochemical analyses (Zhang et al. 2012). After fixation of tissues, brains were processed in a Leica-2125 automatic tissue processor (Leica, Germany) to prepare specimens for sectioning, hematoxylin and eosin (H & E) as well as cresyl violet staining. A Terminal dUTP Nick-End Labeling (TUNEL) assay was also conducted using the *in situ* DNA Fragmentation Assay Kit (BioVision, U.S.A.) for assessment of apoptosis following the manufacturer's instruction.

Morphologic and morphometric studies

Morphological and morphometric analysis was carried out on the H & E, cresyl violet and TUNEL-stained cornus ammonis 1 (CA1) region of the hippocampus on images captured at a magnification of ×400 using the Zeiss Primo Vert microscope (Zeiss, Germany). In order to cover the area of interest at ×400, three captured images were used with each measuring 1159.4×869.57 μm. Viable pyramidal neurons of the hippocampus were identified

as those exhibiting clear purple cytoplasmic staining with visible nuclei and nucleoli, while ischemic (dead) cells were identified as showing shrunken perikarya, triangular shapes and mostly exhibiting dark-stained nuclei. The data obtained was expressed as percentages of viable or TUNEL-positive cells in total number of cells. Quantification of viable and TUNEL positive cells was done using the NIH Image analysis software (Image J) (Onken et al. 2012).

Neurochemical assays

Homogenization of tissues

Brain tissues stored at –80°C were thawed and homogenized in 10 times (w/v) 0.1M PBS (pH 7.4) in a Teflon glass homogenizer for two periods of 10 seconds each. The homogenate was then centrifuged at 15,000 rpm in a microcentrifuge at 4°C for 10 minutes. The supernatant was collected and transferred into newly marked Eppendorf tubes for further analysis using different biochemical assays (Ahmed et al. 2014).

Lipid peroxidation assay

Malondialdehyde (MDA) is produced as a by-product of the reaction of superoxide (O_2·) and hydroxyl ($^{\cdot}OH$) radicals with unsaturated lipid (Ozkul et al. 2007). MDA levels in tissues could indicate the severity of lipid peroxidation (LPO) (Serteser et al. 2002). In this study, LPO assessment was done as previously described by Wills (1966). Briefly, 100 µl of supernatant was mixed with 12.50 µl of cold ethanol, 100 µl of 0.2 M ortho-phosphoric acid and 12.50 µl of 0.67% TBA (Sigma Aldrich, USA). The reaction mixture was then heated at 90°C for 45 minutes, cooled for 2 minutes before 1000 µl of n-butanol and 100 µl of saturated sodium chloride (NaCl) was added. The mixture was centrifuged at 12,000 rpm at 4°C for 2 minutes before spectrophotometric reading at 532 nm. The results were expressed as µmol of MDA per g of wet brain tissue.

Oxygen radical absorbance capacity (ORAC)

The ORAC assay is one of the most accepted methods for measuring the activity and amount of antioxidants present in biological samples (Cao et al. 1998). Briefly, 2,2'-azobis-2-methyl-propanimidamide, dihydrochloride (AAPH) and fluorescein (Sigma Aldrich, USA) were used as the free radical producing system and fluorescent molecule respectively. The oxidation of fluorescein was measured by initiating a reaction following the addition of 50 µl of AAPH to a mixture of 138 µl of fluorescein and 12 µl of sample in a 96-well black plate and the fluorescence read for 2 hours at every 5 minutes interval at an emission and excitation wavelength of 530 nm and 485 nm using the Fluoroskan Ascent fluorescent plate reader (Thermo Fisher Scientific, Waltham, MA, USA). A standard curve was prepared from a 500 µM stock solution of Trolox, an artificial Vitamin E (Prior et al. 2003). The results obtained were expressed as µM Trolox equivalent (TE)/g of wet brain tissue.

Ferric reducing antioxidant power (FRAP) assay

The FRAP assay uses an oxidation/reduction reaction to measure the ability of antioxidants in a sample to reduce ferric tripyridyltriazine (Fe^{3+} – TPTZ) to a ferrous form (Fe^{2+}) which has an intense blue color and can be quantified spectrophotometrically (Ndhlala et al. 2010). In this study, a mixture of 30 ml acetate buffer (300 mM, pH 3.6), 3 ml TPTZ (10 mM in 100 mMHCl), and 3 ml $FeCl_3$·6 H2O (20 mM) was used to prepare the FRAP reagent, from which 300 µl was added to 10 µl of the sample and incubated for 30 minutes in an oven (Memmert, Germany) at 37°C and then read at a wavelength of 593 nm in the Multiskan Spectrum automated plate reader (Thermo Fisher Scientific, Waltham, USA). A serial dilution was prepared from a stock solution of Ascorbic acid for the preparation of a standard curve and expressed as FRAP mg per g of wet brain tissue (Vakili et al. 2014).

Open field neurobehavioral test

The open field (OF) test is a commonly used neurobehavioral assessment tool that provides simultaneous measurement of locomotion and anxiety in laboratory animals (Kendigelen et al. 2012). The apparatus used for the OF assessment in this study involved a square plexi glass box (72×72×20 cm), with a digital camera (Samsung HMX-F90, South Korea) mounted directly above it. The open-field arena was divided into 16 equal squares, via a 4×4 grid, to assist in data analysis and animals were tested individually. Each session lasted 10 minutes in a single run after which the rat was returned into its home cage and the OF box cleaned with 70% ethanol before testing the next rat. The Smart video tracking software version 3.0, from Panlab Harvard Apparatus (Massachusetts, USA) was used to measure the locomotor activity of each experimental rat by extracting the total distance traveled in the OF arena. As a measure of anxiety, the total distance traveled in the 12 squares near the walls was compared with the distance traveled in the 4 squares at the center of the arena. All analysis was done by "blind" observers.

RESULTS

Effects of FRHT on brain oedema

Twenty four hours after reperfusion, brain water content was assessed to evaluate BCCAO-induced brain oedema. Hemispheric brain water content was significantly higher ($p<0.05$) in the ISCHEMIA group (77.80%±0.27) when compared with the C-SHAM group (76.68%±0.36) (Fig. 1). No significant increase in brain water content was observed in the R+I group compared to the C-SHAM group (77.17%±0.11 and 76.68%±0.36).

Effects of FRHT on the blood-brain barrier (BBB)

Assessment of BBB integrity after 20 minutes BCCAO was done by examining the extravasation of injected Evans blue solution from systemic circulation into the brain tissue 4 days after surgery. The BBB integrity appeared to have been compromised in the ISCHEMIA group as the Evans blue content in the brain specimens was higher than in the C-SHAM group (0.6880 µg/g±0.22 *vs.* 0.9189 µg/g±0.34) (Fig. 2); this difference was however not statistically significant. On the other hand, it does appear that pre-treatment with FRHT for 7 weeks did ameliorate the impairment of BBB integrity as Evans blue extravasation values were similar to the C-SHAM group (0.7341 µg/g±0.25 and 0.6880 µg/g±0.22).

Effects of FRHT on lipid peroxidation

Seven days after BCCAO, the level of lipid peroxidation was determined by measuring malondialdehyde (MDA) levels in the cerebrum. Results show that the level of MDA in the cerebral hemispheres of rats in the ISCHEMIA group was significantly higher (0.029 µmol/g±0.0014) when compared to the C-SHAM (0.017 µmol/g±0.0014), and R-SHAM groups (0.014 µmol/g±0.003) (Fig. 3). Whereas 7 weeks administration of FRHT to the R+I group prior to the induction of BCCAO resulted in significantly lower levels of MDA in the cerebral hemispheres when compared to the ISCHEMIA group (0.017 µmol/g±0.0008 *vs.* 0.029 µmol/g±0.0014) to around sham levels (0.014 µmol/g±0.003 *vs.* 0.017 µmol/g±0.0014).

Effects of FRHT on oxygen radical absorbance capacity (ORAC)

Assessment of total antioxidant capacity is very important in our understanding of how antioxidants protect against reactive oxygen species (ROS). In the human body, peroxyl radicals are the most abundant free radicals, which make the measurement of antioxidant capacity against peroxyl-radical even more biologically relevant. In this study, measurement of antioxidant capacity against peroxyl-radical in the R-SHAM and R+I groups showed that $ORAC_{ROO}$ values were significantly higher (36.68 µmol/g±1.98 and 35.16 µmol/g±1.62 respectively) when compared to the ISCHEMIA group (22.26 µmol/g±3.22) (Fig. 4).

Effects of FRHT on ferric reducing antioxidant power (FRAP)

The FRAP assay (a measure of the ability of compounds to neutralize free radicals by acting as an electron donor) was done. Measurement of FRAP levels in the C-SHAM and R-SHAM groups presented a higher significant difference

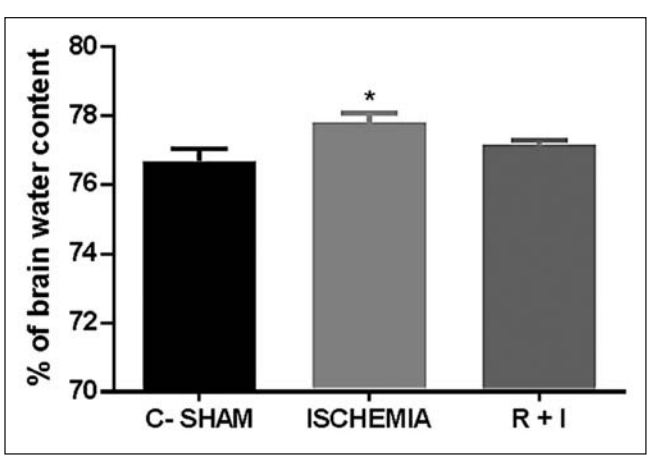

Fig. 1. Effects of FRHT on cerebral oedema in Wistar rats, 24 hours after BCCAO and sham surgery. The ISCHEMIA group had a significantly higher percentage brain water content compared with the C-SHAM and R+I groups. Data is presented as means ±S.E.Ms, n=5, (*)=significant difference at $p<0.05$; wbt=wet brain tissue.

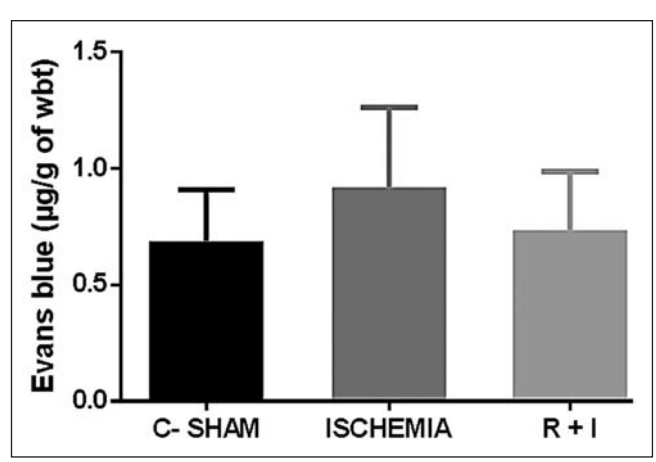

Fig. 2. Blood brain barrier integrity assessment using the Evans blue extravasation method. No significant differences were observed. Data presented as mean ±S.E.M, n=5, wbt=wet brain tissue.

(2.36±0.16 and 1.93±0.37 respectively) when compared to the ISCHEMIA group (0.63±0.11). Similarly, the FRAP values for samples from the R+I group were significantly higher than the ISCHEMIA group (2.12±0.16 *vs.* 0.63±0.11) (Fig. 5).

Effects of FRHT on neuronal loss

Hematoxylin and eosin staining

Seven days after 20 minutes BCCAO or sham surgery, neuronal damage in the CA1 region of the hippocampus was evaluated by staining serial sagittal sections of the right cerebral hemispheres with hematoxylin and eosin stains. No histopathological changes were seen in the C-SHAM and R-SHAM groups (Figs 6A and 6B) whereas the ISCHEMIA group showed marked ischemic neuronal damage with cells exhibiting triangular shapes and deeper staining due to the condensation of cytoplasm and karyoplasm (Fig. 6C). These changes were less frequent in the R+I group (Fig. 6D).

Cresyl violet/nissl staining

Representative photomicrographs of cresyl violet/nissl staining of rat brain sections showed neurodegenerative changes in the cyto-architecture of the CA1 region of the hippocampus in the ISCHEMIA group 7 days post-BCCAO. These changes were identified by the presence of shrunken and darkly stained neurons (Fig. 6G). Quantitative analysis showed a significant decrease in the amount of viable hippocampal neurons in the CA1 region of rats in the ISCHEMIA group when compared with the C-SHAM and R-SHAM groups (Fig. 7A). However, following 7 weeks of pre-treatment with FRHT, there were significantly more cells in the R+I group than in the ISCHEMIA group. The R+I group shows more viable cells in the CA1 region of the hippocampus (Fig. 6H), almost similar to the control groups (Figs 6E and 6F).

Immunohistochemical staining

Terminal deoxynucleotidyltransferase-mediated UTP nick end labeling (TUNEL)-positive cells were identified as dark or light-brown color or with dark brown granules in the cell nucleus. The percentage rate of apoptosis (%RA) in the hippocampal CA1 region was calculated as RA=100×(number of apoptotic neurons/total number of neurons). In this study, the C-SHAM and R-SHAM groups had fewer (8.12%±2.6 and 6.21%±2.47) TUNEL-positive cells respectively in the hippocampal CA1 region (Figs 6I, 6J, and 7B) compared to the ISCHEMIA group with more TUNEL-positive cells (46.72%±12.8). The R+I group also had more TUNEL-positive cells (36.54%±12.2) compared to the C-SHAM and R-SHAM groups but the difference was not statistically significantly.

Effects of FRHT on neurobehaviour

The open field test (OFT) is one of the most widely used measures of animal neurobehavioral deficits. Measures of total distance travelled and rearing events are used as an index of exploratory activity (Lever et al. 2006). In this study, we used a 10-minute OFT to investigate neurobehavioural deficits on days 1, 4, and 7 after BCCAO. Results obtained

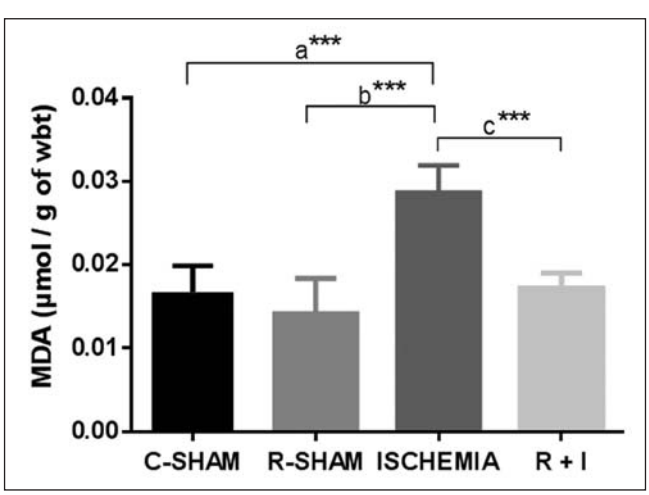

Fig. 3. Effects of FRHT on cerebral lipid peroxidation in Wistar rats, 7 days after BCCAO. MDA levels in the ISCHEMIA group were significantly higher compared to the C-SHAM, R-SHAM and R+I groups. Data is presented as mean ±S.E.M, n=5, wbt=wet brain tissue; (*)=significant difference at P<0.001.

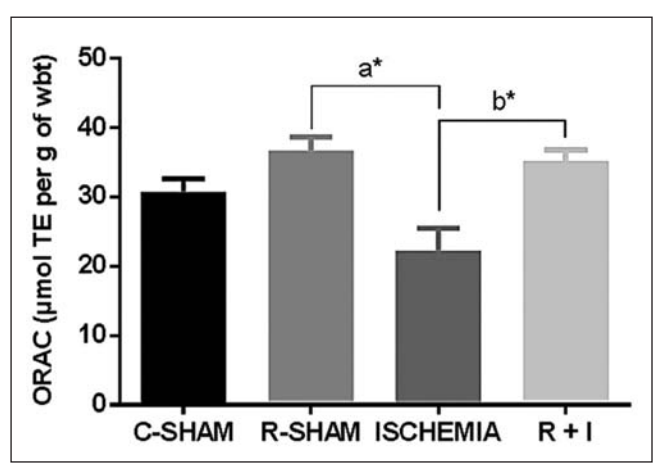

Fig. 4. Effects of 7 weeks intake of FRAP on peroxyl radical absorbance capacity (ORACROO) in the cerebral hemispheres of rats 7 days after BCCAO. Values for the ISCHEMIA group were significantly lower compared with the R-SHAM and R+I groups respectively. Data is presented as mean ±S.E.M, n=5; (*)=significant difference at P<0.01; wbt=wet brain tissue.

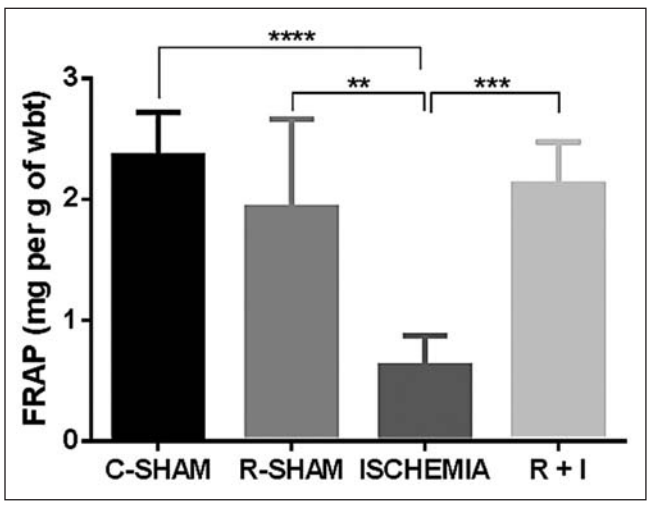

Fig. 5. Effects of 7 weeks intake of FRHT on ferric reducing antioxidant power (FRAP) in the cerebral hemispheres of Wistar rats, 7 days after BCCAO. The ISCHEMIA groups showed significantly lower FRAP levels compared to the R+I and SHAM groups. Values are presented as mean ±S.E.M, n=5; (**, ***, ****)=significant differences at P<0.01, P<0.001, and P<0.0001 respectively; wbt=wet brain tissue.

showed that rats in the R+I and ISCHEMIA groups generally travelled less distance (low locomotor activity) and had fewer rearing events (exploration) compared with the C-SHAM group (Figs 8D and 8E).

DISCUSSION

The clinical manifestations of ischemia reperfusion-induced brain injury are diverse and may include brain oedema formation, BBB disruption, neuronal loss, oxidative stress, inflammation, neurological deficits, etc. some of which have been observed in animal experiments. Knowland and others (2014) reported that the high mortality often associated with ischemic stroke could be linked to BBB damage, one major reason the maintenance of BBB integrity is crucial to the management of ischemic stroke (Zhang et al. 2013). BBB disruption may occur spontaneously during acute stroke or following reperfusion therapy (Nguyen et al. 2013), and the resulting oxidative damage to brain capillary endothelial cells and basement membrane causes damage to the blood-brain barrier and results in cerebral oedema (Cunningham et al. 2005). Such oedema is characterized by the pathological accumulation of fluid in brain tissue and often results in the expansion of brain tissue volume (Ito et al. 1979, Kahle et al. 2009, Bansal et al. 2013).

After a stroke episode, damage to tight junction proteins often results in increased BBB permeability, brain oedema and leukocyte infiltration (Andjelkovic and Keep 2016). Reports on the potential neuroprotective effects of polyphenols against ischemia-induced BBB disruption (and

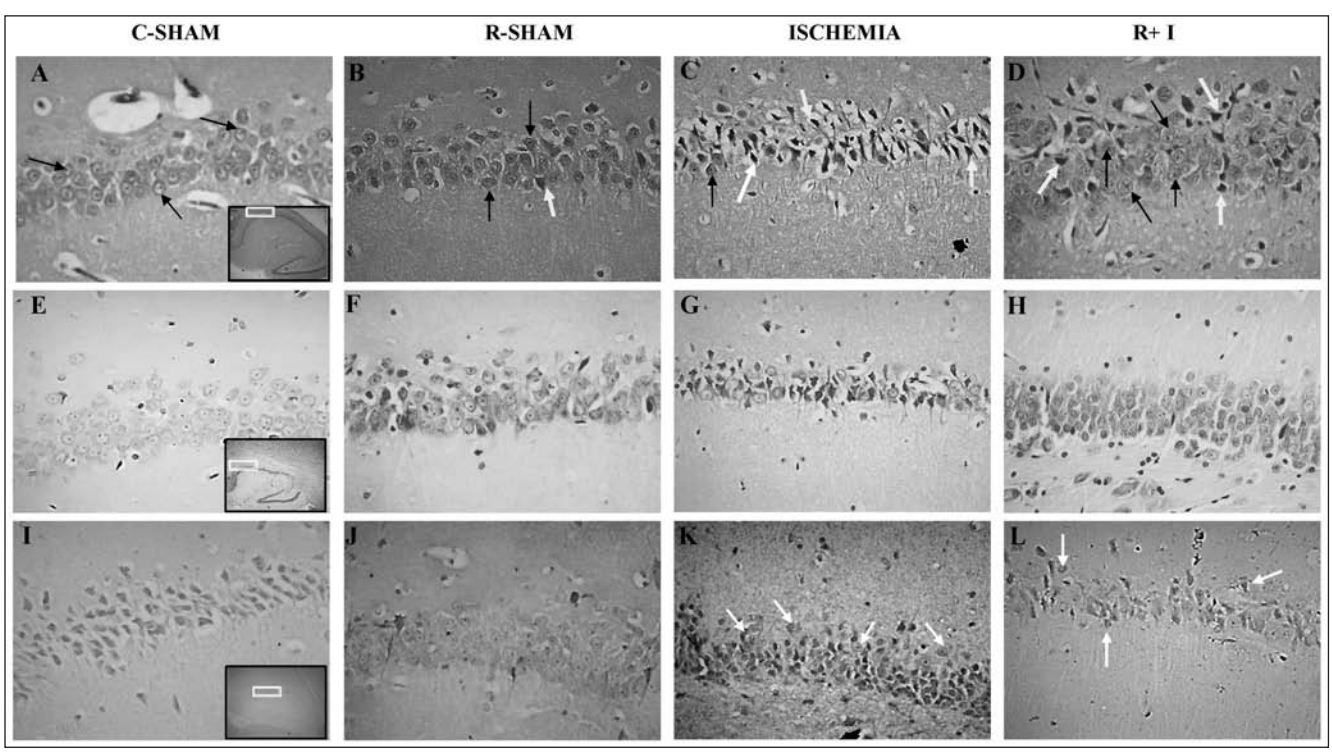

Fig. 6. Effects of 7 weeks intake of FRHT on neuronal loss in the CA1 region of the hippocampus after BCCAO or SHAM surgery. Images A, E and I contain inserts with the hippocampal regions of interest indicated in white rectangular outlines. Figs A–D represent H and E stained sections, with the ISCHEMIA group showing numerous shrunken CA1 pyramidal cells with condensed and deeply stained nuclei (white arrows). Figs E–H show staining with cresyl violet while Figs I–L represent TUNEL staining (for apoptosis).

brain oedema) are scarce in literature, and the associated cellular and molecular mechanisms are poorly understood (Panickar and Anderson 2011). The present study showed that pre-treatment with FRHT resulted in significantly reduced brain oedema (Fig. 1) as well as reduced BBB permeability though not statistically significant (Fig. 2), possibly due to the actions of the rich polyphenol contents in FRHT as previously reported for other teas (Lee et al. 2004, ArunaDevi et al. 2010). Tea polyphenols (Xue et al. 2013), *Lycium barbarum* extracts (Yang et al. 2012), dietary olive leaf extract (Mohagheghi et al. 2011) and lavender extract (Rabiei and Rafieian-Kopaei 2014) have all been shown to potentially protect the BBB against ischemic damage albeit via poorly understood mechanisms of action. We suggest that the observed BBB disruption and brain oedema protection effects of FRHT could have occurred either due to attenuation of oxidative stress damage to capillary endothelial cells and basement membrane (Panickar et al. 2013, Krueger et al. 2015), reversal of the ischemia-induced reduction in the expression of the tight junction proteins claudin-5, occludin and ZO-1 as seen with green tea polyphenols (Liu et al. 2013) or modulation of paracellular permeability to prevent disruption of the tight junctions between brain microvascular endothelial cells (Yang et al. 2016).

I/R-induced injury is known to be associated with neuronal death (Woodruff et al. 2011, Baron et al. 2014). Following an ischemic stroke, the brain loses as many neurons as it does in almost 3.6 years of normal aging (Saver 2006). Our study showed that FRHT pre-treatment significantly attenuated I/R-induced neuronal death in the hippocampal CA1 region (Fig. 7), consistent with previous findings (Abe et al. 1995, Koponen et al. 2000, Wang et al. 2005, Nikonenko et al. 2009) possibly through its antioxidant properties.

The role of oxidative stress in the pathophysiology of ischemic stroke has been well documented (Ozkul et al. 2007, Manzanero et al. 2013, Cichoń et al. 2015). Cellular damage during and after I/R-induced brain injury has been suggested to be due to oxidative damage caused by harmful free radicals (e.g. superoxide anions (O_2^-) (Kinuta et al. 1989, Tsai et al. 2014). Results from this study showed that MDA levels increased markedly in the ISCHEMIA group, indicative of lipid peroxidation but were maintained at physiologic values in the R+I group (Fig. 3). This could be due to the antioxidant properties of FRHT mediated by its polyphenolic compounds, particularly aspalathin, as previously reported (Inanami et al. 1995, Fukasawa et al. 2009, Marnewick et al. 2011, Awoniyi et al. 2012, Hong et al. 2014). The low levels of ORAC and FRAP (indicative of the high levels of oxidative stress) observed in this study for the ISCHEMIC group appeared to have been modulated by long-term pre-treatment with FRHT. Similar findings by Cao and colleagues (1998), Vergely and others (1998), Jung and colleagues (2011), and Akinmoladun and others (2015), all partly support the involvement of the antioxidant actions of FRHT.

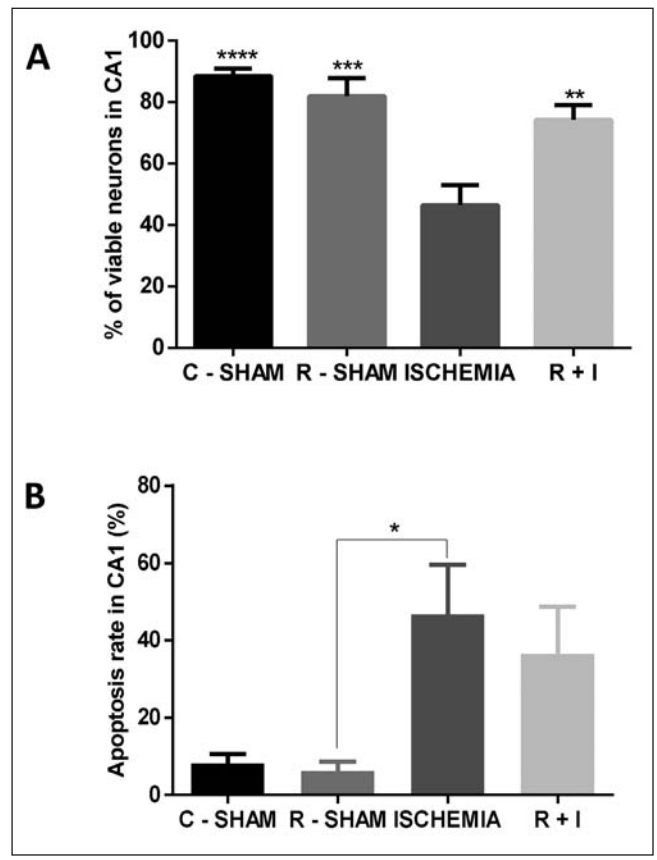

Fig. 7. Neuronal survival (CV staining) and percentage rate of apoptosis (%RA) (TUNEL staining) in the CA1 region of the hippocampus 7 days after BCCAO or sham surgery. (A) ISCHEMIA group had the least viable cells. (B) ISCHEMIA group had the highest (%RA). Data is expressed as mean ±SEM; n=5 rats per group. Significant difference for 7A: ****P < 0.0001 *vs.* control-sham, ***P < 0.001 *vs.* R-sham and **P<0.01 *vs.* R+I); for 7B: *P<0.05 R-sham *vs.* ISCHEMIA.

The severity of neurologic impairment after I/R-induced brain injury has previously been correlated with histomorphological findings (Furlan et al. 1996, Hong et al. 2000, Schiavon et al. 2014) and the present study showed similar relations. Rats in the R+I group generally showed better neurologic outcomes and less histomorphological damage when compared to the ISCHEMIA group (Fig. 6 *vs.* Fig. 8) probably due to the beneficial effects of rooibos tea. This is in line with findings from a study by Inanami and others (1995) which showed that rooibos tea could prevent age-related changes in the brain. Another study by Dal-Pan and colleagues (2017) showed that high polyphenol concentrations in berries could prevent neuropathological damage and cognitive impairment in an animal model of Alzheimer's disease. In our study, only changes that took place within the first 7 days post-surgery were reported although neuronal death and associated neurobehavioural deficits are known to occur months after I/R-induced

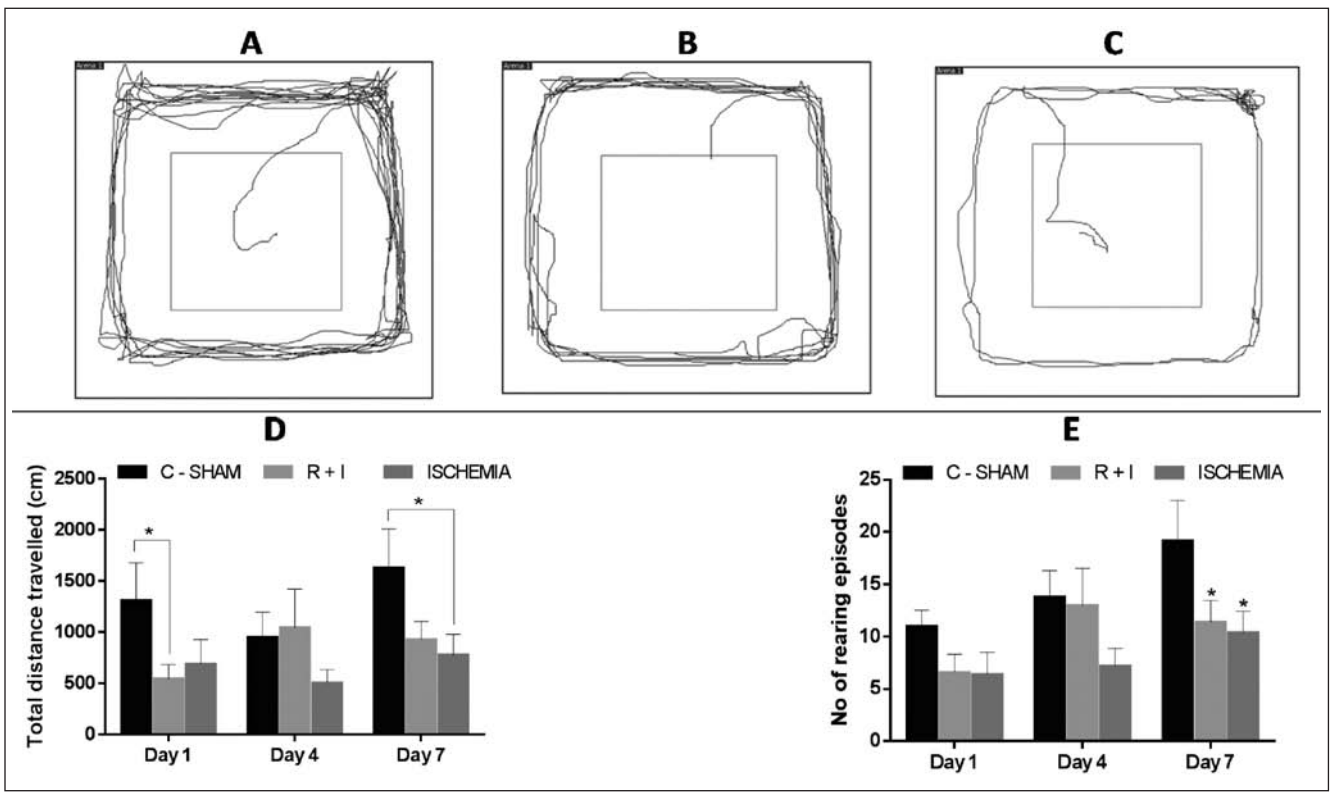

Fig. 8. Effects of 7 weeks pre-treatment with FRHT on neurobehavioural outcomes following the 10-minutes Open Field Test (OFT). Images A, B and C represent the paths travelled by the C-SHAM, R+I and ISCHEMIA rats respectively in the OFT. Image D shows that the R+I and ISCHEMIA rats travelled less than the C-SHAM rats while image E shows that the rearing episodes during the OFT were fewer in the R+I and ISCHEMIA rats compared with the C-SHAM rats. n=5, (*)=significant difference at P<0.05.

injury (Shin et al. 2010). The potential role of FRHT and other botanicals in post-stroke recovery especially in terms of improvements in cognitive and motor impairments, require further investigation.

CONCLUSION

Our results provide some evidence of multifactorial neuroprotection against I/R-induced injury, conferred by long-term consumption of FRHT. A possible reason for this effect is the action of the many polyphenols in FRHT in modulating the oxidative stress and neuroinflammatory pathways which cause neuronal death, brain oedema and BBB disruption. The findings from this study will contribute to existing information on the role of botanicals in stroke prevention and management.

STATISTICAL ANALYSIS

Results were compared using one-way analysis of variance (ANOVA) test. Tukey's *post-hoc* test was conducted for further comparison among groups if a statistically significant difference was obtained. A two-way ANOVA followed by Fisher's protected least significance difference (*post-hoc* LSD) test was used for analysis of open field measurements. Values were expressed as means ±standard error of mean (SEM). $P<0.05$ was considered as statistically significant.

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