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The Effects of Rooibos (*Aspalathus linearis*), Green Tea (*Camellia sinensis*) and Commercial Rooibos and Green Tea Supplements on Epididymal Sperm in Oxidative Stress-induced Rats

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Reactive oxygen species (ROS) are involved in many physiological functions of mammalian sperm. Numerous endogenous antioxidants belonging to both enzymatic and non-enzymatic groups can remove excess ROS and prevent oxidative stress (OS). This study compares the modulation of OS by rooibos, Chinese green tea and commercial rooibos and green tea supplements in rat sperm. Male Wistar rats ($n = 60$) were supplemented with fermented rooibos, 'green' rooibos, Chinese green tea, rooibos supplement, green tea supplement or water for 10 weeks while OS was induced during the last 2 weeks. Sperm count and motility were significantly higher for rats consuming fermented rooibos and 'green' rooibos when compared with the other groups. Catalase activity was significantly higher in the sperm of rats consuming fermented rooibos, 'green' rooibos and both the rooibos and green tea supplements. Superoxide dismutase concentration in the sperm of rats supplemented with fermented rooibos, 'green' rooibos and green tea was higher. Sperm glutathione levels of rats consuming the fermented and 'green' rooibos were also significantly higher. Rooibos fermented and 'green' rooibos showed a tendency to lower the levels of ROS and lipid peroxidation when compared with the control group. In conclusion, both rooibos extracts could offer a measure of protection against induced oxidative damage by increasing the antioxidant defence mechanisms and thereby improving the sperm quality and function.

Keywords: sperm; catalase; glutathione; green tea (*Camellia sinensis*); oxidative stress; rooibos (*Aspalathus linearis*); superoxide dismutase.

INTRODUCTION

Oxidative stress (OS) appears to play a major role in the development and progression of numerous disease states such as cancer, neurodegenerative disorders, ischemia and male infertility (Butterfield, 2002; Cocuzza *et al*., 2007). However, antioxidants may be useful in the prevention and treatment of these conditions. Flavonoids, a class of secondary plant phenolics, display antioxidant and chelating properties and are mostly concentrated in fruits, vegetables, wines and teas (Heim *et al*., 2002). Their cardioprotective effects stem from the ability to inhibit lipid peroxidation, chelate redox-active metals and attenuate other processes involving reactive oxygen species (ROS) (Heim *et al*., 2002). Reactive oxygen species, when in excess, are capable of causing oxidative damage to cellular proteins, nucleic acids and lipids. Lipid peroxidation (LPO) is a free-radical mediated propagation of oxidative insults to mainly polyunsaturated fatty acids (PUFA) involving several types of free radicals and termination may occur through enzymatic means or by free radical scavenging by antioxidants (Korkina and Afans'ev, 1997). Free radicals may have beneficial or detrimental effects on sperm functions depending on their nature and concentration (Baker *et al*., 2003). Oxidative stress plays an important role in human reproduction and it arises as a consequence of excessive ROS production and/or impaired antioxidant defence mechanisms (Novotný *et al*., 2003). Due to their deleterious effects on human spermatozoa, excessive ROS must be continuously inactivated to keep only a small amount necessary to maintain normal cell function (Sharma and Agarwal, 1996; Agarwal *et al*., 2003).

Rooibos (*Aspalathus linearis*) is a shrubby legume indigenous to the Cedarberg region of the Western Cape of South Africa. Traditionally, rooibos has been consumed as a health beverage for more than a century in South Africa and in Europe (Morton, 1983; Nakano *et al*., 1997; Marnewick, 2009). The colour of the unfermented rooibos product is green and referred to

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as 'green' rooibos while during fermentation the colour changes from green to red with oxidation of the constituent polyphenols and referred to as fermented or 'red' rooibos (Mckay and Blumberg, 2007). Rooibos contains unique phenolic compounds with aspalathin and nothofagin, β-hydroxy-dihydrochalcone glucosides, being the most abundant (Schulz *et al*., 2003). The oxidative cyclization of aspalathin results in the formation of flavones, (S)- and (R)-eriodictyol-6-C-β-D-glucopyranoside and further oxidation could result in the formation of the corresponding flavones orientin and isoorientin, vitexin and isovitexin and the flavanones, dihydro-orientin, dihydro-iso-orientin and hemiphlorin (Rabe *et al*., 1994; Marais *et al*., 2000; Bramati *et al*., 2002; Shimamura *et al*., 2006). Other flavones found in rooibos include chrysoeriol, luteolin and luteolin-7-*O*-glucoside, while the flavonols present are quercetin and its *O*-linked glycosides, quercetin-3-robinobioside, hyperoside, isoquercitrin and rutin (Snyckers and Salemi, 1974; Rabe *et al*., 1994; Bramati *et al*., 2002; Kazuno *et al*., 2005; Shimamura *et al*., 2006). The effectiveness of rooibos as an antioxidant on the liver antioxidant status and hepatoprotector in liver diseases has been demonstrated (Kucharska *et al*., 2004). Marnewick *et al*. (2000) showed the protective effects of fermented and 'green' rooibos against mutagenesis using the *Salmonella* mutagenicity assay (Marnewick *et al*., 2009). Other potential health promoting properties of rooibos include antispasmodic effects, immune system modulation, antimicrobial, antiviral and antiageing properties (Marnewick *et al*., 2000; Scheepers, 2001; Khan and Gilani, 2006; Ichiyama *et al*., 2007).

Tea, a product made from the leaves and buds of *Camellia sinensis* is the second most consumed beverage in the world (Costa *et al*., 2002; Juráni *et al*., 2008). Green, oolong and black teas are the three major types of tea produced from this plant. The 'non-fermented' green tea is produced by drying and steaming the fresh leaves to inactivate the polyphenol oxidase, thus avoiding oxidation (Rietveld and Wiseman, 2003). Previous studies have shown green tea as an important source of polyphenols, flavanols and flavonoids (Cabrera *et al*., 2006). There are several polyphenolic catechins in green tea, namely: (−) epicatechin (EC), (−) epicatechin-3-gallate (ECG), (−) epigallocatechin (EGC), (−) epigallocatechin-3-gallate (EGCG), (+) catechin and (+) gallocatechin with EGCG the most abundant catechin (Chu and Juneja, 1997). Green tea also contains gallic acid (GA) and other phenolic acids such as chlorogenic acid, caffeic acid and flavonols such as kaempferol, myricetin and quercetin (USDA, 2003). The health benefits of green tea are attributed mainly to their antioxidant properties and the ability of its polyphenolic catechins to scavenge ROS, which has led to their evaluation in a number of diseases associated with ROS such as cancer (Yang *et al*., 2002), cardiovascular and neurodegenerative diseases (Yang, 1999; Mukhtar and Ahmad, 2000). The present study investigates the modulatory effect of rooibos, green tea and commercial rooibos and green tea antioxidant supplements on various rat epididymal sperm parameters including sperm concentration and motility as well as redox status. The supplements were included in this study to elucidate the possible role of the purified components they contain, in this study model.

MATERIALS AND METHODS

Chemicals. Phosphate buffer saline (PBS), thiobarbituric acid (TBA), gallic acid, (+) catechin, quercetin, *p*-dimethylaminocinnamaldehyde (DAC), sodium chloride (NaCl), hydrogen peroxide (H_2O_2), trypan blue, sodium carbonate (Na_2CO_3), trolox, fluorescein (FL), 2,2'–azobis (2-amidinopropane) dihydrochloride (AAPH), t-butyl hydroperoxide (tbHP), 2,7 dichlorofluorescin diacetate (DCFH-DA), butylated hydroxide toluene/ethanol (BHT, C_2H_2OH) and Folin's reagent were purchased from Sigma-Aldrich (Johannesburg, South Africa). Hydrochloric acid, orthophosphoric acid, *n*-butanol and methanol were purchased from Merck Chemicals (Johannesburg, South Africa).

Preparation of aqueous herbal tea and tea extracts. Aqueous extracts of fermented and 'green' rooibos as well as green tea were prepared by the addition of freshly boiled tap water to the leaves and stems (2 g/100 mL). The herbal tea concentrations used are customary for tea making purposes in South Africa (Joubert, 1998; Marnewick *et al*., 2000). The mixture was allowed to stand for 30 min at room temperature, cooled, filtered (Whatman no. 1) and dispensed into water bottles. Individual batches of fermented and 'green' rooibos of superior grade were supplied by Rooibos Ltd (Clanwilliam, South Africa). Commercial rooibos and green tea supplements were prepared by dissolving finely crushed rooibos tablets (two tablets ~1 g) and green tea capsules (two capsules ~ 1 g) in 100 mL of lukewarm (40 °C) tap water (1 g/100 mL). The mixture was allowed to stand for 30 min at room temperature, to cool and was then dispensed into water bottles. All the extracts were freshly prepared every second day. The green tea and two commercial antioxidant supplements were bought from a local drug store in Cape Town, South Africa. Each rooibos tablet contained 175 mg of a 20% aspalathin-rich extract, 500 µg vitamin A, 150 mg vitamin C, 5 mg vitamin E and 25 µg selenium, and each green tea capsule contained 100 mg epigallocatechin gallate (EGCG) according to the manufacturers label. At the time of the study no commercial supplement was available that contained only the main polyphenols of rooibos, namely aspalathin.

Treatment of animals. Sixty male Wistar rats (120–150 g) were obtained from the Animal Unit of the University of Cape Town (South Africa). They were divided randomly into five ($n = 10$) supplementation groups and one control group. The rats were housed individually in perspex houses with stainless wire-bottomed cages in a closed environment 24–25 °C, with a 12 h ligh–-dark cycle and 50% humidity. The animals were kept under these conditions for 1 week prior to experimentation to acclimatize. The rats had free access to the various aqueous rooibos herbal teas, green tea and commercial supplement extracts (freshly prepared every second day) for 10 weeks as their sole source of drinking fluid while the control group received tap water. The rats were fed *ad libitum* with standard rat food and the fluid intake was monitored at an interval of 2 days for the duration of the study period. The general condition of the rats was monitored daily throughout the experiment. Body weights were recorded

twice a week as well as at the end of the study. Oxidative stress was induced in all rats with intraperitoneal (i.p.) injections of 30 μM *t*-butyl hydroperoxide (tbHP) per 100 g body weight in the last 2 weeks of the 10-week study (Kumar and Muralidhara, 2007). Ethical approval was obtained from CPUT's Faculty of Health and Wellness Sciences Research Ethics Committee. The rats (non-fasting) were sacrificed under pentobarbital anaesthesia by i.p. injection at 0.4 mL/kg body weight. Immediately thereafter, the testes and epididymis were excised and their weights recorded.

Soluble solids, total polyphenols, flavanol, flavonol content and antioxidant capacity determination of the herbal tea extracts and supplements. The soluble solid content of the fermented and 'green' rooibos and green tea were determined gravimetrically (six repetitions) after drying 1 mL aliquots at 110 °C for 12 h. The Folin–Ciocalteu method, with gallic acid as the standard, was used to determine the total polyphenol (TP) content of the rooibos herbal teas, green tea and both rooibos and green tea supplement samples (Singleton and Rossi, 1965). Briefly, the reaction was initiated by the addition of 125 μL of Folin reagent (0.2 N) and 100 μL of sodium carbonate (7.5% Na_2CO_3) to 25 μL of sample into a clear 96-well plate. A blue colour was formed and measured at 765 nm after 2 h incubation at room temperature in a Multiskan Spectrum (Thermo Electron Corporation – USA). The flavanol and flavonol/flavone contents were determined colorimetrically (640 nm) using *p*-dimethylaminocinnamaldehyde (0.5 g/L DAC in 1:4 hydrochloric acid:methanol) with (+)-catechin and spectrophotometrically (360 nm) using quercetin, as the standards, respectively (McMurrough and McDowell, 1978; Mazza *et al*., 1999). The results were expressed as mg catechin or mg quercetin equivalents/mg soluble solids, respectively. The oxygen radical absorbance capacity (ORAC) assay was based on the procedure described by Prior *et al*. (2003). Free radicals were produced by 2,2' azobis(2-amidinopropane) dihydrochloride (AAPH) and the oxidation of the fluorescent indicator, fluorescein (FL) was measured. Both reagents were prepared in 75 mM phosphate buffer (pH 7.4) and 500 μM Trolox (diluted to 5, 10, 15, 20, 25 μM) was used as the standard. The reaction was initiated by the addition of 50 μL of AAPH (25 mg/mL) to 12 μL sample/standard and 138 μL fluorescein in a 96-well black plate and the fluorescence (emission 530 nm, excitation 485 nm) was recorded every 5 min for 2 h (Fluoroskan Ascent: Thermo Electron Corporation, USA).

Epididymal sperm count and motility. The caudal epididymis was rinsed and gently homogenized in 1.5 mL of phosphate buffered saline (PBS, Sigma Chemical Co, South Africa) using Thomas homogenizer. The fragments were allowed to sediment for 5 min and 1 mL of the supernatant containing the sperm was filtered (Whatman no. 1) and collected for sperm count and motility. After collection of epididymal sperm, the concentration was determined using the method described in the World Health Organisation Manual (WHO, 1999). Briefly, a 50 μL aliquot of epididymal sperm was diluted with 95 μL trypan blue solution (Sigma, South Africa). A cover slip was secured to the counting chamber of a Neubauer type hemocytometer. Approximately 10 μL of the thoroughly mixed diluted specimen was transferred to the counting chamber of the hemocytometer, which was allowed to stand for 5 min in a humid chamber to prevent drying. During this time, the cells settled and viable cells were counted using a light microscope at 40× magnification. For sperm motility determination, one drop of fresh epididymal sperm was placed onto a glass slide and a coverslip was placed on top. Ten random fields were scored for number of motile and non-motile sperm. The sperm motility was expressed as percentage motile sperm. Sperm motility was assessed according to the guidelines of the WHO (1999).

Biochemical parameters

Assessment of reactive oxygen species and lipid peroxidation. Intracellular production of ROS in epididymal sperm was measured using 2'7'-dichlorofluorescin diacetate (DCFH-DA) as the probe (Driver *et al*., 2000). The non-fluorescent DCFH in the presence of ROS is rapidly oxidized to the highly fluorescent dichlorofluorescent (DCF). Ten μmol of DCF-DA was added to 100 μL of homogenized sperm samples and incubated for 45 min at room temperature (in dark) in a 96-well microplate. Fluorescence was measured at 530 nm emission and 485 nm excitation using the GloMax® Multidetection System. DCF production was expressed as relative fluorescence unit (RFU). The assay was conducted according to the protocol provided by the manufacturer (Promega, UK).

Lipid peroxidation (LPO) was quantified by measuring the formation of thiobarbituric acid reactive substances (TBARS) (Draper *et al*., 1993). Briefly, 50 μL of epididymal sperm 2×10^6 sperm were added to 6.25 μL of 4 nM cold BHT/C_2H_5OH and 50 μL of (0.2 M) orthophosphoric acid in a microfuge tube. After 10 s of vortexing, 6.25 μL of freshly prepared (0.11 M) thiobarbituric acid reagent was added and heated at 90 °C for 45 min. The samples were cooled down for 2 min on ice and 5 min at room temperature. This was followed by the addition of 500 μL of *n*-butanol and 50 μL of saturated NaCl to each sample, thereafter the reaction mixtures were centrifuged at 12000 rpm for 2 min at 4 °C. The supernatants (300 μL) were transferred into a 96-well microplate and absorbance was measured at 532 and 572 nm at room temperature using the GloMax® Multi detection System. Lipid peroxidation was expressed as nmol malondialdehyde (MDA) per 2×10^6 sperm.

Assessment of antioxidant enzymes and glutathione. Activities of antioxidant enzymes: superoxide dismutase (SOD), catalase (CAT) and glutathione levels were determined in epididymal sperm. Catalase activity was assessed by measuring the amount of substrate (hydrogen peroxide) remaining after sample addition (Zhou *et al*., 1997). Briefly, 50 μL of 40 μM hydrogen peroxide (H_2O_2) solution was added to 50 μL of sample in a 96-well microplate. This was followed by the addition of 100 μL of the reaction cocktail (detection reagent, reaction buffer and horseradish peroxidase) and incubated at room temperature for 15 min. Fluorescence was measured at 590–600 nm with excitation of 530–570 nm using the GloMax® Multidetection System (96-well plate spectrophotometer) according to the manufacturer's technical bulletin (Assay designs, USA). Catalase activity was expressed as relative fluorescent units (RFU). Superoxide dismutase activity was determined from the conversion of xanthine and

oxygen to uric acid and H_2O_2 by xanthine oxidase to form superoxide anion. The superoxide anion then converts tetrazolium salt (WST-1) to WST-1 formazan, a coloured product that absorbs light at 450 nm. The relative SOD activity of the experimental sperm samples was determined from the percentage inhibition of the rate of formation of WST-1 formazan. The reaction was initiated by the addition of 25 μL 1X xanthine solution and absorbance readings were measured at 450 nm every minute for 10 min at room temperature using the GloMax® Multidetection System (Promega, USA). The assay was conducted according to the protocol provided by the manufacturer (Assay designs, USA) using 2×10^6 epididymal sperm cells. The SOD activity was expressed as unit per microlitre (U/μL) sperm cells.

The levels of glutathione (GSH) were determined in epididymal sperm based on the conversion of a luciferin-derivative into luciferin in the presence of glutathione, catalysed by glutathione S-transferase (GST). The signal generated in a coupled reaction with firefly luciferase is proportional to the amount of glutathione present in the sample. The addition of 25 μL of the sperm sample to 50 μL of GSH-Glo™ Reagent (2X) was followed by adding 100 μL of luciferin detection reagent in a 96-well microplate and luminescence was measured by GloMax® Multidetection System. The assay was conducted according to the protocol provided by the manufacturer (Promega, UK) using 20000 cells. The GSH levels were expressed as relative luminescence units (RLU).

Statistical analysis. Data were analysed by two-way ANOVA using the general linear model according to SPSS version 17. The Bonferroni pairwise adjustment was used to determine whether the means differed statistically. Values were considered significant if $p < 0.05$. Data are expressed as mean ± standard deviation (SD).

RESULTS

Study beverages, antioxidant profiles and daily intakes

The soluble solids were significantly higher ($p < 0.05$) in the green tea when compared with fermented and 'green' rooibos. The soluble solids obtained from the fermented and 'green' rooibos constituted approximately 50% of the green tea (Table 1). The total polyphenol content of the rooibos supplement was higher than the rest of the treatment groups while fermented rooibos had the lowest polyphenol content and constituted about half the polyphenol content of the green tea supplement. The flavanol intake of rats that consumed green tea and green tea supplements were higher compared with the rest of the tea treatment groups (Table 1). Rats that consumed fermented rooibos and 'green' rooibos had a higher intake of flavonol than the rest of the tea treatment groups. The ORAC values of the green tea and rooibos supplement groups were higher than the rest of the tea treatment groups while green tea supplement produced the lowest ORAC value (Table 1).

The daily tea intake of rats that consumed fermented rooibos and 'green' rooibos did not differ significantly when compared with the water intake of the control group (Table 2), while the daily tea intake of rats that consumed green tea, the green tea supplement and

Table 1. Antioxidant profile of the beverages and supplements

Treatment	Soluble solids (mg/mL)	Polyphenols (mg/L)	Flavanol (mg/L)	Flavonol (mg/L)	ORAC (mmol tea/L)
Control	ND	ND	ND	ND	ND
Rf	5 ± 1.2^a	981.16 ± 117.69^a	38.66 ± 8.06^a	299.33 ± 49.44^a	14556.81 ± 904.60^a
Rg	7.1 ± 0.8^b	1354.33 ± 61.99^b	92.00 ± 2.60^b	247.00 ± 19.45^b	20888.75 ± 1281.03^b
Gt	12.6 ± 1.2^c	2723.16 ± 204.04^c	896.50 ± 25.44^c	108.66 ± 15.98^c	33350.68 ± 311.77^c
Rs	ND	4836.33 ± 243.92^d	17.66 ± 9.85^a	180.66 ± 22.47^d	33768.22 ± 2322.16^c
Gs	ND	1920.33 ± 195.75^e	822.83 ± 37.73^d	93.00 ± 10.48^c	9755.33 ± 645.04^d

Values in columns are means ± SD of 10 rats per group. Means followed by the same letter do not differ significantly ($p > 0.05$). If letters differ, then $p < 0.05$ versus control. Aqueous solutions (2%) were prepared for fermented rooibos (Rf), 'green' rooibos (Rg), green tea (Gt), rooibos supplements (Rs) and green tea supplements (Gs). ORAC, oxygen radical absorbance capacity; ND, not done.

Table 2. Daily intake of the herbal teas, green tea and commercial tea supplements and various antioxidant tea constituents

Treatment	Tea intake (mL/day)	Total polyphenol intake (mg/day)	Flavanol intake (mg/day)	Flavonol intake (mg/day)	ORAC (μmole/day)
Control	69.40 ± 8.61^a	ND	ND	ND	ND
Rf	74.50 ± 12.93^a	73.1	2.8	21.8	1084.4
Rg	69.00 ± 14.73^a	93.4	6.3	17.0	1441.2
Gt	55.10 ± 13.06^b	150.0	49.4	6.0	1837.6
Rs	35.10 ± 4.33^c	169.7	0.6	6.3	1185.3
Gs	38.70 ± 3.65^c	74.3	31.8	3.6	377.5

The daily intake of polyphenols, flavanols, flavonols and oxygen radical absorbance capacity (ORAC) equivalents are based on the daily tea intake volume and the beverage profile in Table 1.

Means followed by the same letter do not differ significantly ($p > 0.05$). If letters differ, then $p < 0.05$ versus control. Fermented rooibos (Rf), 'green' rooibos (Rg), green tea (Gt), rooibos supplement (Rs) and green tea supplement (Gs). ND, not done.

rooibos supplement were significantly ($p < 0.05$) less when compared with the control group (Table 2). The daily tea intake of rats that consumed the rooibos supplement and green tea supplement represent about 50% of the tea intake of the rats that consumed fermented rooibos. Rats that consumed green tea and green tea supplement had a higher intake of flavanols than the rats that consumed fermented rooibos, 'green' rooibos and the rooibos supplement. The flavonol intake of rats that consumed fermented rooibos and 'green' rooibos were higher than the rest of the treatment groups (Table 2). The ORAC values of the daily tea intake of 'green' rooibos and green tea were higher than the rest of the tea treatment groups while green tea supplement produced the lowest (Table 2).

Body weight gains, testicular weights and epididymis weights

The effects of the different tea preparations and supplements on body weights, testicular weights and epididymal weights are presented in Table 3. Although the various herbal teas, green tea and supplement preparations had no adverse effects on the body weight gain, testes weights and epididymis weights, the rats consuming green tea showed a lower, although not significant, body weight gain. Testicular and epididymis weights for this group were also the lowest compared with all other treatment groups.

Epididymal sperm count and motility

The effects of the rooibos herbal teas, green tea and two supplements on the motility and epididymal sperm concentration are presented in Table 3. A significant increase ($p < 0.05$) was recorded in the epididymal sperm concentration and motility of rats consuming fermented rooibos and 'green' rooibos when compared with the control group and other experimental groups. The groups that consumed the rooibos supplement, green tea and green tea supplement also showed an increase in sperm concentration and motility when compared with the control animals consuming water, although not significant.

Oxidative stress parameters

Reactive oxygen species production and lipid peroxidation. The ROS production (DCFH-DA) and TBARS levels of all groups are shown in Table 4. The ROS levels in epididymal sperm of rats consuming the fermented rooibos, 'green' rooibos and rooibos supplements showed a modest decrease, although, not significant. The rats consuming green tea and the green tea supplement did not show any difference when compared with the control group. Lipid peroxidation, measured as TBARS levels in epididymal sperm of rats, consuming the different tea preparations and supplements did not exert any significant effects, although a trend to lower TBARS levels was evident in all the treated groups when compared with the control group.

Antioxidants enzymes. Data on the activities of epididymal sperm antioxidant enzymes in rats consuming the rooibos herbal teas, green tea and commercial supplements are presented in Table 4. The CAT activity was significantly enhanced ($p < 0.05$) in the sperm of rats consuming fermented rooibos, 'green' rooibos and the rooibos and green tea supplements when compared with

Table 3. Body weight gain, testicular weights, epididymis weights, motility and sperm concentration

Treatment	Body weight gain (g)	Testis weight (g)	Epididymis weight (g)	Sperm motility (%)	Sperm count ($\times 10^6$)
Control	125.80 ± 24.96^a	3.67 ± 0.70^a	0.42 ± 0.07^a	54.50 ± 24.20^a	56.90 ± 27.11^a
Rf	112.10 ± 33.84^a	3.68 ± 0.19^a	0.47 ± 0.05^a	82.00 ± 12.95^b	79.60 ± 27.49^b
Rg	135.11 ± 35.56^a	3.75 ± 0.34^a	0.48 ± 0.06^a	81.67 ± 10.90^b	84.33 ± 26.26^b
Gt	92.00 ± 26.45^a	3.21 ± 0.57^a	0.40 ± 0.08^a	59.22 ± 23.57^a	70.70 ± 49.79^a
Rs	123.40 ± 35.82^a	3.34 ± 0.94^a	0.42 ± 0.10^a	69.44 ± 17.58^a	76.78 ± 21.94^a
Gs	100.70 ± 37.66^a	3.50 ± 0.59^a	0.43 ± 0.09^a	56.50 ± 23.22^a	60.20 ± 25.44^a

Values in columns are means ± SD of 10 rats per group. Means followed by the same letter do not differ significantly ($p > 0.05$). If letters differ, then $p < 0.05$ versus control.

Rats fed with fermented rooibos (Rf), 'green' rooibos (Rg), green tea (Gt), rooibos supplements (Rs) and green tea supplements (Gs).

Table 4. Effect of rooibos herbal teas, green tea and supplements on reactive oxygen species production, thiobarbituric acid reactive substances, catalase, superoxide dismutase and glutathione in epididymal sperm of rats

Treatment	DCFH–DA fluorescence (RFU)	TBARS nmol MDA/ 2×10^6 sperm	Catalase fluorescence (RFU) $\times 10^3$	Superoxide dismutase U/μL	Glutathione luminescence (RLU) $\times 10^4$
Control	3341.24 ± 1199.86^a	5.12 ± 0.80^a	54.84 ± 26.22^a	0.71 ± 0.14^a	64.52 ± 7.70^a
Rf	2970.47 ± 352.13^a	4.50 ± 1.32^a	73.33 ± 16.64^b	0.79 ± 0.17^b	70.96 ± 9.89^b
Rg	3061.82 ± 1021.66^a	4.72 ± 1.18^a	72.11 ± 17.66^b	0.80 ± 0.16^b	68.74 ± 7.48^b
Gt	3598.84 ± 990.76^a	4.73 ± 0.79^a	56.81 ± 24.63^a	0.76 ± 0.17^b	64.52 ± 8.21^a
Rs	3048.26 ± 828.01^a	4.93 ± 0.51^a	70.99 ± 18.57^b	0.75 ± 0.14^a	64.17 ± 8.37^a
Gs	3604.76 ± 1600.56^a	4.94 ± 0.67^a	59.18 ± 21.64^c	0.74 ± 0.15^a	64.09 ± 8.68^a

Values in columns are means ± SD of 10 rats per group. Means followed by the same letter do not differ significantly ($p > 0.05$).
If letters differ, then $p < 0.05$ versus control. Fermented rooibos (Rf), 'green' rooibos (Rg), green tea (Gt), rooibos supplements (Rs) and green tea supplements (Gs).

the control group consuming water. When considering the SOD activity, rats consuming fermented rooibos, 'green 'rooibos and green tea caused a significant ($p < 0.05$) increase when compared with the control group. None of the other groups showed any significant effect on SOD activity.

Glutathione level. The sperm GSH levels are shown in Table 4. Consumption of fermented rooibos and 'green' rooibos resulted in a significant ($p < 0.05$) increase in sperm GSH levels when compared with the control group with green tea and both supplements that showed no modulation of GSH levels when compared with the control group.

DISCUSSION

Certain dietary constituents such as antioxidants may influence the incidence and development of chronic diseases by improving the oxidative stress status and modulating several genes (Kaliora *et al*., 2006). Due to the differences in the phenolic constituents of rooibos and green tea, it is essential to investigate the possible impact of them, on the oxidative stress status of sperm and its effect on male infertility. Generally, an imbalance in pro-oxidant and oxidant status could produce oxidative stress; however, a change in antioxidant activities is frequently used as an important indicator while the antioxidant defence status determines the extent to which oxidative damage occurs in the sperm (Sikka, 2001; Ong *et al*., 2002). Sperm are susceptible to peroxidative damage due to the large amount of polyunsaturated fatty acids which are involved in the regulation of spermatogenesis, sperm maturation, capacitation, acrosome reaction, membrane fusion and low antioxidant capacity (Sikka, 1996; Sanocka and Kurpisz, 2004; Vernet *et al*., 2004).

In the present study where OS was induced with tbHP, it was observed that epididymal sperm concentration and motility of rats that consumed fermented and 'green' rooibos were significantly higher compared with the control. The improvements observed in the sperm quality may be attributed to the prevention of excessive generation of free radicals produced by sperm by means of the antioxidant properties of rooibos. Previously, Purdy *et al*. (2004) demonstrated that flavonoids caused an increase in motility in caprine sperm (Purdy *et al*., 2004). Similarly, Chung *et al*. (2005) found that Maca extract increased the epididymal sperm count of a rat. The metabolism of many compounds by cells caused an increase in the levels of electrophilic radicals, which react with oxygen giving rise to ROS, one of the main sources of free radicals such as hydrogen peroxide (H_2O_2), singlet-oxygen (O_2), hydroxyl radical (OH) or peroxynitrite (Gurib-Fakim, 2006). Cells exhibit a defensive mechanism using various antioxidant enzymes when ROS begin to accumulate.

Our study shows that rats consuming fermented rooibos, 'green' rooibos and to a certain extent the rooibos supplement, protected the sperm against oxidative stress when compared with the green tea and green tea supplement groups. This protection was shown by a tendency to reduce the levels of LPO, DCFH-DA and significantly enhance the levels of the antioxidant enzymes SOD and CAT as well as GSH levels. The potential mechanisms by which this protection is achieved could involve one or more of several different antioxidant properties exhibited by these extracts. Polyphenols, a major class of bioactive phytochemicals in rooibos, not only binds lipid peroxides, but can also impede the lipid peroxidation cascade, either by acting as a sacrificial antioxidant or as a chelator of transition metals (Cd, Cu and Fe) that promotes this process (Halliwell and Gutteridge, 1999; Nijveldt *et al*., 2001). Another intriguing possibility is that polyphenols have the potential to up-regulate the expression of β-glutamylcysteine synthetase, the rate-limiting enzyme in the biosynthesis of GSH (Moskaug *et al*., 2005). This may explain in our findings the enhancement of GSH in rats consuming the rooibos extracts. Reduced glutathione is a powerful intracellular antioxidant that plays a vital role in stabilizing various enzymes and could also be regarded as a good marker for tissue redox status (Wang and Jiao, 2000; Van Acker *et al*., 2000). The significant increase in the GSH levels of the epididymal sperm in the rooibos treated rats may suggest decreased oxidative stress or an increase in the antioxidant capacity of the cell, thereby lowering the vulnerability to oxidative damage. In this present study, the increase in GSH levels obtained with rooibos consumption suggests that the phenolic components of this herbal tea were more effective in increasing the redox/antioxidant status in the epididymal sperm. This may result in an enhanced endogenous detoxification capacity, as glutathione is known to, either directly or via the glutathione *S*-transferases, interact with reactive toxic metabolites, thus reducing the risk of oxidative damage to biomolecules (Siess *et al*., 2000). Previously, Türk *et al*. (2008) reported increased GSH levels in rat epididymal sperm caused by pomegranate juice which is highly rich in polyphenolic compounds.

Lipid peroxidation (LPO) is one of the main manifestations of oxidative damage and it has been found to play a major role in the toxicity of many xenobiotics (Yousef, 2004). Rooibos has been previously reported to reduce age-related LPO accumulation (measured as TBARS) in brains of rats consuming the herbal tea for 21 months (Ulicna *et al*., 2006). Similarly, fermented rooibos and 'green' rooibos were also found to be highly protective against lipid peroxidation in rat liver (Marnewick *et al*., 2005). The effects of other phenolic-rich beverages, especially *C. sinensis* on LPO, have been studied more extensively and several studies support an inhibitory role for green and black tea against LPO (Bursill *et al*., 2007; Koo and Noh, 2007). These proposed mechanisms include inhibition of lipid absorption and cholesterol synthesis as well as up-regulation of the LDL receptor (Bursill *et al*., 2007; Koo and Noh, 2007). The present study showed a moderate decrease, although not significant, in TBARS by the different teas and supplements, which could be attributed to the flavonoid contents. Although rooibos flavonoids are known to be strong antioxidants *in vitro* data regarding their antioxidant potential, bioavailability and metabolism *ex vivo* are still insufficient (Snijman *et al*., 2009). To evaluate the bioavailability and metabolism of flavonoids from rooibos *in vivo*, it is important to identify its metabolites. Bioavailability studies conducted in pigs and humans showed that rooibos flavonoids are

absorbed only to a very low extent (Kreuz *et al*., 2008; Courts and Williamson, 2009; Stalmach *et al*., 2009a, 2009b). A more recent study by Breiter *et al*. (2011) in humans confirmed the trace levels of green rooibos metabolites as well as unchanged rooibos flavonoids in the plasma, accounting for only 0.26% of the total flavonoid intake. The bulk of the rooibos flavonoids reach the large intestines directly, where they are exposed to intestinal bacteria (Breiter *et al*., 2011). Bioavailability studies with green tea or green tea extracts have shown diverse and controversial results with urinary excretion ranging from unquantifiable traces to values close to 10% of the ingested amount (Chow *et al*., 2005; Manach *et al*., 2005; Stalmach *et al*., 2009a, 2009b). However, most of these studies failed to establish the whole pattern of molecules deriving from the catechins originally present in tea. Polyphenolic compounds in green tea and rooibos undergo different chemical modifications along the gastrointestinal tract and inside the human body, before and after consumption and this may contribute to the differences in the results observed in our study between rooibos and green tea. Flavonoids are well documented for their antioxidant properties and their ability to act as antioxidants is determined by its structure particularly its ability to donate a hydrogen ion to the peroxyl radical produced as a result of LPO (Saija, 1995; Kashima, 1999). The decrease in LPO could also be a reflection of the improved redox state as previously discussed. The redox state of cells is known to impact profoundly on cellular functions such as the glutathione S-transferase-mediated elimination of electrophilic xenobiotics and some of the end-products of lipid peroxidation (Rebrin *et al*., 2005). Previously, Yousef and Salama (2009) reported that propolis, a flavonoid containing substance, caused a reduction in TBARS. With the exception of polyphenols and flavanols, the daily flavonol intake was significantly higher in the rooibos herbal tea groups compared with the green tea and green tea supplement groups. This could account for the differences shown in the modulation of the oxidative stress status and improved sperm characteristics of the different groups of rats in this study. Because the phenolic constituents of the South African herbal tea differ from that of green tea, the mechanisms involved in protection against oxidative damage could also differ.

Superoxide dismutase is an enzyme that converts superoxide to hydrogen peroxide and oxygen and CAT converts hydrogen peroxide to oxygen and water. These enzymes are responsible for the antioxidant properties and all have been confirmed to exist in the epididymis (Vernet *et al*., 2004). Our study showed a significant increase in the activities of SOD and CAT of rats consuming fermented rooibos and green rooibos. Antioxidant enzymes with radical scavenging and repair activities counteract reactive oxygen species and reactive oxygen species-induced damage triggered by oxidative stress. The combined effects of SOD and CAT are supposedly sufficient to eliminate oxygen and hydrogen peroxide and protect cellular components against the more reactive hydroxyl radical (Posmyk *et al*., 2005). Previously, Suresh *et al*. (2010) reported that *Mucuna pruriens*, a flavonoid-rich plant reversed and caused a significant increase in the activities of SOD and CAT. Transcription of antioxidant genes could be induced by endogenous and/or exogenous factors, which may result in the increased synthesis or production of antioxidant enzymes (Nilakantan *et al*., 2005). We therefore propose that the flavonoids (and more specifically the flavonols) in rooibos could have caused an increase in the transcription of these antioxidant enzyme genes thereby increasing the availability of these enzymes that play a major role in protecting against oxidative stress and improving sperm quality as one of the mechanisms of protection. The dismutation of superoxide anion, a negatively charged species and membrane-impermeable to H_2O_2 and oxygen, facilitates both the distribution of ROS via diffusion between cellular compartments and the removal of containing enzymes. A decrease in SOD and the H_2O_2 consuming enzyme, CAT, may result in an increased production of H_2O_2 and this may facilitate the production of hydroxyl radicals and consequently damage biological macromolecules. We propose that the increased catalase and superoxide dismutase activity could be the protective leverage for the reactive hydroxyl radicals produced due to oxidative stress induction, which can cause damage to macromolecules such as DNA, proteins and cell membranes.

CONCLUSION

The results of this study suggest that the wide spectrums of polyphenolic constituents present in rooibos (*Aspalathus linearis*) are effective as antioxidants. In addition, rooibos contains a plethora of other components different from that of green tea (*Camellia sinensis*) that aided its protective effect and could offer a protective role against sperm oxidative damage, thereby possibly offering a therapeutic treatment for infertility. In the rat model, our study has shown that rooibos improved sperm quality, protected sperm against oxidative damage, increased antioxidant enzyme activities and increased the levels of GSH in oxidative stress-induced rats. The significantly better protection observed by rooibos and green tea when compared with their commercial supplements may be due to the unique composition and levels of antioxidants and other compounds present in the rooibos and green teas. The effect is not only as a result of the main polyphenolic compounds, but is proposed to be a synergistic effect of all compounds. It is suggested that rooibos can generally be used as a supportive therapy, however, a series of well-controlled clinical intervention trials are needed to explore this possibility further. The exact mechanisms involved in the protective roles of this South African herbal tea also need to be elucidated further in future studies.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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