

Full Length Research Paper

Protective effects of rooibos (*Aspalathus linearis*), green tea (*Camellia sinensis*) and commercial supplements on testicular tissue of oxidative stress-induced rats

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Accepted 17 October, 2011

This study compares the modulation of oxidative stress by an indigenous herbal tea, rooibos, Chinese green tea and commercial rooibos and green tea supplements in rat testicular tissue. Male Wistar rats (n = 60) were fed with either fermented rooibos, “green” rooibos, Chinese green tea, commercial rooibos or green tea supplements for ten weeks. Oxidative stress (OS) was induced in all animals by an intraperitoneal t-butyl hydroperoxide injection in the last two weeks of the study. The superoxide dismutase (SOD) activity increased significantly (P < 0.05) in the testicular tissue of rats that consumed fermented rooibos, green tea and rooibos supplement as compared to the control. The glutathione levels of rats that consumed the green tea supplement was also significantly (P < 0.05) increased when compared with the control. Reactive oxygen species (ROS) levels were significantly (P < 0.05) decreased in rats that consumed the rooibos supplement, while lipid peroxidation measured as thiobarbituric acid reactive substances (TBARS) was significantly (P < 0.05) decreased in rats that consumed fermented rooibos and green tea. In conclusion, both extracts of fermented rooibos and green tea could be effective in the protection of testicular tissue against oxidative damage by possibly increasing the antioxidant defense mechanisms in rats, while reducing lipid peroxidation.

Key words: Antioxidants, epididymal sperm, catalase, glutathione, green tea (*Camellia sinensis*), lipid peroxidation, oxidative stress, reactive oxygen species, rooibos (*Aspalathus linearis*), superoxide dismutase.

INTRODUCTION

Reactive oxygen species (ROS) play a major role in the pathogenesis of several reproductive processes and has

particularly been linked to male infertility (Abdallah et al., 2009). Oxidative stress (OS) is one of the prominent causes of defective sperm which has been demonstrated by the unwarranted ROS generation by sperm and the depletion of antioxidant defenses in the male reproductive tract (Abdallah et al., 2009). Endogenous protective mechanisms may not be enough to limit ROS and their harmful effects as a result of disproportionate production of ROS (Sies, 1993; Kulkarni et al., 2007). Many artificial and natural agents possessing antioxidant

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Abbreviations: OS, Oxidative stress; SOD, superoxide dismutase; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substances.

and radical scavenging properties, such as dietary antioxidants, may be of great importance as additional protective measures and have been proposed to prevent and/or treat oxidative damage induced by ROS (Martinez-Cayuela, 1995; Furst, 1996; Kucharska et al., 2004). Along with a wide range of synthetic antioxidants, several natural occurring compounds in plants and fruits have been studied for the prevention of OS of different aetiologies (Lampe, 1999; Riso et al., 2009). The popularity of these compounds is due to their advantages such as low or no toxicity, as well as containing a plethora of antioxidants which covers dismutation and trapping of almost all types of ROS. Most popular amongst the dietary antioxidants are different types of teas and herbal teas widely used as non-alcoholic health beverages (Benzie and Szeto, 1999; Trevisanato and Kim, 2000). The therapeutic and medicinal values of rooibos (*Aspalathus linearis*) and green tea (*Camellia sinensis*) is the subject of many studies and several researchers have described their functional health benefits (Yang et al., 2002; Marnewick et al., 2000, 2003, 2005, 2009a, b). The aim of this study was to evaluate the possible protective effects of fermented and "green" rooibos (*A. linearis*), green tea (*C. sinensis*) and commercially available rooibos supplement and green tea supplement on biochemical parameters related to induced oxidative stress in the testes of rats. The supplements were included in this study because of the active components of the various herbal teas and tea which they contain.

MATERIALS AND METHODS

Treatment of animals

60 male Wistar rats (120 to 150 g) were obtained from the Animal Unit of the University of Cape Town (South Africa). They were randomly divided into five supplementation groups and one control group (n = 10 per group). Over a 10 week period, the animals received aqueous extracts of superior grade fermented and "green" rooibos (2% w/v) supplied by Rooibos Ltd (Clanwilliam, South Africa), green tea (2% w/v), rooibos or green tea commercial supplements (1% w/v) freshly prepared every second day as their main source of fluid intake. 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, while each green tea capsule contained 100 mg epigallocatechin gallate (EGCG) according to the manufacturers label. The green tea, rooibos and green tea commercial antioxidant supplements were bought from a local drug store in Cape Town, South Africa. Control animals received tap water only. Body weights were recorded twice a week as well as at the end of the study. During the last two weeks of the 10 week study, OS was induced in all animals by a daily intraperitoneal injection (i.p) of t-butyl hydroperoxide (Kumar and Muralidhara, 2007; Aboua et al., 2009). The rats (non-fasting) were sacrificed under pentobarbital anaesthesia (i.p. 0.4 ml/kg body weight). The testes were excised, their weights were recorded, rinsed and gently homogenized in 1.5 ml of phosphate buffered saline using a Thomas homogenizer. The tissue homogenates were collected for measurement of various biochemical parameters. Ethical approval was obtained from Faculty of Health and Wellness Sciences Research Ethics Committee, Cape Peninsula University

of Technology.

Assessment of reactive oxygen species and lipid peroxidation

Intracellular production of ROS in the testis was measured using 2',7'-dichlorofluorescein diacetate (DCFH) as the probe (Driver et al., 2000). The non fluorescent DCFH in the presence of ROS is rapidly oxidized to highly fluorescent dichlorofluorescent (DCF). Briefly, 10 µmol of DCFH was added to 100 µl of testis homogenate and incubated for 45 min at room temperature (in dark) in a 96 well microplate. Fluorescence was measured at 530 nm emission (485 nm excitation) using the GloMax[®] Multi detection System. DCF production was expressed as relative fluorescence units (RFU).

Lipid peroxidation (LPO) was quantified by measuring the formation of thiobarbituric acid reactive substances (TBARS) (Draper et al., 1993). Fifty microliters of testis homogenate were added to 6.25 µl of cold butylated hydroxyl toluene/ethanol (4 nM) and 50 µl of ortho-phosphoric (0.2 M) acid in a microcentrifuge tube. After 10 s of vortexing, 6.25 µl of freshly prepared thiobarbituric acid reagent (0.11 M) was added and the mixture was heated to 90°C for 45 min. The samples were cooled on ice for 2 min and kept at room temperature for 5 min before adding n-butanol (500 µl) and saturated NaCl (50 µl). The reaction mixtures were centrifuged (12000 rpm, 2 min, 4°C) and 300 µl supernatant (top butanol) were transferred into a 96 well microplate. Absorbance was measured at 532 and 572 nm at room temperature (GloMax[®] Multi detection System). Lipid peroxidation was expressed as nmol malondialdehyde per milligram tissue (MDA/mg tissue).

Assessment of antioxidant enzymes

The activities of the antioxidant enzymes: SOD and catalase (CAT) were determined in testes homogenates by means of a plate reader (GloMax[®] Multi detection System, Promega, UK). Catalase activity was measured by catalase fluorometric detection kit (Assay designs, USA). The CAT activity was assessed by measuring the amount of substrate, hydrogen peroxide (H₂O₂), remaining after sample addition (Zhou et al., 1997). Briefly, 50 µl of H₂O₂ (40 µM) solution was added to 50 µl of testes homogenates. This was followed by the addition of 100 µl of the reaction cocktail (detection reagent, reaction buffer and horseradish peroxidase) and incubation at room temperature (15 min). Readings were determined at fluorescence of 590 to 600 nm and excitation at 530 to 570 nm. The CAT activity was expressed as relative fluorescent units (RFU).

Superoxide dismutase activity was measured using superoxide dismutase activity kit (Assay designs, USA). The SOD activity was determined from the conversion of xanthine and oxygen to uric acid and H₂O₂ by xanthine oxidase to form superoxide anion (O₂^{-•}). The O₂^{-•} then converts tetrazolium salt (WST-1) to WST-1 formazan, a colored product that absorbs light at 450 nm. The relative SOD activity of the experimental sample is 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 minutes at room temperature. The assay was conducted according to the protocol provided by the manufacturer (Assay designs, USA). The SOD activity was expressed as U/mg of tissue.

Glutathione (GSH) analysis

The GSH level was measured using a commercial kit GSH-Glo[™]

Table 1. Effect of rooibos herbal teas, green tea, and commercial rooibos and green tea supplements on reactive oxygen species production, thiobarbituric acid reactive substances, catalase, superoxide dismutase and glutathione in the testicular tissue of rats.

Treatment	DCFH-DA	TBARS	Catalase	Superoxide dismutase	Glutathione
	Fluorescence (RFU) x 10 ³	nmolMDA/mg tissue	Fluorescence (RFU) x 10 ³	U/mg of tissue	Luminescence (RLU) x 10 ³
Control	104.96 ± 46.40 ^a	15.73 ± 6.46 ^a	40.37 ± 8.14 ^a	2.38 ± 0.07 ^a	13.38 ± 3.87 ^a
Rf	75.14 ± 26.51 ^a	7.99 ± 3.89 ^b	42.62 ± 4.15 ^a	3.80 ± 0.05 ^b	15.16 ± 1.19 ^a
Rg	79.54 ± 28.08 ^a	13.04 ± 6.83 ^a	40.06 ± 4.53 ^a	2.20 ± 0.09 ^c	14.07 ± 2.11 ^a
Gt	105.07 ± 40.08 ^a	9.17 ± 4.78 ^b	45.30 ± 14.43 ^a	4.27 ± 0.13 ^b	14.40 ± 3.07 ^a
Rs	70.90 ± 12.69 ^b	13.59 ± 5.27 ^a	44.60 ± 7.33 ^a	2.83 ± 0.09 ^b	14.18 ± 2.02 ^a
Gs	96.67 ± 32.23 ^a	14.30 ± 4.53 ^a	44.06 ± 5.24 ^a	2.12 ± 0.13 ^c	16.16 ± 3.61 ^b

Values in columns are means ± STD of 10 rats per group. Means followed by the same letter do not differ significantly ($P > 0.05$). If letters differ, then $P < 0.05$ vs. control. Fermented rooibos (Rf), “green” rooibos (Rg), green tea (Gt), rooibos supplement (Rs) and green tea supplement (Gs).

Glutathione Assay (Promega, UK). Determination of the level of GSH was based on the conversion of a luciferin derivative into luciferin in the presence of glutathione, catalyzed by glutathione S-transferase (GST). The signal generated in a coupled reaction with firefly luciferase is proportional to the amount of GSH present in the sample. Tissue homogenates (25 μ l) were added to GSH-Glo™ Reagent 2X (50 μ l) and incubated at room temperature for 30 min. Subsequently, 100 μ l of luciferin detection reagent was added in a 96 well microplate and the mixture was incubated for 15 min before reading the luminescence using the GloMax® Multi detection System. The assay was conducted according to the protocol provided by the manufacturer (Promega, UK). The GSH level was expressed as relative luminescence units (RLU).

Statistical analysis

Data were analyzed by two-way ANOVA using the general linear model procedure 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 (STD).

RESULTS

There were no differences in the body weight gain, testes weights and epididymis weights of all the tea treated rats (results not shown, recorded in previous study).

Reactive oxygen species production and lipid peroxidation

The ROS production (DCFH-DA) and TBARS levels of all groups are shown in Table 1. Although, all the rats consuming the two rooibos extracts and commercial rooibos supplement showed a decreased in ROS levels; only the rooibos supplement group showed a significant ($P < 0.05$) decrease when compared when the control group. Fermented rooibos and green tea caused a significant ($P < 0.05$) decrease in the LPO, measured as TBARS levels, as compared to the group that received

water. “Green” rooibos and both commercial supplements did not show any significant effects on TBARS levels. A trend to reduce the TBARS levels by “green” rooibos and the two commercial supplements were shown when compared with the control group, although not significant.

Antioxidant enzymes

Data on the activities of antioxidant enzymes in the testes of rats that consumed the rooibos herbal teas, green tea and commercial supplements are presented in Table 1. None of the herbal tea and tea treatment groups showed any significant differences in CAT activity, but a trend of increase in the activity was shown by all the groups, except “green” rooibos as compared to the control group. Rats that consumed fermented rooibos, green tea and the rooibos supplement had a significant ($P < 0.05$) increase in the SOD activity while rats that received “green rooibos” and the green tea supplement had decreased SOD activity significantly ($P < 0.05$) when compared with the control group that consumed only water.

Glutathione level

The GSH levels in the testicular tissue of the different tea treated groups are shown in Table 1. Rats that consumed the commercial green tea supplement had significantly ($P < 0.05$) increased GSH levels when compared with the control group. All other treatment groups showed only a marked, though not significant increase in testicular GSH levels.

DISCUSSION

Oxidative stress is associated with the aetiology of

chronic diseases such as atherosclerosis, diabetes, liver damage, rheumatoid arthritis, cataracts, cancers (Agarwal and Prabakaran, 2005) and can also play a vital role in male infertility (Ong et al., 2002; Makker et al., 2009). Diets rich in fruits and vegetables are linked to the protective effects against these diseases (Segasothy and Phillips, 1999; Riso et al., 2009). These protective effects are likely to be modified by antioxidants which employ a series of redox reactions (Szeto and Benzie, 2002; Blokhina et al., 2003). From the present study, it was observed that fermented rooibos, the commercial rooibos supplement and green tea significantly increased the SOD activity in the testicular tissue, while fermented rooibos and green tea also significantly decreased lipid peroxidation (TBARS), measured as MDA, in this tissue. The commercial rooibos supplement significantly decreased the ROS levels while the commercial green tea supplement caused a significant increase in GSH levels. It has been reported that CAT, SOD, peroxidase activity (POD) and glutathione peroxidase (GSH-Px) constitute a mutually supportive team of defense against ROS (Bandyopadhyay et al., 1999; Khan and Ahmed, 2009).

The main detoxifying systems for peroxides are CAT and GSH (Türk et al., 2008). Glutathione exists in cells in both a reduced (GSH) and an oxidized (GSSG) form and it may also be covalently bound to proteins through a process called glutathionylation (Thomas et al., 1995; Huang and Huang, 2002). The GSH level in the testicular tissue of rats that consumed the commercial green tea supplement increased significantly in this study. Previously, Khan and Ahmed (2009) demonstrated the enhancement of GSH content in the testicular tissue of rats by *Digera muricata*, a plant rich in flavonoids. An important task for cellular GSH is to scavenge free radicals and peroxides produced during normal cellular respiration, which would otherwise oxidize proteins, lipids and nucleic acids (Hayes and Pulford, 1995; Wild and Mulcahy, 2000). Because GSH is the fundamental redox regulator in eukaryotic cells, it is conceivable that the principles of GSH-mediated redox switching of transcription factors can be extrapolated from single cells to multicellular organisms (Moskaug et al., 2005). By participating in the GSH redox cycle, GSH together with glutathione peroxidase (GSH-Px) converts H_2O_2 and lipid peroxides to non-toxic products (Calvin et al., 1981; Sikka, 1996; Sanocka and Kurpisz, 2004).

Superoxide dismutase is a highly specific scavenging enzyme for $O_2^{\cdot-}$ and MDA is a reactive end product of lipid peroxidation. The SOD activity and MDA level can reflect the degree of damage of testicular tissues induced by ROS example, $O_2^{\cdot-}$ and OH^{\cdot} . ROS-induced injury normally causes a decrease of SOD activity and increase of MDA level in tissues (Luo et al., 2006). It is well known that SOD depletion in spermatozoa is thought to be associated with male infertility (Luo et al., 2006). In this study, rats that consumed fermented rooibos, the rooibos

supplement and green tea showed significantly increased SOD activity when compared with the control animals, while the (TBARS) levels showed a decreasing trend only. Rats that consumed fermented rooibos and green tea showed significantly decreased (TBARS) levels in their testicular tissue. This indicated that the antioxidant system of the testicular tissues protected against the induced-OS and could effectively scavenge free radicals and alleviate the damage to spermatogenic cells. Our results are similar to the findings of Luo et al. (2006) who reported that *Lycium barbarum* polysaccharides (LBP), a famous Chinese medicinal herb, significantly increased the SOD activity and decreased the MDA levels in rat testicular tissue damaged due to heat exposure. The present study and the first part of our study showed that rooibos and green tea could significantly increase the SOD activity and decrease the MDA level to protect the testicular tissue and spermatozoa against induced oxidative damage.

Certain enzymes play an important role in antioxidant defense and to maintain viable reproductive ability, a protective mechanism against oxidative stress is of importance. These enzymes include SOD, GSH-Px, glutathione reductase and CAT (Fuji et al., 2003; Türk et al., 2008). Our results show an increase trend in CAT activity of all tea and herbal tea treated groups with the exception of "green" rooibos. Ola-Mudathir et al. (2008) demonstrated that aqueous extracts of onion and garlic which contain dietary phytochemicals with proven antioxidant properties caused an increase in the CAT activity against cadmium-induced testicular oxidative damage. Hydrogen peroxide is often metabolized by CAT and GSH-Px, when CAT activity is decreased; H_2O_2 is reduced to a highly oxidizing OH radical in the presence of Fe^{2+} or other transition metals (Pierrefiche and Laborit, 1995; Da Rosa Araujo et al., 2010). The OH radical cannot be enzymatically removed from cells but a free radical scavenger can detoxify it. We proposed that the trend in the increase of CAT activity could be as a result of the flavonoids, thereby increasing the availability of these enzymes that play an important role in protecting against oxidative damage. Secondly, it can be speculated that the effect is mediated by increased mitochondrial production of H_2O_2 and $O_2^{\cdot-}$ (Moskaug et al., 2005; Miguel et al., 2009).

Many dietary polyphenols are antioxidants of which flavonoids are the most studied and the possibility exists that they protect against oxidative damage by directly neutralizing reactive oxidants (Moskaug et al., 2005). Flavonoids are well documented for their antioxidant properties and their ability to act as antioxidants is determined by their structure, particularly their ability to donate a hydrogen ion to the peroxy radical produced as a result of LPO (Saija, 1995; Kashima, 1999; Procházková et al., 2011). Results from our previous study (unpublished data, chapter 3) showed that the daily flavonol intake was significantly higher in the rats that

consumed the rooibos herbal teas, while rats that consumed the green tea, commercial rooibos and green tea supplement, had significantly higher flavanol intakes. The chemical properties of some antioxidants example, flavanols and flavonols, may give them prooxidant properties at certain levels and this should be considered with respect to mechanisms for induction of cellular antioxidant defenses (Kessler et al., 2003). It is therefore also possible that repeated mild cellular OS induced by flavonoids at higher levels through the diet, boosts cellular antioxidant defense systems and in the long term, shifts these defense systems to a higher steady state, which prevents disease development or reduces the impact of OS when disease occurs (Huang et al., 2000).

Conclusion

The present results show that aqueous extracts of rooibos (*A. linearis*) and green tea (*C. sinensis*) are effective as antioxidants and protected testicular tissue against induced oxidative damage. Fermented rooibos, green tea and the commercial rooibos supplement caused an increase in the activity of the important cellular enzyme, SOD, while fermented rooibos and green tea decreased lipid peroxidation in the rat testicular tissue. Additionally, the rooibos supplement caused a decrease in ROS activity while the GSH level was also decreased by green tea in the respective animals. None of the tea treatments showed an effect on CAT activity, although, a tendency to increase this enzyme was shown by rooibos fermented, green tea and both supplements. The observed differences between the rooibos herbal tea and green tea and their respective commercial supplements, may be due to the unique composition of antioxidants and other compounds of the herbal teas/tea and this might result in the improved protective effect when compared with the supplements. The effect is not only because of the main polyphenolic compounds, but is proposed to be as a synergistic effect of all compounds. These observations suggest that fermented rooibos and green tea could be excellent adjuvant support in the therapy of male infertility and can generally be used as a supportive therapy in cases where oxidative stress is involved. However, further studies are required in order to confirm these effects in humans.

ACKNOWLEDGEMENTS

The authors would like to thank the staff of the Biomedical Sciences Department, Faculty of Health and Wellness Sciences as well as Mr. Fanie Rautenbach of the Oxidative Stress Research Centre, Cape Peninsula University of Technology for their technical assistance. We would like to acknowledge the Cape Peninsula University of Technology and the National Research

Foundation of South Africa for their financial support.

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