THE ROLE OF ROOIBOS (*ASPALATHUS LINEARIS*), GREEN TEA (*CAMELLIA SINENSIS*) AND COMMERCIALLY AVAILABLE ROOIBOS AND GREEN TEA ANTIOXIDANT SUPPLEMENTS ON RAT TESTICULAR AND EPIDIDYMAL FUNCTION

By

DOLAPO OLAITAN AWONIYI

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Supervisor: Dr. Nicole L. Brooks

Co-supervisor: Prof. Jeanine L. Marnewick

Bellville

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DECLARATION

I, Dolapo Olaitan Awoniyi declare that the content of this thesis is my original work, and that the thesis has not been previously submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

Signed   Date

ABSTRACT

Defective sperm function is the most prevalent cause of male infertility. Mammalian sperm are rich in polyunsaturated fatty acids (PUFA) and are susceptible to attack by reactive oxygen species (ROS), an important mediator of sperm function. Normally, a balance is maintained between the amount of ROS produced and the antioxidant defense system. When an imbalance exists between ROS production and antioxidants protection in the favour of ROS activity, oxidative stress (OS) occurs which could lead to cellular damage. A shift in the levels of ROS towards pro-oxidation in semen can induce oxidative stress on sperm.

This study compares the modulation of OS by an indigenous herbal tea (rooibos), Chinese green tea, commercial rooibos and green tea supplements in rat epididymal sperm and testicular function. Sixty male Wistar rats were supplemented with fermented or “green” rooibos, Chinese green tea, rooibos supplement, green tea supplement or water for ten weeks while inducing OS during the last two weeks. Rats consuming fermented rooibos and “green” rooibos showed a significant higher sperm concentration and motility. The superoxide dismutase (SOD) activity was significantly higher in the sperm of rats that consumed fermented rooibos, “green rooibos” and green tea compared to the control. Also, the superoxide dismutase activity of the groups that received fermented rooibos, rooibos supplement and green tea increased significantly in the testicular tissue compared to control. Catalase (CAT) activity in the epididymal sperm was significantly increased in the rats consuming fermented rooibos, “green rooibos” and both rooibos and green tea supplements compared to the control. None of the tea treatments showed a significant effect on catalase activity in the testicular tissue. A tendency to increase this activity was observed in fermented rooibos, green tea, rooibos and green tea supplements. The glutathione levels of rats fed with fermented rooibos and “green” rooibos were significantly higher in the rat epididymal sperm compared to the control while the glutathione level of rats consuming green tea supplement also increased significantly in the testicular tissue compared to

the control. None of the tea treatments showed a significant effect on lipid peroxidation and ROS levels in the rat sperm. Although rooibos fermented, “green” rooibos and rooibos supplements showed a tendency to lower the levels of these biomarkers when compared with the control group in epididymal sperm. However, the rooibos supplement showed a significantly reduced ROS levels in the testicular tissue.

In conclusion, our study demonstrated that rooibos extract and green tea are effective as antioxidants by increasing the antioxidant enzyme activities and glutathione levels in oxidative stress-induced rats and could offer a measure of protection against oxidative damage, thereby improving sperm quality and function.

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To God be the glory for his mercies endureth forever.

DEDICATION

This thesis is dedicated to the glory of God and to my family.

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GLOSSARY OF ABRREVIATIONS

TERMS	DEFINITION
AAF:	2-acetylaminofluorene
AAPH:	2,2'-Azobis (2-methylpropionamidine) dihydrochloride
ACE:	Angiotensin-converting enzyme
ADP:	Adenine diphosphate
AFB_1:	Aflatoxin B1
ANOVA:	Analysis of variance
AOS:	Active oxygen species
Apaf-1:	Apoptotic protease activating factor 1
ARE:	Antioxidant response elements
ART:	Assisted reproductive techniques
ATP:	Adenosine-5'-triphosphate
BHT:	Butylated hydroxy toluene
CAT:	Catalase
CCl_4 :	Carbon tetra chloride
C_2H_2OH:	Ethanol
CPUT:	Cape Peninsula University of Technology
Cys:	Cysteine
DAC:	p-dimethylaminocinnamaldehyde
DCF:	Dichlorofluorescent
DEP:	Diesel exhaust particles
DCFH-DA:	2, 7 dichlorofluorescin diacetate
DPPH:	1,1-diphenyl-2-picryhydrazyl
DNA:	Deoxyribonucleic acid
DV:	Daily Value

EC:	Epicatechin
ECG:	Epicatechin gallate
EGC:	Epigallocatechin
EGCG:	Epigallocatechin gallate
Fas:	Type I membrane protein
FL:	Fluorescein
GA:	Gallic acid
GSH:	Glutathione reduced
GSSG:	Glutathione oxidised
GPx:	Glutathione peroxidase
GST-α:	Glutathione S-transferase alpha
GTP:	Green tea polyphenols
H_2O_2 :	Hydrogen peroxide
HOs:	Heme oxygenases
IP:	Intra peritoneal
Keap1	Kelch-like ECH-associated protein 1
LBP:	Lycium barbarum polysaccharides
LDL:	Low density lipoprotein
LPO:	Lipid peroxidation
MDA:	Malondialdehyde
NaCl:	Sodium chloride
Na_2CO_3:	Sodium carbonate
NADH:	Nicotinamide adenine dinucleotide
NADPH:	β- Nicotinamide adenine dinucleotide phosphate
NO:	Nitric oxide
NQO1:	Quinone oxidoreductase 1
NQO2:	Quinone oxidoreductase 2

Nrf2:	Nuclear factor-E2-related factor
$O_2^{-\cdot}$:	Superoxide anion
OH^- :	Reactive hydroxyl
$ONOO^-$:	Peroxylnitrite anion
ORAC:	Oxygen radical absorbance capacity
OS:	Oxidative stress
PARP:	Poly ADP-ribose polymerase
PBS:	Phosphate buffered saline
PCB	Polychlorinated biphenyl
Prxs:	Peroxiredoxins
PUFA:	Polyunsaturated fatty acids
RFU:	Relative fluorescence unit
RLU:	Relative luminescence unit
$RNOO^-$:	Peroxyl
RNS:	Reactive nitrogen species
ROS:	Reactive oxygen species
RR:	Relative risk
SeCys:	Selenocysteine
SOD:	Superoxide dismutase
SRC:	Standard rat chow
STD:	Standard deviation
TAC:	Total antioxidant capacity
TBA:	Thiobarbituric acid
TBARS:	Thiobarbituric acid reactive substances
tbHP:	t-butyl hydroperoxide
TPA:	Tetradecanoylphorbol-13-acetate
TP:	Total polyphenol

tRNA:	Transfer Ribonucleic acid
UDP-GT:	Glucuronosyl transferase
UK:	United Kingdom
USA:	United States of America
USDA:	US Department of Agriculture
WHO:	World Health Organisation
WST-1:	Tetrazolium salt
ZP:	Zona pellucida

CHAPTER ONE

INTRODUCTION

Infertility is defined as the inability to conceive after 12 months of unprotected sexual intercourse (Agarwal *et al*., 2005). Approximately 15% of all couples trying to conceive are affected by infertility and male factor infertility contributes to approximately half of these cases, with no identifiable cause found in over 25% of infertile males (Sharlip *et al*., 2002). The involvement of reactive oxygen species (ROS) has been linked to infertility in previous studies (Agarwal and Saleh, 2002; Saleh and Agarwal, 2002; Agarwal *et al*., 2003; de Lamirande and Lamothe, 2010). Reactive oxygen species are highly reactive oxidizing agents belonging to the class of free-radicals. A free radical is any compound (not necessarily derived from oxygen) which contains one or more unpaired electrons (Agarwal *et al*., 2003). Excessive production of ROS or free radicals can damage sperm and therefore ROS have been extensively studied as one of the possible causes of infertility (Agarwal and Prabakaran, 2005).

Oxidative stress plays a prominent role in human reproduction and it develops as a result of excessive production of ROS and/or impaired antioxidant defence mechanisms (Novotný *et al*., 2003). Oxidative stress is also involved in many other diseases and several studies have suggested it may be alleviated by the intake of dietary antioxidants originating from plant foods and beverages (Borek, 2004; Lau *et al*., 2005; Scalbert *et al*., 2005). A study by Sato *et al*., (2010) linked dietary intake of green tea extracts to the attenuation of the testicular toxicity and suggested that the protective effects may be attributed to the polyphenolic antioxidants in this tea. Polyphenolic compounds which are often abundant in beverages derived from plant origin, such as herbal teas and teas, may contribute to the inhibitory effect of diets on oxidative stress.

At present no information is available on the possible modulating properties rooibos (*Aspalathus linearis*) herbal tea may have on induced oxidative stress (OS) and infertility. Since

there is a difference in the phenolic profile of this herbal tea and that of the well studied green and black teas (*Camellia sinensis*), the possibility exists that the mechanisms involved in protection against OS could also differ. Thus, it is important to investigate the protective properties of rooibos, green tea and their commercial available antioxidant supplements on testicular function. Previous studies have shown rooibos to have a unique composition of antioxidant and other compounds *in vitro* (Joubert *et al*., 2008) as well as *in vivo* in experimental animals (Marnewick *et al*., 2003) but the *in vivo* protective effects against oxidative stress and infertility are yet to be established. Rooibos has also been previously shown to exhibit *in vitro* and *ex vivo* antimutagenic activity (Marnewick *et al*., 2000) and to regenerate coenzyme Q10 and subsequently inhibit lipid peroxidation in rat liver (Kucharská *et al*., 2004).

Therefore, the aim of this study was to determine if rooibos, green tea and commercially available rooibos and green tea supplements can modulate induced-oxidative stress in rats with a resultant improved sperm motility and function.

The focuses of this study were the following:

1. 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.
2. Protective effects of rooibos (*Aspalathus linearis),* green tea (*Camellia sinensis*) and their commercial supplements on testicular tissue of oxidative stress-induced rats.

This thesis is written in an article based format and comprises of five chapters. The first chapter is the Introduction. Chapter two is the Literature review which highlights reactive oxygen species, oxidative stress and antioxidants. Chapter three is the first article titled “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” which is under review for publication. Chapter four is the second article titled “Protective effects of rooibos (*Aspalathus*

linearis), green tea (*Camellia sinensis*) and their commercial supplements on testicular tissue of oxidative stress-induced rats" which is also under review for publication. The fifth chapter is the general discussion and chapter six concludes the study.

CHAPTER TWO

LITERATURE REVIEW

Superoxide anion, hydroxyl radical and hydrogen peroxide are some of the major ROS present in seminal plasma (Agarwal and Prabakaran, 2005). Sperm (specifically the membrane) are rich in PUFA and thus susceptible to attacks by ROS or membrane lipid peroxide ions (Cerolini *et al*., 2000; Sanocka and Kurpisz, 2004). Sperm produce small amounts of ROS that play a significant role in many of the physiological processes such as capacitation, hyperactivation and sperm oocyte fusion (Sies, 1993; Lewis *et al*., 1995; Cocuzza *et al*., 2007). Any excess ROS must be continuously removed in order to maintain normal cell function. This role is played by the defence mechanism which includes antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase and reductase), vitamins (E, C and carotenoids) and biomolecules (glutathione and ubiquinol) all involved in biological systems (Liu and Lee, 1998; Hamilton *et al*, 2000; Ognjanović *et al.,* 2010). When there is an imbalance between the amount of ROS and antioxidant defence mechanisms, oxidative stress (OS) occurs, which is harmful to sperm cells (Agarwal and Prabakaran, 2005).

2.1 Oxidative stress and reactive oxygen species

Oxidative stress is commonly referred to as the status of oxidative damage within a biological system (Kuhnt *et al*., 2006). In a biological system like the human body, vital macromolecules like proteins, lipids and deoxyribonucleic acid (DNA) may be oxidatively modified resulting in cell or tissue damage, hence the myriad of diseases linked to an excess of free radicals (Wilcox *et al*., 2004). The oxidised or nitrosylated products that are produced in the development of these human diseases may show decreased biological activity resulting in cellular loss of energy metabolism, cell signalling and cell transport, increased cell injury and death via necrotic or apoptotic mechanisms and negative effects on other major cellular

functions (Vincent *et al*., 2004). The human body undergoes a physiological metabolism in maintaining a state of equilibrium between the level of oxidants and antioxidants. Oxidants such as the free radicals, ROS and reactive nitrogen species (RNS) are products of normal cellular metabolism and they play a dual role of being deleterious as well as beneficial to living systems (Buonocore and Groenendaal, 2007).

Oxidative stress activates a specific stress response, an adaptive mechanism aimed to protect cells against ROS-mediated toxicity and to maintain tissue redox balance. This stress response includes enhanced protein expression of endogenous antioxidant enzymes. Gene transcription of most antioxidant enzymes is regulated through the transcription factor, nuclear factor-E2-related factor (Nrf2) and antioxidant response elements (ARE) in the genes encoding enzymatic antioxidants (Schreibelt *et al*., 2007). Under physiological conditions, Nrf2 is linked to the actin-bound Kelch-like ECH-associated protein 1 (Keap1) and located in the cytoplasm. However, upon oxidative stress, Nrf2 dissociates from Keap1 and translocates to the nucleus, where it activates ARE-mediated gene transcription and induces the coordinate transcription of ARE-regulated genes (Schreibelt *et al*., 2007). To date, over 200 Nrf2–ARE-driven genes involved in detoxification and antioxidant defence have been identified, including superoxide dismutases (SODs), peroxiredoxins (Prxs), heme oxygenases (HOs), glutathione peroxidases (GPxs), catalase (CAT), NAD(P)H:quinone oxidoreductase 1 (NQO1) and NHR:quinone oxidoreductase 2 (NQO2) (Schreibelt *et al*., 2007; Nagaraju and Belur, 2008). Endogenous antioxidant enzymes such as SOD, CAT and GPx defend the host against the damaging effects of the free radicals species (Schreibelt *et al*., 2007; Nagaraju and Belur, 2008). Figure 2.1 illustrates a schematic overview of Nrf2/ARE activation by ROS.

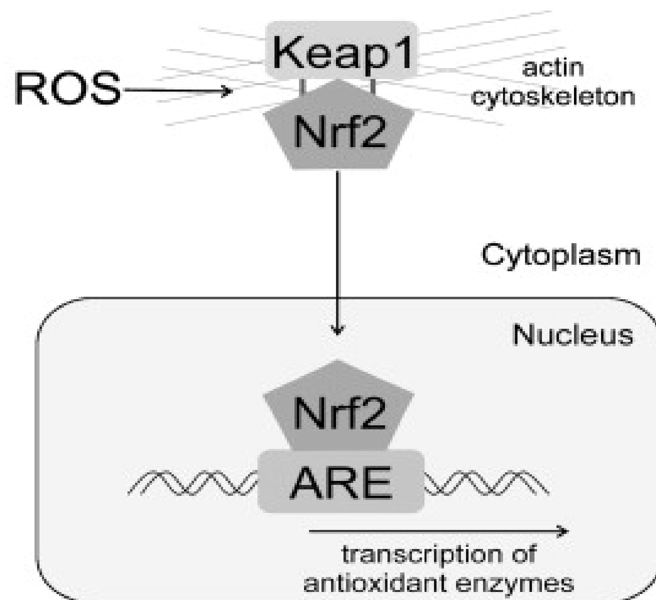


Figure 2.1 Schematic overview of Nrf2/ARE activation by ROS. (Reproduced from Schreibelt *et al.*, 2007)

Reactive oxygen species, also known as free radicals, are oxidizing agents formed as a result of oxygen metabolism and have at least one unpaired electron that makes them reactive species. Free radicals normally attack the nearest stable molecule which becomes a free radical itself, and begins a cascade of chain reactions. Rapidly, they can oxidize biomolecules which they encounter in their vicinity thus exerting either a positive or a negative influence on normal cell function (Warren *et al.*, 1987; Cocuzza *et al.*, 2007). The most common ROS with potential implications in reproductive biology include superoxide ($O_2^{-}\bullet$) anion, hydrogen peroxide (H_2O_2), peroxyl ($RNOO^{-}$) radicals and the very reactive hydroxyl (OH^{-}) radicals (Ford, 2004; Maneesh and Jayalekshmi, 2006) (Figure 2.2). The nitrogen derived free radicals, nitric oxide (NO) and peroxylnitrite anion ($ONOO^{-}$), also appear to play a significant role in reproduction and fertilization (Maneesh and Jayalekshmi, 2006). Nitric oxide's effect depends mainly on its concentration and interactions with H_2O_2. *In vivo*, $ONOO^{-}$ may be formed from superoxide and NO and this can actively react with glutathione, cysteine, deoxyribose and other thiols and thioethers (Koppenol *et al.*, 1992). Reactive oxygen species is involved in cell growth, differentiation, progression and cell death (Mates *et al.*, 1999). Low levels of ROS may be

beneficial in terms of intracellular signalling and defence against micro-organisms (Mates *et al*., 1999).

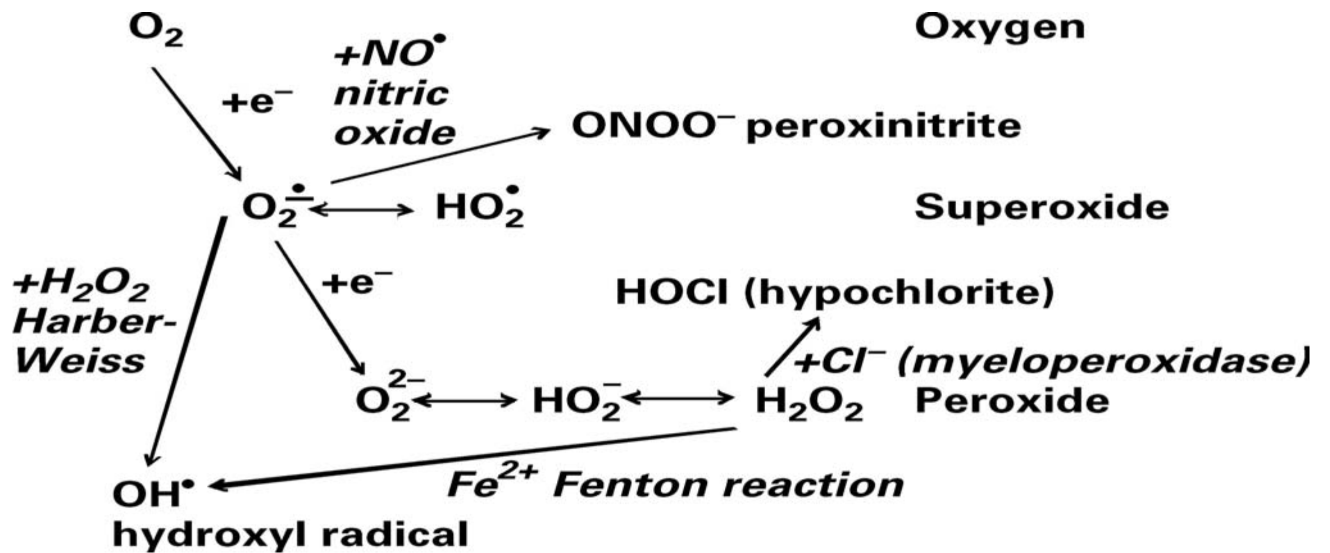


Figure 2.2. Derivation of reactive oxygen species (Reproduced from Ford, 2004)

2.2 Origin of reactive oxygen species in seminal plasma

The sources of ROS are broadly dispersed between external and internal sources in sperm cells. External production of ROS, particularly $O_2^{-\cdot}$ and H_2O_2 can be as a result of leukocyte contamination within the semen which is associated with sub-fertility or even infertility in patients (Aitken *et al*., 1996; Griveau and Le Lannou, 1997; Henkel *et al*., 2005). Leukocytes are present throughout the male reproductive tract and are found in almost every human ejaculate (Sharma and Agarwal, 1996). The origin of leukocytes in semen, their mode of action, and the role that bacteria, viruses and subsequent genitourinary-inflammation might have on sperm function are not clear. However, it has been shown that ROS production by human sperm and contaminating leukocytes can be stimulated by phorbol esters and certain formyl peptides with deleterious effects on sperm motility and fertilization (Pasqualotto *et al*., 2000; Hammadeh *et al*., 2009). The presence of leukocytes in semen did not diminish the *in vitro* fertilizing capacity of sperm, but the introduction of leukocytes into washed sperm preparations reduced sperm

function due to the production of ROS (Aitken *et al.*, 1994; Henkel *et al.*, 2005). Reactive oxygen species in human ejaculate samples originate mainly from seminal leukocytes and leukocytospermia is characterized by abnormally high seminal leukocyte, polymorphonuclear neutrophils and macrophages (Alavarez, 2003). Seminal leukocyte ROS production induces sperm damage during assisted reproductive techniques (ART) procedures (Pasqualotto *et al.*, 2001; Sharma *et al.*, 2001). Similarly, patients with accessory gland infection demonstrated both leukocytospermia and elevated ROS levels (Ochsendorf, 1999).

The production of ROS is also increased by life style factors such as smoking and pollution (Fraga *et al.*, 1996; Mello *et al.*, 2001). An association between cigarette smoking and reduced seminal quality has been identified (Kunzle *et al.*, 2003). Smoking increases ROS production and causes sperm DNA damage and suppresses antioxidants in both semen and serum (Fraga *et al.*, 1996; Mello *et al.*, 2001; Saleh *et al.*, 2002). Alkaloids, nitrosamines, nicotine and hydroxycotinine are harmful substances present in cigarettes and produce free radicals (Traber *et al.*, 2000). Saleh *et al.* (2002) reported that infertile men who smoked cigarettes presented higher seminal ROS levels than infertile non-smokers, possibly due to the significant increase in leukocyte concentration in their semen. Due to a lack of a pro-oxidant defence system, infections in the testes and epididymis produce ROS that are particularly harmful to sperm and may also indirectly affect sperm function by stimulating the presence of ROS in the prostate gland and seminal vesicles (Pasqualotto *et al.*, 2000). An association between prostatitis and male infertility has been reported, but its mechanism is still poorly understood. Prostatitis is associated with the presence of granulocytes in prostatic fluid (Pasqualotto *et al.*, 2000).

Environmental pollution is another major source of ROS production and has been implicated in the pathogenesis of poor sperm quality (Gate *et al.*, 1999; De Rosa *et al.*, 2003). Oxidative stress is hypothesized to play an important role in the development and progression of adverse health effects due to such environmental exposure (Fowler *et al.*, 2004). In a study

conducted by De Rosa *et al.* (2003), tollgate workers exposed continuously to environmental pollutants had inversely correlated blood methaemoglobin and lead levels to sperm parameters when compared to the local inhabitants not exposed to comparable automobile pollution levels.

Immature and morphologically abnormal sperm are another important source of ROS (Agarwal *et al.*, 2003). Potential endogenous sources include mitochondria, cytochrome P450 metabolism, peroxisomes and inflammatory cell activation (Inoue *et al.*, 2003). Mitochondria generate approximately 2-3 nmol of superoxide/min per mg of protein which indicates it to be the highest physiological source of this radical in living organisms (Inoue *et al.*, 2003). Since mitochondria are the major source of free radicals, they are highly enriched with antioxidants including the endogenous antioxidant, glutathione (GSH) and enzymes such as SOD and GPx present on both sides of their membranes in order to minimise OS in this organelle.

Varicocele is the abnormal tortuosity and dilatation of the veins of the pampiniform plexus within the spermatic cord and it is one of the major causes of male infertility (Schoor *et al.*, 2001). About 15% of adult males are believed to either have clinical or subclinical varicocele, with a prevalence as high as 40% in infertile males (Schoor *et al.*, 2001). Irrespective of the fertility status, ROS production in varicocele patients is enhanced with a reduction in the total antioxidant capacity (TAC) (Agarwal *et al.*, 2006a). Several studies have reported OS in testicular tissue, spermatic vein blood and seminal plasma from infertile varicocele patients (Benoff *et al.*, 2004). The presence of OS could cause molecular and genetic defects leading to infertility (Benoff *et al.*, 2004).

Drugs such as the chemotherapy agent cyclophosphamide have been linked to sperm oxidative stress (Das *et al.*, 2002; Ghosh *et al.*, 2002). Cyclophosphamide administration to animals was reported to increase testicular malondialdehyde (MDA) levels (product of lipid peroxidation) and produce a decrease in testicular CAT implying the presence of OS (Das *et al.*, 2002; Ghosh *et al.*, 2002). Oxidative stress can also be produced by drugs such as aspirin and

paracetamol (acetaminophen) by increasing cytochrome P450 activity thereby increasing ROS generation (Agarwal and Said, 2005).

The cause of DNA damage in sperm can be attributed to various pathological conditions including cancer (O'Donovan, 2005) which may not only disrupt hormone levels, but may also induce OS (Sharma *et al*., 2004). Cancer has long been established to have a depressive effect on sperm number and quality (Meirow and Schenker, 1995). A study by Hoei Hansen *et al*. (2003) also supports the hypothesis that testicular germ cell cancer may be aetiologically linked to other male reproductive abnormalities as part of the so-called testicular dysgenesis syndrome. The disruption of spermatogenesis by the cancer process has been described (Thomson *et al*., 2002; Agarwal and Allamaneni, 2004; Van Carsteren *et al*., 2009). Infertility is a significant and frustrating problem for many men after spinal cord injury (Linsenmeyer and Perkash, 1991; Brackett *et al*., 2010) and about 95% of patients with spinal cord injury are known to have elevated seminal levels of ROS (Padron *et al*., 1997). Poor sperm quality and ejaculatory dysfunction are two of the major causes and stasis of prostatic fluid is one of the factors attributed to poor sperm quality (Linsenmeyer and Perkash, 1991). Figure 2.3 illustrates the association of ROS production and effects on sperm resulting in infertility.

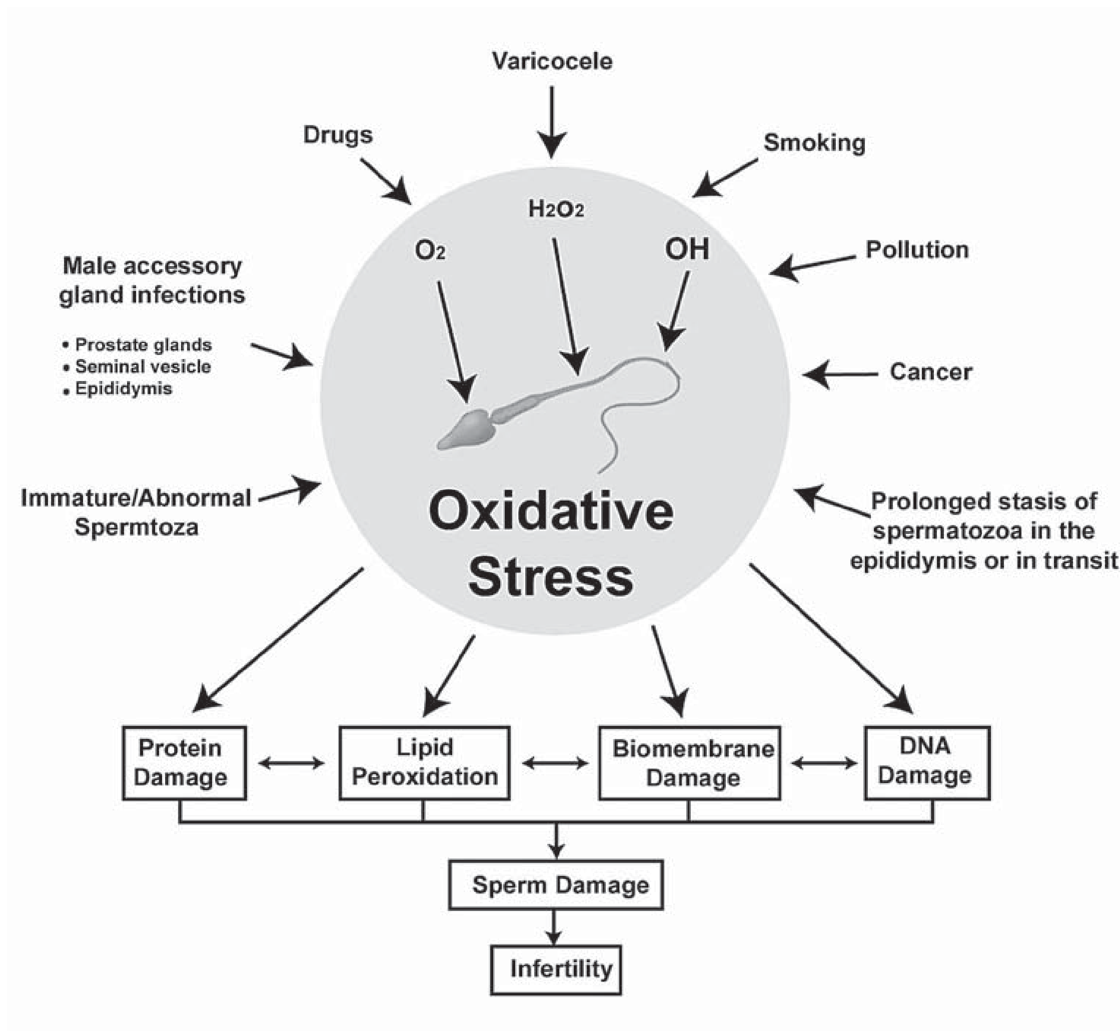


Figure 2.3 Association of increasing reactive oxygen species (ROS) production with infertility (Reproduced from Cocuzza *et al*., 2007)

2.3 Effects of reactive oxygen species on sperm function

Reactive oxygen species, including oxygen ions, free radicals and peroxides cause infertility by two principal mechanisms (Tremellen, 2008). Firstly, ROS damages the sperm membrane which in turn reduces the sperm motility and ability to fuse with the oocyte. Secondly, ROS directly damage sperm DNA compromising the paternal genomic contribution to the embryo (Tremellen, 2008). Reactive oxygen species have been demonstrated to have a spectrum of variable effects on sperm, depending on the extent of OS (Allamaneni *et al*., 2004). Aitken and co-workers (1993) reported that hydrogen peroxide did not have any effect on sperm

motility at a low concentration, but suppressed sperm-egg fusion, implying that patients with normal semen parameters may also suffer from infertility. The ROS levels in such patients are not high enough to impair basic semen parameters, but can cause defects in other fertilization processes such as sperm-oocyte interaction. However, Garg *et al.* (2008) reported that hydrogen peroxide, when incubated with sperm at different concentrations, decreased sperm motility *in vitro*.

Excessive ROS levels are related to an increase in lipid peroxidation (LPO) in the plasma membrane and it had been shown that a correlation exists between ROS and sperm concentration, motility and morphology (Alvarez *et al.,* 1987; Sikka, 1996; Agarwal *et al.,* 2006b; 2009). Garner and co-workers (1997) argued that mitochondrial activity, viability and acrosomal integrity of sperm cells correlated positively with infertility. Sperm may generate ROS in two ways: (i) via the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system at the level of the sperm plasma membrane and (ii) via the nicotinamide adenine dinucleotide (NADH)-dependent oxido-reductase (diphorase) system at the mitochondrial level (Aitken *et al.*, 1997; Cocuzza *et al.*, 2007). A strong positive correlation exists between immature sperm and ROS production which in turn is negatively correlated with sperm quality (Gomez *et al.*, 1998; Cocuzza *et al.*, 2007). Figure 2.4 illustrates a mechanistic pathway showing sperm DNA damage due to oxidative stress.

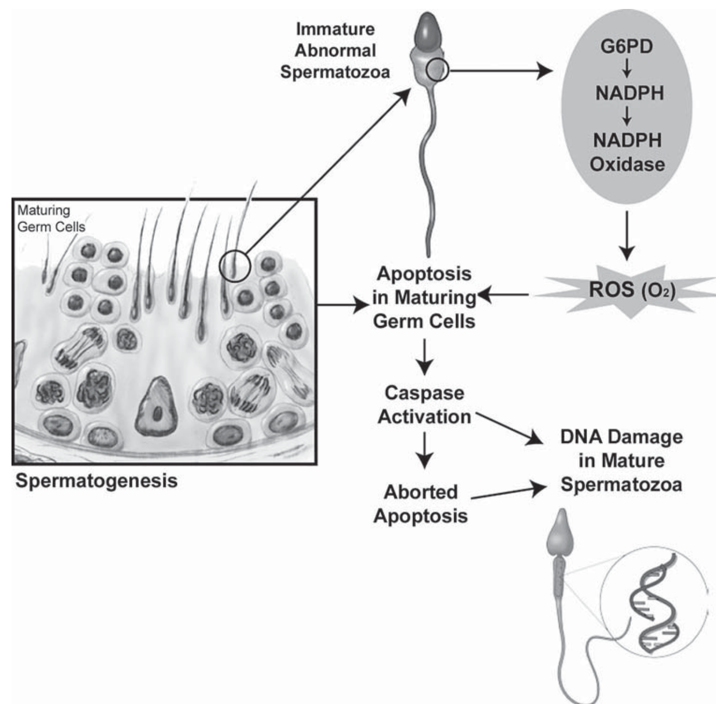


Figure 2.4 Pathway showing sperm DNA damage due to oxidative stress. (Reproduced from Cocuzza *et al.*, 2007)

There is an inverse correlation in the ability of the sperm to produce ROS with their maturational state. During spermatogenesis, there is a loss of cytoplasm to allow the sperm to form its condensed, elongated shape and immature teratozoospermic sperm are often characterized by the presence of excess cytoplasmic residues in the mid-piece (Gomez *et al.*, 1996; Fisher and Aitken, 1997; Said *et al.*, 2005). These residues are rich in the enzyme glucose-6-phosphate dehydrogenase, an enzyme which controls the rate of glucose flux and intracellular production of NADPH through the hexose monophosphate shunt. NADPH is used to fuel the generation of ROS via NADPH oxidase located within the sperm membrane (Gomez *et al.*, 1996; Fisher and Aitken, 1997; Said *et al.*, 2005). Figure 2.5 illustrates the mechanism of OS linked to sperm DNA damage.

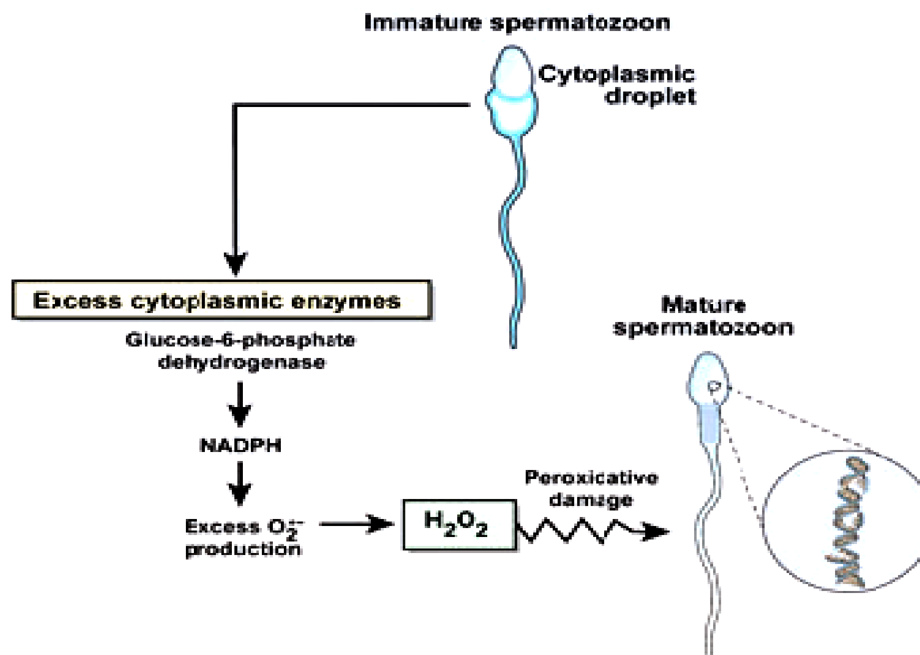


Figure 2.5 The mechanism for the link between OS and sperm DNA damage. (Reproduced from Agarwal and Said, 2005)

Reactive oxygen species may affect the integrity of the sperm genome and induce high frequencies of single and double DNA strand breaks (Twigg *et al.*, 1998; Sallmyr *et al.*, 2008). Studies in which sperm was exposed to artificially produced ROS culminated in a significant increase in DNA damage in the form of modification of all bases, production of free base-sites, deletions, frameshifts, DNA cross-links and chromosomal rearrangements (Duru *et al.*, 2000).

In their basal state, fertile sperm produce very low net amounts of ROS. However, when they are incubated in capacitating conditions, there is stimulation of $O_2^{-\cdot}$ production through the action of an 'oxidase'. Depending on the conditions, the superoxide anion and hydrogen peroxide formed by the dismutation of the superoxide anion can induce human sperm capacitation. Induction of the acrosome reaction in capacitated sperm further stimulates production of $O_2^{-\cdot}$ which causes the release of unesterified fatty acids from the plasma membrane of these cells (de Lamirande *et al.*, 1997)? Hydrogen peroxide appears also to be involved in the acrosome reaction (de Lamirande *et al.*, 1997). Figure 2.6 is a schematic representation of the involvement of ROS in sperm capacitation and acrosome reaction.

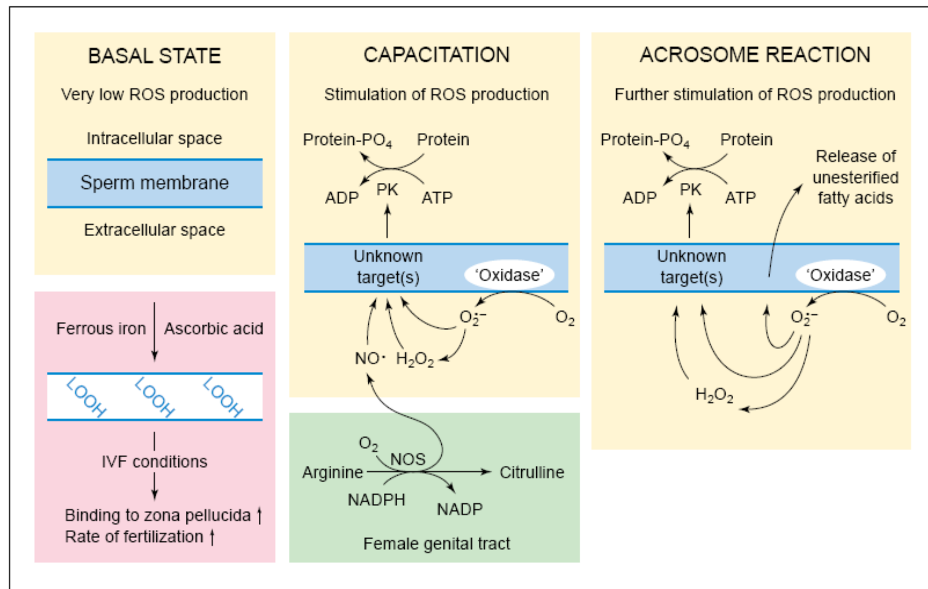


Figure 2.6 Schematic representation of the involvement of reactive oxygen species (ROS) in sperm capacitation and acrosome reaction. (Reproduced from de Lamirande *et al.*, 1997)

Capacitation confers upon the sperm an ability to gain hyperactive motility, interact with oocyte zona pellucida (ZP), undergo acrosome reaction and initiate oocyte plasma membrane fusion (Yanagimachi, 1994; Gaboriau *et al.*, 2007). Nitric oxide also plays a part in the sperm's ability to fuse with the oocyte, but it has no action in ZP binding (Francavilla *et al.*, 2000). Capacitation of a sperm cell is required before fertilization, but the molecular mechanisms and signal transduction pathways involved in this process are not clearly understood in every mammalian species studied (Naz and Rajesh, 2004). Capacitation involves an increase in membrane fluidity, cholesterol efflux, ion fluxes resulting in alteration of sperm membrane potential, increased tyrosine phosphorylation of proteins, induction of hyperactivation and the acrosome reaction (de Lamirande and Gagnon, 1993; Naz and Rajesh, 2004). The acrosome reaction is an exocytic process which involves multiple fusions of the plasma membrane and outer acrosomal structures of the acrosome, resulting in the release of the acrosomal content and exposure of the inner acrosomal membrane (Zaneveld *et al.*, 1991; Gadella *et al.*, 2001). It

is a prerequisite for penetration of sperm through the ZP for fertilization of the oocyte (Köhn *et al*., 1997; Flesch and Gadella 2000; Gadella *et al*., 2001).

Lack of cytoplasm by sperm cells results in a decreased antioxidant defence which is the link between poor sperm quality and elevated ROS. Furthermore, sperm are also susceptible to damage induced by excessive ROS as their plasma membrane contain large quantities of PUFA, which results in LPO and a loss of membrane integrity (Buettner, 1993; Cocuzza *et al*., 2007; Prasad *et al*., 2010). Sperm, unlike other cells are unique in structure, function and susceptibility to damage by lipid peroxidation (Lee, 1996; Garg *et al*., 2000).

Lipid peroxidation is the oxidative deterioration of PUFA's which are fatty acids that contain more than two carbon carbon double bonds (Halliwell, 1984; Agarwal and Saleh, 2002). The most common types of LPO are: non-enzymatic membrane LPO and enzymatic (NADPH and adenine diphosphate (ADP) dependent) LPO. The enzymatic reaction involves NADPH cytochrome P-450 reductase and proceeds via an ADP -Fe^{3+} $O_2^{\cdot -}$ (perferryl) complex (Ernster, 1993). Lipid peroxidation of the sperm membrane is considered to be the key mechanism of ROS-induced sperm damage resulting in infertility. Lipid peroxidation in biological membranes causes impairment of membrane functioning decreased fluidity, inactivation of membrane-bound receptors and enzymes and increased non-specific permeability to ions (Sanocka and Kurpisz, 2004). The proposed roles of ROS in sperm functions are indicated in Table 2.1.

Table 2.1 Proposed roles of reactive oxygen species (ROS) in sperm functions (reproduced from de Lamirande *et al*., 1997)

Reactive oxygen species	Sperm function	Species
Superoxide anion	Hyperactivation	Human
	Capacitation	Human
	A23187-induced	
	Acrosome reaction	Human
Hydrogen peroxide	Hyperactivation	Human
	Capacitation	Hamster
		Human
		Human
	A23187-induced	Human
	Acrosome reaction	
	LPC-induced	Human
	Acrosome reaction	
	Zona pellucida	Human
	Binding and penetration	
Nitric oxide	Hyperactivation	Hamster
	Capacitation	Human

2.4 Apoptosis

Apoptosis is a physiological phenomenon in the body that helps in the elimination of abnormal cells, including sperm. Oxidants or stimulators of cellular oxidative metabolism are agents which induce apoptosis (Buttke and Sandstrom, 1994; Said *et al*., 2004). Many inhibitors of apoptosis have antioxidant activities or enhance cellular antioxidant defences. Therefore,

mammalian cells exist in a state of oxidative siege in which an optimum balance of oxidants and antioxidants is required for survival (Buttke and Sandstrom, 1994). The apoptotic mechanisms remain greatly obscure as it is seen as an integral part of the developmental program which is the end result of a temporal course of cellular events and this is referred to as programmed cell death. While there is considerable variation in the signals and requisite cellular metabolic events necessary to induce apoptosis in diverse cell types, the morphological features associated with apoptosis are highly conserved (Haddad, 2004).

Initiation of a chain of reactions by ROS is done by activating caspases that ultimately lead to apoptosis (Said *et al*., 2004; Agarwal *et al*., 2007). Caspases belong to a family of cysteine proteases involved in apoptotic cell death and can be divided into two major groups: initiator caspases and effector caspases (Riedl and Shi, 2004). Once activated, initiator caspases (8, 9 and 10) cleave downstream procaspases to generate an amplification cascade. Effector caspases (3, 6 and 7) degrade many cell substrates like actin, poly ADP-ribose polymerase (PARP) and Golgi apparatus proteins like Golgin-160 or p115, resulting in classical features of apoptosis (i.e. nucleosomal DNA fragmentation and apoptotic bodies) (Stroh and Schulze-Osthoff, 1998; Hengartner, 2000; Chiu *et al*., 2002). Two distinct pathways (intrinsic and extrinsic) exist in the process of caspase activation in mammalian cells. The intrinsic pathway involves the proapoptotic Bcl-2 protein-dependent release of cytochrome c from the mitochondria into the cytosol, where it binds to the apoptotic protease activating factor 1 (Apaf-1), dATP and procaspase-9 (Shi, 2002; Newmeyer and Ferguson-Miller, 2003). The fusion of this multiprotein complex named apoptosome results in the activation of initiator caspase-9 and the subsequent proteolytic processing and activation of effector caspases-3, -6 and -7 (Riedl and Shi, 2004) while the extrinsic pathway involves the binding of a death receptor such as Type I membrane protein (Fas) to its ligand FasL (Algeciras-Schimnich *et al*., 2002; Holler *et al*., 2003; Danial and Korsmeyer, 2004).

Fas activation facilitates intracellular formation of a protein complex where either caspase-8 or caspase-9 is fully activated which also proteolytically activates downstream effector caspases. Fas can also trigger activation of procaspase-2 and its activity correlates with apoptosis in some cell types such as in germ cells (Giampietri *et al.*, 2003; Milhas *et al.*, 2005; Lavrik *et al.*, 2006; Zheng *et al.*, 2006; Darwish, 2010). When ROS levels are raised in the seminal fluid, the process of apoptosis is also initiated in mature sperm. This is accelerated by ROS-induced DNA damage, which inadvertently leads to a decline in the sperm count. Oxidative stress due to excessive generation of ROS is presumed to cause sperm DNA damage and has correlated positively with apoptosis (Wang *et al.*, 2003).

The extent of sperm DNA damage has been closely related to impaired sperm function as well as male infertility (Sakkas *et al.*, 1999; Cocuzza *et al.*, 2007). Sun *et al.* (1997) and Avendaño *et al.* (2010) found a negative correlation between the percentage of sperm with damaged DNA and the fertilization rate. However, the exact mechanisms responsible for chromatin abnormalities in human sperm are likely to be multi-faceted and are not completely understood (Aitken, 1999). Oxidative stress-induced DNA damage causes a pro-mutagenic change affecting the quality of the germ line preventing fertilization. Fertilization can occur when there is less oxidative damage, but the oocyte must repair the DNA strand breaks before the initiation of the first cleavage. Apoptosis and OS are involved in mediating DNA damage in the germ line (Agarwal and Said, 2005).

2.5 Antioxidants

Antioxidants are compounds which dispose, scavenge and suppress the formation of ROS or oppose their actions (Dandekar *et al.*, 2002). Their components are most important in food because of their ability to reduce free radical-mediated degradation of cells and tissues in an organism (Jin *et al.*, 2004; Wongkham *et al.*, 2001). Sources of antioxidants include teas, fruits, vegetables, legumes and whole-grain cereals. Herbal infusions are also important sources

of antioxidants (Karakaya and Kanvas, 1999; Marongiu *et al.*, 2004; Wu *et al.*, 2004; Nihal *et al.*, 2005). Humans have a highly sophisticated and complex antioxidant protection system to protect the cells and organ systems of the body against the overproduction of ROS. These protection systems include prevention antioxidants which block the formation of new ROS and scavenger antioxidants which remove the formed ROS (Jacob, 1995; Agarwal *et al.*, 2005). Other components include:

- Nutrient-derived antioxidants like ascorbic acid (vitamin C), tocopherols and tocotrienols (vitamin E), carotenoids and other low molecular weight compounds such as glutathione and lipoic acid.
- Antioxidant enzymes, e.g. SOD, GPx and glutathione reductase which catalyse free radical quenching reactions.
- Metal binding proteins such as ferritin, lactoferrin, albumin and ceruloplasmin that sequester free iron and copper ions that are capable of catalysing oxidative reactions.
- Numerous other antioxidant phytonutrients present in a wide variety of plant foods.

2.5.1 Endogenous antioxidant defence system

Antioxidants that are synthesised in the human body include antioxidant enzymes, metal binding proteins and other small molecule antioxidants (Evans and Halliwell, 2001). Antioxidant enzymes which include SOD, CAT and GPx defend the host against the damaging effects of the free radical species (Nagaraju and Belur, 2008). Transition metal-binding proteins such as ceruloplasmin, transferin and ferritin prevent transition metals such as iron and copper from interacting with hydrogen peroxide and superoxide producing the highly reactive hydroxyl radical in what is called the “Fenton reaction” (Young and Woodside, 2001).

2.5.1.1 Superoxide dismutase

Superoxide dismutase is a group of metal-containing enzymes that catalyse the dismutation of the highly reactive superoxide anion to O_2 and to the less reactive species H_2O_2 (Johnson and Giulivi, 2005). There are three forms of SOD in humans differing in structure, active metal center, and number of subunits (Miller, 2004). These are: copper and zinc-containing cytosolic SOD (Cu/ZnSOD, SOD1), which is a homodimer of 32 kDa, manganese-containing mitochondrial SOD (MnSOD, SOD2), an 89 kDa homotetramer, and extracellular Cu/ZnSOD (SOD3), a tetrameric glycoprotein of 135 kDa (Schreibelt *et al.*, 2007). Superoxide dismutase destroys $O_2^{\cdot -}$ by the successive oxidation and reduction of the transition metal ion at the active site in a "ding dong" mechanism with remarkably high reaction rates (Meier *et al.*, 1998). Every type of SOD binds single charged anions such as azide and fluoride. Distinctly, differences have been noted in the susceptibilities of Fe-, Mn^- or Cu/Zn-SODs. Cu/Zn-SOD is competitively inhibited by azide (N_3^-), cyanide (CN^-) and fluoride (F^-) (Leone *et al.*, 1998; Vance and Miller, 1998). Both spermatozoa and oocyte have mechanisms which inhibit excessive generation of ROS at the time of sperm-oocyte fusion and this may be achieved by the release of SOD (Maiorino and Ursini, 2002). An abnormality in SOD production can result in an uninterrupted generation of ROS and damage both spermatozoa and oocyte (Maiorino and Ursini, 2002).

2.5.1.2 Catalase

Catalase is an intracellular antioxidant enzyme that is mainly located in cellular peroxisomes and to some extent in the cytosol of mammalian cells. Catalase is a tetrameric enzyme consisting of four identical, tetrahedrally arranged subunits of 60 kDa, each containing a heme group and NADPH in its active center (Schreibelt *et al.*, 2007). Catalase has two enzymatic activities depending on the concentration of H_2O_2, a powerful oxidizing agent. If the concentration of H_2O_2 is high, CAT catalyzes the conversion of hydrogen peroxide into water and

molecular oxygen. Catalase is particularly important in the case of limited glutathione availability and plays a significant role in the development of tolerance to cellular oxidative stress (Schreibelt *et al.*, 2007). A number of studies have shown beneficial effects of catalase. Studies by Alkan *et al.* (1997) and Pasqualotto *et al.* (2008) reported a higher ROS production (88.8%) in 16 of the 18 patients with a significantly lower seminal plasma SOD, CAT, GPx and total sulphydryl-group concentrations in infertile patients.

2.5.1.3 Glutathione peroxidase

Glutathione peroxidase was discovered in 1957 as an enzyme that protects erythrocytes against oxidative damage (Mills, 1957; Herbette *et al.*, 2007). Together with SOD and CAT, GPx constitute the enzymatic antioxidant system which recycles active oxygen species (AOS) and limits their toxicity in mammals (Herbette *et al.*, 2007). They are selenium-containing enzymes that detoxify cellular organic peroxides and hydrogen peroxide by oxidizing two molecules of glutathione (Brigelius-Flohe, 1999; Herbette *et al.*, 2007). Six types of GPxs have been identified in mammalian cells according to their amino acid sequence, substrate specificity and subcellular localization with GPx1 generally expressed in the cytosol and mitochondrial matrix of virtually all cell types, GPx2 in the gastrointestinal tract, GPx3 in plasma, phospholipid hydroperoxide, PHGPx or GPx4 in epididymal, GPx5 and GPx6 in olfactory epithelium (Brigelius-Flohe, 1999; Herbette *et al.*, 2007). The first three types, GPx1, GPx2, and GPx3, are expressed as homotetramers, whereas GPx4 is a monomer (Arthur, 2000). Except for GPx5 and GPx6, all mammalian GPx proteins contain a selenocysteine (SeCys) residue instead of a Cys residue. SeCys is considered to be the 21st amino acid and its co-translational incorporation into proteins is mediated by a SeCy tRNA (Herbette *et al.*, 2007). Rikans and Hornbook (1997) showed that glutathione metabolism is one of the most essential antioxidant defence mechanisms. Failure of the expression of a GPx in the spermatozoa was correlated with infertility in human (Imai *et al.*, 2001; Foresta *et al.*, 2002).

2.5.2 Glutathione

Reduced glutathione (GSH) is an important water-soluble endogenous antioxidant and is synthesized from the amino acids glycine, glutamate and cysteine. It directly quenches ROS such as lipid peroxides and also plays a prominent role in xenobiotic metabolism (Percival, 1998). Due to its abundance in the human body, GSH provides reducing equivalents to the GPx-catalysed reduction of hydrogen peroxide and lipid hydroperoxides to water and alcohol. In the process, GSH becomes oxidised to GSSG (oxidised glutathione) which is then recycled back to GSH in the presence of reduced NADPH (Young and Woodside, 2001). Exposure of mammalian cells to increased oxidative stress leads to a decrease in the ratio of GSH/GSSG due to GSSG accumulation or reduction in GSH levels. A high GSH/GSSG ratio will help spermatozoa in combating oxidative insults (Agarwal and Sharma, 1996; Bansal and Kaur, 2009). Previous studies have demonstrated the administration of glutathione in improving sperm motility and morphology in infertile men with abnormal semen quality (Lenzi *et al*., 1994). These authors observed that the administration of reduced glutathione to selected infertile patients resulted in an improvement in both sperm parameters and cell membrane characteristics.

2.6 Dietary antioxidants

Dietary antioxidants play a prominent role in the human antioxidant defence system. Potential sources of various antioxidants include fruits and vegetables as well as daily dietary supplements (Agarwal *et al*., 2005). Other sources of essential antioxidants include vitamins such as vitamins C and E as well as antioxidant phytochemicals like carotenoids and polyphenols (Lotito and Frei, 2006). The protective system includes chain-breaking antioxidants capable of reducing oxidant radical levels and blunting the propagation of free radical chain reactions (Sheweita *et al*., 2005). The use of chain breaking antioxidants such as vitamin C and vitamin E as drug supplements may limit OS.

2.6.1 Antioxidant vitamins

Vitamin C (ascorbic acid) is regarded as the most important water-soluble antioxidant in extracellular fluids and has the ability to neutralize hydroxyl, superoxide and hydrogen peroxide radicals and prevents sperm agglutination (Agarwal *et al*., 2004). Vitamin C has received much attention besides polyphenols and carotenoids. It is essential for protection of humans against scurvy and its activity lies in its role as an essential co-factor in numerous enzymatic hydroxylation reactions derived from its strong reducing potential (Jialal *et al*., 1990). It also acts indirectly as an antioxidant by regenerating the lipophilic vitamin E at the aqueous-lipid interphase (May *et al*., 1998).

Vitamin E on the other hand is the principal chain-breaking antioxidant and is present within the cell membrane. It neutralizes hydrogen peroxide and protects the plasma membrane from lipid peroxidation (Agarwal *et al*., 2004). Studies suggest vitamin C to be more effective than vitamin E in reversing ROS-induced mouse embryo toxicity and improve the blastocyst development rate (Wang *et al*., 2002). Eight forms of vitamin E exists, namely α-, β-, γ- and δ-tocotrienols and α-, β-, γ- and δ-tocopherols (Langseth, 1995). Although all have been reported to possess antioxidant activity, α-tocopherol, at plasma concentrations of 15-40 μmol, is the most abundant form and the most potent lipophilic, chain breaking antioxidant in the human body (Chopra and Bhagavan, 1999).

2.6.2 Carotenoids

Carotenoids belong to a group of naturally occurring fat-soluble compounds primarily found in plants, algae and photosynthetic bacteria (Palace *et al*., 1999). Over 600 of these pigments occur naturally and are responsible for the bright colours of plant leaves, fruits and flowers (Palace *et al*., 1999). β-carotene has been best studied and with other carotenoids, they have antioxidant properties *in vitro* and in animal models (Krinsky, 2001, Krishnamoorthy *et al*., 2007). Mixtures of carotenoids or associations with other antioxidants (e.g. vitamin E) can

increase their activity against free radicals. Their antioxidant actions are based on their singlet oxygen quenching properties and their ability to trap peroxyl radicals (Stahl and Sies, 1996). The quenching activity of carotenoids depend mainly on the number of conjugated double bonds of the molecule and is influenced to a lesser extent by carotenoid end groups (cyclic or acyclic) or the nature of substituents in carotenoids containing cyclic end groups. The prevention of lipid peroxidation by carotenoids has been suggested to be mainly via singlet oxygen quenching (Stahl and Sies, 1996). Carotenoids such as β-carotene are also believed to provide an important antioxidant defence (Gupta and Kumar, 2002).

Other antioxidants such as alpha lipoic acid and carnitines may also protect against OS. Alpha lipoic acid regenerates other antioxidants such as vitamin C, E and glutathione through redox cycling by undergoing reduction to form dihydrolipoic acid (Biewenga *et al*., 1997; Agarwal *et al*., 2005). On the other hand, carnitines lower ROS by eliminating excess intracellular toxic acetyl-CoA that are responsible for mitochondria ROS production and also improved sperm motility (Vicari and Calogero, 2001; Agarwal and Said, 2004). The diet should consist of an adequate amount of chain-breaking antioxidants such as vitamin C or vitamin E in addition to beta-carotenes, carotenoids and flavonoids. Various physiological antioxidants are listed in Table 2.2.

Table 2.2 Antioxidant protection systems (reproduced from Percival, 1998)

Endogenous	antioxidants
	Bilirubin
	Thiols, e. g. glutathione, lipoic acid, N-acetyl cysteine
	NADPH and NADH
	Ubiquinone (coenzyme Q10)
	Uric acid
	Enzymes:
	Copper/Zinc and manganese-dependent superoxide dismutase
	Iron-dependent catalase
	Selenium-dependent glutathione peroxidase
Dietary	**antioxidants**
	Vitamin C
	Vitamin E
	β-carotene and other carotenoids and oxycarotenoids, e.g. lycopene and lutein
	Polyphenols, e.g. flavonoids, flavones, flavonols and proanthocyanidins
Metal	**binding proteins**
	Albumin (Copper)
	Ceruloplasmin (Copper)
	Metallothionein (Copper)
	Ferritin (Iron)
	Myoglobin (Iron)
	Transferrin (Iron)

2.6.3 Polyphenols

Plant phenolic compounds have attracted increasing attention in the past decade because of their antioxidant properties and are the major antioxidant components of rooibos herbal tea. Polyphenols are ubiquitous vascular plants and serve to protect against parasites, herbivores and oxidative cell damage (Scalbert *et al.*, 2002). Additionally, they play a part in pollination by producing colours which attract insects to their food sources e.g. anthocyanins

produce the mauve, blue, red, pink and violet colours of fruits and vegetables (Coultate, 1990). Phenolic compounds such as salicylic acid also act as signalling molecules while lignin provide mechanical support (Parr and Bolwell, 2000). Over 8 000 phenolic structures have been identified and are categorized into several classes (Cohen *et al*., 2000).

2.6.3.1 Flavonoids

Besides the traditional vitamins mentioned above, many plant derived substances collectively termed “phytonutrients” or “phytochemicals” are gradually gaining recognition for their antioxidant activity (Briviba and Sies, 1994). Flavonoids are a class of secondary plant metabolites with significant antioxidant and chelating properties (Heim *et al*., 2002). They are widely distributed in the leaves, seeds, bark and flowers of plants with over 4 000 flavonoids identified to date (Harborne and Williams, 2000). In plants, flavonoids serve as protectors against a wide variety of environmental stresses, while in humans they appear to function as biological response modifiers (Percival, 1998). Their cardioprotective effects stem from the ability to inhibit lipid peroxidation, chelate redox-active metals and attenuate other processes involving ROS (Heim *et al*., 2002). Flavonoids, the most abundant and most extensively studied are a subclass of polyphenols that have a C6-C3-C6 backbone structure with two phenolic rings joined by an oxygen-containing pyran ring (Hammerstone *et al.*, 2000). Their antioxidant activities are said to be dependent on the structure especially the degree of hydroxylation of the B ring and the presence of unsaturated double bonds on the C ring (Brown *et al*., 1998). In food, flavonoids exist primarily as 3-O-glycosides and polymers (Hammerstone *et al.*, 2000).

2.6.3.2 Other types of polyphenols

Based on the C-ring variations, flavonoids are further classified as flavanols, flavonols, flavones, flavanones, anthocyanins and isoflavonoids. The flavones apigenin and luteonin are common in cereal grains and aromatic herbs (parsley, rosemary, thyme). Their hydrogenated

analogues hesperin and naringin are exclusively found in citrus fruits (Pietta, 2000). The flavonols quercetin and kaempferol are common in vegetables and fruits with the exception of onions. Isoflavones are mostly found in legumes including soybeans, black beans, green beans, chick peas and sunflower seeds (Pietta, 2000). The flavan-3-ols (+)-catechin, (-)-epicatechin, (-)-epigallocatechin and their gallate esters are predominantly distributed in plants and are also rich in tea leaves (Pietta, 2000). Anthocyanidins and their glycosides (anthocyanins) occur as natural pigments and are abundant in berries and red grape (Pietta, 2000).

The presence of the phenolic acids caffeic acid, ferulic acid, p-coumaric acid, p-hydroxybenzoic acid, vanillic acid and protocatechuic acid has been reported in fermented rooibos tea (McKay and Blumberg, 2007). Ninety nine (99) compounds are present in the volatile oil of rooibos and the major components include quaiacol (24.0%), 6-methyl-3, 5-heptadien-2-one isomer (5.2%), damascenone (5.0%) geranylacetone (4.2%), β-phenylethyl alcohol (4.1%) and 6-methyl-5-hepten-2-one (4.0%) (Habu *et al.*, 1985; McKay and Blumberg, 2007).

2.7 Antioxidants and infertility

Human sperm exposure to extracellularly-generated ROS induces a loss of motility which is directly correlated with the level of lipid peroxidation experienced by the sperm (Gomez *et al.*, 1998; Aitken and Baker, 2006). However, the ability of antioxidants such as α-tocopherol to rescue sperm motility *in vivo* and *in vitro* is an indication that loss of motility in human sperm is caused mainly by lipid peroxidation (Aitken *et al.*, 1989; Suleiman *et al.*, 1996; Aitken and Baker 2006). Hydrogen peroxide is the major ROS involved in peroxidation-induced motility loss and this was first demonstrated by McLeod (1943), however, the addition of catalase restored motility of sperm in their study. A recent study has also demonstrated decreased sperm motility when hydrogen peroxide was incubated with sperm (Garg *et al.*, 2008). Semen contains a variety of non-enzymatic antioxidant molecules such as vitamin C, vitamin E, pyruvate,

glutathione and carnitine (Agarwal and Saleh, 2002). *In vitro* studies have shown vitamin E as a major chain-breaking antioxidant in the sperm membranes and it appears to have a dose dependent protective effect (Hull *et al.*, 2000). Similarly, Krishnamoorthy *et al.* (2007) showed that α-tocopherol and ascorbic acid exhibited protective effects on sperm by inhibiting polychlorinated biphenyl (PCB), an environmental endocrine disruptor (Aroclor 1254)-induced ROS generation. One of the important mechanisms of antioxidant action is to scavenge free radicals, thereby inhibiting oxidation. Since ROS has both physiological and pathological roles, an array of antioxidants is needed to maintain a steady state of ROS in the seminal plasma (Agarwal, *et al.*, 2005).

2.8 Rooibos (*Aspalathus linearis*)

Rooibos (*Aspalathus linearis)* is a shrubby leguminous plant cultivated in the Cedarberg region of South Africa and other names include rooibos herbal tea, rooibosch, rooitea or rooitee (McKay and Blumberg, 2007; Joubert *et al.*, 2008). There are about 278 species of the genus *Aspalathus* which is endemic to South Africa (van Heerden *et al.*, 2003). These species are largely concentrated in the Western Cape Province with a few spreading to southern KwaZulu-Natal. The production of rooibos only occurs in the Clanwilliam area, a region located about 200 to 300 km north of Cape Town, within and adjacent to the Cedarberg and Olifantsrivier mountain ranges, and on the Bokkeveld plateau (Nel *et al.*, 2007). The indigenous people, the Khoikhoi, have been using rooibos since 1772 (Marais *et al.*, 2000) and its leaves and stems are used for the manufacturing of rooibos herbal tea (Bramati *et al.*, 2002). Due to the demand for this South African indigenous herbal beverage by the international market, the tonnage, to an extent has significantly increased, exceeding its domestic use (van der Merwe *et al.*, 2006). The beneficial properties of rooibos are partly ascribed to the unique phenolic constituents in the plant, which are modified enzymatically during the fermentation process (van Heerden *et al.*, 2003). Several phenolic compounds are known to occur in *Aspalathus linearis* and its predominant contents are

dihydrochalcones, flavonols and flavones (Rabe *et al.,* 1994). Figure 2.7 shows a rooibos field in Clanwilliam, Western Cape.

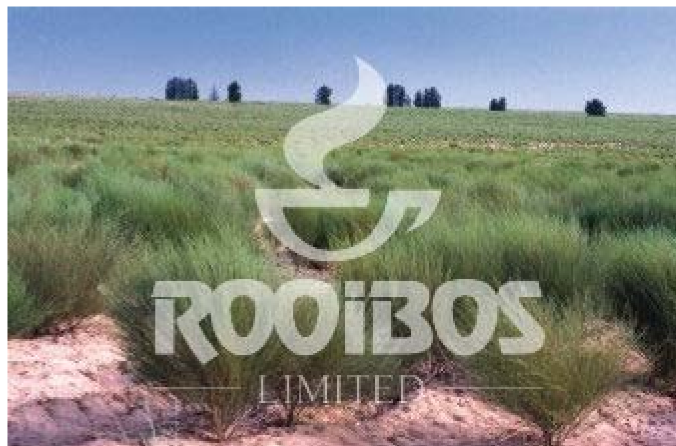


Figure 2.7 Rooibos field in Clanwilliam; Western Cape (Image courtesy of Rooibos Limited)

Two types of rooibos are manufactured based on the fermentation process, namely fermented or “red” rooibos and unfermented or “green” rooibos as shown in Figure 2.8. Fermented rooibos is produced during the hot summer period and early autumn between January and April after the rooibos plant is harvested (Joubert *et al*., 2008). During fermentation, the colour of the unfermented rooibos (“green” rooibos) changes to red and is referred to as “red” rooibos (McKay and Blumberg, 2007). The leaves turn redbrown when the shredded plant material is fermented in heaps at ambient temperature for about 12-14 hours, before being sun-dried (Joubert *et al*., 2008). Unfermented rooibos or “green” rooibos is obtained by preserving the green leaves and stem and ensuring oxidative changes are kept to a minimum (Joubert *et al*., 2008). This is achieved by either drying of shredding plant material under vacuum without delay, or drying of whole shoots to critical moisture content before shredding or steaming of the fresh shoots to inactivate enzymes (Joubert *et al*., 2008).

Figure 2.8 (a) Fermented rooibos plant material **(b)** “Green” rooibos plant material

http://www.rooibosltd.co.za/ background/index.html

2.8.1 Phytochemical content and other components

There are several phenolic compounds present in the brews of both “green” and fermented rooibos but the total concentration of flavonoids in each can differ by more than 100-fold (Bramati *et al.,* 2002; Bramati, *et al*., 2003). The major monomeric polyphenols in rooibos comprises the dihydrochalcones, aspalathin and nothofagin, the flavones, orientin and iso-orientin, vitexin and isovitexin and the flavanones (McKay and Blumberg, 2007). Marnewick *et al.* (2000) found a significantly higher percentage of total polyphenols (41.2% vs 29.7%), flavonoids (28.1% vs 18.8%) and non-flavonoids (13.1% vs 10.9%) in “green” compared to fermented rooibos respectively, however no differences in soluble solid matter was found. Standley *et al*. (2001) also found a significant difference in total polyphenols between “green” and fermented rooibos (41.0% vs 35.0%), and a difference in soluble solids (2.3% vs 1.6%). These disparities were attributed to the enzymatic and chemical modifications that occurred during the fermentation and processing methods such as sun drying and controlled drying (Joubert, 1996; Standley *et al*., 2001). Aspalathin, a dihydrochalcone present in “green” rooibos

is oxidized extensively to dihydro-iso-orientin during fermentation. Bramati *et al.* (2003) reported the concentration of aspalathin in "green" rooibos decreased from 49.9 to 1.2 mg/g with fermentation. The C-glycosyl flavones isoorientin (3.6 mg/g), orientin (2.3 mg/g), isovitexin (0.7 mg/g) and vitexin (0.5 mg/g) were also degraded to a lesser extent (range of differences post-fermentation is 2.7-0.2 mg/g). Nothofagin, a dihydrochalcone similar to aspalathin in structure is degraded as well (McKay and Blumberg, 2007; Joubert, 1996). Aspalalinin is the newly discovered component of rooibos (Joubert *et al.*, 2008). Figure 2.9 shows the structure of aspalathin found in rooibos.

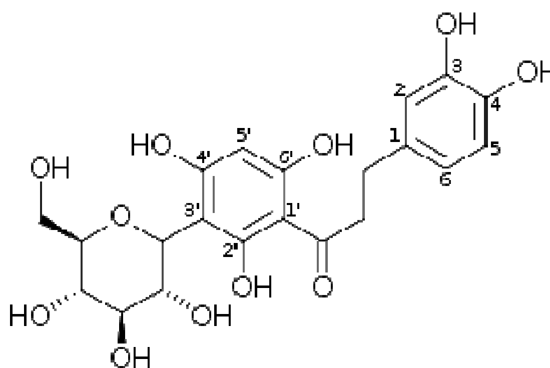


Figure 2.9 Structure of aspalathin. commons.wikimedia.org/wiki/File:Aspalathin.svg

The other predominant flavonoids detected in both types of rooibos are rutin (1.3-1.7 mg/g), isoquercetin and hyperoside (0.3-0.4 mg/g), quercetin (0.04-0.11 mg/g), luteolin (0.02-0.03 mg/g) and chrysoeriol (0.01-0.02 mg/g) (McKay and Blumberg, 2007). Approximately 19 g/kg of dried, "green" rooibos consists of these dihydrochalcones (aspalathin and nothofagin) combined, while fermented rooibos contains only 7% of the amount present in the "green" plant material (McKay and Blumberg, 2007). Table 2.3 illustrates the phytochemicals present in rooibos.

Table 2.3 Phytochemicals present in rooibos (reproduced from Joubert *et al.*, 2008)

Class of phytochemical	Individual component
Monomeric flavan-3-ols	(+)-Catechin
Flavanones	Dihydro-orientin[(R)/(S)-eriodictyol-8-glucoside
	Dihydro-iso-orientin[(R)/(S)-eriodictyol-6-glucoside
	Hemiphlorin
Flavones	Chrysoeriol
	Iso-orientin
	Isovitexin
	Luteolin
	Luteolin-7-O-glucoside
	Orientin
	Vitexin
Flavonols	Rutin
	Quercetin
	Isoquercetin
	Quercetin-3-O-β-D-robinoside
	Hyperoside
Chromone	5,7-Dihydroxy-6-C-glucosyl-chromone
Oligomeric flavan-3-ol	Procyanidin
	Bis-fisetinidol-(4β,6:4β,8)-catechin
Lignan	Secoisolariciresinol
	Secoisolariciresinol-O-glucoside
	Vladinol
	3-Hydroxy-1-(4-hydroxy-3-methoxyphenyl) -2-[4-(3-hydroxy-1-(E)-propenyl)-2,6- dimeth- -oxyphenoxyl] propyl-β-D-glucopyranoside
Dihydrochalcones	Nothofagin
	Aspalathin
Coumarin	Esculetin
Cyclic dihydrochalcones	Aspalalinin
Glycol derivative	p-Hydroxyphenylglycol
	Vanylglycol
Phenylpyruvic acid derivative	3-phenyl-2-glucopyranosyloxypropenoic acid
Phenolic carboxylic acids	3,5-Dihydrobenzoic acid
	Gentisic acid
	Salicylic acid
	p-Hydrobenzoic acid

	Protocatechuic acid
	Vanillic acid
	Gallic acid
	Syringic acid
Hydroxycinnamic acid and derivative	3,4,5-Trihydroxycinnamic acid
	p-Coumaric acid
	Caffeic acid
	Ferulic acid
	Sinapic acid
	Chlorogenic acid
Aldehyde	Syringin
Inositols	(+)-Pinitol

2.8.2 *In vitro* and *in vivo* antioxidant activity

Previous studies have demonstrated the roles of fermentation, processing and preparation conditions on the antioxidant activity of rooibos. Standley *et al.* (2001) showed the ability of rooibos at different stages of processing to scavenge 1,1-diphenyl-2-picryhydrazyl (DPPH) and superoxide ($O_2^{\cdot -}$) radicals. Joubert *et al.* (2004) also compared the capacity of flavonoids from "green" and fermented rooibos to scavenge DPPH and $O_2^{\cdot -}$ radicals, while quercetin was found to be the most potent scavenger. Aspalathin, orientin, luteolin and isoquercetin were slightly less active than quercetin towards DPPH albeit aspalathin was as effective as quercetin towards $O_2^{\cdot -}$. The protective effect of rooibos against lipid peroxidation in the presence of Fe^{2+} and absence of H_2O_2 using a liver microsomal preparation has also been demonstrated (Marnewick *et al.*, 2005). The formation of thiobarbituric acid reactive substances (TBARS) measured as malondialdehyde (MDA) was inhibited 91% and 65% by "green" and fermented rooibos, respectively (Marnewick *et al.*, 2005). Similarly, rooibos also significantly increased the ratio of reduced to oxidised glutathione (GSH/GSSG).

The activities of cytosolic glutathione S-transferase alpha (GST-α) and microsomal UDP-glucuronosyl transferase (UDP-GT), both phase II hepatic drug metabolizing enzymes, were

also enhanced in male Fischer rats that consumed rooibos (Marnewick *et al.*, 2003). Ulicna *et al.* (2003) also examined the hepatoprotective effects of rooibos in rats exposed to carbon tetra chloride (CCl_4), a potent pro-oxidant. Histological analysis revealed that rooibos treatment resulted in a regression of CCl_4 –induced hepatic steatosis and cirrhosis and reduced the production of hepatic MDA, triacyglycerols and cholesterol as well as plasma aminotransferase, alkaline phosphatase and bilirubin (Ulicna *et al.*, 2003). A growing body of evidence suggests that moderate consumption of herbal teas, including rooibos, may have protection against several forms of disease where oxidative stress plays a role (Breet *et al.*, 2005).

In a study by Persson *et al.* (2006) the effect of rooibos on the angiotensin-converting enzyme (ACE), which also catalyses the conversion of angiotensin I to angiotensin II, a potent vasoconstrictor was investigated. The effect of rooibos on the production of the vasodilator, nitric oxide (NO), in cultured endothelial cells from human umbilical veins was investigated. The authors found no significant inhibition of ACE but found a significant dose dependent increase in NO production after incubation of the cells with rooibos for 24 hours. In a similar vein, Persson *et al.* (2010) suggest that rooibos tea may have a cardiovascular effect through the inhibition of ACE activity. The authors observed that oral intake of a single dose (400 ml) of rooibos tea significantly inhibited ACE II genotype activity in healthy volunteers after 60 minutes intake. Similarly, intake of this herbal tea decreased the incidence of viral *Herpes simplex* within 2-3 days in patients who received a dilute infusion of rooibos at least once a week and patients with atopic dermatitis were relieved of their itchy sensation (Shindo and Kato, 1991). In a similar vein, the *in vitro* antioxidant activity of rooibos in cellular systems against various oxidants has also been demonstrated in human polymorphonuclear leukocytes (Yoshikawa *et al.*, 1990). A possible role for rooibos in cardiovascular health was also implied when the herbal tea was observed to inhibit *in vitro* lipid peroxidation (Marnewick *et al.*, 2005). Similarly, Marnewick *et al.* (2010) confirmed that the consumption of fermented, traditional rooibos significantly improved the lipid profile as well as redox status which are relevant to heart disease in adults at risk for

developing cardiovascular disease. In the study, the authors observed a significant increase in plasma total polyphenols levels and GSH:GSSG ratio and a significant decrease in plasma markers of lipid peroxidation after a daily consumption of six cups of rooibos for six weeks. In a more recent study, Villaño *et al.* (2010) observed that plasma antioxidant capacity increased significantly in 15 healthy volunteers after consumption of 500 ml of rooibos tea. The effect of rooibos on the antioxidant status in men occupationally exposed to lead was investigated and consumption of rooibos for 8-week period was reported to significantly enhance plasma GSH levels by 48% and to reduce LPO measured as malondialdehyde (MDA) (Nikolova *et al.*, 2007). Additionally, quercetin a flavonoid and active component of rooibos was demonstrated to alleviate male reproductive toxicity induced by diesel exhaust particles (DEP) (Izawa *et al.*, 2008).

2.8.3 Chemopreventive and other health properties

Although cancer studies is not the main focus of this study, it is important to note that more studies have revealed the involvement of rooibos herbal tea in cancer prevention than in oxidative stress. Rooibos was demonstrated to confer *in vitro* protection against genotoxicity induced by various agents in Chinese hamster ovary cells (Sasaki *et al.*, 1993), mouse embryo fibroblast cells (Komatsu *et al.*, 1994), Chinese hamster lung fibroblasts (Edenharder *et al.*, 2002), and more recently liver cancer (Marnewick *et al.*, 2009). Using the *Salmonella typhimurium* mutagenicity assay, Marnewick *et al.* (2000) examined the antimutagenic properties of fermented and "green" rooibos tea and determined that they were both significantly effective against 2-acetylaminofluorene (AAF) and aflatoxin B_1 (AFB_1)-induced mutagenesis in tester strains TA98 and TA100. Similarly, Marnewick *et al.* (2005) demonstrated that the mean number of tumours per mouse was significantly reduced by both fermented and "green" rooibos. In this study, the effects of a topical application of methanol fractions of fermented and "green" rooibos were examined in a two-stage mouse skin carcinogenesis assay. The authors demonstrated in

their study that topical application of these herbal tea fractions prior to the tumour promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA) significantly suppressed skin tumorigenesis.

Furthermore, rooibos has been shown to protect membrane lipids against peroxidation thereby partially preventing oxidative stress (Ulicna *et al*., 2006). Rooibos has also been used as an anti-hypertensive, immune stimulant, laxative, sedative and spasmolytic agent as well as for the treatment of atherosclerosis and diabetes (McKay and Blumberg, 2007).

2.9 Green tea (*Camellia sinensis*)

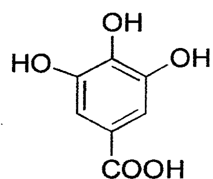
Tea is the second most common beverage consumed in the world with an estimated 18-20 billion cups consumed daily. Tea is produced from the leaves of the plant *Camellia sinensis* (Costa *et al*., 2002). Tea which represents about 80% of worldwide beverage consumption is classified into three major types depending on their manufacturing processes (Zuo *et al.,* 2002). Green tea is produced by drying and steaming the fresh leaves and no fermentation or oxidation occurs. Semi-fermented or oolong tea is produced when the fresh leaves are subjected to a partial fermentation stage before drying, while fermented black and pu-erh teas undergo a full fermentation stage before drying and steaming (Zuo *et al.,* 2002). The “fermentation” of black tea is a chemical oxidation while that of pu-erh tea is attained using microorganisms (Zuo *et al.,* 2002). Tea is commonly used with milk or lemon. Milk proteins might complex with tea polyphenols reducing their antioxidant activity *in vitro,* but not *in vivo* (Leenen *et al.,* 2000). Lemon contains vitamin C (ascorbic acid) which has antioxidative properties and can positively influence the antioxidant potential of tea (Majchrzak *et al.,* 2004). For the purpose of this review, attention will be focused on green tea.

2.9.1 Phytochemical and mineral content

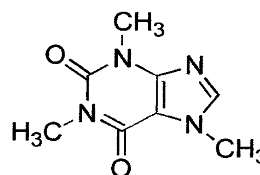
The chemical composition of green tea is complex and includes many proteins (15-20% dry weight) of which enzymes constitute an important fraction. Also included are amino acids (1-

4% dry weight), including teanine or 5-N-ethylglutamine, glutamic acid, tryptophan, glycine, serine, aspartic acid, tyrosine, valine, leucine, threonine, arginine and lysine (Cabrera *et al.*, 2006). Carbohydrates (5-7% dry weight) present include cellulose, pectins, glucose, fructose and sucrose also form part of the composition. Green tea also contain lipids such as linoleic and α-linolenic acids; sterols such as stigmasterol; vitamins (B, C, E); xanthic bases such as caffeine and theophylline; pigments such as chlorophyll and carotenoids. Volatile compounds as aldehydes, alcohols, esters, lactones and hydrocarbons are also included. The composition also includes minerals and trace elements (5% dry weight) such as Ca, Mg, Cr, Mn, Fe, Cu, Zn, Mo, Se, Na, P, Co, Sr, Ni, K, F, and Al (Cabrera *et al.*, 2006). In a previous study, Costa *et al.* (2002) observed large variations of the mineral content (Al, Ca, Mg, and Mn) in green tea from different origins. The polyphenols constitute the most interesting among these components and consequently green tea can be considered an important dietary source of polyphenols particularly flavonoids (Cherubini *et al.,* 1999). Green tea contains a high content of flavanols, i.e. catechins, which can exist in two different configurations, with the "epi" configuration being the more common (Cherubini *et al.,* 1999).

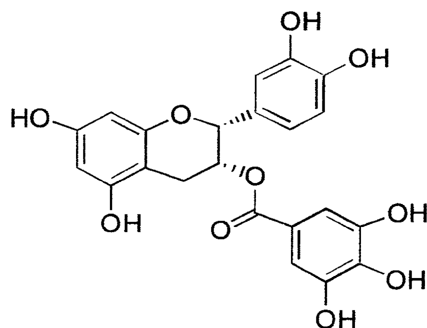
The gallocatechins are catechins containing three hydroxyl groups on the B-ring while the catechins gallates are esterified on the pyran ring with gallic acid (Cherubini *et al.,* 1999). The four major catechins are (-)-epigallocatechin gallate (EGCG) that represents approximately 59% of the total catechins; (-)-epigallocatechin (EGC) (19% approximately); (-)-epicatechin gallate (ECG) (13.6% approximately) and (-)-epicatechin (EC) (6.4% approximately). Green tea also contains gallic acid (GA) and other phenolic acids such as chlorogenic acid and caffeic acid, and flavanols such as kaempferol, myricetin and quercetin and certain amount of caffeine, a plant alkaloid present in some popular beverages such as coffee (USDA, 2003; Zuo *et al.*, 2002). Figure 2.10 shows the structure of catechin, gallic acid and caffeine found in green tea.



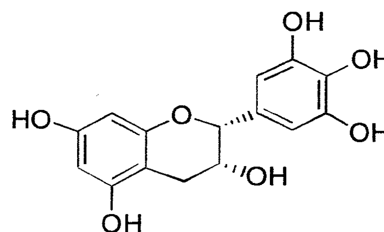
Gallic acid



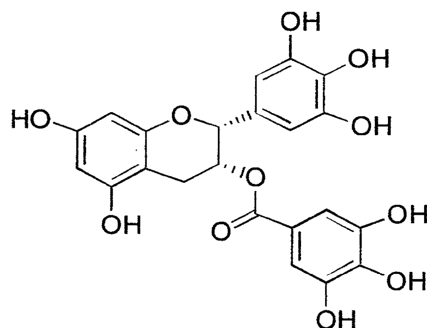
Caffeine



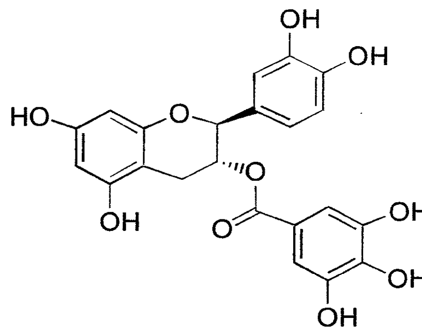
(-)-Epicatechin gallate (ECG)



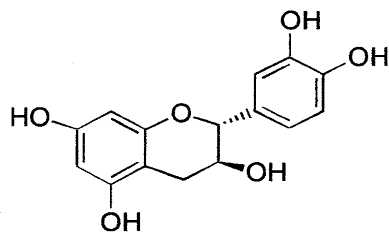
(-)-Epigallocatechin (EGC)



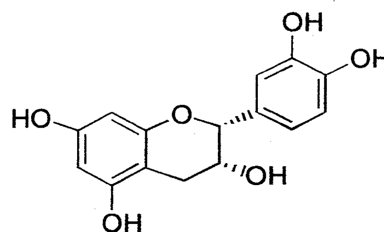
(-)-Epigallocatechin gallate (EGCG)



(-)-Catechin gallate (CG)



(+)-Catechin



(-)-Epicatechin (EC)

Figure 2.10 Structure of catechins, gallic acid and caffeine (reproduced from Zuo *et al.*, 2002)

2.9.2 Antioxidant activity

Green tea is considered an important dietary source of antioxidant nutrients rich in polyphenols and certain phytochemical compounds (Mckay and Blumberg, 2002; Kim *et al.*, 2003). These compounds could increase the green tea polyphenols (GTP) antioxidant potential. Green tea polyphenols present antioxidant activity *in vitro* by scavenging reactive oxygen and nitrogen species and chelating redox active transition metal ions. Green tea polyphenols can chelate metal ions like iron and copper to prevent their participation in Fenton and Haber-Weiss reactions (Mckay and Blumberg, 2002; Kim *et al.*, 2003). They may also function indirectly as antioxidants through the inhibition of the redox sensitive transcription factors; inhibition of 'pro-oxidant' enzymes such as lipoxygenoases, xanthine oxidase; and induction of antioxidant enzymes such as glutathione-S-tranferases and superoxide dismutases (Cabrera *et al.*, 2006). Using erythrocyte membrane-bound ATPases as a model, Saffari and Sadrzadeh (2003) indicated that EGCG is a powerful antioxidant that is capable of protecting erythrocyte membrane-bound ATPases against oxidative stress. Similarly, the repeated consumption of green tea and encapsulated green tea extracts for one to four weeks has decreased the biomarkers of oxidative status (McKay and Blumberg, 2002). Furthermore, oxidative DNA damage, lipid peroxidation and free radical generation were reduced after consuming ~6 cups/day of green tea for seven days in a study with 40 male smokers in China and 27 men and women (smokers and non-smokers) in the United States (Klaunig *et al.*, 1999; Cabrera *et al.*, 2006).

A substantial number of intervention studies in humans with green tea have demonstrated a significant increase in plasma antioxidant capacity after consumption of 1-6 cups per day (Rietveld and Wiseman, 2003; Mckay and Blumberg, 2002; Henning *et al.*, 2003; Higdon and Frei, 2003; Xu *et al.*, 2004). Initial indications also showed that the enhanced blood antioxidant potential results in a reduced oxidative damage in macromolecules such as DNA and lipids (Mckay and Blumberg, 2002; Henning *et al.*, 2003; Higdon and Frei, 2003; Rietveld and

Wiseman, 2003; Xu *et al.*, 2004). However, these authors asserted that the measurement of oxidative damage through biomarkers needs to be further explored. Similarly, Klaunig *et al.* (1999) observed that oxidative DNA damage, lipid peroxidation and free radical generation were reduced after consuming approximately 6 cups per day of green tea for seven days. This study was carried out with forty male smokers in China and twenty seven men and women (smokers and non smokers) in the United States. Therefore, green tea polyphenols may contribute immensely to defenses against oxidative damage (Wu and Wei, 2002). Recently, some epidemiological studies suggest that green tea consumption slightly reduces blood pressure (Matsuyama *et al.*, 2008; Nantz *et al.*, 2009).

2.9.3 Chemopreventive potential and other activities

The chemopreventive effects of green tea depend on its antioxidant action; the specific induction of detoxifying enzymes; its molecular regulatory functions on cellular growth, development and apoptosis; and a selective improvement in the function of the intestinal flora (Cabrera *et al.*, 2006). According to Wu *et al.* (2003), green tea drinkers showed a significantly reduced risk for breast cancer compared to women who did not drink green tea regularly (less than once in a month). Similarly, ovarian cancer risk has been reported to have declined with increasing frequency and duration of green tea consumption (Zhang *et al.*, 2002). Green tea has also been demonstrated to be an effective chemopreventive agent to human prostate cancer. Yu *et al.* (2004) reported that EGCG inhibited the growth of prostate cancer adenoma cells and induced apoptosis. Epidemiological studies have also associated the reduced risk of cardiovascular disease with green tea consumption (Nakachi *et al.*, 2000). In a prospective cohort study of 8522 men and women, Nakachi *et al.* (2000) concluded that consuming >10 cups / day was linked with a decreased relative risk (RR) of death from cardiovascular disease in men and women. Green tea catechins have also been reported to have antibacterial and

antiviral activity. Yee *et al*. (2002) and Takabayashi *et al*. (2004) reported an inhibitory effect of green tea catechins on *Helicobacter pylori* infection.

Furthermore, the *in vitro* inhibition of adenovirus infection by green tea catechins and antifungal activity of green tea catechins against *Candida albicans* have been demonstrated (Weber *et al*., 2003; Hirasawa and Takada, 2004). Yang *et al*. (2004) came to a conclusion that habitual consumption of green tea or oolong tea (120 ml per day or more for one year) significantly reduces the risk of developing hypertension in the Chinese population. In a more recent study, Sato *et al*. (2010) observed that green tea extracts could effectively prevent doxorubicin-induced testicular toxicity. The authors administered green tea extracts orally with intraperitoneal coadministration of doxorubicin, an anticancer agent, to generate toxic reactive oxygen species. From their results, telomerase activity significantly increased in association with the coadministration of green tea extracts when compared to that of doxorubicin groups only.

In order to assess the mechanism and effect of protection offered by antioxidants against oxidative stress various biochemical assays determining the antioxidant enzyme activities, glutathione level and oxidative stress status were used to determine whether aqueous extract of rooibos, green tea and both rooibos and green tea commercial available supplements were associated with changes that could modulate oxidative stress in oxidative stress-induced rats.

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CHAPTER THREE

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

[1]Dolapo Olaitan Awoniyi, [1]Yapo Guillaume Aboua, [2]Jeanine Marnewick, [3]Nicole Brooks.

[1]Department of Biomedical Sciences, Faculty of Health and Wellness Sciences,

Cape Peninsula University of Technology, PO Box 1906, Bellville, 7535, South Africa.

[2]Oxidative Stress Research Centre, Faculty of Health and Wellness Sciences,

Cape Peninsula University of Technology, PO Box 1906, Bellville, 7535, South Africa.

[3] Department of Wellness Sciences, Faculty of Health and Wellness Sciences,

Cape Peninsula University of Technology, PO Box 652, Cape Town, 8000, South Africa.

Corresponding Author

Dr. NL Brooks

Telephone: +27 214603436

E-mail: brooksn@cput.ac.za

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Abstract

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 an indigenous herbal tea rooibos, Chinese green tea and commercial rooibos and green tea supplements in rat epididymal sperm. Male Wistar rats (n=60) were supplemented with fermented and “green” rooibos, Chinese green tea, rooibos supplement, green tea supplement or water for ten weeks while OS was induced during the last two weeks of the study. Rats consuming fermented rooibos and “green” rooibos showed a significant higher sperm concentration and motility when compared with the other study groups. The catalase activity was significantly higher in the sperm of rats consuming fermented rooibos, “green” rooibos and both the rooibos and green tea supplements. The activity of superoxide dismutase in the sperm of rats supplemented with fermented rooibos, “green” rooibos and green tea was higher compared to the control. The sperm glutathione level of rats consuming the fermented rooibos and “green” rooibos were also significantly higher. None of the tea treatments showed an effect on lipid peroxidation and ROS levels, although fermented rooibos and “green” rooibos showed a tendency to lower the levels of these two biomarkers 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*), lipid peroxidation, oxygen radical absorbance capacity, reactive oxygen species, 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[1,2]. 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[3]. Their cardioprotective effects stem from the ability to inhibit lipid peroxidation, chelate redox-active metals and attenuate other processes involving reactive oxygen species (ROS)[3.] ROS, 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[4.] Free radicals may have beneficial or detrimental effects on sperm functions depending on their nature and concentration[5]. 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[6]. 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[7, 8].

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[9-11]. The colour of the unfermented rooibos product is green and referred to 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[12]. Rooibos contains unique phenolic compounds with aspalathin and nothofagin, β-hydroxy-dihydrochalcone glucosides, being the most abundant[13]. The oxidative cyclisation 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[14-17]. 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[15-19]. The effectiveness of rooibos as an antioxidant on the liver antioxidant status and hepatoprotector in liver diseases has been demonstrated[20]. Marnewick *et al.* (2000) showed the protective effects of fermented and ”green” rooibos against mutagenesis using the *Salmonella* mutagenicity assay[21]. Other potential health promoting properties of rooibos include antispasmodic effects, immune system modulation, antimicrobial, antiviral and anti-ageing properties[22-25].

Tea, a product made from the leaves and buds of *Camellia sinensis* is the second most consumed beverage in the world[26,27]. 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[28]. Previous studies have shown green tea as an important source of polyphenols, flavanols and flavonoids[29]. 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[30]. 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[31.] The health benefits of green tea are mainly attributed 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[32], cardiovascular and neurodegenerative diseases[33,34]. 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.

Materials and methods

Chemicals

Phosphate buffered 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 tolueme/ethanol (BHT, C_2H_2OH) and Folin's reagent were purchased from Sigma-Aldrich (Johannesburg, South Africa). Hydrochloric acid, ortho-phosphoric 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[22,35]. The mixture was allowed to stand for 30 minutes 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 minutes 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, while 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 contain 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 randomly divided 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 ^{o}C, with a 12 hour light-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 chow (SRC) and the fluid intake was monitored at an interval of two 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 two weeks of the ten week study[36]. 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. The caudal epididymis was removed, rinsed and gently homogenized in 1.5 mL of phosphate buffered saline using a Thomas homogenizer. The fragments were allowed to sediment for 10 min at room temperature, sperm was filtered (Whatman no. 1) and collected for the measurement of various biochemical parameters.

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 hours. 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[37]. 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 hours incubation at room temperature in a Multiskan Spectrum (Thermo Electron Corporation – U.S.A). 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[38,39]. 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)[40]. 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 minutes for 2 hours (Fluoroskan Ascent: Thermo Electron Corporation, USA).

Epididymal sperm count and motility

After collection of epididymal sperm, the concentration was determined using the method described in the World Health Organisation (WHO) Manual[41]. 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 minutes in a humid chamber to prevent drying. During this time, the cells settled and viable cells were counted using a light microscope at 40X 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 manually 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[41].

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[42]. 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 homogenised sperm samples and incubated for 45 minutes at room temperature (in dark) in a 96 well microplate. Fluorescence was measured at 530 nm emission and 485 nm excitation using the GloMax® Multi Detection 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)[43]. Briefly, 50 µl of epididymal sperm ($2x10^{6)}$ sperm were added to 6.25 µl of 4 nM cold BHT/C_2H_5OH and 50 µl of (0.2 M) ortho-phosphoric acid in a microfuge tube. After 10 seconds of vortexing, 6.25 µl of freshly prepared (0.11 M) thiobarbituric acid reagent was added and heated at 90 ^{o}C for 45 minutes. The samples were cooled down for 2 minutes on ice and 5 minutes at room temperature. This was followed by the addition of 500 µl of n-butanol and 50 µl of saturated NaCl to each sample whereafter the reaction mixtures were

centrifuged at 12 000 rpm for 2 minutes at 4 ^{o}C. The supernatant (300 µl) was 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 $2x10^6$ 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[44]. 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 minutes. Fluorescence was measured at 590 nm-600 nm with excitation of 530 nm-570 nm using the GloMax® Multi Detection 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 minutes at room temperature using the GloMax® Multi Detection System (Promega, USA). The assay was conducted according to the protocol provided by the manufacturer (Assay designs, USA) using $2x10^6$ 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, catalyzed 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® Multi Detection System. The assay was conducted according to the protocol provided by the manufacturer (Promega, UK) using 20 000 cells. The GSH levels were expressed as relative luminescence units (RLU).

Statistical analysis

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

Results

Study beverages, antioxidant profiles and daily intakes

The soluble solids were significantly higher ($P < 0.05$) in the green tea when compared to fermented and “green” rooibos. The soluble solids obtained from the fermented and “green” rooibos constituted approximately 50% of the green tea. The soluble solids of “green” rooibos increased significantly compared to fermented rooibos (Table 1). The total polyphenol content of the rooibos supplement was the highest of the treatment groups while fermented rooibos had the lowest polyphenol content and constituted about half of the polyphenol content of the green tea supplement. The flavanol intake of rats that consumed green tea and green tea supplements were higher compared to 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 rooibos supplement were significantly ($P < 0.05$) less when compared to 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 the lowest, though not significant, body weight gain. Testicular and epididymis weights for this group were also the lowest compared to all other treatment groups.

Sperm concentration 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 to the control animals consuming water, although not significant.

Table 1. Antioxidant profile of the beverages and supplements.

Treatment	SOLUBLE SOLIDS mg/mL	POLYPHENOLS mg/L	FLAVANOL mg/L	FLAVONOL mg/L	ORAC µmole TE/L
Control	ND	ND	ND	ND	ND
Rf	5 ± 1.2a	981.16 ± 117.69a	38.66 ± 8.06a	299.33 ± 49.44a	14556.81 ± 904.60a
Rg	7.1 ± 0.8b	1354.33 ± 61.99b	92.00 ± 2.60b	247.00 ± 19.45b	20888.75 ± 1281.03b
Gt	12.6 ± 1.2c	2723.16 ± 204.04c	896.50 ± 25.44c	108.66 ± 15.98c	33350.68 ± 311.77c
Rs	ND	4836.33 ± 243.92d	17.66 ± 9.85a	180.66 ± 22.47d	33768.22 ± 2322.16c
Gs	ND	1920.33 ± 195.75e	822.83 ± 37.73d	93.00 ± 10.48c	9755.33 ± 645.04d

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. Aqueous solutions (2%) were prepared for fermented rooibos (Rf), “green” rooibos (Rg), green tea (Gt), rooibos supplements (Rs) and green tea supplements (Gs). 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 ± 8.61a	ND	ND	ND	ND
Rf	74.50 ± 12.93a	73.1	2.8	21.8	1084.4
Rg	69.00 ± 14.73a	93.4	6.3	17.0	1441.2
Gt	55.10 ± 13.06b	150.0	49.4	6.0	1837.6
Rs	35.10 ± 4.33c	169.7	0.6	6.3	1185.3
Gs	38.70 ± 3.65c	74.3	31.8	3.6	377.5

The daily intake of polyphenols, flavanols, flavonols and ORAC equivalents are based on the daily tea intake volume and the beverage profile in Table 1. 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). ND-Not done

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

Treatment	Body weight gain (g)	Testis weight (g)	Epididymis weight (g)	Sperm motility %	Sperm concentration X10^6
			Parameters		
Control	125.80 ± 24.96a	3.67 ± 0.70a	0.42 ± 0.07a	54.50 ± 24.20a	56.90±27.11a
Rf	112.10 ± 33.84a	3.68 ± 0.19a	0.47 ± 0.05a	82.00 ± 12.95b	79.60 ± 27.49b
Rg	135.11 ± 35.56a	3.75 ± 0.34a	0.48 ± 0.06a	81.67 ± 10.90b	84.33 ± 26.26b
Gt	92.00 ± 26.45a	3.21 ± 0.57a	0.40 ± 0.08a	59.22 ± 23.57a	70.70 ± 49.79a
Rs	123.40 ± 35.82a	3.34 ± 0.94a	0.42 ± 0.10a	69.44 ± 17.58a	76.78 ± 21.94a
Gs	100.70 ± 37.66a	3.50 ± 0.59a	0.43 ± 0.09a	56.50 ± 23.22a	60.20 ± 25.44a

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.

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

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 demonstrated an insignificant increase when compared to 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, though 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 to 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 to 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 to the control group with green tea and both supplements that showed no modulation of GSH levels when compared to the control group.

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 x10^6 sperm	CATALASE Fluorescence (RFU) x 10^3	SUPEROXIDE DISMUTASE U/µl	GLUTATHIONE Luminescence (RLU) x 10^4
Control	3341.24 ± 1199.86a	5.12 ± 0.80a	54.84 ± 26.22a	0.71 ± 0.14a	64.52 ± 7.70a
Rf	2970.47 ± 352.13a	4.50 ± 1.32a	73.33 ± 16.64b	0.79 ± 0.17b	70.96 ± 9.89b
Rg	3061.82 ± 1021.66a	4.72 ± 1.18a	72.11 ± 17.66b	0.80 ± 0.16b	68.74 ± 7.48b
Gt	3598.84 ± 990.76a	4.73 ± 0.79a	56.81 ± 24.63a	0.76 ± 0.17b	64.52 ± 8.21a
Rs	3048.26 ± 828.01a	4.93 ± 0.51a	70.99 ± 18.57b	0.75 ± 0.14a	64.17 ± 8.37a
Gs	3604.76 ± 1600.56a	4.94 ± 0.67a	59.18 ± 21.64c	0.74 ± 0.15a	64.09 ± 8.68a

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 supplements (Rs) and green tea supplements (Gs).

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[45]. Due to the differences in the phenolic constituents of rooibos and green tea, it is essential to investigate the possible impact of these teas, 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[46, 47]. 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[48-50].

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 to 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[51]. Similarly, Chung *et al.* (2005) found that Maca extract increased the epididymal sperm count of a rat[52]. 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 like hydrogen peroxide (H_2O_2), singlet-oxygen (O_2) hydroxyl radical (OH) or peroxynitrite[53.] Cells exhibit a defensive mechanism using various antioxidant enzymes when ROS begin to accumulate.

Our study showed that rats consuming fermented rooibos, "green" rooibos and to a certain extent the rooibos supplement, protected the sperm against oxidative stress when compared to 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 bind 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[54,55]. Another intriguing possibility is that polyphenols have the potential to up-regulate the expression of beta-glutamylcysteine synthetase, the rate-limiting enzyme in the biosynthesis of GSH[56.] This may explain in our findings the enhancement of GSH in rats consuming the rooibos extracts in our results. 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[57, 58]. 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 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 [59]. Previously, TÜrk *et al.* (2007) reported increased GSH levels in rat epididymal sperm caused by pomegranate juice which is highly rich in polyphenolic compounds [60].

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 [61]. 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 [62]. Similarly, fermented rooibos and “green” rooibos

were also found to be highly protective against lipid peroxidation in rat liver [63]. 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[64, 65]. These proposed mechanisms include inhibition of lipid absorption and cholesterol synthesis as well as up-regulation of the low density pipoprotein (LDL) receptor[64,65]. The present study showed a moderate decrease though not significant in TBARS by the different teas and supplements which could be attributed to the flavonoid contents. 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[66,67]. 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[68]. Previously, Yousef and Salama (2009) reported that propolis, a flavonoid containing substance caused a reduction in TBARS[69]. With the exception of polyphenols and flavanols, the daily flavonol intake was significantly higher in the rooibos herbal tea groups compared to 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 [50]. 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 [70]. Previously, Suresh *et al.* (2009) reported that *Mucuna pruriens,* a flavonoid rich plant reversed and caused a significant increase in the activities of SOD and CAT[71]. 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 [72]. 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 spectrum of polyphenolic constituents present in rooibos (*Aspalathus linearis*) could be effective as potential antioxidants. In addition, rooibos contains a plethora of other components different from that of green tea (*Camellia sinensis*) that enhances 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 to 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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CHAPTER FOUR

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

[1]D. O. Awoniyi, [1]Y. G. Aboua, [2]J. L. Marnewick, [3]S. S. du Plesis, [4]N. L. Brooks.

[1]Department of Biomedical Sciences, Faculty of Health and Wellness Sciences, Cape Peninsula University of Technology, Bellville, South Africa, 7535

[2]Oxidative Stress Research Centre, Faculty of Health and Wellness Sciences, Cape Peninsula University of Technology, Bellville, South Africa, 7535

[3]Division of Medical Physiology, Faculty of Health Sciences, Stellenbosch University, Tygerberg, South Africa, 7505

[4]Department of Wellness Sciences, Faculty of Health and Wellness Sciences, Cape Peninsula University of Technology, Cape Town, South Africa, 8000

Corresponding Author

Dr. NL Brooks

Telephone: +27 214603436

E-mail: brooksn@cput.ac.za

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Abstract

The aim of this study is to compare 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. The drinking water of male Wistar rats (n=60) were supplemented with either fermented rooibos, “green” rooibos, Chinese green tea, a commercial rooibos supplement or a green tea supplement for a period of ten weeks. During the last two weeks of the study (weeks 9-10) oxidative stress was induced in all animals by an intraperitoneal t-butyl hydroperoxide injection. The superoxide dismutase activity increased significantly ($P < 0.05$) in the testicular tissue of rats that consumed fermented rooibos, green tea and rooibos supplement compared to the control. The glutathione levels of rats consuming the green tea supplement was furthermore significantly ($P < 0.05$) increased when compared to the control. Reactive oxygen species levels were significantly ($P < 0.05$) decreased in rats consuming the rooibos supplement while lipid peroxidation measured as TBARS was significantly ($P < 0.05$) decreased in rats consuming fermented rooibos and green tea. Our study demonstrated that extracts of both 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. The “green” rooibos and commercial supplements showed varied effects of protection.

Keywords: 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 defences 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). Many artificial and natural agents possessing antioxidant 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). 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*) have been 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, 2009b). The aim of this study was to evaluate the possible protective effects of fermented and "green" rooibos (*Aspalathus linearis*), green tea (*Camellia 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 they contain the active components of the various herbal teas and tea.

Materials and Methods

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 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 (30 µM/100 g body weight) (Kumar and Muralidhara, 2007). The rats (non-fasting) were sacrificed under pentobarbital anaesthesia (i.p. 0.4 ml/kg body weight). The testes were excised, their weights 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 CPUT's Faculty of Health and Wellness Sciences Research Ethics Committee.

Assessment of reactive oxygen species and lipid peroxidation

Intracellular production of ROS in the testis was measured using 2'7'-dichlorofluorescin 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 minutes 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 µl 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 seconds of vortexing, 6.25 µl of freshly prepared thiobarbituric acid reagent (0.11 M) was added and the mixture heated to 90 ^{o}C for 45 minutes. The samples were cooled on ice for 2 minutes and kept at room temperature for 5 minutes before adding n-butanol (500 µl) and saturated NaCl (50 µl). The reaction mixtures were centrifuged (12000 rpm, 2 minutes, 4 ^{o}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 superoxide dismutase (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 (H_2O_2), remaining after sample addition (Zhou *et al.*, 1997). Briefly, 50 µl of H_2O_2 (40 µM) solution was added to 50 µl of testes homogenate. 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 minutes). Readings were determined at fluorescence 590-600 nm and excitation at 530-570 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 H_2O_2 by xanthine oxidase to form superoxide anion ($O_2^{-}\bullet$). The $O_2^{-}\bullet$ then converts teterazolium 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™ 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 µl) were added to GSH-Glo™ Reagent 2X (50 µl) and incubated at room temperature for 30 minutes. Subsequently, 100 µl of luciferin detection reagent was added in a 96 well microplate and the mixture incubated for 15 minutes 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, chapter 3).

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 to the control group. Fermented rooibos and green tea caused a significant ($P < 0.05$) decrease in the LPO, measured as TBARS levels, 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 consuming 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 increasing the activity was shown by all the groups except "green" rooibos when compared to the control group. Rats that consumed fermented rooibos, green tea and the rooibos supplement caused a significant ($P < 0.05$) increase in the SOD activity while rats that received "green rooibos" and the green tea supplement decreased SOD activity significantly ($P < 0.05$) when compared with the control group consuming water only.

Glutathione level

The GSH levels in the testicular tissue of the different tea treated groups are shown in Table 1. Rats consuming the commercial green tea supplement had significantly ($P < 0.05$) increased GSH levels when compared with the control group. All other treatment groups showed an insignificant increase in testicular GSH levels.

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) x10^3	nmol MDA/mg tissue	Fluorescence (RFU) x10^3	U/mg of tissue	Luminescence (RLU) x10^3
Control	104.96 ± 46.40a	15.73 ± 6.46a	40.37 ± 8.14a	2.38 ± 0.07a	13.38 ± 3.87a
Rf	75.14 ± 26.51a	7.99 ± 3.89b	42.62 ± 4.15a	3.80 ± 0.05b	15.16 ± 1.19a
Rg	79.54 ± 28.08a	13.04 ± 6.83a	40.06 ± 4.53a	2.20 ± 0.09c	14.07 ± 2.11a
Gt	105.07 ± 40.08a	9.17 ± 4.78b	45.30 ± 14.43a	4.27 ± 0.13b	14.40 ± 3.07a
Rs	70.90 ± 12.69b	13.59 ± 5.27a	44.60 ± 7.33a	2.83 ± 0.09b	14.18 ± 2.02a
Gs	96.67 ± 32.23a	14.30 ± 4.53a	44.06 ± 5.24a	2.12 ± 0.13c	16.16 ± 3.61b

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).

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). 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).

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 consuming 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 e.g., $O_2^{-\cdot}$ and OH^- . 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 consuming fermented rooibos, the rooibos supplement and green tea showed significantly increased SOD activity when compared to the control animals, while the TBARS levels showed a decreasing trend only. Rats consuming 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 were 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 (Fujii *et al*., 2003; Türk *et al*., 2008). Our results showed 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). The OH radical cannot be enzymatically removed from cells but a free radical scavenger can detoxify it. We propose that the trend in increasing the 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).

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 its structure, particularly its ability to donate a hydrogen ion to the peroxyl radical produced as a result of LPO (Saija, 1995; Kashima, 1999). Results from our previous study (unpublished data, chapter 3) showed that the daily flavonol intake was significantly higher in the rats consuming the rooibos herbal teas while rats consuming the green tea, commercial rooibos and green tea supplement, had significantly higher flavanol intakes. The chemical properties of some antioxidants e.g. flavanols and flavonols, may give them pro-oxidant 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 showed that aqueous extracts of rooibos (*Aspalathus linearis*) and green tea (*Camellia 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 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 fermented rooibos and green tea could be 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.

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CHAPTER FIVE

GENERAL DISCUSSION

Oxidative stress plays a prominent role in pathophysiology of reproductive malfunction and infertility in animals and humans (Levy *et al.*, 1999; Feldman *et al.*, 2000). The beneficial health effects of rooibos and green tea in human and animal models with respect to their antioxidants, especially flavonoids, in protecting against various diseases have been reported. As a result of the differences in the phenolic constituents of rooibos and green tea, it is imperative to investigate the possible effect of rooibos herbal teas compared with green tea on the oxidative stress status of sperm, testes and its effect on male infertility in an animal model.

A shift in pro-oxidant and oxidant status could lead to OS, but a change in antioxidant activities is frequently used as an important indicator of OS and the antioxidant defence status determines the extent to which oxidative damage occurs in the sperm (Sikka, 2001; Ong *et al.*, 2002). The generation of ROS by sperm is a normal physiological process, however a shift between ROS production and scavenging activity is deleterious to sperm and it has been shown to be associated with male infertility (Sharma and Agarwal, 1996). In mammals, the epididymis is known to play a major role in the final development of motility, fertilizing ability and sperm storage. Sperm concentration can increase up to 10^{10} cells/ml during epididymal transit with a simultaneous increase in sperm metabolism and the possibility of OS threatens the survival of these male gametes (Dacheux *et al.*, 2003).

Defective sperm function frequently causes male infertility which accounts for about 24% of infertile couples (Lipschultz and Howards, 1983; Hull *et al.,* 1985). In the present study where OS was induced by intraperitoneal injection of tbHP, the epididymal sperm concentration and motility of rats that consumed fermented and "green" rooibos were significantly increased compared to the control rate after a short term (10 weeks) exposure to this herbal tea. The

formation of ROS decreases when motility is greater than 60% and this suggests that in infertile men, a sperm suspension with a high concentration of immotile spermatozoa has a greater possibility of producing ROS than a highly motile sperm suspension (Iwasaki and Gagnon, 1992). This observed improvement 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 the herbal tea. Purdy *et al*. (2004) demonstrated that flavonoids caused an increase in motility in caprine sperm. In a similar study, Chung *et al*. (2005) observed that a Maca extract increased the epididymal sperm count of a rat. However, since OS has been established to play a key role in the cause of many diseases especially infertility, the results of our study showed that it is possible that the consumption of rooibos and green tea may have a reducing or lowering capacity on OS thereby improving fertility.

Alterations of GSH metabolism are associated with several diseases that may result in a lowered cellular redox potential (Roth *et al*., 2002). In the present study, where OS was induced in experimental rats, the rooibos herbal teas, the rooibos supplement, green tea and green tea supplement showed a protection against OS compared to the control group due to their varying degrees of tendency to reduce the levels of LPO, ROS and enhance the levels of the antioxidant enzymes SOD and CAT as well as GSH levels. An imbalance of pro-oxidants in the semen and vaginal secretions can induce OS in spermatozoa and decrease the antioxidant activities, which concomitantly correlate with idiopathic infertility (Sharma and Agarwal, 1996).

The significant increased activity of SOD in rat sperm and an increasing trend in CAT activity trend in testicular tissue suggest that both rooibos herbal teas, green tea and the rooibos and green tea supplements could have varying protective synergistic effect on enzyme induction and removal of hydroxyl radicals and subsequent removal of the product produced by the activities of SOD (hydrogen peroxide). In biological systems, it is expected that superoxide anion, catalyzed by SOD, instantly becomes a substrate for catalase and other hydrogen peroxide-catalyzing enzymes such as glutathione peroxidase. Our findings corroborate that of

Ramiro-Puig *et al.* (2007) who reported increased activities of certain antioxidant enzymes following flavonoids supplementation in Wistar rats. The present results support the findings that flavonoids and its other constituents with antioxidant properties and its resultant enhanced catalase activities, could be important in protecting against induced-oxidative stress.

Free radical-induced oxidative damage to spermatozoa is a condition that has gained favourable attention for its role in inducing poor sperm function and infertility (Russo *et al.*, 2006). Factors that can offer protection against oxidative damage are therefore of immense importance. In this study, we recorded an increase in the GSH level of the rats that consumed fermented rooibos, "green" rooibos and the commercial green tea supplement when compared to the control group. Glutathione is a powerful intracellular antioxidant and plays a prominent role in stabilizing various enzymes and could also be regarded as a good marker for tissue antioxidant capacity (Van Acker *et al.*, 2000; Wang and Jiao, 2000). Glutathione protects cells against OS and other types of damage, which may originate from compounds of endogenous and exogenous sources. One major function of cellular glutathione 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).

The continued activity of glutathione peroxidase depends on the regeneration of reduced glutathione by glutathione reductase which in return relies on NADPH. Reduced glutathione can neutralize hydroxyl radicals and thereby function in the detoxification of peroxides through its interaction with sperm glutathione peroxidase and may also facilitate the antioxidant action of alpha-tocopherol in the sperm plasma membrane by participating in the regeneration of this vitamin from tocopheryl radicals (Sharma and Agarwal, 1996). On the other hand, polyphenols have the potential to up-regulate the expression of ß-glutamylcysteine synthetase, the rate-limiting enzyme in the biosynthesis of GSH (Moskaug *et al.*, 2005) and this may explain the increase of GSH level in our findings. Our data is similar to Türk *et al.* (2008) who reported an increase in the GSH level in the epididymal sperm of rats treated with pomegranate juice which

is known for its flavonoid and polyphenolic compounds. Similarly, Khan and Ahmed (2009) demonstrated that *Digera muricata,* a plant which is rich in flavonoids, also enhanced the GSH content in the testis of rats against OS.

The protective role of antioxidants, more specifically flavonoids, have been demonstrated in several studies (Ola-Mudathir *et al*., 2008; Khan and Ahmed, 2009; Suresh *et al*., 2009). According to Halliwell and Gutteridge (1999), mechanisms of antioxidant action can include: (1) suppressing ROS formation either by inhibition of enzymes or chelating trace elements involved in free radical production; (2) scavenging reactive oxygen species; and (3) upregulating and protecting antioxidant defenses. Under physiological conditions, normal levels of antioxidants SOD, CAT, GSH peroxidase and reductase maintain the ROS scavenging capacity in the male reproductive tract and seminal fluid. When these scavengers are monitored objectively, they can be used as a good indicator of sperm damage and infertility caused by OS (Sikka *et al*., 1995). In the present study, two rooibos herbal teas and green tea aqueous preparations reduced the induced- oxidative damage in rat epididymal sperm and testicular tissue. This was observed by the varying increase in SOD, CAT and GSH levels with a decrease in the DCF and LPO compared to the control. The mechanisms by which oxidative damage are reduced by flavonoids, polyphenols and other constituents are unclear. Previous studies have shown rooibos and green teas as important sources of polyphenols and flavonoids (Cabrera *et al*., 2006; Ligor *et al*., 2008). We have shown that a 10 week consumption of rooibos or green tea increased some of the antioxidant enzyme activities and GSH levels in sperm and testicular tissue, possibly due to the protection from oxidation exerted by flavonoids, polyphenols and other components of rooibos with antioxidant activity and as such, act as effective lipid peroxidation inhibitors.

The modification in the antioxidant enzymes activity and GSH level may be as a result of a cascade involving endogenous antioxidants which react differently according to their polarity and redox potential (Palozza and Krinsky 1992; Pietta *et al*., 1998). More specifically, flavonoids

and their metabolites are capable of reducing the highly oxidizing ROS, becoming less aggressive aroxyl radicals. The results of this study indicate that the GSH level increased significantly in the epididymal sperm and/or testes of rats that consumed fermented rooibos, “green” rooibos and the green tea supplement. The GSH peroxidase activity depends highly on GSH concentration and the increase in the GSH level may be as a result of increased synthesis or elevated activation of this enzyme. Glutathione levels in cells are dependent upon the rates of biosynthesis and utilization in oxidation and/or reduction reactions.

The activity of the enzyme glutamate-cysteine ligase and the amounts of the precursor amino acids are the key factors affecting GSH synthesis (Rebrin *et al*., 2005). Furthermore, depletion of GSH levels results from its various reduction reactions, including direct interaction with radical species, enzymatic reduction of hydroperoxides, metabolism of xenobiotics and regeneration of reduced forms of redox pairs such as cysteine/cystine, $NADPH/NADP^+$ and $thioredoxin_{reduced}/thioredoxin_{oxidized}$ (Rebrin *et al*., 2005). Glutathione can scavenge peroxynitrite and HO• as well as convert H_2O_2 to water with the help of GSH peroxidase (Krishnamoorthy *et al.,* 2007). The increased level of GSH in the rat sperm and testes to a large extent reveal the participation of reduced glutathione in the detoxification of H_2O_2 . This enhanced intracellular transport of GSH was important in sustaining the redox state to cope with the OS in the different tea treated groups. Glutathione radicals (GS•) are formed during scavenging of ROS. Two GS• produce GSSG that can be converted back to GSH by NADPH-dependent GSH reductase (Krishnamoorthy *et al.,* 2007). In a previous study, Tϋrk *et al.* (2008) observed marked increases in GSH level and CAT activities in the epididymal sperm of rats treated with different doses of pomegranate (*Punica granatum*) juice known for its antioxidant activity.

Similarly, Ola-Mudathir *et al.* (2008) reported cadmium to decrease GSH levels, SOD and CAT activities and this was reversed and attenuated by aqueous extracts of onion and garlic which contain dietary phytochemicals with proven antioxidant properties. Polyphenols from rooibos and green tea are suggested to account for the effects in this study but other

components cannot be excluded. Phytochemicals are known for inducing phase II antioxidant enzymes and thereby increasing the synthesis of antioxidants and detoxification enzymes and major cellular antioxidants especially GSH (Rebrin *et al.*, 2005). The substantial increase in the GSH level in the sperm and testicular tissue of rats that consumed fermented rooibos, “green” rooibos and green tea supplement may suggest a decreased oxidative stress or an increased antioxidant capacity in the cell, thereby lowering the risk of oxidative damage.

The intraperitoneal administration of tbHP negatively affects the male reproductive system and our results shows the possible active involvement of SOD, CAT and GSH in the scavenging of ROS, demonstrating its effectiveness in increasing the antioxidant status in both epididymal sperm and testicular tissue. The current findings suggest that rooibos and green tea have significant value in improving the antioxidant status as shown by the increased antioxidant enzymes activity and GSH levels and could serve as a supportive therapy in the nutritional management of infertility patients with OS diseases. The exact intracellular mechanisms mediating the induction of antioxidant enzymes by tea flavonoids are not known.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

Increasing the intake of certain dietary antioxidants may help in maintaining an adequate antioxidant status, and as a result enhances normal physiological function of a living system. The main objective of this study was to assess the modulation of oxidative stress by the South African herbal tea, rooibos, green tea and their commercially available supplements on oxidative stress-induced rats. Since oxidative stress has been implicated in the aetiology of infertility, it is therefore imperative to assess the role that antioxidants play in protection against OS. Rooibos and green tea have been previously shown to possess strong antioxidant properties in *in vitro* and in experimental animal studies.

In the present study, rooibos increased sperm concentration and sperm motility. The GSH level and antioxidant activities of SOD and CAT were enhanced by rooibos and green tea in sperm, while a decreased lipid peroxidation and ROS, and increase CAT activity in testicular tissue were shown. Our findings support the involvement of dietary antioxidants, especially flavonoids, in the scavenging mechanism to support the activities of SOD, CAT and GSH in order to maintain a balance between ROS production and the antioxidant systems. There are two possible antioxidant pathways. Firstly, flavonoids, which are the major constituents of rooibos and green tea, directly removed H_2O_2 or scavenged OH^- induced by H_2O_2, suppressed lipid peroxidation and protected the cell membrane from oxidative damage. Secondly, the flavonoids indirectly scavenged the free radicals by activating antioxidant enzyme systems to alleviate the protective effect induced by H_2O_2 .

We therefore propose that the polyphenolic constituents of rooibos (*Aspalathus linearis*) and green tea (*Camellia sinensis*) could be effective as antioxidants in protecting against sperm oxidative damage which possibly improves male fertility. The mechanisms by which this

protection was achieved could involve one or several diverse antioxidant properties displayed by its components. Hence, we support the consumption of dietary antioxidants rich in flavonoids.

This research has mainly focussed on the possible modulating effects of rooibos herbal teas, green tea and their commercially available supplements as dietary antioxidant components on oxidative stress-induced rats. Although, available information reveal the use of rooibos and green tea in oxidative stress studies, the type or combination of antioxidants to be used in order to alleviate oxidative stress and infertility, especially in humans requires further investigation and remains to be elucidated. Studies using greater sample numbers, measuring more oxidative parameters and antioxidant enzymes are pertinent to further substantiate and shed more light on the results from this study. A series of well controlled clinical intervention trials are needed to further explore this possibility in humans. From the results of this study, it is recommended that rooibos and green tea are cost effective and be consumed as a “whole” tea or water tisane rather than a supplement containing only certain components of these plants.

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CHAPTER SEVEN

SCIENTIFIC OUTPUT

NATIONAL

Authors: D. O. Awoniyi, Y. G. Aboua, J. L. Marnewick, N. L. Brooks

Title: 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.

Conference: Pathtech 2009 congress, Durban, South Africa.

Date: 6-10 September, 2009

Presentation: Poster

INTERNATIONAL

Authors: D. O. Awoniyi, Y. G. Aboua, J. L. Marnewick, N. L. Brooks

Title: 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.

Conference: 20th World congress on fertility and sterility (IFFS 2010) Munich, Germany.

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