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The effects of dietary Buriti oil (*Mauritia flexuosa)* supplementation on rat reproductive function

By

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DECLARATION

I, Rosemary Boitumelo Mosito, declare that the contents of this dissertation/thesis represent my own unaided work, and that the dissertation/thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

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ABSTRACT

Oxidative stress (OS) plays a major role in the pathogenesis of different conditions including male infertility. OS is caused by high amounts of reactive oxygen species (ROS) that exceed the antioxidant ability of a system. The sperm membrane is rich in polyunsaturated fatty acids and is prone to damage by ROS. Sperm damage decreases motility, concentration and viability. Testicular oxidative stress impairs Leydig cell function and leads to decreased hormonal control as the cells secrete testosterone.

Studies have shown the role of antioxidants in the fight against OS. Recently more studies have been focused on the use of natural antioxidants derived from fruits, vegetables, nuts and oils for this purpose. The effects of Buriti oil supplementation have been investigated in the diet and it had been shown that it is rich in carotenoids and vitamin E.

This study explored the antioxidant effects of Buriti oil on testicular tissue, epidymal tissue and hormonal function in male Wistar rats. Experiments were conducted for 6 weeks and male adult Wistar rats (10 weeks) were divided into two groups (n=30) for each group. The control group received standard rat chow and water while the experimental group received Buriti oil, rat chow and water daily. Both groups were exposed to natural physiological OS. The plasma, testicular and epididymal tissue samples of both groups were analysed for various parameters.

Testicular weight and epididymal weight of rats fed with Buriti oil were significantly increased compared to the control group. Testicular and epididymal MDA levels were decreased in rats fed with Buriti oil compared to the control group. Superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) activities were increased in both epididymal and testicular tissue of the Buriti oil fed group than the control group. Data were expressed in mean ± SEM.

In conclusion, our findings suggest that Buriti oil supplementation could prevent OS damage in the male reproductive system.

Keywords: oxidative stress, antioxidants, Buriti oil, testicular tissue, epididymal tissue, testosterone, estradiol, superoxide dismutase, catalase, glutathione peroxidase

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DEDICATION

This thesis is dedicated to the faithfulness of

The Lord God Almighty

LIST OF OPERATIONAL TERMS AND CONCEPTS

Antioxidants:	Compounds that act as scavengers of free radicals. Molecules that prevent oxidation of other molecules
Endogenous	Originating from within an organism
Exogenous	Taken up from external environment
Free radical	Any molecule capable of independent existence that contains one or more unpaired electrons
In vivo	A measurement or a process taking place in the living body
In vitro	A study taking place outside its normal biological context
Infertility	State of humans being unable to produce offspring after twelve months of unprotected sexual intercourse where the female is <36years old
Lipid peroxidation	Oxidative degradation of lipids and propagating lipid chain breaking reaction initiated by the attack of free radicals
Oxidation	The combination of a substance with oxygen with subsequent loss of electrons
Oxidative stress	Metabolic imbalance between the production of free radicals and their scavenging counteract antioxidants in favour of free radical overload and subsequent cellular damages
Reactive oxygen species	Any compound derived from oxygen which contains one or more unpaired electrons
Testicular function	Production of testosterone and spermatozoa

LIST OF ABBREVIATIONS

1,25(OH)2D3	1,25-dihydroxyvitamin D3
ABCA1	ATP-binding cassette, sub-family A
ATP	Adenosine triphosphate
BH4	Tetrahydrobiopterin C
BCA	Bicinchoninic acid assay
BPA	Bisphenol A
cAMP	Cyclic adenosine monophosphate
CAT	Catalase
CoQ10	Coenzyme Q_{10}
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FSH	Follicle stimulating hormone
g	Gram
GGTP	Gamma-Glutamyl Transpeptidase
GnRH	Gonadotropin-releasing hormone
GPDH	Glycerol 3-phosphate dehydrogenase
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
GSHt	Total glutathione
GSSG	Oxidised glutathione
H_2O_2	Hydrogen peroxide
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HDL-C	High-density lipoprotein cholesterol
HPLC	High performance liquid chromatography
HPV	Human papilloma virus
LH	Luteinizing hormone

LPO	Lipid peroxidation
MDA	Malondialdehyde
mg	Milligram
min	Minute
ml	Millilitre
MUFAs	Monounsaturated fatty acids
NAD(P)H	Nicotine adenine dinucleotide phosphate
NO	Nitric oxide
NOS	Nitric oxide synthase
ºC	Degrees Celsius
OGDH 2-	Oxoglutarate dehydrogenase
ORAC	Oxygen radical absorbance capacity
OS	Oxidative stress
PAHs	Polycyclic aromatic hydrocarbons
PAs	Proanthocyanidins
PBS	Phosphate buffered saline
PDH	Pyruvate dehydrogenase
PhGpx	Phospholipids glutathione peroxidase
PUFAs	Polyunsaturated fatty acids
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPO	Red palm oil
SOD	Superoxide dismutase
SRC	Standard rat chow
TAC	Total antioxidant capacity
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
t-BHP	tertiary-butyl hydroperoxide

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CHAPTER 1
INTRODUCTION

1.1 Statement of research problem

Male reproductive function can be impaired by advanced aging, chronic diseases, medication and the exposure to pollutants which leads to the accumulation of free radicals in the body causing oxidative stress. The build up of free radicals affects sperm quality and sperm count. Previous studies have shown that antioxidants or antioxidant-rich foods have the ability to protect human health (Bolling *et al*., 2011; Awoyini *et al*., 2012: Nemzer *et al*., 2014). Buriti oil is known to be one of the richest natural plant sources of antioxidants such as carotenoids, tocopherols and metalloporpheryns. It is therefore important to look at the reported benefits of anti-oxidant rich oils such as Buriti oil (*Mauritia flexuosa*) and its effects on reproductive function.

1.2 Research problem

Male factor infertility is usually responsible for infertility in approximately 15% of all infertile couples (Sharlip *et al*., 2002; Mahendra, 2012). Among the many causes of infertility, excessive exposure to certain environmental factors seems to negatively affect sperm production, quality and count (Aleksander and Yvonne, 2011; Sengupta, 2013). Age, environmental pollution and chronic disease can cause oxidative stress which often leads to infertility (Agarwal and Prabakaran, 2005; Agarwal *et al*., 2014). Oxygen is required by cells living under aerobic conditions in order to support life (Agarwal and Prabakaran*,* 2005; Mills and Canfield, 2014). However, metabolites, such as reactive oxygen species (ROS), can modify cell functions and endanger cell survival if in excess. Male germ cells at various stages of differentiation have the potential to generate ROS. Physiological levels of ROS are needed to regulate sperm capacitation, acrosome reaction and sperm–oocyte fusion (Aitken and Curry, 2011; Du Plessis *et al*., 2015). Any over production or accumulation of ROS is harmful to the male reproductive function. Valko *et al*. (2006) showed that seminal plasma antioxidants must continuously inactivate excess ROS in order to maintain normal cell function. Many types of antioxidants have been used to alleviate the over production of ROS.

1.3 Research aim and objectives

Buriti oil is very rich in antioxidants and can lower oxidative stress hence this study investigated the use of Buriti oil as a treatment strategy for male infertility (Santos *et al*., 2013). This baseline study aimed at investigating the effects of Buriti oil on rat testicular and epididymal tissues. Furthermore, we investigated the effects of Buriti oil supplementation on the oxidative stress markers and enzymes of the testicular and epididymal tissues. Finally we assessed the effects of Buriti oil on plasma hormonal content. Red palm oil is known to

be rich in vitamin E and carotenoids beneficial to male fertility; therefore the vitamin E and carotene concentrations of Buriti oil and red palm oil were compared. In order to achieve our aims, the following objectives were set:

- Analysis of carotenoids and vitamin E content of Buriti oil
- Analysis of carotenoids and vitamin E content of Red Palm Oil
- Measurement of antioxidant enzymes such as glutathione peroxidase, catalase, superoxide dismutase in testicular and epididymal tissue
- Measurement of oxidative stress biomarkers such as thiobarbituric acid (TBA) in testicular and epididymal tissue.
- Measurement of testosterone and estradiol concentrations in blood plasma.

CHAPTER 2
LITERATURE REVIEW

2.1 Epidemiology

The male reproductive system performs three major functions; it produces, maintains and transports sperm and semen, it produces and secretes hormones responsible for reproductive function and it discharges sperm to fertilize the ovum (Brandes, 2012). Any impediment in these three main functions can lead to male infertility. Infertile couples cannot have children due to different factors such as congenital, environmental, physiological, and physical stress. Environmental, physiological and genetic factors have been implicated in poor sperm function and infertility (Desai *et al*, 2010; Guerrero-Bosagna and Skinner, 2014). Male factor infertility is usually the cause of infertility in approximately 15% of all infertile couples, and about 25% of these cases do not have an idiopathic cause (Sharlip *et al*., 2002). A study conducted by Louis *et al.* (2013) concluded that in the USA approximately 9-14% of reproductive-aged men may be experiencing couple infertility.

2.2 Male reproductive system and function

The male reproductive system consists of the penis, testes and epididymis amongst accessory organs. The function of the testes is spermatogenesis and this is regulated by hormones (Jones, 1999; Easley *et al*., 2012; Tibary *et al*., 2014). The epididymis temporarily stores the sperm and ensures that it gains motility after production. The penis delivers the sperm into the female during coitus (Chen, 2013; Meccariello *et al.,* 2014).

The male reproductive system also consists of accessory organs such as the vas deferens, seminal vesicle and prostate gland. The prostate gland is a walnut-sized gland located between the penis and the inferior surface of the bladder. The prostate secretes fluid that nourishes the sperm into the urethra during ejaculation (Mangera *et al.,* 2013). Seminal vesicles are two tube-like glands found behind the bladder in males. About 50-70% of the fluid that makes up the seminal fluid is secreted by the seminal vesicles. Each seminal gland has an excretory duct which opens into the corresponding vas deferens as it enters the prostate gland (Cherullo *et al*., 2002). Fluid secreted by the seminal gland is alkaline and helps to protect sperm against acids produced by the vagina. The vas deferens is a thick smooth muscled walled tube that contracts during ejaculation to transfer sperm to the urethra where it combines with secretions from seminal vesicles (Mangera *et al*., 2013).

2.2.1 Testes and sperm maturation

The production of male haploid germ cells from diploid spermatogonial stem cells results from a complex biological process called spermatogenesis (Hess and de Franca, 2008; Easley *et al.,* 2012). Spermatozoa are produced in the testes and matured in the epididymis. Spermatogenesis takes place in the seminiferous tubules of the testes under the control of endocrine secretions (Michael and Griswold, 1998; Hogarth and Griswold, 2010). In humans, sperm production begins at puberty and usually continues until death (Moore *et al.,* 2007; Chen, 2013). Spermatogenesis occurs in cycles necessary for the continuous production of sperm.

Sperm production in mammals is a stepwise process and it takes about 60 days in a rat and 72 days in humans to complete, and this clearly shows that the process is species-specific (Franca and Russel, 1998; Kurowicka *et al*., 2015). Spermatogenesis is divided into two phases in mammals, of which the first stage starts at birth while the second stage starts at puberty (Moore *et al*., 2011; Borgers *et al.,* 2014). Spermatogenesis involves three major vital biological processes known as mitosis; meiosis; and spermiogenesis. Early progenitor cells known as spermatogonia are defined as “undifferentiated” because they have the potential to become gametes but have not yet committed to the process. Undifferentiated spermatogonia divide mitotically for two important reasons; firstly to repopulate the testicular stem cell population and secondly to provide progenitor cells that undergo spermatogenesis (Griswold and Oatley, 2013; Guo *et al*., 2014). During this process germ cells multiply first by repeated mitotic divisions and then by meiosis, which involves the duplication of chromosomes or DNA, genetic recombination, and then reduction of chromosomes through two cell divisions to produce spherical haploid spermatids that differentiate into highly compacted spermatozoa for release into the tubule lumen (Hess and França, 2005; Hogarth and Griswold, 2010). Figure 2.1 illustrates different stages in spermatogenesis. It illustrates the production of the spermatogonium (46, XY) from testis and the differentiation of the primary spermatocyte (46, XY) into secondary spermatocytes (23, X and 23, Y) after undergoing the first meiotic division. These secondary spermatocytes undergo a second meiotic division to produce the two 23, X and two 23, Y spermatids. This entire process results in four haploid sperm cells after spermiogenesis is completed (Guo *et al.,* 2014).

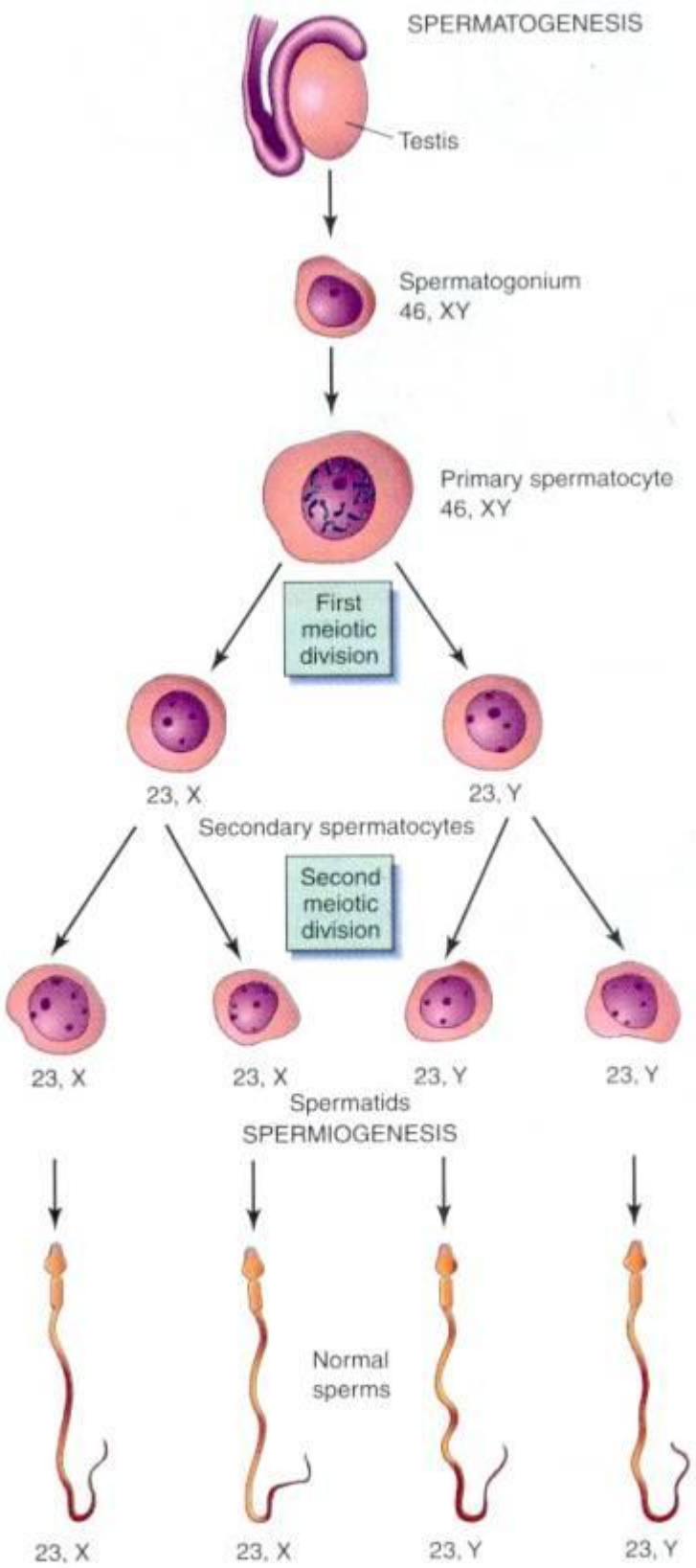


Figure 2.1: Spermatogenesis (Adapted from Clouthier *et al*., 1996)

2.2.2 The epididymis and its role

The anatomical structure of the epididymis includes the caput (head), corpus (body) and the cauda (tail). Each of the three different parts plays a unique role. Tubules from the testes enter the caput of the epididymis and collectively join to form the epididymal duct. The main function of the epididymal duct is to store sperm and to absorb testicular fluid (Tibary *et al*., 2014). Absorbed testicular fluid is replaced by rete epididymal epithelium secretions which help to activate the sperm motility. Sperm membrane alterations occur during the passage of sperm through the cauda of the epididymis which renders them fully mature to be stored until ejaculation (Gadea *et al*., 2013). During epididymis transit, the sperm motility, metabolism and the ability to gain capacitation is aquired (Sullivan *et al.,* 2007). The sperm experiences oxidative stress during transit in the different parts of the epididymis. However, the epididymis has been enriched with an antioxidant defence system that protects the sperm during their passage through the caput to the caudal region of epididymis and thereby facilitating their maturation process (Vernet *et al*., 2001; Gadea *et al*., 2013).

2.2.3 Hormonal control of spermatogenesis

Spermatogenesis is controlled by testosterone and the follicle stimulating hormone (FSH) (Hess and França, 2005; Grigorova *et al*., 2011; Alves *et al.,* 2013). The hypothalamus is responsible for the control of fertility through the release of gonadotropin-releasing hormone (GnRH). This hormone then triggers the anterior pituitary gland to stimulate the secretion of FSH. The normal progression of spermatogenesis is regulated by testosterone which acts on Sertoli cells via signalling through the peritubular myoid cells (Walker, 2011; Smith and Walker, 2014). Any forms of mutations in the genes that encode for FSH and testosterone or their receptors therefore can cause errors in sperm production (Garcia *et al.,* 2014). Estradiol is produced in small amounts in the testes and is vital for bone density and maintance of the epithelium. Thyroid hormone has also been linked to the process of spermatogenesis (Lisboa *et al*., 2015).

2.2.3.1 Testosterone

Testosterone is a steroid hormone from the androgen group synthesized by the Leydig cells in the testes (in males), the ovaries (in females) and adrenal glands in both sexes (Marshall, 2011). Testosterone is required for prostate, penis and scrotum development. It controls the development of Wolffian ducts in the fetus leading to the formation of the epididymis, vas deferens and seminal vesicles. Testosterone is considered to be the key androgen responsible for spermatogenesis regulation (Smith and Walker, 2014). It also exerts a wide range of influences over sexual behaviour, muscle mass and strength, energy production, cardiovascular health and bone integrity (Menke *et al.,* 2010). The biosynthesis of testosterone coincides with spermatogenesis, and foetal Leydig cell differentiation in the

male rat. Several *in vivo* models including hormone suppression, hormone restoration and hypophysectomy were established for the study of the hormonal regulation of spermatogenesis by testosterone (Parent *et al*., 2015). In the Brown Norway rat, serum testosterone levels decreased with aging, accompanied by increases in serum FSH (Bouvattier *et al*., 2012; de Vries *et al*., 2014). The capacity of Leydig cells to produce testosterone is higher in young rats as compared to old rats (Zambrano *et al.*, 2014).

The absence of testosterone signalling can cause a hindrance in spermatogenesis during the process of meiosis to an extent that elongated spermatids will not be formed (Walker *et al*., 2011. Protein analysis indicated that meiotic cells experience oxidative damage and DNA damage due to the loss of testosterone signalling from somatic cells (Stanton *et al*., 2012; Smith and Walker, 2014). Round spermatids are separated from Sertoli cells at an early stage in transgenic mice with decreased testosterone, therefore resulting in the non production of elongated spermatids (Smith and Walker, 2014). Furthermore, studies of mice with reduced androgen receptor activity have shown that germ cells were prematurely released due to the fact that the attachment of Sertoli cells to elongated spermatids could not be maintained. The absence of testosterone signalling is also detrimental to spermatogenesis because human mature spermatozoa that are usually released during the later stages are retained instead of being released and are eventually phagocytised by Sertoli cells (Smith and Walker, 2014).

2.2.3.2 Estradiol

Estradiol is the most natural occurring estrogen produced mainly by the Graffian follicle of the female ovary, the placenta and in smaller amounts by the adrenals and testes. The estradiol hormone is secreted and circulated bound to the sex hormone binding globulin (Kahn *et al.,* 2002; Wildman *et al.,* 2013). Estrogens are necessary in moderate amounts for bone density. In males, estrogens regulate the release of pituitary gonadotropins, sexual behavior and reabsorption of fluid in the efferent ducts of the testes (Mariotti and Mawhinney, 2013). van den Beld *et al*. (2000) conducted a study which proved that decreases in estradiol affects bone density not muscle strength or body composition, while decreases in testosterone affect bone density, muscle strength and body composition (Shaha *et al.,* 2010). Estradiol levels have been reported to be increased in patients with testicular tumours (Esposito *et al*., 2012).

2.2.3.3 Thyroid hormone

Thyroid hormones are produced by the thyroid gland and are responsible for the maintenance of a well functioning metabolism and development of testes (Mullur *et al*., 2014; Lisboa *et al*., 2015). It has two forms namely; thyroxine (3,5,3', 5-tetraiodothyronine, T4)

which is known as a pro-hormone (inactive form), and (3, 5, 3'-tri-iodothyronine, T3) which is the active form. T4 is converted into T3 in the kidney and liver. Thyroid hormone plays an important role in the maturation of testes (Alves *et al.,* 2013). T3 regulates the proliferation of Sertoli cells by preventing the release of immature Sertoli cells therefore regulating the functional growth of the testes (Cooke, 2004). The effect of T4 on the reproductive function differs from species to species and is dependent on the stage of development of the testis. The administration of T4 in mature rats and rams did not have any effect on reproductive function. However, the administration of T4 to immature male mice aged less than 4 weeks caused early maturation and shortened the development period of the sperm (Gao et al., 2014).

Hypothyroidism is a condition in which thyroid gland does not release enough thyroid hormone (Maran and Aruldhas*,* 2002; Almandoz and Gharib, 2012). Sridharan *et al.* (2007) concluded in a study that the altered thyroid function in adult males resulted in poor spermatogenesis that could later lead to infertility. In addition, Wagner *et al.* (2008) concluded that decreased levels of thyroid hormone can cause a decline in the proliferative capacity of Sertoli cells leading to a decreased testicular size. Furthermore, studies conducted on testicular development in foetal rats, neonatal rats and pubertal rats concluded that hypothyroidism does not affect foetal testicular development, but it does decrease neonatal and pubertal testicular development (van Haaster *et al*., 1993; Wagner *et al*., 2008; Fortes *et al*., 2012). Sakai *et al*. (2004) investigated the effect of hypothyroism on adult rats and found that the normal testicular development on adult rats was not affected; however spermatogenesis became abnormal due to the decreased level of thyroid hormone.

Hyperthyroidism is a condition in which abnormally high levels of thyroid hormone are released in the blood stream (Klein and Ojama, 2001; Danzi and Klein, 2012; Richards, 2014). Zamoner *et al.* (2007) induced hyperthyroidism in rats by injecting them with T3 for seven days until thyroid stimulating hormone (TSH) levels were beneath detectable levels. These hyperthyroid rats presented with increased testicular weights and decreased body weights. These hyperthyroid rats also had elevated testicular GSH levels, which was released in order to balance the oxidative stress caused by hyperthyroidism (Sahoo*,* 2013).

2.2.3.4 Follicle stimulating hormone

FSH is one of the main hormones produced by the anterior pituitary gland (Sharma *et al.,* 2012; Gulyani *et al.,* 2014). For many years, researchers thought it was only important in females because its role in male sexual development had not yet been established. Several studies have been conducted in order to investigate the role of FSH in spermatogenesis and

they all concluded that FSH is responsible for the activation of Sertoli cell proliferation (O'shaughnessy *et al.*, 2010; Wells *et al.,* 2013). FSH must first attach to receptors found on the surface of Sertoli cells in order to be active hence, this means the rate of Sertoli cell proliferation depends on the number of FSH receptors present on the cell (Pitetti *et al.,* 2013). It has been shown that there was a direct correlation between the total number of adult Sertoli cells and the testis size (Orth *et al.,* 1988; Oatley *et al*., 2011).

Figure 2.2 below illustrates the relationship between FSH and Sertoli cell number at different stages starting from gestation to adulthood. It indicates that the amount of FSH released is directly proportional to the Sertoli cell number. During the gestation period; FSH is responsible for the proliferation of Sertoli cells hence the cells are at a minimal amount. During the neonatal stage, FSH is responsible for the differentiation of Sertoli cells and the number of these cells begins to increase exponentially. During puberty FSH is responsible for the functional maturation of Sertoli cells and during adulthood, FSH is responsible for the support of spermatogenesis. The image on the right in Figure 2.2 also indicates that during adulthood; daily sperm production increases as Sertoli cell numbers increase (Sharpe, 2001).

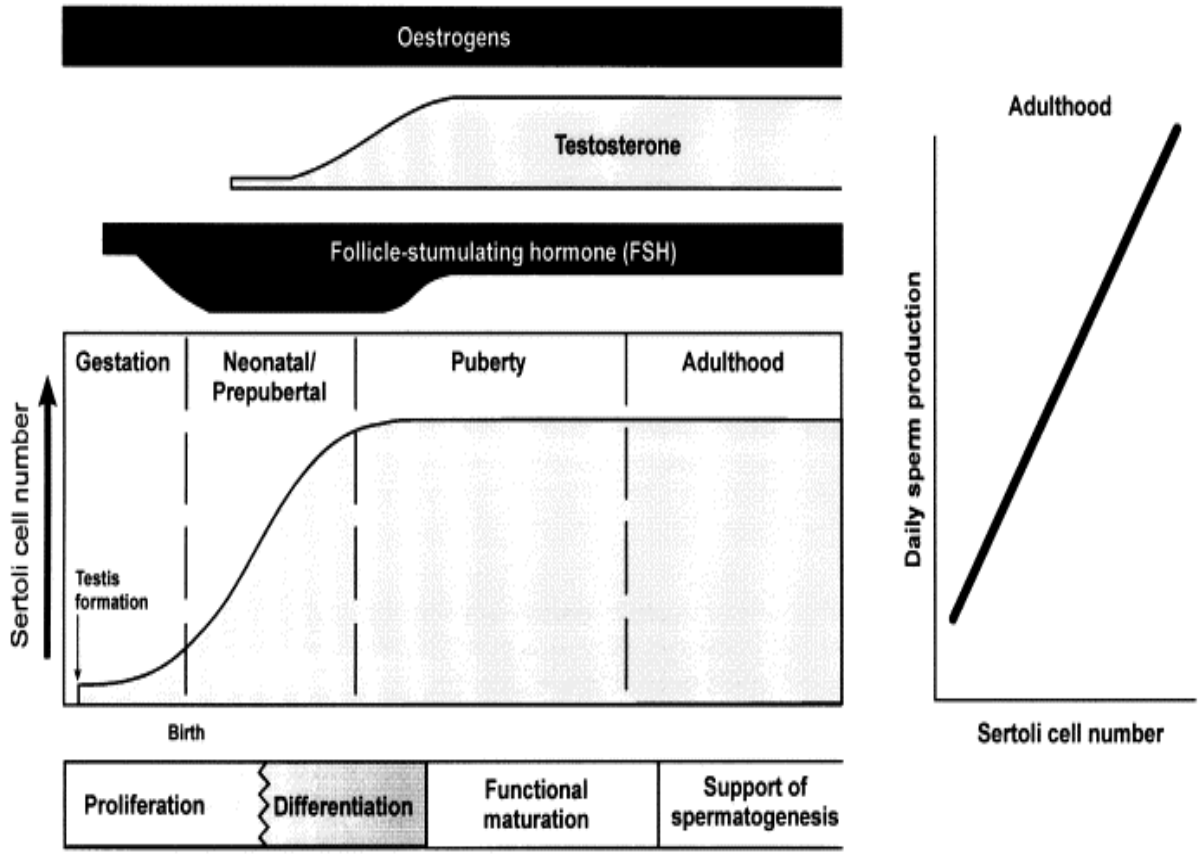


Figure 2.2: Effect of FSH on Sertoli cell proliferation (Adapted from Sharpe 2001)

2.3 Causes of the decline in male fertility

Male fertility can decline in a period of time in an individual due to several factors such as environmental pollution, advanced aging, chronic diseases and prolonged use of certain medication which cause excessive production of ROS (Agarwal and Prabakaran, 2005; Covarrubias *et al*., 2008; Nazıroğlu and Yurekli, 2013). The excessive production causes oxidative stress which can contribute to male infertility.

Figure 2.3 illustrates the different states of balance between ROS and antioxidants defence systems. The first model shows a well balanced ideal model of how ROS and antioxidants (AOX) should be in equilibrium, this is the normal state. The second model shows an inclined imbalanced model whereby there is an excessive production of ROS and less antioxidant defence systems which results in oxidative stress.

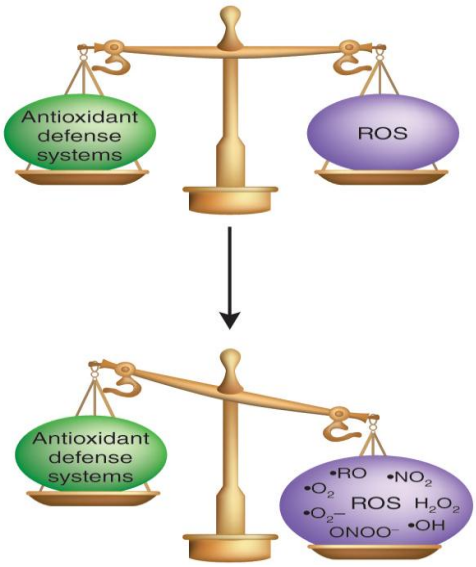


Figure 2.3: Oxidative stress caused by an imbalance of ROS and antioxidants. (Adapted from Tomaselli and Barth, 2010)

2.3.1 Environmental pollution

Environmental pollution has been identified as one of the most important factors causing male infertility. Both industrialised and rural areas are at high risk of pollution, but rural areas are relatively at a lower risk (Civan *et al*., 2015; Yang *et al.,* 2015). Rural area populations are likely to develop diseases due to indoor pollution that results from the use of biomass fuels. These biomass fuels emit air pollutants such as sulphur dioxide, and prolonged exposure can affect sperm quality (Qasim *et al*., 2014).

Populations of industrialised countries are prone to diseases caused by exposure to environmental pollutants such as cadmium, mercury, bisphenol A (BPA) and dioxin (Weck *et al*., 2008). Environmental pollutants carry trace elements and polycyclic aromatic hydrocarbons (PAHs) that can produce harmful effects on reproductive health. Studies show that PAHs have the ability to interact with the estrogen receptor and initiate signalling pathways *in vitro* and *in vivo* and cause endocrine and developmental toxicity in males (Rubes *et al*., 2005; Devine *et al.,* 2012; Jeng *et al*., 2013). A toxicological study was done on mice and it was shown that sulphur dioxide is toxic to the reproductive system of mammals which causes oxidative stress in the testes (Hsu *et al*., 2014; Zhou *et al*., 2014). Men in areas with high concentrations of metal pollution such as lead were shown to have reduced semen quality (Zafar *et al.,* 2014).

In both rural and industrialised areas; the overexposure to environmental factors such as heat and toxins have been shown to impair sperm function, lower sperm count and has been identified as a cause of male infertility (Aleksander and Yvonne, 2011). Environmental toxicant-induced oxidative stress role in mediating disruption to cell junctions was illustrated in the testes and other organs (Wong and Cheng, 2011; Castellanos *et al*., 2015).

2.3.2 Aging

Aging is one of the major causes of decreased sexual functions. Several authors demonstrate that semen volume, sperm motility, normal morphology and sperm viability all decline as a man's age increases (Kidd *et al*., 2001; Hassan and Killick, 2003; Dain *et al*., 2011). The largest effect of male aging is on sperm motility. A quantitative analysis of sperm motion suggests that as men age, they produce fewer motile sperm which cover less forward distance per unit time and this may decrease chances of fertilization for men who delay fatherhood (Sloter *et al*., 2006; Aitken *et al*., 2014; Johnson *et al*., 2015).

Numerous studies show that sperm DNA fragmentation increases as men increase in age (Wyrobek *et al*., 2006; Rybar *et al*., 2011; Humm and Sakas, 2013). Bellver *et al.* (2008) conducted a retrospective study on assisted reproduction whereby they concluded that there is a significant relationship between male age and the rate of implantation. Moreover, age also has a negative impact on the genetic integrity of the sperm. Increased time to pregnancy is associated with advanced male age. Older males have a decrease in serum testosterone levels, therefore this results in a decline in the number of Sertoli and Leydig cells produced (Auger and Jouannet, 2005; Borst *et al*., 2014).

2.3.3 Chronic diseases

Male infertility has also been linked to chronic diseases. Chronic diseases such as prostate cancer cause an over production of leukocytes (Kumar *et al*., 2008). High concentrations of leukocytes in semen induce ROS production and decreases activity of SOD therefore leading to oxidative stress (Hofmann *et al*., 2014). Cardiovascular disease, renal disease and diabetes are also examples of diseases that are associated with male infertility. There is a high production of ROS in patients with such diseases causing oxidative stress which can lead to male infertility (Aitken *et al*., 2014).

The most extensively studied chronic disease that has been associated with male infertility is diabetes. For many years diabetes was proved to be a cause of erectile dysfunction, but not as a cause for male infertility (Sexton and Jarow, 1997; Agbaje *et al*., 2007). Currently more researchers are exploring the link between diabetes and male infertility (Tobias *et al*., 2015). Alves *et al*. (2013) conducted molecular studies which show molecular mechanisms of glucose transport in testicular cells. Testicular cells detect glucose levels and respond to fluctuations in hormones; hence are able to counteract hyperglycemic and hypoglycemic conditions. Sertoli cells are responsible for producing lactate which is metabolized by developing germ cells (Thompson and Tassone, 2014).

The metabolic function of many tissues and organs depends on blood glucose levels, and the metabolism of glucose is also very important in the testes (Sherwood, 2015). *In vivo* maintenance of spermatogenesis depends on glucose metabolism (Alves *et al*., 2013). Glucose levels are found in the tubular fluid and the blood-testes barrier controls blood-germ cells' transportation of glucose. Sertoli cells are the most important cells for the functions of the blood testes barrier (Mital *et al*., 2011). When glucose levels drop; Sertoli cells adapt their glucose transport systems to ensure appropriate lactate production. This lactate is converted to energy in the form of ATP by lactate dehydrogenase (Rato *et al*., 2012; Dupont *et al*., 2014). Low levels of ATP due to glucose deficiency can affect capacitation, sperm motility and concentration. Diabetic men are therefore prone to subfertility and infertility (Alves *et al.*, 2013). A study conducted by La Vignera *et al*. (2009) concluded that a high prevalence of 51% of diabetic patients were either subfertile or infertile. Kim and Moley (2008) concluded from their study that diabetes mellitus causes subfertility by altering steroidogenesis.

2.3.4 Medication

The prolonged usage of medications that control chronic illnesses such as depression, diabetes and/or other metabolic and endocrine disorders may lead to erectile dysfunction, decreased sperm motility and concentration (Gitlin, 2004; Alves *et al*., 2013). Several studies

have been conducted to demonstrate the toxicity of chronic disease medications such as aspirin, paracetamol, and cyclophosphamide on testicular function (Kristensen *et al*., 2012; Mazaud-Guittot *et al*., 2013; Comish *et al*., 2014). All these studies concluded that the use of medication can increase ROS production and eventually cause oxidative stress (Agarwal and Prabakaran, 2005; Nazıroğlu and Yurekli, 2013).

2.4 Male infertility

When a couple cannot fall pregnant after 12 months of unprotected sexual intercourse and the female is less than 36 years old; they are said to be infertile and in most cases it has been shown that the reason why such couples cannot conceive is because in 15% of the cases the male is infertile (Menning, 1980; Poongothai *et al*., 2009; Bang *et al*., 2013). Jorgensen *et al.* (2012) conducted an epidemiology study which concluded that one in twenty men of the general population was affected by male infertility. In order to diagnose a patient as infertile, several steps have to be taken, including careful assessment of reproductive history, a physical exam and semen analysis (Cakiroglu *et al*., 2013). The assessment of a couple's reproductive history consists of asking questions about duration of infertility, impaired libido, the decrease of ejaculate volume, frequency of coitus, testicular trauma that might have happened in the past, any previous or current sexually transmitted diseases and repeated urogenital infections (Krausz, 2011). A physical examination also has to be done in order to rule out all other conditions and correctly diagnose male infertility. This particular examination consists of the assessment of secondary sex characteristics such as body proportions, voice and hair distribution and a rectal exam (Lotti *et al*., 2012). When body hair is nearly absent, it could indicate congenital deficiency of gonadotropins. Organs are evaluated for size and any abnormalities that could possibly lead to infertility. The testes are palpated for size and testicular volume while the penis is examined for the location of the urethral meatus, small firm testes with eunocoid features may indicate Klinefelter syndrome (Pitteloud and Dwyer, 2014). In addition to that, the epididymis is palpated for cysts, vas deferens for segmental absence while the scrotum is palpated for varicocele (Liguori *et al*., 2012).

A physical examination and reproductive history are not enough to lead to the diagnosis of male infertility. Semen has to be analysed for sperm motility, morphology, density, and total sperm count (Franken and Oehninger, 2012). In addition to that, semen has to be analysed for its pH, viscosity and volume and this analysis is done based on a consistent guide that has been set out by the World Health Organisation (Murray *et al*., 2012). Moreover, sperm is analysed for ROS to determine oxidative stress damage, this is done because ROS are able to attack PUFA in the sperm cell membrane and this leads to lipid peroxidation which could

result in sperm damage. Research has shown that a highly fertile female can compensate for abnormal semen parameters of her partner and this can cause a successful pregnancy (Krausz, 2011). The sperm parameters' reference ranges have last been updated by the WHO manual in 2010 (Makker *et al*., 2009).

2.5 ROS and male reproductive function

ROS are highly reactive chemical molecules which contain O_2 (Migdal and Serres, 2011). They include the hydroxyl ion, superoxide ion, nitric oxide, lipid peroxide and hydrogen peroxide. ROS are beneficial to cells in physiological amounts. However, in excess amounts; ROS can be very harmful to cells and lead to oxidative stress (Sena and Chandel, 2012; Holmstrom and Finkel*,* 2014).

2.5.1 Advantages of ROS on male reproductive function

Studies have shown that effects of ROS are sometimes not detrimental to the cell as ROS is needed at very low concentrations in order to regulate male reproductive function (Tandon *et al.,* 2005; Paiva and Bozza, 2014; Schieber and Chandel, 2014). In normal circumstances, there is equilibrium between the generation of ROS and antioxidant strategies of the male reproductive tract. Only a critical amount of ROS is required for normal sperm functions such as capacitation, acrosome reaction and fusion with the oocyte membrane (Agarwal and Saleh, 2002; Aitken *et al*., 2012).

2.5.1.1 Capacitation

Capacitation is the change spermatozoa undergo in order to gain the ability to penetrate the zona pellucida of the ovum (Ickowicz *et al.,* 2012; Chiu *et al.,* 2014). Researchers have shown that O_2^- regulates the propelling movement of spermatozoa. During capacitation, sperm becomes hyperactive and this hyperactivation is represented by a change in the beating pattern of the sperm tail becoming more vigorous. During capacitation an increase in various intracellular conditions such as pH levels, intracellular cAMP, calcium influx and tyrosine phosphorylation are responsible for the initiation of hyperactivation (Chung *et al.,* 2014). Mammalian spermatozoa generate physiological amounts of ROS as by-products of reactions; this ROS is useful as it initiates capacitation (Aitken and Curry, 2011). The induction of moderate amounts of LPO by ferrous ions and ascorbic acids has been shown to increase fertilization in mice. In this study, ROS stimulated sperm activity hence resulting in an increase in fertilization (De Lamirande *et al*., 1997).

2.5.1.2 Acrosome reaction

The acrosome reaction involves the release of enzymes such as acrosine by acrosomal cap on top of the head of mature sperm to digest the cumulus cells and break through the zona

pellucida (Fouriki, 2013). Often this results in an excessive production of ROS which then has to be removed by the action of seminal plasma antioxidants in order to create and maintain normal cell conditions (Valko *et al*., 2006; Aitken *et al*., 2012).

2.5.2 Disadvantages of ROS on male reproductive function

In very small amounts ROS are required for certain reactions and processes to take place in the sperm cells. However, when in excess these ROS can overpower antioxidants present and cause oxidative stress damage to the cells (De Lamirande and Gagnon, 1995; Schieber and Chandel, 2014).

2.5.2.1 Testicular tissue

The rate of ROS generation in the testes is dependent on temperature and heat stress has been proven to compromise sperm quality and increase the risk of infertility (Luo *et al*., 2006; Kheradmand *et al.,* 2011; Durairajanayagam *et al*., 2015.). A study by Koksal *et al.* (2003) showed that the testes were more susceptible to damage by ROS than the liver and they found that testicular antioxidant enzyme activity was significantly decreased compared to liver antioxidant enzymes. The histological findings of Koksal *et al.* (2003) compared somatic cells to testicular germ cells and concluded that the testicular germ cells showed a composition characterized by low glutathione-dependent enzyme activity. Testicular tissue is rich in poly unsaturated fatty acids and exposure to ROS can cause lipid peroxidation and lead to extensive tissue damage (Chandra *et al.,* 2013). When testicular tissue had high concentrations of ROS; the rate of spermatogenesis decreased due to damage to the Sertoli cells (Mathur and D'Cruz, 2011). Sertoli cells also determine the testicular size and if damaged by ROS this could lead to the testes having hindered development and appearing smaller (Sharpe *et al*., 2003; Rebourcet *et al*., 2014). The testes are responsible for the production of hormones such as testosterone therefore any damage by ROS can lead to poor hormone regulation and function (Scott *et al*., 2008).

2.5.2.2 Epididymal tissue and epididymal sperm

A rat model study conducted by Aboua *et al.* (2009) concluded that ROS impaired activity of antioxidant enzymes in epididymal tissue. ROS had been proven to cause damage to polyunsaturated fatty acids of cells therefore leading to cell injury. The epididymis has a high content of polyunsaturated fatty acids and this makes it prone to oxidative stress. In mammals the epididymis is known to play an important role in the maturation and storage of sperm (Pasqualotto *et al*., 2000; Rozman and Horvat, 2013). During the epididymal transit, the sperm concentration reaches 10^{10} cells/ml at the same time as the sperm motility and metabolism increases, with the threat of oxidative stress for gametes (Dacheux *et al*., 2003).

ROS impairs sperm function by causing lipid peroxidation of the polyunsaturated fatty acids in the head and mid-piece (Henkel, 2011). Lipid peroxidation impairs the integrity of the membrane and causes alterations in sperm morphology which may lead to poor motility and spermatozoon-oocyte fusion (Roos and Kaina, 2013; Berghe *et al*., 2014). The epididymis has an advantage of having high concentrations of endogenic antioxidants that naturally function to counteract ROS and protect sperm as they travel through the caput to the caudal region of the epididymis for maturation (Vernet *et al*., 2004; Aboua *et al*., 2009).

2.5.2.3 Hormonal function

Testosterone is produced in the testis and high levels of ROS in the testes can affect its production. Exposure to pollutants such as lead can lead to increased levels of ROS in the testes; hence disrupting spermatogenesis and hormone regulation (Zafar *et al*., 2014). FSH regulates the rate of germ cell apoptosis and can increase spermatogonial differentiation, hence when ROS impairs FSH production; the rate of germ cell apoptosis accelerates (Pitteloud and Dwyer, 2014).

2.5.3 Endogenous and exogenous sources of ROS

Reactive oxygen species (ROS) are endogenously or exogenously produced from either enzymatic or non enzymatic reactions (Wiseman and Halliwell, 1996; Franco and Panayiotidis, 2009). Exogenous ROS are produced from radiation, cigarette smoke, ozone and redox cycling agents while endogenous ROS are derived from the process of mitochondrial oxidative phosphorylation reactions that occur *in vivo* during aerobic reactions (Sena and Chandel, 2012). ROS produced endogenously is from several sources such as the mitochondrial transport chain, NADPH, NO synthase, peroxidases, xanthine oxidases, phagocyte derived myeloperoxidase and arachidonic acid pathway enzymes known as lipoxygenase and cyclooxygenase (Cai and Harrison, 2000; Szeto, 2006; Schulz *et al.,* 2014). Excess free radicals cause errors in spermatogenesis that can result in the release of spermatozoa that has abnormally high levels of cytoplasmic retention (Sakkas *et al*., 2004; Bansal and Bilaspuri, 2010). These excess ROS have an ability to cause further cellular damage such as lipid peroxidation by readily combining with other molecules due to their unpaired electrons that are donated to neighboring cellular structures (Sikka, 2001; Valko *et al*., 2006; El-Beltagi and Mohamed, 2013).

Infections in the male reproductive tract induce inflammation in the seminal tract and activate leukocytes. Activated leukocytes release large amounts of superoxide radicals into phagocytic vesicles in order to kill pathogens. Leukocytes physiologically produce more ROS than spermatozoa and may overwhelm the system’s antioxidant mechanisms (Henkel, 2011).

Figure 2.4 illustrates how exogenous and endogenous sources of ROS affect cells. Exogenous sources of ROS can lead to accumulation of molecular injury therefore causing DNA, lipid and protein damage. This molecular injury leads to damage to organelles such as the mitochondria. Organelle damage can create cellular responses such as inflammation, survival, proliferation and eventually cell death. All these cellular response mechanisms can contribute to endogenous sources of ROS. Systematic responses to cellular responses are characterised by aging. Exogenous ROS also activate the modulation of signal transduction pathways. These pathways can either initiate changes in gene expression and cause cellular responses or contribute to endogenous sources. Endogenous sources of ROS can be from changes in gene expression, organelle damage and cellular responses (Pandey and Rozvi, 2011).

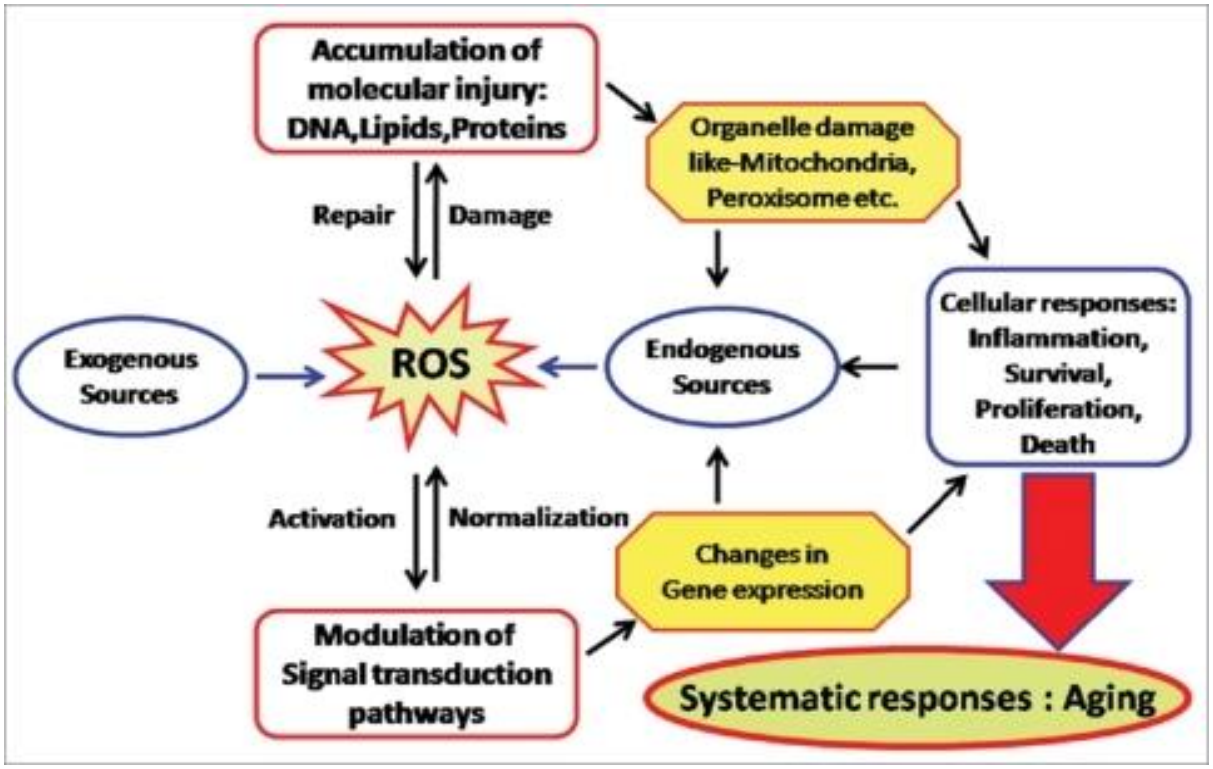


Figure 2.4: Endogenous and exogenous sources of ROS (Adapted from Pandey and Rizvi, 2011)

2.6 Oxidative stress and the male reproductive function

Oxidative stress is a cellular condition caused by an imbalance between the production of free radicals and the ability of antioxidants to scavenge them (Lee 1996; Garg and Bansal, 2000; Bansal and Bilaspuri, 2010; Onaran *et al*., 2013). Sperm is particularly susceptible to oxidative damage due to its unique structural composition (Lee, 1996; Garg and Bansal, 2000; del Barco and Roldan, 2014). Oxidative stress has been implicated in the pathogenesis of all major diseases (Valavanidis *et al*., 2013; Bhattacharyya *et al*., 2014).

Figure 2.5 below shows different diseases and pathologies that may cause an increase in ROS and put sperm under oxidative stress. ROS can result from systemic pathologies or environmental lifestyle factors. Drugs, smoking and pollution are environmental lifestyle factors while the systemic pathologies consist mainly of diabetes, cancer, systemic infections, and male accessory gland infections. All these factors create free radicals which may overpower antioxidants and cause oxidative stress. Oxidative stress eventually causes spermatozoa dysfunction followed by infertility in most cases. However, this type of infertility can be reversed by treating the underlying pathology, supplementing with antioxidants or avoiding factors that promote ROS (Agarwal and Said, 2004).

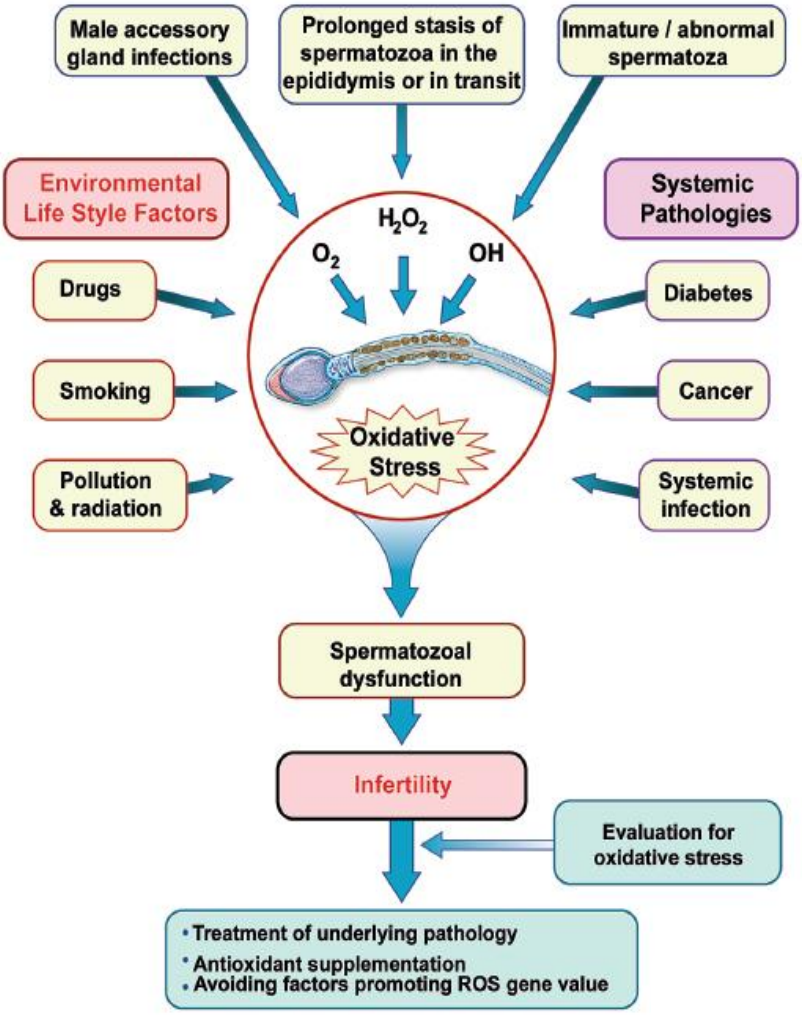


Figure 2.5: Pathological and environmental sources of ROS on sperm (Adapted from Agarwal and Said, 2004)

2.6.1 Causes of oxidative stress in the male reproductive system

The male reproductive function is affected by oxidative stress caused by different factors such as chronic diseases, smoking and seminal infections. Researchers have proved the relationship between these factors and their role in increasing oxidative stress on the male reproductive function (Alves *et al*., 2013; Zhang *et al*., 2013)

2.6.1.1 Chronic disease

Over the years, male infertility has been associated with chronic diseases such as kidney disease, cancer, diabetes and prostatitis (Alshahrani *et al*., 2013; Alves *et al*., 2013; Wasilewski-Masker *et al*., 2014). Researchers have proved that the link between chronic diseases and male infertility is their ability to increase oxidative stress. In 2010, Du Plessis and co-workers conducted a study in which they showed that diabetes impaired male fertility by its interference with spermatogenesis and erectile dysfunction. Shrilatha and Muralidhara (2007) proved that streptozotocin which induced diabetes can increase oxidative stress in male rats after six weeks. Sperm from diabetic men were shown to possess relatively high levels of sperm DNA fragmentation when compared to the control (Agbaje *et al*., 2007). Poldasek *et al*. (2001) investigated the effects of diabetes on sperm DNA damage. Although their study did not particularly measure oxidative stress as a cause of the elevated sperm DNA damage, the authors concluded that the most likely cause was oxidative stress because it had been proven to be the leading underlying cause of several chronic complications of diabetes.

Chronic kidney disease and renal disease patients have been proven to have high levels of oxidative stress (Oberg *et al*., 2004). Pupim *et al*. (2004) concluded that the use of haemodialysis to treat uraemia causes an increase of oxidative stress and chronic inflammation. Celis (1999) reported that kidney transplant patients showed a decrease in sperm motility and concluded that this could be due to oxidative stress. Moreover, there were also elevated levels of oxidative stress in renal transplant patients even though they had stable renal function and no obvious signs of immune rejection of their graft (Moreno *et al*., 2005). End stage renal disease male patients can eventually be infertile due to alterations in the sex hormones and oxidative stress (Eckersten *et al*., 2015).

2.6.1.2 Smoking

Free radicals from cigarette smoking arise from uncoupled endothelial reactions of NO synthase, xanthone oxidase and reactions involved in energy synthesis and transportation of electrons (Ambrose and Barua*,* 2004). When substances are either inhaled or ingested, the first body fluid they are exposed to is blood, therefore reactive oxidants from cigarette smoke enter the blood stream and change blood function (Csiszar *et al*., 2009; Charlet *et al*., 2012).

Künzle (2003) concluded that toxins from cigarette smoke reach the male reproductive system and their effects are mainly by direct interaction with seminal fluid components and the accessory glands leading to increased viscosity, reduced seminal volume and delayed liquefaction time. The increased viscosity of seminal fluid causes a reduction in the motility of spermatozoa; this decreases the chances of sperm fertilizing the egg (Kunzle, 2003). A number of studies reported a reduction in semen volume in smokers in comparison to non-smokers (Zhang *et al*., 2000; Mostafa, 2010; Sankako *et al*., 2013). Zhang *et al*. (2013) also showed that smokers had a significant decrease in semen volume, motility and sperm viability. Their study also proved that sperm morphology worsens with increasing degree of smoking. Direct exposure of spermatozoa to the toxins in cigarette smoke increases the amount of ROS produced by spermatozoa and decreases special functions such as capacitation (Pasqualotto *et al*., 2006; Taha *et al*., 2012; La Maestra *et al*., 2015). Excessive amounts of ROS have been shown to damage DNA of spermatozoa, therefore distorting morphology of spermatozoa (Koksal *et al*., 2003; La Maestra *et al.,* 2015).

Zhu *et al*. (2013) proved that cigarette smoking caused infertility in male mice due to the increase in oxidative stress. They found that the protein profile of the mouse epididymis was altered by cigarette smoking and identified 27 proteins from the most abundant and differentially expressed spots in the two-dimensional electrophoresis (2-DE) gels of epididymal samples. These proteins were classified into groups according to their functions such as energy metabolism, reproduction and structural molecule activity. Through pathway analysis, the proteins were associated with the glutathione metabolism and protein processing in the endoplasmic reticulum (Zhu *et al*., 2013).

2.6.1.3 Seminal infection

The male genito-urinary system can be infected by micro organisms such as viruses and bacteria (Olayemi, 2010). Most male genital tract infections may lead to infertility if left untreated (Rusz *et al*., 2012). It was reported that 15-20% of infertile subjects were affected by semen infection (Weidner *et al*., 2013). Sperm abnormality and DNA fragmentation has been associated with virus infected semen (Moretti *et al*., 2008). Lorusso *et al*. (2010) found that sperm concentration, motility, morphology, and viability were significantly impaired in hepatitis B virus (HBV) seropositive patients. Levy *et al*. (2012) demonstrated semen alterations in 30% of Hepatitis C virus (HCV) infected males before antiviral treatment for high serum viral load. Moreover, these studies showed that when HCV RNA was present in the seminal fluid, concurrently with sperm alterations, fertility was also very poor (Foresta *et al*., 2010). A study carried out by Pal *et al*. (2010), showed that Hepatitis virus (HPV) infection caused an increase in ROS therefore increasing oxidative stress which could possibly lead to male infertility. *Chlamydia trachomatis* is the most prevalent sexually

transmitted bacterial infection in men which can lead to serious problems such as infertility if left untreated (Hosseinzadeh *et al.,* 2004; Satterwhite *et al.,* 2013).

2.7 Antioxidants

Antioxidants are compounds that protect biological systems against the potentially harmful effects of processes or reactions that can cause excessive oxidation (Krinsky, 2002; Lobo *et al*., 2010). The reproductive tract contains a highly effective variety of naturally occurring antioxidants which are either enzymatic or non enzymatic and work together to make sure sperm cells are well protected against high concentrations of ROS (Gharagozloo and Aitken, 2011).

Antioxidants can act against free radical induced oxidative stress by catalyzing reactions between reactive species in order to remove free radicals (Halliwell, 2012; Nemzer *et al*., 2014). Thus, as suggested by Frankel and Meyer (2000), the targeting of antioxidants to prevent particular free radical formation steps and oxidative deterioration processes requires a detailed understanding of the mechanism of oxidation.

Antioxidants have a huge effect on sperm as they carry out different duties that involve the protection of spermatozoa from ROS that produce abnormal spermatozoa and scavenge ROS produced by leukocytes (Valente *et al*., 2014; Sonmez and Tascioglu, 2015). They also prevent DNA fragmentation, improve semen quality in smokers, block premature sperm maturation and improve assisted reproductive techniques (ART) outcome (Sikka, 2004; Hammani and El May, 2013; Aboua *et al*., 2014).

Antioxidants can be categorized as endogenous or exogenous depending on their source. Endogenous antioxidants are those that are generated by the body while exogenous antioxidants are those that are incorporated in the diet and taken in orally (Mancuso *et al*., 2012; Murphy, 2014). Endogenous antioxidants include catalase, superoxide dismutase, and glutathione peroxidise. Unlike other cells, sperm have a unique structure and function and are more susceptible to damage by ROS (Agarwal and Prabakaran, 2005; Murphy, 2014). Therefore there is a need for supplementation with exogenous antioxidants.

2.7.1 Endogenous antioxidants

CAT, SOD, GPx and thiol specific antioxidants use the catalytic removal of free radicals as a defence mechanism (Halliwell, 2012; Eguchi *et al*., 2014). Another mechanism of defense against free radicals is the binding of proteins such as transferrine, metallothionein and haptoglobins to pro-oxidant metal ions such as iron and copper. Moreover, antioxidants such

as GSH, tocopherols, and ascorbic acid reduce free radicals by their ability to donate electrons (Halliwel and Gutteridge, 1999; Eguchi *et al*., 2014).

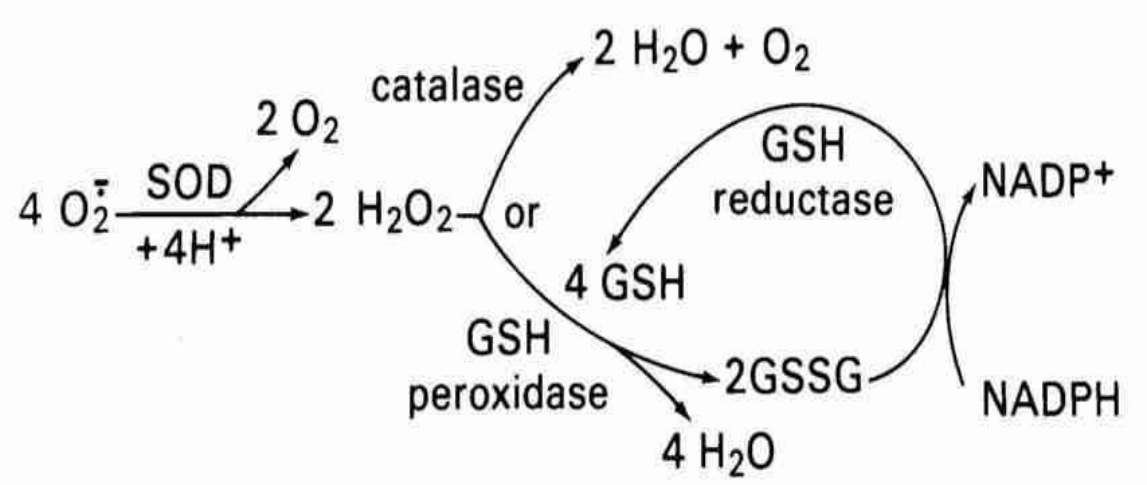


Figure 2.6: Antioxidant defence enzymes (Adapted from Proctor and Reynolds, 1983)

Figure 2.6 illustrates the manner in which antioxidant enzymes function together; SOD catalyzes the dismutation of superoxide, catalase catalyzes the conversion of hydrogen peroxide to H_2O and O_2, while GSH peroxidase transfers electrons from GSH to reduce peroxides to water. The oxidized glutathione produced (GSSG) is re-reduced back to GSH by glutathione reductase utilizing NADPH acting as an enzyme co-factor.

2.7.1.1 Superoxide dismutase

O_2^- is the main form of ROS and is formed by the addition of one electron to dioxygen (O_2). SOD is the main antioxidant enzyme that acts against toxicity that is produced from the superoxide radical by forming hydrogen peroxide as a final product of the dismutation (Johnson and Giulivi, 2005). The superoxide anion is produced *in vivo* as a result of aerobic mechanisms in cells (Zhang *et al*., 2013). SOD is divided into three forms according to the ion it is bound to and their origin (Miller, 2004). These are copper and zinc SOD (Cu/Zn-SOD) found in the cytoplasm, nucleus and plasma membrane; manganese or iron SOD (Mn-SOD or Fe-SOD) present in mitochondria and nickel SOD (Ni-SOD) restricted to the prokaryotic cells. Three types of SOD found in mammals are the dimeric cytosolic Cu/Zn SOD (SOD-1), tetrameric mitochondrial Mn SOD (SOD-2) and tetrameric extracellular SOD (SOD-3) (Brand, 2010). Each type of SOD has a unique way of binding single charged anions due to their differences in susceptibility. To protect against lipid peroxidation of the plasma membrane SOD must be conjugated to catalase or GPx (Su *et al*., 2010). In the absence or decreased production of SOD in the male reproductive system the testicular tissue and spermatozoa can be damaged by ROS (Aitken and Roman, 2008). SOD also

protects against premature sperm hyperactivation and capacitation that can be induced by superoxide radicals before ejaculation.

2.7.1.2 Catalase

Catalase is a heme-containing enzyme found in the brain, kidney and heart in animals, and its highest concentration is in the liver (Halliwel and Gutteridge*,* 1999). Catalase enzymes function in catalytic and peroxidic activity whereby they either decompose hydrogen peroxide into molecules of oxygen and water or oxidise hydrogen donors such as alcohols and phenol formic acids. Catalase enzymes are largely localized in subcellular organelles such as peroxisomes while mitochondria and the endoplasmic reticulum contain small amounts of catalase and this is why intracellular hydrogen peroxide cannot be eliminated unless it diffuses into the peroxisomes (Rodriguez *et al.,* 2000; Devasagayam *et al*., 2004).

2.7.1.3 Glutathione

Glutathione the most abundant non-protein cellular antioxidant and it is made up of a tripeptidic chain (L-γ-glutamyl-L-cysteinylglycine). Glutathione is involved in protein and DNA synthesis, amino acid transportation and most importantly protection of cells against oxidants, electrophiles and free radicals (Chaudhari *et al*., 2008). Glutathione can be represented in two ways namely GSH which represents reduced monomeric glutathione and GSSG which represents oxidised glutathione. Szasz *et al*. (2007) reported that oxidised glutathione disulphide gets reverted to reduced glutathione by glutathione reductase and the ratio between the oxidised and reduced glutathione is very important when determining toxicity in the cells. The enzyme glutathione reductase is therefore responsible for the recycling of GSH.

2.7.1.4 Glutathione peroxidase (GPx)

Glutathione peroxidise is a group of selenoproteins which require selenium as a cofactor. GPx consists of a tetramer of four identical subunits (monomer 22-23KDa) and contains selenocysteine amino acid residue in the active side of each monomer. The general function of GPx is to detoxify peroxides in the cell in order to prevent destruction of cell membranes by free radicals. This enzyme is also considered as the main scavenger of H_2O_2 therefore it is required to repair LPO initiated by superoxide in the membrane to maintain its integrity (Saraswathi and Devaraj, 2013). There are four different subgroups of GPx found in mammals; GPx1 is found predominantly in the kidneys, liver and erythrocytes. GPx2 is found predominantly in the gastrointestinal tract, GPx3 is found in plasma and the membrane associated GPx4 is called phospholipid GPx (PHGPx) (Margis *et al*., 2008). An example of the importance of such enzymes was shown by deletion of GPx4 gene in male mice, which was found to generate a state of oxidative stress that influenced the incidence of miscarriage

and birth defects in mated wild-type female mice, thus demonstrating the protection that this enzyme normally affords (Chabory *et al*., 2009). It can be concluded that exogenous antioxidants are beneficial, however if they are taken in very high concentrations they can be harmful.

2.7.2 Exogenous antioxidants

The first line of defence against ROS that spermatozoa have is in the form of endogenous antioxidants such as SOD, GPx and CAT. These are however not sufficient to totally eliminate free radicals due to the increased exposure to external sources of free radicals in today's world such as from environmental pollution, smoke, ultraviolet radiation and high-fat diet (Yang and Omaye, 2009; Hamid *et al*., 2010; Poljsak and Fink, 2014).

The supplementation with exogenous antioxidants found in food such as nuts, fruits, teas, oils, vegetables and legumes is very important in the protection against oxidative stress. Forman *et al*. (2014) proposed that antioxidants present in fruit and vegetables act together to produce an additive increase in electrophilic signaling that results in the induction of the protective phase II enzymes and increased nucleophilic substrates, such as glutathione, thioredoxin and NADPH.

Exogenous antioxidants can be classified as lipophilic, hydrophilic, synthetic compounds or as natural extracts (Christodouleas *et al*., 2014). Vitamin E and β-carotene are examples of lipophilic antioxidant components, vitamin C and polyphenols are examples of hydrophilic antioxidant components, natural extracts can be from plants such as *Ginko biloba* and *Mentha spicata* while synthetic compound examples are sodium selenite and zinc sulphate (Makker *et al.,* 2009; Agarwal *et al*., 2014). Exogenous antioxidants have both harmful and beneficial effects depending on their dose. Figure 2.7 indicates the advantages and disadvantages of exogenous antioxidants. The regular consumption of fruits and vegetables protect against oxidative stress. However, the consumption of synthetic antioxidants over a long period of time may cause cell toxicity. Synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are preservatives and are incorporated in many food products to prolong their shelf life. A study was done on rats and monkeys to demonstrate the negative effects of synthetic exogenous antioxidants on consumer health and it concluded that they may cause cancer if consumed in high doses (Branen, 1975; Ito *et al.,* 1983).

High doses of exogenous antioxidants such as quercetin can decrease cell viability, total antioxidant capacity and activity of SOD, CAT and glutathione (Robaskiwic *et al*., 2007).

Administration of high concentrations of flavonoids has been shown to generate ROS by autoxidation and redox-cycling (Watjen *et al.,* 2005; Prochazkova *et al*., 2011). β-carotene has been shown to cause prooxidant activity and pro-inflammatory effects when administered in high doses (Yeh *et al*., 2009; Kiokias and Varsakas*,* 2014). Figure 2.7 also shows that physiological doses of exogenous antioxidants provide beneficial effects while high doses provide harmful effects. Both physiological and high doses have an impact on cellular mechanisms such as oxidative metabolism, nitrosative metabolism, dicarbonyl metabolism, inflammatory response and estrogenic effects.

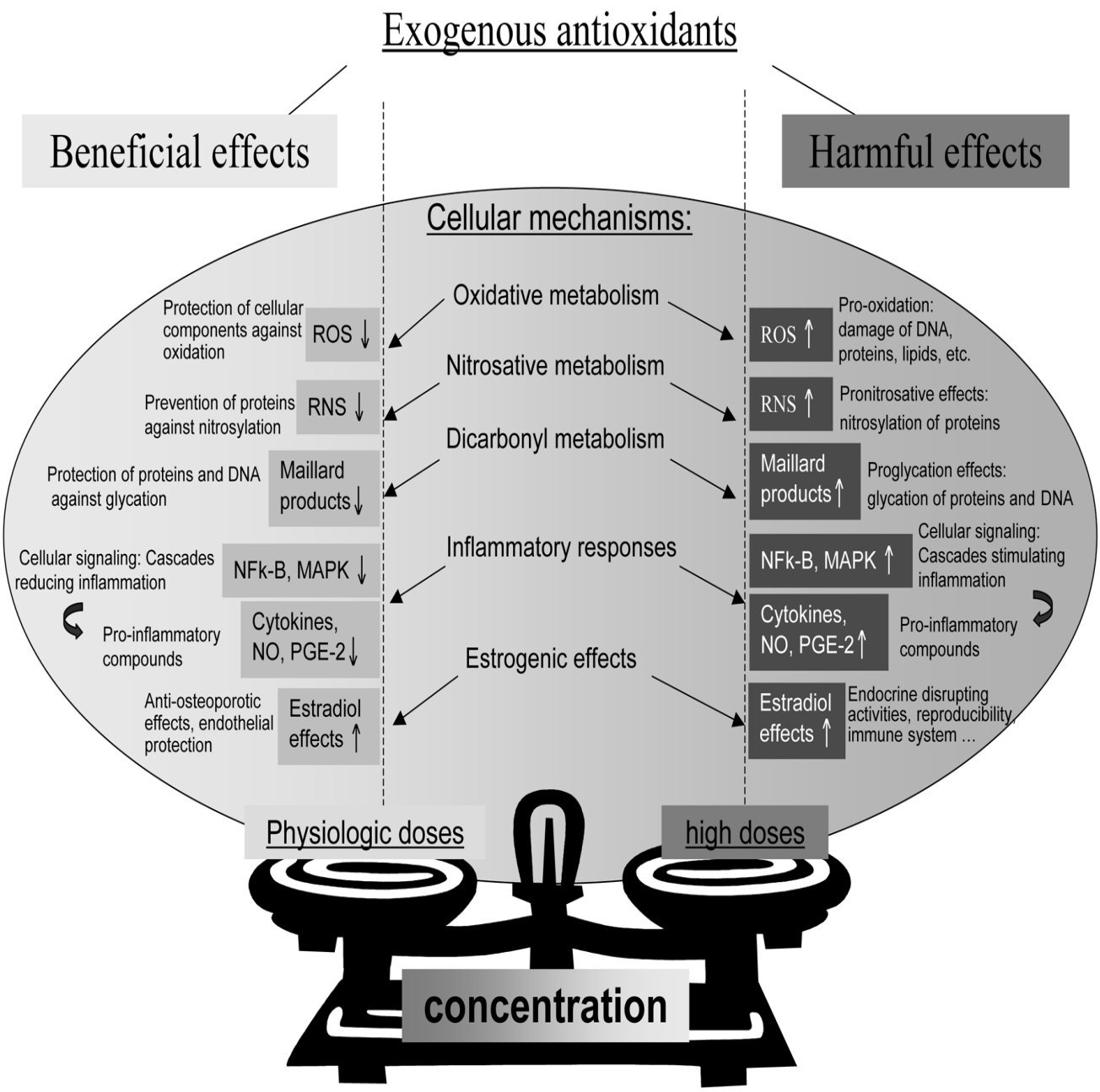


Figure 2.7: Beneficial and harmful effects of exogenous antioxidants (Adapted from Bouayed and Bohn, 2010)

2.8 Management of male reproductive function with exogenous antioxidants

Spermatozoa, due to the reduced size of cytoplasmic enzymes, are unable to repair oxidative damage, therefore antioxidants are required for prevention of oxidative stress (Agarwal and Said, 2004; Agarwal *et al*., 2014). Antioxidants reduce oxidative stress and catalyse redox reactions with reactive oxidants (Blomhoff, 2005; Sahiner and Cansin-Sackesen, 2012). Antioxidants inhibit the decomposition of hydroperoxides by acting as radical scavengers, metal chelators or reducers of hydroperoxides to more stable hydroxyl compounds (Halliwell *et al*., 1995; Valko *et al*., 2006; Waheed *et al.,* 2014).

However, these natural antioxidants are insufficient due to the high production of ROS resulting from factors such as pollution, therefore oral doses of antioxidants should be taken (Dawson *et al.,* 1993; Poljsak and Fink*,* 2014). Moreover, observational epidemiological studies generally support the hypothesis that the intake of foods rich in antioxidants is associated with reduced oxidative stress-related diseases (Stanner *et al*., 2004). Orally administered antioxidants can either be pharmacological or from antioxidant rich foods (Wang *et al*., 2011; Kaume *et al*., 2014).

2.8.1 Pharmacological antioxidants

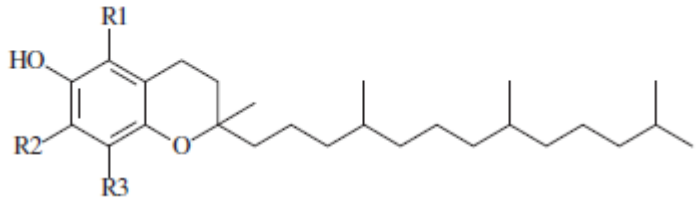
Pharmacological antioxidants can either be used individually or paired together to treat male infertility caused by oxidative stress. Different authors have proved that a balanced mixture of antioxidants such as the use of both vitamin E and C is more effective than high levels of one antioxidant (Liu, 2004; Wang *et al.,* 2011). The supplementary intake of vitamins A, vitamin E and/or vitamin C improved reproductive function in laboratory and farm animals (Baldi *et al*., 2000, Tan *et al*., 2003; Pontes *et al.,* 2015). Coenzyme Q-10 (CoQ10) is recognized as an intracellular antioxidant that protects membrane phospholipids, mitochondrial membrane proteins, and low-density lipoproteins from free radical-induced oxidative damage (Singh and Devaraj, 2007). These antioxidants scavenged ROS produced by leucocytes, prevented DNA fragmentation, improved semen quality in smokers, reduced damage to spermatozoa, and blocked premature sperm maturation (Machando *et al*., 2013).

2.8.1.1 Vitamin E

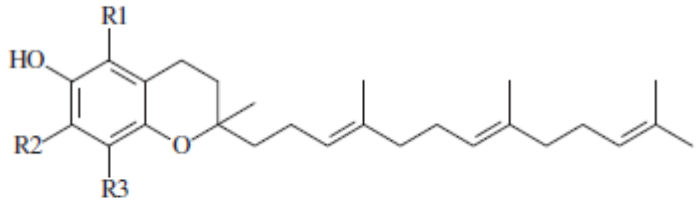
Vitamin E (α-tocopherol) and Vitamin C (ascorbic acid) are antioxidants that are thought to have a protective effect by either reducing or preventing oxidative damage (McCall and Frei, 1999; Martinez-Paramo *et al*., 2012). Supplementation with selenium and vitamin E in infertile men improved sperm quality and had protective effects especially on sperm motility (Moslemi and Tavanbakhsh, 2011). The form of vitamin E that scavenges lipid peroxyl radicals *in vivo* and *in vitro* systems is the α-tocopherol (Niki, 2014). Lipid soluble Vitamin E prevents lipid peroxidation chain reactions in cellular membranes by interfering with the propagation of lipid radicals (Halliwel, 1994; Niki, 2014). Vitamin E is a major chain-breaking antioxidant in the sperm membranes and appears to have a dose-dependent effect (Makker *et al.,* 2009; Said and Agarwal, 2012).

Tocopherols and tocotrienols are vitamin E isomers and are strong antioxidants that give oxidative stability to the oil. Tocotrienols are the less known form of vitamin E while tocopherols are well known. Tocopherols and tocotrienols are divided into alpha, beta, gamma and delta according to the location of the double bonds in the fatty acid chain. Figure 2.8.1 illustrates that tocochromanols contain a polar chromanol head group with a long

isoprenoid side chain. Depending on the nature of the isoprenoid chain, a distinction is made between tocopherols (containing a saturated phytyl chain) or tocotrienols (unsaturated geranylgeranyl chain) (Dörmann, 2007).



Tocopherols



Tocotrienols

Figure 2.8.1: Structures of tocotrienol and tocopherol (Adapted from Dörmann, 2007)

Makker *et al.* (2009) also showed that vitamin E scavenges superoxide, H_2O_2 and hydroxyl radicals. Suleiman *et al*., (1996) showed that the administration of 100 mg of vitamin E three times a day for six months in a group of asthenozoospermic patients with normal female partners led to a significant decrease in lipid peroxidation and an increase in sperm motility (Agarwal *et al*., 2004).

2.8.1.2 Vitamin C

Vitamin C is a water-soluble antioxidant found in the cytosol and extracellular fluid that can interact directly with free radicals (Rinne *et al*., 2000; Pugliese *et al*., 2013). Vitamin C is another important chain-breaking antioxidant, contributing up to 65 per cent of the total antioxidant capacity of seminal plasma (Saleh and Hcld, 2002; Akbari and Jelodar, 2013; Agarwal *et al*., 2014). It neutralizes hydroxyl, superoxide, and hydrogen peroxide radicals and prevents sperm agglutination (Argawal *et al*., 2004). Vitamin C also prevents lipid peroxidation, recycles vitamin E and protects against DNA damage induced by the H_2O_2 radical (Rinne *et al*., 2000). The administration of 200 mg of vitamin C orally along with vitamin E and glutathione for two months significantly reduced 8-hydroxy-2'-deoxyguanosine (8-OH-dG) levels in sperm cells and also improved the sperm count, sperm motility and sperm morphology in oligozoospermic patients (Akmal *et al*., 2006; Agarwal *et al*., 2014).

Due to their different subcellular locations, a combination of Vitamin E and C has been shown to have a better antioxidant effect than either of the two vitamins alone (Rinne *et al*., 2000; Suhail *et al*., 2012).

2.8.1.3 Coenzyme Q10

CoQ10 is a non enzymatic antioxidant that is related to low-density lipoproteins and protects against peroxidative damage (Littarru and Tiano, 2007; Lee *et al*., 2012). CoQ10 is an energy promoting agent and several studies have been conducted to investigate the possibility of using it as a supplement for chronic fatigued patients (Aliev *et al*., 2014; Castro-Marrero *et al*., 2014). Since it is an energy-promoting agent, it also enhances sperm motility. CoQ10 recycles vitamin E and prevents its pro-oxidant activity (Karbownik *et al*., 2001; Tvrda *et al*., 2011; Tremellen, 2012). It has been shown that oral supplementation of 60 mg/day of CoQ10 improved the fertilization rate using intracytoplasmic sperm injection (ICSI) in normospermic infertile males (Lewin and Lavon, 1997). CoQ10 serves as an antioxidant in both mitochondrial and lipid membranes directly scavenging free radicals in the inner mitochondrial membrane by mediating uncoupling through superoxide production (Echtay *et al*., 2003; Brandmeyer *et al*., 2014). A study has shown that the incubation of sperm samples from asthenozoospermic infertile males for 24 hours in Ham's F-10 medium with 50 µM CoQ10 improved sperm motility (Lenzi *et al*., 2004).

2.9 Natural antioxidant rich foods and male infertility

Natural antioxidant-rich foods can be divided into fruits, vegetables, nuts, dried fruits and natural oils (Wu *et al*., 2004). These food sources have been shown to exhibit antioxidant activity and their consumption improves human health (Gharagozloo and Aitken, 2011; Valente *et al*., 2014). Research shows that antioxidant rich foods have been used to treat cardiovascular diseases, erectile dysfunction and diabetes (Song and Juang, 2012; Pantsi *et al*., 2014; Valente *et al*., 2014).

Studies have been done to investigate the positive effects of oral antioxidants in improving sperm health and thus improve fertility (Gharagozloo and Aitken, 2011; Durairajanayagam *et al.,* 2014; Nadjarzadeh *et al.,* 2014). To date, there have been approximately a dozen antioxidants evaluated clinically, either individually or in combination (Klein and Ojama, 2001; Niki 2014). However, most trials are small in size and differ in the target population selected as well as the type, dose and duration of antioxidant therapy. Several reviews of clinical studies addressing the effect of oral antioxidants on male infertility have been published (Lanzafame *et al*., 2009; Zini *et al*., 2009; Ross *et al*., 2010).

A review by Ross *et al.* (2010) gave a detailed account of some 17 studies selected strictly on the basis of randomization, with unselected infertile men as the target population taking oral antioxidants. The review stated that of the 17 studies conducted, 13 reported improvements in at least one semen variable, following a varied regimen of oral antioxidant therapy. The review also found that 6 out of 10 studies reported improvements in the pregnancy rates and all of the studies showed a marked reduction of oxidative stress and/or DNA damage (Ross *et al*., 2010). A quantitave study indicated the effectiveness of oral antioxidant supplementation in male infertility, quadrupling the probability of spontaneous pregnancies within 3 months, and reducing the cost per pregnancy by 60% (Comhaire and Wim, 2011).

Figure 2.8.2 compares the total antioxidant capacity of 28 food sources grouped as fruits, vegetables, nuts and dried fruits. Each group shows food sources from the lowest oxygen radical absorbance capacity (ORAC) concentration to the highest. Nuts (pecan nuts) and dried fruits (rice bran) were found to have the highest total antioxidant capacity (Wu *et al*., 2004).

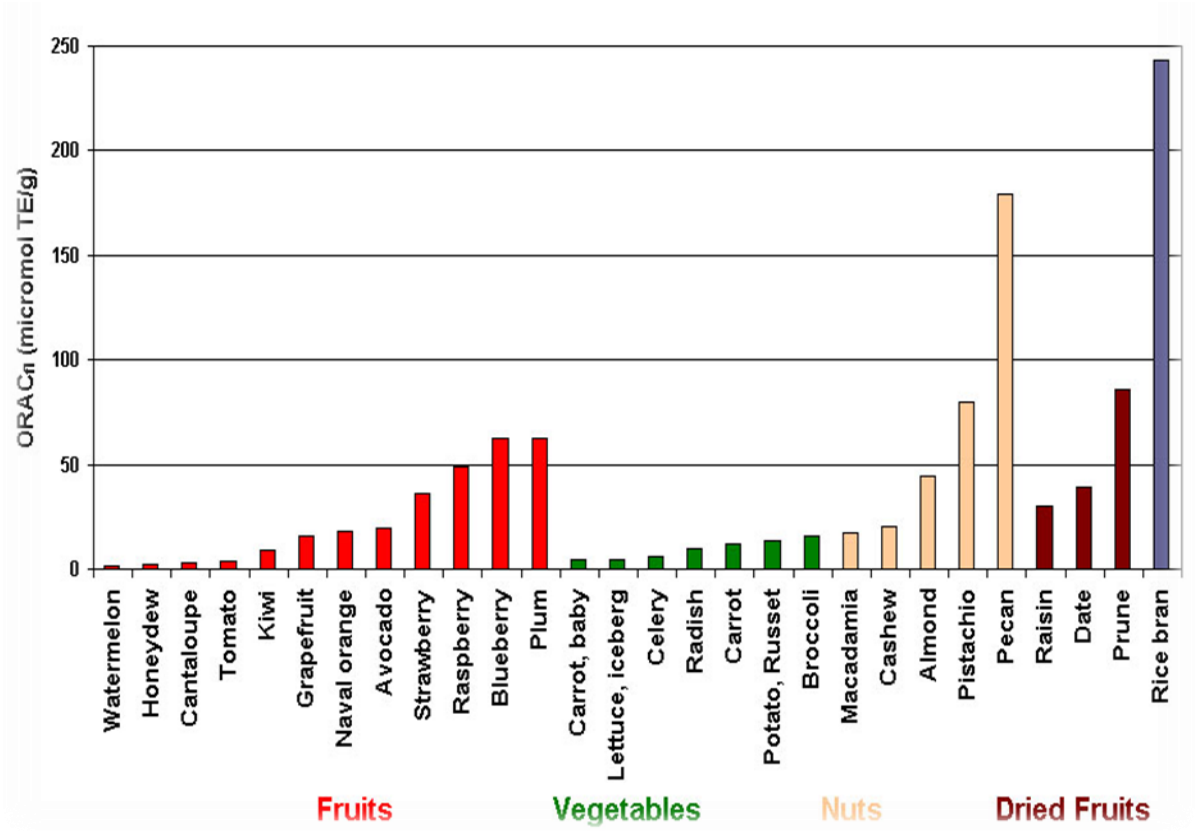


Figure 2.8.2: Total antioxidant capacity of 28 food sources (Adapted from Wu *et al*., 2004)

2.9.1 Carotenoids

Carotenoids are natural pigments that are synthesized by plants and are responsible for the bright colours of various fruits and vegetables (Aberoumand, 2011; Murador *et al.,* 2014). Carotenoids can only be incorporated into the human body through dietary consumption. Carotenoids possess antioxidant activity and are omnipresent in the plant kingdom, and as many as 1000 naturally occurring variants have been identified (Rice-Evans *et al.,* 1997; Stahl and Sies, 2003; Charbert *et al*., 2014). At least sixty carotenoids occur in fruits and vegetables commonly consumed by humans (Lindsay and Astley, 2002; You *et al*., 2015). Mixtures of carotenoids or associations with other antioxidants (e.g. vitamin E) can increase their activity against free radicals (Krinsky, 2002; Krishnamoorthy *et al.*, 2011; Johnson *et al.,* 2013). Carotenoids have a strong ability to react with ROS hence they play an important role as singlet oxygen scavengers and lipid antioxidants (Stahl and Sies, 1997).

Carotene is also a scavenger of peroxyl radicals, especially at low oxygen tension. The interactions of carotenoids with peroxyl radicals may precede via an unstable carotene radical adduct (Rice-Evans *et al.,* 1997; Karuppanapandian *et al.,* 2011). Carotenoid adduct radicals have been shown to be highly resonance stabilized and are predicted to be relatively unreactive (Lombardo *et al.,* 2011). Carotenoids were listed by Schlegel (2013) as one of the supplements that enrich male fertility. A study to investigate the effects of vitamin E and beta-carotene on sperm competitiveness conducted by Almbro *et al*. (2011) suggested that a combination of beta-carotene and vitamin E improved capacitation and chances of fertility. Orazizadeh *et al*. (2014) demonstrated the protective effects of beta-carotene against testicular toxicity when inducing mice with titanium oxide and treating them with beta-carotene thereby protecting testicular tissue.

2.9.2 Nuts

Nuts are highly nutritious and of prime importance for people in several regions in Asia and Africa. Most nuts contain a high of fat percentage: pecan 70 %, macadamia nut 66 %, Brazil nut 65 %, walnut 60 %, almonds 55% and peanut butter 55 % (Blomhoff *et al.,* 2006; Pantsi *et al*., 2014). Most nuts have high protein content, and only a few have very high starch content (Davidson, 1999). Recently, many nuts have also been identified as especially rich in antioxidants (Wu *et al.,* 2004; Vinson and Cai, 2012; Regueiro *et al.,* 2014).

Robbins *et al*. (2012) found that 75 g of walnuts per day added to a Western-style diet improved sperm vitality, motility, and morphology in a group of healthy young men compared to those in a control group of men consuming a usual diet but avoiding tree nuts. A second study was conducted by Carpenter *et al*. (2012) on male college students at the peak

reproductive age (20-35) to investigate the effects of walnut supplementation on male fertility. A group of 117 male students consumed 75g of whole shelled walnuts daily for three months and the control group avoided nuts for the trial period. Walnut was shown to improve sperm parameters in the group that consumed walnuts (Carpenter *et al*., 2012). Improved semen quality was associated with increases in blood serum omega-3 fatty acids and antioxidants both present in walnuts (Robbins *et al*., 2012; Kaur *et al.,* 2014).

2.9.3 Lemon

Lemon fruit contains many compounds such as flavonoids and phenolic compounds. The lemon flavonoids eriocitrin and hesperidin may function to increase the concentration of antioxidant enzymes as well as the antioxidative activity *in vivo (*Huang *et al*., 2012). Lemon juice is a good source of vitamin C (Girones-Vilaplana *et al.,* 2013). A study conducted by Ashamu *et al*. (2010) demonstrated the ability of vitamin C to increase sperm concentration. These findings were in line with the outcomes of the study by Comhaire *et al.* (2000) that showed that antioxidants are able to increase sperm concentration.

2.9.4 Proanthocyanidins

Proanthocyanidins (PA's) are potent polyphenolic antioxidants that occur naturally in fruits, seeds, nuts and flowers which possess anti-bacterial, antiviral and anti-inflammatory properties. Grapes contain high amounts of PA's which are among the most abundant phenolic compounds in grape seeds (Bagchi *et al.,* 2000; Serrano *et al.,* 2009). Sonmez and Tascioglu (2015) conducted a study in which they exposed rat testes with cadmium and later supplemented the rats with grape seed PA's. Cadmium caused oxidative stress damage to testicular tissue as expected and the grape seed PA's decreased the immunoexpression of cadmium-induced testicular stress. Hassan *et al.* (2013) conducted a study on rats that proved that the oral administration of PA's caused a significant histopathological improvement in testicular tissues. This result was due to the reduction of lipid peroxidation by PA's through scavenging ROS and its antioxidative activity.

In addition, the administration of PA's increased serum testosterone, epididymyal fructose and sperm count (Hassan *et al*., 2013). These beneficial effects were attributed to the reduction in lipid peroxidation potential that subsequently led to the improvement of the testicular function (Hassan 2005).

2.9.5 Garlic

The garlic plant's bulb is the most commonly used part of the plant while the cloves are believed to have many medicinal properties (Hammami and El-May, 2012). Garlic has been considered as a disease preventative food and its effects on human health have been

studied. Kasuga *et al*. (2001) investigated the pharmacological activities of four garlic preparations (raw garlic juice, garlic powder, heated garlic juice and aged garlic extract) on male rats with testicular hypospermatogenesis and impotence induced by warm water treatment. Their study reported that different garlic preparations have different pharmacological properties, and aged garlic juice is the most consistent in recovery of spermatogenesis (Kasuga *et al*., 2001). Feeding a higher garlic supplementation over a longer period of time to the adult rats caused an increase in concentration of epididymal spermatozoa (Hammani and El May, 2013).

There has been an interesting debate in the Andrology and reproduction field about the effects of garlic on the male reproductive system. This is due to some researchers believing garlic is beneficial to male reproductive health while others argue that it has detrimental effects on the male reproductive system (Hammani and El May, 2013; Radia *et al*., 2014; Valente *et al.,* 2014).

2.10 The use of natural herbs and oils for treatment of male infertility

The ancient treatment of male infertility consists mainly of the consumption of roots, barks and leaves of certain plants which were believed to have medicinal properties (Gurib-Fakim, 2006; Van Andel *et al*., 2012; Thomas, 2013). Today, those plants are still used in most parts of the world, predominantly in Africa, China and France, and research has since been done to determine their benefits and toxicity. Studies on plants and oils such as *E. longifolia, L. meyenii, M. prureins* and red palm oil (RPO) have investigated their effect on male infertility and only these will be elaborated on below.

2.10.1 Mucona prureins (Leguminoseae)

Mucona prureins (*M. prureins)* is found in tropical countries in Africa and its seeds have been tested and proven to contain dopamine, epinephrine, and norepinephrine (Rahmatullah *et al*., 2012). A study by Ahmad *et al.* (2008) on asthenozoospermic infertile men reported that when these men orally consumed *M. prureins* seed powder for 3 months this resulted in tremendous improvement of their sperm concentration, sperm count and sperm motility. Shukla *et al*. (2009) conducted a similar study on normozoospermic, oligozoospermic and asthenozoospermic infertile men in order to investigate the mechanism of action of *M. prureins* and they found that serum testosterone and luteinizing hormone levels increased after the oral consumption of *M. prureins* seed powder for 3 months. Chauhan and co-workers (2014) reviewed the safety of different plants used for erectile dysfunction and male reproductive function and *M. prureins* was listed as a safe and efficient plant to use. These studies therefore indicate that *M. prureins* improves male fertility in general.

2.10.2 Eurycoma longifolia

The roots of *Eurycoma longifolia (E. longifolia)* has been used traditionally to cure erectile dysfunction and male infertility (Tambi *et al.,* 2012). Sambandan *et al.* (2006) conducted a study involving the administration of root powder extract from *E. longifolia* to male rats. This powder was shown to be effective as it elevated the production of testosterone and sperm parameters in male rats and it was concluded that its effectivity is attributed to the high glycopeptide content of the roots. *E. longifolia* root powder was administered orally to male rats which resulted in an improvement of their sexual performance in rats that were classified as sexually sluggish (Tambi *et al*., 2012). Furthermore, a human study was conducted by Tambi and Imran (2010) to investigate the effects of *E.longifolia* on fertility where subjects were men of subfertile couples due to idiopathic male infertility. This study reported spontaneous pregnancies after the oral consumption o*f E. longifolia*. George and Henkel (2014) conducted a study in which they proposed the use of *E. longifolia* as testosterone replacement therapy. It was concluded that this plant offered a safer and more natural alternative with the same effect as testosterone therapy.

2.10.3 Lepidium meyenii (Maca)

Lepidium meyenii (L. meyenii) has been used as a traditional herb to increase energy levels, promote mental clarity, as an aphrodisiac for both men and women for treating male impotence to improve fertility and sexuality (Cicero *et al*., 2001). Studies investigating the effect of *L. meyenii* on fertility concluded that consumption of this herb was able to improve fertility and increased the chances of pregnancy in rats (Bogani *et al*., 2006; Shin *et al*., 2010). This improvement in fertility is due to its ability to regulate testosterone secretions by stimulating the action of the pituitary gland. *L. meyenii* also improved epididymal sperm count and seminal volume as well as decreased oxidative stress levels in a study that was conducted on male rats over a period of four months (Gasco *et al*., 2007).

2.10.4 Red palm oil

RPO is an antioxidant rich oil found in the rain forest region of West Africa and is obtained from the tropical plant *Elaeis guineensis*. RPO has a relatively high fatty acid content compared to its low content of phytonutrients. Several studies have documented and proved that RPO provides protection against oxidative stress related damage to cells, tissues and organs (Esterhuyse *et al*., 2005; Esterhyuse *et al.,* 2006; Aboua *et al.,* 2009).

RPO has been used in combination with other antioxidant rich foods such as rooibos tea to improve heart function (Bester *et al*., 2006). Burri *et al*. (2012) proposed the global use of RPO in food in order to prevent vitamin E deficiency. Patients with cystic fibrosis showed an increase of vitamin A when supplemented with RPO (Sommerburg *et al.,* 2015). Aboua *et al.*

(2012) conducted a study which showed that rats fed with RPO had improved sperm quality. Ayeleso *et al*. (2014) investigated the effects of red palm oil and rooibos supplementation on diabetic male Wistar rats and concluded that antioxidant enzyme activity was increased and sperm motility was improved.

2.10.5 Buriti oil

Buriti (*Mauritia flexuosa*) is an abundant palm in the Amazonian parts of Southern America (Goulding and Smith, 2007; Saraiva *et al*., 2009). It falls under the genus *Mauritia* which is comprised of solitary palms, with tall, robust, erect stems and palmate leaves. *Mauritia flexuosa*, also known as the moriche palm, ité palm, buriti, or *aguaje* (Peru), is a palm tree. It grows near swamps and other wet areas (Silva *et al.,* 2014).

The oil extracted from the Buriti fruit is of great interest because of its physical and chemical characteristics (Albuquerque *et al*., 2003; Garcia-Quiroz *et al*., 2003; Albuquerque *et al*., 2005). Its fruit has a hard, red and squamous shell that covers a soft and oily pulp, with color variations ranging from dark yellow to reddish after complete ripening (Albuquerque *et al*., 2005; Silva *et al.,* 2014). The average composition of Buriti fruit is 20% shell and pulp, 30% of white cellulose layer and 50% seed (França *et al*., 1999).

Amazonian fruits are rich in fats and more than 61% of these fats are unsaturated. Research has shown that Buriti oil has a high concentration of monounsaturated fatty acids (MUFA's) and a low concentration of PUFA's (Santos *et al.,* 2013). The low concentration of PUFA's gives Buriti oil a high oxidative stability (Aquino *et al.,* 2012). The MUFA's in Buriti oil are higher than those in olive and Brazil nut oils. The latter oils are known to have high nutritional qualities with blood pressure and cholesterol lowering properties (França *et al*., 1999; Albuquerque *et al*., 2005).

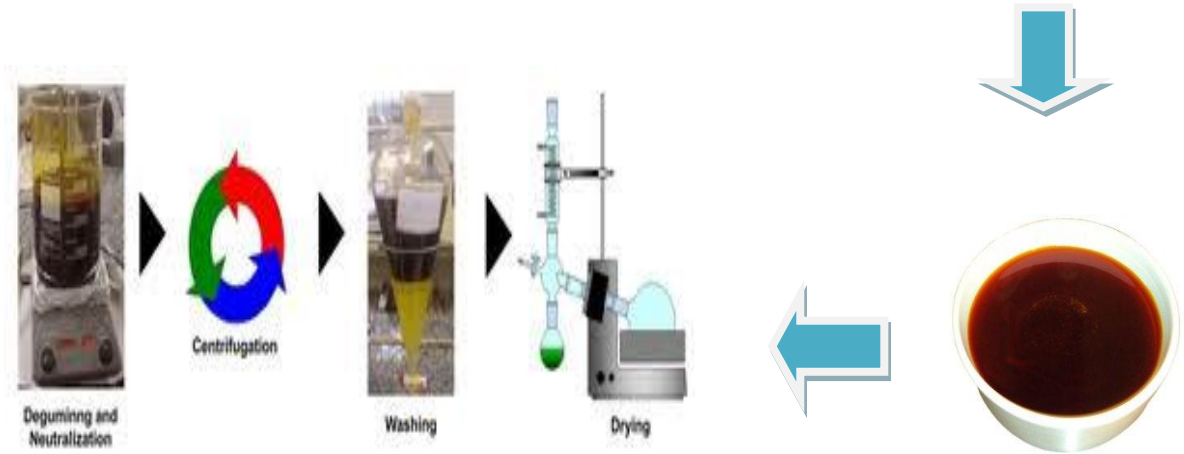
The nutritional value of Buriti oil may vary depending on the season it is harvested and the manner in which the oil is extracted. Buriti oil's stability can be reduced by the improper handling of the fruit after harvesting therefore it is very important to follow the correct post-harvesting procedure in order to get the best value out of it. Crude Buriti oil is more valuable than refined Buriti oil because flavonoids, tocopherols and vitamin A are lost during the refining process (Rosa *et al.,* 2014).

Although refined Buriti oil contains less carotenoids than crude Buriti oil, this level is still far greater than that of other Brazilian palm fruits such as *tucuma, umari* and *bocaiuva bacuri* (Ribeiro *et al.,* 2012; Prado *et al.* 2014). Aquino *et al.* (2012) commented that Buriti contains the highest concentration of β-carotene amongst Brazilian foods that have already been

studied. de Rosso and Mercedante (2007) conducted a study which concluded that Buriti oil contains 1706 ± 54 g of total carotenoids/g of which β-carotene is the most abundant.

Buriti palm tree

Buriti fruit



Crude Buriti oil

Refined Buriti oil

Figure 2.9: Schematic illustration of the extraction and processing of Buriti oil

Figure 2.9 highlights the entire process from the Buriti fruit and pressing of the fruit to extract crude Buriti oil. The crude oil then undergoes a series of centrifugation and distillatory steps in a refinery machine that yields refined Buriti oil.

Silva *et al*. (2009) tested Buriti oil and found it to be very stable to oxidation therefore concluding that it has high oxidative stability equivalent to that of olive oil. They further suggested that the high oxidative stability is due to the high percentage of oleic acid while the rest of the antioxidant activity was due to the minor constituents of the oil such as the tocopherols and pigments (Przybylski and Zambiazi, 2000). Tocopherols are natural antioxidants forming vitamin E (Albuquerque *et al*., 2005; Smolarek and Suh, 2011; Vrolijk *et al.,* 2015). Danet *et al.* (2011) compared the tocopherol contents of Buriti and patawa fruit and they reported that the antioxidant content of Buriti was higher than that of other palm fruits such as patawa. Danet *et al.* (2011)'s study also indicated that both Buriti and patawa are rich in fat, protein and vitamin E, but Buriti contains more vitamin E. Santos *et al.* (2013) identified Buriti pulp as rich in antioxidants when compared to other fruits.

The most active form of vitamin E is the α-tocopherol and Buriti oil contains this form in abundance. Vitamin E consists of both tocopherols and tocotrienols, of which tocopherols are saturated while tocotrienols are unsaturated (Patel *et al*., 2011; Mocchegiani *et al.,* 2014). The structural difference between the two is that tocopherols have a chromanol ring and a 15-ring carbon tail (Qureshi *et al.,* 2001). This difference in structure is responsible for their difference in oxidants scavenging ability both *in vivo* and *in vitro* (Sen *et al.,* 2000; Yoshida *et al*., 2003). Tocopherols are well known as effective chain breaking antioxidants due to their ability to produce stable antioxidant radicals. Agarwal and Sekhon (2010) have conducted numerous animal studies to observe the effects of tocopherols and tocotrienols against chronic diseases. Tocopherols have the ability to protect against photo-oxidation by reacting with singlet oxygen by physical quenching (Fahrenholtz *et al*., 1974; Karuppanapandian *et al*., 2011). Antioxidant properties of tocopherols enable them to prevent oxidative stress capable of causing chronic diseases (Darnet *et al*., 2011).

Several clinical trials have examined the potential of antioxidant supplementation to treat oxidative stress- induced male factor infertility (Ross *et al*., 2010; Said *et al*., 2012; Durairajanayagam *et al*., 2014). These antioxidants mostly derived from the human diet maintain the production of healthy sperm. Studies have recently been carried out to demonstrate the benefits of dietary Buriti oil on human health (Aquino *et al*., 2012; Becker *et al.,* 2014). Animal studies with tocopherols and tocotrienols that investigated these compounds' potential against chronic diseases have been extensively reviewed by Agarwal and Sekhon (2010). These authors argue that evidence is overwhelming to suggest that

tocotrienols may be more superior in their biological properties than tocopherols, and that their anti-inflammatory and antioxidant activities could prevent chronic diseases. Carotenoids found in Buriti oil are able to protect against lipid peroxidation caused by oxygen reactive species such as superoxide radical, peroxide radical and hydroxyl radical, which can be generated by cytotoxic compounds (Cantrell *et al.,* 2003; Rao and Rao, 2007).

2.11 Conclusion

Research has shown previously that the body may need additional antioxidants in the form of dietary supplements. It is therefore important to look into natural ways of dietary supplementation of plants and their products. In this study, the vitamin E and carotenoid content of Buriti oil was compared to that of RPO because previous studies had shown that both oils are rich in vitamin E and carotenoids and Aboua *et al*. (2012) had shown that RPO reduces oxidative stress in testicular and epididymal tissue. Furthermore, antioxidant enzymes and lipid peroxidation in both epididymal and testicular tissue were measured in order to show that Buriti oil protected against oxidative stress. Plasma concentrations of testosterone and estradiol were also measured in order to determine whether antioxidants in Buriti oil increased the hormonal concentrations.

CHAPTER 3
METHODOLOGY AND DESIGN

3.1 Ethical considerations

Care was given to animals according to the doctrine of Laboratory Animal Care of the National Society of Medical Research and the National Institutes of Health Guide for the Care and Use of Laboratory Animals of the National Academy of Sciences (National Institutes of Health publication no. 80-23, revised 1978). An ethics clearance certificate was obtained from the Cape Peninsula University of Technology Faculty of Health and Wellness Ethics Committee (NHREC: 230408-014).

3.2 Place of study

All laboratory activities took place at the Cape Peninsula University of Technology, Health and Wellness Sciences, Biomedical Sciences. Rats were housed at Stellenbosch University animal facility. The Oxidative Stress Research Centre (OSRC) facility was used for the determination of antioxidant content and activity of Buriti oil as well as blood sample analysis under supervision of Mr F Rautenbach (OSRC Laboratory Manager).

3.3 Experimental groups

Adult male Wistar rats weighing about 250g-280g were randomly allocated into two groups according to the dietary supplementation they received. The rats were individually housed to ensure that each animal receive equal amounts of supplements, which were prepared fresh on a daily basis. The rats (n=30) in the experimental group received 200 µL Buriti oil mixed with standard rat chow and free access to water daily. The control group (n =30) received standard rat chow and water only. The antioxidant and oxidative stress biomarkers were assessed. Total number of rats used for the entire study is 60. The feeding period was 6 weeks for both groups. The study design is illustrated by Figure 3.3 below.

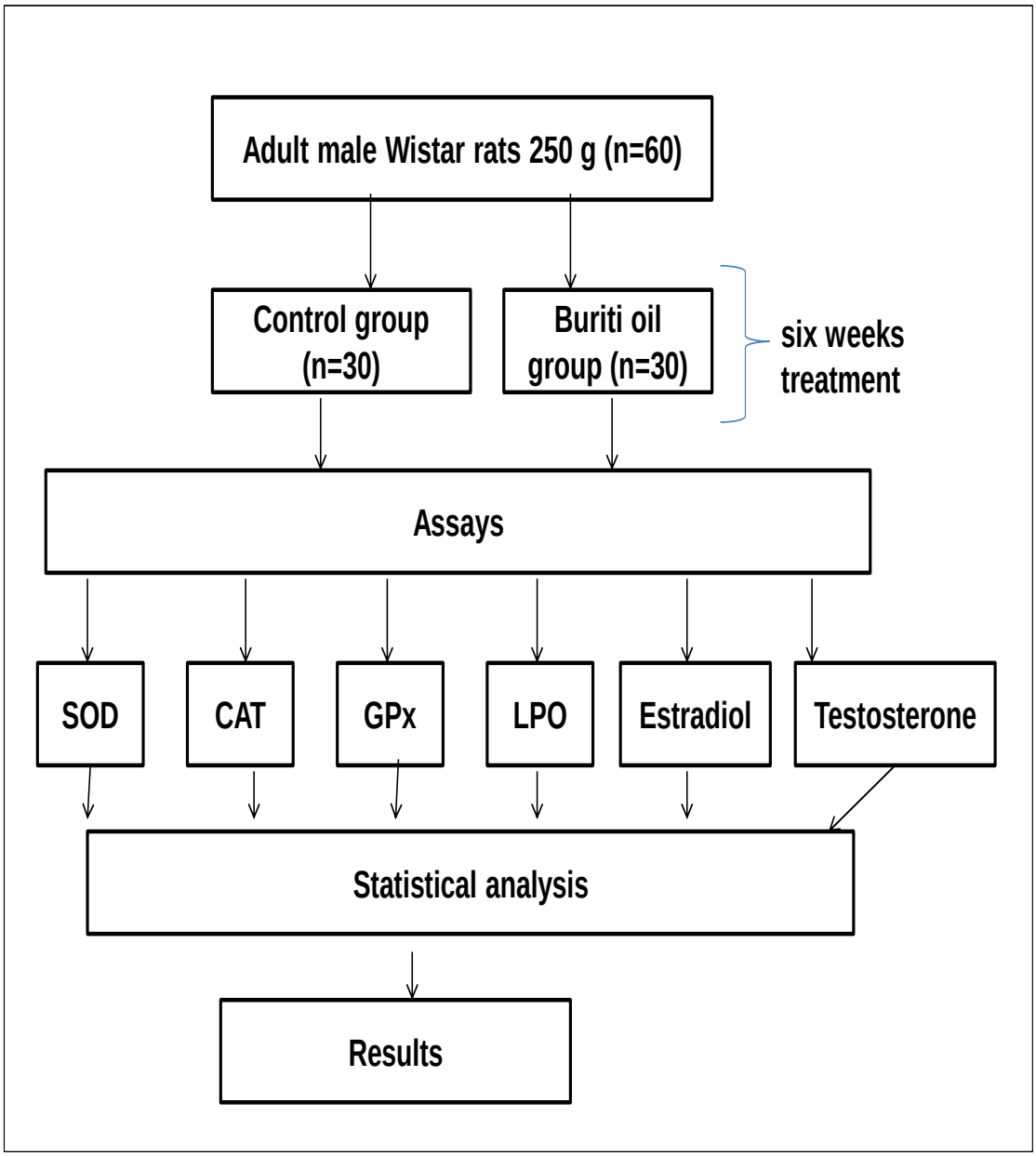


Figure 3.3 Schematic representation of the experimental design

Abbreviations: SOD: superoxide dismutase, CAT: catalase, GPx: glutathione peroxidase, LPO: lipid peroxidation

3.4 Organ and tissue harvesting

At the end of the 6 weeks treatment, rats were anaesthetized using 1ml (± 60 mg/kg) of sodium pentabarbitone. Once anaesthetized rats were weighed, body weights were recorded and 5 mL blood samples were collected from the animal's left ventricle aorta using sterile 10ml disposable syringes with 21G sterile hypodermic needles. Blood samples obtained were collected in EDTA containing tubes and in serum separator clot activator tubes (BD Vacutainers, Plymouth, UK) and placed on ice. Plasma and serum were obtained following centrifugation at 4000 rpm at 4°C for 10

min within 6 hours of collection and stored at -80°C until further analysis. Testes and epididymis were harvested, weighed and washed in phosphate buffered saline before being frozen in liquid nitrogen and stored at -80^{o}C. Testes/epididymis weights were determined by adding up both left and right testes/epididymis.

3.5 Consumables

Purchased from Sigma-Aldrich (Johannesburg, SA):

2,2□Œ-azobis (2-methylpropionamidine) dihydrochloride (AAPH), 6-hydroxydopamine (6-HD), diethylenetriaminepentaacetic acid (DETAPAC), 5,5• f-Dithio-bis-(2-nitrobenzoic acid) reagent (DTNB), ethylenediaminetetraacetic acid (EDTA), fluorescein sodium salt, glacial metaphosphoric acid (MPA), glutathione reduced (GSH), glutathione reductase (GR), malondialdehyde (MDA) standard, orthophosphoric acid (O-PA), perchloric acid (PCA), potassium phosphate (KH2PO4), reduced nicotinamide adenine dinucleotide phosphate (NAD(P)H), bicinchoninic acid (BCA), sodium azide, sodium hydroxide (NaOH), sulphuric acid, superoxide dismutase standard, tertiary-butyl hydroperoxide (t-BHP), thiobarbituric acid (TBA) and trisodium citrate. Greiner 96-well flat bottom and Costar 96-well UV flat bottom microplates

Purchased from Merck (Johannesburg, SA):

Hydrochloric acid (HCl), isopropanol, methanol, perchloric acid (PCA) 70%, sodium acetate and trifluoroacetic acid (TFA).

Purchased from various suppliers

Atlas Animal Foods (Cape Town, SA) supplied the standard rat chow (SRC). Red palm oil (Elaeis guineensis) was kindly donated by Carotino SDN BDH (Malaysia). Buriti oil was purchased from Phytoterapica (Brasil). Ultrapure MilliQ water (Millipore) was used throughout the study. Estradiol ELISA kit (DRG Diagnostics, Germany). Testosterone rat/mouse ELISA kit (DEMEDITEC Diagnostics, Germany).

3.6 Homogenate preparation

Homogenates were prepared using 200 mg of tissue added to 2 mL of PBS in a glass homogenizer on ice water. The tissue was homogenized in a Potter-Elvehjam homogenizer (Sigma-Aldrich Johannesburg, SA) for five strokes and transferred to new tubes and sonicated for 15 seconds on ice. The homogenates were then centrifuged for 10 minutes at 15 000 g in a microcentrifuge at 4^{o}C. The supernatant was transferred to new marked tubes and protein determination was done using a 10X dilution using BCA method.

3.7 Experimental Assays

3.7.1 Protein determination

The bicinchoninic acid (BCA) assay was used to determine the protein concentration in tissue samples. This protein determination was done before all enzyme assays were performed in order to assess the amount of protein available for enzyme analysis. Five albumin standards of different concentrations were prepared and 25 µL of each standard or sample was added in duplicates to a miroplate well. Working reagent of 200 µL was added to each well and mixed thoroughly on a plate shaker for 30 seconds then incubated at 37^{o}C for 30 minutes. The plate was cooled at room temperature and absorbance was measured at 562 nm on a plate reader (Thermo Electron Corporation, Multiskan spectrum, USA). The protein concentrations were quantified by using the standard curve and expressed as µg/ml. The protein concentration result in this study was used for determining the optimum protein concentration required for the catalase assay.

3.7.2 Lipid peroxidation

Testicular and epididymal tissue malondialhyde (MDA) concentration was measured based on the TBA assay method. A sample of 250 µL homogenate was combined with 31.25 µL 4 mM of cold BHT/ethanol and 250 µL of 0.2M ortho-phosphoric acid in eppindorff tubes. The tubes were then vortexed for 10 seconds to mix all components. Thereafter, 31.25 µL of TBA reagent (0.11M in 0.1M NaOH) was added and the tube was vortexed again for 10 seconds. The tubes were then placed in a water-bath that was heated up to 100^{o}C for 2 minutes then lids were opened and closed in order to prevent them from popping and left in the water bath for an hour to allow for the reaction to take place (which results in pink colour). After exactly one hour, the tubes were placed on ice for 2 minutes to allow rapid cooling. Tubes were then placed at room temperature for 5 minutes followed by the addition of 750 µL of n-butanol and 100 µL of saturated NaCl to aid in separation of phases. The tubes were vortexed for 10 seconds and microfuged at 12 000 rpm for 2 minutes at 4^{o}C. The supernatant (200 µL) of the butanol phase was placed in triplicates on a 96 well plate and read at A_{532}-A_{572} using a microplate reader (Thermo Electron Corporation, Multiskan spectrum, USA).

3.7.3 Testosterone

Plasma testosterone concentrations were measured using the DEMEDITEC Testosterone rat/mouse ELISA kit (DEMEDITEC Diagnostics, Germany). All reagents were brought to room temperature before use. An amount of 10 µL of each calibrator, control and sample was dispensed into appropriate wells. A 100 µL of incubation buffer was then added to each well followed by an addition of 50 µL of enzyme conjugate and the microplate was incubated for 60 minutes at room temperature. The wells' contents were discarded and were rinsed 4

times by using diluted wash solution of 300 µL per well. Wash solution was removed thoroughly by beating the plate on absorbent paper. Substrate solution (200 µL) was then added to each well and incubated for 30 minutes at room temperature in the dark and 50µL of stop solution was then added to stop the reaction. The absorbance of each well was determined at 450 nm using a microplate reader (Thermo Electron Corporation, Multiskan spectrum, USA). Testosterone concentrations were expressed as ng/ml.

3.7.4 Estradiol

The DRG Estradiol ELISA kit (DRG Diagnostics, Germany) is a solid phase enzyme-linked immunosorbent assay (ELISA), based on the principle of competitive binding. The microtiter wells are coated with polyclonal antibodies directed towards an antigenic site on the estradiol molecule. Endogenous estradiol of a patient's sample competes with an estradiol-horseradish peroxidise conjugate for binding to the coated antibody. After incubation the unbound conjugate is washed off. The amount of bound peroxidise conjugate is inversely proportional to the concentration of estradiol in the sample. After addition of the substrate solution, the intensity of colour developed is inversely proportional to the concentration of estradiol in the patient sample.

The concentration of estradiol in the plasma was measured using an ELISA kit provided by DRG Diagnostics, Germany. All reagents were brought to room temperature before use. An amount of 25 µL of each standard, control and sample was dispensed into appropriate wells. Thereafter, 200 µL of enzyme conjugate was added to each well, mixed well and the microtiter plate was incubated for 120 minutes at room temperature. The wells' contents were discarded and were rinsed 3 times by using diluted wash solution of 400 µL per well. Wash solution was removed thoroughly by beating the plate on absorbent paper. An amount of 100 µL of substrate solution was then added to each well and incubated for 15 minutes at room temperature. Finally, an amount of 50 µL of stop solution of was then added to each well to stop the reaction. The absorbance of each well was determined at 450 nm using a microplate reader (Thermo Electron Corporation, Multiskan spectrum, USA). Plasma estradiol concentrations were expressed in ng/ml.

3.8 Assessment of antioxidant activities

Superoxide dismutase (SOD) catalase (CAT) and glutathione peroxidase (GPx) activities was determined in testes and epididymal tissue using kits and assessed using a microplate reader (GloMax® Multi Detection System; Promega, UK). The above mentioned enzyme kits were purchased from Sigma-Aldrich Johannesburg, SA.

3.8.1 Superoxide dismutase

SOD activity was determined from the conversion of xanthine and oxygen to uric acid and hydrogen peroxide (H_2O_2) by xanthine oxidase to form superoxide anion (O_2^-). Superoxide dismutase activity was determined by a modified method from Ellerby and Bredesen (2000) using a superoxide dismutase assay kit (Sigma-Aldrich Johannesburg, SA). In a 96-well plate, 170 µL DETAPAC solution (0.1mM) was added to 6 µL lysate and 24 µL of superoxide dismutase buffer was added to each well. Each sample was run in triplicate. Fifteen microliters of stock 6-HD was finally added to the previous mixture and read immediately at 490 nm for 4minutes at 1minute intervals. The activity of SOD was calculated from a linear calibration curve, in the range of 2-20 U/mg.

3.8.2 Catalase

Catalase activity is measured by the ability of catalase enzyme to catalyze the decomposition of H_2O_2 to water and oxygen. The catalase assay was carried out at room temperature in UV microplates using the catalase assay kit (Sigma-Aldrich Johannesburg, SA). The method used was modified from Ellerby (1996), 170 µL of phosphate buffer was added to a 96 well plate followed by 75 µL of H_2O_2 stock solution. The first triplicate contained distilled water for the blank and the following triplicates contained 10 µL of sample homogenates. The plate contents were mixed well and a linear A_{240} decrease/minute was recorded for at least 1 minute in 15 seconds intervals.

3.8.3 Glutathione peroxidase (GPx)

The activity of glutathione peroxidase (GPx) is derived from the oxidation of reduced β-Nicotinamide adenine dinucleotide phosphate (NAD(P)H) in a conjugated glutathione reductase (GR) system using t-BHP (12mM) as a substrate. The cellular glutathione peroxidase activity assay kit purchased from Sigma-Aldrich (Johannesburg, SA) was used. In a 96-well UV Costar plate, 215 µL assay buffer (AB: 50mM potassium phosphate, 1mM EDTA, pH 7.0), 5 µL GSH (30.7mg/ml in water), 5 µL GR (0.1U/ml in AB), and 20µl sample were read before adding 5 µL NAD(P)H. Two readings were recorded (modified method of Ellerby and Bredensen, 2000). The first reading recorded the t-BHP non-dependent NAD(P)H oxidation at 340nm for 3minutes in 30second intervals for samples (A1) and blank (A1b). The second reading was performed after adding 50 µL of t-BHP. This reading monitored the decrease of t-BHP due to NAD(P)H oxidation at 340 nm for 2minutes in 30second intervals for the same samples (A2) and blank (A2b). Samples were ran in triplicate.

3.9 HPLC determination of antioxidant concentrations in oils

Carotenoid, tocopherol and tocotrienol concentrations in Buriti oil and RPO were analysed by an HPLC system (Thermo Fischer Scientific, South Africa).

3.9.1 HPLC determination for carotenoid concentration

The HPLC system (Thermo Fischer Scientific, South Africa) consisted of Spectra system P2000 pump, equipped with HPLC column C18, 150 x 4.6 mm, 5 µm particle size (Agilent Zorbax, South Africa) and a HPLC spectra system FL3000 fluorescence detector. The column and detector array temperature was maintained at room temperature (25±1°C). The following carotenoids were quantitated by this method using a C30 column: lutein, zeaxanthin, α-cryptoxanthin, β-cryptoxanthin, α-carotene, β-carotene, lycopene and their *cis*-isomers. Analysis time was 65 minutes per sample and detection took place at 476 nm. A C-30 column and a mobile phase of 100% methanol (A) and 100% dichloromethane (B) with the following gradient elution was used: 90% A and 10% B in the beginning, maintained for 5 minutes, decreased to 78% A at 15 minutes, 62% A at 30 minutes, 52% A at 40 minutes, 41% A at 50 minutes, 38% A at 55 minutes, maintained for 3 minutes, and returned to 100% A at 65 minutes.

3.9.2 HPLC determination for vitamin E concentration

The components of vitamin E analysed with the HPLC system were tocopherols and tocotrienols. The HPLC system (Thermo Fischer Scientific, South Africa) consisted of Spectra system P2000 pump, equipped with HPLC column C18, 150 x 4.6mm, 5 µm particle size (Agilent Zorbax, South Africa) and a Spectra system FL3000 fluorescence detector. The chromatographic conditions were: flow rate 1mL/min, 15minutes run time, sample injection volume of 20 µL. The mobile phase consisted of ethanol (45/45/5/5), with a flowrate of 1.0 ml/min. Detection was performed at a 292 nm wavelength with a run time of 30 minutes. A gradient program was used as follows: from 60% methanol (A) in 2minutes, from 60 to 30% A in 8minutes, then back to 60% chloromethane (B) at 12minutes; 40% B in 2minutes, from 40 to 70% B in 8minutes, then back to 40% B at 12minutes and 3minutes of reconditioning before the next injection. The column and detector array temperature was maintained at room temperature (25±1°C). The analytical signals were monitored at 2-20mV potentials applied. The method described here is based on direct injection in HPLC with UV detection.

3.10 Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). One way analyisis variance (ANOVA) was used to test for significance between groups. Differences were considered significant at $P<0.05$. Statistical analysis of control and Buriti oil fed group was

perfomed by using unpaired Student's t-test. The statistical software package used for all statistical evaluations and graphical representations is GraphPad ™PRISM5.

CHAPTER 4
RESULTS

4.1 Comparitive assessment of carotenoid concentration in Buriti oil and RPO

The carotenoid concentrations of both RPO and Buriti oil were assayed by using HPLC. The concentration of both alpha carotene and beta carotene were determined by comparing our result to known alpha and beta carotene standards. These standards were injected into the HPLC machine and the area under the peaks was obtained. Equation 4.1 shows the calculations that determined the alpha and beta carotene concentrations of RPO and Buriti oil. The concentration was expressed in mg/100g of oil.

The area under each peak from the chromatogram was determined and substituted into equation 4.1. The highest peak in Buriti oil as seen on figure 4.1.1 was beta carotene. Figure 4.1.1 also shows that Buriti oil has a low alpha carotene peak. The chromatogram in figure 4.1.2 represents the alpha and carotene peaks of RPO. It can be seen from figure 4.1.2 that RPO had higher beta carotene content than the alpha carotene. After substituting peak area values into equation 4.1; Buriti oil alpha carotene was 90.41 mg/100g and its beta carotene was 929.35 mg/100g. RPO alpha carotene was 403.73 mg/100g and its beta carotene was 735.63 mg/100g. Table 4.1 illustrates the comparison between the carotene content of the two oils.

Equation 4.1

$$[C] = \frac{A \text{ of sample} \times [S]}{A \text{ of standard}}$$

Where A= area under α/β carotene peak
[S]= standard concentration
[C]= sample carotene concentration
Sample= Buriti oil or RPO
Standard= α/β carotene with known concentration and peak area

Table 4.1: Comparison of carotene concentration in Red palm oil (RPO) and Buriti oil

	Alpha carotene mg/100g	Beta carotene mg/100g	Total mg/100g
RPO	403.73	735.63	1139.36
Buriti oil	90.41	929.35	1019.76

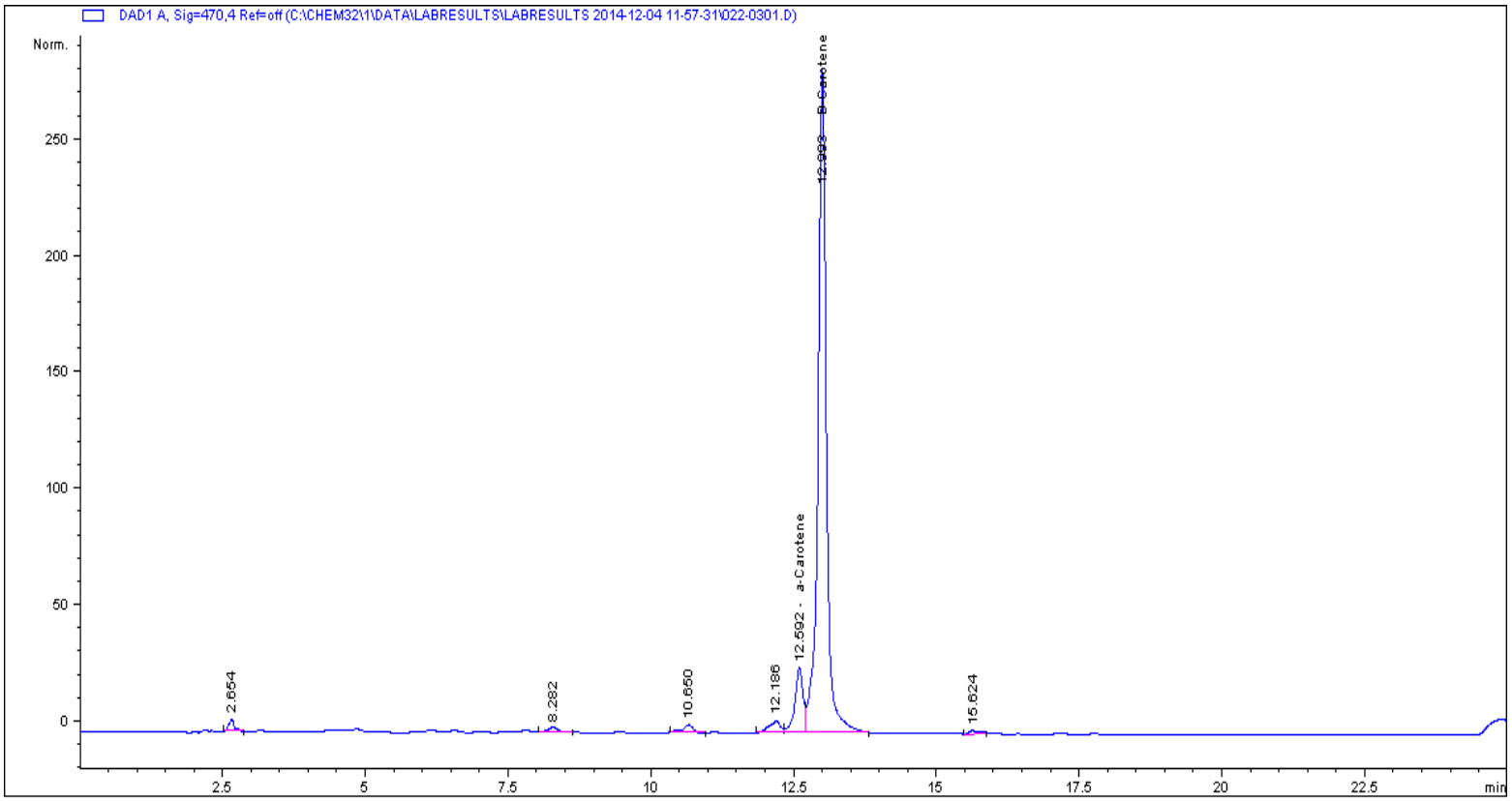


Figure 4.1.1: Carotenoid concentrations of Buriti oil.

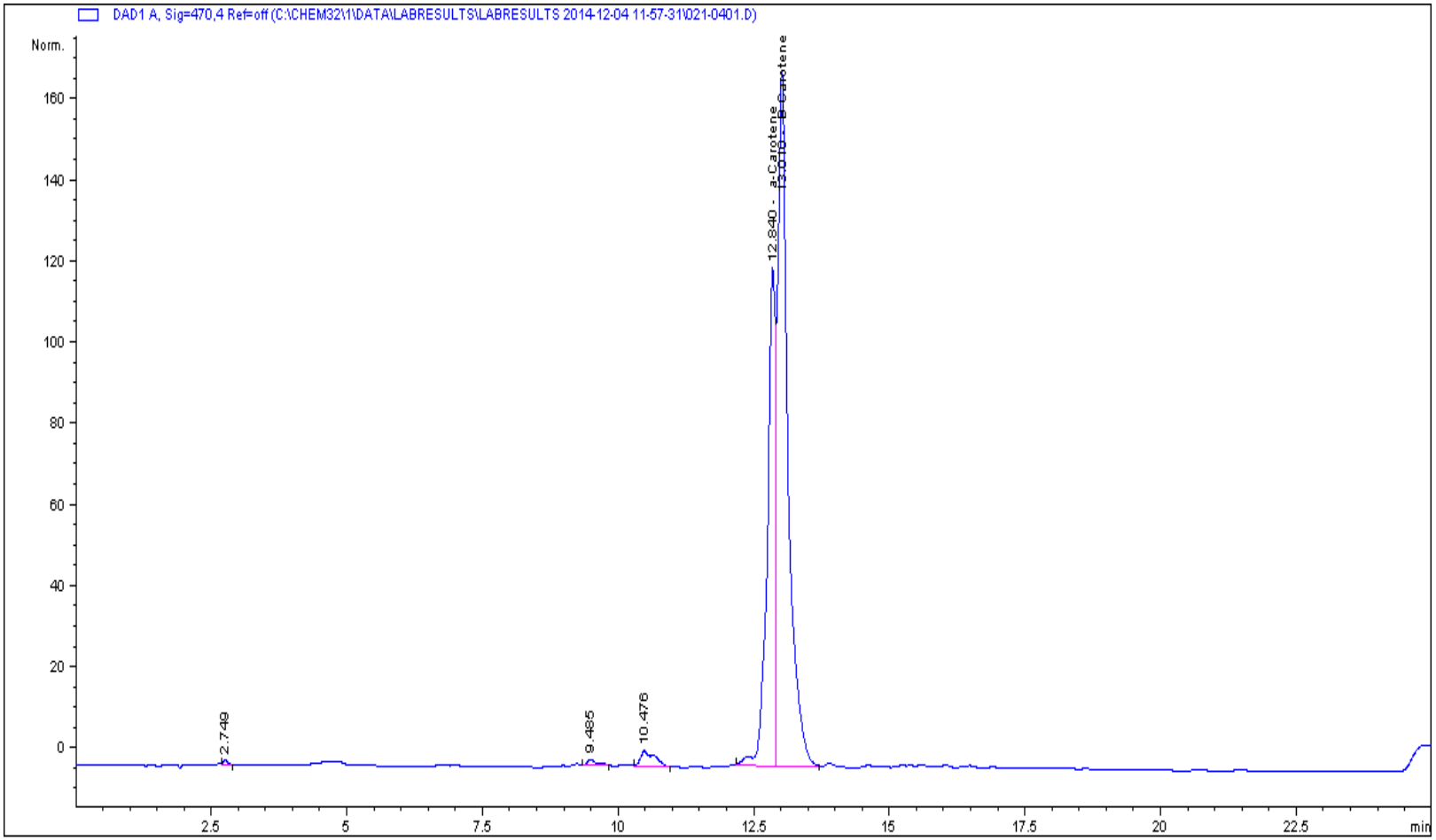


Figure 4.1.2: Carotenoid concentrations of RPO

4.2 Comparative assessment of vitamin E concentration of Buriti oil and RPO

Vitamin E consists of tocopherol and tocotrienols. Tocopherol and tocotrienol concentrations in RPO and Buriti oil were analysed using a HPLC system. Tocopherol concentration was presented as T4 and tocotrienol concentration was represented by T3. Both tocopherol and tocotrienol were separated into alpha, beta and delta peaks. Tocopherol and tocotrienol concentrations of RPO and Buriti oil were calculated using equation 4.2. The concentrations of both alpha, and beta tocopherols were determined by comparing our result to known standard values. The tocotrienol alpha, beta and delta peaks were however too small for area to be calculated. Equation 4.2 shows the calculations that determined the tocopherol and tocotrienol concentrations of RPO and Buriti oil. Figure 4.2.1 shows the different tocopherol and tocotrienol concentration peaks in Buriti oil. Figure 4.2.2 shows the different peaks of tocopherol and tocotrienol in RPO and it can be seen that beta tocotrienol has the highest peak while delta tocopherol has the lowest peak. The peaks in Buriti oil show that beta tocopherol was the highest peak and tocotrienol peaks were too small to measure.

Table 4.2 compares the vitamin E content of RPO and Buriti oil after calculation using Equation 4.2. Alpha tocopherols were higher in Buriti oil than in RPO, (122.65 ug/g versus 35.61 ug/g). Beta tocopherols in RPO were extremely low compared to Buriti oil content, 3.18 ug/g versus 172.35 ug/g. Delta RPO tocopherols were 1.60 ug/g and Buriti oil had 60.19 ug/g. RPO's alpha, beta, and delta tocotrienols were 54.31 ug/g, 84.36 ug/g and 21.88 ug/g respectively. The tocotrienol peaks representing Buriti oil were too low to be measured hence the area could not be determined, the concentrations for tocotrienol were therefore taken to be 0.00 ug/g. Buriti oil had 355.19 ug/g total vitamin E concentration and RPO had 200 ug/g.

Equation 4.2

$$[T] = \frac{A \text{ of sample} \times [S]}{A \text{ of Standard}}$$

Where A= area under α/β/δ tocopherol/tocotrienol peak
[S]= standard concentration
[T]= sample tocopherol/tocotrienol concentration
Sample= Buriti oil or RPO
Standard= tocopherol/tocotrienol with known concentration and peak area

Table 4.2 Comparison of vitamin E concentration in Red palm oil (RPO) of Buriti oil

	Alpha T4	Beta T4	Delta T4	Alpha T3	Beta T3	Delta T3	Total (ug/g)
RPO	35.61	3.18	1.60	54.31	84.36	21.88	200.95
Buriti	122.65	172.35	60.19	0.00	0.00	0.00	355.19

T4: tocopherols
T3: tocotrienols

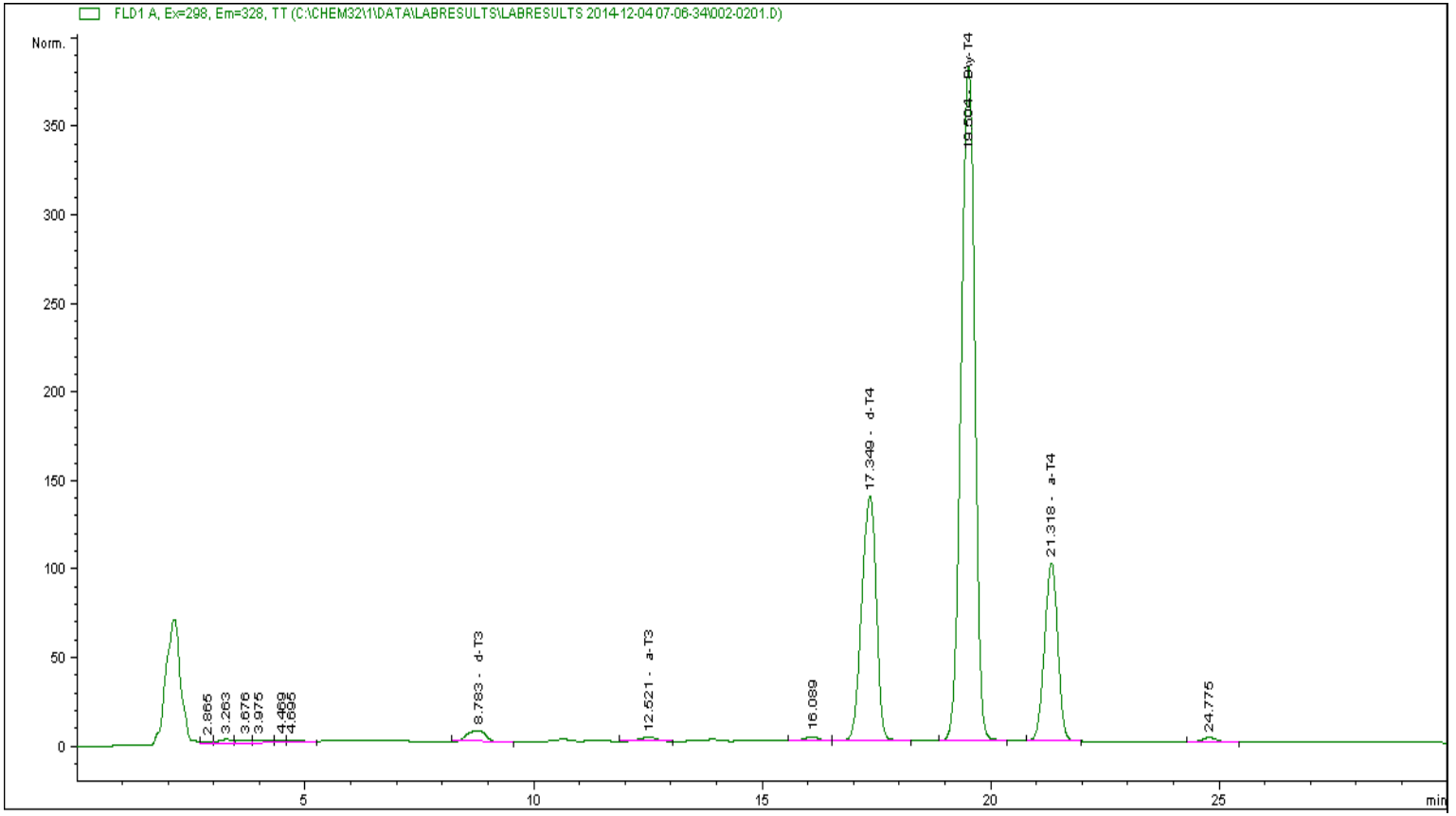


Figure 4.2.1: Vitamin E concentrations of Buriti oil.

Where
d-T3=delta tocotrienols
a-T3=alpha tocotrienols
d-T4=delta tocopherols
a-T4=alpha tocopherols
β-T4= beta tocopherols

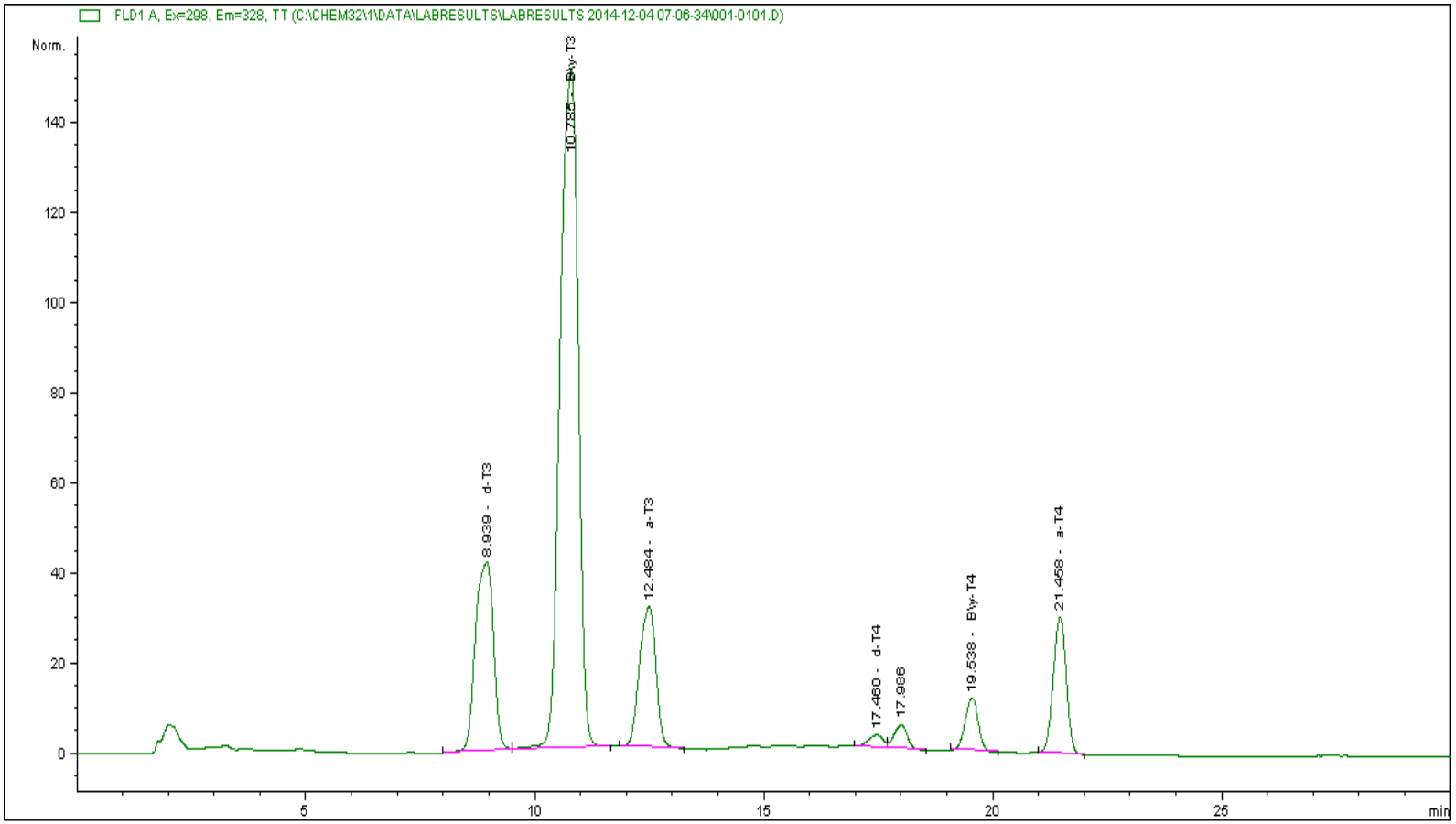


Figure 4.2.2: Vitamin E concentrations of RPO

Where
d-T3=delta tocotrienols
a-T3=alpha tocotrienols
d-T4=delta tocopherols
a-T4=alpha tocopherols
β-T4= beta tocopherols

4.3 Evaluation of body weights

From figure 4.3, it can be seen that on the day of sacrifice no significant difference was found between the control group (338.2 g ± 7.307 g) and the Buriti oil supplemented group (336.1 g ± 6.538 g) in body weights.

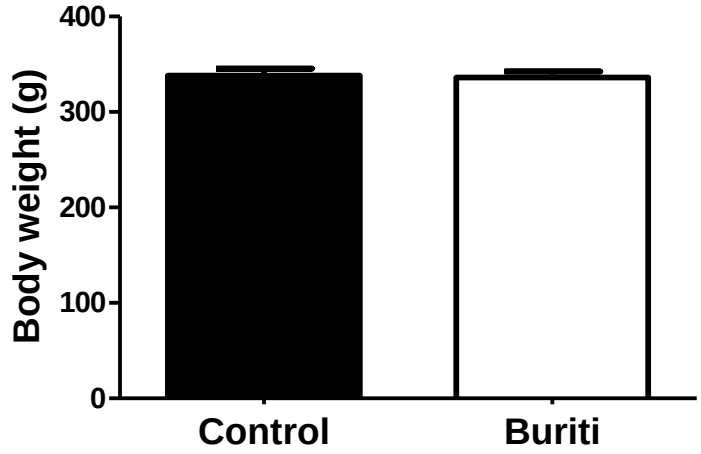


Figure 4.3: Evaluation of body weights of control and Buriti oil group after treatment
Data are represented as mean ± SEM
Control group: received water and standard rat chow diet (n=30)
Experimental group: received water, standard rat chow and Buriti oil diet (n=30)

4.4 Evaluation of testicular and epididymal weights

From figure 4.4.1 it can be seen that rats fed with Buriti oil had a higher testicular weight (3.577 g ± 0.082 g) compared to the control group (3.328 g ± 0.077 g). Likewise, the epididymis of rats fed with Buriti oil was heavier (0.789 g ± 0.013 g) compared to the control group (0.733 g ± 0.023 g) as indicated on figure 4.4.2. The increase in both testicular and epididymal weights in rats fed with Buriti oil was significant ($p<0.05$).

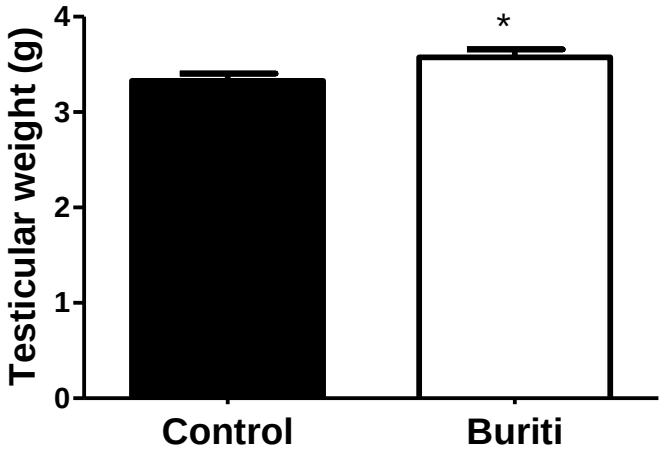


Figure 4.4.1: Evaluation of testicular weights of control and Buriti oil group after treatment
Data are represented as mean ± SEM; (*) indicates significant difference with p<0.05
Control group: received water and standard rat chow diet (n=30)
Experimental group: received water, standard rat chow and Buriti oil diet (n=30)

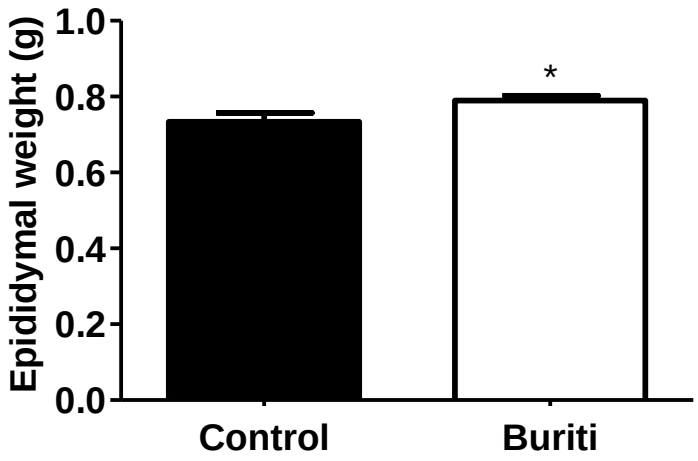


Figure 4.4.2: Evaluation of epididymal weights of control and Buriti oil group after treatment
Data are represented as mean ± SEM; (*) indicates significant difference with p<0.05
Control group: received water and standard rat chow diet (n=30)
Experimental group: received water, standard rat chow and Buriti oil diet (n=30)

4.5 Assessment of testicular and epididymal lipid peroxidation

Malondialdehyde (MDA) is a product of lipid peroxidation and the increased levels are an indication of oxidative stress damage. Although this study is a baseline study, the concentration of MDA in the testis from the Buriti oil fed group in figure 4.5.1 (0.103 µmol/g ± 0.004 µmol/g) was significantly lower than the control group (0.122 µmol/g ± 0.006 µmol/g). The concentration of MDA in the epididymis of the Buriti oil fed group was significantly lower

than the control group. Figure 4.5.2 shows that the epididymal MDA concentration in the control group was 0.028 µmol/g ± 0.003 µmol/g and the Buriti oil fed group was 0.016 µmol/g ± 0.004 µmol/g.

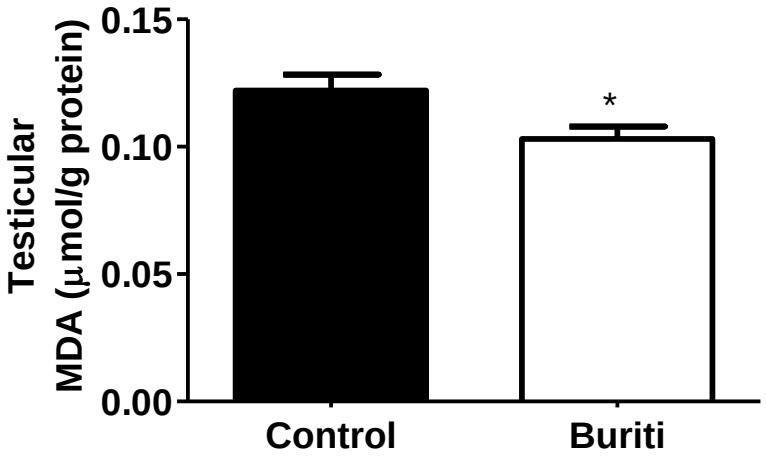


Figure 4.5.1: Evaluation of testicular MDA concentration of control and Buriti oil group after treatment

Data are represented as mean ± SEM; (*) indicates significant difference with p<0.05
Control group: received water and standard rat chow diet (n=30)
Experimental group: received water, standard rat chow and Buriti oil diet (n=30)

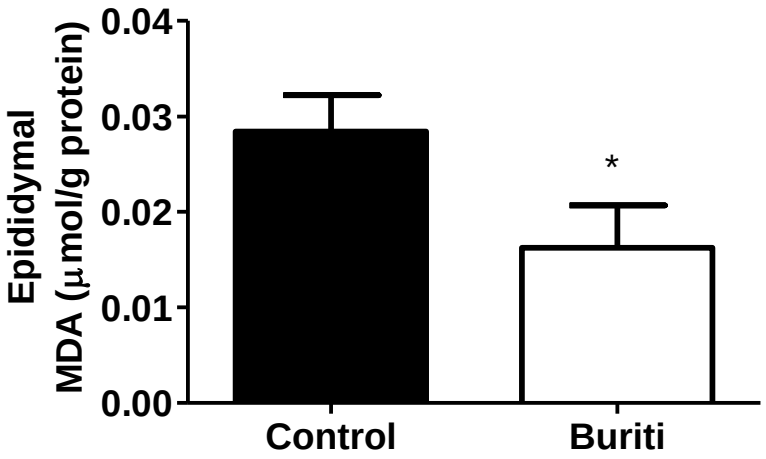


Figure 4.5.2: Evaluation of epididymal MDA concentration of control and Buriti oil group after treatment

Data are represented as mean ± SEM; (*) indicates significant difference with p<0.05
Control group: received water and standard rat chow diet (n=30)
Experimental group: received water, standard rat chow and Buriti oil diet (n=30)

4.6 Assessment of testicular and epididymal SOD activity

Testicular SOD activities are shown in figure 4.6.1. The control group (47.20 µmol/mg ± 0.671 µmol/mg) had a significantly lower SOD activity compared to the Buriti oil group (54.77 µmol/mg ± 0.466 µmol/mg). The epididymal SOD activity is represented by figure 4.6.2, the control group (42.67 µmol/mg ± 1.260 µmol/mg) has significantly lower SOD activity compared to the Buriti oil fed group (46.11 µmol/mg ± 0.714 µmol/mg). The difference in the concentrations between the control and Buriti oil fed group in both testicular and epididymal tissue was significant ($p<0.05$).

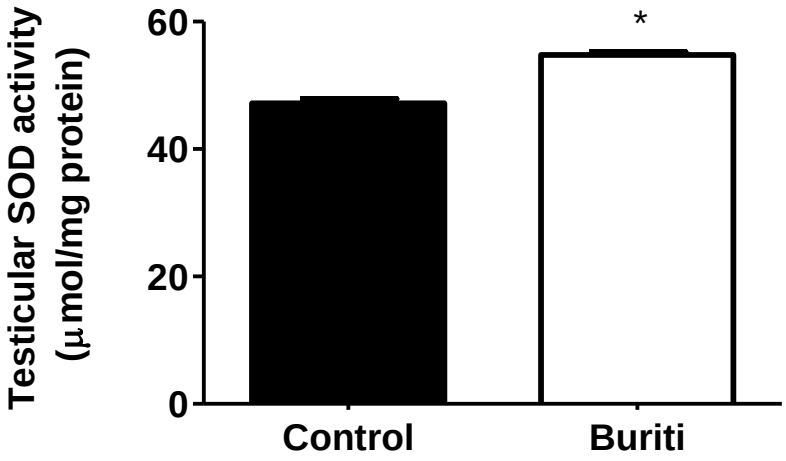


Figure 4.6.1: Evaluation of testicular SOD activity of control and Buriti oil group after treatment

Data are represented as mean ± SEM; (*) indicates significant difference with $p<0.05$
Control group: received water and standard rat chow diet (n=30)
Experimental group: received water, standard rat chow and Buriti oil diet (n=30)

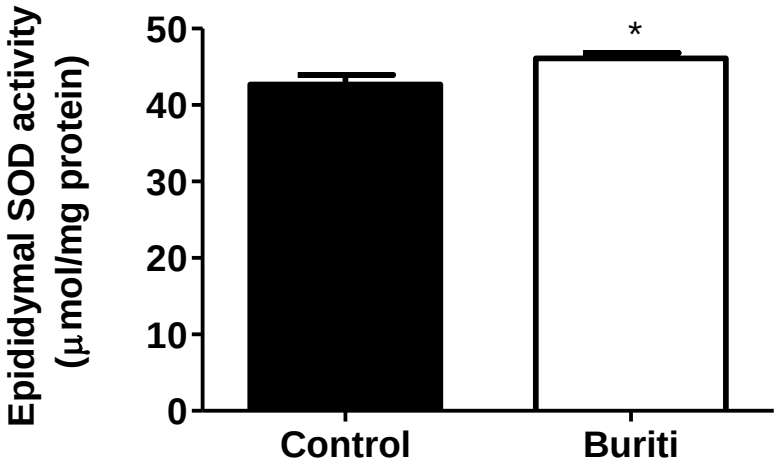


Figure 4.6.2: Evaluation of epididymal SOD activity of control and Buriti oil group after treatment

Data are represented as mean ± SEM; (*) indicates significant difference with $p<0.05$
Control group: received water and standard rat chow diet (n=30)
Experimental group: received water, standard rat chow and Buriti oil diet (n=30)

4.7 Evaluation of testicular and epididymal catalase activity

Figure 4.7.1 and 4.7.2 represent testicular and epididymal catalase activity respectively. It can be seen from figure 4.7.1 that the control group (34.76 µmol/mg ± 0.730 µmol/mg) had lower testicular catalase activity compared to the Buriti oil fed group (40.53 µmol/mg ± 1.749 µmol/mg). Figure 4.7.2 shows that the epididymal catalase activity was higher in the Buriti oil fed group (45.82 µmol/mg ± 2.296 µmol/mg) than in the control group (33.30 µmol/mg ± 1.223 µmol/mg). In both the epididymal and testicular tissue; there was a significant increase in catalase activity in Buriti oil fed group compared to the control, ($p<0.05$).

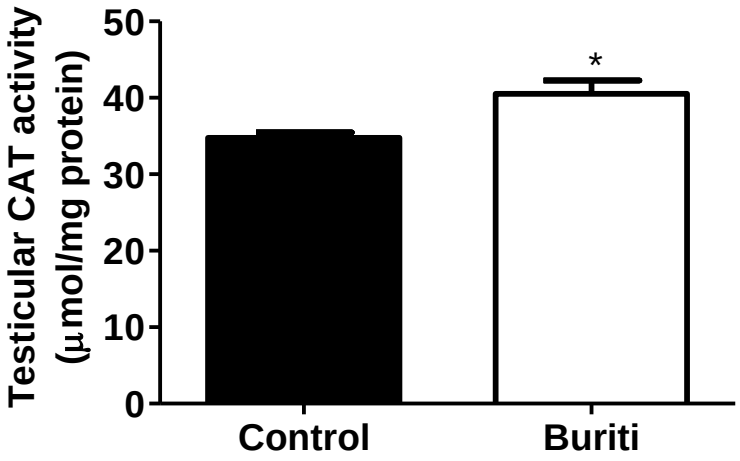


Figure 4.7.1: Evaluation of testicular catalase activity of control and Buriti oil group after treatment

Data are represented as mean ± SEM; (*) indicates significant difference with $p<0.05$
Control group: received water and standard rat chow diet (n=30)
Experimental group: received water, standard rat chow and Buriti oil diet (n=30)

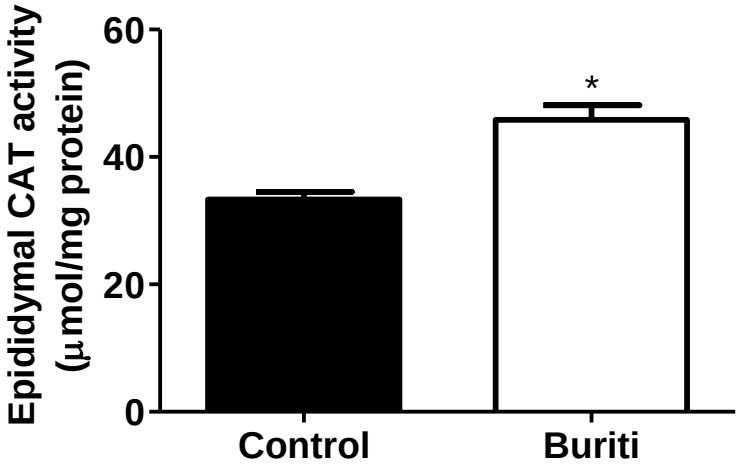


Figure 4.7.2: Evaluation of epididymal catalase activity of control and Buriti oil group after treatment
Data are represented as mean ± SEM; (*) indicates significant difference with p<0.05
Control group: received water and standard rat chow diet (n=30)
Experimental group: received water, standard rat chow and Buriti oil diet (n=30)

4.8 Assessment of testicular and epididymal GSH activity

Figure 4.8.1 and 4.8.2 show testicular and epididymal GSH activity respectively. From figure 4.8.1 it can be seen that the control group (57.79 µmol/mg ± 1.671 µmol/mg) had a lower GSH concentration than in the testicular tissue of the Buriti oil fed group (74.67 µmol/mg ± 4.281 µmol/mg). The epididymal GSH concentrations represented on figure 4.8.2 indicate that the control group (39.97 µmol/mg ± 1.194 µmol/mg) had a lower GSH concentration than the Buriti oil fed group (43.09 µmol/mg ± 1.468 µmol/mg) but the difference in the two groups was insignificant

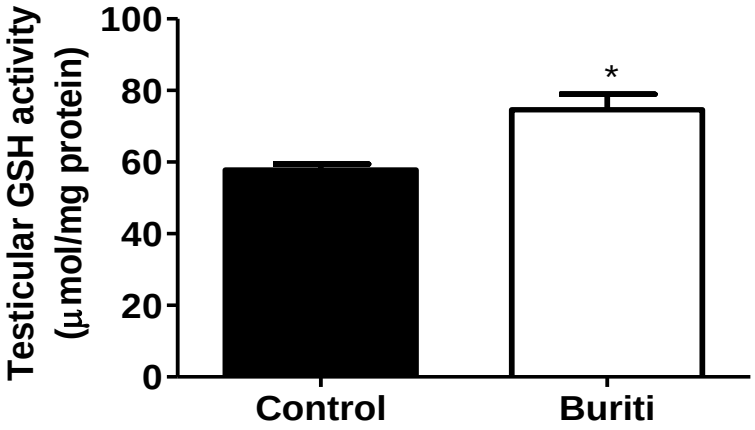


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Figure 4.8.1: Evaluation of testicular GSH activity of control and Buriti oil group after treatment
Data are represented as mean ± SEM; (*) indicates significant difference with p<0.05
Control group: received water and standard rat chow diet (n=30)
Experimental group: received water, standard rat chow and Buriti oil diet (n=30)

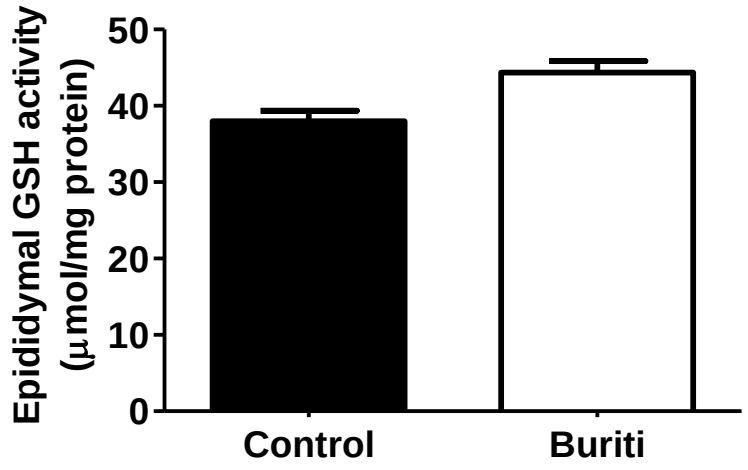

Figure 4.8.2: Evaluation of epididymal GSH activity of control and Buriti oil group after treatment

Data are represented as mean ± SEM
Control group: received water and standard rat chow diet (n=30)
Experimental group: received water, standard rat chow and Buriti oil diet (n=30)

4.9 Evaluation of plasma testosterone concentrations

The concentrations of plasma testosterone in the control group and Buriti oil supplemented group are shown in Figure 4.9. The plasma testosterone of the control group (1.443 ng/ml ± 0.051 ng/ml) was lower than the Buriti oil group (1.829 ng/ml ± 0.074 ng/ml) and the difference was significant ($p<0.05$).

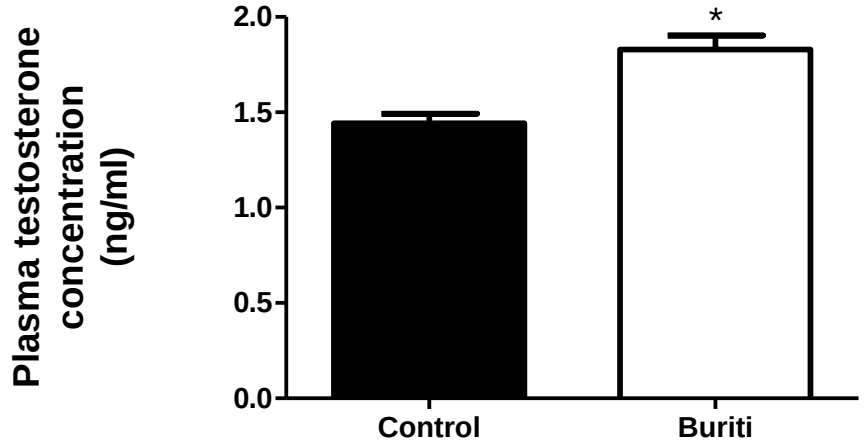

Figure 4.9: Evaluation of plasma testosterone concentration
Data are represented as mean ± SEM; (*) indicates significant difference with $p<0.05$
Control group: received water and standard rat chow diet (n=15)
Experimental group: received water, standard rat chow and Buriti oil diet (n=15)

4.10 Evaluation of plasma estradiol concentrations

The estradiol concentration of control and Buriti oil fed group is illustrated by figure 4.10. The Buriti oil fed group had a concentration of 1.723 ng/ml ± 0.114 ng/ml while the control was 1.672 ng/ml ± 0.141 ng/ml. The results show that the difference in plasma estradiol concentrations between the control and Buriti oil fed group was insignificant ($p>0.05$).

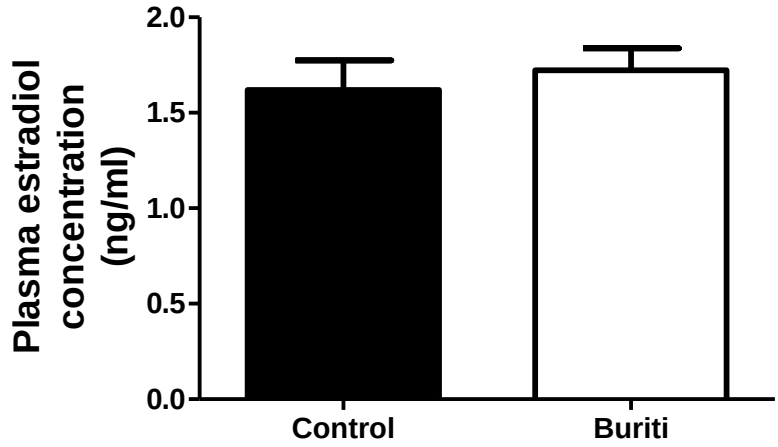

Figure 4.10: Evaluation of plasma estradiol concentration
Data are represented as mean ± SEM
Control group: received water and standard rat chow diet (n=15)
Experimental group: received water, standard rat chow and Buriti oil diet (n=15)

CHAPTER 5
DISCUSSION

Several studies have investigated the effects of oral antioxidant supplementation on male reproductive function. Aboua *et al*. (2012) investigated the effect of red palm oil supplementation on male reproductive function while Awoniyi *et al.* (2013) observed the effects of rooibos tea and green tea on male reproductive function. There are numerous studies conducted on Buriti oil consumption however, no documented studies on the effects of Buriti oil on male reproductive function have been reported (Darnet *et al*., 2011; Aquino *et al*., 2012). To our knowledge, this study might be the first to shed light on the effects of Buriti oil on male Wistar rat reproductive function. Our study was a baseline study therefore no external sources of ROS contributed to oxidative stress state. Both groups experienced physiological oxidative stress from metabolic reactions that took place in the body. The body has antioxidant enzymes in place to combat ROS; however, these antioxidants are usually overpowered by ROS that continues to increase due to poor nutrition, exposure to pollutants and aging. It is therefore important to supplement with dietary antioxidants such as Buriti oil.

5.1 Comparison of the carotenoid content between RPO and Buriti oil

Buriti oil's carotenoid content was compared to that of RPO because previous studies have indicated that RPO was also rich in carotenoids and was found to improve male reproductive function. This comparison of carotenoid content of the two oils was done in order to determine which oil has more α or β-carotene. Carotenoids, in particular α and β-carotene, are converted into vitamin A *in vivo*. Several researchers have compared the carotenoid content of different oils such as coconut oil, Buriti oil and RPO (Kemp *et al.,* 2013; Santos *et al.*, 2013). The protective properties offered by carotenoids on different types of tissue were shown by several authors (Almbro *et al*., 2011; Kemp *et al*., 2013; Orazizadeh *et al*., 2014). Studies on both RPO and Buriti oil indicated that they contain high concentrations of carotenoids (Albuquerque *et al*., 2005; Ribeiro *et al*., 2012; Santos *et al*., 2013).

Carotenoids present in RPO have been shown to protect cardiac tissue in a study conducted by Alinde *et al*. (2012). They concluded that the high concentration of carotenoids in RPO protected tissues against lipid peroxidation and increased activity of antioxidant enzymes. Alinde *et al*. (2012) data was similar to findings from a study by Aboua *et al.* (2012). These authors supplemented oxidative stress induced rats with RPO. They found that the supplementation of RPO scavenged ROS produced, allevieted the oxidative stress status, reversed and increased the testicular and epididymal sperm antioxidant activity. They concluded that RPO protected the sperm against oxidative stress. Orazizadeh *et al*. (2014)

investigated the protective effect of β-carotene on oxidative stress induced testicular damage and found that treatment with β-carotene increased testicular weight, serum testosterone concentration and improved semen quality.

In our study, the different concentrations of carotenoids in RPO and Buriti oil were assessed by an HPLC system. Table 4.1 compares the carotene concentrations in RPO and Buriti oil and showed that Buriti oil contains less α-carotene than β-carotene. Taipina *et al.* (2013) analysed carotene content of Buriti oil and concluded that beta carotene was the major carotene in Buriti oil and our study confirmed their findings. RPO in our study had alpha carotene of 403.73mg/100g which was more than that of Buriti oil (90.41mg/100g). Buriti oil had more beta carotene (929.35mg/100g) than RPO (735.63mg/100g). These results confirmed existing literature as they conclude that beta carotene is the most abundant carotenoid in both RPO and Buriti oil (Saraiva *et al*., 2009; Santos *et al*., 2013; Prado *et al*., 2014). This study showed that Buriti oil has more beta carotene than RPO and to our knowledge no study had ever compared these two oils before in terms of carotenoid content.

5.2 Comparative assessment of vitamin E concentration of Buriti oil and RPO

Alpha tocopherol is the most active form of vitamin E (Singh and Devaraj, 2007). It has been shown that RPO contains high vitamin E content. Several studies illustrated the protective effects of RPO against oxidative stress (Oguntibeju *et al.,* 2008; Aboua *et al*., 2009; Thamahane-Katengua *et al.,* 2013). Although these authors investigated the effects of RPO on different tissues and organ systems they all came to a conclusion that RPO offered protection to tissues by reducing, inhibiting and alleviating oxidative stress in the different models used. Numerous authors reported that Buriti oil is rich in vitamin E which gives it a high oxidative stability (Darnet *et al*., 2011; Santos *et al*., 2013).

Effects of vitamin E deficiency on spermatogenesis were demonstrated by Rossato and Mariotti (2014). They concluded that vitamin E was vital for normal spermatogenesis and its deficiency led to testicular lipid peroxidation. Our results confirmed a study by Rossato and Mariotti (2014) as Buriti oil had a high content of vitamin E concentration which probably protected testicular tissue from oxidative stress damage.

The results in Table 4.2 further indicate a steep difference in the tocotrienol concentrations between Buriti oil and RPO. The alpha, beta and delta tocotrienol concentrations of RPO were higher than Buriti oil tocotrienols. Santos *et al.* (2013) also concluded that Buriti oil has the lowest tocotrienol concentrations amongst oils obtained from Amazonian fruits.

Darnet *et al.* (2011) compared the tocopherol content of Buriti and patawa fruit pulps and concluded that Buriti had a higher content than patawa. In our study we compared Buriti oil to RPO and found that Buriti oil has a higher tocopherol concentration that RPO. Our findings were similar to Santos *et al.* (2013) who compared the tocopherol content of various Amozonian palm fruits and concluded that Buriti oil has the highest tocopherol content amongst oils obtained from Amazonian palm fruits. The total tocopherol and tocotrienol concentration of Buriti oil was 355.19ug/g while that of RPO was 200.95ug/g and this indicates that the total vitamin E content for Buriti oil is higher than that of RPO.

5.3 Effect of Buriti oil supplementation on body weight

There was no significant difference in weight between the Buriti oil supplemented rats and the control group. Before treatment the rats weighed 250g - 280g. After the 6 weeks feeding period, the control group weighed 338.2 g ± 7.307 g while the Buriti oil group weighed 336.1 g ± 6.538 g. There was also no particular trend in the manner in which the rats grew that could indicate that they had two different diets.

Jargar *et al.* (2014) investigated the effects of vitamin E supplementation on nickel-induced oxidative stress in rats. They concluded that nickel induction increased oxidative stress and decreased body weights. Rats supplemented with vitamin E displayed an increase in body weight due to the antioxidative and protective effects of vitamin E. Moreover, Aboua *et al.* (2012) investigated the effects of RPO supplementation on sperm parameters, testicular and epididymal tissue and body weights of male Wistar rats. They found that supplementation with RPO to rats improved sperm motility, protected against tissue lipid peroxidation and increased the body weights. Although Buriti oil contains a high vitamin E concentration, our results did not confirm these above mentioned studies.

Buriti oil fed rats in our study did not gain more weight than the control because after receiving Buriti oil by oral gavaging; they seemed not to consume as much rat chow as the control group. This could explain why there was no significant difference in weight gained between the experimental and control group eventhough the experimental group received extra energy from the oil. We also postulate that the non increase in body weight may be due to the very little to inexistent concentration of tocotrienols in Buriti oil (Ima-Nirwana and Suhaniza, 2004).

5.4 Effect of Buriti oil supplementation on testicular and epididymal weight

The testes and epididymis of experimental rats fed with Buriti oil were significantly larger compared to the control. These findings were similar to the findings of Yuce *et al*. (2013) who demonstrated that supplementation with cinnamon oil causes an increase in

reproductive organ weights of rats. Jargar *et al*. (2014) supplemented diabetic rats with vitamin E and they concluded that the supplemented group gained both body weight and organ weight because vitamin E protected tissues against lipid peroxidation. The significant increase in epididymal and testicular weight of rats supplemented with Buriti oil indicated that the high vitamin E content of Buriti oil enabled it to protect against oxidative stress damage.

Our study was a baseline study and both the control and Buriti oil group had no externally induced ROS. Santos *et al.* (2013) reported that Buriti oil is rich in carotenoids and tocopherols. Therefore the significantly higher epididymal and testicular weight in the experimental Buriti oil fed group compared to the control group indicated that Buriti oil was able to protect organ tissues against oxidative damage. We therefore postulate that antioxidant enzymes in the control group might have alleviated ROS that was produced from metabolic reactions in the body and the Buriti oil supplemented group was supplied extra protection with carotenoids and tocopherols and caused the cells to produce more endogenous antioxidant enzymes.

Aboua *et al*. (2012) found that the group that was fed with RPO had heavier epididymal and testicular weights than the group that was induced with oxidative stress. Our study confirmed the data as rats supplemented with Buriti oil showed increase in weight of testis and epididymis compared to the control group.

5.5 Effect of Buriti oil supplementation on lipid peroxidation of testicular and epididymal tissue

In this study LPO was quantified by measuring the formation of thiobarbituric acid reactive substances (TBARS) and expressed as nmol malondialdehyde (MDA) formed/mg in testicular/epididymal tissue. The amount of MDA produced was significantly lower in epididymal and testicular tissue of the experimental group compared to the control group.

The level of MDA reflects the amount of testicular and epididymal damage caused by ROS. Studies have demonstrated that LPO disrupts lipid membranes and tissues in organs such as the heart, testis and lung (Kharwar and Haldar, 2012; Thamahane-Katengua *et al*., 2013; Ibrahim and Ghoneim, 2014). Antioxidants and antioxidant rich foods have been used to lower LPO caused by oxidative stress. Alinde *et al.* (2012) showed that dietary supplementation with RPO can decrease LPO in cardiac tissue.

Buriti oil is rich in beta carotene. Carotenoids remove free radicals before they interact with the cell membrane by transferring a hydrogen atom with a single electron to a free radical.

Carotenoids have a high free radical scavenging rate and carotenoid-derived radicals have been shown to be stable and relatively unreactive. Carotenoid-derived radicals may further undergo bimolecular decay to generate non radical products (Everett *et al*., 1996; Bohm *et al*., 2012).

Heikal *et al.* (2014) concluded from their study that green tea extract protects against LPO caused by oxidative stress. Rats used in their study were exposed to pesticides which caused LPO, decreased testicular weight and impaired sperm motility. Their results indicated that soluble polyphenols in green tea extract protected testicular and epididymal tissue by scavenging ROS that can potentially cause LPO. Our results support Heikal *et al.* (2014) study because tocopherols and carotenoids found in Buriti oil possibly provided protection against ROS and LPO.

Buriti oil treated rats presented a significant decrease of LPO in their testicular and epididymal tissue than the control group, this indicates that carotenoids present in Buriti oil were able to protect against oxidative stress by scavenging free radicals hence protecting the tissue from lipid peroxidation. Our findings were similar to Aboua *et al*. (2012) who concluded that supplementation with carotenoid and tocopherol rich RPO protected testicular and epididymal tissues from LPO. The decrease in MDA production in the experimental group indicated that the group had more protection against free radicals hence experiencing less LPO than the control group.

5.6 Effect of Buriti oil supplementation on GSH, SOD and Catalase in testicular and epididymal tissue

SOD, CAT and GSH are able to maintain ROS scavenging potential in the male reproductive tract and protect against oxidative stress. The level of production and activity of these antioxidant enzymes is affected by physiological and pathological conditions. Decreased levels of these antioxidant enzymes can lead to decreased protection of tissues against oxidative stress damage. Each antioxidant enzyme has a unique mechanism of action against free radicals (Agarwal *et al*., 2006).

Several researchers have demonstrated that dietary supplementation with antioxidant rich oils and foods can increase antioxidant enzyme activity. Aboua *et al*. (2012), showed that dietary supplementation with red palm oil improved antioxidant enzyme activities in the male reproductive function of oxidative stress induced rats. Alinde (2012) also showed that supplementation with red palm oil increased SOD, CAT and GSH activities in cardiac tissue. Awoyini *et al*. (2013) concluded that supplementation with green tea and rooibos tea

improved the epididymal sperm quality and increased activity of antioxidant enzymes in oxidative stress induced rats. Our study confirmed all the above studies. However, Luo *et al*. (2006) showed that SOD concentrations were significantly decreased in ROS injured rats.

SOD is the main enzyme involved in the dismutation of O_2^- to H_2O_2 and oxygen. In our study, there was a significant increase in epididymal and testicular SOD concentration in rats supplemented with dietary Buriti oil compared to the control group. This indicated that in physiological conditions, the SOD available was not enough to scavenge all ROS present. The increased SOD concentration in testicular and epididymal tissue of the Buriti oil fed rats indicated that antioxidants in Buriti oil increased antioxidant activity of SOD.

Catalase neutralizes H_2O_2 to water and superoxide. In our study, catalase activity was significantly increased in rats supplemented with Buriti oil. Both testicular and epididymal catalase activity were increased. The experimental group had significantly higher catalase activity than the control and this could be due protection offered by Buriti oil. The increase in catalase activity indicated that Buriti oil has antioxidant properties that enable it to scavenge free radicals that caused oxidative stress and boost enzyme activity. Our study confirmed Ayeleso *et al*. (2014) study which concluded supplementation with RPO and rooibos tea increased testicular catalase enzyme activity.

The function of GPx is to remove H_2O_2 generated by metabolic action. The activity of GPx depends on GSH concentration. GSH scavenges peroxynitrite to $HO^{\cdot}$ and convert H_2O_2 to water with the help of GPx. Our study indicated that dietary Buriti oil supplementation increased GSH activity in testicular but no significant increase was seen in epididymal tissue. In a previous study, the reduced GSH concentration in the control group of testicular tissue indicated a high participation of reduced glutathione in H_2O_2 peroxidation (Krishnamoorthy *et al.,* 2011). Buriti oil is rich in tocopherols which elevated GSH in testicular tissue. Our findings were similar to those of Aboua *et al*. (2012) who proved that RPO supplementation improved GSH activity in testicular tissue of rats induced with oxidative rats.

Bester *et al*. (2006) found that RPO supplementation had no effect on biological parameters when the experimental model was not challenged with oxidative stress. Our epididymal GSH results confirmed Bester *et al.* (2006) because Buriti oil supplementation to our non-oxidative stress challenged model did not affect biological parameters in this organ. Our data confirmed a study by Kanter *et al*. (2012). These researchers investigated the protective effects of quercetin against oxidative stress in diabetic rat and concluded that quercetin

increased GSH activity in testis. Alinde *et al*. (2012) showed that supplementation with RPO decreased oxidative stress and increased GSH activity in cardiovascular tissue.

Oxidative stress in this study was not induced therefore it indicates that dietary Buriti oil supplementation can increase activity of SOD, CAT and GSH in a baseline state. We therefore concluded that Buriti oil improved activity of antioxidant enzymes and protected against oxidative stress.

5.7 Effect of Buriti oil supplementation on testosterone and estradiol

Normal concentrations of testosterone in the body influence sexual behaviour, muscle mass, energy, cardiovascular health and bone integrity (Menke *et al*., 2010). Buriti oil is rich in vitamin E and many studies have shown the importance of vitamin E supplementation in increasing testosterone levels (Turk *et al*., 2008; Garcia *et al*., 2014). Estradiol is a natural estrogen found in high concentrations in females and very low concentrations in males (Wildman *et al*., 2013). Testosterone and estrogen are highly involved in the regulation of fertility. The enzyme aromatase converts testosterone to estradiol (Smith and Saunders, 2011). Estrogens were considered to be female hormones and their presence in males remained questionable but researchers like Dorrington *et al.* (1978) later demonstrated the ability of testis to produce estrogens.

Several studies investigated the effects of vitamin E supplementation on testosterone, estrogen or both and they all concluded that it increases concentration of the hormones. Jalili *et al*. (2014) supplemented male rats with vitamin E and found that it protected testicular tissue and significantly increased testosterone levels. They concluded that tocopherols in vitamin E protected and increase Leydig cell numbers hence increasing testosterone release. Our study supported Jalili *et al.* (2014) study as supplementation with vitamin E rich Buriti oil significantly increased testosterone concentration and protected testicular tissue.

Hartman *et al*. (1999) conducted a study which proved that an increase in serum α-tocopherol results in an increase in serum androgens and estrogens in older men. However; the Alpha-Tocopherol Beta-Carotene (ATBC) cancer prevention study conducted by Tornwall *et al*. (2004) contradicted Hartman *et al*. (1999) study as it concluded that serum α-tocopherol is inversely associated with testosterone levels among men who received vitamin E supplements. The current study however, partially agrees with Hartman *et al.* (1999) study as we found that the Buriti oil supplemented group had significant increase in testosterone

compared to the control group but the estradiol concentration was not significantly increased.

Turk *et al*. (2008) supplemented male rats with antioxidant rich pomegranate juice and found that plasma testosterone levels were increased in the group that consumed pomegranate juice instead of water. In our study, the Buriti oil supplemented group showed an increase in testosterone levels and this shows that tocopherols in the oil offered protection to testicular tissue and Leydig cells which secrete testosterone (Santos *et al.,* 2013).

Animal studies show that estrogen deficiency or estrogen resistance impairs sperm motility and can cause infertility (Antal *et al*. 2008; Joseph *et al.,* 2011). The effect of antioxidant supplementation on estradiol in our study was not as we hypothesized. Buriti oil supplementation did not have a significant effect on the estradiol hormone concentrations. The difference in estradiol concentrations between the control group and Buriti oil group was not significant ($p>0.05$). Our study contradicted Selvakumar *et al*. (2011) who showed that alpha-tocopherol supplementation in male rats increased estradiol concentrations.

5.8 Possible mechanism of Buriti oil action

Buriti oil has a high content of tocopherols, carotenoids, and mono unsaturated fatty acids which enabled it to provide protection against oxidative stress in testicular and epididymal tissue. There are different mechanisms in which these components act in order to protect tissues.

We postulate that Buriti oil was able to protect against LPO and to increase antioxidant enzyme activity due to its high oxidative stability. Patel *et al.* (2011) reported that Buriti oil consists of both tocopherols and carotenoids which increased its oxidative stability. Sen *et al.* (2000) stated that tocopherols are saturated while tocotrienols are unsaturated. The ability of tocopherols to interact with free radicals is increased by their structure which consists of a chromatol ring and a 15-carbon tail. Nishio *et al.* (2013) compared the rate of tocopherol and tocotrienol uptake by cells and concluded that tocopherols have a slower uptake rate and provide more protection against LPO than tocotrienols despite the slow rate of uptake by cells.

5.8.1 Mechanism of absorption of Buriti oil antioxidants

The high antioxidant capacity of Buriti oil is due to its content of carotenoids and tocopherols. These antioxidants are absorbed into the body at different rates and different metabolic routes.

5.8.1.1 Tocopherols and tocotrienols

Tocopherols and tocotrienols have different absorption rates (Abuasal *et al*., 2012). Fairus *et al*. (2006) conducted a study in which they administered equal amounts of tocotrienol and tocopherol extracted from palm oil to healthy subjects. They found that unlike tocopherols which were distributed equally in all the lipoprotein fractions, tocotrienols were mainly detected in the high density lipoprotein (HDL) cholesterol at 4 to 8 hours before clearance. Tocopherols in Fairus *et al.* (2006) study were detected in plasma even after 24 hours. This observation suggests that tocotrienols are absorbed faster than tocopherols and that tocotrienols may go through alternative metabolism pathways which require further investigation. Another possible explanation of the low tocotrienol concentration in plasma is the low affinity of α- tocopherol transport protein (α-TTP) for tocotrienols (Gee, 2011). We therefore postulate that supplementation with Buriti oil offers longer protection against free radicals because it is rich in tocopherols which can be detected in plasma for a longer period of time compared to tocotrienols.

Packer *et al*. (2001) suggested that tocotrienols are more effective than tocopherols at reducing lipid peroxidation because they are absorbed faster and have higher intramembrane activities. However, Singh *et al*., (2013) demonstrated that tocotrienols disappear in the plasma within 24 hours of consumption while tocopherols increased and they suggested that tocotrienols were converted to tocopherols by hydrogenation. Alpha-tocotrienol was suggested to be secreted by small HDL particles whilst alpha-tocopherol is exclusively secreted in chylomicrons. The small HDL particles selectively distributed α-tocotrienol to organs and tissues high in adipose content: epididymal fat, perirenal fat and skin. In contrast, α-tocopherol was reported to be more evenly distributed based on the fact that LDL receptors are available in all tissues. The even distribution of alpha tocopherols makes Buriti oil used in our study a powerful free radical scavenging oil in a variety of tissues and this was shown by protection of lipid membranes in testicular and epididymal tissues.

5.8.1.2 Carotenoids

β-carotene is a dietary source of vitamin A for humans and its bioavailability depends on food processing techniques, size and dose. In humans the absorption of β-carotene from plant sources ranges from 5% to 65% (Haskell, 2012). Intestinal conversion of β-carotene to vitamin A decreases as the oral dose increases and this makes β-carotene a safe source of vitamin A. Buriti oil is therefore a safe source of vitamin A as it contains a high concentration of β-carotene (Karuppanapandian *et al*., 2011).

The *in vivo* intestinal absorption of carotenoids takes place through a series of steps. Carotenoids are uptaken by intestinal mucosal cells and incorporated into chylomicrons

where they are transported to various cells in the body. β-carotene is fat soluble and it scavenges free radicals in lipid membranes better than non lipid soluble antioxidants such as vitamin C (Harrison, 2012). The high concentration of B-carotene in Buriti oil increased its ability to protect against lipid peroxidation and this was characterized by the low MDA concentration in the experimental group.

5.8.2 Mechanism of Buriti oil action on Lipid peroxidation

There is a high concentration of mono unsaturated fatty acids (MUFAs) in Buriti oil (Santos *et al*., 2013). MUFAs play an important role in the maintance of serum lipid profiles and cell membrane (Aquino *et al*., 2012; Pantsi *et al*., 2014). Consumption of mono unsaturated fatty acids reduces systemic and cellular oxidative stress.

Buriti oil is rich in carotenoids. Carotenoids are fat soluble phytochemicals with provitamin A and antioxidant roles. Carotenoids have a long-chain conjugated polyene structure which makes them susceptible to oxidation therefore increasing their interactions with free radicals. Carotenoids in Buriti oil were able to react with free radicals before they interacted with testicular and epididymal lipid membranes therefore protecting against oxidative stress (Karuppanapandian *et al*., 2011).

5.8.3 Mechanism of Buriti oil action on body weight

Bone density and muscle mass both influence body weight (Mithal *et al*., 2013). A study conducted by Maniam *et al.* (2008) concluded that palm oils are rich in tocotrienols which are known to decrease osteoporosis therefore increasing bone density. Increased bone density contributes to body weight increase. A previous study by Aboua *et al*. (2012) showed that RPO supplementation to rats increased their body weights. Our outcome was different from theirs because RPO is rich in both tocopherols and tocotrienols while Buriti oil is rich in tocopherols but low on tocotrienols.

Our study showed that testosterone concentrations increased after supplementation with tocopherol-rich Buriti oil. Supplementation of rats with tocopherol for one month before exposure to oxidative stress significantly prevents stress induced decreases of testosterone (Lodhi *et al*., 2014). As rats age, they lose their muscle mass. Sinha *et al*. (2014) supplemented aged rats with testosterone and found that testosterone supplementation increased muscle mass. Testosterone concentration in our study was increased and this means that muscle mass was also increased. From our findings and previous literature, we can safely postulate that adequate levels of both testosterone and estradiol are necessary for an increase in body weights.

5.8.4 Mechanism of Buriti oil action on hormones

Santos *et al.* (2013) reported that Buriti oil has a low concentration of tocotrienols and a high concentration of tocopherols, as confirmed by Muhammad *et al*. (2013) tocotrienol supplementation was able to increase estrogen levels in rats that suffered from severe osteoporosis; their study therefore suggested that tocotrienol deficiency can decrease estrogen levels.

Alpha tocopherol has a direct stimulatory effect on enzymes of gonadal steroid biosynthesis and can exert modulatory action on gonadotropins synthesis and secretion (Al-Damegh, 2014). We found that rats from the Buriti oil supplemented group had increased testosterone concentrations in the plasma and we suggest that this could be due to the high concentrations of alpha tocopherols in Buriti oil. Tocopherols protected cells against oxidative stress by scavenging free radicals. We postulate that Leydig cells in the Buriti oil fed group were protected against oxidative stress hence were able to secrete more testosterone compared to the control group.

CHAPTER 6
CONCLUSION AND RECOMMENDATIONS

In this study we explored how Buriti oil could modulate oxidative stress biomarkers and hormonal function in an *in vitro* experimental animal model using male Wistar rats. Our control groups only experienced normal physiological oxidative stress therefore were not oxidative stress induced. The findings of this study indicate that dietary supplementation of Buriti oil could have a protective effect against physiological oxidative stress and increase testosterone secretion.

Additionally in this study, the effect of Buriti oil supplementation on testicular and epididymal tissue was investigated. The dietary Buriti oil supplementation could have protective effects against LPO damages by restoring MDA levels of oxidative stress and increasing antioxidant enzyme activities (SOD, CAT and GSH). Our investigations of Buriti oil's effect on testosterone and estradiol indicated that antioxidants found in Buriti oil can influence Leydig cell function hence cause an increase of testosterone in the plasma. Our studies, however, showed that estradiol concentration in plasma was not increased by Buriti oil consumption.

We suggest that dietary Buriti oil consumption could have provided protective effect against testicular and epididymal lipid peroxidation damage by scavenging free radicals before they caused any damage.

To our knowledge, this is the first time an investigation focused on measuring testicular and epididymal status in rats supplemented with Buriti oil as well as plasma testosterone and estradiol concentrations.

Functional data such as sperm motility and sperm count was not conducted due to lack of proper infrastructure and this limited our data interpretation to oxidative stress enzymes, MDA and hormonal analysis only. More studies with advanced technologies are recommended to investigate effects of Buriti oil supplementation on all male reproduction parameters. We recommend that effects of Buriti oil on the male reproductive function of an oxidative stress induced rat model should be conducted.

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