The impact of organic hydroperoxides and a red palm oil supplemented diet on spermatogenesis, sperm function and sperm apoptosis

by

YAPO GUILLAUME ABOUA

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Supervisor: Dr Nicole BROOKS

Co-supervisor: Dr Stefan S du PLESSIS

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DECLARATION

I, Yapo Guillaume Aboua, declare that the contents of this thesis represent my own unaided work, and that the 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.

Signed **Date**

ABSTRACT

Many environmental, physiological, and genetic factors have been shown to impair sperm function through oxidative damage. Oxidative stress (OS) arises as a consequence of excessive reactive oxygen species (ROS) production and/or impaired antioxidant defence mechanisms. The decline in male reproductive health generated considerable public and scientific concerns about the possible role of environmental contaminants. A better understanding of how OS affects sperm function will be beneficial as it might help in the design of new and effective treatment strategies to combat the problem of increasing male subfertility. Studies have suggested that antioxidant nutrients and/or medicines play a protective role in human health. Crude red palm oil (RPO) is known to be the richest natural plant source of antioxidants such as carotenoids, tocopherols and metalloporpheryns. The aims of this study were twofold: (i) To establish an *in vivo* animal model of OS by exposing rat to organic hydroperoxide such as t-butyl hydroperoxide (tbHP) and cumene hydroperoxide (cHP) through repeated intraperitoneal injections that can be used for studying these effects on testicular tissue, epididymal sperm and sperm function as well as male reproductive parameters in general. (ii) To investigate the effects of a RPO supplemented diet on male reproductive parameters and tissue in animals exposed to OS.

In the first part of the study, male Wistar rats aged 10-12 weeks were randomly placed in groups and received standard rat chow (SRC) and water *ad lib*. Animals were injected intraperitoneally with saline (0.5 ml), t-butyl hydroperoxide (5μM, 10μM, 20μM and 40μM; 0.5 ml) or cumene hydroperoxide cHP (2.5μM, 5μM, 10μM and

20μM; 0.5 ml) over a 60 day period. In the second part, male Wistar rats aged 10-12 weeks were placed randomly in three groups and fed with SRC. Group 1 received no supplement while the food of groups 2 and 3 were supplemented with 2 mL and 4 mL RPO (in 25 gm SRC/day) respectively. Each group was further divided into 3 subgroups and injected intraperitoneally daily with either saline, 10μM cHP or 20μM tbHP respectively. This was done for 5 consecutive days per week over a 60 day period. Sperm concentrations, and motility, lipid peroxidation, superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) activities as well as apoptosis were assessed.

From the first part of the study, the long term *in vivo* intraperitoneal administration of organic hydroperoxides negatively affected the male reproductive system. It significantly lowered sperm concentration, sperm motility, and impaired antioxidant activities in both epididymal sperm and testicular tissue. The exposure to these hydroperoxides has also led to apoptosis in the sperm cells. We therefore, successfully developed an animal model to test the adverse effects of OS on male reproductive parameters. In the quest of finding possible treatments to the *in vivo* OS caused by organic peroxides, RPO was found to be an excellent dietary supplement in reversing the enzymatic and non-enzymatic antioxidants during the second part of the study. The RPO might actively be involved in the scavenging mechanism to reverse the reduced activities of SOD, CAT and GSH in order to maintain the balance between ROS and the antioxidant systems. In addition RPO might block the OS pathway (caused by H_2O_2) that led to DNA damage and apoptosis. The mechanisms by which RPO protection was achieved involves one or several different antioxidant properties exhibited by its components. We therefore, propose that a

daily intake of RPO supplement to the diet might be helpful to protect males against the adverse effects of ROS in sperm function and possibly assist to preserve fertility.

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DEDICATION

This dissertation is dedicated to

Justin Dorgeles ABOUA

Julianne Albertha KEYSER

And the

COTE D'IVOIRE

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GLOSSARY

Abbreviations	Definition/Explanation
AC	Adenylyl cyclase
APAF1	Adaptor that form apoptosome to activate caspases 9
•CH	Unpaired electron on the carbon atom
Ca^{2+}	Calcium cation
CAT	Catalase
cAMP	Cyclic adenosine monophosphate
cHP	Cumene hydroperoxide
CH_2	Methylene group
CYPs	Cytochrome P450
DAG	Diacylglycerol
DCF	Dichlorofluorescein
DCFH	2'7'-dichlorofluorescin
DIABLO	Direct inhibitor of apoptosis-binding protein
DISC	Death-inducing signalling complex
DNA	Deoxyribonucleic acid
EGFR	Epidermal growth factor R
ER	Estrogen receptor
FSH	Follicle-stimulating hormone
GS•	Glutathione radicals
G6PD	Glucose-6-phosphate-dehydrogenase
GPx	Glutathione peroxidase
GR	Glutathione reductase

GSH	Glutathione
GSSG	Glutathione disulfide
GST	Glutathione S-transferase
H_2O_2	Hydrogen peroxide
IAP	Inhibitor of apoptosis
IP3	Inositol 1,4,5-trisphosphate
KCl	Potassium chloride
LH	luteinizing hormone
LOO•	Lipid peroxyl radical
LOOH	Lipid hydroperoxides
LPO	Lipid peroxidation
MDA	Malondialdehyde
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NaCl	Sodium chloride
NK cells	Neutral Killer cells
NF-kB	Nuclear factor kB
8-OhdG	8- hydroxy-2-deoxyguanosine
O_2	Oxygen
O_2^{-}•	Superoxide anion
OAM	Outer acrosomal membrane
OH^-	Hydroxyl radicals
$ONOO^-$	Peroxynitrite anion
OS	Oxidative stress
PBS	Phosphate buffered saline

PCB	Aroclor 1254
PKA	Protein kinase A
PKC	Protein kinase C
$PLC\beta_1$	Phospholipase C beta
PLCγ	Phospholipase C gama
PM	Plasma membrane
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acids
RFU	Relative fluorescence unit
RLU	Relative luminescence unit
$RNOO^-$	Peroxyl radicals
ROS	Reactive oxygen species
RPO	Red palm oil
SMAC	Second mitochondria-derived activator of caspase
SOC	Store operated Ca^{2+}
SOD	Superoxide dismutase
SRC	Standard rat chow
TBARS	Thiobarbituric acid reactive substances
tbHP	T-butyl hydroperoxide
TK	Tyrosine kinase
TNF-α	Tumor necrosis factor alpha
UV	Ultraviolet
WHO	World Health Organization
ZP	Zona pellucida

Chapter I

Introduction

In recent years, there has been an increasing concern regarding the potential adverse effects of various environmental contaminants designated as endocrine disrupters or hormonally active agents (Saradha and Mathur, 2006). These concerns have originated in part, from observations of developmental and reproductive derangement in wildlife populations exposed to a wide range of synthetic chemicals and their by-products that have been released into the environment in large amounts since World War II. Prenatal and postnatal exposure to these endocrine disrupting chemicals could result in permanent and irreversible damage in both humans and animals (Colborn *et al*., 1993). The costs of environmental injury to reproduction (in both animals and humans) include subfertility, intra-uterine growth retardation, spontaneous abortion, and various birth defects (Fox, 2001, Yousef *et al*., 2005, Yousef *et al*., 2007, Newairy *et al*., 2009).

Many environmental, physiological, and genetic factors have been implicated in poor sperm function and infertility (Kovacic and Jacintho, 2001, Saradha and Mathur, 2006). The decline in male reproductive health might be caused by some common environmental factors and generated considerable public and scientific concerns about the possible role of environmental contaminants as endocrine disrupters (Irvine, 2000).

Epidemiological studies indicated a causal connection between human exposure to contaminants and endocrine disrupting effects such as poor sperm

quality (Swan *et al*., 2003) and increased incidence of cryptochidism (Weidner *et al*., 1998). Studies conducted on occupational pesticide workers have shown that exposure to various contaminants led to abortion, stillbirth, male infertility, neonatal deaths, congenital defects, testicular dysfunction and abnormalities (Xia *et al*., 2004, Xia *et al*., 2005).

The decrease of epididymal and caudal sperm density in rats exposed to environmental toxicants correlated with testicular spermatogenic arrest and fragmentation of Sertoli cells (Dalsenter *et al*., 1996, Pages *et al*., 2002, Kumar *et al*., 2004, Saradha and Mathur, 2006). Various environmental contaminants have been shown to impair sperm function through oxidative damage to sperm membrane. Organic hydroperoxides have been extensively used as model pro-oxidants to generate reactive oxygen species (Latour *et al*., 1995, Chen *et al*., 2000, Rajesh Kumar *et al*., 2002, Kaur *et al*., 2006b, Kumar and Muralidhara, 2007). ROS-mediated damage of sperm membranes have been reported to be responsible for impaired sperm motility (de Lamirande and Gagnon, 1992). The effects of environmental contaminants on the epididymis have been shown to result in sperm DNA damage (Saleh *et al*., 2002), sperm head abnormalities (Kumar *et al*., 2002), altered sperm function and their fertilizing ability (Kumar and Muralidhara, 2007).

ROS generated from abnormal sperm and leukocytes stimulate the process of apoptosis, resulting in the controlled death of sperm. ROS initiates a chain of reactions by activating caspases that ultimately lead to apoptosis (Said *et al*., 2004). Two factors protect the sperm DNA from oxidative insult: the characteristic tight packaging of the DNA and the antioxidants present in seminal plasma (Twigg *et al*., 1998). A better understanding of how oxidative stress (OS) affects sperm function will

be beneficial as it might help in the design of new and effective treatment strategies to combat the problem of increasing male subfertility.

Red palm oil (RPO) is a natural source of multiple macro- and micronutrients, trace elements, vitamins, as well as cocktail antioxidants (tocopherols and Carotene) (Sundram *et al.*, 2003). Carotene and tocopherols are natural antioxidants with important roles as inhibitors of oxidation in biological systems. Tocopherols are effective chain-breaking antioxidants because they produce stable and relatively unreactive antioxidant radicals. The interaction of carotenoids with peroxyl radicals may proceed via an unstable β-carotene radical adduct (Burton and Ingold, 1984, Rice-Evans *et al.*, 1997). The antioxidant potency of a chain-breaking antioxidant is determined by several factors such as chemical reactivity toward radicals, site of radical generation, site of the antioxidant, fate of antioxidant-derived radicals, concentration and mobility of the antioxidant at the microenvironment, and interactions with other antioxidants (Niki *et al.*, 1995).

The aims of this study were twofold:

(i) To establish an *in vivo* animal model of OS by exposing rats to organic hydroperoxides such as t-butyl hydroperoxide (tbHP) and cumene hydroperoxide (cHP) through repeated intraperitoneal injections that can be used for studding these effects on testicular tissue, epididymal sperm and sperm function as well as male reproductive parameters in general.

(ii) To investigate the effects of a RPO supplemented diet on male reproductive parameters and tissue in animals exposed to OS.

The thesis has been written in an article-based format and is composed of six chapters. Chapter I is a brief introduction which highlights the effects of environmental contaminants such as organic hydroperoxides on sperm function and male infertility. Furthermore, the aims of this study are mentioned. Chapter II focuses on the oxidative stress stature and apoptosis due to an imbalance between ROS and antioxidants during and after spermatogenesis. Red palm oil (RPO) is proposed as a possible treatment strategy. Chapter III is the first article titled "Impact of organic hydroperoxides on rat testicular tissue and epididymal sperm". This article has been accepted by the African Journal of Biotechnology. Chapter IV is the second article titled "Can a Red Palm Oil (RPO) diet reduce the effects of oxidative stress in rat spermatozoa?" This article is currently in review. Chapter V is the final article for the thesis titled "Red Palm Oil: A Natural Good Samaritan for Sperm Apoptosis?" which has been accepted by the Medical Technology SA. Finally, Chapter VI is the general discussion and conclusion chapter.

Chapter II

Literature Review

Cells living under aerobic conditions constantly face the oxygen paradox i.e. oxygen is indispensable for supporting life; however, its metabolites such as ROS can modify cell function. OS arises as a consequence of excessive ROS production and/or impaired antioxidant defence mechanisms (Agarwal *et al*., 2003). ROS are highly reactive oxidizing agents belonging to the class of free radicals. A free radical is any compound (not necessarily derived from oxygen), which contains one, or more unpaired electrons. The most common ROS that have potential implications in reproductive biology include superoxide ($O_2^{-}\bullet$) anion, hydrogen peroxide (H_2O_2), peroxyl ($RNOO^{\cdot}$) radicals, and the very reactive hydroxyl ($OH^{\cdot}$) radicals (Sikka, 1996, Ford, 2004, Henkel *et al*., 2005).

2.1 Organic hydroperoxides: A concern for mammalian fertility?

Organic hydroperoxides are widely used in the chemical industry as initiators of oxidation for the production of polymers and fibre-reinforced plastics, in the manufacturing of polyester resin coatings, and pharmaceuticals (Wang *et al*., 1993). cHP is produced at ~130°C via the oxidation of cumene with air in the presence of aqueous sodium carbonate as a catalyst. Cumene hydroperoxide may enter the environment from industrial discharges and spills, and also as a by-product of fuel oil

slicks exposed to ultraviolet (UV) light. It can cause cytotoxic effects including intracellular OS and cell necrosis (Tice and Brevard, 1998). Tb-HP is manufactured in a closed system and is used as an initiator or precursor of other initiators, which are used in polymerization reactions in the plastics industry (Tice and Brevard, 1998). Exposure occurs due to fugitive emission from equipment leakage and emission from shipment (tank truck and drum loading) (Tice and Brevard, 1998). Studies had shown that exposure to tb-HP dramatically increases the level of lipid peroxidation and enhances ROS generation in the testes of rats (Rajesh Kumar *et al*., 2002, Kaur *et al*., 2006a, Kumar and Muralidhara, 2007). Organic hydroperoxides have been extensively used as model prooxidants to induce oxidative stress in various *in vitro* systems (Latour *et al*., 1995, Chen *et al*., 2000, Rajesh Kumar *et al*., 2002, Kaur *et al*., 2006b, Kumar and Muralidhara, 2007).

2.2 Mammalian spermatogenesis and epidymal transit

Spermatogenesis is the process by which a complex, interdependent population of germ cells produces spermatozoa. Mammalian spermatogenesis can be divided into two phases (Rodriguez *et al*., 1997). The first phase of spermatogenesis starts after birth. It is characterized by the sequential appearance of cells within the seminiferous tubules, which corresponds to each stage of the germinal cell. The second phase starts during puberty (ongoing phase), characterized by the concomitant presence of stem cells and early spermatogonia to fully differentiated spermatozoa (Rodriguez *et al*., 1997).

Spermatogenesis is a chronological process that takes about 60 days in the rat and 72 days in man to complete (Hess, 1990). During this period, the immature germ cells, cyclically develop into highly specialized spermatozoa (Rodriguez *et al*., 1997). Spermatogonia undergo several mitotic divisions to generate a large population of cells called primary spermatocytes, which produce haploid germ cells during meiotic cell divisions. After passing the Sertoli cell barrier, spermatocytes reach the adluminal compartment and continue with the further prophase stages. During prophase the reduplication of DNA, the condensation of chromosomes, the pairing of homologous chromosomes and the "crossing over" takes place. After division the germ cells become secondary spermatocytes. They undergo no DNA replication and divide quickly to become spermatids. The two maturation divisions of each spermatocyte result in four haploid cells, namely the spermatids. More differentiations occur thereafter. Sperm released from the testis are unable to exhibit progressive motility or to capacitate, but acquire these abilities during their passage through the epididymis. These processes are referred to as maturation. Other maturational changes include the completion of nuclear condensation and changes in the expression and distribution of molecules on the sperm surface (Cooper *et al*.,1995, Yeung *et al*., 1995, Kirchnoff *et al*., 1997, Bone *et al.,* 2000).

In mammals, the epididymis is known to play an important role in the maturation and storage of sperm. During 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 OS for gametes (Dacheux *et al*., 2003). The sperm plasma membrane, being rich in polyunsaturated fatty acids (PUFA), is highly susceptible to ROS. Fortunately, the epididymis has been enriched with an antioxidant defence system that protects the sperm during their voyage through the

caput to the caudal region of epididymis and thereby facilitates their maturation process (Vernet *et al*., 2001).

2.2.1 Disturbances of spermatogenesis

Proliferation and differentiation of the male germ cells and the intratesticular and extratesticular mechanisms of regulation of spermatogenesis can be disturbed at every level (Holstein *et al*., 2003). This may occur as a result of environmental influences or may be due to diseases that directly or indirectly affect spermatogenesis (Holstein *et al*., 2003, Agarwal and Allamaneni, 2005). In addition, different nutritive substances, therapeutics, drugs, hormones and their metabolites, different toxic substances or x-radiation may reduce or destroy spermatogenesis (Holstein *et al*., 2003, Sheiner *et al*., 2003). Under these negative influences the testis responds by a reduction of spermatogenesis. This may be expressed in the reduced number of mature spermatids, in malformation of spermatids, missing spermiation, disturbance of meiosis, arrest of spermatogenesis at the stage of primary spermatocytes, reduced multiplication or apoptosis of spermatogonia (Bustos-Obergon and Gonzalez-Hormazabal, 2003, Holstein *et al*., 2003, Saradha and Mathur, 2006). If spermatogonia survive then spermatogenesis may be rescued. Otherwise spermatogenesis ceases and “shadows” of seminiferous tubules remain.

2.2.2 Effect of environmental contaminants on testicular function

Several environmental contaminants are known to impair germ cell development at various stages and in so doing reduce sperm count. Van Pelt and co-workers (1999) argued that the estrogen receptor (ER) expressed in Sertoli cells as well as in gonocytes, in the fetal rat testis, demonstrates the possible role of estradiol in testicular development and spermatogenesis (van Pelt *et al.*, 1999). Sertoli cells promote the developing germ cells by producing vital factors essential for germ cell development (Griswold, 1995). In addition, Sertoli cells of the seminiferous tubules govern the daily sperm production (Amann, 1970); therefore, any agent that impairs the viability and the function of Sertoli cells may have profound effects on spermatogenesis. Bustos-Obergon and Gonzalez-Hormazabal (2003) had shown that a single dose of 240 mg/kg malathion administered to male mice (10–12 weeks old) was toxic to the Leydig, Sertoli and spermatogenic cells (Bustos-Obergon and Gonzalez-Hormazabal, 2003). It also caused an early depletion of the seminiferous epithelium with reduction of epithelial height and tubular diameter. Disruption of Sertoli cell junctional proteins (Fiorini *et al.*, 2004) and tight junction molecules (Cheng and Mruk, 2002) may affect Sertoli cell function. Consequently, alterations of one of these proteins after toxicant exposure could impair either their classical functions (cell adhesion, formation of tight or gap junctions) or signal transduction, leading to disturbed germ cell development and infertility.

Apart from the production of spermatozoa, the testis is involved in the production of hormones that are required for various functions in the body, including maintenance of secondary sexual functions, and feedback on the hypothalamus and the pituitary to control the secretion of the gonadotropins luteinizing hormone (LH)

and follicle-stimulating hormone (FSH). The Leydig cells are primarily involved in the secretion of androgens, notably testosterone, as well as other steroids including estrogen (Sharpe, 1994). LH induced Leydig cell secretion of androgens, namely testosterone, is involved in regulation of spermatogenesis by targeting androgen receptors in the seminiferous epithelium. FSH targets receptors within the Sertoli cell to regulate spermatogenesis by stimulating the production of numerous Sertoli cell factors. Therefore, any factor affecting the LH stimulated Leydig cell function, in turn, can interrupt the endocrine regulation of spermatogenesis and consequently affects the reproductive performance. The testis is the major site for testosterone production, which plays a crucial role in the development of secondary sexual characteristics and in initiation as well as regulation of spermatogenesis. The reductions in testosterone levels due to the exposure to environmental toxicants have the potential to adversely affect normal sexual development in humans and wildlife.

It has been reported that human cytochrome P450 (CYPs), the enzyme involved in the conversion of various contaminants into metabolites have the ability to induce ROS production (Bondy and Naderi, 1994). The CYP enzymes of the steroidogenic pathway are also known to produce free radicals. These free radicals are produced as a result of electron leakage due to the interaction of steroid products or other pseudosubstrate with the enzymes. The inability of the pseudosubstrate to be oxygenated promotes the release of ROS (Peltola *et al.*, 1996). Some environmental contaminants are reported to produce ROS through the above-mentioned mechanism and thereby produce oxidative changes in the testis (Bustos-Obergon and Gonzalez-Hormazabal, 2003, Holstein *et al.*, 2003, Yousef *et al.*, 2005, Saradha and Mathur, 2006, Yousef *et al.*, 2007, Newairy *et al.*, 2009).

2.2.3 Effect of environmental contaminants on epididymis

As the spermatozoa progress through the epididymis, to attain progressive motility and fertilizing ability, various environmental toxicants target the efferent ducts and seminiferous epithelium (Hess, 1998). Several environmental toxicants have been reported to decrease the number of sperm in the caudal epididymis significantly with little or no decrease in testicular sperm number (Klinefelter and Suarez, 1997, Anway *et al.*, 2006, Saradha and Mathur, 2006). Compromised sperm plasma membrane after ROS exposure, led to the initiation of a lipid peroxidation cascade. Subsequently, sperm lose their capability for movement, acrosome reaction and penetration of the ova (Aitken and Clarkson, 1987). Toxicant-induced accelerated sperm conversion through the epididymis. It not only affects the number of sperm available for ejaculation, but also the quality by compromising the process of epididymal sperm maturation and the fertilizing capacity of sperm (Saradha and Mathur, 2006, Archibong *et al.*, 2008).

The decrease of epididymal and caudal sperm density of environment toxicant treated rats correlated with the testicular spermatogenic arrest and fragmentation of Sertoli cells (Dalsenter *et al.*, 1996, Pages *et al.*, 2002, Saradha and Mathur, 2006). Various environmental contaminants have been shown to impair sperm function through oxidative damage to sperm membranes. ROS-mediated damage of sperm membranes has been reported to be responsible for impaired sperm motility (de Lamirande and Gagnon, 1992, Archibong *et al.*, 2008). DNA damage and sperm head abnormalities (Kumar *et al.*, 2002) as well as sperm function (Archibong *et al.*, 2008) and sperm DNA integrity (Saleh *et al.*, 2002) have been proven to be due to the effects of environmental contaminants on the epididymis.

2.3 Sources of reactive oxygen species and free radicals in sperm

Apart from the conventional causes for primary pathologies of male reproductive system, environmental life style factors, systemic pathology, varicocoele, cryptorchidism, infections, obstructive lesions, cystic fibrosis, trauma, and tumours, oxidative stress has been identified as important causes of infertility (Agarwal *et al*., 2008, Makker *et al*., 2009) (Figure 2.1). Biologically, free radicals are formed from two sources (Aitken *et al*., 1996b, Griveau and Le Lannou, 1997, (Kovacic and Jacintho, 2001). One source common to all eukaryotic cells is the electron transport chain in the mitochondria. In the process of reducing O_2 to H_2O, the first step involves the addition of one electron to O_2 to form superoxide, $O_2^{-}\bullet$. Usually, superoxide is reduced to water via the addition of more electrons and hydrogen. However, there is a slow and basal level of leakage of superoxide anions, and this contributes to the level of free radicals biologically present.

In sperm cells, the sources of ROS are broadly dispersed between external and internal sources. External production of ROS, particularly $O_2^{-}\bullet$ and H_2O_2 can be the result of leukocyte contamination within the semen. This generation of ROS has been associated with subfertility or even infertility in patients (Aitken *et al*., 1996b, Griveau and Le Lannou, 1997, Henkel *et al*., 2005, Agarwal *et al*., 2007). Leukocytes are present throughout the male reproductive tract and are found in almost every human ejaculate (Tomlinson *et al*., 1992). However, the clinical significance of increased leukocyte infiltration in semen, that is, leukocytospermia, has been linked with poor sperm quality, reduced sperm hyperactivation, and defective sperm

function (Wolff, 1995). The World Health Organization (WHO) defines leukocytospermia as the presence of peroxidase positive leukocytes in concentrations of $>1x10^6$ per milliliter of semen (WHO, 1999). Morphologically abnormal spermatozoa and seminal leukocytes have been established as the main sources of high ROS production in human ejaculates (Aitken *et al.*, 1994, Ochsendorf, 1999). Gomez and colleagues (1998) have indicated that levels of ROS production by pure sperm populations were negatively correlated with the quality of sperm in the original semen (Gomez *et al.*, 1998). The link between poor semen quality and increased ROS generation lies in the presence of excess residual cytoplasm (cytoplasmic droplet).

When spermatogenesis is impaired, the cytoplasmic extrusion mechanisms are defective, and spermatozoa are released from the germinal epithelium carrying surplus residual cytoplasm. Under these circumstances, the spermatozoa that are released during spermiation are believed to be immature and functionally defective (Huszar *et al.*, 1997, Agarwal *et al.*, 2003). Retention of residual cytoplasm by spermatozoa is positively correlated with ROS generation.

Regarding ROS generated by the spermatozoa *per se*, recently, two mechanisms involved in ROS generation have been characterized in rat epididymal sperm (Vernet *et al.*, 2001). One mechanism depends on the mitochondrial respiratory chain (Gavella and Lipovac, 1992) while the other mechanism relies on an enzymatic system related to the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase family found bound to the sperm plasma membrane

(Vernet *et al.*, 2001). The mitochondrial electron transport chain is known to produce ROS during physiological, but also during pathological conditions.

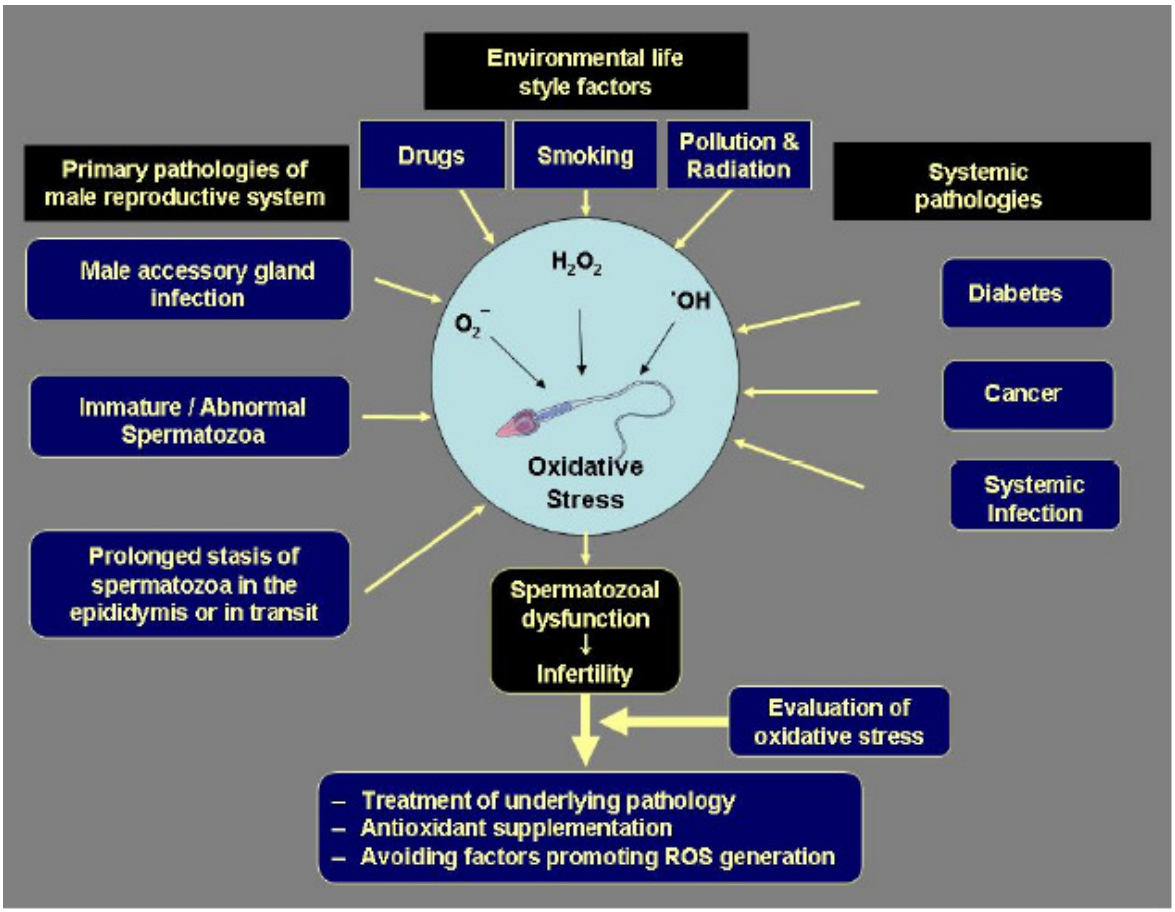

Figure 2.1: Sources of reactive oxygen species and free radicals in sperm (Reproduced and modified from Cocuzza *et al.*, 2007).

2.4 Reactive oxygen species and sperm function

Free radicals may have beneficial or detrimental effects on sperm functions, depending on their nature and concentration (Baker *et al.*, 2003). At low concentrations, ROS have biopositive effects and act selectively. They are metabolic intermediates in the metabolism of prostanoids (Cosentino *et al.*, 2003, Manabe *et al.*, 2004), in the regulation of vasotonus (Ignarro, 1990), in gene regulation (Schreck

et al., 1992, Allen and Tresini, 2000), in the regulation of cellular growth, and the function of extracellular as well as intercellular signal transduction (Demple *et al*., 1991, Joseph and Cutler, 1994). Furthermore, they are involved in antimicrobial defence and immunological surveillance (Test and Weiss, 1986, Klebanoff, 1992). At high concentrations, ROS react non-specifically and exert bionegative effects and damage all major classes of biomolecules such as unsaturated lipids in membranes (Kappus, 1986), proteins (Pacifici and Davies, 1990), nucleic acids (Gandini *et al*., 2000, Irvine, 2000, Sikka, 2001, Allamaneni *et al*., 2004) and carbohydrates (Gracy *et al*., 1999).

2.4.1 Sperm motility and viability

ROS have been shown to have a spectrum of variable effects on spermatozoa depending on the extent of oxidative stress (Allamaneni *et al*., 2004). Aitken and co-workers (1993) reported that a low concentration of hydrogen peroxide did not have any effect on sperm motility, but did suppress sperm-egg fusion (Aitken *et al*., 1993). This may also explain why patients with normal semen parameters can still experience infertility. In such patients, the ROS levels are not high enough to impair basic semen analysis parameters, but can cause defects in other processes that are required for fertilization, such as sperm-oocyte interaction. Excessive ROS levels are related to an increase in lipid peroxidation of the sperm plasma membrane. It had previously been shown that a correlation exists between ROS and sperm concentration, motility, and morphology (Sikka, 1996, Agarwal *et al*., 2006).

The link between ROS and reduced motility may be due to a cascade of events that result in a decrease in axonemal protein phosphorylation and sperm immobilization both of which are associated with a reduction in membrane fluidity that is necessary for sperm-oocyte fusion (de Lamirande and Gagnon, 1995). Another hypothesis is that H_2O_2 can diffuse across the membranes into the cells and inhibit the activity of some enzymes such as glucose-6-phosphate-dehydrogenase (G6PD). This enzyme controls the rate of glucose flux through the hexose monophosphate shunt, which in turn, controls the intracellular availability of NADPH. This in turn is used as a source of electrons by spermatozoa to fuel the generation of ROS by an enzyme system known as NADPH oxidase (Aitken *et al.*, 1997). Inhibition of G6PD leads to a decrease in the availability of NADPH and a concomitant accumulation of oxidized glutathione and reduced glutathione. This can reduce the antioxidant defenses of the spermatozoa and increase peroxidation of membrane phospholipids (Griveau *et al.*, 1995a). Garner and co-workers (1997) argued that the mitochondrial activity, viability and acrosomal integrity of sperm cells correlate positively with fertility (Garner *et al.*, 1997).

2.4.2 Capacitation

Capacitation confers upon the spermatozoon an ability to gain hyperactive motility, interact with oocyte zona pellucida (ZP), undergo the acrosome reaction and initiate oocyte plasma membrane fusion (Yanagimachi, 1994). Capacitation of a sperm cell is required before fertilisation. In every mammalian species studied, the molecular mechanisms and signal transduction pathways involved in this process are not clearly understood (Naz and Rajesh, 2004). Capacitation involves an increase in

membrane fluidity, cholesterol efflux, ion fluxes resulting in alteration of sperm membrane potential, increased tyrosine phosphorylation of proteins, induction of hyperactivation and the acrosome reaction (de Lamirande *et al.*, 1993, de Lamirande and Gagnon, 1993a) (Figure 2.1). Other studies have found that incubating spermatozoa with low concentration of H_2O_2 stimulates sperm capacitation, hyperactivation, and the ability of the spermatozoa to undergo the acrosome reaction and oocyte fusion (de Lamirande *et al.*, 1993, de Lamirande and Gagnon, 1993a, Griveau *et al.*, 1994, Aitken, 1995, Aitken *et al.*, 1995, Aitken, 1997).

During the initial stages of capacitation, intracellular calcium concentrations start to rise, ROS generation is initiated, cAMP concentrations increase and sperm develop a highly vigorous form of motility known as hyperactivation. Capacitation is also associated with a global increase in tyrosine phosphorylation, as a consequence of ROS-induced changes in the redox status of the cells (Aitken, 1995, Aitken *et al.*, 1995), and an increase in cAMP generation (Visconti *et al.*, 1995). The induction of tyrosine phosphorylation is one of the most important events in capacitation, since if it is blocked by tyrosine kinase inhibitors such as genistein, capacitation cannot occur (Aitken *et al.*, 1996a). The redox regulation of tyrosine phosphorylation is a key component of the mechanisms controlling sperm capacitation. However, it is not the only condition (Aitken, 1997). According to Zini and co-workers, superoxide and nitric oxide also take part in these processes (Zini *et al.*, 1995). Free radicals are also involved in the fusion of spermatozoa with the oocyte (de Lamirande and Gagnon, 1993a, Gadella *et al.*, 2001). Nitric oxide plays a role in the sperm's ability to fuse with the oocyte, but it has no action in ZP binding (Francavilla *et al.*, 2000).

During capacitation, efflux of cholesterol from the sperm plasma membrane will enhance permeability to $HCO3^-$ and Ca^2+ leading to activation of adenylyl cyclase (AC) resulting in cAMP production and protein kinase A (PKA) activation leading to protein tyrosine phosphorylation. The tyrosine phosphorylation can also occur via activating epidermal growth factor R (EGFR). High degree of phosphorylation will cause F-actin polymerization and translocation of PLC to the plasma membrane (Breitbart, 2002).

2.4.3 Acrosome reaction

The acrosome reaction involves multiple fusions of the plasma membrane and outer acrosomal structures of the acrosome, resulting in release of the acrosomal content and exposure of the inner acrosomal membrane (Zaneveld *et al*., 1991) (Figure 2.1). It is a prerequisite for penetration of spermatozoa through the ZP for fertilisation of the oocyte (Kohn *et al*., 1997, Gadella *et al*., 2001). The acrosome reaction is defective in spermatozoa with a high concentration of ROS. Griveau and Le Lannou (1997) reported that the acrosome reaction in human spermatozoa appeared susceptible to ROS and that H_2O_2 inactivates several enzymes, including glutathione peroxidase, superoxide dismutase, and glucose-6-phosphate (Griveau and Le Lannou, 1997). Studies have shown that O_2^{-}• serves an extremely important regulatory role in the spermatozoon, facilitating both hyperactivated movement and the induction of the acrosome reaction (de Lamirande and Gagnon, 1993b, Griveau *et al*., 1995b, Flesch and Gadella, 2000, Gadella *et al*., 2001) (Figure 2.1).

During the acrosomal reaction, ZP binds to at least two different receptors in the plasma membrane. One (R) is a Gi-coupled receptor that activates

phospholipase C beta ($PLC\beta_1$) and may regulate AC activity to produce cAMP and activate PKA. The other is a tyrosine kinase (TK) receptor coupled to phospholipase C gamma (PLCγ). Activation of the phospholipases would generate inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). PKA and IP3 would activate $[Ca^{2+}]_i^-$ channels in the outer acrosomal membrane (OAM) and DAG would activate PKC to open $[Ca^{2+}]_i^-$ channels in the plasma membrane (PM). As a result, cytosolic $[Ca^{2+}]$ increases and acrosomal $[Ca^{2+}]$ is decreased leading to store operated Ca^{2+} (SOC) activation and a sustained enhancement of cytosolic $[Ca^{2+}]$. The relatively high $[Ca^{2+}]$ (about 500 nM) can activate actin severing protein to disperse F-actin barrier intervening between the outer acrosomal membrane and the overlying plasma membrane. The two membranes would then be able to come into contact and fuse (Breitbart, 2002).

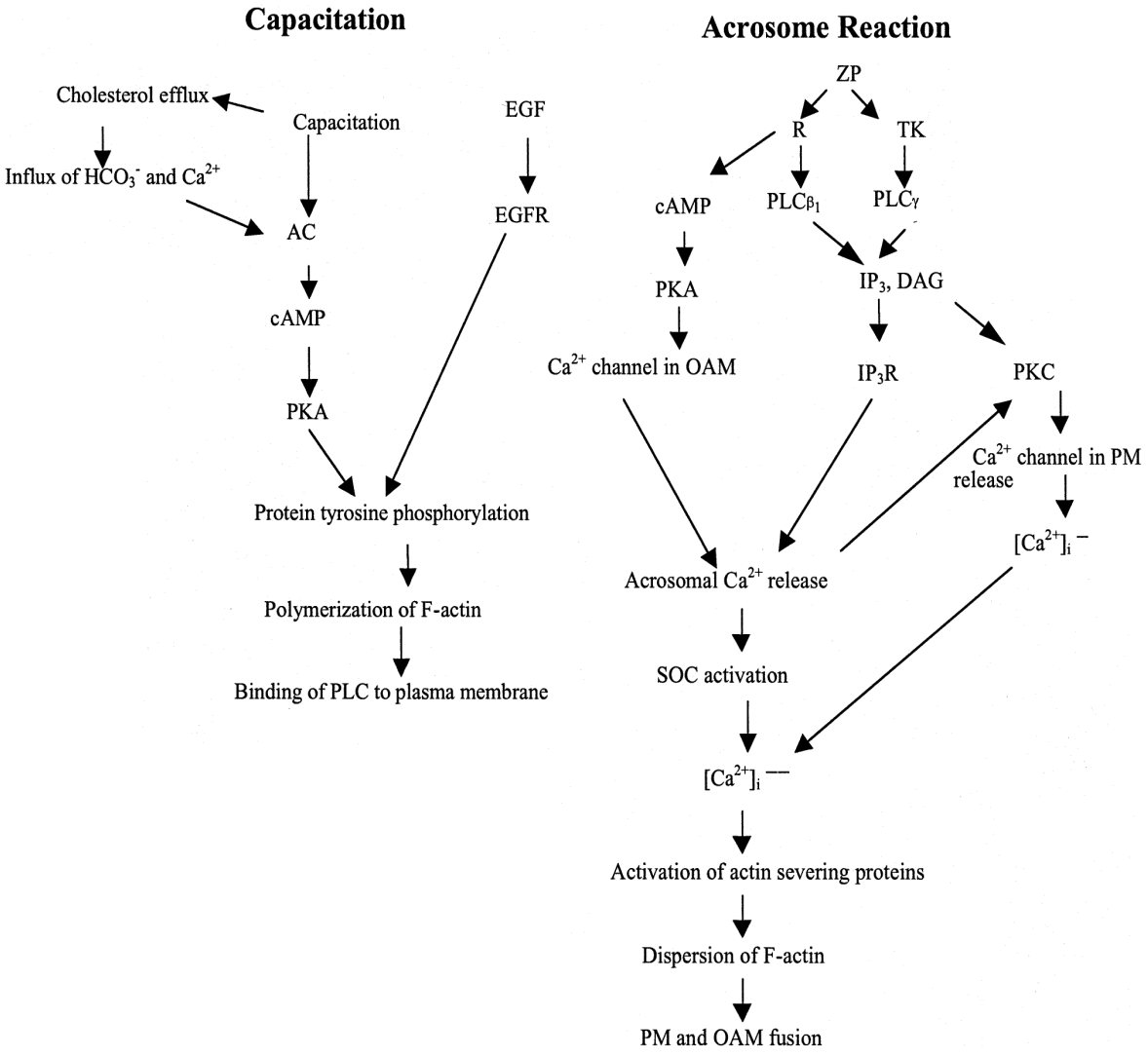

Figure 2.2: Possible signaling in sperm capacitation and acrosome reaction (Reproduced from Breitbart, 2002).

2.5 Reactive oxygen species and apoptosis

Although apoptosis shares certain common mechanisms with necrosis, necrosis is referred to as an “accidental”, non-physiological or passive type of cell death that occurs when cells die from severe and sudden injury, such as ischemia, sustained hyperthermia and physical or chemical trauma (Cohen, 1993, Samali *et al.*, 1996). On the other hand apoptosis is a genetically controlled active cell death process implicated as being a critical physiological mechanism involved in

development and tissue homeostasis (Wilson, 1998, Joza *et al*., 2002). Apoptosis is a protective mechanism in multicellular organisms whereby infected, excessive, potentially dangerous or seriously damaged cells are eliminated or removed (Turk *et al*., 2002). A cell will undergo apoptosis as a result of information received from its environment interpreted in the context of internal information, such as its cell type, state of maturity and developmental history (Hale *et al.*, 1996).

Two distinct pathways exist in the initiation of apoptosis. In the extrinsic or receptor-linked apoptotic pathway, the induction of apoptosis occurs via death receptors (cell surface receptors) that transmit apoptotic signals initiated by specific ligands (Turk *et al*., 2002, Sinha Hikim *et al*., 2003, LaCasse *et al*, 2004, Fadeel and Orrenius, 2005, Jin and El-Deiry, 2005). The intrinsic pathway is triggered by stress stimuli, including growth factor deprivation and DNA damage (Schuler and Green, 2001). This pathway involves the release of an extrinsic protein, cytochrome c on the outer surface of the inner mitochondrial membrane from the mitochondria during apoptosis (Sinha Hikim *et al.,* 2003, LaCasse *et al*, 2004, Fadeel and Orrenius, 2005, Jin and El-Deiry, 2005). The activation of the apoptosis-signalling pathway occurs in response to regulatory factors such as bcl-2 (Joza *et al*., 2002, Burlacu, 2003) and p53 (Chang, 2002). The cell intrinsic pathway triggers apoptosis in response to DNA damage, defective cell cycle, detachment from the extracellular matrix, hypoxia, loss of survival factors or other types of severe cell distress (Figure 2.3) (Ashkenazi, 2002).This pathway involves activation of the pro-apoptotic arm of the bcl-2 gene superfamily, which, in turn, engages the mitochondria to cause the release of apoptogenic factors such as cytochrome c and second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein (SMAC/DIABLO) into the cytosol (Adams and Cory, 1998, Green, 2000, Hunt and Evan, 2001). In the

cytosol, cytochrome c binds the adaptor APAF1, forming an 'apoptosome' that activates the apoptosis-initiating protease caspase-9 (Figure 2.3). In turn, caspase-9 activates 'executioner' proteases caspase-3, -6 and -7. SMAC/DIABLO promotes apoptosis by binding to inhibitor of apoptosis (IAP) proteins and preventing these factors from attenuating caspase activation.

The cell-extrinsic pathway triggers apoptosis in response to engagement of death receptors by their ligands. This pathway stimulates the apoptotic caspase machinery independently of p53. Ligand-induced activation of cell-surface death receptors leads to rapid assembly of a death-inducing signalling complex (DISC) and activation of the apoptosis-initiating proteases caspase-8 and caspase-10.These caspases, in turn, activate the same set of executioner caspases that are activated by the cell intrinsic pathway through caspase-9. The cell-extrinsic pathway is becoming recognized as an important mechanism that is used by NK cells and cytotoxic T lymphocytes to trigger apoptosis in virus-infected cells and in tumour cells (Ashkenazi, 2002) (Figure 2.3). The induction of apoptosis via the intrinsic or extrinsic apoptotic pathways result in the activation of an initiator caspase, which activates a cascade of events leading to the activation of effector caspases, responsible for the cleavage of key cellular proteins that lead to the typical morphological changes observed in cells undergoing apoptosis. Caspase-8 and caspase-10 are inhibitor caspases in death receptor-mediated apoptosis, while caspase-9 is the initiator caspase in mitochondrion-dependent apoptosis (Chen and Wang, 2002). These pathways differ in one fundamental aspect: one is "external" as it is promoted by a series of specific external ligands operating through defined transmembrane receptors; the other is an internal system where activation of the effector enzymes is induced by intracellular changes, involving the mitochondria (Garland, 2000, Joza *et*

al., 2002, Turk *et al*., 2002). Despite the difference in the manner of initiation, the extrinsic and intrinsic pathways merge at the level of caspases-3 and 7 and once activated, they cleave intracellular targets, ultimately leading to the manifestation of apoptosis.

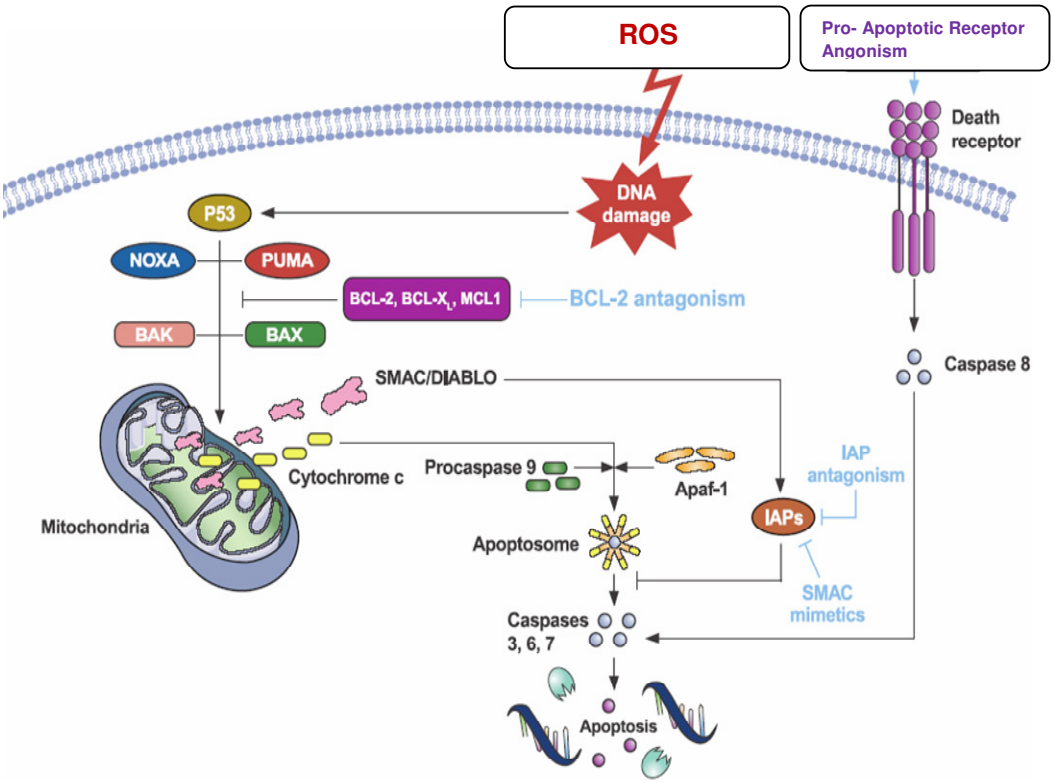

Figure 2.3: The two signalling pathways initiating the apoptotic suicide programme in mammalian cells (Reproduced and modified from Ashkenazi, 2002).

Shrinkage in total cell volume, an increase in cell density, compaction of some cytoplasmic organelles and dilatation of the endoplasmic reticulum accompany morphological changes during apoptosis (Gewies and Grimm, 2003). Chromatin condensation and nuclear envelope breakdown occur during apoptosis however, the

exact mechanisms are unclear (Hale *et al*., 1996). During apoptosis lamin disassembly occurs by proteolysis, which may promote the formation of fragments of DNA by allowing the release of matrix attachment regions to give access to the endonucleases. The nucleus shrinks and its chromatin becomes very dense, collapsing into patches, then into crescents in tight opposition to the nuclear envelope, and finally in many cells into one or several dense spheres (Ormerod, 1998). Concomitant with these early nuclear changes, the cytoplasm shows signs of condensation, microvilli (if present) disappear and blunt protuberances form on the cell surface (Kerr and Harmon, 1991). There are numbers of biochemical changes, which occur in the plasma membrane of apoptotic cells. One of these changes is an alteration of carbohydrates on the plasma membrane of the apoptotic cell, which could play a role in the preferential binding of macrophages to apoptotic cells (Samali *et al*., 1996).

In the early stages of apoptosis, changes occur at the cell surface and one of these changes is the translocation of phosphatidylserine (PS) from the inner surface of the plasma membrane to the outside. Annexin is a calcium dependant phospholipid binding protein with high affinity for PS offering the possibility of detecting cells in the early phase of apoptosis before the loss of cell membrane integrity and permits the discrimination between live, necrotic and apoptotic cell populations. A key protease, caspase-3, activated during the early stages of apoptosis is present in cells undergoing apoptosis, which in turn cleaves and activates other caspases triggering a cascade of proteolytic cleavage events (Green and Kroemer, 1998, Stennicke and Salvesen, 1998, Porter and Janicke, 1999, Slee *et al*., 1999). Antibodies against caspase-3 in cells, which have undergone apoptosis serves to differentiate and quantify cell populations into apoptotic and non-apoptotic

cells (Weil *et al.*, 1998). A characteristic feature of apoptosis is DNA fragmentation, one of the later steps of apoptosis, which results from the activation of endonucleases during the apoptotic process (Gandini *et al.*, 2000, Nagata, 2000). Intracellular stress results in the activation of the mitochondrial, or intrinsic pathway which leads to cytochrome c release, apoptosome formation, and caspase activation. Extracellular ligand binding to death receptors triggers the extrinsic pathways that can either directly result in the activation of the caspases, or require further amplification through the mitochondrial pathway dependant on the cell type. Both apoptotic signalling pathways converge at the level of effector caspases, such as caspases- 3 and - 7 (LaCasse *et al.*, 2004).

With regard to male reproduction, apoptosis is a physiological phenomenon in the body that helps to discard abnormal sperm. ROS generated from abnormal sperm stimulate the process of apoptosis, resulting in the death of sperm. ROS initiates a chain of reactions by activating caspases that ultimately lead to apoptosis (Said *et al.*, 2004, Cocuzza *et al.*, 2007). When ROS levels are raised pathologically, the process of apoptosis is also initiated in mature sperm. Two factors protect the sperm DNA from oxidative insult: the characteristic tight packaging of the DNA and the antioxidants present in seminal plasma (Twigg *et al.*, 1998). However, oxidative stress (OS) may develop as a result of an imbalance between ROS generation and antioxidant scavenging activities (Sikka, 2001). In general, DNA bases and phosphodiester backbones are very susceptible to peroxidation. In addition, spermatozoa are particularly susceptible to OS-induced damage because their plasma membranes contain large quantities of polyunsaturated fatty acids and their cytoplasm contains low concentrations of scavenging enzymes (Sharma and Agarwal, 1996). Strong evidence suggests that high levels of ROS mediate the

occurrence of high frequencies of single- and double-strand DNA breaks commonly observed in the spermatozoa of infertile men (Fraga *et al.*, 1996, Kodama *et al.*, 1996, Kodama *et al.*, 1997, Sun *et al.*, 1997, Aitken and Krausz, 2001). The formation of 8- hydroxy-2-deoxyguanosine (8-OhdG) has been considered as a key biomarker for this oxidative DNA damage (Ames *et al.*, 1993). A significant positive correlation between ROS and DNA fragmentation was reported (Barroso *et al.*, 2000). Furthermore, studies in which the sperm was exposed to artificially produced ROS resulted in a significant increase in DNA damage in the form of modification of all bases, production of base-free sites, deletions, frame shifts, DNA cross-links and chromosomal rearrangements (Twigg *et al.*, 1998). The process of apoptosis is accelerated by ROS-induced DNA damage, which ultimately leads to a decline in the sperm count. Wang and co-workers found a positive relationship between increased sperm damage, as indicated by increased oxidative stress, and caspase-mediated apoptosis in patients with male factor infertility (Wang *et al.*, 2003). The significant positive correlation of ROS with cytochrome c and caspases 9 and 3 suggests possible DNA damage through increased ROS production. Apoptosis was also correlated negatively with fertilisation rates (Host *et al.*, 2002).

2.6 Reactive oxygen species and their potential scavengers

Lipid peroxidation (LPO) of the sperm membrane is considered to be the key mechanism of ROS-induced sperm damage leading to infertility. Sperm, unlike other cells, are unique in structure, function, and susceptibility to damage by LPO (Agarwal *et al.*, 2005a, Agarwal *et al.*, 2005b). A variety of defence mechanisms encompassing antioxidant enzymes (SOD, catalase, glutathione peroxidase and reductase),

vitamins (E, C, and carotenoids), and biomolecules (glutathione and ubiquinol) are involved in biological systems (Liu and Lee, 1998, Hamilton *et al*., 2000). The presence of ROS in the immediate environment of a sperm cell represents an imbalance between the cellular production of these molecules and their destruction by scavengers. These scavengers are localized within sperm and in seminal plasma. Some of these compounds are molecules with intrinsic radical-scavenging activity such as α-tocopherol, ascorbic acid, uric acid, glutathione (Gutteridge and Halliwell, 1989, Sikka, 1996, Bilodeau *et al*., 2001), pyruvate (de Lamirande and Gagnon, 1992, Upreti *et al*., 1998), taurine, hypotaurine and albumin (Eckert and Niemann, 1996, Jaakma *et al*., 1997) superoxide dismutase (SOD) and the glutathione–peroxidase–reductase system (Alvarez *et al*., 1987, Alvarez and Storey, 1992). Although the presence of these enzymatic protectants is extensive intracellularly, their protective roles extracellularly are limited as their levels are low in extracellular compartments. Instead, small molecule scavengers like vitamin C (ascorbate) and vitamin E (tocopherols) seem to play a more important role in the extracellular milieu. The supplemental intake of vitamins A, vitamin E and/or vitamin C improved reproductive function in laboratory and farm animals (Luck *et al*., 1995, Baldi *et al*., 2000, Tan *et al*., 2003). It was also shown to improve sperm quality in heavy smokers (Dawson *et al*., 1992, Mello *et al*., 2001) and in male factor infertility patients (Lenzi *et al*., 1993b, Dalvit *et al*., 1998). However, there is also some uncertainty as to whether oral administration of vitamin E increases its concentration in seminal plasma (Dalvit *et al*., 1998, Ford and Whittington, 1998).

The natural balance of essential nutrients in foods minimizes the risk of adverse nutrient interactions and toxicity. A great number of studies have suggested

that antioxidant nutrients and/or medicines play a protective role in human health (Aruoma, 1999, Fang *et al.,* 2002, Devasagayam *et al.*, 2004). Because of the limitations of enzymatic antioxidants in infertile men (Lewis *et al.*, 1995), it is more practical (from a nutritional perspective) to consider studies aimed at the beneficial effects of dietary-derived antioxidants on spermatozoa function. Favorable attributes of natural diets include multiple macro- and micronutrients, trace elements, vitamins and antioxidants.

2.7 Red palm oil as sources of antioxidants

Over 95% of palm oil consists of mixtures of triacylglycerols (glycerol molecules esterified with three fatty acids) (Sambanthamurthi *et al*., 2000, Sundram *et al*., 2003). The triacylglycerols in palm oil partially define most of the physical characteristics of the palm oil such as melting point and crystallisation behaviour. The minor constituents of RPO can be divided into two groups. The first group consists of fatty acid derivatives, such as partial glycerides (MGs, DGs), phosphatides, esters and sterols. The second group includes classes of compounds not related chemically to fatty acids. These are the hydrocarbons, aliphatic alcohols, free sterols, tocopherols and pigments. Trace metals reported to be present in palm oil are iron, zinc, manganese, cadmium and lead (Sambanthamurthi *et al*., 2000). The nonglyceride fraction of palm oil consists of sterols, triterpene alcohols, tocopherols, phospholipids, chlorophylls, carotenoids and volatile flavour components, such as aldehydes and ketones. Crude RPO is known to be the richest natural plant source of carotenoids in terms of provitamin A equivalents, such as α-carotene and β-carotene (Sundram *et al.*, 2003). The colour of RPO is due to the presence of tocopherols and tocotrienols. Crude palm oil is considered the world's richest natural plant source of

carotenoids. Its retinol (provitamin A) equivalent content has been estimated at 15 times that of carrots and 300 times that of tomatoes (Sundram *et al.*, 2003). Since metabolic processes regulate the *in vivo* conversion of β-carotene to vitamin A, the possibility of toxicity or hypervitaminosis arising from the continued consumption of red palm oil should not occur. The absorption and conversion of β-carotene to vitamin A, which is metabolically regulated, also declines with increasing dietary intake (Chandrasekharan and Sundram, 1997). Apart from this major nutritional implication, carotenoids have significant antioxidant properties. α- and β-carotene, as well as lycopene, are important antioxidants because of their ability to act as effective quenchers of singlet oxygen (Krishnamoorthy *et al.*, 2007). These compounds may act as synergists with chain-breaking antioxidants. Pokorny (1987) defined the synergists as compounds that have little or no antioxidant activity of their own but which can enhance the activity of chain-breaking antioxidants (Pokorny,1987).

2.7.1 RPO antioxidants and lipid hydroperoxides

Antioxidants are compounds that protect biological systems against the potentially harmful effects of processes or reactions that can cause excessive oxidation (Krinsky, 1993). When considering the inhibition of lipid oxidation, antioxidants are classically divided into two types: chain-breaking antioxidants and preventive antioxidants (Frankel, 1980, Wayner *et al.*, 1986, Niki *et al.*, 1995). Chain-breaking antioxidants are substances inhibiting the propagation step i.e. they interrupt the autoxidation chains. This period of strong inhibition of lipid oxidation is called the induction period or lag time. The antioxidant potency of a chain-breaking antioxidant is determined by several factors such as chemical reactivity toward

radicals, site of radical generation, site of the antioxidant, fate of antioxidant-derived radicals, concentration and mobility of the antioxidant at the microenvironment, and interactions with other antioxidants (Niki *et al.,* 1995). Preventive inhibitors decrease the rate of autoxidation by suppressing the rate of initiation reactions. Metal chelators are preventive antioxidants by complexing with transition metal ions, thereby inhibiting the metal-catalyzed initiation and decomposition of hydroperoxides. Other mechanisms of preventive antioxidants include singlet oxygen quenching, oxygen scavenging and hydroperoxide reduction (Frankel, 1995, Frankel and Meyer, 2000).

2.7.1.1 Tocopherols and tocotrienols

A tocopherol molecule has three chiral centers in its phytyl side chain making a total of eight stereoisomeric forms possible (Frankel, 1995). The radical scavenging properties of tocopherols reside in the fused chroman ring system. The phytyl side chain has an effect on the location of tocopherols in different systems and thus on the antioxidant properties of tocopherols (Frankel, 1995). Tocopherols are effective chain-breaking antioxidants because they produce stable and relatively unreactive antioxidant radicals. The tocopheroxyl radicals (TO•) are stable due to the resonance stabilization of their phenoxyl structure (Burton and Ingold, 1984). In addition to their activity as chain-breaking antioxidants, tocopherols have other mechanisms of action, which may influence the initiation of oxidation reactions and thus the formation of hydroperoxides. Tocopherols are effective inhibitors of photo-oxidation by reacting with singlet oxygen either by physical quenching or by chemical reactions (Kamal-Eldin and Appelqvist, 1996). They may also inhibit the enzymatic oxidation catalyzed by lipoxygenase.

2.7.1.2 Carotenoids

Carotenoids are natural pigments that are synthesized by plants and are responsible for the bright colours of various fruits and vegetables. There are several dozen carotenoids in the foods that we eat, and most of these carotenoids have antioxidant activity (Chandrasekharan and Sundram, 1997, Rice-Evans *et al.,* 1997, Sundram *et al*., 2003). β-carotene has been best studied since. β-carotene and others carotenoids have antioxidant properties *in vitro* and in animal models (Krinsky, 2001, Krishnamoorthy *et al*., 2007). Mixtures of carotenoids or associations with others antioxidants (e.g. vitamin E) can increase their activity against free radicals. The antioxidant actions of carotenoids are based on their singlet oxygen quenching properties and their ability to trap peroxyl radicals (Stahl and Sies, 1996). This results in an excited carotenoid, which has the ability to dissipate newly acquired energy through a series of interactions with the solvent, thus regenerating the original unexcited carotenoid. The unexcited carotenoid can be reused for further cycles of singlet oxygen quenching. The quenching activity of a carotenoid mainly depends on the number of conjugated double bonds of the molecule and is influenced to a lesser extent by carotenoid end groups (cyclic or acyclic) or the nature of substituents in carotenoids containing cyclic end groups. The prevention of lipid peroxidation by carotenoids has been suggested to be mainly via singlet oxygen quenching (Stahl and Sies, 1996). β -Carotene is also a scavenger of peroxyl radicals, especially at low oxygen tension (Burton and Ingold, 1984). This activity may be also exhibited by other carotenoids. The interactions of carotenoids with peroxyl radicals may proceed via an unstable β-carotene radical adduct (Burton and Ingold, 1984, Rice-Evans *et al.,* 1997). Carotenoid adduct radicals have been shown to be highly resonance stabilized and are predicted to be relatively unreactive. They may further undergo

decay to generate nonradical products and may terminate radical reactions by binding to the attacking free radicals (Rice-Evans *et al*., 1997). Carotenoids act as antioxidants by reacting more rapidly with peroxyl radicals than do unsatured acyl chains.

Antioxidants may also inhibit the decomposition of hydroperoxides by acting as radical scavengers, metal chelators or reducers of hydroperoxides to more stable hydroxyl compounds. In addition, there is increased evidence that antioxidants have different effects on hydroperoxide formation and decomposition (Frankel, 1995). 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 (Frankel and Meyer, 2000).

2.7.1.3 Chlorophylls

Besides the carotenoids, the other important groups of pigments in palm oil are the chlorophylls. Structurally, the chlorophyll molecule contains a porphyrin (tetrapyrrole) nucleus with a chelated magnesium atom in the centre. Chlorophylls are fat-soluble as a result of a phytol chain attached to one of the porphyrin rings (Sambanthamurthi *et al*., 2000). Metalloporphyrins are a unique class of stable catalytic antioxidants possessing a broad range of antioxidant capacities that include the dismutation of superoxide (Pasternack *et al*., 1981, Faulkner *et al*., 1994, Day *et al*., 1995), hydrogen peroxide (Day *et al*., 1997) and scavenging of peroxynitrite (Szabo *et al.,* 1996, Salvemini *et al*., 1998). In addition to superoxide dismuting activity such as catalase-like activity, *In vitro*, metalloporphyrins display other

antioxidant capacities (Day et al., 1997), inhibition of lipid peroxidation (Day *et al.*, 1999). *In vitro* models of oxidative stress have been useful both in terms of confirming the antioxidant activities of metalloporhyrins obtained in cell-free systems and predicting their use as antioxidants in more complex *in vivo* models of human disease. Metalloporphyrins have been shown to be protective in a wide variety of *in vitro* oxidative stress models involving the generation of O_2, H_2O_2 and peroxynitrite (ONOO) alone or in concert.

2.7.1.4 Coenzyme Q_{-10}

Coenzyme Q_{-10} (CoQ_{10}) is a non-enzymatic antioxidant that is related to low density lipoproteins and protects against peroxidative damage (Frei *et al.*, 1990). CoQ_{10} is a lipid-soluble benzoquinone derivative that resides in the inner mitochondrial membrane and is an essential co-activator in shuttling electrons from complexes I and II to complex III of the electron transport chain during oxidative phosphorylation. CoQ10 plays a vital role in ATP production and serves as an anti-oxidant in both mitochondrial and lipid membranes (Beyer, 1992, Noack *et al.*, 1994, Weber *et al.*, 1994), directly scavenging free radicals in the inner mitochondrial membrane by mediating uncoupling through superoxide production (Echtay *et al.*, 2002).

The advances made in understanding the role of free radicals in the pathogenesis of male infertility suggest a potential health-promoting role of natural dietary such as RPO in order to provide a clear insight on the mechanisms of antioxidants on male reproduction.

Chapter III

Impact of organic hydroperoxides on rat testicular tissue and epididymal sperm

Yapo G Aboua[1*], Stefan S du Plessis[2], Nicole Brooks[1]

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

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

Corresponding author:

*YG Aboua

Tel: +27 21 938 9388

Fax: +27 21 938 9476

guillaboua@gmail.com

Abstract

Organic hydroperoxides such as t-butyl hydroperoxide and cumene hydroperoxide have been implicated to cause oxidative stress leading to damage in membrane lipids, proteins, carbohydrates and DNA. This study was aimed to develop an *in vivo* animal model. The effects of hydroperoxides on testicular tissue and epididymal sperm were investigated. Male Wistar rats aged 10-12 weeks were randomly placed in groups and received standard rat chow and water *ad lib*. Animals were injected intraperitoneally with saline (0.5 ml), t-butyl hydroperoxide (5µM, 10µM, 20µM and 40µM; 0.5 ml) or cumene hydroperoxide cHP (2.5µM, 5µM, 10µM and 20µM; 0.5 ml) over a 60 day period. It was found that cumene hydroperoxide cHP (10µM and 20µM) and t-butyl hydroperoxide tbHP (20µM and 40µM) led to significantly lower epididymal sperm concentrations and motility. Superoxide dismutase and glutathione activities were also higher with an accompanying increase in lipid peroxidation in both testicular tissue and epididymal sperm. It can be concluded that *in vivo* intraperitoneal administration of organic hydroperoxides negatively affect the male reproductive system. We have therefore successfully created an animal model to test the adverse effects of oxidative stress on male reproductive parameters, thereby, enabling us to study possible *in vivo* treatments.

Keywords: Hydroperoxide; sperm; motility; lipid peroxidation; superoxide dismutase; glutathione

Abbreviations

cHP	cumene hydroperoxide
GSH	glutathione
LPO	lipid peroxidation
MDA	malondialdehyde
OS	oxidative stress
RLU	relative luminescence units
SOD	superoxide dismutase
tbHP	t-butyl hydroperoxide

Introduction

Many environmental, physiological, and genetic factors have been implicated in poor sperm function and infertility (Kovacic and Jacintho, 2001). Free radical-induced oxidative damage to sperm is one such condition and it is gaining considerable attention due to its contribution to sperm damage (Agarwal *et al.*, 2003). A better understanding of how these conditions affect sperm function will be beneficial as it might help in the design of new and effective treatment strategies to combat the problem of increasing male subfertility.

In mammals, the epididymis is known to play an important role in the maturation and storage of sperm. During epididymal transit, sperm metabolism increases, accompanied by the threat of oxidative stress (OS) (Dacheux *et al.*, 2003). OS is a cellular condition associated with an imbalance between the production of free radicals, mainly reactive oxygen species (ROS), and their scavenging capacity by antioxidants. When the production of ROS exceeds the available antioxidant defence, significant oxidative damage occurs to many cellular organelles due to damage to lipids, proteins, DNA and carbohydrates. These processes can ultimately lead to cell death. Sperm is particularly susceptible to oxidative damage due to its unique structural composition of high polyunsaturated fatty acid content in its plasma membrane (Garg *et al.*, 2000, Lenzi *et al.*, 2000, Sanocka and Kurpisz, 2004).

Some chemical, physical, or biological agents that alter physiological control processes and affect the normal functioning of the gonads will cause gonadal toxicity (Kelce *et al.*, 1994, Schrader and Kanitz, 1994). Any potential gonadotoxic agent can interrupt the normal functioning of the male reproductive system in the following

ways: (a) at the hypothalamic pituitary- gonadal axis level, (b) directly at the gonadal level, or (c) by altering post-testicular events, such as sperm motility or function or both (Sokol, 1987). Disruption of such physiological events may lead to hypogonadism, infertility, decreased libido, and/or sexual dysfunction (Sokol, 1987). Organic hydroperoxides such as t-butyl hydroperoxide (tbHP) and cumene hydroperoxide (cHP) are widely used in the chemical industry as initiators of oxidation for the production of polymers and fibre-reinforced plastics, in the manufacturing of polyester resin coatings, and pharmaceuticals. Short term studies have shown that exposure to hydroperoxides (intraperitoneal injection) dramatically increases the level of lipid peroxidation (LPO) and enhances ROS generation in the testes of rats (Kumar *et al.*, 2002, Kaur *et al.*, 2006a, Kumar and Muralidhara, 2007).

Organic hydroperoxides have been extensively used as model pro-oxidants to induce OS in various *in vitro* systems (Chen *et al.*, 2000, Kumar *et al.*, 2002, Kumar and Muralidhara, 2007). However, these pro-oxidants have not been used in long-term *in vivo* animal model studies to investigate the underlying biochemical mechanisms by which organic hydroperoxides induce oxidative damage in the testis and spermatozoa. This study aimed at developing an *in vivo* animal model to investigate the effect of OS on male reproduction by studying the *in vivo* effects of intraperitoneal administration of tbHP and cHP over a 60 day period on epididymal sperm and testicular tissue in order for these hydroperoxides to manifest itself during the process of complete spermatogenesis.

Materials and Methods

Animal treatment and research design

This study received institutional review board clearance and rats were housed in an ethically approved animal facility. Male Wistar rats aged between 10 to 12 weeks were randomly placed in 3 groups. Animals were fed *ad lib* with standard rat chow (SRC) and water while their beddings were changed three times per week. Rats (n=54) were randomly allocated to either a placebo group receiving an intraperitoneal injection of saline (Control) or cHP (2.5µM, 5µM, 10µM and 20µM; 80% aqueous, Sigma Chemical Co, South Africa) or tbHP (5µM, 10 µM, 20µM and 40 µM; 70% aqueous, Sigma Chemical Co, South Africa). Six rats were included in each individual treatment respectively. Injections (0.5 ml) were administered on 5 consecutive days per week up to 60 days in order to target, at least one complete cycle of spermatogenesis as it takes 58 days in rats (Franca *et al.*, 1998). The concentrations of cHP and tbHP were adopted and modified from the study of Kumar and Muralidhara, 2007. During the experiments, maximum care was taken to minimize animal suffering. Body weights were recorded at both the onset and completion of the study period. Immediately after euthanization, the testes and epididymis were excised and their weights recorded. One epididymis was rinsed and gently minced in 1.5 ml of phosphate buffered saline (PBS, Sigma Chemical Co, South Africa). The fragments were allowed to sediment for 5 minutes and 1 ml of the supernatant containing the sperm was filtered and collected for further analysis. One of the testes was snap freezed in liquid nitrogen (-196 ^{o}C) and stored at -80 ^{o}C.

Sperm parameters

One drop of sperm suspended in PBS was placed on a glass slide and 10 random fields were manually scored for the number of motile and non-motile sperm. Motility was expressed as a percentage of motile sperm compared to total cells. Epididymal sperm concentration was determined as per the method described in the WHO Manual (WHO, 1999). Briefly, a 50µl aliquot of epididymal sperm was diluted with 950 µl of diluents (50 g sodium bicarbonate, 10 ml formalin (35%), and 0.25 g trypan blue were added and made up to a final volume of 1L with distilled water). A cover slip was secured to the counting chambers of a Neubauer type hemocytometer. Approximately 10 µl of the thoroughly mixed diluted specimen was transferred to each of the counting chambers of the hemocytometer, which was allowed to stand for 5 min in a humid chamber in order to prevent drying. The cells settled during this time and were subsequently counted with a light microscope at 40 X magnification.

Assessment of Lipid Peroxidation

Lipid peroxidation (LPO) was quantified by measuring the formation of thiobarbituric acid reactive substances (TBARS) and expressed as nmol malondialdehyde (MDA) formed / mg testicular tissue or $2x10^6$ sperm (Draper *et al.*, 1993). In short, 50µl of epididymal sperm ($2x10^6$/ml) or 50 µl of homogenized testicular tissue (50 mg frozen testis homogenized in 10 volumes of 1.15% KCl, tris-HCl, 10 mM, pH 7.4, at 4 ^oC, protease inhibitor: P8340 Sigma Chemical Co, South Africa) were added to 6.25µl cold butulated hydroxyl toluene / ethanol (4nM) and 50µl of ortho-phosphoric acid (0.2M) in an Eppendorf tube. After mixing for 10 seconds,

6.25µl of thiobarbituric acid reagent (0.11M), was added, and then heated to 90 ^{o}C (45 minutes). Samples were subsequently first cooled on ice (2 minutes) and thereafter at room temperature (5 minutes) before the addition of n-butanol (500µl) and saturated NaCl (50µl). Eppendorfs were centrifuged (12000 rpm, 2 minutes, 4 ^{o}C) and 300µl of the supernatants (top butanol) was transferred to a 96 well plate. Absorbance was measured (532 and 572 nm) by a GloMax® Multi Detection System (Promega, UK).

Assessment of antioxidant activities

50 mg of frozen testis were homogenized (15000 rpm, 20 minutes) in 10 volumes of 1.15% KCl, tris-HCl (10 mM, pH 7.4) at 4 ^{o}C. The activity of the antioxidant enzymes glutathione (GSH) and Superoxide dismutase (SOD) concentration were assayed in both epididymal sperm and testicular homogenates using a plate reader GloMax® Multi Detection System (Promega, UK).

The GSH assay is based on the conversion of a luciferin derivative into luciferin in the presence of glutathione, catalyzed by glutathione S-transferase. The signal generated in a coupled reaction with firefly luciferase is proportional to the amount of glutathione present in the sample. The assay was conducted according to the protocol provided by the manufacturer (Promega, UK). 50µl of prepared GSH-Glo™ Reagent 2X was added to 50µl of 20000 epididymal sperm cells or 50µl supernatant of testicular tissue homogenate on a 96-well plate and incubated at room temperature (30 minutes). Subsequently, 100µl of reconstituted luciferin detection reagent was added to each well, mixed, and the luminescence was read after incubation (15 minutes).

SOD activity was determined from the conversion of xanthine and oxygen to uric acid and hydrogen peroxide by xanthine oxidase to form superoxide anion. The superoxide anion then converts WST-1 to WST-1 formazan, a colored product that absorbs light at 450 nm. The relative SOD activity of the experimental sample is determined from the percentage inhibition of the rate of formation of WST-1 formazan. The assay was conducted according to the protocol provided by the manufacturer (Assay Designs, USA) using 20000 sperm cells or supernatant of testicular tissue homogenate.

Statistical analyses

GaphPad^{TM} PRISM 4 was used for all statistical evaluations and graphical representations. Data are expressed as mean ± S.E.M. A one-way analysis of variance (ANOVA) test (with Bonferroni *post test* if $P < 0.05$) and Pearson correlation were used for statistical analyses. Differences were regarded as statistically significant if $P < 0.05$ and highly significant if $P < 0.001$.

Results

Body, testicular and epididymal weights

From Table 1, it can be seen that the weight of the animals did not differ significantly at the onset or at the end of the experiment. All animals gained weight during the 60 day treatment period. Both testicular and epididymal weights of the treated animals did not differ from the control values at the end of the experimental period (Table 1).

Sperm count and motility

A significantly (P<0.001) lower epididymal sperm concentration was observed in animals injected with 10µM (67.00 ± 5.73 x 10^6/ml) and 20µM (58.67 ± 4.80 x 10^6/ml) of cHP and 20µM (65.50 ± 4.05 x 10^6/ml) and 40µM (59.17 ± 4.10 x 10^6/ml) of tbHP when compared to the control group (109 ± 10.88 x 10^6/ml) (Table 1). Furthermore, sperm from animals injected with cHP (10µM: 26 ± 4.30 %; 20µM: 18 ± 4.63 %) and tbHP (20µM: 20 ± 5.24 %; 40µM: 17 ± 3.39 %) showed a significant (P<0.001) lower motility when compared to sperm from control animals (66.60 ± 3.36 %) (Figures 1 A and B).

Lipid Peroxidation

The amount of LPO in epididymal sperm and testicular tissue were significantly higher when exposed to higher dosages of hydroperoxide. Figures 2 A and B show that the production of MDA after administration of 20µM cHP (30.94 ± 3.55 nmol MDA / 2x10^6 sperm, P<0.01) or 20µM and 40µM tbHP (27.38 ± 1.07 and 25.92 ± 0.91 nmol MDA / 2x10^6 sperm, P<0.05) were significantly elevated above control values (19.77 ± 1.07 nmol MDA / 2x10^6 sperm) in epididymal sperm. Moreover, there was significantly higher formation of MDA in the testicular tissue of animals receiving 10µM and 20µM cHP (37.05 ± 2.59 and 33.70 ± 8.05 nmol MDA / mg tissue, P<0.01 and P<0.05 respectively) or 40µM tbHP (58.09 ± 1.98 nmol MDA / mg tissue, P<0.001) compared to control (16.63 ± 1.20 nmol MDA / mg tissue) (Figures 3 A and B). Interestingly very strong negative/inverse correlations were found between epididymal sperm motility and LPO in animals treated with cHP (r = - 0.9640, p = 0.0082) or tbHP (r = - 0.9725, p = 0.0055) as well as epididymal sperm

motility and testicular tissue LPO (cHP: r = -0.9682, p = 0.0068 and tbHP: r = -0.9254, p = 0.0242) (Table 2).

Antioxidant activities

- ***GSH***

The activity of GSH is expressed in Relative Luminescence Units (RLU). From Figures 4 (A and B), it can be seen that the formation of luciferase in the epididymal sperm of animals injected with cHP (10µM: 47662 ± 4243 RLU, *P*<0.001 and 20µM: 51118 ± 4776 RLU, *P*<0.01) or tbHP (20µM: 53671 ± 4112 RLU and 40µM: 46548 ± 2949 RLU, *P*<0.001) was significantly lower compared to the control group (9589 ± 2982 RLU). A similar trend was observed in testicular tissue with significantly lowered luciferase fluorescence at 10µM (31456 ± 3742, *P*<0.05) and 20µM (31922 ± 7776 RLU, *P*<0.05) cHP and 40µM tbHP (26094± 6409 RLU, *P*<0.05) compared to control (58077± 5482 RLU) (Figures 5 A and B). A negative/inverse correlation was found between epididymal sperm LPO and GSH activity in animals treated with cHP (r = - 0.9542, p = 0.0117) or tbHP (r = - 0.9749, p = 0.0047) as well as testicular tissue LPO and GSH activity (cHP: r = -0.9631, p = 0.0085 and tbHP: r = - 0.8483, p = 0.0693: not significant) (Table 3). However, a positive correlation was found between epididymal sperm motility and GSH activity in animals treated with cHP (r = 0.9543, p = 0.0116) or tbHP (r = 0.9750, p = 0.0047) as well as testicular tissue and GSH activity (cHP: r = 0.9631, p = 0.0085 and tbHP: r = - 0.8488, p = 0.0689: not significant) (Table 3).

- ***SOD***

The concentrations of SOD were significantly lower (P<0.001) in the epididymal sperm of animals treated with cHP (10µM: 0.20 ± 0.01 U/µl; 20µM: 0.18 ± 0.02 U/µl) or tbHP (20µM: 0.21 ± 0.01 U/µl; 40µM: 0.17 ± 0.01 U/µl) when compared to the control (0.51 ± 0.21 U/µl) (Figures 6 A and B). A similar trend was observed in SOD concentration of testicular tissue (P<0.001) in animals treated with cHP (10µM: 0.21 ± 0.08 U/µl; 20µM: 0.18 ± 0.02 U/µl) or tbHP (20µM: 0.21 ± 0.02 U/µl; 40µM: 0.16 ± 0.03 U/µl) when compared to control (0.56 ± 0.21 U/µl) (Figures 7 A and B). Table 3 shows that a negative/inverse correlation exist between epididymal sperm LPO and SOD concentration in animals treated with cHP (r = - 0.9502, p = 0.0132) or tbHP (r = - 0.8930, p = 0.0413) as well as testicular tissue LPO and SOD concentration (cHP: r = -0.8236, p = 0.0865: not significant and tbHP: r = - 0.7994, p = 0.1045: not significant). However, a positive correlation was found between sperm motility and epididymal sperm SOD concentration in animals treated with cHP (r = 0.9800, p = 0.0034) or tbHP (r = 0.9619, p = 0.0089) as well as testicular tissue and SOD concentration (cHP: r = 0.9805, p = 0.0033 and tbHP: r = 0.9619, p = 0.0089) (Table 3).

Discussion

The process of spermatogenesis, from germ cell recruitment to spermiation, takes a couple of weeks and renders the developing male gametes extremely vulnerable to any form of oxidative insult (Dacheux *et al.*, 2003). In mammals, the epididymis is known to play an important role in the final development of motility and fertilizing ability as well as storage of sperm. During the period of epididymal transit, sperm concentration can increase up to 10^{10} cells/ml. Sperm metabolism also

increases simultaneously and the possibility of OS generations threatens the survival of these male gametes (Dacheux *et al.*, 2003).

In this study, the long-term exposure of male rats to organic hyroperoxides (cHP and tbHP) via intraperitoneal injection did not lead to mortality or any clinical signs of general toxicity. However, it negatively affected sperm parameters by decreasing both sperm concentration and sperm motility as measured at the end of the study. Furthermore, both cHP and tbHP treatments significantly increased LPO while simultaneously lowered GSH activity and SOD concentration. These effects were observed in epididymal sperm and testicular tissue. The higher doses of cHP (10 and 20 µM) and tbHP (20 and 40 µM) might affect the spermatogenesis process and particularly sperm transition through the epididymis not only by decreasing the number of sperm available but also by compromising the quality through the process of epididymal sperm maturation (Dacheux *et al.*, 2003). Previous studies have shown a correlation between high levels of ROS (superoxide, hydroxyl, hydrogen peroxide, nitric oxide, peroxynitrile) and sperm motility (Lenzi *et al.*, 1993b, Armstrong *et al.,* 1999, Bilodeau *et al.*, 2002, Agarwal *et al.*, 2003). De Lamirande and Gagnon (de Lamirande and Gagnon, 1992) also reported that ROS causes sperm immotility within 5-30 minutes, depending on the concentration. The H_2O_2 might diffuse across the membranes into the cells and inhibit the activity of enzymes such as glucose-6-phosphate dehydrogenase (G6PD), which led to a decrease in the availability of reduced nicotinamide adenine dinucleotide phosphate (NADPH). In turn this decreased the formation of ATP, which is an important metabolite for sperm motility. This was confirmed by our findings and could explain the decrease in sperm motility observed. The peroxidative process initiated by the high doses of cHP and tbHP may

lead to ROS-mediated protein oxidation (Ong *et al.*, 2002) which reduced the sperm counts (Agarwal *et al.*, 2003) possibly due to cell death. H_2O_2 can penetrate the plasma membrane, cause protein oxidation and increase LPO production. In the light of the membrane permeability of H_2O_2, the external production or addition of this oxidant must have a negative effect on sperm motility, LPO accumulation, antioxidant (SOD, GSH) activities and DNA integrity. Ramos and Wetzels found DNA fragmentation in human sperm after addition of H_2O_2 (Ramos and Wetzels, 2001). These authors as well as Giwercman and co-workers (2003) emphasized the correlation between sperm motility and DNA integrity (Giwercman *et al.*, 2003). Negative correlations were also observed between DNA fragmentation and semen quality as reflected by sperm motility, morphology, and concentration (Sun *et al.*, 1997). Furthermore, a strong correlation was found between DNA strand breaks and the susceptibility of sperm to low pH-induced DNA denaturation (Aravindan *et al.*, 1997).

Mammalian sperm membranes are rich in polyunsaturated fatty acids (PUFA), which make them very fluid but at the same time very susceptible to free radicals and ROS. Griveau and co-workers (1995a) have shown that reactive oxygen species cause a decrease in sperm motility, an increase in lipid peroxidation, and a loss of membrane PUFA (Griveau *et al.*, 1995a). In the sperm plasma membrane, PUFAs are required to give the plasma membrane the fluidity needed for sperm motility. Spontaneous lipid peroxidation was also shown in rabbit and mouse sperm, and a close linear correlation existed between the extent of peroxidation and the loss of sperm motility (Alvarez and Storey, 1992). Our results show that the amounts of MDA

in the epididymal sperm and in the testicular tissue negatively correlate with the percentages of sperm motility in rats, which is in agreement with the literature.

The principal antioxidant defenses in sperm are SOD and GSH peroxidase (Storey, 1997). The physiological role of GSH is an essential intracellular reducing agent for maintenance of thiol groups on intracellular proteins and for antioxidant molecules. GSH protects cells against oxidative stress and other types of damage, which may arise from compounds of endogenous and exogenous sources. The continued activity of glutathione peroxidase depends on the regeneration of reduced glutathione by glutathione reductase, which in turn relies on NADPH, the principal source of which in sperm is the pentose phosphate shunt. The activity of G6PD, which is the first enzyme in the pentose phosphate pathway, may limit the rate of NADPH production and, hence, the ability of the glutathione peroxidase system to detoxify peroxides (Storey *et al.*, 1998). The high levels of cHP (10 µM and 20µM) and tbHP (20µM and 40µM) overwhelmed the antioxidant capacity of both SOD and GSH. The lower SOD concentration in sperm cells could be attributed to the assault of the high doses of cHP and tbHP. SOD presents the first line of defense against superoxide, as it dismutases the superoxide anion to H_2O_2 and O_2 (Nehru and Anand, 2005). Organic hydroperoxides (cHP and tbHP) might affect the GSH synthesis by decreasing the activity of glutathione-synthase thus leading to a reduced GSH content. On the other hand, the decreased GSH level could be also ascribed to insufficient supply of NADPH. This could explain the correlations found, in this study, between epididymal sperm motility, epididymal sperm LPO, SOD concentration and GSH activity as well as in the testicular tissue.

Conclusion

Intraperitoneal injection of organic hydroperoxides (cHP and tbHP) lowered sperm concentration and sperm motility. It furthermore, impaired antioxidant activities in both epididymal sperm and testicular tissue. We can therefore conclude that we have successfully created an animal model to test the adverse effects of OS on male reproductive parameters, which will also allow us to study possible treatments *in vivo*. For future reference, 60 days of 10µM cHP and 20µM tbHP treatment can be used as doses of organic hydroperoxides to successfully induce OS in the rat model in order to target the complete process of spermatogenesis.

Acknowledgments

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Table 1: Mean (± SEM) body, testicular and epididymal weights as well as epididymal sperm concentration of rats treated (n=6 per treatment) with different concentrations of cumene hydroperoxide (cHP) and t-butyl hydroperoxide (tbHP).

Parameters	Control	cHP (2.5µM)	cHP (5µM)	cHP (10µM)	cHP (20µM)	tbHP (5µM)	tbHP (10µM)	tbHP (20µM)	tbHP(40µM)
Initial Body Weight (g)	284.5 ± 18.85	260.8 ± 8.48	282.8 ± 18.09	279.8 ± 17.16	307.7 ± 4.04	276.5± 12.80	295 ± 5.20	298 ± 15.64	299 ± 6.99
Final Body Weight (g)	420.6 ± 26.09	408.2 ± 14.72	412.5 ± 6.26	394.7±12.78	414.7 ± 12.58	416.5 ± 6.14	434.3 ±13.43	416.7 ± 8.25	381.8 ± 9.93
Weight gain (%)	30.6 ± 22.47	35.6 ± 11.16	31.29 ± 12.17	29.35 ± 14.97	25.55 ± 8.31	33.43 ± 9.47	31.73 ± 9.31	28.41 ± 11.94	21.44 ± 8.46
Testes Weight (g)	4.09 ± 0.15	3.72 ± 0.05	3.61 ± 0.09	3.75 ± 0.16	3.78 ± 0.23	3.69 ± 0.06	3.79 ± 0.10	3.85 ± 0.17	3.55 ± 0.07
Epididymis Weight (g)	0.54 ± 0.03	0.45 ± 0.01	0.49 ± 0.02	0.48 ± 0.03	0.50 ± 0.01	0.48 ± 0.02	0.52 ±0.03	0.50 ± 0.04	0.49 ± 0.01
Sperm concentration (10^6/ml)	109.0 ± 10.88	104.8 ± 7.68	93.00 ± 7.53	67.00 ± 5.73***	58.67± 4.80***	113.3± 11.26	101.2 ± 7.89	65.50 ± 4.05***	59.17 ± 4.10***

*** P<0.001 vs. Control

Table 2: Correlation between lipid peroxidation (LPO) in epididymal sperm and testicular tissue and sperm motility after cumene hydroperoxide (cHP) and t-butyl hydroperoxide (tbHP) exposure

		Lipid peroxidation (LPO)			
		Epididymal sperm		Testicular tissue	
Treatment group		**cHP**	**tbHP**	**cHP**	**tbHP**
Motility	***r***	-0.9640	-0.9725	-0.9682	-0.9254
	p	0.0082	0.0055	0.0068	0.0242

Table 3: Correlation of glutathione (GSH) activity as well as superoxide dismutase (SOD) concentration in epididymal sperm and testicular tissue with sperm motility and lipid peroxidation (LPO) after cumene hydroperoxide (cHP) and t-butyl hydroperoxide (tbHP) exposure

		Glutathione (GSH)				Superoxide dismutase (SOD)			
		Epididymal sperm		Testicular tissue		Epididymal sperm		Testicular tissue	
Treatment group		**cHP**	**tbHP**	**cHP**	**tbHP**	**cHP**	**tbHP**	**cHP**	**tbHP**
Motility	***r***	0.9543	0.9750	0.9631	0.8488	0.9800	0.9619	0.9805	0.9619
	p	0.0116	0.0047	0.0085	0.0689	0.0034	0.0089	0.0033	0.0089
LPO	***r***	-0.9542	-0.9749	-0.9631	-0.8483	-0.9502	-0.8930	-0.8236	-0.7994
	p	0.0117	0.0047	0.0085	0.0693	0.0132	0.0413	0.0865	0.1045

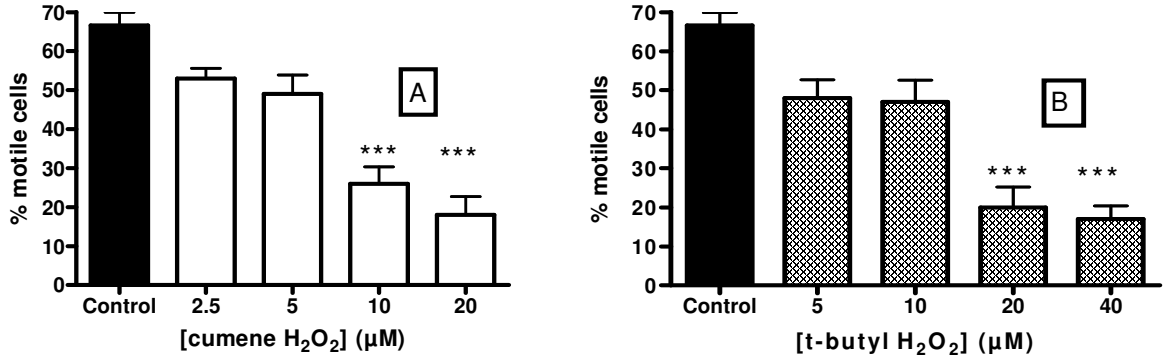

Figure 1: Epididymal sperm motility of rats treated (n=6 per treatment) with (A) cumene hydroperoxide and (B) t-butyl hydroperoxide (*** p<0.001 vs. control).

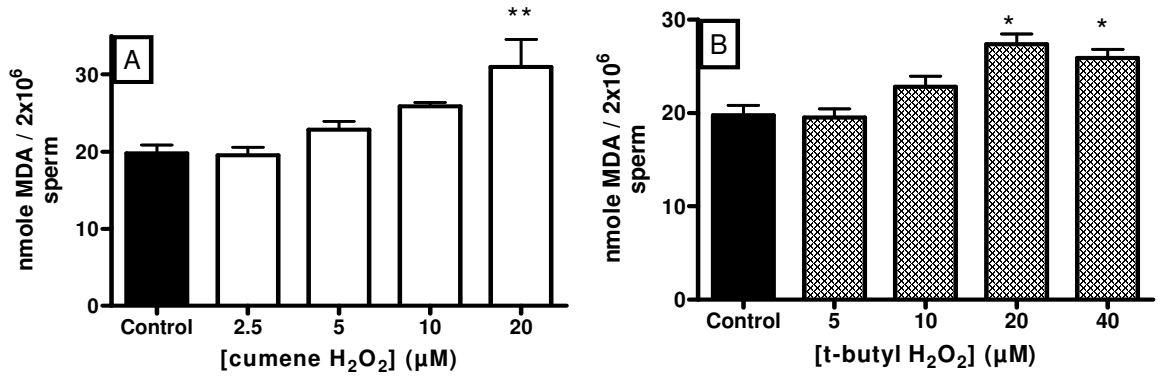

Figure 2: Lipid peroxidation in epididymal sperm of rats treated (n=6 per treatment) with (A) cumene hydroperoxide and (B) t-butyl hydroperoxide (* p<0.05 vs. control, ** p<0.01 vs. control).

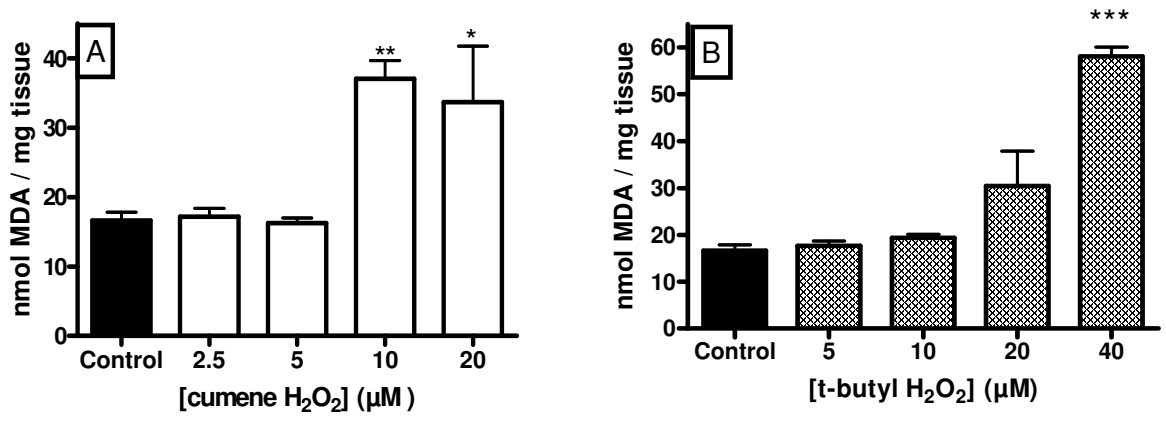

Figure 3: Lipid peroxidation in testicular tissue of rats treated (n=6 per treatment) with (A) cumene hydroperoxide and (B) t-butyl hydroperoxide (* $p<0.05$ vs. control, ** $p<0.01$ vs. control, *** $p<0.001$ vs. control).

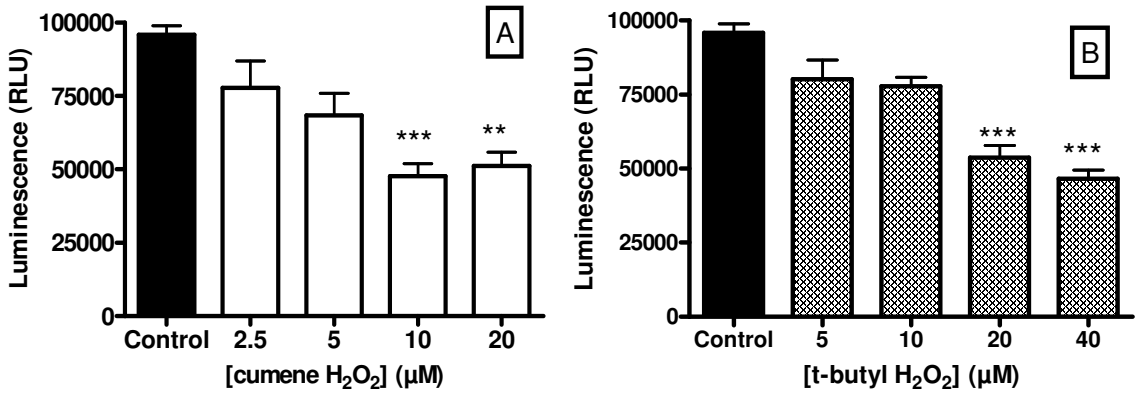

Figure 4: Effects of (A) cumene hydroperoxide and (B) t-butyl hydroperoxide on epididymal rat sperm glutathione (GSH expressed as Relative Luminescence Units RLU) (n=6 per treatment, ** $p<0.01$ vs. control, *** $p<0.001$ vs. control).

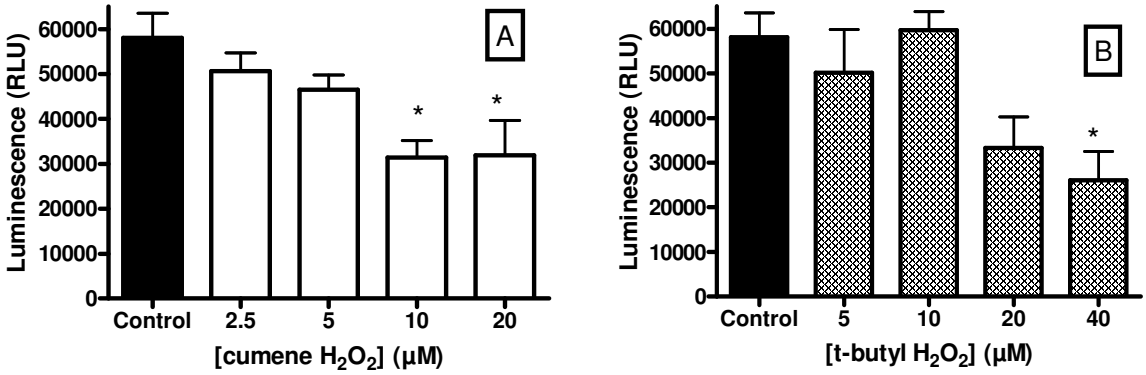

Figure 5: Effects of (A) cumene hydroperoxide and (B) t-butyl hydroperoxide on rat testicular glutathione (GSH expressed as Relative Luminescence Units RLU) (n=6 per treatment, * $p<0.05$ vs. control).

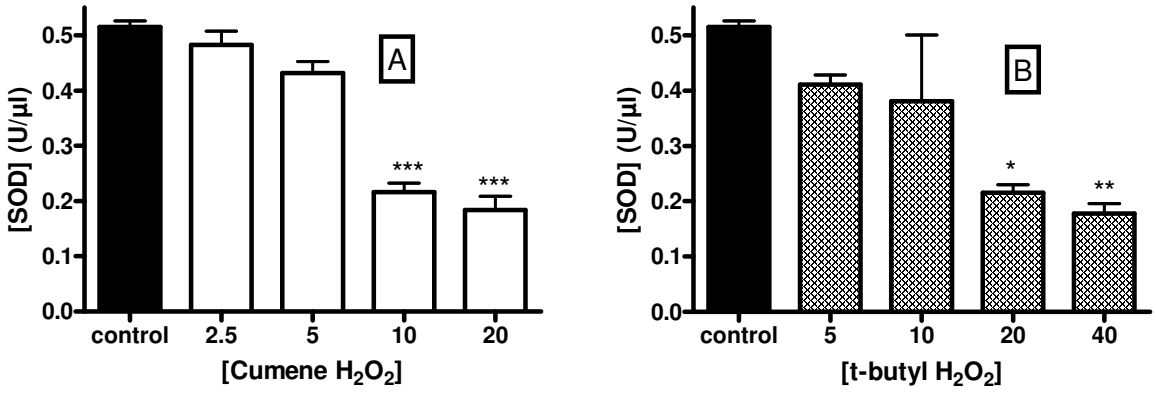

Figure 6: Effects of (A) cumene hydroperoxide and (B) t-butyl hydroperoxide on superoxide dismutase (SOD) concentration of epididymal rat sperm (n=6 per treatment, * $p<0.05$ vs. control, ** $p<0.01$ vs. control, *** $p<0.001$ vs. control).

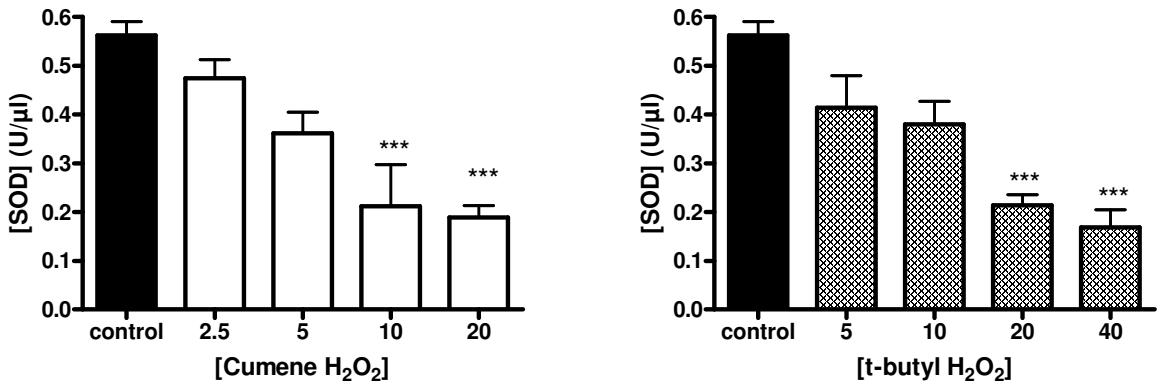

Figure 7: Effects of (A) cumene hydroperoxide and (B) t-butyl hydroperoxide on superoxide dismutase (SOD) concentration of rat testicular tissue (n=6, *** p<0.0 01 vs. control).

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Chapter IV

Can a Red Palm Oil (RPO) diet reduce the effects of oxidative stress in rat spermatozoa?

Y.G. Aboua[1], N. Brooks[1], R.Z. Mahfouz[2], A. Agarwal[2], S.S. du Plessis[3]

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

[2]Center for Reproductive Medicine, Cleveland Clinic, Cleveland, Ohio, USA

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

Author for correspondence and reprints:

Dr SS du Plessis

Division of Medical Physiology Stellenbosch University

PO Box 19063

Tygerberg, 7505

South Africa

Tel: +27 21 938 9388

Fax: +27 21 938 9476

ssdp@sun.ac.za

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ABSTRACT

Male Wistar rats aged 10-12 weeks were randomly placed in three groups and fed with standard rat chow (SRC). Group 1 received no supplement while the food of groups 2 and 3 were supplemented with 2 mL and 4 mL Red Palm oil (RPO) (in 25 gm SRC/day) respectively. Each group was divided into 3 subgroups and injected intraperitoneally daily with either saline, 10µM cumene hydroperoxide (cHP) or 20µM t-butyl hydroperoxide (tbHP) respectively. This was done for 5 consecutive days per week over a 60 day period. Sperm motility, sperm concentration, superoxide dismutase (SOD) concentration, glutathione (GSH) and catalase (CAT) activities in the sperm of animals injected with cHP and tbHP were significantly reduced compared to control while DCF and MDA increased. Interestingly, there was a significant decline in the amounts of DCF and MDA in the sperm of rats fed with 2 mL or 4 mL supplementation of RPO in combination with cHP or tbHP injection compared to rats receiving SRC only and injected with cHP or tbHP. Moreover, the SOD, CAT and GSH in the sperm of these animals increased. The levels of DCF in rat sperm fed with 2 mL or 4 mL supplementation of RPO in combination with cHP or tbHP injection, as well as SOD, CAT and GSH showed no differences when compared to control rats. We conclude that RPO supplementation to the diet can successfully attenuate the OS induced sperm damage induced by organic hydroperoxides. We therefore, propose that a daily intake of RPO supplement to the diet might be helpful in protecting males against the adverse effects of high ROS in sperm function and help preserve fertility.

Keywords: organic hydroperoxides, oxidative stress, red palm oil, sperm, Wistar rat

INTRODUCTION

Oxidative stress (OS) is associated with an imbalance between the production of free radicals, mainly reactive oxygen species (ROS), and scavenging capacity of antioxidants. When ROS production exceeds the available antioxidant defence, significant OS induced damage occurs to many cellular organelles due to damage of lipids, proteins, and DNA molecules, ultimately leading to cell death (Irvine *et al.*, 2000, Moustafa *et al.*, 2004). Recently two mechanisms involved in ROS generation in sperm, *per se,* have been characterized in rat epididymal sperm. One mechanism depends on the mitochondrial respiratory chain (Gavella and Lipovac, 1992), while the other relies on an enzymatic system related to reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase located near the sperm plasma membrane (Aitken *et al.*, 1992, Vernet *et al.*, 2001).

ROS production is critical for spermatozoa function in physiological as well as pathological conditions (Agarwal *et al.*, 2003, Holstein *et al.*, 2003, Agarwal *et al.*, 2006). However, mammalian cells are equipped with antioxidant mechanisms capable of restoring the balance between ROS production and their metabolism. In addition, the body possesses defense mechanisms to reduce the OS induced damage, and such mechanisms use both enzymes and antioxidant nutrients or medicine to arrest the damaging properties of excited oxygen species (Weisburger, 1991, Hwang *et al.*, 2002). Irreversible OS induced sperm damage may occur only when its protective mechanisms break down, or when the effectiveness of antioxidant sources is reduced.

A large number of studies have suggested that antioxidant nutrients and/or medicines play a protective role in human health (Aruoma, 1999, Fang *et al.*, 2002,

Devasagayam *et al.*, 2004). Crude red palm oil (RPO) is known to be the richest natural plant source of carotenoids in terms of provitamin A equivalents, such as α-carotene and β-carotene (Sundram *et al.*, 2003). Isong and co-workers (Isong *et al.*, 1997) had shown that RPO exerted effects on reproductive capacity through improving the efficiency of protein biosynthesis or utilization in a way that was favourable to sex hormone function in rats fed with a RPO supplemented diet. It is also likely that RPO provided vitamin A, which is known to play a part in reproduction through the synthesis of sexual steroids (Alais C, 1991), embryogenesis and spermatogenesis (McArdle and Ashworth, 1999).

Studies have shown that exposure to organic hydroperoxides such as t-butyl hydroperoxide (tbHP) and cumene hydroperoxide (cHP) dramatically increases the level of lipid peroxidation (LPO) and enhances ROS generation in rats. Organic hydroperoxides have been extensively used as model prooxidants to induce OS in various *in vivo* systems (Sestili *et al.*, 1998, Chen *et al.*, 2000, Kumar *et al.*, 2002, Kaur *et al.*, 2006a, Kaur *et al.*, 2006b Kumar and Muralidhara, 2007).

The aim of this study was twofold, 1) To create an *in vivo* animal model to study the effects of OS on rat sperm caused by organic hydroperoxides and 2) To study the effect of RPO supplementation on this induced OS model.

MATERIALS AND METHODS

Animal care and experimental protocol

Ethical approval was obtained from the Institutional Review Board at Cape Peninsula University of Technology. Male Wistar rats aged 10-12 weeks were randomly placed in 3 groups and fed daily 25 gm standard rat chow (SRC) (Epol Ltd.,

Johannesburg, South Africa) with *ad lib* access to water. Group 1 received no supplement while the food of groups 2 and 3 were supplemented with 2 mL and 4 mL RPO (Carotino SDN BHD Co: 69046-T, Johar-Bahru, Malaysia) in 25 gm SRC/day respectively. Each group was further divided into 3 subgroups. These subgroups were injected intra-peritoneally with either saline (0.5 mL), 10µM cHP (0.5 mL, 80% aqueous, Sigma Chemical Co, South Africa) or 20µM tbHP (0.5 mL, 70% aqueous, Sigma Chemical Co, South Africa) respectively. The injections were performed daily for 5 consecutive days per week over a 60 day period in order to target at least one complete cycle of spermatogenesis. Body weights were recorded at onset and completion of the experimental period. Immediately after sacrificing the animals, the testes and epididymis were excised and weighed. The caudal epididymis was subsequently rinsed, and gently minced in 1.5 mL of phosphate buffered saline (PBS, Sigma Chemical Co, South Africa) containing no calcium and magnesium. The fragments were allowed to sediment, and the supernatant containing epididymal sperm retrieved for further analysis. Rats fed with SRC and injected with saline are referred to as control.

Sperm count and motility

Epididymal sperm count was determined by the method as described in the WHO Manual (WHO, 1999). Briefly a 50µL aliquot of epididymal sperm was diluted with 95 µl diluent (50 gm sodium bicarbonate, 10 mL 35% formalin, and 0.25 gm trypan blue were added and made up to a final volume of 1000 mL with distilled water). A cover slip was secured to the counting chambers of a Neubauer type hemocytometer (Marienfeld, Germany). Approximately 10 µl of the thoroughly mixed diluted specimen was transferred to each of the counting chambers of the hemocytometer, which was allowed to stand for 5 min in a humid chamber to prevent

drying. The cells sediment during this time and were counted with a light microscope at 40 X magnification.

For the manual determination of sperm motility, one drop of sperm suspended in PBS, was placed on a glass microscope slide. Ten random fields were scored for the number of motile and non-motile sperm. Motility was expressed as a percentage of motile sperm compared to the total number of cells observed.

Assessment of ROS

Both ROS and lipid peroxidation (LPO) levels were assessed in epididymal sperm using a plate reader (GloMax® Multi Detection System, Promega, UK).

ROS were measured using 2'7'-dichlorofluorescin (DCFH, Sigma Chemical Co, South Africa) as the probe (Driver *et al.*, 2000). The non fluorescent DCFH oxidizes rapidly in the presence of ROS to the highly fluorescent dichlorofluorescein (DCF). DCFH (10 µmol) was added to 100 µl of medium or samples and incubated for 45 minutes at room temperature (in dark) in a 96 well multiplate. Fluorescence intensity was measured at 530 nm emission (485 nm excitation). DCF production was expressed as relative fluorescence units (RFU).

Assessment of lipid peroxidation

Malondialdehyde (MDA) levels indicate the amount of cellular damage secondary to LPO and have been widely adopted as a measure of free radical formation. In this study, LPO was quantified by measuring the formation of thiobarbituric acid reactive substances (TBARS) and expressed as nmol MDA formed (Draper *et al.*, 1993). In short, 50µL of epididymal sperm ($0.1x10^6$) were added to 6.25µL cold butylated hydroxyl toluene/ethanol (4nM) and 50µL of ortho-phosphoric acid (0.2M) in an Eppendorf tube. After mixing for 10 seconds, 6.25µL of

thiobarbituric acid reagent (0.11M) was added, and then heated to 90^{o}C (45 minutes). Samples were subsequently cooled on ice (2 minutes) and then at room temperature (5 minutes) before addition of n-butanol (500µl) and saturated NaCl (50µl). The Eppendorf tubes were centrifuged (12,000 rpm, 2 minutes, 4 ^{o}C) and 300µL supernatant (top butanol) was transferred to 96 well plates and absorbance was measured (532 and 572 nm).

Assessment of antioxidant activities

Superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) activities were determined in epididymal sperm using kits and assessed using a microplate reader (GloMax® Multi Detection System; Promega, UK).

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 ($O_2^{-\bullet}$). The $O_2^{-\bullet}$ then converts WST-1 to WST-1 formazan, a colored product that absorbs light at 450 nm. The relative SOD activity of the experimental sample is determined from percent inhibition of the rate of formation of WST-1 formazan. The assay was conducted according to the protocol provided by the manufacturer (Assay Designs, Michigan, USA) using $0.1x10^6$ cells.

Catalase fluorometric detection Kit is a sensitive assay that utilizes a non-fluorescent detection reagent that is converted to resorufin (excitation 530-571 nm, and emission 590-600 nm) in the presence of peroxidase and H_2O_2 substrate left over from the CAT reaction (Zhou *et al*., 1997). In brief, 50 µL of H_2O_2 (40 µM) solution was added to 50 µL of standard or samples in a 96 well plate and incubated at room temperature for 45 minutes. Subsequently, 100 µL of the reaction cocktail (freshly prepared) was added to each well according to the manufacturer's protocol

(Assay Designs, Michigan, USA). CAT activity was expressed as relative fluorescence units (RFU).

The GSH assay is based on the conversion of a luciferin derivative into luciferin in the presence of GSH, catalyzed by glutathione S-transferase (GST). The signal generated in a coupled reaction with firefly luciferase is proportional to the amount of GSH present in the sample. In short, 50µl of epididymal sperm (50000 cells) were added to 50µl of prepared GSH-Glo™ Reagent 2X and incubated at room temperature for 30 minutes. Subsequently, 100µL of prepared Luciferin Detection Reagent were added and incubated for 15 minutes before reading the luminescence (Promega, UK). The activity of GSH was expressed as relative luminescence units (RLU).

Statistical analyses

GaphPad™ PRISM version 4 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com) was used for all statistical evaluations and graphical representations. Data are expressed as mean ± S.E.M. For comparative studies, Student's *t*-test (2 groups' comparisons) or one-way analysis of variance (ANOVA, > 2 groups' comparisons) test with Bonferroni correction as a *post-hoc test* for base pair comparisons (if $P<0.05$) were used for statistical analyses. Differences were regarded as statistically significant if $P<0.05$ and highly significant if $P<0.001$.

RESULTS

Comparison of animal related parameters

Body, testicular and epididymal weights were comparable in all study groups ($P>0.05$ for all, Table 1). However, the epididymal sperm concentration in animals

injected with 10 µM cHP (13.22 ± 0. 74 x 10^7/mL, P<0.01) and 20 µM tbHP (14.22 ± 1.10 x 10^7/mL, P<0.05) was significantly reduced compared to control (18.85 ± 1.34 x 10^7/mL). Similarly, the sperm motility in animals injected with 10 µM cHP (65.00 ± 3.65 %) and 20 µM tbHP (65.83 ± 5.06 %) showed a significant decrease (P<0.01) compared to the control (86.67 ± 3.33 %). Interestingly, the sperm concentration and sperm motility of animals supplemented with (2mL and 4mL) RPO were in the normal range when compared to the control group despite the hydroperoxide treatment.

Intracellular sperm ROS production

Animals injected with 10 µM of cHP (31585 ± 1851 RFU) showed a significant (P<0.001) increase in DCF levels in their sperm compared to those injected with saline (19308 ± 682 RLU) (Figure 1). The amount of DCF were significantly lower in rats injected with cHP and receiving the 2 mL (22434 ± 1774 RFU, P<0.01) and 4 mL (19329 ± 2313 RFU, P<0.001) RPO supplementation with their diet vs.the group injected with cHP and fed only with SRC (31585 ± 1851 RFU) (Figure 1). The production of DFC in sperm of rats injected with 20 µM tbHP (30500 ± 1630 RFU, P<0.001) was significantly increased compared to those injected with saline (19308 ± 682 RFU) (Figure 1). Interestingly, the amount of DCF produced in the sperm of rats fed with 2 mL (19849 ± 1798 RFU, P<0.001) or 4 mL (20424 ± 1184 RFU, P <0.001) supplementation of RPO in addition to 20 µM tbHP injection showed a significant decrease when compared to rats receiving SRC only and injected with tbHP (30500 ± 1630 RFU). RPO supplementation alone did not affect DCF production (data not shown).

Lipid peroxidation of sperm

Rats injected with 10µM cHP and receiving SRC only, showed a significant increase in MDA in their sperm (23.91± 0.70 nmol, $P<0.001$) vs. those injected with saline (21.12 ± 0.48 nmol) (Figure 3). On the other hand, the amount of MDA produced in the sperm of rats fed with 2 mL (19.82 ± 0.31 nmol, $P<0.001$) or 4 mL (16.42 ± 0.32 nmol, $P<0.001$) supplemented with RPO and 10µM cHP injection also decreased significantly when compared to rats receiving SRC only and injected with 10µM cHP (23.91± 0.70 nmol) as well as those fed with only SRC and injected with saline (Figure 3). However, there were no significant differences in the amount of MDA in the sperm of rats injected with saline and receiving 2 or 4 mL RPO supplementation when compared to those receiving only SRC and injected with saline (data not shown). Figure 4 shows that rats injected with 20µM tbHP and receiving SRC only, had a significant increase in MDA in their sperm (23.45 ± 0.58 nmol, $P<0.05$) compared to those injected with saline (21.12 ± 0.48 nmol). The amount of MDA produced in the sperm of rats supplemented with 2 mL (19.89 ± 0.32 nmol, $P<0.001$) or 4 mL (16.42 ± 0.32 nmol, $P<0.001$) RPO in addition to the 20µM tbHP injection also decreased significantly when compared to rats receiving SRC only and injected with 20µM tbHP. Moreover, the levels of MDA in the sperm of rats injected with 20µM tbHP and receiving 4 mL RPO supplementation in SRC also significantly decreased (16.42 ± 0.32 nmol, $P<0.01$) compared to those fed with only SRC and injected with saline (21.12 ± 0.48 nmol) (Figure 4). The supplementation of RPO alone did not affect the amount of MDA in rats injected with saline (data not shown).

Antioxidant activities of sperm

Superoxide dismutase

Figures 5 and 6 show that the concentrations of SOD in the sperm of rats injected with cHP (0.7762 ± 0.0091 U/µl, P <0.05) and tbHP (0.7750 ± 0.0066 U/µl, P<0.05) had decreased significantly compared to the control (0.8126 ± 0.0108 U/µl). However, the concentrations of SOD in the epididymal sperm of rats injected with cHP or tbHP and fed with RPO did not differ from the control. The supplementation of RPO alone did not affect the production of SOD in rats (data not shown).

Catalase

The fluorescence in the sperm of rats injected with cHP (52499 ± 2002 RFU, P<0.001) decreased significantly compared to those injected with saline (72605 ± 2080 RFU). However, the sperm of rats fed with 4 mL RPO and injected with cHP (65310 ± 3140 RFU, P<0.05) had a significant increase in fluorescence compared to those injected with cHP and fed with SRC only (52499 ± 2002 RFU) (Figure 7). The sperm of rats fed with 4 mL RPO and injected with cHP did not show a significant difference in fluorescence when compared to the control. Similarly, from Figure 8, it can be seen that rats injected with tbHP (49218 ± 6647 RFU, P<0.05) had a significant decrease in their sperm fluorescence compared to those injected with saline (72605 ± 2080 RFU). However, the sperm of rats fed with 2mL (67533 ± 3905 RFU, P<0.05) or 4 mL (72559 ± 2094 RFU, P<0.01) RPO and injected with cHP showed a significant increase in fluorescence when compared to those of animals injected with cHP and fed with SRC only (49218 ± 6647 RFU). The sperm of rats receiving 2mL or 4 mL RPO supplementation and simultaneously injected with cHP

had no increase in fluorescence compared to the control. The supplementation of RPO did not affect the catalase activity (data not shown).

Glutathione

From Figures 9 and 10, it can be seen that the formation of luciferase in the sperm of animals injected with cHP (15192 ± 806.6 RLU, $P<0.05$) and tbHP (15126 ± 598.6 RLU, $P<0.001$) respectively was significantly decreased compared to the control group (19669 ± 1697 RLU). The sperm of rats receiving 2mL or 4 mL RPO supplementation and simultaneously injected with either cHP or tbHP did not differ in luminescence from the control. The supplementation of RPO did not affect the GSH production expressed as RLU in the sperm (data not shown).

Correlation studies

The correlations performed between various parameters are reported in Table 2. DCF fluorescence i.e. ROS production correlated negatively to sperm concentration (r = -0.312), sperm motility (r = - 0.371) as well as catalase activity (r = -0.535), while a positive correlation was observed with TBARS i.e. LPO (r = 0.3799). Catalase on the other hand showed a strong positive correlation with sperm concentration (r = 0.6107) and negative correlation with LPO (r = - 0.0353). Sperm motility also correlated negatively with LPO (r = -0.311).

DISCUSSION

Organic hydroperoxides have been used in various short term studies as an inducer of OS in *in vivo* systems (Younes and Strubelt, 1990, Kaur *et al.*, 2006a, Kaur *et al.*, 2006b, Kumar and Muralidhara, 2007). In this study, longer exposure of animals to hydroperoxides (60 days) via intraperitoneal injection of cHP or tbHP

clearly shows that sperm parameters were negatively influenced as indicated by low epididymal sperm concentration and low sperm motility. It is evident from the correlations done in this study that both sperm concentration and motility correlated negatively to intracellular ROS levels. These results are in support of findings previously reported from our group (Aboua *et al.*, 2009). Our data is also confirmed by reports from other studies (Armstrong *et al.*, 1999, Lenzi *et al.*, 1993b, Bilodeau *et al.*, 2002, Agarwal *et al.*, 2003) which showed a correlation between high levels of ROS (superoxide, hydroxyl, hydrogen peroxide, nitric oxide, peroxynitrile) and decreased sperm motility. We speculate that the injection of organic hydroperoxides might have led to H_2O_2 diffusing across the membranes into the sperm cells thereby inhibiting the activity of some enzymes such as glucose-6-phosphate dehydrogenase (G6PD). This can lead to a decrease in the availability of reduced nicotinamide adenine dinucleotide phosphate (NADPH). In turn, this subsequently decreased the formation of ATP, which is an important metabolite for sperm motility. In the process, cHP and tbHP created an imbalance between ROS and the scavenging capacity of the enzymes in the sperm leading to OS (Aboua *et al.*, 2009). ROS -mediated damage of sperm membranes has also been reported to be responsible for impaired sperm motility (de Lamirande and Gagnon, 1992). This can be attributed to H_2O_2 attacking the lipids in the sperm plasma membrane, thereby initiating a LPO cascade and subsequently, leading to the sperm losing their capability for movement.

The correlation found between LPO and DCFH (positive) can be ascribed to the peroxidative process initiated by cHP and tbHP ultimately leading to ROS-mediated protein oxidation (Ong *et al.*, 2002). The reduced sperm motility observed can also be attributed to LPO as seen from the negative correlation. It was previously reported that LPO led to reduced sperm counts possibly due to cell death (Agarwal *et*

al., 2003). H_2O_2 can penetrate the plasma membrane, cause protein oxidation and increase LPO production. Griveau and co-workers have shown that ROS causes a decrease in sperm motility, an increase in LPO, and a loss of membrane polyunsaturated fatty acids (Griveau *et al*., 1995a). Our data further confirm the results from those studies.

Under physiological situations, adequate levels of antioxidants, SOD, CAT, GSH peroxidase and reductase maintain the ROS scavenging potential in the male reproductive tract and seminal fluid. When monitored more objectively, these scavengers can be used as a good indicator of sperm damage and infertility caused by oxidative stress (Sikka *et al*., 1995). SOD is involved in dismutation of the O_2^- to H_2O_2 and oxygen. In this study, a significant decrease in the SOD concentration in the epididymal sperm of rats treated with 10µM cHP or 20µM tbHP indicated a reduced synthesis the enzyme. However, the treatment with RPO increased SOD concentration to levels comparable to the control. Catalase is known to neutralize H_2O_2 to water and superoxide. There is a significant decline in the activity of catalase in the epididymal sperm of rats treated with 10µM cHP or 20µM tbHP. From the correlations performed in this study it is evident that increased ROS levels is accompanied by decreased catalase, more than likely due to the depleting effect caused by catalase's scavenging activity. LPO also correlated negatively with catalase, yet again confirming that in the absence of catalase or the depletion thereof it can lead to an imbalance in prooxidants and antioxidants, allowing ROS to cause OS with accompanying LPO. The positive correlation between catalase and sperm concentration furthermore confirm these findings. However, the regain in the catalase activity in animals fed with RPO showed that RPO was actively involved in the free radical scavenging mechanism caused by the H_2O_2. This is evident from the reduced

amount of DCF in the sperm of rats injected with cHP or tbHP and receiving RPO supplementation compared to those injected with cHP or tbHP and receiving only SRC. Our data showed that GSH content was significantly decreased in the sperm of rats treated with 10µM cHP or 20µM tbHP. The lower levels of GSH content in the rat sperm indicated a greater participation of reduced glutathione in H_2O_2 detoxification (Krishnamoorthy *et al*., 2007). However, the supplementation of RPO diet increased the level of GSH in sperm. This increased intracellular transport of GSH is essential for maintaining the redox state during OS.

Due to its content in (α and β) carotene, (α and β) tocopherol and phenolic-flavonoid-rich antioxidant complex, RPO was used in this study, to investigate its scavenging and antioxidant capacity on hydroperoxide induced OS in rat epididymal sperm *in vivo.* Tocopherol can transfer a hydrogen atom with a single electron to a free radical, thus removing the radical before it can interact with the cell membrane. Besides playing a beneficial biological role as radical quenchers *in vivo*, tocopherols and tocotrienols are also antioxidants, which contribute to the stability of RPO. Tocopherols can interrupt lipid oxidation by inhibiting hydroperoxide formation in the chain-propagation step, or the decomposition process by inhibiting aldehyde formation (van Rooyen *et al*., 2008). The main function of α-tocopherol is to prevent the peroxidation of membrane phospholipids and prevent cell membrane damage through its antioxidant action (Sundram *et al*., 2003). The antioxidant properties of carotenoids have been suggested to reflect not only the rates of free radical scavenging, but also the reactivity of the resultant carotenoid-derived radicals. Carotenoid radical-cation or adduct radicals have been shown to be highly resonance stabilised and predicted to be relatively unreactive. They may further undergo bimolecular decay to generate non-radical products or, in the case of carotene

radical-adducts, may terminate radical reactions by binding to the attacking free radical (Everett *et al.*, 1996). Both β carotene and lycopene (found in tomatoes) have shown membrane protection against lipid peroxidation and form components of antioxidant defences (Gupta and Kumar, 2002).

Several studies have suggested that tocopherols at higher levels accelerate the formation of hydroperoxides especially at the early stage of oxidation. Jung and Min (Jung and Min, 1990) showed that tocopherols, above their optimum level, had a pro-oxidant effect on hydroperoxide formation. At higher tocopherol concentrations and higher oxidation rates, dihydroperoxides will be formed at the expense of hydroperoxy epidioxides. Kamal-Eldin and Appelqvist (1996) argued that the best antioxidant should be the one that is active so that the concentration of the antioxidant radical will be at a minimum (Kamal-Eldin and Appelqvist, 1996). High concentrations of antioxidant radicals will certainly be problematic at some stage of the oxidation reaction. Our results did not show any sign of toxicity regarding the amount of RPO (2 mL or 4 mL) used in this study.

Increasing intake of dietary antioxidants may help to maintain an adequate antioxidant status and, therefore, the normal physiological function of a living system (Record *et al.*, 2001). Some functional foods and vegetables are the important sources of exogenous antioxidants. RPO was able to reverse the negative effects of the organic hydroperoxides in this animal model. Moreover, the sperm of animals receiving RPO had increased SOD, CAT and GSH activities and sperm motility while the amount of MDA and LPO was reduced.

Conclusion

Recently, much attention has been focused on the protective biochemical function of naturally occurring antioxidants in biological systems, and on the mechanisms of their action. Many natural antioxidant products are capable of preventing or inhibiting the process of OS. RPO supplementation protected the epididymal sperm *in vivo* against the adverse effects of organic hydroperoxide i.e. loss of sperm motility by preventing lipid peroxidation, scavenging the formation of ROS, increasing SOD synthesis and the activities of catalase and GSH via antioxidant potential. We therefore, propose that a daily intake of RPO supplement to the diet might be helpful to protect males against the adverse effects of ROS in sperm function and help to preserve fertility.

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Table1: Body weights, testicular weights, epididymal weights and sperm concentrations of rats treated with organic hydroperoxides (n=6).

	Group 1 SRC			**Group 2** SRC + 2 ml RPO			**Group 3** SRC + 4 ml RPO		
	Saline	**cHP**	**tbHP**	**Saline**	**cHP**	**tbHP**	**Saline**	**cHP**	**tbHP**
Body weight (gm)	404.7±20.13	397.0±20.69	413.8±16.41	411.0±14.23	370.5±12.99	404.7±20.64	393.8±9.65	374.3±10.61	394.0±8.74
Testicular weight (gm)	3.88±0.06	3.94±0.06	3.77±0.12	3.92±0.12	3.84±0.13	3.53±0.26	3.82±0.12	3.87±0.12	3.75±0.11
Epididymal weight (gm)	0.53±0.01	0.49±0.01	0.51±0.01	0.49±0.02	0.47±0.01	0.49±0.01	0.48±0.03	0.48±0.01	0.49±0.02
Sperm concentration (10^7 / ml)	18.85±1.34	13.22± 0.74**	14.22± 1.10*	17.27± 2.04	15.00±1.67	15.70±1.76	14.33±1.81	16.40±2.31	15.43±1.84
Motility (%)	86.67±3.33	65.00±3.65**	65.83±5.06**	82.50±3.81	82.50±3.81	79.17±3.51	79.17±7.00	75.00±4.835	75.00±5.00

Values are expressed as mean ± SEM. * $P<0.05$ vs. saline, ** $P<0.01$ vs. saline

Table 2: Correlations between sperm concentration, motility, ROS production, lipid peroxidation (LPO), superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) activities after cumene hydroperoxide (cHP), t-butyl hydroperoxide (tbHP) and red palm oil (RPO) exposure.

		DCF	TBARS	SOD	CAT	GSH
[Sperm]	r	-0.312	-0.175	-0.095	0.6107	0.1456
	P	0.0474	0.2731	0.5544	<0.0001	0.3766
Motility	r	-0.371	-0.311	0.1476	0.2079	0.0851
	P	0.0155	0.0452	0.3508	0.1864	0.6014
DCFH	r		0.3799	-0.186	-0.535	-0.18
	P		0.0131	0.2387	0.0003	0.2668
TBARS	r			-0.15	-0.326	0.0517
	P			0.3421	0.0353	0.7512

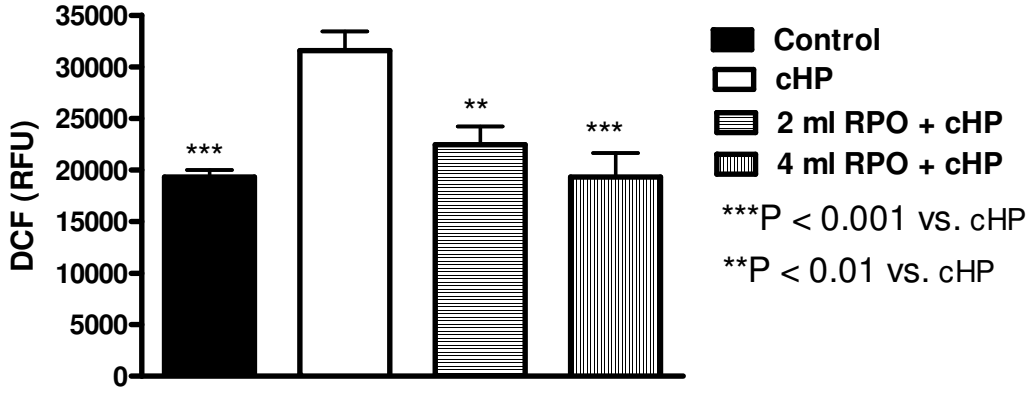

Figure 1: The effects of cumene hydroperoxide (cHP) and red palm oil (RPO) on dichlorofluorescein (DCF) fluorescence (ROS levels) in rat sperm (n=6).

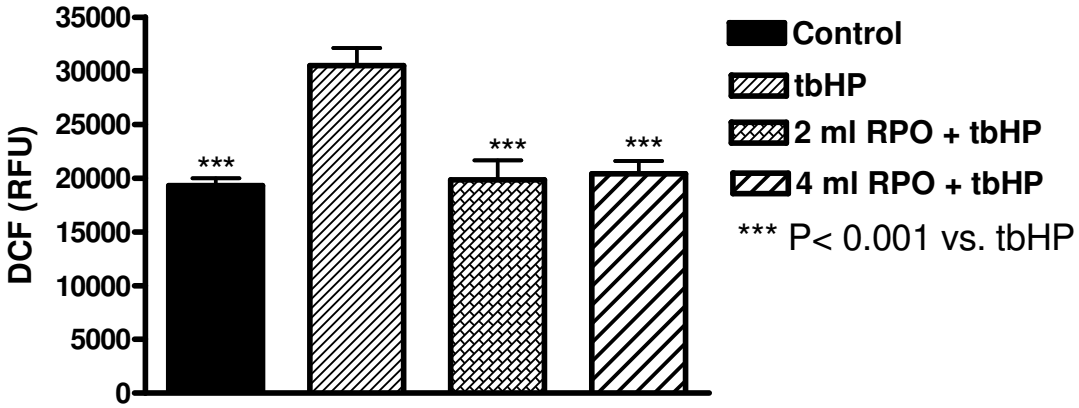

Figure 2: The effects of t-butyl hydroperoxide (tbHP) and red palm oil (RPO) on dichlorofluorescein (DCF) fluorescence (ROS levels) in rat sperm (n=6).

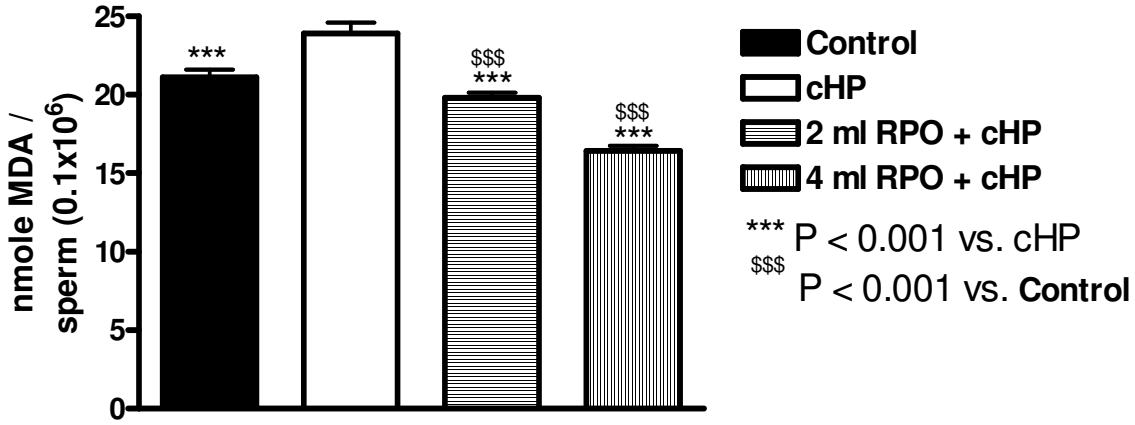

Figure 3: The effects of cumene hydroperoxide (cHP) and red palm oil (RPO) on malondialdehyde (MDA) formation and thus lipid peroxidation in rat sperm (n=6).

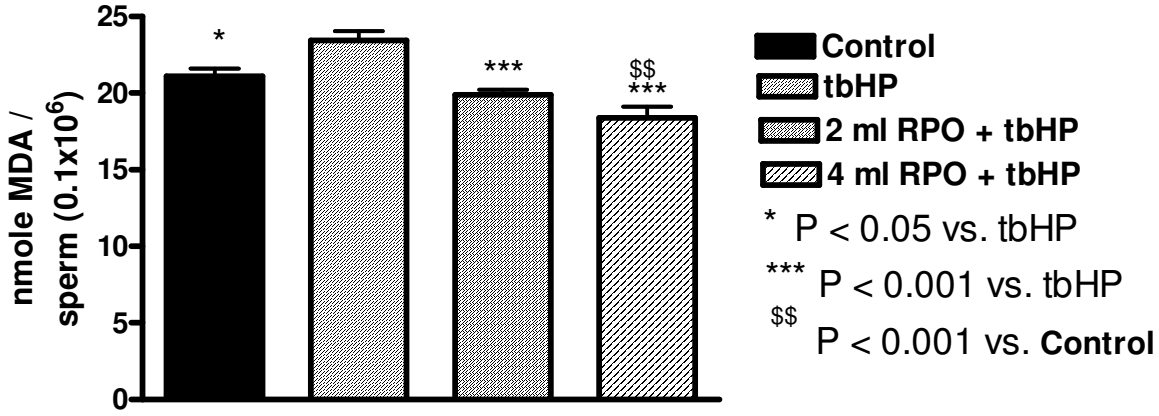

Figure 4: The effects of t-butyl hydroperoxide (tbHP) and red palm oil (RPO) on malondialdehyde (MDA) formation and thus lipid peroxidation in rat sperm (n=6).

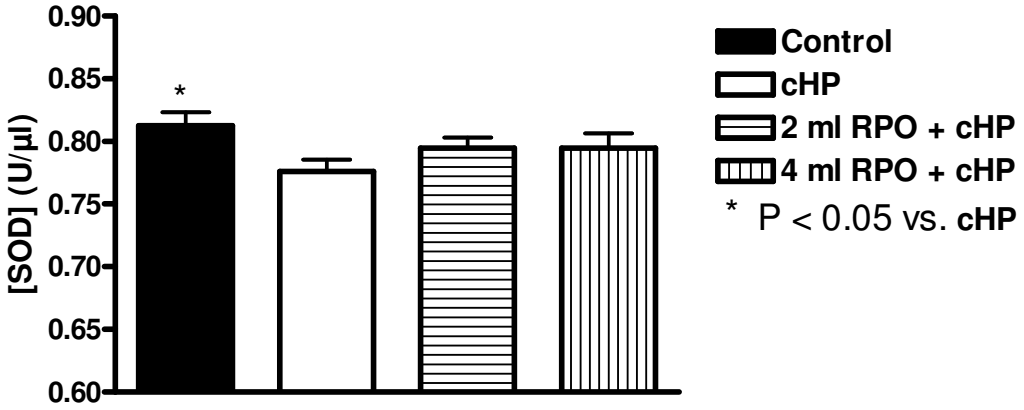

Figure 5: The effects of cumene hydroperoxide (cHP) and red palm oil (RPO) on superoxide dismutase (SOD) production in rat sperm (n=6).

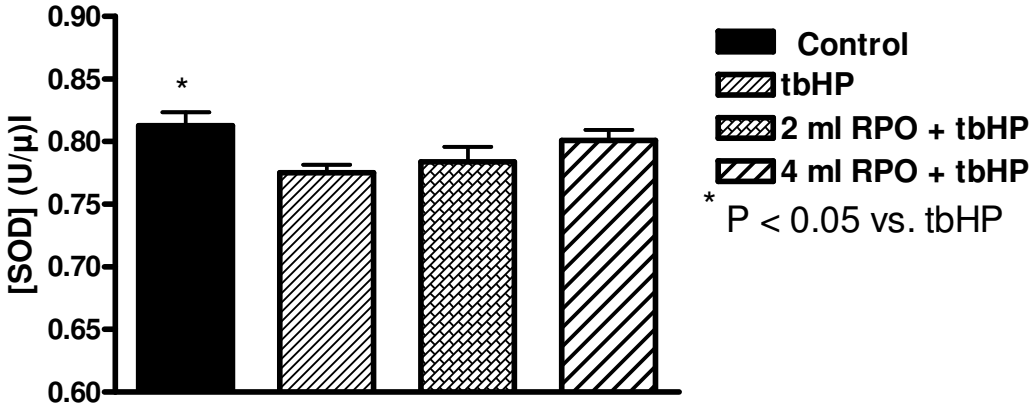

Figure 6: The effects of t-butyl hydroperoxide (tbHP) and red palm oil (RPO) on superoxide dismutase (SOD) production in rat sperm (n=6).

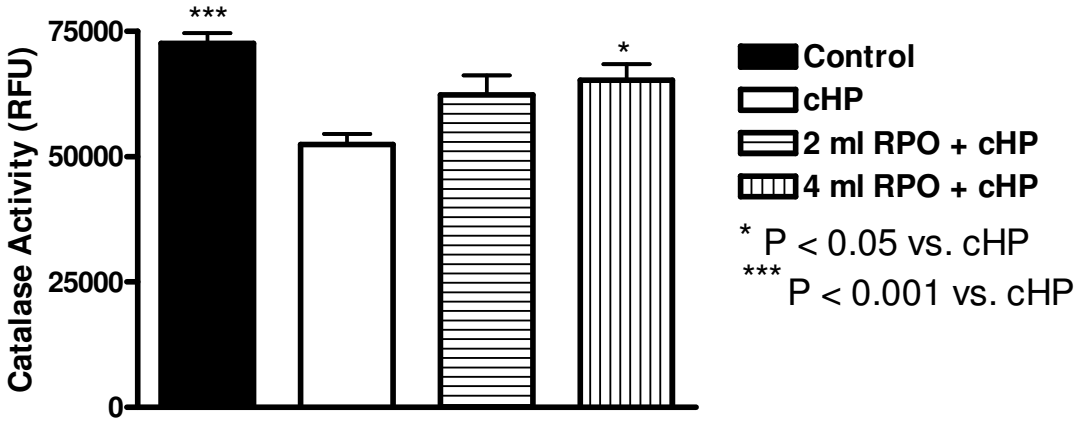

Figure 7: The effects of cumene hydroperoxide (cHP) and red palm oil (RPO) on catalase production in rat sperm (n=6).

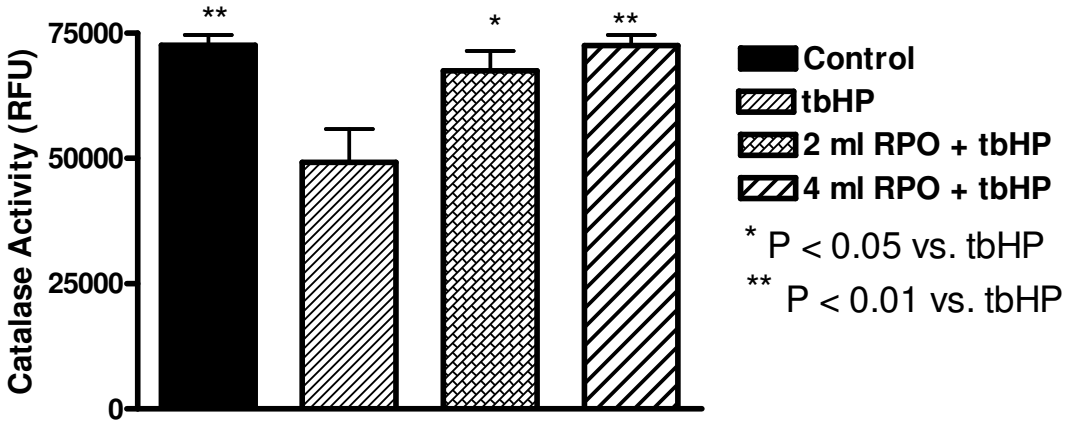

Figure 8: The effects of t-butyl hydroperoxide (tbHP) and red palm oil (RPO) on catalase production in rat sperm (n=6).

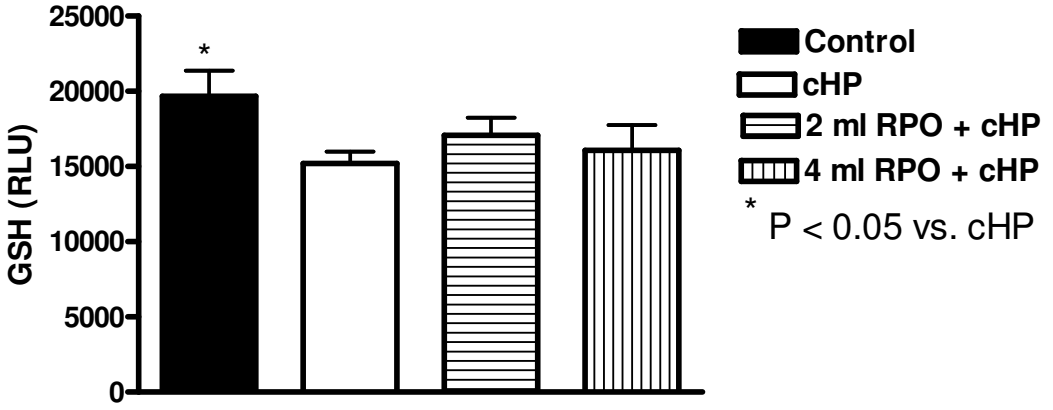

Figure 9: The effects of cumene hydroperoxide (cHP) and red palm oil (RPO) on glutathione (GSH) production in rat sperm (n=6).

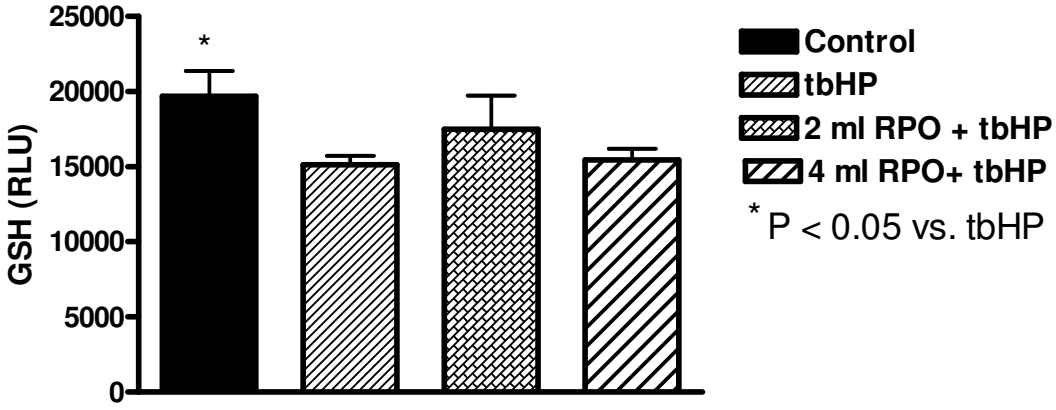

Figure 10: The effects of t-butyl hydroperoxide (tbHP) and red palm oil (RPO) on glutathione (GSH) production in rat sperm (n=6).

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CHAPTER V

Red Palm Oil: A Natural Good Samaritan for Sperm Apoptosis?

Y.G. Aboua[1], N. Brooks[1], D.O. Awoniyi[1]., S.S. du Plessis[2]

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

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

Corresponding author:

*YG Aboua

Tel: +27 21 938 9388

Fax: +27 21 938 9476

guillaboua@gmail.com

Abstract

Cumene hydroperoxide (cHP) and t-butyl hydroperoxide (tbHP) have been implicated in lipid peroxidation of sperm plasma membranes, DNA damage and apoptosis. This study aimed to investigate the *in vivo* effects of these hydroxides on rat sperm apoptosis, specifically caspase 3/7, and the possible protective effect offered by red palm oil (RPO). Rats were divided into three groups receiving either standard rat chow (SRC), 2ml RPO (in 25 g SRC / day) and 4ml RPO (in 25 g SRC / day) respectively. Furthermore, each group was divided into three subgroups. These subgroups consisted of rats injected with saline (control), 10µM cHP or 20µM tbHP. Rats fed with SRC and injected with 10µM of cHP or 20µM of tbHP showed a significant increase in caspase 3/7 activity compared to the control group (injected with 0.5 ml saline). On the other hand, animals fed with SRC in addition to 2 ml or 4 ml of RPO and injected with 10µM of cHP or 20µM of tbHP showed a significant decrease in the production of caspase 3/7 activity compared to those fed with SRC only. It can be concluded that RPO possibly reduces caspase 3/7 activity thereby inhibiting apoptosis caused in rat sperm by the *in vivo* induction of hydroperoxide.

Introduction

Apoptosis, or programmed cell death due to DNA fragmentation, is a distinctive form of eukaryotic cell death characterized by a series of morphologic and biochemical changes that result in elimination of cells from the tissues without eliciting an inflammatory response (Wyllie, 1980). Apoptosis is a genetico-

physiological process that leads to discard abnormal or damage spermatozoa to ensure cellular homeostasis during spermatogenesis in the form of cell suicide.

Reactive oxygen species (ROS) generated from abnormal sperm can stimulate the process of apoptosis, resulting in the death of sperm. ROS initiates a chain of reactions by activating caspases, ultimately leading to apoptosis (Said *et al.*, 2004). When ROS levels are raised pathologically, the process of apoptosis is also initiated in mature sperm. The process of apoptosis is accelerated by ROS-induced DNA damage, which in due course leads to a decline in the sperm count. Oxidative stress, due to excessive generation of ROS, is presumed to cause DNA damage in spermatozoa and has been correlated positively with apoptosis (Wang *et al.*, 2003) and negatively with the fertilisation rate (Host *et al.*, 2002, Agarwal *et al.*, 2005a, Sun *et al.*, 1997). Two distinct pathways exist in the initiation of apoptosis. In the extrinsic or receptor-linked apoptotic pathway, the induction of apoptosis occurs via death receptors (cell surface receptors) that transmit apoptotic signals initiated by specific ligands (Fadeel and Orrenius, 2005, Jin and El-Deiry, 2005). The intrinsic pathway is triggered by stress stimuli, including growth factor deprivation and DNA damage (Schuler and Green, 2001). This pathway involves the release of an extrinsic protein, cytochrome c on the outer surface of the inner mitochondrial membrane of the mitochondria during apoptosis (Fadeel and Orrenius, 2005, Jin and El-Deiry, 2005). The activation of the apoptosis-signalling pathway occurs in response to regulatory factors such as bcl-2 (Joza *et al.*, 2002, Burlacu, 2003) and p53 (Chang, 2002) .

The induction of apoptosis via intrinsic or extrinsic apoptotic pathways result in the activation of an initiator caspase, which activates a cascade of events leading to the activation of effector caspases, responsible for the cleavage of key cellular

proteins that lead to the typical morphological changes observed in cells undergoing apoptosis. Caspase-8 and caspase-10 are inhibitor caspases in death receptor-mediated apoptosis, while caspase-9 is the initiator caspase in mitochondrion-dependent apoptosis (Chen and Wang, 2002). These pathways differ in one fundamental aspect: one is "external" as it is promoted by a series of specific external ligands operating through defined transmembrane receptors; the other is an internal system where activation of the effector enzymes is induced by intracellular changes, involving the mitochondria (Garland, 2000,Joza *et al*., 2002). Despite the difference in the manner of initiation, the extrinsic and intrinsic pathways merge at the level of caspases-3 and 7 and once activated, they cleave intracellular targets, ultimately leading to the manifestation of apoptosis (LaCasse, 2004).

The supplemental intake of vitamins A, vitamin E and/or vitamin C improved reproductive function in laboratory and farm animals (Luck *et al*., 1995, Baldi *et al*., 2000, Tan *et al*., 2003). *In vitro* administration of vitamin C to patients suffering from congestive heart failure have been shown to suppress apoptosis in endothelial cells (Rossig *et al*., 2001). It was also reported that the intake of vitamin C improves sperm quality in heavy smokers (Dawson *et al*., 1992, Mello *et al*., 2001) and in male factor infertility patients (Lenzi *et al*., 1993a, Dalvit *et al*., 1998). Red palm oil (RPO) is the only vegetable oil with a balanced composition of saturated and unsaturated fatty acids both in processed and unprocessed forms (Sundram *et al*., 2003). RPO contains carotenoids, phosphatides, sterols, tocopherols and trace metals (Sundram *et al*., 2003), was shown to be effective against oxidative stress *in vitro* and *in vivo* (Serbinova *et al*., 1992).

Isong and co-workers (Isong *et al.*, 1997) had shown that RPO exerted effects on reproductive capacity by improving the efficiency of protein biosynthesis or utilization in such a way that was favourable to sex hormone function in rats fed with RPO (Alais C, 1991). It is also likely that RPO provided vitamin A, which is known to play a part in reproduction through the synthesis of sexual steroids (Alais C, 1991), embryogenesis and spermatogenesis (McArdle and Ashworth, 1999). Findings from animal models have highlighted the protective/therapeutic role of caspase inhibitors in many systemic diseases such as cardiac arrest, neurological, and rheumatoid diseases or in cases undergoing organ transplantation (Nicholson, 2000). In this study, we explored the effect of RPO, a natural cocktail of antioxidants, on caspase 3/7 activities in rat sperm apoptosis *in vivo*.

Materials and Methods

Ethical approval was obtained from the institutional Review Board. Male Wistar rats aged 10-12 weeks were placed randomly in three groups and fed *ad lib* with SRC in an ethical approved animal facility. Group 1 received no supplement while the food of groups 2 and 3 were supplemented with 2 ml and 4 ml RPO (Carotino SDN BHD Co: 69046-T, Johar-Bahru, Malaysia) in 25 g SRC / day respectively. Each group was divided into 3 subgroups. These subgroups were injected with saline (0.5 ml), 10µM cHP (0.5 ml, 80% aqueous, Sigma Chemical Co, South Africa) or 20µM tbHP (0.5 ml, 70% aqueous, Sigma Chemical Co, South Africa) respectively. The injections were performed daily for 5 consecutive days per week on 8 weeks period in order to target at least one complete cycle of spermatogenesis. Animals were sacrificed and epididymis were immediately excised and rinsed, followed by gentle mincing in 1.5 ml of phosphate buffered saline (PBS,

Sigma Chemical Co, South Africa). Sperm cells were retrieved, divided into aliquots and concentration adjusted to 2 x 10^6 cells / ml. The caspase-Glo® 3/7 Assay was subsequently performed according to the protocol provided by the manufacturer (Promega, UK).

Statistical analyses

GaphPad™ PRISM 4 was used for all statistical evaluations and graphical representations. Data are expressed as mean ± S.E.M. A one-way analysis of variance (ANOVA) test (with Bonferroni *post test* if $P<0.05$) was used for statistical analyses. Differences were regarded statistically significant if $P<0.05$.

Results

Caspase 3/7 activity is expressed as Relative Luminescence Units (RLU). Animals injected with 20 µM of tbHP (Figure 1) had significantly increased caspase 3/7 activity in their sperm compared to those injected with saline alone (4298 ± 308.2 vs.3161 ± 236.5, $P<0.05$). The amount of caspase 3/7 produced by sperm after *in vivo* tbHP administration were significantly lower in rats receiving the 2 ml (2803 ± 368.4, $P<0.05$) and 4 ml (3021 ± 365.9, $P<0.05$) RPO supplementation to their food when compared to the tbHP group fed only on SRC (Figure 1). From Figure 2, it is evident that rats injected with 10 µM of cHP had a significant higher production of caspase 3/7 in their sperm compared to those injected with saline only (4183 ± 289.6 vs. 3161 ± 236.5, $P<0.05$). Interestingly, the amount of caspase 3/7 produced in the sperm of rats fed with 2 ml (2966 ± 306.1, $P<0.05$) or 4 ml (3017 ± 333.6, $P<0.05$) supplementation of RPO in addition to cHP injection also significantly lower when

compared to rats receiving SRC only and injected with cHP (4183 ± 289.6). RPO supplementation alone did not affect caspase 3/7 activity (Data not shown).

Discussion

ROS and its role in male infertility have been researched extensively (Vernet *et al.*, 2001, Agarwal *et al.*, 2003, Ford, 2004, Henkel *et al.*, 2005). Many studies have shown the adverse effects of ROS on the different cellular compartments of spermatozoa including the DNA. High quantities of hydrogen peroxide (H_2O_2) can result in single- and double-strand DNA breaks and apoptosis. Thus, there is a cause to effect relation between apoptosis and DNA damage. This explains the positive correlation between H_2O_2, DNA damage and apoptosis (Moustafa *et al.*, 2004). H_2O_2 may trigger other signaling cascades related to apoptosis via oxidation of amino acid residues and other cell constituents. However, the role of caspases and apoptosis in ejaculated sperm remain unanswered. Caspase, c-jun, p53 and p21 are present in a restricted site for apoptosis (cytoplasmic droplets) in spermatids and immature spermatozoa (Weil *et al.*, 1998). Inactive and active forms of caspase markers have been detected in human sperm cells (Weng *et al.*, 2002, de Vries *et al.*, 2003) in both low and high motility fractions of donors and patients. A significant positive correlation has been shown between in situ-active caspase 3 in the sperm midpiece and DNA fragmentation in the low motility fractions of patients. This suggests that caspase dependent apoptotic mechanisms could originate in the cytoplasmic droplet or within mitochondria, and function in the nucleus (Weng *et al.*, 2002). Mature sperm do not have efficient operative mechanisms for protein synthesis. Both active and inactive forms of caspases (caspase 3) are absent in mature sperm cells (de Vries *et al.*, 2003). They do not show bicarbonate/PKA

dependent signs of apoptosis such as fractionation of DNA or mitochondrial inner membrane depolarization, but do show rapid amino phospholipids exposure (de Vries *et al.*, 2003). Paasch and co-workers (2003) reported that active caspases were present in subpopulations of mature sperm and to a greater extent in sperm from infertile patients (Paasch *et al.*, 2003). This shows that sperm cells with immature appearance and/or cytoplasmic droplets fail to expose phosphatidylserine (PS) and also shows no phosphotyrosine labeling (de Vries *et al.*, 2003). Alternatively, triggering of PS externalization and DNA fragmentation could be due to activation of other caspases or cellular pathways. It also leaves open the possibility that sperm apoptosis may, to some extent, be caspase independent. It can be seen from our result that despite the in *vivo* long-term hydroperoxide administration, both 2 ml and 4 ml of RPO supplementation was able to prevent the induction of caspase 3/7 activation and thus apoptosis.

Since RPO is a natural rich cocktail of antioxidants, it might act by interfering with the activation of the intrinsic or extrinsic apoptotic pathways. Pentikainen *et al.* (2001) postulated that the expression of the Fas ligand, a known inductor of testicular apoptosis, is down-regulated by TNF-α (Pentikainen *et al.*, 2001). Thus, in the seminiferous tubules, germ cell-derived TNF-α may regulate the level of the Fas ligand and thereby control physiological germ cell apoptosis (Pentikainen *et al.*, 2001). In another study Ghosh *et al.* have shown that the transcription activator nuclear factor NF-kB is a transcription factor expressed in the testis. When activated, NF-kB suppresses apoptosis through the transcriptional activation of genes whose products block apoptosis (Ghosh *et al.*, 1998). In normal growing cells, p53 is activated if DNA is damaged. If the DNA has been irreversibly damaged, the cellular p53 may initiate the elimination of programmed cell death and may stop the cell

cycle from starting DNA repair (Gupta *et al*., 2001). From these studies, we postulate that RPO might trigger the production of TNF, NF-kB (or mimic them) or suppress p53 in order to reduce or block apoptosis. Also RPO might block the oxidative stress pathway (caused by H_2O_2) that leads to DNA damage and apoptosis. In conclusion, the long term oral supplementation of RPO prevented apoptosis of rat sperm caused by hydroperoxides. It is therefore recommended as a useful supplement to prevent male germ cells against oxidative stress and subsequent cell death.

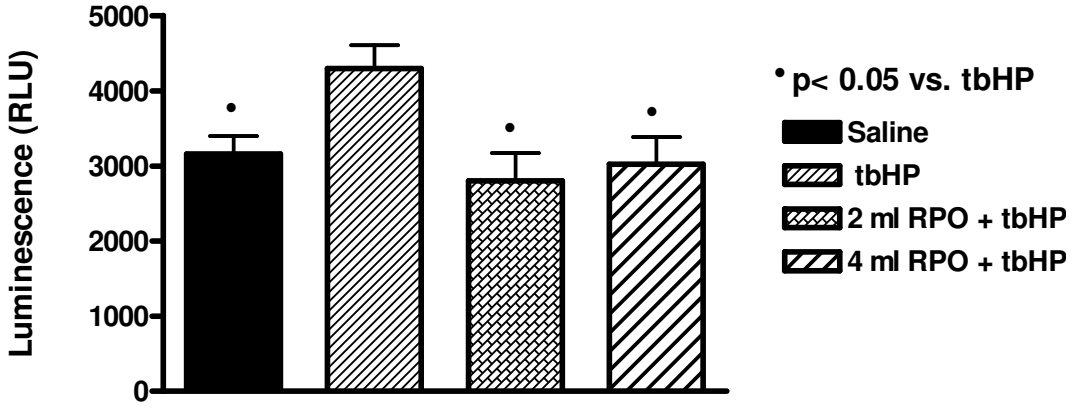

Figure 1: The effects of t-butyl hydroperoxide (tbHP) and red palm oil (RPO) on caspase 3/7 production in rat sperm (*n*=6 per subgroup).

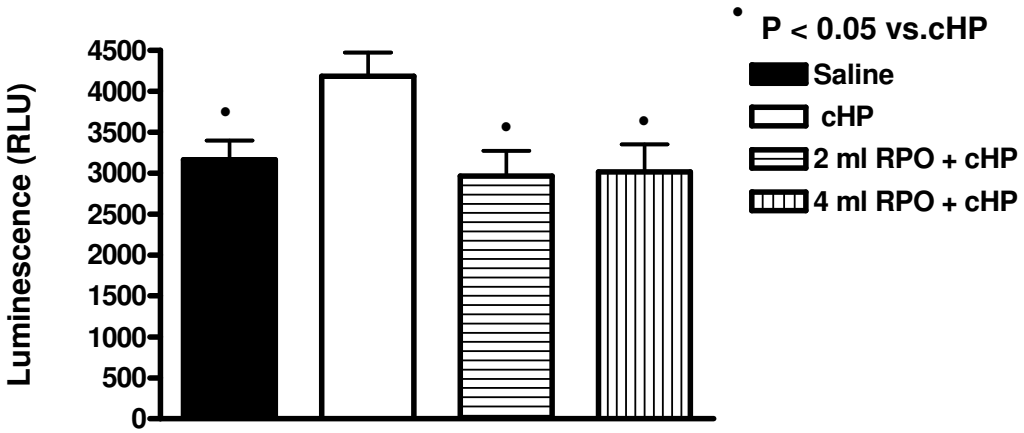

Figure 2: The effects of cumene hydroperoxide (cHP) and red palm oil (RPO) on caspase 3/7 production in rat sperm (*n*=6 per subgroup).

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Chapter VI

General discussion and conclusions

6.1. Establishing an *in vivo* model to study oxidative stress using t-butyl hydroperoxide (tbHP) and cumene hydroperoxide (cHP)

The long-term exposure of male rats to organic hyroperoxides (cHP and tbHP) via intraperitoneal injection had disturbed the cellular environment. CHP (10µM and 20µM) and tbHP (20µM and 40µM) had significantly decreased epididymal sperm concentration and motility. One hypothesis suggests that H_2O_2 diffuses across the membranes into the cells and inhibits the activity of some vital enzymes such as glucose-6-phosphate dehydrogenase (G6PD) and the availability of NADPH, which is then used as a source of electrons by spermatozoa to fuel the generation of ROS by the NADPH oxidase enzyme system (Aitken *et al.*, 1997). Another hypothesis involves a series of interrelated events resulting in a decrease in protein phosphorylation and sperm immobilization, both of which are associated with a reduction in membrane fluidity that is necessary for sperm-oocyte fusion (de Lamirande and Gagnon, 1992). Griveau and co-workers have shown that reactive oxygen species cause a decrease in sperm motility, an increase in lipid peroxidation, and a loss of membrane PUFA (Griveau *et al.,* 1995a) (Figure 6.1).

In the sperm plasma membrane, PUFAs are required to give the plasma membrane the fluidity needed for sperm motility as well as participation in the

membrane fusion events associated with fertilization, and the structural integrity required for viability. Loss of integrity can also lead to an increase in membrane permeability and a loss in the capacity to regulate the intracellular concentrations of ions involved in the control of sperm movement. These results emphasize the inherent vulnerability of the male germ cell to oxidative stress, particularly during late spermatogenesis and the early stages of epididymal sperm maturation. Membrane lipids present in subcellular organelles are highly susceptible to free radical damage. Lipids, when reacted with free radicals can undergo the highly damaging chain reaction of LPO leading to both direct and indirect effects (Devasagayam *et al.,* 2004). During LPO a large number of toxic byproducts are also formed that can have effects at a site away from the area of generation, behaving as 'second messengers'. The damage caused by LPO is highly detrimental to the functioning of the cell (Devasagayam *et al.,* 2004). Initiation of a peroxidative sequence is due to the attack by any species, which can extract a hydrogen atom from a methylene group (CH_2), leaving behind an unpaired electron on the carbon atom (•CH). The resultant carbon radical is stabilized by molecular rearrangement to produce a conjugated diene, which then can react with an oxygen molecule to form a lipid peroxyl radical (LOO•). These radicals can further remove hydrogen atoms from other lipid molecules to form lipid hydroperoxides (LOOH) and at the same time propagate LPO further. The peroxidation reaction can be terminated by a number of reactions. The process of LPO, gives rise to many products of toxicological interest like MDA, 4-hydroxynonenal and various 2-alkenals. Our results showed that the amounts of MDA in the epididymal sperm and in the testicular tissue negatively correlated with the percentages of sperm motility in rats, which is in agreement with the literature (Agarwal *et al*., 2003). The peroxidative process initiated by cHP and tbHP may lead

to ROS-mediated protein oxidation (Ong *et al.,* 2002) which reduced the sperm counts (Agarwal *et al*., 2003) possibly due to cell death (Figure 6.1).

The high levels of cHP (10 µM and 20µM) and tbHP (20µM and 40µM) overwhelmed the antioxidant capacity of SOD, GSH and CAT. The lower SOD concentration in sperm cells could be attributed to the assault of the high doses of cHP and tbHP. SOD presents the first line of defence against superoxide, as it dismutases the superoxide anion to H_2O_2 and O_2 (Nehru and Anand, 2005). This decreased in the SOD activity indicates either reduced synthesis or elevated degradation or inactivation of the enzyme. Both H_2O_2 and HO• were increased in sperm due to cHP or tbHP exposure, which in turn inhibited SOD activity. CAT is known to neutralize H_2O_2 to H_2O and O_2. There was a significant decline in the activity of CAT observed in cHP or tbHP treated rats and it might be due to the reduced conversion of $O_2^{\bullet-}$ to H_2O_2 by SOD led to the accumulation of $O_2^{\bullet-}$. This accumulation of $O_2^{\bullet-}$ inhibited the activity of CAT (Kono and Fridovich 1982, Thomas *et al*., 2006). Organic hydroperoxides (cHP and tbHP) might affect the GSH synthesis by decreasing the activity of glutathione-synthase thus leading to a reduced GSH content. On the other hand, the decreased GSH level could be also ascribed to insufficient supply of NADPH. The principal antioxidant defences in sperm are SOD, GSH peroxidase and CAT (Storey, 1997). The physiological role of GSH is an essential intracellular reducing agent for maintenance of thiol groups on intracellular proteins and for antioxidant molecules. GSH protects cells against oxidative stress and other types of damage, which may arise from compounds of endogenous and exogenous sources. The continued activity of glutathione peroxidase depends on the regeneration of reduced glutathione by glutathione reductase, which in turn relies on NADPH, the principal source of which in sperm is

the pentose phosphate shunt. The activity of G6PD, which is the first enzyme in the pentose phosphate pathway, may limit the rate of NADPH production and, hence, the ability of the glutathione peroxidase system to detoxify peroxides (Storey *et al.*, 1998). Our results showed the active involvement of SOD, CAT and GSH in the scavenging of ROS. The *in vivo* intraperitoneal administration of organic hydroperoxides negatively affects the male reproductive system.

In this study, rats fed with SRC and injected with 10µM of cHP or 20µM of tbHP showed a significant increase in caspase 3/7 activity compared to the control group (injected with 0.5 ml saline). The exposure to cHP and tbHP overwhelmed the sperm defences and possibly induces DNA damage. The high doses of cHP and tbHP could lead to single and double strand DNA breaks and apoptosis. The generation of OH^- by reaction of H_2O_2 with the transition metal ions already bound onto the DNA leads to strand breakage, base modification, and deoxyribose fragmentation. In the nuclease activation mechanism, oxidative stress led to inactivation of calcium (Ca^{2+}) binding by the endoplasmic reticulum, inhibition of plasma membrane Ca^{2+} extrusion systems, and the release of Ca^{2+} from mitochondria (Aruoma, 1998). This sequence of events led to increases in the levels of intracellular free calcium ions. The endonuclease activation resulted to DNA fragmentation without the base modification observed in the Fenton mechanism (Aruoma, 1998). At this point of the process of spermatogenesis, the DNA repair mechanisms might be turned off. Unfortunately, sperm cells are unable to repair the damage induced by excessive amounts of hydroperoxide because they lack the cytoplasmic enzyme systems that are required to accomplish this repair (Agarwal *et al.*, 2005b). Apoptosis is one of the possible causes of DNA fragmentation. Other causes include (i) chromatin remodelling during the process of spermiogenesis; (ii)

DNA damage during sperm transport through the seminiferous tubules; (iii) activation of sperm caspases and endonucleases. The activation of caspases and endonucleases in sperm is different from that described for the classic apoptosis pathway (Sakkas *et al.*, 1999). Agarwal and co-workers (2003) reported that the levels of apoptosis in mature sperm were significantly correlated with the levels of seminal ROS, as determined by the chemiluminescence assay. The levels of caspase 3 and caspase 9 in ejaculated spermatozoa from infertility patients were significantly higher than that from the normal healthy sperm donors (Agarwal *et al.*, 2003). In addition, levels of seminal ROS were positively correlated with levels of caspase 3 and caspase 9. The caspase gene family encodes a set of proteases responsible for carrying out programmed cell death. A ROS-dependent pathway for apoptosis was suggested based on the finding that H_2O_2 induces apoptosis in cell cultures (Sentman *et al.,* 1991). It was shown that ROS can initiate apoptosis (Thompson, 1995, Moustafa *et al.*, 2004). At the molecular level, ROS directly affect DNA, and also alters an intracellular Ca^2+ level, which is shown to be one of the most powerful ways of inducing apoptosis. We found a positive relationship between increased sperm damage and ROS. The positive relationship of ROS and caspases 3/7 suggests possible DNA damage through increased ROS production; however, DNA damage was not measured. During the scavenging process of ROS, two molecule of GS• produce GSSG that can be converted back to GSH by NADPH-dependent GSH reductase (Figure 6.1).

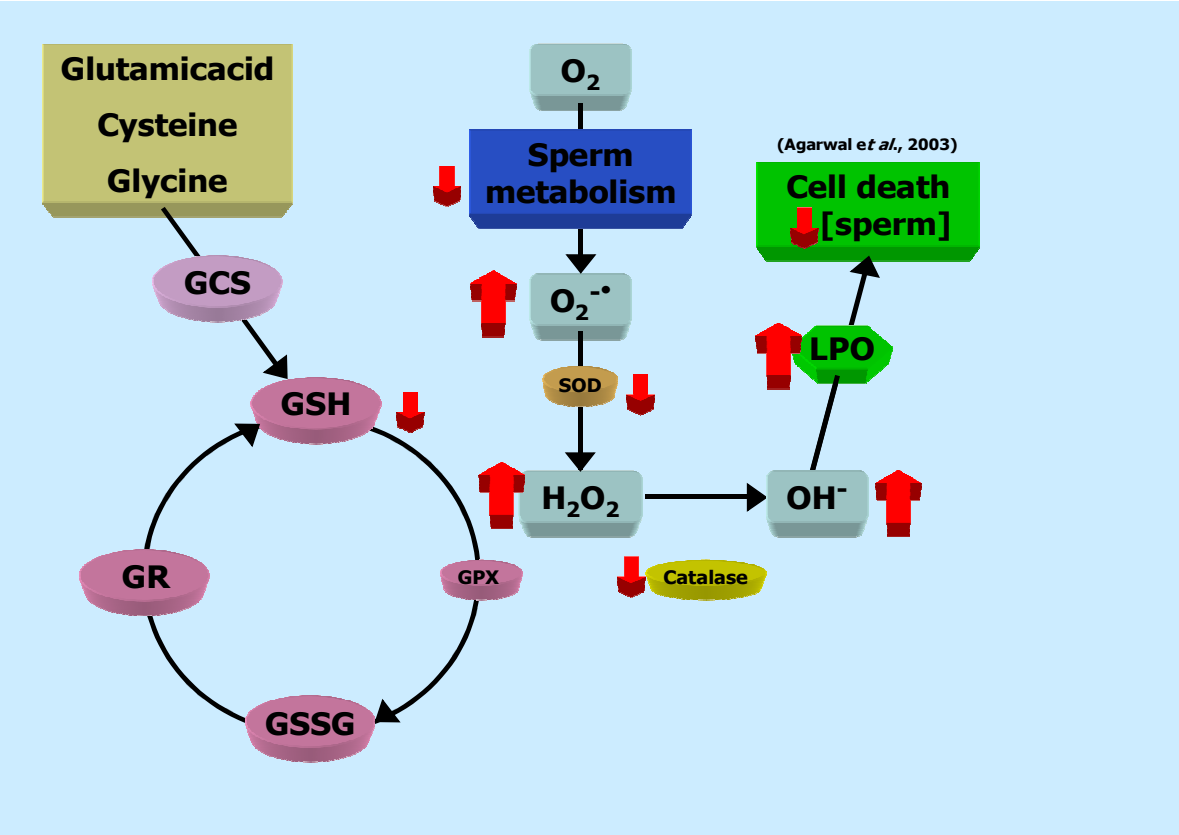

Figure 6.1: The effects of organic hydroperoxide on sperm parameters.

6.2 Evaluation of the effects of RPO supplemented diet on induced oxidative stress in epididymal sperm and apoptosis

A great number of studies have suggested that antioxidant nutrients and/or medicines play a protective role in human health (Sies and Stahl, 1995, Krinsky 1993 & 2001, Paiva and Russell, 1999). In the present study, RPO was observed to reduce/reverse the oxidative damage caused in the rat epididymal sperm and testicular tissue. RPO was able to reverse the negative effects of the organic hydroperoxides in this animal model of OS. Moreover, the sperm of animals receiving RPO displayed higher SOD, CAT and GSH activities and sperm motility, while the amounts of DCF, MDA, LPO and caspases 3/7 were reduced compared to those injected with cHP or tbHP and receiving only SRC. Antioxidants are the main defence against OS induced by free radicals. There are two kinds: prevention antioxidants and scavenger antioxidants. Prevention antioxidants block the formation of new ROS, whereas scavenger antioxidants remove the ROS that have already formed. Prevention antioxidants (transition metal ions) for example iron, are involved in the generation of the highly reactive alcohol by Fenton's reaction (Biemond *et al.*, 1984, Ochsendorf, 1999). Scavenger antioxidants (dietary antioxidants) form an essential part of the human antioxidant defence system.

RPO is a natural rich cocktail of antioxidants containing (α and β) carotene, (α and β) tocopherol (Sundram *et al.*, 2003). α-tocopherol is the most important radical scavenger that protects membrane phospholipids and proteins from oxidative damage (Niki *et al.*, 1995, Takenaka *et al.*, 1991). These functions account for its protective effect against membrane morphological changes. The simultaneous administration of cHP or tbHP and RPO treatment significantly reduced the

generation of ROS and LPO when compared with cHP or tbHP treated rats alone. The main function of RPO was to prevent the peroxidation of membrane phospholipids and prevent cell membrane damage through its antioxidant action (Figure 6.2). The lipophilic character of tocopherol as content of RPO was able to locate itself in the interior of the cell membrane bilayer (Wang and Quinn, 1999, Krishnamoorthy *et al.,* 2007). Tocopherol-OH can transfer a hydrogen atom with a single electron to a free radical, thus removing the radical before it can interact with the cell membrane. In response to RPO supplementation, SOD and CAT levels were higher in these animals compared to those treated with hydroperoxide only. The activity of GSH peroxidase is highly dependent on GSH concentration. The decrease in the activity of GSH peroxidase may be due to the decreased synthesis or elevated degradation or inactivation of the enzyme. GSH can scavenge peroxynitrite and HO• as well as convert H_2O_2 to water with the help of GSH peroxidase. In this study, GSH content was significantly decreased in the sperm of cHP or tbHP treated rats. The lower levels of GSH content in the rat sperm indicate a greater participation of reduced glutathione in H_2O_2 detoxification. Simultaneous RPO supplementation elevated the level of GSH in sperm. This enhanced intracellular transport of GSH was essential in maintaining the redox state and cope with the oxidative stress in the RPO supplemented rats. Glutathione radicals (GS•) are formed during scavenging of ROS process. Two GS• produce GSSG that can be converted back to GSH by NADPH-dependent GSH reductase. Previous studies reported that PCB (Aroclor 1254) decreased the activities of SOD, CAT and GSH and it was reversed by α tocopherol and ascorbic acid in ventral prostate and testicular tissue (Venkataraman *et al.,* 2004, Murugesan *et al*., 2005, Krishnamoorthy *et al*., 2007).

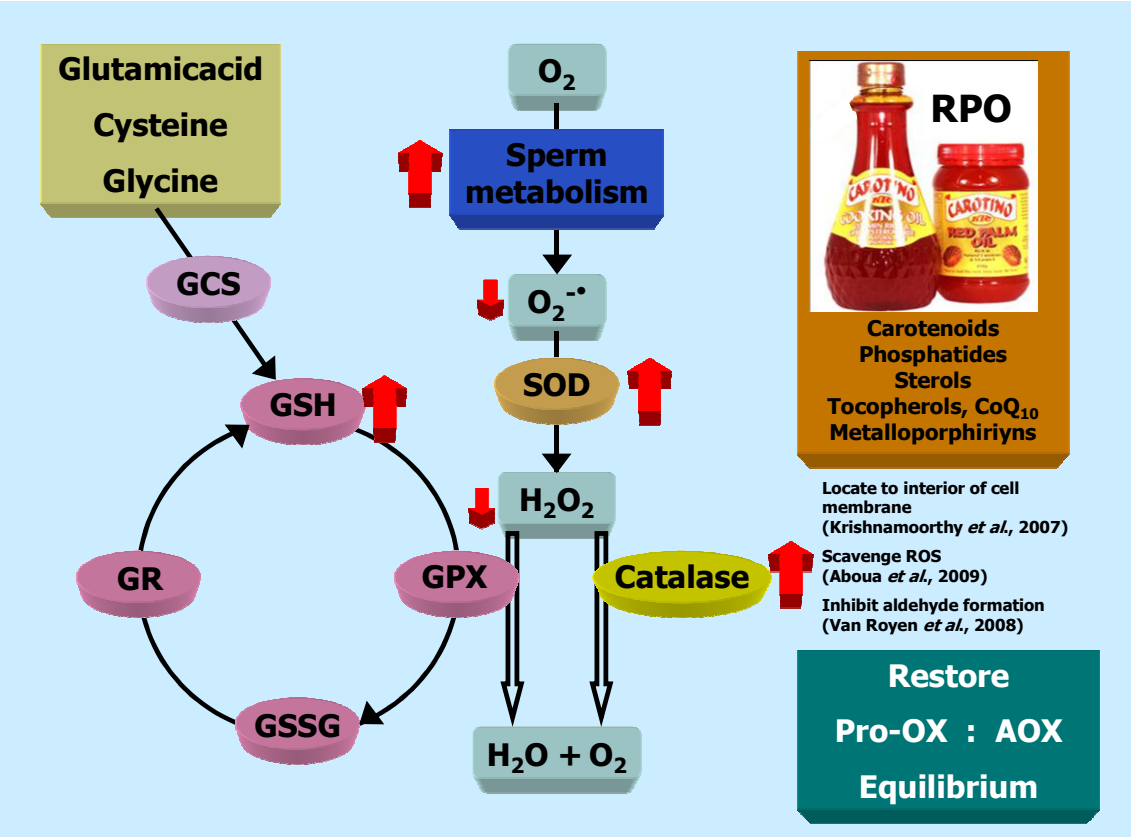

Figure 6.2: The effects of RPO supplemented diet on induced oxidative stress in epididymal sperm and apoptosis.

Conclusion

This thesis reports two congruent studies which provide new insights into the field of andrology. In the first part, the long term *in vivo* intraperitoneal administration of organic hydroperoxides negatively affects the male reproductive system. It has lowered sperm concentration, sperm motility, and impaired antioxidant activities in both epididymal sperm and testicular tissue, showing the active involvement of SOD, CAT and GSH in the scavenging of ROS. We therefore, successfully created an animal model to test the adverse effects of OS on male reproductive parameters. In the quest of finding possible treatments to the *in vivo* OS caused by organic

peroxides, RPO was found to be an excellent dietery supplement in reversing the enzymatic and non-enzymatic antioxidants during the second part of the study. The RPO might actively be involved in the scavenging mechanism to support the reduced activities of SOD, CAT and GSH in order to maintain the balance between ROS and the antioxidant systems. In addition RPO might block the oxidative stress pathway (caused by H_2O_2) that led to DNA damage and apoptosis. The mechanisms by which RPO protection was achieved could involve one or more of several different antioxidant properties exhibited by its components (tocopherols, carotenoids, fatty acids, trace elements, CoQ_{10} and porphyrin molecules). We therefore, propose that a daily intake of RPO supplement to the diet might be helpful to protect males against the adverse effects of ROS in sperm function and possibly assit to preserve fertility.

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