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

by

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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.
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 H₂O₂) 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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<tbody>
<tr>
<td>AC</td>
<td>Adenylyl cyclase</td>
</tr>
<tr>
<td>APAF1</td>
<td>Adaptor that form apoptosome to activate caspases 9</td>
</tr>
<tr>
<td>•CH</td>
<td>Unpaired electron on the carbon atom</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Calcium cation</td>
</tr>
<tr>
<td>CAT</td>
<td>Catalase</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cHP</td>
<td>Cumene hydroperoxide</td>
</tr>
<tr>
<td>CH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Methylene group</td>
</tr>
<tr>
<td>CYPs</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DCF</td>
<td>Dichlorofluorescein</td>
</tr>
<tr>
<td>DCFH</td>
<td>2′7′-dichlorofluorescin</td>
</tr>
<tr>
<td>DIABLO</td>
<td>Direct inhibitor of apoptosis-binding protein</td>
</tr>
<tr>
<td>DISC</td>
<td>Death-inducing signalling complex</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor R</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle-stimulating hormone</td>
</tr>
<tr>
<td>GS•</td>
<td>Glutathione radicals</td>
</tr>
<tr>
<td>G6PD</td>
<td>Glucose-6-phosphate-dehydrogenase</td>
</tr>
<tr>
<td>GPx</td>
<td>Glutathione peroxidase</td>
</tr>
<tr>
<td>GR</td>
<td>Glutathione reductase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>GSSG</td>
<td>Glutathione disulfide</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>IAP</td>
<td>Inhibitor of apoptosis</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol 1,4,5-trisphosphate</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium chloride</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinizing hormone</td>
</tr>
<tr>
<td>LOO•</td>
<td>Lipid peroxyl radical</td>
</tr>
<tr>
<td>LOOH</td>
<td>Lipid hydroperoxides</td>
</tr>
<tr>
<td>LPO</td>
<td>Lipid peroxidation</td>
</tr>
<tr>
<td>MDA</td>
<td>Malondialdehyde</td>
</tr>
<tr>
<td>NADPH</td>
<td>Reduced nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NK cells</td>
<td>Neutral Killer cells</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor κB</td>
</tr>
<tr>
<td>8-OhdG</td>
<td>8-hydroxy-2-deoxyguanosine</td>
</tr>
<tr>
<td>O₂</td>
<td>Oxygen</td>
</tr>
<tr>
<td>O₂−</td>
<td>Superoxide anion</td>
</tr>
<tr>
<td>OAM</td>
<td>Outer acrosomal membrane</td>
</tr>
<tr>
<td>OH⁻</td>
<td>Hydroxyl radicals</td>
</tr>
<tr>
<td>ONOO⁻</td>
<td>Peroxynitrite anion</td>
</tr>
<tr>
<td>OS</td>
<td>Oxidative stress</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PCB</td>
<td>Aroclor 1254</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLCβ₁</td>
<td>Phospholipase C beta</td>
</tr>
<tr>
<td>PLCγ</td>
<td>Phospholipase C gama</td>
</tr>
<tr>
<td>PM</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acids</td>
</tr>
<tr>
<td>RFU</td>
<td>Relative fluorescence unit</td>
</tr>
<tr>
<td>RLU</td>
<td>Relative luminescence unit</td>
</tr>
<tr>
<td>RNOO⁻</td>
<td>Peroxyl radicals</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPO</td>
<td>Red palm oil</td>
</tr>
<tr>
<td>SMAC</td>
<td>Second mitochondria-derived activator of caspase</td>
</tr>
<tr>
<td>SOC</td>
<td>Store operated Ca²⁺</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>SRC</td>
<td>Standard rat chow</td>
</tr>
<tr>
<td>TBARS</td>
<td>Thiobarbituric acid reactive substances</td>
</tr>
<tr>
<td>tbHP</td>
<td>T-butyl hydroperoxide</td>
</tr>
<tr>
<td>TK</td>
<td>Tyrosine kinase</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>ZP</td>
<td>Zona pellucida</td>
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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 cryptorchidism (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 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.
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$_2$•-) anion, hydrogen peroxide (H$_2$O$_2$), peroxyl (RNOO•) radicals, and the very reactive hydroxyl (OH•) 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 lifestyle factors, systemic pathology, varicoceles, 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, $O_2\cdot^-$. 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 $O_2\cdot^-$ 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 $>1 \times 10^6$ 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.
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 \( \text{H}_2\text{O}_2 \) 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 $\text{H}_2\text{O}_2$ 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 HCO$_3^-$ 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$_2$O$_2$ 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 (PLCβ₁) 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 [Ca²⁺]ᵢ channels in the outer acrosomal membrane (OAM) and DAG would activate PKC to open [Ca²⁺]ᵢ channels in the plasma membrane (PM). As a result, cytosolic [Ca²⁺] increases and acrosomal [Ca²⁺] is decreased leading to store operated Ca²⁺ (SOC) activation and a sustained enhancement of cytosolic [Ca²⁺]. The relatively high [Ca²⁺] (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).
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
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.

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 phytol 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 phytol 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 phenoxy 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 unsaturated 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 metalloporphyrins 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 \( \text{O}_2^* \), \( \text{H}_2\text{O}_2 \) and peroxynitrite (ONOO) alone or in concert.

### 2.7.1.4 Coenzyme Q\(_\text{10}\)

Coenzyme Q\(_\text{10}\) (CoQ\(_\text{10}\)) is a non-enzymatic antioxidant that is related to low density lipoproteins and protects against peroxidative damage (Frei et al., 1990). CoQ\(_\text{10}\) 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. CoQ\(_\text{10}\) plays a vital role in ATP production and serves as an antioxidant 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

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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 °C) and stored at -80 °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 °C, 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 °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 °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 °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

GraphPad™ 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 ($67.00 \pm 5.73 \times 10^6$/ml) and 20µM ($58.67 \pm 4.80 \times 10^6$/ml) of cHP and 20µM ($65.50 \pm 4.05 \times 10^6$/ml) and 40µM ($59.17 \pm 4.10 \times 10^6$/ml) of tbHP when compared to the control group ($109 \pm 10.88 \times 10^6$/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 ($30.94 \pm 3.55$ nmol MDA / 2x10$^6$ sperm, $P<0.01$) or 20µM and 40µM tbHP ($27.38 \pm 1.07$ and $25.92 \pm 0.91$ nmol MDA / 2x10$^6$ sperm, $P<0.05$) were significantly elevated above control values ($19.77 \pm 1.07$ 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 ($37.05 \pm 2.59$ and $33.70 \pm 8.05$ nmol MDA / mg tissue, $P<0.01$ and $P<0.05$ respectively) or 40µM tbHP ($58.09 \pm 1.98$ nmol MDA / mg tissue, $P<0.001$) compared to control ($16.63 \pm 1.20$ 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 (10\( \mu \)M: \( 47662 \pm 4243 \) RLU, \( P<0.001 \) and 20\( \mu \)M: \( 51118 \pm 4776 \) RLU, \( P<0.01 \)) or tbHP (20\( \mu \)M: \( 53671 \pm 4112 \) RLU and 40\( \mu \)M: \( 46548 \pm 2949 \) 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 10\( \mu \)M (31456 ± 3742, \( P<0.05 \)) and 20\( \mu \)M (31922 ± 7776 RLU, \( P<0.05 \)) cHP and 40\( \mu \)M 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 \( (10\mu M: 0.20 \pm 0.01 \text{ U/µl}; 20\mu M: 0.18 \pm 0.02 \text{ U/µl}) \) or tbHP \( (20\mu M: 0.21 \pm 0.01 \text{ U/µl}; 40\mu M: 0.17 \pm 0.01 \text{ U/µl}) \) when compared to the control \( (0.51 \pm 0.21 \text{ U/µl}) \) (Figures 6 A and B). A similar trend was observed in SOD concentration of testicular tissue \( (P<0.001) \) in animals treated with cHP \( (10\mu M: 0.21 \pm 0.08 \text{ U/µl}; 20\mu M: 0.18 \pm 0.02 \text{ U/µl}) \) or tbHP \( (20\mu M: 0.21 \pm 0.02 \text{ U/µl}; 40\mu M: 0.16 \pm 0.03 \text{ U/µl}) \) when compared to control \( (0.56 \pm 0.21 \text{ U/µl}) \) (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 \( (\text{cHP: } r = -0.8236, p = 0.0865: \text{ not significant and } \text{tbHP: } r = -0.7994, p = 0.1045: \text{ not significant}) \). 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 \( (\text{cHP: } r = 0.9805, p = 0.0033 \text{ and } \text{tbHP: } r = 0.9619, p = 0.0089) \) (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 \( \text{Dacheux et al., 2003} \). 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 \( 10^{10} \text{ cells/ml} \). 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 hyropheroxides (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$_2$O$_2$ 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$_2$O$_2$ can penetrate the plasma membrane, cause protein oxidation and increase LPO production. In the light of the membrane permeability of H$_2$O$_2$, 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$_2$O$_2$ (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 $\text{H}_2\text{O}_2$ and $\text{O}_2$ (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).

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

*** 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

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Lipid peroxidation (LPO)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Epididymal sperm</td>
</tr>
<tr>
<td>Motility</td>
<td>cHP</td>
</tr>
<tr>
<td>Motility r</td>
<td>-0.9640</td>
</tr>
<tr>
<td>p</td>
<td>0.0082</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Glutathione (GSH)</th>
<th>Superoxide dismutase (SOD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Epididymal sperm</td>
<td>Testicular tissue</td>
</tr>
<tr>
<td>Motility r</td>
<td>0.9543</td>
<td>0.9750</td>
</tr>
<tr>
<td>p</td>
<td>0.0116</td>
<td>0.0047</td>
</tr>
<tr>
<td>LPO r</td>
<td>-0.9542</td>
<td>-0.9749</td>
</tr>
<tr>
<td>p</td>
<td>0.0117</td>
<td>0.0047</td>
</tr>
</tbody>
</table>
**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<0.001 vs. control).
References


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

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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 tBP (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°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°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 (H₂O₂) by xanthine oxidase to form superoxide anion (O₂⁻). The O₂⁻ 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 0.1x10⁶ 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 H₂O₂ substrate left over from the CAT reaction (Zhou *et al.*, 1997). In brief, 50 µL of H₂O₂ (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 $\text{H}_2\text{O}_2$ 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 $\text{H}_2\text{O}_2$ 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
\( \text{H}_2\text{O}_2 \) 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 \( \text{O}_2^- \) to \( \text{H}_2\text{O}_2 \) and oxygen. In this study, a significant decrease in the SOD concentration in the epididymal sperm of rats treated with 10\( \mu \text{M} \) cHP or 20\( \mu \text{M} \) 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 \( \text{H}_2\text{O}_2 \) to water and superoxide. There is a significant decline in the activity of catalase in the epididymal sperm of rats treated with 10\( \mu \text{M} \) cHP or 20\( \mu \text{M} \) 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 \( \text{H}_2\text{O}_2 \). 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$_2$O$_2$ 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.

Acknowledgements: The authors wish to express their gratitude to Mr D.O. Awoniyi for technical assistance.
Table 1: Body weights, testicular weights, epididymal weights and sperm concentrations of rats treated with organic hydroperoxides (n=6).

<table>
<thead>
<tr>
<th></th>
<th>SRC</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Saline</td>
<td>cHP</td>
<td>tbHP</td>
</tr>
<tr>
<td><strong>Body weight</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(gm)</td>
<td></td>
<td>404.7±20.13</td>
<td>397.0±20.69</td>
<td>413.8±16.41</td>
</tr>
<tr>
<td><strong>Testicular weight</strong></td>
<td></td>
<td>3.88±0.06</td>
<td>3.94±0.06</td>
<td>3.77±0.12</td>
</tr>
<tr>
<td>(gm)</td>
<td></td>
<td>0.53±0.01</td>
<td>0.49±0.01</td>
<td>0.51±0.01</td>
</tr>
<tr>
<td><strong>Sperm concentration</strong></td>
<td></td>
<td>18.85±1.34</td>
<td>13.22±0.74**</td>
<td>14.22± 1.10*</td>
</tr>
<tr>
<td>(10^7 / ml)</td>
<td></td>
<td>86.67±3.33</td>
<td>65.00±3.65**</td>
<td>65.83±5.06**</td>
</tr>
<tr>
<td><strong>Motility</strong></td>
<td></td>
<td>86.67±3.33</td>
<td>65.00±3.65**</td>
<td>65.83±5.06**</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th></th>
<th>DCF</th>
<th>TBARS</th>
<th>SOD</th>
<th>CAT</th>
<th>GSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Sperm]</td>
<td>r</td>
<td>-0.312</td>
<td>-0.175</td>
<td>-0.095</td>
<td>0.6107</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.0474</td>
<td>0.2731</td>
<td>0.5544</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Motility</td>
<td>r</td>
<td>-0.371</td>
<td>-0.311</td>
<td>0.1476</td>
<td>0.2079</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.0155</td>
<td>0.0452</td>
<td>0.3508</td>
<td>0.1864</td>
</tr>
<tr>
<td>DCFH</td>
<td>r</td>
<td>0.3799</td>
<td>-0.186</td>
<td>-0.535</td>
<td>-0.18</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.0131</td>
<td>0.2387</td>
<td>0.0003</td>
<td>0.2668</td>
</tr>
<tr>
<td>TBARS</td>
<td>r</td>
<td></td>
<td>-0.15</td>
<td>-0.326</td>
<td>0.0517</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td></td>
<td>0.3421</td>
<td>0.0353</td>
<td>0.7512</td>
</tr>
</tbody>
</table>
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?

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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 hydroperoxides 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, Johor-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**

GraphPad™ 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$_2$O$_2$) 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$_2$O$_2$, DNA damage and apoptosis (Moustafa *et al.*, 2004). H$_2$O$_2$ 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$_2$O$_2$) 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).
References


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 hydroperoxides (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$_2$O$_2$ 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 \( \text{H}_2\text{O}_2 \) and \( \text{O}_2 \) (Nehru and Anand, 2005). This decreased in the SOD activity indicates either reduced synthesis or elevated degradation or inactivation of the enzyme. Both \( \text{H}_2\text{O}_2 \) and \( \text{HO}^\bullet \) were increased in sperm due to cHP or tbHP exposure, which in turn inhibited SOD activity. CAT is known to neutralize \( \text{H}_2\text{O}_2 \) to \( \text{H}_2\text{O} \) and \( \text{O}_2 \). 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 \( \text{O}_2^- \) to \( \text{H}_2\text{O}_2 \) by SOD led to the accumulation of \( \text{O}_2^- \). This accumulation of \( \text{O}_2^- \) 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\(_2\)O\(_2\) 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$_2$O$_2$ 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
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 H₂O₂ 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 H₂O₂ 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 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 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$_2$O$_2$) 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 assist to preserve fertility.
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