The influence of superoxide and anti-oxidants on human sperm function and apoptosis with special reference to the role of Red Palm Oil

by

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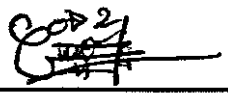
DECLARATION

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



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ABSTRACT

Cells living under aerobic conditions constantly face the oxygen paradox i.e. oxygen is indispensable for supporting life; however, its metabolites such as reactive oxygen species (ROS) can modify cell function. Oxidative stress (OS) arises as a consequence of excessive ROS production and/or impaired antioxidant defence mechanisms. Environmental and physiological factors have been implicated in poor sperm function. Excessive ROS generation results in oxidative damage and consequently decreased sperm function. The objectives of this study are threefold: (i) To measure the production of $O_2^{-\bullet}$ by sperm by means of flow cytometry. (ii) To determine effects of $O_2^{-\bullet}$ on sperm motility and viability in the presence or absence of commercially available scavengers and RPO. (iii) To determine effects of $O_2^{-\bullet}$ on selective apoptotic markers in ejaculated sperm in the presence of absence of commercially available scavengers and RPO.

In the first part of the study, suitable solvents were investigated in order to introduce RPO (because of its hydrophobic nature) as a possible scavenger of ROS in human spermatozoa. Secondly, the $O_2^{-\bullet}$ donor; 2, 3-dimetoxy-1-naphthoquinone (DMNQ) (2.5µM-100µM, 60 min.) was added to normozoospermic post swim-up samples in the absence or presence of Mn(III)TMPyP (50µM) or SOD (50IU) or RPO at 0.1% and 0.5%. CASA was used to analyse motility parameters, while FACS was used to determine viability (PI, 1mM, 15 min.) and $O_2^{-\bullet}$ levels (DHE, 30µM, 15min.). In the last part of the study, three apoptotic markers (Annexin-V, Caspase-3 and DNA fragmentation) were used to investigate sperm apoptosis. Paired *Student's t-test* and ANOVA were used to analyse results.

This study showed that high concentration of superoxide is deleterious to sperm motility, viability and sperm function. The addition of Mn(III)TMPyP (50µM) offered some form of protection with regards to sperm motility parameters. Mn(III)TMPyP, however, had shown contradicting results on sperm viability. The addition of SOD had shown contradictive effects as it exhibited anti-oxidant as well as pro-oxidant characteristics on sperm motility and viability depending on the concentrations of superoxide present. The effect of RPO on sperm motility, viability and possible protection against superoxide is ambiguous as under certain conditions it contributes to serve negative outcomes.

The higher percentage of live cells found was due to the swim-up procedure, a technique that eliminates non-viable and apoptotic spermatozoa. There were higher percentages of necrotic spermatozoa at any concentration of DMNQ compared to those of apoptotic spermatozoa in all groups (DMNQ, DMNQ+RPO, DMNQ+Mn(III)TMPyP and DMNQ+SOD). This implies that the death of sperm cells during *in vitro* incubation (at 37°C) occurs by necrosis rather than by apoptosis. Because of certain limitations (amount of DMNQ, small sample size and short incubation duration) in this study, it might be possible that the amount of superoxide administered was not enough to trigger apoptosis significantly.

The use of dihydroethidium (DHE) as a probe for detecting the generation of superoxide anion by human spermatozoa and the examination of the scavenging effects of Mn(III)TMPyP, SOD and RPO showed that the amount of DHE fluorescence produced by human spermatozoa was minimal. Finally, Mn(III)TMPyP, SOD and RPO respectively showed good, partial and no superoxide scavenging effects.

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Finally, I could not complete this study without the help of **Jesus Christ** my LORD.

This study was financially supported by the **National Research Foundation** and the **Cape Peninsula University of Technology.**

DEDICATION

This dissertation is dedicated to

All the victims and those who lost their life during the war of

COTE D'IVOIRE

particularly

Jonas ABOUA

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GLOSSARY

Abbreviations	Definition/Explanation
ALH	Amplitude of lateral head
ART	artificial reproduction technologies
BCF	Beat-cross frequency
BSA	Bovine Serum Albumin
CASA	Computer aided sperm analysis
DHE	Dihydroethidium
DMNQ	2, 3-dimetoxy-1-naphthoquinone
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
FACS	Flow cytometric analysis
FITC	Fluorescein isothiocinate
FSC	Forward scatter
H_2O_2	Hydrogen peroxide
LIN	Linearity
LPO	Lipid peroxidation
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
$NO^{\bullet}$	Nitric oxide radical
$NO_2^{\bullet}$	Peroxynitrite radical
$O_2^{-\bullet}$	Superoxide anion
OH^-	Hydroxyl radicals
$ONOO^-$	Peroxynitrite anion
OS	Oxidative stress
PBS	Phosphate buffered saline
PI	Propidium Iodide
PGly	Propanol Glycol
PS	Phosphatidylserine
$RNOO^-$	Peroxyl radicals
RNS	Reactive nitrogen species
ROS	Reactive oxygen species

SOD	superoxide dismutase
SSC	Side scatter
STR	Straightness
Mn(III)TMPyP	tetrakis (1-methyl-4-pyridyl) porphyrin
VAP	Average path velocity
VCL	Curvilinear velocity
VSL	Straight line velocity
WHO	World Health Organization
ZP	Zona pellucida

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Cells living under aerobic conditions constantly face the oxygen paradox i.e. oxygen is indispensable for supporting life; however, its metabolites such as reactive oxygen species (ROS) can modify cell function. Oxidative stress (OS) arises as a consequence of excessive ROS production and/or impaired antioxidant defence mechanisms (Agarwal *et al.*, 2003). ROS are highly reactive oxidizing agents belonging to the class of free-radicals. A free radical is any compound (not necessarily derived from oxygen) which contains one or more unpaired electrons.

The most common ROS that have potential implications in reproductive biology include superoxide ($O_2^{-\bullet}$) anion, hydrogen peroxide (H_2O_2), peroxyl ($RNOO^-$) radicals, and the very reactive hydroxyl (OH^-) radicals (Sikka, 1996; Ford, 2004; Henkel *et al.*, 2005a) (Figure 1.1). "Abortive apoptosis" as described by Sakkas *et al.* (2002), improper deoxyribonucleic acid (DNA) packaging during spermatogenesis (Barroso *et al.*, 2000; Sakkas *et al.*, 2002; Oehninger *et al.*, 2003) and excessive production of ROS associated with peroxidative damage to sperm plasma and DNA result in poor sperm quality and male factor infertility (Aitken, 1999; Aitken and Krausz, 2001). Previous studies have demonstrated an increase in free radical production during the *in vitro* preparation of spermatozoa for use in assisted reproductive technologies (Agarwal *et al.*, 1994).

As early as 1943 it was observed that spermatozoa lose their motility more rapidly when incubated with oxygen (MacLeod, 1943). Due to a high content of polyunsaturated fatty acids, the sperm membranes are prone to oxidation (Cerolini *et al.*, 2000; Sanocka and Kurpisz, 2004). Biologically, free radicals are formed from many sources. However, one source common to all eukaryotic cells is the electron transport chain in the mitochondria. In the process of reducing O_2 to H_2O, the first step involves the addition of one electron to O_2 to form superoxide, $O_2^{-\bullet}$. Usually, superoxide is reduced to water via 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 (Lee, 1996).

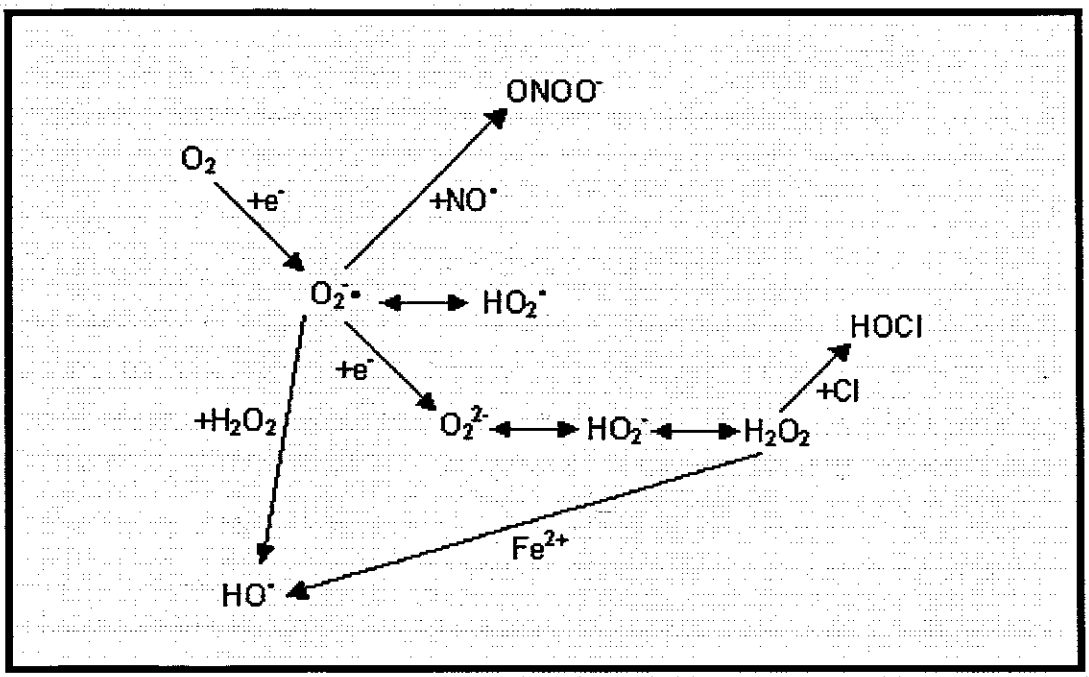

Figure 1.1 Derivation of reactive oxide species from oxygen (Reproduced from Ford, 2004)

1.2 Sources of free radicals in sperm

In sperm cells, the sources of ROS are broadly dispersed between external and internal sources. External production of ROS, particularly O_{2^-} • and H_2O_2 can be the result of leukocyte contamination within the semen. This generation of ROS has been associated with sub-fertility or even infertility in patients (Aitken *et al.*, 1996; Griveau and Le Lannou, 1997; Henkel *et al.*, 2005a). Another important source of ROS is immature and morphologically abnormal spermatozoa (Agarwal *et al.*, 2003). Regarding ROS generated by the spermatozoa per se, recently, two mechanisms involved in ROS generation have been characterized in rat epididymal spermatozoa (Vernet *et al.*, 2001). One mechanism depends on the mitochondrial respiratory chain while the other mechanism relies on an enzymatic system related to the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase family found bound to the sperm plasma membrane (Vernet *et al.*, 2001). The mitochondrial electron transport chain is known to produce ROS during physiological, but also during pathological conditions. This production of ROS is correlated with physical activity. Recently, the topology of superoxide production has been determined in the different complexes of the respiratory chain. Interestingly, complexes I, II and III were found to produce hydrogen peroxide albeit at very low levels (Saint-Pierre *et al.*, 2002).

The production of ROS is also increased by lifestyle factors such as smoking and pollution. Smoking increases ROS production, causing sperm DNA damage, and suppresses antioxidants in both semen and serum (Fraga *et al.,* 1996; Mello *et al.*, 2001).

1.3 Leukocytes as source of free radicals in semen

The exact site of origin of these leukocytes in semen, their mode of action, and the role that bacteria, viruses and subsequent genitourinary-inflammation might have on sperm function are not clear. Experimentally, ROS production by human spermatozoa and contaminating leukocytes can be stimulated by phorbol esters and certain formyl peptides with deleterious effects on sperm motility and fertilisation (Krausz *et al.*, 1994; Henkel *et al.*, 2005a). The presence of leukocytes in semen did not diminish the *in vitro* fertilizing capacity of spermatozoa while the introduction of leukocytes into washed sperm preparations had been shown to reduce sperm function by the production of ROS (Aitken *et al.*, 1994, Henkel *et al.*, 2005a).

1.4 Mode of action of ROS and their potential scavengers

Lipid peroxidation (LPO) of the sperm membrane is considered to be the key mechanism of this ROS-induced sperm damage leading to infertility. Spermatozoa, unlike other cells, are unique in structure, function, and susceptibility to damage by LPO (Lee, 1996; Garg *et al.*, 2000). 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 a balance between the cellular production of these molecules and their destruction by scavengers. These scavengers are localized within spermatozoa and in seminal plasma.

Some of these compounds are molecules with intrinsic radical-scavenging activity such as α-tocopherol, ascorbic acid, uric acid, glutathione (Sikka 1996; Halliwell and Gutteridge, 1989; Bilodeau *et al.*, 2001), pyruvate (de Lamirande and Gagnon, 1992; Upreti *et al.*,1998), taurine, hypotaurine and albumin (Alvarez and Storey, 1983; Eckert and Niemann, 1996; Jaakma *et al.*, 1997; Fukui *et al.*, 2000), superoxide dismutase (SOD) and the glutathione–peroxidase–reductase system (Alvarez *et al.*, 1987; Alvarez and Storey, 1989; Taylor, 2001). Although the presence of these enzymatic protectants is extensive intracellularly, their protective

(Alvarez *et al.*, 1987; Alvarez and Storey, 1989; Taylor, 2001). 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 (Hurley and Deane, 1987; 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.*, 1993; 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).

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 (Lee, 1996). RPO is the only vegetable oil with a balanced composition of saturated and unsaturated fatty acids both in processed and unprocessed forms (Pryor *et al.*, 1987). RPO contains carotenoids, phosphatides, sterols, tocopherols and trace metals (Krinsky, 1993; Sundram *et al.*, 2003). These agents are natural antioxidants and act as scavengers of oxygen free radicals (Rossell *et al.*, 1985; Guo *et al.*, 1999). Triglycerides constitute the major component of palm oil whereas carotenoids, tocopherols, tocotrienols, sterols, phospholipids and aliphatic hydrocarbons constitute the minor components of palm oil (Pryor *et al.*, 1987; Krinsky 1993; Nagendran *et al.*, 2000). The tocotrienols have been reported to be natural inhibitors of cholesterol synthesis and protect the heart from ischemia/reperfusion injury (Qureshi *et al.*, 1991; Pearce *et al.*, 1992, Ong and Gog, 2002; Esterhuyse *et al.*, 2005 & 2006, Engelbrecht *et al.*, 2006). The tocopherols and tocotrienols promote an antithrombotic state by reducing platelet aggregation and modulating prostanoid synthesis (Qureshi *et al.*, 1991; Pearce *et al.*, 1992). They have also been highlighted in the prevention and therapy of certain forms of cancers (Qureshi *et al.*, 1991; Murakoshi *et al.*, 1992; Nesaretnam *et al.*, 1995; Guthrie *et al.*, 1997). Sundram and co-workers (1989) have shown that crude palm oil was effective in increasing the tumour latency period in 7, 12 dimethylbenz (a) anthracene (DMBA) treated rats. This was attributed to the presence of tocotrienols and carotenoids in the crude oil. When the vitamin E content in palm oil was

removed, significantly more tumours became apparent (Nesaretnam *et al.*, 1992). *In vitro* studies on human breast cancer cells have shown that palm tocotrienols have greater inhibitory effects on these cells and at much lower concentrations (180ug/ml) (Nesaretnam *et al.*, 1995 & 1998; Guthrie *et al.*, 1997).

1.5 Biological and pathological roles of reactive oxygen species and nitrogen species

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 (Lands, 1985; Constantino *et al.*, 2003; Manabe *et al.*, 2004), in the regulation of vasotonus (Ignarro, 1990), in gene regulation (Scheck *et al.*, 1992; Allen and Tresini, 2000), in the regulation of cellular growth, and the function of extracellular as well as intercellular signal transduction (Saran and Bors, 1989; 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 (Ames, 1989; Gandini *et al.*, 2000; Irvine *et al.*, 2000; Sakkas *et al.*, 2000; Zini *et al.*, 2000; Allamaneni *et al.*, 2004; Henkel, *et al.*, 2004) and carbohydrates (Gracy *et al.*, 1999).

Nitric oxide radical (NO•) and reactive nitrogen species (RNS) have been found to have biological roles in inflammation and in mediating many cytotoxic and pathological events (Darley-Usmar *et al.*, 1995; Fujisawa *et al.*, 2001). Synthesis of NO• in response to infection and inflammation could contribute to poor sperm motility and function which may lead to infertility (Rosselli *et al.*, 1995). RNS (e.g., NO•) like ROS, may normally be useful for maintaining sperm motility but can be toxic in excess (Hellstrom et *al.*, 1994). Other RNS such as nitrogen dioxide (NO_2•) radical and peroxynitrite ($ONOO^-$) anion are considered to be damaging to sperm cells. The primary mechanism of nitric oxide induced sperm damage is likely to be the inhibition of mitochondrial respiration and DNA synthesis (Hibbs *et al.*, 1987). Nitric oxide-induced toxicity is also mediated indirectly through its interaction with superoxide anions and formation of peroxynitrite anion, which when protonated, decomposes to form OH^- and NO_2^-, both of which are cytotoxic agents (Beckman *et al.*, 1990).

apoptosis, resulting in the death of spermatozoa. ROS initiates a chain of reactions by activating caspases that ultimately lead to apoptosis (Said *et al.*, 2004; Agarwal *et al.*, 2007). When ROS levels are raised pathologically, the process of apoptosis is also initiated in mature spermatozoa. The process of apoptosis is accelerated by ROS-induced DNA damage, which ultimately leads to a decline in the sperm count (Figure 1.2). Oxidative stress due to excessive generation of ROS is presumed to cause spermatozoa DNA damage and has correlated positively with apoptosis (Wang *et al.*, 2003a). According to Host and co-workers (2002) apoptosis correlated negatively with the fertilisation rate.

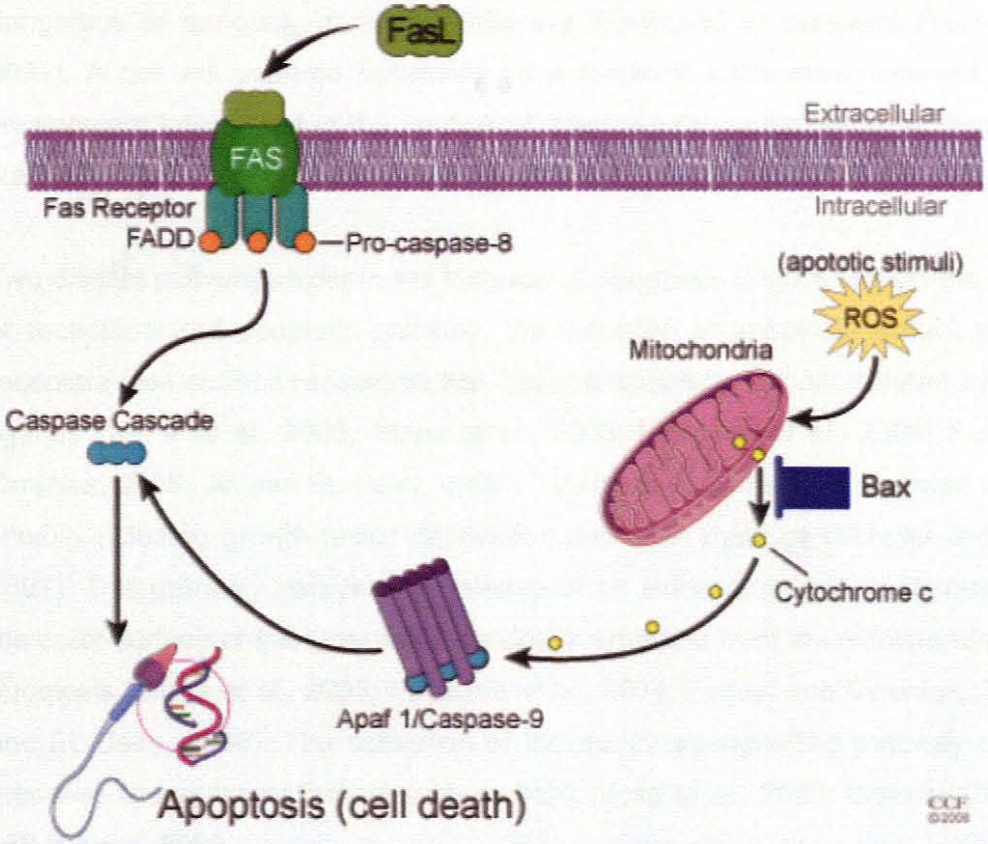

Figure 1.2.: ROS induced apoptosis. ROS (apoptotic stimulus) trigger mitochondria to release cytochrome C initiating caspase cascade. Interaction between Fas and Fas ligand is also necessary in apoptotic mechanism. DNA fragmentation occurs as result of activation of effector caspases (caspases 3 and 6) eventually causing apoptosis (Reproduced from Agarwal *et al.*, 2007)

1.6 Apoptosis

Although apoptosis shares certain common mechanisms with necrosis, necrosis is referred to as an "accidental", non-physiological or passive type of cell death that occurs when cells die from severe and sudden injury, such as ischemia, sustained hyperthermia and physical or chemical trauma (Cohen, 1993; Samali *et al.*, 1996). On the other hand apoptosis is a genetically controlled active cell death process implicated as being a critical physiological mechanism involved in development and tissue homeostasis (Wilson, 1998; Joza *et al.,* 2002). Apoptosis is a protective mechanism in multicellular organisms whereby infected, excessive, potentially dangerous or seriously damaged cells are eliminated or removed (Turke *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 (Figure 1.3). 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 (Turke *et al.,* 2002; Sinha *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 *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 induction of apoptosis via the intrinsic or extrinsic apoptotic pathways result in the activation of an initiator caspase, which activates a cascade of events leading to the activation of effector caspases, responsible for the cleavage of key cellular proteins that lead to the typical morphological changes observed in cells undergoing apoptosis. Caspase-8 and caspase-10 are inhibitor caspases in death receptor-mediated apoptosis, while caspase-9 is the initiator caspase in mitochondrion-dependent apoptosis (Chen and Wang, 2002). These pathways differ in one fundamental aspect: one is "external" as it is promoted by a series of specific external ligands operating through defined transmembrane receptors; the other is an internal system where activation of the effector enzymes is induced by intracellular

changes, involving the mitochondria (Garland, 2000; Joza *et al.*, 2002; Turke *et al.*, 2002). Despite the difference in the manner of initiation, the extrinsic and intrinsic pathways merge at the level of caspases-3 and 7 and once activated, they cleave intracellular targets, ultimately leading to the manifestation of apoptosis (Figure 1.3).

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 (Ramachandra and Studzinski, 1995; Gewies, 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 apposition to the nuclear envelope, and finally in many cells into one or several dense spheres (Ormerod, 1998; Gewies, 2003). 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; Bowen *et al.*, 1998).

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 Jänicke, 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).

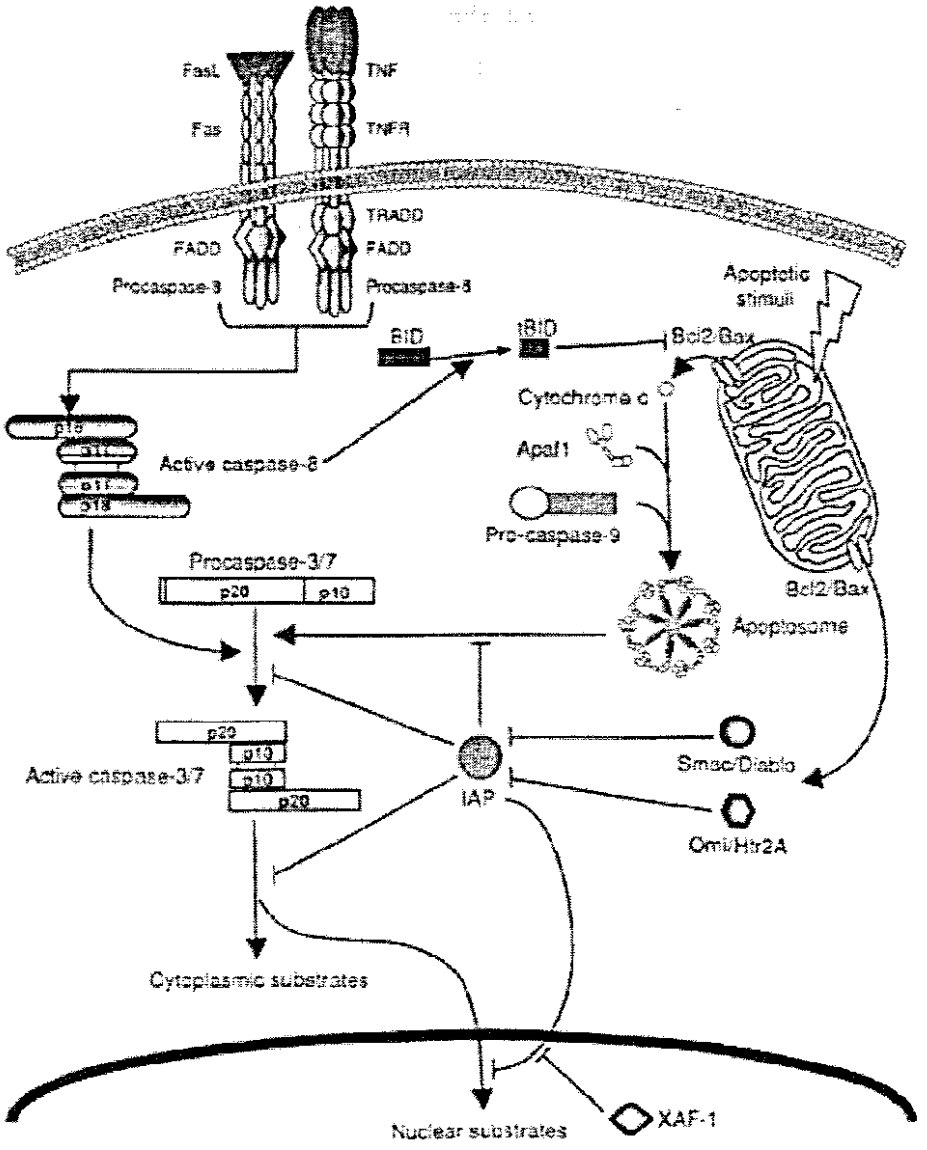

Figure 1.3: Intrinsic and extrinsic apoptotic pathways in the mammalian cell. Intracellular stress results in the activation of the mitochondrial, or intrinsic pathway which leads to cytochrome c release, apoptosome formation, and caspase activation. Extracellular ligand binding to death receptors triggers 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 (Reproduced from LaCasse *et al.*, 2004)

1.7 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. 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 fertilisation, 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; Alvarez *et al.,* 1987; Agarwal *et al.,* 2006b). Garner and co-workers (1997) argued that the mitochondrial activity, viability and acrosomal integrity of sperm cells correlate positively with fertility. However, Caisson *et al.*, (1999), warned that trying to correlate the proportion of live spermatozoa with motility and/or fertilisation rates present a potential source of bias. The category of live spermatozoa may not only involve live spermatozoa which are motile, but also those which have their ATP content already exhausted (Caisson *et al.*, 1999) and therefore are immotile.

1.8 Sperm functional tests

In this literature review, only two sperm functional tests will be briefly discussed, namely; capacitation and acrosome reaction.

1.8.1 Capacitation

Capacitation confers upon the spermatozoon an ability to gain hyperactive motility, interact with oocyte zone pellucida (ZP), undergo 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 and Gagnon, 1993a; Naz and Rajesh, 2004). Low concentration of hydrogen peroxide stimulates sperm capacitation (de Lamirande and Gagnon, 1993a). According to Zini and co-workers, (1995) superoxide and nitric oxide also take part in these processes.

Free radicals are also involved in the fusion of spermatozoa with the oocyte (de Lamirande and Gagnon, 1993a; Flesch and Gadella, 2000; 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 zona pellucida binding (Francavilla *et al.,* 2000). Low concentrations of hydrogen peroxide cause tyrosine phosphorylation, which in turn results in the

binding of the spermatozoal membrane proteins with ZP-3 proteins on the zona pellucida and ultimately, spermatozoa-oocyte fusion (Aitken *et al.*, 1993).

1.8.2 Acrosome reaction (AR)

The acrosome reaction is an exocytotic process which 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). It is a prerequisite for penetration of spermatozoa through the zona pellucida for fertilisation of the oocyte (Köhn *et al.*, 1997; Flesch and Gadella 2000; 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 hydrogen peroxide inactivates several enzymes, including glutathione peroxidase, superoxide dismutase, and glucose-6-phosphate. Studies have shown that $O_2^{-}\bullet$ 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).

1.9 Objectives of this study

Oxygen toxicity is an inherent challenge to cells which live under aerobic conditions including spermatozoa. The increase in oxidative damage to the sperm membrane, proteins and DNA is associated with defective sperm function. A variety of defensive mechanisms encompassing antioxidant enzymes are involved in biological systems. A balance between the benefits and risks of free radicals and antioxidants appears to be necessary for the survival and functioning of spermatozoa.

The objectives of this study are threefold:

(i) To measure the production of $O_2^{-}\bullet$ by sperm by means of flow cytometry.

(ii) To determine effects of $O_2^{-}\bullet$ on sperm motility and viability in the presence or absence of commercially available scavengers and RPO.

(iii) To determine effects of $O_2^{-}\bullet$ on selective apoptotic markers in ejaculated sperm in the presence of absence of commercially available scavengers and RPO.

1.10 Plan of study

This study was undertaken in two parts. Firstly, the effect of tetrakis (1-methyl-4-pyridyl) porphyrin (Mn(III)TMPyP), superoxide dismutase (SOD) and RPO on sperm function (motility and viability) was determined and also $O_2^{-\bullet}$ was measured. Secondly, the effect of Mn(III)TMPyP, SOD and RPO on sperm apoptosis was evaluated.

CHAPTER 2

MATERIALS AND METHODS

2.1 Introduction

This chapter will describe the detailed protocols and methods that were employed during this study. A brief outline of the experimental procedure is given in Figure 2.1.

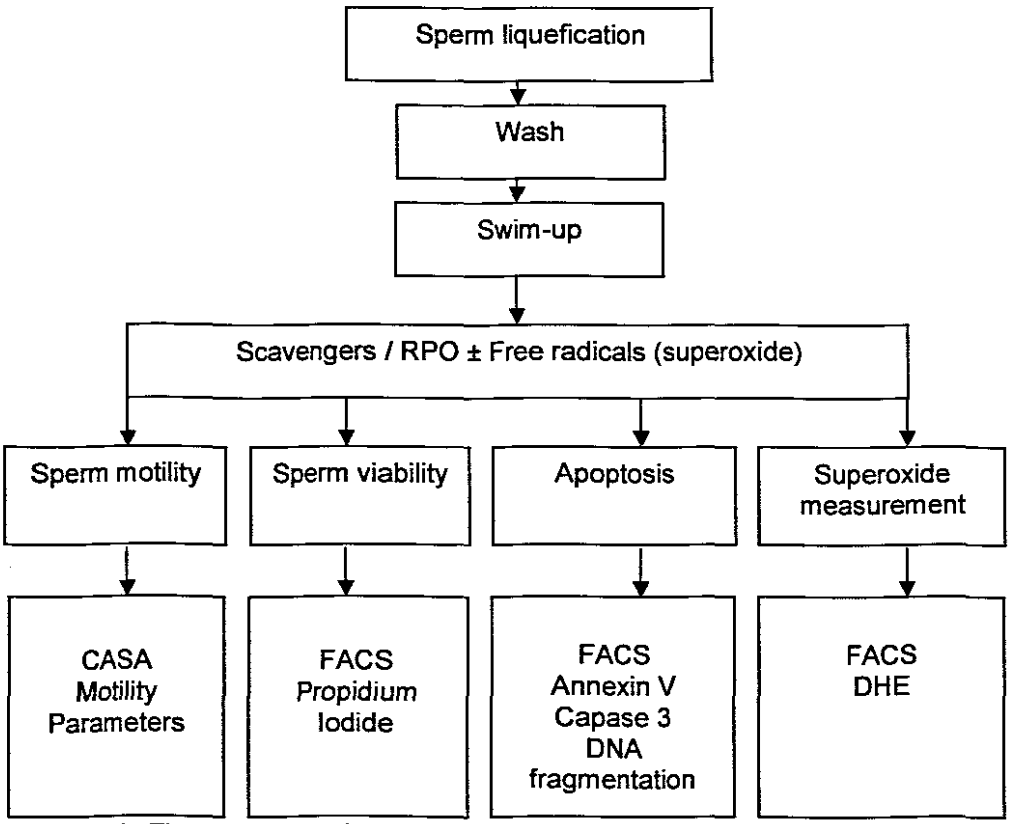

Figure 2.1: Flow chart showing the generalized experimental protocol

2.2 Preparation of Ham's culture medium

1. Measure 1000 ml of millipore water (temperature between) 15-20° C in a volumetric flask.
2. Dissolve Ham nutrient mixture F-10 (Ham's, Sigma Chemical Co. St. Louis, MO, USA), in 90 % of the water in a beaker.
3. *While gently stirring the water, add the powdered medium. Stir until dissolved. Do* NOT heat.
4. Rinse original package with small amount of water to remove all traces of powder. Add to solution in step 3.

5. To the solution in step 4, add 1.2 g sodium bicarbonate for each litre of final volume of medium being prepared. Stir until dissolved.
6. While stirring, adjust the pH of the medium to 0.1-0.3 pH units below the desired pH (7.4) since it may rise during the filtration. The use of 1N HCl or 1N NaOH is recommended.
7. Add additional millipore water to bring the solution to final volume.
8. Sterilize immediately by filtration using a membrane with a porosity of 0.22 microns (Corning Incorporated, N.Y., USA).
9. Aseptically dispense medium into sterile containers.
10. Store at 4° C.
11. Warm to 37° C before use.
Add 0.03 g of Bovine Serum Albumin (BSA, Sigma Chemical Co. St. Louis, MO, USA) in 10 ml of Ham's for the swim-up.

2.3 Sperm collection

Semen samples were obtained from 24 normozoospermic healthy volunteer donors studying at the Tygerberg Campus, University of Stellenbosch, aged between 19-23 years. All semen samples were collected by masturbation after 2-3 days of sexual abstinence according to the World Health Organization criteria (WHO, 1999). Semen samples were collected in sterile wide mouthed containers after which the semen was allowed to liquefy for 30 minutes (30') at 37°C. Ethical approval from our institution was obtained and donors have provided consent to partake in this study.

2.4 Semen preparation

Fresh semen was placed in a 5 ml tube and an equal amount of Ham's culture medium was added. The tube was centrifuged for 5 minutes at 400xg. The supernatant was discarded leaving a pellet at the bottom which was resuspended in fresh Ham's culture medium and centrifuged again for 5 minutes at 400xg. The supernatant was carefully removed by aspiration without disturbing the pellet and 1.2 ml of Ham's culture medium mixed with 3% bovine serum albumin (BSA) was layered on top of the pellet. The tube was placed in a rack inclined at 45 degrees and incubated (37°C, 5% CO_2, 60 minutes) to allow motile sperm to swim up into the BSA media. After 1 hour the upper media containing a homogenous motile sperm population was collected.

2.5 Computer aided sperm analysis (CASA)

Sperm motility was determined with the Hamilton-Thorne IVOS analyzer (Hamilton-Thorne Research, Beverly, MA). The settings of the analyzer were as follows: 30 frames/60 Hz; minimum contrast, 80; minimum cell size, 2; minimum static contrast, 30; low average path velocity (VAP) cut-off, 5 μm/s; low straight-line velocity (VSL) cut-off, 11 μm/s; head size, non-motile, 3; head intensity, non-motile, 160; static head size, 1.01-2.91; static head intensity, 0.60-1.40; slow cells not motile; magnification, 2.01, and temperature, 37°C. Sperm motility is assessed by several parameters when analysed using computer aided sperm analysis (CASA) as illustrated in Figure 2.2.

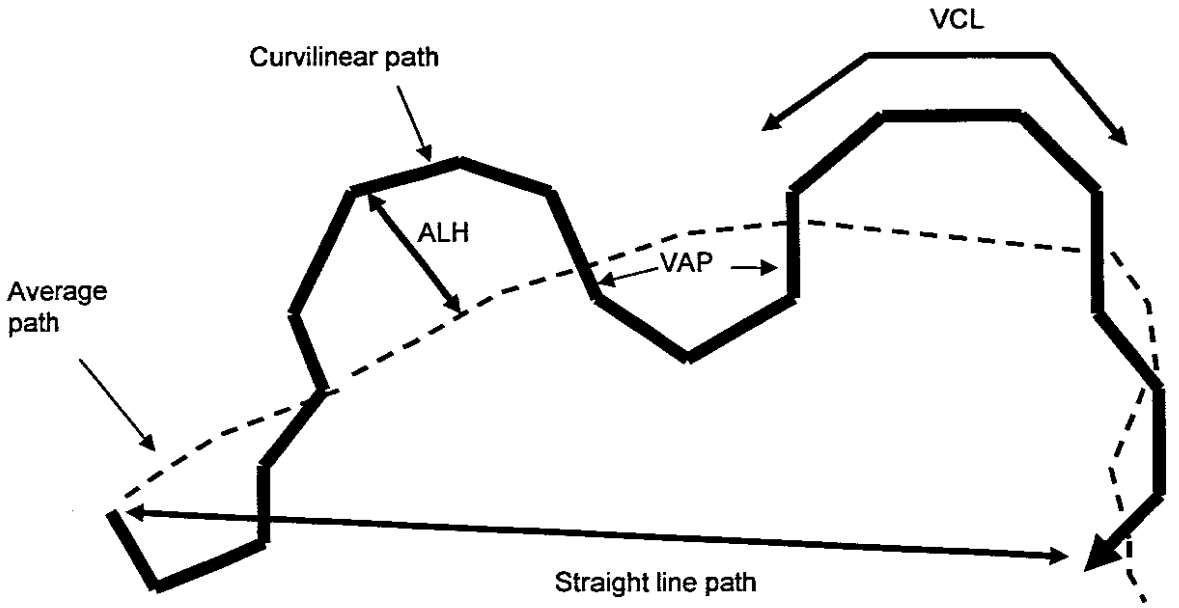

Figure 2.2: An illustration of different sperm motility parameters measured using CASA (Reproduced from WHO, 1999)

Motility parameters analysed by means of CASA include the following:

(i) Motility: the percentage of motile spermatozoa.

(ii) Progressive motility: the percentage of progressively motile cells.

(iii) Curvilinear velocity (VCL) (μm/s): time average velocity of sperm head along its actual curvilinear path, as perceived in two dimensions in the microscope.

(iv) Straight line velocity (VSL) (μm/s): time-average velocity of a sperm head along the straight line between its first detected position and its last.

(v) Average path velocity (VAP) (μm/s): time-average velocity of a sperm head along its average path.

(vi) Amplitude of lateral head displacement (ALH) (μm): magnitude of lateral displacement of sperm head about its average path.

(vii) Linearity (LIN): the linearity of a curvilinear path VSL/VCL.

(viii) Straightness (STR): linearity of the average path, VSL/VCL.

(ix) Beat-cross frequency (BCF) (Hz): the average rate at which the sperm's curvilinear path crosses its average path.

(x) Rapid cells: the percentage of rapidly moving cells.

(xi) Static cells: a percentage of static/motion-less cells.

2.6 Flow cytometry

Flow cytometry is a single cell technique by which individual cells are analyzed as they pass through the laser in single file. In a few minutes, the flow cytometer can acquire data on all subpopulations within a sample, making it ideal for assessment of homogenous populations, such as spermatozoa. The adaptation of flow cytometry to sperm assessment began when it was used for measuring their DNA content (Evenson *et al.*, 1980) and its application to semen analysis has gradually increased over the last 10–15 years. Flow cytometry is now applied to semen evaluation to determine parameters such as cell viability, acrosomal integrity, mitochondrial function, capacitation status, membrane fluidity and DNA status. New fluorescent stains and techniques are continuously being developed that have potential application to the flow cytometric evaluation of spermatozoa. Flow cytometry permits the observation of physical characteristics, such as cell size, shape and internal complexity, and any component or function of the spermatozoon that can be detected by a fluorochrome or fluorescently labelled compound. The analysis is objective, has a high level of experimental repeatability and has the advantage of being able to work with small sample sizes. Flow cytometry also has the capacity to detect labelling by multiple fluorochromes associated with individual spermatozoa, meaning more than one sperm attribute can be assessed simultaneously (Almid, and Johnson, 1988; Harrison, and Vickers, 1990; Gillan *et al.*, 2005).

2.6.1. Assessment of cell viability using propidium iodide (PI)

Sperm cell viability was measured by flow cytometric analysis (FACS: fluorescence-activated cell sorter). A Becton Dickinson FACSCalibur™ analyser (BD, San Jose, CA, USA) was used to quantify fluorescence at the single-cell level and data was analysed using CellQuest™ version 3.3 (Becton Dickinson, San Jose, CA, USA) software. In each sample, the mean fluorescence intensity of the analysed cells was determined after gating the cell population by forward and side light scatter signals as recorded on a dot plot (Figure 2.3). In total, 100,000 events were acquired, but non-sperm particles and debris (located at the bottom left corner of the dot plot)

were excluded by prior gating, thereby limiting undesired effects on overall fluorescence. Final gated populations usually contained 12,000-15,000 sperm cells. Fluorescence signals were recorded on a frequency histogram (Figure 2.4) using logarithmic amplification. Fluorescence data are expressed as mean fluorescence (percentage of control, control adjusted to 100%). In all experiments, light was avoided by working in the dark to prevent the light sensitivity of the fluorescence used.

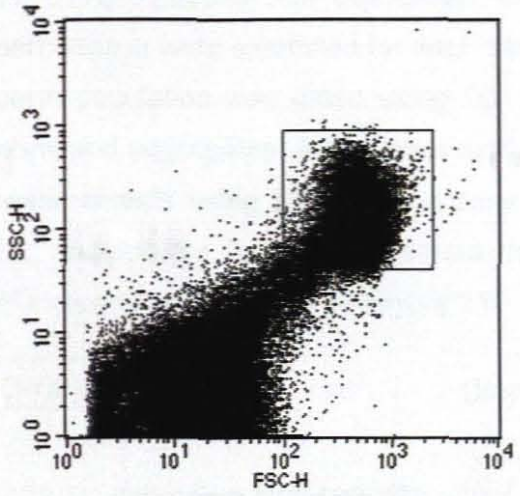

Figure 2.3: A representative dot plot of sperm cells showing the spread of the total recorded "events." Gated population (top right): sperm cells and bottom left: non-sperm particles, and debris

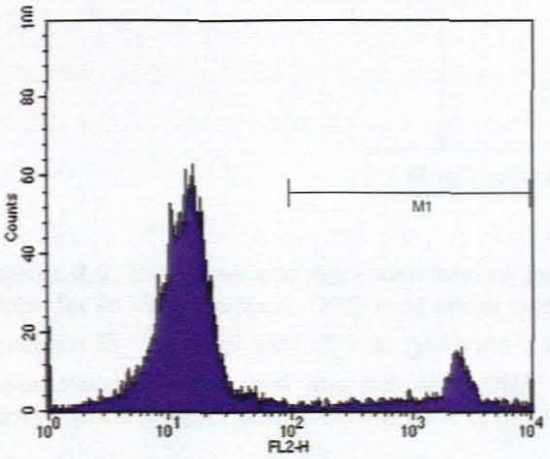

Figure 2.4: A frequency histogram of PI fluorescence with two peaks. Cells possessing a damaged membrane will permit PI to enter into the cell and bind to DNA causing the cells to fluoresce red. The peak to the left is depicting viable cells which are able to exclude PI while that to the right is non-viable cells which had absorbed PI

2.6.2 Assessment of superoxide production using dihydroethidium (DHE)

De Iuliis and co-workers (2006) validated the use of dihydroethidium (DHE) as a probe for detecting the generation of $O_2^{-\bullet}$ by human spermatozoa and examined the relationship between this activity and defective sperm function (Figure 2.5). In this study 2.3-dimetoxy-1-naphtoquinone (DMNQ) has been used to generate $O_2^{-\bullet}$ (Navarro-Antolin *et al.*, 2001; Redondo-Horcajo and Lamas, 2005). The DHE fluorescence was then measured using flow cytometry (Becton Dickinson, San Jose, CA, USA) equipped with Cell Quest version 3.3 software. A minimum of 10,000 spermatozoa were examined for each assay at a flow rate of <100 cells/second. The sperm population was gated using 90° and forward-angle light scatter to exclude debris and aggregates. Argon laser excitation at 488 nm was coupled with emission measurements using 530/30 band pass and 585/42 band pass filters for FL1 and FL2, respectively. As more superoxide is produced by the cells, DHE fluorescence will increase as explained by Figure 2.6.

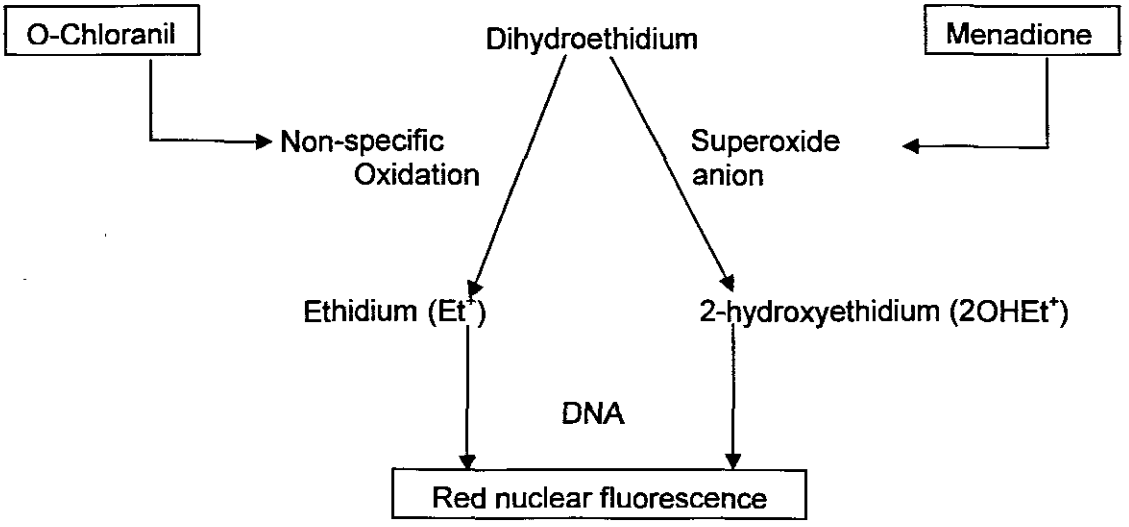

Figure 2.5: Diagrammatic representation of the chemistry underlying the use of DHE as a probe for ROS generation. DHE may either undergo a non-specific two-electron oxidation to generate Et_ or react with $O_2^{-\bullet}$ to generate the specific reaction product, 2OHEt_. Both of these fluorochromes will interact with DNA in the sperm nucleus to generate a red fluorescence (Reproduced from de Iuliis *et al.*, 2006)

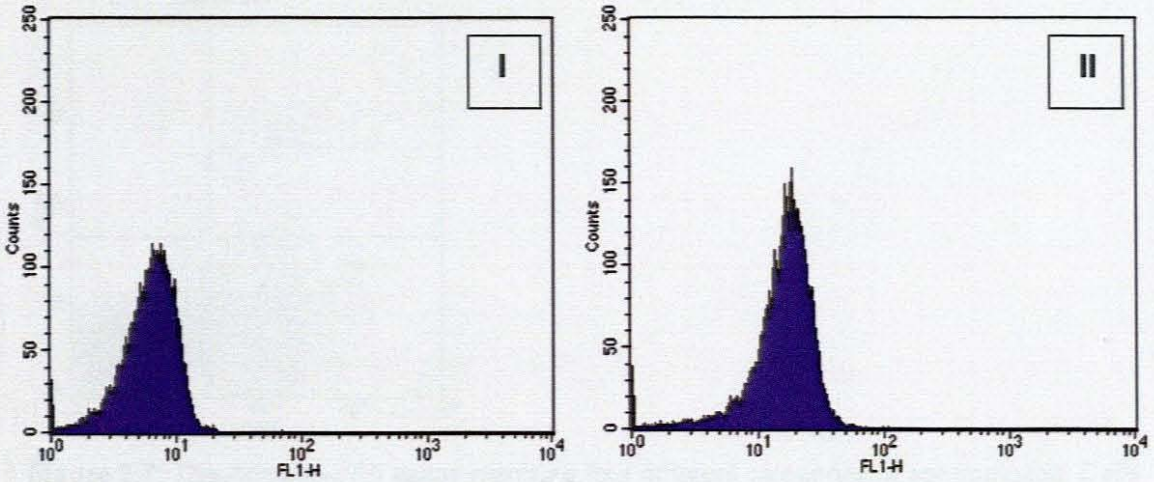

Figure 2.6 A representative frequency histogram showing baseline fluorescence (log) of DHE on x-axis (I); a shift to right depicting an increase in fluorescence intensity (II)

2.6.3 Assessment of apoptosis using Annexin-V

In apoptotic cells, the membrane phospholipid, phosphaltidylserine (PS) is translocated from the inner to outer leaflet of the plasma membrane, thereby exposing PS to the external environment (Barroso *et al.*, 2000; Oosterhuis *et al.*, 2000; Anzar *et al.*, 2002). Annexin–V is a Ca^{2+} dependant phospholipid-binding protein that has a high affinity for PS and binds to cells with exposed PS.

Apoptotic cells become Annexin–V positive after nuclear condensation has started, but before the cell becomes permeable to PI indicative of the loss of plasma integrity. Staining for Annexin-V was performed using an apoptosis detection kit (Roche Diagnostics, Mannheim, Germany). Annexin-V conjugated with fluorescein isothiocinate (FITC) and simultaneously stained with PI (Sigma-Aldrich Co., UK) allows the discrimination between apoptotic, necrotic and live cell populations. The samples were analysed using a FACSCalibur™ (Becton Dickinson, San Jose, CA, USA) equipped with standard optics. An argon-ion laser (INNOVA 90, Coherent, Santa Clara, CA, USA) tuned at 488 nm and running at 200 mW was used as light source. For each sample 10000 events were counted. Forward light scatter (FSC), side scatter (SSC), A-FITC fluorescence (FL1), and PI fluorescence (FL3) were evaluated using CellQuest™ version 3.3 (Becton Dickinson, San Jose, CA, USA). A gate was applied in the FSC/SSC dot-plot to restrict the analysis to spermatozoa. For the gated cells, the percentages of Annexin-V negative or positive (A_ or Aþ), and PI negative or positive (PI_ or PIþ), as well as double positive cells were evaluated, based on quadrants determined from single-stained and unstained control samples (Figure 2.7).

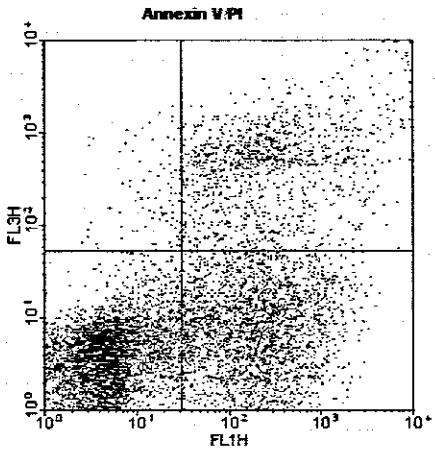

Figure 2.7: The Annexin-V/PI assay identified four different categories of spermatozoa. Cells in the lower left quadrant were not fluorescent (A_/PI_) and were recorded as live cells, e.g. without membrane dysfunction. Apoptotic, but viable spermatozoa (Aþ/PI_) were labelled with Annexin-V but not with PI and fell in the lower right quadrant. Early necrotic spermatozoa (Aþ/PIþ) that bound both Annexin-V and PI (upper right quadrant) are assumed to maintain some degree of membrane integrity although having damaged permeable membranes and thus still bind Annexin-V. Late necrotic spermatozoa (A_/PIþ) however were labelled by PI but not Annexin-V (upper left quadrant). It is assumed that these latter spermatozoa have completely lost sperm membrane integrity and are thus unable to bind Annexin-V

2.6.4 Assessment of apoptosis using Apo-direct

A characteristic feature of apoptosis is DNA fragmentation, one of the later steps of apoptosis, which result from the activation of endonucleases during the apoptotic process (Gandini *et al.*, 2000; Nagata, 2000). The detection of fragmented DNA, has been quantified using the APO-DIRECT™ (BD Biosciences, Pharmingen, USA) assay which is a single step method for labelling DNA breaks with FITC- dUTP, followed by flow cytometric analysis (fluorescence-activated cell sorting, Becton Dickinson, San Jose, CA) equipped with software Cell Quest version 3.3. A minimum of 10,000 spermatozoa were examined for each assay at a flow rate of <100 cells/second. The sperm population was gated using 90° and forward-angle light scatter to exclude debris and aggregates. An argon laser at 15 mW was used, and green fluorescence (480–530 nm) was measured in the FHL1 channel (Figure 2.8). Two dyes are used; PI stained total DNA and fluorescence about 623 nm. FITC-dUTP stained apoptotic cells and fluorescence at 520 nm.

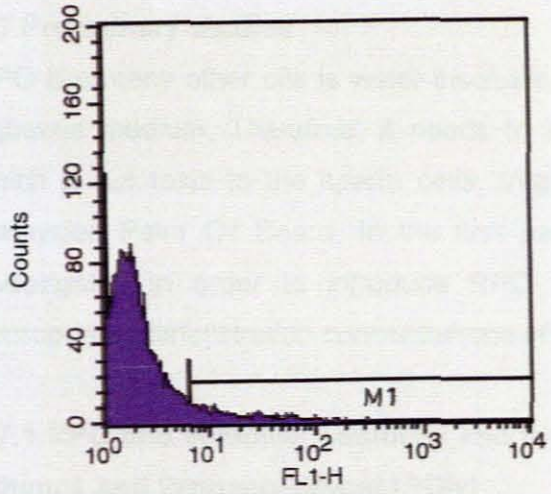

Figure 2.8: Frequency histogram showing DNA fragmentation using Apo-direct assay. The percentage of cell with fragmented DNA is indicated with marker M1

2.6.5 Assessment of apoptosis using Caspase-3

Antibodies against caspase-3 in cells which are undergoing apoptosis serve to differentiate and quantify cell populations into apoptotic and non-apoptotic cells as measured by flow cytometry (fluorescence-activated cell sorting, Becton Dickinson, San Jose, CA) equipped with software Cell Quest version 3.3. A minimum of 10,000 spermatozoa were examined for each assay at a flow rate of <100 cells/second. The sperm population was gated using 90° and forward-angle light scatter to exclude debris and aggregates. An argon laser at 15 mW was used, and green fluorescence (480–530 nm) was measured in the FHL1 channel (Figure 2.9).

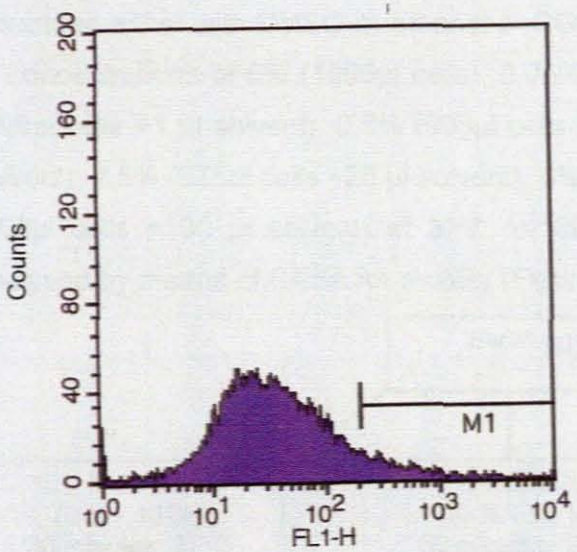

Figure 2.9: A frequency histogram show cells expressing caspase-3 which is indicated by the marker M1

2.7 Preliminary studies

RPO like many other oils is water insoluble and is difficult to be introduced into an aqueous medium. Therefore, it needs to be dissolved in an appropriate solvent which is not toxic to the sperm cells. Virgin red palm oil was obtained from the Malaysian Palm Oil Board. In the first part of the study suitable solvents were investigated in order to introduce RPO to the sperm media. Thereafter, the appropriate administration concentrations of RPO for sperm were determined.

2.7.1 RPO and vehicles: solubility and toxicity of dimethylsulphoxide (DMSO), Ethanol, and Propanol Glycol (PGly)

- ***Solubility of RPO into dimethylsulphoxide (DMSO), Ethanol, and Propanol Glycol (PGly)***

DMSO (Merck, South Africa), ethanol (Burdick & Jackson, USA) and PGly (Merck, South Africa) are commercially available solvents and commonly used to dissolve lipids. In this study, different volumes of DMSO, ethanol and PGly were used to try to dissolve RPO (87.5%; 75%; 50%).

- ***Investigating the toxicity of DMSO, Ethanol and PGly to sperm cells***

After collection through swim-up, sperm cells were counted and the concentration determined by means of CASA. Cells were subsequently divided into aliquots and incubated either with DMSO or ethanol or PGly (also known as polyethylene glycol) at concentrations of 0% (1000µl cells), 0.05% (999,5µl cells +0.5 µl solvent), 0.1% (999µl cells +1 µl solvent), 0.5% (995µl cells +5 µl solvent), 1% (990µl cells +10 µl solvent), 2.5% (975µl cells +25 µl solvent), 5% (950 µl cells +50 µl solvent) and 10% (900µl cells +100 µl solvent) at 37°C for 30 minutes after which samples were analysed by means of CASA for motility (Figure 2.10).

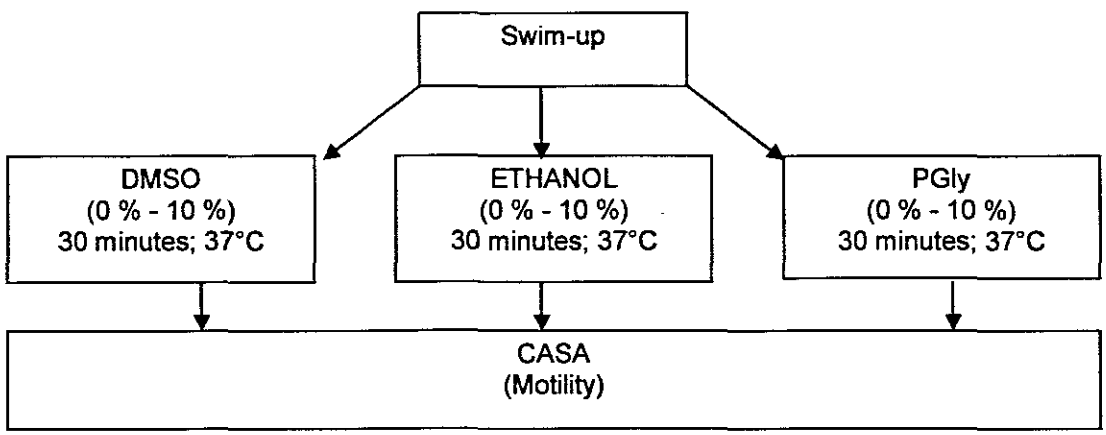

Figure 2.10: Protocol to investigate the toxicity of DMSO, ethanol and PGly

2.7.2 Determining the appropriate administration concentrations of RPO/PGly for sperm motility

After collection through swim-up, sperm cells were counted and their concentration determined by means of CASA. Cells were subsequently divided into aliquots at a concentration of $2x10^6$/ml each. As shown in Figure 2.11, cells were incubated with PG only at 0.1% (999µl cells + 1µl PG), 0.5% (995µl cells + 5µl PG) and 2.5% (975µl cells + 25µl PG). Furthermore, cells were incubated with RPO/PGly at a ratio of 1:1 and included 0.1% (998 µl cells + 2µl RPO/PGly), 0.5% (990 µl cells + 10µl RPO/PGly) and 2.5% (950µl cells + 50µl RPO/PGly) for 30 minutes at 37°C. Samples were immediately analysed by means of CASA for motility.

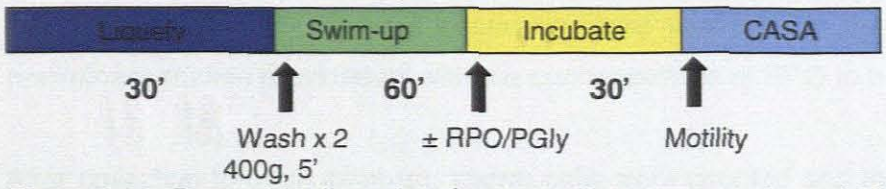

Figure 2.11: Protocol to determine the appropriate concentrations of RPO/PGly for sperm motility

2.7.3 Dihydroethidium (DHE)-specificity for superoxide studies in the absence or presence of Mn(III)TMPyP, SOD and RPO

After collection through swim-up, sperm cells were counted and their concentration determined by means of CASA. Cells were subsequently divided into aliquots at a concentration of $2x10^6$/ml each. As shown in Figure 2.12, cells were incubated without or with Mn(III)TMPyP (50µM; Chemical Co., St Louis, MO, USA) or SOD (50 IU; Chemical Co., St Louis, MO, USA) or RPO at 0.1%; 0.5%; 2.5% for 30 minutes at 37°C. After 30 minutes DMNQ (Calbiochem, San Diego, USA) was added at concentrations of 0µM; 2.5µM; 5µM; 10µM; 25µM; 50µM; 100µM and incubated for a further 60 minutes at 37°C. Samples were immediately analysed by means of CASA for motility parameters (Figure 2.12). DHE (30µM) (Sigma Chemicals Co., St. Louis, MO) was added to cells and then incubated at 37°C for 15 minutes before assessment by means of FACS analysis (Figure 2.12).

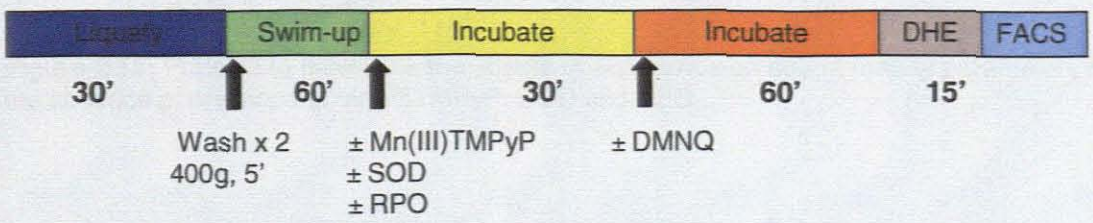

Figure 2.12: Protocol to determine the scavenging effects of Mn(III)TMPyP, SOD and RPO on superoxide by monitoring DHE fluorescence

2.8 The effects of superoxide on sperm motility parameters in the absence or presence of Mn(III)TMPyP, SOD and RPO

Mn(III)TMPyP ,SOD and RPO are used as superoxide scavengers, therefore, they needed to be added to the aliquots prior the addition of DMNQ in order to provide protection to the sperm cells. In previous studies (Faulkner *et al.,* 1994; Gardner *et al.,* 1996; Navarro-Antolin *et al.*, 2001), Mn(III)TMPyP (2.5 µM to 25µM and 50µM in mammalian cells and in *E.coli*), had been used to scavenge superoxide. It has also been reported previously in the literature (Zini *et al.,* 1993; Griveau *et al.*, 1995a) that the mean values of SOD used on human spermatozoa ranged between 42-46 IU/10^9 and 65 IU/10^9 of SOD. Therefore, the concentrations for the scavengers used (Mn(III)TMPyP and SOD) were based on these findings. On the other hand, the preliminary studies provided us with the concentrations of RPO to be used.

After collection through swim-up, sperm cells were counted and their concentration determined by means of CASA. Cells were subsequently divided into aliquots at a concentration of 2x10^6/ml each. As shown in Figure 2.13, cells were incubated without or with Mn(III)TMPyP (50µM; Chemical Co., St Louis, MO, USA) or SOD (50 IU/ml; Navarro-Antolin *et al.*, 2001; Chen *et al.,* 2003) or RPO at 0.1%; 0.5%; 2.5% for 30 minutes at 37°C. After 30 minutes DMNQ (Calbiochem, San Diego, USA) was added at concentrations of 0µM; 2.5µM; 5µM; 10µM; 25µM; 50µM; 100µM and incubated for a further 60 minutes at 37°C. Samples were immediately analysed by means of CASA for motility parameters (Figure 2.13). The motility parameters of interest were total motility, progressive motility, VAP, VSL, VCL, ALH, BCF, STR, LIN, rapid cells, and static cells of which motile cells, progressive motility; rapid cells and static cells seemed to be the more important motility parameters.

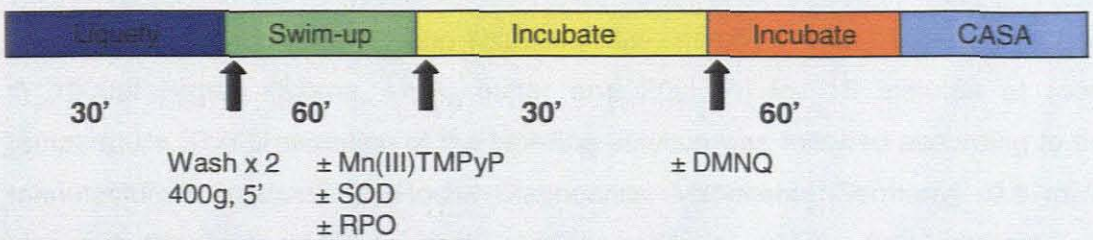

Figure 2.13: Protocol to determine the effects of superoxide on sperm motility parameters in the absence or presence of Mn(III)TMPyP, SOD and RPO

2.9 The effects of superoxide on PI fluorescence in the absence or presence of Mn(III)TMPyP, SOD and RPO

Following the description under 2.8 (after incubation in DMNQ for 60 minutes at 37°C), cells were incubated with PI, (Sigma, St. Louis, MO, USA) (1µM, 15 min) (Pena *et al.*, 1998), a fluorescent marker of non-viable cells, and analysed using FACS (Figure 2.14). For this analysis, viable sperm are defined as cells that possess an intact plasma membrane. This attribute is evaluated by staining with PI, a fluorescent probe that binds to DNA. Cells having an intact plasma membrane will prevent PI from entering into the cell and staining the nucleus (Gillan *et al.*, 2005).

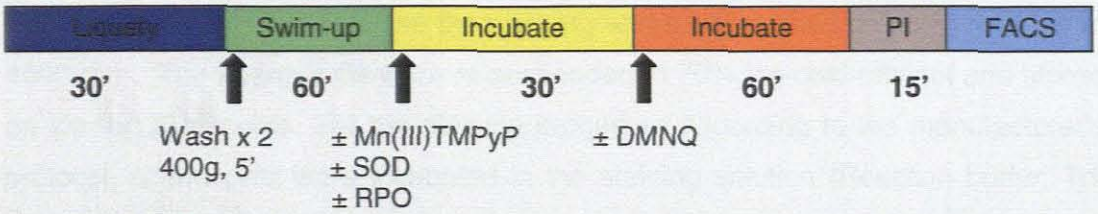

Figure 2.14: Protocol to determine the effects of superoxide on PI fluorescence in the absence or presence of Mn(III)TMPyP, SOD and RPO

2.10 The effects of superoxide on sperm apoptosis parameters in the absence or presence of Mn(III)TMPyP, SOD and RPO

For the effect of RPO on apoptosis and DHE fluorescence 0.1% of RPO was used because it is less detrimental to the cells. Three apoptotic markers (Annexin-V, Caspase-3 and DNA fragmentation) were used to investigate sperm apoptosis.

2.10.1 Annexin-V Assay

After incubation of aliquots containing $2x10^6$ sperm/ml with DMNQ (refer to section 2.8), sperm suspensions were centrifuged at 4000 rpm for 5 minutes. These cells were washed twice in phosphate buffered saline (PBS) and the pellets were resuspended in 100µl of labelling solution [20µl Annexin-V–Fluos labelling reagent in 1000µl Hepes (Sigma, USA) buffer and 20µl PI] for 15 minutes at room temperature. The preparation of the labelling solution was followed according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). 0.5 ml of Hepes buffer was added to each sample and analysed by flow cytometry as mentioned under the heading 2.6.3 .The protocol is outlined in Figure 2.15.

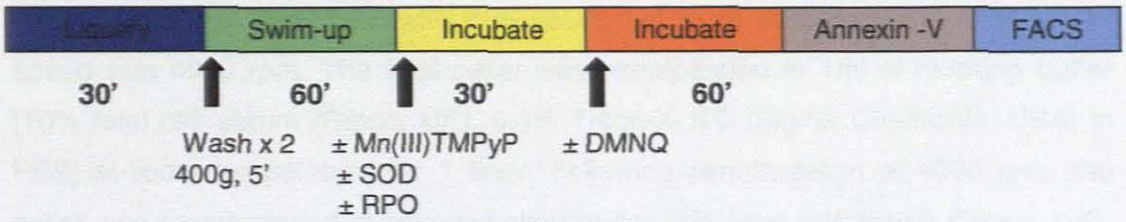

Figure 2.15: Protocol to investigate the effects of superoxide on sperm apoptosis in the absence or presence of Mn(III)TMPyP, SOD and RPO using Annexin-V

2.10.2 Apo-direct assay

After incubation of aliquots with DMNQ (refer to section 2.8), sperm cells were fixed in 1% paraformaldehyde (Sigma Aldrich Co., UK) in PBS solution and this was followed by incubation on ice for 30-60 minutes. The cells were washed twice in PBS and centrifugation between the washing steps was carried out for 5 minutes at 4500 rpm. The sperm cells were re-suspended in 70% ice-cold ethanol and stored on ice for 30 minutes. For the staining procedure according to the manufacturer's protocol, sperm cells were incubated in the staining solution (Reaction buffer, Tdt enzyme, FITC-dUTP suspended in distilled water) for 60 minutes at 37°C. Following the staining procedure, the sperm cells were rinsed twice in rinsing buffer with centrifugation (4500 rpm for 5 minutes) between washing steps. The cell pellets were re-suspended in a propidium iodide/RNase (PI/RNase) staining buffer and incubated in the dark for 30 minutes at room temperature. The sperm cells were analysed within 3 hours of staining by flow cytometry for the detection of labelled DNA strand breaks to detect apoptotic sperm cells in the ejaculate sperm samples. Positive and negative control cells (purchased components of the Apo-direct kit; BD Biosciences, Pharmingen, USA) were stained in the same manner according to the manufacturer's protocol and samples were analysed by flow cytometry as mentioned under 2.6.4. The protocol is outlined in Figure 2.16.

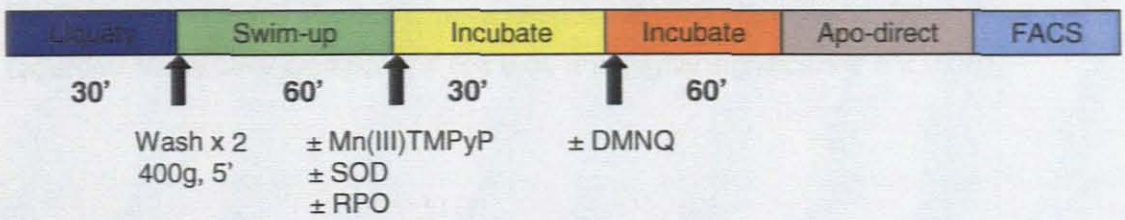

Figure 2.16: Protocol to investigate the effects of superoxide on sperm apoptosis in the absence or presence of Mn(III)TMPyP, SOD and RPO using Apo-direct

2.10.3 Caspase-3 Assay

After incubation of aliquots with DMNQ (refer to section 2.8), spermatozoa were fixed in a 4% paraformaldeldehyde solution (Sigma Aldrich Co., UK) at 37°C for 30 minutes. The pellet was obtained following centrifugation at 4000 rpm and then a

double wash was performed with PBS solution at room temperature and centrifuge speed was 4000 rpm. The final pellet was resuspended in 1ml of blocking buffer [10% fetal calf serum (Gibco, UK), 0.4% Triton-X-100 (Sigma Chemicals, USA) in PBS] at room temperature for 1 hour. Following centrifugation at 4000 rpm, the pellet was re-suspended in an incubation buffer [2% fetal calf serum (Gibco, UK), 0.4% Triton-X-100 (Sigma Chemicals, USA) in PBS] with 20µl of FITC-conjugated polyclonal rabbit active anti-caspase-3 antibodies (Phamingen, USA) for 1 hour. These spermatozoa were washed three times with 0.1% Tween- 20 in PBS (Merck, Schuchardt, Germany), re-suspended in PBS and filtered though a nylon filter to reduce any clumping of cells, thus ensuring a single cell suspension. In all the analyses, spermatozoa unlabelled with FITC caspase-3 antibody served as the negative control. Samples were analysed by flow cytomety as mentioned under 2.6.5. Furthermore, the protocol is outlined in Figure 2.17.

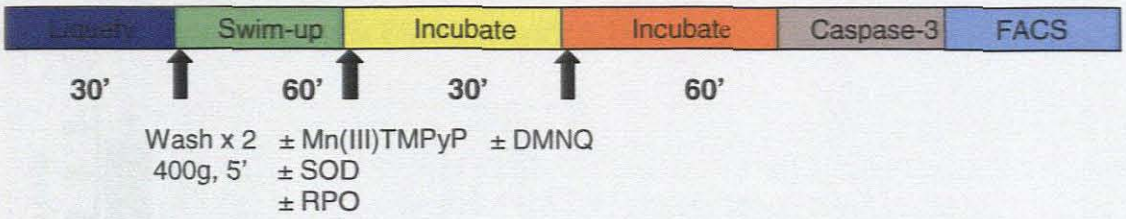

Figure 2.17: Protocol to investigate the effects of superoxide on sperm apoptosis in the absence or presence of Mn(III)TMPyP, SOD and RPO using Caspase-3

2.11 Statistical analyses

GaphPad™ PRISM 4 was used for all statistical evaluations and graphical representations. Some data are expressed as percentages of the control (mean ± S.E.M), and control values were adjusted to 100%. For comparative studies, Student *t*-test (unpaired) or one-way analysis of variance (ANOVA) test (with Bonferroni *post test* if $P < 0.05$) were used for statistical analyses. Differences were regarded statistically significant if $P < 0.05$ and highly significant if $P < 0.001$.

CHAPTER 3

RESULTS

3.1 Preliminary studies

This initial study evaluated DMSO, ethanol and PGly in the attempt to determine the appropriate solvent capable of dissolving RPO with the least toxic effects on sperm cells.

3.1.1 Solubility and toxicity of dimethylsulphoxide (DMSO), Ethanol, and Propanol Glycol (PGly)

RPO started to dissolve in a solution of 87.5% of DMSO. However, a significant decrease in motility was observed if spermatozoa were incubated in concentrations equal to or higher than 0.1% DMSO (Figure 3.1).

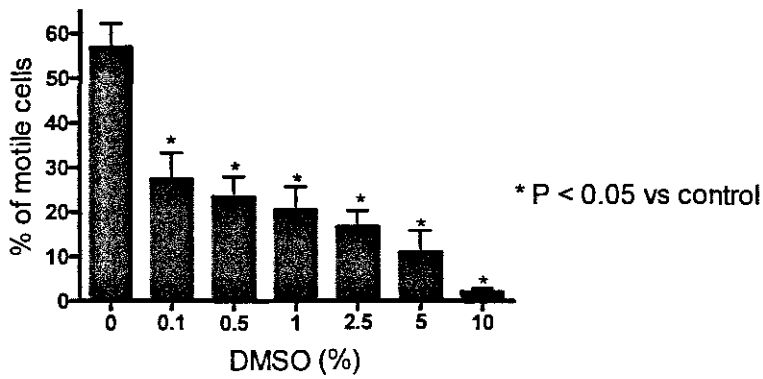

Figure 3.1: Solubility of RPO in DMSO and the toxic effect of DMSO on sperm motility (n=5)

RPO dissolved in solution of 75% of ethanol. From Figure 3.2 it can be seen that ethanol concentrations of 0.05% and higher significantly decreased sperm motility when compared to control.

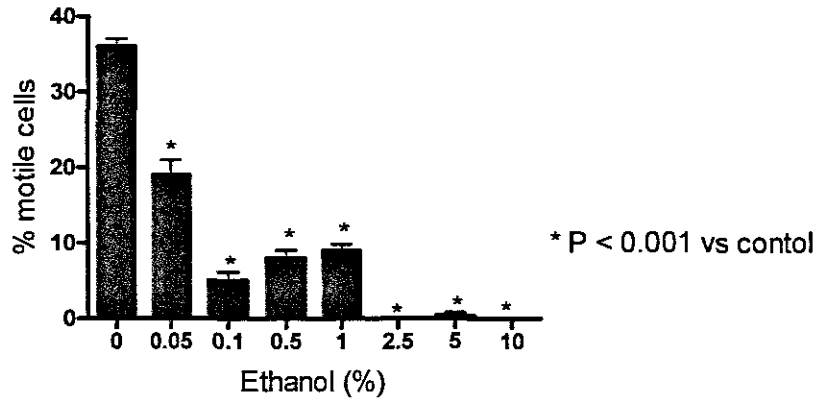

Figure 3.2: Solubility of RPO in ethanol and the toxic effect of ethanol on sperm motility (n=6)

RPO already dissolved in a solution of 50% of PGly. However, PGly only decreased sperm motility at concentrations equal or higher to 0. 5%.

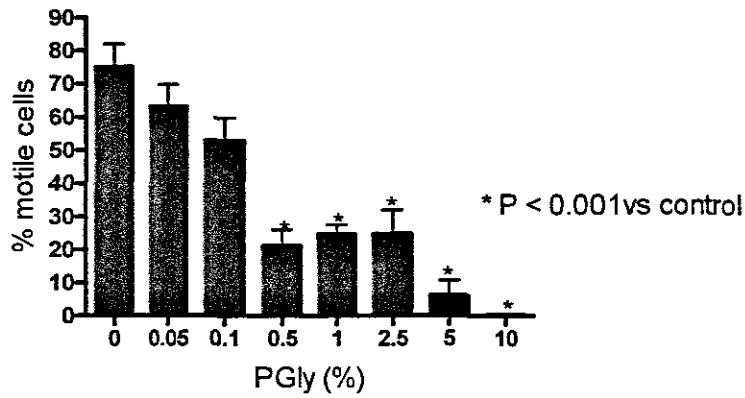

Figure 3.3: Solubility of RPO in PGly and the toxic effect of PGly on sperm motility (n=6)

It was subsequently decided to use PGly as the vehicle of preference to administer RPO because it is less detrimental to sperm motility (0.5%) and already dissolves RPO at low concentration (50%).

3.1.2 Determining the appropriate administration concentrations of RPO/PGly for sperm motility

Figure 3.4 shows that 2.5% RPO+PGly (51.44±5.26%) and 2.5% PGly (28.78 ±6.3%) significantly decreased the number of motile cells when compared to control (69.00±3.09%). Furthermore, it can be seen that at concentrations of 2.5% the addition of RPO significantly increased the number of motile cells when compared to PGly alone.

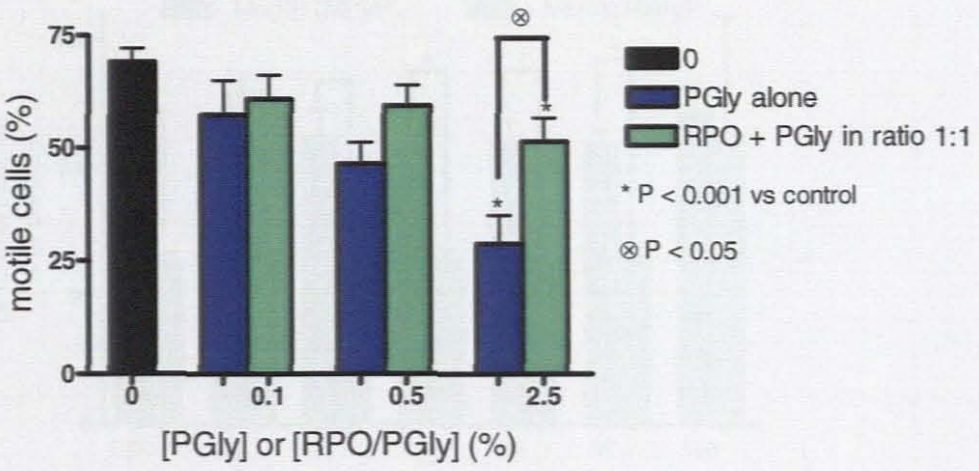

Figure 3.4: Effect of PGly or RPO + PGly on sperm motility (n=13)

Due to these findings, the administration of RPO+PGly shall be limited to the concentrations of 0.1% and 0.5% because these have less toxic effect on the cells. Furthermore, RPO+PGly shall be simply referred as to RPO.

3.1.3. Dihydroethidium (DHE)-specificity for superoxide studies in the absence or presence of Mn(III)TMPyP, SOD and RPO

3.1.3.1 Effect of exogenous superoxide on dihydroethidium (DHE) fluorescence in sperm

From Figures 3.5, 3.6 and 3.7 it can be observed that the addition of DMNQ (up to 100µM) did not statistically affect DHE fluorescence.

3.1.3.2 The effects of superoxide on dihydroethidium (DHE) fluorescence in the absence or presence of Mn(III)TMPyP in sperm

From Figure 3.5 it is evident that the treatment with Mn(III)TMPyP significantly reduced DHE fluorescence in samples containing no DMNQ up to 50µM DMNQ.

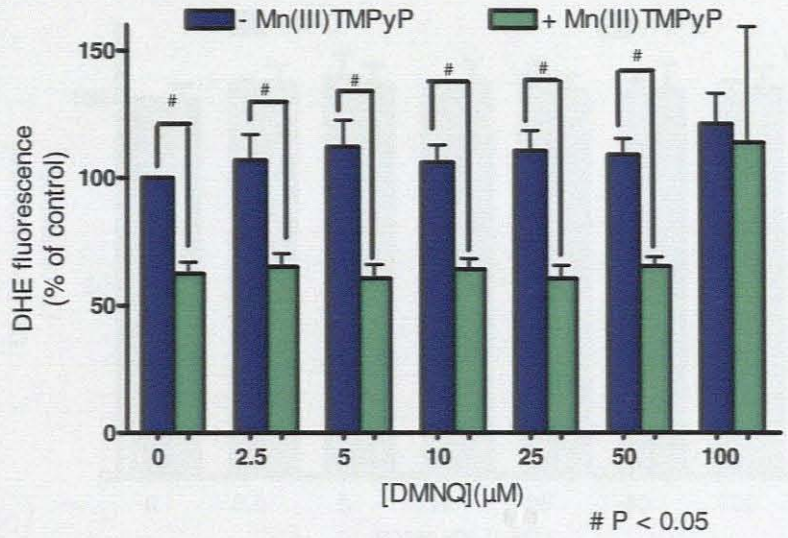

Figure 3.5: The scavenging effect of Mn(III)TMPyP on superoxide production of sperm (n=6)

3.1.3.3 The effects of superoxide on dihydroethidium (DHE) fluorescence in the absence or presence of SOD in sperm

Figure 3.6 shows that the addition of SOD significantly decreased DHE fluorescence in samples to which 25μM to 100μM DMNQ was added.

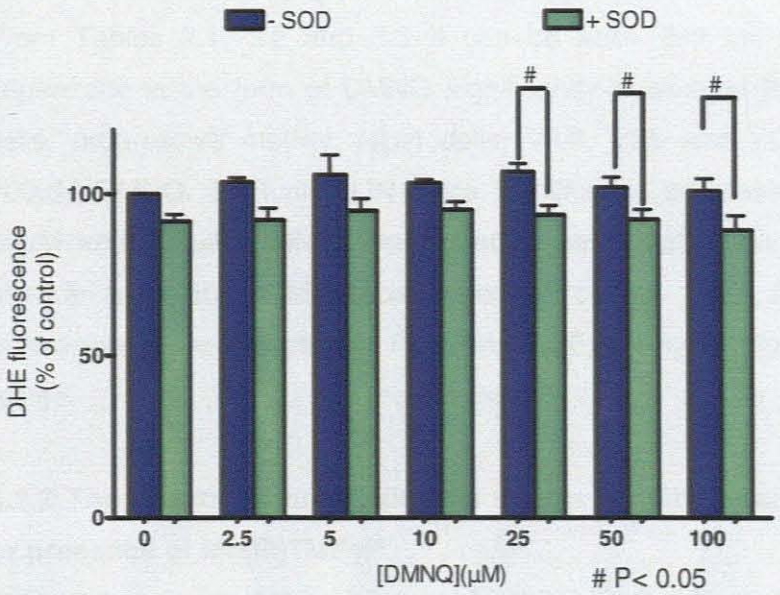

Figure 3.6: The scavenging effect of SOD on superoxide production of sperm (n=6)

3.1.3.4 The effects of superoxide on dihydroethidium (DHE) fluorescence in the absence or presence of RPO in sperm

From Figure 3.7 it can be seen that RPO did not lead to any significant differences in DHE fluorescence ($P > 0.05$) for any of the DMNQ concentrations.

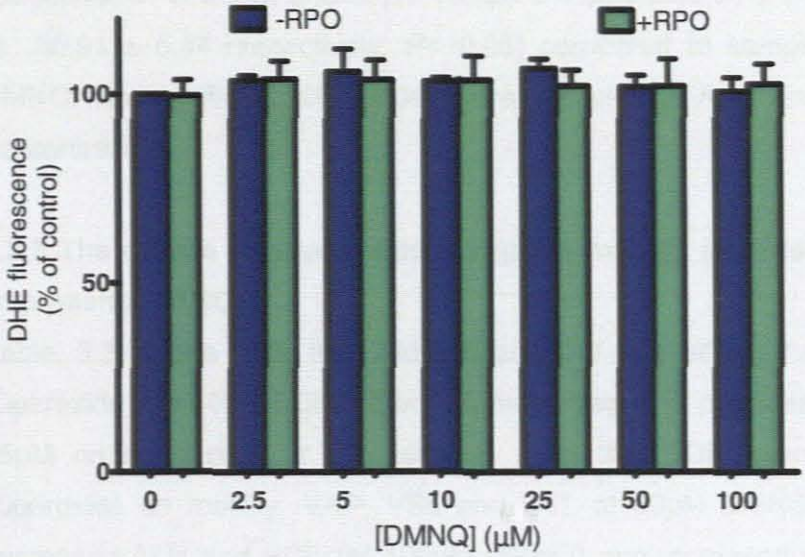

Figure 3.7: The scavenging effect of 0.1% RPO on superoxide (n=6)

3.2 The effects of superoxide on sperm motility parameters in the absence or presence of Mn(III)TMPyP, SOD and RPO

3.2.1 The effects of exogenous superoxide on sperm motility parameters

From Tables 3.1, 3.2 and 3.3 it can be seen that the addition of exogenous superoxide in the form of DMNQ significantly decreased the percentage of motile cells, progressive motility, rapid cells, VAP, VSL and VCL, at concentration of 100μM DMNQ. STR and LIN were significantly decreased at concentrations of 50μM and 100μM DMNQ. On the other hand static cells significantly increased already from 5μM DMNQ compared to control (0μM DMNQ). No significant differences were detected in ALH and BCF at any of the DMNQ concentrations tested.

3.2.2 The effects of superoxide on sperm motility parameters in the absence or presence of Mn(III)TMPyP

Table 3.1 shows that the addition of Mn(III)TMPyP did not significantly attenuate the effect of superoxide on percentages of progressive motility, rapid cells, VAP, VSL and VCL at 100μM DMNQ when compared to control (0μM DMNQ). However, Mn(III)TMPyP attenuated the negative effect of superoxide on static cells at 5μM and 10μM DMNQ. Meanwhile, Mn(III)TMPyP significantly decreased the percentages of motility and static cells at 100μM DMNQ (27.91 ± 3.09 vs. 16.36 ± 2.06 and 82.70 ± 3.17 vs. 57.82 ± 5.54 respectively; $P< 0.05$). Furthermore, the addition of Mn(III)TMPyP decreased the number of static cells already at DMNQ

concentration of 25μM and 50μM (48.20 ± 4.32 vs. 30.91 ± 2.83 and 61.20 ± 5.08 vs. 35.91 ± 5.44 respectively; $P< 0.05$) compared to samples treated with only DMNQ. No significant differences were detected in ALH and BCF at all DMNQ concentrations.

3.2.3 The effects of superoxide on sperm motility parameters in the absence or presence of SOD

Table 3.2 shows that the addition of SOD intensified the harmful effects of superoxide from 10μM DMNQ on the percentages of progressive motility and from 25μM on rapid cells. It can also be seen that SOD intensified the effects of superoxide on motility, VAP, VSL and VCL at 50μM DMNQ. Furthermore, SOD decreased ALH and BCF (at 100μM DMNQ) and increased static cells (at 2.5μM DMNQ) when compared to control (0μM DMNQ). However, SOD reversed the negative effect of superoxide on STR (50μM DMNQ) and static cells (10μM DMNQ). Samples treated with DMNQ in combination with SOD showed significantly less rapid cells at concentration from 2.5μM to 100μM DMNQ when compared to those treated with DMNQ alone. This was also observed with the percentage of progressive motility (from 10μM DMNQ) and motile cells (from 50μM DMNQ).

3.2.4 The effects of superoxide on sperm motility parameters in the absence or presence of RPO

The addition of 0.1% or 0.5% RPO (Table 3.3) intensified the negative effects of superoxide on motility, progressive motility and rapid cells at 50μM DMNQ. Similarly, RPO intensified the effects of superoxide on VAP, VSL and VCL at 5μM and 50μM DMNQ. However, RPO attenuated the effect of superoxide on static cells from 5μM to 25μM DMNQ. Moreover, it reversed the effect of superoxide on STR and LIN at 50μM. No significant differences were detected in ALH and BCF at all DMNQ concentrations. In the control group 0.1% and 0.5% RPO significantly increased the percentage of motility compared to the superoxide control (0μM DMNQ). In addition 0.5% RPO increased rapid cells, VAP and LIN. No significant difference was detected in the 2.5μM DMNQ group. In the 5μM DMNQ group, 0.1% and 0.5% RPO significantly reduced VAP, VSL, VCL and percentage of static cells. 0.1% and 0.5% RPO significantly decreased the percentage of static cells at 5μM DMNQ. Moreover, 0.1% RPO decreased VCL at 5μM DMNQ. In the 50μM DMNQ group, 0.1% and 0.5% RPO significantly reduced motility, progressive motility and VCL. 0.1% and 0.5% RPO significantly decreased percentages of motility and

progressive motility at 100μM DMNQ. In addition 0.1% RPO reduced BCF while 0.5% RPO reduced static cells at 100μM DMNQ.

Table 3.1: The effects of superoxide on sperm motility parameters in the presence or absence of Mn(III)TMPyP (50μM) (n=12)

Parameters	Treatment	0μM DMNQ	2.5μM DMNQ	5μM DMNQ	10μM DMNQ	25μM DMNQ	50μM DMNQ	100μM DMNQ
MOTILE		54.64 ± 6.20	58.18 ± 4.11	55.09 ± 4.12	59.45 ± 5.98	55.36 ± 5.58	52.91 ± 3.10	27.91 ± 3.09*
(%)	+Mn(III)TMPyP	59.82 ± 3.26	53.27 ± 5.06	49.64 ± 3.94	54.73 ± 3.08	54.27 ± 2.50	46.82 ± 3.74	16.36 ±2.06*$
PROGRESSIVE		34.00 ± 4.30	31.18 ± 3.66	26.00 ± 2.42	36.64 ± 4.06	34.91± 5.78	25.27 ± 2.26	05.36 ± 1.03*
MOTILITY (%)	+Mn(III)TMPyP	35.82 ± 2.55	31.64 ± 4.26	25.27 ± 3.23	32.00 ± 3.25	30.36 ± 2.20	23.36 ± 1.68	03.36 ± 0.52*
RAPID		44.91 ± 5.64	46.55 ± 4.60	42.36 ± 3.91	50.55 ± 5.39	43.82 ± 5.02	40.73 ± 2.84	13.00 ± 1.81*
CELLS (%)	+Mn(III)TMPyP	49.45 ± 3.21	43.73 ± 5.45	38.18 ± 3.75	43.73 ± 3.78	43.73 ± 2.90	33.18 ± 3.17	09.27 ± 1.74*
VAP		53.33 ± 2.85	50.93 ± 3.34	47.88 ± 2.82	54.98 ± 2.98	51.17 ± 2.71	43.25 ± 2.72	31.87 ± 1.91*
(μm/s)	+Mn(III)TMPyP	55.45 ± 2.16	56.38 ± 3.71	50.51 ± 2.41	56.62 ± 3.64	55.22 ± 3.57	48.85 ±1.86	29.96 ± 1.49*
VSL		46.65 ± 2.91	43.38 ± 3.06	39.86 ± 2.49	47.91 ± 3.10	43.72 ± 2.81	35.83 ± 2.45	25.59 ± 1.84*
(μm/s)	+Mn(III)TMPyP	47.62 ± 2.13	48.66 ± 3.26	42.18 ± 2.30	48.83 ± 3.20	46.88 ± 3.04	40.97 ± 1.76	22.74 ± 1.12*
VCL		80.24 ± 2.87	79.44 ± 3.91	73.20 ± 2.35	83.27 ± 3.13	77.18 ± 3.03	73.27 ± 2.21	53.08 ± 2.74*
(μm/s)	+Mn(III)TMPyP	79.65 ± 3.19	79.55 ± 6.25	75.99 ± 2.92	80.88 ± 4.80	81.43 ± 4.84	74.12 ± 2.42	51.91 ± 2.19*
ALH		03.89 ± 0.20	04.11 ± 0.20	04.33 ± 0.15	04.06 ± 0.19	04.25 ± 0.13	04.34 ± 0.12	04.04 ± 0.16
(μm/s)	+Mn(III)TMPyP	03.84 ± 0.19	04.11 ± 0.16	04.28± 0.13	03.92 ± 0.18	04.22 ± 0.13	04.15 ± 0.11	03.42 ± 0.24
BCF		23.74 ± 0.81	23.22 ± 1.00	22.09± 0.96	23.29 ± 1.08	23.22 ± 1.72	23.30 ± 0.72	24.99 ± 1.30
(Hz)	+Mn(III)TMPyP	23.68 ± 0.68	23.49 ± 0.70	22.68± 0.26	24.31 ± 0.66	22.62 ± 0.70	22.58 ± 0.46	24.51 ± 0.99
STR		82.20 ± 1.11	78.30 ± 1.25	74.80 ± 0.55	79.90 ± 0.60	79.20 ± 1.14	72.70 ± 3.59*	73.30 ± 1.30*
(%)	+Mn(III)TMPyP	83.45 ± 1.03	83.64 ± 0.62	80.45 ± 0.69	83.27 ± 0.83	82.18 ± 0.55	80.91 ± 0.47*	76.55 ± 1.51*
LIN		56.60 ± 1.62	50.10 ± 1.14	49.90 ± 0.82	52.80 ± 0.69	51.00 ±1.26	47.20 ± 1.05*	45.20 ± 3.34*
(%)	+Mn(III)TMPyP	58.36 ± 1.37	56.73 ± 0.92	55.73 ± 0.99	57.82 ± 1.33	55.60 ± 1.01	52.91 ± 0.68*	45.09 ± 1.25*
STATIC		24.60 ± 3.29	33.90 ± 4.55	42.90± 3.01*	44.67 ± 4.13*	48.20 ± 4.32*	61.20 ± 5.08*	82.70 ± 3.17*
CELLS (%)	+Mn(III)TMPyP	24.09 ± 3.36	31.09 ± 5.16	31.36 ± 3.53	28.27 ± 3.33	30.91 ± 2.83$	35.91± 5.44$	57.82± 5.54*$

Note: Values are represented as means ± S.E.M. Differences were regarded statistically significant if $P < 0.05$ and highly significant if $P < 0.001$.

* $P < 0.001$ vs. control (0μM DMNQ and no Mn(III)TMPyP); $ $P < 0.05$ vs parameter of same [DMNQ]

Table 3.2: The effects of superoxide on sperm motility parameters in the presence or absence of SOD (n=12)

Parameters	Treatment	0μM DMNQ	2.5μ MDMNQ	5μM DMNQ	10μM DMNQ	25μM DMNQ	50μM DMNQ	100μM DMNQ
MOTILE		54.64 ± 6.20	58.18 ± 4.11	55.09 ± 4.12	59.45 ± 5.98	55.36 ± 5.58	52.91 ± 3.10	27.91 ± 3.09*
(%)	+SOD	56.10 ± 3.99	49.90 ± 3.97	46.60 ± 3.62	42.30 ± 3.01	38.60 ± 3.13	20.80±4.22*$	03.70± 0.96*$
PROGRESSIVE		34.00 ± 4.30	31.18 ± 3.66	26.00 ± 2.42	36.64 ± 4.06	34.91 ± 5.78	25.27 ± 2.26	05.36 ± 1.03*
MOTILITY (%)	+SOD	30.30 ± 2.73	23.90 ±1.82	22.70 ± 2.70	21.40± 2.16*$	18.20±2.02*$	7.00 ± 1.80*$	01.00± 0.42*$
RAPID		44.91 ± 5.64	46.55 ± 4.60	42.36 ± 3.91	50.55 ± 5.39	43.82 ± 5.02	40.73 ± 2.84	13.00 ± 1.81*
CELLS (%)	+SOD	42.40 ± 3.64	31.70 ± 1.64$	32.50 ± 1.77$	33.10 ± 1.56$	30.70±2.96*$	14.50±3.61*$	02.60± 0.77*$
VAP		53.33 ± 2.85	50.93 ± 3.34	47.88 ± 2.82	54.98 ± 2.98	51.17 ± 2.71	43.25 ± 2.72	31.87 ± 1.91*
(μm/s)	+SOD	54.00 ± 2.11	46.06 ±1.77	46.03 ± 2.63	49.05 ± 3.66	49.70 ± 2.22	40.40 ±1.95*	30.62 ±2.56*
VSL		46.65 ± 2.91	43.38 ± 3.06	39.86 ± 2.49	47.91 ± 3.10	43.72 ± 2.81	35.83 ± 2.45	25.59 ± 1.84*
(μm/s)	+SOD	44.34 ± 1.64	36.61 ±1.72	37.13 ± 1.30	42.11 ± 2.15	40.25 ± 2.13	31.38 ±1.82*	22.15± 2.18*
VCL		80.24 ± 2.87	79.44 ± 3.91	73.20 ± 2.35	83.27 ± 3.13	77.18 ± 3.03	73.27 ± 2.21	53.08 ± 2.74*
(μm/s)	+SOD	81.71 ± 3.81	74.79 ± 3.35	79.16 ± 3.84	78.85 ± 4.61	78.07 ± 3.27	64.75 ±2.50*	51.36 ±3.07*
ALH		03.89 ± 0.20	04.11 ± 0.20	04.33 ± 0.15	04.06 ± 0.19	04.25 ± 0.13	04.34 ± 0.12	04.04 ± 0.16
(μm/s)	+SOD	04.21 ± 0.15	04.36 ± 0.14	04.23 ± 0.18	04.60 ± 0.19	04.45 ± 0.19	03.99 ± 0.12	02.20 ± 0.54*
BCF		23.74 ± 0.81	23.22 ± 1.00	22.09 ± 0.96	23.29 ± 1.08	23.22 ± 1.72	23.30 ± 0.72	24.99 ± 1.30
(Hz)	+SOD	22.03 ± 0.75	21.77± 0.59	22.40 ± 1.10	21.98 ± 0.77	21.78 ± 0.51	21.06± 0.84	15.65 ±2.06*
STR		82.20 ± 1.11	78.30 ± 1.25	74.80 ± 0.55	79.90 ± 0.60	79.20 ± 1.14	72.70 ± 3.59*	73.30 ± 1.30*
(%)	+SOD	79.90 ± 0.75	77.40 ± 0.84	75.60 ± 1.77	80.50 ± 0.92	78.30 ± 1.01	76.00 ± 1.73	72.50 ± 2.67*
LIN		56.60 ±1.62	50.10 ± 1.14	49.90 ± 0.82	52.80 ± 0.69	51.00 ± 1.26	47.20 ± 1.05*	45.20 ± 3.34*
(%)	+SOD	53.50 ±1.302	48.40 ± 1.35	47.13 ± 0.91	52.30 ± 1.23	50.10 ± 1.47	46.40 ±1.815*	44.60 ±1.93*
STATIC		24.60 ± 3.29	33.90 ± 4.55	42.90 ± 3.01*	44.67 ± 4.13*	48.20 ± 4.32*	61.20 ± 5.08*	82.70 ± 3.17*
CELLS (%)	+SOD	31.20 ± 4.66	44.90 ± 3.55*	46.90 ± 4.87*	41.10± 3.75	44.90 ± 3.37*	60.10± 5.80*	90.56±1.82*

Note: Values are represented as means ± S.E.M. Differences were regarded statistically significant if $P < 0.05$ and highly significant if $P < 0.001$.

* $P < 0.001$ vs. control (0μM DMNQ and no SOD); $ $P < 0.05$ vs parameter of the same [DMNQ]

Table 3.3: The effects of superoxide on sperm motility parameters in the absence or presence of different concentrations of RPO (n=12)

Parameters	Treatment	0uM DMNQ	2.5uM DMNQ	5uM DMNQ	10uM DMNQ	25uM DMNQ	50uM DMNQ	100uMDMNQ
MOTILE (%)		54.64 ± 6.20 $	58.18 ± 4.11	55.09 ± 4.12	59.45 ± 5.98	55.36 ± 5.58	52.91 ± 3.10	27.91 ± 3.09*
	0.1% RPO	60.89 ± 5.32 $	48.56 ± 6.66	43.29 ± 6.48	48.22 ± 5.91	44.56 ± 5.97	25.67 ± 6.11* $	09.33±3.62* $
	0.5% RPO	60.62 ± 3.70 $	49.46 ± 4.28	49.80 ± 2.81	56.31 ± 4.18	48.23 ± 3.48	33.23 ± 7.71* $	13.46±2.53* $
PROGRESSIVE MOTILITY (%)		34.00 ± 4.30	31.18 ± 3.66	26.00 ± 2.42	36.64 ± 4.06	34.91 ± 5.78	25.27 ± 2.26	05.36 ±1.03*
	0.1% RPO	43.00 ± 6.01	29.00 ± 7.65	26.50 ± 6.23	27.33 ± 5.42	27.67 ± 5.81	12.33 ± 4.40* $	02.50±1.14* $
	0.5% RPO	40.46 ± 3.79	29.27 ± 3.87	25.88 ± 2.81	32.38 ± 3.22	30.50 ± 3.71	14.15 ± 3.90* $	02.53±0.63* $
RAPID CELLS (%)		44.90 ± 5.64	46.55 ± 4.60	42.36 ± 3.91	50.55 ± 5.39	43.82 ± 5.02	40.73 ± 2.84	13.00 ± 1.81*
	0.1% RPO	51.89 ± 5.40	40.11 ± 6.56	34.78 ± 3.62	38.56 ± 6.04	35.11 ± 6.00	18.22 ± 4.70*	05.22 ± 2.58*
	0.5% RPO	52.08 ± 3.93 $	37.46 ± 3.88	33.33 ± 3.35	46.00 ± 4.03	35.15 ± 3.85	24.77 ± 6.68*	06.84 ± 1.63*
VAP (um/s)		53.33 ± 2.85	50.93 ± 3.34	47.88 ± 2.82	54.98 ± 2.98	51.17 ± 2.71	43.25 ± 2.72	31.87 ± 1.91*
	0.1% RPO	51.82 ± 5.75	45.59 ± 3.89	36.46 ± 3.24* $	45.43 ± 4.69	44.11 ± 4.04	36.92 ± 2.46*	31.22 ± 3.29*
	0.5% RPO	62.32 ± 2.08 $	49.10 ± 2.73	38.52 ± 2.13* $	55.18 ± 2.33	46.16 ± 2.84	39.22 ± 2.44*	29.43 ± 3.36*
VSL (um/s)		46.65 ± 2.91	43.38 ± 3.06	39.86 ± 2.49	47.91 ± 3.10	43.72 ± 2.81	35.83 ± 2.45	25.59 ± 1.84*
	0.1% RPO	42.78 ± 6.00	36.53 ± 3.94	28.24 ± 3.13* $	36.31 ± 4.74	35.26 ± 4.08	27.67 ± 2.68*	22.90 ± 2.73*
	0.5% RPO	54.62 ± 2.00	41.09 ± 2.61	30.79 ± 1.86* $	47.15 ± 2.16	38.65 ± 2.57	31.64 ± 2.02*	22.81 ± 2.47*
VCL (um/s)		80.24 ± 2.87	79.44 ± 3.91	73.20 ± 2.35	83.27 ± 3.13 $	77.18 ± 3.03	73.27 ± 2.21	53.08 ± 2.74*
	0.1% RPO	74.88 ± 6.34	68.86 ± 4.94	57.83 ± 3.96* $	69.32 ± 5.32 $	66.68 ± 4.93	58.60 ± 3.09* $	49.14 ± 4.63*
	0.5% RPO	86.24 ± 2.35	74.13 ± 3.12	62.43 ± 2.78* $	79.95 ± 2.95	69.60 ± 3.63	63.08 ± 3.18* $	51.92 ± 5.52*
ALH (um/s)		03.89 ± 0.20	04.11 ± 0.20	04.33 ± 0.15	04.06 ± 0.19	04.25 ± 0.13	04.34 ± 0.12	04.04 ± 0.16
	0.1% RPO	03.90 ± 0.10	03.82 ± 0.22	03.70 ± 0.25	03.95 ± 0.18	03.76 ± 0.22	03.71 ± 0.18	02.76 ± 0.81
	0.5% RPO	03.97 ± 0.08	04.19 ± 0.11	04.17 ± 0.10	04.10 ±0.13	04.03 ± 0.18	04.03 ± 0.18	03.40 ± 0.48
BCF (Hz)		23.74 ± 0.81	23.22 ± 1.00	22.09 ± 0.96	23.29 ± 1.08	23.22 ± 1.72	23.30 ± 0.72	24.99 ± 1.30 $
	0.1% RPO	20.38 ± 0.85	20.47 ± 0.38	22.88 ± 1.46	20.76 ± 0.94	20.67 ± 0.91	20.86 ± 1.38	19.11 ± 1.57 $
	0.5% RPO	23.12 ± 0.45	22.61 ± 0.98	23.58 ± 1.26	22.44 ± 0.41	24.12 ± 0.93	21.82 ± 1.25	23.03 ± 2.77
STR (%)		82.20 ± 1.11	78.30 ± 1.25	74.80 ± 0.55	79.90 ± 0.60	79.20 ± 1.14	72.70 ± 3.59*	73.30 ± 1.30*
	0.1% RPO	78.67 ± 2.72	76.56 ± 2.10	76.33 ± 1.93	76.33 ± 2.01	76.78 ± 2.14	74.38 ± 2.58	76.44 ± 3.70
	0.5% RPO	84.69 ± 0.71	81.15 ± 0.79	78.85 ± 0.97	82.62 ± 0.72	81.54 ± 1.09	79.23 ± 0.89	72.00 ± 6.18
LIN (%)		56.60 ± 1.62	50.10 ± 1.14	49.90 ± 0.82	52.80 ± 0.69	51.00 ± 1.26	47.20 ± 1.05*	45.20 ± 3.34*
	0.1% RPO	54.22 ± 3.37	51.22 ± 2.49	47.78 ± 1.73	49.78 ± 2.42	50.67 ± 2.42	46.44 ± 1.77	50.56 ± 3.97
	0.5% RPO	60.92 ± 0.99 $	53.15 ± 1.36	51.98 ± 1.04	52.62 ± 1.19	53.92 ± 2.13	49.46 ± 1.04	41.46 ± 3.50*
STATIC CELL (%)		24.60 ± 3.29	33.90 ± 4.55	42.90 ± 3.01*	44.67± 4.13*	48.20 ± 4.32*	61.20 ± 5.08*	82.70 ± 3.17*
	0.1% RPO	20.89 ± 3.95	36.22 ± 6.24	35.89 ± 7.28 $	33.22 ±6.30 $	31.50 ± 5.10 $	53.13 ± 8.73*	79.11 ± 7.31*
	0.5% RPO	23.92 ± 3.24	29.08 ± 3.96	34.15 ± 4.68 $	26.23 ± 3.56 $	28.08 ± 3.38 $	49.77± 7.79*	63.69±6.64* $

Note: Values are represented as means ± S.E.M. Differences were regarded statistically significant if $P < 0.05$ and highly significant if $P < 0.001$

* $P < 0.001$ vs control (0μM DMNQ and no RPO); $ $P < 0.001$ vs parameter of the same [DMNQ] and no RPO.

3.3 The effects of superoxide on PI fluorescence in the absence or presence of Mn(III)TMPyP, SOD and RPO

Non-viable cells can be determined using membrane-impermeable nucleic acid stains which positively identify dead spermatozoa by penetrating cells with damaged membranes. An intact plasma membrane will prevent these products from entering the spermatozoa and thereby staining the nucleus. Commonly used examples include phenanthridines, for example propidium iodide (Gillan *et al.*, 2005).

3.3.1 The effects of superoxide on PI fluorescence

Figures 3.8, 3.9 and 3.10 show that superoxide had no effect on PI fluorescence for any concentration of $O_2^{-\bullet}$ equal to or less than 50µM DMNQ. The addition of 100µM DMNQ led to a significant increase ($P < 0.05$) in PI fluorescence.

3.3.2 The effects of superoxide on PI fluorescence in the absence or presence of Mn(III)TMPyP

Figure 3.8 shows that Mn(III)TMPyP treatment prior to DMNQ addition significantly changed PI fluorescence when compared to samples not treated with Mn(III)TMPyP. PI fluorescence significantly increased in samples stimulated with 50 and 100µM DMNQ in the presence of Mn(III)TMPyP (206.80 ± 34.45 vs.118.3 ± 12.63 and 306.70 ± 61.67 vs. 194.6 ± 31.36; $P < 0.001$) while samples treated with lower concentrations of DMNQ (2.5µM, 5µM and 10µM), Mn(III)TMPyP addition significantly reduced PI fluorescence (110.9 ± 9.28 vs. 79.32 ± 12.62; 100.6 ± 9.15 vs. 72.40 ± 9.24; 148.00 ± 23.90 vs. 89.88 ± 15.55; $P < 0.001$).

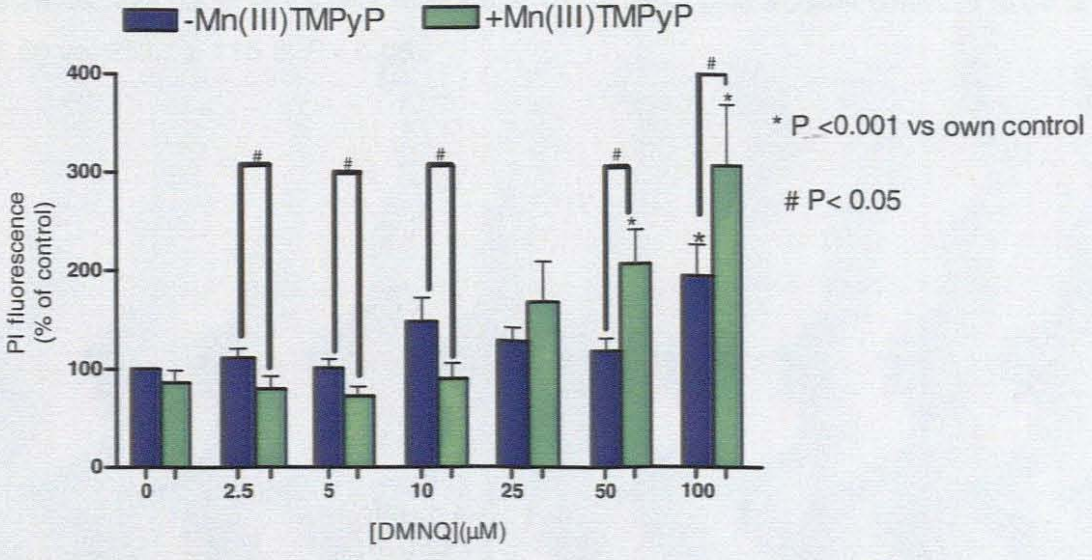

Figure 3.8: The effect of superoxide on PI fluorescence in absence or presence of Mn(III)TMPyP (n=12)

3.3.3 The effects of superoxide on PI fluorescence in the absence or presence of SOD

Figure 3.9 showed that SOD had no effect on PI fluorescence at any given concentration of DMNQ.

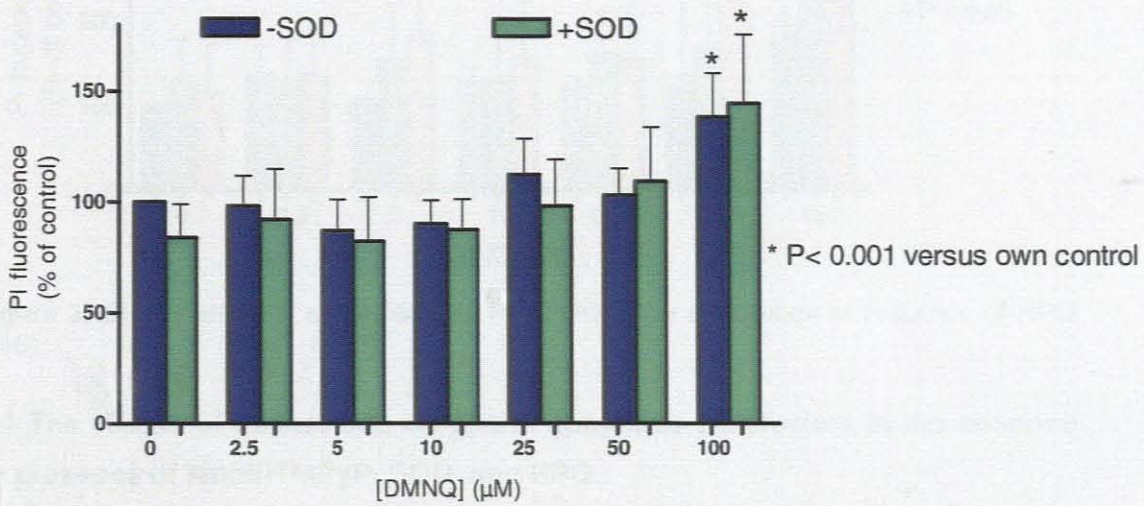

Figure 3.9: The effect of superoxide on PI fluorescence in absence or presence of SOD (n=11)

3.3.4 The effects of superoxide on PI fluorescence in the absence or presence of RPO

Figure 3.10 showed that RPO treatment prior to DMNQ addition of up to 25μM had no effect on PI fluorescence. However, RPO treatment combined with higher dosage of DMNQ significantly increased PI fluorescence: 0.1% RPO plus 100μM (181.5 ± 15.60 vs. 2.49.1 ± 70.28; $P < 0.05$), 0.5% RPO plus 50μM DMNQ (122.7 ± 11.76 vs. 227.00 ± 101.1; $P < 0.05$) and 0.5% RPO plus 100μM DMNQ (181.5 ± 15.60 vs. 252.1 ± 115.8; $P < 0.05$).

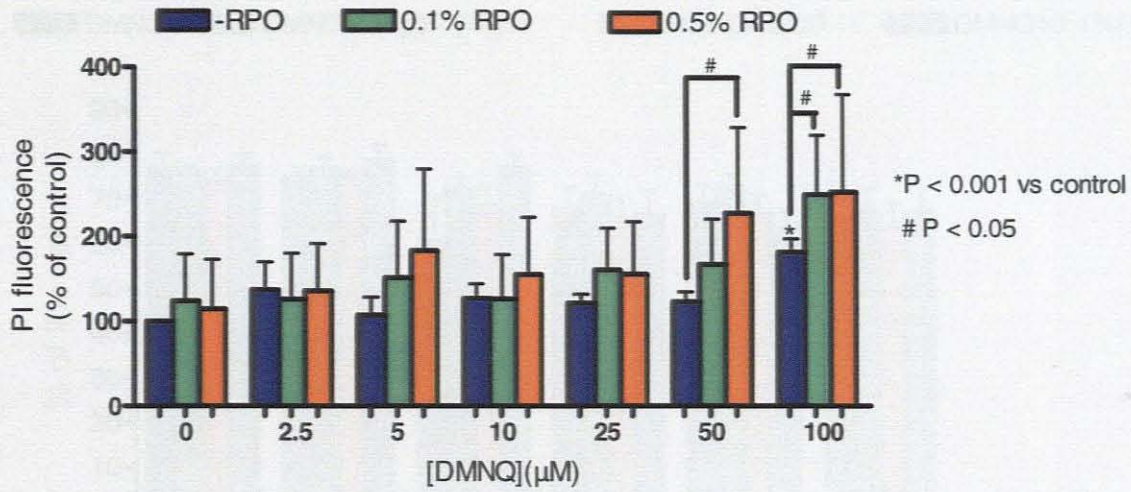

Figure 3.10: The effect of superoxide on PI fluorescence in absence or presence of RPO (n=6)

3.4 The effects of superoxide on sperm apoptosis parameters in the absence or presence of Mn(III)TMPyP, SOD and RPO

For the apoptosis studies, the concentrations of DMNQ were limited to 50μM and 0.1% RPO was used because it was less detrimental to sperm (Refer to 3.1.2. and Table 3.3).

3.4.1 Annexin –V Assay

Figures 3.11, 3.12, .13 and Table 3.4 show three parameters (% of live cells, % of apoptotic cells, and % of necrotic cells) as determined by the Annexin-V assay. No statistically significant differences ($P > 0.05$) were observed on addition of increasing concentrations of DMNQ (up to 50μM) in the absence or presence of Mn(III)TMPyP, or SOD or RPO for any of the three cell populations.

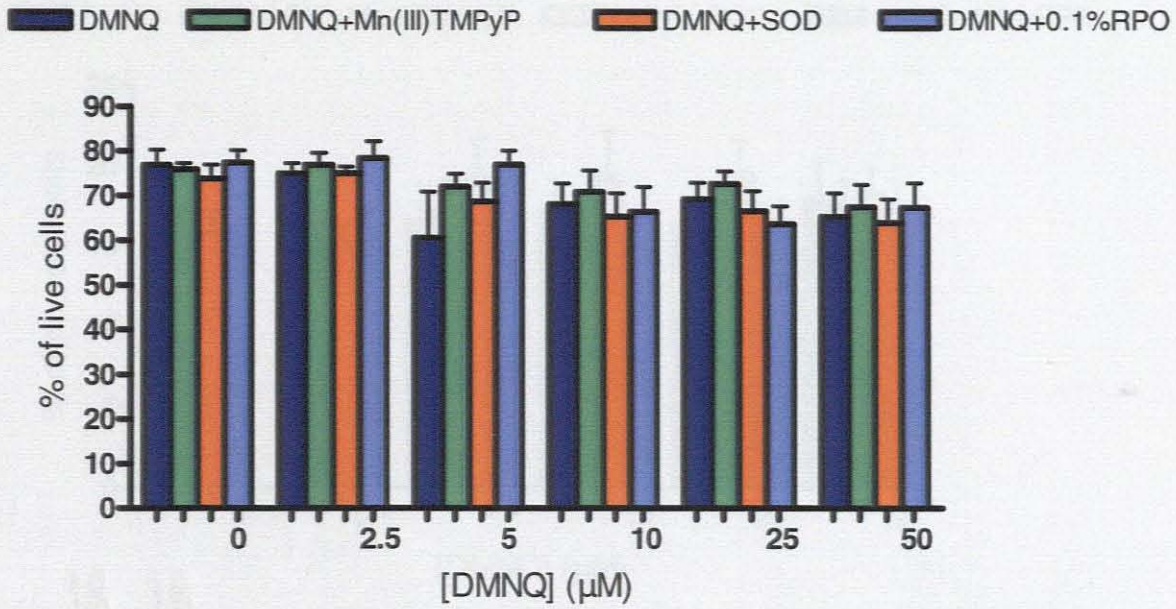

Figure 3.11: Annexin-V Assay: Percentage of live cells (n=6)

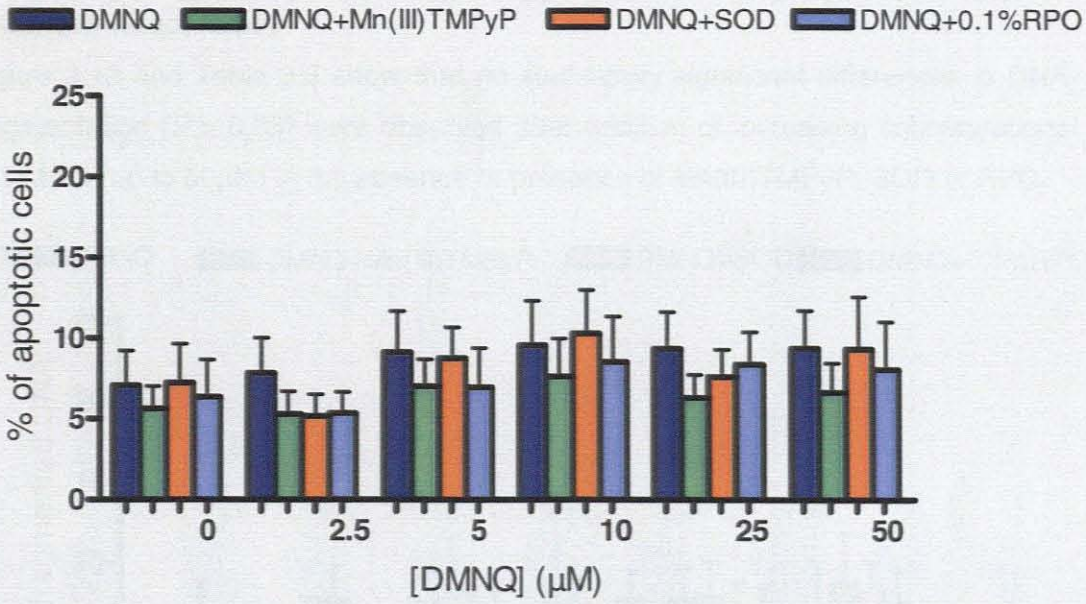

Figure 3.12: Annexin-V Assay percentage of apoptotic cells (n=6)

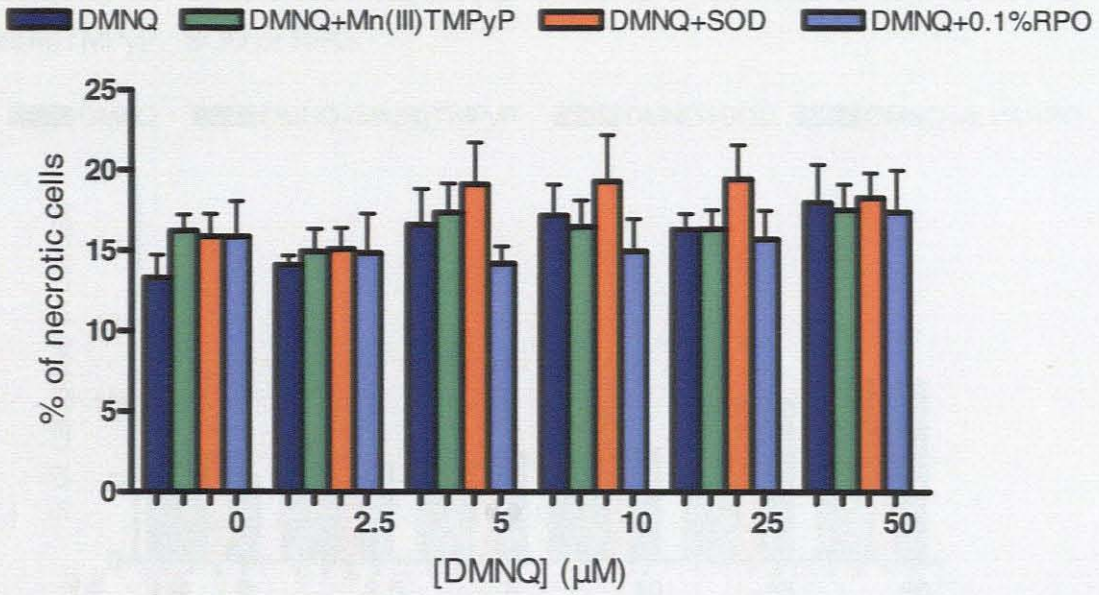

Figure 3.13: Annexin –V Assay percentage of necrotic cells (n=6)

3.4.2 Apo-direct Assay

Figure 3.13 and Table 3.5 show that no statistically significant differences in DNA fragmentation ($P > 0.05$) were observed after addition of increasing concentrations of DMNQ (up to 50μM) in the absence or presence of Mn(III)TMPyP, SOD or RPO.

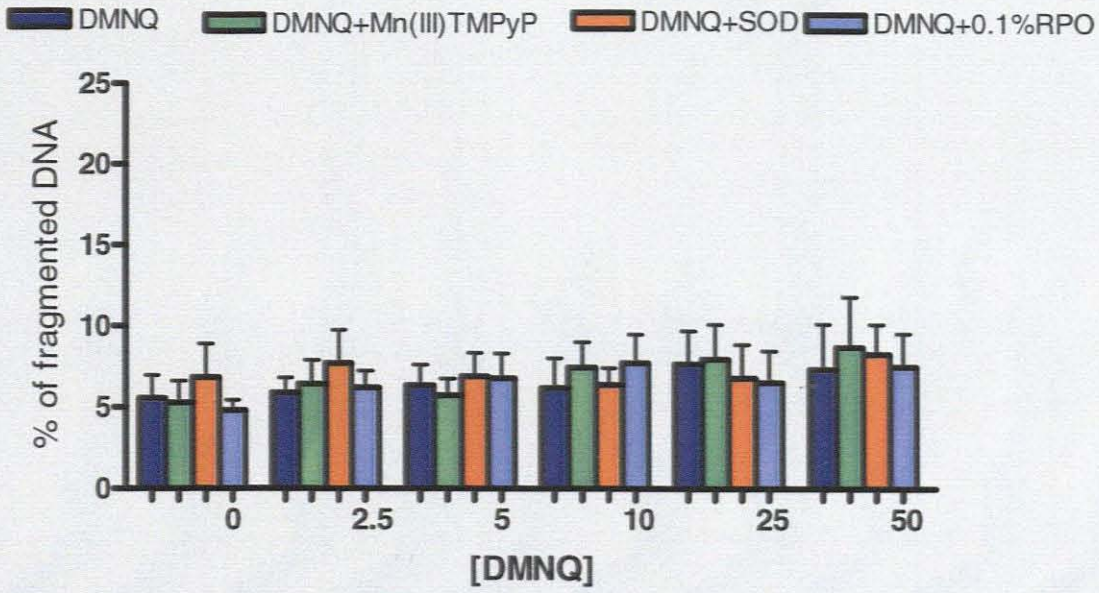

Figure 3.14: Apo-direct Assay illustrating the percentage of fragmented DNA (n=6)

3.4.3 Caspase-3 Assay

Figure 3.15 and Table 3.6 shows that no statistically significant differences in percentage of caspase protease ($P > 0.05$) were observed after addition of

increasing concentrations of DMNQ (up to 50μM) in the absence or presence of Mn(III)TMPyP, SOD or RPO.

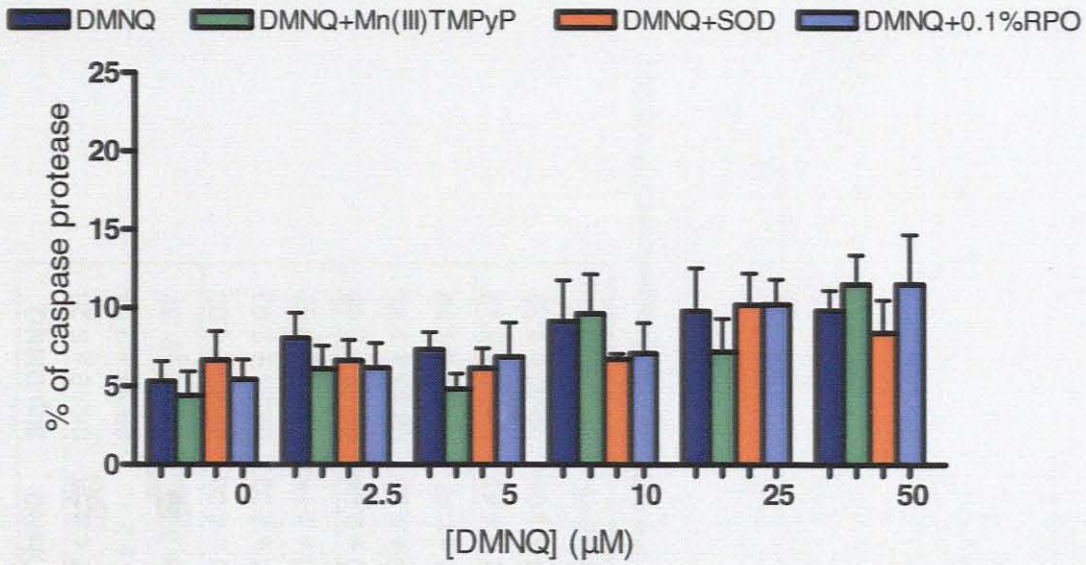

Figure 3.15: Caspase-3 Assay illustrating the percentage of caspase protease cells (n=6)

Table 3.4: Effects of superoxide, Mn(III)TMPyP, SOD and RPO on sperm apoptosis parameters using the Annexin-V Assay

	parameters	0μ DMNQ	2.5 μ DMNQ	5μ DMNQ	10μ DMNQ	25μ DMNQ	50μ DMNQ
DMNQ	% LIVE	76.85 ± 3.37	74.90 ± 2.30	60.51 ±10.21	68.1 ± 4.575	69.12 ± 3.64	65.19 ± 5.28
	% APOPTOTIC	07.07± 2.15	07.81 ± 2.19	11.21 ± 2.10	10.68 ± 2.50	08.31± 2.10	09.33 ± 2.34
	% NECROTIC	13.3 ± 1.40	14.10 ± 0.56	16.59 ± 2.24	17.17 ± 1.91	16.3 ± 0.98	17.98 ±2 .37
RPO	% LIVE	77.33 ± 2.71	78.33 ± 3.76	76.93 ± 3.04	66.33 ± 5.53	63.53 ± 4.00	67.27 ± 4.00
(0.1%)	% APOPTOTIC	06.34 ± 2.31	04.66 ± 1.22	06.94 ± 2.42	08.51 ± 2.78	08.27 ± 2.02	08.05 ± 2.93
	% NECROTIC	15.86 ± 2.18	14.84 ± 2.43	14.19 ± 1.06	14.95 ± 1.98	15.70 ± 1.78	17.41 ± 2.57
Mn	% LIVE	75.81 ± 1.36	76.83 ± 2.65	71.98 ± 2.80	70.74 ± 4.79	72.50 ± 2.84	67.38 ± 1.16
(50μM)	% APOPTOTIC	05.62 ± 1.38	05.28 ± 1.40	07.00 ± 1.66	07.61 ± 2.32	05.53 ± 1.33	06.63 ± 1.79
	% NECROTIC	16.21 ± 1.00	14.92 ± 1.42	17.37 ± 1.77	16.47 ± 1.65	16.34 ±1.16	17.51 ± 1.59
SOD	% LIVE	73.78 ± 3.06	74.94± 11.44	68.58 ± 4.21	65.25 ± 5.13	66.52 ± 4.41	63.89 ± 5.23
(50IU)	% APOPTOTIC	07.21 ± 2.42	05.17 ± 1.33	08.74 ± 1.90	10.27± 2.68	07.59 ± 1.65	09.30 ± 3.22
	% NECROTIC	15.87 ± 1.39	15.09 ± 1.31	19.11± 2.60	19.31 ± 2.86	19.45± 2.10	18.27±1.55

Note: Values are represented as means ± S.E.M. Differences were regarded statistically significant if $P < 0.05$ and highly significant if $P < 0.001$

Table 3.5: Effects of superoxide, Mn(III)TMPyP, SOD and RPO on sperm apoptosis using the Apo-direct assay

Apo-direct	0μDMNQ	2.5μ DMNQ	5μ DMNQ	10μ DMNQ	25μ DMNQ	50μ DMNQ
DMNQ	05.55 ± 1.38	05.87 ± 0.92	06.34 ± 1.26	06.16 ± 1.83	07.65 ± 2.03	07.35 ± 2.78
RPO	04.81±0.62	06.20 ± 1.01	06.79 ±1.50	07.71 ± 1.77	06.53 ±1.93	07.50 ± 2.05
Mn	05.25 ±1.35	06.43 ± 1.45	05.71 ±1.03	07.45 ± 1.55	07.96 ± 2.14	09.5 0± 3.21
SOD	06.82 ±2.06	07.69 ±2.04	06.91 ± 1.43	06.37 ± 1.03	06.80 ± 2.06	08.28 ± 1.82

Note: Values are represented as means ± S.E.M. Differences were regarded statistically significant if $P < 0.05$ and highly significant if $P < 0.001$

Table 3.6: Effects of superoxide Mn(III)TMPyP, SOD and RPO on sperm apoptosis using the Caspase-3 assay

Caspase-3	0μ DMNQ	2.5μ DMNQ	5μ DMNQ	10μ DMNQ	25μ DMNQ	50μ DMNQ
DMNQ	05.30 ±1.29	08.06 ±1.60	07.34 ± 1.10	09.11 ± 2.62	09.78 ± 2.73	09.82 ±1.28
RPO	05.41±1.28	06.14 ±1.59	06.86 ± 2.18	07.08 ± 1.95	10.22 ± 1.59	11.51± 3.14
Mn	04.40 ±1.55	06.09 ±1.49	09.15 ± 4.43	09.61 ± 2.51	07.19 ± 2.10	11.51±1.85
SOD	06.65 ±1.85	06.63 ±1.31	06.13 ± 1.28	06.71 ± 0.351	10.21± 1.98	08.40 ± 2.09

Note: Values are represented as means ± S.E.M. Differences were regarded statistically significant if $P < 0.05$ and highly significant if $P < 0.001$

CHAPTER 4

DISCUSSION

4.1 Preliminary studies

4.1.1 Solubility and toxicity of dimethylsulphoxide (DMSO), Ethanol, and Propanol Glycol (PGly)

This study is the first to our knowledge to investigate the use of RPO as a possible scavenger of ROS in human spermatozoa. Due to the hydrophobic nature of RPO it was imperative to find a suitable solvent in order to act as a vehicle for RPO administration to sperm solutions. From the results, it is evident that PGly was able to dissolve RPO at much lower concentrations than both ethanol and DMSO. Furthermore, when the effects of these three solvents were tested on sperm motility, it was observed that PGly had the least detrimental effects. It was thus decided to use PGly as the solvent for RPO at concentrations of 0.1%, 0.5% and 2.5%.

4.1.2 Determining the appropriate administration concentrations of RPO for sperm motility

From our results, it was evident that concentrations of PGly and PGly in combination with RPO higher than 0.5% significantly reduced sperm motility. It was therefore decided to use concentrations of 0.1% and 0.5% RPO in this study. Furthermore, it was observed that RPO had the ability to attenuate the deleterious effect of PGly on sperm motility. It can be postulated that this might be due to the hydrophobic nature of RPO and to its chemical composition.

4.1.3. Dihydroethidium (DHE)-specificity for superoxide studies in the absence or presence of Mn(III)TMPyP, SOD and RPO

In this part of the study used DHE as a probe for detecting the generation of the superoxide anion by DMNQ and to examine the scavenging effects of Mn(III)TMPyP, SOD and RPO on superoxide and thus DHE fluorescence.

4.1.3.1 The effects of superoxide on dihydroethidium (DHE) fluorescence in sperm

From our results, it can be seen that the increase in DMNQ did not affect the intensity in DHE fluorescence produced. It can therefore be speculated that the amount of DHE used might be too minimal and might have been depleted, thereby not leading to an increase in DHE fluorescence.

4.1.3.2 The effects of superoxide on dihydroethidium (DHE) fluorescence in the absence or presence Mn(III)TMPyP in sperm

Mn has some undefined antioxidant properties, in contrast with iron, which is clearly a pro-oxidant *in vivo*. Lin and co workers (2005) proposed that manganese might directly scavenge radicals and/or up-regulate several key antioxidant enzyme defences including manganese containing SODs to exert its action. Studies have shown that manganese homeostasis results in elevated Mn levels that suppress superoxide generator (Antebi and Fink, 1992, Lapinskas *et al.*, 1995). Our data have shown that Mn(III)TMPyP could not only scavenge endogenously produced superoxide, but also exogenously added superoxide with concentrations up to 50μM DMNQ. It can be argued that no scavenging effects were observed at 100μM DMNQ due to the Mn(III)TMPyP concentration being too low, had been depleted and could therefore not perform significant scavenging shown by a reduction in DHE fluorescence at all concentrations of DMNQ except 100μM.

4.1.3.3 The effects of superoxide on dihydroethidium (DHE) fluorescence in the absence or presence of SOD in sperm

Our results showed that when SOD was added the samples stimulated with 25μM to100μM DMNQ, DHE fluorescence was reduced. This shows that SOD was able to act as scavenger and it could significantly reduce superoxide concentrations in those samples. It likely that those concentrations (25μM to 100μM DMNQ) up-regulated the SOD scavenging pathway. Our results confirmed the findings of Dimmeler *et al.* (1999) who showed that Cu/ZnSOD up-regulation inhibited the activation of the caspase cascade by shear stress in an increased superoxide medium.

4.1.3.4 The effects of superoxide on DHE fluorescence in the absence or presence of RPO in sperm

Crude red palm oil is a rich source of antioxidant vitamin A precursors (α- and β-carotene) and vitamin E [tocopherols, and tocotrienols] (Sundram *et al.*, 2003). Supplementation of culture media with antioxidants such as vitamin E neutralized loss of motility caused by ROS generation. The addition of vitamin E is known to remove ROS-producing cells by density gradient sperm preparation methods or to remove leukocytes by antibodies against the common leukocyte antigen; (Alvarez and Storey, 1989; de Lamirande and Gagnon, 1992; Aitken *et al.*, 1996). However, our data showed that RPO had no superoxide scavenging effects as it did not decrease DHE fluorescence.

4.2 The effects of superoxide on sperm motility parameters in the absence or presence of Mn(III)TMPyP, SOD and RPO

Because of the damaging potential of ROS, cells depend on defence mechanisms to effectively neutralise or metabolise these toxic effects to prevent significant free radical injury. In this study, two commercially available superoxide scavengers (Mn(III)TMPyP and SOD) as well as RPO had been used to investigate their possible protective effect against superoxide toxic effects in spermatozoa. In this study, a decrease in percentage of motile cells, progressive motility and rapid cells, as well as an increase in the percentage of static cells were regarded as negative or a decrease in sperm function as these parameters are indicative of the ability of spermatozoa to progress towards the oocyte *in vitro*.

4.2.1 The effects of superoxide on sperm motility parameters

In this study it was found that the addition of exogenous superoxide in the form of DMNQ at 100μM significantly reduced the percentage of motile cells, progressive motility, and rapid cells. It also decreased the values of the following motility parameters: VAP, VSL, STR and LIN. Similarly, exogenous superoxide significantly increased static cells from concentrations as little as 5μM DMNQ. It is therefore clear that superoxide is deleterious to sperm motility and sperm function at higher concentrations. These results are in agreement with de Iuliis and co-workers (2006) who demonstrated that human spermatozoa are capable of generating $O_2^{-}\bullet$ and that the production of this oxygen radical is inversely correlated with defective sperm function. Gil-Guzman and co-workers (2001) showed that levels of ROS production were negatively correlated with teratozoospermia and spermatozoa developmental stages. ROS production was found to be highest in the immature fraction of

ejaculated sperm, which also contained sperm with abnormal head morphology and cytoplasmic retention. It has been suggested that ROS induces membrane lipid peroxidation in sperm and that the toxicity of generated fatty acid peroxides are important causes of decreased sperm function (Armstrong *et al.*, 1999; Agarwal *et al.*, 2006a). Baker and co-workers (2003) argued that spermatozoa were the first cell type suggested to generate highly reactive oxygen derived free radicals and ROS. In their investigation they found that elevated ROS levels indicated a combination of either semen contamination by leukocytes (especially granulocytes) or the occurrence of defective and/or immature spermatozoa. It has been shown that spermatozoa are only a minor source of ROS production with leukocytes contributing to the major ROS production (Aitken *et al.*, 1994; Henkel *et al.*, 2005b).

4.2.2 The effects of superoxide on sperm motility parameters in the absence or presence of Mn(III)TMPyP

Manganese (Mn) is an essential ultra trace element similar to chromium, molybdenum, and cobalt. It is needed for a wide variety of physiological processes ranging from the regulation of reproduction to normal brain function (Lin *et al.*, 2005). Mn can exist in various oxidation states ranging from −3 to +7, with +2 oxidation state being the most predominant in biological systems (Lin *et al.*, 2005). While redox active metals such as Fe(II) can accelerate lipid peroxidation, ionic manganese (10–100 μM) has been shown to inhibit lipid peroxidation in rat liver microsomes (Coassin *et al.*, 1992). Also, several known manganese complexes including the manganese salen and manganese bis(cyclohexylpyridine)-substituted macro cyclic ligand have shown promise as a possible SOD mimic (Beyer, and Fridovich 1990; Doctrow *et al.*, 1997; Salvemini *et al.*, 1999). Studies have shown that these complexes are as effective as SOD enzymes in detoxifying superoxide under some experimental conditions (Melov *et al.*, 2001).

The addition of Mn(III)TMPyP (50μM) attenuated the effects of superoxide on the number of static cells (5μM and 10μM DMNQ). Therefore, it offered some form of protection to spermatozoa against the harmful effects of superoxide. However, Mn(III)TMPyP decreased the percentages of motile and static cells. This finding is interesting or rather contradictive to the protective properties ascribed to Mn(III)TMPyP. However, this finding is in agreement with Lin *et al.*, 2005 who found that manganese exhibits pro- and anti –oxidant characteristics in their study done on worms. Despite several reports suggesting the beneficial effects of manganese in

unicellular organisms, it is well known that chronic exposure to high atmospheric levels of manganese is toxic (Lin *et al.*, 2005). Studies have shown that an overload of manganese causes the disease "manganism," which has Parkinson's-like symptoms (Roth and Garrick, 2003; Wang and Zhu, 2003b).

4.2.3 The effects of superoxide on sperm motility parameters in the absence or presence of SOD

The addition of SOD (50IU) exacerbated the harmful effect of superoxide by significantly reducing motility, progressive motility, rapid cells and LIN at concentrations equal or higher to 25μM DMNQ. Similarly, SOD amplified the reduction of VAP, VSL and VCL at higher DMNQ concentrations (≥50μM). Moreover samples treated with SOD in combination with superoxide had shown significant differences in rapid cells (≥ 2.5μM DMNQ), progressive motility (≥ 10μM DMNQ) and static cells (at 100μM DMNQ). Nevertheless, the addition of SOD attenuated the harmful effects of superoxide by reducing the percentages of STR (50μM DMNQ) and static cells (10μM DMNQ). Therefore, it offered some form of protection. Contradictory reports exists regarding the effect of antioxidant supplementation on sperm motility in both fresh liquefied and frozen-thawed semen from various species, including human (Askari *et al.*, 1994; Maxwell and Stojanov, 1996; Sinha *et al.*, 1996; Aurich *et al.*, 1997; Upreti *et al.*, 1997, Donnelly *et al.*, 1999), the effect being dependent of the antioxidant employed and the dose used (Peña *et al*, 2003). Johnson and Giulivi (2005) argued that the conversion of superoxide anion to hydrogen peroxide by SOD may have anti-oxidant and pro-oxidant consequences. On one hand, the dismutation of superoxide anion to H_2O_2 and oxygen facilitates both the distribution of ROS, i.e. diluting their effects via diffusion between cellular compartments, and the removal of H_2O_2 by H_2O_2-consuming enzymes (anti-oxidant). On the other hand, if the actions of SOD and H_2O_2-consuming enzymes are not in concert, an increased production of H_2O_2 is expected from SOD activity. Sikka (1996) has shown that a relationship exists between an increase in ROS induced oxidative stress, lipid peroxidation, decreased level of SOD and motility in spermatozoa. De Lamirande and Gagnon (1992) argued that motility is impaired because of adenosine triphosphate depletion during lipid peroxidation of the sperm plasma membrane. Peroxidation increased in proportion to a decrease in SOD effects (Alvarez *et al*, 1987). Therefore, the time of complete motility loss is determined by the rate of peroxidation (Alvarez *et al.*, 1987) and so would correlate with SOD activity (Alvarez and Storey, 1992). On the other hand the dismutation of

$O_2^{-}\bullet$ by SOD converts it into a powerful oxidant, H_2O_2, that can readily penetrate sperm cells, lower their motility and cause irreparable damage to both DNA and membranes (Alvarez *et al.*, 1987). All these arguments might explain our findings.

4.2.4 The effects of superoxide on sperm motility parameters in the absence or presence of RPO

Crude palm oil 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; Yoshida *et al.*, 2003). The dietary intake of foods rich in carotenoids was associated with a reduced risk of some types of cancer (Portakel *et al.*, 2000; Cameron *et al.*, 2003) and cardiovascular diseases (Ferdinandy and Schulz, 2003), presumably affording antioxidant properties to reduce oxidative stress when tested in both endothelial and non-endothelial cells. Palm oil containing these antioxidants was shown to be effective against oxidative stress *in vitro* and *in vivo* (Serbinova *et al.*, 1992). Vitamin E and related compounds are also abundant in red palm oil. Chow and Hong (2002) reported that dietary vitamin E is capable of reducing the production and / or availability of not only $O_2^{-}\bullet$, but also NO and $ONOO^{-}$. However, it is not clear if the action of vitamin E to reduce the generation of $O_2^{-}\bullet$ and other ROS is independent of its antioxidant function. Our results showed that cells treated with RPO in the absence of DMNQ had beneficial effects, as the number of motile cells as well as the percentage of motile cells increased. When RPO was administered in combination with superoxide it exacerbated the negative effects of superoxide on motility, progressive motility, rapid cells, VAP, VSL and VCL. However, RPO was found to effectively reverse the effects of superoxide on STR and LIN (50µM DMNQ) and attenuate the percentage of static cells (5µM to 25µM DMNQ). The effect of RPO on sperm motility and possible protection against superoxide is therefore rather ambiguous as under certain conditions it contributes to serve negative outcomes. It was therefore speculated that this might be due to its chemical composition.

4.3 The effects of superoxide on PI fluorescence in the absence or presence of Mn(III)TMPyP, SOD and RPO

PI is a fluorescent probe that binds to DNA. Cells having an intact plasma membrane will prevent PI from entering into the cell and staining the nucleus (Gillan *et al.*, 2005). However, cells possessing a damaged plasma membrane will permit PI to enter into the cell and bind to the DNA causing the cells to fluoresce red. PI

can therefore be used as a viability marker and an increase in PI fluorescence can be interpreted as an increase in non- viable cells.

4.3.1 The effects of superoxide on PI fluorescence

Viability data showed a similar trend when compared to that observed during motility experiments. The addition of 100μM DMNQ led to an increase in non- viable cells. At 100μM DMNQ, a more likely metabolic fate would be to undergo dismutation in order to form H_2O_2. The H_2O_2 generated as a consequence of this reaction appears to be the cause of the susceptible increase in PI fluorescence. It has been speculate that high concentrations of superoxide might be responsible for the decrease in cell viability. Our data confirmed the relationship between increased ROS, oxidative stress, LPO and decreased motility and viability of sperm cells as previously reported by Sikka (1996).

4.3.2 The effects of superoxide on PI fluorescence in the absence or presence of Mn(III)TMPyP

When Mn(III)TMPyP was added to cells exposed to low concentrations of superoxide (10μM DMNQ) it led to a decrease in PI fluorescence. This can be interpreted that Mn(III)TMPyP was able to scavenge some of the endogenously produced as well as the exogenously added superoxide and therefore, could protect these cells against the harmful effects of superoxide and cell death caused thereby. However, when Mn(III)TMPyP was added to cells exposed to higher concentration of superoxide (≥ 50μM DMNQ), it led to a significant increase in non- viable cells. Therefore, it seems that Mn(III)TMPyP exacerbated the deleterious effects of superoxide in instances where superoxide concentrations are too high to be scavenged by the available amount of Mn(III)TMPyP. Our data confirmed the finding of previous studies (Lin *et al.*, 2005) who found that, depending on the species of worms and the dosages of manganese used, it can be beneficial or toxic for their development and reproduction. Moreover, Gardner and co-workers (1996) had shown that Mn(III)TMPyP appears to be an effective $O_2^{-\bullet}$ scavenger in the cytoplasm of mammalian cells. However, Mn(III)TMPyP or other metalloporphyrins may be useful as therapeutic agents for catalytically increasing $O_2^{-\bullet}$ /H_2O_2 production of natural or synthetic redox-active compounds like menadione. It has been therefore speculate that Mn(III)TMPyP might have pro- or anti-oxidant properties depending on the concentration and the availability of superoxide in the medium.

4.3.3 The effects of superoxide on PI fluorescence in the absence or presence of SOD

The SOD content (50 IU/ml) in this study was very minimal compared to the mean values of 42-46 IU/10^9 spermatozoa reported by Zini *et al.*, (1993) and 65 IU/10^9 spermatozoa as reported by Griveau *et al.*, (1995a). The administration of SOD did not statistically alter the effects of superoxide. Our data confirmed previous findings (Symonyan and Nalbandyan 1972; Nordberg and Arn'er 2001; Johnson and Giulivi, 2005) where SOD lost its protective character due to its low concentration. It has been therefore speculate that all the SOD administered had performed its scavenging activities and been depleted while the remaining superoxide that had not been scavenged had been converted to H_2O_2 possibly leading to the decrease in viability.

4.3.4 The effects of superoxide on PI fluorescence in the absence or presence of RPO

Isong and co-workers (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. 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 and Linden 1991), embryogenesis and spermatogenesis (McArdle and Ashworth, 1999). Our *in vitro* investigation contrasts their finding and shows that both 0.1% and 0.5% RPO when combined with 100μM DMNQ increased the number of non-viable cells thereby showing that *in vitro* administration of RPO might be harmful to spermatozoa.

4.4 The effects of superoxide on sperm apoptosis in the absence or presence of Mn(III)TMPyP, SOD and RPO

Apoptosis is a mechanism that enables metazoans to control cell number in tissues and to eliminate individual cells that threaten the animal's survival. Apoptosis also plays an essential role in the processes of gamete maturation and embryogenesis, contributing to the appropriate formation of various organs and structures (Weng *et al.*, 2002). Spermatogenesis is a dynamic process of germ cell proliferation and differentiation from stem spermatogonia to mature spermatozoa. In the mammalian testes, germ cells expand clonally through many rounds of mitosis before undergoing the differentiation steps that result in mature spermatozoa (Sakkas *et al.*, 1999a). Mature sperm cells have been reported to present distinct signs of apoptosis-related cell damage (Sun *et al.*, 1997; Sakkas *et al.*, 1999b; Barroso *et al.*,

2000; Gandini *et al.*, 2000; Irvine *et al.*, 2000; Muratori *et al.*, 2000; Oosterhuis *et al.*, 2000; Shen *et al.*, 2002), although they lack transcriptional activity and have a very small amount of cytoplasm (Weil *et al.*, 1998). It is not clear whether the apoptotic markers detected in spermatozoa are residues of an abortive apoptotic process started before ejaculation (Sakkas *et al.*, 1999a & b; Tesarik *et al.*, 2002) or whether they result from apoptosis initiated in the post-ejaculation period. In this study, three apoptotic marker assays were conducted to investigate the presence of these specific markers on sperm in presence of selected scavengers and RPO.

4.4.1 Annexin–V Assay

The higher percentage of live cells in our results is due to the swim-up procedure that has been employed. This is in agreement with previous studies demonstrating a high efficiency of this technique in terms of elimination of non-viable and apoptotic spermatozoa (Sakkas *et al.*, 2000; Zini *et al.*, 2000; Younglai *et al.*, 2001). Several authors have described the presence of apoptotic markers (phosphatidylserine externalization) in spermatozoa from men with normal and abnormal spermatogenesis (Sakkas *et al.*, 1999a; Gandini *et al.*, 2000; Oosterhuis *et al.*, 2000; Tesarik *et al.*, 2002; Ricci *et al.*, 2002). However, it is not clear whether ejaculated spermatozoa still retain the ability to activate the apoptotic signalling cascade or whether the apoptotic markers detected in ejaculated spermatozoa are the reflection of an abortive apoptotic process started before ejaculation (Sakkas *et al.*, 1999a). The outcome of the apoptotic process may be related to a premature release of spermatids at early stages of apoptosis from Sertoli cells which normally should actively participate in the elimination of such cells (Lachaud *et al.*, 2004). From our results, there were no significant differences for the Annexin-V parameters and across all of the different DMNQ, M(III)TMPyP, SOD and RPO concentrations. The percentage of necrotic spermatozoa in any same concentration of DMNQ, is higher than that of apoptotic in all groups (DMNQ, DMNQ+RPO, DMNQ+M(III)TMPyP and DMNQ+SOD). Thus, it is likely that the death of sperm cells during *in vitro* incubation (at 37°C) occurs by necrosis rather than by apoptosis. Since our investigation has been limited to 50μM DMNQ, it might be possible that the amount of superoxide produced was not enough to trigger significant apoptosis. However, it has been shown that Hams-F10 culture medium possesses antioxidant properties which may act by metal chelation and/or free radical scavenging (Faure *et al.*, 2004). Other attributing factors could be the small sample size (n=6) as well as the short incubation duration and therefore no significant differences were detected.

4.4.2 Apo-direct Assay

DNA fragmentation is one of the later steps of apoptosis. The detection of fragmented DNA has been quantified by using the Apo-direct assay which is a single step method for labelling DNA breaks with FITC- dUTP, followed by flow cytometric analysis. Although, no significant differences were found, (regarding the different assays used) a trend of increased DNA fragmentation was observed when DMNQ concentrations were increased. Henkel and co-workers (2005a) reported that an increase in DNA damage in sperm is possibly due to an increase of ROS production, produced by the sperm cells themselves or by the leukocytes. Henkel *et al.* (2003) found that spermatozoa are only a minor source of ROS production in the ejaculate. However, the impact of these cells and the production of ROS on male fertility potential are still poorly understood. Lopes and co-workers (1998) have established that poor-quality semen samples have a greater percentage of spermatozoa with DNA fragmentation than normal fertile samples (Sun *et al.*, 1997; Lopes *et al.*, 1998). In addition, these authors have demonstrated a negative correlation between the percentage of spermatozoa with fragmented DNA and fertilisation rates in *in vitro* fertilisation (IVF) (Sun *et al.*, 1997) and in intracytoplasmic sperm injection (ICSI) (Lopes *et al.*, 1998). They had shown that free radicals, such as $O_2^{-\bullet}$, H_2O_2 and OH^- can cause DNA damage in human spermatozoa when exposed for time periods consistent with clinical sperm preparation techniques for ICSI or IVF.

4.4.3 Caspase-3 Assay

Caspases are expressed as inactive proenzymes that participate in a cascade triggered in response to pro-apoptotic signals resulting in death of the cell. Caspase-3 is the main executor within this apoptotic cascade. Irrespective of the assays used, the percentage of apoptotic cells did not show significant differences. However, there was a trend of an increase in the caspase-3 marker, with increasing DMNQ concentrations in the absence of presence of M(III)TMPyP, SOD and RPO. Since the swim-up did eliminate the non-viable cells, this finding is in accordance with the association of apoptotic markers and the death of human spermatozoa, observed by different authors (Sakkas *et al.*, 1999a & b; Blanc-Layrac *et al.*, 2000; Ramos and Wetzels, 2001).

Summary

In this study, RPO has been used due to its biochemical composition as a possible scavenger of superoxide expecting that it will reverse the damage caused by superoxide on spermatozoa. Because of the hydrophobic nature of RPO it was imperative to find a suitable solvent to act as vehicle for RPO administration to sperm solutions. PGly was found to be most appropriate solvent, but with some limitations regarding its detrimental effects at concentration higher than 0.5% on sperm motility. However, it was observed that RPO had the ability to attenuate the deleterious effect of PGly on sperm motility.

This study showed that high concentrations of superoxide are deleterious to sperm motility, viability and sperm function. The addition of Mn(III)TMPyP (50μM) offered protection to some of sperm motility parameters. However, Mn(III)TMPyP had shown contradicting characteristics on sperm cell viability. The addition of SOD also showed contradictory effects as it exhibited both pro-oxidant and anti-oxidant characteristics on sperm motility and viability depending on superoxide concentrations. The effect of RPO on sperm motility, viability and possible protection against superoxide is ambiguous as under certain conditions it contributes to serve negative outcomes.

ROS causes lipid peroxidation of sperm plasma membranes, resulting in alteration of sperm function and DNA fragmentation in ejaculated spermatozoa. In this study three apoptotic markers (Annexin-V, Caspase-3 and DNA fragmentation) were used to investigate the presence of apoptotic markers on sperm. The higher percentage of live cells found was due to the swim-up procedure, a technique that eliminates non-viable and apoptotic spermatozoa. There were higher percentages of necrotic spermatozoa at any concentration of DMNQ compared to those of apoptotic spermatozoa in all groups (DMNQ, DMNQ+RPO, DMNQ+Mn(III)TMPyP and DMNQ+SOD). This implies that the death of sperm cells during *in vitro* incubation (at 37°C) occurred by necrosis rather than by apoptosis. Because of certain limitations (amount of DMNQ, small sample size and short incubation duration) in this study, it might be possible that the amount of superoxide administered was not enough to trigger significant apoptosis.

Dihydroethidium (DHE) was found not to be a highly specific probe for superoxide as increased levels of DMNQ did not give rise to any increase in DHE fluorescence. Finally, Mn(III)TMPyP, SOD and RPO respectively showed good, partial and no superoxide scavenging effects.

CONCLUSION

Reactive oxygen species (ROS) are free radicals implicated in many human diseases. Superoxide ($O_2^{-}\bullet$) anion, hydrogen peroxide (H_2O_2), and hydroxyl (OH^-) radicals are the most common types of ROS. Even though ROS are toxic to human cells, they are normally produced in the body as by-products during oxygen metabolism. In addition, some cells may have inherent mechanisms that produce ROS for physiological purposes such as leukocytes and spermatozoa. Several systemic diseases, such as cancer, cardiovascular problems, infections and diabetes mellitus are known to increase the production of ROS. Environmental stressors such as radiation and pollution, as well as cigarette smoking also increase the production of ROS. When the balance between ROS and antioxidants is tipped towards an overabundance of ROS, oxidative stress (OS) occurs. Therefore, there is increased interest to examine possible therapeutic treatments to combat the deleterious role of ROS in male reproduction.

Antioxidants are the main defence against OS induced by free radicals. These can be preventive antioxidants, such as metal chelators and metal binding proteins that block the formation of new ROS, whereas scavenger antioxidants, such as vitamin E and C, β-carotene and other dietary supplements, glutathione and enzymes, remove ROS already generated by cellular oxidation.

Once oxidative stress is diagnosed and implicated as the cause of male infertility, treatment plans must focus on identifying and eliminating the source of ROS. This could be done in one of two ways (a) avoiding factors promoting *in vivo* ROS generation (b) *in vitro* scavenging of ROS during artificial reproduction technologies (ART) from the male gametes.

In this study, it has been shown that high concentrations of superoxide on its own (one of the family members of ROS) proved to be harmful to spermatozoa *in vitro*. The study further focussed on possible *in vitro* treatment regimes to decrease these deleterious effects of superoxide on spermatozoa.

Our results showed that Mn(III)TMPyP and SOD have superoxide scavenging effects *in vitro*. However, more studies determining the correct dosages of these two

scavengers in combination with metal chelators need to be done to ensure greater success during ART. With regards to RPO, our *in vitro* study did not show any positive results. It can be suggested that, due to its hydrophobic nature, RPO cannot be effectively introduced to ART media containing spermatozoa and should rather be supplemented daily to the diet of those patients in order to perform its preventive anti-oxidant properties. Animal studies are planned, in which these animals receive RPO in their diet in order to shed light on RPO's effects on spermatogenesis and its capacity to protect the male reproductive system from ROS damage.

It was thus shown that DHE is not a superoxide probe per se. However, it can be used to monitor the level of ROS during sperm preparation techniques necessary to enhance and maintain sperm quality and function after ejaculation and before the semen specimen is used for ART. Monitoring superoxide levels can also help to select a suitable sperm preparation technique that separate mature motile spermatozoa and thus minimise the interaction between ROS producing cells in semen (such leukocytes and immature abnormal spermatozoa) and normal spermatozoa. It would be advisable to obtain an appropriate and very specific superoxide probe.

As excessive levels of ROS can lead to apoptosis an attempt was made to determine if superoxide on its own could also evoke the same cellular response. With regards to our apoptotic studies, no significant results were obtained and it is recommend that concentrations of the superoxide donor, scavengers and exposure times should be varied more. It is also recommend that future studies should include the oral administration of different concentrations of RPO to animals for a period long enough to target complete spermatogenesis and epididymal maturation. This could give a much better assessment of the possible protective effects RPO might lend to ejaculated sperm regarding these apoptotic markers.

REFERENCES

Agarwal, A., Prabakaran, S. and Sikka, S.C. (2007) Clinical Relevance of Oxidative Stress in Patients with Male Factor Infertility: Evidence-Based Analysis. *American Urological Association, Education and Research,* 26, 2-11.

Agarwal, A., Prabakaran, S. and Allamanen, S. (2006a) What andrologist/urologist should know about free radicals and why. *Urology,* 67, 2–8.

Agarwal, A., Sharma, A., Nallella, R.K., Thomas, K.P., Alvarez, A. Jr. and Sikka, S.C. (2006b) Reactive oxygen species as an independent marker of male factor infertility. *Fertility and Sterility,* 86, 878 – 885.

Agarwal, A., Saleh, R.A. and Bedaiwy, M.A. (2003) Role of reactive oxygen species in the pathophysiology of human reproduction. *Fertility and Sterility,* 79, 829–843.

Agarwal, A., Ikemoto, I. and Loughlin, K.R. (1994) Effect of sperm washing on levels of reactive oxygen species in semen. *Archives of Andrology,* 33, 157–62.

Aitken, R.J. and Krausz, C. (2001) Oxidative stress, DNA damage and the Y chromosome. *Reproduction,* 122, 497-506.

Aitken, R.J., West, K.M. and Buckingham, D.W. (1994) Leukocyte infiltration into the human ejaculate and its association with semen quality, oxidative stress, and sperm function. *Journal of Andrology,* 15, 343- 352.

Aitken, R.J. (1999) The Amoroso lecture. The human spermatozoa cell in crisis. *Journal of Reproduction and Fertility,* 115, 1–7.

Aitken, R.J., Buckingham, D. and Harkiss, D. (1993) Use of a xanthine oxidase free radical generating system to investigate the cytotoxic effects of reactive oxygen species on human spermatozoa. *Journal of Reproduction and Fertility,* 97, 441-450.

Aitken, R.J., Buckingham, D.W., West, K. and Brindle, J. (1996) The use of paramagnetic beads and Ferro fluids to assess and eliminate the leukocytic contribution to oxygen radical generation by human sperm suspensions. *American Journal of Reproduction and Immunology,* 35(6), 541–551.

Alais, C. and Linden, G. (1991) *Biochimie alimentaire*, 2nd edition. Paris: Mason, 110– 116.

Allamaneni, S.S., Naughton, C.K. and Sharma, R.K. (2004) Increased seminal reactive oxygen species levels in patients with varicoceles correlate with varicocele grade but not with testis size. *Fertility and Sterility,* 82, 1684–1686.

Allen, R.G. and Tresini, M. (2000) Oxidative stress and gene regulation. *Free Radical Biology and Medicine*, 28, 463-99.

Almid, T. and Johnson, L.A. (1988) Effects of glycerol concentration, equilibration time and temperature of glycerol addition on post-thaw viability of boar spermatozoa frozen in straws. *Journal of Animal Sciences*, 66,2899–2905.

Alvarez, J.G. and Storey, B.T. (1992) Evidence for increased lipid peroxidative damage and loss of superoxide dismutase as a mode of sublethal damage to human sperm during cryopreservation. *Journal of Andrology,* 13, 232-241.

Alvarez, J.G. and Storey, B.T. (1983) Taurine, hypotaurine, epinephrine and albumin inhibit lipid peroxidation in rabbit spermatozoa and protect against loss of motility. *Biology of Reproduction,* 29, 548–555.

Alvarez, J.G. and Storey, B.T. (1989) Role of glutathione peroxidase in protecting mammalian spermatozoa from loss of motility caused by spontaneous lipid peroxidation. *Gamete Research,* 23, 77–90.

Alvarez, J.G., Touchstone, J.C., Blasco, L. and Storey, B.T. (1987) Spontaneous lipid peroxidation and production of hydrogen peroxide and superoxide in human spermatozoa: superoxide dismutase as a major enzyme protectant against oxygen toxicity. *Journal of Andrology,* 8, 338-348.

Ames, B.N. (1989) Endogenous oxidative DNA damage, aging and cancer. *Free Radical Research Communication,* 7, 121-128.

Antebi, A. and Fink, G. R. (1992) The yeast Ca(2+)-ATPase homologue, PMR1, is required for normal Golgi function and localizes in a novel Golgi-like distribution. *Molecular Biology of Cellular,* 3, 633–654.

Anzar, M., He, L., Buhr, M.M., Kroetsh, T.G. and Pauls, K.P. (2002) Sperm apoptosis in fresh and cryopreserved bull semen detected by flow cytometry and its relationship with fertility. *Biology of Reproduction,* 66, 354-360.

Armstrong, J.S., Rajasekaran, M., Chamulitrat, W., Gatti, P., Hellstrom, W.J. and Sikka, S.C. (1999) Characterization of reaction of reaction species induced effects on human spermatozoa movement and energy metabolism. *Free Radical Biology and Medicine,* 26, 869-880.

Askari, H.A., Check, J.H., Peymer, N. and Bollendorf, A. (1994) Effect of natural antioxidants tocopherol and ascorbic acids in maintenance of sperm activity during freeze thaw process. *Archives of Andrology,* 33, 11–15.

Aurich, J.E., Schönherr, U., Hoppe, H. and Aurich, C. (1997) Effects of antioxidants on motility and membrane integrity of chilled stored stallion semen. *Theriogenology,* 48, 185–192.

Baldi, A., Savoini, G., Pinotti, L., Monfardini, E., Cheli, F. and Orto, V.D. (2000) Effects of Vitamin E and Different Energy Sources on Vitamin E Status, Milk Quality and Reproduction in Transition Cows. *Journal of Veterinary Medicine,* 47, 599.

Baker, M.A., Krutskikh, A. and Aitken, R.J. (2003) Biochemical entities involved in reactive oxygen species generation by human spermatozoa. *Journal of Reproduction and Fertility*, 81, 459–469.

Barroso, G., Morshedi, M. and Oehninger, S. (2000) Analysis of DNA fragmentation, plasma membrane translocation of phosphatidylserine and oxidative stress in human spermatozoa. *Human Reproduction,* 15, 1338-1344.

Beckman, J.S., Beckman, T.W. and Chen, J. (1990) Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Natural Academic of Science USA*, 87, 1620-1624.

Beyer, W. F. and Fridovich, I. (1990) Superoxide dismutase mimic prepared from desferrioxamine and manganese dioxide. *Methods of Enzymology,* 186, 242–249.

Bilodeau, J.F., Blanchette, S., Gagnon, C. and Sirard, M.A. (2001) Thiols prevent H_2O_2-mediated loss of sperm motility in cryopreserved bull semen. *Theriogenology,* 56, 275–86.

Blanc-Layrac, G., Bringuier, A.F., Guillot, R. and Feldmann, G. (2000) Morphological and biochemical analysis of cell death in human ejaculated spermatozoa. *Cell Molecular Biology,* 46, 187-197.

Bowen, I.D., Bowen, S.M. and Jones, A.H. (1998) *Mitosis and apoptosis: matter of life and death*, Chapman and Hall.

Burlacu, A. (2003) Regulation of apoptosis by Bcl-2 family proteins. *Journal of Cellular Molecule and Medicine,* 7, 249-257.

Cameron, I.L., Munoz, J. and Barnes, C.J. (2003) High dietary level of synthetic vitamin E on lipid peroxidation, membrane fatty acid composition and cytotoxicity in breast cancer xenograft and in mouse host tissue. *Cancer Cell International,* 3, 181-187.

Cerolini, S., Maldjian, A., Surai, P. and Noble, R. (2000) Viability susceptibility to peroxidation and fatty acid composition of boar semen during liquid storage. *Animal Reproduction Science,* 58, 99–111.

Chang, N. (2002) A potential role of p53 and WOX1 in mitochondrial apoptosis. *International Journal of Medicine,* 9, 19-24.

Chen, M. and Wang, J. (2002) initiator caspases in apoptosis signalling pathways. *Apoptosis*, 7, 313-319.

Chen, H., Chow, P.H., Cheng, S.K., Cheung, A.L.M., Cheng, L.Y.L. and Wai-sum, O. (2003) Male Genital Tract Antioxidant Enzymes: Their Source, Function in the Female, and Ability to Preserve Sperm DNA Integrity in the Golden Hamster. *American Society of Andrology*, 24,704–711.

Chow, C.K. and Hong, C.B. (2002) Dietary vitamin E and selenium and toxicity of nitrite and nitrate. *Toxicology,* 180, 195-207.

Coassin, M., Ursini, F. and Bindoli, A. (1992) Antioxidant effect of manganese. *Archives of Biochemistry and Biophysiology,* 299, 330–333.

Cohen, J.J. (1993) Apoptosis. *Immunology Today,* 14,126-130.

Cosentino, F., Eto, M., De Paolis, P., van der Loo, B., Bachschmid, M., Ullrich, V., Kouroedov, A., Gatti, C.D., Joch, H., Volpe M. and Lüscher, T.F. (2003) High Glucose Causes Up regulation of Cyclooxygenase-2 and Alters Prostanoid Profile in Human Endothelial Cells: Role of Protein Kinase C and Reactive Oxygen Species. *Circulation*, 107, 1017-1023.

Cosson, J., Ballard, R., Cyber, C., Deanne, C. and Squat, M. (1999) Ionic factors regulating the motility of fish sperm, in: Gagnon, C. (Ed.), *From Basic Science the Male Gamete to Clinical Application*, Cache River Press, Vienna, IL, USA, 161–186.

Dalvit, G.C., Cetica, P.D. and Beconi, M.T. (1998) Effect of (α-tocopherol and Ascorbic acid on bovine *in vitro* fertilisation. *Theriogenology,* 49, 619-627.

Darley-Usmar, V., Wiseman, H. and Halliwell, B. (1995) Nitric oxide and oxygen radicals: a question of balance. *FEBS Letters* 369, 131-135.

Dawson, C.B., Harris, W.A. and Teter, M.C. (1992) Effect of ascorbic acid supplementation on the sperm quality of smokers. *Fertility and Sterility*, 58, 1034.

de Iuliis, G.N., Wingate, J.K., Koppers, A.J., McLaughlin, E.A. and Aitken, R.J. (2006) Definitive Evidence for the Nonmitochondrial Production of Superoxide Anion by Human Spermatozoa. *Journal of Clinical Endocrinology and Metabolism,* 91, 1968–1975.

de Lamirande, E. and Gagnon, C. (1993a) Human sperm hyperactivation and capacitation as parts of an oxidative process. *Free Radical Biology and Medicine,* 14, 157–166.

de Lamirande, E. and Gagnon, C. (1992) Reactive oxygen species and human spermatozoa. Part II. Depletion of adenosine triphosphate plays an important role in the inhibition of sperm motility. *International Journal of Andrology,* 13, 379– 386.

de Lamirande, E. and Gagnon, C. (1993b) A positive role for superoxide anion in triggering hyperactivation and capacitation of human spermatozoa. *International Journal of Andrology,* 16, 21-25.

Demple, B., Herman, T. and Chen, D.S. (1991) Cloning and expression of APE, the cDNA encoding the major human apurinic endonuclease: Definition of a family of DNA repair enzymes. *Biochemistry*, 88, 11450-11454.

Dimmeler, S., Hermann, C., Galle, J. and Zeiher, A.M. (1999) Up regulation of Superoxide Dismutase and Nitric Oxide Synthase Mediates the Apoptosis Suppressive Effects of Shear Stress on Endothelial Cells. *Arteriosclerosis and Thrombosclerosis Vascular Biology,* 19, 656-664.

Doctrow, S. R., Huffman, K., Marcus, C. B., Musleh, W., Bruce, A., Baudry, M. and Malfroy, B. (1997) Salen-manganese complexes: combined superoxide dismutase/catalase mimics with broad pharmacological efficacy. *Advance Pharmacology, 38, 247–269.*

Donnelly, E.T., McClure, N. and Lewis, S.E.M. (1999) Antioxidant supplementation in vitro does not improve human sperm motility. *Fertility and Sterility*, 72, 484–486.

Eckert, J. and Niemann, H. (1996) Effects of platelet-derived growth factor (PDGF) on the in vitro production of bovine embryos in protein-free media *Theriogenology,* 46 (2) 307-320.

Engelbrecht, A.M., Esterhuyse, J.,du Toit, E.F., Lochner, A. and van Rooyen, J. (2006) p38-MAPK and PKB/Akt, possible role players in red palm oil-induced protection of the isolated perfused rat heart? *Journal of Nutrition Biochemistry*, 17(4), 265-71.

Esterhuyse, J., du Toit, E.F., Bernadé, A.J.S. and van Rooyen, J. (2005) Dietary red palm oil improves reperfusion cardiac function in the isolated perfused rat heart of animals fed a high cholesterol diet. *Prostaglandins Leukotrienes and Essential Fatty Acids*, 72(3), 153-161.

Esterhuyse,J., van Rooyen, J., Strijdom, H., Bester, D. and du Toit, E.F. (2006) Proposed mechanisms for red palm oil induced cardioprotection in a hyperlipidiaemic perfused rat heart model. *Prostaglandins Leukotrienes and Essential Fatty Acids*, 75(6), 375-84.

Evenson, D.P., Darzynkiewicz, Z. and Melamed, M.R. (1980) Relation of mammalian sperm chromatin heterogeneity. *Fertility Science,* 210, 1131–3.

Fadeel, B. and Orrenius, S. (2005) Apoptosis: a basic biological phenomenon with wide-ranging implications in human disease. *Journal of International Medicine,* 258, 479-517.

Faulkner, K., Liochev, S.I. and Fridovich, I. (1994) Stable Mn(III) porphyrins mimic superoxide dismutase *in vitro* and substitute for it *in vivo. Journal of Biological Chemistry*,269,23471-23476.

Faure, P., Oziol, L., Le Bihan, M. and Chombard, P. (2004) Cell culture media are potent antioxidants that interfere during LDL oxidation experiments. *Biochimie,* 86, 373-378.

Ferdinandy, P. and Schulz, R. (2003) Review Nitric oxide, superoxide, and peroxynitrite in myocardial ischemia-reperfusion injury and preconditioning. *British Journal of Phamacology,* 138, 532-543.

Flesch, F.M. and Gadella, B.M. (2000) Dynamics of the mammalian sperm plasma membrane in the process of fertilisation *Biochimica et Biophysica Acta,* 1469, 197-235.

Ford, W.C.L. (2004) Regulation of sperm functions by reactive oxygen species. *Human Reproduction Update,* 10, 387-399.

Ford, W.C.L. and Whittington, K. (1998) Debate: is antioxidant therapy a promising strategy to improve human reproduction? Antioxidant therapy for male subfertility: a promise that remains unfulfilled. *Human Reproduction,* 13, 1416.

Fraga, C.G., Motchnik, P.A. and Wyrobek, A.J. (1996) Smoking and low antioxidant levels increase oxidative damage to sperm DNA. *Mutation Research,* 351,199–203.

Francavilla, F., Santucci, R., Macerola, B. Ruvolo,G. and Romano, R. (2000) Nitric oxide synthase inhibition in human sperm affects sperm-oocyte fusion but not zona pellucida binding. *Biology of Reproduction,* 63,425–429.

Fujisawa, M., Yamanaka, K., Amanaka, H., Tamanaka, Tanaka, H. Okada, H.S. Arakawa, S. and Kamidono, S. (2001) Expression of endothelial nitric oxide synthase in the Sertoli cells of men with infertility of various causes. *BJU International*, 87, 85-88.

Fukui, Y., Kikuchi, Kondo, H. and Mizushima, S. (2000) Fertilizability and developmental capacity of individually cultured bovine oocytes. *Theriogenology*, 53 (8), 1553-1565.

Gandini, L., Lombardo, F., Paoli, D., Caponecchia, L., Familiari, G., Verlengia, C., Dondero, F. and Lenzi, A. (2000) Study of apoptotic DNA fragmentation in human spermatozoa. *Human Reproduction,* 15, 830-839.

Gadella, B.M., Rathi, R., Brouwers, J.F.H.M., Stout, T.A.E. and Colenbrander, B. (2001) Capacitation and the acrosome reaction in equine sperm. *Animal Reproduction Science,* 68, 249–265.

Gardner, P.R., Nguyen, D.D.H. and White, C.W. (1996) Superoxide Scavenging by Mn(II/III) Tetrakis (1-Methyl-4-pyridyl) Porphyrin in Mammalian Cells. *Archives of Biochemistry and Biophysics,* 325, 20–28.

Garg, R., Kumbkarni, Y., Aljada, A., Mohanty, P., Ghanim, H., Hamouda, W. and Dandona, P. (2000) Troglitazone Reduces Reactive Oxygen Species Generation by Leukocytes and Lipid Peroxidation and Improves Flow-Mediated Vasodilatation in Obese Subjects. *Hypertension*, 36, 430-435.

Garner, D.L. Thomas, C.A. Joerg, H.W. DeJarnette; J.M. and Marshall, C.E. (1997) Fluorometric assessments of mitochondrial function and viability in cryopreserved bovine spermatozoa. *Biology of Reproduction*, 57, 1401–6.

Garland, J.M. (2000) Regulation of apoptosis by cell metabolism, cytochrome and the cytoskeleton. In: *Programmed Cell Death in Animals and Plants.* (Eds. J.A. Bryant, S.G. Hughes and J.M. Garland) BIOS Scientific Publishers Ltd, Oxford.

Gewies, A. (2003) Aporeview: Introduction to Apoptosis. http://www.celldeath.de/encyclo/aporev/apointro.df

Gillan, L., Evans, G. and Maxwell, W.M.C. (2005) Flow cytometric evaluation of sperm parameters in relation to fertility potential. *Theriogenology*, 63, 445-457.

Gil-Guzman, E., Ollero, M., Lopez, M.C., Sharma, R.K., Alvarez, J.G., Thomas, A.J. Jr. and Agarwal A. (2001) Differential production of reactive oxygen species by subsets of human spermatozoa at different stages of maturation. *Human Reproduction,* 16, 1922–1930.

Gracy, R.W., Talent, J.M., Kong, Y. and Conrad, C.C. (1999) Reactive oxygen species: the unavoidable environmental insult? *Mutation Research,* 428, 17-22.

Green, D. and Kroemer, G. (1998) The central executioners of apoptosis: caspases or mitochondria? *Trends in Cell Biology*, 8, 202-205.

Griveau, J.F., Dumont, E., Renard, P., Callegari, J. P. and Le Lannou, D. (1995a) Reactive oxygen species, lipid peroxidation and enzymatic defence systems in human spermatozoa. *Journal of Reproduction and Fertility,* 103, 17 -26.

Griveau, J.F. and Le Lannou, D. (1997) Reactive oxygen species and human spermatozoa: physiology and pathology. *International Journal of Andrology,* 20, 61–69.

Griveau, J. F., Renard, P. and Le Lannou, D. (1995b) Superoxide anion production by human spermatozoa as a part of the ionophore-induced acrosome reaction process. *International Journal of Andrology,* 18, 67-74.

Guo, Q., Sebastian, L., Sopher, B.L., Miller, M.W., Ware, C.B., Martin, G.M. and Mattson, M.P. (1999) Increased Vulnerability of Hippocampal Neurons from Presenilin-1 Mutant Knock-In Mice to Amyloid b-Peptide Toxicity: Central Roles of Superoxide Production and Caspase Activation. *Journal of Neurochemistry*, 72, 1019–1029.

Guthrie, N., Gapor, A., Chambers, A.F. and Carroll, K.K. (1997) Inhibition of proliferation of oestrogen receptor MDA-MB-435 and positive MCF-7 human breast cancer cells by palm tocotrienols and tamoxifen alone and in combination. *Journal of Nutrition*, 127, 544S-548S.

Hale, A.J., Smith, C.A., Sutherland, L.C., Stoneman, V.E.A., Longthome, V.L., Culhane, A.C. and Williams, G.T. (1996) Apoptosis: molecular regulation of cell death. *European Journal of Biochemistry*, 236, 1-26.

Halliwell, B. and Gutteridge, J.M.C. (1989) *Free Radicals in Biology and Medicine*, 2nd edition. Clarendon Press, Oxford.

Hamilton, I.M.J., Gilmore, W.S., Benzie, I.F., Mulholland, C.W. and Strain, J.J. (2000) Interaction between vitamins C and E in human subjects. *British Journal of Nutrition,* 84, 261–267.

Harrison, R.A. and Vickers, S.E. (1990) Use of fluorescent probes to assess membrane integrity in mammalian spermatozoa. *Journal of Reproduction and Fertility*; 88:343–52.

Hellstrom, W.J.G., Bell, M., Wang R. and Sikka, S.C. (1994) Effect of sodium nitroprusside on sperm motility, viability, and lipid peroxidation. *Fertility and Sterility,* 61, 1117- 1122.

Henkel, R., Hajimohammad, M., Stalf, T., Hoogendijik, C., Menhert, C., Menkveld, R., Gips, H., Schill, W. and Kruger, T.F. (2004) Influence of deoxyribonucleic acid damage on fertilisation and pregnancy. *Fertility and Sterility*, 81, 965-972.

Henkel, R., Kierspel, E., Hajimohammad, M., Stalf, T., Hoogendjik, C., Mehnert, C., Menkveld, R., Schill, W.B. and Kruger, T.F. (2003) DNA fragmentation of spermatozoa and assisted reproduction technology. *Reproductive Biomedicine*, 7, 477-484.

Henkel, R., Maass, G., Schuppe, H.C., Jung, A., Schubert, J. and Schill, W. (2005a) Molecular aspects of declining sperm motility in older men. *Fertility and Sterility,* 84, 1430-1437.

Henkel, R., Kierspel, E., Stalf, T., Menhert, C., Menkveld, R., Tinneberg, H., Schill, W. and Kruger, T.F. (2005b) Effect of reactive oxygen species produced by spermatozoa and leukocytes on sperm functions in non-leukocytospermic patients. *Fertility and Sterility,* 83, 635-642.

Hibbs, J.B, Vavrin, Z. and Taintor, R.R. (1987) L-arginine is required for the expression of the activated macrophage effector's mechanism causing selective metabolic inhibition in target cells. *Journal of Immunology,* 138, 550-65.

Host, E., Gabrielsen, A. and Lindenberg, (2002) Apoptosis in human cumulus cells in relation to zona pellucida thickness variation, maturation stage, and cleavage of the corresponding oocyte after intracytoplasmic sperm injection. *Fertility and Sterility,* 77, 511–15.

Hurley, W.L. and Deane, R.M. (1987) Recent developments in the roles of vitamins and minerals in reproduction. *Journal Dairy of Science*, 72,785.

Ignarro, I. (1990) Biosynthesis and metabolism of endothelium derived nitric oxide. *Annual Review of Pharmacology and Toxicology*, 30, 535-544.

Irvine, D.S., Twigg, J.P., Gordon, E.L., Fulton, N., Milne, P.A. and Aitken, R.J. (2000) DNA integrity in human spermatozoa: relationships with semen quality. *Journal of Andrology*, 21, 33-44.

Isong, E.U., Ebong, P.E., Ifon, E.T., Umoh, I.B. and Eka, O.U. (1997) Thermoxidised palm oil induces reproductive toxicity in healthy and malnourished rats. *Plant Foods for Human Nutrition,* 51, 159–166.

Jaakma, Ü., Zhang, B. R., Larsson, B., Niwa, K. and Rodriguez-Martinez, H. (1997) Effects of sperm treatments on the in vitro development of bovine oocytes in semidefined and defined media. *Theriogenology*, 48 (5), 711-720.

Jin, Z. and El-Deiry, W.S. (2005) Overview of cell death signalling pathways. *Cancer Biology and Therapy*, 4, 139-163.

Johnson, F. and Giulivi, C. (2005) Superoxide dismutases and their impact upon human health. *Molecular Aspects of Medicine,* 26, 340–352.

Joseph, J.A. and Culter, R.C. (1994) The role of oxidative stress in signal transduction changes and cell loss in senescence. *Annals of New York Academy of Science*, 738, 37.

Joza, N., Kroemer, G. and Penninger, J.M. (2002) Genetic analysis of the mammalian cell death machinery. *Trends in genetics*, 18, 142-149.

Kappus, H. (1986) Lipid peroxidation: mechanism, analysis, enzymology and biological relevance. (Ed In Sies), *Oxidative stress*. Academic Press, New York, 273-282.

Kerr, J.F.R. and Harmon, B.V. (1991) Definition and Incidence of Apoptosis: An Historical Perspective. In: *Apoptosis: The Molecular Basis of Cell Death.* (Eds. L.D. Tomei and F.O. Cope), Cold Spring Harbour Laboratory Press.

Klebanoff, S.J. (1992) Oxygen metabolites from phagocytes. In Gallin, J.J., Goldstie, I.M. and Syndeman, R. (Eds), *Inflammation Basic Principles and Clinical Correlates.* Raven Press, New York, 541-555.

Köhn, F.M., Mack, S.R., Schill, W.B. and Zaneveld, L.J.D. (1997) Detection of human sperm acrosome reaction: comparison between methods using double staining, *Pisum sativum* agglutinin, concanavalin A and transmission electron microscopy. *Human Reproduction,* 12, 714–721.

Krausz, C., Mills, C., Rogers, S., Tan, S.L. and Aitken, R.J. (1994), Stimulation of oxidant generation by human sperm suspensions using phorbol esters and formyl peptides: relationships with motility and fertilisation *in vitro*. *Fertility and Sterility,* 62, 599-605.

Krinsky N.I., (1993) Actions of carotenoids in biological systems. *Reviews of Nutrition,* 13, 561–587.

LaCasse, E.C., Holcik, M., Komeluk, R.G. and Mackenzie, A.E. (2004) Apoptosis in health, disease, and therapy: overview and methodology. In: Apoptosis in health and Disease: Clinical and Therapeutic Aspects. (Eds. M. Holcik, A.E. Mackenzie, R.G. and E.C. LaCasse), Cambridge University Press.

Lachaud, C., Tesarik, J., CanÃadas, M.L. and Mendoza, C. (2004) Apoptosis and necrosis in human ejaculated spermatozoa. *Human Reproduction,* 19, 607-610.

Lands, W.E.M. (1985) Interactions of lipid hydroperoxides with eicosanoid biosynthesis. *Journal of Free Radical Biology,* 1, 97-102.

Lapinskas, P. J., Cunningham, K.W., Liu, X. F., Fink, G. R. and Culotta, V. C. (1995) Mutations in PMR1 suppress oxidative damage in yeast cells lacking superoxide dismutase. *Molecular Cellular Biology,* 15, 1382–1388.

Lee, H. (1996) The Protective Roles of Vitamin E and Vitamin C as Antioxidants in Semen. *Nutrition Bytes.* http://repositories.cdlib.org/uclabiolchem/nutritionbytes/vol2/iss1/art4

Lenzi, A., Culasso, F. and Gandini, L. (1993) Placebo-controlled, double-blind cross-over trial of glutathione therapy in male infertility. *Human Reproduction*, 8, 1657.

Lewis, S., Boyle, P.M., McKinney, K.A., Young, I.S. and Thompson, W. (1995) Total Antioxidant Capacity of Seminal Plasma is Different in Fertile and Infertile Men. *Fertility and Sterility*, 64, 868-70.

Lin, Y.T., Hoang, H., Hsieh, S.I., Rangel, N., Foster, A.L., Sampayo, J.N., Lithgow, G.J., and Srinivasan, C. (2005) Manganous ion supplementation accelerates wild type development, enhances stress resistance, and rescues the life span of a short–lived *Caenorhabditis elegans* mutant. *Free Radical Biology and Medicine*, 8, 1-9.

Liu, J.F. and Lee, Y.W. (1998). Vitamin C supplementation restores the impaired vitamin E status of guinea pigs fed oxidized frying oil. *Journal of Nutrition,* 128, 116–122.

Lopes, S., Jurisicova, A., Sun, J.G. and Robert F. C. (1998) Reactive oxygen species: potential cause for DNA fragmentation in human spermatozoa. *Human Reproduction,* 13, 896–900.

Luck, M.R., Jeyaseelam, I. and Scholes, R.A. (1995) Ascorbic acid and fertility. *Biology of Reproduction* 52, 262.

Macleod, J. (1943) The role of oxygen in the metabolism and motility of human spermatozoa. *American journal of Physiology* 138: 512-518.

McArdle, H.J. and Ashworth, C.J. (1999) Micronutrients in foetal growth and development. *British Medical Bulletin,* 55, 499–510.

Manabe, Y., Anrather, J., Kawano, T., Niwa, K., Zhou, P., Ross, E. and Iadecola, C. (2004) Prostanoids, not reactive oxygen species, mediate COX-2-dependent neurotoxicity. *Annals of Neurology*, 55,668–675.

Maxwell, W.M.C. and Stojanov, T. (1996) Liquid storage of ram semen in the absence or presence of some antioxidants. *Reproduction and Fertility Development,* 8, 1013–1020.

Mello, P.R., Pinto, G.R. and Botelho, C. (2001) The influence of smoking on fertility, pregnancy and lactation. *Journal de Pediatria*, 77,257-64.

Melov, S., Doctrow, S. R., Schneider, J. A., Haberson, J., Patel, M., Coskun, P. E., Huffman, K., Wallace, D. C. and Malfroy, B. (2001) Lifespan extension and rescue of spongiform encephalopathy in superoxide dismutase 2 nullizygous mice treated with superoxide dismutase-catalase mimetics. *Journal of Neuroscience*, 21, 8348–8353.

Murakoshi, M., Nishino, H. and Satomi, Y. (1992) Potent preventive action of alpha-carotene against carcinogenesis: spontaneous lever carcinogenesis and promoting stage of lung and skin carcinogenesis in mice are suppressed more effectively by alpha-carotene than by beta-carotene. *Cancer Research*, 52, 6583-6587.

Muratori, M., Piombon, P., Baldi, E., Filimberti, E., Pecchioli, P., Moretti, E., Gambera L, Baccetti B, Biagiotti, R. and Forti, G. (2000) Functional and ultrastructural features of DNA-fragmented human sperm. *Journal of Andrology, 21,903*-912.

Nagata, S. (2000) Apoptotic DNA Fragmentation. *Experimental Cell Research,* 256, 12-18.

Nagendran, B., Unnithan, U.R., Choo, Y.M. and Sundram, K. (2000) Characteristics of red palm oil, a carotene and vitamin E- rich refined oil for food uses. *Food and Nutrition Bulletin,* 21, 189-194.

Navarro-Antolin, J., Lopez-Munoz, M.J., Klatt, P., Soria, J., Michel, T. and Lamas S. (2001) Formation of peroxynitrite in vascular endothelial cells exposed to cyclosporine A. *Faseb Journal,* 15, 1291-3.

Nesaretnam, K., Guthrie, N., Chambers, A.F. and Carroll, K.K. (1995) Effect of tocotrienols on the growth of human breast cancer cell line in culture. *Lipids*, 30: 1139-1143.

Nesaretnam, K., Khor, H.T., Ganseon, J., Chong, Y.H., Sundram, K. and Gapor, A. (1992) The effect of vitamin E tocotrienols from palm oil on chemically induced mammary carcinogenesis in female rats. *Nutrition Research*, 12: 63-75.

Naz, R.K. and Rajesh, P.B. (2004) Role of tyrosine phosphorylation in sperm capacitation / Acrosome reaction. *Reproductive Biology and Endocrinology*, 2, 75.

Nordberg, J. and Arn'er, E.J. (2001) Reactive oxygen species, antiosxidant and the mammalian thioredoxin system. *Free Radical Biology and Medicine*, 31, 1287–1312.

Oehninger, S., Morshedi, M., Weng S.L., Taylor, S., Duran, H. and Beebe, S. (2003) Presence and significance of somatic cell apoptosis makers in human ejaculated spermatozoa. *Reproductive Biomedicine Online,* 7, 469-476.

Ong, A.S.H. and Goh, S.H. (2002) Palm oil: A healthful and cost-effective dietary component. *Food Nutrition Bulletin*, 23 (1), 11-22.

Oosterhuis, G.J.E., Mulder, A.B., Kralsbeek-Batenberg, E., Lambalk, C.B., Schoemaker, J. and Vermes, I. (2000) Measuring apoptosis in human spermatozoa: a biological assay for semen quality? *Fertility and Sterility,* 74, 245-250.

Ormerod, M.G. (1998) The study of apoptotic cells by flow cytometry. *Leukaemia*, 12, 1013-1025.

Pacifici, R.E. and Davies, K.J.A. (1990) Protein degradation as an index of oxidative stress. *Methods Enzymol*ogy, 186, 485-501.

Pearce, B.C., Parker, R.A., Deason, M.E., Qureshi, A.A. and Wright, J.J (1992) Hypocholesterolemic activity of synthetic and natural tocotrienols. *Journal of Medical Chemistry,* 35, 3595–3606.

Pena, A.I., Quintela, L.A. and Herradon, P.G. (1998) Viability assessment of dog spermatozoa using flow cytometry. T*hriogenology* 50, 1211-1220.

Peña, F.J., Johannisson, A., Wallgren, M. and Martinez, H.R. (2003) Antioxidant supplementation *in vitro* improves boar sperm motility and mitochondrial membrane potential after cryopreservation of different fractions of the ejaculate. *Animal Reproduction Science,* 78, 85–98.

Portakel, O., Ozkaya, O. and Erden, I.M. (2000) Coenzyme Q_{10} concentrations and antioxidant status in tissues of breast cancer patients. *Clinical Biochemistry* 33, 279-284.

Porter, A.G. and Jänicke, R.U. (1999) Emerging roles of caspase-3 in apoptosis. *Cell Death and Differentiation,* 6, 99-104.

Pryor, W.A., Ames, B.N., Saul, R.C., McCord, J.M. and Harman, D. (1987), Oxygen radicals and human disease. *Annals of International Medicine,* 107, 526–545.

Qureshi, A.A., Qureshi, N., Wright, J.J.K., Shens, S., Kromer, G., Gapor, A., Chong, Y.H., Dewitt, G., Ong, A.S.H., Peterson, D. and Bradlow, B.A. (1991) Lowering of serum cholesterol in hypercholesterolemic humans by tocotrienols (palm vitee). *American Journal of Clinical Nutrition*, 53, 1021–1026.

Ramachandra, S. and Studzinski, G.P. (1995) Morphological and biochemical criteria of apoptosis. In: *Cell Growth and Apoptosis.* (Ed. G.P.Studzinski), Oxford University Press.

Ramos, L. and Wetzels, A.M.M. (2001) Low rates of DNA fragmentation in selected motile human spermatozoa assessed by the TUNEL assay. *Human Reproduction,* 16, 1703-1707.

Redondo-Horcajo, M. and Lamas, S. (2005) Oxidative and nitrosative stress in kidney disease: A case for cyclosporine A. *Journal of Nephrology*, 18, 453-457.

Ricci, G., Perticarari, S., Fragonas, E., Giolo, E., Canova, S., Pozzobon, C., Guaschino, S. and Presani, G. (2002) Apoptosis in human sperm: its correlation with semen quality and the presence of leukocytes. *Human Reproduction*, 17, 2665-2672.

Rossell, J.B., King, B. and Downes, M.J. (1985) Composition of oils. *Journal of American Oil and Chemistry Society,* 62, 221–229.

Rosselli, M., Dubey, R.K., Imthurn, B., Macas, E. and Keller, P.J. (1995) Effects of nitric oxide on human spermatozoa: evidence that nitric oxide decreases sperm motility and induces sperm toxicity. *Human Reproduction,* 10, 1786-1790.

Roth, J. A. and Garrick, M. D. (2003) Iron interactions and other biological reactions mediating the physiological and toxic actions of manganese. *Biochemistry and Pharmacology*, 66,1–13.

Said, T.M., Paasch, U., Glander, H.J. and Agarwal, A. (2004) Role of caspases in male infertility. *Human Reproduction Update,* 10, 39–51.

Saint-Pierre, J., Buckingham, J.A., Roebuck, S.J. and Brand, M.D., (2002) Topology of superoxide production from different sites in the mitochondrial electron transport chain. *Journal of Biology and Chemistry,* 277, 44784–44790.

Sakkas, D., Mariethoz, E. and Saint John, J.C. (1999a) Abnormal Sperm Parameters in Humans are Indicative of an Abortive Apoptotic Mechanism Linked to the Fas-Mediated Pathway. *Experimental Cell Research,* 241, 350–355.

Sakkas, D., Mariethoz, E., Manicardi, G., Bizzarro, D., Bianchi, P.G. and Bianchi, U. (1999b) Origin of DNA damage in ejaculated human spermatozoa. *Reviews of Reproduction,* 4, 31-37.

Sakkas, D., Manicardi, G.C., Tomlinson, M., Mandrioli, M., Bizzaro, D., Bianchi, P.G. and Bianchi, U. (2000) The use of two density gradient centrifugation techniques and the swim-up method to separate spermatozoa with chromatin and nuclear DNA anomalies. *Human Reproduction,* 15, 1112-1116.

Sakkas, D., Moffatt, O., Manicardi, G.C., Mariethoz, E., Tarozzi, N. and Bizzaro, D. (2002) Nature of DNA damage in ejaculated human spermatozoa and the possible involvement of apoptosis. *Biology of Reproduction,* 66, 1061-1067.

Salvemini, D., Wang, Z. Q., Zweier, J. L., Samouilov, A., Macarthur, H., Misko, T. P., Currie, M. G., Cuzzocrea, S., Sikorski, J. A. and Riley, D. P. (1999) A nonpeptidyl mimic of superoxide dismutase with therapeutic activity in rats. *Science,* 286, 304-306.

Samali, A., Gorman, A.M. and Cotter, T.G. (1996) Apoptosis: The story so far. *Experentia*, 52, 933-941.

Sanocka, D. and Kurpisz, M. (2004) Reactive oxygen species and sperm cells Reproductive. *Biology and Endocrinology.* http://www.rbej.com/content/2/1/12

Saran, M. and Bors, W. (1989) Oxygen radicals acting as chemical messengers: a hypothesis. *Free Radical Research Communication*, 7, 3-6.

Scheck, R., Albermann, K. and Baeuerde, P.A. (1992) Nuclear transcription factor kB: an Oxidative stress responsive transcription factor of eukaryotic cells. *Free Radical Research Community*, 7, 3-10.

Schuler, M. and Green, D.R. (2001) Mechanism of p53-dependant apoptosis. *Biochemical Society Transcription*, 29, 684-688.

Serbinova, E., Choo, M. and Packer, L. (1992) Distribution and antioxidant activity of a palm oil carotene fraction in rats. *Biochemistry International*, 28,881– 6.

Shen, H.M., Dai, J., Chia, S.E., Lim, A. and Ong, C.N. (2002) Detection of apoptotic alterations in sperm in subfertile patients and their correlations with sperm quality. *Human Reproduction,* 17, 1266 -1273.

Sikka, S.C. (1996) Oxidative stress and role of antioxidants in normal an abnormal sperm function. *Frontiers in Bioscience* 1, 78-86.

Sinha, H. A.P., Lue, Y., Diaz-Romera, M., Yen, P.H., Wang, C. and Swerdloff, R.S. (2003) Deciphering the pathways of germ cell apoptosis in the testis. *Journal of Steroid Biochemistry and Molecular Biology*, 85, 175-182.

Sinha, M.P., Sinha, A.K., Singh, B.K. and Prasad, R.L (1996) The effect of glutathione on the motility, enzyme leakage and fertility of frozen goat semen. *Animal Reproduction Science,* 41,237-243.

Slee, E.A., Adrain, C. and Martin, S.J. (1999) Serial killers: ordering caspase activation events in apoptosis. *Cell Death and Differentiation,* 6, 1067-1074.

Stennicke, H.R. and Salvesen, G.S. (1998) Review: Properties of the caspases. *Biochemical and Biophysical Acta,* 1387, 17-31.

Sundram, K., Sambanthamurthi, R. and Tan, Y.A. (2003) Palm fruit chemistry. *Asia Pac Journal of Clinical Nutrition,* 12, 355-362.

Sundram, K., Khor, H.T., Ong, A.S.H. and Pathmanathan, R. (1989) Effect of different palm oils on mammary carcinogenesis in female rats induced by 7,12-dimethylbenz-α-anthracene. *Cancer Research*, 49, 1447-1451.

Sun, J.G., Jurisicova, A. and Casper, R.F. (1997) Detection of deoxyribonucleic acid fragmentation in human sperm: correlation with fertilisation in vitro. *Biology of Reproduction*, 56,602-607.

Symonyan, M. and Nalbandyan, R. (1972) Interaction of Hydrogen Peroxide with Superoxide Dismutase from Erythrocytes. *FEBS Letters, 28,* 22.

Tan, D-X., Manchester, L.C., Hardeland, R., Lopez-Burillo, S., Mayo, J.C., Sainz, R.M. and Reiter, R.J. (2003) Melatonin: a hormone, a tissue factor, an autocoid, a paracoid, and an antioxidant vitamin. *Journal of Pineal Research*, 34, 75-78.

Taylor C.T. (2001) Antioxidants and reactive oxygen species in human fertility. *Environmental Toxicology and Pharmacology,* 10, 189–198.

Tesarik, J., Martinez, F., Rienzi, L., Iacobelli, M., Ubaldi, F., Mendoza, C. and Greco, E. (2002) *In vitro* effects of FSH and testosterone withdrawal on caspase activation and DNA fragmentation in different cell types of human seminiferous epithelium. *Human Reproduction*, 17, 1811-1819.

Test, S.T. and Weiss, S.J. (1986) The generation or utilisation of chlorinated oxidants by human neutrophils. *Advance Free Radical Biology and Medicine*, 2, 91-96.

Turke, B., Stoka, V., Rozman-Pungerčar, J., Cirman, T., Droga-Mazovec, G., Orešić, K. and Turk, V. (2002) Apoptotic pathways: involvement of lysosomal proteases. *Biology and Chemistry*, 383, 1035-1044.

Upreti, G.C., Jensen, K., Munday, R., Duganzich, D.M., Vishwanath, R. and Smith, J.F. (1998) Studies on aromatic amino acid oxidase activity in ram spermatozoa: role of pyruvate as an antioxidant. *Animal Reproduction Science*, 51, 275–287.

Upreti, G.C., Jensen, K., Oliver, J.E., Duganzich, D.M., Munday, R. and Smith, J.F. (1997) Motility of ram spermatozoa during storage in chemically-defined diluents containing antioxidants. *Animal Reproduction Science,* 48, 269–278.

Vernet, P., Fulton, N., Wallace, C. and Aitken, R.J. (2001) Analysis of reactive oxygen species generating systems in rat epididymal spermatozoa. *Biology of Reproduction,* 65, 1102–1113.

Wang, X., Sharma, R.K., Sikka, S.C., Thomas, A J., Falcone, T. and Agarwal, A. (2003a) Oxidative stress is associated with increased apoptosis leading to spermatozoa DNA damage in patients with male factor infertility. *Fertility and Sterility,* 80, 531–535.

Wang, R. G. and Zhu, X. Z. (2003b) Subtoxic concentration of manganese synergistically potentiates 1-methyl-4-phenylpyridinium-induced neurotoxicity in PC12 cells. *Brain Research,* 961,131–138.

Weng, S.L., Taylor, S.T., Morshedi, M., Schuffner, A., Duran, E., Beebe, S. and Oehninger, S. (2002) Caspase activity and apoptotic markers in ejaculated human sperm. *Molecular Human Reproduction*, 8, 984–991.

Weil, M., Jacobson, M.D. and Raff, M.C. (1998) Are caspases involved in the death of cells with a transcriptionally inactive nucleus? Sperm and chicken erythrocytes. *Journal of Cell Science,* 111, 2707-2715.

Wilson, M.R. (1998) Apoptosis: unmasking the executioner. *Cell Death and Differentiation*, 5, 646-652.

World Health Organization. (1999) WHO laboratory manual for the examination of human semen and sperm- cervical mucus interaction. Cambridge University Press, Cambridge.

Yanagimachi, R. (1994) *Mammalian Fertilization.* In Physiology of Reproduction, Knobil ENJ. New York, Raven Press, 189-317.

Yoshida, Y., Niki, E. and Noguchi, N. (2003) Comparative study on the action of tocopherols and tocotrienols as antioxidant: chemical and physical effects. *Chemical Physiological Lipids,* 123, 63-75.

Younglai, E.V., Holt, D., Brown, P., Jurisicova, A. and Casper, R.F. (2001) Sperm swim-up techniques and DNA fragmentation. *Human Reproduction*, 16, 1950-1953.

Zaneveld, L.J.D., de Jonge, C.J., Anderson, R.A. and Mack, S.R. (1991) Human sperm capacitation and the acrosome reaction. *Human Reproduction,* 6, 1265–1274.

Zini, A., de Lamirande, E., and Gagnon, C. (1995) Low levels of nitric oxide promote human sperm capacitation *in vitro. Journal of Andrology,* 16, 424–431.

Zini, A., de Lamirande, E. and Gagnon, C. (1993) Reactive oxygen species in semen of infertile patients: Levels of superoxide dismutase and catalase-like activities in seminal plasma and spermatozoa. *International Journal of Andrology*, 16,183-188.

Zini, A., Finelli, A., Phang, D. and Jarvi, K. (2000) Influence of semen processing technique on human sperm DNA integrity. *Urology,* 56, 1081-1084.