The Relationship between Male Genital Tract Infection, Oxidative Status in the Ejaculate, and Apoptotic Markers in Human Spermatozoa

by

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ABSTRACT

Aim: Leukocytes are the major source of reactive oxygen species (ROS) in the ejaculate and contribute to up to 30% of male infertility. ROS have been associated with markers of apoptosis such as sperm DNA damage, externalization of phosphatidylserine and caspase-3 activation. Therefore, this study aimed at investigating the impact male genital tract infections/inflammations on the induction of apoptosis in spermatozoa.

Materials and Methods: Semen samples were obtained from 60 men consulting for fertility problems at the Reproductive Biology Unit, University of Stellenbosch at Tygerberg Academic Hospital, and Vincent Pallotti Hospital (Cape Town, South Africa). To investigate the relationship between male genital tract infection and sperm apoptosis, the following were measured: semen parameters including sperm count, motility and forward progression; oxidative status in the ejaculate by evaluating the concentration of seminal leukocytes, ROS production in the ejaculate, generation of $O_2^{\cdot}$ and H_2O_2 by spermatozoa, and the activity of reduced glutathione (GSH) in sperm; sperm apoptotic markers by measuring mitochondrial membrane potential ($\Delta\psi_m$), caspase-3/7 activation, and DNA fragmentation (TUNEL).

Results: The concentration of seminal leukocytes had a significant positive correlation with ROS production in the ejaculate (ρ=0.378; P=0.0064), sperm $O_2^{\cdot}$ production (ρ=0.336; P=0.0098), and caspase-3/7 activation in sperm (ρ=0.527; P<0.0001). Furthermore, at the cutoff value of ≥0.25×10^6 leukocytes/mL of semen, the concentration of peroxidase-positive cells correlated significantly with sperm GSH activity (ρ=0.718; P=0.008), the percentage of sperm with disrupted $\Delta\psi m$ (ρ=0.465; P=0.043), caspase-3/7 activation in sperm (ρ=0.794; P=0.001), and the percentage of sperm with fragmented DNA (ρ=0.563; P=0.017). ROS production in the ejaculate, besides the association with seminal leukocytes, was also correlated with the sperm count (ρ= -0.296; P=0.033), sperm GSH activity (ρ=0.577; P<0.0001), caspase-3/7 activation in sperm (ρ=0.487; P=0.0005), and sperm DNA fragmentation (ρ=0.331; P=0.0171). Caspase-3/7 activation was strongly correlated with oxidative stress in both, the ejaculate and in spermatozoa; although this parameter was not correlated with sperm $\Delta\psi_m$ and DNA fragmentation. Sperm $O_2^{\cdot}$, which had a link

with seminal leukocyte concentration, was significantly correlated to sperm $\Delta\psi_m$ (P=0.0098), as was sperm GSH activity (P=0.0055). Sperm DNA fragmentation was positively correlated with ROS in the ejaculate and sperm H_2O_2-production (P=0.039).

Conclusions: Excessive ROS in the ejaculate, mainly a consequence of seminal leukocytes, is not only linked to internal generation of $O_2^{-\bullet}$, but also to sperm DNA fragmentation and the activation of effector caspases. Moreover, even in non-leukocytospermic patients with $\geq 0.25 \times 10^6$ leukocytes/mL of semen, oxidative stress can occur which can trigger apoptosis, caspase-3/7 activation, and induce sperm DNA fragmentation. Therefore, it is possible that male genital tract infection, the major cause of leukocyte infiltration in the male reproductive tract, can induce apoptosis, of which the observed sperm DNA fragmentation is a late feature.

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Declaration

I, the undersigned, hereby declare that '*The Relationship between Male Genital Tract Infection, Oxidative Status in the Ejaculate, and Apoptotic Markers in Human Spermatozoa*' is my own work, and has not been previously submitted, in its entirety or in part, for any degree or examination in any other university, and that all the sources I have used quoted have been indicated and acknowledged by complete references.

Cleyson Mupfiga   **May 2009**

Signed: _____

Acknowledgements

"If I have seen further, it is by standing on the shoulders of giants"

Sir Isaac Newton (1643-1727)

TABLE OF CONTENTS

CHAPTER ONE

INTRODUCTION

1.1 Background

Infertility is defined as the failure of a couple to induce pregnancy after one year of regular unprotected intercourse during the fertile phase of the menstrual cycle (Nieschlag et al. 2000; Evers, 2002). This inability to conceive affects up to 15% of all couples of reproductive age (Bruckert, 1991; Juul et al., 1999; Boivin et al., 2007) and in up to half of these cases the male partner is responsible for the infertility (Hull et al., 1985; de Kretser and Baker, 1999). Therefore, about 7% of men may have impaired fertility during the course of their lives (Purvis and Christiansen, 1992; Nieschlag et al., 2000); a prevalence that apparently exceeds that of diabetes mellitus which is considered to be a common condition (Nieschlag et al., 2000; Wild et al., 2004). Moreover, human semen quality seems to have progressively declined over the past few decades (Carlsen et al., 1992; Auger et al., 1995; van Waeleghem et al., 1996; Skakkebaek, 2006). Considering that up to 50% of the cases of male infertility are classified as idiopathic (Sherins, 1995), it is clear that the aetiology of male infertility is poorly understood and, consequently, there are limited interventions to address its causes and prevent its onset. Therefore, despite the successes of assisted reproductive techniques (ART) such as *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI), it remains imperative to investigate the causes and understand the mechanisms of infertility in the human male.

Investigations of male infertility begin with a semen analysis which provides descriptive information on the numbers of sperm present in the ejaculate, the proportion that are motile, and the percentage that are morphologically normal (WHO, 1999). Many studies have clearly demonstrated that the probability of conception is related to sperm motility (Alper et al., 1985; Ron-el et al., 1991; Robinson et al., 1994), sperm concentration (Biljan et al., 1994; Calvo et al., 1994) and morphology (Kruger et al., 1986, 1987; Kobayashi et al., 1991; Menkveld et al., 1996). However, many men who demonstrate normal parameters on standard semen

analysis remain unable to induce pregnancy (Hull et al., 1985; WHO, 1992; Baker, 1994). This suggests that conventional semen analysis does not provide complete diagnostic information. Furthermore, many studies show that it is not so much the absolute number of sperm that determines fertility, but their functional competence *in vitro* such as the ability to penetrate cervical mucus (Eggert-Kruse et al., 1989; Sharara et al., 1995), capacitation (Sukcharoen et al., 1995), acrosome reaction or sperm-oocyte fusion (Liu and Baker, 2000; 2003). Indeed, defective sperm function is recognized as a major cause of the loss of fertilizing potential in infertile men (Hull et al., 1985; McLachlan and de Kretser, 2001). In the proposed mechanism for the loss of sperm function, oxidative damage may play a key role (Alvarez et al., 1987; Iwasaki and Gagnon, 1992, Aitken, 1994) and evidence now suggests that the damage to sperm, caused by oxygen-derived oxidants called reactive oxygen species, is a significant contributing factor in up to 30% of the cases of male infertility (Iwasaki and Gagnon, 1992; Ochsendorf et al., 1994; Shekarriz et al., 1995).

Infection of the male genital tract is a known major cause of the generation of reactive oxygen species (ROS) in the human ejaculate (Comhaire et al., 1999; Ochsendorf, 1999), and it has been strongly associated with sperm damage and male infertility (Fraczek and Kurpisz, 2007; Tremellen, 2008). The following review discusses the generation of ROS in the ejaculate and their role in the pathology of human sperm, with particular interest in the relationship between male genital tract infection and the mechanisms of sperm damage.

1.2 Reactive Oxygen Species

Reactive oxygen species (ROS) are byproducts of the metabolism of oxygen by cells and they include free radicals and peroxides (Valko et al., 2007). The most common ROS that have effects on human reproductive biology include the superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), peroxyl radicals ($ROOH^{\bullet}$), and the hydroxyl radical ($OH^{\bullet}$) (de Lamirande et al., 1997). Free radicals are molecules that have one or more unpaired electrons in their outer orbitals. The unpaired electrons confer a high oxidative potential to these ROS and a short half-life of nanoseconds, which means they practically react at the site of generation. As a consequence of their high reactivity, free radicals readily take part in the oxidation of biomolecules such as

lipids, proteins, nucleic acids and hyaluronic acid, and this oxidative damage is a key component of pathological processes such as inflammation, carcinogenesis and ageing (Halliwell and Gutteridge, 1989; Sies, 1991; Fuchs et al., 1997). The most commonly produced free radicals are illustrated in Table 1.

Non-radical ROS that are either oxidizing agents or easily converted into free radicals include hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl). Hydrogen peroxide is uncharged and, unlike the $O_2^{\bullet}$ anion and other free radicals, it can cross cell membranes. Perhaps its most damaging quality is that it can be split into the very dangerous $OH^{\bullet}$ radical when it accepts an electron from ferrous or cuprous ions in a reaction known as Fenton reaction (Table 1) (Ochsendorf, 1999). In addition, hydrogen peroxide is a major secretory product of white blood cells (Klebanoff and Clark, 1978), and these cells are a feature of male genital tract infection. Table 2 describes the most common non-radical ROS; hydrogen peroxide and hypochlorous acid.

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Table 1: Reactive oxygen species: most common free radicals.

Species	Generation	Half-life	Distance$^{\Psi}$	Effect
Hydroxyl radical (OH$^{\bullet}$)	Metal ion-dependent breakdown of hydrogen peroxide. Fenton reaction: $H_2O_2 + 1e^-(Fe^{2+}/Cu^+) \rightarrow Fe^{3+}/Cu^{2+} + OH^{\bullet} + OH^-$ Haber-Weiss reaction: $O_2^{-\bullet} + H_2O_2 \rightarrow O_2 + H_2O + OH^{\bullet}$	0.3ns	1.8nm	Very strong oxidant; responsible for initiation of lipid peroxidation and protein oxidation.
Superoxide anion (O$_2^{-\bullet}$)	In mitochondria and endoplasmic reticulum during electron transport; first intermediate in the reduction of molecular O_2 by the respiratory chain. $O_2 + 1e^- \rightarrow O_2^{-\bullet}$ Generated through NADPH oxidase during phagocytosis. $NADPH + 2O_2 \rightarrow NADP^+ + H^+ + 2O_2^{-\bullet}$	0.4μs-1ms	55nm-3μm	Reducing or oxidizing dependent on the redox properties of its reactant; dismutates to hydrogen peroxide.

$^{\Psi}$ distance ROS can travel.

Table 2: Reactive oxygen species: most common non-radical oxidants.

Species	Generation	Half-life	Distance$^{\psi}$	Effect
Hydrogen peroxide (H_2O_2)	Mitochondria during oxidative phosphorylation; in peroxisomes in fatty acid metabolism; by-product of SOD-catalyzed dismutation. $$2\ O_2^{\cdot-} + 2\ H^+ \rightarrow H_2O_2 + O_2$$	Persistent		Very strong oxidant; responsible for initiation of lipid peroxidation and protein oxidation.
Hypochlorous acid (HOCl)	$H_2O_2 + Cl^- \rightarrow HOCl + OH^-$ **(hypochlorous acid formation)** $HOCl + O_2^{\cdot-} \rightarrow O_2 + Cl^- + OH^{\cdot}$ **or** $HOCl + Fe^{2+} \rightarrow Fe^{3+} + Cl^- + OH^{\cdot}$ **(hydroxyl formation)** Activated neutrophils in response to infectious agents; reaction is catalyzed by peroxidase-myeloperoxidase (MPO); reacts with $O_2^{\cdot-}$ to form more ROS.	Persistent		Highly toxic with detrimental effects for neighboring cells; able to degrade biomolecules either directly or by decomposition into chlorine.

$^{\psi}$ distance ROS can travel; **SOD** superoxide dismutase.

1.3 ROS production by Spermatozoa

Spermatozoa, like all cells living under aerobic conditions, produce ROS mostly as products of normal metabolism. These ROS, mostly free radicals, are produced in mitochondria during a stepwise reduction of oxygen to generate energy. However, mitochondria are not the only source of ROS in the mammalian germ line. Immature sperm are reported to generate significantly higher levels of ROS (Gil-Guzman et al., 2001; Ollero et al., 2001; Moustafa et al., 2004). This is because immature sperm are often characterized by the presence of excess cytoplasmic residues around the midpiece region (Aitken, 1999) and these residues are rich in the enzyme glucose-6-phosphate dehydrogenase, an enzyme which controls the intracellular production of β-nicotinamide adenine dinucleotide phosphate (NADPH) through the hexose monophosphate shunt. NADPH fuels the generation of ROS via the NADPH oxidase which is located in the plasma membrane (Gomez et al., 1996; Fisher and Aitken, 1997).

However, even normal human sperm are thought to have a sperm-specific and calcium-dependant NADPH oxidoreductase that is located in the mitochondrial respiration system in the sperm midpiece (Banfi et al., 2001; Armstrong et al., 2002) which produces ROS. Other ROS-generating systems that have been described in mammalian sperm include lipoxygenase (Oliw and Sprecher, 1989) and cytochrome P450 (Baker et al., 2004).

1.4 ROS production by Leukocytes

Sperm are not the only source of ROS in seminal plasma. White blood cells (leukocytes) are the major source of free radicals in the ejaculate and they are reported to produce up to 1000 times more ROS than sperm (Plante et al., 1994; de Lamirande and Gagnon, 1995). Leukocytes, which are attracted by pathogens and tissue damage to the site of infection, release 'bursts' of the highly aggressive ROS as a first-line of defence against invading organisms (Klebanoff and Clark, 1978; Depuydt et al., 1996). However, even under physiological conditions, these immune cells are a common feature of most parts of the male reproductive tract and the ejaculate (Aitken et al., 1994; Aitken and Baker, 1995), of which 50-60% are

polymorphonuclear granulocytes, 20-30% are monocytes, and 2-5% are lymphocytes (Wolff and Anderson, 1988; Wolff, 1995).

The presence of leukocytes in the ejaculate is not pathological in itself. The mean number of leukocytes in the seminal fluid of healthy men has been reported to be 17×10^6/mL of semen (Wolff and Anderson, 1988). According to the World Health Organization (WHO, 1999), a condition in which the concentration of leukocytes in the ejaculate is greater than 1×10^6 cells/mL is defined as leukocytospermia. This is generally considered as a clinical sign of infection or inflammation of the accessory sex organs, or of the lower genitourinary tract (Wolff and Anderson, 1988; Barratt et al., 1990). However, this value is empirical (Aitken and Baker, 1995) and was regarded as too high by some authors (Menkveld and Kruger, 1998; Sharma et al., 2001; Punab et al., 2003; Henkel et al., 2005).

The role played by leukocytes in semen is subject to much controversy (Wolff, 1995). As the production of ROS is one of the principal mechanisms by which white blood cells destroy pathogens, it is not surprising that seminal leukocytes have been associated with excessive generation of ROS in the ejaculate (Aitken et al., 1994; Whittington et al., 1999; Sharma et al., 2001; Henkel et al., 2003a; 2005). Furthermore, the production of ROS by leukocytes in the ejaculate is non-specific, and leukocytospermia has been associated with poor sperm morphology (Menkveld and Kruger, 1998; Menkveld et al., 2003), increased abnormalities of the sperm midpiece (Thomas et al., 1997), and decreased sperm motility (Diemer et al., 2003a). However, some studies have found no significant negative influence of seminal leukocytes on semen quality or sperm function (Christiansen et al., 1991; Tomlinson et al., 1992; 1993; Rodin et al., 2003).

In addition, the activation status of leukocytes appears to have an important influence on ROS production in the ejaculate (Tremellen, 2008). The activation of leukocytes is regulated by peptides called cytokines that are produced by leukocytes and other cells to influence the growth, differentiation or function of cells via autocrine or parachrine mechanisms (Ochsendorf, 1999). Recent data suggest that cytokines and ROS may interact and bring about the toxic effects of infection/inflammation in the male genital tract (Tremellen, 2008). This is supported by the positive correlation observed between seminal ROS and pro-inflammatory cytokines such as interleukin-6

(Camejo et al., 2001; Nandipati et al., 2005), interleukin-8 (Rajasekaran et al., 1995; Martinez et al., 2007), and tumour necrosis factor (Sanocka et al., 2003; Martinez et al., 2007). Interleukin-1 is reported to stimulate granulocytes and macrophages to produce ROS and secrete other cytokines (Depuydt et al., 1998), while interleukin-8 is thought to exert a negative effect on sperm fertilizing potential (Rajasekaran et al., 1995). Interleukin-8 has also been considered to be a potential marker for male genital tract infection (Koumantakis et al., 1998).

Other possible mechanisms of the development of infection-related infertility are: obstruction of the seminal tract (Keck et al., 1998), deterioration of spermatogenesis (Diemer et al., 2003b; Fraczek et al., 2007), and dysfunction of the accessory glands (Comhaire et al., 1999). These mechanisms are strongly associated with leukocytospermia, in general, and the high production of ROS by the seminal leukocytes, in particular (Berger et al., 1982; Sharma et al., 2001; Henkel et al., 2005).

The basic method of detecting leukocytes in semen samples is by direct observation with a bright field microscope (Menkveld and Kruger, 1998; Menkveld et al., 2003). However, this method is inadequate because it does not distinguish between leukocytes and immature germinal epithelial cells (Wolff, 1995). Hence, other techniques that differentiate leukocytes from germ cells such as the Papanicolaou method (Menkveld and Kruger, 1998), monoclonal antibodies (WHO, 1999), and polymorphonuclear elastase (Henkel et al., 2007), are considered to be reliable. However, the peroxidase stain (Endtz test) is more suitable for routine use (Wolff, 1998) because it is less time consuming and not labour intensive. Furthermore, the peroxidase stain allows for the detection of polymorphonuclear granulocytes and its results are strongly correlated to ROS production in semen (Shekarriz et al., 1995).

1.5 Antioxidants

The human body has developed several antioxidant strategies to protect itself from cellular damage induced by ROS (Sies, 1991). Antioxidants are compounds that scavenge, suppress the formation of ROS, and oppose their actions (Sikka et al., 1995). This allows for normal oxidative metabolism to occur without damage to the cells, while still allowing for normal ROS-mediated cellular responses such as destruction of infectious pathogens and intracellular signaling (Valko et al., 2007). Antioxidants play a significant protective role in reproductive biology and among the well-known enzymatic antioxidants are superoxide dismutase (SOD) and catalase which inactivate the $O_2^{\cdot-}$ radical and H_2O_2 by converting them into water and oxygen (Table 3). SOD is present within both sperm and seminal plasma (Zini et al., 1993) while catalase is lacking in spermatozoa (Alvarez and Storey, 1992). The addition of SOD to sperm in culture has been confirmed to protect the sperm from oxidative attack (Kobayashi et al., 1991), and its deficiency has been reported in infertile men (Sanocka et al., 1997; Pasqualotto et al., 2000). However, other authors have found no significant differences in SOD activity between infertile men and their fertile counterparts (Zini et al., 2000; Hsieh et al., 2002). Although catalase is only found in seminal plasma, the reported evidence supports a link between reduced seminal catalase activity and male infertility (Miesel et al., 1997; Sanocka et al., 1997; Zini et al., 2000).

Table 3: Enzymatic antioxidants: superoxide dismutase and catalase.

Enzyme	Reaction
Superoxide dismutase (SOD)	$2\,O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$ Superoxide dismutase can have 3 isoforms: cytosolic copper/zinc superoxide dismutase (Cu/ZnSOD), intra-mitochondrial manganese superoxide dismutase (MnSOD), and extracellular Cu/Zn SOD. Cytosolic SOD constitutes the major proportion of total SOD activity and probably is responsible for removing $O_2^{\cdot-}$ radicals from the cytosol. MnSOD is responsible for removing $O_2^{\cdot-}$ generated by oxidative metabolism in the mitochondria.
Catalase	$2\,H_2O_2 \rightarrow 2H_2O + O_2$ Catalase is a hydrogen peroxide detoxifier; removes this membrane permeable molecule from the media and inhibits NADPH oxidase and thus inhibits superoxide production.

Glutathione peroxidase (GPx) is another important enzymatic antioxidant. This antioxidant is found in the testis, prostate, seminal vesicles, vas deferens, epididymis, seminal plasma and spermatozoa (Vernet et al., 2004). GPx, using reduced glutathione (GSH; L-γ-glutamyl-L-cysteinyl-glycine) as an electron donor, protects sperm against ROS attack and its inhibition, *in vivo*, using mercaptosuccinate leads to a large increase in sperm lipid peroxidation (Twigg et al., 1998). Male infertility has been associated with a reduction of GPx in seminal plasma (Giannattasio et al., 2002) and spermatozoa (Garrido et al., 2004). Furthermore, men with excessive seminal ROS production that is caused by leukocytospermia, have demonstrated significantly reduced GPx activity within their spermatozoa (Therond et al., 1996). Therefore, reduced glutathione is necessary to detoxify oxidants and free radicals in sperm, and a high cellular concentration of GSH is maintained by the enzyme glutathione reductase

which catalyzes the reduction of oxidized glutathione (GSSG) (Ochsendorf et al., 1998), as illustrated in Figure 1.

(From: http//:lpi.oregonstate.edu/infocenter/vitamins/riboflavin/ribogsh.jpg)
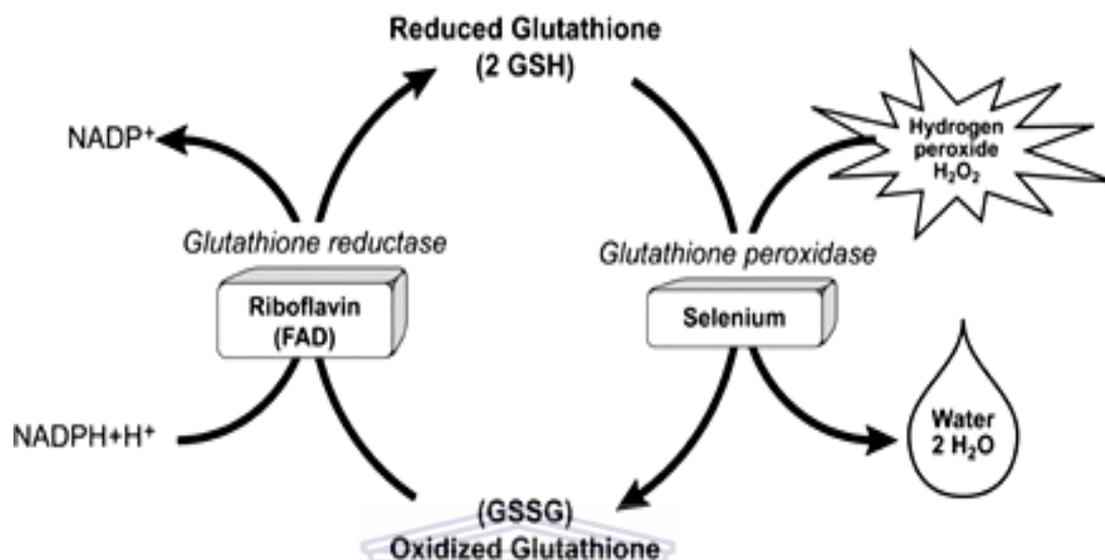
Figure 1   One molecule of hydrogen peroxide (H_2O_2) is reduced to two molecules of water while two molecules of reduced glutathione (GSH) are oxidized in a reaction catalyzed by glutathione peroxidase (a selenium-dependent enzyme). Oxidized glutathione (GSSH) is recycled by the flavin adenine dinucleotide (FAD)-dependent enzyme, glutathione reductase, using NADPH which itself is regenerated in a reaction catalyzed by glucose-6-phosphate dehydrogenase in the hexose monophosphate shunt. The restored GSH is ready to detoxify more H_2O_2. When the antioxidant system is functioning properly, most of its components are present in the reduced form; H_2O, GSH and $NADP^+$. If there is a malfunction, then the oxidized components (H_2O_2, GSSG and NADPH) tend to accumulate.

Besides the enzymatic antioxidants, small molecules that are present in semen act as ROS scavengers, and these include ascorbic acid (vitamin C), α-tocopherol (vitamin E), amino acids (taurine and hypotaurine), albumin and carnitine (Tremellen, 2008). These agents principally act to directly neutralize free radical activity. A number of

researchers have reported a significant reduction in the activity of non-enzymatic antioxidants in the seminal plasma of infertile men (Fraga et al., 1991, 1996; Smith et al., 1996; Therond et al., 1996; Lewis et al., 1997; Mostafa et al., 2006; Song et al., 2006).

The antioxidant capacity of spermatozoa is insufficient by virtue of the limited volume and the distribution of sperm cytoplasmic space. Therefore, antioxidants in the seminal plasma are essential in protecting sperm from oxidative attack. This is evident in the increased ROS production in washed sperm, especially in preparation for assisted reproductive techniques (Plante et al., 1994; Twigg et al., 1998). Furthermore, repeated centrifugation separates spermatozoa from seminal plasma and this leads to a 20- to 50-fold increase in levels of ROS (Aitken and Clarkson, 1987; Iwasaki and Gagnon, 1992). However, during spermatogenesis and epididymal storage, sperm are not in contact with seminal antioxidants and must rely on epididymal and their intrinsic antioxidant capacity. Sperm are therefore vulnerable to oxidative damage during epididymal transit, especially in the presence of epididymal inflammation caused by male genital tract infection.
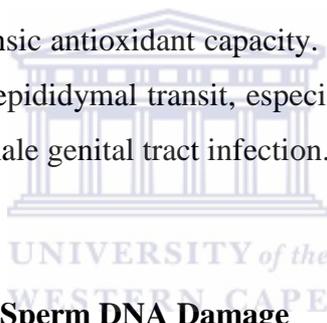
1.6 Oxidative Stress and Sperm DNA Damage

As described above, aerobic metabolism entails the production of ROS even under basal conditions. Hence, there is a continuous requirement for the inactivation of these ROS (Sies, 1991). However, at physiological concentrations, ROS are beneficial to normal sperm function, and they are associated with sperm capacitation and hyperactivation (de Lamirande and Gagnon, 1993; Aitken and Fisher, 1994; de Lamirande et al., 1997), thereby assisting sperm in their transit through the cumulus and zona pellucida. It is obvious, then, that in the male reproductive system, a balance has to be maintained between the generation of ROS and their scavenging by the battery of antioxidants in the seminal plasma and in the sperm (Alvarez et al., 1987; Halliwell and Gutteridge, 1989; Sikka et al., 1995). However, increased ROS production or deficient antioxidant protection causes this balance to shift in favour of oxidants, potentially leading to cellular or genetic damage - this condition is called 'oxidative stress'.

The underlying mechanism of sperm damage, which is induced by oxidative stress, is peroxidation of the sperm plasma membrane (Jones et al., 1979). The plasma membrane of human sperm is particularly vulnerable to ROS attack because it has an exceptionally high content of polyunsaturated fatty acids (Alvarez et al., 1987; Zalata et al., 1998). The ROS-induced peroxidation reduces sperm membrane fluidity and tail motion and, therefore, less sperm reach the oocyte for fertilization (Whittington et al., 1999; Kao et al., 2008). Moreover, ROS not only damage the sperm membrane, but are also known to attack the sperm genome (Twigg et al., 1998).

Several investigators have directly linked ROS production to sperm nuclear DNA damage (Kodama et al., 1997; Lopes et al., 1998; Saleh et al., 2002a; Wang et al., 2003; Henkel et al., 2005). Normally, sperm DNA is tightly packaged by protamines and this protects its purine and pyrimidine bases and the deoxyribose backbone from free radical attack (Ward and Coffey, 1991). However, this protamination is deficient in some infertile men (Balhorn et al., 1988; Aoki and Carrell, 2003; Oliva, 2006), and their sperm DNA is exposed to ROS-induced oxidation. Furthermore, DNA damage is significantly greater in leukocytospermic semen samples (Alvarez et al., 2002; Henkel et al., 2003a; 2003b) and in infertile men who may have reduced antioxidant protection (Pasqualotto et al., 2000).

The negative impact of sperm DNA fragmentation on fertilization and pregnancy has been repeatedly and clearly demonstrated (Figure 2). Sperm DNA damage was associated with poor motility and abnormal morphology (Huang et al., 2005; Sergerie et al., 2005), and reduced acrosome reaction (Ozmen et al., 2007). Furthermore, high incidence of sperm DNA fragmentation has been observed among couples with unexplained infertility and recurrent pregnancy failure (Host et al., 2000; Carrell et al., 2003). This negative influence of sperm nuclear DNA damage has been demonstrated for intrauterine insemination (IUI) (Duran et al., 2002) and *in vitro* fertilization (IVF) (Henkel et al., 2004). Even in natural conception, oxidative sperm DNA damage adversely affects human fertility and prolongs the time to pregnancy (Spanò et al., 2000; Loft et al., 2003). Despite this, there is evidence to suggest that sperm with fragmented DNA are still able to fertilize an oocyte but further development stops once the defective paternal genome is switched on, resulting in failed pregnancy (Twigg et al., 1998; Ahmadi and Ng, 1999; Henkel et al., 2004). Fragmented DNA appears to cause of poor embryo quality, poor blastocyst

development and even early embryo death (Tomsu et al., 2002; Zorn et al., 2003; Seli et al., 2004; Meseguer et al., 2008). Assisted reproductive techniques, such as intracytoplasmic sperm injection (ICSI), circumvent natural barriers to fertilization and allow sperm with damaged DNA to achieve blastocyst formation and even full-term pregnancy (Gandini et al., 2004). However, there is rising concern that the genetic damage may be transmitted to the offspring, causing chromosomal abnormalities, birth defects, childhood cancer (Aitken and Krausz, 2001; Aitken and De Iuliis, 2007), and even infertility (Silber and Repping, 2002).
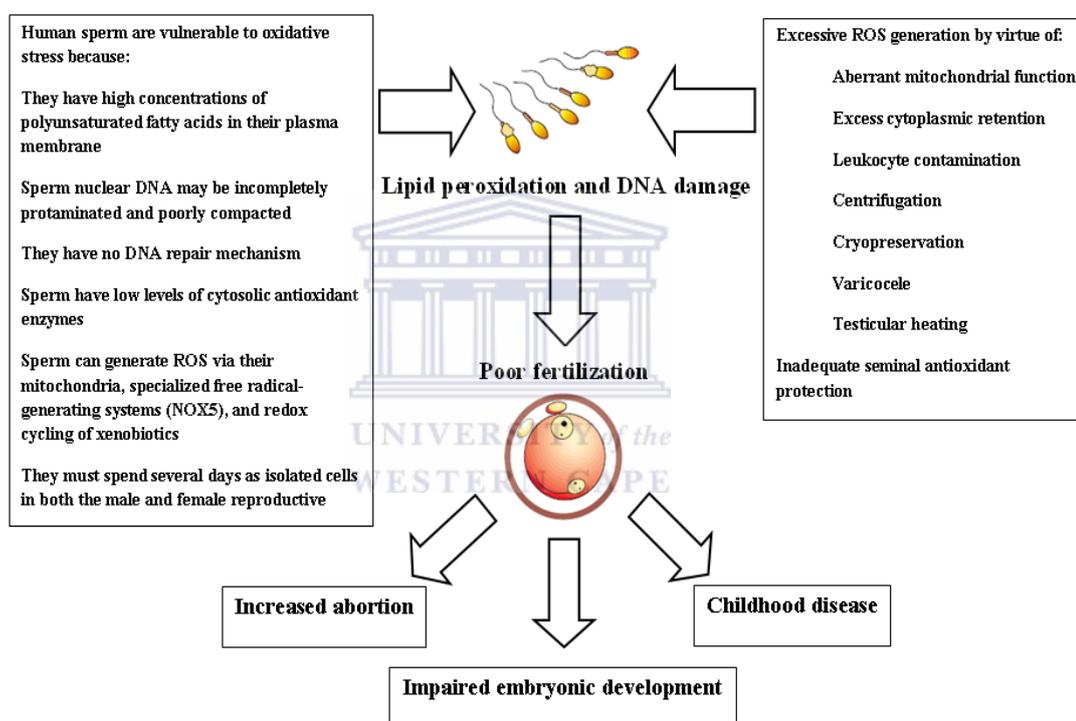
Figure 2 An assortment of factors can conspire to induce oxidative stress and nuclear DNA damage in spermatozoa emerging from the male reproductive tract. Studies have demonstrated the consequences of damage to the male genome, and these include infertility, poor blastocyst formation and embryo development, and increased abortion. Moreover, there is rising concern that genetic damage may be transmitted to the offspring and may cause childhood disease such as cancer. (Adapted from Aitken and Krausz, 2001).

Several investigators have confirmed the damaging effect of oxidative stress on the sperm genome (Kodama et al., 1997; Aitken et al., 1998; Saleh et al., 2002a; Wang et al., 2003; Moustafa et al., 2004; Henkel et al., 2005). Furthermore, studies show that sperm DNA damage is predictive of fertilization and pregnancy after natural conception and following the use of assisted reproduction such as IUI (Duran et al., 2002), IVF (Host et al., 2000; Henkel et al., 2004), and ICSI (Lopes et al., 1998; Henkel et al., 2003b; Tesarik et al., 2004). For these reasons, various authors have suggested that tests for DNA integrity or damage should be introduced into the routine andrological work-up (De Jonge, 2002; Perreault et al., 2003; Sharma et al., 2004; Tesarik et al., 2004; Henkel et al., 2005). A number of tests have been developed to assess sperm DNA damage, and these include sperm chromatin structure assay (SCSA), terminal deoxynucleotide transferase-mediated dUTP nick-end labelling (TUNEL) assay, and measurement of 8-hydroxydeoxyguanosine (8-OHdG). The TUNEL assay is highly specific and easily reproducible and, therefore, is one of the most frequently used to test to investigate sperm DNA fragmentation (Henkel, 2007).
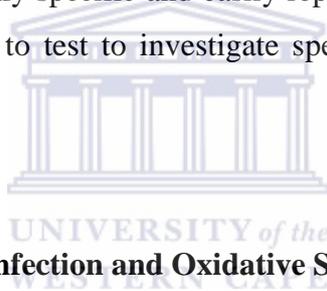
1.7 Male Genital Tract Infection and Oxidative Stress

The causes of oxidative stress are manifold (Figure 2). They include smoking (Fraga et al., 1996), excessive alcohol consumption (Maneesh et al., 2006), environmental pollution (Chitra et al., 2001); chronic systemic disease (Oberg et al., 2004; Agbaje et al., 2007), and infection (Mazzilli et al., 1994; Depuydt et al., 1996; Ochsendorf, 1999; Potts et al., 2000; Brackett et al., 2008). Among the latter, male genital tract and accessory gland infections play a significant, and yet potentially correctable, role in male infertility (Cunningham and Beagley, 2008; Pellati et al., 2008; Schuppe et al., 2008; Tremellen, 2008). The prevalence of infertility that is related to male genital tract infection varies from 10% to 20% in randomly selected subjects (WHO, 1999), and up to 35% in a study of over 4000 patients consulting for infertility (Henkel et al., 2007). Reportedly, up to 50% of men suffer from prostatitis at some point in their lives, with the condition becoming chronic in 10% of men (Schaeffer, 2003). The majority of these infections are self-limiting (Weidner et al., 1999), and

the men do no present with clear symptoms of genital tract infection (Gonzales et al., 2004), so most these men will not seek treatment.

In male genital tract infection, activated leukocytes generate excessive ROS and secrete cytokines that have been negatively correlated with fertility (Depuydt et al., 1998) and semen quality (Eggert-Krause et al., 2001). The highly oxidant ROS cocktail released by leukocytes, such as $OH^{\bullet}$, $O_2^{-\bullet}$ and the persistent H_2O_2, can induce lipid peroxidation (Zalata et al., 1998) and substantial sperm DNA fragmentation (Alvarez et al, 2002), especially in infertile patients with deficient antioxidant capacity (Pasqualotto et al., 2000). It is also worth considering that, in cases of longstanding male genital tract infection and inflammation such as orchitis, epididymitis (Weidner et al., 1999), and even prostatitis (Henkel et al., 2006), spermatozoa are exposed to activated leukocytes and their products for long periods during their maturation. Therefore, causality between leukocytes in the ejaculate and DNA fragmentation in spermatozoa should not be underestimated (Figure 3).

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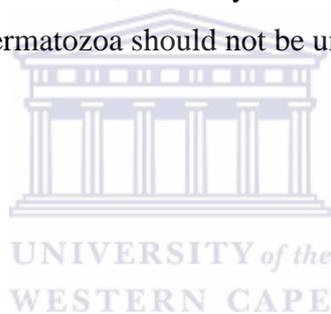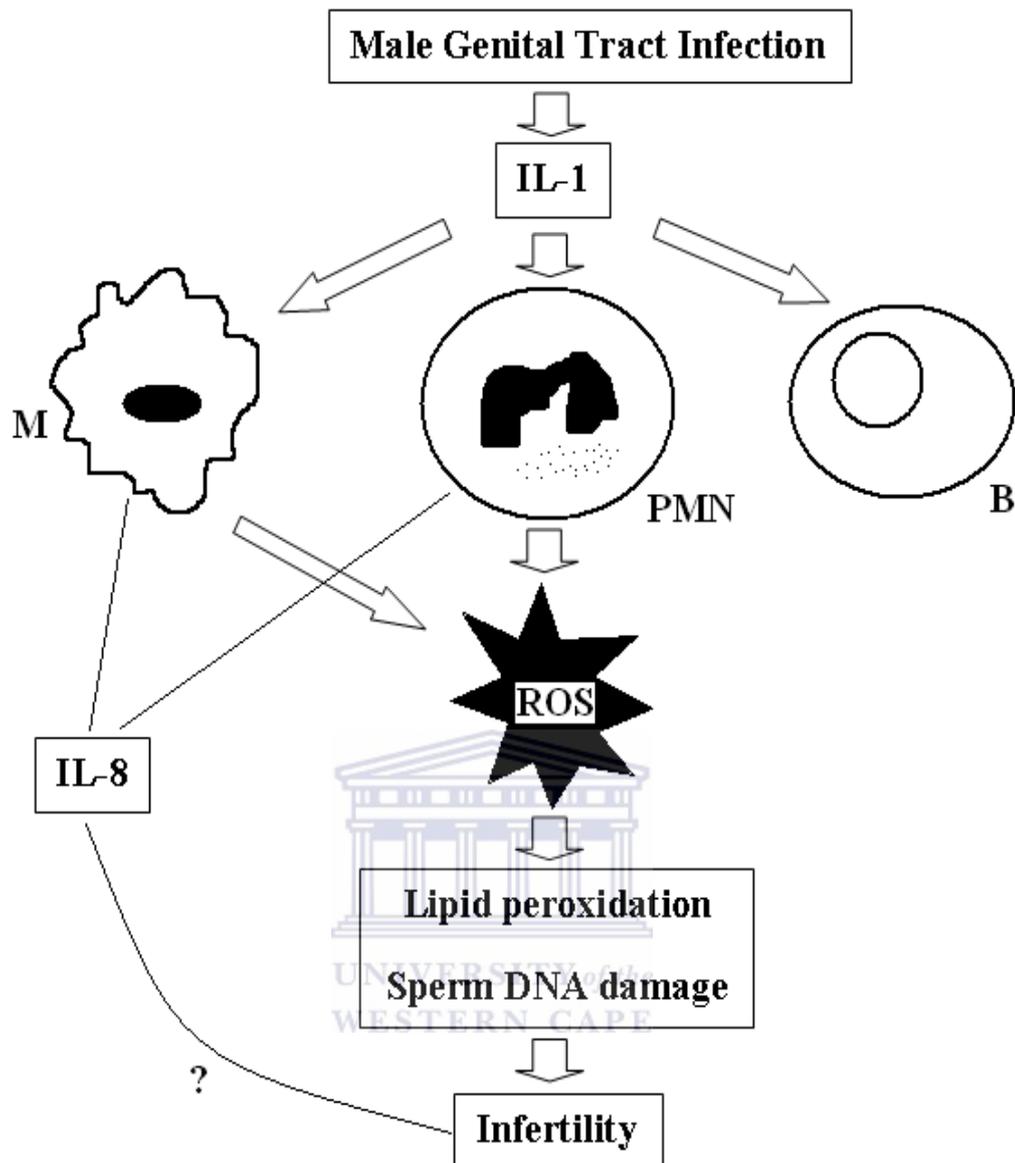
Figure 3   Overview of some of the harmful effects of male genital tract infection on leukocytes in the ejaculate, cytokines, and sperm function. Interleukin-1 (IL-1) is reported to stimulate seminal leukocytes, among which activated polymorphonuclear (PMN) leukocytes and macrophages (M) release reactive oxygen species (ROS) into the seminal plasma. These ROS, such as hydrogen peroxide and superoxide anion, cause lipid peroxidation and sperm DNA damage which contribute to infertility. Interleukin-8 (IL-8) is also reported to have a negative effect on sperm fertilizing potential. Lymphocytes (B) have not been directly associated with male infertility.

1.8 Apoptosis

Apoptosis is a mode of cell death that involves a series of genetically-regulated morphological and biochemical alterations leading the cell to controlled death (Kerr et al., 1972). In the male reproductive tract, it seems clear that this programmed cell death is a key phenomenon during spermatogenesis (Gorczyca et al., 1993; Sun et al., 1997; Lopes et al., 1998; Sakkas et al., 1999; Barroso et al., 2000; Irvine et al., 2000). Recently, several features that are characteristic of apoptosis in somatic cells have been described in human spermatozoa. Ejaculated sperm, particularly from infertile men, have been reported to show Fas expression (Sakkas et al., 2002; Taylor et al., 2004), translocation of phosphatidylserine (Henkel et al., 2004), and reduction in mitochondrial membrane potential (MMP; $\Delta\psi_m$) (Paasch et al., 2004). Caspases (aspartic acid-directed cysteine proteases) are also intimately involved in apoptosis and they have been described in human spermatozoa (Wang et al., 2003). To date, 14 caspases have been identified in humans, and they have been divided into two functional subgroups; initiating caspases (caspase-6, -8, -9, and -10), and effector caspases (caspase-2, -3, and -7) (Nicholson, 1999). Caspases, initially inactive, are activated by a cascade of apoptotic events which, ultimately, converge on caspase-3; the executioner. This caspase then irreversibly activates specific enzymes that degrade the nuclear DNA (Earnshaw et al., 1999). In sperm, as in somatic cells, DNA fragmentation is regarded as a late feature and a hallmark of apoptosis, and it is often preceded by early markers of programmed cell death, such as caspase activation, Fas expression or $\Delta\psi_m$ disruption (Oosterhuis et al., 2000).

Although a number of pathways for the activation of the apoptotic cascade have been cited, the evidence strongly supports two: the death receptor (extrinsic) pathway, and the mitochondrial (intrinsic) pathway (Reed, 2000; Green, 2003). In the extrinsic pathway, death receptors of the tumour necrosis factor family, including Fas, transmit signals via the Fas-associated death domain (FADD) and this triggers caspase-8 which in turn activates caspase-3 (Nagata, 2000). In the intrinsic pathway, on the other hand, apoptosis is induced by signals which include a variety of proteins (Bak, Bax or BH1) that activate the mitochondria to release cytochrome c (Liu et al., 1996). ROS can also trigger apoptosis via the intrinsic pathway by destabilizing the mitochondrial membrane and disrupting the electron transfer chain (Green and Reed, 1998). Cytocrome c enters the cytosol and cleaves caspase-9 which in turn activates

caspase-3. Friedlander (2003) demonstrated a significant positive correlation of endogenous generation of ROS with oxidative stress-induced sperm damage. They proposed that the exposure of mitochondria to ROS leads to the release of an apoptosis-inducing factor (AIF), which directly interacts with DNA and leads to DNA fragmentation (Cande et al., 2002; Friedlander, 2003). In addition, positive correlation was reported to exist between increased ROS in the ejaculate and high levels of cytochrome c, caspase-3 and -9 (Wang et al., 2003). Therefore, it is conceivable that exogenous ROS, such as from activated seminal leukocytes in the presence of male genital tract infection, can induce mitochondrial damage which can lead to sperm DNA fragmentation and apoptosis via the mitochondrial apoptotic pathway (Figure 4).
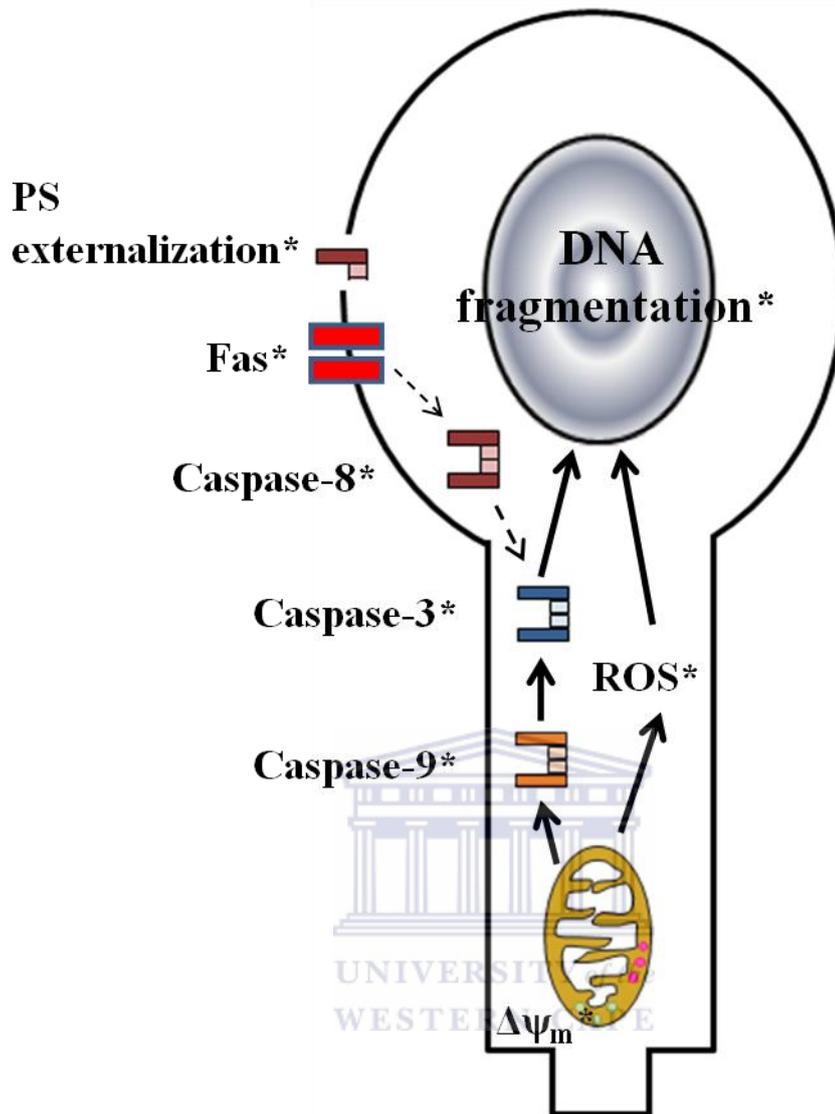
Figure 4   Proposed apoptotic signaling pathways in ejaculated spermatozoa of infertile men. Apoptotic signaling pathways in sperm are extrapolated from current knowledge of apoptosis in somatic cells. (* indicates apoptosis-related markers found in spermatozoa of infertile men). $\Delta\psi_m$ = mitochondrial membrane potential; ROS = reactive oxygen species; Fas = fibroblast-associated death receptor; PS = phosphatidylserine. (Figure adapted from Marchetti and Marchetti, 2007).

1.9 Aim of the Study

From the literature, it is clear that ROS play an important role in the normal physiology and pathology of the male germ cell. Furthermore, the human sperm is particularly susceptible to oxidative attack, not only for the high lipid content of its plasma membrane, but also because of their inadequate intrinsic antioxidant store. This vulnerability of sperm becomes important when the more robust seminal antioxidant system either malfunctions, or is overwhelmed by excessive ROS generation; as may be the case in the presence of activated seminal leukocytes. Considering that oxidative stress has been strongly and repeatedly linked to DNA fragmentation; a late event of apoptosis, it is possible that male genital tract infection and the attendant infiltration of activated leukocytes into the reproductive tract and the ejaculate, may induce apoptosis in human sperm.

The aim of this study is to investigate the relationship between:

1. male genital tract infection as indicated by the presence of leukocytes in the ejaculate;

2. the oxidative status in sperm and the ejaculate by evaluating the generation of ROS by sperm and in the ejaculate, and assessing the antioxidant activity in sperm by measuring the concentration of reduced glutathione;

3. and the markers of sperm apoptosis: $\Delta\psi_m$ disruption and caspase-3/7 activation in sperm as early markers of apoptosis, and sperm nuclear DNA fragmentation as a late apoptotic event.

CHAPTER TWO

MATERIALS AND METHODS

Figure 5 illustrates the experimental set-up of the study.
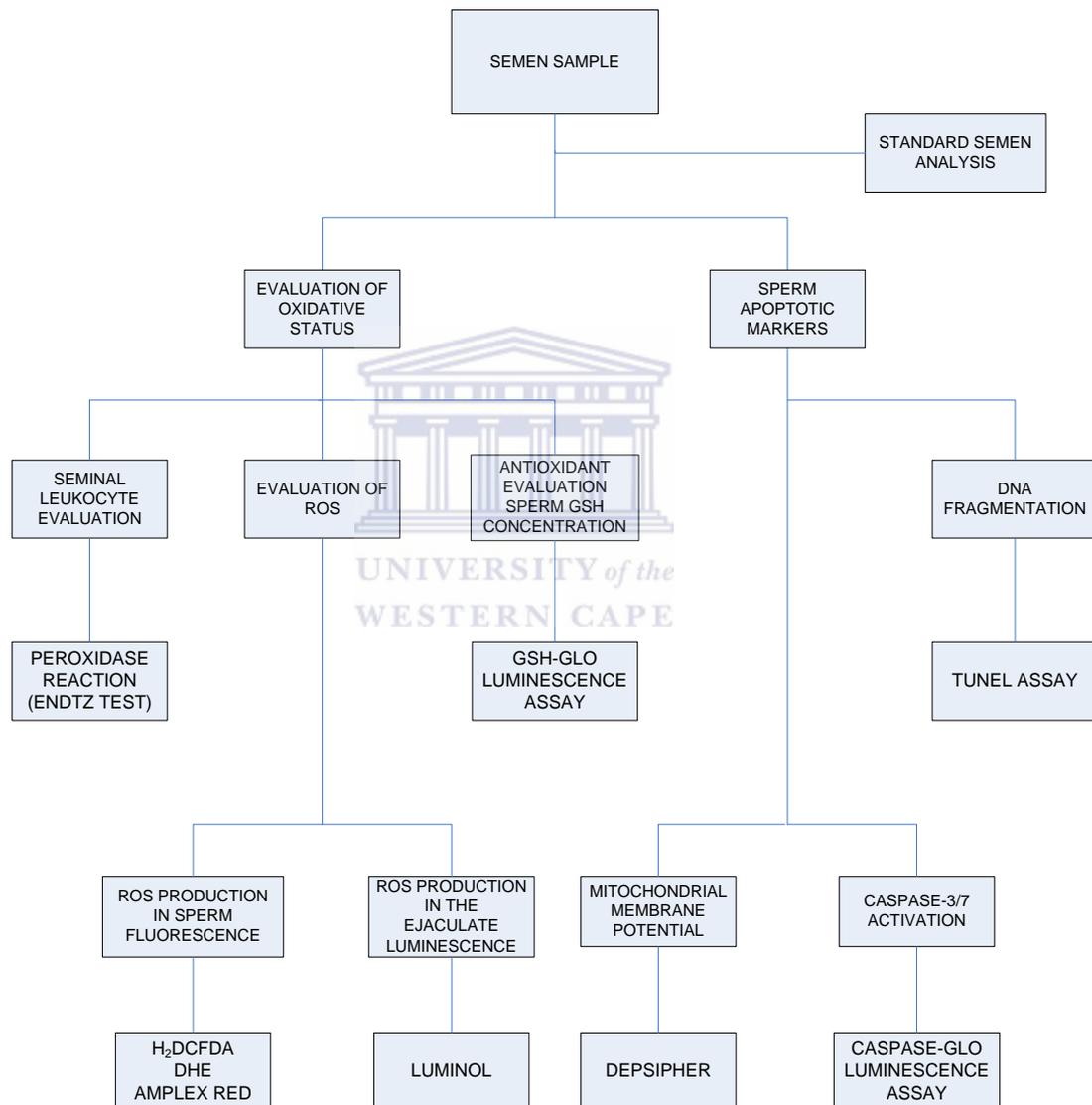
Figure 5 The study had three experimental sub-groups: a standard semen analysis; evaluation of oxidative status, in spermatozoa and in the ejaculate; and assessment of sperm apoptotic markers. ROS = reactive oxygen species; GSH = reduced glutathione; DHE = dihydroethidium; H_2DCFDA = 2,7-dichlorofluorescein diacetate.

2.1 Sample Collection

Semen samples were obtained from 60 men who attended the Reproductive Biology Unit in the Department of Obstetrics and Gynaecology, University of Stellenbosch at the Tygerberg Academic Hospital, Tygerberg, South Africa; and Vincent Pallotti Hospital, Pinelands, South Africa, for fertility problems or assisted reproduction treatment. The samples were collected by masturbation into sterile plastic cups following 2-4 days of sexual abstinence. This study was approved by the Research Committee at the Faculty of Science, University of the Western Cape, and ethical clearance was obtained by the relevant Institutional Review Boards.

2.2 Conventional Sperm Parameters

Semen analysis was performed by experienced scientists to determine the sperm concentration, the percentage of motile sperm, and the sperm forward progression according to the World Health Organization criteria (WHO, 1999). For the sperm forward progression, a grading system on a scale of 1-4 was used (Hotchkiss et al., 1938) which was then assigned arbitrary values to allow for statistical analysis (Table 4).

Table 4: Forward progression grading system.

Hotchkiss Scale	Arbitrary grading	Description of movement
0	0	No movement
1	1	Movement – no forward
1+	1.33	Movement – forward now and then
2	2	Movement – slow and undirected
2+	2.33	Movement – slow but directed forward
3-	2.66	Movement – fast but undirected
3	3	Movement – fast and directed forward
3+	3.33	Movement – very fast and directed forward
4	4	Movement – extremely fast and directed forward

2.3 Oxidative Status in the Ejaculate and in Sperm

2.3.1 Concentration of Peroxidase-positive Leukocytes

The concentration of leukocytes in the ejaculate was determined using the peroxidase stain (Shekarriz et al., 1995). A benzidine stock was prepared by dissolving 125 mg of benzidine (4,4-diaminobiphenyl; Sigma Chemical Co., St Louis, MO, USA) in a mixture of 50 mL of distilled water, and 50 mL of 96% ethanol (Merck, Johannesburg, South Africa) at room temperature (RT). This solution was prepared in 4 mL aliquots and stored away from light at 4°C. To prepare a working solution, 5 µL of 30% hydrogen peroxide (Chemical and Laboratory Suppliers, Cape Town, South Africa) was added to the 4 mL aliquots of the stock solution. In the staining procedure, 20 µL of liquefied semen were mixed with 20 µL of the benzidine working

solution in a 1.5 mL reaction tube (Greiner Bio-One GmbH, Frickenhausen, Germany), and allowed to stand for 5 minutes at RT. Peroxidase-positive leukocytes stained brown (Figure 6) and these were counted in a Makler Chamber (Sefi-Medical Instruments, Haifa, Israel) using a light microscope at ×400 magnification. The results were corrected for dilution and recorded as the number of peroxidase-positive cells ($\times 10^6$)/mL of semen. Ejaculates with leukocyte counts of greater than 1×10^6/mL were classified as leukocytospermic in accordance with the WHO (1999).
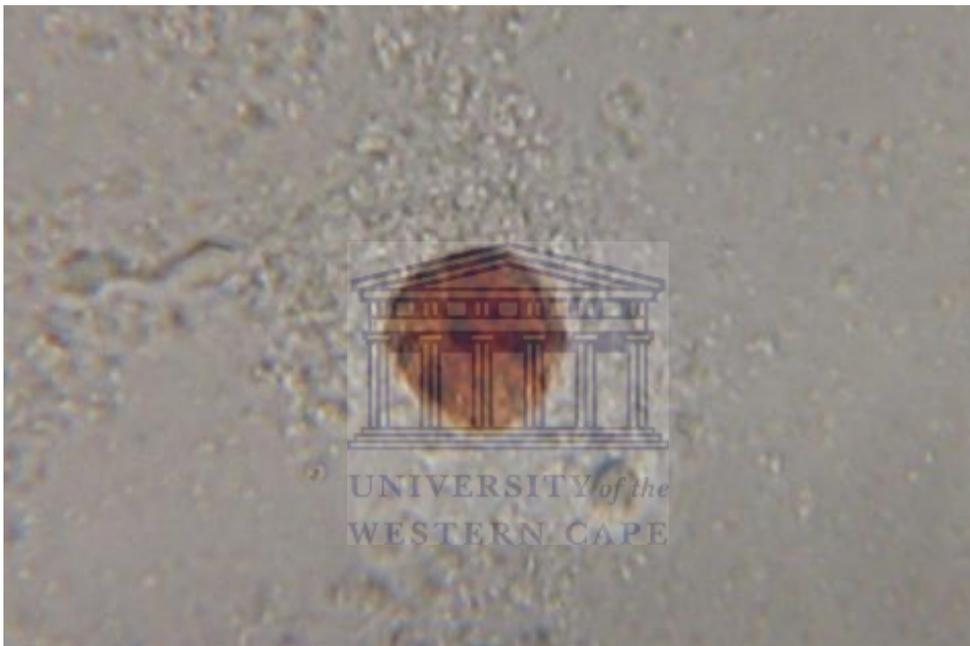
Figure 6 A seminal leukocyte after the peroxidase stain. (×1000 magnification)

2.3.2 Measurement of ROS production in the Ejaculate

The generation of ROS in the ejaculate was measured by using a chemiluminescence assay with 4 mmol/L luminol (5-amino-2,3 dihydro-1,4 phalazinedione; Sigma) as described by Shekarriz et al. (1995). Luminol is a sensitive chemiluminescence probe that reacts with a variety of ROS including hydrogen peroxide, hydroxyl radicals and the superoxide anion (Aitken and West, 1990). A Berthold luminometer (MicroLumat *Plus* LB 96 V, Bad-Wildbad, Germany) was used to inject the luminol and to read the luminescence. Liquefied semen was diluted 1:5 in human tubal fluid (Quinn et al., 1985) medium supplemented with 10 mg/mL human serum albumin

(HTF-HSA), and then centrifuged for 10 minutes at 300×g at RT. After discarding the supernatant, the resultant pellet was re-suspended in 750 µL of HTF-HSA medium and then 250 µL of suspension were transferred to 2 wells of a white microtitre plate (Dynatech MicroFLUOR™, Chantilly, VA, USA). The first well in each row was designated a negative control and contained 250 µL of HTF-HSA medium only. The luminometer was set in the integrated mode to inject 25 µL of luminol into each well and read the luminescence for 10 minutes. Background luminescence which was obtained from the negative control reading was subtracted from all test values. The concentration of ROS was expressed as relative light units ($\times 10^4$ RLU) per 20×10^6 sperm. All readings were performed in duplicate.

2.3.3 Measurement of Hydrogen Peroxide

Hydrogen peroxide (H_2O_2) is cell permeable and plays an important role in sperm mitochondrial membrane potential disruption and induction of DNA strand breaks. However, the methods of measuring this highly reactive oxidant are controversial. For this reason, 2 fluorescent probes were used to measure intracellular production of H_2O_2 by sperm.

2.3.3.1 2,7-Dihydrofluorescein diacetate.

2,7-dihydrofluorescein diacetate (H_2DCFDA, Molecular Probes, Eugene, Oregon, USA) was reconstituted to 50 µM in PBS and stored in 100 µL aliquots at -80°C protected from light. H_2DCFDA is a stable, non-fluorescent cell-permeable probe which de-esterifies in the presence of intracellular H_2O_2 to form a fluorescent 2,7-dichlorofluorescein (DCF).

Semen samples were washed in PBS and centrifuged at 300×g for 10 minutes. After centrifugation, the supernatant was discarded and the pellet was re-suspended in PBS. Following this, 20 µL of H_2DCFDA was added to 400 µL of the washed semen (final concentration of H_2DCFDA is 2.5 µM) and incubated for 45 minutes at 37°C protected from light. The mixture was then centrifuged again for 10 minutes at 300×g and the pellet was re-suspended in 400 µL of PBS. A fluorescence microscope

(Zeiss), fitted with a 488 nm excitation filter and 510-530 nm emission filter, was used to analyze the sperm at ×400 magnification. All sperm fluorescing bright green (Figure 7) were H_2O_2-positive and expressed as a percentage of the total sperm observed in that field [H_2O_2-positive (DCF) (%)].
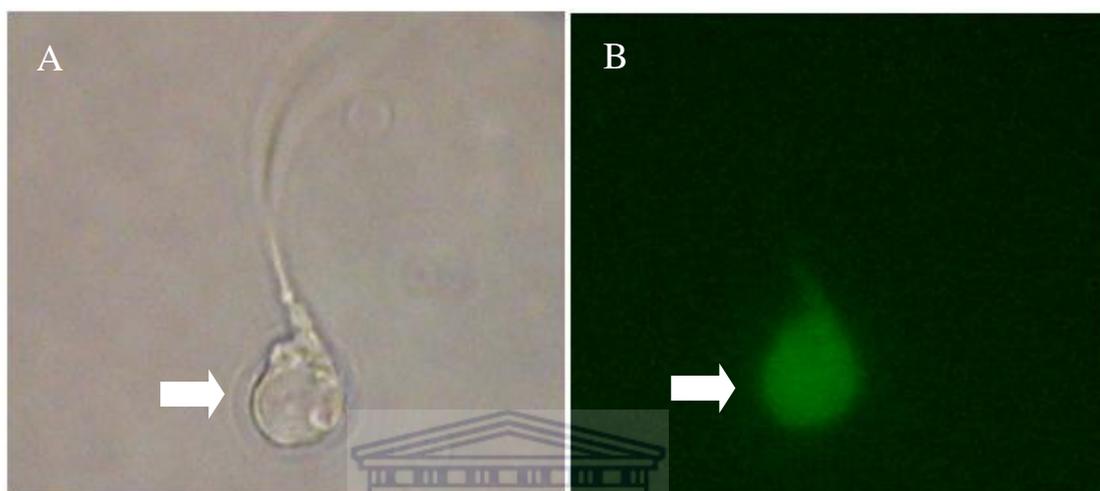
Figure 7   A sperm with a head deformity (arrow, A) was H_2O_2-positive and fluoresced bright green (arrow, B) after staining with H_2DCFDA. (×1000 magnification)

2.3.3.2 Amplex UltraRed™ and Horseradish Peroxidase (HRP)

A working solution of 100 µM Amplex UltraRed™ reagent (10-acetyl-3,7-dihydrophenoxazine, Molecular Probes), and 0.2U/mL HRP (Sigma) was prepared by mixing 50 µL of 10 mM Amplex UltraRed™ reagent stock solution, 100 µL of 10 U/mL HRP and 4.85 mL of 1X reaction buffer (50 mM sodium phosphate, Merck). This 5 mL volume was stored in 50 µL aliquots at -80°C protected from light. For each semen sample, 100 µL ejaculate was washed in PBS and centrifuged for 10 minutes at 300×g, the resultant supernatant was discarded and the pellet was re-suspended in PBS. Thereafter, 50 µL of the washed semen was mixed with 50 µL of Amplex UltraRed™ working solution, and incubated for 30 minutes at RT away from light. The Amplex UltraRed™ reagent reacted with H_2O_2 to produce a red fluorescence oxidation product called resofurin. Therefore, the sperm fluorescing red

(Figure 8) when analyzed at ×400 magnification with a fluorescence microscope (Zeiss) equipped with 488 nm excitation and 590 emission filters were considered to be H_2O_2-positive and recorded as a percentage of the total number of sperm under observation [H_2O_2-positive (Amplex) (%)].
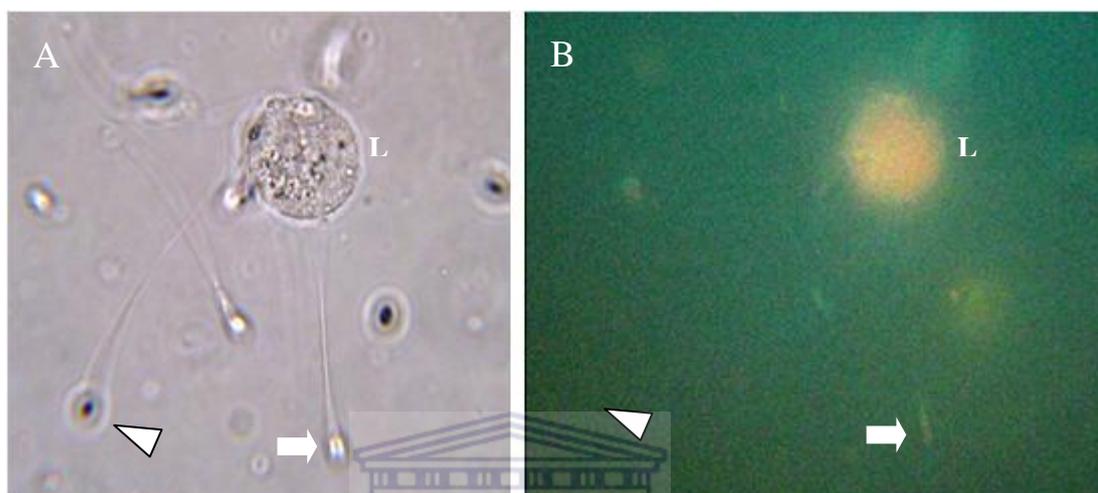
Figure 8 H_2O_2-positive sperm (white arrows) showed a red fluorescence (B) after staining with Amplex UltraRed™ and HRP. The arrow head (A) shows a H_2O_2-negative sperm which showed no fluorescence (B). The polymorphonuclear leukocyte (L) also stained positive for H_2O_2. The pictures were difficult to take because the fluorescence faded quickly after exposure to UV light. (×1000 magnification)

2.3.4 Measurement of Superoxide Anion ($O_2^{-\bullet}$)

Intracellular generation of $O_2^{-\bullet}$ was estimated using dihydroethidium (DHE, Molecular Probes), an uncharged probe that reports overall cellular $O_2^{-\bullet}$ production. When DHE is oxidized by $O_2^{-\bullet}$ within the cell, it produces DNA sensitive fluorochromes that generate a orange/red nuclear fluorescence when excited at 488 nm (Rothe and Valet, 1990). For this assay, a 20 μM DHE stock solution was prepared by dissolving 6.3 mg of DHE in 1 mL of DMSO (Sigma) and then adding 999 mL of distilled water. A 100 μL volume of ejaculate was diluted (1:2) with PBS and then centrifuged at 300g for 10 minutes. After discarding the supernatant, the

pellet was re-suspended with PBS. Following this, 20 µL of DHE stock was added to 180 µL of sperm suspension, and then incubated for 15 minutes at 37°C in the dark. After incubation, a wet smear was made on a slide and then analyzed under a fluorescence microscope (Zeiss) at ×400 magnification. The excitation wavelength was 488 nm and the emission filter was 590 nm. Orange/red fluorescing sperm [$O_2^{-\bullet}$-positive sperm (DHE) (%); Figure 9] were recorded as a percentage of all the sperm observed.
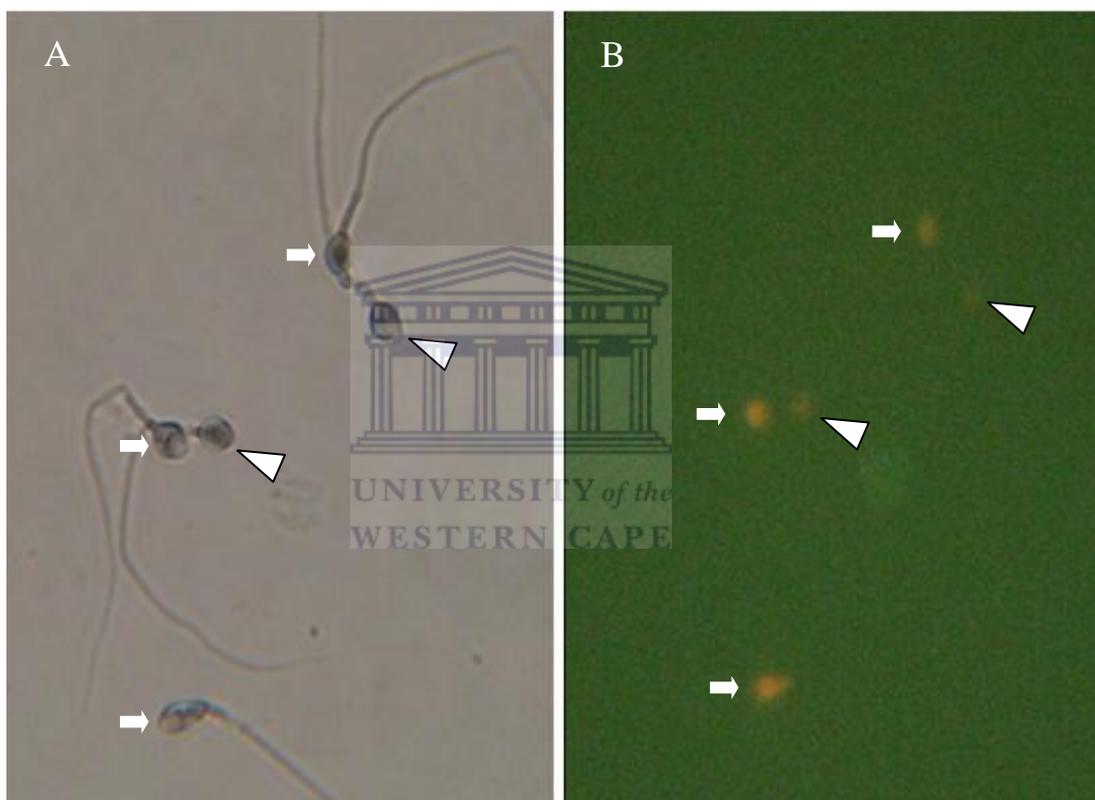
Figure 9 Spermatozoa after staining with DHE; $O_2^{-\bullet}$-positive sperm (arrows) fluoresced orange/red, and $O_2^{-\bullet}$-negative sperm (arrow heads). (×1000 magnification)

2.3.5 Measurement of Reduced Glutathione (GSH) activity in Sperm

The GSH-Glo™ luminescence assay (Promega) was used to estimate the activity of reduced glutathione (GSH) in the ejaculate. GSH, a substrate of glutathione peroxidase, is part of an antioxidant enzymatic scavenging system that forms an important intracellular defence against oxidative stress. The GSH-Glo™ assay is

based on the conversion of a luciferin derivative in the presence of GSH which is catalyzed glutathione S-transferase (GST). The signal generated in the reaction is proportional to the amount of GSH present in the sample.

For the assay, a GSH-Glo™ reagent 2X was prepared according to manufacturer's instructions. In brief, 50 µL GSH-Glo™ reagent 2X contains 0.5µL Luciferin-NT substrate, 0.5 µL GST, and 49 µL of reaction buffer. A reconstituted Luciferin detection reagent stock solution was also prepared by adding Luciferin detection reagent to Luciferin reaction buffer as per manufacturer's instructions. The 50 µL GSH-Glo™ reagent 2X was added to 50 µL of washed semen (diluted with PBS, centrifuged for 10 minutes at 300×g, and re-suspended with PBS) and then incubated for 30 minutes at RT protected from light in a 1.5 mL reaction tube. Subsequently, 50 µL reconstituted Luciferin detection reagent was added and the reaction mixture was incubated for 15 minutes at RT away from light. Following this, 100 µL of the reaction mixture was transferred to a microtitre plate (Dynatech MicroFLUOR™). Luminescence was measured using a Berthold luminometer (MicroLumat *Plus* LB 96 P) set at integration mode to measure luminescence for 30 seconds for each well. The first well in each row contained a negative control which was prepared and incubated in the same way as the test, but with 50 µL PBS replacing 50 µL washed semen. The negative control luminescence was subtracted from the test result and the outcome was recorded as $\times 10^4$ RLU/20×10^6 sperm.

2.4 Sperm Apoptotic Markers

2.4.1 Determination of the Integrity of MMP ($\Delta\psi_m$)

The membrane potential of the mitochondria ($\Delta\psi_m$) in sperm was determined using a lipophilic cationic dye DePsipher™ (Trevigen, Minneapolis, USA). Each aliquot of DePsipher™ contained 1 µg of the dye diluted in 1 mL of PBS. After washing with PBS, a sample was centrifuged for 10 minutes at 300×g. The pellet was then re-suspended with 50-200 µL of DePsipher™ and incubated at 37°C in 5% CO_2 for 20 minutes protected from light. After incubation, the sample was centrifuged and the pellet was re-suspended with a reaction buffer. The sample was immediately observed using a fluorescence microscope with a 488 nm excitation filter (Zeiss) at

×400 magnification.   Sperm with intact $\Delta\psi_m$ excited an intense red fluorescence (590 nm emission filter) due to the formation of dye aggregates inside mitochondria (Figure 10, A).   In the sperm with disrupted $\Delta\psi_m$ ($\Delta\psi_m$-negative sperm), the monomer dye fluoresced green (530 nm emission filter) (Figure 10, B).   The number of $\Delta\psi_m$-negative sperm was recorded as a percentage of the total number of sperm counted.
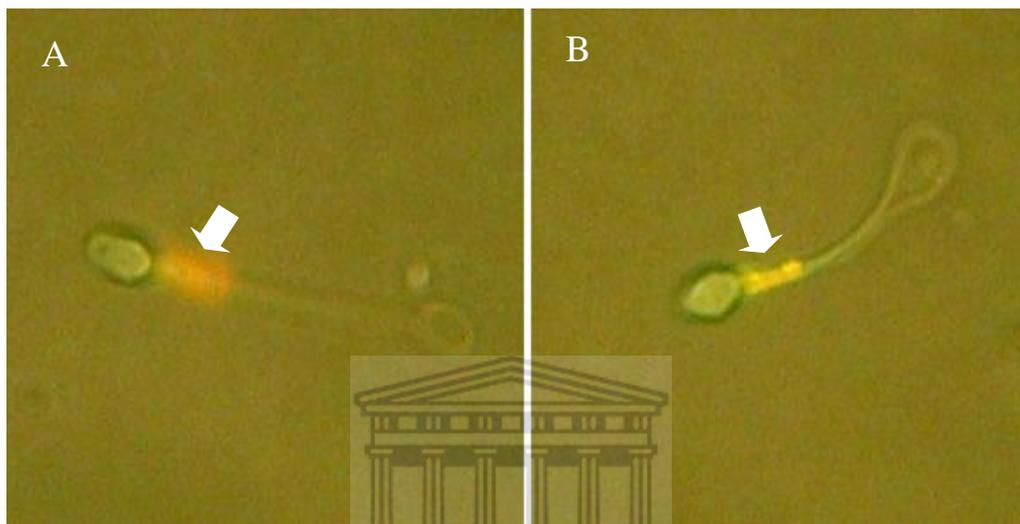
Figure 10   After staining with DePsipher™, sperm with intact $\Delta\psi_m$ showed red fluorescence (A), while those with disrupted $\Delta\psi_m$ had a green fluorescence (white arrow; B). (×1000 magnification)

2.4.2 Measurement of Caspase-3/7 activation in Sperm

Caspase activation was determined using the Caspase-Glo™3/7 assay (Promega). This luminescent assay measures the activities of caspase-3 and -7 which are members of the cysteine aspartic acid-specific protease (caspase) family that play key effector roles in apoptosis in mammalian cells.

The Caspase-Glo™ reagent was prepared by mixing the Caspase-Glo™ substrate and Caspase-Glo™ buffer at RT protected from light.   The resultant reagent was then stored in 100 μL aliquots and at -80°C.   Semen samples were washed in PBS and centrifuged at 300×g for 10 minutes.   Following this, the supernatant was discarded and the pellet was re-suspended in PBS.   A 100 μL aliquot of Caspase-Glo™ reagent

was then equilibrated to RT and mixed with 100 µL of the washed semen sample in a reaction tube. The mixture was then incubated for 60 minutes away from light at RT. After incubation, the contents of the reaction tube were homogenized, and then 100 µL of the reaction mixture were transferred into a white microtitre plate (Dynatech MicroFLUOR™) as the test. The first well of each row was used as a negative control and it contained 100 µL of PBS mixed with 100 µL of Caspase-Glo™ reagent which had been incubated for 60 minutes. Luminescence was measured using a Berthold luminometer (MicroLumat *Plus* LB 96 P) set at integration mode to measure luminescence for 30 seconds for each well. The Caspase-Glo™ reagent contains the tetrapeptide sequence DEVD which causes cell lysis followed by caspase cleavage resulting in a luminescent signal that is proportional to the caspase activity present in the reaction. The negative control luminescence was subtracted from the test result and the outcome was recorded as $\times 10^4$ RLU/20×10^6 sperm.

2.4.3 Measurement of Sperm DNA Fragmentation

DNA fragmentation was measured by using the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay (Henkel et al., 2003b). The TUNEL assay quantifies the incorporation of deoxyuridine triphosphate (dUTP) at single and double strand DNA breaks in an enzymatic reaction by labeling the free 3'-OH and the modified nucleotides with terminal deoxynucleotidyl transferase.

After washing samples in phosphate buffered saline (PBS) (Oxoid Ltd., Hampshire, England), the resultant pellet was re-suspended, and a wet smear was made on a StarFrost™ slide (Knittel Gläser, Braunschweig, Germany) and left to air-dry at RT. The slides were then fixed in 4% formaldehyde in PBS (pH 7.4) for 25 minutes at 4°C. After fixation, the slides were washed in PBS for 5 minutes at RT and permeabilized in 0.2% Triton™ X-100 (Sigma) in PBS for 5 minutes at RT. The slides were then rinsed twice in PBS for 5 minutes at RT. A 100 µL of equilibration buffer was added to each slide and allowed to equilibrate for 10 minutes. After this, 20 µL of TUNEL reagent (DeadEnd™, Promega, Madison, WI, USA) was added to each slide and then a plastic cover slip (Promega) was applied. Following incubation for 60 minutes at 37°C in a humidified chamber away from light, the reaction was

terminated by immersion in 2×SSC (Promega) for 15 minutes. The slides were then washed in PBS three times and more than 200 randomly selected sperm were immediately analyzed using a fluorescence microscope (Zeiss, Oberkochen, Germany) equipped with a 50W mercury lamp, a 488 nm excitation filter and a 510-530 nm emission filter, at ×400 magnification with an oil immersion objective. Sperm with normal DNA only had background staining (TUNEL-negative), while those with fragmented DNA had a bright green fluorescence (TUNEL-positive) (Figure 11). TUNEL-positive sperm were recorded as a percentage of the total number of sperm per field.
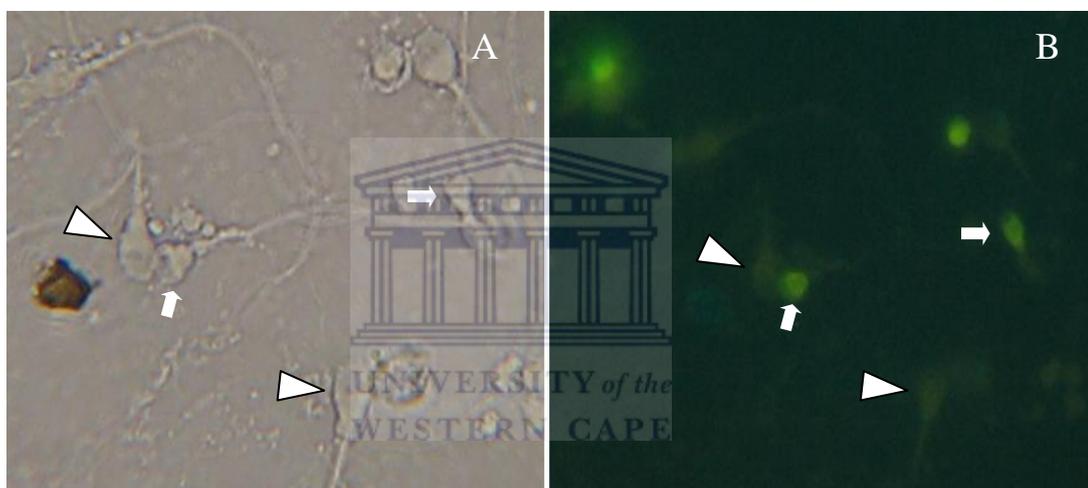
Figure 11   Sperm with nuclear DNA damage (TUNEL-positive sperm) fluoresced bright green (white arrows) after TUNEL assay. TUNEL-negative sperm (white arrow heads) showed background staining only. (×1000 magnification)

2.5 Statistical Analysis

Normal distribution was tested by means of the D'Agostino-Pearson test (D'Agostino and Pearson, 1973), and following this parametric and nonparametric tests were performed accordingly. The results were presented as mean±SD, median and range. A P-value of less than 0.05 (P < 0.05) was considered for statistical significance. In order to compare the Amplex UltraRed™ and H_2DCFDA assays which were used to determine H_2O_2 generation is sperm, Bland and Altman (Bland and Altman, 1986), and Mountain plots (Krouwer and Monti, 1995) were performed as well as

concordance correlation coefficients (Lin, 1989) were calculated. The latter evaluates the degree to which pairs of observations fall on a 45º line through the origin, and it contains a measure of precision (Pearson correlation coefficient: ρ) and accuracy (Bias correlation factor: C_b).

MedCalc® statistical software (version 9.3.8.0; Mariakerke, Belgium) was used to perform all statistical tests and analyses.

CHAPTER THREE

RESULTS

3.1 Summary Statistics

The summary statistics of the parameters that were analyzed in this study are shown in Table 5. Following the D'Agostino-Pearson test for normality, the data from all parameters were not normally distributed except for the percentage of motile spermatozoa (P = 0.177). The data for the parameters in this study shows high standard deviation, which is a common finding in biological data (Table 5).

3.2 Semen Parameters

The sperm count ($\times 10^6$/mL) was negatively correlated with GSH activity in spermatozoa (ρ = -0.435; P = 0.0017), and ROS production in the ejaculate (ρ = -0.296; P = 0.033). This parameter also showed a tendency for negative correlation with caspase-3/7 activity in sperm (ρ = -0.247; P = 0.0668). Sperm motility was positively correlated with sperm forward progression (ρ = 0.288; P = 0.0283).

3.3 Oxidative Status in the Ejaculate and in Spermatozoa

3.3.1 Peroxidase-positive Leukocytes

The concentration of peroxidase-positive cells ($\times 10^6$/mL of ejaculate) was positively correlated with ROS production in the ejaculate (ρ = 0.378; P = 0.0064), the percentage of $O_2^{\cdot-}$-positive sperm (ρ = 0.366; P = 0.0098) (Table 6b), and caspase-3/7 activation in sperm (ρ = 0.527; P < 0.0001). There was no correlation between the concentration of peroxidase-positive cells and semen parameters (Table 6a), the percentage of TUNEL-positive sperm (ρ = 0.0195; P = 0.8817), and the percentage of $\Delta\psi_m$-negative sperm (ρ = 0.0854; P = 0.5121) (Table 6c).

Table 5: Summary statistics of all parameters measured in this study.

	n	Mean±SD	Median	Range	P[‡]
Sperm count ($\times 10^6$/mL)	60	76.8±57.6	80	8-338	<0.0001
Sperm motility (%)	59	52.5±10.6	50	20-70	0.177
Forward progression	59	2.53±0.32	2.66	1.33-3.3	0.0049
Peroxidase-positive cells ($\times 10^6$/mL)	60	0.52±1.28	0.14	0.02-8.9	<0.0001
ROS production in the ejaculate†	53	10.6±41.7	0.43	-0.5-248	<0.0001
H_2O_2-positive sperm (Amplex) (%)	59	21.7±13.9	17.6	2-72	<0.0001
H_2O_2-positive sperm (DCF) (%)	60	23.5±13.7	20.4	5.4-89.4	<0.0001
$O_2^{-\bullet}$-positive sperm (DHE) (%)	60	25.9±15.8	21.0	4.1-76.9	0.003
GSH activity in sperm†	53	73.2±182	18.2	-21-1187	<0.0001
$\Delta\psi_m$-negative sperm (%)	60	25.2-20.1	16.8	4.5-83.9	<0.0001
Caspase[a] activation in sperm†	58	247±353	133.8	44-1842	<0.0001
TUNEL-positive sperm (%)	59	10.3±6.6	9.2	2.3-37.1	<0.0001

[‡] P-value for D'Agostino-Pearson test for normality; **SD**: standard deviation; **ROS**: reactive oxygen species; † $\times 10^4$ RLU/20$\times 10^6$ sperm; **Amplex**: Amplex UltraRed™; **DCF**: 2,7-dihydrofluorescein diacetate; [a] caspase-3/7; **GSH**: reduced glutathione.

Table 6a: Relationship between the concentration of peroxidase-positive cells and semen parameters.

	n	ρ	P-value
Sperm count ($\times 10^6$/mL)	60	0.0165	0.8989
Sperm motility	59	0.148	0.2608
Forward progression	59	0.0043	0.9738

Table 6b: Relationship between the concentration of peroxidase-positive cells and oxidative status.

	n	ρ	P-value
ROS production in the ejaculate†	53	0.378	0.0064
H_2O_2-positive sperm (Amplex) (%)	59	0.210	0.1092
H_2O_2-positive sperm (DCF) (%)	60	0.085	0.5162
$O_2^{-\bullet}$-positive sperm (DHE) (%)	60	0.336	0.0098
GSH activity in sperm†	53	0.211	0.1275

† $\times 10^4$ RLU/20×10^6 sperm.

Table 6c: Relationship between the concentration of peroxidase-positive cells and sperm apoptotic markers.

	n	ρ	P-value
$\Delta\psi_m$-negative sperm (%)	60	0.0854	0.5121
Caspase[a] activation in sperm†	58	0.527	<0.0001
TUNEL-positive sperm (%)	59	0.0195	0.8817

[a] caspase-3/7; † $\times 10^4$ RLU/20×10^6 sperm.

Three cut-off values for leukocyte count in the ejaculate were used to evaluate the relationship between the peroxidase-positive cells and other parameters under investigation. These cutoff values ($\times 10^6$/mL) were ≥ 0.25, ≥ 0.5, and ≥ 1.0 (Tables 7a-7c). At the cut-off value of $\geq 0.25\times 10^6$/mL, the concentration of peroxidase-positive cells had strong positive correlations with GSH activity in sperm ($\rho = 0.718$; $P = 0.0072$), the percentage of $\Delta\psi_m$-negative sperm ($\rho = 0.465$; $P = 0.0427$), caspase-3/7 activation in sperm ($\rho = 0.794$; $P = 0.0008$), and the percentage of TUNEL-negative sperm ($\rho = 0.563$; $P = 0.017$). At the cut-off value of $\geq 0.5\times 10^6$/mL, peroxidase-positive cell count was positively correlated with ROS production in the ejaculate ($\rho = 0.817$; $P = 0.0209$) and caspase-3/7 activation in sperm ($\rho = 0.729$; $P = 0.0157$). At $\geq 1.0\times 10^6$ leukocytes/mL of ejaculate, the concentration of peroxidase-positive cells was positively correlated with sperm forward progression ($\rho = 0.756$; $P = 0.0455$) and ROS production in the ejaculate ($\rho = 0.943$; $P = 0.035$).

As Tables 7a-7c show, the strength of the correlation between peroxidase-positive cell concentration and the following parameters had a tendency to increase from lower to higher cut-off value; sperm count, sperm motility, forward progression, ROS production in the ejaculate, and the percentage of $O_2^{-\bullet}$-positive sperm. Conversely, the strength of the correlation decreased from lower cut-off value to higher with other parameters (the percentage of H_2O_2-positive sperm, GSH activity in sperm, the percentage of $\Delta\psi_m$-negative sperm, caspase-3/7 activation in sperm, and the percentage of TUNEL-positive sperm).

Table 7a: Relationship between the concentration of peroxidase-positive cells at different cut-off values with semen parameters.

	$\geq 0.25 \times 10^6$/mL			$\geq 0.5 \times 10^6$/mL			$\geq 1.0 \times 10^6$/mL		
	n	ρ	P	n	ρ	P	n	ρ	P
Sperm count‡	20	-0.097	0.6739	12	-0.204	0.4989	8	-0.530	0.1607
Sperm motility	20	-0.114	0.6196	12	0.107	0.7231	8	0.396	0.2949
Forward progression	20	0.072	0.7539	12	0.302	0.3173	8	0.756	0.046

‡ $\times 10^6$/mL of semen.

Table 7b: Relationship between the concentration of peroxidase-positive cells at different cut-off values with oxidative status.

	$\geq 0.25 \times 10^6$/mL			$\geq 0.5 \times 10^6$/mL			$\geq 1.0 \times 10^6$/mL		
	n	ρ	P	n	ρ	P	n	ρ	P
ROS production in the ejaculate†	20	0.234	0.3077	9	0.817	0.021	6	0.943	0.035
H_2O_2-positive sperm (A) (%)	20	0.374	0.1031	12	0.263	0.3836	8	0.310	0.4128
H_2O_2-positive sperm (D) (%)	20	0.234	0.3077	12	-0.01	0.7538	8	-0.05	0.9068
$O_2^{-\bullet}$-positive sperm (DHE) (%)	20	0.072	0.7539	12	0.302	0.3173	8	0.405	0.2842
GSH activity in sperm†	15	0.718	0.008	9	0.483	0.1716	6	0.314	0.4822

† $\times 10^4$ RLU/20×10^6 sperm; **A**: Amplex UltraRed; **D**: 2,7-dihydrofluorescein diacetate.

Table 7c: Relationship between the concentration of peroxidase-positive cells at different cut-off values with sperm apoptotic markers.

	$\geq 0.25 \times 10^6$/mL			$\geq 0.5 \times 10^6$/mL			$\geq 1.0 \times 10^6$/mL		
	n	ρ	P	n	ρ	P	n	ρ	P
$\Delta\psi_m$-negative sperm (%)	20	0.465	0.043	12	0.399	0.1854	8	0.214	0.5708
Caspase activation in sperm†‡	19	0.794	0.001	12	0.729	0.016	8	0.571	0.1306
TUNEL-positive sperm (%)	19	0.563	0.017	12	0.0525	0.8617	8	0.238	0.5287

† $\times 10^4$ RLU/20×10^6 sperm; ‡ caspase-3/7.

Furthermore, patients were grouped according to cut-off values for seminal leukocytes, and comparisons were made using the Mann-Whitney test between the following groups: $\geq 0.25 \times 10^6$ and $<0.25 \times 10^6$ leukocytes/mL of semen; $\geq 0.5 \times 10^6$ and $<0.5 \times 10^6$/mL, and $\geq 1 \times 10^6$ and $<1 \times 10^6$ leukocytes/mL of semen for the different parameters. For the comparison between the subgroups $\geq 0.25 \times 10^6$ and $<0.25 \times 10^6$ leukocytes/mL, there were significant differences for peroxidase-positive cell concentration ($P < 0.0001$), ROS production in the ejaculate ($P = 0.0188$), the percentage of $O_2^{-\bullet}$-positive sperm ($P = 0.023$), and caspase-3/7 activation in sperm ($P = 0.0004$). The Mann-Whitney test between the $\geq 0.5 \times 10^6$ and $<0.5 \times 10^6$/mL groups revealed significant differences for peroxidase-positive cells ($P < 0.0001$), ROS production in the ejaculate ($P = 0.0004$), the percentage of $O_2^{-\bullet}$-positive sperm ($P = 0.0306$), GSH activity in sperm ($P = 0.0086$), caspase-3/7 activation in sperm ($P = 0.0001$), and the percentage of TUNEL-positive sperm ($P = 0.0195$). There was significance difference between $\geq 1 \times 10^6$ and $<1 \times 10^6$ leukocytes/mL groups for peroxidase-positive cells ($P < 0.0001$), ROS production in the ejaculate ($P = 0.0004$), the percentage of $O_2^{-\bullet}$-positive sperm ($P = 0.0168$), GSH activity in sperm ($P = 0.0083$), and caspase-3/7 activation in sperm ($P = 0.0002$). Table 7d shows the Mann-Whitney test for differences between the above groups.

Table 7d: Mann-Whitney test for differences between the medians of the subgroups according to peroxidase-positive cell concentration ($\times 10^6$ leukocytes/mL of semen) for the parameters.

	Comparison			Comparison			Comparison		
	≥0.25	<0.25		≥0.5	<0.5		≥1.0	<1.0	
	X±SD	X±SD	P	X±SD	X±SD	P	X±SD	X±SD	P
Sperm count ($\times 10^6$/mL)	25.8±18.3	73.7±57.1	0.7301	86.5±62.7	74.4±56.7	0.6309	90.8±69.3	74.6±56.0	0.6170
Sperm motility (%)	55.5±9.4	51.0±11.0	0.2146	54.2±7.9	52.1±11.2	0.6311	53.8±9.2	52.4±10.9	0.8500
Forward progression	2.5±0.4	2.5±0.3	0.3327	2.6±0.3	2.5±0.3	0.2960	2.5±0.4	2.5±0.3	0.3300
ROS production†	346±747	11.1±19.6	0.0188	573±911	10.2±18.5	0.0004	771±1074	20.9±76.6	0.0004
H_2O_2-positive cells (A) (%)	25.8±18.3	19.5±10.8	0.3247	31.2±21.0	19.2±10.5	0.0919	36.0±23.9	19.4±10.4	0.0924
H_2O_2-positive cells (D) (%)	25.8±18.8	22.4±10.4	0.8754	30.2±21.6	21.9±10.6	0.3183	32.1±26.3	22.2±10.5	0.6093
$O_2^{\cdot-}$-positive sperm (E) (%)	33.6±19.1	22.0±12.5	0.0230	36.6±20.5	23.2±13.4	0.0306	41.0±21.8	23.5±13.5	0.0168
GSH activity in sperm†	1588±3064	393±821	0.1284	2547±3714	361±773	0.0086	3391±4367	393±789	0.0083
$\Delta\psi_m$-negative sperm (%)	28.1±23.3	23.7±18.5	0.7717	35.6±26.8	22.6±17.5	0.1251	39.0±30.1	23.1±17.6	0.0984
Caspase[a] activity in sperm†	4758±5400	1352±970	0.0004	6593±6106	1392±958	0.0001	18421±6608	1488±1061	0.0002
TUNEL-positive sperm (%)	11.3±8.6	9.8±5.5	0.7518	14.9±8.9	9.1±5.4	0.0195	14.7±10.7	9.6±5.5	0.1597

‡ $\times 10^6$ cells/mL of semen; † ROS production in the ejaculate $\times 10^4$ RLU/20×10^6 sperm; [a] caspase-3/7; **A**: Amplex UltraRed; **D**: DCF; **E**: DHE.

3.3.2 ROS production in the Ejaculate

ROS production in the ejaculate was positively correlated with the concentration of peroxidase-positive cells ($\rho = 0.378$; P = 0.0064), GSH activity in sperm ($\rho = 0.577$; $P < 0.0001$), caspase-3/7 activation in sperm ($\rho = 0.487$; P = 0.0005), and the percentage of TUNEL-positive sperm ($\rho = 0.331$; P = 0.0171). In addition, ROS production in the ejaculate was negatively correlated with sperm count ($\rho = -0.296$; P = 0.033). Tables 8a to 8c demonstrate the relationship between ROS production in the ejaculate and other parameters.

Table 8a: Relationship between ROS production in the ejaculate and semen parameters.

	n	ρ	P-value
Sperm count ($\times 10^6$/mL)	53	-0.296	0.033
Sperm motility	52	0.0264	0.8502
Forward progression	52	0.0846	0.5458

Table 8b: Relationship between ROS production in the ejaculate and oxidative status.

	n	ρ	P-value
Peroxidase-positive cells ($\times 10^6$/mL)	53	0.378	0.0064
H_2O_2-positive sperm (Amplex) (%)	52	0.120	0.3899
H_2O_2-positive sperm (DCF) (%)	53	0.169	0.2221
$O_2^{-\bullet}$-positive sperm (DHE) (%)	53	-0.011	0.9358
GSH activity in sperm†	53	0.577	<0.0001

† $\times 10^4$ RLU/20×10^6 sperm

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Table 8c: Relationship between ROS production in the ejaculate and sperm apoptotic markers.

	n	ρ	P-value
$\Delta\psi_m$-negative sperm (%)	53	0.175	0.2072
Caspase[a] activation in sperm†	52	0.487	0.0005
TUNEL-positive sperm (%)	53	0.331	0.0171

[a] caspase-3/7; † $\times 10^4$ RLU/20×10^6 sperm.

3.3.3 The percentage of H_2O_2-positive Sperm

Hydrogen peroxide is potentially the most harmful ROS, particularly to spermatozoa of infertile patients, and two methods were used in this study to detect H_2O_2-positive sperm. Tables 9a to 9b show the relationship between the percentage of H_2O_2-positive sperm as measured by Amplex UltraRed (Amplex) and 2,7-dihydrofluorescein diacetate (DCF). The percentage of H_2O_2-positive sperm as measured by the two methods had a weaker positive correlation than expected

($\rho = 0.306$; P = 0.0199). The percentage of H_2O_2-positive sperm (Amplex) was positively correlated with the percentage of TUNEL-positive sperm ($\rho = 0.274$; P = 0.0388). Notably, there was no correlation between the percentage of H_2O_2-positive sperm and sperm motility (Table 9a), or the percentage of $O_2^{\cdot-}$-positive sperm (Table 9b).

Table 9a: Relationship between the percentage of H_2O_2-positive sperm and semen parameters.

	Amplex			DCF		
	n	ρ	P-value	n	ρ	P-value
Sperm count ($\times 10^6$/mL)	59	0.0621	0.6360	60	-0.075	0.5658
Sperm motility	58	-0.169	0.2050	59	-0.033	0.7994
Forward progression	58	-0.044	0.7413	59	-0.162	0.2161

Table 9b: Relationship between the percentage of H_2O_2-positive sperm and oxidative status.

	Amplex			DCF		
	n	ρ	P-value	n	ρ	P-value
Peroxidase-positive cells ($\times 10^6$/mL)	58	0.210	0.1092	60	0.0845	0.5162
ROS production in the ejaculate†	52	0.120	0.3899	53	0.169	0.2221
H_2O_2-positive sperm (Amplex) (%)				59	0.306	0.0199
H_2O_2-positive sperm (DCF) (%)	59	0.306	0.0199			
$O_2^{-\bullet}$-positive sperm (DHE) (%)	59	0.201	0.1322	60	0.0273	0.8340
GSH activity in sperm†	52	0.220	0.1168	53	0.198	0.1527

† $\times 10^4$ RLU/20$\times 10^6$ sperm.

Table 9c: Relationship between the percentage of H_2O_2-positive sperm and sperm apoptotic markers.

	Amplex			DCF		
	n	ρ	P-value	n	ρ	P-value
$\Delta\psi_m$-negative sperm (%)	59	0.0562	0.6685	60	0.203	0.1198
Caspase[a] activation in sperm†	57	0.201	0.1322	58	0.218	0.100
TUNEL-positive sperm (%)	58	0.274	0.039	59	0.146	0.2649

Despite the significant correlation, the Wilcoxon test (Figure 12) revealed no significant difference between the medians of the two methods employed to determine the percentage of H_2O_2-positive sperm (p = 0.1891). Furthermore, Bland and Altman (Figure 13) and Mountain plots (Figure 14) revealed no significant graphical differences between the Amplex and DCF methods.
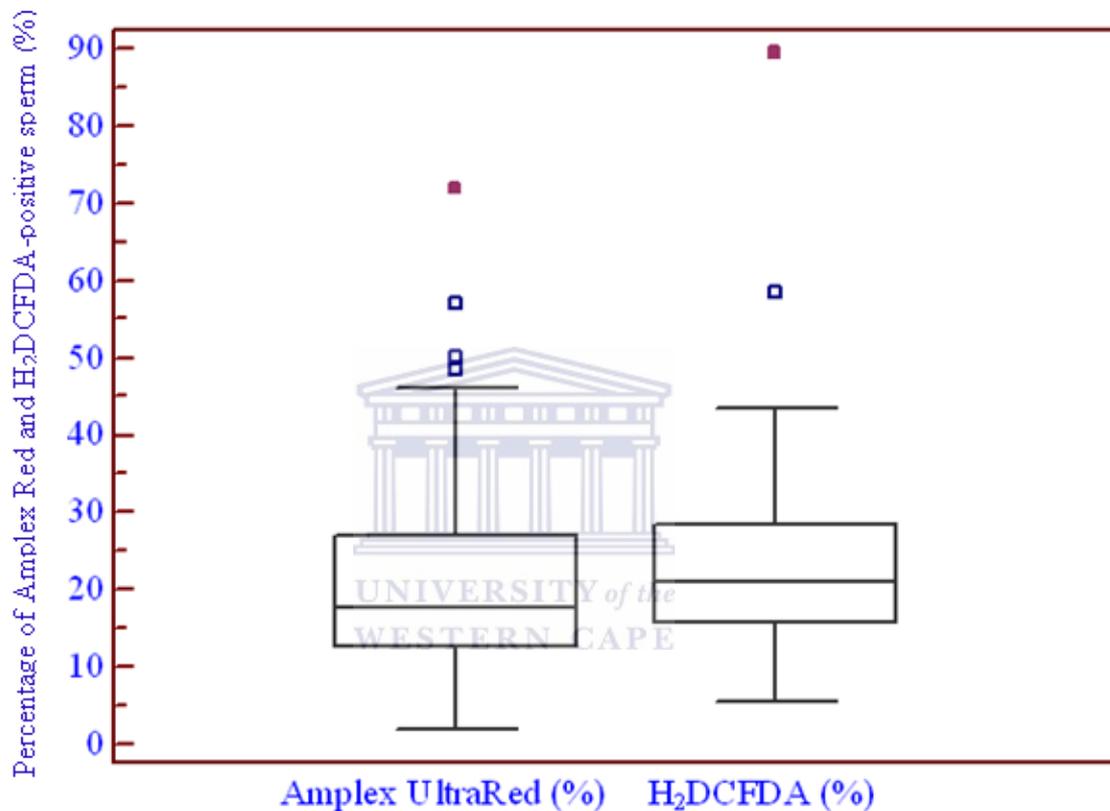
Figure 12   Comparison of the two fluorescence techniques for measuring H_2O_2 in sperm; Amplex UltraRed™ and HRP (Amplex UltraRed, n = 59) and 2,7-dihydrofluorescein diacetate (H_2DCFDA, n = 59). The Wilcoxon test for paired samples revealed no significant differences between the two techniques (P = 0.1891).
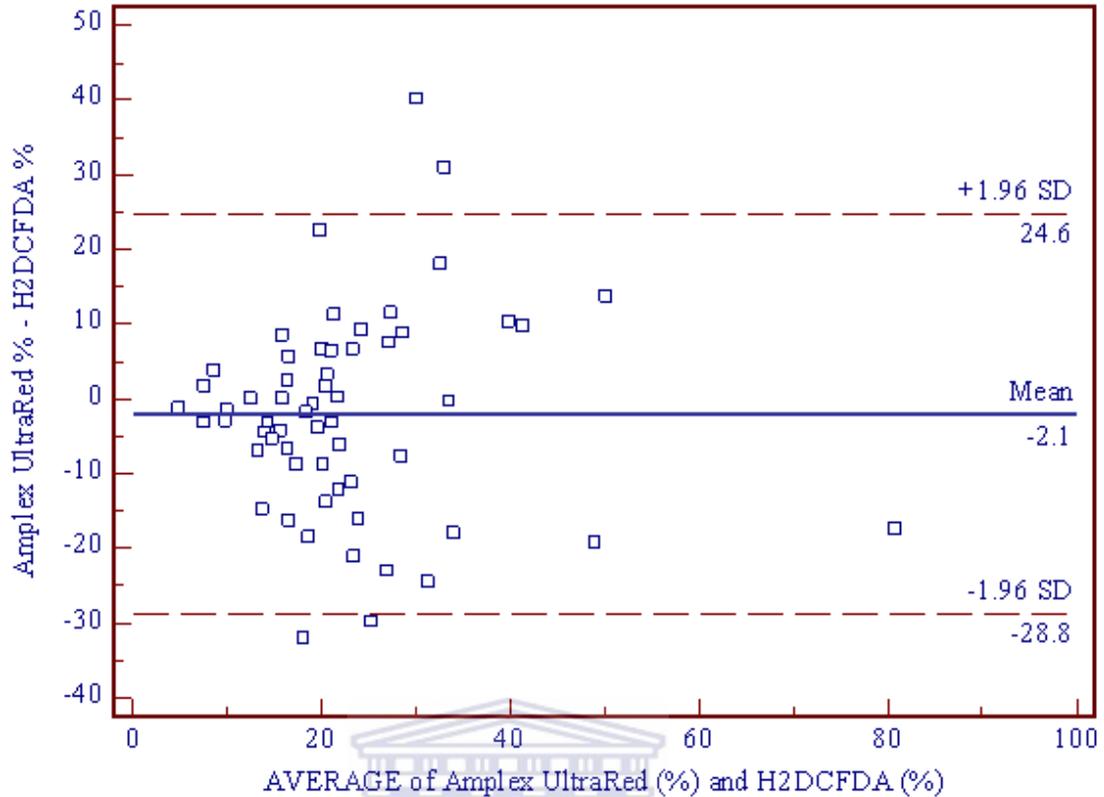
Figure 13   Bland and Altman plot showing the comparison of the two fluorescence methods for measuring H_2O_2 in spermatozoa. The differences between the two methods are sufficiently centred around zero to conclude that the methods have satisfactory agreement. A triangular shape of the distribution is visible. This means that the variation of the data depends on the magnitude of the measurement Concordance correlation coefficient was 0.5088 (Pearson ρ = 0.5747; Bias correlation factor C_b = 0.9886).
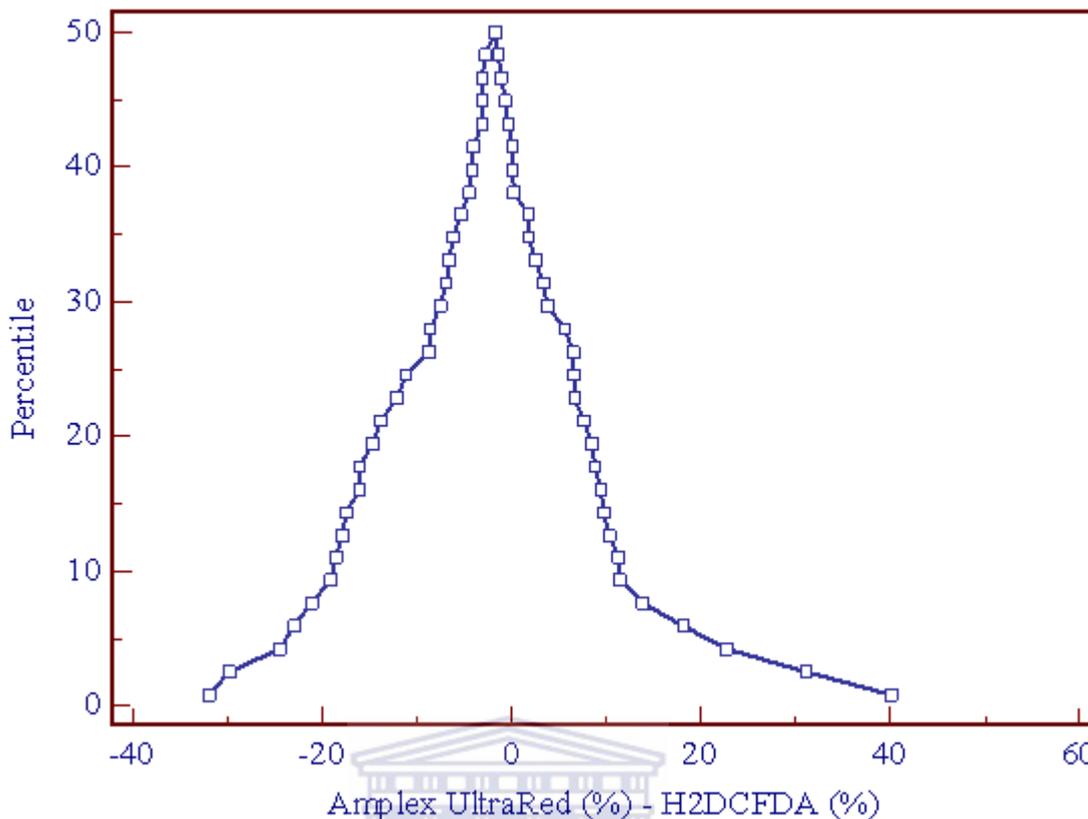
Figure 14   Mountain plot showing the comparison of the Amplex and DCF fluorescence methods used to detect H_2O_2 in sperm. The peak of the graph is close to zero, revealing that there is no significant difference between the two methods.

3.3.4 The percentage of $O_2^{\cdot-}$-positive Sperm

The percentage of $O_2^{\cdot-}$-positive sperm was positively correlated with the concentration of peroxidase-positive cells ($\rho = 0.336$; $P = 0.0098$), the percentage of $\Delta\psi_m$-negative sperm ($\rho = 0.261$; $P = 0.0446$), and caspase-3/7 activity in sperm ($\rho = 0.457$; $P < 0.0001$).   Despite the association with other sperm apoptotic markers, the percentage of $O_2^{\cdot-}$-positive sperm did not have a significant correlation with the percentage of TUNEL-positive sperm ($\rho = 0.205$; $P = 0.205$). Furthermore, there was no significant correlation between the percentage of $O_2^{\cdot-}$-positive sperm and the percentage of H_2O_2-positive sperm (Amplex, $\rho = 0.220$; $P = 0.1168$; DCF, $\rho = 0.198$; $P = 0.1527$).   In addition, this parameter had a generally non-significant negative correlation with semen parameters. Tables 10a to 10c show the relationship between the percentage of $O_2^{\cdot-}$-positive sperm and other parameters.

Table 10a: Relationship between the percentage of $O_2^{-\bullet}$-positive sperm and semen parameters.

	n	ρ	P-value
Sperm count ($\times 10^6$/mL)	60	-0.150	0.2487
Sperm motility	59	0.005	0.9674
Forward progression	59	-0.109	0.4054

Table 10b: Relationship between the percentage of $O_2^{-\bullet}$-positive sperm and oxidative status.

	n	ρ	P-value
Peroxidase-positive cells ($\times 10^6$/mL)	60	0.336	0.0098
ROS production in the ejaculate†	53	-0.011	0.9358
H_2O_2-positive sperm (Amplex) (%)	59	0.140	0.2863
H_2O_2-positive sperm (DCF) (%)	60	0.027	0.8340
GSH activity in sperm†	53	0.093	0.5013

† $\times 10^4$ RLU/20×10^6 sperm.

Table 10c: Relationship between the percentage of $O_2^{-\bullet}$-positive sperm and sperm apoptotic markers.

	n	ρ	P-value
$\Delta\psi_m$-negative sperm (%)	60	0.261	0.0446
Caspase[a] activation in sperm†	58	0.457	<0.0001
TUNEL-positive sperm (%)	59	0.204	0.1207

[a] caspase-3/7; † $\times 10^4$ RLU/20×10^6 sperm.

3.3.5 GSH activity in sperm

GSH activity in sperm was positively correlated with ROS production in the ejaculate ($\rho = 0.577$; $P < 0.0001$), the percentage of $\Delta\psi_m$-negative sperm ($\rho = 0.385$; $P = 0.0055$), and caspase-3/7 activation in sperm ($\rho = 0.529$; $P = 0.0002$). This parameter also had a significant negative correlation with the sperm count ($\rho = -0.435$; $P = 0.0017$). In general, GSH activity in sperm had a negative correlation with semen parameters, a non-significant positive correlation with oxidative status parameters, and a significant positive correlation with sperm apoptotic markers (Tables 11a to 11c).

Table 11a: Relationship between GSH activity in sperm and semen parameters.

	n	ρ	P-value
Sperm count ($\times 10^6$/mL)	53	-0.435	0.0017*
Sperm motility	52	0.049	0.7287
Forward progression	52	-0.118	0.3996

Table 11b: Relationship between GSH activity in sperm and oxidative status.

	n	ρ	P-value
Peroxidase-positive cells ($\times 10^6$/mL)	53	0.211	0.1275
ROS production in the ejaculate†	53	0.577	<0.0001
H_2O_2-positive sperm (Amplex) (%)	52	0.220	0.1168
H_2O_2-positive sperm (DCF) (%)	53	0.198	0.1527
$O_2^{-\bullet}$-positive sperm (DHE) (%)	53	0.0933	0.5013

† $\times 10^4$ RLU/20×10^6 sperm.

Table 11c: Relationship between GSH activity in sperm and sperm apoptotic markers.

	n	ρ	P-value
$\Delta\psi_m$-negative sperm (%)	53	0.385	0.0055
Caspase[a] activation in sperm†	53	0.529	0.0002
TUNEL-positive sperm (%)	53	0.205	0.1399

[a] caspase-3/7; † $\times 10^4$ RLU/20×10^6 sperm.

3.4 Sperm Apoptotic Markers

3.4.1 The Percentage of $\Delta\psi_m$-negative Sperm

Tables 12a to 12c illustrate the relationship between the percentage of $\Delta\psi_m$-negative sperm and other parameters. The percentage of $\Delta\psi_m$-negative sperm had a weak positive correlation with the percentage of $O_2^{-\bullet}$-positive sperm ($\rho = 0.261$; $P = 0.0446$) and a stronger positive correlation with GSH activity in sperm ($\rho = 0.385$; $P = 0.0055$). This parameter, even though it is an early marker of apoptosis, did not

correlate significantly with either caspase activation in sperm or the percentage of TUNEL-positive sperm.

Table 12a: Relationship between the percentage of $\Delta\psi_m$-negative sperm and semen parameters.

	n	**ρ**	**P-value**
Sperm count ($\times 10^6$/mL)	60	-0.150	0.2487
Sperm motility	59	0.005	0.9674
Forward progression	59	0.084	0.9493
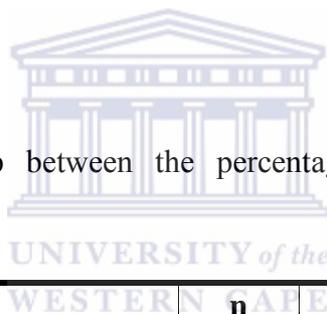
Table 12b: Relationship between the percentage of $\Delta\psi_m$-negative sperm and oxidative status.

	n	**ρ**	**P-value**
Peroxidase-positive cells ($\times 10^6$/mL)	60	0.085	0.5121
ROS production in the ejaculate†	60	0.175	0.2072
H_2O_2-positive sperm (Amplex) (%)	59	0.056	0.6685
H_2O_2-positive sperm (DCF) (%)	60	0.203	0.1198
$O_2^{-\bullet}$-positive sperm (DHE) (%)	60	0.261	0.0446
GSH activity in sperm†	53	0.385	0.0055

† $\times 10^4$ RLU/20×10^6 sperm.

Table 12c: Relationship of the percentage of $\Delta\psi_m$-negative sperm with apoptotic markers.

	n	ρ	P-value
Caspase[a] activation in sperm†	58	0.205	0.1210
TUNEL-positive sperm (%)	59	0.204	0.1207

[a] caspase-3/7; † $\times 10^4$ RLU/20×10^6 sperm.

3.4.2 Caspase-3/7 Activation in Sperm

There was no significant correlation between caspase-3/7 activity in sperm and semen parameters (Table 13a). However, caspase-3/7 activation in sperm had a significant positive correlation with the concentration of peroxidase-positive cells ($\rho = 0.527$; $P = 0.0001$), ROS production in the ejaculate ($\rho = 0.487$; $P = 0.0005$), the percentage of $O_2^{-\bullet}$-positive sperm ($\rho = 0.457$; $P = 0.0006$), and GSH activity in sperm ($\rho = 0.529$; $P = 0.0005$) (Table 13b). Despite the strong correlation with the oxidative status in sperm and in the ejaculate, there was no significant correlation between this parameter and either the percentage of $\Delta\psi_m$-negative sperm or the percentage of TUNEL-positive sperm (Table 13c).

Table 13a: Relationship between caspase-3/7 activation in sperm and semen parameters.

	n	ρ	P-value
Sperm count ($\times 10^6$/mL)	58	-0.243	0.067
Sperm motility	58	0.157	0.2345
Forward progression	58	-0.0996	0.4522

Table 13b: Relationship between caspase-3/7 activation and oxidative status.

	n	ρ	P-value
Peroxidase-positive cells ($\times 10^6$/mL)	58	0.527	0.0001
ROS production in the ejaculate†	52	0.487	0.0005
H_2O_2-positive sperm (Amplex) (%)	57	0.201	0.1322
H_2O_2-positive sperm (DCF) (%)	58	0.218	0.100
$O_2^{-\bullet}$-positive sperm (DHE) (%)	58	0.457	0.0006
GSH activity in sperm†	52	0.529	0.0002

† $\times 10^4$ RLU/20×10^6 sperm.

Table 13c: Relationship between caspase-3/7 activation and apoptotic markers.

	n	ρ	P-value
$\Delta\psi_m$-negative sperm (%)	57	0.0087	0.9484
TUNEL-positive sperm (%)	57	0.0087	0.9484

[a] caspase-3/7; † $\times 10^4$ RLU/20×10^6 sperm.

3.4.3 The percentage of TUNEL-positive Sperm

The percentage of TUNEL-positive sperm was positively correlated with ROS production in the ejaculate ($\rho = 0.331$; $P = 0.017$), and the percentage of H_2O_2-positive sperm (Amplex) ($\rho = 0.274$; $P = 0.039$). The relationship between the percentage of TUNEL-positive sperm and the other parameters is depicted in Tables 14a to 14c.

Table 14a: Relationship between the percentage of TUNEL-positive sperm and semen parameters.

	n	ρ	P-value
Sperm count ($\times 10^6$/mL)	59	-0.133	0.3098
Sperm motility	58	-0.057	0.6689
Forward progression	58	0.160	0.2277

Table 14b: Relationship between the percentage of TUNEL-positive sperm and oxidative status.

	n	ρ	P-value
Peroxidase-positive cells ($\times 10^6$/mL)	59	0.02	0.8817
ROS production in the ejaculate†	53	0.205	0.1399
H_2O_2-positive sperm (Amplex) (%)	58	0.274	0.039
H_2O_2-positive sperm (DCF) (%)	59	0.146	0.2649
$O_2^{-\bullet}$-positive sperm (DHE) (%)	59	-0.034	0.7946
GSH activity in sperm†	53	0.205	0.1399

† $\times 10^4$ RLU/20×10^6 sperm.

Table 14c: Relationship between the percentage of TUNEL-positive sperm and sperm apoptotic markers.

	n	ρ	P-value
$\Delta\psi_m$-negative sperm (%)	59	0.204	0.1207
Caspase[a] activation in sperm†	57	0.0087	0.9484

[a] caspase-3/7; † $\times 10^4$ RLU/20×10^6 sperm.

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CHAPTER FOUR

DISCUSSION

It is now generally accepted that ROS-mediated damage to human sperm is a significant contributing factor in the aetiology of impaired sperm function and male infertility (Henkel et al., 2003b; Moustafa et al., 2004; Nallela et al., 2005), and extensive studies have been conducted to establish the link between oxidative stress and defective sperm function by measuring; ROS production by sperm and by contaminating leukocytes in the ejaculate (Henkel et al., 2003a); sperm DNA damage and its effect on fertilizing potential of the male germ cell (Sun et al., 1997; Alvarez et al., 2002; Erenpreiss et al., 2002; Henkel et al., 2003b); lipid peroxidation (Aitken et al., 2007; Cosci et al., 2008); and seminal antioxidant capacity (Pasqualotto et al., 2000; Sharma et al., 2001). Furthermore, ROS have been recently implicated as possible mediators of apoptosis in human spermatozoa (Ramos and Wetzels, 2001; Moustafa et al., 2004). However, data on the effect of male genital tract infection, as a significant cause of oxidative stress in the male reproductive tract, on the function of spermatozoa and the integrity of the sperm genome is scant.

Hence, in this study, selected biological assays were performed in order to investigate the relationship between male genital tract infection, the oxidative status in spermatozoa and the ejaculate, and markers of apoptosis in the male gamete.

4.1 Conventional Semen Parameters

The influence of ROS on the quality of semen is underpinned by lipid peroxidation (Aitken et al., 1993), the induction of which results in loss of sperm plasma membrane fluidity (Whittington et al., 1999), decreased axonemal protein phosphorylation (de Lamirande and Gagnon, 1992), which leads to impaired sperm motility (Agarwal et al., 2003). Furthermore, the prolonged exposure of the seminiferous epithelium to high levels of ROS – produced by either immature sperm

or activated leukocytes – damages these tubules and contributes to testicular atrophy, reduction of gamete production, and low sperm count (Aziz et al., 2004).

In this study, sperm count was negatively correlated with ROS in the ejaculate (ρ = -0.296; P = 0.033), and with sperm GSH activity (ρ = -0.435; P = 0.0017). This inverse relationship between sperm count and ROS in the ejaculate was demonstrated in previous studies (Ochsendorf et al., 1994; Henkel and Schill, 1998; Henkel et al., 2005). These results confirm that excessive ROS impairs, not only sperm function, but also sperm production, depending on the site of ROS production. The negative correlation of sperm count with GSH activity is sperm indicates that reduced glutathione may be more active in sperm from men with poor semen parameters.

4.2 Oxidative Status in the Ejaculate and Sperm

4.2.1 Peroxidase-positive Leukocytes

The biological role of leukocytes in the ejaculate is subject to on-going controversy despite the high incidence of these white blood cells among infertile men (WHO, 1999; Zorn et al., 2000; Henkel et al., 2007). In the male reproductive tract, increased numbers of seminal leukocytes may result from disturbed spermatogenesis (Wolff et al., 1990; Thomas et al., 1997), the harmful influence of environmental factors such as smoking (Close et al., 1990), and atypical sexual behavior or prolonged sexual abstinence (Blackwell and Zaneveld, 1992; Anderson, 1995). However, the most common reason for infiltration of leukocytes into the ejaculate is genitourinary infection/inflammation (Fraczek and Kurpisz, 2007).

Although the role of seminal leukocytes is in question, it is without doubt that leukocytes are the main source of ROS in the ejaculate (Plante et al., 1994). Not surprisingly, this study revealed that the concentration of peroxidase-positive cells was positively correlated with ROS in the ejaculate (ρ = 0.378; P = 0.0064). Furthermore, peroxidase-positive cells were also positively correlated with the percentage of $O_2^{\cdot-}$-positive sperm (ρ = 0.336; P = 0.0098) as determined using dihydroethidium. Previously, other studies have demonstrated a similar association between seminal leukocytes and ROS production in sperm (Saleh et al., 2002b; Henkel et al., 2003a; 2005).

The present study also demonstrated a strong positive correlation between the number of peroxidase-positive cells and caspase-3/7 activity in the spermatozoa ($\rho = 0.527$; $P < 0.0001$). Active caspase-3 in human sperm was reported by Weng et al. (2002) but this was not correlated to the number of leukocytes. As effector caspases, caspase-3 and -7 play a central role in apoptosis and, considering that seminal leukocytes are associated with excessive ROS production, it is conceivable that ROS derived from leukocytes may induce apoptosis in sperm by triggering caspase activation. Indeed, a positive correlation between ROS production in the ejaculate and caspase-3 and -9 has been demonstrated (Wang et al., 2003).

Interestingly, in this study there was no correlation between the number of peroxidase-positive cells and the percentage of TUNEL-positive spermatozoa ($\rho = 0.0195$; $P = 0.8817$). DNA fragmentation in spermatozoa may be caused by internal influences like apoptosis or ROS production in the sperm (Henkel et al., 2003b), or external inducers like activated leukocytes (Erenpreiss et al., 2002). However, ROS production in the ejaculate by leukocytes seems to have a lesser direct influence on sperm DNA fragmentation (Henkel et al., 2003b). Nevertheless, since even low levels of ROS can cause DNA damage in spermatozoa, the potential of leukocyte-derived oxidative stress to damage the male genome should not be ruled out or underestimated. Seminal leukocytes, activated by stimuli such as inflammation or pathogens, respond with an oxidative burst characterized by the production of high amounts of oxidants which include hydrogen peroxide (Aitken et al., 1994) and this uncharged oxygen derivative can easily permeate plasma membranes and damage sperm DNA.

Some reports indicate that the presence of leukocytes in the ejaculate has no adverse influence on semen quality or fertilization potential of spermatozoa (el-Demiry et al., 1986; Tomlinson et al, 1992; Tomlinson et al., 1993), and some studies even suggest positive roles for these cells in the removal of dead or defective sperm from the male reproductive tract (Tomlinson et al., 1992; Ricci et al., 2002). However, most studies demonstrate the relationship between the increased number of leukocytes in semen and the deterioration of semen parameters (Wolff et al., 1990; Yanushpolsky et al., 1996) or fertilizing ability of sperm (Vogelpoel et al., 1991, Krausz et al., 1994; Sukcharoen et al., 1995), mainly by reducing sperm motility and damaging the potential for sperm to penetrate the oocyte (Maruyama et al., 1985; Kovalski et al.,

1992). Decreased sperm fertilizing ability may then be an early result of excessive ROS production by leukocytes, and prolonged oxidative stress in the male reproductive tract may result in impaired spermatogenesis and damaged sperm DNA (Fraczek and Kurpisz, 2007).

4.2.2 Cutoff Values for Leukocytospermia

According to the World Health Organization (WHO, 1999), a condition in which the number of leukocytes exceeds 1×10^6/mL of ejaculate is defined as leukocytospermia, and this is considered to be the threshold value above which sperm morphology and function are impaired (Menkveld and Kruger, 1998). However, this value is empirical and recent reports suggest that at much lower cut-off values the number of leukocytes in the ejaculate is significantly correlated with sperm dysfunction (Henkel and Schill, 1998; Menkveld and Kruger, 1998; Saleh et al., 2001; Punab et al., 2003; Henkel et al., 2005). Data from the current study showed that at the cut-off value of $\geq0.25\times10^6$ leukocytes/mL of semen there was a strongly significant correlation between the number of leukocytes in the ejaculate and caspase-3/7 activation in sperm, GSH activity in sperm, ROS in the ejaculate, the percentage of sperm with disrupted mitochondrial membrane potential ($\Delta\psi_m$), and the percentage of TUNEL-positive sperm (Tables 7a-7c). Thus, this study confirms that even in non-leukocytospermic patients, oxidative stress can occur which can trigger apoptosis, caspase-3/7 activation, and induce sperm DNA fragmentation.

Plante et al. (1994) demonstrated that seminal leukocyte counts of 5×10^6/mL of ejaculate did not impair sperm motility unless the leukocytes were activated. On the other hand, Aitken et al. (1995) reported that leukocytes were harmful to Percoll-separated sperm at counts of greater than 0.2×10^6/mL of semen. This highlights two points regarding leukocytes in the ejaculate: firstly, it is not only the leukocyte concentration in semen but rather the degree of their activation that determines the final effect of oxidative stress on spermatozoa; secondly, that seminal plasma affords massive antioxidant protection to spermatozoa. These factors are important during assisted reproduction when spermatozoa are separated from seminal plasma but remain proximal to leukocytes in antioxidant-depleted media. Therefore, sperm separation methods should not only include the removal of leukocytes from seminal

fluid (Henkel et al., 2005), but also the addition of the antioxidants to sperm separation media (Baker et al., 1996).

4.2.3 ROS production in the Ejaculate and in Spermatozoa

In this study, ROS production in the ejaculate was correlated positively with the number of peroxidase-positive cells (ρ = 0.378; P = 0.0064), sperm GSH activity (ρ = 0.577; P < 0.0001), caspase-3/7 activity in sperm (ρ = 0.487; P = 0.0005), and the percentage of TUNEL-positive sperm (ρ = 0.331; P = 0.0171). These findings confirm previous reports that ROS production in the ejaculate, mostly a consequence of activated leukocytes (Aitken et al., 1994), is a possible inducer of apoptosis in human sperm; a process in which DNA fragmentation is a hallmark (Wang et al., 2003).

Reactive oxygen species play a significant role in the pathophysiology of sperm dysfunction (Aitken, 1999; Agarwal et al., 2003) and seminal oxidative stress status could be an important diagnostic and prognostic tool in infertility treatment. Levels of ROS production in the ejaculate can be measured by a chemiluminescence assay using luminol (5-amino-2,5-dihydro-1,4-phthalazinedione) and a luminometer (Shekarriz et al., 1995). ROS production in the ejaculate is reported to be higher in infertile men than in their fertile counterparts (Allamaneni et al., 2005; Nallella et al., 2005; Athayde et al., 2007; Das et al., 2008) and, not surprisingly, the highest ROS production was found in infertile leukocytospermic patients (Allamaneni et al., 2005; Athayde et al., 2007).

Although no receiver operated characteristic (ROC) analysis was performed to determine the threshold values for oxidative stress in this study, other authors have suggested seminal ROS cut-off values. Allamaneni et al. (2005) described all seminal ROS levels of 18.5×10^4 RLU per 20×10^6 sperm as the optimal cut-off value for oxidative stress, while other studies reported a optimal cut-off of 10×10^4 RLU per 20×10^6 sperm for washed semen (Shekarriz et al., 1995; Athayde et al., 2007) and 51.5×10^4 RLU per 20×10^6 sperm for semen with leukocytes (Athayde et al., 2007). Undoubtedly, the accurate assessment of ROS production levels in the ejaculate may

augment the diagnosis of infertility in cases where oxidative stress plays a major role and, to this regard, a consensus on normal levels of seminal ROS is essential.

In order to detect ROS production from spermatozoa, it is necessary to separate these cells from contaminating leukocytes, and this can be readily achieved using magnetic beads coated with a monoclonal antibody directed against the common leukocyte antigen, CD45 (Aitken et al., 1996). Previous studies have demonstrated that ROS production by the spermatozoa of oligozoospermic patients is greater than that generated by spermatozoa of fertile donors (Aitken et al., 1992), and the level of ROS generation was strongly correlated with the quality of the original semen sample as reflected in the movement characteristics of the spermatozoa and their morphology, as well as sperm count (Gil-Guzman et al., 2001; Aziz et al., 2004).

Immature spermatozoa are well characterized sources of ROS in the ejaculate and a negative correlation between ROS production and semen quality has been documented (Gil-Guzman et al., 2001; Aziz et al., 2004). It has been hypothesized that abnormal spermatozoa with cytoplasmic retentions are significant generators of ROS, since they retain an excess of cytoplasmic enzymes that are involved in glucose metabolism such as glucose-6-phosphate dehydrogenase (Aitken, 1999). Moreover, it has been confirmed that, when semen is fractionated by a density gradient method, the layer with the highest percentage of immature sperm produces the most ROS (Gil-Guzman et al., 2001) and this ROS generation is significantly correlated with the extent of DNA damage in mature spermatozoa (Ollero et al., 2001; O'Brien and Zini, 2005). Spermatozoa also generate ROS from the mitochondrial electron transport chain (ETC) during cellular respiration, with an estimated 2% of consumed oxygen being converted into the superoxide anion via this route (Boveris and Chance, 1973; Koppers et al., 2008).

In this study, the percentage of $O_2^{-\bullet}$-positive sperm was positively correlated with the percentage of sperm with disrupted $\Delta\psi_m$ ($\rho = 0.261$; $P = 0.0446$), and caspase-3/7 activation in sperm ($\rho = 0.457$; $P < 0.0001$). These parameters are indicative of apoptosis in spermatozoa and, since the most likely source of $O_2^{-\bullet}$ in sperm is mitochondrial oxidative phosphorylation, programmed death of the human male germ cell is executed via the mitochondrial (intrinsic) apoptotic pathway.

This study revealed that the percentage of H_2O_2-positive sperm (Amplex) was positively correlated with the percentage of TUNEL-positive sperm ($\rho = 0.274$; $P = 0.0388$). Although no correlation between sperm $O_2^{-\bullet}$ production and the generation of H_2O_2 could be shown here, it is known that $O_2^{-\bullet}$ is dismutated to H_2O_2 by the enzyme superoxide dismutase. A possible explanation for the lack of correlation, in this study, between the generation of these two reactive oxygen species by sperm, is that the membrane-permeating H_2O_2 was produced by seminal leukocytes. Furthermore, the activity of sperm antioxidants, such as glutathione, may also distort the relationship between mitochondria-derived $O_2^{-\bullet}$ and H_2O_2 in spermatozoa.

4.2.4 GSH activity in Sperm

Given the potentially toxic nature of ROS to spermatozoa, it is important that the oxidants are rapidly scavenged before they trigger lipid peroxidation or DNA damage. One of the most significant antioxidants in the modulation of ROS levels in mammalian cells is reduced glutathione (Droge et al., 2002). Data from the present study revealed that the activity of GSH in sperm was positively correlated with ROS production in the ejaculate ($\rho = 0.577$; $P < 0.0001$), the percentage of sperm with disrupted $\Delta\psi_m$ ($\rho = 0.385$; $P = 0.0055$), and caspase-3/7 activation in sperm ($\rho = 0.529$; $P = 0.0002$). The association of this antioxidant with both, oxidative status and sperm apoptotic markers, points to an up-regulation of glutathione metabolism to counter the damaging effects of ROS, a relationship demonstrated in previous studies (Lewis et al., 1997; Ebisch et al., 2006). In addition, glutathione has been suggested as a global marker for sperm fertilization capacity (Foresta et al., 2002).

Glutathione therapy has been reported to be beneficial in some andrological pathologies. Lenzi et al. (1992) administered glutathione to 11 infertile patients, via intramuscular injections, and reported a significant improvement in sperm motility pattern. However, Donnelly et al. (2000) reported no significant effect of glutathione on sperm motility and DNA damage *in vitro*. Ochsendorf et al. (1998) demonstrated that men with oligozoospermia had significantly lower sperm glutathione concentration than normozoospermic men. In contrast, Garrido et al. (2004) reported

that there was no significant difference between glutathione concentration in sperm from fertile and subfertile patients. Therefore, there is need for more investigations to improve our understanding of the role of glutathione in the function of spermatozoa.

4.3 Apoptotic Markers in Spermatozoa

Apoptosis is the underlying process for maintaining homeostasis during spermatogenesis (Sakkas et al., 2003; Giampietri et al., 2005) and, recently, several reports have suggested that the apoptotic process also occurs in mature sperm (Oehninger et al., 2003; Lachaud et al., 2004). These studies were based on the presence of biochemical markers of apoptosis which include mitochondrial membrane potential disruption (Barroso et al., 2006), plasma membrane translocation of phosphatidylserine (Kotwicka et al., 2008), the activity of aspartic acid-derived cysteine proteases (caspases) (Grunewald et al., 2005), DNA fragmentation (Sakkas et al., 2002; Tesarik et al., 2006), and the presence of pro- and anti-apoptotic proteins (Paasch et al., 2003).

In this study, $\Delta\psi_m$ disruption, caspase-3/7 activation and DNA fragmentation, were investigated as suitable markers of apoptosis in spermatozoa: the first parameter as an early event of this process, and the latter two as executioner and late event, respectively.

4.3.1 Disruption of the MMP ($\Delta\psi_m$)

The sperm mitochondria are an important focus of ROS-induced damage and, since intact $\Delta\psi_m$ is required for mitochondrial ATP production, this damage can result in the loss of sperm motility (de Lamirande and Gagnon, 1992). The disruption of $\Delta\psi_m$ is also a general feature of early stage apoptosis that precedes other manifestations of this process such as DNA fragmentation (Kroemer et al., 1997).

In the present study, the percentage of sperm with $\Delta\psi_m$ disruption was positively correlated with the percentage of $O_2^{-\bullet}$-positive sperm ($\rho = 0.261$; $P = 0.0045$) and the activity of GSH in sperm ($\rho = 0.385$; $P = 0.0055$). This appears to indicate that sperm $\Delta\psi_m$ can be disrupted by excessive $O_2^{-\bullet}$ production. Schulz et al. (2009) reported that

incubation of sperm from healthy donors with *E. coli* can induce significant $\Delta\psi_m$ disruption which was associated with diminished sperm motility and vitality. This demonstrates that ROS generated from outside the spermatozoa, for example, in patients with male genital tract infection/inflammation or leukocytospermia, can destabilize mitochondrial function and trigger more internal ROS production which can lead to apoptosis in spermatozoa. This is in agreement with reports by several authors that externally-derived ROS may act as apoptosis mediators (Armstrong et al., 1999; Vicari, 1999; Agarwal and Said, 2005; Villegas et al., 2005). Other studies have reported significant correlation between sperm $\Delta\psi_m$ and sperm motility (Marchetti et al., 2002; Wang et al., 2003; Zhang et al., 2008), and caspase-3 activity (Said et al., 2004; Angelopoulou et al., 2007), and sperm mitochondrial membrane potential has been recommended as a sensitive evaluation for sperm quality (Marchetti et al., 2002; 2004).

The significance of the positive correlation between the percentage of sperm with $\Delta\psi_m$ disruption and sperm GSH activity is not clear. However, given the association of sperm GSH activity with oxidative status in the ejaculate and sperm caspase-3/7 activity, the correlation with sperm $\Delta\psi_m$ suggests an increase in GSH activity in the response to oxidative stimuli in the sperm or in seminal plasma.

4.3.2 Caspase-3/7 activation in Sperm

It is now established that the central component of the apoptotic machinery involves one or more members of a family of aspartic acid-directed cysteine proteases called caspases (Thornberry and Lazebnik, 1998). The activity of caspases in human spermatozoa has been positively correlated with oxidative stress (Wang et al., 2003) and DNA fragmentation (Weng et al., 2002) in infertile patients.

In this study, sperm caspase-3/7 activation was positively correlated with the percentage of peroxidase-positive cells ($\rho = 0.527$; $P = 0.0001$), ROS production in the ejaculate ($\rho = 0.487$; $P = 0.0005$), the percentage of $O_2^{\cdot-}$-positive sperm ($\rho = 0.457$; $P = 0.0006$), and GSH activity in sperm ($\rho = 0.529$; $P = 0.0002$). Clearly, there is a significant strong relationship between the activity of caspase-3/7 and the oxidative status in the ejaculate and in spermatozoa. Seminal leukocytes are the

principal source of ROS in the ejaculate, and they have been linked with oxidative stress even in non-leukocytospermic patients (Henkel et al., 2003a). Therefore, it can be hypothesized that these cells can induce apoptosis via the caspase cascade. Recent studies have revealed that activated caspase-3 was localized exclusively to the midpiece area of positively-stained sperm (Weng et al., 2002; Almeida et al., 2005; Kotwicka et al., 2008), indicating that caspase-dependant apoptosis in sperm may be sequestered in a region where mitochondria and cytoplasmic droplets would be located. Significant correlation between caspase-3 activity and DNA fragmentation has been reported (Weng et al., 2002) but this association may not be consistent, as reflected by the current study. Some studies have demonstrated that apoptosis may not significantly contribute to sperm DNA damage (Moustafa et al., 2004) while others have even reported that oxidative stress-induced apoptosis may be caspase-independent (Grunewald et al., 2005).

Understanding the mechanism of caspase regulation is intimately linked to the ability to manipulate apoptosis for therapeutic gain (Thornberry and Lazebnik, 1998; Brill et al., 1999). Therefore, given the conflicting reports in this and other studies on the relationship between caspase activity and sperm function, further investigation is indicated.

4.3.3 Sperm DNA Fragmentation

The success of fertilization and the subsequent development of the embryo in humans depend, partly, on the integrity of the sperm DNA (Ahmadi and Ng, 1999). Spermatozoa of infertile men exhibit substantially more DNA damage compared to sperm of fertile men (Evenson et al., 1999; Irvine et al., 2000), and clinical evidence shows that damage to the male genome may adversely affect reproductive outcomes (Zini et al., 2001; Duran et al., 2002; Henkel et al., 2004). However, our understanding of the causes of sperm DNA damage remains rudimentary and, of the several proposed hypotheses of causes of DNA damage in human spermatozoa, the major theories are; poor chromatin packaging, abortive apoptosis, and oxidative stress.

Poor protamination of sperm DNA during spermatogenesis is clearly a major factor in determining the susceptibility of sperm chromatin to damage (Nasr-Esfahani et al., 2005; Carrell et al., 2006). This incomplete packaging of sperm chromatin has been reported in 5-15% of infertile men (Carrell and Liu, 2001; Oliva, 2006), and it has been linked with poor fertilizing potential and DNA fragmentation (Cho et al., 2003; Aoki et al., 2006). Furthermore, protamination of sperm chromatin appears to protect sperm DNA from oxidative stress by sequestering transition metals that are capable of promoting sperm DNA fragmentation (Liang et al., 1999). Therefore, this function may account for the extensive DNA damage observed in spermatozoa of with incomplete protamine-histone transition (Bianchi et al., 1993).

In addition to poor chromatin packaging, DNA fragmentation in spermatozoa of infertile men may involve an incomplete apoptotic pathway that is mediated by the cell surface protein; fibroblast associated (Fas) death receptor (Sakkas et al., 1999). The induction of apoptosis via the Fas pathway is an important mechanism by which Sertoli cells regulate the number and quality of germ cells, particularly under stressful conditions (Boekelheide et al., 2000). Sakkas et al. (1999) proposed that some of the spermatozoa with DNA damage exhibited Fas because they had been earmarked for elimination, but had escaped apoptosis; hence this process is called 'abortive apoptosis'. However, despite reports of higher incidence of Fas in sperm of men with poor semen parameters (Sakkas et al., 2002), there is no consensus on the percentage of Fas-positive sperm in the human ejaculate (Henkel et al., 2004). In addition, Castro et al. (2004) found no significant Fas in ejaculated sperm of both normozoospermic and non-normozoospermic men, while McVicar et al. (2004) could demonstrate Fas in sperm of infertile men only. Furthermore, the presence of Fas in human ejaculated sperm is related to, neither DNA damage as detected by the Comet assay, nor fertilization and pregnancy (Henkel et al., 2004; McVicar et al., 2004).

Alternatively, sperm DNA damage has been associated with high levels of reactive oxygen species (Barroso et al., 2000; Irvine et al., 2000). Sperm DNA fragmentation has been correlated with sperm-derived ROS (Henkel et al., 2003b) and this may be reflective of an underlying defect of spermatogenesis as it is sperm with cytoplasmic retentions that are largely responsible for sperm free radical generation (Gomez et al., 1996). Male genital tract infection associated with leukocytospermia has also been associated with DNA damage in spermatozoa (Sharma et al., 2001; Alvarez et al.,

2002; Erenpreiss et al., 2002) although this relationship is not always demonstrated (Henkel et al., 2003b). The negative effects of seminal leukocytes are mediated by oxidative stress, of which the stable H_2O_2 molecule is the most pernicious reactive oxygen species, particularly to spermatozoa of infertile men (Hughes et al., 1996).

In the current study, the percentage of TUNEL-positive sperm was positively correlated with ROS production in the ejaculate ($\rho = 0.331$; $P = 0.017$) and the percentage of H_2O_2-positive sperm (Amplex UltraRed) ($\rho = 0.274$; $P = 0.039$). Data from this study lends some support to the oxidative stress hypothesis of sperm DNA damage but the evidence is inconclusive as there is no correlation of sperm DNA damage with $\Delta\psi_m$, caspase activation or the concentration of seminal leukocytes. Some studies have also demonstrated that TUNEL positivity and apoptotic markers may not always correlate (Sakkas et al., 2002; Moustafa et al., 2004). This suggests that DNA fragmentation may be independent of classical apoptotic pathways, and that ROS may directly damage nuclear DNA in sperm.

4.4 Conclusions and Future Outlook

This study confirms that leukocytes are the major source of ROS production in the ejaculate, and that they may be related to the generation of $O_2^{-\bullet}$ in spermatozoa. Despite the positive correlation between ROS production in the ejaculate and sperm caspase-3/7 activity, the association between seminal oxidative stress and sperm apoptotic markers is rather tenuous. However, there is a relationship between sperm-derived $O_2^{-\bullet}$ and sperm $\Delta\psi_m$. In the light of these links, it would be important to investigate further what is going on in sperm mitochondria under the influence extrinsic ROS generated by leukocytes in the course of male genital tract infection/inflammation.

In addition, the superoxide anion may have a more prominent contribution to apoptosis than previously reported. The generation of this free radical in somatic cells is thought to involve electron leakage from the mitochondrial electron transfer chain (ETC) during normal cellular respiration, with an estimated 2% of consumed oxygen being converted to $O_2^{-\bullet}$ in this manner (Boveris and Chance, 1973). Normally, this ROS production is balanced by the antioxidant store within the cytoplasm and the

mitochondrial matrix (Andreyev et al., 2005). However, excessive mitochondrial ROS generation may outstrip the limited antioxidant defences, resulting in oxidative stress. This intrinsic generation of $O_2^{-\bullet}$ has been described in human spermatozoa by Koppers et al. (2008) and it has been positively correlated with poor motility by the same authors.

Commonly overlooked is the fact that sperm carry two different kinds of DNA; nuclear and mitochondrial. Mitochondria, which are located in the sperm mid-piece, are the energy generators of spermatozoa and supply adenosine triphosphate which helps propel the sperm towards the oocyte for fertilization. Mitochondrial DNA (mtDNA) encodes for two ribosomal RNAs, 22 transfer RNAs, and 13 polypeptides that are essential for mitochondrial respiration and oxidative phosphorylation associated with the electron transfer chain. Current understanding of the role of mitochondria in disease is expanding, and it is becoming clear that mitochondrial dysfunction may be a significant factor in some cases of male infertility and asthenozoospermia (Følgero et al., 1993; Ruiz-Pesini et al., 2000). In the context of sperm function mitochondria are important, not only because they are the powerhouse of the germ cell, but because as the site of oxidative phosphorylation they are a major source of electron leakage from the ETC (Chance et al., 1979; Giulivi et al., 1995) which is a significant contributor to internal ROS production by sperm (Koppers et al., 2008). In addition, mitochondria are involved in the regulation of apoptosis by means of the mitochondrial or intrinsic pathway (Gogvadze and Orrenius, 2006), and in sperm this process is characterized by the loss of mitochondrial membrane potential (Marchetti et al., 2002; Zhang et al., 2008), caspase-3 activity (Weng et al., 2002), and nuclear DNA fragmentation (Wang et al., 2003). Furthermore, mtDNA is located on the matrix side of the mitochondrial inner membrane (Antunes et al., 1996) and consequently it is vulnerable to oxidative damage by mitochondrial-derived ROS. Mitochondrial DNA is more susceptible to oxidation by ROS because, unlike nuclear DNA, it lacks protective packaging proteins such as protamines and histones (Marchesi and Feng, 2007). Moreover, mtDNA replicates at a faster rate without proper proofreading and only has a basic repair mechanism (Croteau et al., 1999) and this makes the genome prone to mutations and deletions in spermatozoa (O'Connell et al., 2002). Since mtDNA is essential to the energy generation role of mitochondria in

spermatozoa, it is indispensable for successful fertilization and there is need to gather more data on the effects of mtDNA damage on male infertility.

In conclusion, this study has demonstrated a clear association between the concentration of seminal leukocytes and ROS production in the ejaculate and in spermatozoa. Moreover, seminal leukocyte concentration was strongly correlated with the effector caspases; -3 and -7. Therefore, there is a case for a relationship between male genital tract infection and sperm oxidative stress, which may lead to sperm apoptosis via the mitochondrial pathway.

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CHAPTER FIVE

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