CHAPTER 1

INTRODUCTION AND AIM OF STUDY

1.1 Introduction

The history of oxidative stress and male infertility dates back to an article published in the American Journal of Physiology in 1943 (Macleod, 1943). Male fertility markers have been scrutinized in order to comprehend the molecular events that can lead to subfertility and permit an accurate diagnosis and design of therapeutic protocols. Among these markers, the study of oxidative stress (OS) status in semen has emerged as a promising field (Agarwal *et al*., 2003). OS can be defined as the imbalance between pro-oxidative and anti-oxidative molecules in a biological system which arises as a consequence of excessive production of free radicals and impaired antioxidant defense mechanisms (Agarwal *et al*., 2003). Those free radicals derived from oxygen are called reactive oxygen species (ROS).

ROS are produced primarily by the physiological metabolism of oxygen in cells under aerobic conditions. These molecules are very reactive with cellular structures, undermining or eliminating their biological functions and properties. On the other hand small amounts of ROS and other free radicals such as nitric oxide (NO) are necessary to maintain normal sperm function (Agarwal *et al*., 2003) such as motility, capacitation, and acrosome reaction (AR). Previous studies have demonstrated an increase in free radical production during the *in vitro* preparation of spermatozoa for use in assisted reproductive technologies, especially during the obligatory centrifugation steps (Agarwal *et al*., 1994).

Even though much is known about the presence and role of free radicals in human spermatozoa, there is little evidence of studies that have measured specific types of free radicals and if measured, indirect, insensitive and non-specific approaches are used.

1.2 Objective and statement of the problem

The aim of this study is threefold: (i) to standardize and establish flow cytometry as an accurate technique to directly measure specific free radicals in human spermatozoa, (ii) to investigate the effects of sperm centrifugation on free radical generation and sperm function, (iii) to investigate the effects of NO and H_2O_2 (a member of the ROS family) on sperm function.

1.3 Plan of study

To serve as a background to the study, a broad overview of current literature on the role of reactive oxygen species and other free radicals in human spermatozoa is provided in chapter two. This is followed by the basic materials and methods in chapter 3. Chapters 4 and 5 comprise of results, and the discussion respectively.

1.4 Conclusion

Resolving the various factors contributing to the creation of excessive free radical generation is strategically important because such data will help design methods for the prevention of pathologies involving oxidative stress. This will be particularly important for the future of assisted reproductive technology.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

A free radical is any compound (not necessarily derived from oxygen) which contains one or more unpaired electron(s) (Halliwell and Gutteridge, 1999). Free radicals derived from oxygen are called reactive oxygen species (ROS) and examples include superoxide ($O_2^{-\cdot}$) anion, hydrogen peroxide (H_2O_2), peroxyl (ROO^-) radicals, and hydroxyl (OH^-) radicals (Ford, 2004). Those derived from nitrogen are called reactive nitrogen species (RNS) and include nitric oxide (NO^-) and peroxynitrite anion ($ONOO^-$) (Armstrong, *et al*., 1999). Figure 1 shows how these free radicals are interrelated with each other. The assumption that free radicals can influence male infertility has received substantial scientific support (Gagnon and de Lamirande, 2003). Some studies have shown that almost 40% of infertile males display abnormally increased ROS levels (Agarwal *et al*., 2003).

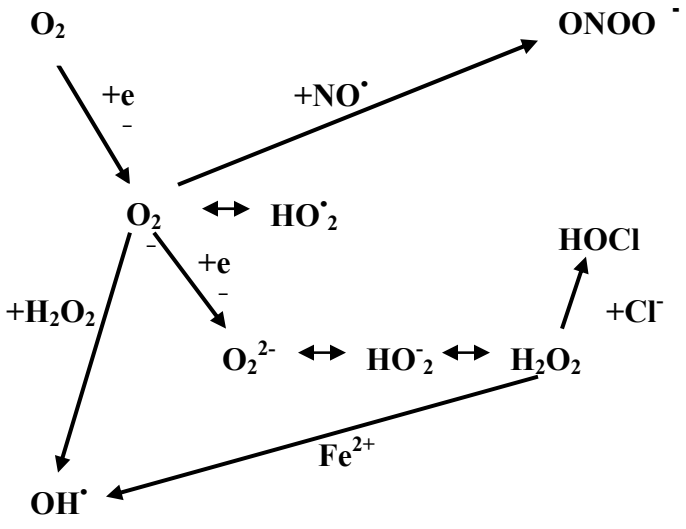

Figure 1. *Derivation of reactive oxygen species from oxygen (Ford, 2004)*

Human spermatozoa are extremely susceptible to free radical induced damage due to their plasma membrane composition (Padron *et al*., 1997). The plasma membranes of spermatozoa contain large quantities of polyunsaturated fatty acids (PUFA) which make them very susceptible to OS-induced damage (Alvarez and Storey, 1995). This is exacerbated by low concentrations of scavenging enzymes in their cytoplasm such as superoxide dismutase (SOD), glutathione peroxidase (GPX), vitamin E and catalase (de Lamirande and Gagnon, 1995).

2.2 Sources of free radicals in semen

Human semen is a complex mixture consisting of a combination of diverse products synthesized along the whole male genital tract, including the seminiferous tubules and accessory glands. Seminal cells include both mature and immature spermatozoa, round cells from different stages of the spermatogenic process, leukocytes and other occasional cell types such as epithelial cells.

2.2.1 Spermatozoa

The source of ROS generation by spermatozoa is a subject of intense speculation (Aitken and Baker, 2004). However all actively respiring cells generate ROS as a consequence of electron leakage from intracellular redox activities, such as the mitochondrial electron transport chain. Under physiological O_2 tensions, it has been calculated that 1-3% of the O_2 reduced in mitochondria may form superoxide anions ($O_2^{-\cdot}$) (Halliwell and Gutteridge, 1999).

Studies have shown that immature spermatozoa are also a source of ROS (Gil-Guzman *et al*., 2001). Hypotheses have been put forward that immature and abnormal sperm with large cytoplasmic droplet retention are important ROS producers, since they retain an excess of cytoplasmic enzymes that are involved in glucose metabolism, such as glucose-6-phosphate dehydrogenase, the NADPH oxidase system, and NADH dependent oxidoreductase. These metabolic processes can occur at two different sites: the plasma membrane and the mitochondria (Gil-Guzman *et al*., 2001).

2.2.2 Leukocytes

The presence of leukocytes (predominantly granulocytes) in semen has been associated with severe male factor infertility cases (Aitken *et al*., 1987). Increased leukocyte infiltration in semen, that is, leukocytospermia, has been linked with poor sperm quality, reduced sperm hyper-activation, and defective sperm function (Wolff, 1995). On the other hand, no correlation was found between seminal leukocyte concentrations and impaired sperm quality (Tomlinson *et al*., 1993) or defective sperm function (Aitken *et al*., 1994). The World Health Organization (WHO) defines leukocytospermia as the presence of peroxidase-positive leukocytes in concentrations of $>1x10^6$ per milliliter of semen (WHO, 1999).

Peroxidase-positive leukocytes in semen originate to a large extent from the prostate and the seminal vesicles (Wolff, 1995). They are found to be the major source of high ROS production in semen (Rajasekaran *et al*., 1995). Activated leukocytes can produce 100-fold higher amounts of ROS than non-activated leukocytes (Plante *et al*., 1994).

Leukocytes may be activated in response to a variety of stimuli including inflammation and infection (Pasqualotto *et al*., 2000). Sperm damage from leukocyte-derived ROS occurs if seminal leukocyte concentrations are abnormally high, such as in leukocytospermia (Shekarriz *et al*., 1995[a]).

Sharma *et al*., (2001) observed that seminal leukocytes might cause OS even at concentrations below the WHO cutoff value for leukocytospermia. This may be due to the fact that seminal plasma contains large amounts of ROS scavengers but confers a very variable (10% to 100%) protection against ROS generated by leukocytes (Kovalski *et al*., 1992). It is however not yet clear from the existing literature whether the interaction between leukocytes and spermatozoa implies a direct or indirect stimulatory effect, which may enhance the capacity of spermatozoa to generate excessive ROS. Agarwal, *et al*., (2003) indicated that levels of ROS production by pure sperm suspensions from infertile men with a laboratory diagnosis of leukocytospermia were significantly higher than were those from infertile men without leukocytospermia. In addition, seminal leukocyte concentrations were strongly correlated with levels of ROS in the original cell suspensions containing sperm and leukocytes (basal ROS); in the leukocyte-free sperm suspensions (pure sperm ROS); and in the leukocyte-free sperm suspensions (phorbol ester-induced ROS). From this observation we can postulate that seminal leukocytes play a role in enhancing sperm capacity for excessive ROS production either by direct sperm-leukocyte contact or by soluble products released by the leukocytes.

2.3 Biological roles of free radicals

Free radicals can react with a wide range of biological molecules, some of which include fatty acids, sulphydryl proteins and nucleic acids, and are implicated in a large number of diseases, e.g. arthritis, atherosclerosis, and degenerative diseases of ageing (Halliwell and Getteridge, 1999). However, free radicals also have physiological roles. They are produced by leukocytes as part of the phagocytotic process to kill engulfed bacteria but also in smaller amounts by other cell types to act as cell-to-cell and intracellular messengers (Babior, 1999).

2.3.1 Sperm capacitation

Freshly ejaculated sperm cannot fertilize until they have spent some time in a suitable environment in order to capacitate. Although numerous hypotheses have been developed, the precise nature of capacitation is still obscure (Yamaguchi, 1994). Changes associated with sperm capacitation include an increase in respiration and subsequent changes in the motility pattern, called hyper-activation, which is characterized by pronounced flagellar movements and a marked lateral excursion of sperm head in a non-linear trajectory (Ehrenwald et al., 1990), removal of cholesterol from the plasma membrane, destabilization of the sperm membrane, an increase in intracellular pH and calcium levels, activation of second messenger systems and removal of zinc (Andrews and Bavister, 1989).

The most important change in sperm after capacitation is its ability to undergo the acrosome reaction in response to the *zona pellucida* protein 3 (ZP3), progesterone and

calcium ionophore (Russel *et al*., 1978). Capacitation is also associated with changes in sperm plasma membrane fluidity, intracellular changes in ionic concentration, and sperm cell metabolism (Yamaguchi, 1994).

2.3.1.1 Role of free radicals during sperm capacitation

Superoxide anion radical plays an important role during maturation of spermatozoa (Kumar *et al*., 1991) and in the control of sperm function through the redox regulation of tyrosine phosphorylation (Aitken *et al*., 1995). Superoxide has been shown to promote the capacitation of human spermatozoa (Zhang and Zheng, 1996[b]) and there is a superoxide surge in the capacitated spermatozoa during the process (Purohit et al., 1998). It has been reported that (i) exogenously generated superoxide through xanthine/xanthine oxidase system induced hyper-activation and capacitation, (ii) capacitating sperm produced elevated concentrations of superoxide over prolonged periods of time and (iii) removal of this ROS by superoxide dismutase (SOD) prevented hyper-activation and capacitation (de Lamirande and Gagnon, 1995). Hydrogen peroxide was also shown to promote capacitation of human spermatozoa (Griveau *et al*., 1994). The mechanisms and targets of action of hydrogen peroxide are still unknown.

Nitric oxide is a free radical synthesized *in vivo* during the conversion of L-arginine to L-citrulline by the enzyme nitric oxide synthase (NOS). Recent reports suggested the expression of NOS in mouse and human spermatozoa (Lewis *et al*., 1995). NO appears to be involved in sperm hyper-activation (Herrero *et al*., 1994) and *zona pellucida* binding

(Sengoku *et al*, 1998). However, the role of endogenous NO in human sperm capacitation still remains to be elucidated.

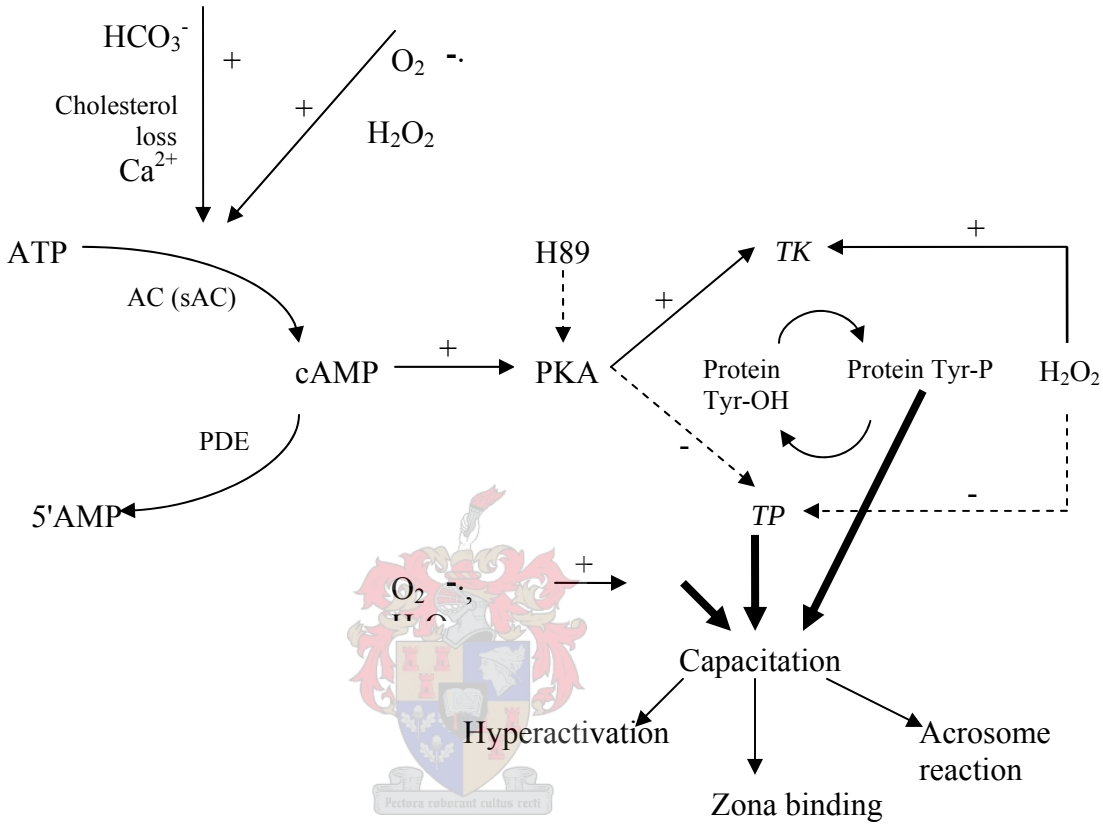

Figure 2. *Postulated effects of reactive oxygen species on intracellular signaling during sperm capacitation (Ford, 2004)*

2.3.2 Free radicals and sperm cell signaling pathways

There is general agreement that the potentiation of capacitation by ROS is associated with increased protein tyrosine phosphorylation and that it shares features with the cAMP-dependent capacitation pathway (Thundathil *et al*., 2003). Physiological concentrations of ROS have been proposed to enhance sperm capacitation by increasing cAMP synthesis and by inhibiting protein tyrosine phosphatases whilst activating

tyrosine kinase (Fig. 2). Evidence that they increase intracellular cAMP concentrations includes the observations that addition of a phosphodiesterase inhibitor or addition of dibutyryl cAMP had a similar potency to stimulating ROS production (Aitken et al., 1998). Exposure to superoxide increased sperm cAMP concentrations (Zhang and Zheng, 1996[b]) and exposure to NADPH produced a larger increase in intracellular cAMP than the phosphodiesterase inhibitor pentoxifylline (Aitken *et al*., 1998). Double phosphorylation of the threonine-glutamine-tyrosine motif characteristic of ERK 1/2 activation is regulated by NO (Thundathil *et al*., 2003). Stimulation of this event by fetal cord serum ultrafiltrate was blocked by the nitric oxide synthase inhibitor N^W-nitro-L-arginine methyl ester (L-NAME), but not SOD or catalase. However superoxide did influence phosphorylation of the Thr-Glu-Tyr motif in proteins of lighter molecular weight (16-33 kDa) and regulated phosphorylation of some insoluble ERK 1/2 substrates in parallel with the ERK pathway (Gagnon and de Lamirande, 2003).

2.3.3 Acrosome reaction (AR)

The acrosome is a cap-like membrane limited organelle which covers the anterior part of the nucleus on the sperm head (Meizel, 1984). As illustrated in figure 3, the acrosome reaction involves the fusion, vesiculation and loss of the outer acrosomal membrane and its overlying sperm plasma membrane and the release of acrosomal matrix material (Meizel, 1984). During this process hybrid membrane vesicles are formed (Fedder and Ellerman-Erickson, 1995). This organized membrane fusion and vesiculation is required for sperm penetration through the acellular coating enclosing the egg.

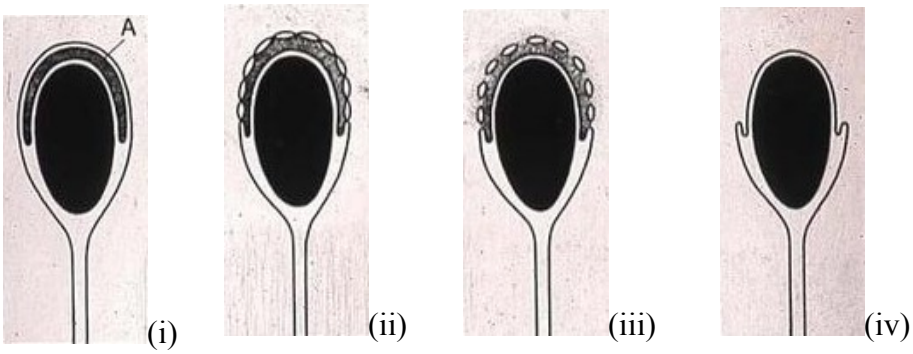

Figure 3. *An illustration of the sperm acrosome reaction (i) location of the acrosome, at the anterior part of the sperm head, (ii) fusion of the outer acrosomal membrane and its overlying sperm plasma membrane, (iii) vesiculation and loss of the outer acrosomal membrane and its overlying sperm plasma membrane (iv) acrosome reacted sperm head which has released its acrosomal content.*

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Under physiological and *in vitro* conditions, the egg-specific extracellular matrix, i.e. the *zona pellucida*, stimulates acrosomal exocytosis in sperm (Yamaguchi, 1994 and Florman *et al*., 1992). One of the *zona pellucida* glycoproteins, ZP3, a sulphated glycoprotein, stimulates AR (Yamaguchi, 1994, and Bleil and Wassarman, 1983). Progesterone, a major component of follicular fluid, has been found to induce AR in spermatozoa (Osman *et al*., 1989). AR can be induced *in vitro* by ionophores which exchanges Ca^{+2} for other ions such as H^{+} and Na^{+} (Russell *et al*., 1979).

2.3.3.1 Role of free radicals in the acrosome reaction

The role of ROS in sperm capacitation is very well documented, but reports on their involvement in the acrosome reaction are rather scanty. Superoxide anion production is

shown to be associated with ionophore induced acrosome reaction (Aitken *et al*., 1995 and Griveau *et al*., 1995). Superoxide production drops suddenly after addition of acrosome reaction inducers (Purohit *et al*., 1998), but is highest during the capacitation process rather than at the time of acrosome reaction. H_2O_2 is known to induce hyper-activation and promote capacitation, but is not involved in the acrosome reaction of the spermatozoa (Griveau *et al*., 1994).

2.4 Pathological effects of increased free radicals

A characteristic feature of most, if not all, biological membranes is an asymmetrical arrangement of lipids within the bilayer. The lipid composition of plasma membranes of mammalian spermatozoa is markedly different from those of mammalian somatic cells. They have very high levels of phospholipids, sterols, saturated and polyunsaturated fatty acids, therefore sperm cells are particularly susceptible to damage induced by excessive ROS release (Alvarez and Storey, 1995). Lipids are major substances responsible for the fluidity of membrane lipid bilayers, and changes in composition of plasma membranes of sperm cells from their epididymal maturation to their capacitation in the female reproductive tract. They are also involved as intermediates in cell fusion (Yeagle, 1994). Lipid peroxidation of sperm plasma membranes is considered to be the key mechanism of ROS-induced sperm damage leading to infertility.

2.4.1 Lipid peroxidation (LPO) of spermatozoa

Peroxidation of polyunsaturated fatty acids (PUFAs) in sperm cell membranes is an autocatalytic, self-propagating reaction (Halliwell, 1990) which can give rise to cell

dysfunction associated with loss of membrane function and integrity. It is divided into two steps: initiation and propagation (Aitken and Fisher, 1994). Initiation is the removal of the hydrogen atom from an unsaturated fatty acid. The second step, propagation, is the formation of a lipid alkyl radical followed by its rapid reaction with oxygen to form a lipid peroxyl radical. The peroxyl radical is capable of removing a hydrogen atom from an unsaturated fatty acid resulting in the formation of a lipid radical and lipid hydroperoxide (Halliwell, 1990). Since the alkyl and peroxyl radicals are regenerated, the cycle of propagation could continue indefinitely or end when one of the substrates is consumed or terminated in the radical-radical reaction.

2.4.2 Impairment of sperm motility

Excessive ROS production in semen has been correlated with a reduction of sperm motility (Lenzi et al., 1993). This link between ROS and reduction in sperm motility may be due to a cascade of events that result in a decrease in axonemal protein phosphorylation and sperm immobilization, both of which are associated with a reduced membrane fluidity that is necessary for sperm oocyte fusion (de Lamirande and Gagnon, 1995). Another hypothesis is that H_2O_2 can diffuse across the membranes into the cells and inhibit the activity of some enzymes such as glucose-6-phospate dehydrogenase (G_6PD). This enzyme controls the rate of glucose flux through the hexose monophosphate shunt, which in turn controls the intracellular availability of NADPH. This in turn, is used as a source of electrons by spermatozoa to fuel the generation of ROS by an enzyme system known as NADPH oxidase (Aitken *et al*., 1997).

2.4.3 Deoxyribonucleic acid (DNA) damage

Two factors protect the sperm DNA from an oxidative insult: (i) the characteristic tight packaging of the DNA, and (ii) the antioxidants present in the seminal plasma (Twigg *et al*., 1998). Studies in which sperm was exposed to artificially produced ROS resulted in a significant increase in DNA damage in the form of modification of all bases, production of base-free sites, deletions, frameshifts, DNA cross-links, and chromosomal arrangements (Duru *et al*., 2000). Oxidative stress has also been correlated with high frequencies of single and double DNA strand breaks (Twigg *et al*., 1998).

2.4.4 Sperm apoptosis

Apoptosis is a process of programmed cell death. It is a physiological phenomenon characterized by cellular morphological and biochemical alterations that cause a cell to commit suicide (Vaux, and Flavell, 2000). It is genetically determined and takes place to help discard cells that have an altered function or no function at all (Vaux and Korsmeyer, 1999). In the male reproductive system, apoptosis may be responsible for controlling the overproduction of male gametes (Sakkas *et al*., 1999). Apoptosis appears to be strictly regulated by extrinsic and intrinsic factors and can be triggered by a wide variety of stimuli. Examples of extrinsic stimuli that are potentially important in testicular apoptosis are irradiation, chemotherapy, and toxin exposure (Lee *et al*., 1997). Figure 4 shows the events that take place in a cell undergoing apoptosis. Apoptosis-inducing genes such as p53, Bax, and Fas and apoptosis-suppressing genes such as Bcl-2 and c-kit play a prominent role in the genetic control of apoptosis (Sinha, 1999).

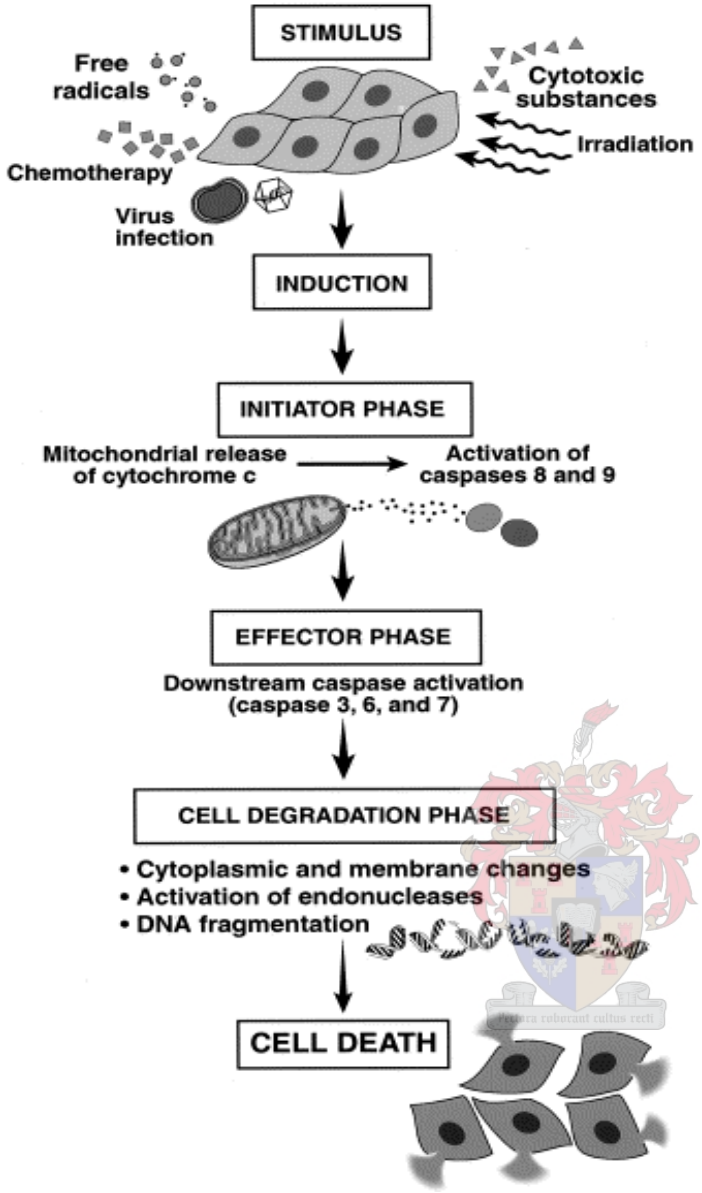


Figure 4. *Events that take place in human cells undergoing apoptosis (Agarwal et al., 2003)*

Spontaneous germ cell apoptosis has been shown in spermatogonia, spermatocytes, and spermatids in the testis of normal men and in patients with nonobstructive azoospermia (Agarwal *et al*., 2003). Ejaculated spermatozoa have also been shown to demonstrate changes consistent with apoptosis (Lee *et al*., 1997). It has been shown that the levels of

apoptosis in mature spermatozoa were significantly correlated with levels of seminal ROS (Agarwal *et al*., 2003). They also found that levels of caspase 3 and caspase 9 in the ejaculated spermatozoa from infertile patients were significantly higher than in normal healthy sperm donors. In addition, levels of seminal ROS were positively correlated with levels of caspase 3 and caspase 9. The caspase gene family encodes a set of proteases responsible for carrying out programmed cell death (Agarwal *et al*. 2003).

2.5 Conclusion

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

CHAPTER 3

MATERIALS AND METHODS

3.1 Introduction

The rest of this chapter will outline the detailed protocols and methods that were employed in this study. A brief outline of the experimental procedure followed is given in figure 5.

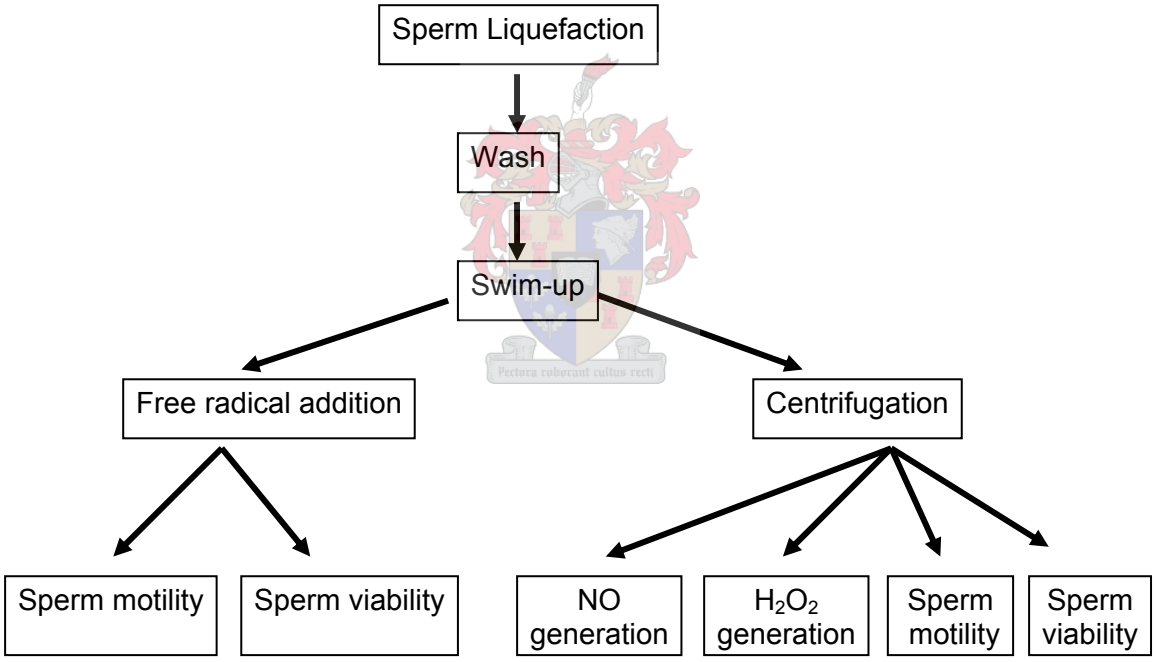


Figure 5*. Flow chart showing the generalized experimental protocol*

3.2 Preparation of human tubal fluid (HTF) culture medium

HTF culture medium was prepared as follows:

1. Dissolve the following chemicals in about 600ml tissue culture grade water in a 1000ml volumetric flask: 5.938g NaCl; 0.350g KCl; 0.049g $MgSO_4.7H_2O$; 0.050g KH_2PO_4; 2.100g $NaHCO_3$; 0.036g Na pyruvate; 0.501g Glucose; 0.003g Phenol red; 3.136 ml Na lactate (60% syrup)
2. Separately dissolve 0.300g $CaCl_2.H_2O$ in 100ml tissue culture grade water and add slowly to the rest.
3. Add penicillin/streptopen (75mg)
4. Make up to 1000ml with additional culture grade water and mix thoroughly.
5. Adjust pH to 7.5-7.6
6. Check the osmolarity is between 280-290 mOsm.
7. Filter-sterilize into plastic containers under positive pressure.
8. Store at 4˚C.
9. Warm to 37˚C before use.

Add 3% BSA if the medium is to be used as a capacitation medium.

3.3 Semen collection

Semen samples were obtained from 24 normozoospermic healthy volunteer donors studying at the Tygerberg Campus, University of Stellenbosch, aged between 19-23 years. All semen samples were collected by masturbation after 2-3 days of sexual abstinence according to the World Health Organization criteria (WHO, 1999). Semen

samples were collected in sterile wide mouthed containers after which the semen was allowed to liquefy for 30 minutes at 37˚C. Ethical approval from our institution was obtained.

3.4 Semen preparation

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

3.5 Computer assisted semen analysis (CASA)

Sperm motility was determined with the Hamilton-Thorne IVOS analyzer (Hamilton-Thorne Research, Beverly, MA). The settings of the analyzer were as follows: 30 frames/60 Hz; minimum contrast, 80; minimum cell size, 2; minimum static contrast, 30; low average path velocity (VAP) cut-off, 5 μm/s; low straight-line velocity (VSL)cut-off, 11 μm/s; head size, non-motile, 3; head intensity, non-motile, 160; static head size, 1.01-2.91; static head intensity, 0.60-1.40; slow cells not motile; magnification, 2.01, and temperature, 37˚C. Sperm motility is assessed by several parameters when analyzed using computer assisted semen analysis (CASA) as illustrated in figure 6.

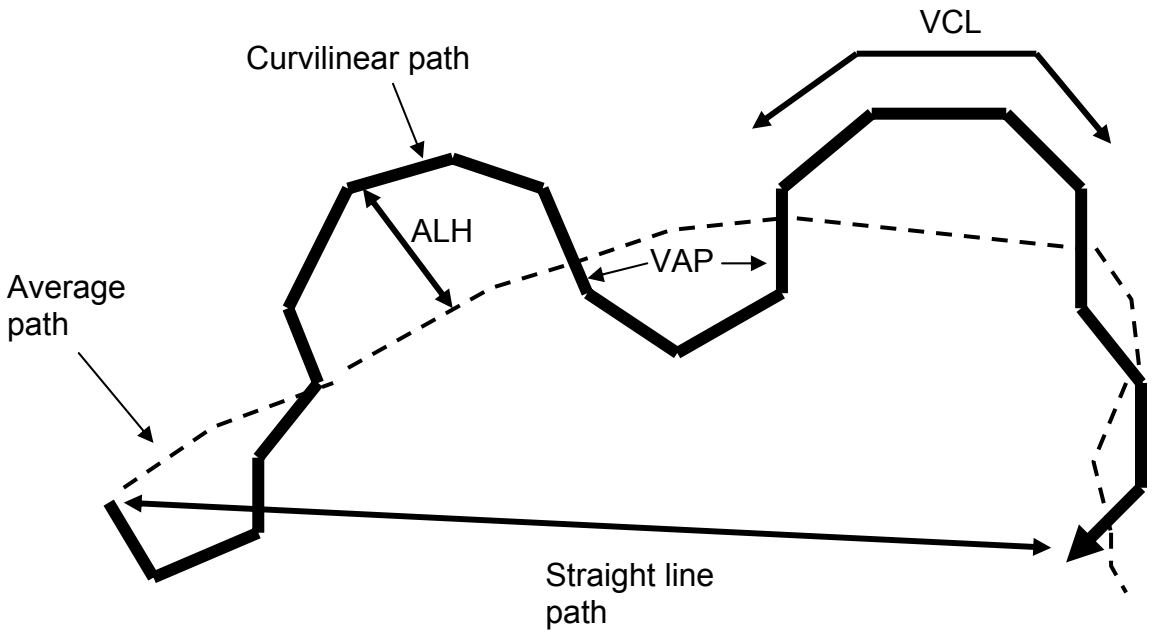


Figure 6. *An illustration of different sperm motility parameters measured using CASA (WHO, 1999)*

Motility parameters analyzed by means of CASA include the following:

(i) Motility: the percentage of motile spermatozoa.

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

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

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

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

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

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

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

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

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

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

3.6 Flow cytometry

Free radicals and sperm cell viability were measured by flow cytometric analysis (FACS: fluorescence-activated cell sorter). A Becton Dickinson FACSCalibur™ analyzer (BD, Sanjose, CA, USA) was used to quantify fluorescence at the single-cell level and data was analyzed using CellQuest™ version 3.3 (Becton Dickinson, Sanjose, CA, USA) software. In each sample, the mean fluorescence intensity of the analyzed cells was determined after gating the cell population by forward and side light scatter signals as recorded on a dot plot (Fig 7). In total, 100,000 events were acquired, but non-sperm particles and debris (located at the bottom left corner of the dot plot) were excluded by prior gating, thereby limiting undesired effects on overall fluorescence. Final gated populations usually contained 12,000-15,000 sperm cells. Fluorescence signals were recorded on a frequency histogram (Fig. 8A and B; Fig 9) using logarithmic amplification. Fluorescence data are expressed as mean fluorescence (percentage of control, control adjusted to 100%).

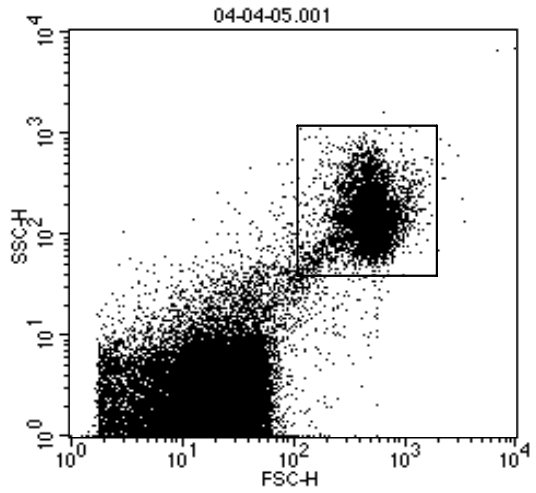


Figure 7. *A representative dot plot of sperm cells showing the spread of the total recorded "events". Gated population (top right): sperm cells and bottom left: non-sperm particles, debris.*

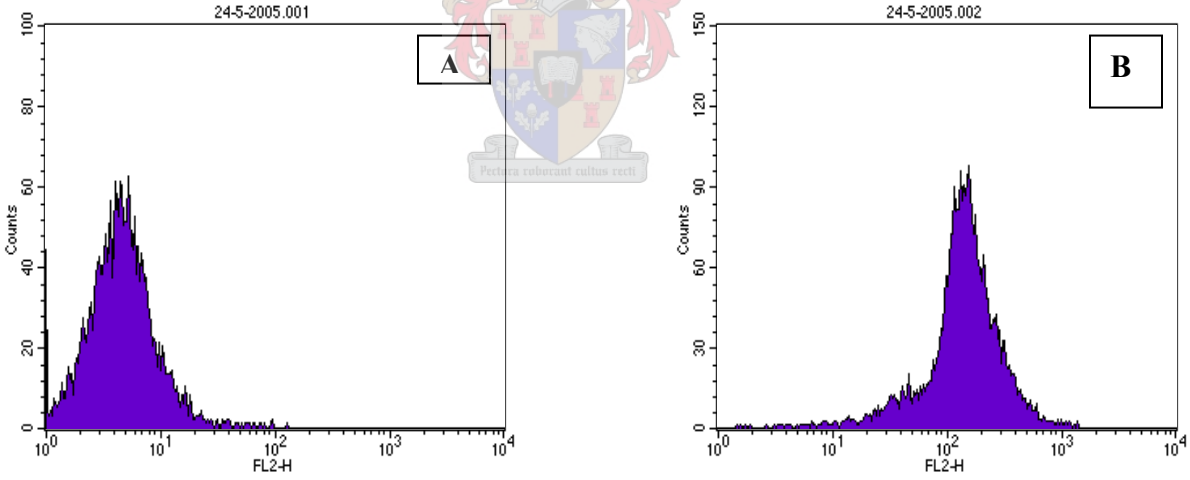


Figure 8*. A representative frequency histogram showing baseline fluorescence (log) of DAF-2/DA or DCFH on x-axis (A); a shift to right depicting an increase in fluorescence intensity (B)*

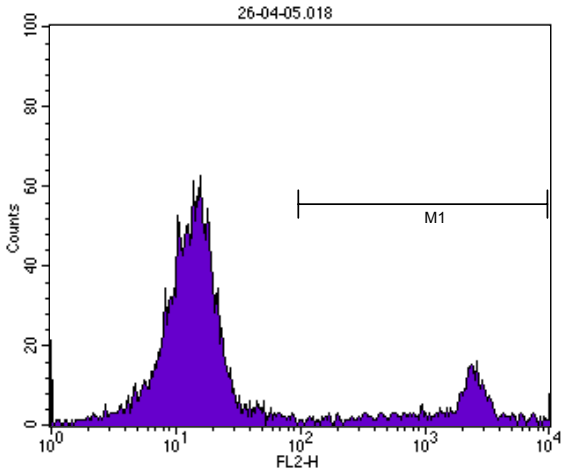


Figure 9. *A frequency histogram of PI fluorescence with two peaks. Cells possessing a damaged membrane will permit PI to enter into the cell and bind to DNA causing the cells to fluoresce red. The peak to the left is depicting viable cells which are able to exclude PI while that to the right is non-viable cells which had absorbed PI.*

3.7 Protocols

This section will outline in detail all the protocols that were employed in this study.

3.7.1 Standardization and establishment of flow cytometry as an accurate technique to directly measure specific free radicals

3.7.1.1 Probe specificity of DAF-2/DA for NO

After collection through swim-up, sperm cells were counted and concentration determined by means of CASA. Cells were subsequently divided into aliquots at a concentration of $2x10^6$/ml each. As shown in figure 10, cells were treated with the NO-donor sodium nitroprusside (SNP) (Sigma-Aldrich Co. Ltd, St Louis, USA), with

increasing concentrations. Subsequently, cells were incubated with non-limiting concentrations of 4,5-diaminofluorescein-2/diacetate (DAF-2/DA, 10µM, 37˚C) (Calbiochem, San Diego, CA, USA) for 120 min, modified from a technique previously described in isolated cardiomyocytes (Strijdom *et al*., 2004). Fluorescence in these cells was produced by oxidation of DAF-2/DA to its highly green-fluorescent DAF-2T form, and signals were recorded on a frequency histogram (Fig. 8A) using logarithmic amplification. A right-shift of fluorescence (Fig. 8B) indicated increased NO generation. In all experiments, light was avoided by working in the dark since the probe is known to be light sensitive.

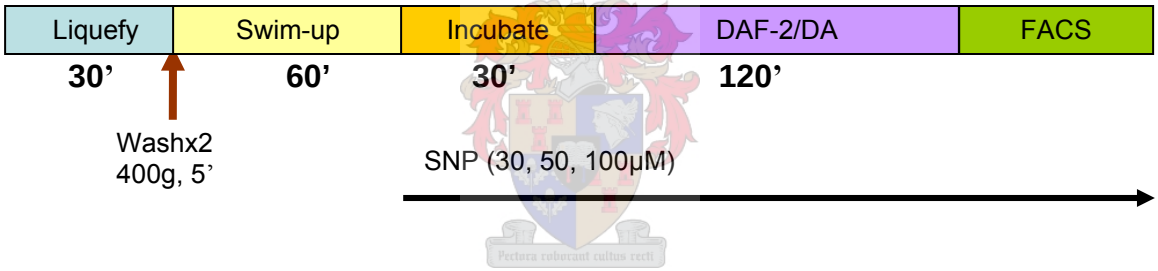


Figure 10. *Protocol to validate probe specificity of DAF-2/DA*

3.7.1.2 Probe specificity of DCFH for ROS

Sperm cells were collected via swim-up method, counted and concentration determined by means of CASA. Subsequently, the cells were divided into aliquots at a concentration of $2x10^6$ cells/ml each. As shown in figure 11, the sperm cells were incubated with or without the non-specific ROS scavenger, N-(2-mercaptopropionyl)Glycine, (MPG, 50µM) (Sigma-Aldrich Co. Ltd, St Louis, USA) for 45 min until wash-out. This was followed by the administration of non-limiting concentrations of the non-specific ROS

probe 2,7-dichlorofluorescein diacetate (DCFH; 5µM, 37˚C) (Sigma Chemicals CO., St. Louis, MO) for 15 min (Benedi *et al*., 2004). The sperm cells were then washed twice and further incubated in probe free medium (37˚C, 30 min) before FACS analysis. Fluorescence signals were recorded on a frequency histogram (Fig. 8A) using logarithmic amplification. A right shift of fluorescence (Fig. 8B) indicates increased ROS generation. In all experiments, light was avoided by working in the dark since the probe is known to be light sensitive.

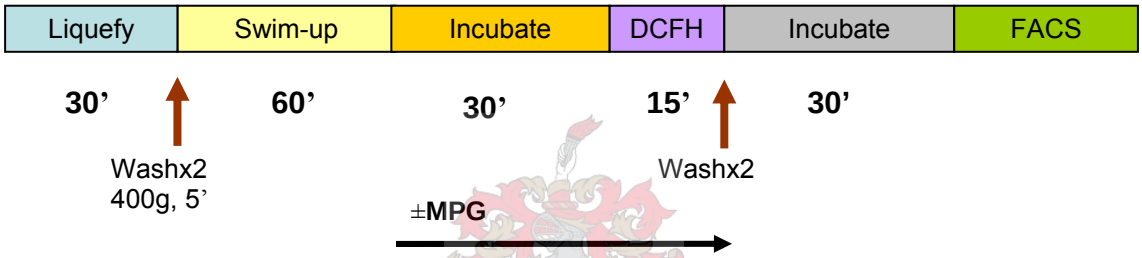


Figure 11. *Protocol to validate probe specificity of DCFH*

3.7.2 Investigation of the effects of sperm centrifugation on free radical generation and sperm function

3.7.2.1 Effects of centrifugation on DAF-2/DA fluorescence

After collection through swim-up, sperm cells were counted using CASA. Cells were subsequently divided into aliquots at a concentration of $2x10^6$/ml each. As shown in figure 12, cells were incubated in the presence or absence of the NOS inhibitor, L-NAME, (0.7mM, Sigma Chemical Co., St Louis, MO, USA) for 15 min prior to

centrifugation (10 or 30 min) at 400xg. L-NAME remained present until FACS analysis started. The rest of the experiment was done as outlined in section 3.7.1.1.

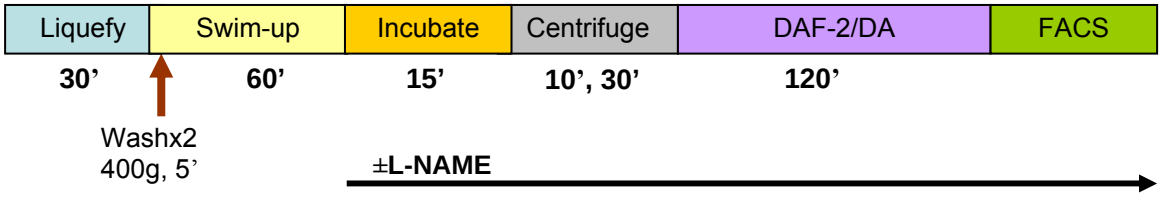


Figure 12. *Protocol to determine the effects of centrifugation DAF-2/DA fluorescence*

3.7.2.2 Effects of centrifugation on DCFH fluorescence

After collecting the sperm cells via swim-up method, they were counted and concentration determined by means of CASA. Subsequently, the cells were divided into aliquots of concentration $2x10^6$ cells/ml each. As shown in figure 13, the sperm cells were incubated in the presence or absence of the non-specific ROS scavenger, MPG, (50µM) 15 min prior to centrifugation (10 or 30 min). MPG remained present until wash-out. After centrifugation cells were incubated with non-specific ROS probe DCFH (5µM, 37˚C) for 15 min and the rest of the experiment was done as outlined in section 3.7.1.2.

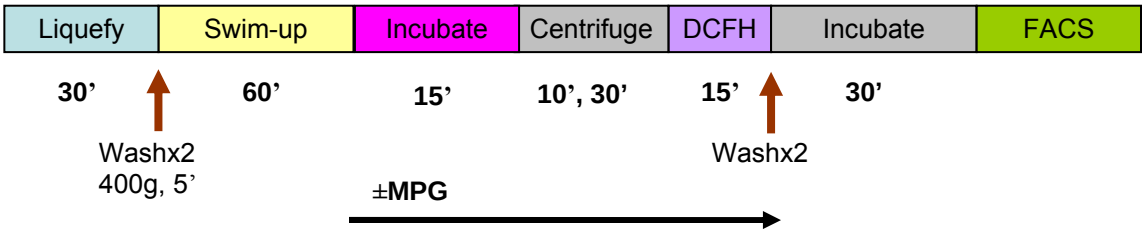


Figure 13. *Protocol to determine the effects of centrifugation on DCFH fluorescence*

3.7.2.3 Effects of centrifugation on sperm motility parameters

After collection through swim-up, sperm cells were counted and concentration determined by means of CASA. Cells were subsequently divided into aliquots at a concentration of $2x10^6$/ml each. Some of the cells as shown in figure 14, were incubated with either the NOS inhibitor L-NAME (0.7mM), ROS scavenger MPG (50µM), or a combination of both 15 minutes prior to centrifugation, remaining present until the end. The cells were centrifuged (10 or 30 min), and then incubated for 120 minutes before motility was measured using CASA. The motility parameters of interest were total motility, progressive motility, VAP, VSL, VCL, ALH, BCF, STR, LIN, rapid cells, and static cells of which motile cells, progressive motility, rapid cells and static cells seemed to be the more important features.

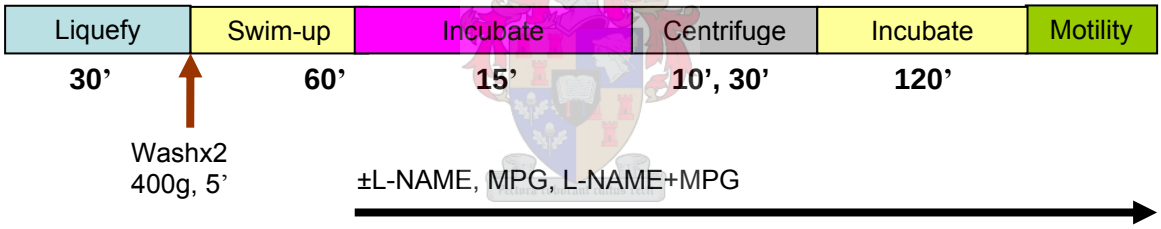


Figure 14. *Protocol to determine the effects of centrifugation on sperm motility*

3.7.2.4 Effects of centrifugation on PI fluorescence

After collecting the sperm cells via swim-up method, they were counted and concentration determined by means of CASA. Thereafter, the cells were divided into aliquots at a concentration of $2x10^6$ cells/ml each. As shown in figure 15, some of the cells were incubated with either NOS inhibitor L-NAME (0.7mM), ROS scavenger MPG (50µM), or a combination of both. The cells were centrifuged (10 or 30 min) then left to

capacitate for 120 minutes before incubated with propidium iodide (PI), (Sigma, St. Louis, MO, USA) (1µM, 15 min) (Pena, *et al*., 1998), a fluorescent marker of non-viable cells, and analyzed using FACS. For this analysis, viable sperm are defined as cells that possess an intact plasma membrane. This attribute is evaluated by staining with PI, a fluorescent probe that binds to DNA. Cells having an intact plasma membrane will prevent PI from entering into the cell and staining the nucleus (Cardelli *et al*., 2005).

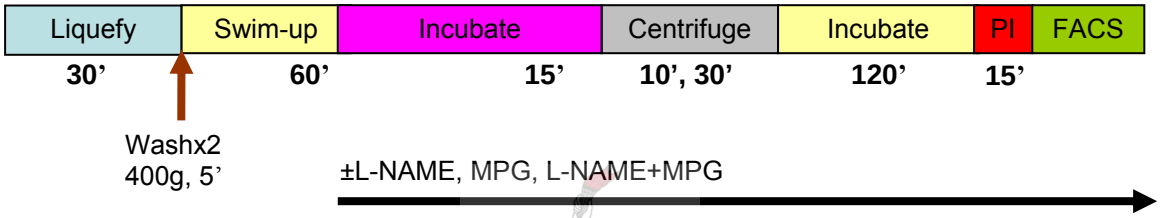


Figure 15*. Protocol to determine the effects of centrifugation on PI fluorescence*

3.7.3 Investigating the effects of NO and H_2O_2 on sperm function

3.7.3.1 Effects of NO on sperm motility parameters

After collecting the sperm cells via swim-up method, they were counted and concentration determined by means of CASA. Subsequently, the cells were divided into aliquots at a concentration of $2x10^6$ cells/ml each. As shown in figure 16, the cells were incubated with freshly prepared NO donor, SNP, with increasing concentrations (10-150µM) for 30, 90 or 120 minutes, after which motility parameters were measured by means of CASA.

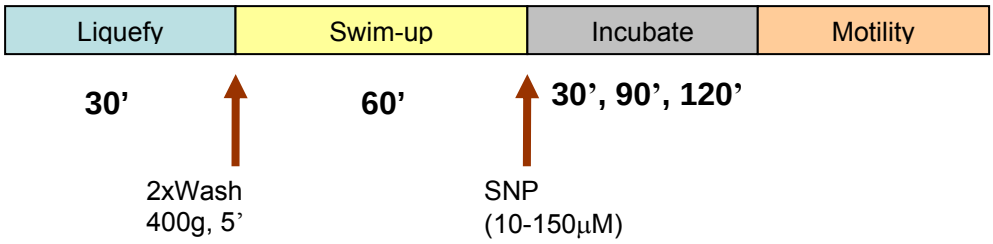


Figure 16. *Protocol to determine the effects of exogenously applied NO on sperm motility*

3.7.3.2 Effects of NO on PI fluorescence

After collecting the sperm cells via swim-up method, they were counted and concentration determined by means of CASA. Thereafter, the cells were divided into aliquots at a concentration of $2x10^6$ cells/ml each. As shown in figure 17, the cells were incubated with freshly prepared NO donor, SNP with increasing concentrations (10-150µM) for 30, 90 or 120 min, after which PI (1µM) was added and cells were incubated for 15 minutes before viability assessment using FACS analysis.

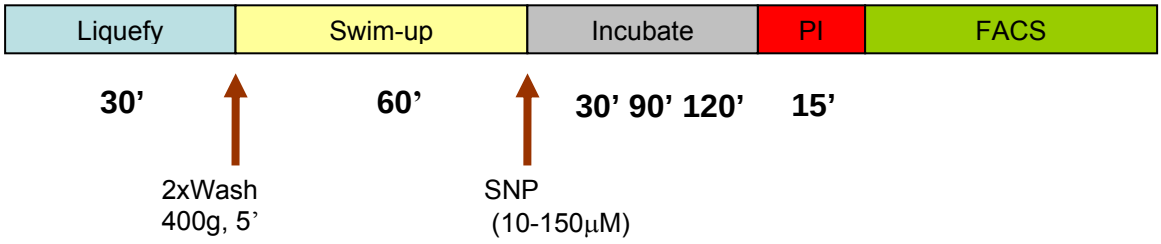


Figure 17. *Protocol to determine the effects of exogenously applied NO on PI fluorescence*

3.7.3.3 Effects of H_2O_2 on sperm motility parameters

After collection through swim-up, sperm cells were counted and concentration determined by means of CASA. Cells were subsequently divided into aliquots at a concentration of $2x10^6$/ml each. As shown in figure 18, the sperm cells were incubated with hydrogen peroxide (10, 30, 50, 70 and 100µM) in the presence or absence of a H_2O_2 scavenger, catalase (100U/ml). Motility parameters were measured at 30 and 60 minutes after hydrogen peroxide incubation by means of CASA.

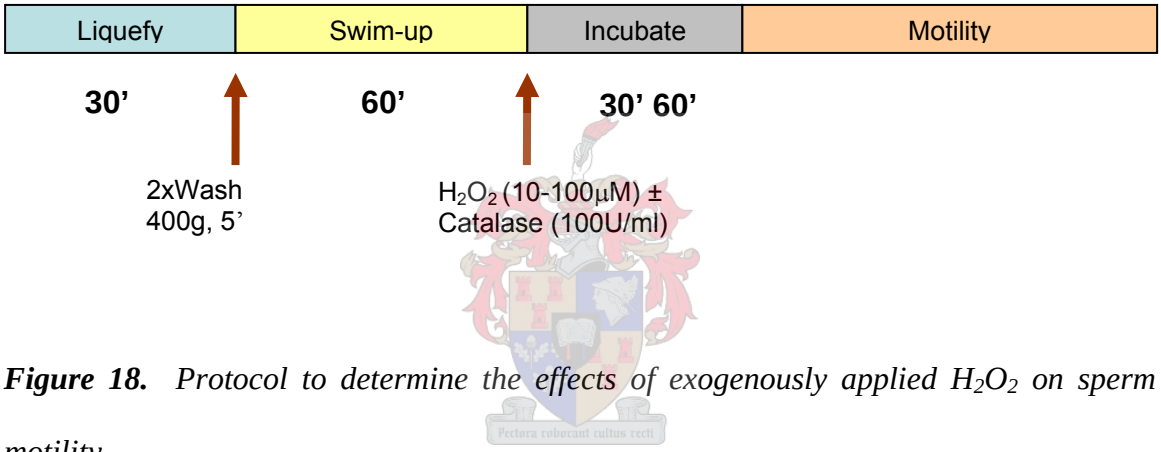


Figure 18. *Protocol to determine the effects of exogenously applied H_2O_2 on sperm motility*

3.7.3.4 Effects of H_2O_2 on PI fluorescence

After collection through swim-up, sperm cells were counted and concentration determined by means of CASA. Cells were subsequently divided into aliquots at a concentration of $2x10^6$/ml each. As shown in figure 19, the cells were incubated with H_2O_2 (10-100 µM) in the presence or absence of its scavenger, catalase (100U/ml). PI (1 µM) was added after 30 and 60 min of H_2O_2 incubation, after which the cells were incubated for 15 minutes before viability assessment using FACS analysis.

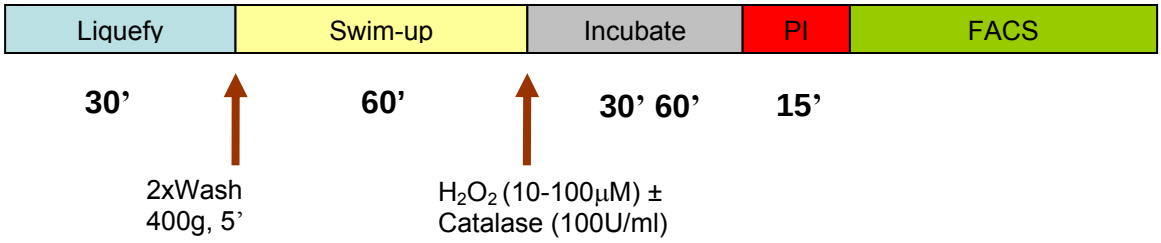


Figure 19. *Protocol to determine the effects of exogenously applied H_2O_2 on PI fluorescence*

3.8 Statistical analyses

GraphPad™ Prism 4 was used for all statistical evaluations. Some data are expressed as percentages of the control (mean ± S.E.M), and control values were adjusted to 100%. For comparative studies, student's *t*-test (unpaired) or one-way analysis of variance (ANOVA) tests (with Bonferroni *post test* if $P < 0.05$) were used for statistical analyses. Differences were regarded statistically significant if $P < 0.05$.

Chapter 4

Results

4.1 Standardization and establishment of flow cytometry as an accurate technique to directly measure specific free radicals

4.1.1 Probe specificity of DAF-2/DA for NO

Figure 20 shows that there was a significant increase in mean DAF-2/DA fluorescence in cells treated with 30μM SNP (170.10±17.40% vs. control; control adjusted to 100%; P<0.05); 50μM SNP (292.20±31.45% vs. control; *P*=0.001) and 100μM SNP (387.80±24.35% vs. control; *P*<0.001).

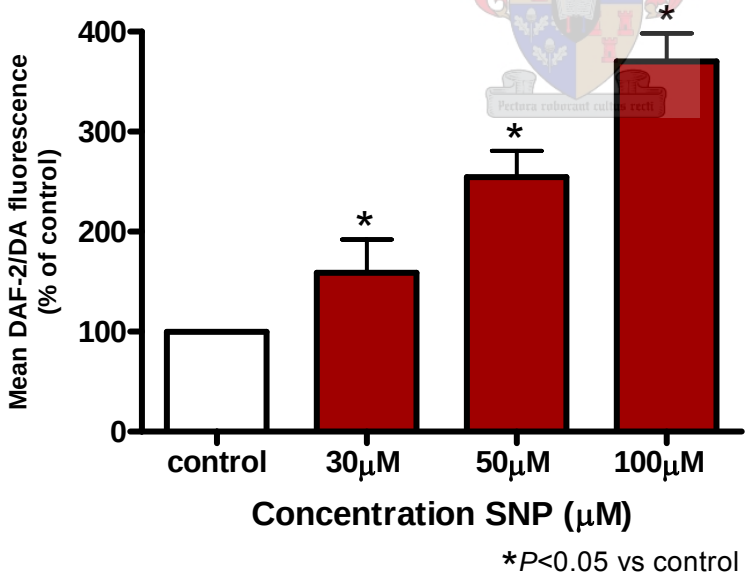


Figure 20*. Effects of SNP on DAF-2/DA fluorescence (n=12)*

4.1.2 Probe specificity of DCFH for ROS

To verify that our probe was measuring ROS, we added a ROS scavenger MPG (50μM), which significantly reduced DCFH fluorescence in MPG loaded control cells (60.41±3.36%) vs. MPG free control cells (P<0.05) (Fig. 21).

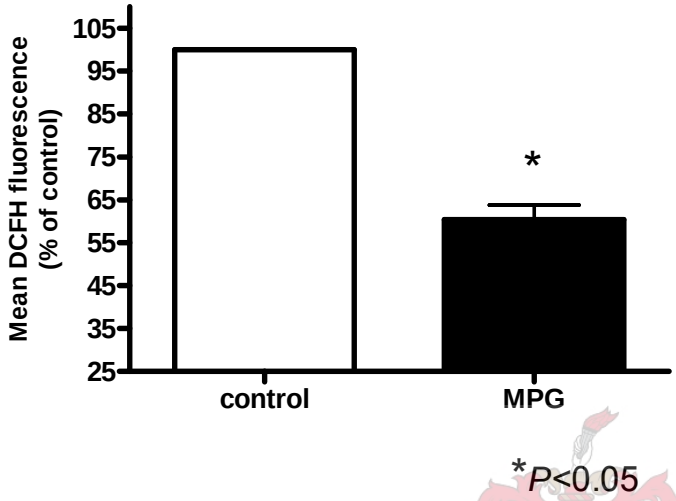


Figure 21. *Effects of MPG on DCFH fluorescence (n=12)*

4.2 Investigation of the effects of sperm centrifugation on free radical generation and sperm function

4.2.1 Effects of centrifugation on DAF-2/DA fluorescence

Centrifugation of sperm cells for 10 minutes significantly increased DAF-2/DA fluorescence when compared to the control (mean DAF-2/DA fluorescence in control: 100% vs. 10 min centrifugation: 119.35±7.53%; P=0.01). On the other hand 30 min of centrifugation significantly reduced fluorescence (90.68±4.5%; P<0.05) compared to control (Fig 22). L-NAME (0.7mM) significantly inhibited fluorescence in the 10 min

group (119.35±7.53% vs. 78.23±2.50%; *P*<0.05) and 30 min group (90.68±4.56% vs. 71.90±7.61%; *P*<0.05) (Fig. 23).

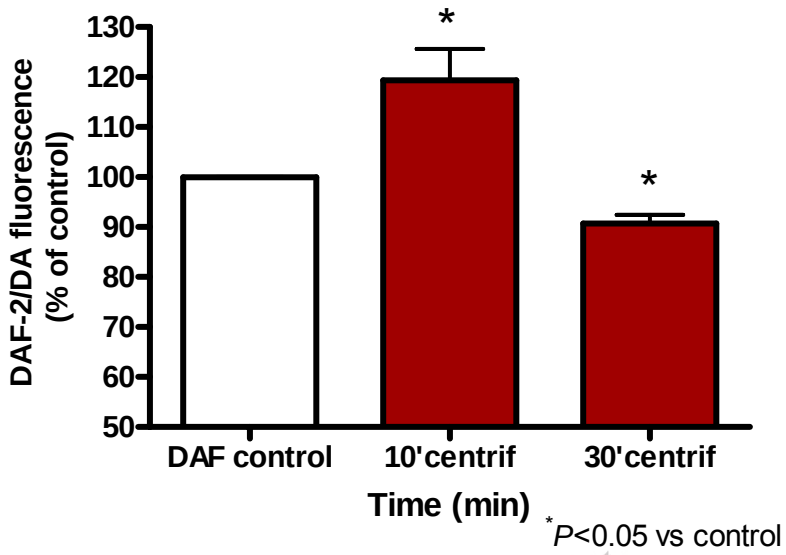


***Figure 22**. Effects of centrifugation on DAF-2/DA fluorescence (n=12)*

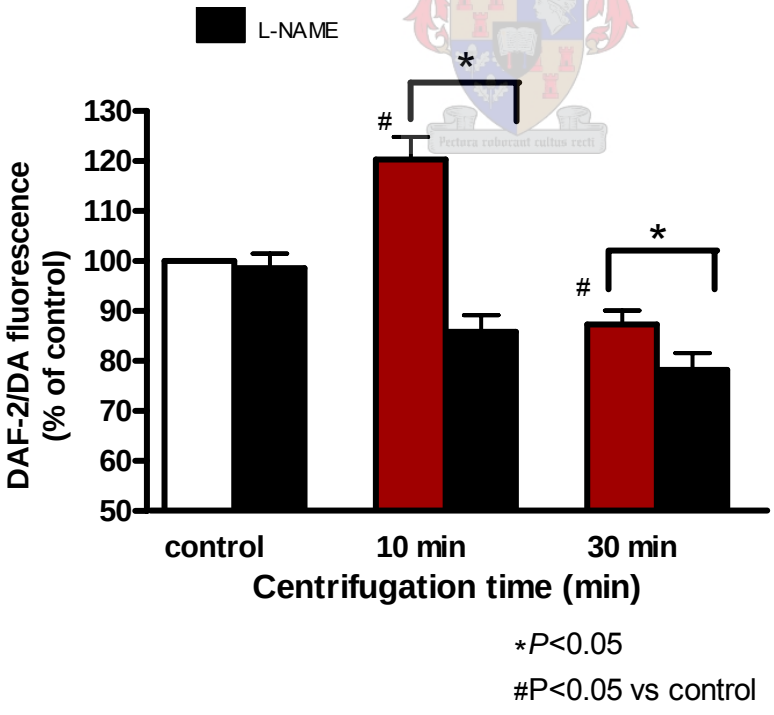


***Figure 23**. Effects of L-NAME on DAF-2/DA fluorescence (n=12)*

4.2.2 Effects of centrifugation on DCFH fluorescence

Figure 24 shows that centrifugation of sperm cells for 10 min and 30 min respectively increased DCFH fluorescence significantly when compared to the control (144.50±10.73%; 153.60±10.73; respectively, $P<0.05$). MPG significantly inhibited fluorescence in the control, 10 min, and 30 min groups (60.41±3.36%, 74.38±4.86%, 75.79±9.80%, respectively; $P<0.05$ in all groups) (Fig. 25).

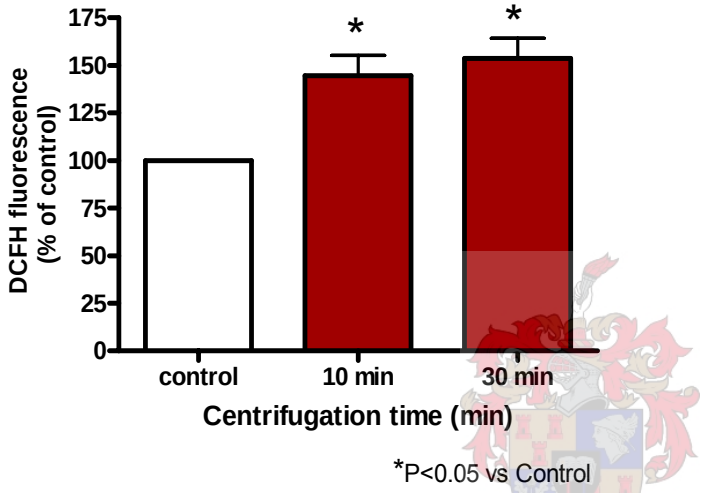


***Figure 24**. Effects of centrifugation on DCFH fluorescence (n=12)*

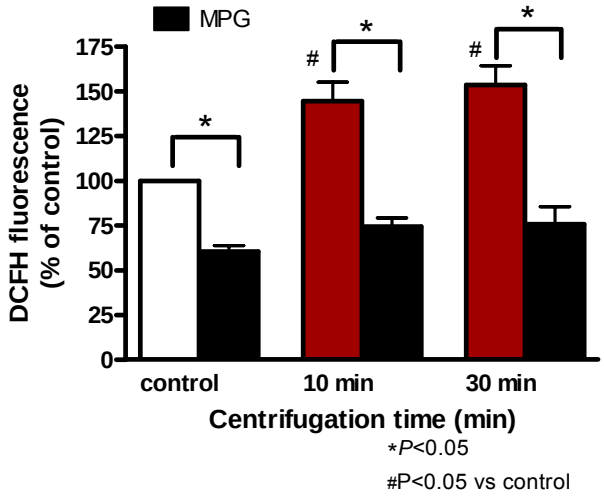


***Figure 25**. Effects of MPG on DCFH fluorescence (n=12)*

4.2.3 Effects of centrifugation on sperm motility parameters

Table I shows the effects of centrifugation on various sperm motility parameters in the absence or presence of the NOS inhibitor L-NAME, the ROS scavenger, MPG, or a combination of both. Ten minutes of centrifugation significantly decreased progressive motility, VCL, and STR compared to non-centrifuged control cells. Static cells were significantly increased in untreated cells centrifuged for 10 min compared to the control. No significant differences in motility parameters were observed between L-NAME treated 10 min centrifuged cells and untreated 10 min centrifuged cells. Addition of MPG to the 10 min centrifuged cells significantly increased progressive motility compared to the untreated 10 min centrifuged cells. The author also observed a significant decrease in static cells when MPG was added to the 10 min centrifuged cells compared to the 10 min centrifuged untreated group. No significant differences were observed between MPG treated and L-NAME treated groups. Addition of L-NAME + MPG to the 10 minutes centrifuged cells significantly increased progressive motility and STR compared to untreated 10 min centrifuged cells. On the other hand, static cells were significantly decreased in the L-NAME + MPG treated group compared to the untreated 10 min centrifuged cells. VAP and VCL improved significantly in the L-NAME + MPG treated group whereas, static cells were significantly decreased compared to L-NAME treated cells. No significant differences were observed between the L-NAME + MPG treated group compared to the MPG treated group. We also did not observe significant differences between L-NAME, MPG and L-NAME + MPG treated cells centrifuged for 10 min when respectively compared to non-centrifuged control.

Table I also shows the effects of 30 min centrifugation on various sperm motility parameters in the absence or presence of the NOS inhibitor L-NAME, ROS scavenger MPG or a combination of both. Thirty minutes of centrifugation significantly decreased all motility parameters except static cells, which were significantly increased when compared to the non-centrifuged control. Motile cells, progressive motility, VAP, VCL, BCF and rapid cells were significantly decreased in L-NAME treated 30 min centrifuged cells compared to the non-centrifuged control. Addition of L-NAME significantly increased VAP, VSL and STR compared to untreated 30 min centrifuged cells. L-NAME treated cells showed a significant reduction in static cells compared to untreated 30 min centrifuged cells. Addition of MPG to the 30 min centrifuged cells significantly increased motile cells, VAP, VSL, VCL and STR compared to untreated 30 min centrifuged cells. Static cells were significantly decreased in the MPG treated group compared to the untreated 30 min centrifuged group. There was a significant increase in motile cells in the MPG treated group compared to the L-NAME treated group. Addition of L-NAME + MPG significantly increased most motility parameters except significantly decreasing static cells when compared to untreated 30 min centrifuged cells. We also observed a significant increase in motile cells and progressive motility in the L-NAME + MPG group compared to the L-NAME treated group, while static cells were significantly decreased in the L-NAME + MPG group compared to the L-NAME treated group. No significant differences were observed between the L-NAME + MPG treated group compared to the MPG treated group. No significant differences were found between MPG and L-NAME + MPG treated 30 minutes centrifuged cells when respectively compared to the non-centrifuged control.

Table I further shows that 30 min of centrifugation significantly decreased motile cells, VAP, VSL, VCL, and ALH while increasing static cells when compared to 10 min of centrifugation. Motile cells were significantly decreased in 30 min centrifuged L-NAME treated cells compared to 10 min centrifuged L-NAME treated cells. No significant differences were observed between 30 and 10 min centrifuged groups treated with MPG. We also did not observe significant differences between 30 and 10 min centrifuged groups treated with L-NAME + MPG.

Table I. *The effects of 10 and 30 min of centrifugation on motility parameters (n=12)*

	CONTROL	10 minutes Centrifugation				30 minutes Centrifugation			
		Untreated	L-Name	MPG	L-Name + MPG	Untreated	L-Name	MPG	L-Name + MPG
Motile (%)	76.17±3.57	67.00±2.65	69.98±3.11	71.04±3.90	74.43±2.76	57.67±2.76 * €	58.39±2.56 * €	67.00±3.76 @ &	70.23±4.76 @ &
Progr. Mot (%)	41.67±1.53	32.83±1.62 *	37.34±2.03	40.32±3.23 #	41.89±2.34 #	31.67±1.62 *	33.21±1.87 *	36.87±2.78	41.76±3.05 @ &
VAP (μm/s)	57.45±2.60	51.13±2.64	49.45±2.98	54.56±4.78	58.32±3.76 $	40.45±1.74 * €	48.34±2.04 * @	51.54±3.23 @	55.67±4.17 @
VSL (μm/s)	45.90±1.66	40.43±1.64	47.06±1.43	44.78±2.07	46.28±2.76	30.08±1.80 * €	43.21±2.01 @	39.34±1.76 @	40.05±1.87 @
VCL (μm)	86.85±3.80	71.68±1.83 *	68.56±2.36	69.90±3.17	79.34±3.25 $	60.18±2.97 * €	66.08±2.54 *	72.54±2.78 @	70.56±3.47 @
ALH (μm/s)	3.68±0.23	2.58±0.20	3.12±0.35	3.56±0.24	3.58±0.32	1.78±0.24 * €	2.89±0.21	3.13±0.33	3.24±0.46 @
BCF (Hz)	19.88±0.41	15.65±0.33	16.69±0.55	20.06±0.35	21.67±1.23	12.35±0.37 *	13.35±0.21 *	15.37±0.17	19.06±1.02 @
STR (%)	80.50±3.59	70.00±2.86 *	75.37±3.78	74.65±4.21	81.23±5.23 #	63.25±2.85 *	72.98±4.87 @	71.98±1.98 @	78.23±4.23 @
LIN (%)	56.25±1.60	53.50±1.49	52.98±3.29	55.45±5.23	58.45±3.28	46.25±1.63 *	52.65±3.97	50.37±2.18	55.87±3.80 @
Rapid cells (%)	62.67±2.60	55.17±1.69	54.34±4.03	56.87±3.09	58.45±4.12	49.83±1.92 *	52.23±3.90 *	55.24±2.96	58.02±3.21 @
Static cells (%)	24.34±1.65	35.65±1.34 *	30.23±2.13	26.84±1.56 #	22.12±1.89 # $	47.34±1.45 * €	31.19±2.86 @	27.23±3.23 @	24.24±2.01 @ &

*$P<0.05$ vs Control; [#]$P<0.05$ vs. Untreated 10 min Centrifugation; [$]$P<0.05$ vs. 10min Centrifugation + L-Name; [@]$P<0.05$ vs. 30min Centrifugation Untreated;

[&]$P<0.05$ vs. 30min Centrifugation + L-Name; [€]$P<0.05$ vs. 10min Centrifugation + Corresponding group

4.2.4 Effects of centrifugation on PI fluorescence

Figure 26 shows that there were no significant differences in PI fluorescence among control groups (Untreated, L-NAME, MPG, and L-NAME + MPG treated). There was a significant increase in PI fluorescence in untreated cells centrifuged for 10 min compared to the untreated control (165.10±19.99 vs. untreated control, adjusted to 100%; $P<0.05$). Ten min centrifuged L-NAME treated cells showed a significant increase in PI fluorescence (157.50±12.21) compared to the L-NAME treated control (106.30±8.44; $P<0.05$). No significant difference was observed between L-NAME treated 10 min centrifuged cells and untreated 10 min centrifuged cells. Ten minutes centrifuged MPG treated cells showed no significant difference compared to the MPG treated control. However, PI fluorescence was significantly decreased in MPG treated 10 min centrifuged cells compared to untreated 10 min centrifuged cells (113.60±2.21 vs. 165.10±19.99; $P<0.001$). The author also observed a significant decrease in PI fluorescence in MPG treated 10 min centrifuged cells compared to the L-NAME treated 10 min centrifuged cells (113.60±2.21 vs. 157.50±12.21; $P<0.01$). L-NAME + MPG treated 10 min centrifuged cells did not show any significant difference to the L-NAME + MPG treated control. However, PI fluorescence was significantly decreased in the L-NAME + MPG 10 min centrifuged cells compared to untreated 10 min centrifuged cells (106.90±2.50 vs. 165.10±19.99; $P<0.001$). PI fluorescence was significantly decreased in L-NAME + MPG treated 10 min centrifuged cells compared to L-NAME treated 10 min centrifuged cells (106.90±2.50 vs. 157.50±12.21; $P<0.01$). No significant difference was observed between L-NAME + MPG treated 10 min centrifuged cells and MPG treated 10 min centrifuged cells.

There was a significant increase in PI fluorescence in untreated cells centrifuged for 30 min compared to the untreated control (239.40±27.78 vs untreated control; $P<0.001$). Thirty minutes centrifuged L-NAME treated cells showed a significant increase in PI fluorescence compared to the L-NAME treated control (179.10±15.99 vs. 106.30±8.44; $P<0.001$). No significant difference was observed between L-NAME treated 30 min centrifuged cells and untreated 30 min centrifuged cells. Thirty minutes centrifuged MPG treated cells showed no significant difference compared to the MPG treated control. However, PI fluorescence was significantly decreased in MPG treated 30 min centrifuged cells compared to untreated 30 min centrifuged cells (127.20±2.35 vs. 239.40±27.78; $P<0.001$). We also observed a significant decrease in PI fluorescence in MPG treated 30 min centrifuged cells compared to the L-NAME treated 30 min centrifuged cells (127.20±2.35 vs. 179.10±15.99; $P<0.01$). L-NAME + MPG treated 30 min centrifuged cells did not show any significant difference to L-NAME + MPG treated control. However, PI fluorescence was significantly decreased in the L-NAME + MPG 30 min centrifuged cells compared to untreated 30 min centrifuged cells (125.90±4.97 vs. 239.40±27.78; $P<0.001$). PI fluorescence was significantly decreased in L-NAME + MPG treated 30 min centrifuged cells compared to L-NAME treated 30 min centrifuged cells (125.90±4.97 vs. 179.10±15.99; $P<0.01$). No significant difference was observed between L-NAME + MPG treated 30 min centrifuged cells and MPG treated 30 min centrifuged cells. Thirty minutes of centrifugation significantly increased PI fluorescence compared to 10 min of centrifugation in the untreated cells (239.40±27.78 vs. 165.10±19.99; $P<0.01$).

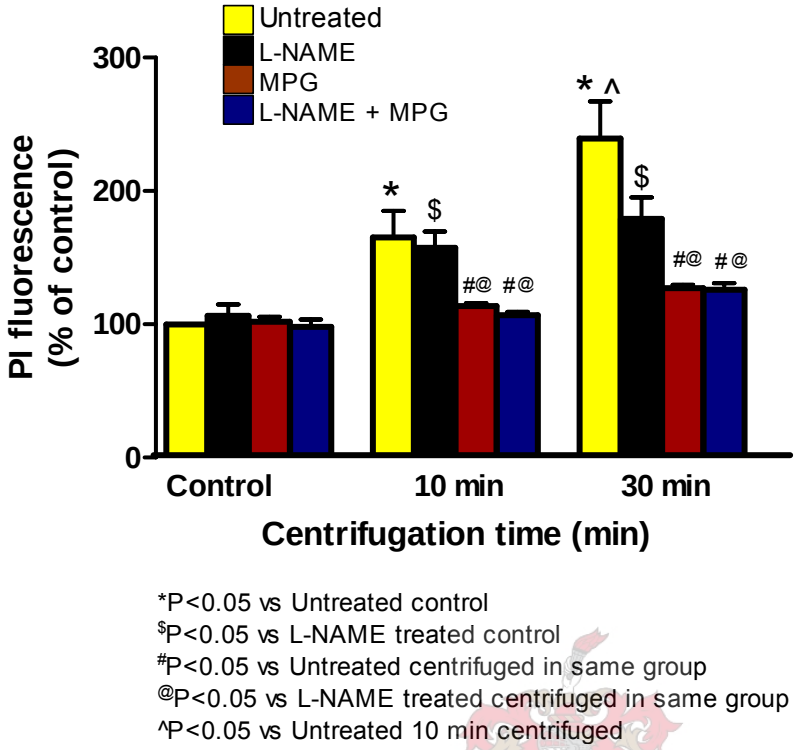


Figure 26. *Effects of centrifugation on sperm viability in the presence of L-NAME, MPG or L-NAME + MPG (n=12)*

4.3. Investigating the effects of NO and H_2O_2 on sperm function

4.3.1 Effects of NO on sperm motility parameters

Table II shows the effects of different concentrations of SNP on sperm motility parameters after 30 min of incubation. In the 10µM SNP group, no differences were observed compared to the control. In the 30µM SNP group only the static cells were significantly decreased compared to the control. At concentrations of 50µM, 70µM, 100µM and 120µM of SNP, no differences were observed compared to the control. Both VAP and VSL were significantly decreased with 150µM SNP administration compared

to the control. Table III shows the effects of different concentrations of SNP on sperm motility parameters after 90 min of incubation. In the 10μM SNP group, no differences were observed compared to the control. There was a significant increase in motile cells, progressive motility, VAP, VSL and rapid cells in sperm cells treated with 30μM SNP, while static cells were significantly decreased compared to the control. Concentrations of 50μM, 70μM and 100μM did not show any differences compared to the control. On the other hand, SNP concentrations of 120μM and 150μM significantly decreased motile cells, progressive motility, VAP, VSL and rapid cells compared to the control.

Table IV shows the effects of different concentrations of SNP on sperm motility parameters after 120 min of incubation. In the 10μM SNP group, no differences were observed compared to the control. 30μM SNP significantly increased progressive motility and rapid cells compared to the control. Static cells were significantly decreased with 30μM SNP compared to the control. Concentrations of 50μM, 70μM and 100μM did not show any differences compared to the control. On the other hand, SNP concentrations of 120μM and 150μM significantly decreased motile cells, progressive motility, VAP, VSL, VCL and rapid cells compared to the control. SNP concentrations of 120μM and 150μM also significantly increased static cells compared to the control.

.

***Table II**. Effects of SNP on sperm motility parameters after 30 min of incubation (n=12)*

Parameter	Control	10μM	30μM	50μM	70μM	100μM	120μM	150μM
Motile (%)	71.00±2.35	70.00±3.36	78.86±0.30	67.43±0.45	66.71±0.41	58.43±0.57	58.14±2.56	62.43±1.56
Progr. Mot (%)	44.71±1.39	42.71±1.49	43.57±1.36	43.00±1.54	45.00±1.50	33.00±1.68	36.43±1.59	33.71±1.53
VAP (μm/s)	61.86±2.56	69.26±3.66	70.14±2.79	60.41±3.34	59.68±2.58	54.88±2.44	44.06±1.62	43.46±1.69*
VSL (μm/s)	54.85±2.63	59.27±2.61	60.11±2.75	52.53±2.41	49.12±2.57	48.37±2.49	35.78±1.54	35.43±1.42*
VCL (μm)	90.97±3.64	94.32±4.54	95.09±4.46	89.75±4.54	87.10±3.67	82.15±4.66	69.37±3.86	68.55±1.87
ALH (μm/s)	4.73±0.17	4.88±0.18	4.80±0.23	4.35±0.18	4.13±0.20	3.73±0.24	3.00±0.23	2.90±0.24
BCF (Hz)	21.26±1.33	19.52±1.33	21.83±1.31	19.91±0.30	20.38±1.33	18.15±0.40	18.80±0.47	16.76±0.47
STR (%)	81.75±3.61	82.50±3.54	85.00±3.47	81.00±3.48	78.50±3.60	80.50±4.69	75.00±3.71	72.50±2.75
LIN (%)	55.25±2.42	57.50±2.47	60.50±2.54	55.00±2.35	54.75±2.51	56.50±2.53	51.00±2.49	49.25±1.52
Rapid cells (%)	66.14±2.44	60.86±2.45	63.00±2.38	60.57±2.50	59.57±2.46	53.43±2.59	52.29±1.57	51.29±2.51
Static cells (%)	27.00±1.59	26.00±1.59	19.50±1.28*	24.50±1.50	27.50±1.62	27.75±1.45	28.75±1.45	30.00±1.47

* *P<0.05 vs. control*

***Table III**. Effects of SNP on sperm motility parameters after 90 min of incubation (n=12)*

Parameter	Control	10μM	30μM	50μM	70μM	100μM	120μM	150μM
Motile (%)	62.86±2.43	69.00±0.51	72.86±3.47*	62.86±2.51	64.14±1.59	58.57±0.56	53.57±2.68*	47.00±1.69*
Progr. Mot (%)	40.14±2.52	43.00±1.47	46.29±2.52*	38.48±1.51	39.86±1.58	37.14±1.61	32.14±2.59*	29.43±1.62*
VAP (μm/s)	61.15±4.54	68.48±2.64	69.88±1.91*	60.30±3.25	57.53±2.57	54.75±2.57	42.53±2.68*	40.50±1.72*
VSL (μm/s)	52.45±2.63	57.53±2.58	58.60±2.80*	50.70±2.33	47.03±1.52	46.50±2.54	34.98±1.54*	33.98±1.47*
VCL (μm)	87.69±4.59	92.52±3.60	94.84±4.37	88.73±3.43	85.25±2.69	81.45±3.70	68.25±2.79	66.13±2.91
ALH (μm/s)	4.60±0.20	4.675±0.21	4.575±0.26	4.10±0.22	3.88±0.22	3.40±0.28	2.775±0.26	2.68±0.28
BCF (Hz)	20.28±1.30	18.53±2.25	21.38±1.30	19.20±1.27	19.25±1.34	17.50±1.41	18.80±1.54	16.13±0.48
STR (%)	79.50±3.60	81.75±2.50	83.75±3.53	79.00±3.62	77.50±2.66	77.25±2.81	73.00±3.86	70.00±3.93
LIN (%)	54.00±2.46	56.25±2.45	58.75±1.50	53.75±3.41	53.75±2.47	55.00±2.57	50.00±2.51	48.50±1.70
Rapid cells (%)	57.00±2.48	63.14±2.50	64.29±2.50*	56.71±2.52	58.29±2.60	53.29±2.57	45.71±2.66*	42.29±1.69*
Static cells (%)	28.50±1.46	26.25±2.57	20.75±1.53*	25.25±1.41	30.25±1.59	31.75±1.54	33.50±2.61	32.50±1.56

**P<0.05 vs. Control*

***Table IV**. Effects of SNP on sperm motility parameters after 120 min of incubation (n=12)*

Parameter	Control	10μM	30μM	50μM	70μM	100μM	120μM	150μM
Motile (%)	60.14±2.51	59.71±3.53	66.57±2.59	57.71±2.59	59.86±2.68	51.86±2.63	38.86±2.65*	43.14±3.74*
Progr. Mot (%)	33.43±2.58	35.43±2.61	41.57±2.61*	35.43±2.62	36.29±1.61	28.14±2.65	20.29±1.63*	18.71±2.51*
VAP (μm/s)	62.20±3.67	61.16±3.78	66.14±4.30	57.95±2.80	53.85±2.49	51.73±3.45	40.01±1.61*	36.58±2.63*
VSL (μm/s)	48.61±2.61	51.08±3.58	55.79±2.39	47.89±2.78	43.60±2.48	41.95±2.42	31.22±1.34*	31.35±2.39*
VCL (μm)	87.33±3.55	90.62±4.48	90.95±5.58	83.41±4.45	80.40±4.30	76.79±4.42	63.70±2.63*	63.39±3.86*
ALH (μm/s)	3.95±0.17	3.35±0.19	4.05±0.17	3.55±0.19	3.00±0.16	2.83±0.19	2.18±0.18	2.20±0.12
BCF (Hz)	19.28±1.27	18.36±2.22	19.14±2.32	17.46±1.35	18.24±1.64	15.97±1.37	15.33±1.36	15.46±1.47
STR (%)	76.75±4.62	78.25±4.59	80.05±4.58	75.75±3.54	74.50±3.36	73.75±3.71	71.25±2.81	67.25±4.75
LIN (%)	51.75±2.41	53.75±3.45	55.00±2.28	51.25±3.34	51.25±2.20	49.50±2.61	47.25±1.42	43.75±2.43
Rapid cells (%)	52.71±2.56	52.00±2.59	61.43±4.62*	50.86±2.63	51.29±2.68	44.00±2.68	29.86±1.68*	28.00±2.66*
Static cells (%)	30.25±1.55	27.25±2.54	23.50±1.28*	25.50±1.57	32.50±1.54	37.00±2.34	38.00±1.51	37.25±1.61

**P<0.05 vs. Control*

4.3.2 Effects of NO on PI fluorescence

Figure 27 A shows that there was no significant difference in PI fluorescence in cells incubated with SNP concentrations ranging from 10-150μM after 30 min of incubation. A significant increase in PI fluorescence was observed after 90 minutes in cells incubated with 120μM SNP (182.10±15.87 vs. control; $P<0.05$) and 150μM SNP (187.30±24.00 vs. control; $P<0.05$) (Fig. 27 B). Figure 27 C shows a significant increase in PI fluorescence in cells incubated for 120 min with 100μM, 120μM and 150μM SNP (146.10±12.52, 187.80±25.15 and 230.07±10.99 respectively; $P<0.05$) compared to the control.

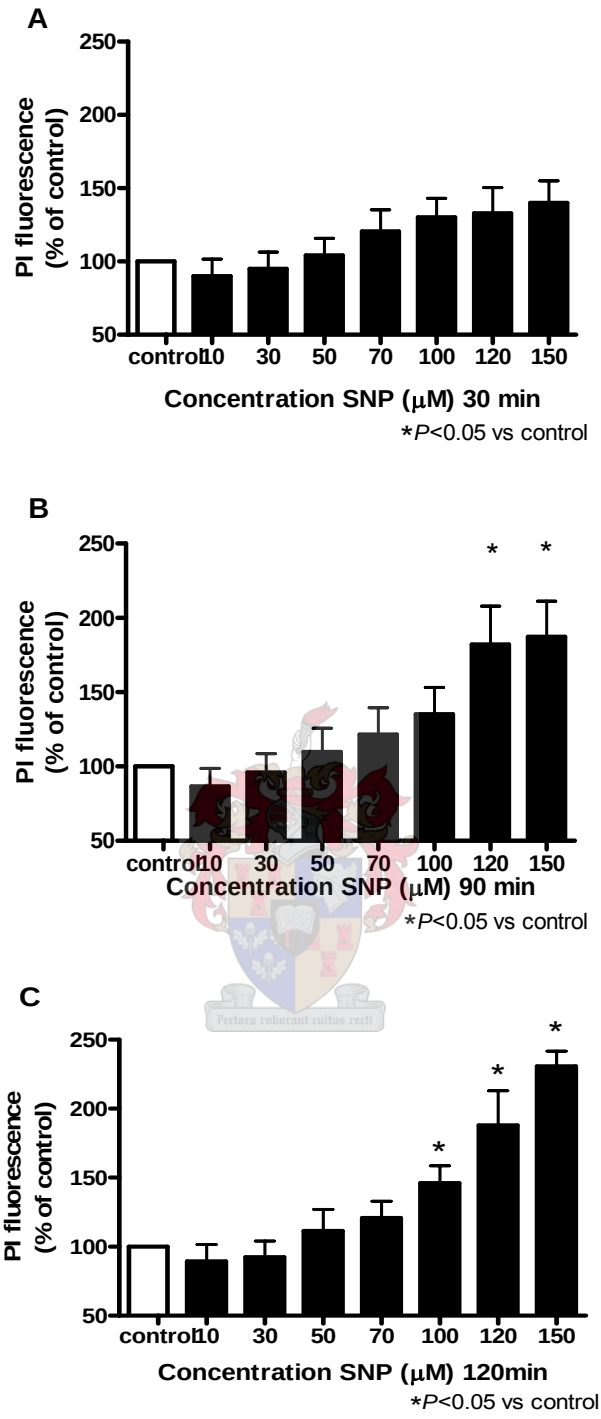


Figure 27*. Sperm viability as measured after 30 (A), 90 (B), and 120 minutes (C) of incubation with the NO donor, SNP at various concentrations (n=12)*

4.3.3 Effects of H_2O_2 on sperm motility parameters

Table V shows the effects of H_2O_2 on sperm motility parameters in the presence or absence of its scavenger, catalase, (100U/ml) after 30 min of incubation. No significant differences were observed when cells were incubated with 10μM H_2O_2 compared to the control. H_2O_2 significantly reduced most sperm motility parameters starting from the concentration of 30μM in a dose dependent manner after 30 minutes of incubation. 100μM of H_2O_2 reduced VAP, VSL, VCL, ALH, BCF and LIN to zero. Static cells were significantly increased starting from 30μM H_2O_2. Catalase maintained all motility parameters at every concentration of H_2O_2 after 30 min of exposure.

Table VI shows the effects of H_2O_2 on sperm motility parameters in the presence or absence of its scavenger, catalase (100U/ml) after 60 minutes of H_2O_2 incubation. No significant differences were observed when cells were incubated with 10μM H_2O_2 compared to the control. Static cells were significantly increased at all H_2O_2 concentrations except at 10μM. All the other motility parameters were significantly reduced at all H_2O_2 concentrations higher than 10μM compared to the control. Catalase significantly maintained motility parameters at all H_2O_2 concentrations even after 60 minutes of exposure.

***Table V**. Effects of H_2O_2 on motility parameters after 30 min of incubation in the presence or absence of catalase (n=12)*

Parameter	Treatment	Control	10μM	30μM	50μM	70μM	100μM
Motile (%)	H_2O_2	77.13±0.32	73.67±2.12	48.13±0.43*	31.13±0.42*	15.25±0.39*	4.13±0.28*
	H_2O_2 + Catalase	72.25±0.33	70.03±1.87	68.00±0.45$^{\$}$	69.63±0.32$^{\$}$	65.63±0.41$^{\$}$	70.38±0.28$^{\$}$
Progr. Mot (%)	H_2O_2	56.00±0.35	49.03±1.24	12.63±0.25*	9.75±0.26*	5.25±0.25*	0.50±0.12*
	H_2O_2 + Catalase	52.63±0.20	50.76±2.63	48.63±0.38$^{\$}$	49.75±0.30$^{\$}$	47.25±0.33$^{\$}$	48.13±0.36$^{\$}$
VAP (μm/s)	H_2O_2	74.63±0.83	67.87±0.98	33.00±0.36*	28.75±0.82*	11.33±0.71*	0.00
	H_2O_2 + Catalase	66.50±0.58	68.12±1.06	70.70±0.68$^{\$}$	64.30±0.46$^{\$}$	76.00±0.87$^{\$}$	66.50±0.20
VSL (μm/s)	H_2O_2	65.28±0.85	61.08±0.67	24.30±0.24*	24.90±0.79*	10.83±0.69*	0.00
	H_2O_2 + Catalase	60.80±0.61	62.36±1.53	60.83±0.66$^{\$}$	55.57±0.54$^{\$}$	66.73±0.83$^{\$}$	56.77±0.34
VCL (μm)	H_2O_2	96.00±0.44	88.65±3.02	54.63±0.54*	43.68±0.63*	16.30±0.89*	0.00
	H_2O_2 + Catalase	90.15±0.49	91.34±2.12	92.47±0.63$^{\$}$	90.77±0.71$^{\$}$	98.07±0.76$^{\$}$	96.20±0.53
ALH (μm/s)	H_2O_2	4.15±0.14	3.78±0.12	2.03±0.15*	1.95±0.16*	0.28±0.18*	0.00
	H_2O_2 + Catalase	3.73±0.15	3.87±0.21	4.40±0.15$^{\$}$	4.03±0.18$^{\$}$	4.53±0.08$^{\$}$	4.03±0.24
BCF (Hz)	H_2O_2	22.03±0.25	17.37±0.43	12.98±0.25*	11.88±0.41*	17.08±0.91*	0.00
	H_2O_2 + Catalase	21.40±0.21	20.90±0.65	19.70±0.23$^{\$}$	20.20±0.32$^{\$}$	18.93±0.37	19.83±0.35
STR (%)	H_2O_2	85.50±0.40	80.03±2.94	73.50±0.39	76.25±0.43	72.00±0.68	0.00
	H_2O_2 + Catalase	87.25±0.30	82.13±2.82	84.67±0.11$^{\$}$	85.33±0.29	85.67±0.21$^{\$}$	83.67±0.28
LIN (%)	H_2O_2	63.50±0.42	57.67±1.87	46.25±0.42	56.50±0.70	53.50±0.47	0.00
	H_2O_2 + Catalase	64.25±0.30	59.94±2.54	60.33±0.36$^{\$}$	61.00±0.58$^{\$}$	62.00±0.31$^{\$}$	57.67±0.22
Rapid cells (%)	H_2O_2	70.88±0.37	65.53±3.98	31.75±0.36*	16.50±0.29*	7.25±0.26*	0.25±0.11*
	H_2O_2 + Catalase	66.13±0.31	67.23±2.08	61.25±0.41$^{\$}$	64.00±0.33$^{\$}$	56.75±0.35$^{\$}$	59.88±0.33$^{\$}$
Static cells (%)	H_2O_2	13.25±0.41	17.28±0.45	37.00±0.48*	45.75±0.77*	45.50±0.56*	59.75±0.79*
	H_2O_2 + Catalase	13.50±0.58	15.29±0.78	14.00±0.43$^{\$}$	14.00±0.36$^{\$}$	10.50±0.38$^{\$}$	13.00±0.34$^{\$}$

*P<0.05 vs. Control; $^{\$}P$<0.05 vs. H_2O_2 only

Table VI. *Effects of H_2O_2 on motility parameters after 60 min of incubation in the presence or absence of catalase (n=12)*

Parameter	Treatment	Control	10μM	30μM	50μM	70μM	100μM
Motile (%)	H_2O_2	68.13±0.33	62.76±0.24	44.88±0.35*	11.58±0.51*	3.75±0.45*	0.00
	H_2O_2 + Catalase	62.50±0.44	63.78±0.49	61.75±0.43$^{\$}$	58.88±0.34$^{\$}$	60.88±0.41$^{\$}$	61.38±0.29
Progr. Mot (%)	H_2O_2	43.38±0.23	38.98±0.21	15.38±0.21*	3.38±0.26*	0.00	0.00
	H_2O_2 + Catalase	39.98±0.32	3923±0.24	39.63±0.28$^{\$}$	38.25±0.37$^{\$}$	40.38±0.33	35.13±0.32
VAP (μm/s)	H_2O_2	70.73±0.54	65.29±0.34	33.00±0.31*	23.67±0.45*	9.87±0.43*	0.00
	H_2O_2 + Catalase	64.54±0.43	67.90±0.50	66.78±0.78$^{\$}$	60.34±0.53$^{\$}$	53.34±0.87$^{\$}$	61.43±0.30
VSL (μm/s)	H_2O_2	61.45±0.32	58.23±0.38	22.56±0.24*	20.54±0.75*	7.89±0.54*	0.00
	H_2O_2 + Catalase	58.57±0.54	59.23±0.58	61.56±0.66$^{\$}$	54.76±0.30$^{\$}$	59.12±0.83$^{\$}$	55.55±0.21
VCL (μm)	H_2O_2	89.34±0.56	85.21±1.78	49.67±0.51*	41.89±0.62*	14.45±0.43*	0.00
	H_2O_2 + Catalase	86.87±0.23	87.90±2.34	88.05±0.46$^{\$}$	85.23±0.76$^{\$}$	89.45±0.76$^{\$}$	87.61±0.47
ALH (μm/s)	H_2O_2	3.98±0.15	3.23±0.18	2.04±0.28*	1.82±0.18*	0.30±0.19*	0.00
	H_2O_2 + Catalase	3.34±0.19	3.56±0.23	4.12±0.23$^{\$}$	3.88±0.28$^{\$}$	4.02±0.08$^{\$}$	4.00±0.26
BCF (Hz)	H_2O_2	20.43±0.25	18.46±0.32	10.94±0.46*	9.54±0.52*	9.34±0.85*	0.00
	H_2O_2 + Catalase	20.76±0.30	20.07±0.43	17.45±0.43$^{\$}$	19.01±0.32$^{\$}$	17.54±0.37$^{\$}$	18.34±0.35
STR (%)	H_2O_2	81.34±0.40	77.98±0.54	68.90±0.35*	76.76±0.43*	69.56±0.43*	0.00
	H_2O_2 + Catalase	83.45±0.46	80.96±1.23	80.67±0.11$^{\$}$	81.98±0.37	78.34±0.21$^{\$}$	80.06±0.32
LIN (%)	H_2O_2	60.07±0.32	56.34±0.65	44.43±0.43*	46.45±0.60*	45.87±0.64*	0.00
	H_2O_2 + Catalase	61.32±0.13	64.23±0.48	58.79±0.39$^{\$}$	60.12±0.49$^{\$}$	57.34±0.31$^{\$}$	54.32±0.22
Rapid cells (%)	H_2O_2	60.13±0.13	57.87±0.78	31.38±0.56*	5.50±0.30*	0.00	0.00
	H_2O_2 + Catalase	50.75±0.31	55.67±1.63	51.75±0.23$^{\$}$	49.25±0.22$^{\$}$	50.38±0.35	46.38±0.41
Static cells (%)	H_2O_2	16.67±0.54	18.25±0.78	35.78±0.25*	51.78±0.56*	67.89±0.45*	86.87±0.54*
	H_2O_2 + Catalase	17.56±0.48	16.23±0.67	16.65±0.41$^{\$}$	13.12±0.38$^{\$}$	12.34±0.28$^{\$}$	12.98±0.45$^{\$}$

*$P<0.05$ vs. Control; $^{\$}P<0.05$ vs. H_2O_2 only

4.3.4 Effects of hydrogen peroxide on PI fluorescence

Figure 28 A shows the effects of H_2O_2 on sperm viability after 30 min of incubation. There was a significant increase in PI fluorescence for cells incubated with 100µM H_2O_2 (210.20±26.49 vs. control; $P<0.05$), as well as cells incubated with 150µM H_2O_2 (246.80±34.58; $P<0.001$) compared to the control.

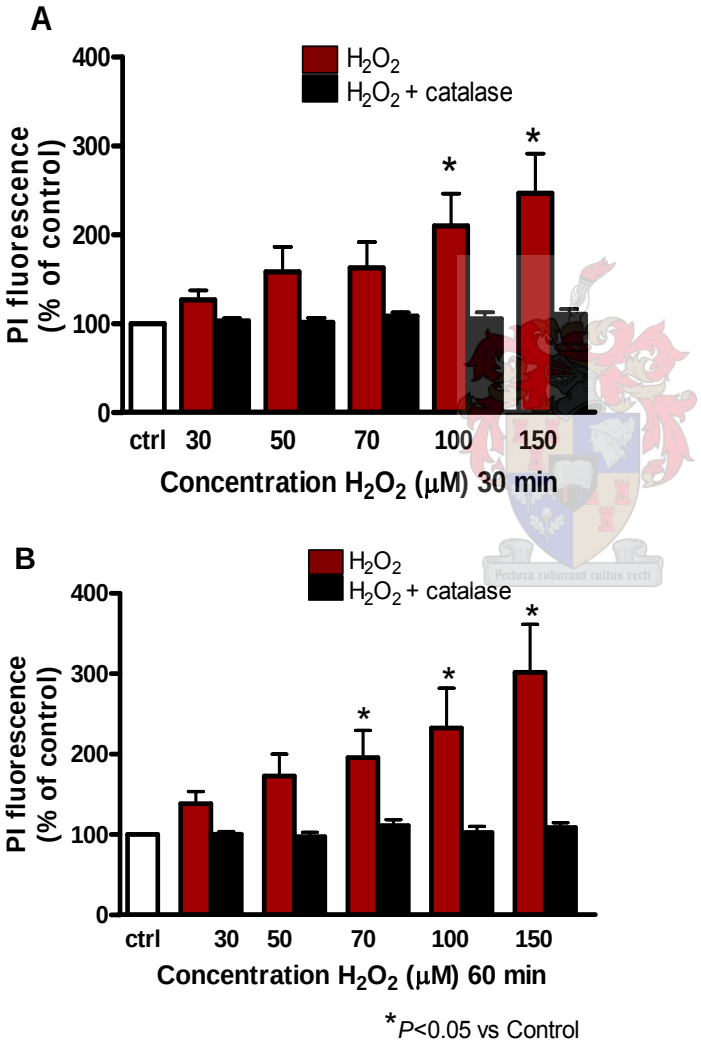


Figure 28. *Effects of H_2O_2 on PI fluorescence in the presence or absence of catalase after 30 minutes (A) and 60 minutes (B) of incubation (n=10)*

Figure 28 B shows a significant increase in PI fluorescence after 60 minutes of incubation starting from the concentration of 70μM H_2O_2 (195.70±33.84 vs. control; $P<0.05$), 100μM H_2O_2 (232.50±39.59 vs. control; $P<0.01$) and 150μM H_2O_2 (301.90±49.55 vs. control; $P<0.001$). At both time points (30 and 60 min), the effects of H_2O_2 on increased PI fluorescence could be completely reversed by the addition of the specific H_2O_2 scavenger, catalase.

CHAPTER 5

DISCUSSION

5.1 Standardization and establishment of flow cytometry as an accurate technique to directly measure specific free radicals

5.1.1 Probe specificity of DAF-2/DA for NO

This study was the first to directly measure intracellular NO in human spermatozoa using the nitric oxide specific probe, DAF-2/DA as measured by flow cytometry. As of yet, the ability of human spermatozoa to synthesize NO during in vitro capacitation has been demonstrated indirectly by measuring nitrite accumulation (Lewis *et al*., 1996), as well as L-[^{3}H]citrulline generation (Revelli *et al*., 1999). Specificity of DAF-2/DA was validated by the administration of a NO-donor SNP, in increasing concentrations to the cells, and subsequently measuring changes in mean fluorescence by FACS analysis. We observed a significant increase in mean DAF-2/DA fluorescence with an increase in SNP concentration (Fig. 20). We interpreted the increase in DAF-2/DA fluorescence as an increase in NO generation.

5.1.2 Probe specificity of DCFH for ROS

Measuring ROS using the probe DCFH has been shown in PC12 cells (Benedi et al., 2004). As far as we are aware, our study was the first to measure ROS in human

spermatozoa by FACS analysis using the fluorescent probe DCFH. To verify that our probe was indeed measuring ROS, we administered a non-specific ROS scavenger, MPG, which significantly reduced DCFH fluorescence in the control cells (Fig. 21). This reduction in the DCFH fluorescence signal was interpreted as a decrease in ROS generation, and thus supporting its use as a ROS probe.

5.2 Investigation of the effects of sperm centrifugation on free radical generation and sperm function

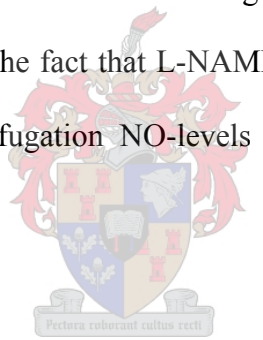
Wu et al., (2004) demonstrated increased NO production in sperm cells exposed to centrifugation. It has been shown that the duration of centrifugation is more important than the g-force for inducing free radical formation in semen (Shekarriz *et al*., 1995[b]), thus in this study only centrifugation duration was varied.

5.2.1 Effects of centrifugation on NO generation

The author established in this study that sperm centrifugation has an effect on DAF-2/DA fluorescence. Ten minutes centrifugation led to increased NO production whereas 30 minutes centrifugation caused a reduction (Fig. 21). Centrifugation causes pelleting of the sperm cells at the bottom of the tube making them hypoxic and increasing their temperature (Agarwal *et al*., 1994). Furthermore, Santoro *et al*., (2001) reported up-regulation of iNOS in patients with varicocele due to testicular hypoxia and increased scrotal temperature. We therefore speculate that the increase in NO generation in our short centrifugation samples was possibly via NOS up-regulation due to the brief hypoxia period and temperature increase. Agarwal *et al*., (1994) observed that washing procedures

involving excessive manipulation i.e. prolonged centrifugation may cause harm to the motile sperm population. Our results demonstrate that 30 min centrifugation of spermatozoa significantly decreased NO production as indicated by an attenuation of the DAF-2/DA fluorescence signal. This might have been as a result of NOS enzyme down-regulation or loss. An alternative explanation could be that the prolonged period of hypoxia caused a decrease in the substrates needed for NO production such as L-arginine and oxygen.

The NOS inhibitor, L-NAME, significantly decreased NO production in the 10 min centrifuged cells, indicating that the increased NO generation was derived from NOS activation during centrifugation. The fact that L-NAME caused a further decrease in the already attenuated 30 min centrifugation NO-levels is difficult to explain (Fig. 23). Further investigation is necessary.



5.2.2 Effects of centrifugation on ROS generation

The data in this study has shown that centrifugation caused an increase in DCFH fluorescence (Fig. 24). This was shown for both 10 min and 30 min centrifugation. These results are in agreement with the observation of Agarwal et al., (1994) in which ROS was measured by chemiluminescence using the probe luminol. No significant difference in ROS generation was observed between cells centrifuged for 10 min and 30 min. A non-specific ROS scavenger, MPG, was able to significantly attenuate the fluorescence signal in control, 10 min and 30 min centrifuged cells. We therefore speculate that MPG was able to scavenge the ROS generated during centrifugation.

5.2.3 Effects of centrifugation on sperm motility parameters

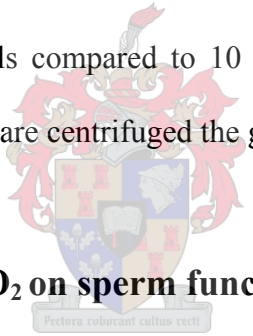
In this section an increase in the value of a motility parameter is regarded as an improvement except for static cells, where a decrease would be regarded as an improvement. Our results show that 10 min of centrifugation led to impairment of 4 of the 11 motility parameters measured (Table I). The inhibition of NO generation with L-NAME in the 10 min centrifuged sperm had no further effect on the said motility parameters. The data in this study demonstrate that scavenging the ROS generated by 10 min centrifugation with MPG, improved 2 of the 4 affected motility parameters compared to untreated centrifuged cells; the other parameters remained unchanged. We speculate that ROS may have been partly responsible for the impairment of these sperm motility parameters, since we observed increased ROS generation after 10 min centrifugation (see 5.2.2) and subsequent to scavenging the loss of motility parameters was prevented. It has been suggested that ROS induce membrane lipid peroxidation in sperm and that the toxicity of generated fatty acid peroxides are important causes of decreased sperm function (Armstrong, *et al*., 1999). The addition of both the NOS inhibitor, L-NAME and the ROS scavenger, MPG, improved 3 of the 4 affected motility parameters that were impaired by 10 min centrifugation. Interestingly, all 3 treatment group parameters returned to baseline (uncentrifuged) values, suggesting that harmful effects of 10 min centrifugation could be reversed by NOS inhibition, ROS scavenging and/or a combination of both.

Our data show that 30 min of centrifugation impaired all the motility parameters (Table I). Four of the 11 affected parameters were significantly improved when L-NAME was added, suggesting that NO had a possible detrimental role. Addition of MPG improved 6 out of the 11 motility parameters, compared to untreated centrifuged cells. This suggests that ROS generated played a relatively greater role in the impairment of the sperm motility parameters compared to NO. The combination of both L-NAME and MPG improved all the affected motility parameters. Interestingly all the parameters returned to baseline (uncentrifuged) values in the MPG only group as well as the combination L-NAME + MPG group. However in the L-NAME only group, only 4 motility parameters returned to baseline, suggesting that NO-production is relatively less detrimental compared to ROS. We observed that 30 min untreated centrifugation caused relatively more impairment to the motility parameters compared to the 10 min untreated centrifugation: (6 out of the 11 parameters measured), thereby confirming previous findings that prolonged centrifugation is more detrimental to sperm function (Agarwal et al., 1994).

5.2.4 Effects of centrifugation on sperm viability

PI is a fluorescent probe that binds to DNA. Cells having an intact plasma membrane will prevent PI from entering into the cell and staining the nucleus (Cardelli *et al*., 2005). However, cells possessing a damaged plasma membrane will permit PI to enter into the cell and bind to the DNA causing the cells to fluoresce red. We interpreted the increase in PI fluorescence as an increase in non-viable cells. The viability data showed a similar trend compared to that observed with motility parameter findings. Ten and thirty minutes

of centrifugation led to an increase in non-viable cells. The inhibition of NO generation with L-NAME in centrifuged sperm (10 and 30 min) could not maintain sperm viability (Fig. 26). We speculate that ROS may have been partly responsible for the increase in non-viable cells since we observed increased ROS generation due to centrifugation. However, the author does not rule out the role of NO levels generated due to centrifugation on sperm viability. The addition of the ROS scavenger, MPG or a combination of MPG and L-NAME was able to maintain viability to control levels. The author therefore suspected that ROS generated due to centrifugation played a role in the increase in non-viable cells, since when it was scavenged we could maintain sperm cell viability. We have also demonstrated that 30 min of centrifugation caused a significantly greater increase in non-viable cells compared to 10 min of centrifugation. These data suggest that the longer sperm cells are centrifuged the greater the harm.



5.3. Effects of NO and H_2O_2 on sperm function

NO at high concentrations is known to have detrimental effects on fertility in human reproduction (Dimmeler and Zeiher, 1997). In this study NO derived from exogenously administered SNP (at concentrations >100μM) reduced motility parameters (Table III) and decreased viability (Fig. 27) whereas lower concentrations of SNP (30μM) significantly enhanced most motility parameters, but had no effect on viability. This was in agreement with the findings of Hellstrom *et al*. (1994) where NO at low concentrations improved sperm motility and viability after thawing. This confirms the paradoxical role of nitric oxide, i.e. being beneficial at lower concentrations and becoming detrimental at higher concentrations (Wu et al., 2004). In this study the improvements of sperm function

(motility parameters and viability) as well as detrimental effects after exogenous NO treatment only started to show after 90 min of incubation. This indicated that the effects of NO were time and dose dependent. To explain how NO at high concentrations becomes harmful to spermatozoa, Wu *et al*., (2004) demonstrated that addition of ATP counteracted the inhibitory effects of NO on sperm motility and viability. Their results suggested that NO interfered with the energy metabolism of sperm and resulted in a decrease in motility and viability. NO at higher concentrations can form highly toxic peroxynitrite when it reacts with superoxide. Since the cell membrane of the sperm has a higher ratio of unsaturated fatty acids than cells elsewhere in the body, it can be readily damaged by these free radicals (Padron *et al*., 1997).

Our results are in agreement with earlier findings that H_2O_2 is the most cytotoxic ROS to human spermatozoa (Duru, *et al*., 2000). H_2O_2 is an integral compound and a by-product of aerobic metabolism (Armstrong *et al*., 1999). In this study we have shown that all sperm motility parameters and viability decreased with increased H_2O_2 concentrations. Sperm motility is directly dependent on ATP supply from the mitochondrial pool (Chaki and Misro, 2002). It is therefore likely that the availability of ATP for metabolism was affected as a result of H_2O_2 treatment. Unless inactivated by the scavenging enzymes present inside the cell, H_2O_2 has the ability to diffuse along both intracellular compartments and across the membrane to the extracellular medium leading to plasma membrane damage (Chaki and Misro, 2002). The H_2O_2 scavenger, catalase was able to reverse all harmful effects at all H_2O_2 concentrations.

Conclusion

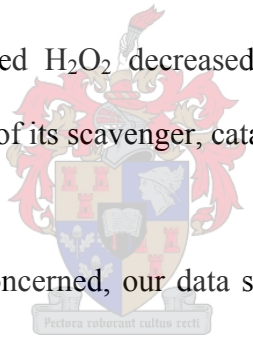
To date, there is increasing interest in all the factors potentially affecting male fertility, since several studies seem to point to a progressive reduction in fertility over the last few decades. Nevertheless, it is undeniable that there is a vast lack of knowledge regarding the molecular events that lead to sub-fertile semen. Assisted reproductive techniques are fast becoming an alternative option. The identification of factors that lead to defective sperm function during *in vitro* semen preparation techniques and ways of avoiding them would play a great role in maintaining normal sperm physiology.

In this study we have standardized and established flow cytometry as an accurate technique to directly measure free radicals in human spermatozoa. This was the first study to directly measure intracellular NO in sperm by FACS analysis. Our findings suggest that the DAF-2/DA-FACS analysis method can be regarded as an objective, relatively easy to perform and a time-saving technique to detect and measure intracellular NO production in human spermatozoa. This study also, for the first time, described the technique of measuring intracellular ROS by FACS analysis using the fluorescence probe DCFH in human spermatozoa.

This study has demonstrated that 10 and 30 min of sperm centrifugation was detrimental to both sperm motility and viability, but generally 30 min centrifugation

was more detrimental to sperm than 10 min. Following the demonstration of the above free radical analysis techniques in sperm, we measured NO and ROS in human spermatozoa exposed to different centrifugation time durations. The study has demonstrated that 10 min sperm centrifugation lead to increased NO generation, whereas 30 min attenuated NO production.

Centrifugation for 10 and 30 min resulted in an increase in ROS production. We have demonstrated that ROS is detrimental to human spermatozoa. This was confirmed by reduction in sperm function (motility and viability) due to ROS generated during 10 and 30 min of centrifugation. The detrimental effects of ROS were further supported when exogenously administered H_2O_2 decreased sperm function which could be reversed by the administration of its scavenger, catalase.



As far as the role of NO is concerned, our data suggest some contradictory results. Ten minutes of sperm centrifugation led to an increase in NO generation whereas 30 min led to a decrease. The inhibition of NO during 10 min centrifugation had no effect on motility parameters compared to untreated 10 min centrifuged group. However, inhibition of NO during 30 min centrifugation had a marginal improvement in motility parameters. Contrary to this, inhibition of NO production in 10 and 30 min had no effect on sperm viability. The administration of a NO donor, SNP, at lower concentrations improved motility parameters whereas at higher concentrations decreased both motility and viability. Given the above contradicting data, further investigations into the role of NO in human spermatozoa are necessary.

Based on the findings of this study, we recommend the use of DAF-2/DA and DCFH-FACS analysis as an accurate and relatively easy technique to detect intracellular NO and ROS respectively in human spermatozoa. We also recommend that sperm separation techniques should avoid using centrifugation or prolonged centrifugation in assisted reproductive technologies. Future studies will indicate whether addition of ROS scavengers prior to centrifugation could improve sperm selection in assisted reproductive technologies.

REFERENCES

Agarwal, A., Ikemoto, I., Loughlin, K.R. Effect of sperm washing on reactive oxygen species level in semen. Archives of Andrology 33:157-162; 1994.

Agarwal, A., Saleh, R.,and Bedaiwy, M.A. Role of reactive oxygen species in the pathophysiology of human reproduction. Fertility and Infertility 79:829-843; 2003.

Aitken, R.J., Hulme, M.J., Henderson, C.J., Hergreave, T.B., Ross, A. Analysis of the surface labeling characteristics of human spermatozoa and the interaction with anti-sperm antibodies. Journal of Reproduction and Fertility 80:473-485; 1987.

Aitken, R.J., Krausz, C., and Buckingham, D. Relationship between biochemical markers for residual sperm cytoplasm, reactive oxygen species generation and the presence of leukocytes and precursor germ cells in human sperm suspension. Molecular Reproduction Development 39:268-279; 1994.

Aitken, R.J., and Fisher, H. Reactive oxygen species generation and human spermatozoa: the balance of benefit and risk. Bioassays 16:259-267; 1994.

Aitken, R.J., Buckingham, D.W, Brindles, J., Gomez, E., Baker, H.W., Irvine, D.S. Analysis of sperm movement in relation to the oxidative stress created by leukocytes in

washed sperm preparations and seminal plasma. Human Reproduction 10:2061-2071; 1995.

Aitken, R.J., Fisher, H., Gomez, E., Knox, W., and Lewis B. Reactive oxygen species generation by human spermatozoa is induced by exogenous NADPH and inhibited by flavoprotein inhibitors diphenylene iodonium and quinacrine. Molecular Reproduction Development 47:468-482; 1997.

Aitken, R.J., Harkiss, D., Knox, W., Peterson, M., and Irvine, D.S. A novel signal transduction cascade in capacitating human spermatozoa characterized by a redox-regurated cAMP-mediated induction of tyrosine phosphorylation. Journal of Cell Science 111:645-656; 1998.

Aitken, R.J., and Baker, M.A. Oxidative stress and male reproductive biology. Reproduction, Fertility and Development 16:581-588; 2004.

Alvarez, J.G., and Storey, B.T. Differential incorporation of fatty acids into and peroxidative loss of fatty acids from phospholipids of human spermatozoa. Molecular Reproduction and Development 42:334-346; 1995.

Andrews, J.C., and Bavister, B.D. Capacitation of hamster spermatozoa with the divalent cation chelators D-penecillamine, L-histidine and L-cysteine in a protein free culture medium. Gamete Research 23:159-170; 1989.

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

Babior, B.M. NADPH oxidase: an update. Blood 93:1464-1476; 1999.

Benedi, J., Arroyo, R., Romero, C., Martin-Aragon, S., and Villar, A.M. Antioxidant properties and protective effects of a standardized extract of Hypericum perforatum on hydrogen peroxide-induced oxidative damage in PC12 cells. Life Sciences 75:1263-1276; 2004.

Bleil, J.D., Wasserman, P.M. Sperm-egg interactions in the mouse: sequence of events and induction of the acrosome reaction by zona pellucida glycoprotein. Developmental Biology 95:317-324; 1983.

Cardelli, P., Lattari, M., Massaro, P., Policila, M., Barlattani, A. Pharmacologic treatment of dysfunctional patient. Minerva Stomatol 54: 265-279; 2005.

Chaki, S.P., and Misro, M.M. Assessment of human sperm function after hydrogen peroxide exposure: development of a viginal contraceptive. Contraception 66:187-192; 2002.

de Lamirande, E., and Gagnon, C. Impact of reactive oxygen species on spermatozoa: a balancing act between beneficial and detrimental effects. Human Reproduction 10:15-21; 1995.

Dimmeller, S., and Zeiher, A.M. Nitric oxide and apoptosis: another paradigm for the double-edged role of nitric oxide. Nitric Oxide 1:275-281; 1997.

Duru, N.K., Morshedi, M., Oehninger, S. Effects of hydrogen peroxide on DNA and plasma membrane integrity of human spermatozoa. Fertility and Sterility 74:1200-1207; 2000.

Ehrenwald, E., Parks, J.E., Foote, R.H. Bovine oviductal fluid components and their potential role in sperm cholesterol efflux. Mol Reprod Dev 25:195-204; 1990.

Fedder, J., Ellerman-Erickson, S. Effects of cytokines on sperm motility and ionophore stimulated acrosome reaction. Archives of Andrology 35:173-185; 1995.

Florman, H.M., Corron, M.E., Kim, T.D.H., Babcock, D.F. Activation of voltage-dependent calcium channels of mammalian sperm is required for zona pellucida induced acrosome exocytosis. Developmental Biology 152:304-314; 1992.

Ford, W.C.L. Regulation of sperm function by reactive oxygen species. Human Reproduction Update 10:387-399; 2004.

Gagnon, C., and de Lamirande E. Redox control of changes in protein sulfhydryl levels during human sperm capacitation. Free Radical Biol Med 35:1271-1285; 2003.

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

Griveau, J.F., Renard, P., and Lannou, D. An *in vitro* promoting role for hydrogen peroxide in human sperm capacitation. Int. J. Androl. 17:300-307; 1994.

Griveau, J.F., Renard, P., and Lannou, D. Superoxide production by human spermatozoa as a part of the inophore induced acrosome reaction. International Journal of Andrology 18:77-74; 1995.

Halliwell, B. How to characterize a biological antioxidant. Free Radicals Research Communication 9:1-32; 1990.

Halliwell, B., and Getturidge J.M. Antioxidant defense mechanisms: from the beginning to the end (of the beginning). Free Radical Research 4:261-272; 1999.

Hellstrom, W.J., Bell, M., Wang, R., Sikka, S.C. Effects of sodium nitroprusside on sperm motility, viability, and lipid peroxidation. Fertility and Sterility 61:1117-1122; 1994.

Herrero, M.B., Cebral, E., Boquet, M., Viggiano, J.M., Vitullo, A., Ginenno, M.A. Effect of nitric oxide on mouse sperm hyperactivation. Acta Physiol Pharmacol ther Latinoam 44: 65-69; 1994.

Kovalski, N.N., de Lamirande, E., Gagnon, C. Reactive oxygen species generated by human neutrophils inhibit sperm motility: protective effects of seminal plasma and scavengers. Fertility and Sterility 58:809-816; 1992.

Kumar, G.P., Laloraya, M.M. Superoxide radical and superoxide dismutase activity changes in maturing mammalian spermatozoa. Andrologia 23:171-175; 1991.

Lee, J., Richburg, J.H., Younkin, S.C., and Boekelbeide, K. The Fas system is a key regulator of germ cell apoptosis in the testis. Endocrinology, 138:2081-2088; 1997.

Lenzi, A., Cualosso, F., Gandini, L., Lombardo, F., Dondero, F. Placebo-controlled, double-blinded, cross-over trial of glutathione therapy, in male infertility. Human Reproduction 2044-2050; 1993.

Lewis, S.E.M., Boyle, P.M., Mc Kinney, K.A. Total antioxidant capacity of seminal plasma is different in fertile and infertile men. Fertility and Sterility 64: 863-869; 1996.

Macleod, J. The role of oxygen in the metabolism and motility of human spermatozoa. American Journal of Physiology 138:512-518; 1943.

Meizel, S. the importance of hydrolytic enzymes to an exocytotic event, the mammalian sperm acrosome reaction. Biology of Reproduction 59:125-157; 1984.

Osman, R.A., Andria, M.L., Jones, A.D., Mezeil, S. Steroid induced exocytosis: the human sperm acrosome reaction. Biochemical and Biophysical Research Communication 160: 828-833; 1989.

Padron, O.F., Brackett, N.L., Sharma, R.K., Lynne, C.M., Thomas, A.J. Jr., Agarwal, A. Seminal reactive oxygen species and sperm motility and morphology in men with spinal cord injury. Fertility and Sterility 67:1115-1120; 1997.

Pasqualotto, F.F., Sharma, R.K., Agarwal, A., Nelson, D.R., Thomas, A.J., and Potts, J.M. Seminal oxidative stress in chronic prostatitis patients. Urology 55:881-885; 2000.

Pena, A.I., Quintela, L.A., and Herradon, P.G. Viability assessment of dog spermatozoa using flow cytometry. Theriogenology 50:1211-1220; 1998.

Plante, M., de Lamirande, E., and Gagnon, C. Reactive oxygen species released by activated neutrophils, but not by deficient spermatozoa, are sufficient to affect normal sperm motility. Fertility and Sterility 62:387-393; 1994.

Purohit, S.B., Laloraya, M., Kumar, G.P. Acrosome reaction inducers impose alterations in repulsive strain and hydration barrier in human sperm membrane. Biochem. Mol. Bio. Intl. 45:227-235; 1998.

Rajasekaran, M., Hellstrom, W.J., Naz, R.K., Sikka, S.C. Oxidative stress and interleukins in seminal plasma during leukocytospermia. Fertility and Sterility 64:166-171; 1995.

Revelli, A., Costamagna, C., Moffa, F., Aldieri, E., Ochetti, S., Bosia, A., Massobrio, M., Lindblom, B., and Ghigo, D. Follicular fluid proteins stimulate nitric oxide (NO) synthesis in human sperm: a possible role of NO in acrosomal reaction. Journal of Cell Physiology 178:85-92; 1999.

Russel, J.H., Hale, A.H., Inbar, D., Fisen, H.N. Loss of reactivity of BAL B/c myeoloma tumor with allogeneic and syngeneic cytotocic T lymphocytes. Eur J Immunol 8:640-645; 1978.

Sakkas, D., Mariethoz, E., Manicardi, G., Bizzaro, D., Bianchi, P., and Bianchi, U. Origin of DNA damage in ejaculated human spermatozoa. Rev. Reprod. 4:31-37; 1999.

Santoro, G., Romeo, C., Impellizzeri, P., Ientile, R., Cutroneo., Trimarchi, F., Pedale, S., Turiaco, N., and Gentile, C. Nitric oxide synthase patterns in normal and varicocele testis in adolescents. BJL International 88:967-973; 2001.

Sengoku, K., Tamate, K., Yoshida, T., Takaoka, Y., Miyamoto, T., and Ishikawa, M. Effects of low concentrations of nitric oxide on zona pellucida binding ability of human spermatozoa. Fertility and Sterility 69:522-527;1998.

Sharma, R., Pasqualotto, F.F., Nelson D.R., Thomas, A.J., and Agarwal A. Relationship between seminal white blood cell counts and oxidative stress in men treated at an infertility clinic. Journal of Andrology 22:575-583; 2001.

Shekarriz, M., Thomas, A.J., and Agarwal, A. Incidence and level of seminal reactive oxygen species in normal men. Adult Urology 45:103-107; 1995[a].

Shekarriz, M., Sharma, R.K., Thomas, A.J., and Agarwal, A. Positive myeloperoxidase staining (Endtz Test) as an indicator of excessive reactive oxygen species formation in semen. Journal of Assististed Reproduction Genetics 12:70-72; 1995[b].

Sinha, H.A.P., and Swerdloff, R.S. Hormonal and genetic control of germ cell apoptosis in the testis. Rev. Reprod. 4:38-47; 1999.

Strijdom, H., Muller, C., & Lochner, A. Direct intracellular nitric oxide detection in isolated adult cardiomyocytes: flow cytometric analysis using the fluorescent probe, diaminofluorescein. Journal of Molecular and Cellular Cardiology 37: 897-902; 2004.

Thundathil, J., Lamirande, E., and Gagnon, C. Nitric oxide regulates the phosphorylation of the threonine-glutamine –tyrosine motif in proteins of human spermatozoa during capacitation. Biology of Reproduction 68:1291-1298; 2003.

Tomlinson, M.J., Barrat, G.L.R., Cooke, I.D. Prospective study of leukocytes and leukocyte subpopulations in semen suggests that they are not a cause of male infertility. Fertility and Sterility 60:1069-1075; 1993.

Twigg, J., Irvine, D.S., and Aitken, R.J. Oxidative damage to DNA in human spermatozoa does not prelude pronucleus formation at intracytoplasmic sperm injection. Human Reproduction 103:1864-1871; 1998.

Vaux, D.L., and Flavell, R.A. Apoptosis genes and autoimmunity. Current Opinions in Immunology 12:719-724; 2000.

Vaux, D.L., and Korsmeyer, S.J. Cell death in development. Cell 96:245-254; 1999. www.137.222.110.150/restricted/gallery/album95/ac.

Wolff, H. The biological significance of white blood cells in semen. Fertility and Sterility 63:1143-1147; 1995.

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

Wu, T.P., Huang, B.M., Tsai, H.C., Lui, M.C., Liu, M.Y. Effects of nitric oxide on human spermatozoa activity, fertilization and mouse embryonic development. Archives of Andrology 50:173-179; 2004.

Wu, G.J., Ding, D.C., Chen, I.G, & Huang, Y.C. Less NO production and better motion parameters in human sperm by swim-up processing. Archives of Andrology 50, 373-377; 2004.

Yamaguchi, R. Mammalian fertilization. In: Knobil, E., Neill, J., editors. Physiology of reproduction. New York: Raven Press; p 189-317; 1994.

Yeagle, P.L. Lipids and lipid-intermediate structures in the fusion of biological membranes. Curr. Top Membr. 4:197-214; 1994.

Zhang, H., and Zheng, R.L. Possible role of nitric oxide on fertile and asthenozoospermic infertile human sperm functions. Free Radical Research 25:347-354; 1996[a].

Zhang, H., and Zheng, R.L. Promotion of human sperm capacitation by superoxide anion. Free Radical Research 24:261-267; 1996[b].