MALE FERTILITY: SIZE, MOTION AND AMOUNT COUNT ... BUT WHAT ABOUT ROS?

Prof Stefan S du Plessis

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BIOGRAPHY

Professor Stefan du Plessis was born in Stellenbosch but grew up in Malmesbury where he completed his schooling at Swartland High School. He obtained his BSc, BScHon (*cum laude*) and MSc degrees in Animal Physiology and his PhD in Medical Physiology from Stellenbosch University.

While pursuing his postgraduate studies, he joined the Department of Human and Animal Physiology in 1992 as a member of staff. In 1997 he was appointed as a lecturer in the Division of Medical Physiology and was later promoted to senior lecturer, associate professor and full professor. He continued studying part time, obtaining an HonsB(B&A) and MBA from the University of Stellenbosch Business School and also a certificate in Human Assisted Reproduction from the American Center for Reproductive Medicine (ACRM) in the United States of America (USA).

Currently Prof du Plessis is the Head of the Division of Medical Physiology in the Faculty of Medicine and Health Sciences where he is actively involved in both undergraduate and postgraduate teaching as well as research. He is furthermore the group leader of the Stellenbosch University Reproductive Research Group (SURRG) in the Department of Biomedical Sciences. Being a reproductive physiologist, his research interests include investigating the impact of various factors (e.g. oxidative stress, lifestyle and non-communicable diseases) on spermatozoa at a functional and molecular level. Numerous postgraduate students have completed their studies under his supervision, and as a National Research Foundation-rated researcher with an h-index of 23, he has been invited to deliver keynote addresses at various institutions and conferences worldwide. To date his research has been presented at more than 50 international and nearly 100 national conferences. He is the editor of 3 subject-specific books and has published more than 70 peer-reviewed papers and 25 book chapters on various aspects of andrology and male infertility.

As a teacher, he is recognised by serving as faculty member, advisor, moderator and examiner to several national and international universities as well as The Colleges of Medicine of South Africa. Nationally he was honoured with the Excellence in Physiology Award by the Physiological Society of Southern Africa (2013), and internationally he received the Dr Edmund Sabanegh Award for Excellence in Male Infertility Research from the Cleveland Clinic Foundation, USA (2012). Prof du Plessis is also a former recipient of the much-coveted Fulbright Fellowship (2014), affording him the opportunity to spend a year at the ACRM in the USA. He serves on the editorial boards of two leading journals and has also completed two terms as member of the Stellenbosch University Council.

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In my journey through life, I have met many wonderful people who not only influenced and shaped me as a person but also unwittingly moulded my career as academic and researcher.

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My close friend and collaborator of the last few years, Gerhard, one of the most interesting humans I have ever known. Your passion for sperm is inspiring. You are a stimulating colleague and great friend.

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Acta non verba

MALE FERTILITY: SIZE, MOTION AND AMOUNT COUNT … BUT WHAT ABOUT ROS?

INTRODUCTION

For most couples, procreating is a natural part of life that involves neither special planning nor intervention. Fertility is a key element of reproductive health, and infertility is documented as a global public health issue by the World Health Organization (WHO) (Boivin et al., 2007; Datta et al., 2016). The American Society for Reproductive Medicine, the American College of Obstetricians and Gynecologists as well as the WHO (2010) recognise infertility as a disease, and it is therefore formally classified as "a disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse" (Zegers-Hochschild et al., 2009: 1522).

Subfertility affects between 15% and 25% of couples, and it was estimated that in 2010, a predicted 48.5 million couples globally were unable to have a child after five years of attempting (Borges, 2016; Mascarenhas et al., 2012;Trussell, 2013). In the United States of America, one in six couples (Thoma et al., 2013) and in the United Kingdom, around one in seven couples (National Collaborating Centre for Women's and Children's Health, 2013) are diagnosed with infertility (Datta et al., 2016). What is surprising and alarming is the fact that an analysis of almost 300 demographic and reproductive health surveys revealed that nearly one in every four couples in developing countries was affected by infertility (Mascarenhas et al., 2012). The experience of infertility can cause those affected personal distress (Schneider and Forthofer, 2005), significant treatment costs (Bell, 2010) and, in some contexts, ostracism and discrimination (Cui, 2010).

In approximately 20% of infertile couples, the male partner is the sole cause, and in a further 30–40%, it is a contributing cause of infertility (Borges, 2016; Thonneau et al., 1991). Male infertility can be due to a variety of conditions. The minimum full evaluation for male infertility should include a complete medical history, physical examination and at least two semen evaluations as infertility is most commonly due to deficiencies in the semen (Cooper et al., 2010).

The WHO proposed reference values (see Table 1) based on several population studies to estimate male reproductive potential by the assessment of sperm parameters (WHO, 2010). Despite the normalisation effects, this approach can only provide an indirect indication of male fertility status and is used as a surrogate measure of male fecundity (Cooper et al., 2010). It is important to understand that while the results may correlate with 'fertility', the assay is not a direct measure of fertility (Guzick et al., 2001; Vasan, 2011). Thus, the predictive power of these parameters remains debateable (De los Rios et al., 2004; Mayorga-Torres et al., 2016).

Parameter (Units)	Lower reference (5^{th} centile)
Semen volume (ml)	1.5
Sperm concentration (10^6/ml)	15
Total number (10^6/ejaculate)	39
Total motility (%)	40
Progressive motility (%)	32
Normal forms (%)	4
Vitality (%)	58

Table 1: *Lower reference limits for semen parameters from fertile men whose partners had a time-to-pregnancy of 12 months or less. (Adapted from Cooper et al., 2010)*

The most basic assessments performed during the examination include the measuring of the semen volume, sperm concentration, sperm motility and sperm morphology. These values when compared to the WHO reference values help to define and classify the patient as either normozoospermic or as possessing abnormal semen values.

'Normozoospermic' refers to when the sperm number, motility and morphology meet the WHO lower reference values. It has been well documented that these three basic sperm parameters correlate with fertilisation ability and can thus predict male fertility to a certain extent.

Sperm number: The amount of sperm can be reported as either sperm concentration or total number of sperm in the ejaculate. Both of these values correlate with fertilising ability as pregnancy rates by intercourse and intrauterine insemination decline as sperm density decreases (Boivin et al., 2007; Bostofte et al., 1990; Smith et al., 1977). 'Oligozoospermia' refers to a sperm concentration of $< 15 \times 10^6$/ml or when the total number of sperm in the ejaculate is $< 39 \times 10^6$ per ejaculate while 'azoospermia' refers to the absence of sperm in the seminal plasma (WHO, 2010).

Sperm motility: In order for spermatozoa to reach the oocyte, they must be motile and display certain motion characteristics. The efficient passage of spermatozoa through cervical mucus is dependent on rapid progressive motility (Bjorndahl, 2010) and is therefore a good predictor of fertilisation failure, an outcome that is actually more important when making decisions regarding a couple's treatment options (Aitken et al., 1985). Manual semen analysis lacks the ability to measure the kinematics of sperm motion. Computer-aided sperm analysis is potentially useful because of its capacity to analyse sperm motion (sperm head and flagellar kinetics), some of which have been shown to be closely related to *in vitro* fertilisation (IVF) outcome (Freour et al., 2010). 'Asthenozoospermia' refers to progressive motility of < 32% (WHO, 2010).

Sperm morphology: Sperm size and sperm form have been shown to seriously affect normal sperm function, including a spermatozoon's ability to undergo the acrosome reaction (Menkveld et al., 2003) and to bind to the zona pellucida (ZP) of the oocyte (Aziz et al., 1998; Garrett et al., 1997; Menkveld, 2010). Consequently, morphology, which encompasses morphometry, has been regarded as one of the most important semen variables for predicting a man's fertility potential (Aziz et al., 1998; Maree et al., 2010; Menkveld, 2010). The clinical implications of poor morphology scores remain highly controversial as pregnancy is possible with low morphology scores (Van Waart et al., 2001). 'Teratozoospermia' refers to a normal morphology of < 4% (WHO, 2010).

Currently, almost 50% of men suffering from infertility cannot be diagnosed with a specific cause for their problem. As the diagnosis of male infertility is predominantly based on the reference values of conventional seminal parameters, as recommended by the WHO (WHO, 2010), many reproductive endocrinologists and andrologists suggest that the prognostic relevance of the 2010 WHO classification system is rather limited and not ideal (Esteves et al., 2012; Esteves, 2015; Hamilton et al., 2015). It is especially inadequate to shed light on male infertility of unknown origin, which can be further classified as idiopathic (33% – when identification of the aetiology of an abnormal semen analysis is not possible) and unexplained (11% – when the reason for infertility is not clear, with a normal semen analysis and partner evaluation) infertility, as is the case in many patients (Cardona Maya, 2010; Gudeloglu et al., 2015; Hamada et al., 2012). One of the reasons might be that the WHO classification system provides no insights into the functional potential of the sperm to undergo certain maturational processes or ability to fertilise the oocyte.

It has been shown that high levels of reactive oxygen species (ROS) are present in the semen of 25–40% of infertile men (De Lamirande and Gagnon, 1995) whereas fertile men do not have a detectable level of semen ROS (Agarwal, Sharma et al., 2006; Aitken et al., 1989; Aitken et al., 1991). These infertile patients also display low levels of antioxidants in their seminal plasma (Mahfouz, Sharma, Sharma et al., 2009; Sharma et al., 1999). When ROS outnumber antioxidants due to either increased ROS levels, decreased antioxidant properties or a combination of both, this imbalance can lead to the development of oxidative stress (OS) (Agarwal, Virk et al., 2014; Du Plessis, Agarwal, Halabi and Tvrda, 2015; Kothari et al., 2010). Currently, it is believed that OS is an important and plausible cause of unexplained male infertility.

The increasing importance of this problem has pushed scientists over the last decade to extensively dwell on the study of the role of ROS as a causative agent in male infertility (Du Plessis, Agarwal, Halabi and Tvrda, 2015).

REACTIVE OXYGEN SPECIES

ROS are chemically reactive chemical species containing oxygen. ROS, also referred to as free radicals, are formed as a by-product of oxygen metabolism.

Free radicals are molecules with one or more unpaired electrons (Agarwal et al., 2006). These highly reactive molecules attack the nearest stable molecule to obtain an electron. Subsequently, the targeted molecule becomes a free radical itself and initiates a cascade of events that can ultimately lead to cellular damage (Agarwal, Makker and Sharma, 2008; Kothari et al., 2010). However, at physiological levels, free radicals also help to preserve homeostasis by acting as signal transducers (De Lamirande et al., 1997).

There are two common forms of free radicals, namely ROS and reactive nitrogen species (RNS). Examples of ROS include the superoxide anion (O_2-), hydrogen peroxide (H_2O_2), the extremely reactive hydroxyl radical ($OH^•$) and the perhydoxyl radical (HO_2-) (Sikka, 2001). RNS are often considered to be a subclass of ROS (Sikka, 2001) and include nitric oxide (NO), nitrous oxide (N_2O), peroxynitrite (NO_3-), nitroxyl anion (HNO) and peroxynitrous acid (HNO_3) (Agarwal et al., 2006a; Sharma and Agarwal, 1996a).

General reactive oxygen species production

The reduction of diatomic oxygen (O_2), which is essential for cellular respiration and survival, results in the creation of a highly reactive oxygen metabolite, O_2-, which is capable of interfering with cellular functions. If this reduction is followed by the gain of another electron, peroxide (O_2^{2-}) is formed. Interestingly, O_2^{2-} is not considered a free radical (Ford, 2004). The endogenous H_2O_2, a weak though abundant free radical, is subsequently generated by numerous metabolic reactions in the human body including the peroxisomal pathway via beta glycolate and monoamine oxidases. It is also produced by O_2- dismutation (Halliwell et al., 2000).

Both O_2- and H_2O_2 can undergo a series of cellular transformations to form the extremely reactive $OH^•$ through the Fenton and Haber Weiss reaction, which involves two steps. The first step consists of a reduction of ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) in the presence of O^{2-}. The second step consists of H_2O_2 conversion to $OH^•$. Ferrous ions act as catalysts in this slow reaction (Koppenol, 2001). O_2- interacts with NO to form peroxynitrite (ONOO-). NO is a reactive radical with an odd number of electrons catalysed by the family of nitric oxide synthase (NOS) enzymes (Kelm, 1999; Lampiao et al., 2006a). Other ROS species including ozone, organic peroxyl and alkoxy radicals may be present too but are not biologically important.

Generation of reactive oxygen species by spermatozoa

ROS production is related to sperm physiology due to its role in various sperm processes (Agarwal et al., 2006a; Du Plessis, Agarwal, Halabi and Tvrda, 2015; Kothari et al., 2010). Spermatozoa rely on both glycolysis and mitochondrial oxidative phosphorylation for energy; the latter is a process that is subsequently accompanied by ROS generation (Du Plessis, Agarwal, Mohanty and Van der Linde, 2015; Du Plessis et al., 2008). Research has shown that ROS cause electron leakage from actively respiring spermatozoa, mediated by intracellular redox activities. The generation of ROS in spermatozoa may occur via two methods: (i) the nicotinamide adenine dinucleotide phosphate oxidase system at the level of the sperm plasma membrane and/or (ii) the nicotinamide adenine dinucleotide-dependent oxido-reductase reaction at the mitochondrial level (see Figure 1). The latter mechanism appears to be the main source of ROS (Agarwal, Virk et al., 2014). Spermatozoa are rich in mitochondria because a constant supply of energy is

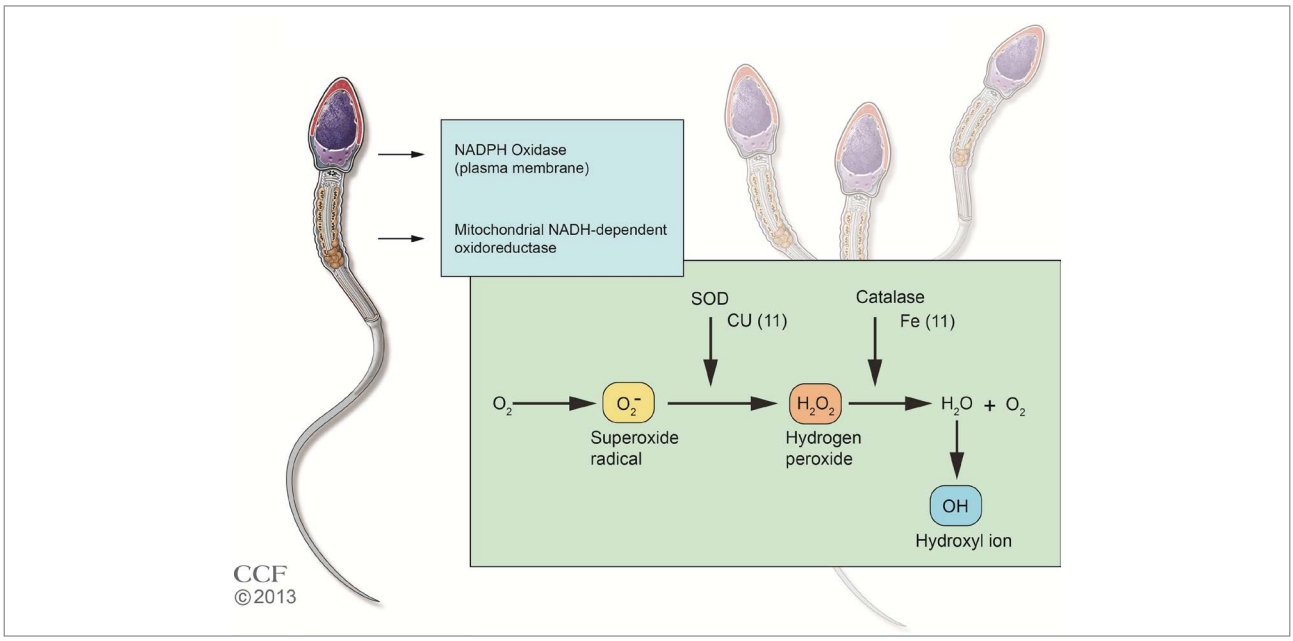

Figure 1: *Generation of reactive oxygen species (NADPH: nicotinamide adenine dinucleotide phosphate; NADH: nicotinamide adenine dinucleotide; SOD: superoxide dismutase; CU: copper; Fe: iron)*

required for their motility (Henkel, 2011). Therefore, the presence of dysfunctional spermatozoa in the semen significantly elevates the production of ROS, which in turn affects mitochondrial function and, subsequently, sperm function such as motility.

Sources of reactive oxygen species in semen

ROS found in semen originate from various endogenous and exogenous sources (Figure 2). The human ejaculate consists of different types of cells, including mature and immature spermatozoa, round cells, leukocytes and epithelial cells. Of these, leukocytes (mainly neutrophils and macrophages) and immature spermatozoa are considered the main endogenous sources of ROS (Du Plessis, Agarwal, Halabi and Tvrda, 2015). The presence of varicocele of a higher grade is also considered as an endogenous source of ROS (Shiraishi et al., 2012). Several lifestyle factors such as excessive smoking and alcohol consumption as well as environmental factors such as radiation and toxins can contribute to exogenous ROS (Gharagozloo and Aitken, 2011; Harlev et al., 2015).

Immature spermatozoa: During spermatogenesis, damaged spermatozoa undergo arrested spermiogenesis. This causes them to maintain excess residual cytoplasm, which can activate the nicotinamide adenine dinucleotide phosphate (NADPH) system. As such, spermatozoa with cytoplasmic droplets act as a potential contributor to ROS production (Aitken et al., 1997). Immature spermatozoa with excess cytoplasm around their midpiece are functionally defective, having impaired motility and abnormal morphology, which impacts negatively on their fertilisation potential (Whittington and Ford, 1999).

Leukocytes: Leukocytes are the predominant source of ROS during spermatozoa preparation as they are able to produce up to 1 000 times more ROS than spermatozoa in human semen (Kessopoulou et al., 1992). Originating from the prostate gland and seminal vesicles, peroxidase-positive leukocytes include polymorphonuclear leukocytes and macrophages (Saleh et al., 2003). During infection or inflammation processes in vivo, leukocytes release large amounts of superoxide when conquering pathogens. Seminal leukocytes also stimulate spermatozoa to produce ROS (Agarwal, Durairajanayagam and Du Plessis, 2014).

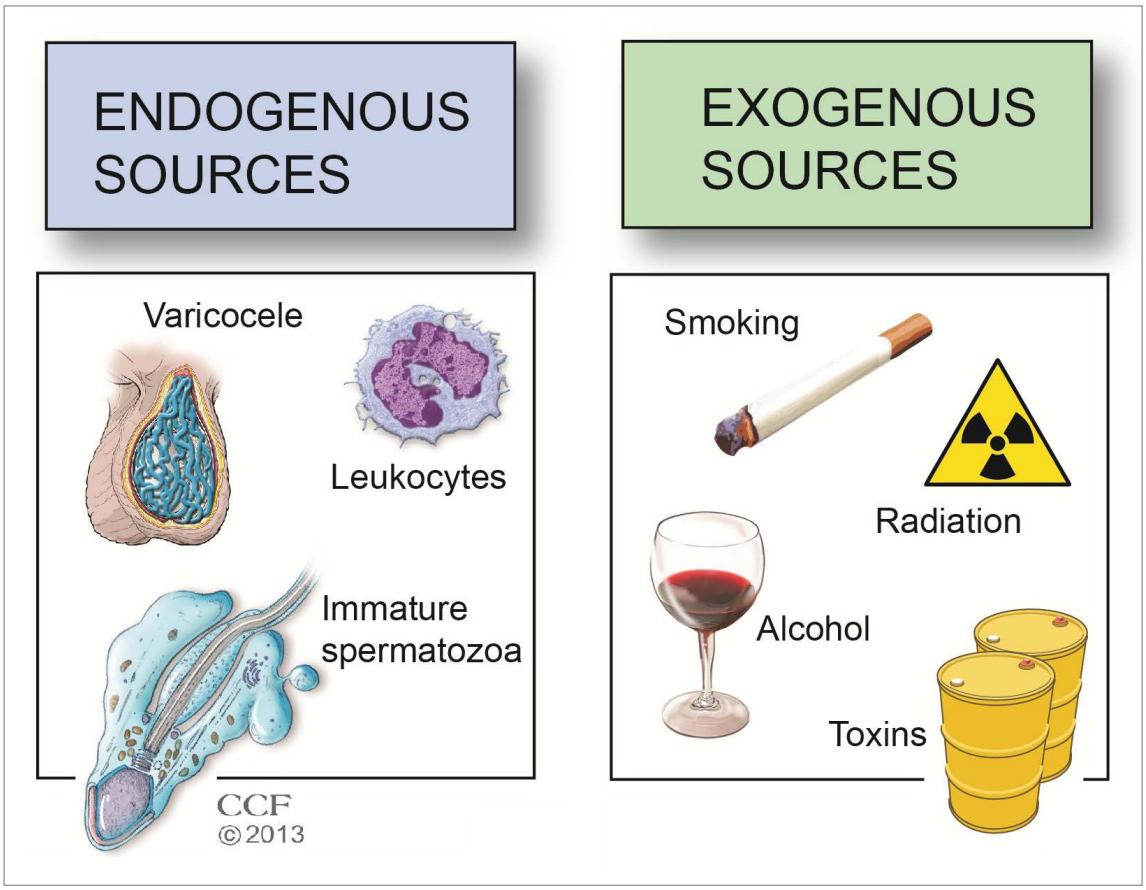

Figure 2: *Sources of reactive oxygen species*

Varicocele: Varicocele is the abnormal dilatation of veins in the pampiniform plexus surrounding the spermatic cord. Varicocele of a higher grade is associated with greater amounts of seminal ROS (Shiraishi et al., 2012). Infertile men with varicocele have increased OS levels and lowered antioxidant concentrations (Agarwal et al., 2006b).

Roles of reactive oxygen species in seminal plasma

Normal human spermatozoa function are facilitated by physiological levels of ROS (De Lamirande and Gagnon, 1993). However, spermatozoa are particularly susceptible to pathological levels of ROS (Tremellen, 2008).

Physiological roles

Physiological levels of ROS are essential for proper sperm function, including maturation, capacitation, hyperactivation, acrosome reaction and sperm oocyte interaction (Figure 3) (Agarwal et al., 2006a ; Du Plessis, Agarwal, Halabi and Tvrda, 2015; Kothari et al., 2010). ROS play important roles in signal transduction processes during the complex molecular cascades of spermatozoa; however, the specific types of ROS involved in mediating these events still remain inconclusive.

Capacitation: Capacitation is the penultimate process in the maturation of spermatozoa and is required

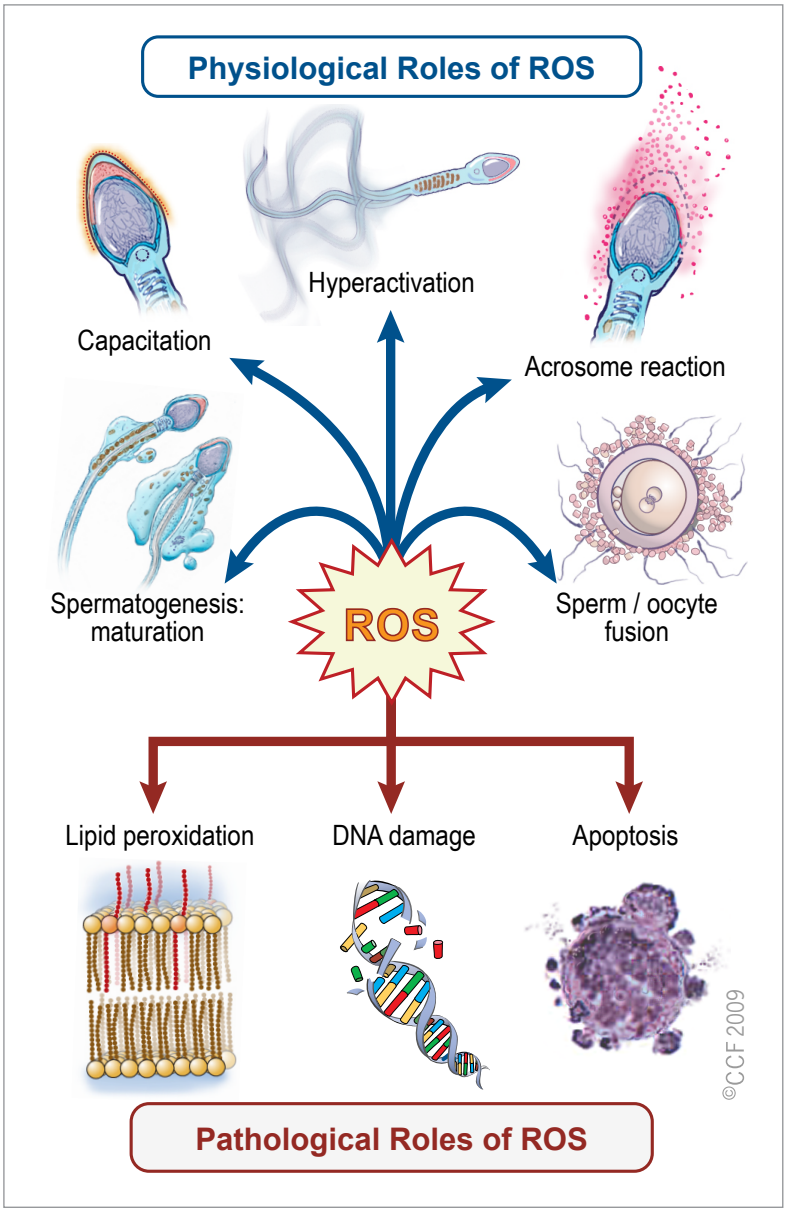

Figure 3: *Physiological and pathological roles of reactive oxygen species*

to render them competent to successfully fertilise the ovum. Controlled ROS production occurs in spermatozoa during the capacitation process, initiating various molecular modifications required for successful fertilisation (refer to Figure 4 for detail signalling pathways) (De Lamirande and O'Flaherty, 2008; Du Plessis, Agarwal, Halabi and Tvrda, 2015; Kothari et al., 2010).

Hyperactivation: Hyperactivation is a specific state of sperm motility when spermatozoa become highly motile and exhibit features of high amplitude, asymmetric flagellar movement, increased side-to-side head displacement and non-linear motility (Suarez, 2008). The role of ROS in the initiation of hyperactivation (see Figure 4) has been well documented in vitro as was shown when spermatozoa were incubated with low concentrations of OH-induced hyperactivation (Makker et al., 2009).

Acrosome reaction: Once the spermatozoon binds to the ZP of the oocyte, it initiates the acrosome reaction marked by an exocytotic release of proteolytic enzymes, creating a pore in the ZP's extracellular matrix (De Lamirande and O'Flaherty, 2008). The molecular events of the acrosome reaction overlap substantially with those of capacitation, including phosphorylation of similar tyrosine proteins, influx of Ca^{2+} and increased cyclic adenosine monophosphate and protein kinase A levels. The role of ROS in the *in vivo* acrosome reaction involves the phosphorylation of three plasma membrane proteins (refer to Figure 5), and this could be mimicked *in vitro* when physiological concentrations of O_2-, H_2O_2 and NO were added to the seminal plasma (Agarwal, Virk et al., 2014).

Sperm-oocyte fusion: For successful fertilisation, the spermatozoa must penetrate the ZP and fuse with the oocyte. High amounts of polyunsaturated fatty acids (PUFAs), particularly docosahexaenoic acid (DHA), play

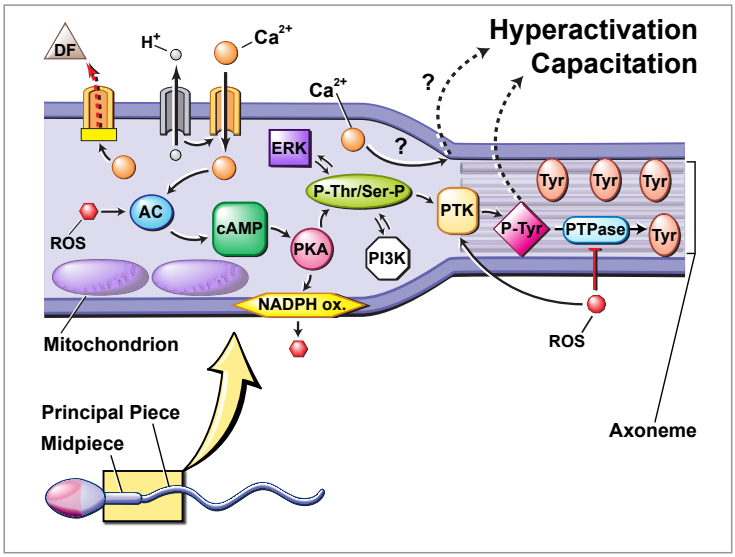

Figure 4: *Biochemical pathway proposed to regulate sperm capacitation and hyperactivation. The process is initiated by an influx of Ca^{2+} and HCO_3-, possibly caused by the inactivation of an ATP-dependent Ca^{2+} regulatory channel (PMCA) and alkalisation of the cytosol. Both Ca^{2+} and ROS, specifically O_2-•, activate adenylate cyclase (AC), which produces cyclic adenosine monophosphate (cAMP). cAMP activates downstream protein kinase A (PKA). PKA triggers a membrane-bound NADPH oxidase to stimulate greater ROS production. In addition, PKA triggers phosphorylation of Ser and Tyr residues that, in addition to other interconnected pathways, lead to the activation of protein tyrosine kinase (PTK). PTK phosphorylates tyrosine residues of the fibrous sheath surrounding the axoneme, the cytoskeletal component of the flagellum. ROS, specifically hydrogen peroxide, increase the amount of tyrosine phosphorylation by promoting PTK activity and inhibiting phosphotyrosine phosphatase (PTPase) activity, which normally dephosphorylates Tyr residues. The enhanced tyrosine phosphorylation observed in capacitation is the last known step in the process, but intermediate steps or other (in)direct methods may be involved (Du Plessis, Agarwal, Halabi and Tvrda, 2015).*

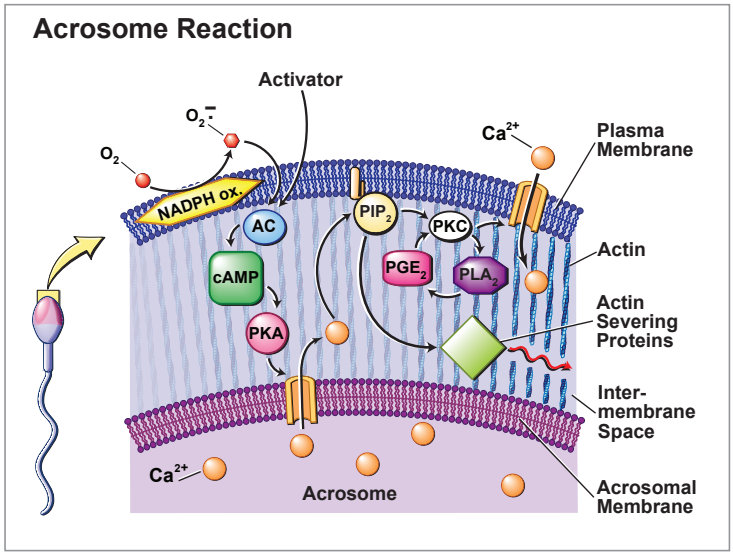

Figure 5: *Biochemical pathway proposed to regulate the acrosome reaction (AR). Induction of the AR can occur by physiological and non-physiological activators, including the zona pellucida (ZP), progesterone or ROS. Subsequent release of Ca^{2+} from the acrosomal calcium store generated during capacitation causes the cleavage of phosphatidylinositol-4,5-biphosphate (PIP2), which forms diacylglycerol (DAG) and inositol triphosphate (IP3). The latter activates actin-severing proteins, which leads to the fusion of the acrosomal and plasma membranes and eventual acrosomal exocytosis. DAG later activates PKC, causing a second, greater influx of Ca^{2+} and activation of PLA2. The release of large amounts of membrane fatty acids increases the fluidity of the plasma membrane necessary for later fusion with the oocyte (Du Plessis, Agarwal, Halabi and Tvrda, 2015).*

a major role in regulating membrane fluidity in sperm. ROS have been shown to increase the membrane fluidity and rates of sperm-oocyte fusion, which occurs during the biochemical cascade of capacitation and acrosome reaction. Throughout capacitation, ROS inhibit protein tyrosine phosphatase activity and prevent dephosphorylation and deactivation of phospholipase A_2 (PLA_2). PLA_2 cleaves the secondary fatty acid from the triglycerol backbone of the membrane phospholipid and increases the membrane's fluidity (Calamera et al., 2003; Khosrowbeygi and Zarghami, 2007).

Pathological roles

Spermatozoa are particularly susceptible to OS as their cell membranes are rich in PUFAs and lack cytoplasmic enzymes and antioxidant defence mechanisms. This makes them more vulnerable to oxygen-induced damage and specifically lipid peroxidation (LPO) at pathological levels of ROS (Tremellen, 2008).

Depending on the nature, amount and duration of the ROS insult, these defects cause significant damage to biomolecules such as lipids, proteins, nucleic acids and sugars (Agarwal, Virk et al., 2014) with a deleterious effect on semen quality, including spermatozoa motility (Aitken and Clarkson, 1987; Khosrowbeygi and Zarghami, 2007), viability, morphology and concentration (Agarwal, Tvrda and Sharma, 2014; Agarwal, Virk et al., 2014).

Lipid peroxidation: Lipids are responsible for the fluidity of membrane layers and the changes that occur during capacitation in the female reproductive tract (Sanocka and Kurpisz, 2004). The plasma membrane of mammalian spermatozoa is markedly different from mammalian somatic cells in terms of its lipid composition. The plasma membrane contains high levels of lipids in the form of PUFAs. These lipids contain unconjugated double bonds separated by methylene groups. The placement of a double bond adjacent to a methylene group weakens the methyl carbon-hydrogen bond, consequently making hydrogen extremely susceptible to abstraction and oxidative damage. When the levels of ROS within the cell are high, ROS will attack PUFAs, causing a cascade of chemical reactions called LPO (Makker et al., 2009). Approximately 50% of the fatty acids in human spermatozoa are composed of DHA with 22-carbon chains and six cis double bonds. DHA is thought to play a major role in regulating spermatogenesis and membrane fluidity (Agarwal, Virk et al., 2014). As the LPO cascade proceeds in the sperm, almost 60% of the fatty acid is lost from the membrane, hence affecting its function by decreasing its fluidity, increasing nonspecific permeability

to ions and inactivating membrane-bound receptors and enzymes. Since LPO is an autocatalytic self-propagating reaction associated with abnormal fertilisation, it is critical to understand the mechanism behind this process, which can be conveniently separated into three main steps, namely initiation, propagation and termination (Tremellen, 2008). Initiation involves the abstraction of hydrogen atoms associated with carbon-carbon double bonds, which results in free radical formation. These free radicals react with fatty acid chains and form lipid radicals, which then react with oxygen to form the peroxyl radicals. These peroxyl radicals, which can abstract hydrogen from lipid molecules, particularly in the presence of metals such as copper and iron, cause an autocatalytic chain reaction. The radicals eventually react with hydrogen to form lipid peroxides. This reaction characterises the propagation stage. These radicals act on additional lipids, forming cytotoxic aldehydes due to hydroperoxide degradation. Peroxyl and alkyl radicals are regenerated in a cyclical fashion in the propagation step until they react with another radical to form a stable end product called malondialdehyde (MDA) during the third step of termination. Thus, MDA is used in biochemical arrays to monitor the degree of peroxidative damage to spermatozoa (Agarwal, Virk et al., 2014; Sikka, 2001; Sanocka and Kurpisz, 2004).

DNA damage: Semen parameters such as concentration, motility and morphology are commonly used to determine the fertilisation potential of sperm from an ejaculate. Although this provides a general overview of the quality of sperm, it does not provide information on one of the most important components of the reproductive outcome, deoxyribonucleic acid (DNA). Single or double-stranded DNA breaks can be a source of differences in reproductive potential between fertile and infertile men (Zribi et al., 2011). It has been reported that chromatin in the sperm nucleus is vulnerable to oxidative damage, leading to base modifications and DNA fragmentation (Zribi et al., 2011). The chromatin of human spermatozoa has a highly condensed and organised structure. This is further packaged into nucleosomes and coiled into a solenoid. During the process of spermiogenesis, sperm chromatin undergoes a series of modifications in which histones are replaced with transition proteins and, subsequently, protamines. DNA strands are condensed by the protamines and form the basic packaging unit of sperm chromatin called toroid. Toroids are further compacted by intra- and intermolecular disulfide cross-links. This DNA compaction and organisation help to protect sperm chromatin from oxidative damage, making them particularly resistant to DNA damage (Schulte et al., 2010). However, in some cases where poor compaction and incomplete protamination of sperm chromatin exist, DNA is more vulnerable to OS and produces base-free sites, deletions, frame-shift mutations, DNA cross-links and chromosomal rearrangements. Damaged DNA has been observed in testicular, epididymal and ejaculated human spermatozoa (Duru et al., 2000). Single- and double-stranded DNA breaks can be detected by using either the TUNEL or the Comet assay. Single-strand breaks are a direct result of oxidative damage on sperm DNA while double-strand breaks may arise from exposure to 4-hydroxy-2-nonenal, a major product of LPO (Badouard et al., 2008). It was discovered that 8-hydroxy-2-deoxyguanosine and two ethenonucleosides (1, N6-ethenoadenosine and 1, N6-ethenoguanosine) are the two major DNA adducts found in human sperm DNA, both of which have been considered key biomarkers of DNA damage caused by OS (Gonzalez-Marin et al., 2012). Despite these findings, DNA damage is not a cause for concern during intrauterine insemination and IVF, because the coexisting LPO damage by ROS eliminates the possibility of fertilisation. However, if normal natural selection is bypassed during intracytoplasmic sperm injection, sperm with significant amounts of DNA damage have the opportunity to fertilise the oocyte (Makker et al., 2009). When DNA is minimally damaged, spermatozoa can undergo self-repair and potentially regain the ability to fertilise the oocyte and proceed with development (Aitken and Koppers, 2011). In fact, the oocyte is also capable of repairing damaged sperm DNA. In cases where the oocyte repair machinery is not sufficient to repair DNA damage, the embryo may fail to develop or implant in the uterus and can be naturally aborted. In other cases, the oocyte may successfully repair sperm DNA-strand breaks before the initiation of the first cleavage division, thereby producing normal offspring. It has been reported that 80% of the structural chromosomal aberrations are of paternal origin in humans (Gonzalez-Marin et al., 2012). DNA damage is a contributory factor to apoptosis, poor fertilisation rate, high frequency of miscarriage and morbidity in offspring (Chen et al., 2013).

Apoptosis: Another theory regarding sperm DNA damage and impaired fertilisation is that of unsuccessful apoptosis. Apoptosis, also known as programmed cell death, is a physiological phenomenon characterised by cellular morphological and biochemical modifications that cause cells to die in a controlled manner (Makker et al., 2009). During early development, apoptosis is

important in the ontogeny of the germ line as a means of regulating the germ cell to Sertoli cell ratio. In adulthood, apoptosis plays a vital role in selectively destroying the premeiotic spermatogonia during the first round of spermatogenesis by preventing the overproduction of germ cells from seminiferous tubules in response to ROS (Tremellen, 2008). During this process, the human ejaculate expresses various apoptotic markers that initiate apoptosis, some of which include Fas, phosphatidylserine (PS), Bcl-Xl and p53. Fas is a type I membrane protein that belongs to the tumour necrosis factor-nerve growth factor receptor family and is secreted by the Sertoli cells located on the germ cell surface. To further support this theory, the same study reported that the percentage of Fas-positive spermatozoa was as high as 50% in men with abnormal sperm parameters (Agarwal et al., 2003). In addition, this apoptotic pathway activates the inner and outer mitochondrial membranes to cause the release of the signalling molecule cytochrome C, which triggers caspases, such as caspases 3 and 9, and annexin-V binding (annexins are calcium-dependent phospholipid-binding proteins, which bind to PS). This pathway eventually leads to sperm apoptosis (Aitken and Baker, 2013). In an earlier study, it was reported that annexin-V staining was used to study the externalisation of PS, a marker for early apoptosis. It was observed that mature spermatozoa from infertile patients with increased ROS levels had significantly higher levels of apoptosis than mature spermatozoa from the control group (Agarwal and Said, 2003).

REACTIVE OXYGEN SPECIES-BASED STUDIES

In vitro laboratory studies

In vitro-based studies exploring the effects of ROS on and interaction with semen and sperm parameters remain important. Not only will such studies help to define the pathological thresholds and establish the specific results/effects of different types of ROS, but they also reiterate the importance of minimising OS introduced during sperm preparation for assisted reproductive technologies (ART).

When we incubated spermatozoa *in vitro* with varying concentrations of 2,3-dimetoxyl-1,4-naphthoquinone (DMNQ), an O_2- generator, we found the percentage motility and progressive motility to be considerably decreased at concentrations equal to and above 25 µmol L-1 DMNQ. (Aboua et al., 2009). We subsequently explored the impact of a different type of ROS, namely H_2O_2, at various concentrations (0, 2.5, 7.5 and 15 µM) on sperm function. Similarly to superoxide, we also observed a marked reduction in total and progressive motility as well as an increase in static cells (Du Plessis, McAllister et al., 2010). An increase in intracellular NO (increased DAF-2DA fluorescence) and ROS (increased DCFH-DA fluorescence) was also detected after H_2O_2 exposure. These results supported the notion that high concentrations of exogenous H_2O_2 not only adversely affected sperm motility parameters but furthermore more than likely damaged the mitochondrial membranes (LPO), leading to leaking of electrons and contributing to elevation in ROS and NO (Du Plessis, McAllister et al., 2010).

Subsequent to this, we set off to measure basal and stimulated (100 µM H_2O_2) intracellular O_2- (dihydroethidium [DHE]) and H_2O_2 (dichlorofluorescin diacetate [DCFH-DA]) levels in neat semen as well as in mature and immature sperm fractions and examined the relationship with viability and apoptosis through flow cytometry. Interestingly, both mature and immature sperm showed reduced intracellular levels of basal O_2- compared with the neat semen. Unstimulated immature spermatozoa showed a significantly higher percentage of DCF fluorescence compared to both neat
($p < 0.001$) and mature ($p = 0.05$) sperm while the mature fraction also displayed higher fluorescence compared to the neat sample ($p < 0.001$). We were the first to report this shift in intracellular H_2O_2 and O_2- levels. We hypothesised that conventional centrifugation might increase the activity of the superoxide dismutase (SOD) enzyme that converts the generated superoxide ion during centrifugation into hydrogen peroxide, which may prove fatal to spermatozoa. This conversion lowers the available intracellular superoxide levels in both immature and mature sperm when compared with the neat unprocessed spermatozoa. However, mature spermatozoa may have higher catalase (CAT) activity/expression when compared with immature spermatozoa, enabling them to scavenge the generated H_2O_2 more effectively.

Stimulation with H_2O_2 caused a significant increase in the percentage of sperm showing DCF (intracellular H_2O_2) and DHE (increase in intracellular O_2-) fluorescence and was associated with an increase in the mean percentage of apoptotic sperm in all three groups (difference not significant) (Figure 6). Interestingly enough, the percentage of apoptotic sperm also positively correlated with DCF fluorescence (intracellular H_2O_2) in neat non-exposed fractions ($r = 0.60$; $p < 0.05$). From the results we concluded that apoptotic changes in sperm were attributed largely to intracellular H_2O_2 levels while dead

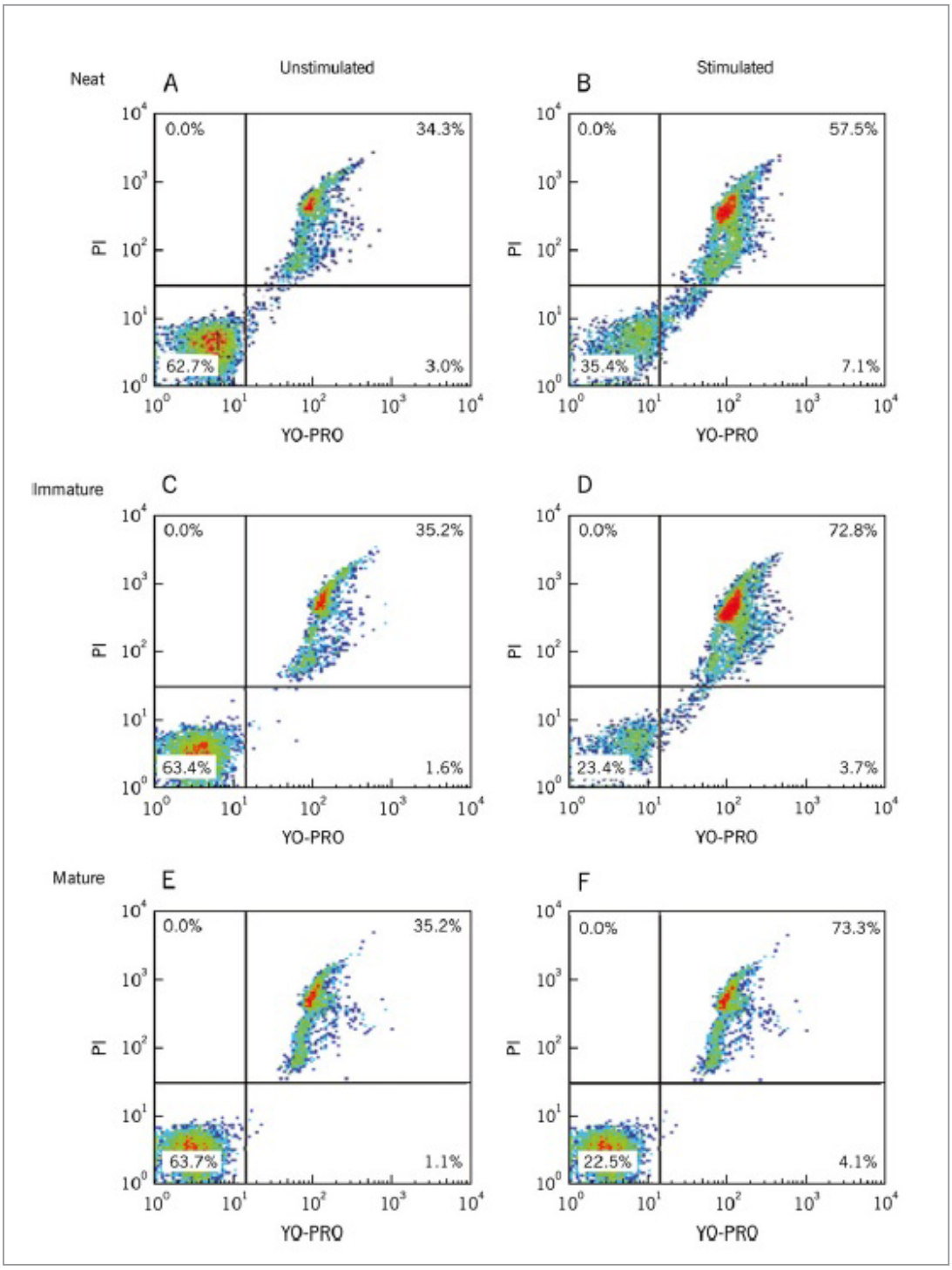

Figure 6: *Representative flow cytometry pseudo-coloured dot plots for unstimulated (left) and stimulated (right) neat (*A, B*), immature (*C, D*) and mature (E, F) sperm fractions. Each quadrant is shown as follows: lower left – viable, nonstained sperm; lower right – apoptotic sperm (Yo-Pro positive only); upper right – dead spermatozoa (positive for Yo-Pro and PI). The numbers in parentheses represent the percentage of sperm population in each quadrant. Only three sperm populations could be identified using Yo-Pro-1/PI for apoptosis (Mahfouz et al., 2010).*

sperm were related to intracellular O_2- levels (Mahfouz et al., 2010).

In a follow-up study, we were able to clearly demonstrate that extended periods of centrifugation increased intracellular ROS and NO levels and simultaneously reduced sperm viability and motility. These effects could be ameliorated by the addition of N-(2-mercaptopropionyl)Glycine (MPG; ROS scavenge) and NG-nitro-L-arginine methyl ester (L-NAME; NOS inhibitor) (Lampiao et al., 2010).

These studies demonstrated the adverse and harmful effects of exogenous and elevated endogenous ROS levels on sperm motility and viability parameters. They furthermore exemplify the importance of minimising ROS introduction during sperm preparation for various ART procedures, especially when spermatozoa are removed from the seminal plasma during washing procedures. Because sperm preparation is necessary for ART procedures to select and maximise superior quality spermatozoa, maintaining sperm integrity is vital to the success of the procedure. We therefore recommend that sperm separation techniques should avoid using centrifugation or prolonged centrifugation in ART. Future studies will indicate whether addition of ROS scavengers prior to centrifugation could improve sperm recovery in ART.

In vivo animal studies

In order to further investigate the *in vivo* effects of supraphysiological systemic levels of ROS on spermatogenesis and the male reproductive system, we established an animal model. Male Wistar rats (n = 54) were randomly stratified into different groups and received daily intraperitoneal injections of either saline, cumene hydroperoxide (cHP, 10 µm) or t-butyl hydroperoxide (tbHP, 20 µm) over a 60-day period. Hydroperoxide exposure significantly decreased the sperm concentration and motility of epididymal spermatozoa. SOD concentration as well as glutathione (GSH) and CAT activities were significantly lower in the epididymal sperm from both hydroperoxide groups compared to controls ($p < 0.05$). At the same time, ROS levels (measured as DCF fluorescence) and LPO (measured as MDA levels) were higher in the sperm from hydroperoxide-treated animals compared to controls ($p < 0.05$) (Aboua et al., 2012). Sperm intracellular ROS correlated inversely with concentration ($r = -0.312$; $p < 0.05$), motility ($r = -0.371$; $p < 0.05$) as well as the antioxidant enzymes CAT ($r = -0.535$; $p < 0.001$). LPO was also positively correlated with increased ROS levels ($p = 0.3799$, $p < 0.01$) (Aboua et al., 2012). We hypothesised that the exogenous hydroperoxides disturbed cell membranes and moved into the spermatozoa, thereby inhibiting the activity of enzymes such as glucose-6-phosphate dehydrogenase. This led to a decrease in NADPH, which in turn reduced the formation of ATP, which is an important metabolite for sperm motility. In the process, these exogenous hydroperoxides caused an imbalance between pro- and antioxidants and led to the development of OS (Aboua et al., 2009) with subsequent onset of LPO and further decline in sperm parameters (Aboua et al., 2012).

In vivo clinical studies

In a study in which we evaluated the conventional semen parameters and functional parameters (i.e. intracellular ROS production, mitochondrial membrane potential, Sperm Chromatin Structure Assay, sperm membrane LPO and antioxidant capacity of seminal plasma) on the semen samples from 40 healthy donors, 23 patients with idiopathic infertility and 34 fertile controls, no significant differences were observed in any of the conventional seminal parameters or functional parameters between the fertile and infertile men apart from ROS. Increased intracellular ROS production (as measured by DCFH-DA and flowcytometry) was observed in the infertile patients (121.2 ± 29.9 MFI) compared to the fertile group (71.7 ± 8.7 MFI; $p < 0.001$) and the healthy controls (94.6 ± 28.5 MFI; $p < 0.05$). Alterations in intracellular ROS production could therefore be associated with male idiopathic infertility, and this functional parameter could eventually distinguish more accurately between semen samples than the conventional parameters (Mayorga-Torres et al., 2016).

In a retrospective clinical study, semen samples from infertile men were analysed for motility and morphology according to seminal leukocyte concentration. The association between leukocytes and sperm quality was found to be concentration dependent. There was a positive association with normal morphology and progressive motility in samples with a concentration of less than 1×10^6/ml leukocytes, but these parameters were significantly reduced in samples presenting with higher leukocyte concentrations (Lackner et al., 2010) Although the study evaluated the effect of leukocyte concentration rather than ROS levels, leukocytes have been described in the literature as the primary source of ROS and thus could be viewed as a surrogate marker for ROS. This clearly shows that the effect of ROS appears to be twofold, and hence any effect on spermatozoa by leukocytes could also be double-edged (Lackner et al., 2010).

In a similar type of clinical study, we stratified 200 semen samples (162 patients undergoing fertility assessment and 38 volunteer donors) according to peroxidase-positive leukocytes. We observed that leukocytospermia (> 1 x 10^6 leukocytes/ml) significantly increased hyperviscosity (9.01 ± 0.49 vs. 7.39 ± 0.23 cP; $p < 0.005$), which could also possibly be attributed to elevated ROS levels (Flint et al., 2014).

In yet another clinical study, we tested semen samples of patients (n = 100) referred to Tygerberg Hospital for male factor fertility assessment and healthy volunteers (n = 20) participating in the donor programme at the Stellenbosch University Reproductive Research Group for the presence of sexually transmitted infections (STIs). The samples were classified as control (n = 65) or positive for Neisseria gonorrhoea (n = 19), *Trichomonas vaginalis* (n = 25) or *Chlamydia trachomatis* (n = 11). When determining ROS levels by measuring the mean percentages of DCFH-DA fluorescence, it was observed that all three STI-positive populations (N. *gonorrhoea:* 95.41 ± 1.64%; T. *vaginalis:* 93.06 ± 4.12%; C. *trachomatis:* 92.80 ± 4.44%) displayed statistically significant higher ROS levels than the control samples (69.50 ± 13.64%; $p < 0.05$). Similarly, DNA fragmentation was also significantly ($p < 0.05$) elevated in the STI groups (N. *gonorrhoea:* 26.99 ± 1.91%; T. *vaginalis:* 26.90 ± 2.13%; C. *trachomatis:* 29.73 ± 1.99%) compared to the control group (11.98 ± 0.76%) (Flint, 2016).

Correlation analysis showed a positive correlation between ROS and DNA fragmentation ($r = 0.494$; $p < 0.05$) and a negative correlation between ROS and total motility ($r = -0.653$; $p < 0.05$) (Flint, 2016). These findings yet again infer that various factors can contribute to pathological/elevated ROS levels in semen, which in turn can impact negatively on sperm basic and functional parameters.

All of our clinical findings correspond well with those of a recent study published by Agarwal and coworkers in which it was shown that infertile men, irrespective of their clinical diagnoses, had reduced semen parameters and elevated ROS levels compared to proven fertile men who had established a pregnancy recently or in the past. Furthermore, ROS were found to be negatively correlated with traditional semen parameters such as concentration, motility and morphology. The cut-off value of ROS in proven donors was determined to be 91.9 RLU/s with a specificity of 68.8% and a sensitivity of 93.8%. Thus, measuring ROS levels in the seminal ejaculates provides clinically relevant information to clinicians (Agarwal, Sharma et al., 2014).

Proteomics studies

Proteins are the dictators of cellular functions, making their study an important aspect of the diagnosis and treatment of different diseases (Gupta et al., 2014). Advances in proteomics, which is the study of the protein profile of a particular cell or tissue, help us to increase our understanding of the structural and functional proteins present in spermatozoa and seminal plasma (Du Plessis et al., 2011). Bioinformatics furthermore helps to connect these identified proteins to their biological significance in various states of disease (Agarwal, Durairajanayagam, Halabi et al., 2014). Proteomics therefore allows for a wider view of investigating the OS response than the conventional biochemical methods.

We therefore embarked on a first-of-a-kind series of proteomic studies to identify differences in sperm and seminal plasma protein expression between ROS-negative (< 20 RLU/s/10^6 sperm) and ROS-positive (> 20 RLU/s/10^6 sperm) semen samples. The median (25th, 75th percentile) values of the two groups were 4 (0, 9) and 2 236 (33, 4 439) RLU/s/10^6 sperm respectively ($p < 0.01$) (Hamada et al., 2013; Sharma, Agarwal, Mohanty, Hamada et al., 2013).

During the first experiment, the isolated spermatozoa were lysed and CyeDye labelling was performed on the extracted proteins prior to 2D-DIGE. Protein spots exhibiting > 1.5-fold statistically significant ($p < 0.05$) difference in intensity between the experimental groups were excised from the preparatory gel and identified by liquid chromatography-tandem mass spectrometry (LC-MS-MS; Finnigan LTQ) (Hamada et al., 2013).

In this first-of-its-kind pilot study, a total of 1 343 protein spots in gel 1 and 1 265 spots in gel 2 were detected by the Decyder software, of which 31 were differentially expressed; of these, 6 spots decreased and 25 spots increased in abundance in the ROS- sample compared with the ROS+ sample. A total of 18 spots were selected for further LC-MS-MS protein sequencing analysis owing to their abundance and position on the gel. The three major proteins that were highly expressed in spermatozoa from ROS+ semen samples included AKAP4, HSP90-β and endoplasmin HSP90-β1. Elevated ROS levels can cause premature capacitation and increased AKAP4 phosphorylation while the latter two proteins typically relate to cellular stressors, such as heat, glucose deprivation and free radical attack (Hamada et al., 2013).

In ROS- sperm, an overabundance of four antioxidant proteins has been identified. These proteins

are lactotransferrin isoform 2, lactotransferrin isoform 1 precursor, peroxiredoxin-1 and Mn-SOD mitochondrial isoform, all of which may exert essential cytoprotective effects against the buildup of ROS levels (Hamada et al., 2013).

Subsequent to this, the same group of samples was subjected to a different proteomics technique as LC-MS/MS was performed after in-solution digestion of proteins. Data were analysed by searching the National Center for Biotechnology Information with MASCOT. A second set of searches was performed with SEQUEST. Furthermore, functional bioinformatics analysis was done using publicly available (gene ontology [GO]) annotations from GO Term Finder and GO Term Mapper, UNIPROT, STRAP and BioGPS and proprietary software packages (Ingenuity Pathway Analysis [IPA]) from IngenuityW Systems [29] and Metacore™ from GeneGo Inc. to identify the differentially affected processes, pathways, interactions and cellular distribution of the proteins in the two study groups. Based on the SEQUEST score, a total of 74 proteins were identified and differential expression was calculated based on the normalised spectral count ratios between the ROS+ and ROS- samples. Of these, 47 were overexpressed (10 > twofold increase) and 27 underexpressed (5 > twofold decrease) in the ROS+ group. The gene ontology annotation comparisons of cellular localisation and biological processes of the differentially expressed proteins in the two samples are provided in figures 7 and 8. Transcriptional regulatory network analysis of the differentially expressed proteins,

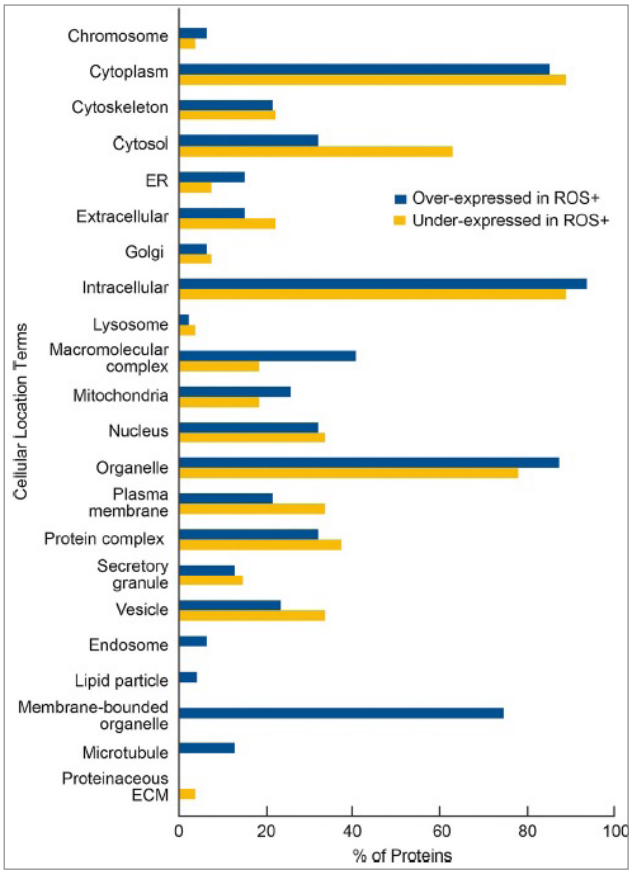

Figure 7: *Cellular distribution showing that the proteins that were significantly overexpressed in the ROS+ group were located in the cytoplasm, intracellular, organelle and membrane-bound organelle and that those underexpressed in the ROS+ group were located in the cytoplasm, cytosol, intracellular and organellar (Sharma, Agarwal, Mohanty, Hamada et al., 2013).*

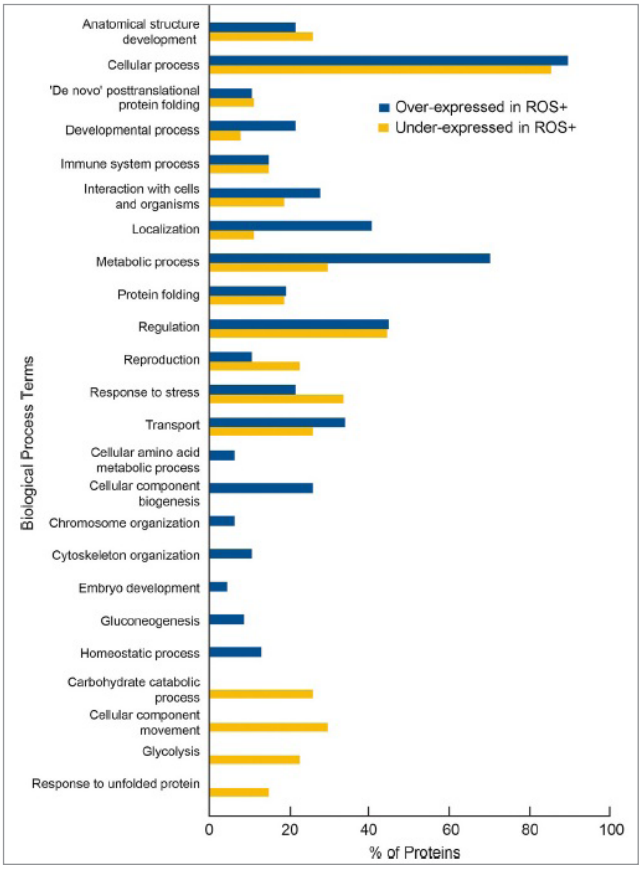

Figure 8: *Biological process distribution of overexpressed proteins consisted of cellular processes, metabolic processes, localisation, regulation and transport in spermatozoa from ROS+ compared to cellular processes, regulation, response to stress, cellular movement and glycolysis in the ROS- group (Sharma, Agarwal, Mohanty, Hamada et al., 2013).*

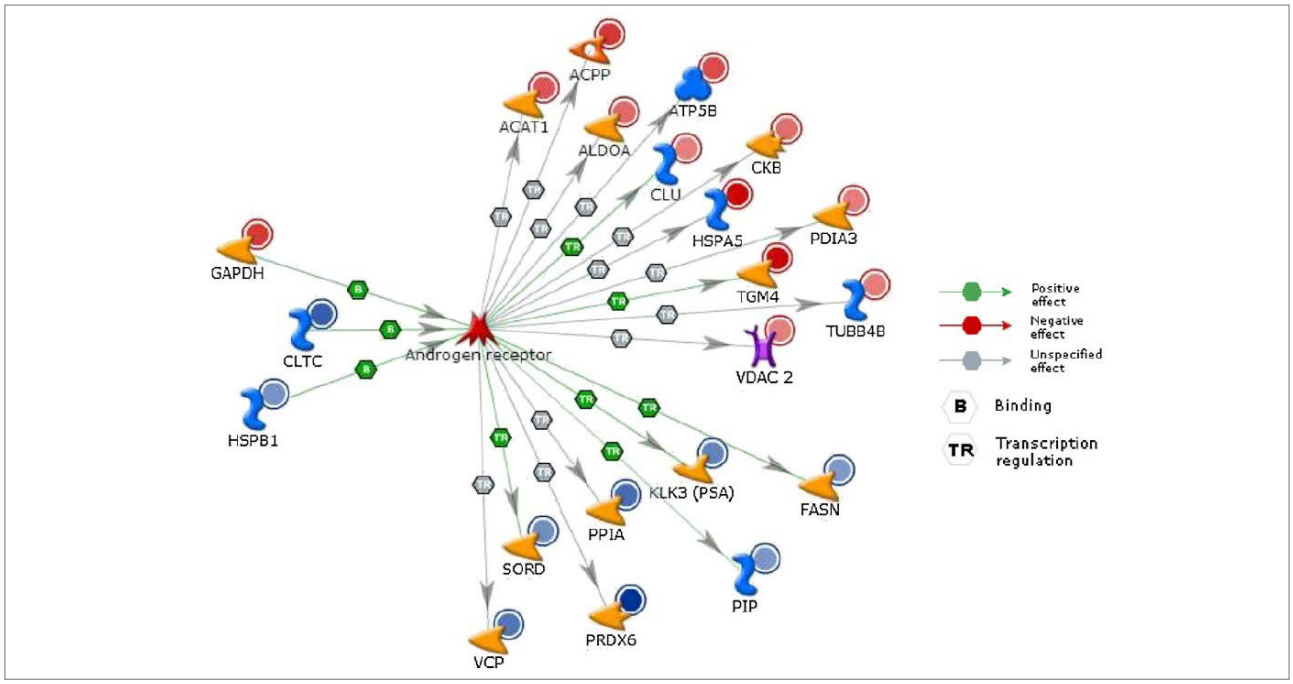

Figure 9: *Transcriptional regulatory network showing interactions between differentially expressed ROS+ proteins and androgen receptor. Proteins with red or blue circles around them are overexpressed (HSPA5 and TGM4) or underexpressed (PRDX6) in spermatozoa from the ROS+ (relative to the ROS-) group. The levels of expression values are reflected in the intensity of red or blue colours. Green arrows with a hexagon indicate a positive effect. TR = transcription regulation; PGK2 = phosphoglycerate kinase 2; GAPD-S = glyceraldehyde phosphate dehydrogenase-S; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; ALDOA = fructose-biphosphate aldolase A; MDH2 = mitochondrial malate dehydrogenase precursor (Sharma, Agarwal, Mohanty, Hamada et al., 2013).*

using Metacore™ , showed that the androgen receptor was one of the topmost regulators with 21 differentially expressed proteins in the ROS+ group interacting with the receptor (Figure 9).

Spermatogenesis and sperm function are dependent on androgen action, and androgens act by stimulating the receptors present on the Sertoli cells and the head area of the spermatozoa (Aquila et al., 2007; Rey et al., 2009). Stanton et al. report that any loss in androgen receptor signalling during meiosis induces changes in the proteins that are associated with various molecular processes such as apoptosis, cell signalling, OS and ribonucleic acid processing and furthermore can also impair sperm function (Aquila et al., 2007; Stanton et al., 2012).

Additionally, various isoforms of histone proteins were also identified, and these included histone cluster 1, H2aa (HIST1H2AA), histone cluster1 H2ae (HISTH1AE) and histone cluster1 and H2ba (HIST1H2BA). Histones are a group of proteins that are replaced by the protamines during sperm maturation in the epididymal region (Luense et al., 2016). Their presence in the ejaculated spermatozoa is indicative of improper packaging of sperm chromatin and subsequent DNA damage. These findings have been attributed to OS (Agarwal, Makker and Sharma, 2008; Luense et al., 2016).

The seminal plasma from each group of ROS- and ROS+ samples was subsequently pooled and subjected to proteomic analysis (Sharma, Agarwal, Mohanty, Du Plessis et al., 2013). In-solution digestion and protein identification with liquid chromatography tandem mass spectrometry (LC-MS/MS) followed by bioinformatics were used to characterise potential biomarker proteins. A total of 14 proteins were identified through MASCOT and SEQUEST. Three of these were unique to the ROS- group (fibronectin 1 isoform 3 preprotein/fibronectin 1 isoform b precursor, macrophage migration inhibitory factor-1 peptide and galectin 3 binding proteins) and four were unique to the ROS+ group (cystatin S precursor, albumin preprotein, lactotransferrin precursor-1 peptide and prostate-specific antigen isoform 4 preprotein). Interestingly, most of the proteins identified as being unique to the ROS+ group represent proteins that are present in their precursor form and are most likely indicative of post-translational problems (Sharma, Agarwal, Mohanty, Du Plessis et al., 2013). Of the seven proteins that were commonly expressed, three were upregulated (prolactin-induced protein, semenogelin II precursor and acid phosphatase prostate short isoform precursor) while four were downregulated (clusterin preprotein, Zinc alpha-2-glycoprotein 1, prostate specific antigen isoform 1 preprotein and semenogelin 1 isoform)

in the ROS+ group. Gene ontology annotations and comparative analysis (using GO annotations) showed extracellular distribution of the bulk of the proteins. These proteins were mostly involved in regulatory processes and the stress response and played a major role in antioxidative and catalytic activity (see Figure 10) (Sharma, Agarwal, Mohanty, Du Plessis et al., 2013).

The 14 proteins were analysed with IPA software to identify the significant pathways and interaction networks. The top network generated was composed of 35 nodes of which only 6 were observed in our dataset (see Figure 11). The common protein that connected 5 out of these 6 proteins was Ubiquitin C, which is involved in conjugation and degradation of proteins impacting major processes and functions. Further process and regulatory networks, pathways and interactions involving the identified proteins are shown in Figure 12 (Sharma, Agarwal, Mohanty, Du Plessis et al., 2013).

The findings of our proteomic studies provide the groundwork for further testing, including the proposition

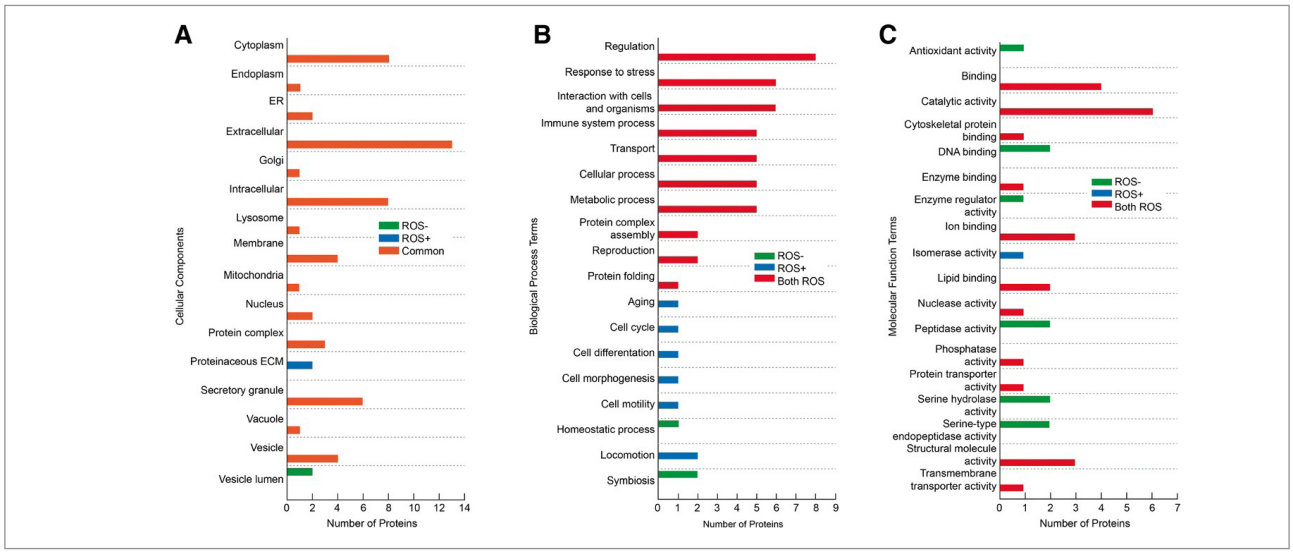

Figure 10: *Comparative analysis was done using GO annotations in three categories either unique to the ROS+ and ROS- groups or common to both. (A) Among the cellular distribution of proteins, the most commonly expressed in the ROS+ and ROS- groups were extracellular in origin; (B) Biological processes of proteins commonly expressed in both the ROS- and ROS+ groups involved in major functions such as regulation, response to stress, cellular and metabolic processes and reproduction; (C) Molecular functions of proteins that were common were involved in catalytic activity (Sharma, Agarwal, Mohanty, Du Plessis et al., 2013).*

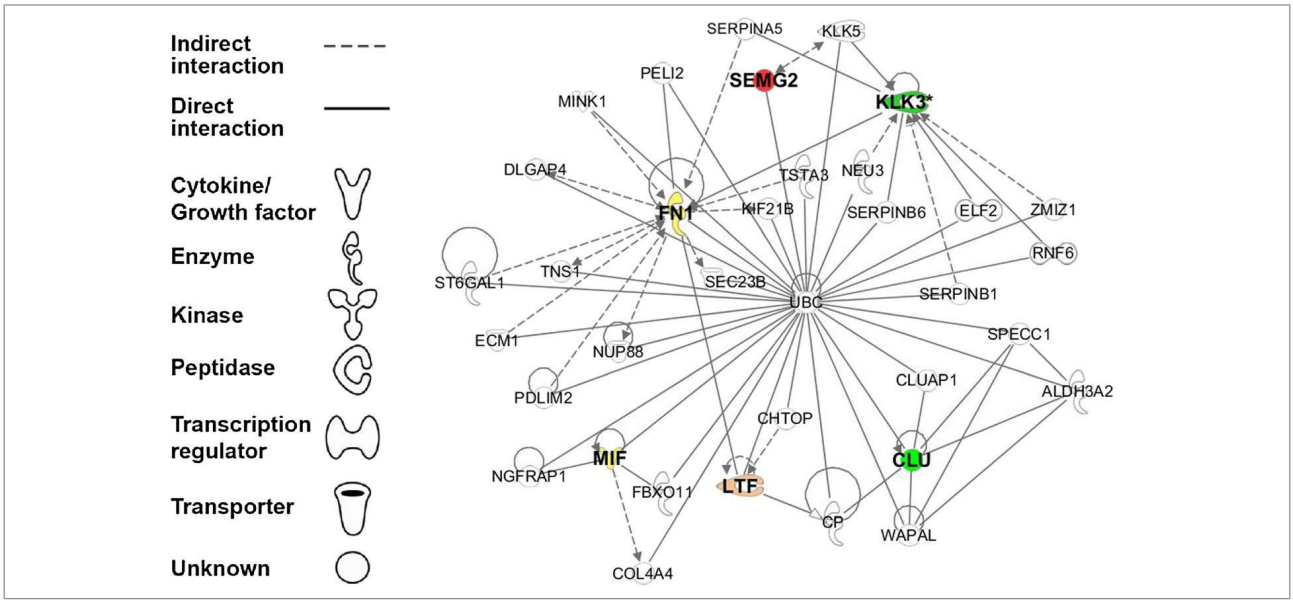

Figure 11: *IPA-generated network for seminal plasma proteins showing six focus molecules highlighted in bold. KLK3 = prostate-specific antigen; SEMG2 = semenogelin-II; FN1 = fibronectin 1 isoform 3 preprotein; LTF = lactoferrin; MIF = macrophage migration inhibitory factor-1 peptide/factor protein; CLU = clusterin (Sharma, Agarwal, Mohanty, Du Plessis et al., 2013).*

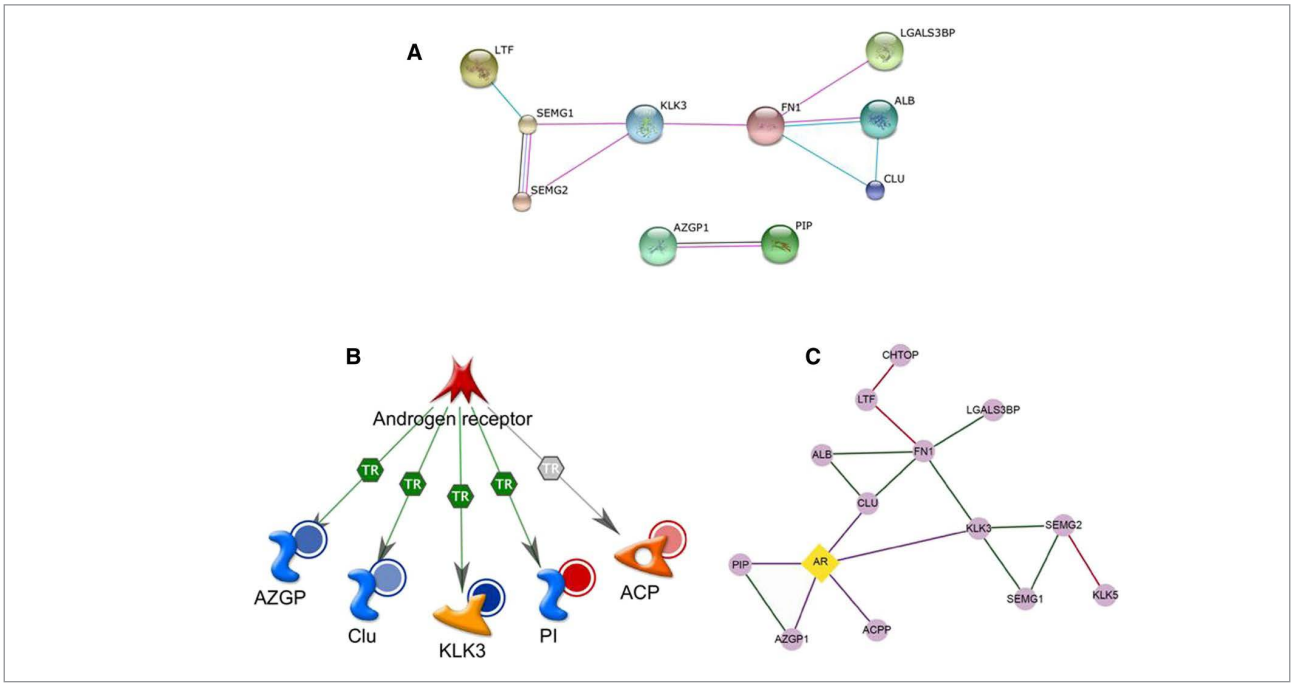

Figure 12: *Known or predicted protein-protein interaction networks for seminal plasma proteins. (A) Eight of 14 protein interactions identified by STRING analyses were based on 'experimental evidence' and/or 'curated pathway databases'; (B) Five proteins regulated by the androgen receptor were identified through MetaCore analysis; red circles = upregulated proteins; blue circles = downregulated proteins – the shading indicates the intensity of the regulation; (C) Consolidated direct interaction network evidence from 3 data sources (IPA, MetaCore and STRING) showing that amongst 12 of 14 proteins, the central players were FN1, KLK3 and AR, all of which are connected to the majority of these proteins (Sharma, Agarwal, Mohanty, Du Plessis et al., 2013).*

that these newly identified sperm proteins have potential clinical implications as they play crucial roles in OS and the pathophysiology of male infertility.

MEASURING OF REACTIVE OXYGEN SPECIES

Currently, a combination of independent assays, both direct and indirect, is used to measure ROS and OS (Sharma and Agarwal, 1996).

The most widely recognised method for measurement of seminal ROS is the chemiluminescence assay. Luminol (5-amino-2, 3, dihydro 1, 4, phthalazinedione) or lucigen can be used for quantification of redox activities of spermatozoa (Agarwal, Cocuzza et al., 2008). Lucigen measures only extracellular superoxide radicals while luminol is used to measure extracellular and intracellular levels of ROS, and therefore the latter is commonly used in the clinical setting.

By employing this technique, various research groups focused on establishing a reference value for seminal ROS in order to help predict fertility outcomes (Du Plessis, Agarwal, Halabi and Tvrda, 2015). Das et al. determined a cutoff value of 0.075 × 10^6 counts per minute (cpm) per million spermatozoa, above which ROS cause a significant drop in fertilisation and pregnancy outcomes (Das et al., 2008). However, Desai et al. concluded that a value of 0.0185 × 10^6 cpm/million spermatozoa was needed to distinguish between fertile and infertile men (Desai et al., 2009). Subsequently, the same group devised a new method of reporting ROS levels in semen. The reference value was initially set at < 20 RLU/s/10^6 spermatozoa (Kashou et al., 2013) and was recently revised to 102.2 RLU/s/10^6 spermatozoa (Agarwal et al., 2015).

Flow cytometry as well as fluorescent microscopy can also be used to detect ROS and RNS. By using different probes, specific types of ROS can be identified, for example DCFH-DA for H_2O_2, DHE for O_2- and DAF-2DA for NO (Henkel et al., 2005; Lampiao et al., 2006a; Lampiao et al., 2006b; Mahfouz, Sharma, Lackner et al., 2009).

A light microscope is required for the nitroblue tetrazolium assay, and it allows differentiation of spermatic and leukocytic ROS. Nitroblue tetrazolium interacts with superoxide radicals by changing to diformazan, a blue pigment that correlates with the concentration of intracellular ROS (Agarwal, Cocuzza et

al., 2008; Tafuri et al., 2015).

As OS results from an imbalance between ROS production and the intracellular and extracellular antioxidants that scavenge ROS, it is also useful to measure this biological event directly or indirectly via markers of the resultant effects. Assays used include measuring of MDA, one of the final products of sperm cell membrane LPO (Shang et al., 2004), as well as quantification of sperm DNA damage (Loft et al., 2003; Sharma, Masaki and Agarwal, 2013).

Another useful marker of OS is to calculate a composite ROS-TAC score by combining the ROS in semen (chemiluminescence assay) and total antioxidant capacity (TAC) in seminal plasma (colorimetric assay) (Agarwal et al., 2015; Mahfouz, Sharma, Sharma et al., 2009;Sharma et al., 1999).

Although useful, these traditional methods of measuring seminal OS are time-sensitive and time-consuming, making them difficult to be used for routine diagnostic purposes. Oxidation reduction potential (ORP) is a direct measurement of OS or redox imbalance in biological samples, and recently we showed that the MiOXSYS System can reliably measure ORP levels in semen and seminal plasma. ORP levels are furthermore not affected by semen age, making this new technology easy to employ in a clinical setting (Agarwal et al., 2016).

MANAGEMENT AND TREATMENT OF OXIDATIVE STRESS

As mentioned earlier, there are innate mechanisms in place to prevent OS from occurring in healthy men. However, in instances where these natural defences fail to maintain the fine balance between ROS and antioxidants, measures must be taken to prevent or alleviate OS; these include lifestyle changes, surgery and antioxidant supplementation. In the management of OS, the first step to take is to ascertain the underlying cause of the imbalance and treat it (Agarwal et al., 2004). For instance, infections can be treated with antibiotics and anti-inflammatory medication while varicocele can be corrected by surgery (Tremellen, 2008). Detrimental behaviour leading to increased ROS production should also be addressed through lifestyle changes. These can include the following of a balanced diet, regular exercise, losing weight, cessation of substance abuse (smoking, drugs and alcohol) as well as minimising exposure to pollution, toxins and heavy metals. Furthermore, it is also vital to prevent activities that may cause elevation of scrotal temperature (e.g. hot baths, extended periods of driving or sedentary office hours).

Thereafter, antioxidant treatment (both enzymatic and non-enzymatic) may be given to supplement the natural antioxidants and increase the ability of the seminal plasma to combat OS (Agarwal et al., 2004; Agarwal, Durairajanayagam and Du Plessis, 2014). However, despite the lack of clinical consensus on the efficacy of antioxidants as a potential therapy, if used, antioxidants need to keep free radicals at levels that are physiologically appropriate.

Antioxidants are able to neutralise pro-oxidants either by preventing their formation via termination of propagative oxidative chain reactions or by scavenging existing ROS, thereby maintaining the delicate pro-oxidant/antioxidant balance and consequently protecting the cell and its microenvironment from oxidative damage (Lampiao, 2012). Enzymatic antioxidants include SOD, CAT and the GSH family of enzymes (GSH reductase and peroxidase). Non-enzymatic antioxidants include vitamins and vitamin-like substances, for example vitamin C (ascorbate), vitamin B (folic acid), vitamin E (α-tocopherol), carnitine, carotenoids, cysteines, pentoxifylline, metals, taurine, albumin and coenzyme Q10. For a more detailed explanation of the various antioxidants and their effects on sperm function, please refer to the following comprehensive reviews of Agarwal (Agarwal, Durairajanayagam and Du Plessis, 2014; Agarwal, Virk et al., 2014) and Tremellen (2012).

The remainder of this section will briefly allude to a few antioxidant studies performed in our laboratory.

We set off exploring the ameliorating effects of an atypical non-enzymatic substance, namely melatonin, on ROS and RNS as well as sperm parameters. Melatonin, a hormone secreted by the pineal gland and responsible for controlling the circadian rhythm, has been reported to have endogenous antioxidant properties (Du Plessis, Cabler et al., 2010; Lampiao and Du Plessis, 2013). In this first-of-a-kind study related to spermatozoa, *in vitro* melatonin-treated samples (n = 12) showed a significantly higher percentage of motile, progressive motile and rapid cells while melatonin simultaneously reduced the number of nonviable spermatozoa when compared with the control. It can be concluded that melatonin was able to directly or indirectly scavenge NO, as indicated by the reduction in DAF-2DA (Du Plessis, Hagenaar and Lampiao, 2010).

In light of the movement to exploit our indigenous knowledge systems, we also investigated the effect of various plant extracts as possible antioxidant therapies. Supplementing the diets of rats injected for a 60-day period with hydroperoxides with red palm oil

successfully attenuated the DCF (ROS) and MDA (LPO) levels while it ameliorated the levels of antioxidants (SOD, CAT and GSH) in epididymal sperm. Crude red palm oil is known to be the richest natural plant source of carotenoids in terms of provitamin A equivalents, such as α-carotene and β-carotene (Sundram et al., 2003). Via these antioxidant properties, it was thus able to successfully attenuate the OS-induced sperm damage due to the organic hydroperoxides (Aboua et al., 2012; Aboua et al., 2009). Similarly, we were able to show that both fermented rooibos (*Aspalathus linearis*) and green tea (*Camellia sinensis*) supplementation, to the same animal model as described previously, were effective in the protection of testicular tissue against oxidative damage. This could be ascribed to the antioxidant properties (high polyphenol/flavonoid content), thereby possibly increasing the antioxidant defence mechanisms in rats while reducing LPO (Awoniyi et al., 2011).

Interestingly enough, a Cochrane review aimed at evaluating the effectiveness and safety of oral supplementation with antioxidants for subfertile male partners in couples seeking fertility assistance only yielded low-quality evidence for four small randomised controlled trials suggesting improvement in live birth rates (Showell et al., 2014). While the results of studies using certain antioxidant agents are promising, the current body of evidence as a whole suggests the need for further well-designed and larger scale randomised placebo-controlled trials in order to shed more light on antioxidants as a clinical treatment option for pathological levels of ROS and OS.

CONCLUSION

An important perspective is that free radicals are not exclusively beneficial or exclusively detrimental to sperm function and male fertility. Rather, they need to be maintained at appropriate levels to ensure physiological function while preventing pathological damage. It is evident that high levels of seminal ROS may be a causative factor of male infertility and could explain a large proportion of unexplained male infertility cases. It is suggested that an accurate assessment of the seminal ROS levels become an integral part of the andrology workup of patients with unexplained and idiopathic infertility in order to assist clinicians in elucidating the underlying reasons, thereby providing an optimal treatment regime for these patients.

A subject of current interest is the mechanisms responsible for removing and regulating ROS as there are still inconsistencies in clinical outcomes in terms of the effect of therapies aimed at reducing seminal ROS.

ROS and ROS-regulated pathways are actively involved in modification of diverse cellular processes involved in reproduction, from hormonal signalling and spermatogenesis through to sperm and metabolism and functional processes such as the acrosome reaction and fertilisation.

Future progress in the field needs identification of the most crucial cellular targets for ROS action as well as discovery of the underlying mechanisms and consequences of the interaction between ROS and cellular components.

Therefore, much remains to be learned about the effects of ROS on biological systems, the adaptive strategies that overcome ROS attack and the natural involvement of ROS in the signalling and regulation of male fertility.

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