IN VITRO EFFECTS OF AQUEOUS LEAF EXTRACT OF *MORINGA OLEIFERA* ON HUMAN SPERM

by

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DISSERTATION

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DEDICATION

I dedicate this work to my family and Mr Jim Smurthwaite for their inestimable sacrifices and support.

DECLARATION

I, Faith Tebatso Moichela, declare that the dissertation titled: **"***In vitro* effects of aqueous leaf extracts of *Moringa oleifera* on human sperm**"** is my own work and that all the sources I have used or quoted have been duly indicated and acknowledged by means of complete references. Furthermore, I declare that this work has not been submitted before for any degree at any other institution.

Faith Tebatso Moichela — 16.02.2021

Full names — **Date**

Signature

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ABSTRACT

Infertility affects nearly 186 million couples globally, with male factors contributing to half of the cases. Oxidative stress is an established cause of declining semen quality. *Moringa oleifera* has proven antioxidants. This study aimed to investigate *in vitro* effects of aqueous leaf extract of *M. oleifera* on human sperm functions. Semen samples from donors (n = 40) and patients (n = 30) were washed with HTF-bovine serum albumin (BSA), and then incubated with various concentrations of *M. oleifera* (0, 0.625, 6.25, 62.5, and 625 µg/ml) at 37°C for 1 hour. Sperm motility, vitality, mitochondrial membrane potential (MMP), reactive oxygen species (ROS), DNA fragmentation, capacitation, and acrosome reaction were assessed. Sperm motility, vitality, MMP, and capacitation were enhanced, while ROS production, and DNA fragmentation decreased after *M. oleifera* treatment. Uncapacitated spermatozoa increased significantly with a reduction in acrosome reaction in donors. *M. oleifera* antioxidant compounds suppressed excessive ROS, preserved mitochondrial membrane, DNA and acrosome integrity, while enhancing sperm motility and viability.

KEY CONCEPTS

Sperm function

Oxidative stress

Infertility

Antioxidants

Moringa oleifera

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RESEARCH OUTPUTS

Parts of the results were presented and submitted for publication as follows:

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LIST OF ABBREVIATIONS

ABP	Androgen binding protein
ALH	Amplitude of lateral head displacement
AMH	Anti-Műllerian hormone
ANOVA	Analysis of variance
ART	Assisted reproductive technologies
AR	Acrosome reaction
ASRM	American Society for Reproductive Medicine
ATP	Adenosine Triphosphate
BCF	Beat cross frequency
BMREC	Biomedical Research Ethics Committee
BTB	Blood testes barrier
BSA	Bovine serum albumin
Ca^{2+}	Calcium
cAMP	cyclic Adenosine monophosphate
CASA	Computer-aided sperm analysis
CAT	Catalase
CP	Capacitation
CTC	Chlortetracycline
DHE	Dihydroethidium
DNA	Deoxyribonucleic acid
DPX	Dibutylphthalate polystyrene xylene
E and N	Eosin and nigrosine
ETC	Electron transport chain
ERK	Extracellular signal kinase
FSH	Follicle stimulating hormone
GABA	Gamma amino butyric acid
GIFT	Gamete intrafallopian transfer
GnRH	Gonadotropin-releasing hormone

GnIH	Gonadotropin inhibiting hormone
GSH	Glutathione
GPx	Glutathione peroxidase
hCG	human Chorionic gonadotropin
HTF	Human tubular fluid
Hyp	Hyper-activation
HPT	Hypothalamic pituitary testicular axis
ICMART	International Committee for Monitoring Assisted Reproductive Techniques
ICSI	Intracytoplasmic sperm injection
IFV	*In vitro* fertilisation
LH	Luteinising hormone
LIN	Linearity index
MDA	Malondialdehyde
MMP	Mitochondrial membrane potential
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
ODF	Outer dense fibres
OSPHOX	Oxidative phosphorylation
OS	Oxidative stress
PBS	Phosphate buffer saline
PKA	Protein kinase A
PKC	Protein kinase C
PMN	Polymorphonuclear
PTK	Phosphorylation of tyrosine kinase
PUFA	Polyunsaturated fatty acids
PVP40	Polyvinylpyrrolidone
RM	Repeated measure
ROS	Reactive oxygen species
SCs	Sertoli cells
SCA	Sperm class analyser

SCD	Sperm chromatin dispersion
SCSA	Sperm chromatin structure assay
SOD	Superoxide dismutase
SSC	Spermatogonial stem cell
STI/(D)	Sexually transmitted infection (disease)
STR	Straightness index
TEM	Transmission electron microscopy
TCM	Traditional Chinese Medicine
TGF-β	Tumour growth factor-beta
TM	Traditional medicine
TNF-α	Tumour necrosis factor-alpha
TREC	Turfloop Research Ethics
TUNEL	Terminal deoxynucleotidyl transferase-mediated nick ending labelling
VAP	Average path velocity
VCL	Curvilinear velocity
VSL	Straight-line velocity
WOB	Wobble/oscillation index
WHO	World Health Organisation
ZP	Zona pellucida

CHAPTER 1

INTRODUCTION

1.1. Background

Infertility and population growth are increasing parad oxical burdens of reproduction and the health care systems. Infertility may not receive as much consideration and remedial intervention as its counterpart, overpopulation, but it has and continues to devastate those suffering from it (Ombelet and Goossens, 2017). It is defined as the disease of the reproductive system, and characterised by failure of a non-contracepting, sexually active couple to establish a pregnancy or carry to term after one year of regular coitus in the absence of any known reproductive pathology (Zegers-Hochschild et al., 2009; Chimbatata and Malimba, 2016). This disease affects one in eight couples who attempt to conceive for the first time, and one in six couples with preceding pregnancies, despite the outcome (Jungwirth et al., 2015). It is also clear that infertility and its complications can no longer be confined to biological function, but presents emotional, psychological, social, and financial distress (Wu et al., 2013).

Although the absolute prevalence of infertility is impossible to estimate, the World Health Organisation (WHO) in 2010 asserted that about 48.5 million (from 42 million in 1992) couples were struggling to fall pregnant (Nagórska et al., 2019). Subsequent estimates in 2017 revealed a steady but increasing trend towards the burden of infertility affecting 186 million couples worldwide (Inhorn and Patrizio, 2015). However, the known prevalence and incidence rates have likely gone up, especially in developing countries where the incidence of genito-urinary tract infections have increased (Chigbu et al., 2012; Solomon and Henkel, 2017). Pelvic inflammatory diseases (Rametse et al., 2018) and metabolic diseases have progressively contributed to infertility (Leisegang et al., 2014). Environmental chemical exposure owing to industrialisation and technological advancements have been indicated to augment the pathogenesis of infertility (Kim, 2011; Nazıroğlu et al., 2013). As a result, there is an overall decline in gamete production and quality in both sexes (Buckett and Tan, 2005; Silva et al., 2019).

At the same time, high rates of infertility have also been reported for developed countries in Europe (Coale and Watkins, 2017), Asia (Sharma et al., 2009), the Middle East (Eldib and Tashani, 2018), and America (Louis et al., 2013), despite assisted reproductive technologies, facilities, specialists, and other contemporary therapies for infertility (Mascarenhas et al., 2012) being available. Africa, on the other hand, is enduring the burden of declining fertility with concomitantly high fertility rates (Ombelet and Onofre, 2019). Reportedly, the prevalence of infertility in Africa was amongst the highest in the world, with about 32% incidence in 2009 (Sharma et al., 2009). This phenomenon came to represent what was termed the “infertility belt”, stretching across from the western, to the central, and the eastern part of the continent (Larsen, 2003; Okonofua and Obi, 2009).

Sub-Saharan Africa region is no exception to high infertility levels and subsequently decreased fertility in both male and females, primarily due to the disproportionately high burden of sexually transmitted diseases (STDs) and lack of access to well-equipped fertility centres (Fledderjohann, 2012; Apari et al., 2014). Previous studies in Nigeria and Uganda revealed that 21.7% and 66% of couples, respectively, could not establish or produce a live birth following their several attempts (Mugisha et al., 2013; Osaikhuomwomwan and Osenmwenka, 2015). The incidence of infertility in South Africa was 15-20% in 2014 (Pedro and Andipatin, 2014).

Despite presumed as innately fertile, especially in Africa (Masuku, 2005), males are accountable for almost half of the global cases of infertility (about 40-50%) (Campagne, 2013; Kumar and Singh, 2015). The remaining 20-30% are shared between men and women, as well as idiopathic causes (Agarwal et al., 2015). Male infertility is multifactorial, with causes classified as non-obstructive testicular spermatogenic failure (Tiseo et al., 2015). Obstructive infertility involves blocked ducts, resulting in a defective transit of testicular secretions and spermatozoa (Cocuzza et al., 2013) into the female reproductive tract. The last category is coital infertility, which is less common, but contributes significantly to the cases of azoospermia (Raheem and Ralph, 2011). Coital infertility occurs in patients whose ductal systems and sperm production are standard, but the difficulty hinges on erectile and ejaculatory function (Gudeloglu and Parekattil, 2013).

In the majority of childless cases where the female factor is ruled out, the male diagnosis starts with basic semen analysis, which gives an overview of the fertilising potential of a man (Andrade-Rocha, 2003). Semen analysis is useful in detecting subtle abnormalities in sperm morphology, motility, and counts (Agarwal and Sharma, 2007), which are negatively associated with successful fertilisation (naturally or using assisted reproductive techniques) (Rogers et al., 1983; Franken and Henkel, 2012), and embryonic development (Shi et al., 2016). Because of the limited predictive value of semen analysis, its sensitivity towards detection of sperm with functional defects creates an opportunity for exploration of advanced sperm functional testing (Aitken, 2006; Franken and Oehninger, 2012).

Comprehensive assays give insight into every segment of sperm that underlie functional fertility (Talwar and Hayatnagarkar, 2015). Therefore, the biological mechanisms governing reproductive processes ensure that morphologically mature and genetically uncompromised is selected for fertilisation in natural conception, but also in medically assisted techniques. Oxidative stress plays a clinical role in differentiating an infertile man from a fertile man in unexplained causes (Agarwal et al., 2006). The diverse aetiology of ROS-induced male infertility significantly impedes the prognostic efficacy of conventional therapies. These reasons further increase the search for holistic and more affordable alternatives such as herbal antioxidants.

Evidence can be found in complementary and alternative methods such as Chinese traditional medicine (Zhou et al., 2019), acupuncture (Pei et al., 2005), Ayurveda (Doddamani et al., 2019) and African medicine (Mahomoodally, 2013) to remedy various aspects of male infertility (Kotta et al., 2013). Alternative and supplementary therapies have been discussed in length in conjunction with male reproductive dysfunctions (Rama Devi et al., 2004; Mahomoodally, 2013). Herbal therapies, an alternative to Western allopathic medicine, have been proven to be the most widely used, commercialised, and investigated forms of fertility enhancers in males (Lampiao et al., 2008; Mohdmmad et al., 2015).

Moringa oleifera of the monogeneric family *Moringaceae* is one the most valuable plants of the 21st century (Sujatha and Patel, 2017). *M. oleifera* is an indigenous Himalayan plant that was naturalised in tropical and subtropical parts of India, Pakistan, Afghanistan, and Bangladesh (Farooq et al., 2012; Abd-Rabou et al., 2017).

M .oleifera has proven relevance in applied fields of nutrition (Yang et al*.,* 2006), the economy (Ajayi et al., 2013), and most importantly, in medicine (Coppin, 2008). *M. oleifera* leaves are endowed with various antioxidant compounds, minerals and vitamins, phenolics, and other phytochemicals with potent free-radical scavenging activity (Melo et al., 2013; Abd Rani, Husain, and Kumolosasi, 2018).

M. oleifera has anti-inflammatory (Mittal et al., 2017), anti-clastogenic (Promkum et al., 2010), antibiotic (Maurya and Singh, 2014), antidiabetic, anti-carcinogenic (Abd-Rabou et al*.,* 2017), hypotensive (Acuram and Chichioco Hernandez, 2019; Goothy and Sudhan, 2019), hypolipidemic (Sugunabai et al., 2014), and antioxidative (Ashok Kumar and Pari, 2003) properties. All these pharmacological properties have led to the commonly accepted notion that *M. oleifera* is the panacea of alternative medicine (Koul and Chase, 2015).

Several studies have been dedicated to the understanding of how *M. oleifera* affects male reproductive functions (Cajuday and Pcsidio, 2010; Afolabi et al., 2013). Aphrodisiac properties of aqueous seed extract of *M. oleifera* was investigated in male albino rats, and results revealed an improvement in sexual performance (Pare and Zade, 2010). Serum follicle stimulating hormone (FSH) and cholesterol increased after *M. oleifera* administration in male Wistar rats (Nwamarah et al., 2015). Androgenic and antiperoxidative effects of *M. oleifera* were proven in male Wistar rats through an increase in serum testosterone and testicular malondialdehyde (MDA) in cadmium-chloride exposed rats (Chatterjee et al., 2017). Harmful effects of electromagnetic radiation on rat testes were reversed by *M. oleifera* (Bin-Meferij and El-kott, 2015). In the same study, sperm count, and morphology improved significantly after treatment with *M. oleifera*. Activities of antioxidant enzymes catalase (CAT) and superoxide dismutase (SOD) increased in *M. oleifera* treatment groups compared to controls. Lastly, histological architecture, spermatogenic maturation and proliferation improved after exposure to *M. oleifera* (Bin-Meferij and El-kott, 2015).

To further highlight the dose- and time-dependency effect of the *M. oleifera* extract on mammalian spermatogenic function, semen of Friesian bulls was extended with crude *M. oleifera* extracts. Afterwards, the percentage of sperm progressive motility, morphology, and plasma membrane increased significantly at low doses (Sokunbi et al., 2015). However, cytotoxic effects were observed with longer periods as evidenced

by a significant decrease in the percentage viability of the bull sperm (Sokunbi et al., 2015). In the same study, moderate doses and durations retained acrosome integrity when compared to untreated groups. According to the best my knowledge, no studies exist to support the ethno-medicinal use of *M. oleifera* for the treatment of male reproductive and sexual dysfunction in humans. This study reports for the first time, the direct effects of aqueous leaf extracts of *M. oleifera* leaf on human sperm functionality.

1.2. Research problem

Having a child is a responsibility that is culturally engraved in the societal norms of many communities. The presence of children not only ensures perpetual preservation and progression of clans or tribes, but affords parents a sense of fulfilment, belonging, and happiness. The inability to fulfil these biological and cultural expectations brings with it feelings of inadequacies and failure. Despite the sustained belief in other societies that women are solely accountable for barrenness, it is firmly established that the causes of infertility are distributed equally amongst both genders.

Demographic reports on deteriorating semen profiles across the globe are substantive of the enormous contribution males have in infertility. By acknowledging the decline in semen quality and quantity, it became imperative to study spermatogenesis, sperm functions, and sperm factors that culminate into infertility. Understanding sperm factors contributing to infertility led to the development of primary and state-of-the-art systems of evaluating infertility.

Assisted reproductive techniques are amongst the advanced methods of evaluating and managing male infertility. These methods allow conception to take place outside the woman’s body. While the successes of assisted reproductive techniques (ARTs) are embraced as medical breakthroughs in modern reproductive medicine, they do not cater for most infertile couples. Availability, affordability, accessibility, and success rates of these modern-day interventions in third world countries are still insufficient. For example, only a small proportion of sub-Saharan countries practices ARTs, and less than 10% of South Africans have access to ARTs. Western therapies such as ARTs are confined to the middle- and upper-income classes, and people residing in the metropolitan / suburban areas with adequate health care centres.

On the other hand, traditional herbal medicines are ubiquitously distributed in all areas; they are affordable, holistic, and reportedly involve little to no adverse effects. *M. oleifera* is an excellent source of phytochemical compounds offering a wide range of pharmacological properties. Most of the medicinally active fractions are found in the leaves; and they have demonstrated potent antioxidative activity *in vivo* and *in vitro* in animal reproduction. Additionally, the few studies on genotoxic effects of *M. oleifera* were mainly confined to basic sperm parameters and yielded contradicting results. Study designs are needed to rationalise and standardise the safe use of *M. oleifera* leaves for treating reproductive and sexual challenges in humans. Accordingly, as far as we are aware, no study to date investigated the effects of *M. oleifera* on the functional parameters of human spermatozoa *in vitro*. Therefore, it was against this background that this study was conducted.

1.3. Purpose of the study

1.3.1 Aim

The aim of the study was to evaluate the *in vitro* effects of aqueous leaf extracts of *M. oleifera* on human sperm parameters.

1.3.2 Objectives

The objectives of the study were to:

1. Determine the effects of aqueous *M. oleifera* leaf extracts on sperm motility;
2. Determine the effects of aqueous *M. oleifera* leaf extracts on sperm vitality;
3. Assess sperm mitochondrial membrane potential after treatment with aqueous *M. oleifera* leaf extracts;
4. Measure the effect of aqueous *M. oleifera* leaf extracts on sperm (reactive oxygen species) ROS production;
5. Determine the effects of aqueous *M. oleifera* leaf extracts on sperm DNA fragmentation;
6. Assess capacitation and acrosome reaction after treatment with aqueous *M. oleifera* leaf extracts;
7. Assess the effects of aqueous *M. oleifera* leaf extracts on asthenozoospermic sperm parameters.
8. Compare the effects of aqueous *M. oleifera* leaf extracts on fertile and infertile human sperm.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of the basic functioning of the male reproductive system

The reproductive system functions to ensure the perpetual existence of a species. The system requires the interconnection of organs, glands, endocrine and paracrine secretions to efficiently carry out this function (Cooke et al., 1991; Foley, 2016). Males contribute a genetically intact spermatozoon, while females produce meiotically-competent oocyte, and additionally provide a site for fertilisation (Bodnar, 1961; Desai et al., 2017). Sperm cells are produced in the seminiferous tubules of the testis, and are released into the epididymis for storage and maturation (Schoysman and Bedford, 1986; Bergmann, 2006). Afterwards, sperm cells are suspended in seminal secretions that serve as the medium and vehicle from three accessory glands: prostate, Cowper's (bulbourethral) and vesicular (Mann and Lutwak-Mann, 1951; Flint et al., 2015). From the testis, spermatozoa are emptied into the vas deferens, which transports them to the ejaculatory duct. Semen (seminal fluid and spermatozoa) is then expelled into the outside through the urethra of the penis during sexual intercourse into the vagina (van der Horst et al., 1999a; Lohiya et al., 2001) (Figure 1).

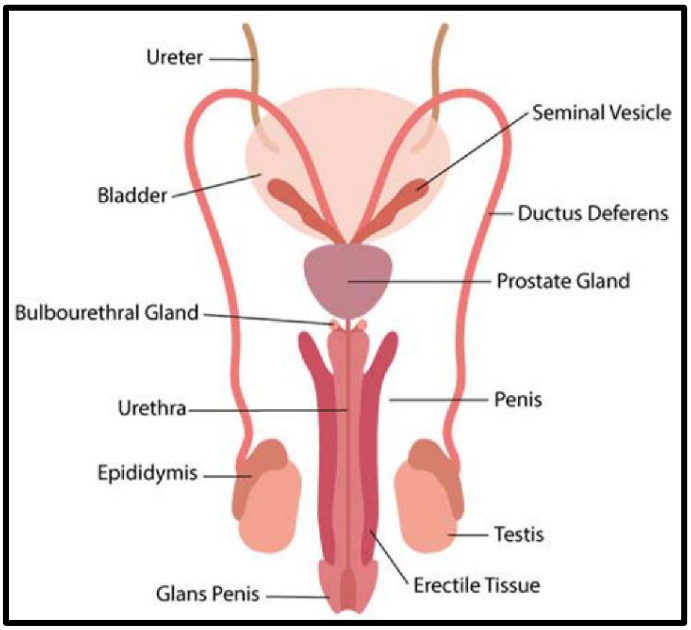

Figure 1: Posterior view of the male reproductive system depicting the testis, ducts, glands, and the external organs [reproduced from Da Silva, 2018].

2.2 The architecture of the male reproductive system

The male reproductive system exists exclusively for the production, development, and maturation of spermatozoon, which in turn serves as a vehicular apparatus for paternal genetic material (Rieth et al., 2000). It can accomplish this due to the anatomical and histological architecture of the internal and external circuits functioning synchronously. Internally, the system comprises accessory glands, the genital duct system, and testes (Stan, 2015). The glandular system of male reproduction includes the prostate, bulbourethral (Cowper's) and seminal vesicles (vesicular glands) (Condorelli et al., 2014).

2.2.1 Male accessory glands and male reproductive function

Secretions of the accessory glands provide a hospitable environment for viability, maturation, and transport of spermatozoa, and contribute enormously to the ejaculate volume (Juyena and Stelletta, 2012). Additionally, glandular products counteract the post-copulatory immune activity of the female tract against spermatozoa.

The prostate produces alkaline mucus, prostasomes, zinc, citric acid, choline, lipids, and coagulating and liquefying factors, which maintain pH and seminal consistency vital for sperm motility, competition, and ultimately, successful fertilisation (Poiani, 2006). The bulbourethral gland (Cowper's) is positioned inferiorly to the prostate, and secretes thick mucus that neutralises acidic residual urine left in the spongy urethra before ejaculation (Chughtai et al., 2005) (Figure 2). Vesicular glands, based superiorly to the prostate (Figure 2) (Hammerich, Ayala, and Wheeler, 2008), secrete fructose mainly for sperm energy requirements (Du Plessis et al., 2013), prostaglandins, and other pro-inflammatory signalling molecules (Bromfield et al., 2014), which facilitate the transit of spermatozoon into the female reproductive tract. Additionally, seminal vesicles also contain vitamin C (Gonzales, 1989; McKay and Sharma, 2020). The glands mentioned above release their secretions into the reproductive tract.

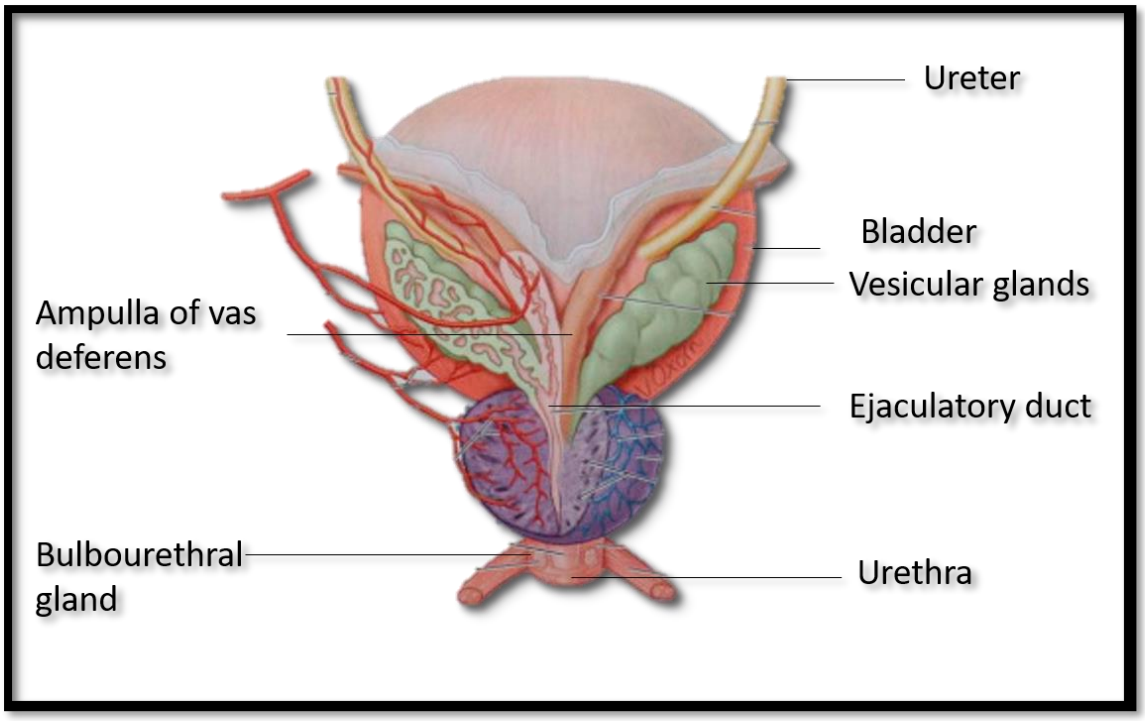

Figure 2. Posterior view of male accessory glands; prostate, vesicular and bulbourethral glands. Reproduced from Moore et al. (2013).

2.2.2 Male reproductive tract function

The entire reproductive elements follow the male development pattern; the Wolffian ducts become virilised and stabilised as testosterone binds to their receptors (MacLeod et al., 2010). The reproductive tract consists of the epididymis and ductus deferens (Jacob et al., 2012).

2.2.2.1 Epididymis

Epididymis attaches superiorly to the testes as it continues from the vasa efferentia (efferent ducts) and terminates at the vas deferens that spans 5-7 m of the entire duct (Arrotéia et al., 2012). The tightly coiled tube divides into the caput (head), corpus (body), and the cauda (tail) segments, each of which is surrounded by the smooth muscle, and is cased within the tunica vaginalis membrane of the testis (Clement and Giuliano, 2015) (Figure 3). The epididymal epithelium proteins play a protective role in preventing premature capacitation of spermatozoa, which in turn ensures a large number of competent sperm cells reaching the ampulla of the oviduct for fertilisation (Bailey, 2010). Spermatozoa exit the epididymis at its distal caudal region into the vasa deferential ducts.

2.2.2.2 Vas deferens

The vas deferens is a muscular continuation of the excurrent tube that conducts spermatozoa and seminal fluids from the epididymal cauda at a length of 30-40 cm to the ejaculatory duct, and ultimately to the urethra (Goldstein and Schlegel, 2013; Kadioglu et al., 2014). Inside the pelvic cavity, each of the ducts runs down the bladder. The terminal of each duct is called an ampulla (Figure 2), which is the reservoir of sperm (Jones and Lopez, 2014). The fundamental physiological importance of the ductus deferens is to capacitate, store, and ensure efficient transit of spermatozoa down the reproductive tract (Mahmud et al., 2015). Additionally, these ducts provide insights into the evaluation of obstructive azoospermia (De Boer et al., 2004; ASRM, 2008).

2.2.2.3 Urethra

The urethra forms part of both the urinary and reproductive tracts. From a clinical perspective, the susceptibility of the urethra to developing retention cysts due to occlusion of accessory glands, trauma, and genito-urinary tract infections are critical in male infertility evaluation (Mittal et al., 2017). These cysts may obstruct ejaculation, thereby leading to infertility.

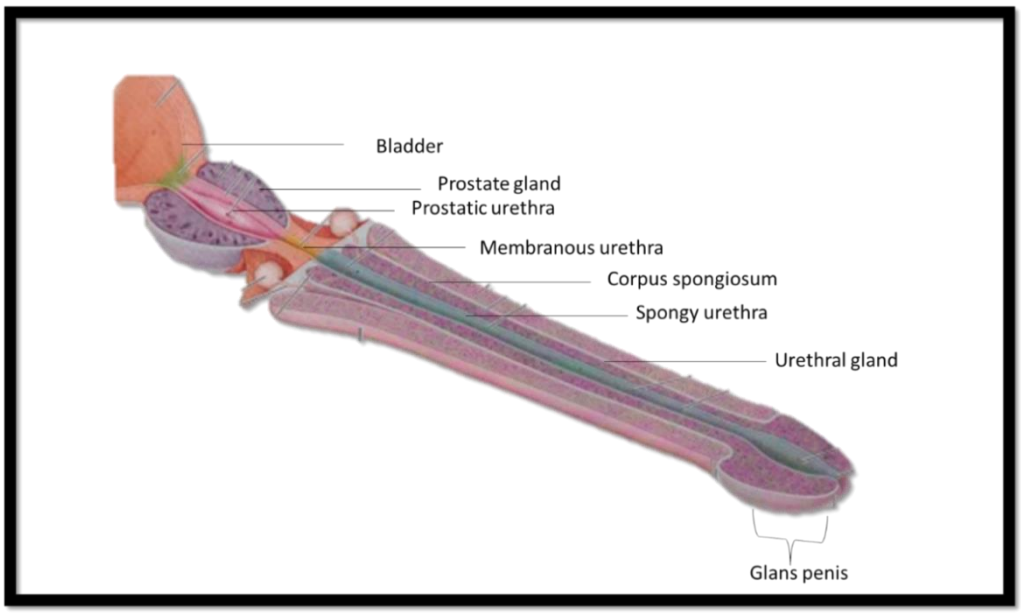

Figure 3: Male urethra depicting prostatic, membranous, and spongy segments. Reproduced from Moore et al. (2013).

2.3 Testicular function

In human adult males, testes appear as a pair of ellipsoidal-shaped testes enveloped in 3 parenchymal layers; tunica vasculosa, tunica vaginalis, and a tough membranous tunica albuginea with a diameter of 2,5 x 4 cm with a variable length ranging between 4.5–5.1 cm each (Holstein et al., 2003; MacLeod et al., 2010). Thin fibrous septum stemming from the tunica albuginea penetrates testes parenchyma, thereby separating the highly circuitous seminiferous tubules (tubular compartment) from the testis interstitial compartment (Basu, 2011). The seminiferous tubules converge into rete testes, which then enjoin to form larger efferent ducts that eventually terminate into the head of the epididymis (Figure 4).

The lymphatic vessels, blood, macrophages, nerves, fibroblasts, lymphocytes, extracellular matrix with collagen, and most interestingly, the Leydig cells constitute the interstitial component. Leydig cells are the steroidogenic powerhouse of testosterone (Weinbauer et al., 2010; Basu, 2011; Desai et al., 2017).

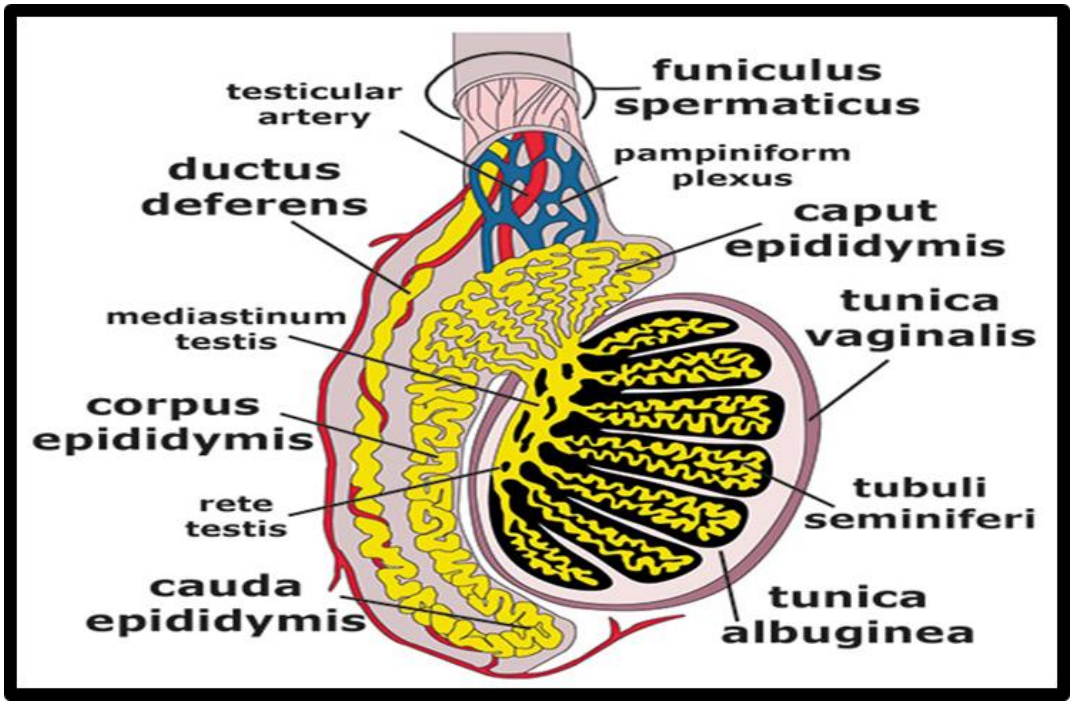

Figure 4: Sagittal view of the human testis with connecting tubules and two tunica membranes (Sudakoff et al., 2002).

2.3.1 Hypothalamic-pituitary testicular axis (HPT-axis)

In contrast to the previously held notion that the hypophysis is the master gland, the hypothalamus arguably qualifies as the master endocrine organ due to its pivotal role in central innervation of the HPT-axis. The hypothalamus is the smallest of the brain; yet, it has an organised circuitry of a specialised population of cells that produce hormones and neurotransmitters (Trachtman, 2010; Saper and Lowell, 2014) (Figure 5). These chemical messengers regulate the reproductive function through innervating pituitary gonadotropin synthesis and release, which exert its effects on the testis. The system is termed the hypothalamic-pituitary-gonadal axis (Dagklis et al., 2015).

The assortment of peripheral, central, and environmental networks ensures a proportionate hypothalamic reproductive activity with various external and internal cues (Wahab et al., 2015) (Figure 5). The responsiveness of the hypothalamus to the internal and external inputs becomes apparent during puberty.

2.3.1.1 Gonadotropin-releasing hormone (GnRH)

Structurally classified as a decapeptide, GnRH is produced by the GnRH neurons found in the hypothalamic arcuate nucleus (infundibulum) and preoptic nucleus next to the median eminence (Dees et al., 1981; Forni and Wray, 2015). Humans have two isoforms of GnRH, which are GnRH-I and GnRH-II; and the first type plays a crucial role in reproduction (Cheng and Leung, 2005). The neurons release the neurohormone GnRH into the hypophyseal portal vasculature, which in turn stimulates the anterior pituitary gland (Zimmer et al., 2010). The beginning of puberty is characterised by increased secretion of another hypothalamic neuropeptide, called kisspeptin, hence termed “gatekeeper of puberty” (Messager, 2005; Terasawa et al., 2013). Kisspeptin triggers hypothalamic secretion and pulsatile release of GnRH into the systemic circulation (Ramaswamy et al., 2008). These populations of cells are not only the prime activators of puberty, but they are also indirectly involved in the pulsatile release of gonadotropins. Also, kisspeptin and its associated neurons (neurokinin B and dynorphin) indirectly facilitate the release of two gonadotropins, released by the anterior pituitary; luteinising hormone (LH) and the follicle stimulating hormone (FSH) (Moore et al., 2019) (Figure 5).

2.3.1.2 Follicle stimulating hormone and luteinising hormone

The anterior pituitary gland is found inferiorly to the hypothalamus (Yeung et al., 2006). The anterior is the second component that is indispensable to the functional cascade of the reproductive axis. The plasma membrane of gonadotropes expresses high-affinity GnRH receptors, upon which the stimulatory effects of the follicle stimulating hormone (FSH) and the LH biosynthesis, and secretion are exerted (Marques et al., 2018). Once released, the GnRH differentially modulates secretory patterns of both gonadotropins with a concomitant pulsatile LH (Mullen et al., 2013) and tonic FSH release (Mullen, Cooke, and Crow, 2013). Following their exocytotic transport into the systemic circulation, FSH and LH affect testicular events central to male fertility, spermatogenesis in the Sertoli cells and testosterone synthesis by the Leydig cells, respectively (Jin et al., 2013).

Gonadotropins secretion is subject to a carefully orchestrated negative feedback system to adequately maintain optimal testicular function (Weinbauer et al., 2010). Accordingly, cellular signalling molecules such as hypothalamic corticotropin-releasing hormone (CRH) and gonadotropin-inhibiting hormone (GnIH) (Kirby et al., 2009), and neurotransmitters (such as GABA and NO) (García-Galiano et al., 2012), activin, inhibin, anti-Műllerian hormone (AMH) (Matuszczak et al., 2013). Contribute to an interplay of all mechanism mentioned above, which contributes significantly to the synthesis and release of gonadotropic hormones (Figure 5). Consequently, any disruptions in either the synthesis, metabolism, or elimination of these intracellular factors distort the reproductive capacity and amplify testicular pathology, which may result in infertility.

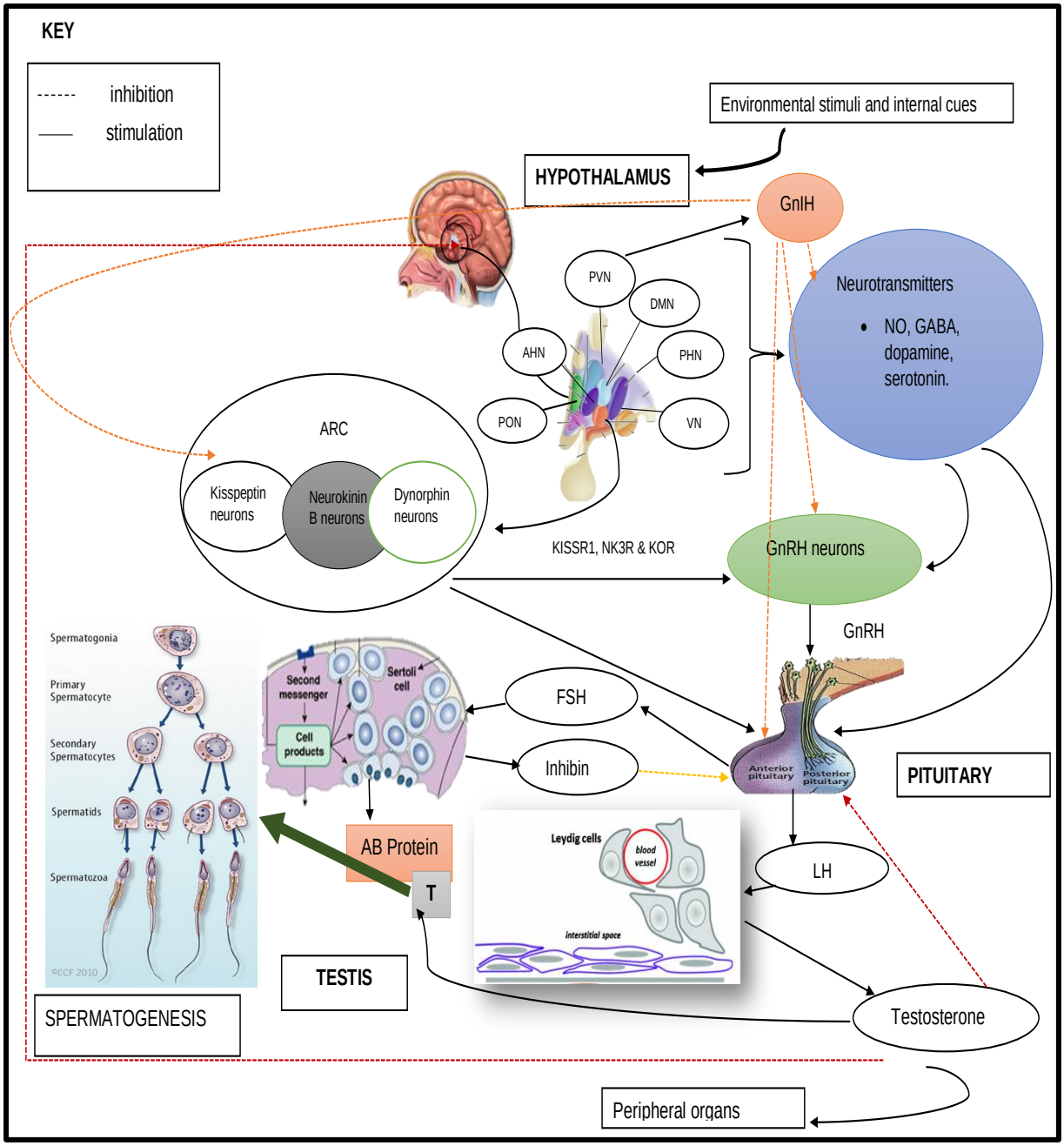

Figure 5: Schematic depiction of neuroendocrine regulation of the hypothalamic pituitary testicular axis in males. The pituitary gland was reproduced from [https://www2.estrellamountain.edu/faculty/farabee/biobk/BioBookENDOCR.html], Brain was accessed from [https://www2.estrellamountain.edu/faculty/farabee/biobk/BioBookENDOCR.html]. Spermatogenesis was reproduced from Sharma and Agarwal (2018).

2.3.2 Cellular composition of the testis

The testis comprises of the seminiferous tubules and the interstitium house the following cells: Between the lobules of the the seminiferous tubules are the diploid cells, Sertoli cells providing nourishment for the developing germ cells (Basu, 2011). Housed inside the interstitial component of the testis ids the Leydig cells (Desai et al., 2017).

2.3.2.1 Sertoli cells function

Residing inside the seminiferous tubules are germ cells in their respective developmental stages (Chaturvedi and Johnson, 1993; Suede et al., 2020). Sertoli cells (SCs), also called "sustentacular or "nurse" provide a cytoarchitectural and immunological environment favourable for spermatogenesis (Rebourcet et al., 2014). The spatial arrangement of spermatozoa into various levels of development is anchored by the testicular somatic cells; (Sertoli cells) interspersed between the seminiferous epithelium (Johnson, Thompson, and Varner, 2008). Sertoli cells bind to testosterone and FSH, and synthesise a glycoprotein androgen binding protein (ABP), which regulates germ cell maturation to spermatozoa (Guitton et al., 2000). Additionally, other SCs transport proteins such as transferrin and ceruloplasmin, which deliver Fe^{3+} and Cu^{2+}, to the germ cells for nutritive purposes (Stallard and Griswold, 1990; Fujisawa, 2006).

Adjacent SCs have tight junctions in between, creating a physical barrier called the blood testes barrier (BTB) in between themselves, and near the basement membrane of the seminiferous tubule (Cheng et al., 2011; Mital et al., 2011). BTB defines functional permeability of the seminiferous epithelium by regulating the entry of electrolytes, sugars, ions, and water into the adluminal compartment (transcellular) (Li et al., 2016).

Also, the barrier restricts the movement of noxious substances such as pesticides, biphenyls, and food additives into the adluminal compartment (Xiao et al., 2014). Under normal conditions, SCs directly augment spontaneous apoptosis (abortive apoptosis) through the Fas-fas ligand paracrine pathway, which is essential in balancing germ cell population with SC supporting capacity (Murphy and Richburg, 2015). Moreover, SCs eliminate the accumulation of apoptotic, residual, and

degenerate cytoskeletal sperm elements through phagocytosis (Baradi and Rao, 1983; Wang and Han, 2019).

By compartmentalising the seminiferous epithelium into basal and adluminal segments (Figure 7), SCs' BTB prevents the autoimmune attack of meiotic and post-meiotic sperm cells from systemic circulation (Cheng and Mruk, 2012). Disruption of the SCs' BTB would not only expose germ cells to xenobiotics but would expose their antigens to the immunologic response from vasculature causing infertility.

2.3.2.2 Leydig cells function

The most fundamental functions of sex organs are the initiation and maintenance of two physiologically and biochemically related processes, referring to gametogenesis and steroidogenesis (McGuire and Bentley, 2010). Leydig cells, together with Sertoli cells, are critical in the proper functions of the former processes. One of the first population of cells to colonise the interstitium of the testes are the Leydig cells (Griswold and Behringer, 2009; O'Shaughnessy and Fowler, 2011).

Leydig cells are self-regulating cells, wherein testosterone exerts inhibitory effects on the upstream tissues of the hypothalamic-anterior pituitary testes axis (Peper et al., 2010). Pulsatile GnRH is secreted into the hypophyseal vascular portal system to stimulate LH biosynthesis and release by the adenohypophysis, which consequently modulates testicular androgen synthesis (Corradi et al, 2016).

2.4 Spermatogenesis

Spermatogenesis encompasses a series of complex stages that halve the genetic material and modify the structural make-up of a primitive germ cell into a fully functional mature spermatozoon (Krausz and Sassone-Corsi, 2005). The purpose of this transformative differentiation is to produce a genetically unique sperm (Sharma and Agarwal, 2011). The cyclical events of this complicated process require highly specific and timely gene expression, hormonal control, and specialised cells such as Leydig and Sertoli cells (Wistuba et al, 2009; Xiao et al., 2014).

Seminiferous tubules are the sperm-manufacturing organelles in the testis (Xiao et al., 2014). The convoluted tubule, making up 80 – 90% of testicular volume, is stacked into lobules defined by the fibrous septa inside testes (Esfandiari and Dehghani, 2010;

Mbaeri et al., 2013). A pluripotent undifferentiated cell mitotically divides for the regeneration of germ cell population, resulting in a spermatogonial stem cell (SSC) that remains at the basement compartment (Desai et al., 2017). However, one of the resulting clone cells is fated to become a mature spermatozoon through mitosis, meiosis, and spermiogenesis (Dimitriadis et al., 2015). It takes approximately 74 days for an undifferentiated sperm to be perfected into a mature spermatid (Stukenborg et al., 2014).

2.4.1 Events at the basal compartment of the seminiferous tubules

During embryogenesis, gonocytes have the potential to either take the female (enter meiosis) or male pathway (enter mitotic arrest); and in the latter pathway, they remain in quiescence until puberty (Stukenborg et al., 2014; Nikolic et al., 2016). Once migrated into the testis, these primordial germ cells become prespermatogonium (prototype stem cells) in the basal membrane (Amann, 2008). At puberty, differentiation of SSCs occurs in the SSC niche at the basement membrane, tunica propria (Cheng and Mruk, 2012). Two types of spermatogonial sub-types have been found in men; type A and type B, both of which are morphologically distinct in terms of nuclear stainability (Holstein, Schulze, and Davidoff, 2003) (Figure 6). Type A further divides into pale type A (A_{pale}), and dark type A (A_{dark}), which is a non-proliferative reserve for the replenishment of the stem cell pool. Pale (A_{pale}) type self-renews and with time, it differentiates into type B spermatogonia, which marks the last mitotic division (Durairajanayagam et al., 2015).

Type B spermatogonia are the precursors of meiotic differentiation (primary spermatocytes), and dividing synchronously, they give rise to preleptotene spermatocytes. The latter cells can migrate the tight junctions (Mays-Hoopes et al., 1995; Mruk and Cheng, 2010; Griswold, 2016) (Figure 6). While in transit, preleptotene would differentiate into leptotene, and then zygotene spermatocytes; and this stage is signalled by chromatin condensation (Cheng and Mruk, 2009).

2.4.2 Events at the adluminal compartment of the seminiferous tubules

It is in this compartment that by meiotically halving of the primary spermatocytes, a population of genetically different daughter cells is created. Behind the BTB, zygotene spermatocytes are committed to the first meiotic division to yield pachytene

spermatocytes through chromosomal pairing (Lui et al., 2003; Cheng and Mruk, 2009; Sharma and Agarwal, 2011b) (Figure 6). The last stage of the first meiotic division is characterised by chromosomal crossing over (chiasma) that yield diplotene spermatocytes (duplication) (Holm and Rasmussen, 1983; Paniagua et al., 2020). Chromatids from the previous division enter meiosis II to yield four haploid gametes with 23 chromosomes (22 autosomes and 1 X or Y sex chromosomes) (Durairajanayagam et al., 2015).

2.4.3 Spermiogenesis and spermiation

These round spermatids are mitotically inert, and will transiently move to the elongation and remodelling differentiation known at spermiogenesis (Weinbauer et al., 2010). The hallmark step of spermiogenesis is the packaging of nuclear DNA, followed by protamination of the histones (Tanaka and Baba, 2005). During spermiogenesis, the round spermatids come into direct contact with the nuclear membrane and flattening of the sperm head (Lehti and Sironen, 2016). Also, Golgi apparatus, mitochondrial sheath as well as acrosomal vesicle appear (de Boer et al., 2015; Lebelo and van der Horst, 2016). Microtubule axoneme of the flagellum appears as elongation ensues (O'Donnell, 2014). Excess cytoplasmic residues are extruded and phagocytosed by Sertoli cells to complete the apical basement maturation (Blanco-Rodríguez and Martínez-García, 1999; O'Donnell, 2014). At approximately day 74 since the differentiation of the primitive spermatogonia, mature sperm cells detach from the Sertoli cells, while exiting the seminiferous epithelium into the lumen through spermiation (O'Donnell et al., 2011) (Figure 6).

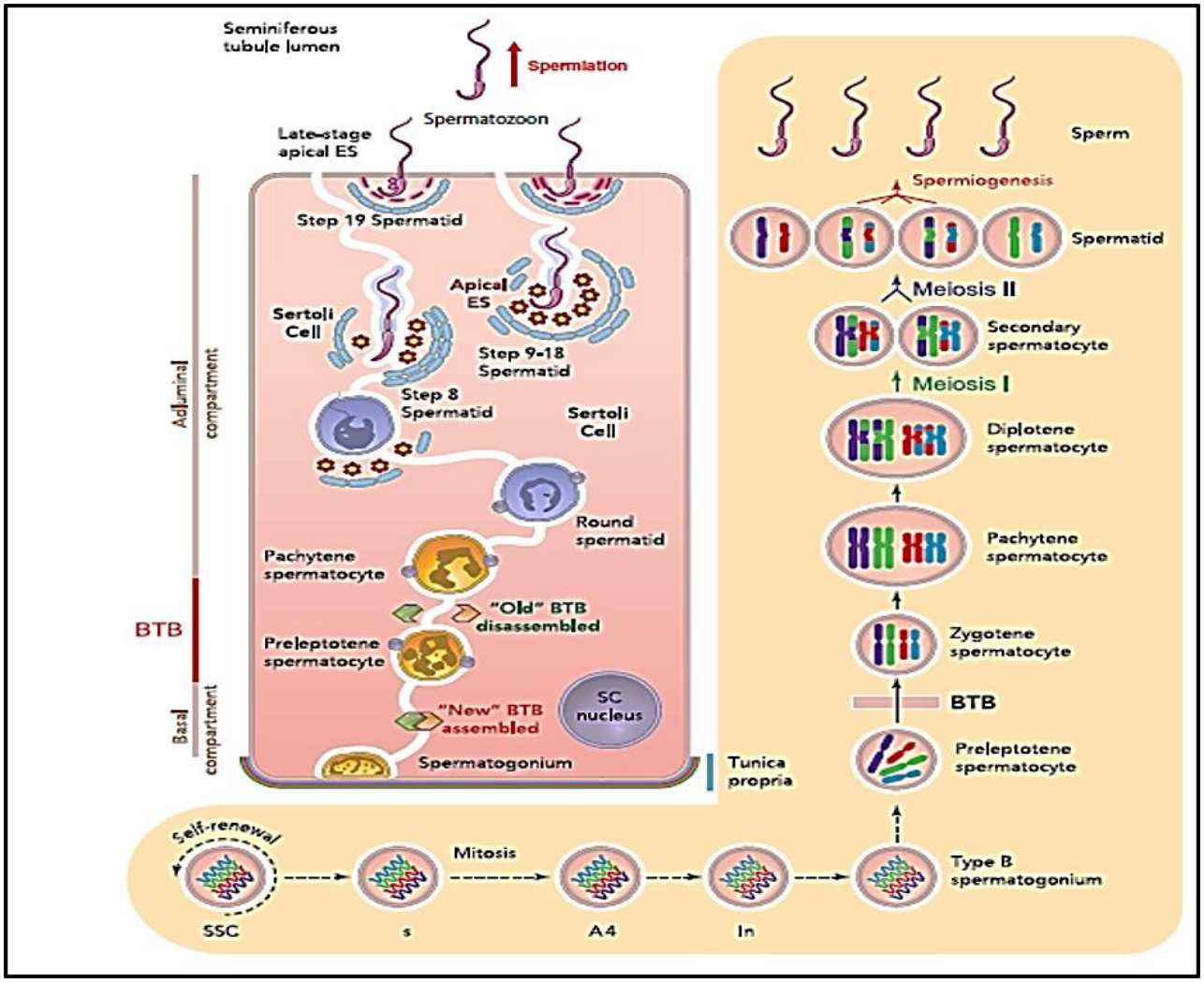

Figure 6: A summary of mammalian spermatogenesis in the seminiferous epithelium showing the Sertoli cell BTB that separates two compartments (Xiao et al., 2014).

2.5 Structure of human sperm

Although mammalian sperm looks deceptively small and simple, it is infrastructurally complex and a terminally developed cell. The principal purpose of sperm is to deliver haploid genomic material for fertilisation of an oocyte (Mortimer, 1997). Interestingly, sperm delivers more than just nuclear DNA; some RNA molecules that are crucial for embryogenesis are also delivered (Bukowska et al., 2013). In order to fulfil this role, spermatozoa need to protect the DNA (in the head) from noxious clastogenic substances (Zini and Libman, 2006), transport it to the oviduct (flagellum), and provide energy for transport through mitochondrial oxidative phosphorylation (OSPHOX) (in the mid-piece) (Pesch and Bergmann, 2006). All functional characteristics crucial for fertility competence are enclosed in the plasma membrane (Ayad, 2018) (Figure 7).

This membrane consists of heterogeneous glycoproteins and lipid profiles known as regional domains (Petrunkina et al., 2001). The domains play specific roles based on the antigen distribution in the recognition and binding of sperm with the oocyte (Prag, 2017).

2.5.1 Head

Most of the sperm head volume is occupied by the nucleus, acrosome, and minimal cytoplasmic elements. Histologically, the head is flattened and oval-shaped, spanning 4.0–5.5 µm and 2.5–3.5 µm of the total sperm length and width (Maree et al., 2010; Desai et al., 2017). The nucleus is condensed into a much smaller structure, and the compaction is thought to be significant to the kinematic dynamics of the sperm and fertilisation (Buffone et al., 2012).

The spermatozoon head is divided into the acrosomal, post-acrosomal, posterior ring and equatorial segments (Varner and Johnson, 2007) (Figure 7). The acrosomal region is directly involved in acrosome reaction, while the post-acrosomal and equatorial regions play a significant role in sperm-egg membrane recognition and fusion (Toshimori, 2009; Ngcauzele, 2018). The acrosomal matrix separates the inner membrane from the outer membrane, and both membranes contain acrosomal contents involved in exocytosomal release and hydrolysis of the zona pellucida of the oocyte for fertilisation (Abou-Haila and Tulsiani, 2000; Ngcauzele, 2018).

The posterior ring prevents head cytosolic contents from mixing with that of the flagellum, and anchors the nuclear envelope (Varner and Johnson, 2007) (Figure 7).

2.5.2 Neck

The neck is the shortest component of the human spermatozoon, measuring only 1 µm (Prag, 2017). It is a junction between the sperm head and flagellum. The neck is more than just an articular piece; it houses a specialised connecting piece consisting of 9 columns of proteins closed by the capitulum, which is attached to the sperm head (Yuan et al., 2015). The essential organelle in the sperm neck is the centriole (Avidor-Reiss et al., 2019) (Figure 7). Centrioles are reportedly involved in early embryonic development, and their impairment has been correlated with male infertility and pregnancy failure (Chemes and Alvarez Sedo, 2012).

2.5.3 Flagellum

A mature mammalian spermatozoon is a characteristically elongated cell, and most of the length is attributed to the flagellum. It is subdivided into the mid-piece, principal, and end-pieces, making it approximately 40–50 µm (van der Horst and Maree, 2009; Desai et al., 2017). The mid-piece is wound helically around the anterior flagellum, which contains a variable number of mitochondria that produce ATP through OSPHOX (Ramalho-Santos et al., 2007) (Figure 7). A mitochondrial sheath surrounds the mid-piece, and it is separated from the principal piece by an annulus, which is a ring-like structure that controls the diffusion of molecules between the two segments (Rawe et al., 2000; Guan et al., 2009) (Figure 7).

The principal piece makes up the majority of the tail with an approximate length of 40 µm. A fibrous sheath covers it; a cytoskeletal structure overlying the plasma membrane is made up of two longitudinal columns ending at the end-piece (Barone et al., 1994; Rawe et al., 2000). The principal piece acts as an anchor to the mid-piece by preventing dislocation of the mitochondria as the sperm bends (Barone et al., 1994) The function of the sheath is mainly structural in that it is a scaffold protein and enables recoiling during flagella beatings (Eddy, 2007; Lindemann and Lesich, 2016).

The axoneme human spermatozoa, just like any mammalian, is arranged in a 9+2 microtubular circle and about 250 accessory structures (Inaba, 2003). The nine represents the outer dense fibres (ODFs) arranged in microtubules adjacent to two large outer and inner dynein motor proteins (Figure 7). These proteins provide active flagellar movement through microtubule sliding mechanism, increasing beat velocity and frequency. The outer dynein arm contains ATPases, whose activity mostly includes chemical energy production through the glycolytic pathway (Goodson et al., 2012). Apart from being responsible for structural flexibility and the flagellar sliding mechanics, the axonemal microtubule dense fibres and the sheath could be playing a role in ATP production for hyperactivated, whiplash movement, capacitation, and acrosome reaction (Eddy et al., 2003; de Krester et al., 2016; Fisher and Henkel, 2020). The end-piece is devoid of the protective sheath and other cytoskeletal components, and consists of only the axoneme (van der Horst et al., 2011) (Figure 7).

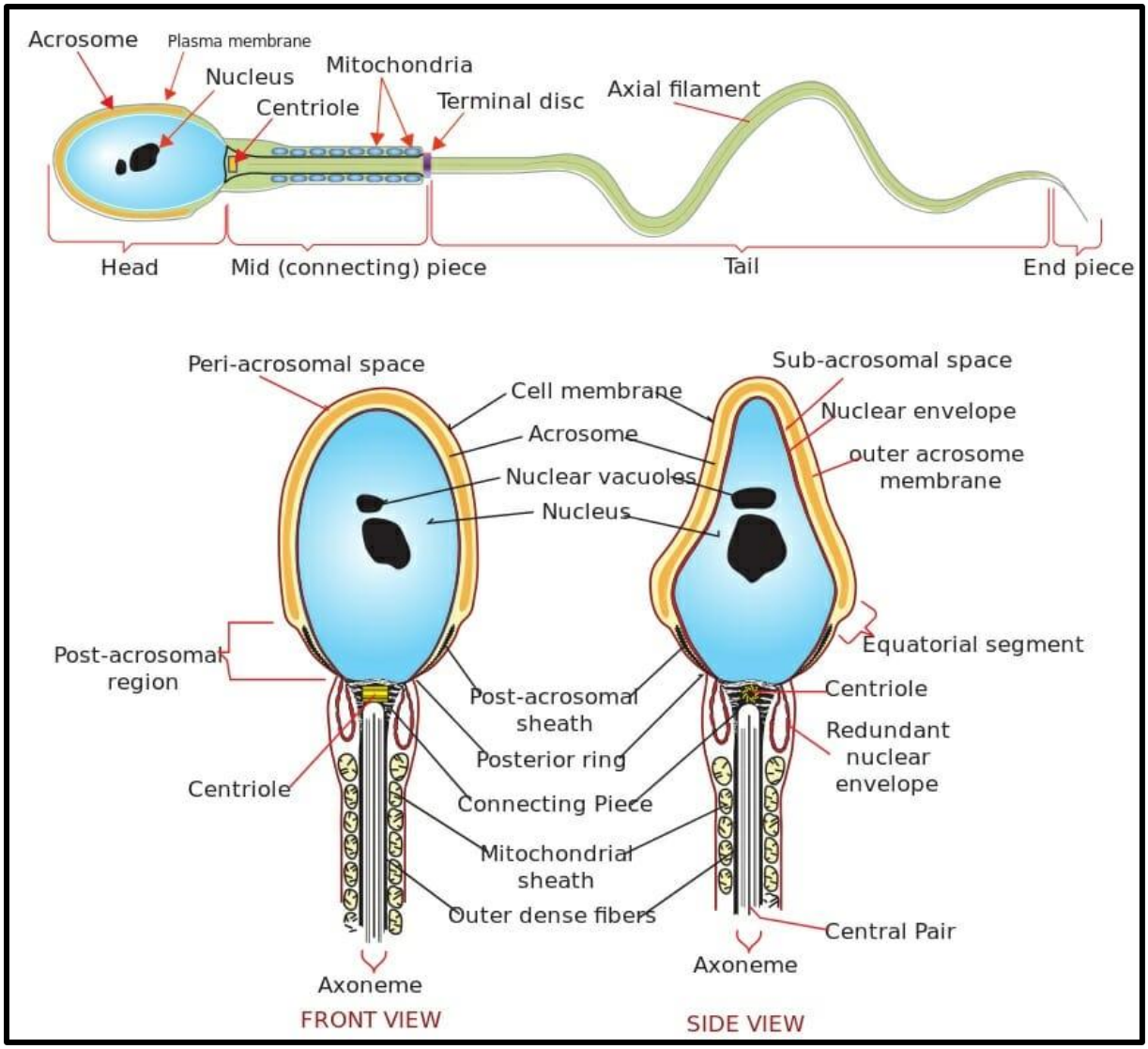

Figure 7: Structure of mammalian spermatozoa (Elgeti et al., 2015).

2.6 Evaluation of sperm functional parameters

Sperm analysis is fundamentally based on the principle of competition and selection of functionally and structurally intactness. Ideally, spermatozoa with normal morphology, motility including swimming velocities, intact MMP, vitality, condensed nuclear material, and controlled physiological ROS stand a better chance of capacitation, acrosome reaction, and ultimately reaching fertilisation (Agarwal et al., 2014; del Barco-Trillo et al., 2016; Sousa et al., 2011). Selection of spermatozoon with fertilising capacity is a stringent and efficient process occurring at critical points along the female genital tract, namely; the cervix, uterus, uterotubal junction, oviduct, cumulus oophorus, and zona pellucida (Henkel, 2012; Sakkas et al., 2015). The

heterogeneous nature of semen is said to be predominantly consisting of the population with suboptimal functionality (Sousa et al., 2011).

Conventional semen analysis remains a surrogate measure of a male fertility potential partner in a sub-fertile couple (Wang and Swerdloff, 2014). Additionally, it is used for evaluation of reproductive toxicity, epidemiological distribution of infertility, and efficacy treatment of therapeutic agents (Guzick et al., 2001; Cooper et al., 2010). Despite providing valuable information on sperm production, count, spermatogenesis, tubal patency, and secretory efficiency of accessory organs, a conventional semen analysis is not a diagnostic of male infertility nor is it a test of fertility (Vasan, 2011; du Plessis et al., 2013; Baskaran et al., 2020). Henkel et al. (2005) further asserted that the standard semen analysis showed high variability and was incapable of providing information on the functional capacity of the spermatozoon.

Routine semen analysis is divided into three quantifiable categories: microscopic, macroscopic, and physiological. The microscopic analysis reveals events illustrative of optimal spermatogenesis such as sperm concentration and count, agglutination, seminal debris, and immature sperm (Comhaire and Vermeulen, 1995; Agarwal et al., 2008). The macroscopic analysis assesses on physical parameters the congenital abnormalities, health, and secretory function of accessory glands, and ejaculatory efficiency. These physical attributes include pH, volume, viscosity, liquefaction (coagulation), and colour/smell (Agarwal et al., 2008; Vasan, 2011; Aziz, 2013). The last physiological category is motility and viability, which are indicative of sperm membrane integrity and maturity.

The World Health Organisation (2010) provides evidence-based standard protocols for handling and examining semen, even though the recommended lower reference values have been met with controversy (Franken and Oehninger, 2012). The lower reference values as provided by the WHO (2010) are as follows; volume ≥ 1.5 ml, concentration ≥ 15 mil/ml, total count ≥ 39 mil/ml, total motility ≥ 40%, progressive motility of ≥ 32%, and vitality of ≥ 58% (WHO, 2010). Because of the limitations of basic semen analysis, it is further advised that analysis results should be advised with caution in the management of male infertility (Henkel, 2015). All these parameters are imperfect tools, and they are limited in discriminating functional sperm from abnormal.

For this reason, it becomes imperative to include advanced functional tests that can accurately predict men's fertilising potential.

2.6.1 Motility

Motility is generally accepted as the definitive proxy of male infertility (Natali and Turek, 2011). However, the consideration of motion or motility as a sole index for sperm life or fertility potential can be erroneous, since not all motile cells are competent for fertilisation, and not all immotile cells are dead (Van Zyl and Alwelien, 2015). Manual semen analysis has been widely applied in clinical male work-up settings and seminology laboratories to determine motility. However, their inability to sufficiently capture sperm head and flagellum kinematic patterns have rendered the introduction of an automated, computational system (CASA) a useful necessity (Vasan, 2011).

2.6.1.1 Computer-aided sperm analysis (CASA)

CASA is an automated, high precision tool that measures sperm motility accurately and objectively for various species (Maree and van der Horst, 2013; van der Horst, Maree and du Plessis, 2018). As with the use of any new computational technology, the reliability of CASA can be significantly diminished by technical and operational faults of the system by the user, by biological variability of samples, poor internal quality control such as inappropriate pipetting, inconsistent dilutions, or centrifugal factors (Talarczyk-Desole et al., 2017; Patel et al., 2018). Other than measuring sperm sub-populations' motilities based on progression and speed (total, progressive, non-progressive motility, and immotility), CASA measures percentage hyperactivation and kinematic characteristics (Alipour et al., 2017). Sperm kinematics include velocities: curvilinear (VCL), straight-line (VSL), and average path (VAP) velocities (μm. s^{-1}), head oscillatory/wobble index (WOB), straightness index (STR) and linearity index (%). Flagellar attributes are: beat cross frequency (Hz), and amplitude of lateral head displacement (μm) (Larsen et al., 2000; World Health Organization, 2010) (Figure 8).

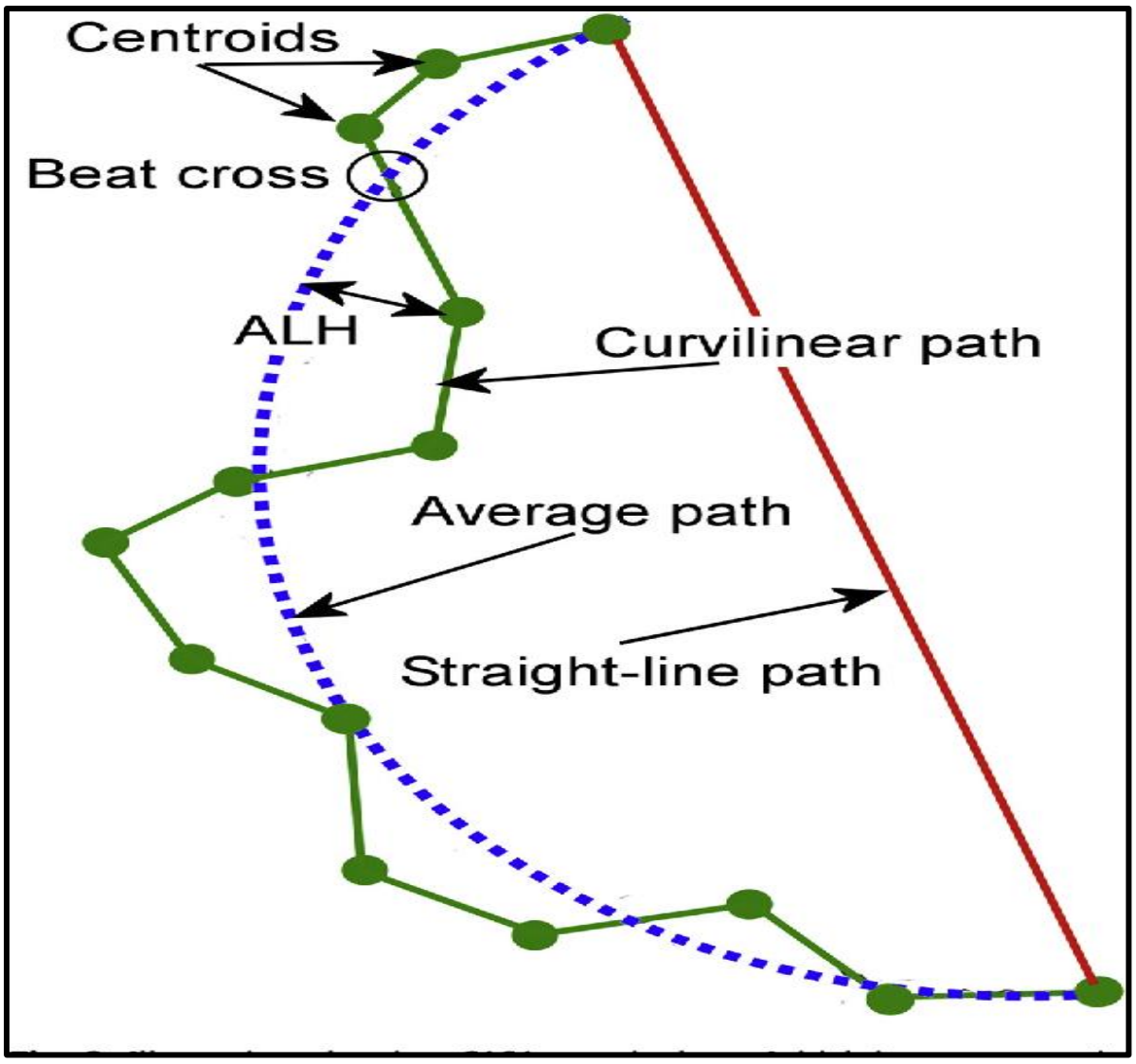

Figure 8: Sperm motility kinematics as seen under the computer-aided sperm analysis (Amann and Waberski, 2014)

2.6.2 Vitality

2.6.2.1 One-step eosin nigrosine staining technique

It is mandatory to distinguish live sperm from dead sperm in investigative settings that handle, examine, and analyse semen for research or clinical assessment of male infertility (Björndahl et al., 2004). Sperm vitality reflects a proportion of dead or live membrane-intact spermatozoa measured through osmotic behaviour (hypotonic conditions) or exclusion dye techniques (Moskovtsev and Librach, 2013). In the last century, several staining techniques have been developed for the assessment of vitality.

However, few have been developed to assess incompatibility with human sperm until the 1990s and the discovery of eosin-nigrosin (Mortimer, 1985; Björndahl et al., 2003). One-step eosin-nigrosin has proven valuable for the prediction of a ratio of live / dead cell in the semen sample in andrology.

It works on the principle that metabolically active sperm, with an intact membrane, is impermeable to eosin stain (Björndahl et al., 2004), while nigrosin dye adjusts the contrast-background ratio of the sperm heads, thereby increasing visibility under a bright field microscope. Dead cells absorb the dye and stain deep pink/purple or red, while live cells appear white (Agarwal et al., 2016). Hence, it had been recommended by the WHO (2010) for viability evaluation in basic semen analysis.

2.6.3 Mitochondrial membrane potential (MMP) ($\Delta\psi_M$)

The mitochondrion is usually referred to as the powerhouse of the cell (Opuwari, 2009). Nutrients are oxidised in the mitochondria to produce ATP through oxidative phosphorylation, which is a series of redox reactions that establishes an electrochemical gradient (Cottet-Rousselle et al., 2011). Generation of the proton motive force establishes a mitochondrial membrane potential (Meyers, Bulkeley, and Foutouhi, 2019). MMP regulates sperm motility and maintains viability (Agnihotri et al., 2016). MMP is not only sensitive to detecting the electron transport chain (ETC) efficiency, but it was indicated as a reliable marker of mitochondrial functionality and as being predictive of sperm fertilising success (Marchetti et al., 2002; Amaral et al., 2013; Henkel, 2015).

Under pathological conditions, the capacity of mitochondria to generate energy is disturbed, resulting in a decline in the electrochemical gradient, which is signified by mitochondrial permeability transition complexes (Shunmugam, 2016). The pores in the outer membrane of the mitochondria release pro-apoptotic proteins (mostly cytochrome c) to facilitate a cascade of events that ultimately lead to the demise of the cell (Bernardi and Di Lisa, 2015). The fluorescent probes for quantifying functional membrane integrity take advantage of the sensitivity of sperm plasma membrane to minute changes in the chemiosmotic intramembrane gradients (Sakamuru et al., 2012).

2.6.3.1 Lipophilic cationic dye

Lipophilic cationic fluorescent dyes can readily diffuse into hydrophobic membranes, and hence, they have the highest binding affinity such that they can distribute equally within the mitochondria and accumulate (Ehrenberg et al., 1988; Perry et al., 2011). The DePsipher dye accumulates in membrane-intact mitochondria and fluoresces red, while in apoptotic cells it cannot aggregate in the mitochondria; it just remains in the cytoplasm and therefore fluoresces green (Sivandzade et al., 2019). One of the lipophilic dyes proven to be working on the principle, as mentioned above, is (5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimiddasolyl-carbocyanine iodide (JC-1) (Marchetti et al., 2004).

2.6.4 Reactive oxygen species (ROS)

2.6.4.1 ROS generation

Diatomic oxygen is relatively inert, even though two of its valence electrons are unpaired (Ford, 2004). However, when oxygen-containing molecules get reduced, their by-products become highly unstable and reactive, as they will remain with the unpaired electron, creating sequelae of oxidative reactions (Wagner, Cheng, and Ko, 2018). Some of the clinically relevant free radical in the human ejaculate are singlet anion ($^1O_2$) and peroxyl radical (Ford, 2004).

Of the known ROS, $O_2^{-\bullet}$ is the most potent intracellularly produced ROS in nearly all biological systems (Zorov et al., 2014). However, because of its size, superoxide has limited permeability to sperm lipid membrane, and therefore becomes changed into hydrogen peroxide (H_2O_2) either spontaneously or by enzymatic dismutation (Mitchell et al., 1990; Dunand et al., 2007). Although H_2O_2 is not precisely a free radical, it is involved in subsequent ROS synthesis (Aprioku, 2013). H_2O_2 and superoxide can be converted to indiscriminately, highly reactive hydroxyl ($OH^{\bullet-}$) through the iron-dependent Haber-Weiss reaction (Kehrer, 2000). Hydroxyl anion initiates membrane damage to spermatozoa by removing electrons from lipid membranes of the sperm. Spermatozoa, similar to any anaerobic cell, are often confronted with what is commonly termed the ROS paradox (Agarwal et al., 2003; Tvrdá et al., 2017). To the same extent that oxygen is indispensable to aerobic life, its intermediates can initiate or/and facilitate cellular demise (Ogbuewu et al., 2010). Extrinsically, ROS generation can be triggered or exacerbated by environmental, lifestyle factors (smoking and

drinking), where medication, and genetics contribute the “oxidative interface” (Lavranos et al., 2012; Ray et al., 2012) (Figure 10).

2.6.4.1 Sources of ROS

Reactive oxygen species (ROS) leaks through mitochondrial OSPHOX and glycolytic systems of ATP production, using nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nascimento et al., 2008). Additionally, immature or defective sperm cells have excess cytoplasm (which would have been customarily shed during spermiation) (O’Donnell, 2014) (Figure 10). This excess cytoplasm contains membrane bound-glucose-6 phosphate dehydrogenase (G-6-DPH) involved in additional ROS generation through the pentose monophosphate-shunt pathway (Gomez et al., 1996; Tremellen, 2008). In this pentose phosphate shunt, the biosynthesis of cytoplasmic 8-docosahexaenoic acid upregulates the availability of NADPH (Said et al., 2005). The putative NADPH subsequently donates electrons for the production of spermatozoal ROS at the sperm membrane level (O’Flaherty, 2015). There is a positive association between abnormally high levels of ROS in oligoasthenoteratozoospermia males with impaired spermatogenesis (Ollero et al., 2001; Hosseinzadeh et al., 2013).

Also, sperm is enveloped in polyunsaturated fatty acids (PUFA) rich membrane, which contains fewer antioxidant capacities, therefore rendering spermatozoa particularly vulnerable to peroxidative and oxidative damage (Hsieh et al., 2006; Parekattil and Agarwal, 2012). It is generally noted that ROS production by seminal polymorphonuclear granulocyte exceeds physiological ROS levels a 1000-fold (Agarwal, et al., 2014; Wagner et al., 2018). In the presence of genitourinary infections, cryptorchidism, inflammation, iatrogenic factors, and varicocele, the activation of PMN leukocytes is often pronounced, with corresponding excess ROS levels (Henkel and Schill, 2003; Henkel, 2011; Lobascio et al., 2015; Cho et al., 2016; Solomon and Henkel, 2017) (Figure 10). The balance between pro-oxidants and anti-oxidants is a margin within which sperm ROS can mediate sperm signal transduction of hyperactivation, motility, capacitation, acrosome reaction, and sperm-egg fusion (Aitken et al., 1989; de Lamirande and Gagnon, 1995; Kothari et al., 2010) (Figure 10). On the other hand, overproduction of sperm ROS results in an imbalance between the

oxidants and antioxidant defences in favour of the former; this phenomenon is termed oxidative stress (OS) (Agarwal et al., 2019). OS is a leading proven cause of male infertility (Agarwal et al., 2019). One of the hallmark clinical manifestations of the testicular OS is sperm DNA damage (Iommiello et al., 2015) (Figure 9).

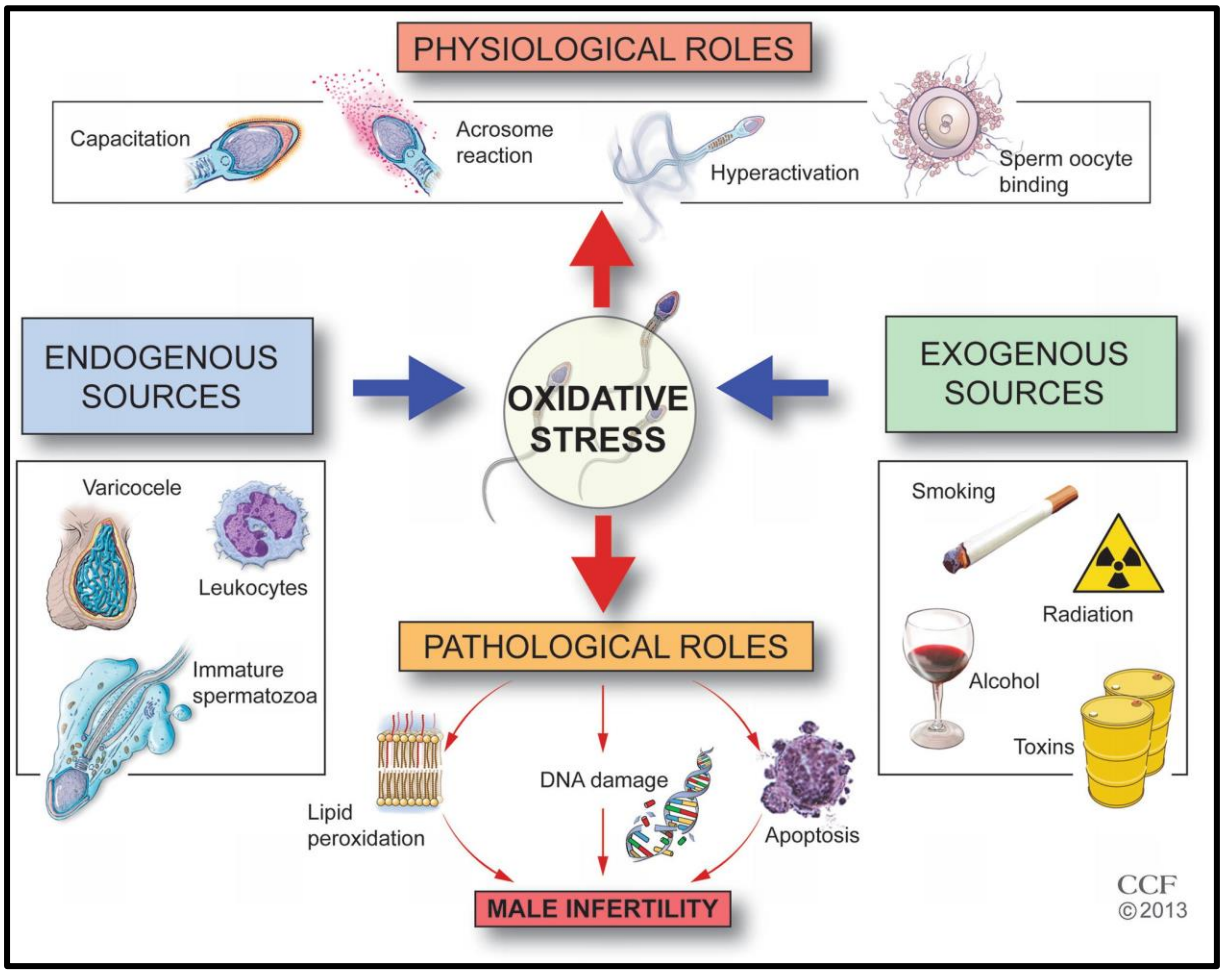

Figure 9: The physiological and pathophysiological roles of ROS in male infertility (Sharma and Agarwal, 2020).

2.6.4.2 Antioxidants

Spermatozoa possess an intrinsic free radical scavenging activity, which is limited by the reduced cytoplasmic volume, rendering them particularly vulnerable to oxidative damage (Henkel et al., 2018). Additionally, sperm cells are bathed in a seminal milieu endowed with enzymatic, non-enzymatic antioxidant systems to combat excessive ROS (Pasqualotto et al., 2004; Papas et al., 2019). Nutritional antioxidants have been prescribed for pathologies, in which oxidative stress was implicated as a cause or modulator, including male infertility (de Young et al., 2004; Henkel et al., 2019). Enzymatic antioxidants found in the seminal plasma are superoxide dismutase (SOD),

catalase (CAT), glutathione peroxidase (GPx), and glutathione (GSH) (Tavilani et al., 2008; Tomar et al., 2017). Non-enzymatic antioxidants such as vitamins C, E, B_{12}, carotenoids, carnitine, bioflavonoids bilirubin, albumin, uric acid, N-cysteine, and folate are exogenously obtained from the diet (Armstrong et al., 1998; Ferreira, 2012; Aprioku, 2013; Adewoyin et al., 2017). Trace elements such as zinc, selenium, and magnesium also have oxidative protectants (Palani and Alshatteri, 2017).

2.6.4.3 ROS measurement using a fluorescent probe

There exists a thin margin, upon which ROS are physiological and pathological for sperm function, further making it necessary to quantify ROS levels essential for normal function (Tomar et al., 2017). Therefore, a variety of methods of detection include chemiluminescent or fluorescent assays that have been developed for direct measurement of ROS production, and which rely on the extent to which superoxide will oxidise the functional groups of reagents in the assay (Gosalvez et al., 2017). Similarly, the measurement of sperm ROS in this study used dihydroethidium (DHE). DHE is a fluorescing probe that fluoresces red when oxidised by O_2-. The intercalation of sperm nuclear DNA with the dye is marked by hydroxylation of DHE into a red or orange 2-hydroxyethidium substance, which fluoresces at excitation and emission wavelengths of 535 nm and 635 nm, respectively (Aitken et al., 2013; Held, 2015).

2.6.5 DNA fragmentation

Intrinsic sperm DNA damage results from incorrect chromatin packaging, post-ejaculatory defective apoptosis, endogenous nuclease activity, or oxidative damage (Agarwal et al., 2014; Mupfiga et al., 2013; Zini and Libman, 2006). These causes can be further aggravated by environmental pollutants, radiation, and chemotherapy (Henkel, 2017). Once membrane peroxidation (by excess ROS) has been initiated, its intermediates such as malondialdehyde proceed to the sperm mid-piece to induce and propagate mitochondrial dysfunction (Hsieh et al., 2006). The cascade, which further exacerbates electron leakage, and one of the free radicals that actively participates in the early DNA base attack is hydrogen peroxide (Aitken and Koppers, 2011).

Initiation of the DNA backbone attack through oxidising nucleotide bases, forming adducts such as 8-hydroxy-2,'-deoxyguanosine (8OHdg), is close to or follows apoptosis (Aitken et al., 2011). The formation of adducts will continue to form single and double strands in sperm nuclear DNA backbone, and ultimately, chromatin cross-

linking (Iommiello et al., 2015). Higher levels of DNA damage have consistently been found in men with abnormal seminology, when compared to healthy normozoospermic men (Wang, 2003; Zhang et al., 2008). More concerning is the coexistence of “normal” with pathology, as some of the normozoospermic men are still experiencing infertility, and their unidentifiable factors are often termed idiopathic (Hamada et al., 2012; Henkel, 2017).

Sperm DNA fragmentation has also been negatively correlated with the percentage of basic semen parameters, viability, quality of the embryo, pregnancy loss, disrupted early development, and therefore neonatal morbidities in both naturally conceived and medically assisted reproduction offsprings (Marchetti et al., 2002; Henkel and Franken, 2011; Sivanarayana et al., 2012; Samplaski et al., 2015; Henkel, 2017). Quantifying DNA fragmentation is further necessitated by the extensive practice of assisted reproductive techniques, in which the natural selection of genetically intact spermatozoa is bypassed, leading to the inadvertent use of oxidatively damaged DNA sperm (Aitken and Koppers, 2011).

2.6.5.1 Sperm DNA fragmentation quantification

DNA fragmentation traditionally does not form part of the routine semen analysis in the andrological diagnostic setup (Cissen et al., 2016). Despite being used less frequently as an indirect tool for quantifying male infertility, sperm DNA fragmentation is gaining considerable attention (Lim et al., 2013). Discrepancies in fertilising tendencies between normozoospermic and oligoasthenoteratozoospermia patients in infertility treatment have led to the appreciation and use of sperm DNA and chromatin integrity assessment techniques (Belloc et al., 2014).

Numerous techniques have been developed for the assessment of DNA fragmentation such as sperm chromatin dispersion (SCD) and sperm chromatin structure assay (SCSA), which detect strand breaks quantitatively (continuous basis)(Larson-Cook et al., 2003; Henkel, 2017). Terminal deoxynucleotidyl transferase-mediated nick ending labelling (TUNEL), on the other hand, is emerging as the golden tool in the diagnosis of unexplained male infertility, as it qualitatively detects single and double breaks (exact ratio of fragmented to non-fragmented sperm DNA), despite a lack of standardisation for human sperm (Sharma and Agarwal, 2011a; Cho and Agarwal,

2017; Ribeiro et al., 2017). TUNEL is based on the enzymatic ability of terminal transferase (TdT) to detect fluoresceinated dUTP single or double-stranded DNA nicks by labelling the resultant free hydroxyl groups (3-OH) (McKenna et al., 1998; Sharma et al., 2016). In these techniques, analysis of DNA fragmented sperm can be done by flow cytometry and fluorescence microscopy (Henkel and Franken, 2011).

2.6.6 Capacitation (CP) and acrosome reaction (AR)

Capacitation (CP) and acrosomal reaction (AR) are indispensable steps in the fertilisation of most mammalian species (de Lamirande, Leclerc, and Gagnon, 1997). CP is a collective term for the physiological and biochemical modifications the sperm undergo as they transit the female reproductive tract to render them competent for fertilisation (O'Flaherty, de Lamirande, and Gagnon, 2006). The oviductal luminal milieu primes sperm for cervical mucus penetration and fusion of sperm with zona pellucida, which in turn activates sperm acrosome for penetration of oocyte vestments (de Lamirande et al., 1997; Badawy et al., 2006). Signalling pathways regulating sperm capacitation and hyperactivation have few similarities, such as all having Ca^{2+} dependency (Visconti et al., 2011; Beltrán et al., 2016). CP is a time-dependent event that depends on lipid composition of the sperm plasma membrane, the redistribution and reorganisation of membrane proteins, ion influxes, and the amount of ROS present (de Lamirande et al., 1997).

An efflux of cholesterol initiates CP in the presence of albumin, which increases membrane fluidity (Ickowicz et al., 2012) (Figure 10). Increase in membrane permeability allows the opening of calcium ion channels (CatSper) and sodium bicarbonate cotransporter protein, which facilitate the influx of Ca^{2+} and bicarbonate (HCO_3^-) ions, leading to a hyperpolarised sperm membrane (Ickowicz et al., 2012; Leemans et al., 2019) (Figure 10). The presence of HCO_3^- increases the activity of soluble adenylyl cyclase, which in turn increases the biosynthesis and the activity of a secondary messenger cyclic adenosine monophosphate (cAMP) (Rivlin et al., 2004; Bailey, 2010) (Figure 10). The formation of cAMP is accompanied by the formation and involvement of ROS, particularly superoxide (de Lamirande and Gagnon, 1995; Jin and Yang, 2016) (Figure 10). Cyclic monophosphate activation induces activation of a protein kinase A (PKA) subunit, which coincides with a whiplash vigorous flagella movement called hyperactivation (Yanagimachi, 1994; Ickowicz et al., 2012). After cAMP-PKA activation, this either inhibits phosphatases or the stimulation of

phosphorylation of kinases (Visconti et al., 1995; Tulsiani and Abou-Haila, 2012; O'Flaherty, 2015) (Figure 10). It has been further demonstrated that ROS also plays a signalling role in downstream phosphorylation of kinases, leading up to CP (Leclerc et al., 1997; Rivlin et al., 2004; O'Flaherty et al., 2006) (Figure 10).

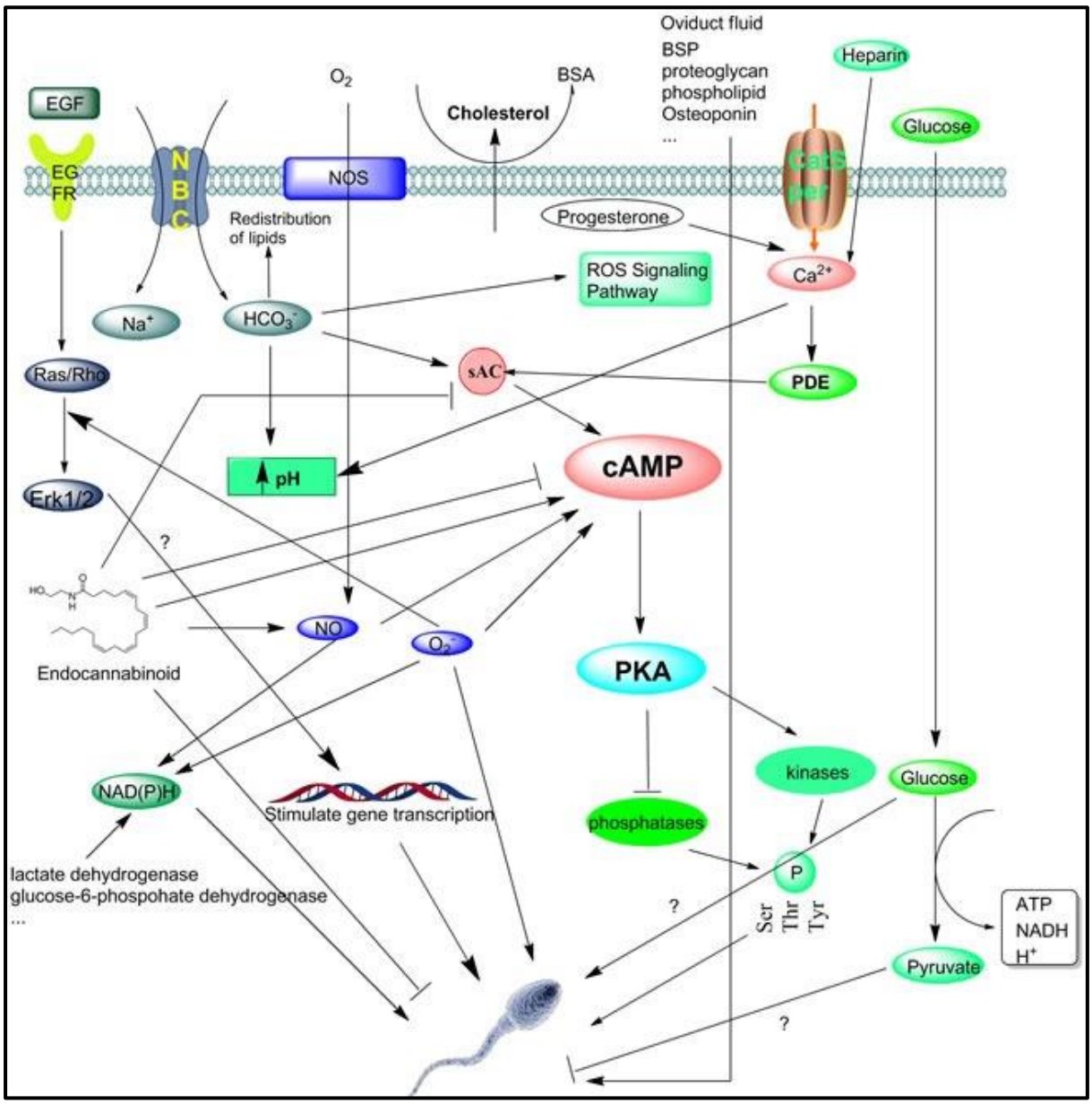

Figure 10: Molecular events involved in the regulation of mammalian sperm capacitation (Jin and Yang, 2016).

The only spermatozoon that underwent capacitation can recognise oocyte signals and undergo AR. An acrosome reaction is an exocytotic event, in which the apical vesicle in the sperm head fuses with the inner plasma membrane to empty acrosomal contents that digest the zona pellucida of the oocyte to facilitate entry into egg

vestments (Yanagimachi, 1994; Fraser, 1998). Hyaluronidase, phospholipase A2 and acrosin are hydrolytic acrosomal contents released first to penetrate the cumulus oophorus, and secondly to disperse the zona pellucida (ZP) of the egg (Tulsiani et al., 1998; C. Esteves and Verza Jr., 2011). Acrosin is a serine protease that not only facilitates protein activation, but clears off steric charges that may hinder egg-sperm recognition and binding at the oolemma (Takano et al., 1993; Cuasnicú et al., 2016).

One of the four ZP glycosylated proteins with sperm binding site is ZP3. It is accepted that the binding of sperm to ZP3 glycoprotein through Gi-receptors triggers a series of events leading up to AR (Fraser, 1998; Tulsiani and Abou-Haila, 2012). Similar to CP and hyperactivation, AR is a ROS-regulated AMP-PKA-dependent signalling pathway that leads to the opening of CatSper ion channels increasing calcium (Fraser, 1998; Sun et al., 2017) (Figure 11). An increase in extracellular Ca^{2+} stimulates an increase in intracellular Ca^{2+} concentration that is crucial for phosphorylation of protein kinase-C (PKC) subunit (Tateno et al., 2013). The phosphorylation of PKC increases membrane fluidity, which in turn mediates phosphorylation of tyrosine kinase (PTK) and eventually AR (Ickowicz et al., 2012) (Figure 11).

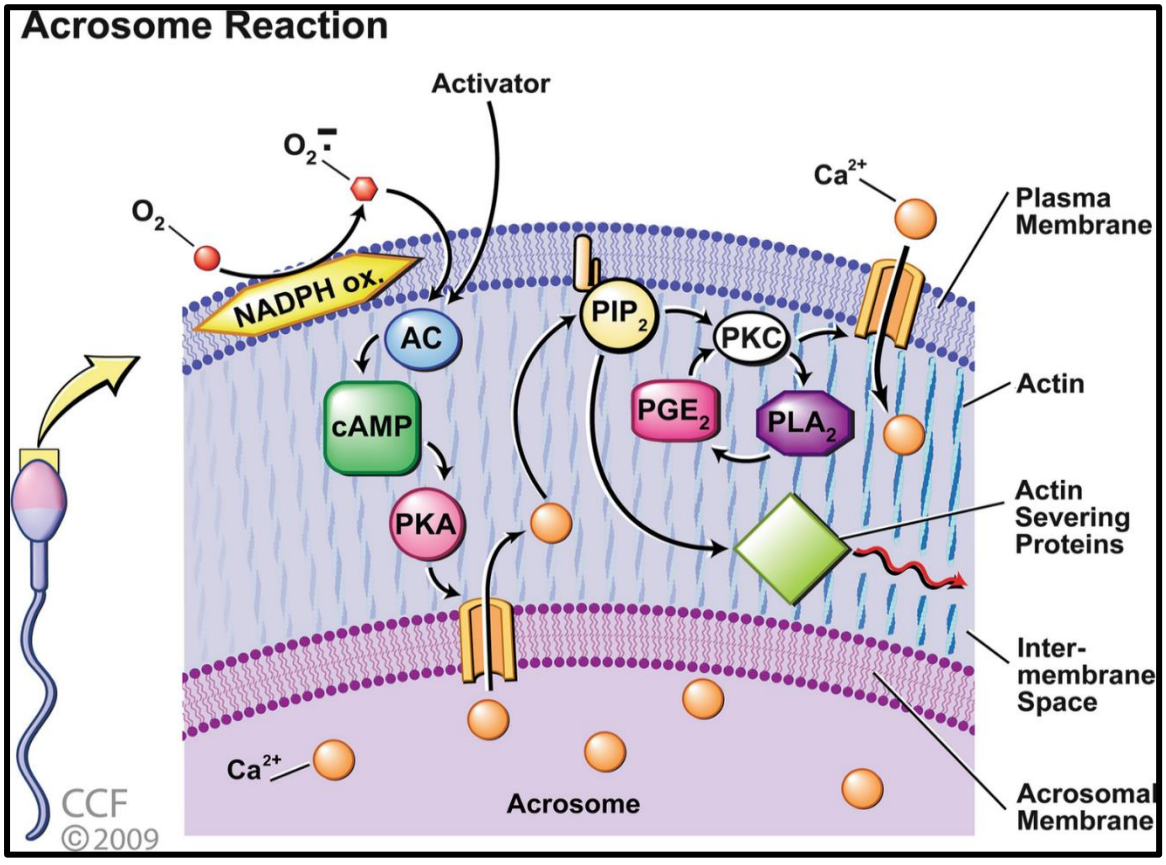

Figure 11: Illustration of molecular events regulating acrosome reaction (Dutta et al., 2020).

2.6.6.1 Quantification of CP and AR

Molecular interactions regulating CP and AR suggest that they are highly coordinated events with complex signalling pathways. Understanding mechanisms that augment CP and AR precedes the development of fertilisation models *in vivo* and *in vitro* (Esteves and Verza, 2011). Therefore, direct or indirect bio-assaying of the two processes as biomarkers of male infertility is essential (Lessard et al., 2011).

Fluorescence labels commonly used in andrology settings are immune-monoclonal localising probes such as antibodies (Grasa et al., 2008), glycan-binding lectins (Xin et al., 2016), and membrane Ca^{2+}-sensitive stains (Gillan et al., 2005). The unbiased labelling using fluorescent dyes in both instances depends on permeability properties of the spermatozoon surface characteristics (Fierro et al., 2013).

Chlortetracycline (CTC) antibiotic has shown to have the highest affinity for bivalent cations such as Ca^{2+}/Mg^{2+}, giving an intense fluorescent emission in living, fixed, or unfixed non-permeabilised cells (Green and Watson, 2001; Pietrobon et al., 2001; Silva and Gadella, 2006). For adjustment of the contrast background ratio of live, membrane-responsive, and acrosome-reacted segments, supravital stains such as Hoechst 33258 or propidium iodide are recommended (Pintado et al., 2000).

Some of the widely accepted methods of quantifying CP and acrosomal patterns are transmission electron microscopy (TEM) (Pesch and Bergmann, 2006), phase-contrast, differential-inferential contrast dyes (Topper et al., 1999), and fluorescent probes (Lee et al., 1987; Fierro et al., 2013). Limitations to analyse only small populations, different culture media of sperm, and tiny acrosomal regions specified against conventional phase-contrast dyes have shifted preference to fluorescence microscopy for evaluation of CP and AR in reproductive biology (Cross and Meizel, 1989; Esteves et al., 2007).

2.7 Infertility

The World Health Organisation passed an operational definition of health as – “the complete state of physical, mental, and social well-being, and not merely the absence of infirmity” in 1948 (Huber et al., 2011). From a reproductive perspective, health includes full utilisation of sexual, reproductive systems, processes, and functions throughout life. In 1994, at an International Conference of Population and Development, the WHO declared that reproductive health implies that people can have

a satisfying and safe sex life, and that they can reproduce and have the freedom to decide if, when, and how often to do so (Jansen, 2007). For an involuntarily childless couple, this state of health is not achievable.

For this reason, the International Committee for Monitoring Assisted Reproductive Technology and the WHO define infertility as the disease of the reproductive system that is characterised by the failure to achieve clinical pregnancy within one year or more of regular unprotected sex in a non-contracepting couple (Zegers-Hochschild et al., 2009). Infertility affects nearly 15 - 20% of couples globally (Agarwal et al., 2015). Infertility − just as any other disease − causes its sufferers a great deal of emotional, physical, social, and financial burden, and mental anguish (Dyer et al., 2004; Chachamovich et al., 2010; Dyer and Patel, 2012). It is worth noting that the consequences of infertility affect women more significantly than men, especially in pronatalist and patriarchal communities (Greil et al., 2011).

Statistically, nearly 85% of couples will fall pregnant within one year of attempting to conceive, and the probability increases to 95% within two years. For couples trying for three or more years to conceive without success, their chances of a successful conception fall below 25% (Eldib and Tashani, 2018). Depending on the length of unprotected intercourse, the female's age and parity, seminal profile, and frequency of intercourse, the couple's infertility status can be classified as either primary or secondary (Anwar and Anwar, 2016).

Primary infertility is diagnosed in cases where nulliparous women have not conceived, but have been in a non-contracepting union for at least five years, while secondary infertility refers to the absence of live birth by women who have previously fallen pregnant, despite the outcome of their previous pregnancy (Mascarenhas et al., 2012; Anwar and Anwar, 2016). Data from Europe, Asia, the Middle East, and Africa suggest that primary infertility is less common than secondary infertility (Mascarenhas et al., 2012; Inhorn and Patrizio, 2015). Sub-Saharan African countries record the unusually high prevalence of infertility of about 30% that coexists with high fertility rates, a phenomenon that is termed "barren amid plenty" (Cui, 2010; Inhorn and Patrizio, 2015). Persistently high infertility rates observed in the region stretching from central Africa to the southern parts of Africa were collectively referred to as the infertility belt (Collet et al., 1988; Inhorn, 2003; Ombelet and Onofre, 2019).

Most of infertility inferential data derives from gynaecological investigations, mainly because women are typically blamed for the inability to have children in marriage (Mohammed-Durosinlorun et al., 2019). In many normative African countries, social status, security, and identity of women is determined or strongly associated with the parity (Dhont et al., 2010; Mumtaz et al., 2013). To apply further pressure and blame on women, in the event of childlessness, men are often protected from the public scrutiny either by remarriage or having a close family member impregnating the wife (Mumtaz et al., 2013; Agarwal et al., 2015). Therefore, the socio-cultural values of infertility have shaped the differences in infertility treatment-seeking behaviour.

The exclusion of men from infertility discourse in some regions does not rule out their contribution. Males independently contribute 20-40% of global cases of infertility, a percentage that is similar to that found among females, while some causes are shared between the two partners (Agarwal et al., 2015; Kumar and Singh, 2015) (Figure 12). About 90% of all male infertility cases are attributed to abnormal semen parameters, specifically total count and concentration. Infertility can be complete or partial, while the latter manifestation is termed subfertility (Zegers-Hochschild et al., 2009). The causes of infertility can be extrinsic such as environmental pollutants, diet, smoking and drinking, medication, and including but not limited to recreational drugs (Braga et al., 2012; Xiao et al., 2014; Anwar and Anwar, 2016; Sharma, 2017). Intrinsic factors can be congenital, medical or result from cryptorchidism, varicocele, genetic disorders, endocrinopathy, infectious diseases, obstruction, metabolic diseases, and immunological factors (Anwar and Anwar, 2016; Opuwari et al., 2017).

Subfertility or infertility can be caused by the absence of sperm (azoospermia), low sperm concentration (oligozoospermia), low sperm motility (asthenozoospermia), abnormal sperm morphology (teratozoospermia), and reduced sperm vitality (necrozoospermia), or a combination of the first three abnormalities (oligoasthenoteratozoospermia) (Guzick et al., 2001; Agarwal and Sekhon, 2011; Aziz, 2013; Sharma, 2017). Deterioration of semen quality can originate or be influenced by pre-testicular, testicular, and post testicular factors (Iwamoto, Nozawa and Yoshiike, 2007).

Secretions from vesicles, prostate, and epididymis are indirectly but strongly linked to unknown causes of infertility (Sharma, 2017). When these factors merge to complicate

the aetiology and pathogenesis of infertility, the type of infertility is termed idiopathic (Hamada et al., 2012). Idiopathic infertility is diagnosed when the cause is unidentifiable; this diagnosis has been reported to affect 30% of couples globally (Sharma, 2017). At the core of idiopathic cases is an excess of ROS production. Oxidative stress has been found to play an independent role in about 30–80% of unexplained causes of infertility (Agarwal et al., 2019).

Idiopathy in male infertility evaluation makes therapy even more difficult. Depending on the cause, treatment of infertility can be non-surgical or surgical (Hall, 2014). Surgical methods may include orchidopexy, varicocelectomy, and testicular sperm aspiration (Hanerhoff and Welliver, 2014; Barekat et al., 2016). These interventions correct anatomical deformities impeding sperm production, transit, or functionality (Lok, Ledger, and Li, 2003; Goldstein and Schlegel, 2013). Non-surgical interventions include hormonal therapy such exogenous human chorionic gonadotropin (hCG), FSH, testosterone, dopamine agonist, oral corticoids, phosphodiesterase inhibitors, and antioxidants (Chehval and Mehan, 1979; Cocuzza and Agarwal, 2007; Henkel, Sandhu, and Agarwal, 2019). Empiric therapies can be used in conjunction with assisted reproductive technologies (ART) (Liu, 2003). The International Committee for Monitoring Assisted Reproductive Techniques (ICMART) (2009) and the WHO defined ARTs as all treatments or procedures that include *in vitro* handling of human oocyte and sperm or embryo to establish a pregnancy. These techniques include *in vitro* fertilisation (IVF), intracytoplasmic sperm injection (ICSI), gamete intrafallopian transfer (GIFT), embryo transfer, zygote intrafallopian transfer, embryo cryopreservation, oocyte and embryo donation, and gestational surrogacy (Zegers-Hochschild et al., 2009). These techniques have made a remarkable contribution to infertility management. However, they are not readily accessible, accepted, or affordable for and by the majority of people who wish to conceive / have a child. For this reason, alternatives have been sought.

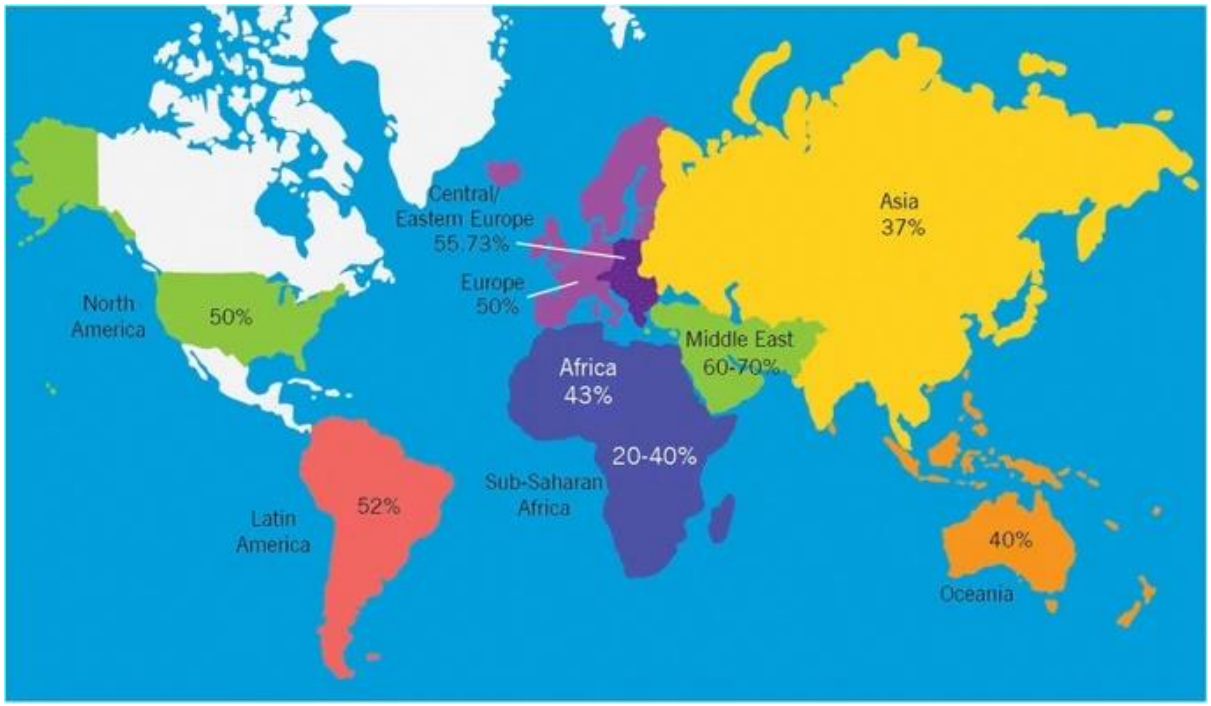

Figure 12: Demographic illustration of global male factor infertility (Agarwal et al., 2015)

2.8 Herbal medicine

Traditional medicine (TM) refers to the sum-total of knowledge, skills, and practices based on beliefs, theories, and experiences indigenous to different cultures, whether explicable or not, and used for the maintenance of health as well as in the prevention, diagnosis, improvement, or treatment of mental or physical illnesses (Mothibe and Sibanda, 2019). Traditional medicines are usually preferred by people believing in their efficacy for being holistic in that they take a person's environment, and mind-body interactions into consideration as opposed to their orthodox medicinal counterparts that are mainly palliative (Roberti di Sarsina, 2007). Central to the holistic methods of healing are herbs (Wachtel-Galor and Benzie, 2011). Herbal medicines may be herbal materials, herbal preparations, or finished herbal products (Mothibe and Sibanda, 2019).

The latter form of herbals provides the raw material for the mass synthesis of pharmaceutical drugs. These conventional / Western medicines have revolutionised the health care system in most parts of the world (Wachtel-Galor and Benzie, 2011). Conventional medicines remain readily recognised as the official form of health care, because they are scientifically proven (Hardy, 2008), while the preference for TM

among a growing number of people across the globe is mostly based on personal positive experience, affordability and accessibility. The evidence-based efficacy of allopathic medicine is most effective in alleviating symptoms and providing a response in emergencies (More, 2016). Allopathic medicine has made breakthroughs in infectious diseases, vaccines, surgery, imaging, organ transplanting, genetics, and proteomics, and sophisticated disease detection technologies, to name a few (Weatherall et al., 2006; Barry et al., 2009; van Rooyen et al., 2015). As the population numbers grow, pathology progresses, and the disease burden overwhelms even the most capacitated health care systems in the world (Singh, 2010). When conventional medicine is insufficient in meeting the rising public health care needs, natural remedies can compensate effectively. Integration of complementary medicine into day-to-day health care provision has therefore gained considerable attention (Bodeker and Kronenberg, 2002; Lampiao et al., 2019).

The inclusion of herbal therapies in everyday healthcare has seen an escalation in trade and export, in a regulatory framework, and research activities geared towards their use (Figure 13). As a result, the use of herbal medicines is now a global phenomenon with nearly 4 billion of the world population (making 80%) relying on them for their primary health care needs (Ekor, 2014). Even progressive nations such as selected parts of Europe (van Andel and Carvalheiro, 2013), the US (Rashrash et al, 2017), and China (McQuade et al., 2012) use traditional natural medicine. The dependence on ethnobotanical medicine increases proportionately with its affordability, accessibility, proximity, sociocultural influences, availability, and lack of possible alternatives in middle- and low-income countries (Abdullahi, 2011; Antwi-Baffour, 2014; Semenya and Potgieter, 2014; Gyasi et al., 2016). In Africa, about 90% of the Ethiopian population primarily uses herbal medicines, and traditional practices to diagnose and treat ailments (Abay, 2009). South Africa reports a similarly high support of herbal medicines, where 80% of South Africans consult a traditional practitioner for the restoration of their well-being before resorting to biomedical medicines (van Rooyen et al., 2015). In 2007, trade in traditional medicines yielded nearly R2.9 billion a year, contributing to 5.6% of the National Health budget (Mander et al., 2007). Additionally, in 2014, it was documented that nearly 35,000–70,000 of plants were harvested for medicinal use (Xego et al., 2016). It has to be noted that the

use of herbal medicine is regulated under the Traditional Practitioners Health Act 22 of 2007 as promulgated in the Government Gazette (Tshehla, 2015).

South African traditional practitioners are often consulted for acute and chronic health conditions (van Rooyen et al., 2015). Common reasons of visitations to the traditional healer include fever, headache, burns, colic, cough, skin problems, diarrhoea, needing a tonic, stomach complaints, dysentery, and parasitic, bacterial and viral infections (Van Wyk, 2011; Mahomoodally, 2013; Ganjhu et al., 2015). The application or use of herbal medicines in various South African provinces is not limited to minor conditions; traditional healers are also involved in managing the symptoms of non-communicable chronic conditions and diseases such as cardiovascular diseases, neurodegenerative diseases, diabetes, cancer, and sexual and reproductive disorders (Semenya and Potgieter, 2014; Odeyemi and Bradley, 2018; Rasethe et al., 2019).

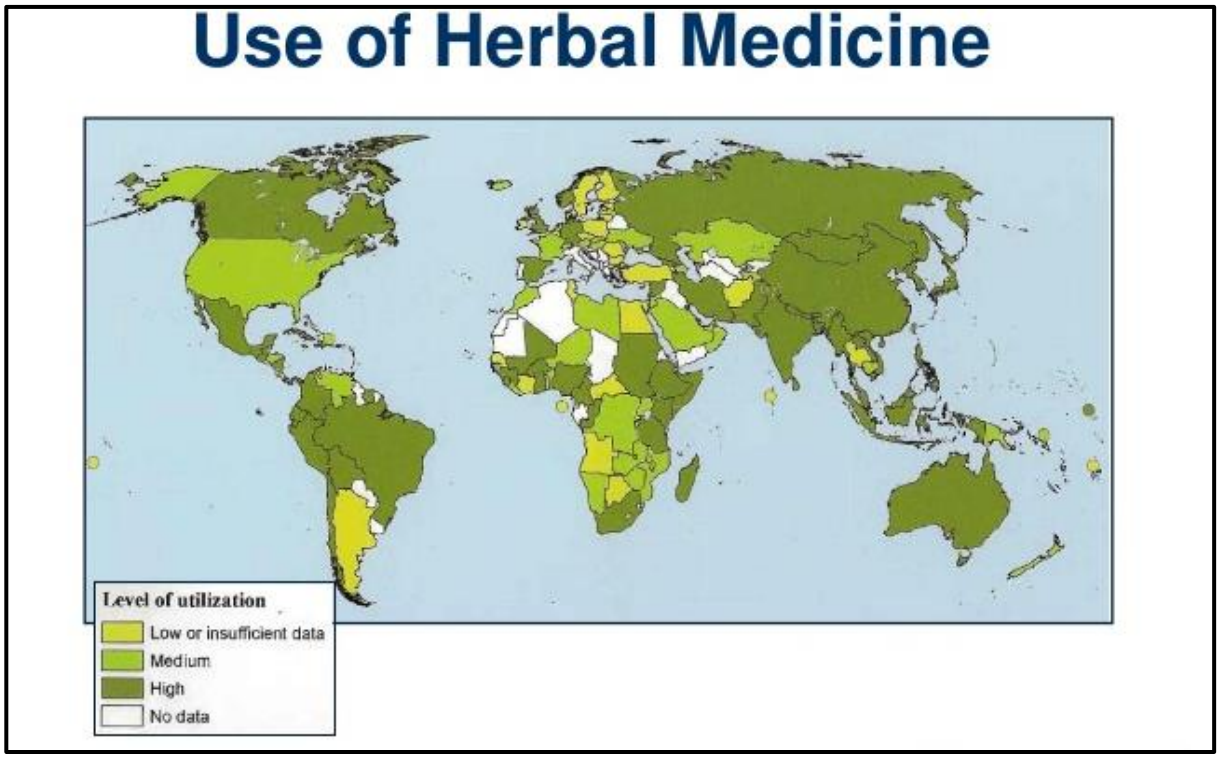

Figure 13: Schematic illustration of global dependence on herbal medicine (Bodeker et al., 2005).

2.8.1 Herbal medicine and male infertility

Natural remedies have gained considerable attention in male infertility management around the world. Different countries have traditional means of improving sexual and reproductive competence that may not form part of the mainstream or conventional

modern-day medical standards. These natural modalities are indicated for infertile men presenting with impotence, hormonal deficiencies, impaired sexual drive, behaviour, and performance, and poor semen (Yao and Mills, 2016). For instance, in India, the Ayurvedic system is one of the oldest forms of using holistic medicine in the world (Chauhan et al., 2014). In terms of male infertility, there exist Ayurvedic Vajikarana (aphrodisiacs) herbs such as Withania somnifera (Ashwagandha or the Indian ginseng), Asparagus racemosus (Shatavari), Cannabis sativa (marijuana), and Mucuna pruriens (velvet bean), which have been used for the enhancement of ejaculatory function, libido, spermatogenesis, and semen quality (Whan et al., 2006; M. Ahmad et al., 2008; Thakuret al., 2009; Dutta and Sengupta, 2018). Traditional Chinese Medicine (TCM) has also proven to be useful in augmenting hypothalamic pituitary testicular function, testosterone production, boosting of seminal antioxidants, and semen parameters in men with idiopathic infertility (Chen et al., 1999; Liu et al., 2004; McQuade et al., 2012; Zhou et al., 2019). Most herbs are found in more than one natural medicine system.

Extracts of *Lycium barbarum* (matrimony vine / wolfberry) has demonstrated pro-fertility effects in both animals and humans (Luo et al., 2006; Wang et al., 2018). Some of the TCM herbs proven to ameliorate male infertility are *Cuscuta chinensis* (dodder) (Jo et al., 2015), *Epimedium brevicornum Maxim* (horny goat weed) (Abdelaziz et al., 2020) and *Panax ginseng* (Asian ginseng) (Salvati et al., 1996).

The African natural medical system comprises approximately 50,000 plant species, 25% of which are readily accessible for ethno-medicinal use, especially in sub-Saharan countries (Iwu, 2014). Because of the limited health care amenities in Africa for the detection and treatment of male infertility, reproductive issues are the second most common health care burden (Gerrits and Shaw, 2010; Idu et al., 2016. Common sexual reproductive complaints reported to the traditional healers by males in African countries include a low sex drive, STDs, erectile and ejaculatory dysfunction, and a low sperm count (Abdillahi and Van Staden, 2012; Agarwal et al., 2015; Bechoua et al., 2016).

An ethnomedicinal survey that was conducted in Nigeria revealed several species such as *Aloe vera* (aloe), *Sesacum indicum* (sesame seed), *Terminalia catapa* (Indian almond), *Allium sativum* (garlic), and *Piper guineense* (Guinea black pepper), but this

list was not limited to *Afromomum melegeuta* (Alligator pepper), where the species were prescribed and prepared to be used as aphrodisiacs, fertility tonics, and sperm enhancers (Yakubu and Afolayan, 2009; Singh et al., 2010; Lawal et al., 2013; Idu et al., 2016). Most of the reports on the native medicines used in African traditional practices, stated that although they were putatively therapeutic, they still remained to be scientifically validated for practical use. *Moringa oleifera* is one of the most extensively used plants with acclaimed healing properties, most of which had unverified.

2.9 *Moringa oleifera*

2.9.1 Physical characterisation

Known colloquially as the horseradish (owing to the root taste), drumstick (due to the shape of its pods) or Ben tree oil, *M. oleifera* is a medium-sized deciduous tree that proliferates to reach a height average of 5-10 m (Fahey, 2005; Shih et al., 2011) (Figure 15 d). It has a softwood perennial trunk with whitish-grey gummy and corky barks (Fahey, 2005; Farooq, 2012; Leone et al., 2016). The branches appear delicate and are widespread (Parrotta, 2014). Its green tripinnately-shaped leaf compounds hold small leaflets that appear yellowish or white (Amabye and Tadesse, 2016) (Figure 15 a and 15 e). *M. oleifera's* mature fruit (pods) is about 50 cm long, carrying 50 ovoid or triangular seeds that are wrapped in a papery sheath (Vlahov, Chepkwony, and Ndalut, 2002; Taher et al., 2017; Salama et al., 2020) (Figure 14 a, 14 b, 14 c and 14 f). All parts of the plant are used for nutritional and medicinal purposes.

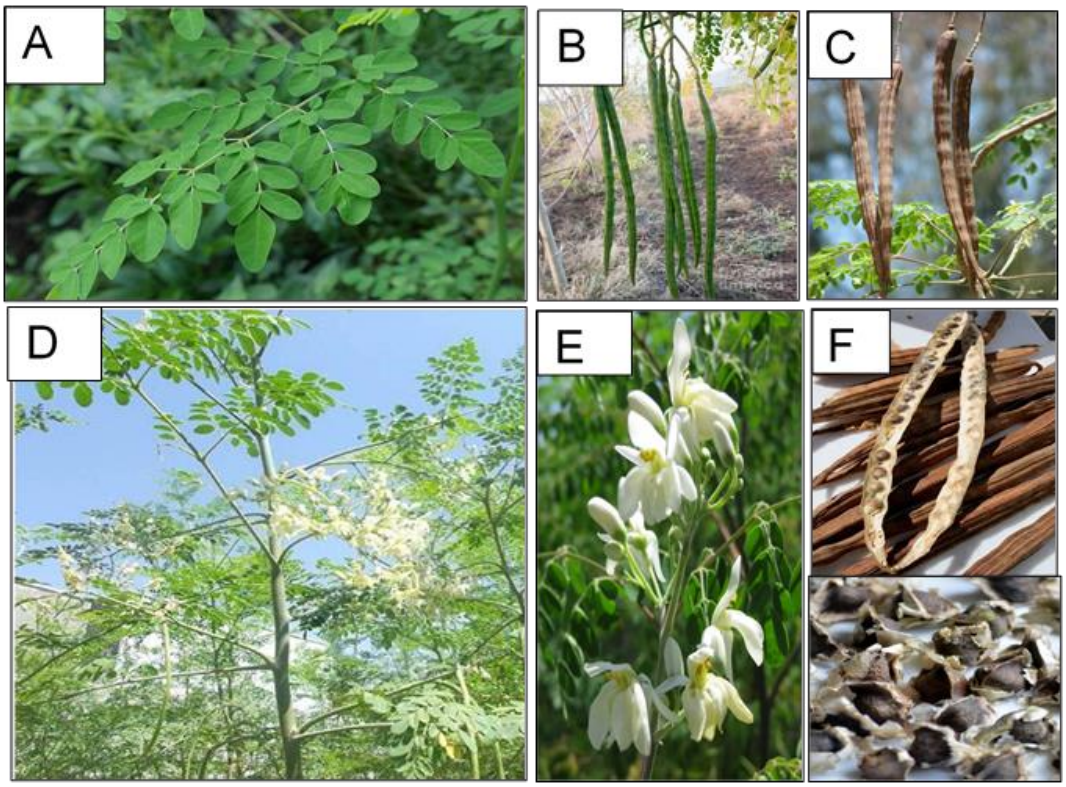

Figure 14: *M. oleifera* aerial parts*:* leaves (**A**), pods or fruit (**B** and **C**), tree (**D**), flowers (**E**) and seeds (**F**). (https://www.freshlymoms.com/blogs/news/moringa-benefits-for-new-mothers).

2.9.2 Distribution

Moringaceae is a monotypic family of order *brassicales,* comprising 33 species; and 14 of them have wide medicinal, nutritional, industrial, and economical applications, earning its popularity as “the miracle tree of life” (Arora et al., 2013). *M. oleifera* is the most versatile, widely accepted, available, and studied species of the genus *Moringa*. *M. oleifera* is native to sub-Himalayan countries; India, Bangladesh, Pakistan, and Afghanistan (Verdcourt, 1985). Because of its high adaptability to grow in various weather conditions, *M. oleifera* is now distributed and naturalised in various parts of the world (Farooq, 2012). It grows well in arid, tropical, and subtropical regions of Central, North and South America, in Nepal, the Philippines, the Pacific Islands, the Caribbean, and in Africa (Morton, 1991; Coote et al., 1997; Alegbeleye, 2018). In Africa, Ghana, Malawi, and Ethiopia are the largest producers of *M. oleifera,* while the South African farmers have also taken an interest in the plant (Pakade et al., 2013).

In South Africa, *M. oleifera* is grown mostly in Limpopo, Mpumalanga, KwaZulu-Natal, the Free State, and Gauteng (Mabapa et al., 2017).

2.9.3 Phytochemistry and pharmacological properties

M. oleifera is enriched with essential nutrients, micro and macro elements, and antioxidant phytochemical constituents. Elemental analyses of *M. oleifera* indicated the presence of zinc, magnesium, potassium, calcium, sulphur, iron, selenium, manganese, and copper, which are integral to the preventative and curative role in pathogenesis (Kasolo et al., 2010; Valdez-Solana et al., 2015; Dilawar et al., 2018). Mineral elements are mainly found in the diet, and their importance in cell-signalling, functioning, and maintenance, while metabolism, detoxification, and oxidative stress-related pathologies have been extensively demonstrated (Soetan et al., 2010; Siddiqui et al., 2014). *M. oleifera* leaves exhibit antidiabetic (Sugunabai et al., 2014), anti-carcinogenic (Tiloke et al., 2013), antibacterial (Amabye and Tadesse, 2016), antihypertensive (Acuram et al., 2019), neuroprotective (Sutalangka et al., 2013), anti-inflammatory (Mittal et al., 2017), and antioxidative properties (Tesfay et al., 2011; Abdelazem, 2019).

M. oleifera leaves are a source of simple and complex carbohydrates such as glucose, galactose, arabinose, mannose, raffinose, fructose, sucrose, and xylose, which can serve as substrates for cellular fuel metabolism (Tesfay et al., 2011; Chen et al., 2017). Except for methionine, *M. oleifera* leaves have been found to contain all essential and non-essential amino acids that play a role in protein biosynthesis in biological systems (Makkar and Becker, 1996; Sánchez-Machado et al., 2010). Furthermore, leaves of *M. oleifera* have variable compositions of polyunsaturated, monounsaturated, saturated and fatty acids such as oleic, arachidonic, stearic, linolenic and palmitic acid, to name a few (Castillo-López et al., 2017). *M. oleifera's* phytochemicals, therefore, play a role in enzyme synthesis and metabolism (Leone et al., 2016; Saini, Sivanesan, and Keum, 2016). The proximate nutritional analysis further confirmed the high fibre content in *M. oleifera* leaves (Valdez-Solana et al., 2015).

Vitamins A, B, C, and E have been identified in *M. oleifera* leaves (Fahey, 2005). Vitamin A (β-carotene) is crucial for cell proliferation, reproduction, and embryonic development (Kidmose et al., 2007; Vergara-Jimenez et al., 2017). Vitamin B complexes found in *M. oleifera* include biotin, folate, nicotinic acid, thiamine, riboflavin,

and pyridoxine (Mbikay, 2012). Vitamin C (ascorbate) in *M. oleifera* is involved in antioxidative protection of cell membranes from peroxidative damage through radical scavenging activity (Fahey, 2005; Sankhyan et al., 2013; Gopalakrishnan et al., 2016; Vergara-Jimenez et al., 2017). Vitamin E (α-tocopherol) is a chain-breaking non-enzymatic antioxidant that plays a stabilising role in the redox regulation status of biological systems through the interference of ROS production (Ajantha et al., 2018). Vitamin deficiencies are often associated with oxidative stress (Ryan et al., 2010).

Also, alkaloids, tannins, saponins, flavonoids, and phenolic compounds with proven antioxidative potential have been isolated and characterised from *M. oleifera* leaves (Valdez-Solana et al., 2015; Gopalakrishnan et al., 2016). Flavonoids identified in *M. oleifera* include quercetin, myricetin, rutin, kaempferol, catechin, isothiocyanates, (Saini et al., 2016). These metabolites boost *M. oleifera's* biological activities such as steroidogenesis, and antimicrobial, anticlastogenic, antitumor, and antiradical activities (Leone et al., 2016). Caffeolic, ferulic, chlorogenic and gallic acids are some of the versatile phenolic acids identified in the *M. oleifera* leaves, and possess free radical scavenging ability or activity (Valdez-Solana et al., 2015; Castillo-López et al., 2017; Lin et al., 2019). The bioavailability of *M. oleifera's* bioactive compounds depend on extraction methods, solvents used, and extract storage.

2.9.4 Previous studies of *M. oleifera* on mammalian reproductive function other than sperm functioning

Reproductive development, function and competency, including fertility, are strictly functions of hypothalamic-pituitary-gonadal (HPG) axis (Dwyer and Quinton, 2019). Therefore, male infertility may occur at any of the three fertility regulating points; neurohormonal imbalances, impairment in reproductive organs and glands, compromised sexual behaviour / activity, and abnormal semen quality and quantity (Dutta and Sengupta, 2018). As a result, medicinal plants or their extracts exert their effects directly or indirectly at any of the sites of interaction mentioned above (Nudell et al., 2002). Assessments of *M. oleifera* on mammalian male reproductive enhancement and aphrodisiac activity have yielded affirmative results (Zade et al., 2013).

2.9.4.1 M. oleifera and hypothalamic regulation of mammalian reproduction

The neuromodulatory effects of *M. oleifera* have been studied in rats (Ray, Hazra, and Guha, 2003; Ray et al., 2004; Abdel-Rahman Mohamed et al., 2019). Concerning the regulating effects of the plant on sexual activities, *M. oleifera* aqueous, alcohol, and chloroform extracts at concentrations of 100, 200, and 500 mg/kg caused significant increases in mounting and intromission frequencies, and ejaculation latency with decreased mounting latency in male albino rats (Zade et al., 2013). In another study, *M. oleifera* attenuated sexual dysfunction in male Wistar rats through inhibition of cleaving enzymes monoamine oxidase type B (MOAB) and phosphodiesterase (PDE-5), thereby increasing mounting and intromission numbers (Prabsattroo et al., 2012). Similarly, libido and sperm parameters of Bali bulls increased significantly in the group supplemented with *M. oleifera* leaves (Syarifuddin et al., 2017).

2.9.4.2 M. oleifera and pituitary regulation of mammalian reproduction

An *in vivo* study showed beneficial effects of *M. oleifera* supplementation on male rabbits, where serum gonadotropin levels (FSH and LH) were dose-dependently increased (Nwamarah et al., 2015; Ajuogu et al., 2019). To further indicate the importance of the variabilities in the *M. oleifera* aerial parts and their toxicities on FSH, LH, testosterone biosynthesis and availability, another *in vivo* study was conducted (Prabsattroo et al., 2012). Similar increases in gonadotropin and testosterone, and a seminal counts increase were noted after *M. oleifera* ingestion. In the same study, *M. oleifera* seeds, flowers, and roots showed contradicting adverse effects on fertility indices of adult male Wistar rats (Ogunsola et al., 2017). A similar study was carried out to assess hyperglycemia-induced reproductive dysfunction in male Sprague Dawley after *M. oleifera* administration, and the findings revealed a significant decrease in glucose levels, accompanied by pronounced improvement in seminal parameters and testicular histological appearance (Kamalrudin et al., 2018).

Still, on the pituitary regulation, another remarkable effect of *M. oleifera* in male fertility was the significant reduction in serum prolactin levels with increased gonadotropin levels in male albino rats (Harrison et al., 2016). The latter observation was indicative that *M. oleifera* could potentially relieve hyperprolactinemia-induced male infertility.

2.9.4.3 M. oleifera and testicular regulation of mammalian reproduction

Studies on testicular effects of *M. oleifera* revealed that the plant affects steroidogenesis (mainly testosterone), spermatogenesis, the architecture of seminiferous tubules and Sertoli cells, and seminal antioxidants activities.

Serum testosterone synthesis and availability were significantly increased in male rats and rabbits in these studies after treatment with *M. oleifera* (Ebong et al., 2014; Prabsattroo et al., 2015; Harrison et al., 2016; Suarni et al., 2019). Testicular weights, rate of spermatogenesis, density and diameter of seminiferous tubules, volume and hyperactive Leydig cells were improved in bucks and rats administered with aqueous leaf extracts of *M. oleifera* (Cajuday and Pocsidio, 2010; Walaa et al., 2015). Chemically-induced histopathologies in rat testes were dose-dependently reversed following *M. oleifera* administration (Bassey et al., 2013; Sadek, 2014; Bin-Meferij and El-kott, 2015; Abarikwu et al., 2017). *M. oleifera* leaves significantly increased seminal SOD, CAT, and glutathione peroxidase in rats' testes (Afolabi et al., 2013; Saalu et al., 2011; Zeng et al., 2019).

CHAPTER 3

RESEARCH METHODOLOGY

3.1. Introduction

This chapter outlines the components of the methodology, the study site (herbal extraction, semen collection, and *in vitro* experiment), sampling, apparatus utilised, testing protocols, statistical analysing tools, and ethical considerations observed to investigate the aim of the study.

3.2 Research Methods

3.2.1 Methods and materials

3.2.1.1 Chemicals and apparatus

Unless otherwise stated, all chemicals and laboratory consumables were purchased from Sigma Aldrich.

3.3 Study design

This study followed an experimental design, in which the effect of the independent or causal variable (*M. oleifera* aqueous leaf extract concentration(s) was / were manipulated to objectively measure dependent variable(s) or outcomes, referring to sperm functional parameters). Semen samples were collected from fertile donors (unproven fertile) and patients (unproven infertile), liquefied for 30 min at 37˚C, washed off of the seminal plasma using Human Tubular Fluid (HTF) supplemented with 1% bovine serum albumin (BSA) through centrifugation at 300 x *g* for 10 min.

3.4 Sampling

3.4.1 Study sites

Fresh *M. oleifera* leaves were harvested from the University's experimental farm upon receipt of permission. Leaves were dried and ground at the School of Agricultural and Environmental Sciences, Department of Plant Production, Soil Sciences and the Agricultural Engineering Unit at the University of Limpopo. Extraction of the leaves was carried out in the Department of Biochemistry, Microbiology and Biotechnology's laboratory, University of Limpopo.

The Reproductive Biology unit, Department of Obstetrics and Gynaecology (Figure 15), of the Tygerberg Academic and Vincent Pallotti Hospitals were the site for sperm

sample collection, being a specialized centre for assisted reproduction technology treatment and clinical management of patients presenting with normo-, oligo- and / or asthenozoospermia. Also, the University of Western Cape was used as a site for fertile sperm collection from the comparative spermatology programme in the Department of Medical Biosciences and the *in vitro* experiment was conducted in this department in the University.

The *in vitro* experiment was conducted in the Department of Medical Biosciences, University of the Western Cape.

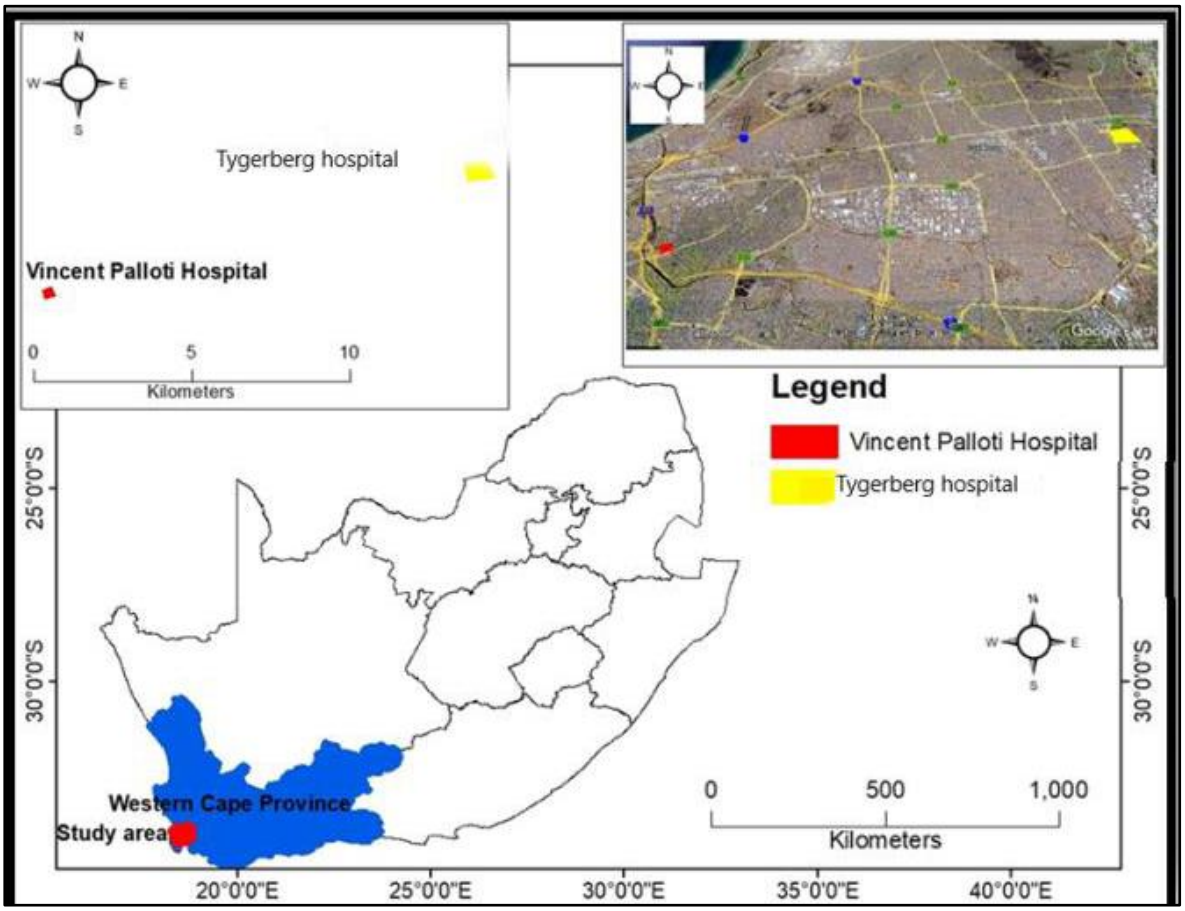

Figure 15: Depiction of the geographical locations where semen samples were obtained.

3.4.2 Study population

In this study, the population comprised male patients aged 18–45 years. These men presented with various cases of infertility, and routinely sought assisted reproductive treatment at Tygerberg and Vincent Pallotti Hospitals; it also included donors in a

comparative spermatology group in the Medical Bioscience Department of the University of the Western Cape.

3.4.3 Sampling procedure

Participants were sampled by convenience based on their convenient accessibility, availability, willingness, and eligibility to the research objectives.

In this study, the population comprised male patients (unproven infertile) aged 18–45 years. These men presented with various cases of infertility, and routinely sought assisted reproductive treatment at Tygerberg and Vincent Pallotti Hospitals; it also included donors (unproven fertile) in a comparative spermatology group in the Medical Bioscience Department of the University of the Western Cape. The donor and patients groups were subsequently combined and analysed separately as a “combined group”. The combined group was included and analysed to firstly, determine the cumulative (overall) effect of the plant extracts on semen parameters. Secondly, the inclusion of the combined group underpins the phenomenon that normal coexist with pathology or idiopathy or unexplained infertility. In unexplained cases, normozoospermic men are still incapable of achieving pregnancy while in idiopathic cases, seminal quality is compromised without any detectable cause. For these reasons, the introduction of the “combined group” was aimed at measuring the overall potency of the plant in defective aspects of “normally functional sperm” such as as excess ROS.

3.4.3.1 Inclusion and exclusion criteria

Inclusion: Normozoospermic men whose seminograms conformed to the World Health Organization’s cut-off values were included in the study. Men whose samples with concentration ≤ 15 million sperm / ml (oligozoospermia) or progressive motility < 32% and / or total motility < 40% (asthenozoospermia) (WHO, 2010) from the patient group were included in the study but were analysed separately.

Exclusion: Participants with low semen volume (<1 ml) were excluded, irrespective of the sperm concentration. Azoospermic samples were also not part of the study. Their samples were discarded as biohazardous waste.

3.5 Data collection

3.5.1 Preparation and extraction of *M. oleifera* leaves

Following their harvest, fresh leaves were allowed to dry for seven days at room temperature. The dried material was ground on the laboratory bench, using a typical grinder at the University of Limpopo. For the preparation of extract, 100 g of *M. oleifera* leaf powder was decocted in 1 L of distilled water for 20 min at a moderate heat. The average extract yield recovered was 25% (w/w). The mixture was decanted and filtered using cheesecloth, followed by centrifugation at 800 x *g* for 10 min at room temperature (Shunmugam, 2016). A further filtration was done using Whatman filter paper (No. 4 and 1), and the filtrate was stored overnight at 4˚C, followed by -80˚C, before freeze-drying (lyophilisation). The dried extract yielded 24.52% (w/w) on average and was stored at -20˚C for further experimental use.

3.5.1.1 The rationale for the determination of therapeutic dose

The calculations for the concentrations were based on the previous assumption that an average healthy man weighs approximately 80 kg (Erasmus et al., 2012). Type 2 diabetes mellitus treatment-naïve patients in a prospective randomised placebo study were administered 500 mg of *M. oleifera,* encapsulated into tablets, for two weeks (Taweerutchana et al., 2017). The therapeutic dose from this study was inferred because of lack of congruent reporting on the prescription of *M. oleifera* leaves to males by practitioners of traditional medicine. Based on the preceding data, the following calculation was used to determine the therapeutic dose:

Since 1 kg = 1 L = 1000 ml

Therefore, conversion of 80 kg (weight of an average man) to *in vitro* dilutions gives:

80 kg = 80,000 ml (*in vivo*)

$$X\ (\text{therapeutic concentration}) = \frac{(\text{estimate traditional dosage})}{(\text{average weight of a man in ml})}$$

$$X\ (\text{therapeutic concentration}) = \frac{500\ \text{mg}}{80\ 000\ \text{ml}}$$

$$\text{X} = \frac{1\ \text{mg}}{160\ \text{ml}}$$

$$\text{X} = 6.25 \times \frac{10^{-3}\text{mg}}{\text{ml}}$$

$$X = 6.25\ \mu g/ml$$

A stock solution containing 62.5 mg/ml of *M. oleifera* in HTF-BSA was prepared, and serial dilutions were performed to obtain final working concentrations of 0, 0.625, 6.25, 62.5, and 625 µg/ml of *M. oleifera.*

3.5.1 Experimental procedures

The *in vitro* experiment was conducted in the Department of Medical Biosciences, University of the Western Cape.

3.5.2 Preparation of culture medium and semen

3.5.2.1 Preparation of Human Tubular Fluid (HTF)

The HTF medium was formulated based on the WHO (2010) prescriptions. Accordingly, HTF-BSA was prepared from 5.931 g NaCl, 0.35 g KCl, 0.05 g $MgSO_4.7H_2O$, 0.05 g KH_2PO_4, 2.1 g $NaHCO_3$, 0.5 g D-glucose, 0.036 g sodium pyruvate, 0.3 g $CaCl_2.2H_2O$, 4.0 g sodium DL-lactate (60% (v/v), 10 µg phenol red, and 20 mmol/l HEPES in distilled water. The medium was prepared under aseptic conditions. The human tubular fluid was supplemented with 1% of bovine serum albumin (BSA).

3.5.2.2 Determination of HTF-BSA volume

The amount of HTF-BSA and used to wash the semen after liquefaction was determined from baseline semen concentration and volume, the number of parameters tested, and the following formula was used:

$$\frac{7.5\ million\ /ml}{Sperm\ concentration\ (\frac{million}{ml})}\ x\ No\ of\ parameters\ tested$$

$$= Semen\ volume\ (ml)\ x\ No\ of\ parameters\ tested$$

$$= Volume\ of\ HTF - BSA\ (ml)$$

3.5.2.3 Source and preparation of semen

Following the receipt of informed consent by participants (Appendix 4, 5, and 6), semen samples were collected in sterile vials from 70 participants by masturbation after 3-5 days of abstinence. Samples were incubated at 37°C for 30 min for liquefaction upon 1 hr of acquisition. After that, 2 µL of liquefied semen was placed on pre-warmed Leja slides® (Delfran Pharmaceuticals, Cape Town) and Sperm Class Analyzer® (SCA, version 6.4: Microptic Barcelona, Spain) to determine sperm concentration. Simultaneously, the time it too semen to fill the chamber was recorded using a stopwatch as a measure of viscosity (sec).

The motile sperm was recovered and reconstituted in human tubular fluid, supplemented with 1% of BSA (1:5), centrifuged at 300 x *g* for 10 min, and the pellet was re-suspended in a fresh HTF-HSA. Subsequently, sperm suspension was exposed to increasing concentrations of aqueous extract of *M. oleifera* (0, 0.625, 6.25, 62.5, and 625 µg/ml) for 1 hr at 37^{o}C, and various sperm parameters were analysed as indicated in Figure 16.

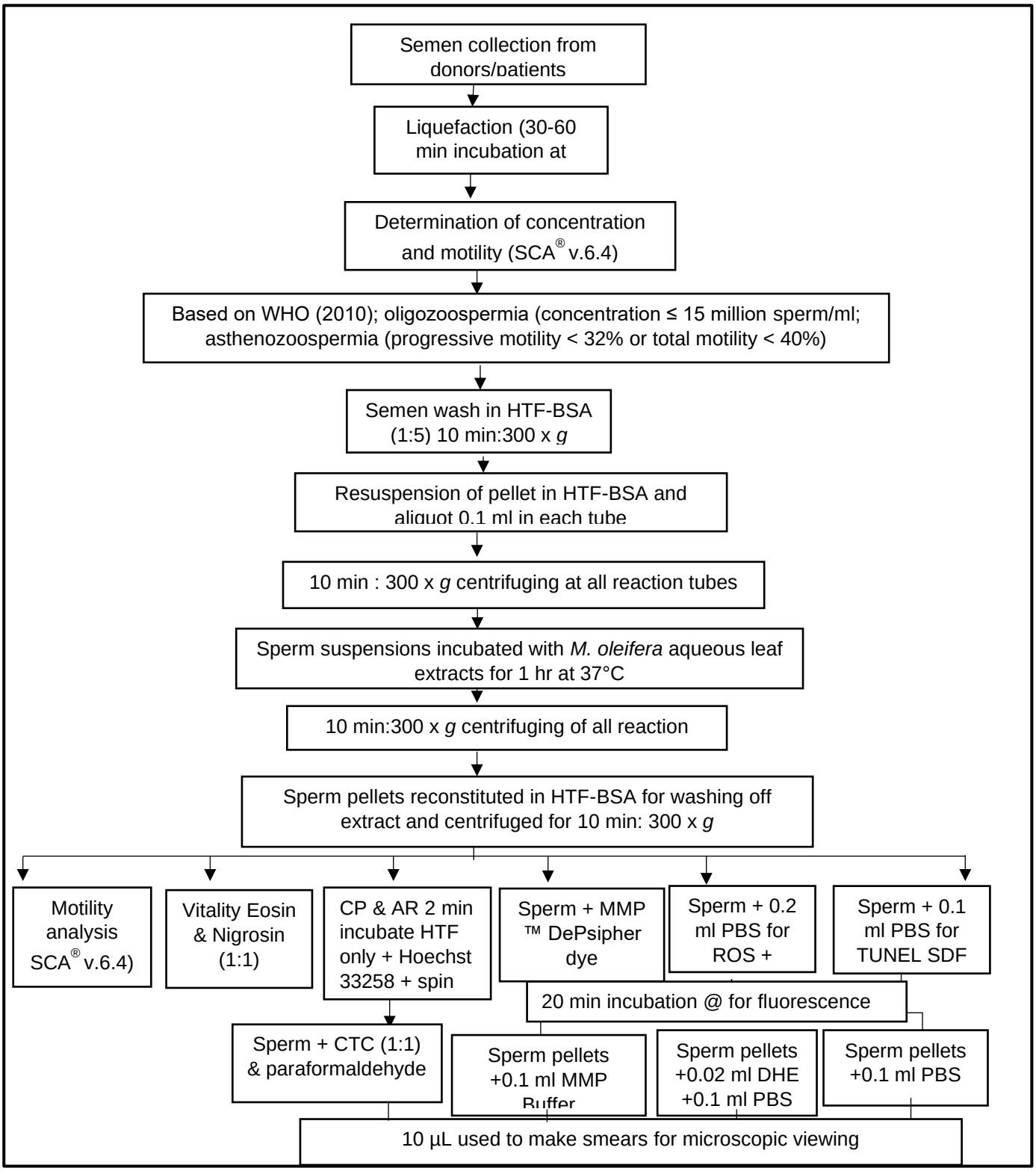

Figure 16: Experimental layout outlining functional assays and computational systems utilised.
SCA: Sperm Class Analyser, WHO: World Health Organization, HTF: Human tubular fluid, BSA: Bovine serum albumin, CP: Capacitation, AR: Acrosome reaction, MMP: Mitochondrial membrane potential, PBS: Phosphate buffer saline, ROS: Reactive oxygen species, TUNEL: Terminal deoxynucleotidyl transferase-mediated nick ending labelling, SDF: Sperm DNA fragmentation, CTC: Chlortetracycline, DHE: Dihydroethidium.

3.6 Laboratory analysis

3.6.1 Determination of effects of *M. oleifera* aqueous leaf extracts on sperm motility

To determine the effects of aqueous leaf extract of *M. oleifera* on sperm motility, washed sperm suspensions were incubated at 37°C with the various concentrations (0, 0.625, 6.25, 62.5, and 625 µg/ml) of the extract for 1 hr. Suspensions were subsequently re-suspended in 100 µl HTF-BSA. Thereafter, 10µl of the sperm suspension was loaded on a pre-warmed Leja slide, and at least 200 spermatozoa were assessed according to the WHO criteria, using Sperm Class Analyzer® (Figure 17) (SCA, Microptic S.L., Barcelona, Spain version 6.4) with a Zeiss photomicroscope using a 10 X objective. The following characteristics of the sperm motility were analysed: Total motility (%), progressive motility (%), beat cross frequency (BCF; Hz), linearity (LIN; %), straightness (STR; %), average path velocity (VAP; µm/s), curvilinear velocity (VCL; µm/s), straight-line velocity (VSL; µm/s), Wobble (WOB; %), and hyperactivation (%) (Tables 1 and 2).

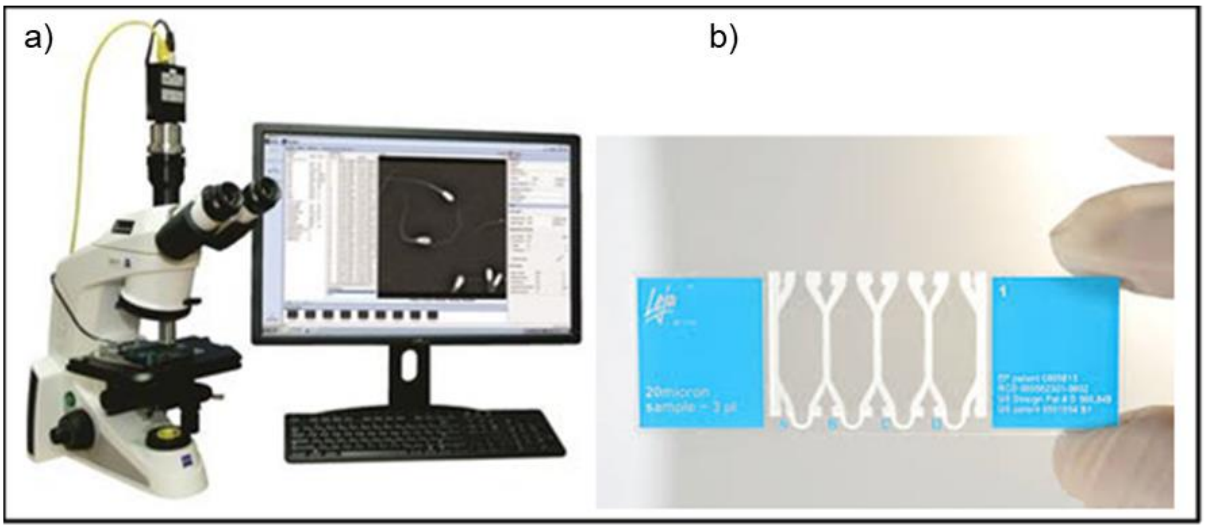

Figure 17: (a) Computer-aided sperm class analyser (CASA) and (b) Leja slides used for determination of sperm motility and concentration.

Table 1: Categories of sperm motility classes defined

Category	Definition	Unit
Total motility	Includes all spermatozoa moving progressively or not in a field. This classification does not take speed into consideration.	%
Non-progressive (grade C)	Exhibited by a population of spermatozoa that do not swim in large circles or linearly, where flagella beat does not change head movements.	%
Progressive motility (grades A + B)	Spermatozoa are moving linearly forward or in large circles.	%
Immotility (grade D)	No sperm movement.	%
Hyperactivation	Asymmetrical, vigorous and random form of motility sperm exhibits following the acquisition of fertilising potential, and evident at the site of fertilisation.	%

Table 2: Sperm motility kinematic parameters

Motility kinematic parameters	**Definition**	Units
Curvilinear velocity (VCL)	Time average velocity of sperm head along its actual curvilinear path.	µm/s
Straight-line velocity (VSL)	Average time velocity of sperm head in a straight line between two points of detection.	µm/s
Average path velocity (VAP)	The average velocity of the sperm head along its average spatial trajectory.	µm/s
Amplitude of lateral head displacement (ALH)	The maximum lateral displacement of sperm head about its average path.	µm
Oscillatory index / Wobble (WOB)	"Wiggling" or oscillatory movements of sperm's actual path about its average.	%
Linearity index (LIN)	The linearity of the average path.	%
Straightness (STR)	Straightness of the average path.	%
Beat cross frequency (BCF)	The time-averaged rate at which sperm curvilinear tracks crosses its average path trajectory.	Hz

3.6.2 Determination of effects of *M. oleifera* aqueous leaf extracts on sperm vitality

After 1 hr incubation at 37°C with *M. oleifera* extracts, an aliquot of 50 µl of E & N dye was transferred into an Eppendorf tube. Then, sperm suspension and E & N were mixed (1:1). The tubes were incubated for 2 minutes. After incubation, 10 µl of the mixture was smeared on slides (Lasec®, Delfran Pharmaceutical, Cape Town). The slides were air-dried at room temperature, mounted with dibutyl phthalate polystyrene

xylene (DPX) medium, and viewed through a 10 X objective using a light microscope (Zeiss, Oberkochen, Germany). Sperm cells stained pink represented dead sperm cells, while those stained white were live cells (Figure 18). Two hundred (200) spermatozoa were assessed for each sample. The percentage of live sperm was calculated.

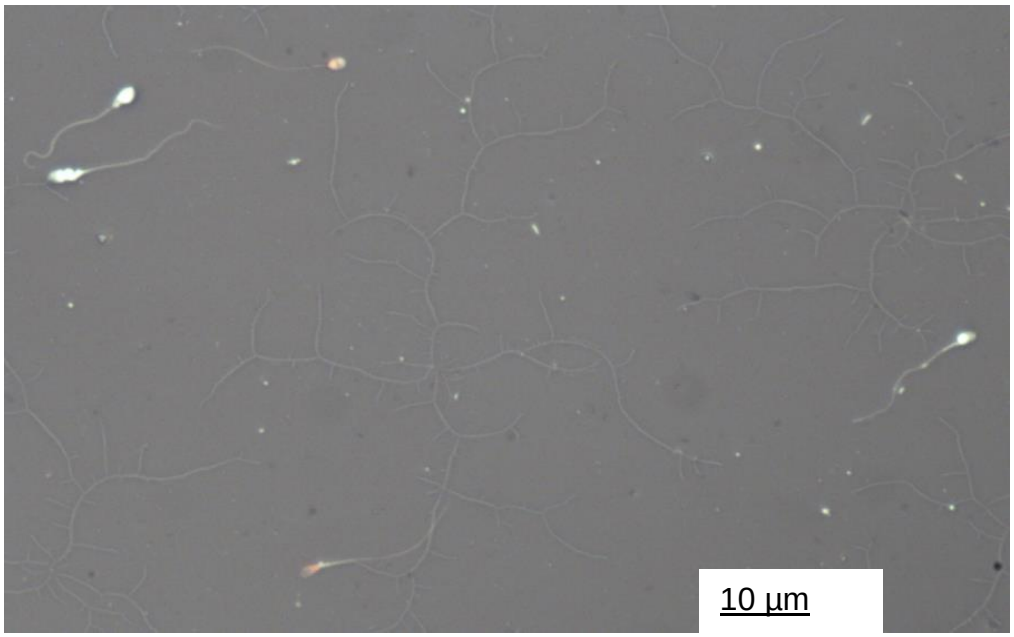

Figure 18: One-step Eosin-nigrosin (E & N) staining of human sperm specimen. The dye excludes viable / live (white) from dead (pink) cells, based on the membrane intactness. Samples were viewed through a 10 X objective.

3.6.3 Determination of effects of *M. oleifera* aqueous extracts on sperm mitochondrial membrane potential (∆ψm)

Sperm mitochondrial membrane potential (MMP) was assessed using the lipophilic cationic dye (DePsipher™, Trevigen, Minneapolis, USA).

The protocol was applied with modifications from the manufacturer's instructions as previously described by Henkel et al. (2011). Briefly, a reaction buffer was diluted with distilled water (1:10), and 20 µl of stabiliser was added per 1 millilitre of the buffer. A volume of 1 µl of DePsipher was added per millilitre of the buffer. After that, the mixture was thoroughly vortexed for 1 min at 10,000 x *g* for homogenisation. The supernatant was transferred into an Eppendorf tube, and covered in foil for later use.

Sperm cells were exposed to various concentrations (0, 0.625, 6.25, 62.5, and 625 µg/ml) of *M. oleifera* aqueous extracts at 37°C for 1 hr, and then washed with HTF-

BSA. The recovered pellet of washed spermatozoa (100 µl) was re-suspended in dilute DePsipher solution (100 µl), and centrifuged for 10 min at 300 x *g*. The pellets were re-suspended in dilute reaction buffer (100 µl), and incubated at 37°C for 20 min, shielded from light, and then centrifuged at 300 x *g* for 10 min. Thereafter, the supernatant was discarded.

The pellet was re-suspended in 100 µl of the pre-warmed reaction buffer. From each sample, 10 µL was used to make smears, and this was viewed at 100 X magnification using epifluorescence. Sperm mid-pieces of the flagella that fluoresced green were regarded as having disturbed / loss MMP, as the dye would have leaked into their cytosol (meaning they became apoptotic). Spermatozoa with red fluorescence were indicative of high ATP, and high MMP with the ability to retain aggregates of DePsipher in their mitochondria (meaning intact MMP). The results were expressed as the percentage of membrane-intact sperm (red), as shown in Figure 19.

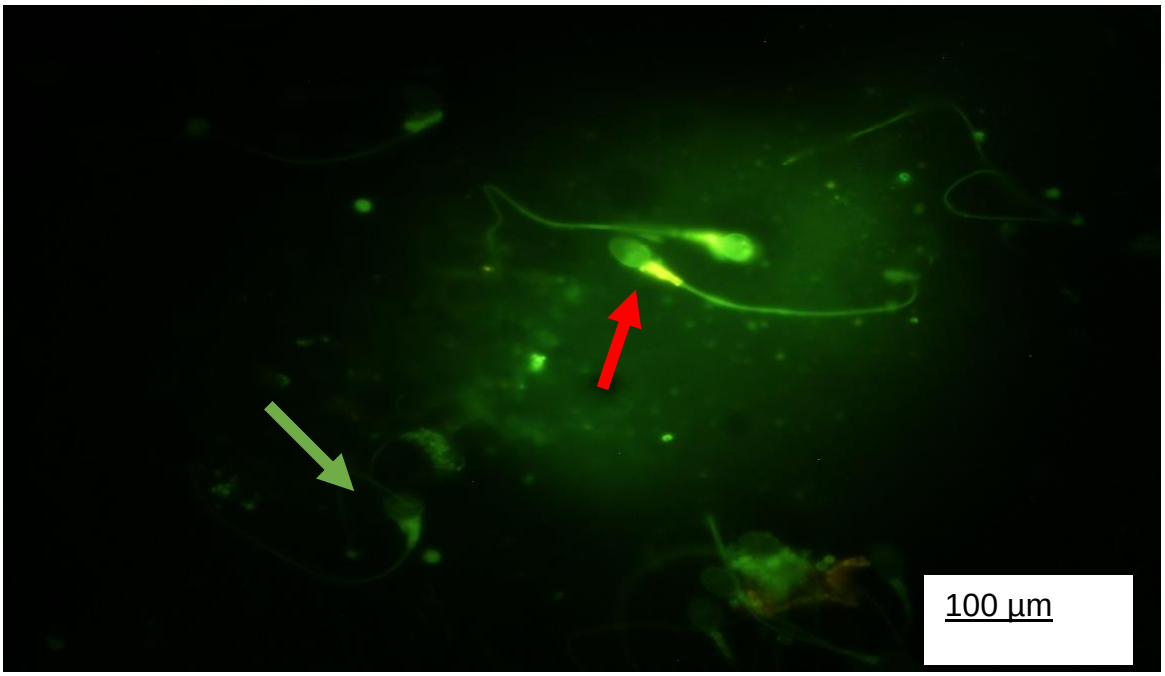

Figure 19: Spermatozoal mitochondrial membrane potential (MMP) integrity status as observed using epifluorescence. Mid-piece fluoresces orange red (red arrow) denotes intact MMP, while the green arrow depicts membrane disrupted spermatozoa. Specimens were viewed at 100 X magnification.

3.6.4 Determination effects of *M. oleifera* aqueous extracts on sperm reactive oxygen species (ROS) production

For the determination of sperm ROS production following *M. oleifera* extracts, the method was performed as previously described by Henkel et al. (2012) and Shalaweh et al. (2015), using dihydroethidium (DHE; Molecular Probes, Eugene, OR, USA). A stock solution of 20 µM DHE in PBS at a pH of 7.4 was prepared. After 1 hr incubation of sperm suspension at 37°C with various *M. oleifera* extract concentrations, HTF-BSA (500 µl) was added to the sperm suspension (100 µl) and centrifuged for 10 min at 300 x *g*.

The pellet was re-suspended in 200 µl of PBS and centrifuged for 10 min at 300 x *g*. After this step, a dilute DHE (20 µl) and PBS (100 µl) was added to the samples, and incubated at 37°C for a further 20 min. Subsequently, 10 µl of each sample was placed on a slide, covered with a coverslip and evaluated under oil immersion (100 X), using an epifluorescent microscope with 488 nm excitation and 590 emission filters (Zeiss, Oberkochen, Germany). Spermatozoa that fluoresced brightly orange (blue arrows) indicated excess ROS production, while those with a faint fluorescence (green arrows) represented little or no ROS production (Figure 20). Results were then expressed as percentage spermatozoa with red fluorescence (indicative of excess ROS production).

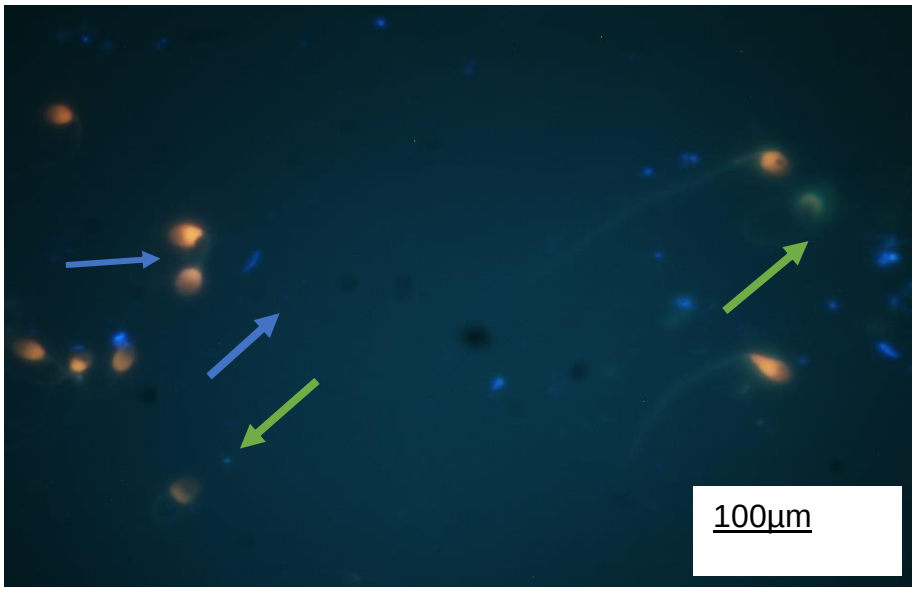

Figure 20: Detection of excessive superoxide anion by human spermatozoa as detected by a DHE fluorescent probe. Green arrows indicate ROS-negative spermatozoa, and red arrows show ROS-positive spermatozoa. Specimens were viewed at 100 X magnification.

3.6.5 Determination of effects of *M. oleifera* aqueous extracts on sperm DNA fragmentation.

Effects of *M. oleifera* extract on sperm DNA integrity were determined using TUNEL assay (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labelling) [Dead End]™ Fluoremetric, Promega, Madison, USA. The TUNEL system is based on the enzymatic ability of terminal transferase (TdT) to detect fluoresceinated dUTP single- or double-stranded DNA nicks by labelling the resultant free hydroxyl groups (3-OH) (Sharma and Agarwal, 2011a). In the study, TUNEL assay was performed for SDF, and at least 200 TUNEL-positive spermatozoa were assessed. After 1 h incubation with different concentrations of MOL, the samples were centrifuged for 10 min at 300 x *g* in HTF-BSA. Thereafter, sperm suspension (100 µl) was re-suspended in PBS and centrifuged again, with this step repeated once. Then, 10 µl of the sample was smeared on Starfrost slides (Knittel Gläser, Braunschweig, Germany).

Slides were left to air-dry, and subsequently fixed with fresh 4% methanol-free formaldehyde (KIMIX chemicals, Eppingdust) in PBS for 25 min at 4˚C. Slides were shortly washed with PBS for another 5 min and allowed to dry at room temperature. The sperm plasma membrane was permeabilised with 0.2% Triton X-100 detergent in PBS for 5 min and rinsed twice with PBS at room temperature. Excess liquid was removed by tapping the slides on the paper towel-layered working counter, and 100 µl of equilibration buffer was added to the slides, and the samples were left to equilibrate for roughly 10 min as per the manufacturer's instructions. Then, 50 µl TdT reagent buffer was added, and the coverslip was placed on slides, and the sample was incubated for 1 h in a light-devoid humidified chamber.

Slides were submerged in 2 X SSC buffer (Promega) for 15 min, washed three times with PBS for 5 min each, and placed back in the humidified chamber to avoid drying while reading. At least 200 spermatozoa were randomly selected and analysed using an epifluorescence microscope (Zeiss, Oberkochen, Germany). Excitation and emission filters were 488 nm and 530 nm, respectively, and viewed at 100 X magnification. DNA intact sperm exhibited a bit of a background staining (TUNEL-negative), whereas DNA-fragmented sperm emitted a bright green fluorescence (TUNEL-positive) as shown in Figure 22. Accordingly, the percentage of TUNEL-positive spermatozoa were calculated from at least 200 cells.

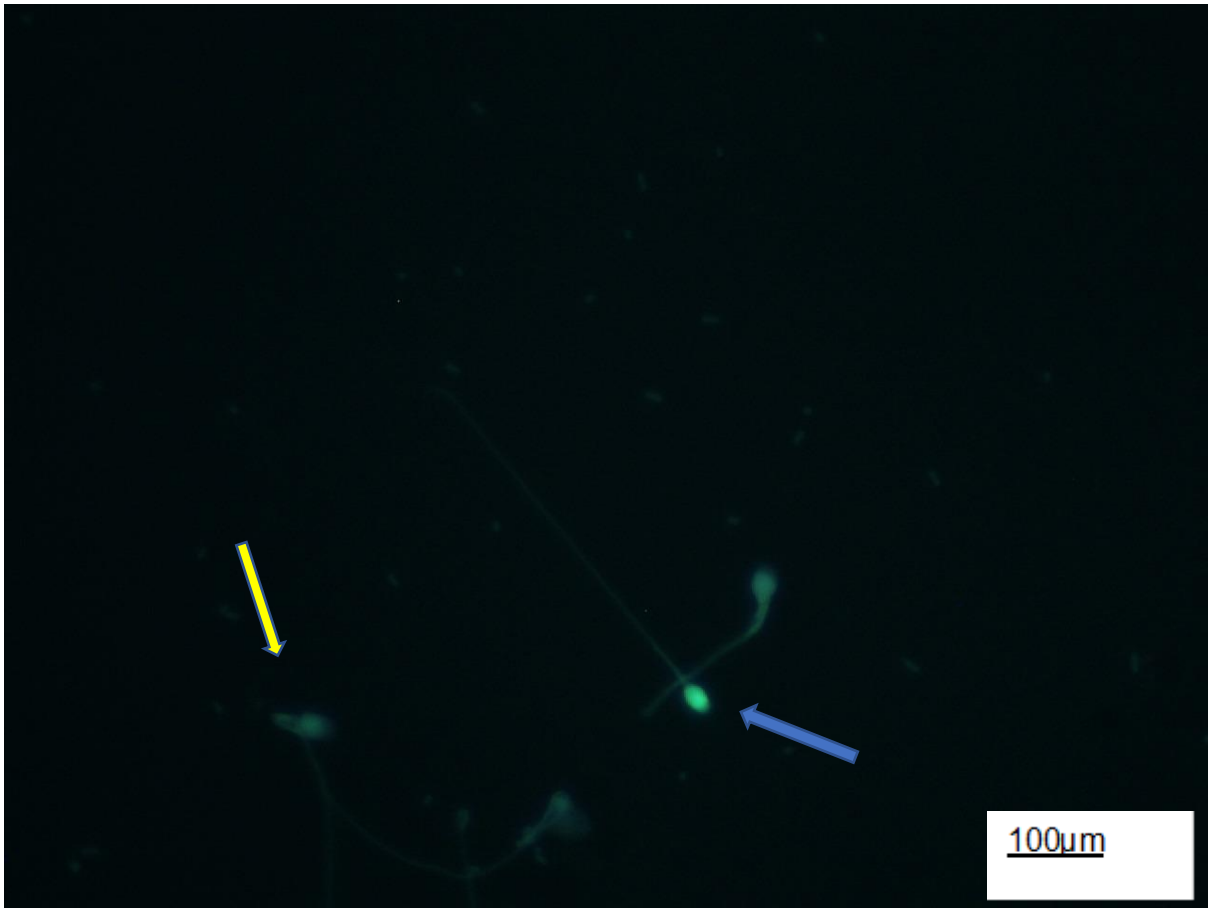

Figure 21: Detection of sperm DNA fragmentation using TUNEL assay. Blue arrow indicates TUNEL-positive sperm, and yellow arrows indicate TUNEL negative sperm. Specimens were viewed at 100 X magnification.

3.6.6 Determination of effects of *M. oleifera* aqueous extracts on sperm capacitation and acrosome reaction

This study adopted the dual staining of Hoechst 32258 / Chlortetracycline (CTC) according to Green et al. (1996) for assessment of capacitation and acrosome status of human spermatozoa, following *M. oleifera* extract exposure. Following 1 hr incubation of sperm with the aqueous extracts, one microliter (1µl) of Hoechst 32258 was mixed with 100 µl sperm suspension in HTF-BSA. The samples were mixed with 4 ml of 2% polyvinylpyrrolidone (PVP40) in HTF only, then incubated at room temperature for 2 min, and centrifuged at 900 x *g* for 5 min.

Chlortetracycline (CTC) buffer (pH 7.8) was prepared on the day of use from the following reagents: 750 µm CTC in a buffer of 130 mM NaCl, 5 mM cysteine, and 20

mM Tris-HCl. The solution was wrapped in a foil and stored at 4˚C for subsequent use for up to 4 weeks. Hoechst-stained sperm suspension (45 µl) was mixed with CTC solution (45 µl) and 12.5% w/v paraformaldehyde in 0.5 Tris-HCl (pH 7.4) (8 µl). Then, 10 µl of the mixture was loaded on a slide, and one drop of 0.22 M 1, 4–diazabicyclo (2.2.2) octane (DABCO) dissolved in glycerol: PBS (9:1) was carefully placed to delay any loss of fluorescence.

Slides were viewed with a 100 X objective, using an epifluorescent Zeiss microscope. From each slide, 200 live (Hoechst-negative) were assessed for CTC fluorescent patterns, where uniform fluorescence over the entire head indicated an F pattern (non-capacitated, acrosome intact). Fluorescence-free band of the post-acrosomal segment, B pattern (capacitated, acrosome-intact cells) (represented by blue arrows), and (indicated by yellow arrows) dull or absent fluorescent over the entire head with / without a fluorescent post-acrosomal region characterised AR pattern (capacitated and acrosome-reacted cells) and corresponded with white arrows as shown in Figure 22).

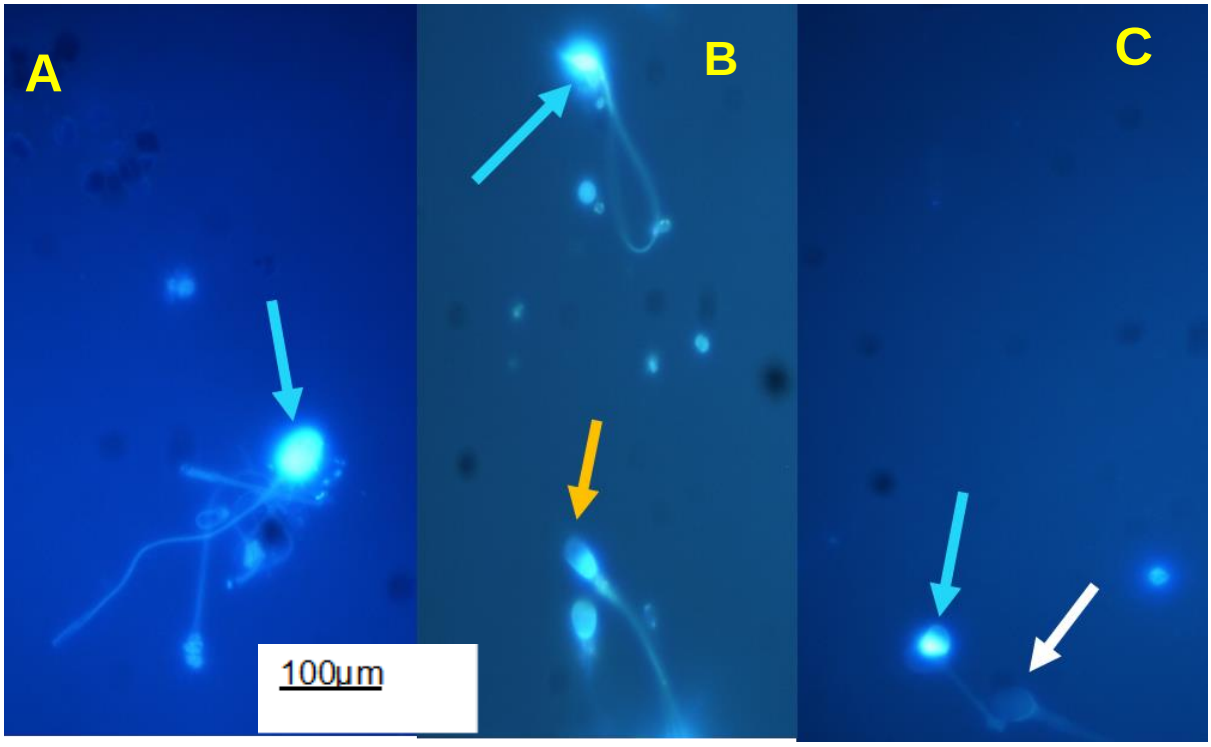

Figure 22: Three staining CTC patterns of human spermatozoa as seen under fluorescence microscope. A) F pattern, B) B pattern and C) AR pattern. Specimens were viewed at 100 X magnification.

3.7 Ethical considerations

3.7.1 Ethical clearance and approval

Ethical clearance was obtained from the Turfloop Research Ethics Committee (TREC) of the University of Limpopo (TREC/45/2019: PG; Appendix 1) and the Medical Biosciences Research Ethics Committee (BMREC) from the University of the Western Cape (BM17/7/14; Appendix 2). Approval to collect samples from the Tygerberg Hospital was granted by the Western Cape Department of Health (Appendix 3). Permission for collection of semen samples from the Vincent Pallotti Hospital was granted by the Aevitas Fertility clinic (Appendix 4).

3.7.2 Harm

The procedural approach during data collection involved no medical risk or harm, because participants were thoroughly briefed before consenting to their participation. After their briefing, they willingly provided semen for experimentation. Participants (especially donors) were assured of their safety, and their choice to withdraw from the study at any time. The University Counselling Services were at the participants' disposal in case they were to experience any distress during the semen provision. As stated previously, there were no risks incurred. In any case where a participant could have misunderstood the handling of the samples, they were briefed.

3.7.3 Disposal of waste

At the end of the experiment, any residual semen samples were disposed of as biohazardous waste in VT Medwaste bins. The waste was later incinerated by the BCL-mediated medical waste company.

3.7.4 Anonymity and confidentiality

No actual participant names were used in the recording of data or disclosed to anyone in the dissemination of results. Participant's results were restricted only to researchers directly involved with the data collection, for confidential reasons. A non-coercive informed consent form was provided for participants to sign as an assurance of their willingness to take part in the research, and that they had been informed that they could withdraw whenever they decided to do so without penalty or risk to their well-being. The informed consent form was available in English, Afrikaans, and Xhosa to eliminate any potential language barriers (Appendices 5-7). Participants were assigned sample numbers or codes as a way of maintaining their confidentiality.

3.8 Data analyses

3.8.1 Reliability and validity

In this study, donors were screened twice before recruitment into the programme to ensure reliability of the study findings. In hospitals, the infertile status of patients was diagnosed by doctors, andrologists or practised reproductive biologists.

All testing kits / protocols were performed strictly according to the manufacturer's instructions.

3.8.2 Quality assurance

It is well known that quality assurance in an andrology-testing laboratory is difficult due to the lack of standardisation of the protocols, and biological variation of semen sample either from an individual or between different samples. One sample was used to test all parameters at a time, and experimental procedures were performed according to the WHO (2010) laboratory manual for examination and processing semen to ensure these limitations did not interfere with the quality of results in this study.

3.8.3 Bias

Convenience sampling was used. Sampling bias could not be avoided in this study, because the sampling frame was poorly defined as it included any available, eligible and willing donor. At the same time, infertile couples were people of any race or nationality who sought fertility treatment at either of the two hospitals (Figure 16). The availability of the researcher or participant, financial, and time constraints justified the type of sampling chosen.

3.8.4 Statistical analysis

Data were analysed using GraphPad Prism 6.0.1 for Windows (GraphPad Software, San Diego, California, USA). Descriptive data were generated using column statistic function, and the D'Agostino-Pearson Omnibus test was used for normality testing. Consequently, further analysis included parametric testing using one-way analysis of variance (ANOVA), while non-parametric analyses were performed using the Kruskal-Wallis test, in which Dunnets' multiple tests were applied for comparisons of group means. Repeated measures one-way ANOVA (RM 1-way ANOVA) were used in non-Gaussian data for trend analyses. Spearman correlation was used to assess

relationships between the sperm parameters. Statistical significance was set at $p < 0.05$.

CHAPTER 4

RESULTS

4.1. Introduction

Findings in this study reported in this chapter consist of two parts. The first part reports on the donor, patient, and combined (donor and patient) groups. The second part entails results on the asthenozoospermic group. The study consisted of 70 samples. Forty donors (40) were recruited based on the WHO (2010) guidelines from the Andrology Research Programme in the comparative spermatology group of the Medical Biosciences Department, University of the Western Cape. Using the same inclusion criteria, semen samples from 30 patients attending the Tygerberg and Vincent Pallotti Hospitals were included. Standard semen parameters of donor and patient groups are summarised in the table below (Table 3).

In an effort to determine the effects of *M. oleifera* aqueous leaf extracts on human sperm functions *in vitro*, sperm suspensions were incubated at 37℃ with various concentrations of *M. oleifera* (0, 0.625, 6.25, 62.5 and 625 µg/ml) for one hour.

4.2. Summary statistics of standard semen parameters according to the WHO (2010)

After 30 to 60 minutes of liquefaction at 37℃ before treatment, ejaculates were assessed for inclusion. The overall descriptive data are summarised in Table 3. The study included donors whose seminograms were above the WHO's (2010) lower reference points: concentration ≥ 15 million/ml, total motility ≥ 40% or progressive motility ≥ 32%. The cut-off values defining male infertility (patients) in this study were set as follows: oligozoospermia (concentration < 15 million/ml), asthenozoospermia (total motility < 40% or progressive motility < 32%), and they formed part of the study.

Table 3: Summary statistics of standard semen parameters after liquefaction before treatment with *M. oleifera* aqueous leaf extracts

Summary statistics	Group	Concentration ($x10^6$/ml)	Viscosity (sec)	Total Motility (%)	Progressive Motility %	Volume (ml)
N	Donor (fertile)	40.0	40.0	40.0	40.0	40.0
	Infertile (patients)	30.0	30.0	30.0	30.0	30.0
	Combined	70.0	70.0	70.0	70.0	70.0
Min	Donor (fertile)	18.5	2.0	15.4	0.4	1.0
	Infertile (patients)	9.6	5.0	11.5	2.6	1.0
	Combined	10.6	2.0	11.5	4.0	1.0
Median	Donor (fertile)	80.1	16.0	58.6	17.4	3.0
	Infertile (patients)	46.8	15.5	52.7	31.7	2.5
	Combined	58.6	16.0	54.2	26.0	3.0
Max	Donor (fertile)	180.5	392.0	96.3	82.6	5.0
	Infertile (patients)	210.0	360.0	92.4	68.4	5.0
	Combined	210.0	392.0	96.3	82.6	5.0
Mean	Donor (fertile)	78.1	39.6	58.4	25.3	2.8
	Infertile (patients)	57.2	36.7	50.4	32.7	2.7
	Combined	69.1	35.2	54.9	28.7	2.8
SD	Donor (fertile)	37.9	67.2	19.4	20.6	1.0
	Infertile (patients)	37.9	67.2	19.4	20.6	1.0
	Combined	41.4	57.9	20.7	20.1	1.0
SEM	Donor (fertile)	6.0	10.6	3.1	3.3	0.2
	Infertile (patients)	7.9	12.5	3.9	3.4	0.2
	Combined	5.0	6.9	2.5	2.4	0.1
Lower 95% CI of mean	Donor (fertile)	66.0	18.1	52.1	18.7	2.5
	Infertile (patients)	41.0	11.1	42.3	25.6	2.4
	Combined	59.2	21.4	50.0	23.9	2.5
Upper 95% CI of mean	Donor (fertile)	90.2	61.1	64.6	31.9	3.1

	Infertile (patients)	73.5	62.4	58.4	39.7	3.1
	Combined	78.9	49.0	59.8	33.5	3.0
Coefficient of variation	Donor (fertile)	48.5	169.6	33.3	81.6	34.8
	Infertile (patients)	76.1	187.0	42.9	57.6	35.2
	Combined	60.0	164.5	37.6	69.8	34.5

Descriptive statistics of the donor (N = 40), patient (N = 30) and combined (N = 70). N = Number of samples, SD = standard deviation, SEM = Standard error of the mean, Min = minimum, Max = maximum, CI = confidence interval

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4.3. Effects of *M. oleifera* leaf extracts on sperm motility parameters *in vitro*

Summary statistics of sperm motility following 1-hr incubation with increasing concentrations (0, 0.625, 6.25, 62.5, and 625 µg/ml) of aqueous leaf extracts of *M. oleifera* are displayed in Table 4.

Following treatment of human sperm with *M. oleifera* leaf extracts for 1 hr, the plant did not significantly affect overall sperm total motility ($p = 0.1212$) in the donor group. The highest concentration (625 µg/ml) produced a significant increase in total motility of the patient and combined groups in comparison with control groups, respectively ($p = 0.005$ and $p = 0.0427$), with no change in the donor group ($p = 0.9714$) (Figure 23).

No differences in total motility were observed in the control and all *M. oleifera* treatments (0.625, 6.25, 62.5, and 625 µg/ml) between donors, patients, and the combined groups ($p > 0.05$) (Figure 23). Repeated measures one-way ANOVA revealed a significant positive trend in sperm total motility ($p < 0.0001$) of patients and combined groups, while no trends could be observed for donors after treatment with increasing concentrations of *M. oleifera.*

(a)

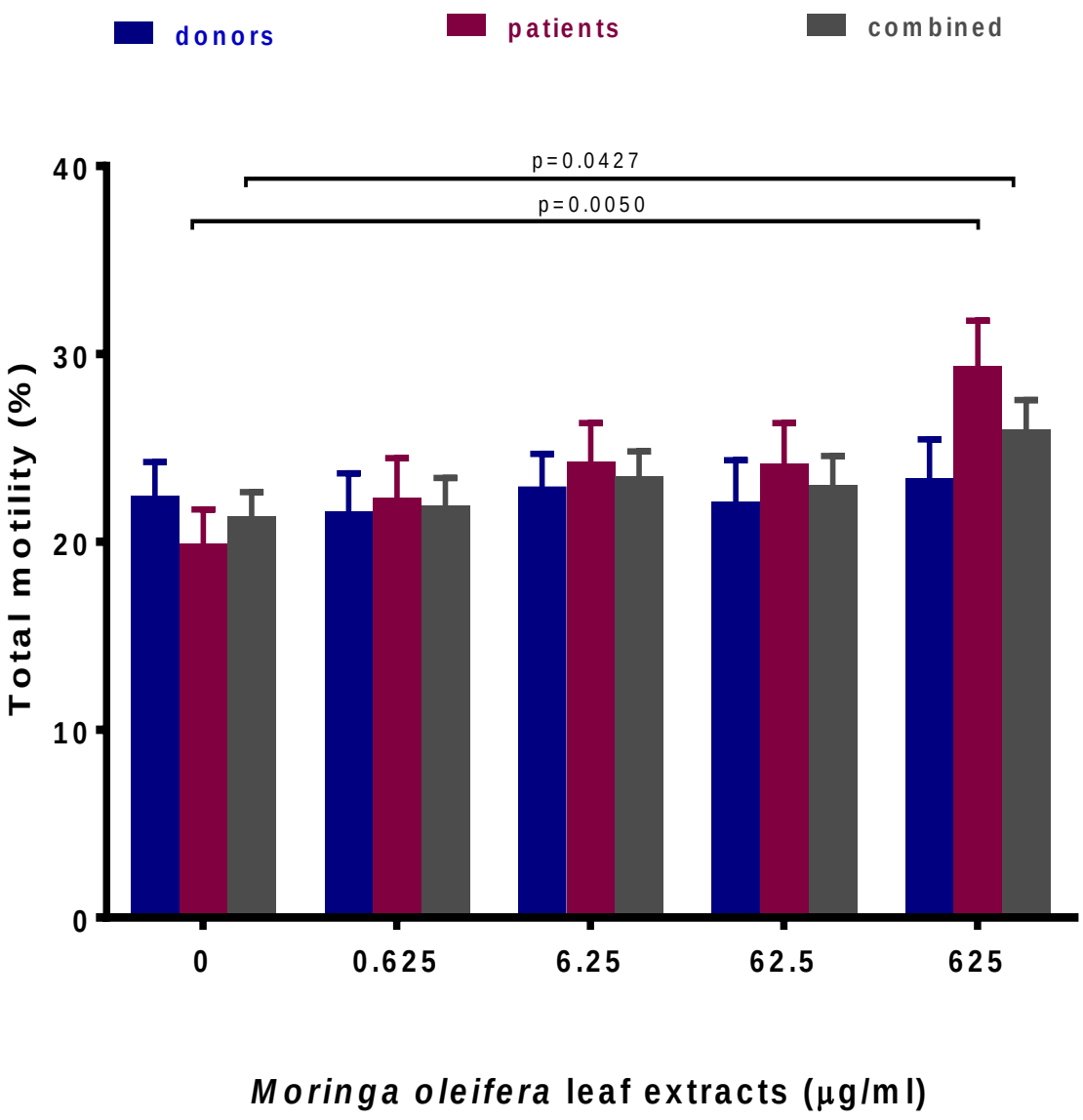


Figure 23: Total motility after 1-hr incubation with *M. oleifera in vitro*. Mean ± SEM. Blue bars represent the mean values of donors (N = 40), purple bars are for patients (N = 30), and grey bars are for combined group (patients and donors) (N = 70).

Increasing concentrations of *M. oleifera* did not change overall percentages of sperm with non-progressive ($p > 0.05$) and progressive motility ($p > 0.05$) in the donor and

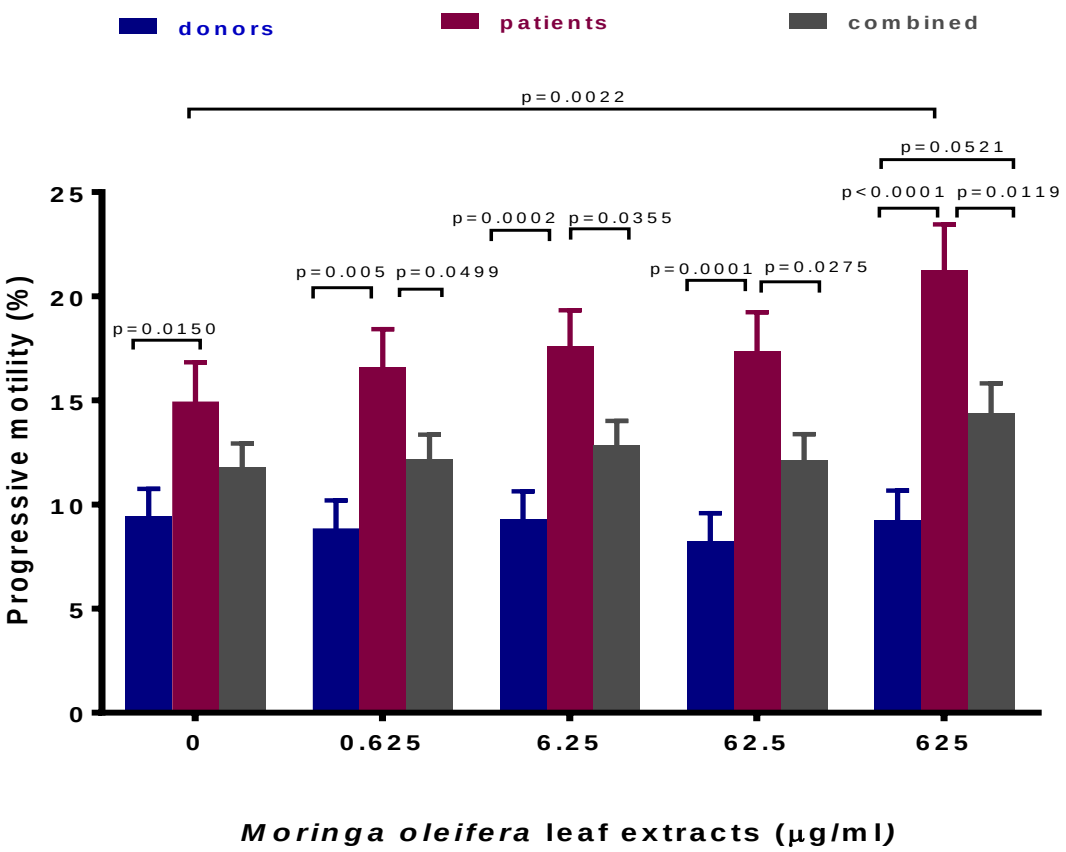
combined groups, respectively. Despite insignificant results with other concentrations of *M. oleifera*, the highest concentration (625 µg/ml) significantly increased the progressive ($p = 0.0022$) and non-progressive sperm motility ($p = 0.0215$) in the patient group when compared to the respective controls (Figures 24a and b). On the contrary, no significant effects were observed in progressive and non-progressive motility when the highest concentration of *M. oleifera* extract was compared to the control in donor ($p > 0.05$).

The control of progressive motility showed no statistical difference between the combined groups and donors ($p > 0.05$). Patients' progressive motility was significantly higher than that of the donors when the controls were compared ($p = 0.0150$). However, non-progressive motility of patients was significantly higher than that of donors ($p = 0.0017$) (Figure 24b). Controls between patients and the combined groups showed no difference ($p > 0.05$)

M. oleifera improved progressive and non-progressive motility significantly more in patients than in donors at doses 0.625 µg/ml ($p = 0.0005$ and $p < 0.0001$), 6.25 µg/ml ($p = 0.0002$ and $p = 0.0002$), 62.5 µg/ml ($p = 0.0001$ and $p = 0.0059$) and 625 µg/ml ($p < 0.0001$ and $p = 0.0083$) (Figures 25a and b). Similar observations were made in progressive and non-progressive motility between patients and the combined groups at concentrations 0.625 µg/ml ($p = 0.0499$ and $p = 0.0246$), 6.25 µg/ml ($p = 0.0335$ and $p = 0.0314$), 62.5 µg/ml ($p = 0.0275$) and 625 µg/ml ($p = 0.0119$) (Figures 24a and b).

Positive linear trends were significant for progressive motility and non-progressive motility ($p = 0.0117$ and $p < 0.0001$) of the patient group. In addition, significant straight-line trends were shown for percentage of progressive ($p = 0.0023$) and non-progressive motility ($p = 0.0013$) in the combined group. However, no significant trends could be recorded with increasing doses of *M. oleifera* in the percentage of progressive or non-progressive motility in the donor group.

a)



b)

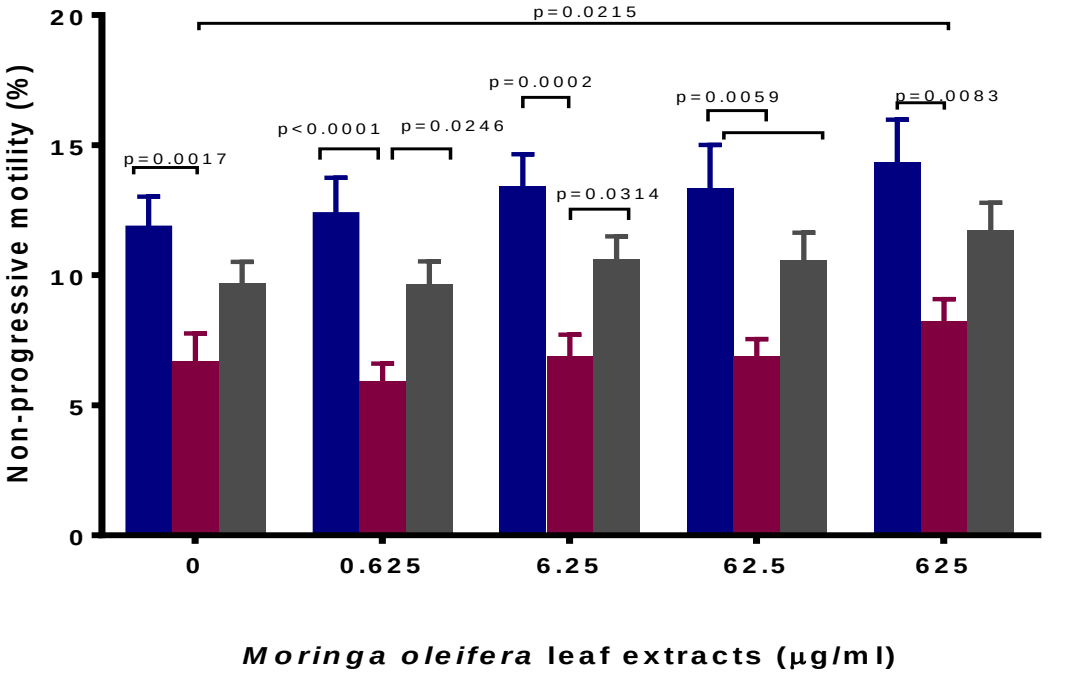


Figure 24: Progressive (a) and non-progressive motility (b) after 1hr incubation with *M. oleifera in vitro*. Mean ± SEM. Blue bars represent the mean values of donors (N = 40), purple bars are for patients (N = 30), and grey bars are for combined group (patients and donors) (N = 70).

After 1-hr incubation of sperm with *M. oleifera* aqueous extracts, sperm kinematic parameters were investigated. Overall, *M. oleifera* leaf extracts did not alter sperm kinematic parameters in the three groups tested: donor, patient, and the combined groups ($p > 0.05$) (Table 4).

Although no specific changes could be seen in sperm kinematics of motility when all groups were exposed to various concentrations of *M. oleifera*, significant trends were recorded in the straightness index (STR), straight-line velocity (VSL), and average path velocity (VAP) in patients and combined groups. The one-way ANOVA repeated measure revealed significant decreasing tendencies in the sperm percentage straightness in patients ($p = 0.0177$), and in combined ($p = 0.0012$) groups. Similarly, a decreasing trend was observed in sperm straight / rectilinear velocity (VSL) with increasing dose of *M. oleifera* in patients ($p = 0.0262$), and in combined ($p = 0.0040$) groups. The linearity of sperm demonstrated dose-dependent significant decreasing trends in the patients' group, following a 1-hour incubation with the plant ($p = 0.0236$).

Table 4: Comparative results on donors, patients, and combined sperm motility kinematic parameters after treatment with aqueous extract of *M. oleifera*.

Kinematics	Group:	***Moringa oleifera* leaf extract concentrations (µg/ml)**				p-value	
		0	**0.625**	**6.25**	**62.5**	**625**	
LIN (%)	donors	37.6 ± 1.8	36.3 ±1.8	37.4 ±1.8	35.7 ±1.1	37.6 ± 2.1	0.93
	patients	50.1 ±1.1	52.1 ±1.8	48.5±2.1	49.3 ±1.8	47.1 ±2.2	0.48
	combined	43 ± 1.5	43.1 ±1.6	42.2 ±1.5	41.5 ±1.5	41.7 ± 1.6	0.21
STR (%)	donors	64.2 ±1.7	62.4 ±1.7	63.3 ±1.9	61.8 ±2.0	60.0 ±2.1	0.60
	patients	73.0±1.7	74.6±1.3	72.9 ±2.1	71.8 ±1.5	70.1 ±2.1	0.54
	combined	68.0 ± 1.3	67.6 ±1.3	67.4 ±1.5	66.1±1.4	64.3 ± 1.6	0.56
WOB (%)	donors	54.5 ±1.4	53.1±1.4	54.9±1.4	53.9 ±1.4	54.5±1.9	0.92
	patients	62.6±1.4	64.5±1.3	62.2±1.8	62.3±1.3	60.2±1.4	0.36
	combined	57.9±1.1	58.5±1.1	58.0±1.2	57.5±1.1	56.9±1.3	0.25
VCL (µm/s^{-1})	donors	70.6±2.91	70.5±3.6	69.4±3.0	67.1 ±2.5	68.7±4.0	0.94
	patients	53.8±1.9	55.5±2.3	55.5±3.0	54.9±2.6	54.9±2.8	0.99

	combined	63.4 ± 2.1	64.1 ± 2.4	63.5 ± 2.3	61.8 ± 1.9	62.6 ± 2.7	0.92
VAP (μm/s^{-1})	donors	37.2±1.4	36.5 ±1.5	36.5±1.3	35.1±1.2	35.5±1.5	0.58
	patients	34.6±1.7	35.8±1.7	34.3±1.7	34.5±1.7	33.0±1.9	0.86
	combined	36.1 ±1.1	36.2 ± 1.1	35.5 ± 1.1	34.8 ± 0.1	34.4 ± 1.2	0.51
VSL (μm/s^{-1})	donor	25.7±1.3	24.3±1.2	25.1 ±1.1	23.5±1.2	22.7±1.2	0.39
	patients	32.4±2.1	33.4±1.1	31.3±1.9	31.9±2.1	29.5±2.1	0.72
	combined	28.6 ± 1.2	28.2 ±1.2	27.7 ± 1.1	27.1 ± 1.2	25.6 ± 1.2	0.28
ALH (μm)	donors	2.4±0.1	2.4±0.1	2.4±0.1	2.3±0.1	2.3±0.1	0.98
	patients	2.2±0.1	2.2±0.1	2.3±0.1	2.3±0.1	2.3±0.1	0.89
	combined	2.3 ±0.1	2.3 ±0.1	2.3 ±0.1	2.3 ±0.1	2.3 ±0.1	1.00
BCF (Hz)	donors	15.7±0.8	14.7±0.9	14.7±0.8	15.5±1.2	13.6±1.0	0.49
	patients	8.3±0.4	8.8±0.1	8.2±0.5	8.4±0.6	8.0±1.0	0.72
	combined	12.5 ± 0.7	12.1±0.7	11.9 ± 0.6	12.5 ± 0.9	11.2 ± 0.6	0.78
Hyperactivation (%)	donors	3.1 ±1.0	4.9 ± 1.4	3.5 ±0.8	2.8 ± 0.8	5.3 ± 1.7	0.49
	patients	0.9± 0.3	0.9 ± 0.3	1.4 ±0.8	1.0 ± 0.4	1.2 ± 0.1	0.90
	combined	2.2 ±0.1	3.2 ±0.8	2.6 ± 0.6	2.0 ±0.5	3.5 ± 1.0	0.98

Data are expressed as means ± SEM of donors (N = 40), patients (N = 30), combined (N = 70) groups. NS = non-significant, LIN (linearity), WOB (oscillation index), VCL (curvilinear velocity), VSL (straight-line velocity), BCF (beat cross frequency), ALH (amplitude of lateral displacement).

4.3.1. Correlations of sperm total motility and functional parameters.

Table 5 depicts various associations between total sperm motility and other functional parameters following treatment of *M. oleifera* for 1-h. Total motility showed no association with percentage of sperm progression in donors (r = 0.50, p = 0.4500), or in combined groups (r = 0.90, p = 0.0800), while a strong positive correlation was observed in patients (r = 1.00, p = 0.0167). Furthermore, no correlation was recorded between total motility and hyperactivation in patients (r = 0.90, p = 0.0833), in combined group (r = 0.50, p = 0.4500), and in the donors (r = 0.40, p = 0.5167). Viable spermatozoa showed no relationship with total motility in the donors (r = 0.50, p = 0.4500). In contrast, the association between total sperm motility and live vitality was significantly and positively correlated in patients and combined groups (r = 1.00, p = 0.0167).

In addition, total sperm motility showed no relationship with MMP-intact spermatozoa in donors (r = 0.73, p = 0.1639), or in patients and the combined group (r = 0.90, p = 0.0833). Similarly, no correlations could be drawn with reactive oxygen species-positive (ROS-positive) sperm in donors (r = -0.59, p = 0.2925), or in patients, and in the combined groups (r = -0.80, p = 0.1333). Again, no relationship was established between total motility with sperm capacitation in the donors (r = -0.56, p = 0.3275), in patients (r = -0.90, p = 0.0833), and in the combined group (r = -0.50, p = 0.4500).

Acrosome reaction demonstrated no relationship in the distinctive groups: donors (r = -0.64, p = 0.2423), and the combined (r = -0.10, p = 0.9500) group. However, contrary results were apparent in patients' total motility when correlated with percentage acrosome reaction, and a significant, strong, and positive association was shown (r = 1.00, p = 0.0167). The degree of sperm DNA fragmentation did not show any relationship with total motility in any of the groups; donors (r = -0.74, p = 0.1491), patients (r = -0.84, p = 0.0761), and the combined group (r = -0.80, p = 0.1333).

Table 5: Relationships between total sperm motility and functional parameters

Parameters	Donors		Patients		Combined group	
	r	p	r	p	r	p
Progressive motility (%)	0.50	0.4500	1.00	0.0167	0.90	0.0833
Hyperactivated motility (%)	0.40	0.5167	0.90	0.0833	0.50	0.4500
Vitality (%)	0.50	0.4500	1.00	0.0167	1.00	0.0167
MMP-intact (%)	0.73	0.1639	0.90	0.0833	0.90	0.0833
ROS-positive (%)	-0.59	0.2925	-0.80	0.1333	-0.80	0.1333
Capacitation (%)	-0.56	0.3275	-0.90	0.0833	-0.50	0.4500
Acrosome reaction (%)	-0.64	0.2423	1.00	0.0167	-0.10	0.9500
DNA fragmentation (%)	-0.74	0.1491	-0.84	0.0761	-0.80	0.1333

4.3.2. Correlation between progressive motility and functional parameters

Regarding the percentage of sperm progression to functional parameters investigated in the donor, combined, and patient group (Table 6), no relationships could be observed with sperm forward-movement in the donor group; hyperactivation, vitality (r = 0.10, p = 0.9500), and capacitation, reactive oxygen species, acrosome reaction, DNA fragmentation (r = 0.02, p = 0.7833), and MMP. (r = 0.70, p = 0.2333).

Similarly, no association with established with hyperactivation and MMP (r = 0.90, p = 0.0833) was present in patients. On the other hand, the relationships with vitality and acrosome reaction were significantly strong (r = 1.00, p = 0.0167) in patients. The percentage of ROS-positive (r = -0.80, p = 0.1333), capacitation, and DNA fragmentation (r = -0.90, p = 0.0833) were not correlated to progressive motility in the patients' group. No relationship was seen between forward-moving sperm and hyperactivation (r = 0.80, p = 0.1333), vitality (r = 0.90, p = 0.0833), and MMP integrity (r = 0.70, p = 0.2333) in the combined group. No correlation was observed between progression, sperm ROS, and DNA fragmentation (r = -0.50, p = 0.4500) and

capacitation (r = -0.20, p = 0.7833), and acrosome reaction (r = -0.30, p = 0.6833), respectively, in the combined group.

Table 6: Correlation between progressive motility and functional parameters

Parameters	Donors		Patient		Combined group	
	r	p	r	p	r	p
Hyperactivated motility (%)	0.10	0.95	0.90	0.0833	0.80	0.1333
Vitality live (%)	0.10	>0.9999	1.00	0.0167	0.90	0.0833
MMP-intact (%)	0.70	0.2333	0.90	0.0833	0.70	0.2333
ROS-positive (%)	0.20	0.7833	-0.80	0.1333	-0.50	0.4500
Capacitation (%)	0.30	0.6833	-0.90	0.0833	-0.20	0.7833
Acrosome reaction (%)	0.20	0.7833	1.00	0.0167	-0.30	0.6833
DNA fragmentation (%)	0.20	0.7833	-0.90	0.0833	-0.50	0.4500

4.3.3 Correlation between sperm hyperactivation and functional parameters

Sperm hyperactivated motility is correlated with other parameters as shown in Table 7. The Spearman correlation test revealed no associations between hyperactivated motility and live sperm, MMP integrity (r = 0.60, p = 0.3500), and DNA fragmentation (r = 0.90, p = 0.0833) in the donor group. In the same group, hyperactivation showed no correlation with respect to ROS-positive, capacitated, and acrosome-reacted sperm (r = -0.10, p = 0.9500). Similarly, no relationship was found between hyperactivated sperm and sperm vitality, DNA fragmentation (r = 0.90, p = 0.0833), and MMP integrity (r = 0.70, p = 0.2333). Contrarily, a significant positive correlation was revealed between hyperactivation and acrosome reaction (r = 1.00, p = 0.0167) in the patient group. When correlated with ROS production and capacitation, hyperactivation showed no significant relationship (r = -0.60, p = 0.3500) and (r = -0.70, p = 0.2333).

When the two groups (patients and donors) were analysed, no correlation was observed between hyperactivation and other parameters. Correlation with live sperm (r = 0.50, p = 0.4500), and MMP-intact (r = 0.30, p = 0.6833) sperm yielded no association. Acrosome reaction, ROS production and DNA fragmentation of the combined group showed similar association as with the patient group (r = -0.10, p = 0.9500.). In addition, no relationship was observed with capacitation in the combined group (r = 0.00, p > 0.9999).

Table 7: Correlation between sperm hyperactivation and functional parameters

Parameters	Donors		Patient		Combined group	
	r	p	r	p	r	p
Vitality live (%)	0.60	0.3500	0.90	0.0833	0.50	0.4500
MMP-intact (%)	0.60	0.3500	0.70	0.2333	0.30	-0.6833
ROS-positive (%)	-0.10	0.9500	-0.60	0.3500	-0.10	0.9500
Capacitation (%)	-0.10	0.9500	-0.70	0.2333	0.00	>0.9999
Acrosome reaction (%)	0.90	0.0833	1.00	0.0167	-0.70	0.2333
DNA fragmentation (%)	-0.10	0.9500	0.90	0.0833	-0.10	0.9500

4.4. Effects of *M. oleifera* leaf extracts on sperm vitality *in vitro*

Sperm suspensions were incubated with various concentrations (0, 0.625. 6.25, 62.5, and 625 µg/ml) of *M. oleifera* leaf extracts for 1 hr. Individual concentrations of *M. oleifera* also did not alter sperm vitality in the donor group when compared to the control group.

Moreover, no significant differences could be observed in the control and all four concentrations between the three groups: donors, patients, and the combined group ($p > 0.05$) (Figure 25).

Dunnett's test showed significant increases in live sperm between the control and 62.5 µg/ml ($p = 0.0264$) and 625 µg/ml ($p = 0.0040$) of *M. oleifera* extract in the patient group (Figure 25). One-way ANOVA repeated measures linear trend analysis showed positive and highly significant trends in vitality, with increasing concentrations of the extract in the patient group ($p > 0.0001$).

Independent analysis showed remarkable improvements when the following concentrations: 6.25 µg/ml ($p = 0.0256$), 62.5 µg/ml ($p = 0.0488$), and 625 µg/ml ($p = 0.0011$) (Figure 25) were compared to their respective controls of the combined group.A significant linear trend was observed in the combined group when a repeated measure trend analysis was performed ($p < 0.0001$).

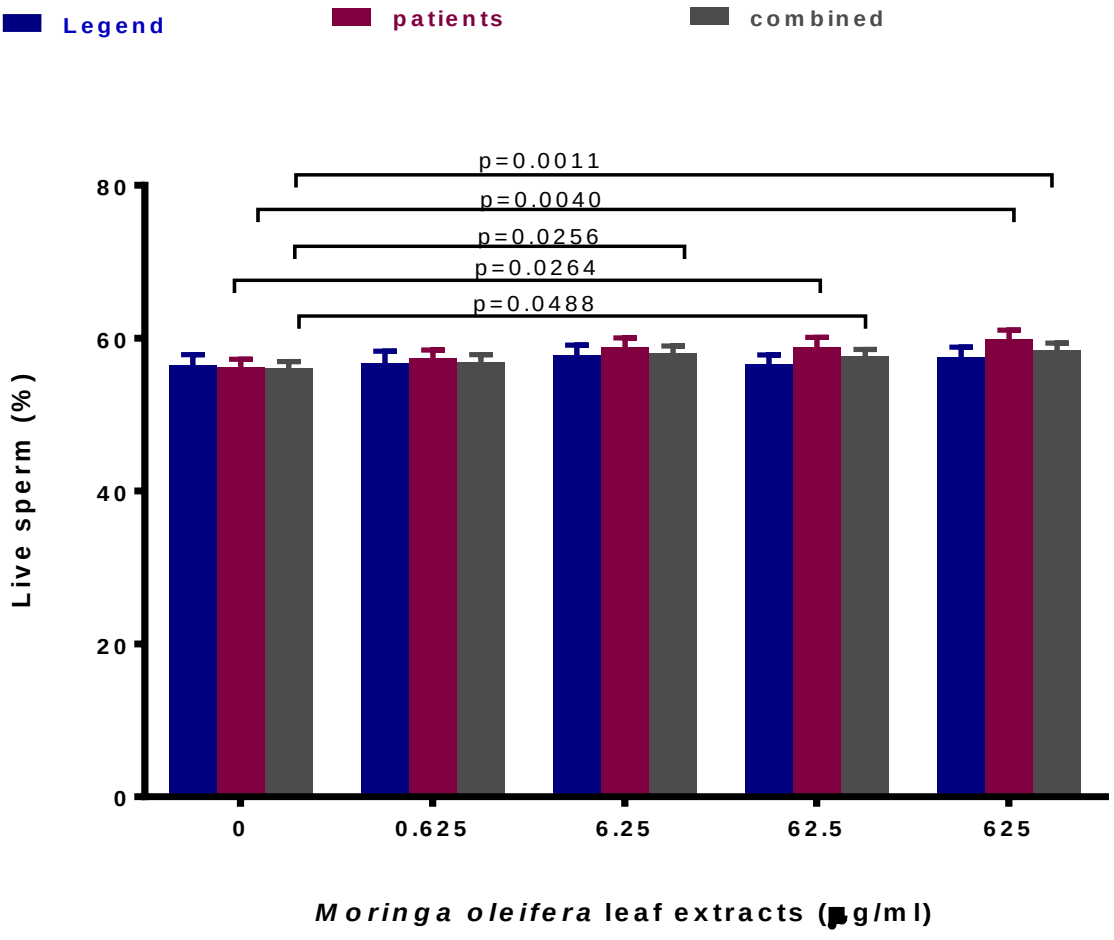


Figure 25: Percentage of live sperm after 1hr incubation with *M. oleifera in vitro*. Mean ± SEM. Blue bars represent the mean values of donors (N = 40), purple bars are for patients (N = 30), and grey bars are for the combined group (patients and donors) (N = 70).

4.4.1. Correlation of sperm vitality with other functional parameters

The relationship between sperm vitality and functional parameters is summarised in Table 8. Vitality and MMP integrity showed no relationship in the donor group (r = 0.20, p = 0.7833). Also, no relationship was noted with percentages of sperm capacitation, acrosome reaction (r = 0.30, p = 0.6833), and ROS and DNA fragmentation (r = -0.30, p = 0.6833) of donors.

No correlation was observed between live sperm with intact MMP in patients and in the combined group (r = 0.90, p = 0.0833). However, a strong, positive and significant relationship was seen with the percentage of the acrosome reaction in the patient (r = 1.00, p = 0.0167). When the percentage of live sperm was correlated with ROS-positive spermatozoa and DNA fragmentation in patients and the combined groups, no link was established (r = -0.80, p = 0.1333). Moreover, correlation analysis of capacitation and DNA-fragmented sperm in the patients showed no relationship (r = -0.90, p = 0.0833). In addition, no association was shown with acrosome-reacted sperm of the combined group (r = -0.10, p = 0.9500).

Table 8: Correlation of sperm vitality (live) and functional parameters

Parameters	Donors		Patient		Combined	
	r	p	r	r	R	r
MMP-intact (%)	0.20	0.7833	0.90	0.0833	0.90	0.0833
ROS-positive (%)	-0.30	0.6833	-0.80	0.1333	-0.80	0.1333
Capacitation (%)	0.30	0.6833	-0.90	0.0833	-0.50	0.4500
Acrosome reaction (%)	0.30	0.6833	1.00	0.0167	-0.10	0.9500
DNA fragmentation (%)	-0.20	0.7833	-0.90	0.0833	-0.80	0.1333

4.5. Effects of *M. oleifera* leaf extracts on sperm mitochondrial membrane potential (∆ψm) *in vitro*

After incubation with increasing concentrations of *M. oleifera* (0, 0.625. 6.25, 62.5 and 625 µg/ml), no significant changes in the percentage of intact mitochondrial membrane potential in sperm were observed when compared to the control ($p > 0.05$) (Figure 26) in the donor group. However, a statistically significant increase in the percentage of MMP-intact sperm of the patients after exposure to *M. oleifera* extracts was observed ($p = 0.0262$). A significant increase in the percentage of intact MMP was observed at 625 µg/ml in the patient group in comparison with the untreated group ($p = 0.0094$) (Figure 26). Similarly, increments in MMP-intact sperm at the highest concentration of the plant were observed in the combined group ($p = 0.0277$) (Figure 26). Conversely, as was the case with the percentage of live sperm, no improvements in MMP were observed between the donor control and the therapeutic dose of 625 µg/ml ($p > 0.05$).

The control and low concentrations of *M. oleifera* did not produce statistically significant differences between donors and patients: 0.625 µg/ml ($p = 0.0904$) and 6.25 µg/ml ($p = 0.0887$), and the combined sperm MMP ($p > 0.05$). In contrast, MMP

in patients was significantly more improved than in donors at concentrations 62.5 µg/ml (p = 0.0052) and 625 µg/ml (p = 0.0077) (Figure 26).

The repeated measure showed a significant positive trend in MMP with increasing concentrations of *M. oleifera* (p < 0.0001) in the patient sperm. No trend was seen in the donor and combined groups.

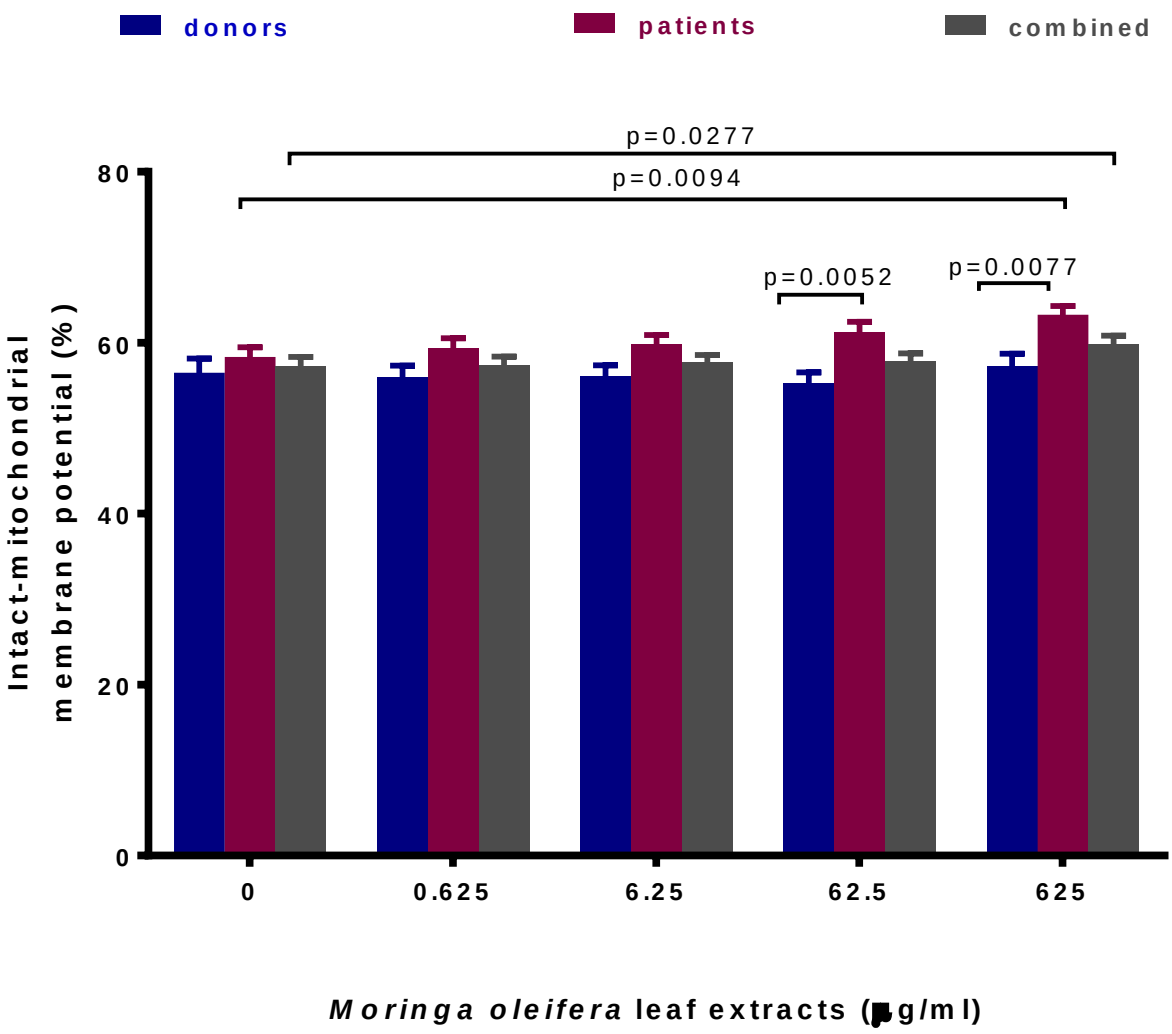


Figure 26. Percentage-intact mitochondrial membrane potential (MMP) spermatozoa after 1 hr incubation with *M. oleifera in vitro*. Means ± SEM. Blue bars represent the mean values of donors (N = 40), purple bars are for patients (N = 30), and grey bars are for the combined group (patients and donors) (N = 70).

4.5.1 Correlation of sperm-intact mitochondrial membrane potential and functional parameters

Mitochondrial membrane potential intactness of sperm was correlated with other functional parameters in three groups, and results are displayed in Table 9. Intactness of sperm MMP showed no relationship with ROS-positive and DNA-fragmented sperm (r = -0.30, p = 0.6833), capacitation (r = 0.30, p = 0.6833), and acrosome reaction (r =-0.70, p = 0.2333), respectively, in the donor's group. In patients, acrosome reaction displayed no significant correlation with MMP (r = 0.90, p = 0.0833). No relationship was seen between MMP and ROS production (r = -0.90, p = 0.0833), while on the contrary, negative strong correlations were observed with capacitation (r = -1.00, p = 0.0167) and DNA fragmentation (r = -1.00, p = 0.0167) in patients and the combined groups. Despite relationship observed with other parameters in the combined group, MMP showed no relationship with capacitation (r = -0.70, p = 0.2333).

Table 9: Correlation of MMP-intact sperm with functional parameters

Parameters	**Donors**		**Patient**		**Combined**	
	r	p	r	p	r	p
ROS-positive (%)	-0.30	0.6833	-0.90	0.0833	-0.90	0.0833
Capacitation (%)	0.30	0.6833	-1.00	0.0167	-0.70	0.2333
Acrosome reaction (%)	-0.70	0.2333	0.90	0.0833	0.00	>0.9999
DNA fragmentation (%)	-0.30	0.6833	-1.00	0.0167	-0.90	0.0833

4.6. Effects of *M. oleifera* on reactive oxygen species-positive (ROS) spermatozoa *in vitro*

M. oleifera leaf extracts showed remarkable changes in sperm superoxide free radicals scavenging, following 1-hour incubation. Overall, *M. oleifera* aqueous extracts induced a significant decrease in sperm intracellular ROS production (ROS-positive

spermatozoa) at higher concentrations of the extract in all groups. On the other hand, *M. oleifera* leaf extracts significantly reduced spermatozoa intracellular reactive oxygen species (ROS) production in the patient group, even at moderate concentrations tested compared to the control as follows; 6.25 µg/ml ($p = 0.0350$) (not displayed on the graph), and high concentration, 62.5 µg/ml ($p < 0.0001$). When the control was compared to concentration 625 µg/ml in all groups, a highly significant reduction was observed ($p < 0.0001$) (Figure 27).

ROS-positive spermatozoa were significantly higher in the control, 0.625 µg/ml, 6.25 µg/ml, 62.5 µg/ml, and 625 µg/ml of patients rather than donors, which were ($p < 0.0001$, $p = 0.0033$, and $p = 0.0043$) and the combined group ($p = 0.0160$, $p = 0.0063$, and $p = 0.0427$) (Figure 27). Correspondingly, ROS-positive sperm in the combined group was slightly higher than in the healthy donor control group ($p = 0.0641$) (Figure 27), 0.625 µg/ml ($p = 0.0331$), 6.25 µg/ml ($p = 0.0033$) and 62.5 µg/ml ($p = 0.0027$). Furthermore, ROS positive spermatozoa in patients was significantly higher that of the combined group treated with 0.625 µg/ml *M. oleifera* ($p = 0.0068$) (Figure 27). The inhibitory effect of *M. oleifera* at the highest dose of 625 µg/ml did not differ between the combined group and donors ($p > 0.05$) as well as patients ($p > 0.05$).

The one-way ANOVA repeated measures trend analysis revealed a highly significant inverse tendency in ROS-positive spermatozoa in a dose-dependent manner, across all three groups ($p < 0.0001$).

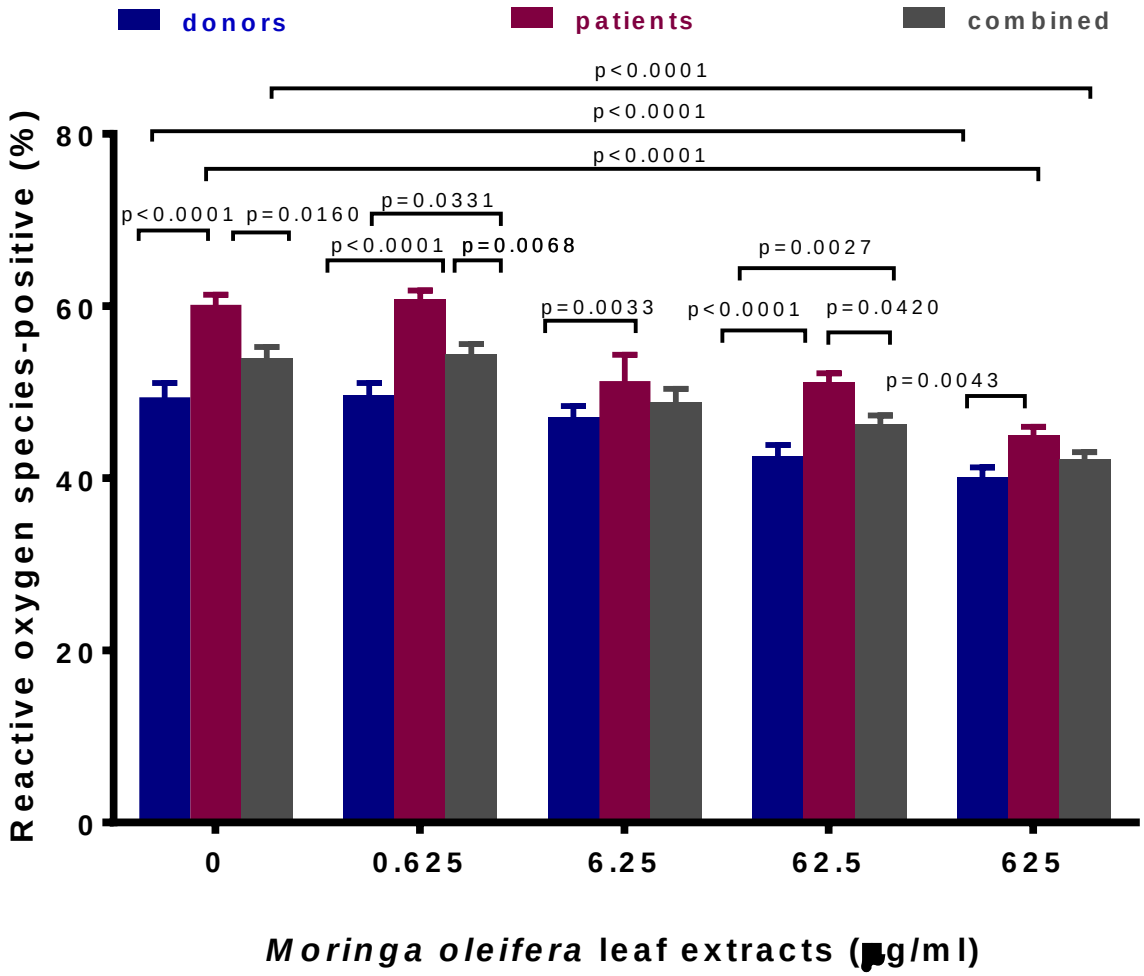


Figure 27: Percentage ROS-positive spermatozoa after 1 hr incubation with *M. oleifera in vitro*. Means ± SEMs. Blue bars represent the mean values of donors (N = 40), purple bars are for patients (N = 30), and grey bars are for combined group (patients and donors) (N = 70).

4.6.1 Correlation of reactive oxygen species with functional parameters

Sperm ROS production were correlated with other sperm functional characteristics as shown in Table 10. The percentage of ROS-positive sperm with capacitated sperm showed no relationship in donors (r = 0.70, p = 0.2333), patients, and the combined group (r = 0.90, p = 0.0833). Similarly, no association could be established between ROS-positive spermatozoa with acrosome reaction in donors (r = 0.00, p > 0.9999), patients, and the combined group (r = 0.10, p = 0.9500), respectively. Interestingly, sperm ROS production was strongly positively correlated with the percentage of sperm with DNA fragmentation in all groups (r = 1.00, p = 0.0167). Patient ROS-positive

sperm displayed no significant correlation with DNA fragmentation (r = 0.90, p = 0.0833).

Table 10: Correlation of reactive oxygen species production with functional parameters

Parameters	Donors		Patient		Combined group	
	r	p	r	p	r	p
Capacitation (%)	0.70	0.2333	0.90	0.0833	0.90	0.0833
Acrosome reaction (%)	0.00	>0.9999	0.10	0.9500	0.10	0.9500
DNA fragmentation (%)	1.00	0.0167	0.90	0.0833	1.00	0.0167

4.7. Effects of *M. oleifera* leaf extracts on sperm DNA fragmentation *in vitro*

Aqueous *M. oleifera* extracts in higher concentrations improved DNA intactness in all groups tested (Figure 28). However, low concentrations (0.625 µg/ml, and 6.25 µg/ml) of *M. oleifera* did not significantly change the percentage of spermatozoa with DNA fragmentation ($p > 0.05$) when compared to their controls (Figure 28).

On the other hand, incubation with higher *M. oleifera* concentrations of 62.5 µg/ml ($p = 0.0167$), and 625 µg/ml) ($p < 0.0001$) of the extract resulted in a significant decrease in the percentage of TUNEL-positive sperm (DNA-fragmented spermatozoa) in comparison with the control (Figure 28).

Percentage of sperm DNA fragmentation in the patient group dropped significantly at doses 62.5 µg/ml ($p = 0.0082$), and 625 µg/ml ($p = 0.0006$) in comparison with the respective controls (Figure 28).

No differences were measured between the control, 6.25 µg/ml, 62.5 µg/ml, and 625 µg/ml of the three groups; donors, patients, and the combined group ($p > 0.05$) (Figure 28). However, marginal significance in sperm with DNA fragmentation could be observed between the donors and patients at the lowest dose of *M. oleifera* (0.625 µg/ml) ($p = 0.0380$). Repeated measures correspondingly showed significant negative linear trends in the percentage of spermatozoa with DNA fragmentation with increasing concentrations of *M. oleifera* extracts after 1-h incubation ($p < 0.0001$) in the donors, patients, and the combined groups.

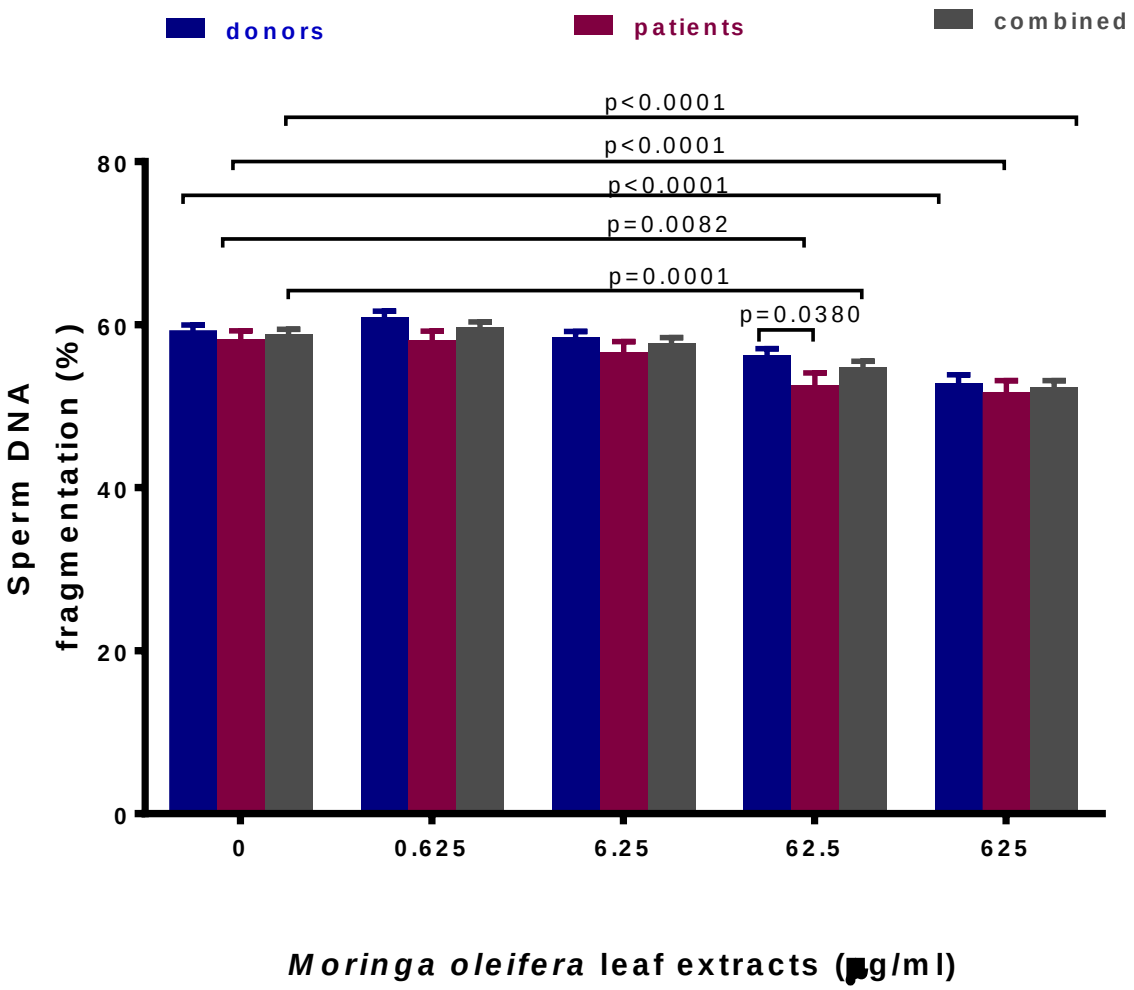


Figure 28. Percentage of sperm DNA fragmentation after 1 hr incubation with *M. oleifera in vitro*. Means ± SEM. Blue bars represent the mean values of donors (N = 40), purple bars are for patients (N = 30), and grey bars are for the combined group (patients and donors) (N = 70).

4.7.1 Correlation of sperm DNA fragmentation and functional parameters

Sperm DNA fragmentation was correlated with functional parameters, capacitation and acrosome reaction and the Spearman analysis results are shown in Table 11. DNA fragmentation showed no relationship with capacitation in donors (r = 0.70, p = 0.2333), patients, and the combined group (r = 0.90, p = 0.0833), respectively. Similarly, no relationship was established between sperm with acrosome reaction in the combined (r = 0.10, p=0.9500) and the donor groups (r = 0.00, p > 0.9999). Contrarily, a significantly positive relationship was demonstrated between patients' DNA fragmentation and the percentage of acrosome-reacted spermatozoa (r = 1.00, p = 0.0167).

Table 11: Correlation of sperm DNA fragmentation and functional parameters

Parameters	Donors		Patient		Combined group	
	r	p	r	p	r	p
Capacitation (%)	0.70	0.2333	0.90	0.0833	0.90	0.0833
Acrosome reaction (%)	0.00	>0.9999	1.00	0.0167	0.10	0.9500

4.8 Effects of *M. oleifera* on sperm capacitation and acrosome reaction *in vitro*

Low concentrations, as observed with previous parameters, did not affect the sperm capacitation-acrosomal status. Based on the Hoechst 33258 counterstain, three statuses could be recorded for capacitation and acrosome reaction: F (uncapacitated-acrosome-intact spermatozoa), B (capacitated acrosome-intact spermatozoa) and AR (capacitated acrosome-reacted spermatozoa) patterns. Percentage of uncapacitated and acrosome-intact spermatozoa (F pattern) significantly increased at 625 µg/ml when compared to the control group (p = 0.0047) (Figure 29a) in the donor group. A significant decrease in the lower treatment groups was observed when compared to the highest concentration (625 µg/ml) as follows (0.625 µg/ml (p = 0.0022), 6.25 µg/ml (p = 0.0009), and 62.5 µg/ml (p = 0.0023), respectively.

No changes in the percentage of uncapacitated (F pattern) spermatozoa were observed in patients after treatment with *M. oleifera* ($p > 0.05$) (Figure 29a). The analysis of the combined group revealed a marginally significant increase in the percentage of F pattern sperm after *M. oleifera* exposure in the combined group ($p = 0.0113$) (Figure 29a).

The control and 62.5 µg/ml in F pattern of the patient group was slightly higher than that of the donor group ($p = 0.0136$ and $p = 0.0095$) (Figure 29a).Furthermore, uncapacitation was consistently higher in the patient group than in combined group in all *M. oleifera* concentrations; control ($p = 0.0356$), 0.625 µg/ml ($p = 0.0050$), 6.25 µg/ml ($p = 0.0067$), 62.5 µg/ml, and 625 µg/ml ($p < 0.0001$) (Figure 29a). Trend analysis showed a significant increase in uncapacitated (F pattern) sperm in the donor ($p = 0.0036$), and the combined group ($p = 0.0032$), and no trends were observed in patients when increasing concentrations were applied.

The extracts did not alter sperm capacitation (B pattern) in the donor, patient, and the combined groups ($p > 0.05$) (Figure 29b). Dunnets test showed a marginally significant decrease in capacitated acrosome-intact sperm at concentration 625 µg/ml in the patient group ($p = 0.0491$) (Figure 29b). Trend analysis showed significant increases in uncapacitated (F pattern) sperm in the donor group ($p = 0.0036$), and in the combined group ($p = 0.0032$), while no trends in patients were observed as the dose increased.

Capacitation in the donors appeared to be significantly higher than in patients in all concentration tested; 0 µg/ml ($p < 0.0001$), 0.625 µg/ml, 6.25 µg/ml, 62.5 µg/ml, and 625 µg/ml ($p < 0.0001$) (Figure 29b). Similarly, percentage of capacitated spermatozoa was significantly higher in the combined group than peatients at the following concentrations: 0.625 µg/ml ($p = 0.0032$), 6.25 µg/ml ($p = 0.0009$) and 62.5 µg/ml ($p = 0.0035$). Furthermore, the same increases in donors' B pattern spermatozoa in comparison with the combined group were observed at concentrations; 0.625 µg/ml ($p = 0.0185$), 6.25 µg/ml ($p = 0.0077$), 62.5 µg/ml ($p = 0.0221$), and 625 µg/ml ($p = 0.0083$) (Figure 29b). The combined group also showed significant differences in B pattern sperm when compared to patients at doses: control ($p = 0.0008$) (Figure 29b), 0.625 µg/ml ($p = 0.0032$), 6.25 µg/ml ($p = 0.0009$), and (625 µg/ml) ($p = 0.0001$) (Figure 29b). However, linear trends were observed in the percentage of capacitated

acrosome-intact spermatozoa (B pattern) in the combined group (p = 0.0270) and patients (p < 0.0001) as the concentrations of *M. oleifera* increased.

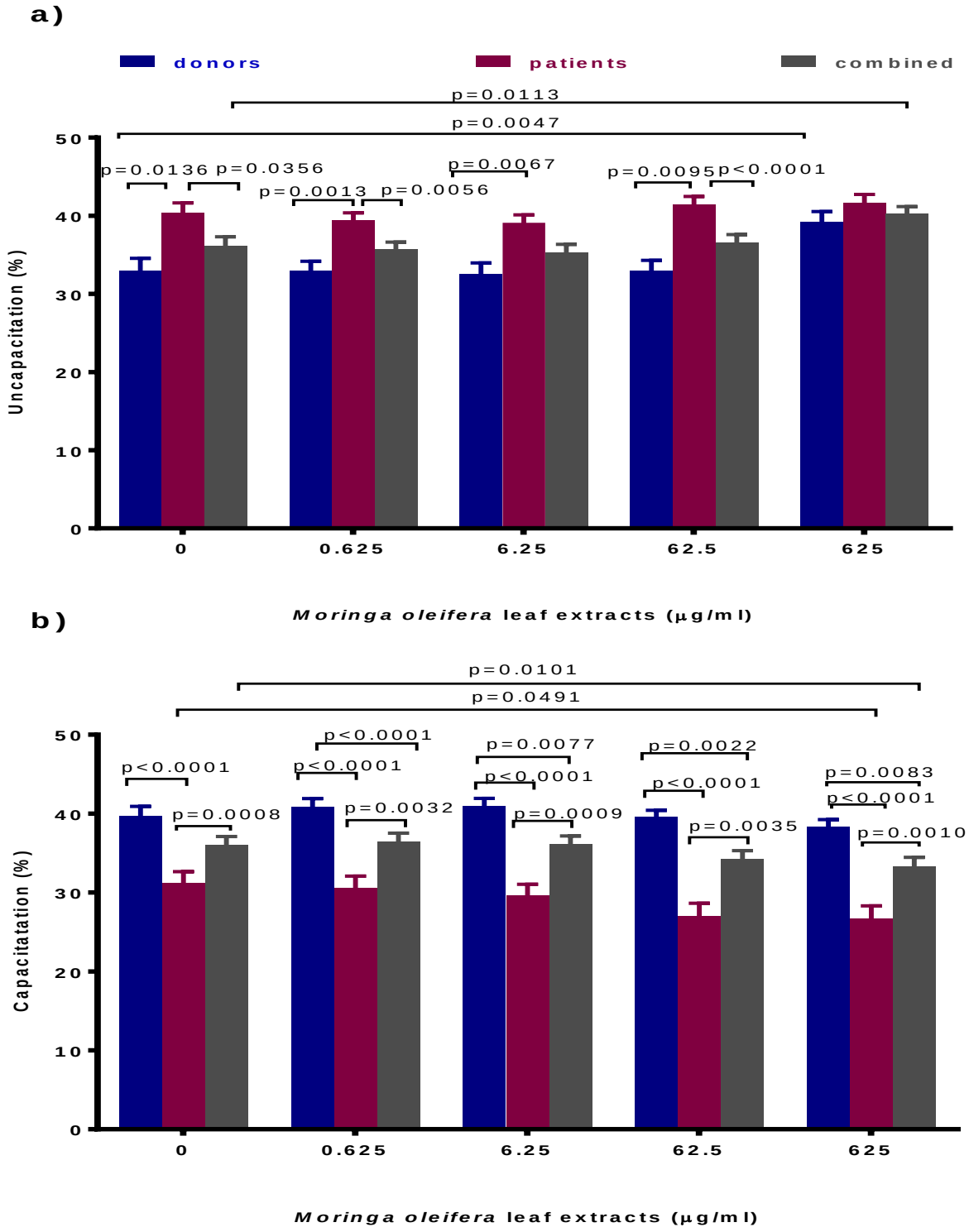


Figure 29: Percentage of uncapacitated (F pattern) **(a)** and capacitated (B pattern) spermatozoa **(b)** after 1 hr incubation with *M. oleifera in vitro*. Means ± SEM. Blue bars represent the mean values of donors (N = 40), purple bars are for patients (N = 30), and grey bars are for the combined group (patients and donors) (N = 70).

The percentage of capacitated acrosome-reacted (AR pattern) spermatozoa was slightly but significantly decreased in the donor group compared to the control p = 0.0378) (Figure 30). Similarly, the decrease in acrosome-reacted sperm at *M. oleifera* highest concentration 625 µg/ml was significantly different compared to the lower concentrations tested of 0.625 µg/ml (p = 0.0035), 6.25 µg/ml (p = 0.0096), and 62.5 µg/ml (p = 0.0079) in the donor group. No changes in sperm acrosome reaction occurred in either the patient or the combined groups (Figure 30) after treatment with *M. oleifera* extracts.

No significant changes could be recorded in percentage acrosome reaction between the donors, patients, and combined groups in the control, 0.625 µg/ml, 6.25 µg/ml, and 62.5 µg/ml (p > 0.05) (Figure 30). On the other hand, treatment with the highest concentration of 625 µg/ml, patient's acrosome reaction was significantly higher compared to the donor (p = 0.0004), and the combined groups (p = 0.0449) (Figure 30).

Furthermore, increasing concentrations of *M. oleifera* extract caused a positive trend in the percentage of acrosome reaction (AR pattern) in donors (p = 0.0208), while no significant trends were observed in the patients and combined groups (p > 0.05) respectively.

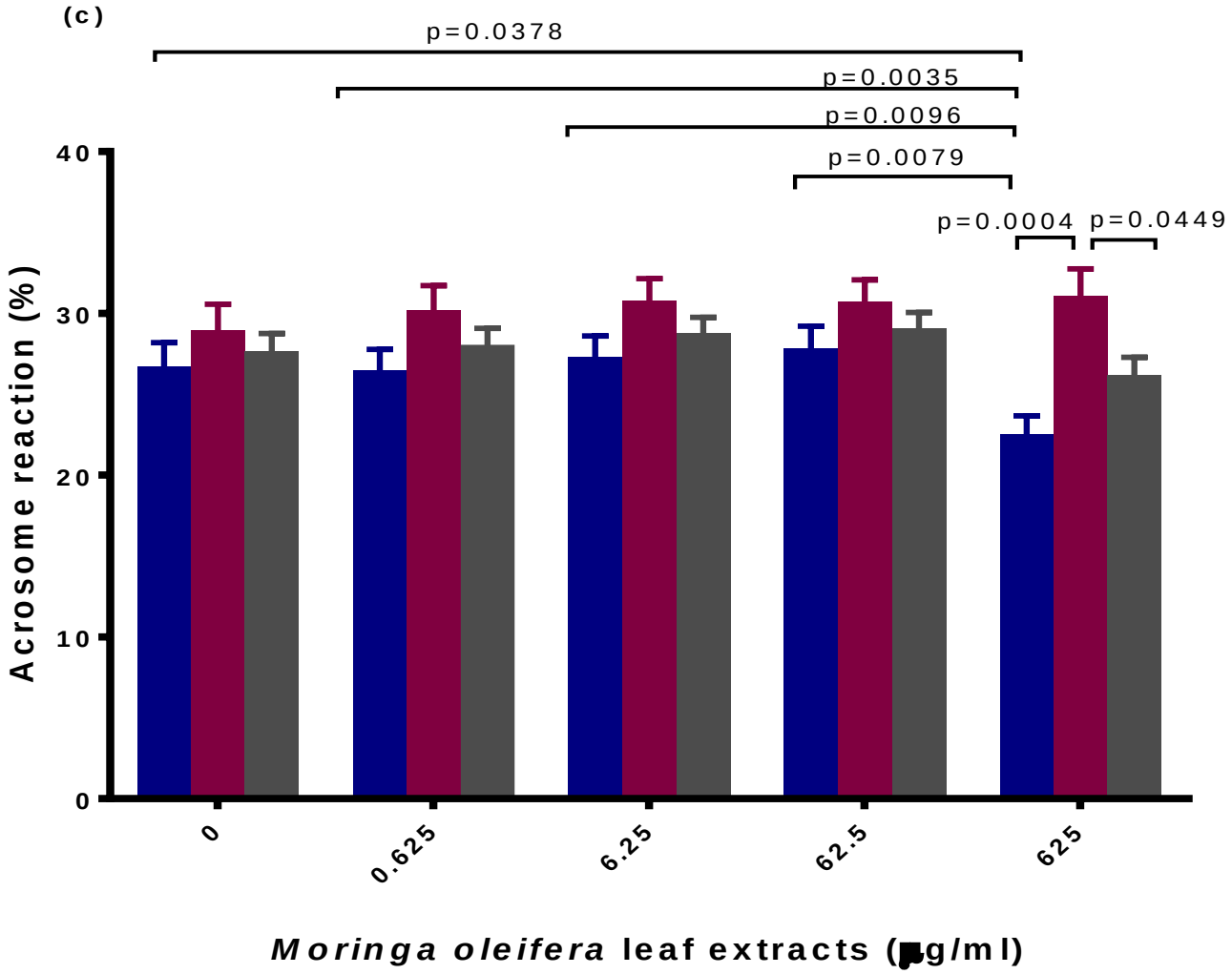


Figure 30. Percentage sperm of acrosome reaction after 1 hr incubation with *M. oleifera in vitro*. Mean ± SEM. Blue bars represent the mean values of donors (N = 40), purple bars are for patients (N = 30), and grey bars are for combined group (patients and donors) (N = 70).

4.8.1 Correlation of sperm capacitation and acrosome reaction

Two interrelated sperm functional processes were correlated as shown in Table 12. Capacitation and acrosome-reacted spermatozoa showed no relationship in the donor and the combined group (r = 0.30, p = 0.6833) as well as (r = -0.90, p = 0.0833).

Table 12. Correlation of sperm capacitation and acrosome reaction

Parameters	Donors		Patient		Combined group	
	r	p	r	p	r	p
Acrosome reaction (%)	0.30	0.6833	-0.90	0.0833	0.30	0.6833

4. 9. Effects of *M. oleifera* on asthenozoospermic sperm *in vitro*

Asthenozoospermic semen samples were identified and analysed separately, selected from both the patient and the donor groups. The cut-off values defining asthenozoospermic samples (total motility < 40% or progressive motility < 32%) (WHO, 2010). Sixteen samples were found to be asthenozoospermic, and the effects of *M. oleifera* on this group were recorded after 1-hr incubation.

4.9.1 Effects of *M. oleifera* on sperm motility from asthenozoospermic samples *in vitro*

After incubation with *M. oleifera* concentrations (0, 0.625, 6.25, 62.5, and 625 µg/ml), no effects were seen in the total sperm motility between the individual treatments and their controls ($p > 0.05$) (Table 13). However, repeated measures ANOVA trend analysis revealed that total motility tended to increase steadily in a concentration-dependent manner ($p = 0.0178$). *M. oleifera* extracts displayed no change in the percentage of progressive and non-progressive motile sperm ($p > 0.05$). Significant linear trends were evident with increasing concentrations of *M. oleifera* in progressive ($p = 0.0445$) and non-progressive ($p = 0.0141$) motile sperm.

Extracts did not yield any changes in the percentage of hyperactivation ($p > 0.05$) of asthenozoospermic samples (Table 13).

Table 13: Effects of *M. oleifera* on sperm motility parameters on asthenozoospermic samples *in vitro*

Sperm motility parameters (%)	***Moringa oleifera* leaf extract concentrations (µg/ml)**					**p-value**
	0	**0.625**	**6.25**	**62.5**	**625**	
Total motility (All grades)	16.0 ± 12.3	16.3 ± 2.0	17.5 ± 1.9	17.5 ± 1.9	22.1 ± 2.7	0.3414
Progressive (grade A+B)	7.1 ± 1.5	9.0 ± 1.8	10.0 ± 1.8	9.0 ± 2.0	13.5±1.4	0.2413
Non-progressive (Grade C)	6.2 ±1.0	6.6 ±1.1	6.9 ±1.1	7.3 ±0.7	8.4 ±1.1	0.4186
Hyperactivation	1.0 ±0.5	2.3 ± 1.4	3.2 ±1.5	2.5 ± 1.2	5.8± 3.5	0.9758

Kinematic parameters investigated in asthenozoospermic samples were sperm percentage linearity index (LIN) (%), straightness index (STR) (%), wobble / oscillation index (WOB) (%), flagella speeds: curvilinear velocity (VCL) (µms^{-1}), straight-line / rectilinear velocity (VSL) (µms-1), and average path velocity (VAP) (µms^{-1}) as well as other essential spatial sperm kinematic characteristics, the amplitude of lateral head displacement (ALH) (µm) and flagella beat cross frequency (BCF) (Hz). Results on the effect of *M. oleifera* extracts on asthenozoospermic kinematics are displayed in Table 13.

Various concentrations of *M. oleifera* extract (0, 0.625, 6.25, 62.5, and 625 µg/ml) did not cause any changes in all of the sperm kinematics in the asthenozoospermic group ($p > 0.05$) (Table 14).

Table 14: Effects of *M. oleifera* sperm motility kinematics from asthenozoospermic samples *in vitro*

Sperm kinematic parameters	***Moringa oleifera* leaf extract concentrations (µg/ml)**					**p-value**
	0	**0.625**	**6.25**	**62.5**	**625**	
LIN (%)	42.0 ± 4.1	44.6 ± 4.0	42.2 ± 3.9	40.9 ± 2.6	41.9 ±2.	0.9693
STR (%)	66.0 ±3.5	67.3 ±3.7	66.4 ±4.0	64.8 ±2.7	64.9 ±3.8	0.9864
WOB (%)	57.9 ±2.7	60.8 ±2.6	57.7±2.9	57.9 ±1.9	58.2 ±3.0	0.9117
VCL (µm. s^{-1})	59.4 ±3.2	62.5 ±4.3	68.2. ± 5.8	60.0 ± 2.8	67.5± 8.5	0.6935
VAP (µm. s^{-1})	34.0 ±2.0	36.6 ±2.5	37.2 ±2.3	33.9 ±2.4	35.2 ±1.6	0.8337
VSL (µm. s^{-1})	29.1 ± 3.1	30.8 ±3.2	30.4±2.2	29.1 ±2.6	29.00±2.3	0.9816
ALH (m.s^{-1})	2.2 ±0.1	2.3 ± 0.1	2.4 ±1.3	2.3 ± 0.1	2.4± 0.2	0.7432
BCF (Hz)	11.3± 1.1	11.8± 1.2	11.7± 0.83	11.0 ±1.3	11.4 ±1.3	0.9885

4.9.2. Effects of *M. oleifera* on asthenozoospermic vitality *in vitro*

Treatment of asthenozoospermic sperm with *M. oleifera* extracts (0, 0.625. 6.25, 62.5, and 625 µg/ml) caused no cytotoxicity nor any enhancement in asthenozoospermic sperm after 1-h exposure ($p > 0.05$) (Figure 31). While no concentration elicited any harmful effects on the percentage of live sperm, linear trends were significant with increasing concentrations ($p = 0.0089$).

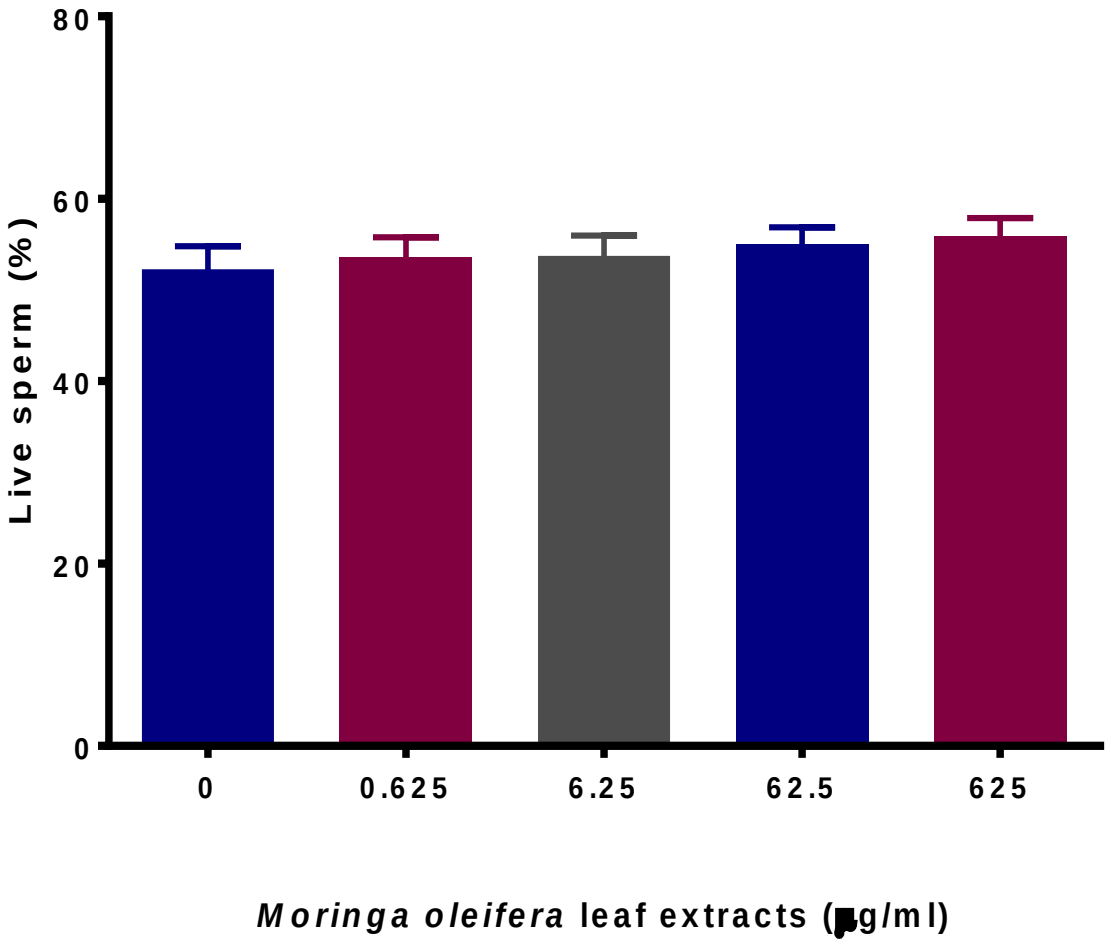


Figure 31: Percentage of live asthenozoospermic sperm after treatment with *M. oleifera* extracts for 1 hr *in vitro*. Mean ± SEM.

4.9.3. Effects of *M. oleifera* extract on mitochondrial membrane potential (MMP-intact) in asthenozoospermic samples *in vitro*

M. oleifera extracts (0, 0,625, 6.25, 62.5, and 625 µg/ml) did not cause any significant change in the percentage of sperm with intact MMP of asthenozoospermic samples ($p > 0.05$) (Figure 32). Repeated measures showed an increase of asthenozoospermic MMP-intact in the post treatment linear trends ($p = 0.0051$).

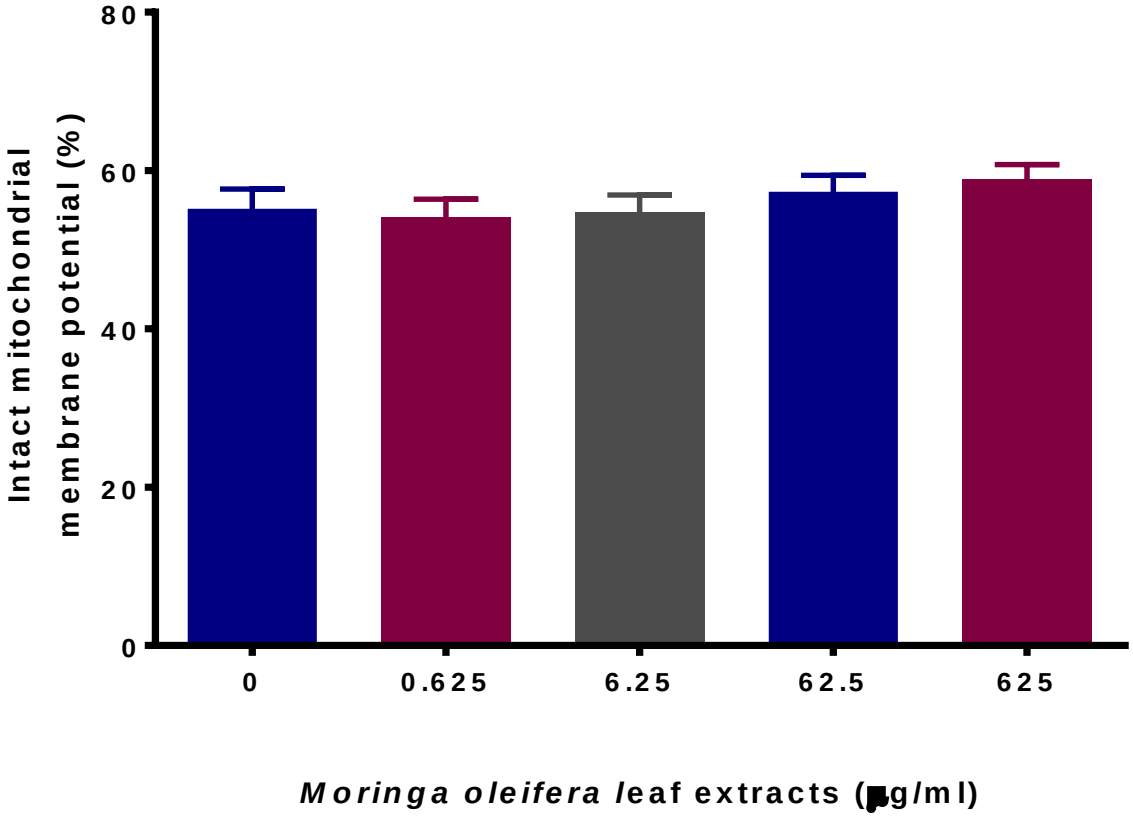


Figure 32: Percentage of asthenozoospermic MMP-intact sperm after 1hr incubation with *M. oleifera in vitro*. Mean ± SEM.

4.9.4. Effects of *M. oleifera* on asthenozoospermic reactive oxygen species-positive spermatozoa *in vitro*

M. oleifera significantly reduced the percentage of ROS-positive sperm in the asthenozoospermic group ($p < 0.0001$) in a concentration-dependent manner (Figure 33). An insignificant decrease in ROS was shown at 62.5 µg/ml ($p > 0.05$), followed by a pronounced significant decrease at 625 µg/ml ($p = 0.0005$) (Figure 33) compared to the control group. Additionally, a significant decrease was observed when comparing the highest (625 µg/ml) with lower concentrations (0.625 µg/ml; $p < 0.0001$) and (6.25 µg/ml; $p = 0.0009$), respectively (Figure 33). ANOVA linear trends also showed a highly significant decrease in ROS-positive asthenozoospermic spermatozoa when the *M. oleifera* dose was increased ($p < 0.0001$).

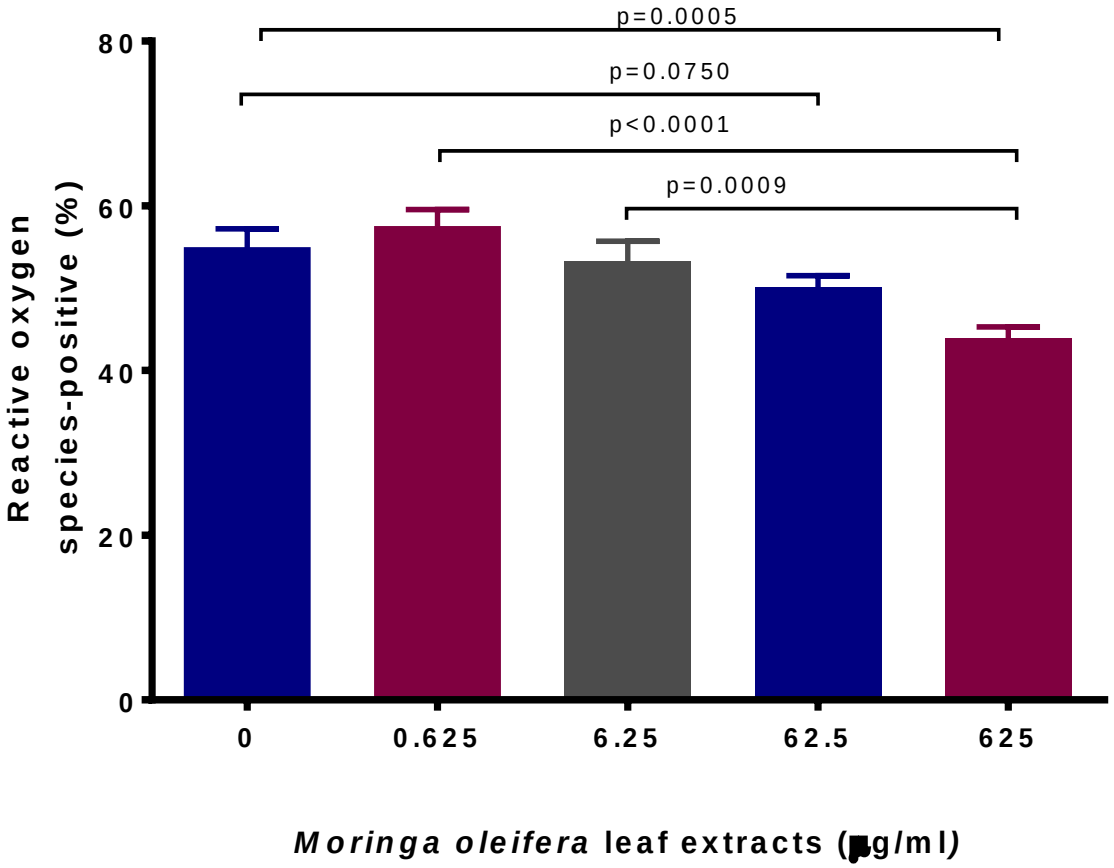


Figure 33: Percentage of ROS-positive asthenozoospermic sperm after 1 hr incubation with *M. oleifera in vitro*. Mean ± SEM.

4.9.5. Effects of *M. oleifera* on DNA-fragmented sperm in asthenozoospermic samples *in vitro*

A significant decrease in asthenozoospermic DNA fragmentation was observed when the two highest concentrations, 62.5 µg/ml (p = 0.0439) and 625 µg/ml (p = 0.0094), were compared to the control (Figure 33). The highest concentration of *M. oleifera* (625 µg/ml) differed significantly when compared to the concentrations of 0.625 µg/ml (p = 0.0065) and non-significantly to 6.25 µg/ml (p > 0.05).Consequently, a highly significant negative linear trend in sperm DNA fragmentation was observed with increasing concentrations of *M. oleifera* extract (p = 0.0007).

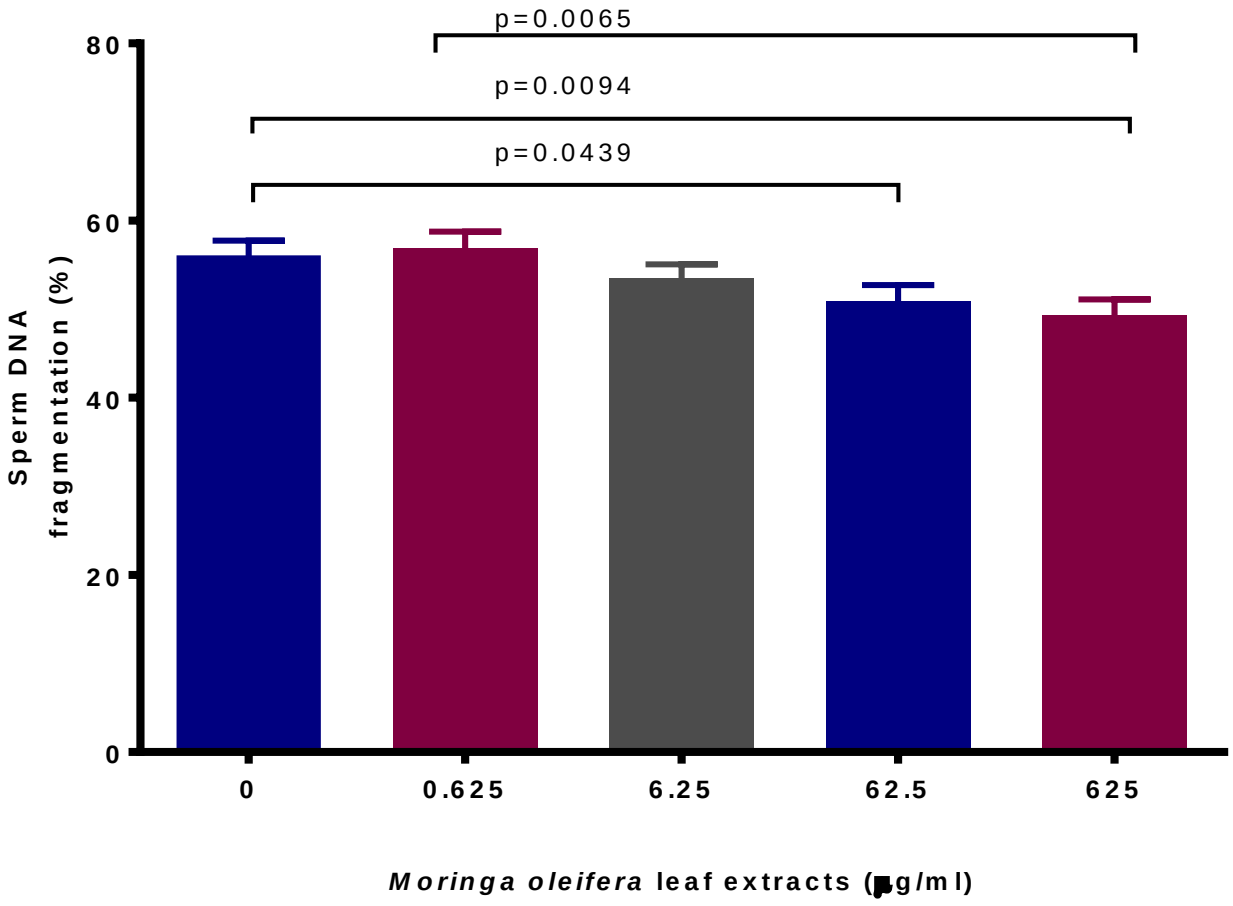


Figure 34: Percentage of asthenozoospermic DNA fragmentation after 1 hr with *M. oleifera in vitro*. Mean ± SEM.

4.9.6. Effects of *M. oleifera* on capacitation and acrosome reaction in asthenozoospermic sperm *in vitro*

Asthenozoospermic spermatozoa treated with varying concentrations of *M. oleifera* aqueous leaf extracts (0, 0,625, 6.25, 62.5, and 625 µg/ml) *in vitro* did not show changes in capacitation and acrosomal staining patterns (F, B, and AR) (Figure 35). Uncapacitation (F pattern) ($p = 0.7898$), capacitation ($p = 0.6933$) (B pattern), and acrosome reaction ($p = 0.9884$) (AR) patterns remained unchanged when the controls were compared to the highest *M. oleifera* concentration (625 µg/ml).

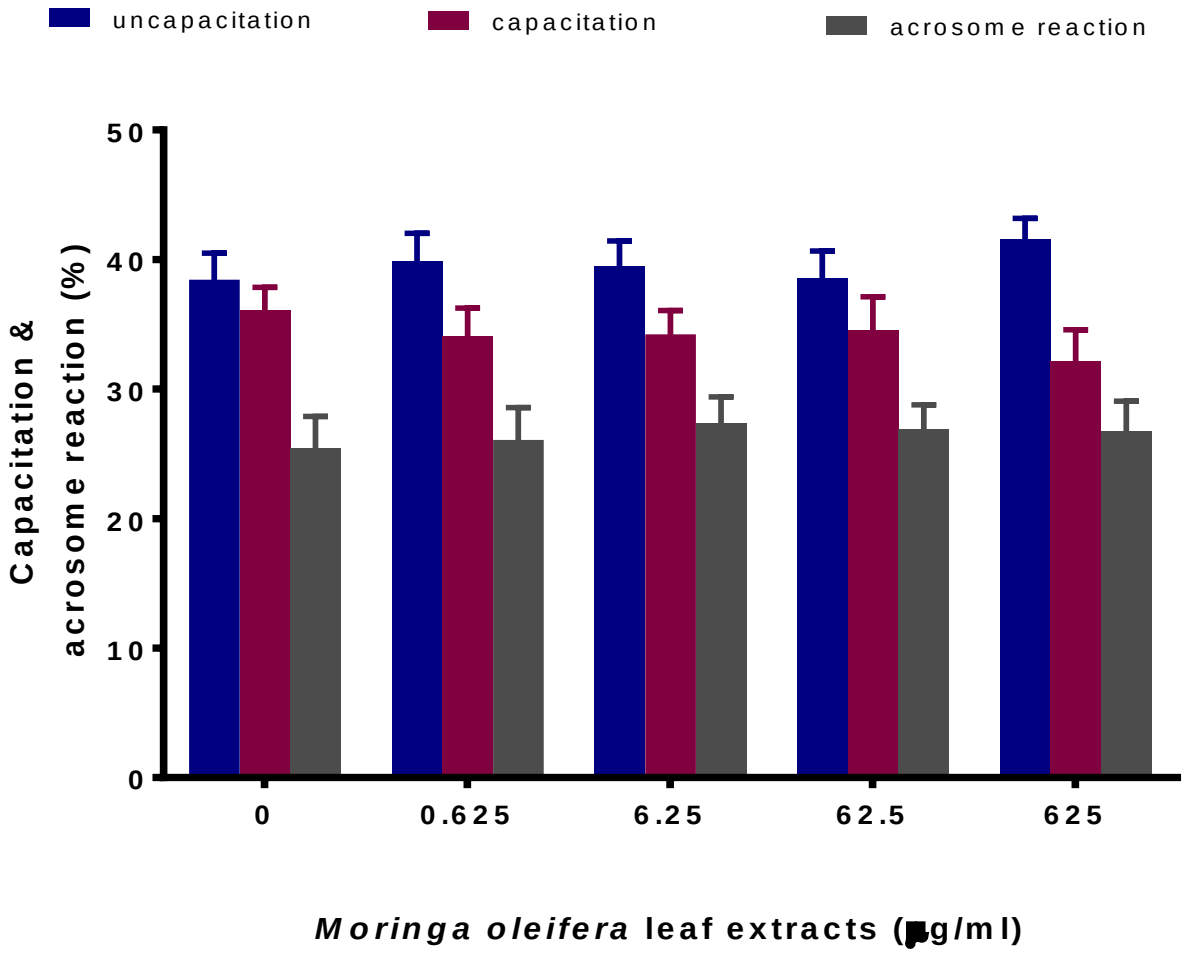


Figure 35: Percentage of asthenozoospermic sperm capacitation and acrosome reaction after 1 hr incubation with *M. oleifera in vitro*. Mean ± SEM.

4.9.7. Correlation of asthenozoospermic sperm parameters

The results of the Spearman analysis for correlations of all parameters is displayed in Table 15. The percentage of total, progressive, and hyperactivated motility grades of asthenozoospermic sperm were significantly and positively correlated (r = 1.00, p = 0.0167). The three types of motility further showed no correlations with vitality (r = 0.90, p = 0.0833), MMP intactness (r = 0.50, p = 0.4500), and acrosome reaction (r = 0.70, p = 0.2333). Similarly, no correlation was observed between three types of motility and sperm ROS production, and DNA fragmentation (r = -0.80, p = 0.1333) and capacitation (r = -0.70, p = 0.2333).

The percentage live sperm and intact MMP in the asthenozoospermic group also did not show any correlation (r = 0.70, p = 0.2333), nor did it show a correlation with acrosome reaction (r = 0.60, p = 0.3500), sperm ROS production (r = -0.90, p = 0.0833), DNA fragmentation (r = -0.90, p = 0.0833), or capacitation (r = -0.60, p = 0.3500). In addition, asthenozoospermic sperm MMP integrity displayed no relationship with acrosome reaction (r = 0.10, p = 0.9500) and capacitation (r = -0.10, p = 0.9500).

Reactive oxygen species (ROS) production by asthenozoospermic sperm showed a significantly strong positive relationship with the percentage DNA fragmentation (r = 1.00, p = 0.0167). No association could be established between ROS production and acrosome reaction (r = -0.50, p = 0.4500) as well as capacitation (r = 0.30, p = 0.6833). Similar to ROS, DNA fragmentation of asthenozoospermic men was not correlated to capacitation (r = 0.30, p = 0.6833) and acrosome reaction (r = -0.50, p = 0.4500). Capacitation also showed no relationship with asthenozoospermic acrosome reaction (r = 0.30, p = 0.6833).

Table 15: Correlation of asthenozoospermic sperm parameters.

Sperm parameters	Total motility (%)		Progressive motility (%)		Hyperactivated motility (%)		Vitality live (%)		MMP-intact (%)		ROS-positive (%)		DNA fragmentation (%)		Capacitation (%)		Acrosome reaction (%)	
	r	p	r	p	r	p	r	p	r	p	r	p	r	p	r	p	r	p
Total motility (%)	-	-	1.00	0.0167	1.00	0.0167	0.90	0.0833	0.50	0.4500	-0.80	0.1333	-0.80	0.1333	-0.70	0.2333	0.70	0.2333
Progressive motility (%)	1.00	0.0167	-	-	-	-	0.90	0.0833	0.50	0.4500	-0.80	0.1333	-0.80	0.1333	-0.70	0.2333	0.70	0.2333
Hyperactivated motility (%)	1.00	0.0167	0.90	0.0833	0.90	0.0833	0.90	0.0833	0.50	0.4500	-0.80	0.1333	-0.80	0.1333	-0.70	0.2333	0.70	0.2333
Vitality live (%)	0.90	0.0833	0.90	0.0833	0.90	0.0833	-	-	0.70	0.2333	-0.90	0.0833	-0.90	0.0833	-0.60	0.3500	0.60	0.3500
MMP-intact (%)	0.50	0.4500	0.50	0.4500	0.50	0.4500	0.70	0.2333	-	-	-0.90	0.0833	-0.90	0.0833	-0.10	0.9500	0.10	0.9500
ROS-positive (%)	-0.80	0.1333	-0.80	0.1333	-0.80	0.1333	-0.90	0.0833	-0.90	0.0833	-	-	1.00	0.0167	0.30	0.6833	-0.50	0.4500
DNA fragmentation (%)	-0.80	0.1333	-0.80	0.1333	-0.80	0.1333	-0.90	0.0833	-0.90	0.0833	1.00	0.0167	-	-	0.30	0.6833	-0.50	0.4500

Capacitation (%)	-0.70	0.2333	-0.70	0.2333	-0.70	0.2333	0.60	0.3500	-0.10	0.9500	0.30	0.6833	0.30	0.6833	-	-	-0.20	0.7833
Acrosome reaction (%)	0.70	0.2333	0.70	0.2333	0.70	0.2333	-0.60	0.3500	0.10	0.9500	-0.50	0.4500	0.30	0.6833	-0.20	0.7833	-	-

CHAPTER 5

DISCUSSION

Plant remedies for sexual and reproductive dysfunctions and fertility improvement are the oldest form of therapy to date., ŧThey are reliable and accessible. The fact that nearly 80% of people rely chiefly on traditional herbal medicine (Yuan et al., 2016) is an indication of the urgency to decipher the mechanisms responsible for the often observed pharmacodynamics and pharmacokinetics of herbal medicines. Within the context of male infertility and related reproductive issues, naturopathic systems such as Ayurveda (Mahdi et al., 2011; Hussain et al., 2018); Siddha (Mohan et al., 2013); Unani (Ahmad et al., 2008), Chinese (Dieterle et al., 2009); and African remedies (Kolahdooz et al., 2014) have been benefitting men all over the world.

These plants have been prepared unempirically as unprocessed materials through decoction, concoction, infusion, ashing, and taken as vegetables or condiments (Abera, 2003; Ozioma and Chinwe, 2019). On the other hand, pharmaceutical formulations have been semi-purified from plants for infertility (Nantia et al., 2009). A review by Maridass and Britto (2008) reported that 50% of natural medicines and their analogues in the pharmacopoeia were said to be botanical-based, while recent reports indicated a proportion of 25% of pharmaceutical infertility therapies are derived from plants (Jaradat and Zaid, 2019). Socio-economic reasons driving the use of traditional herbs for sexual and reproductive restoration are related to the inherent limitations in conventional therapies of fertility treatments such as their prohibitively high costs, adverse side effects, and their limited availability to the majority of third world country populations (Kaadaaga et al., 2014).

South Africans rely almost exclusively on plants for the provision of their health care needs (Mbatha et al., 2012). The rising interest and demand for traditional medicine in South Africa have been met with considerable concerns around legislative regulation (Pefile, 2005.). Fortunately, the South African Government put forward a regulatory framework in the Traditional Health Practitioners Act No 22 of 2007, according to which traditional medical practices can be carried out safely and efficaciously in health care delivery (Tshehla, 2015; Street, 2016) in South Africa.

For the safety of natural medicines to be ensured, thorough scientific validation of claimed properties must be undertaken. Although its widespread use and commercialisation fall under the regulation of a non-profit initiative, the *Moringa* Development Association of South Africa (MDASA) (Mabapa et al., 2017), most of its acclaimed activities still remain to be scientifically proven. However, *M. oleifera's* pharmacological properties have been studied at length, using *in vivo* and *in vitro* models for efficacy assessments in nearly all body systems, and biological activities were ascribed mainly to antioxidants (Gopalakrishnan et al., 2016; Vergara-Jimenez et al., 2017). The effects of *M. oleifera* on LH and FSH, prolactin, testicular antioxidant enzymes and testosterone synthesis had also been investigated (Bin-Meferij and El-kott, 2015; Orji et al., 2016; Ododo et al., 2019). However, most studies that examined how *M. oleifera* affects the male reproductive, sexual, and seminal functions, were limited to animal models. Also, studies on the effects of *M. oleifera* on seminal parameters were confined to basic semen characteristics such as motility, morphology, and concentration (Afolabi et al., 2013), and did not examine advanced sperm functional assays. To the best of my knowledge, no study to date has previously evaluated the effects of *M. oleifera* leaves on the physiological functioning of human spermatozoa *in vitro*. Given the abovementioned evidence, our study aimed to shed light on *in vitro* effects of aqueous leaf extracts of *M. oleifera* on human spermatozoa functioning.

5.1. Effects of *M. oleifera* leaf extracts on sperm motility *in vitro*

Sperm motility remains the lifeblood of male fertility evaluation as it best predicts the fertilising capacity of a man (Shibahara et al., 1997; Turner, 2006). Essentially, sperm requires all forms of motility to be able to fertilise an egg successfully. Complete lack of motility is another form of infertility (Ortega et al., 2011). Immotile sperm cells, alive or not, are incapable of first, penetrating cervical mucus and reaching the site of fertilisation (Suarez and Pacey, 2006). Moreover, sperm needs to first move not in tight circles, but in a straight line to ascent to the oocyte-cumulus complexes (Mansour et al., 1995). Finally, sperm requires vigorous flagellar beating to penetrate the corona radiata surrounding the oocyte, and fuse the oocyte for fertilisation (Wallach et al., 1980; Skinner et al., 2019). Hence, several studies have emphasised the definitive

role of including sperm motility when evaluating the possible effects of medications in the management of male infertility (Esteves, 2014).

The effects of *M. oleifera* leaf extract concentrations (0, 0.625, 6.25, 62.5, and 625 µg/ml) on various grades of motility, including flagellar and head kinematics, were investigated for the donor, patient, and asthenozoospermic groups *in vitro*. In this study, total, progressive, and non-progressive motility were investigated after treatment with aqueous leaf extracts of *M. oleifera in vitro*. Donors and asthenozoospermic groups did not show any alterations in sperm motion parameters after treatment with *M. oleifera.* Similar results were recorded of no alteration to human sperm motility with aqueous extracts of *Cissampelos capensis* rhizome *(dawidjies)* (Shalaweh et al., 2015)*,* roots of *Mondia whitei (*white ginger*)* (Tendwa, 2016)*, ethanolic* leaf extracts of *Custanea sativa (*sweet chestnut) (Biagi et al., 2019) *in vitro.* The ineffectiveness of *M. oleifera* to restore sperm motility of asthenozoospermic to normal in this study was similar to that recorded with *Mucuna pruriens* (velvet bean) (Ahmad et al., 2008). Treatment with *M. oleifera* extracts showed significantly increasing tendencies of forward progression of motile sperm in the asthenozoospermic group.

In contrast*, M. oleifera* leaf extracts significantly improved sperm total, progressive, and non-progressive motilities in the patient group and the combined groups *in vitro*. Consequent to improvements in the enumerated motility grades by the aqueous leaf extracts of *M. oleifera*, a highly significant reduction in non-motile spermatozoa *in vitro* was observed. Although *M. oleifera* extract concentrations (0.625, 6.25, 62.5, and 625 µg/ml) showed no significant improvement in the donor and the combined groups, a significant dose-dependent increasing trend was evident in all grades of motility in the patient group.

Unfortunately, no group exhibited hyperactivated motility in all the treatments. Kinematic motility parameters are useful as relatively objective and quantitative indicators of sperm maturational status. Hence, their analysis assists in discriminating sperm populations with fertilising capacity (van der Horst et al., 1999b; Spiropoulos, 2001). The effects of *M. oleifera* leaf extracts on hyperactivation in all groups was further supported by no effects observed in sperm kinematics after treatment, that is, ALH, VSL, VCL, VAP, and BCF. Tendwa (2016) also recorded no change in sperm

kinematics after treating human sperm with increasing aqueous extracts of *M. whitei in vitro*.

Ideally, a substance that enhances hyperactivated motility would improve kinematics related to sperm vigour such as increased VCL, VAP, ALH, and BCF, and reduce parameters that are characteristic of linear progression such as LIN, STR, and VSL (Mortimer and Mortimer, 1990; Cancel et al., 2000; Adamkovicova et al., 2016). To support this, Shalaweh (2015) demonstrated that increasing concentrations of *C. capensis in vitro* increased BCF and hyperactivation in human spermatozoa. Interestingly, treatment with *M. oleifera* leaf extracts showed significant decreasing trends in the percentage STR in patients and the combined groups.

Although being incomparable models, the effects of *M. oleifera* on sperm motility in this study, were in agreement with previous findings *in vivo*. Increases in sperm motility were observed when *M. oleifera* was administered concomitantly with cyclophosphamide in Swiss albino rats (Nayak et al., 2016). Similarly, a coconut oil and *M. oleifera* leaf extract mixture blocked spermicidal effects of mercury chloride in male rats (Abarikwu et al., 2017). Mean sperm motility of bulls' semen extended with crude extracts of *M. oleifera* was significantly higher than the respective control (Sokunbi et al., 2015). Total and progressive motility, velocities, and amplitude were significantly increased with decreases in linearity in Bali bulls' sperm after *M. oleifera* supplementation (Syarifuddin et al., 2017). The differences in the *in vivo* and *in vitro* effects of *M. oleifera* on sperm motility can in part be explained in terms of the inherent methodological disparities in both models. In addition, *in vivo* models involve complex systemic interactions that may affect pharmacokinetic and pharmacodynamics of the plant or its bioactive compounds. *In vitro* models, on the other hand, provide insight into the direct effect of the plant on a specific cell. These reasons could partially explain why the results from *in vivo* studies and the current study differ.

Although the mechanism through which aqueous leaf extracts of *M. oleifera in vitro* affected sperm motility is not known, the improvement can be surmised from the reported phytochemistry of *M oleifera*. Sperm motility is heavily dependent on oxidative phosphorylation in the mitochondria and glycolytic pathways for provision of ATP in the flagellum (Nascimento et al., 2008). Reports indicated that both pathways use a wide range of substrates, depending on the localisation of the enzymes

(Tourmente et al., 2015). It can be speculated that *M. oleifera's* simple and complex sugars (glucose, mannose, xylose, arabinose, and stachyose) (Teixeira et al., 2014; Chen et al., 2017; Caicedo-Lopez et al., 2019), and its amino acids and fatty acids (Teixeira et al., 2014) served as substrates for ATP production; hence the improvement of sperm motility.

Other possible explanations may be attributed to the presence of the antioxidative trace elements calcium, magnesium, manganese, zinc, iron available in *M. oleifera* leaves (Valdez-Solana et al., 2015). The significance of bivalent cations in sperm function and motility has been confirmed previously (Magnus et al., 1990; Sorensen, 1999). A combination of secondary bioactive compounds in *M. oleifera* might have complemented or synergistically exerted their stimulatory effects on patient sperm motility as found in this study.

Other medicinal plants with proven enhancing effect on human sperm motility are *Astralagus membranaceous* (Mongolian milkvetch), which showed an increase in pure semen and washed semen from 22.6% to 146.6% and 13.8% to 138.2%, respectively, at a concentration of 10 mg/ml (Hong et al., 1992). Additionally, *A. membranaceous* significantly increased the percentage of progressive and total motility in 30 infertile men *in vitro* (Liu et al., 2004), similar to an observation made after 1-h incubation of sperm with *M. oleifera* in this study. *Withania somniferia* (horse's smell) is another herb with proven efficacy in the improvement of sperm motility of oligozoospermic men (Ambiye et al., 2013). *Tribulis terrestris* (puncture vine) significantly increased sperm motility in 40 healthy men after 60 min of treatment (Khaleghi et al., 2017), and this observation was contrastingly made in patient rather than in donor sperm after treatment with *M. oleifera* in the current study. Conversely, *Typha capensis* (bulrush) (Henkel et al., 2012), *Zingiber officinale* (ginger) (Jorsaraei et al., 2008), *Carica papaya* (papaya) (Ghaffarilaleh et al., 2019), *Ruta graveolens* (common rue) (Harat et al., 2008) have been shown to exert immobilising effects on human spermatozoa. The differences in effects are mainly due to the presence of bioactive compounds.

5.3 Effects of *M. oleifera* leaf extracts on sperm vitality *in vitro*

Sperm vitality forms an integral part of the conventional semen analysis, and its assessment is recommended by the WHO (Björndahl et al., 2003; World Health Organization, 2010). Vitality distinguishes the proportion of membrane-viable, live

cells, despite the motility status, and is usually assessed by exclusion dyes and membrane osmo-tolerance (Moskovtsev and Librach, 2013). Viable sperm may be motile or immotile; and that implies that the percentage immotile cells (within a margin of error in a given sample) must exceed that of dead cells (World Health Organization, 2010). Hence, it is advised that a vitality test should be performed when total and progressive motilities are below 15 and 40%, respectively (Agarwal et al., 2016).

The understanding that a substance could affect the liveability of sperm, independent of its motility, has strengthened the inclusion of sperm vitality assessment in the safety testing of therapeutics with the potential to improve male fertility, including herbal medicines. Plants or plant derivatives exert their biological effect on membrane integrity through interacting with surface proteins and phospholipids (Qiu et al., 2011). In this study, aqueous leaf extracts of *M. oleifera in vitro* did not cause any direct cytotoxic effects on sperm vitality in any of the groups; instead, some improvements were apparent. The increase in the percentage of live cells after treatment was evident at 625 µg/ml of the extract. Our results of improvement in the percentage of live sperm are in line with previous *in vivo* studies: Senduro goats semen, supplemented with *M. oleifera,* was found to have significantly increased sperm viability (Wahjuningsih et al., 2019). Also, semen of Friesian bulls, in which the extender was infused with *M. oleifera* leaf extracts, showed an increased proportion of live sperm (El-Nagar, 2017). Similarly, New Zealand rabbit bucks' sperm viability together with motility and MMP were significantly increased after administration of aqueous leaf extracts of *M. oleifera* (El-makawy, 2016). Again, *M. oleifera* leaf extracts dose-dependently increased the sperm viability without affecting the motility of cadmium-exposed Wista rats (Ododo et al., 2019). On the other hand, increasing concentrations of *M. oleifera*-fed rabbit bucks showed no significant effect in the liveability of sperm (Abu et al., 2013). The differences may not only be due to modulatory effects of the chemical constituents of *M. oleifera,* but the apparent disparities inherent to the models used (*in vivo* and *in vitro*), the preparation of the extracts, and the type and the way the viability tests differentiate live from dead sperm.

Sperm longevity and survival are dependent on the presence of seminal electrolytes, which not only provide nutrition, but also serve as a cushion from oxidative injury (Hamad, 2014; Vitku et al., 2017). These could in part speak to the results of *M. oleifera* on spermatogenic function *in vivo,* but not *in vitro*. In the current study, sperm

underwent several wash steps before and after treatment that removed the protective function that seminal plasma affords spermatozoa. However, *M. oleifera* leaves are highly enriched in mineral elements such as calcium, magnesium, cobalt, molybdenum, potassium, iron, zinc, copper, phosphorus, sulphur, and manganese (Biel et al., 2017; Valdez-Solana et al., 2015). The mode of action by which *M. oleifera* maintained and increased the percentage of live sperm *in vitro* in the present study is not clear, since sperm is terminally differentiated. However, the elemental properties may have contributed to the observed results.

Another possible mechanism can be explained in terms of the free radical scavenging potential of *M. oleifera*, particularly of superoxide anion. Excessive ROS is known to disrupt sperm plasma membrane integrity by increasing the oxidation of membrane proteins and lipids (Sanocka and Kurpisz, 2004). These intermediates (peroxides) from oxidation initiate a cascade of events that progress (if not attenuated) to induce mitochondrial injury (Hamilton et al., 2016). The damage is then manifested through apoptotic-like damage in sperm cells such as caspase activation (Mupfiga et al., 2013). Uninterrupted, the culmination of these events will be diminished viability or death of the sperm cell (Mahfouz et al., 2010). Inhibition or absence of such sperm death (non-viability) could be attributed to the presence of antioxidants in *M. oleifera*.

Some of the antioxidant compounds isolated from *M. oleifera* leaves are polyphenols such as rutin, kaempferol, quercetin, caffeine, and gallic acids (Valdez-Solana et al., 2015). Additionally, *M. oleifera* leaves provide a rich source of vitamins C, E, and A (Vergara-Jimenez, et al., 2017). Therefore, observed increases in vital sperm percentages *in vitro* could have been the result of the mentioned interactions between plant extracts and sperm cytoskeletal elements.

Similar increases in human sperm vitality were confirmed for the following medicinal plants: *T. terrestris* (puncture vine) (Asadmobini et al., 2017), *Z. officinale* (ginger), and *Paullinia cupana* (guaraná) (Werner et al., 2017). Interestingly, *Aloe vera* increased sperm viability with decreased motility in asthenozoospermic men *in vitro* (Baqir et al., 2014). There are more plants with proven spermicidal effects on human sperm, and their mode of action involves decreasing sperm membrane integrity and diminished vitality. *Passiflora edulis* (passion fruit) (Alvarez-Gómez et al., 2010), *Stephania hernandifolia* (tape vine), and *Achyranthes aspera* (chaff flower) (Paul et al., 2006)

were found to diminish human sperm vitality. Moreover, *Polygala tenuifolia* (Yuan Zhi) induced death in 100% of the sperm population within 20 minutes of exposure by compromising sperm plasma membrane (Qiu et al., 2011).

5.4 Effects of *M. oleifera* leaf extracts on sperm mitochondrial membrane potential *in vitro*

Mitochondrial membrane potential (MMP) is central in defining functional and fertilising aspects of a sperm (Agnihotri et al., 2016). Mammalian sperm mitochondrion participates in more than just ATP production, but also in ROS production (especially superoxide anion), calcium metabolism, intrinsic apoptotic death, and steroidogenesis (Koppers et al., 2008; Costello et al., 2009; Amaral et al., 2013).

Sperm thrives on the ability of the inner mitochondrial to synthesise ATP energy for function through oxidative phosphorylation, while the electron transport chain (ETC) maintains the flow of electrons across the membrane (Piomboni et al., 2012). By using transmembrane protein complexes, the net movement of electrons creates an electrochemical gradient, which is called to the MMP (Wang et al., 2003). The multifaceted nature of the MMP function makes it a reliable indicator of sperm quality (Espinoza et al., 2009). Because of this diverse functioning of sperm MMP, several studies have shown that it correlates strongly with basic parameters such as motility, viability, and morphology (Gallon et al., 2006; Zhang et al., 2016), and conversely negative with excessive ROS (Wang et al., 2003; Li et al., 2012), and DNA fragmentation (Donnelly et al., 2000). In light of the above, sperm DNA fragmentation and capacitation of the patient group showed a strong inverse relationship with MMP intactness in this study. By contrast, no associations could be observed between vitality, motility, capacitation, and ROS production.

Aqueous leaf extracts of *M. oleifera* did not alter the percentage of sperm with intact MMP in the healthy donors and the asthenozoospermic groups in all concentrations included. These particular findings were in line with a recent study that evaluated the post-thaw effect of *M. oleifera* seed extracts on ram semen, in which it was revealed that seed extracts did not affect sperm MMP significantly (Carrera-Chávez et al., 2020). Perhaps the leaf extracts did not have any deleterious effects on the

permeability of sperm mitochondrial membrane. On the other hand, the patient and combined groups showed a marginal increase in sperm with intact MMP at the concentration of 625 µg/ml. However, in a different cell type, methanolic pod extracts of *M. oleifera* improved MMP of male Wistar rats' liver cells *in vitro*. The researchers attributed these results to the ability of *M. oleifera* extracts to inhibit the opening of mitochondrial permeability transition pore and subsequent lipid peroxidation, which would have led to impaired MMP (Bezi et al., 2018). These findings are in line with our findings that *M. oleifera* has the potential to improve sperm cell MMP.

Also, human spermatozoa have been shown to sustain MMP, viability, and motility when compared to other mammalian spermatozoa (Agnihotri et al., 2016). This may be due to a high volume of mitochondria (50 - 75) (Barbagallo et al., 2020) enveloped in the human spermatozoon mid-piece, compared to other species such as mice (20 - 70 in mice) (Cassina et al., 2015). Moreover, with no alteration on sperm MMP, viability, and motility found when exposed to *M. oleifera* aqueous leaf extracts, this could support the longest standing controversy on what pathway between mitochondrial OXPHOS and flagella glycolysis is most efficient in providing functional energy for sperm (Fisher and Henkel, 2020). In this study, sperm cells that fluoresced green (disrupted MMP) exhibited motility under an epifluorescence microscope, which suggested that sperm in the current study might have resorted to anaerobic glycolytic ATP synthesis, using *M. oleifera* substrates.

M. oleifera elicits different activities in MMPs of various cell types. Most studies, in which the plant affected *in vitro* cellular MMP, were limited to immortalised cancer cell lines, with no study on mammalian spermatozoa. *M. oleifera* aqueous leaf extracts were used to treat human epithelial immortalised liver (HepG2), colorectal (CaCo-2), lymphocyte (Jurkat), and kidney (HEK293) cells *in vitro*. The results revealed a significant time-dependent decrease in MMPs of all cells, followed by increased ROS production, an increase in pro-apoptotic proteins, depletion of GSH, and decreased viability (Madi et al., 2016). Similarly, *M. oleifera* leaf extract-derived gold nanoparticles induced a significantly increased mitochondrial depolarisation, increased caspase activity, and phosphatidylserine externalisation of A549 lung and SNO oesophageal cancer cell lines *in vitro* (Tiloke et al., 2016). These results are in sharp contrast with our findings, for obvious reasons. The mode of cell death induction in immortal and normal cells differs markedly, although it is clear that the basis upon which *M. oleifera*

extracts affect cell MMP *in vitro* depends on OSPHOX enzymes, lipid oxidation and regulation of apoptosis.

Other studies, in which plant extracts improved the percentage of human sperm with intact MMP *in vitro,* were with *M. whitei* (Tendwa, 2016). In another study, human spermatozoal dysfunction was induced by bisphenol-A. Incubation of damaged sperm with *Eruca sativa* (garden rocket) aqueous leaf extracts, which contained polyphenols and flavonoids, was shown to recover sperm MMP at low doses, but impaired both plasma and mitochondrial membrane potentials at high doses (Grami et al., 2018). The effects of polyphenols on MMP are in line with the findings in the current study where *M. oleifera* bioactive compounds (flavonoids and polyphenols with proven fertility properties) improved patient MMP at the highest concentration. In another *in vitro* study, *propolis* (bee gel) maintained human sperm MMP, while increasing activities of OSPHOX enzyme complexes II and IV *in vitro* (Cedikova et al., 2014).

In contrast, most male contraceptive formulations exert their inhibitory effects by destabilising the integrity of sperm inner MMP. One of the main psychoactive constituents of *Cannabis sativa* (marijuana) is cannabinoids, and they have been shown to negatively impact male fertility (Whan et al., 2006). The addition of anandamide to human sperm *in vitro*, dose-dependently diminished mitochondrial activity when compared to the respective control (Rossato et al., 2005). *C. papaya* induced similar effects of decreased MMP *in vitro* (Ghaffarilaleh, Fisher, and Henkel, 2019). Thymoquinone is the main active compound of *Nigella sativa* (black seed), and its spermostatic activities are owed to its ability to mitigate MMP in human sperm *in vitro* (Iranpour et al., 2017). However, thymoquinone significantly ameliorated nicotine-induced MMP damage in male rats *in vivo* (Rosli et al., 2019). The observed discrepancies regarding the effects of plant extracts on mammalian sperm MMP were mostly linked to excessive ROS production. This warrants further critical assessment.

In normal cells such as human spermatozoa, *M. oleifera* decreases excessive endogenous ROS, inhibits lipid peroxidation, and inhibits the opening of the mitochondrial permeability pores. Once again, these activities were possibly directly mediated through the inhibition or reduction of sperm excessive endogenous ROS by antioxidants properties of *M. oleifera* leaves.

5.5 Effects of *M. oleifera* leaf extracts on reactive oxygen species (ROS) *in vitro*

Sperm cells as part of their normal respiration, intrinsically generate ROS in their mitochondrial ETC (Aitken et al., 1997; Cassina et al., 2015). The principal ROS produced by sperm is superoxide, and it precedes all free radical generation, both intrinsically and extrinsically (Henkel et al., 2005; Gosalvez et al., 2017). In their basal state, sperm ROS are non-detrimental; in fact, low regulated ROS play a central role in physiological functions such as fertilisation (Henkel, 2011; Chen et al., 2013). The ability of intracellular superoxide to mediate the functions, as mentioned earlier, is dependent on its amount in the ejaculate or processed sample as well as the concentrations of antioxidants. An imbalance between sperm ROS production and antioxidants systems leads to oxidative stress, which is a well-established cause of infertility (Agarwal et al., 2008).

Reportedly, excessive sperm ROS is present in about 25-88% in idiopathic causes of male infertility (Moein et al., 2014). There have been significant differences in the ROS levels of infertile men compared to their healthy counterparts (Pasqualotto et al., 2008; Agarwal et al., 2014). The same phenomenon was observed in the current study, where the mean of sperm ROS production in the patient group was significantly higher than that from the donor group, the combined (donor and patient) group, and the asthenozoospermic group. Similarly, non-normozoospermic samples were found to have a low percentage of MitoSOX (sperm mitochondrial superoxide production fluorescent probe with little or no staining) when compared to normozoospermic after centrifugation (Marques et al., 2014). The MitoSOX probe uses the same principle as the DHE probe used in the present study. The dihydroethidium probe was used to detect levels of sperm superoxide anion with epifluorescence after treatment with increasing *M. oleifera* leaf extracts *in vitro*.

In the present study, *M. oleifera* leaf extracts showed significant decreases in superoxide radical production in a dose-dependent manner, specifically at concentrations 6.25, 62.5, and 625 µg/ml in donors, patients, the combined group, and the asthenozoospermic group. The first possible explanation for the observed reduction or inhibition of intracellular ROS production after *M. oleifera* treatment is that there were already little or no ROS present in the treatment samples before incubation.

The removal of ROS may in part be attributed to the wash process that removed immature, dead, and abnormal ROS-producing spermatozoa and polymorphonuclear leukocytes; hence, no excess ROS was detected with DHE (Henkel and Schill, 2003; Ricci et al., 2009). The second possible explanation for the observed decrease in intracellular ROS after *M. oleifera* treatment could be attributed to the extract free-radical scavenging activities. Nutritional antioxidants have been prescribed for pathologies, in which oxidative stress was implicated as a cause, including male infertility (Balercia et al., 2005).

Under *in vivo* conditions, sperm cells are bathed in the seminal milieu, containing both enzymatic (SOD, CAT, and GSH) and non-enzymatic antioxidants (vitamin C, E, and glutathione) (Aitken and Roman, 2008; Micheli et al., 2016). *M. oleifera* has been proven to boost enzymatic antioxidants in mammalian semen under oxidative stress *in vivo* (Sadek, 2014; Abarikwu et al., 2017). Additionally, *M. oleifera* leaves are also rich in vitamins C and E (Ajantha et al., 2018), which have been indicated as major chain-breaking antioxidants with strong reducing potential that fine-tune the balance between free radicals and antioxidation scavenging activities (Henkel et al., 2019).

Quercetin and kaempferol are amongst the abundant flavonoids in *M. oleifera* leaves (Verma et al., 2009). Although reports on beneficial and detrimental effects in male reproductive and fertility potential of quercetin are contradictory (Ranawat et al., 2013), the reducing potential observed in this study could be attributed in part to the presence of quercetin. An addition of quercetin to rat sperm suspension significantly improved sperm viability, morphology, motility, and antioxidants, and successfully inhibited lipid peroxidation, following hydrogen peroxide exposure (Abdallah et al., 2011). Polyphenols donate electrons or hydrogen groups in nearly all biological cellular redox reactions (Tsao, 2010). *M. oleifera* is rich in phenolic compounds such as chlorogenic, ferulic, gallic and coumaric acids, which have a potent free-radical quenching ability (Castillo-López et al., 2017). Seminal intracellular enzyme complexes and their involvement in male fertility regulation are highly dependent on co-factors such as Zn, Se, Mg, Mn, and Cu (Dobrakowski et al., 2018; Shquirat et al., 2013); and their presence in *M. oleifera* leaves has been confirmed (Dilawar et al., 2018). It is possible that the superoxide radical scavenging or inhibition we observed at the highest concentrations in the current study could have involved an interplay of the abovementioned mechanisms.

The findings of ROS inhibition or reduction by *M .oleifera* in this study are similar to those found by Kedechi et al. (2017) after human sperm were incubated with hydroxytyrosol extracted from *Olea europaea* (olive) oil *in vitro.* Again, increasing concentrations of methylxanthine and polyphenol compound derived from *P. cupana* (guaraná) protected human sperm from cryogenic oxidative stress by reducing ROS and NO levels post-thaw (Werner et al., 2017).

Contradictory results were reported in similar studies that investigated *in vitro* effects of *Eurycoma longifolia* (Tongkat ali) (Erasmus et al., 2012), and *C. capensis* and *T. capensis* extracts (Henkel et al., 2012; Shalaweh et al., 2015) in human sperm, where increasing concentrations enhanced the intrinsic ROS production by sperm. In similar studies, extracts showed significant increases in endogenous sperm ROS production in sperm with fragmented DNA after incubation.

5.6 Effects of *M. oleifera* leaf extracts on sperm DNA fragmentation *in vitro*

Sperm cells are charged with the responsibility of transporting paternal genetic complement to the oocyte for fertilisation (Zini and Agarwal, 2018). Delivery of such genomic material is dependent upon intact sperm nuclear DNA. The direct causal relationship between oxidative stress and sperm DNA fragmentation is now firmly established (Lopes et al., 1998; Agarwal et al., 2014; Dobrakowski et al., 2018). DNA fragmentation was negatively correlated with motility (Huang et al., 2005), viability (Samplaski et al., 2015), and MMP (Lobascio et al., 2015), and positively correlated with ROS production (Iommiello et al., 2015). Similarly, in the current study, the percentage of sperm with DNA fragmentation showed a strong positive correlation with ROS in donors, patients, the combined group, and the asthenozoospermic group.

Excessive ROS augments the DNA instability through direct modification of bases (Koh et al., 2016), disrupting the maturational stage(s) of sperm (Hamilton et al., 2018), and the dysregulation of abortive apoptosis (Sakkas et al., 2003). Lipid peroxidation and increased membrane fluidity due to PUFA oxidation ((Tvrdá et al., 2017), phosphatidyl-serine translocation, and caspase and endonuclease activation (Wang et al., 2003; Jeng et al., 2016) are hallmark steps indicative of sperm ROS-induced apoptotic DNA damage (Aitken and Koppers, 2011).

Treatment with *M. oleifera* revealed a significant reduction in the percentage of DNA-fragmented (TUNEL-positive) sperm in the donors, patients, the combined group, and the asthenozoospermic group. The first reason for the observed reduction in TUNEL-positive (DNA-fragmented) sperm or the lack of detection thereof in this study, might be related more to the organisational arrangement of mammalian sperm than the effect of *M. oleifera*. Mature sperm is transcriptionally inert (with the exclusion of mitochondrial protein synthesis) (Grunewald et al., 2005; Baker, 2011). This was substantiated by a study by Lachaud et al. (2004), in which the presence of apoptotic markers that resulted from disturbed abortive apoptosis pre-ejaculation was found. This indicates that any chemical substance such as plant extract (in this regard), in the sperm's immediate environment is incapable of directly exerting any significant effect on sperm nuclear DNA. The second possibility that might not have been influenced by *M. oleifera* extract involves the compact nature of sperm nuclear DNA. Unlike somatic cells, the nucleus of mammalian spermatozoa is highly condensed due to chromatin remodelling in spermiogenesis, in which histones are replaced by protamines (Wu et al., 2015). This modification makes sperm DNA 6-7 times more compact than any somatic cell (Bani-Hani, 2011; Champroux et al., 2016), and therefore makes it difficult to detect changes in DNA fragmentation due to *M. oleifera* accurately applied extracts *in vitro*. Despite this tight packaging of sperm DNA, it still is denatured, dispersed, and fragmented.

Since superoxide availability precedes the production of free radicals in the sperm's microenvironment (Gosalves et al., 2017), it is possible that the absence or inhibition of superoxide resulted in a subsequent inhibition effect on the production of peroxidative derivatives. Intermediates such as H_2O_2 and nitric oxide that would initiate and facilitate DNA damage (Verma et al., 2009); hence, a significant reduction.

The loss of mitochondrial membrane integrity and DNA damage are reliable early and late markers of apoptosis, respectively (Aziz et al., 2007; Li et al., 2010). The relationship between sperm mitochondrial function and sperm nuclear DNA integrity has been studied extensively (Marchetti et al., 2002; Zhang et al., 2008), and has been reflected in this study as sperm MMP integrity, correlated inversely to sperm with DNA fragmentation. The correlation between sperm loss of MMP and DNA fragmentation

seems to revolve around the presence, origin, and amount of ROS (Mupfiga et al., 2013; Lobascio et al., 2015). Therefore, it can safely be assumed that through stabilising sperm MMP, *M. oleifera* indirectly conferred protection to sperm oxidative DNA damage.

Vitamins E and C have been shown to mitigate oxidative DNA damage of human sperm both *in vivo* and *in vitro* by neutralising lipid peroxides or interfering with lipid peroxidation (Greco et al., 2005; Li et al., 2010). Besides, N-acetyl cysteine is another potent antioxidant with proven ameliorative effects in DNA damage (Hughes et al., 1998; Barekat et al., 2016; Baetas et al., 2019). Interestingly, *M. oleifera* leaves contain essential amino acids such as cysteine (Okereke and Akaninwor, 2013). Therefore, the presence of cysteine in the leaves might have been one of the ways through which *M. oleifera* extracts decreased free radicals and subsequently TUNEL-positive (DNA-fragmented) percentages after treatment. Zinc and selenium (present in *M .oleifera* leaves) (Dilawar et al., 2018) have yielded positive results towards human sperm DNA integrity in antioxidant therapy of infertile men (Ménézo et al., 2007; Tunc et al., 2009; Pourmasumi et al., 2019). Another vitamin previously shown to be antioxidative in terms of ROS-induced DNA is a vitamin (B9) known as folate or folic acid (synthetic) when combined with other minerals (Agarwal and Sekhon, 2011; Saint et al., 2019). A tetrahydrofolic acid is a form of folate that was confirmed in *M. oleifera* leaves (Saini et al., 2015). The protective effects of *M. oleifera* may have resulted from synergistic actions of any of the antioxidants mentioned above against ROS-induced sperm DNA damage.

M. oleifera has been demonstrated to protect mammalian sperm against oxidative DNA damage *in vivo*. *M. oleifera* leaf aqueous and ethanolic extracts reversed cyclophosphamide-induced sperm DNA damage in male rats (Sathya et al., 2010; Nayak et al., 2016). Similarly, *M. oleifera* significantly reduced testicular lipid peroxidation dose-dependently (indicated by MDA levels) as well as sperm DNA fragmentation in rats exposed to gamma radiation (Eshak and Osman, 2013). The antioxidant properties of *M. oleifera* demonstrated in these studies prove useful in mitigating DNA damage of mammalian sperm mainly through free-radical scavenging mechanisms.

Plant-based antioxidants similarly protected human sperm DNA fragmentation *in vitro* and *in vivo: Terminalia arjuna (Arjun tree)* (Parameswari et al., 2017), *Ceratonia siliqua (*carlob*)* (Faramarzi et al., 2019), *Opuntia ficus-indica (*barbary fig*)* (Meamar et al., 2012) *and Morinda officialis (*mulberry*)* (Chen et al., 2014). On the other hand, moderate to high doses of *Cestrum parqui* (green crestrum) methanolic extracts induced apoptosis and necrosis of human sperm *in vitro* (Chenni et al., 2011). Another study aimed to investigate *in vivo* and *in vitro* the spermicidal effects of *Platycodon* saponin derived from *Platycodon grandiflorum* (balloon flower), where the extract exerted its activity through ultrastructural modification without DNA damage in human and rat sperm (Lu et al., 2013). The dissimilarities on the effects of various phyto-antioxidants on sperm DNA damage indices *in vitro* and *in vivo* are an indication that antioxidants supplementation should always match the presence of pro-oxidants to prevent antioxidative stress (Halliwell, 2000).

5.7 Effects of *M. oleifera* leaf extracts on sperm capacitation and acrosome reaction *in vitro*

M. oleifera extracts neutralised the sperm oxidative damage and consequently, provided protective activities towards sperm functional characteristics, while capacitation and spontaneous acrosome were negatively affected.

Aqueous leaf extract of *M. oleifera* significantly increased the number of non-capacitated (F pattern) and acrosome-intact spermatozoa and caused a corresponding reduction in the number of acrosome-reacted spermatozoa at 625 µg/ml in the donor and patient groups. The extracts showed a decrease in capacitation (B pattern) in the patient group. Low and regulated ROS are prerequisites for induction of processes crucial for fertilisation. For instance, superoxide anion was proven to exert modulatory effects on capacitation (de Lamirande, Lamothe, and Villemure, 2009), hyperactivation (de Lamirande and Gagnon, 1993; Oehninger et al., 1995), acrosome reaction (Ichikawa et al., 1999), and sperm-oocyte fusion (Aitken, Irvine, and Wu, 1991). ROS, especially superoxide, is involved in extracellular processes such as cholesterol efflux, followed by an intracellular increase in Ca^{2+}, an increase in cyclic adenosine monophosphate (cAMP), and downstream phosphorylation of kinases and tyrosines, and subsequent inhibition of phosphatases in protein kinase activation (PKA) and extracellular signal-regulated kinase (ERK) pathways (O'Flaherty

et al., 2006; Jin and Yang, 2016). This may be due to the superoxide scavenging activity elicited by *M. oleifera* at the highest concentrations, as the level of ROS showed significant reductions at the highest concentration. Compounds such as glucosinolates and isothiocyanates in *M. oleifera* (Dinkova-Kostova and Kostov, 2012; Vergara-Jimenez et al., 2017) may inhibit capacitation through antagonising ROS-dependent sulfhydryl proteins (Ford, 2004).

In other *in vivo* studies, where mammalian sperm capacitation and acrosome reaction were studied, ethanolic *M. oleifera* extracts resulted in a significant increase in the percentage of acrosome-intact uncapacitated rabbit sperm ((El-Desoky et al., 2017). Along this line, Sokunbi et al. (2015) found *M. oleifera* crude extracts increased the percentage of uncapacitated bull sperm with intact acrosome after 24 hours of treatment.

Prostatic gland granules rich in vitamin E inhibited capacitation without affecting the acrosome reaction of rabbit sperm (Mourvaki et al*.,* 2010). Vitamins E and C supplementation to a bull semen capacitating medium resulted in significant decreases in the percentages of sperm capacitation (O'Flaherty et al., 1997). Furthermore, progesterone-induced capacitation showed a dependence upon redox regulation, as it was stimulated by ROS generation, but inhibited by the presence of catalase and other membrane-permeant thiols (Aitken et al., 1996). The presence of zinc has been shown to inhibit capacitation (Andrews et al., 1994). Quercetin, present in *M. oleifera* leaves, prevented premature capacitation and acrosome reaction in stored stallion sperm (McNiven and Richardson, 2006). It can be assumed that dose-dependent inhibition of capacitation and ultimately acrosome reaction may have followed the mode of actions involving ROS scavenging activity of *M. oleifera*.

Hyperactivated motility coincides with the onset of capacitation (Buzadzik et al., 2014); accordingly, *M. oleifera* extracts did not affect sperm hyperactivation. A possible explanation for the unaltered number of capacitated spermatozoa could be associated with that finding. Only capacitated sperm can undergo an acrosome reaction. Hyperactivation, capacitation, and acrosome reaction are interrelated processes that ensure successful fertilisation. The strong relationship between these parameters was also confirmed in the current study. Correspondingly, a significant concomitant decrease in sperm acrosome reaction (AR pattern) was recorded at the highest

concentration of 625 µg/ml in the donors. Although most independent, capacitation and AR are transduced by nearly the same biochemical pathways (Ickowicz et al., 2012). It was, therefore, anticipated that an increase in uncapacitation (F pattern) would yield a reduction in acrosome-reacted spermatozoa (AR pattern).

Again, *M. oleifera* could have led to these observations through inhibition or decrease in superoxide anion and other ROS. In the absence of essential ROS that modulates phosphorylation of tyrosine-like/protein kinase, cascades leading to capacitation and acrosome reaction would not occur (Ickowicz et al., 2012; Jin and Yang, 2016). Alternatively, it is probable that aqueous *M. oleifera* leaf extracts may have affected acrosome reaction sperm through altering sperm membrane lipid profiles and fluidity (Breitbart, 1997), Ca^{2+} ion channels (Benoff et al., 1994; Morakinyo et al., 2011), transporter proteins that regulate acrosome reaction (Bastián et al., 2010).

An increase in sperm with intact acrosome in our study was consistent with those reported by El-Harairy et al. (2016) and El-Nagar (2017) where *M. oleifera* leaves were used as semen extenders. Similar observations were made when human spermatozoa were exposed to *Thymus munbyanus* (thyme) essential oil *in vitro* (Chikhoune et al., 2015). Our results were in contrast with reports from studies conducted by with *C. capensis (*Shalaweh et al., 2015) and *E. longofolia* (Erasmus et al., 2012)*,* in which increased intracellular ROS production promoted the spontaneous acrosome reaction of human sperm *in vitro*. Dissimilarities in the latter studies could be accounted for by variabilities in sperm samples, intra-laboratory differences and the obvious phytochemicals in respective plant parts. Although it would generally appear that excess antioxidant activities of extracts mediate their actions through ROS "*trapping*", which eventually interfere with hyperphosphorylation of proteins involved in CP and AR, exact mechanisms are not precise.

5.8 Conclusion

M. oleifera leaves are endowed with vast bio-constituents that span pharmacological spectrum in the management of sexual and reproductive afflictions. Previous studies have alluded to the biological activities of medicinal fractions extracted from the leaves. Antioxidants reportedly play a significant role in ameliorating oxidative stress-related reproductive pathologies, including sperm dysfunction. The existing phytochemical analysis of *M. oleifera* leaves already reveals numerous polyphenols,

trace elements, vitamins, and flavonoids, to name a few that exert significant free-radical scavenging activity in the intracellular and extracellular immediate environment of the sperm. In this study, *M. oleifera* aqueous leaf extracts dose-dependently inhibited excessive endogenous sperm ROS in donors, patients, and the asthenozoospermic samples, possibly through boosting the antioxidative capacities. The differences in effects of the plant on the donors and the patient group can in part be attributed to the difference in biochemical phenomena underlying physiology and pathology respectively. For instance, in healthy donor group the spermatozoal microenvironment balanced redox, was driven towards a reductive state because of the presence of excess antioxidants of *M. oleifera*. By the same token, spermatozoa fom the infertile group are already functionally compromised or their immediate enivrionment mainly due to oxidative stress, incubation with the extract contrastingly reverses the state to “normal”. By neutralising or inhibiting superoxide radical overproduction, *M. oleifera* leaf extracts successfully improved the percentage of live sperm and total motility in patients. The enhancement in sperm vitality was accompanied by a dose-dependent increase in sperm with intact mitochondrial membrane potential, nuclear DNA, and acrosome with the prevention of precocious capacitation and acrosome reaction.In conclusion, *M. oleifera* free-radical scavenging properties successfully boosted sperm functional parameters *in vitro* in all groups by conferring protection against excessive ROS production, nuclear DNA strand break, and premature capacitation and acrosome reaction. Our findings concur with other studies that maintain that *M. oleifera* could be beneficial in the management of male infertility, either as raw remedies or through a pharmaceutically processed plant. Collectively, the indications made from this study provide further justification for pharmacological and toxicological study designs on how *M. oleifera* could be utilised for fertility enhancement in males.

5.9 Future outlooks and recommendations

While the findings from this study reaffirm previous reports on the *in vitro* effects of the plant on mammalian reproductive cells, we have identified experimental shortcomings, which future studies could benefit from, and which can upgrade the validity, reliability, and reproducibility of results. *M. oleifera* is widely distributed and utilised; geographical differences can affect the phytochemicals and nutritional composition of the plant

(Mabapa et al., 2017). This implies that *M. oleifera's* phytochemical characteristics, to which biological activities *in vitro* are attributed, will vary considerably from region to region, and consequently, from study to study. This fact will therefore bring into question the assertions claimed of the effects of the plant on tested cells or tissue. Future studies evaluating the effects of herbal extracts on any tissue *in vitro* may benefit from conducting 2,2′-diphenyl-1-picrylhydrazyl (DDPH) assaying for antioxidant activity, phenolic content, vitamin and mineral content, and simultaneously preliminary phytochemical screening or characterisation of their extract. Inclusion of such analyses will ensure reliable conclusions on the biological effects of extracts as observed in the study.

In studies such as this, where oxidative stress was potentially a significant element linking all sperm parameters, oxidative-reduction potential (ORP) testing would prove useful. Oxidative-reductive potential measures the tendency with which one substance oxidises (loses electrons) or reduces (gains electrons) another. This would provide a baseline redox capacity of the extracts pre-treatment.

Finally, there exist overlaps in basic semen parameters between fertile and infertile groups, as is the case with any biological samples. This brings in high levels of variability between samples. Therefore, the classification of semen as infertile from fertile was not based solely on the source or the WHO-based spermiogram cut-off points, but also on other non-spermatozoidal seminal markers such as bacteriospermia and the presence of polymorphonuclear (PMN) leukocytes (visible in CASA system). Future similar studies would benefit from conducting functional testing such as the Endtz test on raw semen to help properly categorise samples as healthy or infertile before treatment.

CHAPTER 6

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APPENDICES

APPENDIX 1: University of Limpopo TREC approval

University of Limpopo
Department of Research Administration and Development
Private Bag X1106, Sovenga, 0727, South Africa
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TURFLOOP RESEARCH ETHICS COMMITTEE

ETHICS CLEARANCE CERTIFICATE

MEETING: 06 March 2019

PROJECT NUMBER: TREC/45/2019: PG

PROJECT:

Title:	In Vitro Effects of Aqueous Leaf Extract of Moringa Oleifera On Human Sperm.
Researcher:	FT Maichela
Supervisor:	Dr. CS Opuwari
Co-Supervisor/s:	Prof. R Henkel
School:	Health Care Science
Degree:	MSc in Medical Science

PROF P MASOKO
CHAIRPERSON: TURFLOOP RESEARCH ETHICS COMMITTEE

The Turfloop Research Ethics Committee (TREC) is registered with the National Health Research Ethics Council, Registration Number: **REC-0310111-031**

Note:

i) **This Ethics Clearance Certificate will be valid for one (1) year, as from the abovementioned date. Application for annual renewal (or annual review) need to be received by TREC one month before lapse of this period.**

ii) **Should any departure be contemplated from the research procedure as approved, the researcher(s) must re-submit the protocol to the committee, together with the Application for Amendment form.**

iii) **PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES.**

Finding solutions for Africa

APPENDIX 2: University of the Western Cape BMREC approval

UNIVERSITY of the WESTERN CAPE

OFFICE OF THE DIRECTOR: RESEARCH
RESEARCH AND INNOVATION DIVISION

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26 April 2018

Dr C Opuwari
Medical Biosciences
Faculty of Natural Sciences

Ethics Reference Number: BM17/7/14

Project Title: In-vitro effects of aqueous leaf extract of *Moringa oleifera* on TM3 human sperm functions.

Approval Period: 25 April 2018 – 25 April 2019

I hereby certify that the Biomedical Science Research Ethics Committee of the University of the Western Cape approved the scientific methodology and ethics of the above mentioned research project.

Any amendments, extension or other modifications to the protocol must be submitted to the Ethics Committee for approval.

Please remember to submit a progress report in good time for annual renewal.

The Committee must be informed of any serious adverse event and/or termination of the study.

Ms Patricia Josias
Research Ethics Committee Officer
University of the Western Cape

PROVISIONAL REC NUMBER -130416-050

APPENDIX 3: Permission for semen collection from Tygerberg Hospital

Western Cape Government
Health

TYGERBERG HOSPITAL
REFERENCE:
Research Projects
ENQUIRIES: **Dr GG Marinus**
TELEPHONE:**021 938 5752**

PROJECT NUMBER: TREC/45/2019: PG - Renewal

TITLE: **In Vitro Effects of Aqueous Leaf Extract of Moringa Oleifera On Human Sperm.**

Dear FT Moichela [Supervisor: Dr CS Opuwari]

PERMISSION TO CONDUCT YOUR RESEARCH AT TYGERBERG HOSPITAL.

1. In accordance with the Provincial Research Policy and Tygerberg Hospital Notice No 40/2009, permission is hereby granted for you to conduct the above-mentioned research here at Tygerberg Hospital.

2. Researchers, in accessing Provincial health facilities, are expressing consent to provide the Department with an electronic copy of the final feedback within six months of completion of research. This can be submitted to the Provincial Research Co-Ordinator (Health.Research@westerncape.gov.za).

DR GG MARINUS
MANAGER: MEDICAL SERVICES

DR D ERASMUS
CHIEF EXECUTIVE OFFICER
Date: 24 May 2019

Administration Building, Francie van Zijl Avenue, Parow, 7500 Private Bag X3, Tygerberg, 7505
tel: +27 21 938-6267 fax: +27 21 938-4890 www.capegateway.gov.za

APPENDIX 4: Permission for semen collection from Vincent Pallotti Hospital

Aevitas
Fertility Clinic • Cape Town

Dr Gerhard Hanekom [MBChB, MMed (O&G), FCOG(SA), Cert Reproductive Medicine(SA)] • Dr Victor Hulme [MBChB, MMed (O&G)]
• Prof Thinus Kruger [MBChB, MPharMed, MMed (O&G), FCOG (SA), FRCOG (London), MD/PhD]
• Prof Igno Siebert [MBChB, MMed (O&G), LKOG/FCOG (SA), PhD

11 September 2019

Ethics Reference: TREC/45/2019:PG

TITLE: In vitro effects of aqueous leaf extract of Moringa oleifera on human sperm functions

Dear Dr Moichela

PERMISSION TO CONDUCT YOUR RESEARCH AT AEVITAS CLINIC FROM 6.3.2019 TO 6.3.2020

1. In accordance with the Provisional Research Policy and Tygerberg Hospital Notice No 40/2009, permission is hereby granted for you to conduct the abovementioned research here at Aevitas Clinic.

Prof T I Siebert

Centre of Excellence in Fertility, Endometriosis & Endoscopic Surgery
Start Your Family with People Who Care

Drs Aevitas PTY
PR No: 1605925 VAT: 4160266
Life Vincent Pallotti Hospital Park Rd
Pinelands, 7405, South Af
Tel: +27 21 531 6999 Fax: +27 21 531 7
E-mail: info@aevitas.c
Website: www.aevitas.c

APPENDIX 5: Informed consent form (English)

A Written and informed consent form will be given to participants prior to semen collection.

PARTICIPATION INFORMED CONSENT FORM

***In vitro* effects of aqueous leaf extract of *Moringa oleifera* on human sperm functions**

Information sheet and consent to participate in Research

To whom it may concern,

You are requested to consider participating in a scientific study on male sperm functions and medicinal plants. The aim of this study is to investigate the *in vitro* effects of *Moringa oleifera* on human sperm functions.

After collection of your semen through masturbation, it will be exposed to various concentrations of the aqueous leaf extract of *Moringa oleifera*.

The objectives of the study are as follows:

- To determine effects of *M. oleifera* on sperm viability, motility and morphology.
- To measure of the effects of aqueous extracts of *M. oleifera* on ROS production in sperm.
- To determine effects of *M. oleifera* on sperm DNA fragmentation.
- To determine spermatozoan mitochondrial membrane potential, capacitation and acrosome reaction after extract exposure.
- To compare the effects of *M. oleifera* leaf extracts on fertile and infertile men.

For those participating in the assisted reproductive treatment, after your semen has been processed for the treatment you are scheduled for, a small portion of the semen sample (<100 µl) will be taken for these additional tests. This procedure will under no circumstance affect the assisted reproduction treatment or outcome as only the residual samples will be used. This will allow us to determine the possible effects of *Moringa oleifera* on human sperm, that is whether it can improve male fertility or not.

The current study will not involve any additional medical risk or cost. Given the invasive nature of the study it may include little discomfort. Participation in this scientific study is voluntary and participants are allowed to withdraw at any point in time until the data will be submitted for publication without any consequences. Withdrawal from the study

should be done in a formally addressed email. In order to protect confidentiality of the participants, a unique number will be assigned to each sample and will contain no personal information of the participants involved in the study. All data will be confidential and available to the scientific researchers involved as well as to the participants if requested. The samples will be disposed of in the correct medical procedure by incineration once the data is captured.

The UWC Biomedical Research Ethics Committee has ethically received the study (Approval number: 130416-050)

In the event of any problems or concerns, additional information can be obtained from the doctor or scientific investigator directly by email.

Consent

I have been informed about the study titled “In vitro effects of aqueous leaf extract of *Moringa oleifera* in human sperm functions” by the scientific investigator.

I understand the purpose and procedure of the study.

I have been given an opportunity to ask questions about the study and have had answers to my satisfaction.

I declare that my participation in this study is entirely voluntary and uncompensated and that I may withdraw at any point in time until the data will be submitted for publication without any consequences and without affecting any treatment or care that I am entitled to.

If I have any further questions or query related to the study, I understand that I may contact the researchers.

If I have any questions or concerns about my rights as a study participant, or if I am concerned about any aspect of the study or researcher, then I may contact:

_____ _____

Signature of Participant Date

_____ _____

Signature of Investigator Date

APPENDIX 6: Informed consent form (Afrikaans)

DEELNAME INGELIGTE TOESTEMMING VORM

***In vitro* effekte van waterige blaar uittreksel van *Moringa oleifera* o n h Uman sperm funksies**

Inligtingsblad en toestemming om deel te neem aan Navorsing

Aan wie dit mag gaan,

Jy is versoek om te oorweeg om aan 'n wetenskaplike studie deel te neem oor manlike spermfunksies en medisinale plante. Die doel van hierdie studie is om die *in vitro* te ondersoek gevolge van *moringa oleifera* op menslike spermfunksies .

Na die versameling van y ons semen monsters masturbasie , Dit sal blootgestel word aan verskeie konsentrasies van die waterige blaar uittreksel van *Moringa oleifera* .

Die doelstellings van die studie is soos volg:

- Om gevolge van *M. oleifera* op spermvatbaarheid, motiliteit en morfologie te bepaal.
- Om spermatozoan te meet -ROS neutraliserende vermoë van waterige uittreksels van *M. oleifera.*
- Om sperm DNA fragmentering te bepaal.
- Om spermatozoan mitochondriale membraanpotensiaal, kapasitasie en akrosome reaksie na eksponeringsblootstelling na te gaan.
- Om die effekte van *M. oleifera* blaar uittreksels op vrugbare en onvrugbare mans te vergelyk.

Vir diegene wat deelneem aan die assistente reproduktiewe behandeling, nadat jou semen verwerk vir die behandeling wat jy is geskeduleer vir 'n klein gedeelte van die semen monster (<100 µl) geneem sal word vir hierdie addisionele toets s. Hierdie proses sal onder geen omstandighede beïnvloed die bygestaan voortplanting behandeling of uitkoms as net die oorblywende monsters sal gebruik word. Dit sal ons toelaat om die moontlike effek s van *Moringa te bepaal oleifera* op menslike sperm, dit is of dit manlike vrugbaarheid kan verbeter of nie.

Die huidige studie sal geen verdere mediese risiko of koste inhou nie . Gegewe die indringende aard van die studie kan dit min ongemak insluit. Deelname aan hierdie wetenskaplike studie is heeltemal vrywillig en deelnemers mag op enige tydstip

terugtrek totdat die data sonder enige gevolge vir publikasie ingedien sal word. Onttrekking uit die studie moet gedoen word in 'n formele aangespreek e-pos. Ten einde die vertroulikheid van die deelnemers te beskerm, sal 'n unieke nommer aan elke monster toegeken word en sal geen persoonlike inligting van die vrywilligers wat by die studie betrokke is, bevat nie . Alle data sal vertroulik en beskikbaar wees vir die betrokke wetenskaplike navorsers sowel as die deelnemers indien dit aangevra word. Die monsters sal deur die verbranding in die korrekte mediese prosedure weggedoen word sodra die data gevang is.

Die studie is eties ontvang deur die UWC Biomediese Navorsingsetiekkomitee

(Goedkeuringsnommer:130416-050)

In geval van enige probleme of probleme, kan addisionele inligting direk per e-pos by die dokter of wetenskaplike ondersoeker verkry word.

toestemming

Ek is ingelig oor die studie getiteld " *In vitro* effekte van waterige blaar uittreksel van *Moringa oleifera* in menslike spermfunksies "deur die wetenskaplike ondersoeker.

Ek verstaan die doel en prosedure van die studie.

Ek het die geleentheid gekry om vrae oor die studie te vra en antwoorde tot my bevrediging gehad.

Ek verklaar dat my deelname aan hierdie studie heeltemal vrywillig en ongekompenseer is en dat ek op enige tydstip kan terugtrek totdat die data sonder enige gevolge vir publikasie voorgelê sal word sonder om enige behandeling of sorg waarvoor ek geregtig is, te beïnvloed.

As ek verdere vrae of navrae het wat verband hou met die studie, verstaan ek dat ek die navorsers kan kontak.

As ek enige vrae of kommer het oor my regte as studieleier, of as ek bekommerd is oor enige aspek van die studie of navorser, kan ek kontak:

_____ _____

Handtekening van Deelnemer datum

_____ _____

Handtekening van Ondersoeker datum

APPENDIX 7: Informed consent form (IsiXhosa)

INKQUBO YOMSEBENZISWANO OQHUBILEYO

Iimpembelelo ze- *in vitro* ze-extract leaf of *Moringa oleifera* o n h uman imisebenzi yesidoda

Iphepha leenkcukacha kunye nemvume yokuthatha inxaxheba kuPhando

Ngubani onokukhathalela,

Uceliwe ukuba uthathe inxaxheba ekutheni uthathe inxaxheba kwisifundo sezenzululwazi kwimisebenzi yesilisa kunye nemithi yezityalo. Injongo yale sifundo kukuphanda i- *in vitro* nemiphumo *kaMoringa i-oleifera* kwimisebenzi yesintu .

Emva kokuqokelelwa y yethu isampuli isampula, kuya kubonakala kwiindawo ezahlukahlukeneyo ze-extract leaf of *Moringa oleifera* .

Iinjongo zesifundo zilandelayo:

- Ukumisela iziphumo *zikaMnu oleifera* kwi-sperm yokusebenza, i-motility kunye ne-morphology.
- Ukumisela ukuhlukana kwe-DNA yesininzi.
- Ukujonga i- spermatozoan mitochondrial potential membrane, ukuxhotyiswakunye ne-acrosome reaction emva kokukhutshwa kwe-extract.
- Ukuthelekisa nemiphumo ye- *M. oleifera yamacwecwe amacwecwe* kumadoda athile angenasiphelo.

Okanye ngabo abathatha inxaxheba unyango ekuzaleni, emva kokuba imbewu yakho sele ukwenzela unyango ziyacwangciswa, inxalenye encinane kwamadlozi isampula (<100 µl) ziya kuthathwa kula uvavanyo s olongezelelweyo. Le nkqubo iya phantsi akukho iimeko kuchaphazela unyango ekuzaleni okanye isiphumo njengoko kuphela iisampuli intsalela iza kusetyenziswa. Oku kuya kusenza sikwazi ukubona ukuba kunokwenzeka isiphumo s ye *Moringa oleifera* kwisidoda somntu, oko kukuthi ingaba kuphuculwe ukuzala komntu okanye cha.

Uphononongo lwangoku aluyi kubandakanyeka nayiphi na ingozi yonyango okanye eyindleko. Ukubonelelwa ngendalo ebangelwayo isifundo singabandakanyeka kakhulu. Ukuthatha inxaxheba kulolu cwaningo lwezenzululwazi ngokuzithandela kwaye abathathi-nxaxheba bavunyelwe ukuhoxisa naliphi na ixesha ngexesha lokuba idatha ingeniswe ukupapashwa ngaphandle kwemiphumo. Ukurhoxiswa kweso sifundo kufuneka kwenziwe kwikhompyutha echongiweyo. Ukuze ukhusele imfihlo yabathathi-nxaxheba, inombolo ekhethekileyo iya kubelwa isampula nganye kwaye ayiyi kuqulethelwa ngolwazi oluqulethwe ngamavavolontiya abandakanyekayo kwisifundo. Yonke idatha iya kuba yimfihlo kwaye ifumaneke kubaphandi bezesayensi abachaphazelekayo kunye nabachaphazelekayo ukuba bayacelwa. Iisampuli ziya kulahlwa kwinkqubo efanelekileyo yezocwangco ngokutshatyalaliswa xa idatha ithathwa.

Uphononongo luye lwafunyanwa ngokomthetho yiKomidi yeeNkcazo zoBuchule be-UWC

(Inombolo yemvume: 130416-050)

Xa kwenzeka nayiphi na ingxaki okanye ukukhathazeka, ulwazi olongezelelweyo lunokufumaneka kwiregrha okanye uphando loosayensi ngokuchanekileyo nge-imeyile.

Mvume

Ndixelelwe ngophando oluthi " *In vitro* iziphumo zecatshulwa lamagqabi e- *Moringa oleifera* kwimisebenzi yobuninzi babantu "ngumphandi wenzululwazi.

Ndiyayiqonda injongo nenkqubo yesifundo.

Ndinike ithuba lokuba ndibuze imibuzo malunga nokufunda kwaye ndifumene iimpendulo kwaneliseko lwam.

Ndiyaxela ukuba inxaxheba kweso sifundo iphela ngokuzithandela kwaye ingenakuhlawulwa kwaye ndize ndirhoxise naliphi na ixesha ngexesha lokuba idatha ingeniswe ukupapashwa ngaphandle kwemiphumo kwaye ngaphandle kokuchaphazela nayiphi na inyango okanye ukunakekelwa kwam.

Ukuba ndinemibuzo eminye okanye umbuzo ophathelene nesifundo, ndiyaqonda ukuba ndidibana nabaphandi.

Ukuba ngaba nayiphi na imibuzo okanye ukuxhalabisa ngamalungelo am nje njengomfundi othabatha inxaxheba, okanye ukuba ndixhalabele nayiphi na into yesifundo okanye umphandi, ngoko ndiyaqhagamshelana nayo:

_____ _____

U tyikityo lwaBathathi- nxaxheba Umhla

_____ _____

Isayinwe yoMphandi Umhla