EVALUATION OF STANDARD AND DEVELOPMENT OF NEW SPERM FUNCTIONAL TESTS IN SELECTED PRIMATE SPECIES

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KEYWORDS

Male fertility

Non-human primates

Sperm function

Sperm motility

Sperm vitality

Acrosome intactness

Sperm hyperactivation

Validation and sensitivity tests

Heavy metals

Optimized techniques

ABSTRACT

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Male infertility in humans has increased in the last few decades and could be as high as 40%, while up to 50% of these men have "unexplained" (idiopathic) infertility. Although newly developed molecular techniques have great value in detecting subtle causes of male infertility, more detailed sperm functional tests are required to identify compromised fertility, especially in a clinical set-up. Since ethical constraints often preclude the pursuit of many basic research questions in humans, non-human primates (NHPs) have been identified as key models in human-related studies. NHPs are often used in studies on male fertility/infertility, IVF or assisted reproductive technology (ART) procedures, male contraception and reproductive toxicology. However, comparing results of NHP and human studies require that techniques used for assessment must be objective, standardized and sensitive to recognize compromised sperm function. The aim of this study was to evaluate standard sperm functional tests and develop new functional tests using NHP sperm, specifically from vervet monkeys (*Chlorocebus aethiops*), chacma baboons (*Papio ursinus*) and rhesus monkeys (*Macaca mulatta),* for application in human and NHP studies and to ultimately develop a basic primate model. The sperm functions investigated included sperm motility, longevity, vitality, DNA integrity, acrosome reaction, and hyperactivation. The sperm functional tests evaluated were: CASA motility analysis; Sperm Longevity test; Eosin-Nigrosin and Hoechst and Propidium Iodide staining, as well as the use of WST-1 cytotoxicity assay for vitality; the TUNEL assay for DNA integrity; Acrosome Intactness Test; and induction of hyperactivation via stimulants. The validity of each test was investigated by inhibiting sperm function through the use of copper sulphate and cadmium chloride. All functional tests were successfully performed across all three species, except the TUNEL assay for DNA integrity, and was further used for validation testing. Validation testing proved that all sperm functional parameters were significantly affected by the highest concentrations of the chemicals (250 µg/ml $CuSO_4$ and 500 µg/ml $CdCl_2$) and if not significant, trends of reduction were seen. The tests employed were therefore sensitive to the inhibitory effect of the metals. By evaluating these established sperm functional tests we found that primates would serve as good models for research study. Furthermore, we optimized and modified techniques for sperm and functional analysis in these three primate species and this study will standardize protocols for use in future studies on male infertility. Additionally, comparing human and NHP sperm function can possibly reveal or explain the high infertility rates in humans.

JUNE 2017

DECLARATION

I declare that Evaluation Of Standard And Development Of New Sperm Functional Tests In Selected Primate Species is my own work, that it has not been submitted before for any degree or examination at any other university, and that all the sources I have used or quoted have been indicated and acknowledged as complete references.

Farren Chelsea Prag

June 2017

Signed_____

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LIST OF ABBREVIATIONS

4-AP - 4-aminopyridine

$\Delta\Psi_M$ - inner mitochondrial membrane potential

mg - milligram

ml - millilitre

µg - microgram

µl - microlitre

µM - micromolar

A23187 - calcium ionophore

AI - artificial insemination

ALH - amplitude of lateral head displacement

ANOVA - analysis of variance

AR - acrosome reaction

ARIC - acrosome reation after ionophore challenge

ART - assisted reproductive technology

ASRM - American Society of Reproductive Medicine

ATP - adenosine triphosphate

BCF - beat cross frequency

Caf - caffeine

cAMP - cyclic adenosine monophosphate

CASA - computer - aided sperm analysis

CASMA - computer - aided sperm motility analysis

CDC - Centre for Disease Control and Prevention

$CdCl_2$ - cadmium chloride

CO_2 - carbon dioxide

Conc - concentration

$CuSO_4$ - copper suplhate

dbcAMP - dibutryl cyclic AMP

dH_2O - distilled water

DHS - Demographic and Health Surveys

DNA - deoxyribonucleic acid

DPX - distyrene, plasticizer (tricresyl phosphate), xylene

dutp - deoxyuridine triphosphate

ECRA - Ethics Committee for Research on Animals

E-N - eosin-nigrosin

FISH - fluorescence *in situ* hybridization analysis

FITC-PNA - fluorescein-conjugated *Arachis hypogea* (peanut) agglutinin

FITC-PSA - fluorescein-conjugated *Pisum sativum* (edible pea) agglutinin

GSH - reduced glutathione

GSSG - oxidized glutathione

H&P - Hoechst 3342 (trihydrochloride trihydrate) and Propidium Iodide

HA - hyaluronic acid

HIV -Human immunodeficiency virus

HTF - human tubal fluid

HZA - hemizona assay

ICSI - intracytoplasmic sperm injection

IM - immotility

IMSI - intracytoplasmic morphologically selected sperm injection

IUI - intrauterine insemination

IVF - *in vitro* fertlization

JC-1 - 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimiddasolyl-carbocyanine iodide

LIN - linearity

min - minutes

MSOME - motile sperm organelle morphological examination

n - sample size

NaOH - sodium hydroxide

NHP - nonhuman primate

NP - non-progressive motility

ODF - outer dense fibres

PBS - phosphate buffered saline

PCT - postcoital test

PI - propidium iodide

PICSI - picked spermatozoa for ICSI

PID - pelvic inflammatory disease

PNA - peanut agglutinin

PR - progressive motility

PS - PureSperm

PSA - prostate specific antigen

Rh123 - rhodamine 123

ROS - reactive oxygen species

SAGA-1 - sperm agglutination antigen-1

SAMRC - South African Medical Research Council

SCA - Sperm Class Analyzer

SCI - sperm capacitation index

SCSA - sperm chromatine structure assay

SD -standard deviation

SDF - sperm DNA fragmentation

SIAS - superimposed image analysis system

SPA - sperm penetration assay

SSC - saline sodium citrate

STDs - sexually transmitted diseases

STIs - sexually transmitted infections

STR - straightness

SU - Swim-up

TdT - terminaldeoxynucleotidyl transferase

TUNEL - terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling

UTJ - uterotubal junction

VAP - average path velocity

VCL - curvilinear velocity

VSL - straight-line velocity

WHO - World Health Organization

WOB - wobble

CONTENTS

Chapter 1: Introduction to study

1.1 General introduction

Infertility affects up to 15% of couples (Raheem and Ralph, 2011) and male infertility refers to a male's inability to result pregnancy in a fertile female (Kumar and Singh, 2015). A male factor is solely responsible in about 20% of infertile couples and contributory in 30-40% of cases (Jarow et al., 2002). The causes of male infertility can be classified into three major groups, the first being, non-obstructive infertility resulting from inadequate sperm production by the testes. The second is obstructive infertility, where there is normal sperm production but there's a blockage in the genital tract and the third is coital infertility where there is normal sperm production and patent genital tract, however, infertility is secondary to sexual dysfunction, which impairs intromission or ejaculation (Raheem and Ralph, 2011). It is therefore important to identify the class of infertility and provide the appropriate treatment.

Male infertility is commonly due to deficiencies in the semen and semen quality is used as a surrogate measure of male fecundity (Cooper et al., 2010). Sperm abnormalities are a critical factor in male infertility, which include abnormalities related to sperm count, sperm motility and sperm structure and shape (WHO, 1999; Kumar and Singh, 2015). The analysis of retrospective data indicates that sperm counts may have declined in parts of the world but a geographical variation in the semen quality exists (Auger and Jouannet, 1997; Jorgensen et al., 2001; Swan, 2006). The reason for this variation may be due to environmental, nutritional, socioeconomic or other unknown causes (Fisch and Goluboff, 1996).

From the 1980s, an emerging concern has been reported about deteriorating semen quality (Osser et al., 1984; Menkveld et al., 1986; Murature et al., 1987). In 1992, a study including meta-analysis, found that the mean sperm count of healthy men declined by 1% per year between 1938 and 1990 (Carlsen et al., 1992). In 2000, an updated meta-analysis was done, which confirmed the falling trend in sperm count (Swan et al., 2000) and another meta-analysis reported a global decrease in sperm density by about 50% over the past 50-60 years (Carlsen et al., 1992; Fisch, 2008).

The fact that standard semen evaluation only allows for the diagnosis of male infertility without providing evidence for an aetiological or physiopathological origin, the inclusion of sperm functional testing has become necessary (Oehninger et al., 2000). Furthermore, if no therapy is indicated, if treaments fail or if the degree of sperm abnormalities is severe enough to refer the couple to assisted reproductive technology (ART), there is a need to determine the sperm functional capacity (Oehninger et al. 2000). In the light of the

above, it is therefore necessary to employ sperm functional testing in order to further identify the causes and appropriate treament for infertility.

1.2 Aim and objectives

This study involved the use of non-human primate spermatozoa to evaluate sperm function with selected tests and employ the use of chemicals to inhibit sperm function to determine the sensitivity of these tests. This will thereby allow for the possibility of developing a primate model for reproductive studies.

Aim 1: Evaluate and modify standard sperm functional tests

Objectives:

- to determine the appropriate sperm preparation method
- to optimize and modify the sperm functional tests for non-human primates
- to rule out tests that are not successful for further testing

Aim 2: Validate the ability of each test to detect changes in sperm functional parameters

Objectives:

- to inhibit sperm function and evaluate the sensitivity of each test to recognize compromised sperm function
- to determine whether the sperm functional tests would be successful for evaluating the spermatozoa of the selected primate species

1.3 Overview of chapters

Chapter 2:

This chapter contains the introduction of the study by explaining and giving background to the relevant topics of this study such as infertility, the use of non-human primates for research and the effect of heavy metals on the male reproductive system. Male infertility is the focus for this study and the primates selected for

investigation were the vervet monkey, chacma baboon and the rhesus monkey. The heavy metals selected for inhibitory actions were copper sulphate and cadmium chloride.

Chapter 3:

The methods and materials of this study are explained in this chapter. It contains and provides protocols for the techniques used to evaluate sperm function, namely, sperm motility and longevity, vitality, DNA integrity, acrosome intactness and hyperactivation. The preparation of the primate spermatozoa functional for testing and the introduction of the heavy metals are also explained.

Chapter 4:

The results obtained in this study will be found in this chapter and it has two parts, Optimization and Validation. Optimization was the first step of evaluation of all the employed tests used for all three primate species. Validation was the second step, which involved the introduction of the heavy metals to inhibit sperm function and test the sensitivity of each test.

Chapter 5:

This chapter contains the discussion of the results obtained for each test as well as for each species, with the support of results from previous studies. Tests were found to be successful in determining sperm function and negative effects were seen after exposure to heavy metals. The conclusion highlights that we were successful in our aim of evaluating sperm function in these primate species and provide steps and suggestions for future studies.

Chapter 2: Introduction

2.1 Infertility

Infertility, defined as failure to conceive after a year of unrestricted and unprotected intercourse, is a frequent complaint in gynaecological service (Mati, 2004). It has been seen to lead to distress and depression, as well as discrimination and ostracism (Chachamovich et al., 2010; Cui, 2010). The inability to have children affects men and women across the globe (Mascarenhas et al., 2012), affecting 8 to 12 percent of couples worldwide (Reproductive Health Outlook, 2002). In addition, for women with irregular menstrual cycles and over the age of 35 years, infertility is defined as unsuccessful conception after 6 months of trying to conceive (CDC, 2016). Female infertility can futhermore be divided into primary and secondary infertility. Primary infertility is defined as the absence of a live birth for women who desire a child and have been in a union for at least five years, during which they have not used any contraceptives. Secondary infertility is the absence of a live birth for women who desire a child and have been in a union for at least five years since their last live birth, during which they did not use any contraceptives (Mascarenhas et al., 2012).

It is estimated that 1 in 7 couples have problems conceiving and the incidence of infertility is similar in most countries regardless of their level of development (Chan, 2010). In Japan, especially, couples reject insemination or adoption as an alternative method to having a child, meaning that males are most likely to seek infertility evaluations when a couple has difficulty conceiving (Kobayashi et al., 2012). Whereas the impact of infertility is felt mainly at the couple and individual levels, in Africa it is also a matter of major concern to the extended family (Mati, 2004). It is an important problem from health, social/cultural and economic considerations, with patients experiencing considerable social and mental anguish, as well as a heavy financial investment needed for its investigation and treatment (Mati, 2004).

According to the World Health Organization (WHO), the global prevalence of infertility is difficult to determine because of the presence of both male and female factors which complicate any estimate that may only address women and an outcome of pregnancy diagnosis or live birth (WHO, 2015). According to Mati (2004), it is estimated that 50–80 million people world-wide are inflicted with infertility. According to another study it accounts for 40-60% of all gynaecological consultations in developing countries (Giwa-Osagie, 2004), however, the problem is greater in the African region where it affects 20–30% of the population.

A more recent study by Mascarenhas et al. (2012), showed that worldwide 48.5 million couples are unable to have a child, of which 19.2 million couples are unable to have a first child, and 29.3 million couples are

unable to have an additional child (the latter excludes China). Fourteen million four hundred thousand of these couples live in South Asia, and a further 10.0 million live in Sub-Saharan Africa. The number of couples suffering from infertility has increased since 1990, when 42.0 million couples were unable to have a child. Though the number of infertile couples has increased globally and in most regions, it has decreased from 4.2 million in 1990 to 3.6 million in 2010 in the High Income region (Pacific Asia, Australia, North America and Western Europe), and from 4.4 million in 1990 to 3.8 million in 2010 in the Central/Eastern Europe and Central Asia region (Mascarenhas et al., 2012).

Although many people still believe that infertility is a female problem (Kobayashi et al., 2012), the cause of infertility can be present in both male and female partners, both of which should be investigated in all cases (Mati, 2004). It is, however, rarely acknowledged that male infertility contributes to at least half of all cases worldwide and is often the most difficult to treat (Devroey et al., 1998; Irvine, 1998; Kamischke and Nieschlag, 1998). The factors causing high rates of infertility in parts of the developing world are varied, but tubal infertility due to sexually transmitted, post-partum, post-abortive and iatrogenic infections is widely regarded as the primary form of preventable infertility in the world (Sciarra, 1994, 1997; Reproductive Health Outlook, 2002). As a result, secondary infertility is encountered more frequently than primary infertility. The prevalence of primary infertility is about less than 5%, while secondary infertility ranges from 10 to 40% (Mati, 2004).

A high prevalence of HIV infection in countries with high infertility rates calls for evaluation of the relationship between the two. HIV infection may decrease fertility through factors related to reduced coital frequency due to ill-health, erectile dysfunction and increased prevalence of sexually transmitted infections (STIs) among HIV-infected individuals (Mati, 2004). In males, HIV infection is associated with hypergonadotrophic hypogonadism. The changes in semen parameters increase in severity with progression of the disease (Mati, 2004). In females, HIV infection exacerbates the severity of pelvic inflammatory disease (PID) thereby causing greater fallopian tube damage (Mati, 2004). It is also probable that a number of tropical conditions contribute to the low fertility in Africa (Mati, 2004). Filariasis has been associated with abnormal semen parameters, while tuberculosis and schistosomiasis have been shown to cause tubal obstruction (Mati, 2004).

2.1.1 Male Fertility/Infertility

Male infertility is a relatively common condition in humans that affects approximately 1 in 20 of the male population (Aitken et al., 2011). Although the majority of such patients produce sufficient numbers of spermatozoa to achieve conception, fertility is compromised because these cells are functionally deficient (Aitken et al., 2011) or have chromosome abnormalities (Ng et al., 2002). This lack of sperm quality can influence the fertilizing potential of spermatozoa and their ability to promote normal embryonic development as a consequence of high levels of deoxyribonucleic acid (DNA) damage in the paternal genome (Aitken et al., 2011).

Sperm dysfunction has consistently been identified as the single most common cause of male infertility. Men can produce sperm which are dysfunctional even when their semen parameters are 'normal' (Aitken et al., 1991). Currently, there are no drugs a man can take, or add to his spermatozoa *in vitro*, to treat sperm dysfunction. The only option is ART, comprising a range of treatments, which are all invasive (Alasmari et al., 2013). The treatment selected depends on the condition, i.e. intrauterine insemination (IUI) for mild, *in vitro* fertlization (IVF) for moderate and intracytoplasmic sperm injection (ICSI) for men with severe sperm dysfunction (Alasmari et al., 2013).

Although some of the physiological, enviromental and lifestyle drivers that promote defective sperm function have been elucidated, the root causes of the impaired spermatogenesis are only just beginning to be unravelled (Aitken et al., 2011). The major factors that have been identified to contribute to poor semen quality and sperm DNA damage include paternal age, diabetes, obesity, radiotherapy, chemotherapeutic drugs and exposure to lifestyle factors such as cigarette smoke, radiofrequency electromagnetic radiation and alcohol (Aitken et al., 2011). In the last 50 years, there has also been a marked global decline in human sperm concentrations of about 1% per year (reported in western countries) and it is believed that environmental endocrine disrupters may also be one of the factors responsible for the declining human sperm counts and other male reproductive tract disorders (Arima et al., 2009).

One of these factors being tobacco smoking and its effects on reproductive function has been widely studied (Mukhopadhyay et al., 2010). It has been suggested that certain components in the smoke interact directly or indirectly with the male or female gametes, affecting their function (Mukhopadhyay et al., 2010). Tobacco combustion yields over 1000 compounds; the major components known to affect semen parameters and sperm function are lead, cadmium, and nicotine. Cigarette smoking has been found to be significantly related to decreased sperm motility, sperm density, and normal sperm morphology (Mukhopadhyay et al., 2010).

2.1.2 Female Fertility/Infertility

Infertility occurs in 15% of couples with a child wish and may be caused by medical problems in the male, as we have previously mentioned, but also by medical problems in the female partner. Modern medicine is faced with the great challenge of women with reproductive disorders like infertility and endometriosis (Dancet et al., 2011). According to the Demographic and Health Surveys (DHS) comparative reports, from mid-2002, it was estimated that more than 186 million ever-married women age 15-49 in developing countries (excluding China) were infertile because of primary or secondary infertility (Rutstein et al., 2004). About 18 million were involuntarily primarily infertile without having experienced a birth and 168 million were secondarily infertile (Rutstein et al., 2004).

According to a study by Mascarenhas et al. (2012), worldwide, the age-standardized percent of women aged 20–44 years, affected by secondary infertility (Figure 1) has decreased from 3.9% to 3.0%. The proportion of women who want a first child has decreased less over time, meaning that the proportion of women who are affected by primary infertility has changed little, from 1.6% in 1990 to 1.5% in 2010 (Mascarenhas et al., 2012).

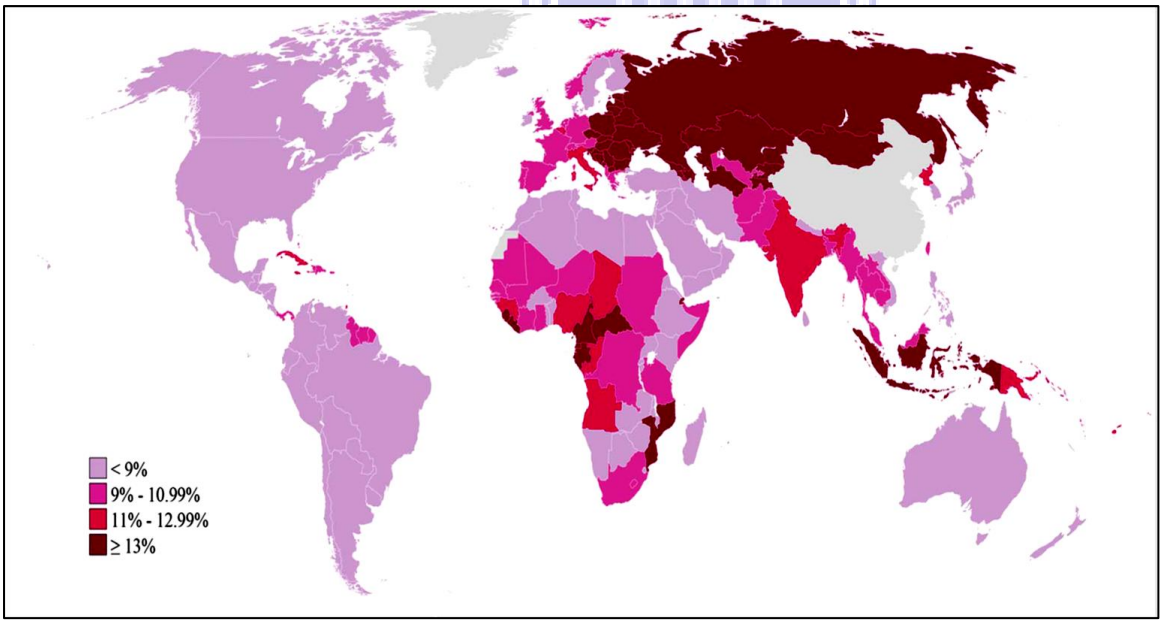


Figure 1: Prevalence of secondary infertility among women whom have had a live birth and seek another (the age-standardized percent of women aged 20–44 years) (Mascarenhas et al., 2012).

There are a number of factors that cause female infertility and increase the risk thereof. One such factor is anovulation, a condition in which follicular development and rupture is impaired and hence the oocyte is not released from its follicle (Brugo-Olmeda et al., 2000). The causes for anovulation include intrinsic ovarian failure as well as ovarian dysfunction secondary to gonadotrophic regulation. The most frequent causes of

anovulation in women with a suspicion of ovulatory failure may derive from conditions such as hyperprolactinaemia, hypogonadotrophic hypogonadism, hypergonadotrophic hypogonadism and polycystic ovaries (Brugo-Olmeda et al., 2000). Other factors that may cause infertility include tubal-peritoneal damage (Brugo-Olmeda et al., 2000) or tubal patency (CDC, 2016) due to pelvic adhesions secondary to infections, PID, prior surgeries or endometriosis (Brugo-Olmeda et al., 2000), however, the main culprits are sexually transmitted diseases (STD). Endometriosis and uterine abnormailites, either congenital or acquired, and altered sperm migration, due to antisperm antibodies and certain pathogenic agents in the cervical mucus, have also been associated with the presence and cause of infertility (Brugo-Olmeda et al., 2000).

The risk of female infertility is increased as a woman ages because her ovaries are less able to release eggs, the egg number declines and are not as healthy, the onset of health conditions which affect fertility and the likeliness of miscarriage rises (CDC, 2016). There are lifestyle factors which may also increase the risk for infertility, they include: smoking, excessive alcohol use, extreme weight gain or loss and excessive physical or emotional stress that result in amenorrhea (Human Fertilisation & Embryology, 2012; CDC, 2016).

The scientific understanding of female infertility is limited because of ethical restrictions on research in women. In the field of ART, there is a need to better understand fertilization, embryonic development, embryonic implantation, and embryonic stem cells (Dancet et al., 2011). Obvious ethical considerations restrict such research on human gametes and embryos. With regards to endometriosis, there is a need to better understand pathogenesis, spontaneous evolution, noninvasive diagnosis, and treatment of endometriosis, as recurrences after surgical therapy or after cessation of medical treatment are common (Dancet et al., 2011).

Concerns have been raised about safety issues in ART, stating that some ART innovations are too quickly introduced in humans without proper prior assessment of safety and efficiency in animal models. Therefore preclinical nonhuman primate (NHP) models are necessary after *in vitro* research and/or research in small animal models are required before clinical application in humans (Dancet et al., 2011).

2.2 Mammalian Spermatozoa

The mammalian spermatozoon is a deceptively simple and terminally differentiated cell (Ramalho-Santos et al., 2002). It seems to have a limited array of functional features, in essence to deliver an intact haploid genome to an oocyte at fertilization. These functions involve many important aspects in physiology, and

cellular and molecular biology in fertility and toxicology (Ramalho-Santos et al., 2007). A functional mammalian spermatozoon consists of a head, neck and tail; its total length varies but is species-specific (Pesch and Bergmann, 2006). The plasma membrane surrounds the spermatozoon in total and is characterized by a regional glycoprotein and lipid constitution. These surface domains are important for the function of the membrane areas (Topfer-Petersen and Waberski, 2001). For example, the equatorial segment of the sperm head's membrane is responsible for the contact to the oocyte membrane during fertilization (Rovan, 2001).

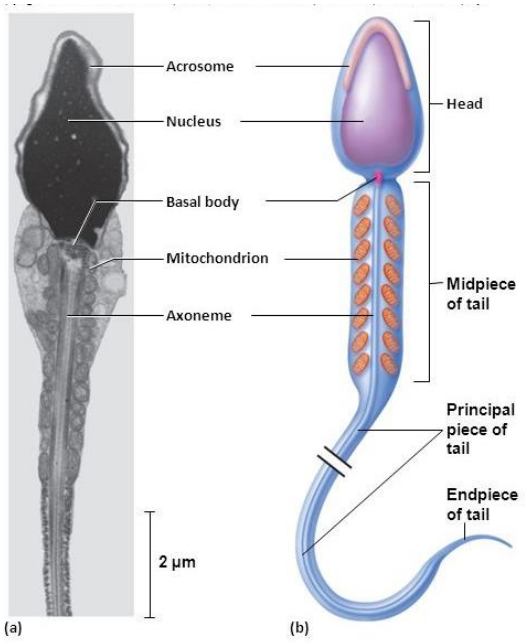


Figure 2: Structure of human spermatozoa as an example of mammalian sperm structure (Saladin, 2006)

2.2.1 The head

The sperm head consists of the acrosome and the nucleus (Figure 2) being surrounded by the plasma membrane (Pesch and Bergmann, 2006). The nucleus contains protamines (sperm-specific DNA binding proteins) which have replaced histones found in somatic cells. The presence of protamines allows for tighter chromatin packaging and has a role in reducing cell volume and increasing the spermatozoon's aerodynamic properties, thus potentially facilitating fertilization (Ramalho-Santos et al., 2007). Overlaying the nucleus is the acrosome, a cap-like structure covering the first two thirds of the sperm head (Pesch and Bergmann, 2006). It contains hydrolytic enzymes to aid in sperm penetration through oocyte-protecting layers, for instance, the translucent glycoprotein-based zona pellucida (Ramalho-Santos et al., 2007). The release of these enzymes causes lysis of the zona pellucida and penetration of the corona radiata of the oocyte. This process of enzymal release is called the acrosome reaction (AR), during which the outer acrosomal membrane coagulates with the plasma membrane (Barross et al., 1976; Rovan, 2001). Therefore, lack of an

acrosome in any circumstance signals that spermatozoa will likely not be fully functional (Ramalho-Santos et al., 2007).

2.2.2 The neck

The neck of human spermatozoa is approximately 1µm long and attached anteriorly to the basal plate and posteriorly to the outer dense fibres (ODF) of the flagellum and serves as a connecting, articular piece (Pesch and Bergmann, 2006). It is a short linking segment between the flagellum and the sperm head, composed of segmented columns and a dense fibrous structure, the capitulum, with the proximal centriole located next to it (Pesch and Bergmann, 2006).

2.2.3 The tail

The tail is the largest part of spermatozoa and consists of the midpiece, principal piece and end piece (Figure 2) (Pesch and Bergmann, 2006). The human sperm midpiece is about 5µm long and is characterized by the mitochondrial sheath that surrounds the axonemal complex and the nine ODF (Pesch and Bergmann, 2006). ODF are characteristic structures of species performing internal fertilization (Fawcett, 1970). There is a positive correlation between survivability and the number of mitochondria; sperm with a higher number of mitochondria have a higher survivability (Rovan, 2001). At the caudal end of the mitochondrial sheath is the annulus that marks the barrier between midpiece and principle piece. The flagellar membrane is firmly adhered to the annulus which has a mechanical significance by preventing caudal displacement of the mitochondria during tail movement (Fawcett, 1975; Si and Okuno, 1993). The principal piece is the longest segment and is enclosed by fibrous sheaths made of two longitudinal columns, a dorsal and ventral one, and connecting ribs halfway around the tail (Fawcett, 1961; Eddy et al., 2003). The sheath ends 9-10 µm from the tip of the tail where the principal piece merges into the end piece (Pesch and Bergmann, 2006). The fibrous sheath also works as a scaffold for proteins in signaling pathways; therefore it might be involved in regulating sperm maturation, motility, capacitation, hyperactivation and AR (Eddy et al., 2003).

2.2.4 The importance of the structure and function of sperm parts

Sperm selection in the female genital tract is an extremely efficient and stringent process (Henkel, 2012 (a)), therefore spermatozoa are required to be functionally efficient. There are several levels of sperm selection in the female genital tract, namely at the cervix, uterus, uterotubal junction (UTJ), oviduct, cumulus oophorus and zona pellucida (Henkel, 2012 (a)). Spermatozoa are required to pass through these levels of selection in order to fertilize, therefore functional testing would prove whether spermatozoa are structurally and functionally fit.

To initiate fertilization, mammalian spermatozoa rely on the propulsive forces generated by their flagella to reach the site of fertilization in the oviduct and to penetrate the investments of the oocyte (Mortimer, 1997). The cytoskeletal compounds are responsible for flagellar motion and modulation of its form. The mitochondria provide energy (ATP) which is mostly used for motility (Afzelius, 1959; Gibbons and Grimstone, 1960; Gibbons, 1961, 1965). Dynein is a motor protein and has been shown to play a very important role in flagella organization and motility (Fossella et al., 1999). Lack of this protein is associated with immotility of the spermatozoa and is described as "immotile-cilia syndrome" (Afzelius, 1976). Only spermatozoa with intact membranes could undergo capacitation and acrosome reaction (Yanagimachi, 1981). Defects of the sperm head itself may concern size and form of the nucleus or chromatin condensation (Pesch and Bergmann, 2006). Cytoplasmic droplets are the most common defect at the neck region but can also be found at the midpiece and the principal piece of the tail. They represent a failure in maturation because the residual cytoplasm is released down the tail during spermiogenesis (Blom, 1950, 1973, 1983; Jasko et al., 1990). The consequence of tail defects are that spermatozoa are mostly immotile and unable to reach the ovum. Simple coiled or broken tails are among the most common sperm defects and double tails are also regularly observed (Koehler et al., 1998).

2.3. Sperm functional tests for evaluation of male fertility

Semen analysis is routinely used to evaluate the male partner in infertile couples as well as to assess the reproductive toxicity of environmental or therapeutic agents (Guzick et al., 2001). It has been referred to as an imperfect tool but remains the cornerstone of the investigation of male infertility (Vasan, 2011). Semen analysis must also be performed at a consistently high level standard in order to evaluate descriptive parameters of the ejaculate (Vasan, 2011). Even though a standard semen analysis provides useful information for the initial evaluation of infertile men, it is not a test of fertility (Jequier, 2010). Furthermore, the assay provides no insights into the functional potential of the spermatozoon to fertilize an ovum or to undergo the maturation processses required to achieve fertilization (Smith et al., 1977; Guzick et al., 2001; Brazil, 2010), which is why it is defined as an imperfect tool. Henkel (2015) mentioned that the results of a standard semen analysis have to be used with caution as it does not necessarily predict the outcome of assisted reproduction treatment (Bonde et al., 1998; Guzick et al., 2001). Reason being, the fertilization process in itself is multifactorial and can therefore be limited by numerous sperm parameters (Amann and Hammerstedt, 1993; Henkel et al., 2005). The quality of ejaculates and the functional parameters of the male

germ cell vary on a daily basis and do not necessarily reflect the situation on the day of insemination in an assisted reproduction program (Henkel et al., 2005).

Standard semen analysis characteristics can be classified into three groups, namely macroscopic, microscopic and physiologic parameters (Agarwal et al., 2008 (a)). The five macroscopic parameters include: pH, coagulation/liquefaction, colour, viscosity and volume (Agarwal et al., 2008 (a)). Microscopic examation in essence assesses spermatogenesis (Agarwal et al., 2008 (a)) and it involves evaluation of sperm agglutination, concentration and morphology. Lastly, physiologic variables include the examination of sperm motility and viability (Agarwal et al., 2008 (a)).

The WHO (2010) has recommended revised evidence-based reference values for measuring human semen parameters to allow decisions to be made about patient management and thresholds for clinical trials or investigations (WHO, 2010). These values were obtained from 1953 men in eight countries whom became fathers with a time to pregnancy of less than 12 months (Franken and Oehninger, 2012). Therefore this is a population of fertile men whose partners were of high or normal fecundity and where pregnancy was established in less than 12 months (Franken and Oehninger, 2012). The lower reference limits for standard semen characteristics according to the WHO (2010) are as follows: volume ≥1.5ml, pH ≥7.2, total sperm number >39x10^6/ejaculate, sperm concentration >15x10^6/ml, total motility >40%, progressive motility >32%, normal sperm morphology ≥4% and vitality ≥58% (WHO, 2010).

Although these widely used thresholds for normal semen measurements have been published by the WHO for routine semen analysis, the incorporation of functional tests to further evaluate sperm and potentially determine fertility is vital since, as previously mentioned, men can have normal semen parameters but still produce dysfunctional sperm. However, our knowledge of the cellular and biochemical basis of sperm dysfunction is still limited (Franken and Oehninger, 2012).

According to Vasan (2011), clinicians are still searching for semen parameter thresholds in the normal fertile populations to be able to define fertility, subfertility, and infertility more accurately. If sperm abnormalities are observed during standard semen analysis or if the couple is diagnosed with “unexplained” (idiopathic) fertility, the workup should proceed to incorporate sperm functional tests (Vasan, 2011). According to Lamb (2010), the real strength of sperm functional tests lies in its ability to identify men with normal semen parameters but who have functionally deficient sperm that will fail to fertilize in routine IVF.

With the advent of ART, sperm separation strategies from seminal plasma were developed (Henkel and Schill, 2003; Henkel, 2012 (b)). In the early years, the focus of these separation techniques was on obtaining motile

spermatozoa, but in the later years the focus shifted to the isolation of functional spermatozoa (Franken and Henkel, 2010). However, the conventional sperm separation techniques have shown distinct limitations in that they do not necessarily select spermatozoa according to their functional competence or genetic quality as it is achieved in the female genital tract (Henkel, 2012 (a)). Therefore scientists and clinicians are increasingly urged to improve sperm separation techniques in order to select the most functional spermatozoa for fertilization (Henkel, 2012 (a)). Hence, further proving the strength of sperm functional tests.

Many advances have been made to evaluate male fertility and potentially diagnose and provide treatment. However, it is essential that an accurate diagnosis is made to determine the appropriate course of treatment (University of Utah, 2014). Therefore, as previously mentioned, due to the fact that the standard semen analysis is incomplete and does neither provide sufficient information about the functional capacity of the male germ cell, nor shows low variability of the individual parameters (sperm count or motility), scientists were urged to find other solutions to the problem of accurately prediciting male fertility (Henkel, 2012 (a)). Sperm functional tests allow for further screening of sperm abnormalities and may give an indication of the suited treatment. Sperm functional testing can for instance indicate that less expensive technologies (such as fertility drugs or opting for a low-cost IVF programme) may assist a couple seeking to conceive, therefore ART such as ICSI or IVF may not always be necessary (Lamb, 2010). In essence these tests allow for couples to make more informed decisions.

Sperm functional tests which may be employed in a clinical setting include:

- *Sperm-mucus interaction*, also known as the postcoital test (PCT), assesses the cervical environment as a cause of infertility (Agarwal et al., 2008 (a)). The test must be conducted when the cervical mucus is thin and clear just before ovulation. The mucus is examined 2-8 hours after normal intercourse. The presence of more than 10-20 spermatozoa per 400 high-power fields, the majority of which demonstrates progressive motility, is usually considered normal (Vasan, 2011). The finding of immobilized spermatozoa with a side-to-side shaking motion suggests the presence of antisperm antibodies either on the spermatozoa or in the cervical mucus (Vasan, 2011). The Practical guidelines of American Society of Reproductive Medicine (ASRM) (2004) recommends the test in the setting of hyperviscous semen, unexplained fertility or low volume semen with normal sperm count (Agarwal et al., 2008 (a)). The test may be useful in patients who are unable to produce an ejaculate or are unwilling because of religious proscriptions (Agarwal et al., 2008 (a)).

- *Acrosome reaction* evaluates whether, after capacitation, spermatozoa will be able to fuse with the ova's plasma membrane and release acrosomal enzymes that will allow sperm penetration and fertilization (Agarwal et al., 2008 (a)). Determining the ability of the acrosome to react is important in the diagnostic and therapeutic approach to couples undergoing conventional ART treatment (Franken and Oehninger, 2012). Therefore, the test may be recommended in cases of profound abnormalities of head morphology or in the setting of unexplained fertility in patients with poor IVF pregnancy rates (Agarwal et al., 2008 (a)). While transmission electron microscopy is the procedure of choice, it is, however, labour-intensive and expensive, and as a result other techniques including fluorescence microscopy and beads coated with antiacrosomal antibodies have also been developed (Agarwal et al., 2008 (a)).
- *Sperm capacitation* is a collection or series of biochemical and structural changes in sperm function in order to undergo acrosome reaction and be able to fertilize (Lamb, 2010; Vasan, 2011). These changes occur in the female genital tract but can be induced *in vitro* by incubating spermatozoa with capacitation-inducing media (Vasan, 2011). It is thought to have a role in preventing the release of lytic enzymes until spermatozoa reach the oocyte (Tesarik, 1989). There is a change in the membrane permeability to calcium ions, which induce a hyperactive motility of the sperm, a process that is thought to aid in penentration of the cumulus and zona pellucida (Lamb, 2010). Hence, one of the signs of capacitation is the display of hyperactivation by spermatozoa (Vasan, 2011). Thus far, the assays developed to directly assess capacitation defects have only limited utility (Lamb, 2010).
- *Sperm penetration assay (SPA), sperm capacitation index (SCI)* (can be induced *in vitro* by incubating spermatozoa with capacitation-inducing media) *or zona-free hamster oocyte penetration assay* yields information regarding the fertilizing capacity of human spermatozoa by testing capacitation, acrosome reaction, sperm/oolemma fusion, sperm incorporation into the ooplasm, and the decondensation of the sperm chromatin during the process (Vasan, 2011). SPA uses zona-free hamster oocytes to measure fertilization capability, where the zona pellucida is stripped to allow cross species fertilization (Agarwal et al., 2008 (a)). SCI assesses the mean number of penetrations per ovum. It has been suggested that ICSI should be offered to couples with a SCI less than 5 instead of doing standard IVF procedures (Ombelet et al., 1997). However, the need for human oocyte supply remains a limitation to the use of this test (Agarwal et al., 2008 (a)).
- *Hemizona and zona pellucida binding* evaluates the interaction between spermatozoa and the zona pellucida, as it is a critical event leading to fertilization and reflects multiple sperm functions (i.e. completion of capacitation as manifested by the ability to bind to the zona pellucida and to undergo

ligand-induced acrosome reaction) (Oehninger et al., 1994; ESHRE, 1996; Liu and Baker, 2003). The most common sperm-zona pellucida binding tests utilized are the hemizona assay (HZA) and a competitive intact-zona binding assay (Fenichel et al., 1991). The HZA uses nonfertilized oocytes and is useful in couples who have failed to fertilize during regular IVF, to determine the cause of the failure (Vasan, 2011). Both bioassays have the advantage of providing a functional homologous test for spermatozoa binding to the zona pellucida, comparing populations of fertile and infertile spermatozoa in the same assay (Franken and Oehninger, 2012), however it is no longer widely offered (Lamb, 2010). The significant factors affecting the validity of both assays include oocyte sources and maturation, inter-assay and intra-assay variability, sperm motility, morphology and acrosomal status (Franken and Oehninger, 2012).

- *Hyaluronic acid (HA) (hyaluronan) binding*- evaluates the spermatozoon's ability to bind to this glycosaminoglycan secreted by the cumulus mass (Kim et al., 2008) which spermatozoa penetrate before reaching the oocyte (Henkel, 2015). Mature human sperm membranes expose HA receptors (Ranganathan et al., 1994) and reports have revealed that the percentage of sperm bound to HA reflects maturational status and function (Huszar et al., 2003). This appears as an essential step in the fertilization process (Henkel, 2015) and this observation can be used for fertility diagnosis as well as for the selection of functional spermatozoa for ICSI (Huszar et al., 2007). To date, there are two methods for HA binding, the picked spermatozoa for ICSI (PICSI) dish (hyaluronan-coated chamber) and a hyaluronan-containing medium (Henkel, 2012). Although sperm bound to HA have lower DNA damage and better chromatin condensation, it has been found that the sperm-hyaluronan binding has no predictive value in a clinical test because the assay failed to predict fertilization, pregnancy and baby take-home rate after IVF and ICSI (Ye et al., 2006; Nijs et al., 2009).
- *Sperm DNA damage* is multifactorial and theories on its etiology include protamine deficiency and mutations that may affect DNA packaging or compaction during spermiogenesis (Agarwal and Said, 2003; Erenpreiss et al., 2006; Zini and Libman, 2006). The factors associated with increased DNA damage are tobacco use, chemotherapy, testicular carcinoma and other systemic cancers (Agarwal et al., 2008 (a)). DNA damage is positively correlated with poor semen parameters (low concentration and low sperm motility), leukocytospermia and high reactive oxygen species (ROS) levels (Agarwal and Said, 2003; Erenpreiss et al., 2002; Trisini et al., 2004; Zini and Libman, 2006). In general, significant DNA damage is rarely found in a proven fertile male, and the incidence of DNA damage is higher in infertile men (Lamb, 2010). A variety of different tests are available and are divided into direct and indirect tests. The direct tests include single cell electrophoresis, Comet assay, TUNEL

(terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling) assay (Agarwal et al., 2008 (a)) and DNA oxidation measurement (Vasan, 2011). The indirect tests include sperm chromatine structure assay (SCSA), which measures sperm chromatin integrity (Agarwal et al., 2008 (a)), sperm chromatin dispersion assay, sperm fluorescence *in situ* hybridization analysis (FISH) (Vasan, 2011) and DNA intercalating dyes (acridine orange) that differentiate single and double stranded DNA (Agarwal et al., 2008 (a)). Although each test measures DNA damage differently, all generally correlate well with each other (with the exception of the acridine orange staining test) and they also generally inversely correlate with sperm concentration (Lamb, 2010).

- *Reactive oxygen species (ROS)* in small amounts are normal and in fact necessary for hyperactivation and capacitation of spermatozoa. However, in large amounts, it causes spermatozoal damage by lipid peroxidation of the plasma membrane, germ cell apoptosis and DNA strand breakage (Agarwal and Said, 2003). Measurement of ROS is done by several methods, the most common being the measuring of cellular probes coupled with flow cytometry by detection of chemiluminescence (Vasan, 2011). This is briefly done by incubating semen or suspensions with a redox-sensitive, light-emitting probe and measuring light emission over time with a light meter (Vasan, 2011). The indirect methods of measurement include the Endtz test, redox potential (reduced glutathione/oxidized glutathione-GSH/GSSG), measurement of lipid peroxidation product levels, chemokines, measurement of oxidative DNA damage, and measurement of reactive nitrogen species by Greiss reaction and fluorescence spectroscopy (Agarwal et al., 2008 (b)). Leukocytospermia also is associated with increased ROS levels and can serve as an indirect measurment of ROS (Saleh et al., 2002).
- *Sperm proteomics* may identify molecular targets implicated in sperm dysfunction (Aitken and Baker, 2008) and it allows for comparison of protein structure of normal and defective sperm (Aitken, 2010). This noninvasive technique not only provides the potential to detect causes of infertility, but may play a role in the development of male contraception (Chu et al., 2006). Seminal fluid has been found to have 923 proteins and at least 20 proteins have altered expressions in infertile men (Agarwal et al., 2008 (a)). Seminal plasma proteins have multiple origins as the seminal fluid is composed of secretions from the testis, seminal vesicles, prostate and the bulbo-urethral glands (Krause and Rothauge, 1991). Therefore, seminal plasma markers might rather reflect pathologies of the respective accessory glands which can also contribute or be a cause of male infertility (Henkel, 2015). While the proteomic analysis of seminal plasma is a good approach for andrological diagnostics as it is non-consumptive of spermatozoa, the methodolgy is still in its infancy and specific marker proteins still have to be validated for their use (Henkel, 2015). The analysis of sperm cells, however, might

give a better idea of the actual fertilizing potential of spermatozoa from a specific male. In contrast to the analysis of seminal plasma, the analysis of spermatozoa is more difficult and might be limited for various reasons (Henkel, 2015). For instance, the protein concentrations for spermatozoa is much less than for seminal plasma but the number of spermatozoa available varies individually and might not even reach the detection limit if the seminal sperm count is very low (Henkel, 2015).

- C*omputer-aided sperm analysis (CASA)* is useful because of its capacity to analyze sperm motion (kinematics of sperm motility), as manual semen analysis lacks the ability to do so (Vasan, 2011). Sperm kinematic parameters include: curvilinear velocity (VCL) (µm/s), straight-line velocity (VSL) (µm/s), average path velocity (VAP) (µm/s), amplitude of lateral head displacement (ALH) (µm), linearity (LIN) (%), wobble (WOB) (%), straightness (STR) (%) and beat-cross frequency (BCF) (Hz) (WHO, 2010). CASA has two main advantages: it is a semiautomated technique that provides both high precision and quantitative assessment of these sperm kinematics (Agarwal et al., 2008 (a)). CASA can accurately be applied in semen analysis (concentration of spermatozoa), washed sperm preparation anaylsis (sperm concentration and motility classes) and sperm fertilizing ability (evaluation of hyperactivation, an essential capability for fertilization, both *in vivo* and *in vitro*) (Mortimer et al., 2015). Many factors may affect the performance of CASA instruments, eg. sample preparation, frame rate, sperm concentration and counting-chamber depth (Davis and Katz, 1992; Mortimer, 1994 (a,b); Kraemer et al., 1998). In using CASA to obtain movement parameters, at least 200 motile spermatozoa per specimen should be analysed and secondly, the system should be linked to computer software that permits data organization and statistical analysis (WHO, 2010).
- *Mitochondrial membrane potential* evaluates the functionality of sperm mitochondria by determining the inner mitochondrial membrane potential ($\Delta\Psi_M$) (Henkel, 2015). The $\Delta\Psi_M$ has been described as a sensitive indicator of mitochondrial function in terms of the functionality of the mitochondrial electron transfer chain (Ly et al., 2003). Several cationic lipophilic dyes have been used to determine the $\Delta\Psi_M$, such as rhodamine 123 (Rh123), however mitochondria have several binding sites for this dye which renders this probe not very useful (Henkel, 2015). In contrast, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimiddasolyl-carbocyanine iodide (JC-1) was found to evaluate the $\Delta\Psi_M$ accurately (Salvioli et al., 1997) and specifically (Marchetti et al., 2004 (a)). A study by Marchetti et al. (2004 (b)) indicated a positive relationship between sperm $\Delta\Psi_M$ and the fertilization rate *in vitro* after IVF (Henkel, 2015). The test have been suggested as a sensitive parameter of sperm functional quality and therefore useful in diagnosis of male infertility and the prediction of fertilization in IVF (Kasai et al., 2002; Marchetti et al., 2002; Marchetti et al., 2004 (b);Marchetti et al., 2012). However,

a study conducted by Zorn et al. (2012) compared sperm parameters (including DNA damage and $\Delta\Psi_M$) and found that DNA damage is a better predictor of the occurrence of natural pregnancy than the other parameters investigated (Henkel, 2015). Therefore, more work is needed to evaluate, standardize and validate this functional parameter ($\Delta\Psi_M$) of spermatozoa (Henkel, 2015).

- *Motile sperm organelle morphological examination (MSOME)* is a method that evaluates sperm morphology at higher, digital magnification (X6300) using Nomarski interference contrast microscopy (Bartov et al., 2002). It examines the morphological status of the acrosome, post-acrosomal lamina, neck, mitochondria, flagellum and sperm nucleus, as well as the observation of the shape, presence and size of vacuoles (Henkel, 2012). MSOME identifies objects undetectable by light microscopy and is therefore a much more stringent method than the evaluation of sperm morphology according to strict criteria (Oliveira et al., 2009 (a)). MSOME is also thought to identify good quality spermatozoa and has therefore been included in ICSI protocols in an increasing number of groups (intracytoplasmic morphologically selected sperm injection: IMSI) (Henkel, 2015). Although the methodology is appealing because it is non-consumptive, the procedure for diagnostic (MSOME) and treatment (IMSI) purposes is time consuming and of little practical value for routine semen testing as MSOME has not been properly validated yet (Henkel, 2015).
- *Birefringence* involves the evaluation of live sperm cells via polarization microscopy (Henkel, 2015). The birefringence (double refraction) of light caused by the ansiotropic properties of the compact texture of the sperm nucleus, acrosome and flagellum permits the evaluation of the organelle structure of the male germ cell (Henkel, 2012). Studies have shown a strong relationship between partial birefringence and acrosome reaction (Magli et al., 2012), yet the patterns of it, total or partial, depends to some extend on motility and normal sperm morphology (Henkel, 2015). A positive relationship between sperm cell birefringence and motility (Collodel et al., 2013), as well as the fertility index (Baccetti and Mirolli, 1994), has also been confirmed, and authors have concluded that polarization microscopy offers several advantages and should be considered in sperm analysis (Collodel et al., 2013). However, clinical evaluation of the technique, in terms of of the establishment of reliable cutoff values, has not been carried out yet (Henkel, 2015).

2.4. Nonhuman primates as research models in reproductive studies

Non-human primates (NHPs) have been identified as key models in human-related studies and NHPs are often used in research on male fertility/infertility, IVF or ART procedures, male contraception and

reproductive toxicology. With respect to infertility and in a biomedical research capacity (Wolf, 2009), NHPs are invaluable animal models due to their phylogenetic closeness to humans regarding reproductive physiology, endocrinology, phylogenetics and anatomy (Bornman et al., 1988; Dancet et al., 2011). Other characteristics of NHPs which make them better research models than rats and mice include their susceptibility to human infectious agents and their responses to experimentally induced diseases (Wolf, 2009).

Among different NHPs, the Old World Primates (e.g. the rhesus monkey, cynomolgus monkey and vervet monkey) generally represent a more relevant/applicable human model than the New World Primates (e.g. common marmoset, capuchin monkey and squirrel monkey) (van der Horst, 2005). The many species of primates most commonly used in repoductive studies include the rhesus monkey (*Macaca mulatta),* the cynomolgus monkey (*Macaca fascicularis)*, the African green or vervet monkey (*Chlorocebus aethiops*), the chacma baboon (*Papio ursinus*), the olive baboon (*Papio anubis*) and the common marmoset (*Callithrix marmoset*).

The rhesus monkey has been used in procedures such as ovarian stimulation, oocyte aspiration, IVF, ICSI, embryo culture and embryonic stem cell derivation (Dancet et al., 2011). The chacma baboon has also been found to be appropriate research models for human reproduction (Dancet et al., 2011), since they are closer to humans than macaques and are readily available as a pedigreed research resource that mirrors many complex disease traits found in humans (Simerly et al., 2010). The vervet monkeys are the most widely distributed of all African monkeys (Eley, 1992). The Primate Unit of the Medical Research Council of South Africa (SAMRC) has established a successful breeding program of vervet monkeys. In the unit, under captive conditions, these monkeys are used for research purposes, including male reproduction (Seier, 1986) and have become increasingly popular as a good model for this purpose (van der Horst, 2005).

Understanding the process of fertilization in humans is vital for the diagnosis and treatment of clinical infertility and birth defects, as well as for discovering our reproductive origins (Simerly et al., 2010). In order to better this understanding, fertilization processes in animals, particularly primates, have been investigated. A study by Hewitson and Schatten, (2002), using rhesus monkeys, showed that the cytoskeletal events during fertilization, by IVF and ICSI, are very similar to those in human fertilization.

In the area of ART, the need for primate models is increasing due to ethical constraints of reproductive research in humans and to increased public awareness about the safety of new developments in reproductive technology (D'Hooghe et al., 2004). Since the development of human ART, clinically discarded specimens have been donated by anonymous infertile patient couples for investigations on the process of

human fertilization. These studies have generated important knowledge, however, questions still remain regarding the normalcy of the human material investigated, since it was developmentally compromised and anonymously donated. To address these concerns and to verify accuracy, NHP studies using Old World macaques were subsequently conducted (Simerly et al., 2010).

Hormonal, immunological, mechanical and other diverse approaches have been used in NHPs to test their suitability as models for human male contraception (van der Horst, 2005). A study by McCauley et al. (2002) displayed sperm agglutination antigen-1 (SAGA-1) is a good candidate contraceptive immunogen. However, when testing primate and human spermatozoa, this immunogen compromises fertility in the chimpanzee and human but not in bonnet monkeys, macaques and the baboon (McCauley et al., 2002).

An overview of primate sperm physiology is essential before considering the applications of artificial insemination (AI) and ARTs in NHPs (Wolf, 2009). A variety of parameters are routinely evaluated in NHPs for male fertility, most of which correspond to clinical endpoints examined in humans. These parameters include testicular volume, ejaculate weight, sperm characteristics (including motility, morphology and sperm count), serum testosterone and histopathology of male reproductive organs such as testis and epididymis (Faqi, 2012). According to a study by Wolf (2009), the sperm population in rhesus monkeys is remarkably uniform in motility and morphology. The conventional semen parameters used to estimate fertility potential in rhesus macaques includes semen volume, sperm concentration, motility and morphology (Wolf, 2009).

Few studies have reported on computer aided sperm motility analysis (CASMA) in non-human primates (van der Horst, 2005). Most studies have used a subjective rating of sperm motility which may vary between laboratories. A definition of the normal quantitative motile status of NHP sperm will provide a baseline for sperm motility as well as assissting in establishing cut-off points of fertility assessment (van der Horst, 2005).

Since male infertility in humans has increased in the last few decades and could be as high as 40%, with up to 50% of these men have “unexplained” (idiopathic) infertility, it has become essential to determine alternative causes of human infertility (Agarwal et al., 2008; Maya, 2010; Mayorga-Torres et al., 2016; WHO, 2010). Therefore the use of functional tests during evaluation of male function could be helpful in the evaluation of infertile couples (Mayorga-Torres et al., 2016). As mentioned before, many physiological, environmental (Aitken et al., 2011) and lifestyle factors such as paternal age (Singh et al., 2003; Schmid et al., 2007), diabetes (Agbaje et al., 2008), obesity (Chavarro et al., 2010), radiotherapy (Smit et al., 2010), chemotherapy (O’Flaherty et al., 2010), cigarette smoke, radiofrequency electromagnetic radiation and alcohol (Aitken et al., 2009; De luliis et al., 2009; Pacey, 2010) could be contributing to poor semen quality

and sperm DNA damage in humans (Aitken et al., 2011). Future studies aiming to investigate the effect of these factors on human male reproduction will necessitate the use of NHPs as research models.

However, comparing results of NHP and human studies require that techniques used for assessment must be objective, standardized and sensitive to recognize compromised sperm function. Therefore, one of the main aims of the present study was to evaluate sperm functional tests and develop new techniques using NHP spermatozoa for application in human and non-human primate studies. This study will potentially standardize protocols for use in future studies to ultimately develop a basic primate model and to possibly explain the high infertility rate in humans.

2.5. Non-human primate species of focus

The focus species for this study were the vervet monkey *(Chlorocebus aethiops)*, also known as the African green monkey, the chacma baboon (*Papio ursinus*) and the rhesus monkey (*Macaca mulatta)*.

2.5.1 Vervet monkey

The African green monkey is the one of the most widely distributed non-human primates of all African monkeys (van der Horst et al., 2004). They are found from southern Ethiopia and Somalia to the Cape region of South Africa. It is also one of very few non-human primates that is successfully bred under controlled captive conditions in the Primate Unit of the SAMRC (van der Horst et al., 2004).

Although the use of macaques has become popular in many areas of research, including human reproduction, there are several factors, such as the high costs of buying and shipping these monkeys from their country of origin, which limits their use in many developing countries (van der Horst et al., 2004). The vervet monkey has therefore emerged as an alternative animal model in research on human reproduction. This species' basic sperm characteristics have been extensively defined in previous studies (van der Horst et al., 2004). Unlike macaques, vervet females are believed to maintain a menstrual cycle year-round and have a straight cervix (Sparman et al., 2007). These characteristics can be advantageous for embryology and embryonic stem cell research, and a greater understanding of early pregnancy in this species could expand options for new investigators to the field of primate reproduction (Bondarenko et al., 2009). The male vervets have also been reported to possess human-like sperm characteristics (e.g. concentration, motility, pH, and acrosomal integrity) (Sparman et al., 2007).

According to previous studies, sperm concentration of vervet monkeys (280.50X10^6/ml) is closer to that of humans than other primate species, i.e. 419.43X10^6/ml for macaques (Harrison, 1980) and 2,56X10^6/ml for chimpanzees (Gould et al., 1993). The same observation is noted when evaluating motility, where the motion parameters of vervet monkey spermatozoa (VCL = 110.69 µm/s, VSL = 75.52 µm /s, LIN = 58.92%) (Mdhluli et al., 2004) are closer to those of humans (VCL = 84 µm/s, VSL = 77.8 µm/s, LIN =87 %) (Morales et al., 1988), than chimpanzees (VCL = 50 µm/s, VSL = 19 µm/s, LIN = 39 %) (Gould et al., 1993). According to the study by Maree and van der Horst (2012), sperm motility of six species were investigated and the following values were obtained when assessing vervet monkey spermatozoa: motility 94.7 ± 4.7%, VCL 328.8 ± 18.6 µm/s, VSL 293.0 ± 24.4 µm/s, VAP 315.4 ± 20.6 µm/s, LIN 89.0 ± 4.4%, STR 92.8 ± 3.0%, WOB 95.9 ± 1.8%, ALH 3.0 ± 0.2 µm and BCF 13.9 ± 1.2 Hz (Maree and van der Horst, 2012).

Mdhluli et al. (2004) also investigated sperm morphology and reported that vervet monkey spermatozoa, like in other non-human primate species, have fewer head defects compared to humans. Their semen pH evaluations revealed that vervet semen are also within normal human pH ranges of 7.2–8.0. The acrosomal staining procedure used in this previous study demonstrated that a finite number of fluorescence patterns occur in vervet monkey spermatozoa, as in humans (Mdhluli et al., 2004). Therefore, it is speculated that the distribution of carbohydrate moieties on the surface of the spermatozoa, responsible for recognition of Peanut agglutinin (PNA) lectin, is similar between human and vervet monkey spermatozoa (Mdhluli et al., 2004). Their results showed an abundance of spermatozoa with intact outer acrosomal membranes, which suggest that vervet monkey spermatozoa placed in the capacitating medium did not readily undergo spontaneous acrosomal loss *in vitro* (Mdhluli et al., 2004).

2.5.2 Baboons

The diversity and duration of field research on wild baboon populations, at sites from Ethiopia through to South Africa, is unparalleled among mammals. The baboon population of Amboseli, Kenya, is the subject of particularly intensive and diverse longterm investigations (Alberts and Altmann, 2004).

Baboons share important evolved characteristics with humans and like humans, baboons have adapted to a very wide range of environments, from near desert to temperate montane grasslands to moist evergreen forest. They therefore have achieved a nearly continental distribution in Africa (Alberts and Altmann, 2004). Since both these species have adapted to diverse habitats, they have broken free of the seasonal constraints of their habitats in major aspects of their life histories (Alberts and Altmann, 2004). As a result, baboons similar to humans, but unlike the large majority of primate species, show virtually no seasonality in

reproduction. Finally, baboons, like humans, exhibit a highly flexible social system, which plays a key role in their adaptability (Alberts and Altmann, 2004).

The only species of baboon studied in any detail and reported in literature is the olive baboon (*Papio anubis*). However, the chacma baboon (*P. ursinus*) is widely distributed in Southern Africa, from Southern Angola and Zambia in the north to the southernmost regions of South Africa (Bornman et al., 1988). Animals in troops of up to 100 raid crops and orchards, particularly during dry seasons and droughts. These animals are either shot or captured in efforts to control their numbers. This species is also a more robust animal than most other primate species of Central Africa (Bornman et al., 1988).

As part of the development of a baboon model for ART, it is essential to document baboon spermatology based on a sufficient number of animals and to assess reproducibility of semen volume, pH concentration, motility and morphology in multiple sample from the same baboon (Nyacheio et al., 2012). Electroejaculation is one of the most commonly used methods to obtain spermatozoa from baboons, similar to squirrel monkeys, rhesus monkeys and marmosets (Nyacheio et al., 2012).

The reference values (mean ± SD) for semen parameters in the chacma baboons, according to a study by Bornman et al. (1988), were found to be 204.8 ± 103.9 x10^6/ml for sperm concentration, 60.9 ± 7.7% for motility, 4.2 ± 0.5 for forward progression, 65.5 ± 6.1% for live sperm count and 50.0 ± 12.5% for morphologically normal sperm. Another study conducted by Maree and van der Horst (2012), indicated the following reference values when swim-up spermatozoa (highly motile) were assessed: motility 93.9 ± 2.9%, VCL 367.7 ± 45.5 µm/s, VSL 337.8 ± 40.2 µm/s, VAP 357.2 ± 44.2 µm/s, LIN 92.0 ± 3.6%, STR 94.7 ± 3.0%, WOB 97.2 ± 1.2%, ALH 3.4 ± 0.4 µm and BCF 15.4 ± 1.4 Hz (Maree and van der Horst, 2012).

2.5.3 Rhesus monkey

The rhesus monkey is an Old World Primate species native to eastern Afghanistan, Pakistan, India, Nepal, and parts of China. Rhesus and cynomolgous macaques are not endangered in the wild and adapt well to captive housing. These monkeys can live up to 40 years in captivity (Shidler and Lenon, 2007).

Macaques are genetically very similar to humans and share analogous neurological, reproductive and immunological systems with humans (Shidler and Lenon, 2007). Research with rhesus and cynomolgous monkeys, as well as with other NHPs, provide a great deal of information about primate biology since they can be better controlled and provide more consistent results than human studies, and are often precursors to human studies (Shidler and Lenon, 2007).

The rhesus macaque has been used extensively as a model for early human development as well as for fertilization and embryonic development in primates (Sparman et al., 2007). The processing of rhesus monkey semen for recovery of high quality spermatozoa has its basis in methods developed more than 30 years ago when IVF was first achieved for rhesus monkey oocytes (VandeVoort, 2004). Over these past 20-30 years, remarkable advancements in ART have been achieved using the rhesus monkey model (Sparman et al., 2007). Several of these accomplishments were described, including multiple follicle stimulation, oocyte fertilization by ICSI and oocyte/embryo manipulation (Sparman et al., 2007).

According to a study by Vandevoort (2004), macaque spermatozoa do not spontaneously capacitate and acquire the ability to bind to the zona pellucida, similar to humans and some other mammalian species (VandeVoort, 2004). This was critical to the development of methods for macaque IVF. A modification in macaque spermatozoa processing was hereby discovered in that cAMP and caffeine is required for sperm to acquire fertilizing ability. This method for capacitation is therefore often called “activation” (VandeVoort, 2004).

In a study by Hung et al. (2009), macaques were used as a NHP model to examine the effect of environmental tobacco smoke *in vivo* on semen quality, sperm function, and sperm metabolism. Motility was assessed by visual observation, however, the motion parameters were analysed with the use of CASA, a system also employed in the current study. Some of the functional tests included evaluation of acrosomal status, sperm viability, sperm-zona binding assessment and TUNEL analysis for apoptotic sperm detection (Hung et al., 2009).

A study by Maree and van der Horst (2012), investigated the cut-off values for sperm swimming speed to identify three subpopulations of spermatozoa. The macaques were one of the six species studied and the Sperm Class Analyzer (SCA) CASA system was used to measure sperm motility. The following values were obtained for sperm motility and kinematic parameters: motility 94.0 ± 5.5%, VCL 292.3 ± 19.2 µm/s, VSL 217.6 ± 34.3 µm/s, VAP 251.6 ± 27.9 µm/s, LIN 74.2 ± 9.3%, STR 86.1 ± 5.4%, WOB 85.9 ± 5.8%, ALH 4.0 ± 0.4 µm and BCF 17.3 ± 1.3 Hz (Maree and van der Horst, 2012).

2.6 Sperm functions for evaluation of non-human primate semen

The sperm functions selected for evaluation in this study are as follows: motility and longevity, vitality, DNA integrity, acrosome intactness and hyperactivation. All these sperm functions are required by spermatozoa in the female reproductive tract to reach and ultimately fertilize the oocyte.

2.6.1 Sperm motility

Sperm motility is described as the ability of spermatozoa to efficiently move towards an oocyte (Krishna, 2012). It is a reflection of the normal development of the flagellar axoneme and the maturation spermatozoa undergo within the epididymis (Agarwal et al., 2008 (a)). The efficient passage of spermatozoa through cervical mucus is dependent on rapid progressive motility (Keel and Webster, 1988), i.e. spermatozoa with a forward progression of at least 25 µm/s (for humans) (Mortimer, 1997; Hirano et al., 2001). Rapid progressive motility (i.e. >25 µm/s at 37°C and >20 µm/s at 20°C) is expressed as a percentage range (0-100%) and determined by the speed at which spermatozoa moves with flagellar movement in a given volume (Vasan, 2011). The motility of each semen sample is usually graded as follows: progressive motility (PR) - spermatozoa moving actively, either linearly or in a large circle, regardless of speed; non-progressive motility (NP) - all other patterns of motility with an absence of progression, e.g. swimming in small circles, the flagellar force hardly displacing the head, or when only a flagellar beat can be observed; and immotility (IM) – no sperm movement (WHO, 2010).

Reduced sperm motility can be a symptom of disorders related to male accessory sex gland secretion and the sequential emptying of these glands (Vasan, 2011). Other reasons for decreased sperm motility include male obesity (Palmer et al., 2012) and age, where motility has been found to decrease by 0.7% per year (Eskenazi et al., 2003). According to a previous study by Guzick et al. (2001), the measurement of motility provides useful information for diagnosing male infertility, along with morphology and sperm concentration. In most clinical set-ups a simple but subjective system for grading motility is recommended that distinguishes spermatozoa with progressive or non-progressive motility from those that are immotile.

There are severeal advantages for using detailed sperm motility analysis such as CASA and analysis of sperm subpopulations. The manual scoring method of the percentage of motile sperm in semen has been and still is the standard method for evaluating sperm motility. However, this scoring method has been subject to large inter-laboratory variation (Jørgensen et al., 1997). CASA has the advantage of increasing the accuracy and reproducibility of measurements of sperm concentration and percentage of motile sperm (Rijsselaere et al., 2005). The technology also allows for the objective analysis of sperm motility kinematics (Mortimer and Mortimer, 2013) and the detection of subtle changes in sperm motion that cannot be identified by conventional, manual sperm motility analysis (Abaigar et al., 2001; Agarwal et al., 2003; Rijsselaere et al., 2005).

CASA analysis of sperm subpopulations with particular patterns of motility has proven invaluable in research (Mortimer, 2000). Many investigators have used CASA to demonstrate the existence of sperm

subpopulations in various mammalian species such as pig, gazelle, horse and many more (Abaigar et al., 1999, 2001; Quintero-Moreno et al., 2003, 2004,; Martinez-Pastor et al., 2005; Nunez-Martinez et al., 2006; Quintero-Moreno et al., 2007). It is now recommended that sperm subpopulations be evaluated rather than relying on mean values for the entire sperm population, because mean values for motility oversimplify the analysis and decrease the usefulness of the data (Abaigar et al., 1999, Martinez-Pastor et al., 2005). The analysis of subpopulations can be used in human infertility diagnosis and pre-assisted conception workup to identify sperm subpopulations with either the appropriate kinematics to penetrate into cervical mucus or that show hyperactivated motility under capacitating conditions (Mortimer and Mortimer, 2005).

2.6.2 Longevity

The length of time spermatozoa are able to remain viable after ejaculation may be of concern in some cases (University of Utah, 2014). According to Denil et al. (1992), the ability of spermatozoa to maintain motility over time, termed motility longevity, may also be an important factor because insemination and ovulation may not coincide exactly (Denil et al., 1992). Therefore, shortened longevity may play an important role in failed fertilization when semen is deposited either naturally in the female tract or by IUI (Ohl and Menge, 1996). The measurement of spermatozoa to maintain motility in media *in vitro* is a simple assay, however, proper preparation and processing of the samples is essential to obtain a motile fraction free from seminal plasma (Ohl and Menge, 1996). According to a clinical study, some sperm may remain alive and fertile in the femal reproductive tract for up to five days (Wilcox et al., 1995). Although most information would be obtained by examining motility longevity directly in the female tract, there is no non-surgical method of obtaining a sample for observation (Ohl and Menge, 1996). Unfortunatley, no defined sperm longevity assessment assay thus has yet been established that could be amenable to routine use in a diagnostic andrology laboratory (Franco et al., 1994; Mortimer, 1994 (a); Monsour et al., 1995; Morimoto et al., 1997; Hossain et al., 2008). According to Himes (2007), sperm longevity has been the subject of many studies as an important factor in the fertilization success of marine broadcast spawners. Sperm are considered motile if they show any sign of movement, including swimming, wiggling back and forth, or movements of the tail or head. Immotile sperm do not show any sign of movement during the recording period (Himes, 2007).

2.6.3 Vitality

The vitality of spermatozoa is the percentage of sperm which is alive; it is not the same as the percentage of sperm which is motile, since non-motile sperm may or may not be alive (Unisonplus, 2013). Sperm vitality, as estimated by assessing the membrane integrity of the cells, may be determined routinely on all samples, but is especially important for samples with less than 40% progressively motile spermatozoa. This test can

also verify the motility evaluation, since the percentage of dead cells should not exceed (within sampling error) the percentage of immotile spermatozoa (WHO, 2010). When the motility is reported as less than 5-10%, viability testing is recommended, as profoundly low motility can result from dead spermatozoa or necrospermia (Agarwal et al., 2008 (a)). Fresh semen obtained from infertile men occasionally exhibits the total absence of motile sperm (100% asthenozoospermia). Vitality tests may reveal that a proportion of the immotile sperm has functional membranes, which are considered viable (Khalili et al., 1999). Men with severe type of teratozoospermia, such as acrosomeless globozoospermia, usually fail ICSI treatment. In addition, the injection of dead spermatozoon into an oocyte generally results in fertilization failure. Therefore, it is necessary to carefully evaluate the viability of the immotile spermatozoon before starting ICSI (Khalili et al., 1999).

There are various methods available for measuring sperm vitality, including membrane integrity and enzyme function. Two specific tests for membrane integrity include eosin-nigrosin staining (dead sperm stain pink and live sperm remain colourless/white) (Reactifs Ral, 2005), and the use of fluorescent DNA-binding supravital stains (Lybaert et al., 2009), for example the FluoVit kit (staining dead sperm red and live sperm blue) (Microptic, 2013). A test for enzyme function, and thus indirectly a cell's viability, involves the use of the WST-1 and XTT cytotoxicity assays. These assays measure the cleavage of tetrazolium salts by active enzymes, producing a formazan dye detectable only in metabolic active cells (Roche Diagnostics, 2006, 2011).

2.6.4 DNA Integrity

In order for a spermatozoon to be fertile, the chromatin must decondense correctly after fertilization (Halotech, 2014). The spermatozoal chromatin is a tightly packed structure due to disulfide cross linkages between protamines that allow compaction of the nuclear head, protecting DNA fragments from stress and breakage (Agarwal et al., 2008 (a)). Nuclear alterations, such as abnormal chromatin structure, micro-deletions in the chromosome, or DNA fragmentation, will all reduce the spermatozoon's ability to produce a viable embryo. Sperm DNA fragmentation (SDF) is thus inversely related to the spermatozoon's ability to fertlize. Numerous scientific publications have demonstrated that a SDF value that exceeds 30% suggests sub-par sperm quality (Halotech, 2014). Numerous causes for SDF exists, including: increased testicular temperature due to tight clothing; bouts of fever or varicocele; the presence of reactive oxygen species in the semen; the metabolic effects of being overweight such as high insulin levels; smoking; certain drugs such as certain serotonin re-uptake inhibitors; and age (Halotech, 2014).

Examples of methods that are used to evaluate DNA integrity include the Halosperm Kit and the terminaldeoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)- nick-end labelling

(TUNEL) assay. Halosperm is based on the sperm chromatin dispersion test (HalotechDNA, 2014) where the acidic treatment allows for the detection of denatured DNA which fluoresces red and non-denatured which fluoresces green. The TUNEL assay allows for single- and double-stranded breaks to be identified through the incorporation of labelled dUTP (WHO, 2010).

2.6.5 Acrosome reaction or intactness

The acrosome cap is a structure within the sperm head surrounding the upper 40 to 60% of the sperm nucleus (University of Utah, 2014). Mammalian fertilization involves capacitation of the spermatozoon and acrosome reaction, the latter process being a major event for sperm penetration through the zona pellucida and subsequent fusion with the egg membrane (Guraya, 2000). During the acrosome reaction, due to fusion of the plasma membrane and outer arosomal membrane, the acrosomal cap gets perforated and leads to the release of the acrosomal contents, e.g. hyaluronidase and acrosine, which help to dissolve the zona pellucida at the site of sperm entry (Mukhopadhyay et al., 2008).

The presence or absence of the acrosome may be assessed by several methods, including electron microscopy, conventional microscopy or phase contrast microscopy (Lybaert et al., 2009). The zona pellucida-induced acrosome reaction can be assessed on spermatozoa removed from the surface of the zona pellucida or exposed to disaggregated human zona pellucida proteins (WHO, 2010). According to a study by Huse et al. (1993), a triple-stain technique was used to investigate the acrosome reaction in infertile and fertile males. It was found that there was no significant difference in the ratio of acrosome-reacted spermatozoa between normozoospermic and fertile males. However, all the cases in the latter group had 15% or more acrosome-reacted sperm, therefore it would appear that a disturbance of the acrosome reaction is one of the causes of reduced fertility potential in normozoospermic males (Huse et al., 1993).

Tests available for evaluation of the acrosome reaction involve the treatment of spermatozoa with calcium ionophore (A 23187) which induces calcium influx and is one way of testing the competence of capacitated spermatozoa to undergo the acrosome reaction (Aitken et al., 1993 (a)). This assay is also called the acrosome reation after ionophore challenge (ARIC) test (WHO, 2010). Second, is the use of fluorescent probes, fluorescein-conjugated *Pisum sativum* (edible pea) agglutinin (FITC-PSA) and *Arachis hypogea* (peanut) agglutinin (FITC-PNA). The ionophore treatment allows for sperm to be assessed under phase contrast microscopy where acrosome reacted sperm would be without acrosome and intact sperm with a dark head (Mukhopadhyay et al., 2008). The fluorescent labelled plant lectins (Aviles et al., 1997), however, requires for the sperm to be viewed via fluorescence microscopy where intact acrosomes appear bright and reacted acrosomes appear dark. According to Lybaert et al. (2009), the FITC-PSA is observed to stain the

whole spermatozoa, the head and flagellum, and the FITC-PNA is restricted to the head and acrosomal cap (Lybaert et al., 2009).

2.6.6 Hyperactivation

Hyperactivation is characterized by the development of asymmetrical, high amplitude flagellar beats, causing vigorous and sometimes non-directed movement of free-swimming spermatozoa (Mortimer et al., 1998). However, the movement of hyperactivated sperm varies under different physical conditions and in different species (Suarez and Ho, 2003). Since hyperactivation is known to occur at or near the site of fertilization, several theories have been proposed for the functional significance of hyperactivated motility, including that it: provides a mechanism to reduce the chance of sperm entrapment in the oviductal crypts; allows detachment of bound spermatozoa from the oviductal epithelium; exists as a 'search' mechanism for the oocyte; stirs the ampullary fluid to ensure biochemical homogeneity; and confers the ability to traverse the cumulus matrix allowing the generation of sufficient thrust for the spermatozoon to penetrate the zona pellucida (Mortimer et al., 1998).

While the signal transduction cascade regulating hyperactivation remains to be completely described, it is clear that calcium ions interact with the axoneme of the flagellum to switch on hyperactivation. Although hyperactivation often occurs during the process of capacitation, the two events are regulated by somewhat different pathways (Suarez and Ho, 2003). According to Munire et al. (2004), impairment of hyperactivation might be related to infertility and the spermatozoa of infertile patients have a decreased capability to be hyperactivated, stressing the importance of hyperactivation in human fertility.

Ntanjana (2015) reported significant differences in percentage hyperactivation among three subpopulations of human spermatozoa, highlighting the fact that subpopulations should be considered in future during clinical assessment of male fertility, especially when assessing hyperactivation.

Hyperactivation may be induced chemically via stimulants, inducers or agonists. Many studies have demonstrated the use of chemicals such as procaine hydrochloride, pentoxifylline (Ortgies et al., 2012), caffeine (Colas, 2010), 4-aminopyridine (4-AP) and progesterone (Alasmari et al., 2013) for this purpose. Hyperactivation in human spermatozoa is detected as a change in swimming pattern, as an increase in amplitude, the star-shape or figure eight pattern, as well as with an increase in ALH, a decrease in linearity (LIN) and straightness (STR). Both naturally occurring hyperactivated motility and chemically induced hyperactivation may be evaluated. Human tubal fluid (HTF) modified as capacitating medium (addition of HEPES and increase in NaOH) may be used to induce natural hyperactivation (Mortimer, 1994(a)).

2.7 The Effect of Heavy Metals on Sperm Function

A study by Carlsen et al. (1992), indicated a significant deterioration in reproductive health of normal men in many countries in the preceeding 50 years. This prompted other researchers to evaluate their own data and several studies established an increasing incidence of various abnormalities of the human reproductive system such as decreased sperm count and increased incidence of testicular and prostate cancer (Adami et al., 1994; Carlsen et al., 1995; Zheng et al., 1997; Swan et al., 2000; Skakkebaek et al., 2001; Richiardi et al., 2004). These results triggered an interest in the possible causes of these reproductive abnormalities as well as factors that contribute to this deterioration, including environmental and lifestyle factors (Saradha and Mathu, 2006; Agarwal et al., 2008 (c); Phillips and Tanphaichitr, 2008; Jurewicz et al., 2009; Mendiola et al., 2009; Wong and Cheng, 2011).

Humans are exposed to various types of environmental contaminants at different stages of their life span and there has been a growing concern about the deleterious effects of chemicals on the developing male reproductive system (Puluputturi and Dayapulae, 2012). Exposure of heavy metals during pregnancy has been associated with adverse effects on development of gonads (Puluputturi and Dayapulae, 2012). These substances may act as testicular toxicants and correspond to different compounds, which are related to social habits, life conditions, working hazards or use of drugs and medicines (Johnson et al., 1970; Monsey and Arrondo, 1993; Bustos-Obregon, 2001). Many heavy metals are classical testicular toxicants, though the mechanism of their action may differ (Puluputturi and Dayapulae, 2012). Metals are present everywhere, in food, dietary supplements, water, air, alcoholic drinks and tobacco (Pizent et al., 2012). Cigarette smoke contains about 30 metals, of which cadmium, arsenic and lead are in the highest concentrations (ATSDR, 2008). Alcoholic beverages including wine can be contaminated with metals in concentrations exceeding the allowable limits (Tariba, 2011, Tariba et al., 2011 (a, b)).

Metals may effect the male reproductive system directly, when they target specific reproductive organs, or indirectly, when they act on the neuroendocrine system (Pizent et al., 2012). Metals can affect the testis size, the semen quality, the secretory function of the prostate and seminal vesicles, the reproductive endocrine function and can lead to the loss of fertility and libido or to impotence (Apostoli et al., 1998; Benoff et al., 2000; Apostoli et al., 2007; Wirth and Mijal, 2010; Apostoli and Catalani, 2011). Moreover, exposure to cadmium, lead, and inorganic arsenic may contribute to prostate cancer development (Chen et al., 1988; Chen and Wang, 1990; Goyer et al., 2004; Telisman et al., 2007; Benbrahim-Tallaa and Waalkes, 2008). Environmental exposure to cadmium and/or lead is associated with increased serum prostate specific antigen (PSA) (Zeng et al., 2004; Pizent et al., 2009).

The disruption of spermatogenesis in men at any stage of cell differentiation can decrease the total sperm count, increase the abnormal sperm count, impair the stability of sperm chromatin or damage sperm DNA (Mangelsdorf et al., 2003). Metals may impair progressive sperm motility by accumulating in the epididymis, prostate, vesicular seminalis or seminal fluid (Hess, 1998). Metals can also cause hormonal imbalance by affecting the neuroendocrine system, disrupting the secretion of androgens from Leydig cells or Inhibin B from Sertoli cells (Jensen et al., 2006).

Due to the adverse effects of metals on sperm quantity and quality, it was decided to use heavy metals in this study in order to verify the sperm functional tests after it was optimised for the NHPs. The metals considered for use were copper suplhate ($CuSO_4$) and cadmium chloride ($CdCl_2$). Cadmium is used in the production of nickel-cadmium batteries, pigments (bright yellow, orange, red and maroon dyes), ceramics, plastic stabilisers and fertilisers (Pizent et al., 2012). The action of cadmium is spermatogenic stage specific. A high dose of $CdCl_2$ exposure causes rapid testicular edema, haemorrhage and necrosis (Puluputturi and Dayapulae, 2012). Copper suplhate may be used as a fungicide and herbicide in both agricultural and non-agricultural settings, as well as an antimicrobial substance (National Pesticide Information Center, 2012). According to a study by Chen et al. (2011), $CuSO_4$ significantly reduced the motility of both moving and resting spermatozoa. The motility of the moving spermatozoa was strongly affected at low concentrations of $CuSO_4$ (10 µM, 30 µM) and the resting spermatozoa at high concentrations (60µM, 100µM) (Chen et al., 2011). Studies by Israel (2013) and Prag (2013), found that these two metals decreased sperm motility at concentrations of 250 µg/ml $CuSO_4$ and 500 µg/ml $CdCl_2$, after 5 hours of exposure. These specific concentrations were therefore considered for use for these two metals to observe a possible change or decrease in sperm function.

2.8 Conclusion

In order to address the large percentage of unexplained male infertility in humans, more detailed investigations using sperm functional tests are needed to identify possible causes for compromised fertility. Since many environmental and lifestyle factors might be contributing to infertility, future studies aiming to elucidate the effect of such factors on male fertility will need the use of appropriate research models. The current study involved an assessment of NHP sperm function in order to establish the possibility of using these species as primate models for reproductive studies. We hypothesise that by evaluating various established sperm functional tests and designing novel techniques for sperm analysis in primates, protocols

will be standardized for use in future studies on male infertility. This will thereby allow comparisons of human and NHP sperm function which may reveal or explain the high infertility rates in humans.

Chapter 3

Materials and Methods

3.1 Ethical Clearance

Ethical clearance for this study was obtained from the Ethics Committee of the University of the Western Cape (Ethical clearance number: 13/10/91) as well as the Ethics Committee for Research on Animals (ECRA) (Project number: 11/13).

3.2 Primate Housing and Care

The three nonhuman-primate species included in this study were: Vervet monkey (*Chlorocebus aethiops),* Chacma baboon (*Papio ursinus*) and Rhesus monkey (*Macaca mulatta*). The primates were housed at the Primate Unit and Delft Animal Centre, Medical Research Council (Cape Town, South Africa). The primates were housed in accordance with the revised South African National Standard for the Care and Use of Animals for Scientific Purposes (South African Bureau of Standards, SANS 10386). All males were identified with a number tattooed on the inner right thigh, as well as cage labelling.

3.2.1 Vervet monkey

Male vervet monkeys (Figure 3a) were selected from a captive-bred, indoor breeding colony, with a history of successful reproductive performance for two generations.

The environmental conditions were maintained at temperatures between 25-27 °C, a humidity of 45%, about 15-air changes/hour and a photoperiod of 12 hours (Seier, 1986). The monkeys were kept on a diet consisting of a stiff porridge (pre-cooked maize meal) enriched with vitamins and minerals, trace elements and a protein supplement. The diet was supplemented with seasonal fruit at noon, as it was reported that the diet supported good reproductive performance and breeding (Seier, 1986). Drinking water was available *ad libitum*. All individuals had regular access to exercise cages and environmental enrichment (foraging and food puzzles) (de Villiers, 2006).

3.2.2 Chacma baboon

Male baboons (Figure 3b) used in this study were originally wild caught and individually housed in single cages, with full visual, olfactory and auditory contact with one another. Grooming was made possible between adjacent individuals through panels of wired mesh to allow for tactile contact. The animals were

fed a standard diet of pelleted feed (Aquafeeds, Cape Town, South Africa) and seasonal fruit or vegetables. The diet was supplemented with bread slices covered with vitamin C syrup (Portfolio Pharmaceuticals, Johannesburg, South Africa). In addition, the baboons were provided with foraging logs three times per week. Drinking water was available *ad libitum* (de Villiers and Seier, 2010).

3.2.3 Rhesus monkey

Male rhesus monkeys (Figure 3c) ranged between the ages of 10-14 years and were selected from an outdoor breeding colony which consisted of 10 adult males. The breeding stock are of Chinese origin and the colony has a fifteen year history of successful second generation reproductive performance.

The males were housed indoors and paired with a compatible breeding female in double galvanised steel cages with temperatures ranging from 25-27 °C and 12-hour light/dark controls. The monkeys were kept on a standard diet of special monkey cubes (Equifeeds, Cape Town, South Africa) fed in the morning (250 g). The diet was supplemented with seasonal fruit or vegetables at noon and the afternoon diet included two slices of bread with added multivitamin syrup (Portfolio Pharmaceuticals, Johannesburg, South Africa). The animals were provided with foraging logs and various other enrichment devices on a daily basis. Drinking water was available *ad libitum*.

(Wildlife Pictures Online, 2016) (Tydon Safaris, 2016) (Monkeyland Primate Sanctuary, 2016)

Figure 3: Three selected primate species. a = vervet monkey, b = chacma baboon and c = rhesus monkey

3.3 Experimental Outline

In order to evaluate the functionality of spermatozoa from these primates, sperm functional tests were performed, including motility, longevity, vitality, DNA integrity, acrosome intactness and hyperactivation. The methods involved for each functional test are elaborated further in this chapter.

Once all functional tests were performed and optimized, a comparison study was made where the samples were treated with heavy metals (such as $CuSO_4$ and $CdCl_2$). The validity of each test was investigated, where the samples were analyzed once again with the functional tests, including positive and negative controls. This determined whether the functional tests were sensitive in detecting any change in functionality of the spermatozoa after treatment therefore allowing us to modulate the tests to develop a basic primate model. Figure 4 provides a basic outline of the experimental procedure of this study.

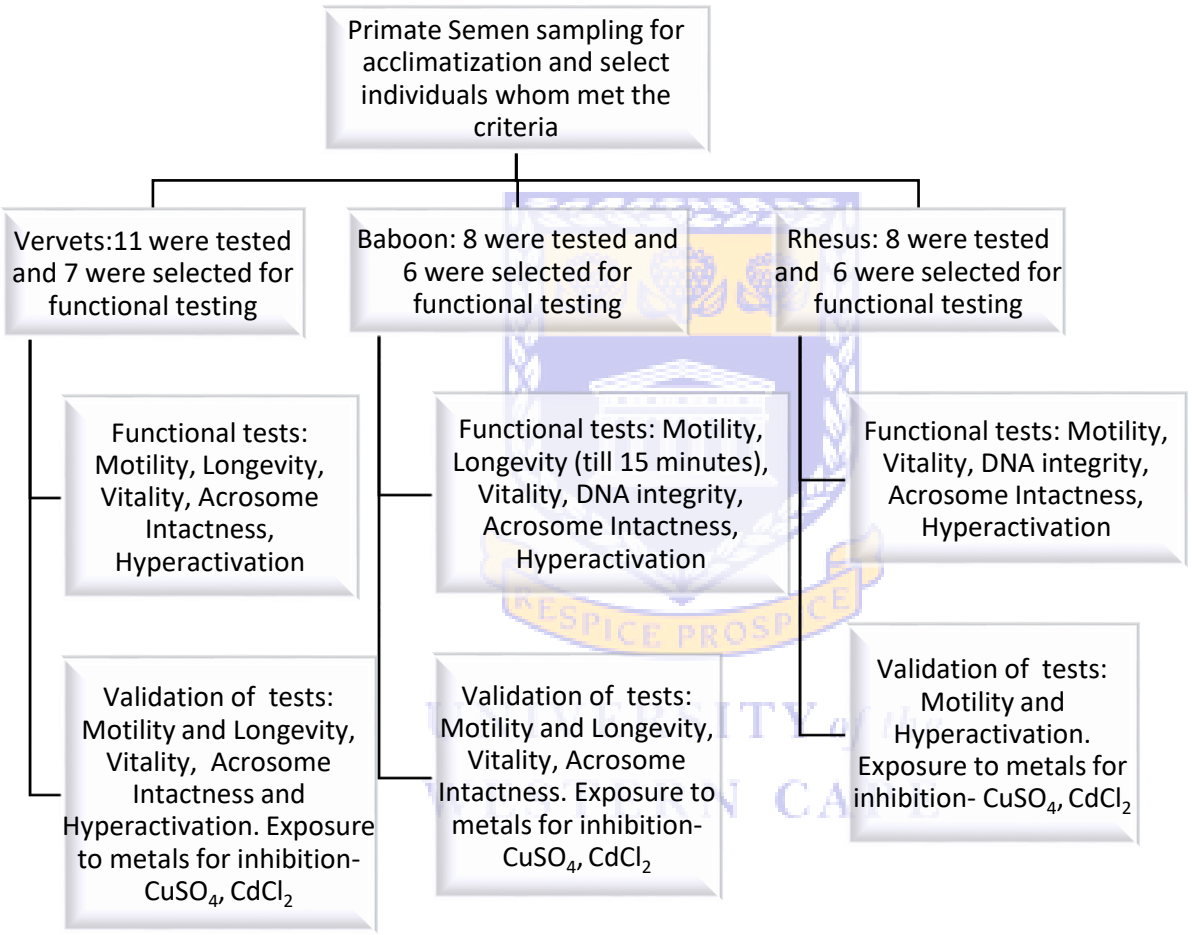


Figure 4: Outline of experimental procedure for each primate species and the functional tests applied

3.4 Acquisition of Semen Samples

Semen samples were collected from the three non-human primate species via rectal probe electro-ejaculation (Figure 5) into sterile, pre-warmed 15 ml or 50 ml plastic containers. A minimum of 20 semen samples were selected from each species based on the semen quantity and quality (volume > 350 µl, sperm concentration > 20 M/ml and percentage total motility > 50%). These cut-offs were selected due to

preliminary testing revealing these standards to be most beneficial for functional testing. The semen samples were kept in an incubator at 37 °C for the first 5 minutes after collection to allow the coagulum within the semen to liquefy. The sampling was performed on a weekly basis, two days a week with four primates per week (two primates per day). Each individual primate was thus sampled from once every two weeks to allow an even rotation among them.

After the initial incubation, semen samples were either used neat or the bottom fraction, generated by means of a density gradient centrifugation technique and containing the most motile spermatozoa, was used for functional testing.

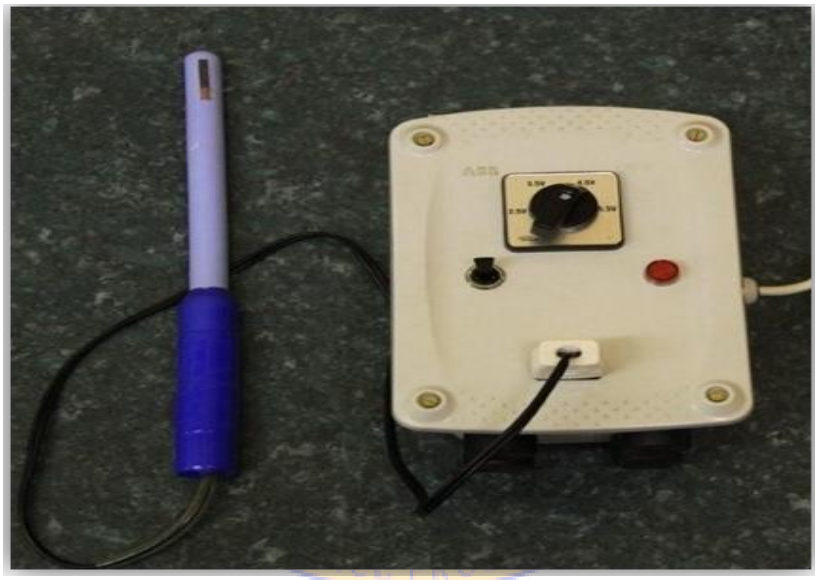


Figure 5: The rectal probe and transformer used for electro-ejaculation

3.5 Preparation of Samples

3.5.1 Density gradient centrifugation

PureSperm (PS) 40/80 (Nicadon, Molndal, Sweden) was used according to the manufacturer's instructions to form a two-layer density gradient preparation for the separation and purification of motile spermatozoa. The density gradient was prepared as follows:

A pipette was used to place 50-300 µl PS 80 in a centrifuge tube and an equal volume of PS 40 was layered on top. The range in volume of PS was due to the low volumes of semen sample obtained after electro-ejaculation of some animals, therefore requiring a large range. Thereafter, the same volume of semen was layered on top of the PS 40. This preparation was centrifuged at 300 *g* for 20 minutes. A pipette was used to aspirate all surface layers (supernatant) and the pellet was used for subsequent experiments.

The pellet was washed in 100 µl HTF and centrifuged at 500 *g* for 10 minutes. After centrifugation, the supernatant was removed and 100 µl HTF was added to the remaining pellet to dilute the sperm concentration. This pellet contained the most motile spermatozoa and this preparation was kept at a temperature of 37 °C before subsequent sperm functional tests were performed.

3.5.2 Swim-up (SU)

For the SU method, aliquots of semen were taken from the sample as soon as it liquefied and placed in tubes underneath a layer of culture medium (Mortimer, 1994 (a)). The medium selected for this method was Ham's F10 (Invitrogen|Thermo Fisher Scientific, Paisley, Scotland). A pipette was used to place 150 µl Ham's F10 in a centrifuge tube and 50 µl semen was carefully layered beneath it. The preparations were incubated for 30 minutes, where after the top layer, containing only motile spermatozoa, was extracted and used for subsequent sperm functional testing.

3.6 Functional Tests:

3.6.1 Sperm Motility

Sperm motility of the baboon and rhesus monkey samples was assessed using the Motility module of the Sperm Class Analyzer (SCA) (Microptic S.L., Barcelona, Spain) CASA system, Version 5.1, in combination with a Nikon Eclipse 50i Phase Contrast Microscope (IMP, Cape Town, South Africa) equipped with a heated stage. A Basler A602fc or Basler A312fc digital camera (Microptic S.L., Barcelona, Spain) was used for capturing data whilst mounted onto the microscope. For the vervet sperm motility analysis, the same CASA system was used in combination with an Olympus CH2 microscope (Wirsam, Cape Town, South Africa), equipped with a heated stage and mounted with a Basler aVA 1000-100gc digital camera (Microptic S.L., Barcelona, Spain).

The properties of the SCA system were set as follows for the three species: Frame rate: 50 images/sec; Optics: Ph- (negative phase contrast); Chamber: Leja 20; Species: Human; Particle area: no less than 5 and no more than 80 µm^2; VCL intervals: 5<slow<80<medium<120<rapid (µm/s); VAP points: 7 and Connectivity: 12.

Motility assessment involved aliquoting 2 µl of semen or sperm preparation into a pre-warmed (37 °C) 8 chamber Leja slide (Leja Products B.V., Nieuw-Vennep, The Netherlands) and analysed using the SCA system and microscope. The percentage total motile, progressive motile, rapid, medium and slow swimming sperm as well as eight kinematic parameters were assessed. The kinematic parameters included: Curvilinear velocity (VCL) (µm/s) - time-averaged velocity of a sperm head along its actual curvilinear path; Average path

velocity (VAP) (µm/s) - time-averaged velocity of a sperm head along its average path; Straight-line velocity (VSL) (µm/s) - time-averaged velocity of a sperm head along the straight line between its first detected position and its last; Linearity (LIN) - the linearity of a curvilinear path, VSL/VCL; Straightness (STR) (%) – the linearity of the average path, VSL/VAP; Wobble (WOB) (%) – a measure of oscillation of the actual path about the average, VAP/VCL; Beat-cross frequency (BCF) (Hz) – the average rate at which the curvilinear path crosses the average path; and Amplitude of Lateral Head Displacement (ALH) (µm) - magnitude of lateral displacement of a sperm head about its average path (WHO, 2010). Fields were captured randomly to avoid bias toward higher sperm motility, however, fields containing debris were disregarded. A total of at least 200 motile sperm were captured during each analysis.

3.6.2 Longevity

Motility was assessed over time until a substantial decrease was observed. The assessment of longevity was therefore incorporated at this point. Sperm motility was examined every 15 minutes until sperm were no longer moving (the baboon samples were, however, only analysed until 15 minutes because they were no longer motile after this time point). Sperm were considered motile if they show any sign of movement, including swimming, wiggling back and forth, or movements of the tail or head. Immotile sperm do not show any sign of movement during the recording period (Himes, 2007).

3.6.3 Vitality

Sperm vitality was assessed using three different techniques.

3.6.3.1 Eosin-Nigrosin Staining Technique:

The Eosin-Nigrosin (E-N) staining technique is used to assess the percentage of dead and alive spermatozoa. Due to their compromised plasma membrane, dead spermatozoa will allow eosin to enter the cell and are resultingly stained pink, while living spermatozoa remain colourless and appear white (Reactifs Ral, 2005). The nigrosin serves as a background stain to provide contrast.

Protocol: The staining solution wass preheated in an incubator at 37 °C before use. The ratio of sperm preparation to staining solution was 1:3.

1. A coverslip was placed on a heated stage (37 °C) and 30 µl stain was placed on it.
2. 10 µl sperm preparation was mixed with the heated solution for 30 seconds.

3. 10 µl mixture was placed on a heated slide and smeared.
4. The slides were left to air-dry overnight.
5. The slides were mounted with two drops of DPX mountant, a coverslip was added and left overnight.
6. The slides were examined with a Nikon Eclipse 50i microscope (IMP, Cape Town, South Africa) using a 40X objective.
7. At least 100 spermatozoa were counted and the percentage live and dead spermatozoa were calculated.

3.6.3.2 Hoechst and Propidium Iodide Staining Technique

The second method involved the use of two fluorescent dyes, namely Hoechst 33342 (trihydrochloride trihydrate) and Propidium Iodide (PI) (H&P) (Sigma, Cape Town, South Africa). Hoechst 33342 is a specific stain for AT-rich regions of double-stranded DNA and fluoresces with a blue colour. The Hoechst enters cell membranes and therefore stains both fixed and vital cells. PI was used as a nuclear counterstain and fluoresces with a red colour. PI cannot enter the cell membrane of vital cells and therefore only stains damaged or dead cells.

Protocol: Stock solutions were prepared before use (1 mg/ml in distilled water). Working solutions (1 mg/ml) were wrapped in aluminium foil and stored in the refrigerator at 4 °C protected from light, ready for use. Aliquots were stored in the freezer at -20 °C and were thawed when refrigerated solutions were completed.

1. Aliquots of the staining solutions were pre-heated in an incubator (37 °C) before use.
2. 10 µl sperm preparation was added to 1 µl Hoechst
3. The mixture was incubated for 5 minutes at 37 °C.
4. 1 µl PI was added to the mixture and incubated for 5 minutes 37 °C.
5. 10 µl of the mixture was placed on a slide, a coverslip was added and viewed under Nikon Eclipse 50i fluorescence microscope (IMP, Cape Town, South Africa) using a 20X objective, with a DAPI filter (Figure 6). A total of at least 100 sperm were captured and counted with the NIS Elements imaging software (IMP, Cape Town, South Africa). A final percentage was then determined of live (blue) and dead (red) sperm.

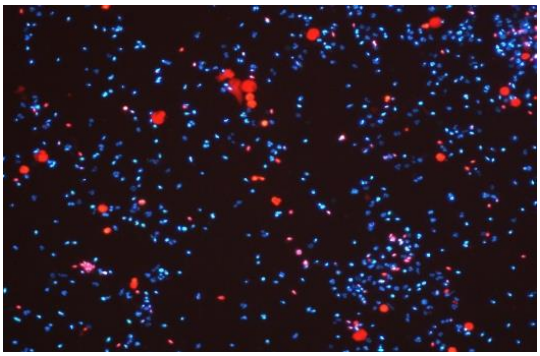
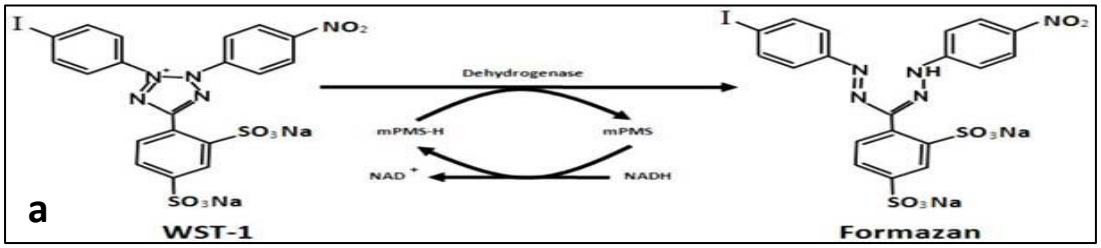


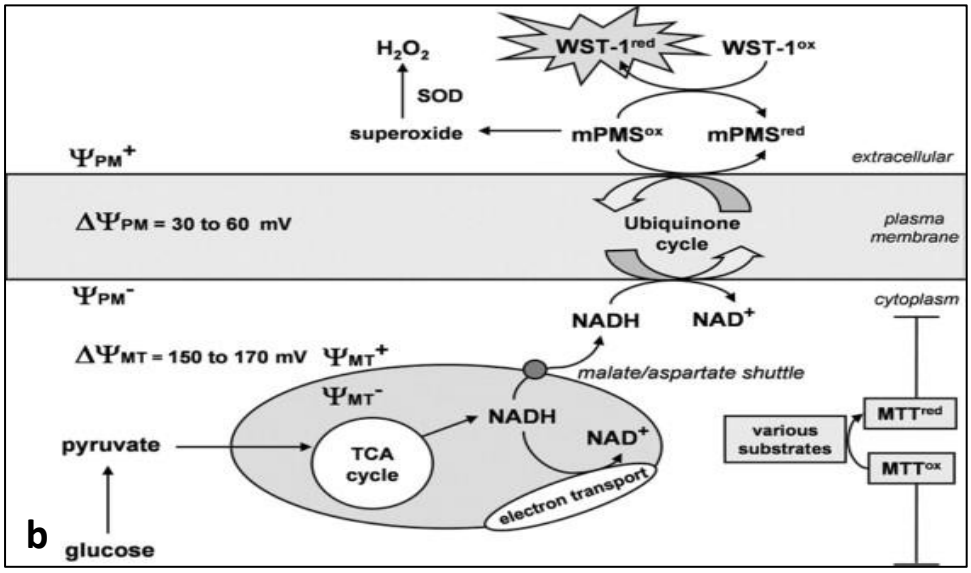
Figure 6: Hoechst and Propidium iodide stained spermatozoa showing live (blue) and dead (red) cells for evaluation of vitality (20x objective).

3.6.3.3 Cell Proliferation Reagent WST-1 Kit

The WST-1 reagent (Roche, Mannheim, Germany) is a clear, slightly red, ready to use solution, containing WST-1 and an electron coupling reagent, diluted in phosphate buffered saline (PBS) (Roche Diagnostics, 2006). This test is based on the principle that the tretrazolium salt WST-1 is cleaved to formazoan (Figure 7a) by cellular enzymes or reduced at the external surface of the plasma membrane by NADH and NADPH oxidase (Berridge and Tan, 1998). An expansion in the number of viable cells results in an increase in the overall activity of mitochodrial dehydrogenases (Figure 7b) in the sample. An augmentation in enzyme activity leads to a increase in formazoan dye which correlates to the number of metabolically active cells. The absorbance of the dye is measured at appropriate wavelengths.



(Yin et al., 2013)



(Berridge et al., 2005)

Figure 7: Diagrams illustrating the principle of the WST-1 assay with (a) depicitng the product formazoan after the cleavage and (b) depicting the action of the mitochodrial dehydrogenases and NADH and NADPH

Protocol:

1. 50 µl Puresperm separated spermatozoa were placed in 96 well microplates in an incubator at 37 °C and 5% CO_2.
2. 200 µl WST-1 stock solution was first diluted with 800 µl PBS, whereafter 50 µl WST-1 reagent was added per well.
3. The plates were incubated for 4 hours in a humidified atmosphere.
4. The absorbance was measured every 1-2 hours using a microplate (ELISA) reader at 420-480 nm (reference wavelength >600 nm).

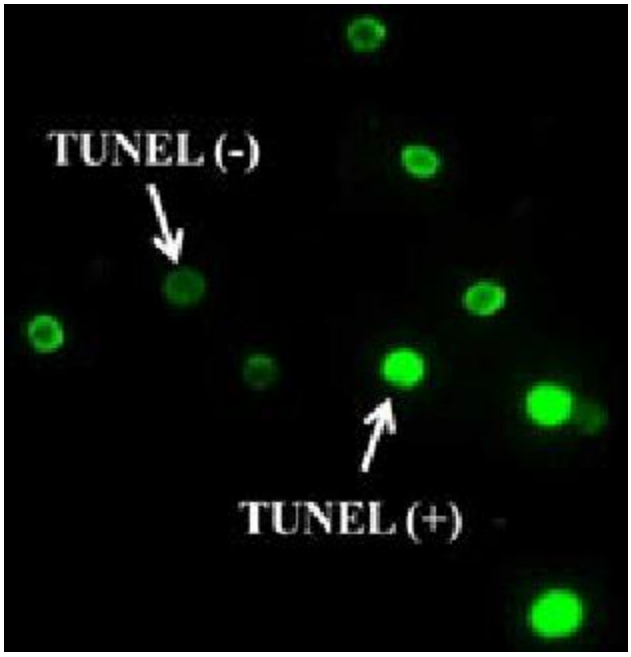
3.6.4 DNA Integrity

The assessment of DNA integrity involved the terminaldeoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-nick-end labelling assay (TUNEL) (WHO, 2010). TUNEL quantifies the incorporation of dUTP at single- and double-stranded DNA breaks in a reaction catalyzed by the template-independent enzyme, TdT. Incorporated dUTP is labeled such that breaks can be quantified either by flow cytometry, fluorescence microscopy, or light microscopy (Evenson et al., 2002).

Protocol: The DeadEnd™ Fluorometric TUNEL System G3250 (Promega, USA) was used to assess DNA integrity.

1. Spermatozoa were prepared to have a final concentraion of 50-100x10^6/ml. After the last centrifugation step in the PureSperm preparation, 50 µl PBS was added.
2. The preparation was centrifuged for 300 *g* at 4 °C for 10 minutes, where after the supernatant was discarded and the pellet was resuspended in 50 µl PBS.
3. Step 2 was repeated.
4. Smears were prepared as follows: 20 µl sperm preparation were placed onto slides and round smears were made or 10 µl was used for making normal smears. The slides were left to air-dry overnight or placed in an incubator for 10 minutes.
5. The slides were fixed by immersion in 4% formaldehyde for 25 minutes at 4 °C.
6. The slides were washed in PBS for 5 minutes, moving slides up and down every minute.
7. The spermatozoa were permeabilized in 0.2% Triton X-100 for 5 minutes, where after 20 µl was added to prepared round smears and 50 µl was added to normal smears.
8. Slides were rinsed twice in fresh PBS for 5 minutes, moving slides every minute. The excess liquid was removed by tapping the slides.
9. 50 µl equilibration buffer was added to the slides and equilibrated for 10 minutes.
10. The TdT incubation buffer was prepared using 90 µl Equilibration buffer, 10 µl Nucleotide mix and 2 µl TdT enzyme. This preparation was enough for covering three slides. Using tissue paper, the spaces around the equilibrated area was blotted.
11. 30 µl TdT incubation buffer was added to round smears and 40 µl to normal smears.
12. The cells were covered with plastic coverslips (halves for round smears and a whole coverslip for normal smears) and placed in a dark humidified chamber (containing paper towel soaked with water at the bottom). It was incubated at 37 °C for 60 minutes and covered with aluminium foil.
13. The 20X Saline Sodium Citrate (SSC) was diluted to 2X SSC and placed in a coplin jar. The coverslips were removed from the slides and slides were immersed in 2X SSC for 15 minutes at room temperature.
14. The slides were washed in PBS for 5 minutes at room temperature. The process was repeated twice, resulting in a total of three washes.
15. The samples were stained with Hoechst (1mg/ml in PBS) for 15 minutes at room temperature in the dark.

16. One drop of Dako anti-fade mounting medium (Diagnostech, Johannesburg, South Africa) was added with the addition of coverslips.
17. The slides were analysed immediately with a Nikon Eclipse 50i fluorescence microscope (IMP, Cape Town, South Africa) using a 20X objective, with a DAPI filter. Images were captured with the NIS Elements imaging software (40X- FI: 200 ms exp., 2X gain, BF: 600 ms exp., 1X gain, autowhite, 100X-FI: 600 ms exp., 1X gain, BF: Autowhite) (Figure 8).
18. Scoring: A total of at least 100 sperm were captured and counted with the NIS Elements imaging software (IMP, Cape Town, South Africa). A final percentage was then determined of spermatozoa with nuclear DNA damage and those without.



(Ghasemzadech et al., 2015)

Figure 8: Example of sperm with nuclear DNA damage (TUNEL-positive sperm) fluorescing bright green and sperm without damage (TUNEL-negative sperm) fluorescing light green after TUNEL assay.

3.6.5 Acrosome Intactness

The assessment of acrosome intactness involved the use of fluorescein-conjugated Pisum sativum agglutinin (FITC-PSA) (Sigma, Cape Town, South Africa), as well as fluorescein-conjugated Peanut agglutinin (FITC-PNA) (Sigma, Cape Town, South Africa), to determine the acrosomal status of a sperm sample. PNA has been shown to bind Galß(1-3)Gal NAC residues (Varki et al., 1999; Sharon, 2007) located on the outer acrosomal membrane (Mortimer et al., 1987; Martínez-Menárguez et al., 1992; Avilés et al., 1997). However, PSA recognizes a-methyl mannoside residues from complex oligosaccharide structures, localized within the acrosome contents (Cross et al., 1986; Varki et al.,

1999; Sharon, 2007). According to Lybaert et al. (2009), FITC-PSA stains the whole spermatozoa (head, acrosomal cap, equatorial segment as well as the flagellum. Whereas, FITC-PNA staining is restricted to the head and the acrosomal cap (Lybaert et al., 2009).

Protocol, FITC-PSA:

1. The FITC-PSA working solution was prepared before use by adding 50 µl FITC-PSA to 450 µl PBS resulting in FITC-PBS. Aliquots were stored at -20 °C and the working solution was stored at 4 °C.
2. The sperm sample was first diluted with Ham's F10 (Invitrogen, Thermo Fisher Scientific, South Africa) medium to yield a concentration of $2x10^6$/ml.
3. Two 5 µl drops of sperm sample was placed on a slide, spread in a circle and left overnight to dry.
4. The slides were then fixed with 95% Ethanol for 30 minutes in a refrigerator at 4 °C and left to air dry for 30 minutes.
5. 5 µl FITC-PSA solution was added to each round smear and kept in the dark for 45 minutes.
6. The slides were then washed in dH_2O for a few seconds and left to dry upright.
7. The slides were mounted by adding 2 drops of Dako fluorescent mounting medium (Diagnostech, Johannesburg, South Africa). Coverslips were added and left for a few minutes.
8. The slides were analysed with a Nikon Eclipse 50i Fluorescence Microscope (IMP, Cape Town, South Africa) using a green fluorescence filter and 100X-objective. A total of at least 100 spermatozoa were captured and counted with the NIS Elements BR. 3.10 (Build 578) on Nikon DS-U2/L2USB Program, Annotations and Measurements: Count and Taxonomy facility. A final percentage was then determined of bright intact acrosomes and (dark or with a centre band) reacted acrosomes.

Protocol, FITC-PNA:

1. The FITC-PNA working solution was prepared before use by adding 5 mg FITC-PNA to 5 ml PBS. Aliquots of 600 µl were stored at -20 °C and kept in the dark.
2. A swim-up preparation was prepared with Ham's F10 (Invitrogen, Thermo Fisher Scientific, South Africa).
3. 20 µl sperm preparation was smeared over one end of the slide and left to air dry.

4. The air dried smears were then immersed in FITC-PNA staining solution (600 µl aliquot added to 15.4 ml PBS) in Coplin jars at room temperature for 15 minutes in the dark.
5. The slides were rinsed twice in PBS and thereafter fixed by immersion in 4% para-formaldehyde at room temperature for 15 minutes.
6. The slides were rinsed twice in PBS, allowed to air dry and mounted with Dako fluorescent mounting medium (Diagnostech, Johannesburg, South Africa). Coverslips were added and left for a few minutes.
7. The slides were analysed with a Nikon Eclipse 50i Fluorescence Microscope (IMP, Cape Town, South Africa) using a green fluorescence filter and 100X-objective. A total of at least 100 spermatozoa were captured and counted with the NIS Elements BR. 3.10 (Build 578) on Nikon DS-U2/L2USB Program, Annotations and Measurements: Count and Taxonomy facility. A final percentage was then determined of bright intact acrosomes and (dark or with a centre band) reacted acrosomes.

3.6.6 Hyperactivation

The Motility module of the SCA system was used to evaluate sperm fractions for percentage hyperactivation. Cut-off values for certain kinematic parameters is used to detect hyperactivated sperm in humans as follows: VCL ≥150 µm/s, linearity ≤50% and ALH ≥7 µm (Mortimer and Mortimer, 2013). However, such values have not been determined for many other mammalian species. The cut-off values for the rhesus monkey were determined by Baumber and Meyers (2006). These thresholds for hyperactivation were applied to this study and were as follows: VCL≥130 µm/s, linearity ≤69% and ALH ≥7.5 µm (≥3.75 µm) (Baumber and Meyers, 2006). Hyperactivation analysis was performed on washed spermatozoa incubated under capacitating conditions (i.e., in sperm medium, HTF, at 37 ^{o}C and 5% CO_2).

The Flush technique was employed to evaluate and analyse sperm hyperactivation. The technique involves the exposure of semen to chemicals or media, immediate or over time using a Leja slide (Leja Products B.V., Nieuw-Vennep, The Netherlands) while being kept or incubated on a pre-warmed microscope stage (Ntanjana, 2015). Semen is displaced from the entrance of the slide towards the furthest end of the chamber by the medium used. The sperm from the semen will swim in all directions but also towards the medium and some may reach the area where the semen and medium

were intially introduced. Sperm motility is then analysed in the area where spermatozoa come into contact with almost pure medium, which is easy to identify as no semen debris or seminal particles are present (Ntanjana, 2015). This technique was introduced by Prof G van der Horst, in spermatozoa from broadcast spawners by flushing with fresh and marine water to activate motility and also during experimentation with African elephant (*Loxodonta africana*) sperm (van der Horst et al., 2010).

The stimulants used to induce hyperactivation were Procaine and Caffeine (Sigma, Cape Town, South Africa). These stimulants were selected as they were demonstrated to be inducers in previous studies at concentrations ranging from 1 to 10 mM. According to a study by Ortgies et al. (2012), procaine has been demonstrated to induce hyperactivation in spermatozoa from different mammalian species. A concentration of 5 mM was used in this study and hyperactivation was induced even in noncapacitating conditions demonstrating the star-shaped motility pattern of stallion spermatozoa (Ortgies et al., 2012). Caffeine has been shown to have inductory effects at a concentration of 1 mM in both rhesus monkeys (Baumber and Meyer, 2006) and the olive baboon, showing significantly higher sperm motility (Nyacheio et al., 2010). Another study by Colas et al. (2010) also demonstrated Caffeine at a concentration of 10 mM, was also able to induce hyperactivation of ram spermatozoa.

Protocol: The concentration range used for Caffeine were 1, 5 and 10 mM, and the concentrations for Procaine were 2, 4 and 8 mM. After preliminary experiments, Caffeine 5 mM was selected for all experiments of induction.

1. Neat semen was used to evaluate sperm hyperactivation.
2. 1 ul semen was added to a 4 chamber Leja slide.
3. 2 ul chemical (stimulant) was added to the semen in the same chamber.
4. The sperm was then analyzed immediately and every 15 minutes thereafter for an hour or until motility fell below WHO (2010) reference limits (motility >40%).
5. Data was captured and analyzed using the phase contrast microscope: the Motility module of the Sperm Class Analyzer (SCA) (Microptic S.L., Barcelona, Spain) CASA system, Version 5.1, in combination with the Nikon Eclipse 50i Phase Contrast Microscope (IMP, Cape Town, South Africa).

Once the data had been captured and analyzed by the CASA system, data sheets were produced and the three kinemtic parameters VCL, LIN and ALH of the spermatozoa for each species were evaluated

according to the rhesus monkey cut values (Baumber and Meyers, 2006). Spermatozoa which presented data that fell into the threshold values were labelled as hyperactivated. The amount of spermatozoa which presented hyperactivation were counted and a percentage was determined.

3.7 Validation of Functional Tests

Once all functional tests have been performed and optimized, these tests were applied in subsequent experiments where spermatozoa were exposed to heavy metals that have been proven to affect sperm function in order to validate the sensitivity of these tests. As previously mentioned, the two metals Copper sulphate ($CuSO_4$) and Cadmium chloride ($CdCl_2$) have been shown to decrease or inhibit human sperm motility at concentrations between 250 µg/ml and 500 µg/ml.

The metal concentrations, 10 µg/ml, 50 µg/ml, 100 µg/ml and 250 µg/ml $CuSO_4$, and 10 µg/ml, 50 µg/ml, 100 µg/ml and 500 µg/ml $CdCl_2$, were prepared in HTF medium before use and stored at 4 °C. Motile spermatozoa were selected with the PureSperm separation (see section 3.5 above), however, at the last centrifugation step 100 µl of the HTF medium containing the metal, was added instead of the neat HTF medium. This last step was applied when exposing the spermatozoa to each concentration of both metals. The resultant suspension was then used for all functional testing and a comparison was made to the initial results determining the sensitivity of the functional tests.

3.8 Statistical analysis/Data interpretation

MedCalC Program Version 12.3.0 (Mariakerke, Gent, Belgium) was used for statistical analysis. ANOVA (one way analysis of variance analysis) was performed for parametric data distributions. Any significant differences ($p \leq 0.05$) indicated in the ANOVA table between groups was analysed further using the Student-Newman-Keuls test for pairwise comparisons. If Levene's test showed $P < 0.05$, the Kruskal-Wallis test was employed. T-tests (independent samples) were employed for comparisons and further analysed with the Welch-test. If the F-test showed $P < 0.05$, the Mann-Whitney test was employed. Data was expressed as mean percentage ± standard deviation (SD) and $P < 0.05$ was considered significant using the analyses above.

Chapter 4: Results

As mentioned in Chapter 2 and 3, the functional tests that were evaluated in this study included: sperm motility and longevity, vitality, DNA integrity, acrosome reaction and hyperactivation. The results are presented in the following order for the three non-human primates: vervet monkey, chacma baboon and rhesus monkey. Due to low quality (baboon) and limited availability of semen samples (rhesus), certain functional tests (longevity, WST-1 assay) could not be completed for both the baboon and rhesus monkey.

4.1 Optimization

4.1.1 Species: Vervet Monkey

4.1.1.1 Preparation method

The vervet monkey semen samples obtained were prepared using two methods, namely the PureSperm (PS) and Swim-up (SU) method. The average motility characteristics of seven semen samples and sperm preparations are presented in Table 1. The two parameters which presented significance between Semen and the preparation methods was the concentration and total motility, and the rest of the parameters presented similar results between methods. Semen was an exception in only presenting significance for concentration, however, the PS method indicated the better result for concentration and total motility. The PS method presented a significantly higher motility average (P=0.049) as well as a higher sperm concentration (P=0.035) compared to the SU method (Figure 9), however, Semen had a significantly higher sperm concentration compared to the PS (P<0.001) and SU (P<0.001) method. Since the subsequent sperm functional testing required a high concentration of spermatozoa as well as a high percentage of total motile spermatozoa, it was therefore decided to use the PS method for all further sample preparation and functional testing, unless stated otherwise.

Table 1: Average concentration and motility parameter measurements ± SD of vervet semen and prepared sperm samples (n=7).

Preparation Method	*Sperm Concentration (X10⁶/ml)*	*Total Motility (%)*	*Progressive Motility (%)*	*Rapid Spermatozoa (%)*	*VCL (µm/s)*
Semen	1422.76 ± 2942.14 [a]	81.23 ± 15.36 [a]	34.64 ± 15.24	28.35 ± 14.62	108.58 ± 33.86
PS	78.88 ± 28.81[b]	88.66 ± 8.37 [a]	33.67 ± 24.88	25.88 ± 26.86	101.96 ± 46.02
SU	16.22 ± 11.11 [c]	83.46 ± 20.24 [b]	58.91 ± 17.45	55.62 ± 18.23	207.50 ± 64.85

PS = PureSperm, SU = swim-up, SD = standard deviation, VCL = curvilinear velocity. a, b and c = values labelled with different superscript letters in the same column were significantly different (P<0.05).

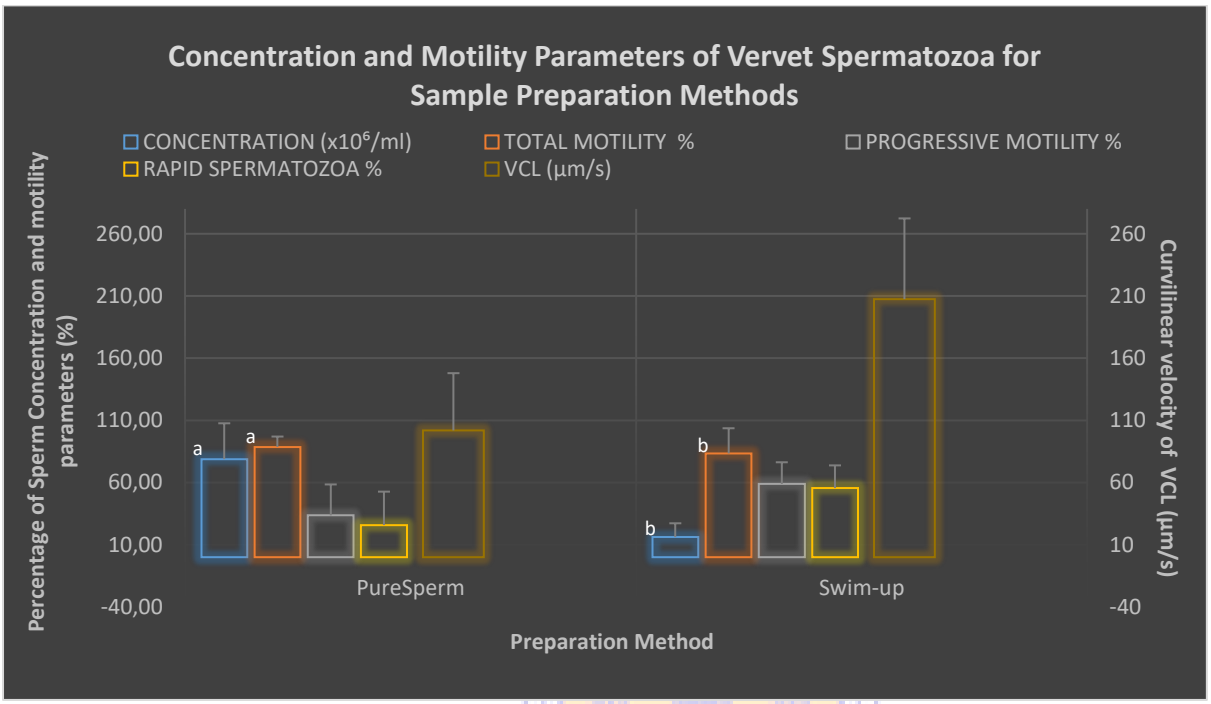


Figure 9: The average vervet monkey sperm concentration, motility and curvilinear velocity analysis of two preparation methods: PS and SU, to verify which preparation of spermatozoa would be best suited for functional testing by demonstrating a high motility reading and sperm concentration. a and b = significantly different ($P<0.05$).

4.1.1.2 Functional Tests

4.1.1.2.1 Motility and longevity

A time-based study was performed to evaluate the motility parameters and longevity of vervet spermatozoa. A total of five samples were prepared with PS and incubated for 45-75 minutes. Sperm motility and kinematic parameters were measured every 15 minutes (Table 2.1 and 2.2). Although there was a trend for percentage total, progressive and rapid sperm motility as well as sperm velocity (VCL, VSL and VAP) to decrease over time, none of the parameters presented a significant difference over the 75 minute period. It should also be noted that the average measurements for the swimming speed and most of the motility parameters already halved after 60 minutes of incubation.

Table 2.1: Average measurements for vervet monkey sperm motility parameters after 15-75 minutes

Time (mins)	*n*	*Total Motility (%)*	*Progressive Motility (%)*	*Rapid Spermatozoa (%)*	*Medium Spermatozoa (%)*	*Slow Spermatozoa (%)*
15	5	86.14 ± 7.51	58.54 ± 21.88	54.29 ± 23.85	11.86 ± 10.29	19.99 ± 14.45
30	5	72.23 ± 18.08	40.99 ± 20.18	35.94 ± 20.11	10.78 ± 7.94	25.50 ± 12.99
45	5	68.46 ± 15.91	35.54 ± 15.49	30.75 ± 14.56	10.90 ± 5.31	26.81 ± 12.86
60	4	67.87 ± 15.81	26.39 ± 16.62	21.16 ± 14.85	11.80 ± 5.53	34.90 ± 14.17
75	4	55.74 ± 23.02	24.38 ± 23.65	19.60 ± 23.68	10.39 ± 7.47	25.75 ± 17.67

Table 2.2: Average measurements for vervet monkey sperm kinematic parameters after 15-75 minutes

Time (mins)	*n*	*VCL (µm/s)*	*VSL (µm/s)*	*VAP (µm/s)*	*LIN (%)*	*STR (%)*	*WOB (%)*	*ALH (µm)*	*BCF (Hz)*
15	5	206.88 ±98.91	140.06 ±94.35	172.63 ±102.86	62.85 ±13.66	78.49 ±6.53	79.44 ±10.96	2.94 ±0.89	12.34 ±2.02
30	5	153.70 ±60.02	95.63 ±56.56	147.87 ±62.28	59.69 ±12.14	76.10 ±7.49	77.88 ±8.73	2.86 ±0.76	14.08 ±2.62
45	5	132.45 ±39.81	78.80 ± 8.54	104.25 ±42.43	57.92 ±12.25	74.60 ±7.93	77.10 ±9.41	2.66 ±0.67	13.60 ±1.80
60	4	114.91 ±42.82	64.66 ±46.01	86.09 ±47.11	52.48 ±19.90	70.57 ±15.05	72.23 ±13.75	2.39 ±0.44	11.32 ±2.72
75	4	113.49 ±72.76	71.18 ±82.68	88.05 ±83.65	50.19 ±29.20	68.51 ±21.47	68.29 ±21.83	1.55 ±1.06	9.81 ±6.64

VCL-curvilinear velocity, VSL- straight-line velocity, VAP –average-path velocity, LIN -linearity, STR –straightness, WOB –wobble, ALH - amplitude of lateral head displacement and BCF –beat-cross frequency.

4.1.1.2.2 Vitality

As mentioned in Chapter 3, three methods were used to assess sperm vitality, namely Eosin-Nigrosin staining, Hoechst and Propidium Iodide staining and WST-1 cytotoxicity assay. Each vitality test involved the use of a different set of samples on different days of testing and thus the three methods could not be compared directly.

4.1.1.2.2.1 Eosin-Nigrosin (E-N)

The prepared vervet monkey sperm samples were used for E-N staining after 15 minutes and 75 or 90 minutes of incubation (37 °C). The calculated average percentages of total motility as well as live and dead spermatozoa are presented in Table 3. A discrepancy in the total motility compared to vitality percentage was noted at the first time point. The total motility of the samples at the 15 minute time point was 78.30 ± 17.73 and the percentage live sperm at 15 minutes was 54.12 ± 17.52. The motility of vervet samples was shown to decline to 23.78 ± 27.08 at the last time point. However, a significant decrease of 35% (P=0.0454)

in the percentage live spermatozoa was found after 75 or 90 minutes of incubation, which was similar to the 36% decrease in total motility over the same time period in Table 2.1 and thus indicated the ability of E-N staining to distinguish between live and dead vervet monkey spermatozoa.

Table 3: Average vitality percentages of the prepared vervet monkey sperm samples after 15 and 75-90 minutes of incubation (n=5) using E-N staining.

Time (mins)	***Total Motility (%)***	***Live Spermatozoa (%)***	***Dead Spermatozoa (%)***
15	78.30 ± 17.73	54.52 ± 17.52 [a]	45.48 ± 17.52
75-90	23.78 ± 27.08	35.02 ± 17.79 [b]	62.98 ± 19.53

a and b = values labelled with different superscript letters in the same column were significantly different (P<0.05).

4.1.1.2.2.2 Hoechst and Propidium Iodide (H&P)

The prepared vervet monkey sperm samples were exposed to H&P staining and allowed to incubate (37 °C) for 45 to 60 minutes. The average percentage live spermatozoa was initially low compared to the percentage total motility at 15 minutes (Table 4) – a similar discrepancy as found in 4.1.2.2.1 above. However, the data showed that percentage live spermatozoa tend to decrease by 46% at last time point readings (similar to the decrease in percentage total motility), but this decrease was not significant (P>0.05). However, the test was sensitive in determining live from dead vervet monkey spermatozoa.

Table 4: Average vitality percentages of the prepared vervet monkey sperm samples after 15 and 45-60 minutes of incubation (n=4) using H&P staining.

Time (mins)	***Total Motility (%)***	***Live Spermatozoa (%)***	***Dead Spermatozoa (%)***
15	61.35 ± 18.32	49.58 ± 10.09	50.42 ± 10.09
45-60	34.25 ± 7.51	27.39 ± 34.13	72.61 ± 34.13

4.1.1.2.2.3 WST-1 assay

This assay was only attempted with the vervet species. To evaluate the WST-1 reagent, semen, PS and SU samples were compared in this assay. The absorbances were read at 15 minute intervals until 45 minutes of incubation (37 °C). Average absorbance and sperm concentration are displayed in Table 5. The data showed a significant difference at the 15 minute time point, where semen had a higher absorbance reading than the SU (P=0.023) but not PS. The SU, however, had a significantly higher absorbance than the PS (P=0.005) (Figure 10). The reason for this difference in absorbance is most probably due to the semen containing a

higher concentration of spermatozoa (145.93x10^6/ml) than the SU samples, as well as the SU containing a higher sperm concentration (12.87x10^6/ml) than the PS samples (6.53x10^6/ml), and therefore there were more spermatozoa to produce a detectable product. These results prove this assay's ability to detect live spermatozoa and its possible sensitivity to determine live from dead, as the absorbance values indicate the detectable formazan dye which is only produced by live cells. It was decided to use the PS samples as it was the preparation method of choice for all other functional tests. The remaining time points showed that the absorbance readings tend to increase over time for semen, SU and PS samples, however, this increase was not significant (P>0.05).

Table 5: Average sperm concentration and absorbance readings of the vervet monkey semen PS and SU samples (n=3) after exposure to the WST-1 reagent for 15-45 minutes during incubation (37 °C).

Time (mins)	***Average Absorbance Readings***		
	Semen (Avg Conc- 145.93M/ml)	***SU (Avg Conc- 12.87 M/ml)***	***PS (Avg Conc- 6.53 M/ml)***
15	0.088 ± 0.046 [a]	0.063 ± 0.005 [b]	0.058 ± 0.099 [ac]
30	0.443 ± 0.052	0.138 ± 0.032	0.131 ± 0.139
45	0.825 ± 0.148	0.200 ± 0.042	0.211 ± 0.239

PS = PureSperm, SU = swim-up, Avg- average, Conc- concentration, M/ml-million/ml. a, b and c = values labelled with different superscript letters in the same row were significantly different (P<0.05).

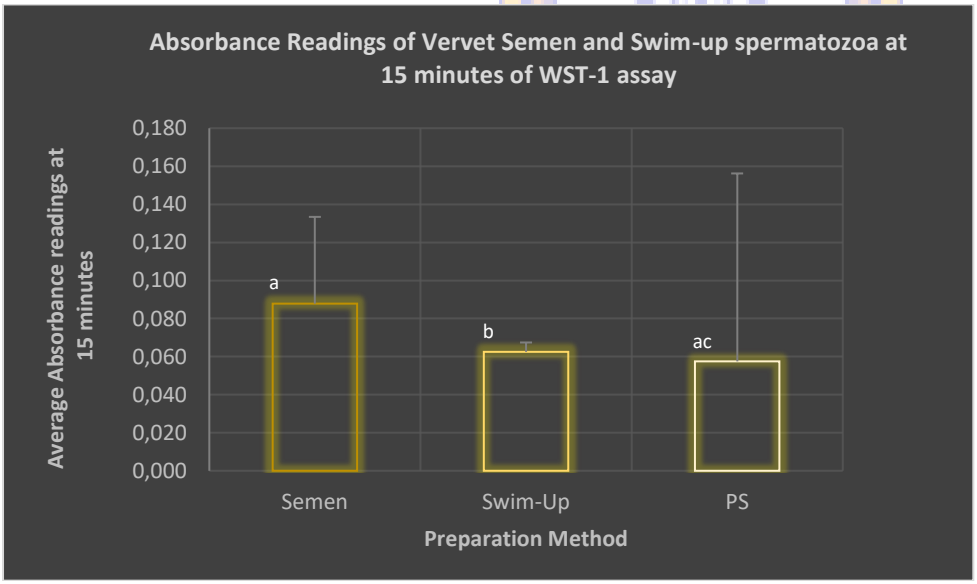


Figure 10: Average absorbance measurement for vervet monkey semen, SU and PS samples at 15 minutes of incubation (37 °C). a, b and c = significantly different (P<0.05).

4.1.1.2.3 DNA integrity

The evaluation of DNA integrity (using the TUNEL system) was not attempted with the vervet species as it proved unsuccessful with the baboon and rhesus monkey. Reasons for this decision are further explained in sections 4.2 and 4.3.

4.1.1.2.4 Acrosome Intactness

Acrosome intactness was evaluated using both the FITC-PSA and FITC-PNA reagents. However, the FITC-PNA reagent proved to be unsuccessful as it produced no measurable results (Figure 11).

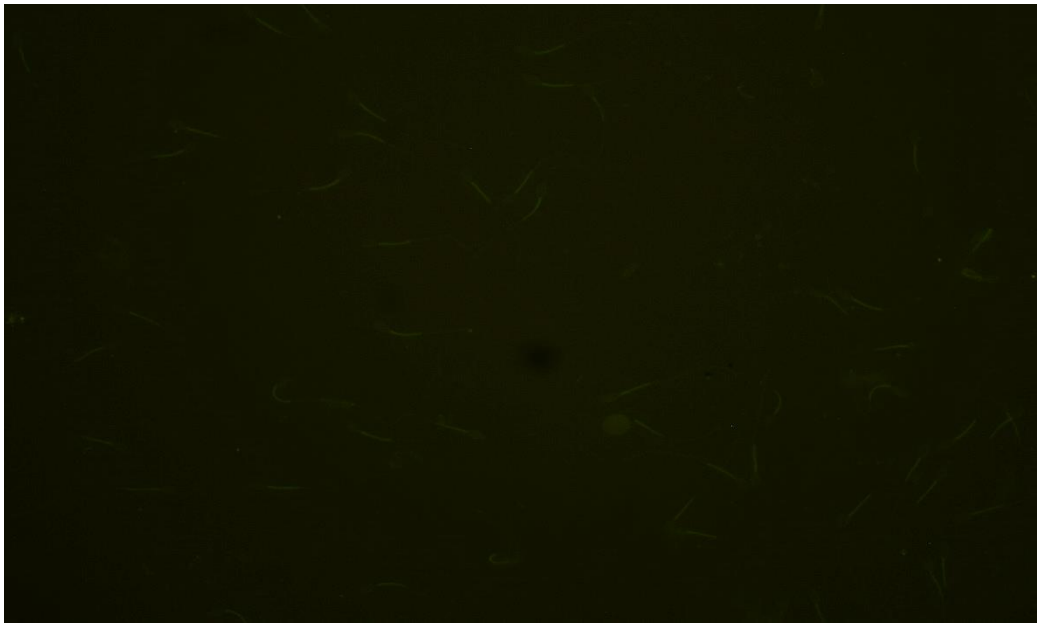


Figure 11: Unstained heads of vervet monkey spermatozoa after exposure to FITC-PNA, the spermatozoa are only visible due to their tails.

Semen and prepared samples of vervet monkeys were thus exposed to the FITC-PSA reagent and compared after 15 and 45 minutes of incubation (37 °C). The average percentage intact and reacted acrosomes over time are displayed in Table 6. The difference in the percentage intact acrosomes between PS samples and semen proved to be insignificant at both time points, 15 and 45 minutes ($P>0.05$). Furthermore the difference in percentage intact and reacted acrosomes of PS as well as the semen samples proved insignificant when the two time points were compared. A tendency for more intact acrosomes in the PureSperm preparation as well as a decrease in percentage intact acrosomes was seen after 45 minutes of exposure for both PS and Semen samples, however, the results were insignificant.

Table 6: Average intact and reacted acrosome percentages of vervet monkey semen and PS samples (n=4) after exposure to FITC-PSA at 15 and 45 minutes of incubation (37 °C).

Time (mins)	***Semen***		***PS***	
	Intact Acrosome (%)	***Reacted Acrosome (%)***	***Intact Acrosome (%)***	***Reacted Acrosome (%)***
15	35.42 ± 29.17	64.58 ± 29.17	90.48 ± 10.67	9.52 ± 10.67
45	16.00 ± 22.63	84.00 ± 22.63	82.76 ± 15.03	17.24 ± 15.03

4.1.1.2.5 Hyperactivation

Hyperactivation was induced via the Flush technique using caffeine at a concentration of 5 mM as previously described in Chapter 3. Samples were exposed to the stimulant and measured at 5, 15 and 30 minutes of incubation (37 °C). The average percentage hyperactivation of vervet spermatozoa is displayed in Table 7. The percentage hyperactivation was significantly higher in the caffeine samples for each time point, 5 minutes (P=0.011), 15 minutes (P=0.001) and 30 minutes (P=0.002), compared to the control samples (Figure 12). However, due to large standard deviations, the percentage hyperactivation proved statistically insignificant (P<0.05) but seem to decrease, for both the control and caffeine samples, over time. The dataset results therefore indicate that caffeine has the ability to induce hyperactivation in vervet spermatozoa and this technique was employed in the second part of the study.

Table 7: Average percentage sperm hyperactivation of the vervet monkey samples after exposure to caffeine (5mM) and 5, 15 and 30 minutes incubation time (n= 9).

Time (mins)	***Hyperactivation (%)***	
	Control	***Caffeine 5 mM***
5	3,56 ± 6,27 [a]	11,57 ± 17,01 [b]
15	0,63 ± 1,43 [a]	3,04 ± 5,96 [b]
30	0,33 ± 0,48 [a]	1,46 ± 3,18 [b]

a and b = values labelled with different superscript letters in the same row were significantly different (P<0.05).

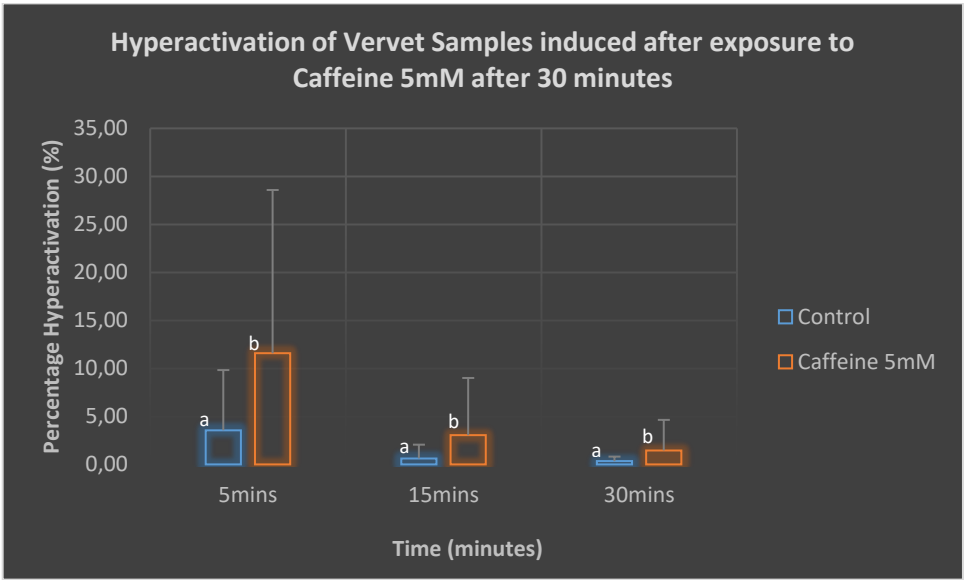


Figure 12: Average percentage sperm hyperactivation of the vervet monkey samples after exposure to caffeine (5mM) after 30 minutes of incubation (37 °C). a and b = significantly different (P<0.05).

4.1.2 Species: Baboon

4.1.2.1 Preparation method

The baboon semen samples obtained were prepared using the same two methods as previously described for vervet monkey. The average motility characteristics of ten semen samples and sperm preparations are presented in Table 8. A comparison between Semen and the two preparation methods were made and the data proved statistically insignificant (P>0.05) when comparing PS and SU, but the Semen presented a significantly higher sperm concentration compared to both PS (P<0.001) and SU (P<0.001). Additionally, the SU presented a significantly higher VCL compared to Semen (P=0.038). However, due to PS seemingly resulted in a higher sperm percentage total motility, as well as the fact that PS was selected as the preparation method of choice for vervet monkey semen samples, it was therefore decided to use this method for all further functional testing of baboon semen samples.

Table 8: Average concentration and motility parameter measurements ± SD of baboon semen and prepared sperm samples (n=10).

Preparation Method	*Sperm concentration* *(x10⁶/ml)*	*Total Motility* *(%)*	*Progressive Motility* *(%)*	*Rapid Spermatozoa* *(%)*	*VCL* *(µm/s)*
Semen	1201.05 ± 1854.76 [a]	55.98 ± 28.37	35.06 ± 22.92	31.28 ± 22.08	137.31 ± 43.86 [a]
PS	36.07 ± 19.23 [b]	67.87 ± 28.24	48.52 ± 32.42	43.95 ± 32.79	170.28 ± 62.09 [ab]
SU	17.5 ± 16.20 [a]	54.55 ± 23.17	41.67 ± 26.19	37.86 ± 27.20	222.06 ± 92.07 [b]

PS = puresperm, SU = swim-up, SD = standard deviation, VCL = curvilinear velocity, a, b and ac = values labelled with different superscript letters in the same column were significantly different (P<0.05).

4.1.2.2 Functional Tests

4.1.2.2.1 Motility and longevity

Evaluating the various motility parameters of baboon spermatozoa was attempted by preparing PS samples from twelve semen samples. The prepared samples were incubated for 15 minutes and compared to the neat semen samples (Table 9.1 and 9.2). Unfortunately, a time-based study could not be performed due to time constraints and therefore longevity could not be evaluated. Only two parameters presented with significant data, percentage Medium spermatozoa (P=0.003) and BCF (P<0.001) were significantly higher in PS samples compared to the semen samples (Figure 13). However, the PS preparations tend to have higher motility and kinematic values than the semen, which could be used as reference values for all subsequent experiments.

Table 9.1: Average measurements for baboon sperm motility parameters after 15 minutes incubation (37 °C) of baboon semen and prepared samples (n=12).

Prep.	***Total Motility (%)***	***Progressive Motility (%)***	***Rapid Spermatozoa (%)***	***Medium Spermatozoa (%)***	***Slow Spermatozoa (%)***
Semen	61.44 ±18.76	32.32 ±16.90	27.88 ± 17.41	8.36 ± 2.46 [a]	25.20 ± 8.96
PS	82.37 ±13.60	55.57 ±19.92	45.63 ± 22.86	16.70 ± 6.51 [b]	20.05 ± 10.98

a and b = values labelled with different superscript letters in the same column were significantly different (P<0.05)

Table 9.2: Average measurements for baboon sperm kinematic parameters after 15 minutes incubation (37 °C) of baboon semen and prepared samples (n=12).

Prep.	***VCL (µm/s)***	***VSL (µm/s)***	***VAP (µm/s)***	***LIN (%)***	***STR (%)***	***WOB (%)***	***ALH (µm)***	***BCF (Hz)***
Semen	121.56 ±28.84	79.14 ±24.79	98.64 ±28.31	64.32 ± 9.51	79.67 ±5.77	80.35 ±7.44	2.20 ± 0.40	13.71 ±2.50 [a]
PS	140.21 ±42.30	100.56 ±40.08	120.34 ±43.70	70.08 ± 5.16	82.76 ±5.16	84.37 ±6.91	2.10 ± 0.26	15.21 ±0.71 [b]

PS –PureSperm, VCL-curvilinear velocity, VSL- straight-line velocity, VAP –average-path velocity, LIN -linearity, STR –straightness, WOB –wobble, ALH –amplitude of lateral head displacement and BCF –beat-cross frequency, a and b = values labelled with different superscript letters in the same column were significantly different (P<0.05).

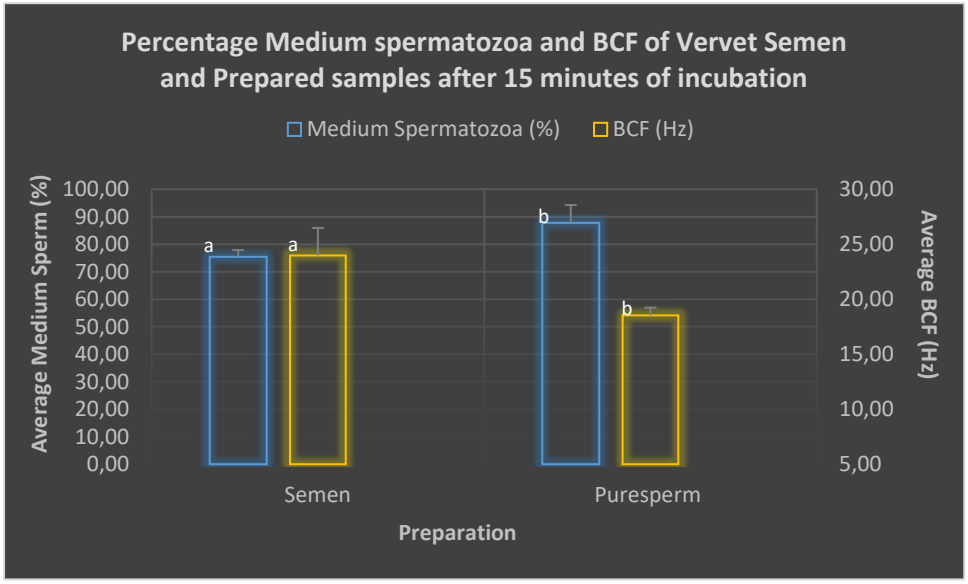


Figure 13: The average percentage medium spermatozoa and BCF of baboon semen and prepared samples (after 15 minutes incubation (37 °C)). a and b = significantly different ($P<0.05$).

4.1.2.2.2 Vitality

Each vitality test involved the use of a different set of samples on different days of testing and thus the three methods could not be compared directly.

4.1.2.2.2.1 Eosin-Nigrosin (E-N)

The prepared baboon sperm samples (n=7) were used for E-N staining at 15 minutes of incubation (37 °C). The calculated average live spermatozoa was 45.75 ± 11.23%, and dead spermatozoa was 54.25 ± 11.23%, while the calculated average total motility for these prepared samples was 52.94 ± 21.21%. These values seemed to be in the same range and thus indicated the ability of E-N staining to distinguish between live and dead baboon spermatozoa.

4.1.2.2.2.2 Hoechst and Propidium Iodide (H&P)

The prepared baboon sperm samples (n=5) were exposed to H&P staining and allowed to incubate (37 °C) for 15 minutes. The calculated average live sperm was 38.49 ± 19.17%, and dead spermatozoa was 61.51 ± 19.17%, while the calculated average total motility was 37.00 ± 13.69%. The low motility clearly indicates why the vitality of these samples were low. Again, these values seemed to be in the same range and thus indicated the ability of H&P staining to distinguish between live and dead baboon spermatozoa.

4.1.2.2.3 DNA Integrity

The evaluation of DNA integrity involved the use of the TUNEL assay (as stipulated in the materials and methods). Prepared samples (n=7) were exposed to the TUNEL System G3250 and slides were analysed with the fluorescence microscope. However, the baboon spermatozoa showed no results of fluorescence and all samples proved the same result. The assay did not distinguish between fragmented and non-fragmented DNA. It was therefore decided to no longer proceed with this method as well as evaluation of DNA integrity.

4.1.2.2.4 Acrosome Intactness

As with the vervet monkeys, the baboon prepared samples (n=3) were evaluated using the FITC-PSA reagent as described in Chapter 3. The prepared samples were exposed to the reagent after 15 minutes of incubation (37°C). The calculated average intact acrosomes was 70.54 ± 32.40%, and reacted acrosomes was 29.46 ± 32.40%.

4.1.2.2.5 Hyperactivation

Hyperactivation was induced via the Flush technique using caffeine at a concentration of 5 mM as previously described in Chapter 3. Samples were exposed to the stimulant and measured at 5, 15, 30, 45 and 60 minutes of incubation (37 °C). The average percentage hyperactivation of baboon sperm is displayed in Table 10. Although the percentage hyperactivation was relatively low in all samples, it was significantly higher in the caffeine samples at the 15 (P=0.003) and 30 minute (P=0.001) time point compared to the control samples (Figure 14). The percentage hyperactivation also proved statistically significant, for both the control (P=0.001) and caffeine (P=0.001) samples, over time. The control samples presented a decrease of percentage hyperactivation after 30 minutes but then showed an increase after 60 minutes. However, the caffeine samples presented an increase in hyperactivation at the 15 minute time point but decreased after 60 minutes. The dataset results therefore indicate the ability of caffeine to induce hyperactivation and the effect of caffeine seems to be at its peak at 15 minutes in baboon spermatozoa.

Table 10: Average percentage sperm hyperactivation of the baboon samples after exposure to caffeine (5 mM) and 5, 15, 30, 45 and 60 minutes incubation time.

Time (mins)	*n*	*Hyperactivation (%)*	
		Control	*Caffeine 5 mM*
5	4	1.70 ± 1.69 [a]	2.21 ± 2.01 [a]
15	5	0.58 ± 0.64 [b*]	4.25 ± 4.30 [b*]
30	5	0.15 ± 0.21 [c*]	1.36 ± 1.80 [bc*]
45	4	0.60 ± 0.89 [abc]	1.13 ± 1.05 [abc]
60	3	0.85 ± 0.97 [cd]	0.63 ± 0.55 [d]

* = significantly different between columns (P<0.05), a, b, c, and d = significant data over time (P<0.05)

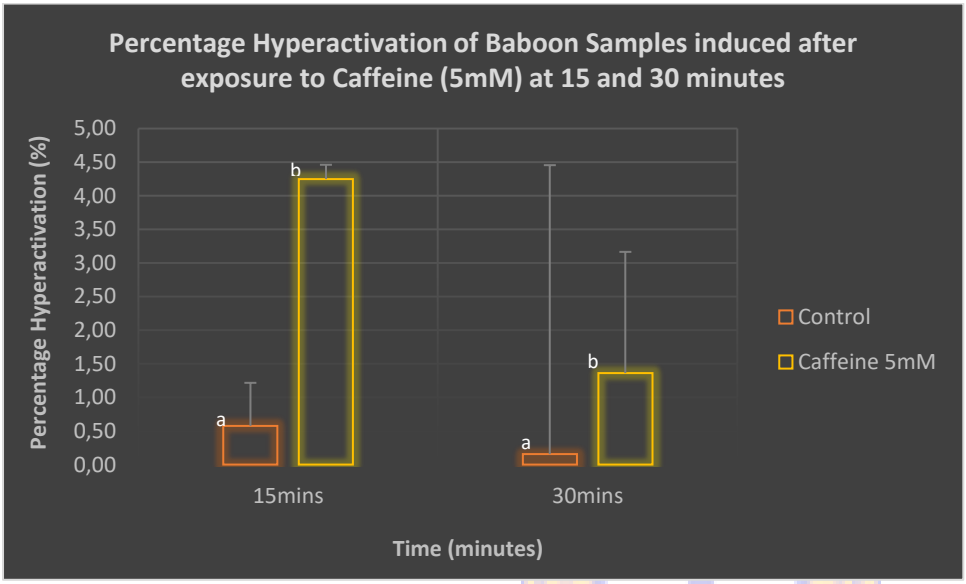


Figure 14: Average percentage sperm hyperactivation of the baboon samples after exposure to caffeine (5 mM) after 15 and 30 minutes of incubation (37 °C). a and b = significantly different (P<0.05).

4.1.3 Species: Rhesus monkey

4.1.3.1. Preparation method

The rhesus monkey semen samples obtained were prepared using the same two methods as previously described for vervet and baboon semen samples. The average motility characteristics of five semen samples and four sperm preparations are presented in Table 11. A comparison between Semen and the two preparation methods were made and the results proved statistically insignificant (P<0.05) for the PS and SU comparison, but the Semen presented statistically higher sperm concentration compared to both PS (P=0.009) and SU (P=0.001). However, due to PS seemingly resulted in a higher sperm percentage total motility, as well as the fact that PS was selected as the preparation method of choice for vervet monkey and baboon semen samples, it was therefore decided to use this method for all further functional testing of rhesus monkey semen samples.

Table 11: Average concentration and motility parameter measurements ± SD of rhesus monkey semen and prepared sperm samples.

Preparation Method	*n*	*Sperm concentration (X10^6/ml)*	*Total Motility (%)*	*Progressive Motility (%)*	*Rapid Spermatozoa (%)*	*VCL (µm/s)*
Semen	5	83,10 ± 85,73 [a]	74,77 ± 15,45	40,90 ± 8,80	37,62 ± 8,75	152,22 ± 43,53
PS	4	24,40 ± 12,31 [b]	90,63 ± 6,10	74,97 ± 18,89	70,50 ± 22,16	204,77 ± 66,58
SU	4	9,70 ± 5,61 [b]	74,92 ± 16,98	68,65 ± 17,36	65,74 ± 18,57	261,72 ± 25,94

PS = PureSperm, SU = swim-up, SD – standard deviation, VCL - curvilinear velocity, a, and b = values labelled with different superscript letters in the same column were significantly different (P<0.05).

4.1.3.2 Functional Tests

4.1.3.2.1 Motility and longevity

The motility parameters and longevity functional testing of rhesus spermatozoa was attempted by preparing PS samples from nine semen samples. The prepared samples were incubated for 15 minutes and compared to the neat semen (Table 12.1 and 12.2). Unfortunately, a time-based study could not be performed due to time constraints and therefore longevity could not be evaluated. Only four parameters presented with significant data, namely percentage LIN (P=0.004), STR (P=0.001), WOB (P=0.016) and ALH (P=0.02) were significantly higher compared to the semen samples (Figure 15). However, the PS preparations tend to have higher motility and kinematic values than the semen, which could be used as reference values for all subsequent experiments.

Table 12.1: Average measurements for rhesus sperm motility parameters of rhesus monkey semen and prepared samples (n=9)

Preparation	*Total Motility (%)*	*Progressive Motility (%)*	*Rapid Spermatozoa (%)*	*Medium Spermatozoa (%)*	*Slow Spermatozoa (%)*
Semen	38.10 ± 27.46	20.52 ± 18.73	17.80 ± 17.19	9.00 ± 9.39	11.30 ± 6.38
PS	54.20 ± 26.17	42.21 ± 27.00	36.94 ± 26.80	8.76 ± 5.19	8.49 ± 5.20

Table 12.2: Average measurements for rhesus sperm kinematic parameters of rhesus monkey semen and prepared samples (n=9)

Preparation	*VCL (µm/s)*	*VSL (µm/s)*	*VAP (µm/s)*	*LIN (%)*	*STR (%)*	*WOB (%)*	*ALH (µm)*	*BCF (Hz)*
Semen	120.45 ± 42.72	62.43 ± 42.45	81.01 ± 45.21	46.66 ± 21.04 [a]	70.24 ± 19.43 [a]	63.17 ± 15.31 [a]	2.66 ± 1.09 [a]	13.13 ± 5.41
PS	166.34 ± 46.32	114.67 ± 34.41	132.01 ± 41.81	68.58 ± 6.65 [b]	87.13 ± 5.21 [b]	78.71 ± 6.02 [b]	2.81 ± 0.45 [b]	18.52 ± 3.60

PS –PureSperm, VCL-curvilinear velocity, VSL- straight-line velocity, VAP –average-path velocity, LIN -linearity, STR –straightness, WOB – wobble, ALH –amplitude of lateral head displacement and BCF –beat-cross frequency, a and b = significantly different (P<0.05).

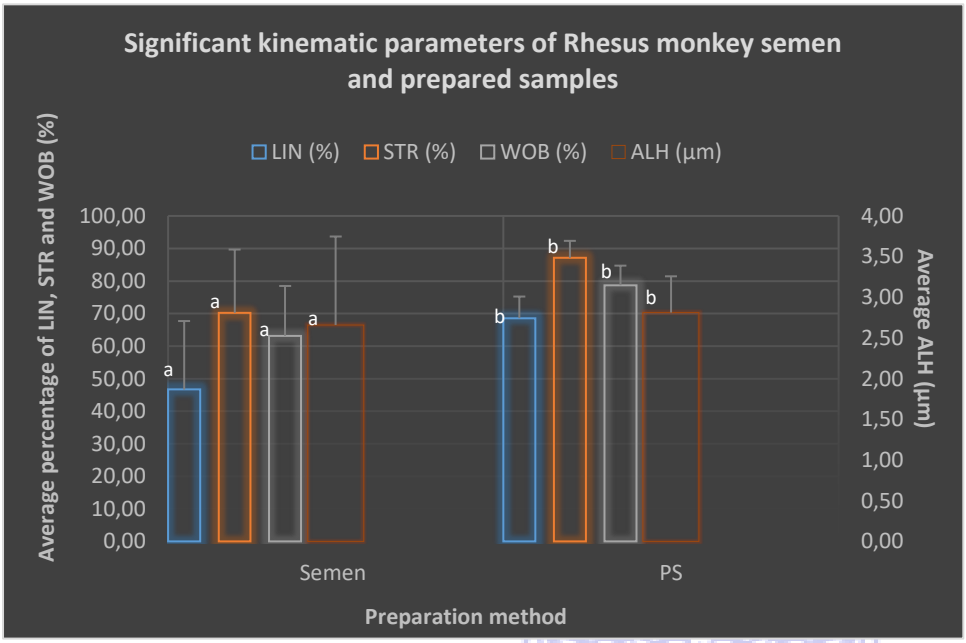


Figure 15: The average percentage kinematic parameters of rhesus monkey semen and prepared samples. a and b = significantly different (P<0.05).

4.1.3.2.2 Vitality

Each vitality test involved the use of a different set of samples on different days of testing and thus the three methods could not be compared directly.

4.1.3.2.2.1 Eosin-Nigrosin (E-N)

The prepared rhesus monkey samples (n=3) were used for E-N staining at 15 minutes of incubation (37 °C). The calculated average live spermatozoa was 51.38 ± 5.06%, and dead spermatozoa was 48.17 ± 5.06%, while the calculated average total motility was 83.77 ± 13.90%. This discrepancy in the percentage total motility and live spermatozoa could have been due to time delays between taking the motility reading and preparing the smears for E-N staining.

4.1.3.2.2.2 Hoechst and Propidium Iodide (H&P)

The prepared rhesus monkey sperm samples (n=3) were exposed to H&P staining and allowed to incubate (37 °C) for 15 minutes. The calculated average live spermatozoa was 38.56 ± 6.91%, and dead spermatozoa was 61.44 ± 6.91%, while the calculated average total motility was 23.47 ± 27.41%. The low motility clearly indicates why the vitality of these samples where low thus verifying the ability of H&P staining to distinguish between live and dead rhesus monkey spermatozoa.

4.1.3.2.3 DNA Integrity

The TUNEL assay was attempted to evaluate DNA integrity for rhesus monkey prepared samples. However, it proved unsuccessful as fluorescence was not present and there was no distinction between fragmented and non-fragmented DNA for all samples. It was therefore concluded to no longer proceed with this method as well as evaluation of DNA integrity.

4.1.3.2.4 Acrosome intactness

The rhesus monkey prepared samples (n=3) were evaluated using the FITC-PSA reagent as described in Chapter 3. The prepared samples were exposed to the reagent after 15 minutes of incubation (37 °C). The calculated average intact acrosomes was 79.71 ± 4.47%, and reacted acrosomes was 20.29 ± 4.47%.

4.1.3.2.5 Hyperactivation

Hyperactivation was induced via the Flush technique using caffeine at a concentration of 5 mM as previously described in Chapter 3. Samples were exposed to the stimulant and measured at 15, 30, 45 and 60 minutes of incubation (37 °C). The average percentage hyperactivation of rhesus monkey spermatozoa is displayed in Table 13. The percentage hyperactivation was significantly higher in the caffeine samples at the 45 (P=0.034) and 60 minute (P=0.025) time points compared to the control samples (Figure 16). However, the percentage hyperactivation proved statistically insignificant (P<0.05), for both the control and caffeine samples, over time. The dataset results therefore indicate the ability of caffeine to induce hyperactivation in rhesus monkey spermatozoa.

Table 13: Average percentage sperm hyperactivation of the rhesus monkey samples after exposure to caffeine (5 mM) and 15, 30, 45 and 60 minutes incubation time.

Time (mins)	***n***	***Hyperactivation (%)***	
		Control	***Caffeine 5 mM***
15	4	7.40 ± 7.44	17.32 ± 15.57
30	3	5.99 ± 4.39	16.19 ± 14.13
45	4	3.44 ± 3.29 [a]	10.49 ± 14.86 [b]
60	4	3.33 ± 4.11 [a]	12.85 ± 20.71 [b]

a and b = significantly different (P<0.05).

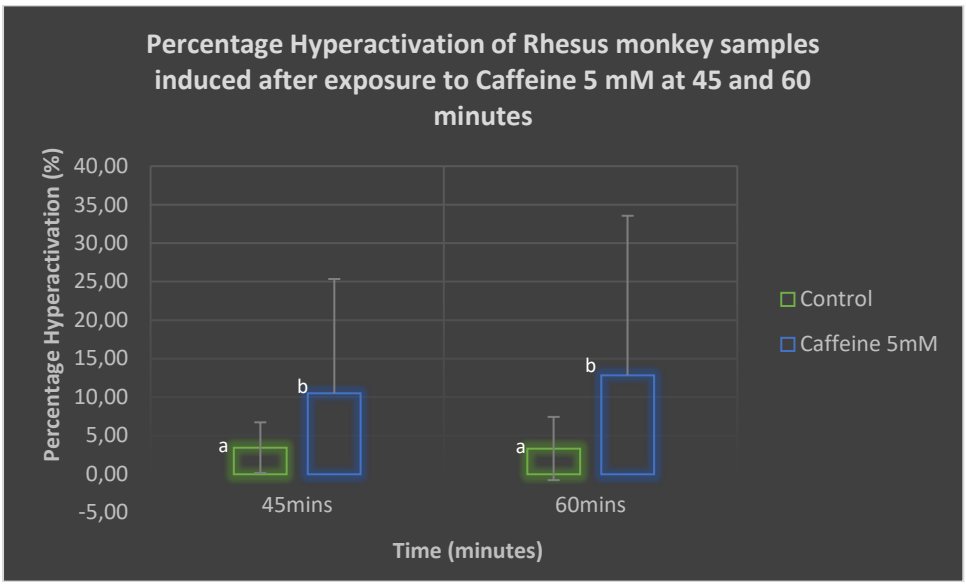


Figure 16: Average percentage sperm hyperactivation of the rhesus monkey samples after exposure to caffeine (5 mM) after 45 and 60 minutes of incubation (37 °C). a and b = significantly different ($P<0.05$).

4.2 Validation of Functional Tests

As described in Chapter 3, once all functional tests were performed and optimised for the three species, subsequent experiments were applied. Spermatozoa were exposed to two heavy metals, namely copper sulphate ($CuSO_4$) and cadmium chloride ($CdCl_2$), which have previously been proven to affect sperm function. Exposure to these metals would validate the sensitivity of the functional tests evaluated and developed in this study. Spermatozoa were selected using the PS method and suspended in the chemical solution (metal) as described in Chapter 3. This suspension was used for all further functional testing. Due to low quality (baboon) and limited availability of semen samples (rhesus monkey), certain functional tests could not be completed for the baboon (WST-1 assay and hyperactivation) and rhesus monkey (vitality and acrosome reaction), respectively.

4.2.1 Vervet monkey

The prepared vervet monkey samples were exposed to the metals $CuSO_4$ and $CdCl_2$, where after the following functional tests were performed: motility and longevity, vitality (E-N, H&P and WST-1 assay), acrosome intactness and hyperactivation.

4.2.1.1 Motility and longevity

A time-based study was performed to evaluate the motility parameters and longevity of the vervet monkey sperm suspension. A total of four samples were exposed to each metal and motility was measured at 15, 60

and 120 minutes. The samples were exposed to the following concentrations of $CuSO_4$: 10, 50, 100 and 250 µg/ml (Table 14.1 and 14.2) and $CdCl_2$: 10, 50, 100 and 500µg/ml (Table 15.1 and 15.2). Reason being for the lower total motility averages in the control samples compared to the values reported in 4.1.1.1 and 4.1.1.2.1, may be due to the low total motility percentages for all samples except one. The percentages ranged from 20-40%, with one sample displaying a total motility of 80%. This resulted in a low total motility average and higher standard deviations compared to what was seen before.

$CuSO_4$ treatment: After 15 minutes of exposure to $CuSO_4$, four out of the thirteen motility parameters presented significantly lower values for the 250 µg/ml concentration when compared to the control and other $CuSO_4$ concentrations (Figure 15 and 16). After 60 minutes, eight out of the thirteen motility parameters presented significant lower values for the 250 µg/ml concentration when compared to the control. Additionally, five of the thirteen motility parameters were also significantly lower compared to the control for the 100 µg/ml concentration at this time point. After 120 minutes, five out of the thirteen parameters presented significantly lower values for both the 100 µg/ml and 250 µg/ml concentrations when compared to the control, 10 µg/ml and 50 µg/ml concentrations. Only the 10 µg/ml concentration did not result in any effect for any of the three time points on the sperm motility parameters when compared to the control (Figure 17 and 18). The decrease in all motility parameters over time clearly indicated the negative effect of the highest metal concentrations (100 µg/ml and 250 µg/ml) on vervet sperm motility.

Table 14.1: Average measurements for vervet monkey sperm motility parameters after exposure to copper sulphate (10-250 µg/ml) at 15, 60 and 120 minutes (n=4).

Time ***(mins)***	***Conc. Of $CuSO_4$ (µg/ml)***	***Total Motility*** ***(%)***	***Progressive Motility*** ***(%)***	***Rapid Spermatozoa*** ***(%)***	***Medium Spermatozoa*** ***(%)***	***Slow Spermatozoa*** ***(%)***
15	Control	46,94 ± 24.54	23,22 ± 15,76 [a]	19,05 ± 14,05 [a]	10,14 ± 6,99	17,73 ± 5,22
	[10]	43,31 ± 20.71	21,46 ± 11,58 [a]	16,97 ± 5,73 [a]	6,52 ± 8,82	19,82 ± 7,20
	[50]	42,65 ± 27.44	25,35 ± 25,95 [a]	20,06 ± 17,40 [a]	7,76 ± 10,38	14,83 ± 4,46
	[100]	32,89 ± 17.93	14,90 ± 9,16 [a]	12,07 ± 6,83 [a]	6,38 ± 3,84	14,45 ± 8,55
	[250]	7,31 ± 7.42	0,63 ± 0,76 [b]	0,48 ± 0,56 [b]	0,48 ± 0,97	6,35 ± 6,44
60	Control	40,82 ± 20.73 [a]	11,56 ± 7,24 [a]	9,15 ± 6,35 [a]	5,39 ± 3,75 [a]	26,29 ± 11,29
	[10]	37,64 ± 12.72 [a]	15,50 ± 3,94 [a]	11,66 ± 5,07 [ab]	5,89 ± 2,29 [a]	20,10 ± 12,20
	[50]	31,72 ± 9.67 [a]	9,38 ± 6,91 [ab]	7,80 ± 6,78 [ac]	3,87 ± 0,42 [a]	20,05 ± 13,41
	[100]	14,55 ± 9.82 [b]	3,71 ± 3,27 [c]	2,58 ± 2,35 [d]	3,51 ± 2,89 [a]	8,46 ± 6,77
	[250]	0,38 ± 0.77 [b]	0,00 ± 0,00 [cd]	0,00 ± 0,00 [de]	0,00 ± 0,00 [b]	0,38 ± 0,77
120	Control	24,76 ± 12.96	5,88 ± 4,15 [a]	5,05 ± 3,57 [a]	1,22 ± 1,07	18,49 ± 9,49
	[10]	28,45 ± 11.66	7,80 ± 2,14 [ab]	5,85 ± 0,59 [a]	3,33 ± 1,48	19,27 ± 10,15
	[50]	15,82 ± 5.72	3,86 ± 2,77 [ac]	2,32 ± 1,56 [ab]	2,79 ± 2,39	10,71 ± 3,00
	[100]	3,70 ± 7.41	0,31 ± 0,62 [d]	0,21 ± 0,41 [c]	0,21 ± 0,41	3,29 ± 6,58
	[250]	0,00 ± 0.00	0,00 ± 0,00 [d]	0,00 ± 0,00 [c]	0,00 ± 0,00	0,00 ± 0,00

Table 14.2: Average measurements for vervet monkey sperm kinematic parameters after exposure to copper sulphate (10-250 µg/ml) at 15, 60 and 120 minutes (n=4).

Time (mins)	*Conc. of $CuSO_4$ (µg/ml)*	*VCL* (µm/s)	*VSL* (µm/s)	*VAP* (µm/s)	*LIN* (%)	*STR* (%)	*WOB* (%)	*ALH* (µm)	*BCF* (Hz)
15	Control	118,89 ± 37,94	56,71 ± 21,27	70,68 ± 24,37	47,22 ± 3,17	79,89 ± 3,76 [a]	59,08 ± 1,79 [a]	2,49 ± 1,02	17,00 ± 5,06
	[10]	125,18 ± 21,19	71,50 ± 13,60	81,68 ± 14,28	57,34 ± 6,68	87,42 ± 2,62 [b]	65,47 ± 5,87 [a]	3,08 ± 0,41	20,35 ± 4,46
	[50]	121,29 ± 20,47	60,52 ± 12,88	74,03 ± 15,50	49,95 ± 7,95	81,70 ± 1,78 [ac]	61,03 ± 8,50 [a]	2,49 ± 0,56	20,81 ± 4,25
	[100]	94,94 ± 21,11	50,97 ± 22,48	61,58 ± 23,23	51,44 ± 15,54	80,40 ± 8,39 [abc]	62,95 ± 13,63 [a]	1,86 ± 0,62	18,78 ± 8,50
	[250]	31,72 ± 30,58	8,97 ± 9,26	14,24 ± 13,72	19,89 ± 14,30	44,00 ± 30,97 [d]	33,76 ± 22,60 [b]	0,55 ± 0,93	4,52 ± 6,84
60	Control	81,47 ± 14,41	36,76 ± 10,90	46,53 ± 11,22	44,76 ± 8,56	78,34 ± 5,27 [a]	56,80 ± 6,82 [a]	1,83 ± 0,76 [a]	14,17 ± 3,63 [a]
	[10]	100,64 ± 23,25	56,27 ± 28,95	67,86 ± 29,08	53,91 ± 16,41	80,77 ± 7,69 [ab]	65,73 ± 14,35 [a]	2,02 ± 0,43 [a]	14,81 ± 3,16 [a]
	[50]	87,05 ± 34,19	44,33 ± 22,36	55,68 ± 23,81	49,08 ± 6,80	77,26 ± 9,47 [ac]	63,48 ± 2,61 [a]	1,52 ± 0,82 [a]	13,87 ± 5,94 [a]
	[100]	56,86 ± 39,73	20,51 ± 14,37	27,97 ± 19,31	27,10 ± 18,25	54,81 ± 36,66 [d]	37,06 ± 24,79 [b]	1,53 ± 1,16 [a]	13,44 ± 9,85 [a]
	[250]	3,46 ± 6,92	0,07 ± 0,15	0,22 ± 0,44	0,53 ± 1,06	8,33 ± 16,67 [e]	1,59 ± 3,19 [b]	0,00 ± 0,00 [b]	0,00 ± 0,00 [b]
120	Control	71,96 ± 11,96	37,56 ± 16,56	46,64 ± 14,57	51,03 ± 18,24 [a]	78,13 ± 10,56 [a]	63,98 ± 13,62	1,68 ± 0,91	15,67 ± 4,42 [a]
	[10]	80,94 ± 6,45	42,58 ± 8,53	52,28 ± 4,51	53,12 ± 12,69 [a]	81,03 ± 12,57 [a]	64,86 ± 6,96	1,47 ± 0,70	14,40 ± 3,62 [a]
	[50]	61,57 ± 28,75	25,20 ± 20,39	34,69 ± 22,57	34,91 ± 20,07 [a]	64,43 ± 20,79 [a]	50,56 ± 17,00	1,21 ± 1,06	10,79 ± 8,26 [a]
	[100]	12,59 ± 25,18	2,06 ± 4,12	4,14 ± 8,28	4,10 ± 8,19 [b]	12,46 ± 24,91 [b]	8,22 ± 16,44	0,13 ± 0,26	1,40 ± 2,80 [b]
	[250]	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00 [b]	0,00 ± 0,00 [b]	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00 [b]

Conc-concentration, CuSO4-copper sulphate, VCL-curvilinear velocity, VSL- straight-line velocity, VAP-average-path velocity, LIN-linearity, STR-straightness, WOB-wobble, ALH-amplitude of lateral head displacement and BCF-beat-cross frequency, a - e = significantly different data relevant to the concentration of CuSO4 (P<0.05).

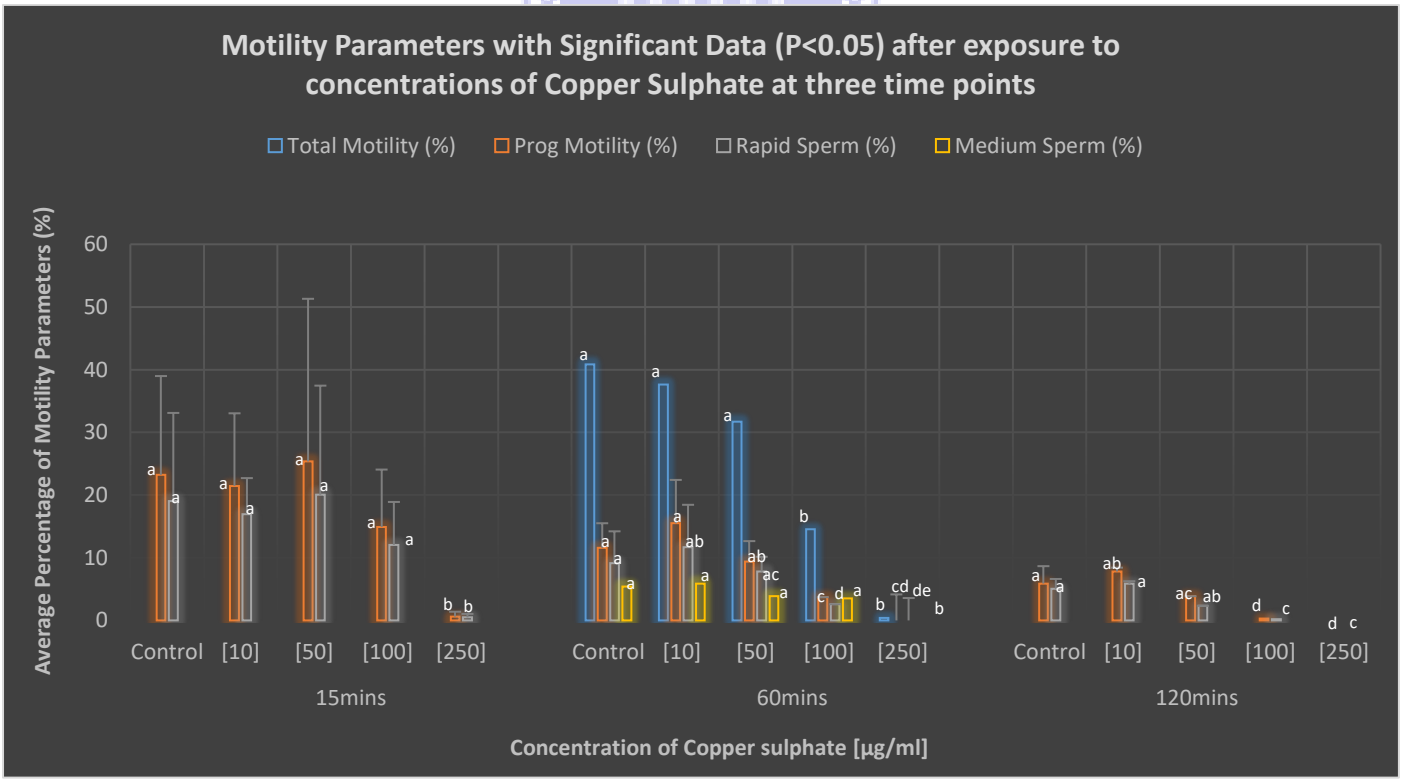


Figure 17: Average vervet monkey motility parameters that presented significant data at 15, 60 and 120 minutes of exposure to Copper Sulphate (10-250 µg/ml). a-e = significantly different values at the same time point (P<0.05)

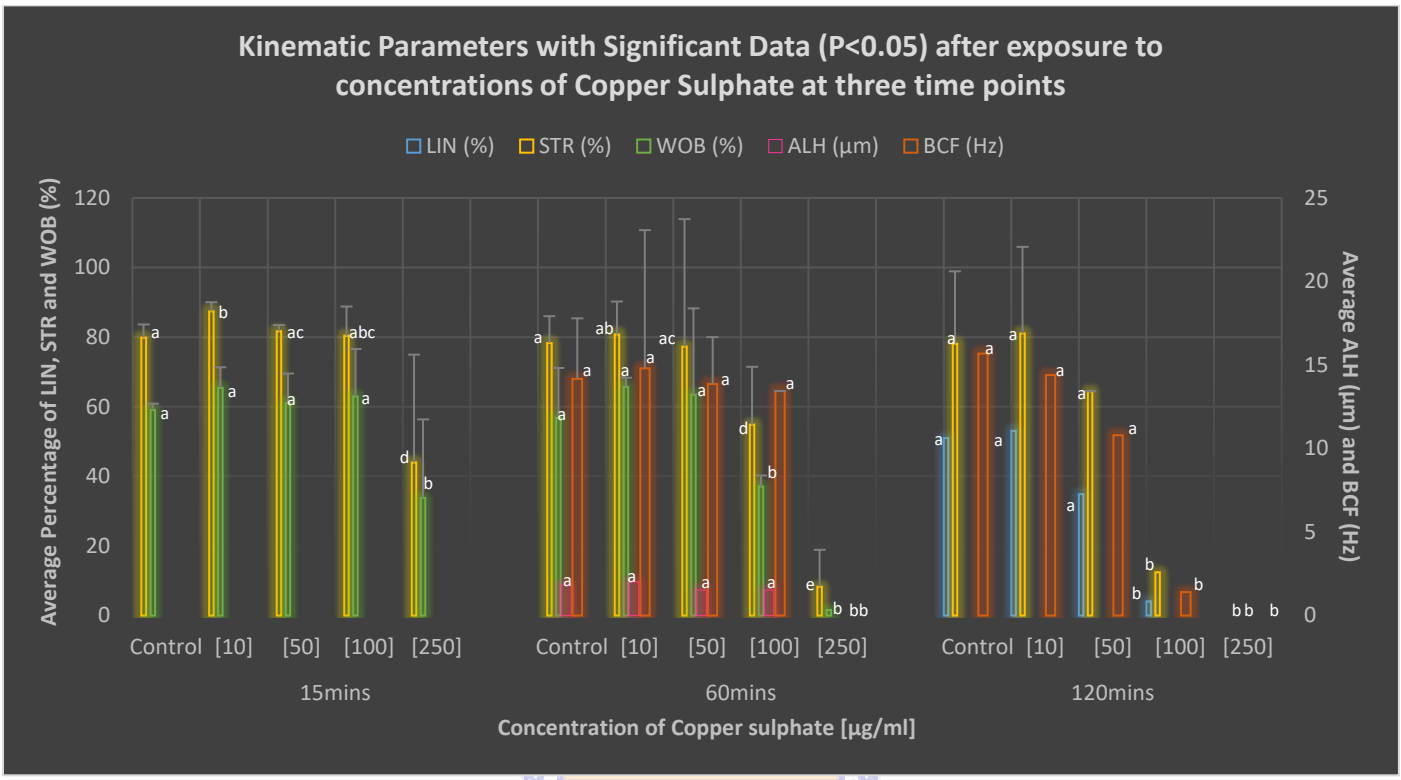


Figure 18: Average vervet monkey kinematic parameters that presented significant data at 15, 60 and 120 minutes of exposure to Copper Sulphate (10-250 µg/ml). a-b = significantly different values at the same time point (P<0.05)

$CdCl_2$ treatment: After 15 minutes of exposure to $CdCl_2$, all motility parameters had a tendency of lower average values when the different metal concentrations were compared to the control. However, due to large standard deviations, only two out of thirteen motility parameters presented significantly lower values for the 100 µg/ml and 500 µg/ml concentrations when compared to the control and other $CdCl_2$ concentrations (Table 15.1 and 15.2). After 60 minutes, a similar trend was observed as for 15 minutes with eight out of the thirteen motility parameters presented with significantly lower values for the 100 µg/ml and 500 µg/ml concentrations when compared to the control and other $CdCl_2$ concentrations. After 120 minutes, nine of the thirteen motility parameters presented with significantly lower values for 100 µg/ml and 500 µg/ml concentrations. Additionally, six of the thirteen motility parameters were also significantly lower compared to the control for the 50 µg/ml concentration at this time point. Only the 10 µg/ml concentration did not result in differences for any of the three time points on the sperm motility parameters when compared to the control (Figure 19 and 20). The decrease in all motility parameters over time clearly indicated the negative effect of the highest metal concentrations (50 µg/ml, 100 µg/ml and 500 µg/ml) on vervet sperm motility.

Table 15.1: Average measurements for vervet monkey sperm motility parameters after exposure to cadmium chloride (10-500 µg/ml) at 15, 60 and 120 minutes (n=4).

Time (mins)	*Conc. of $CdCl_2$* (µg/ml)	*Total Motility* (%)	*Progressive Motility* (%)	*Rapid Spermatozoa* (%)	*Medium Spermatozoa* (%)	*Slow Spermatozoa* (%)
15	Control	45,70 ± 35,36	26,76 ± 31,64	23,19 ± 31,14	6,29 ± 3,53	16,22 ± 3,61
	[10]	42,61 ± 36,69	23,87 ± 32,81	21,76 ± 32,30	3,92 ± 2,31	16,94 ± 5,89
	[50]	41,35 ± 33,80	19,59 ± 31,35	17,56 ± 30,32	4,34 ± 3,22	19,45 ± 3,35
	[100]	29,57 ± 43,88	17,76 ± 34,09	16,62 ± 32,27	3,50 ± 5,86	9,46 ± 6,66
	[500]	1,21 ± 2,41	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	1,21 ± 2,41
60	Control	39,72 ± 40,6	23,75 ± 31,15 [a]	21,52 ± 30,21 [a]	3,68 ± 3,15	14,52 ± 8,74 [a]
	[10]	39,76 ± 35,3	19,13 ± 29,22 [a]	16,96 ± 27,39 [a]	4,36 ± 4,07	18,44 ± 5,77 [a]
	[50]	21,99 ± 33,6	8,22 ± 15,52 [a]	7,10 ± 13,62 [a]	2,30 ± 3,27	12,58 ± 16,94 [a]
	[100]	14,74 ± 28,60	3,04 ± 6,07 [b]	2,09 ± 4,18 [b]	1,94 ± 3,65	10,71 ± 20,73 [b]
	[500]	0,00 ± 0,00	0,00 ±0,00 [c]	0,00 ± 0,00 [c]	0,00 ± 0,00	0,00 ± 0,00 [c]
120	Control	32,82 ± 44,20 [a]	16,50 ± 27,81 [a]	14,78 ± 26,39 [a]	5,93 ± 7,59 [a]	12,11 ± 11,80
	[10]	26,71 ± 34,90 [a]	11,36 ± 21,63 [a]	9,55 ± 18,41 [a]	3,24 ± 6,10 [a]	13,91 ± 10,73
	[50]	10,40 ± 13,50 [a]	0,36 ± 0,42 [b]	0,10 ± 0,12 [b]	0,82 ± 1,01 [b]	9,48 ± 12,79
	[100]	6,82 ± 13,60 [a]	0,28 ± 0,56 [b]	0,14 ± 0,28 [b]	0,97 ± 1,95 [b]	5,71 ± 11,41
	[500]	0,00 ± 0,00 [b]	0,00 ± 0,00 [b]	0,00 ± 0,00 [b]	0,00 ± 0,00 [b]	0,00 ± 0,00

Table 15.2: Average measurements for vervet monkey sperm kinematic parameters after exposure to cadmium chloride (10-500 µg/ml) at 15, 60 and 120 minutes (n=4).

Time (mins)	*Conc. of $CdCl_2$* (µg/ml)	*VCL* (µm/s)	*VSL* (µm/s)	*VAP* (µm/s)	*LIN* (%)	*STR* (%)	*WOB* (%)	*ALH* (µm)	*BCF* (Hz)
15	Control	112,17 ± 56,84	63,95 ± 39,75	79,09 ± 50,72	54,27 ± 12,39	80,08 ± 9,92	67,34 ± 10,84	2,49 ± 0,54 [a]	15,66 ± 3,30 [a]
	[10]	105,76 ± 59,64	59,17 ± 32,56	74,14 ± 45,72	57,16 ± 15,32	81,40 ± 7,29	69,65 ± 13,29	2,26 ± 1,14 [a]	14,52 ± 4,07 [a]
	[50]	93,44 ± 50,15	52,32 ± 31,98	66,41 ± 42,45	54,73 ± 11,88	79,17 ± 6,65	68,73 ± 10,48	2,38 ± 0,65 [a]	15,27 ± 1,85 [a]
	[100]	78,99 ± 67,74	37,21 ± 44,13	50,32 ± 56,24	37,00 ± 19,96	65,67 ± 15,35	52,62 ± 21,95	1,33 ± 1,36 [b]	7,81 ± 7,01 [b]
	[500]	9,20 ± 18,40	2,86 ± 5,72	5,35 ± 10,70	7,77 ± 15,54	13,36 ± 26,71	14,54 ± 29,08	0,00 ± 0,00 [c]	0,00 ± 0,00 [c]
60	Control	103,95 ± 50,99	59,55 ± 38,91	73,53 ± 47,69	52,24 ± 25,09 [a]	75,36 ± 20,41 [a]	65,38 ± 21,72 [a]	1,80 ± 0,99 [a]	10,70 ± 8,50 [a]
	[10]	91,84 ± 48,55	52,76 ± 36,26	64,52 ± 45,21	54,96 ± 12,87 [a]	82,07 ± 4,13 [a]	66,75 ± 14,55 [a]	1,84 ± 0,75 [a]	13,54 ± 3,94 [a]
	[50]	52,15 ± 43,61	28,23 ± 32,77	39,62 ± 36,04	35,66 ± 30,47 [a]	48,20 ± 40,07 [a]	55,59 ± 37,84 [a]	0,94 ± 1,10 [a]	6,90 ± 7,96 [a]
	[100]	32,42 ± 37,57	15,38 ± 18,01	20,49 ± 23,90	23,64 ± 27,39 [b]	37,47 ± 43,28 [b]	31,52 ± 36,46 [b]	0,46 ± 0,93 [b]	3,17 ± 6,35 [b]
	[500]	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00 [c]	0,00 ± 0,00 [c]	0,00 ± 0,00 [c]	0,00 ± 0,00 [c]	0,00 ± 0,00 [c]
120	Control	81,28 ± 63,87	46,71 ± 38,26 [a]	56,68 ± 46,95 [a]	43,44 ± 34,13	62,17 ± 42,36 [a]	51,60 ± 37,58	1,92 ± 1,49 [a]	13,42 ± 9,59 [a]
	[10]	63,77 ± 43,87	28,76 ± 34,87 [a]	38,07 ± 42,15 [a]	35,68 ± 19,15	69,70 ± 10,96 [a]	49,49 ± 20,47	0,98 ± 0,94 [a]	8,39 ± 5,59 [a]
	[50]	25,21 ± 29,28	6,67 ± 7,81 [b]	11,69 ± 13,52 [a]	13,17 ± 15,24	28,41 ± 33,04 [b]	23,26 ± 26,90	1,28 ± 1,49 [a]	3,92 ± 5,43 [b]
	[100]	13,47 ± 26,94	3,22 ± 6,43 [c]	6,61 ± 13,23 [b]	5,97 ± 11,94	12,16 ± 24,32 [c]	12,27 ± 24,55	0,81 ± 1,63 [b]	0,99 ± 1,99 [c]
	[500]	0,00 ± 0,00	0,00 ± 0,00 [c]	0,00 ± 0,00 [b]	0,00 ± 0,00	0,00 ± 0,00 [c]	0,00 ± 0,00	0,00 ± 0,00 [c]	0,00 ± 0,00 [c]

Conc-concentration, CdCl2-cadmium chloride, VCL-curvilinear velocity, VSL-straight-line velocity, VAP-average-path velocity, LIN-linearity, STR-straightness, WOB-wobble, ALH-amplitude of lateral head displacement and BCF-beat-cross frequency, a – c = significantly different data relevant to the concentration of $CdCl_2$ ($P<0.05$).

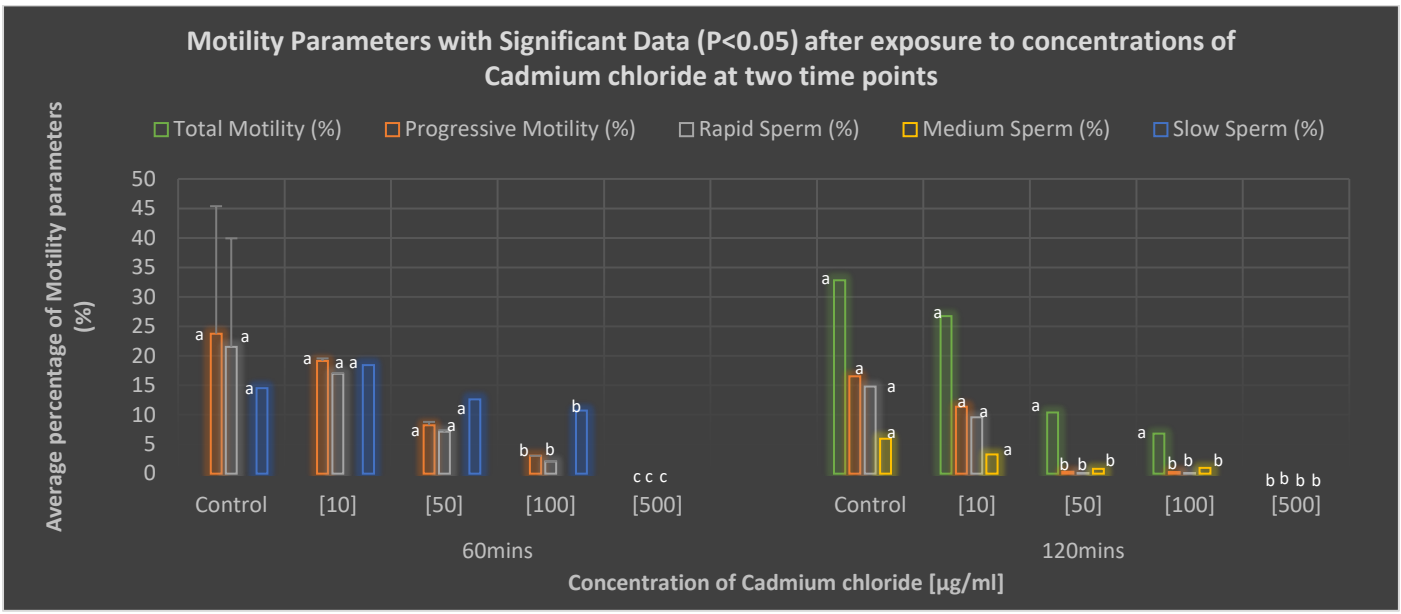


Figure 19: Average vervet monkey motility parameters that presented significant data at 60 and 120 minutes of exposure to Cadmium chloride (10-500 µg/ml). a-c = significantly different values at the same time point (P<0.05)

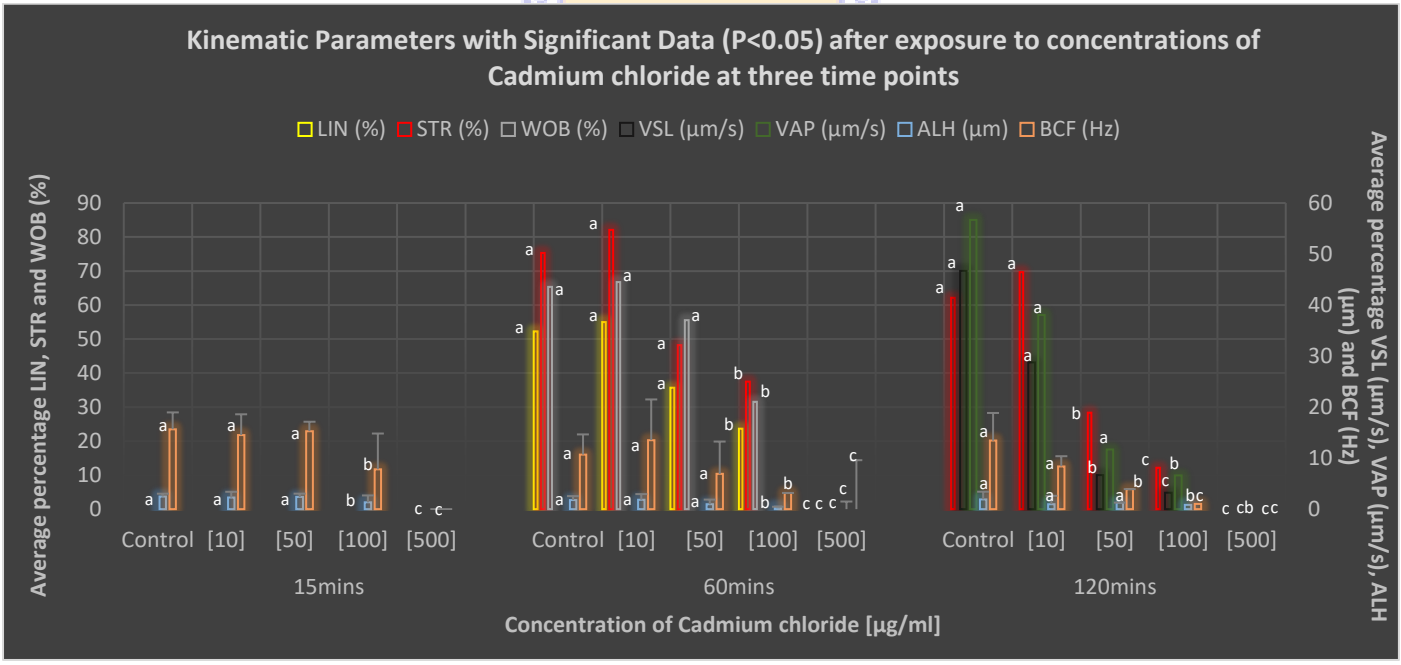


Figure 20: Average vervet monkey kinematic parameters that presented significant data at 15, 60 and 120 minutes of exposure to Cadmium chloride (10-500 µg/ml). a-c = significantly different values at the same time point (P<0.05)

4.2.1.2 Vitality

4.2.1.2.1 Eosin-Nigrosin (E-N)

Although this test indicated a discrepancy between the total motility and percentage live spermatozoa during optimization, it was still employed for validation testing, in order to observe the effect of the metals.

The prepared vervet monkey suspension was exposed to the E-N stain after incubation (37 °C). A total of five samples were exposed to each metal and vitality was measured at 15 and 120 minutes. The samples were exposed to the following concentrations of $CuSO_4$: 10, 50, 100 and 250 µg/ml (Table 16) and $CdCl_2$: 10, 50, 100 and 500 µg/ml (Table 17).

Table 16: Average vitality percentages of the vervet monkey prepared suspensions after exposure to the E-N staining kit at 15 and 120 minutes of incubation (n=5) for $CuSO_4$ treatment.

Time (min)	*$CuSO_4$ (µg/ml)*	*Live Spermatozoa (%)*	*Dead Spermatozoa (%)*
15	Control	46,60 ± 22,96	53,40 ± 22,96
	[10]	41,20 ± 16,24	58,80 ± 16,24
	[50]	52,40 ± 22,86	47,60 ± 22,86
	[100]	42,80 ± 15,44	57,20 ± 15,45
	[250]	43,60 ± 19,50	56,40 ± 19,50
120	Control	27,40 ± 19,37	72,60 ± 19,37
	[10]	29,60 ± 16,47	70,40 ± 16,47
	[50]	29,60 ± 16,95	70,40 ± 16,95
	[100]	32,20 ± 24,36	67,80 ± 24,36
	[250]	28,40 ± 13,33	71,60 ± 13,33

Table 17: Average vitality percentages of the vervet monkey prepared suspensions after exposure to the E-N staining kit at 15 and 120 minutes of incubation (n=5) for $CdCl_2$ treatment.

Time (min)	*$CdCl_2$ (µg/ml)*	*Live Spermatozoa (%)*	*Dead Spermatozoa (%)*
15	Control	40,80 ± 18,29	59,20 ± 12,3
	[10]	44,00 ± 11,79	56,00 ± 11,79
	[50]	39,00 ± 17,61	61,00 ± 17,61
	[100]	33,80 ± 8,5	66,20 ± 8,5
	[500]	30,40 ± 11,13	69,60 ± 11,13
120	Control	32,40 ± 12,46	67,60 ± 12,46
	[10]	29,60 ± 17,64	70,40 ± 17,64
	[50]	39,40 ± 20,44	60,60 ± 20,44
	[100]	37,80 ± 16,18	62,20 ± 16,18
	[500]	31,80 ± 12,66	68,20 ± 12,66

No significant differences were found between the control and metal concentrations for percentage live and dead spermatozoa at 15 and 120 minutes, and over time, for both metals. This either indicates that the metal had no effect on the vitality of the vervet sperm tested or that the E-N stain is unable to detect the effects of the metals on vervet monkey sperm vitality. The control was observed to have a lower value compared to samples exposed to certain concentrations of both $CuSO_4$ and $CdCl_2$ and this may be due to variation and the large standard deviations. The discrepancy in percentage total sperm motility and vitality, as reported in

section 4.1.1.2.2.1 was not seen again; the percentages of total sperm motility (Table 14.1 and 15.1) and vitality (Table 16 and 17) were in the same range for all validation results.

4.2.1.2.2 Hoechst and Propidium Iodide (H&P)

The vervet monkey prepared suspensions were exposed to the H&P stains and allowed to incubate (37 °C). A total of five samples were exposed to each metal and vitality was measured at 15 and 120 minutes. The samples were exposed to the following concentrations of $CuSO_4$: 10, 50, 100 and 250 µg/ml (Table 18) and $CdCl_2$: 10, 50, 100 and 500 µg/ml (Table 19). Once again, the discrepancy in percentage total sperm motility and vitality, as reported in section 4.1.1.2.2.2 was not found with the validation results.

Table 18: Average vitality percentages of the vervet monkey prepared suspensions after exposure to the H&P staining at 15 and 120 minutes of incubation (n=5) for $CuSO_4$ treatment.

Time (min)	***$CuSO_4$ (µg/ml)***	***Live Spermatozoa (%)***	***Dead Spermatozoa (%)***
15	Control	53,96 ± 37,05	46,04 ± 37,05
	[10]	54,52 ± 33,85	45,48 ± 33,85
	[50]	48,59 ± 37,48	51,41 ± 37,48
	[100]	33,20 ± 33,25	66,80 ± 33,25
	[250]	19,30 ± 29,83	80,70 ± 29, 83
120	Control	26,067 ± 28,29 [a]	73,93 ± 28,29 [a]
	[10]	30,02 ± 40,39 [a]	69,98 ± 40,39 [a]
	[50]	22,92 ± 39,55 [a]	77,08 ± 39,55 [a]
	[100]	0,00 ± 0,00 [b]	100,00 ± 0,00 [b]
	[250]	0,00 ± 0,00 [b]	100,00 ± 0,00 [b]

a and b = significantly different data relevant to the concentration of $CuSO_4$ ($P<0.05$)

Table 19: Average vitality percentages of the vervet monkey prepared suspensions after exposure to the H&P staining at 15 and 120 minutes of incubation (n=5) for $CdCl_2$ treatment.

Time (min)	***$CdCl_2$ (µg/ml)***	***Live Spermatozoa (%)***	***Dead Spermatozoa (%)***
15	Control	53,19 ± 30,73	46,81 ± 30,73
	[10]	49,32 ± 29,78	50,68 ± 29,78
	[50]	57,45 ± 24,51	42,49 ± 24,54
	[100]	41,36 ± 29,40	59,31 ± 30,11
	[500]	46,59 ± 38,34	53,41 ± 38,34
120	Control	50,88 ± 36,52 [a]	49,12 ± 36,52 [a]
	[10]	40,89 ± 28,82 [a]	59,11 ± 28,82 [a]
	[50]	38,40 ± 38,82 [a]	61,60 ± 38,82 [a]
	[100]	37,60 ± 36,13 [a]	62,40 ± 36,13 [a]
	[500]	0,22 ± 0,49 [b]	99,78 ± 0,49 [b]

a and b = significantly different data relevant to the concentration of $CdCl_2$ ($P<0.05$).

$CuSO_4$ treatment: Although the average percentage live spermatozoa seems to have decreased with an increase in metal concentration, there was no significant effect on sperm vitality at the 15 minute time point. However, at 120 minutes a significant decrease was found in the percentage live spermatozoa when comparing the 100 µg/ml and 250 µg/ml concentrations to the control as well as the lower concentrations of $CuSO_4$ (P=0.018) (Figure 21).

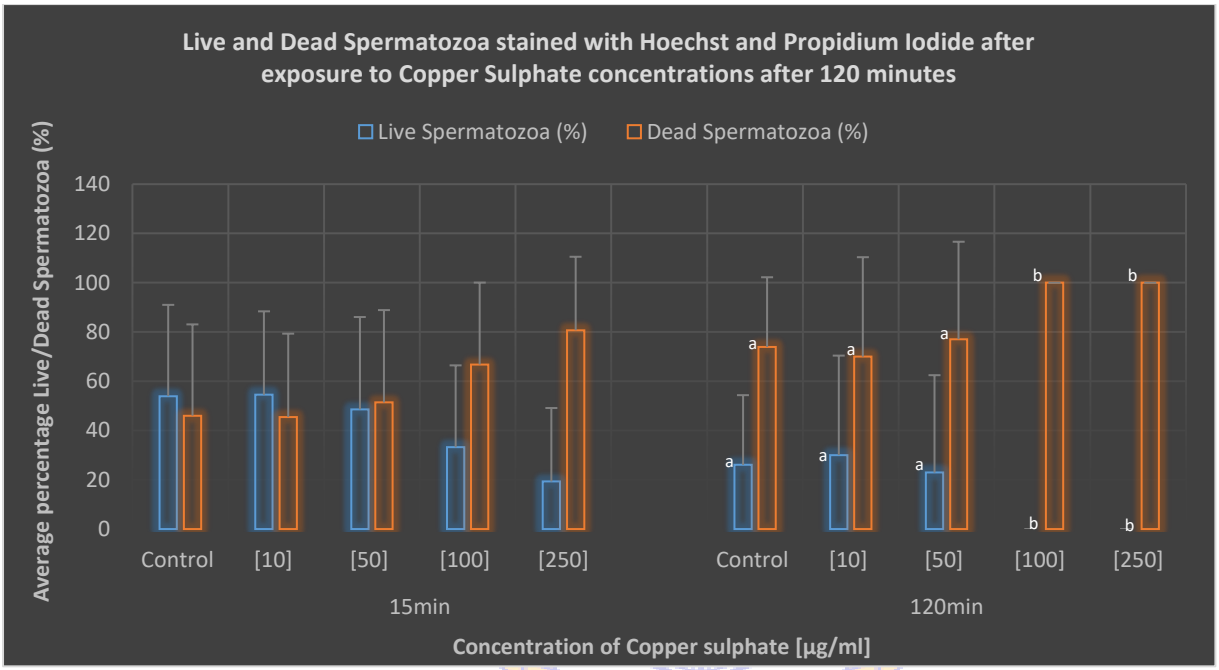


Figure 21: Average percentage live and dead vervet monkey spermatozoa at 15 and 120 minutes of exposure to Copper sulphate concentrations. a and b = significantly different values at the same time point (P<0.05)

$CdCl_2$ treatment: Although the average percentage live spermatozoa seems to have decreased with an increase in metal concentration, there was no significant effect on sperm vitality at the 15 minute time point. However, at 120 minutes a significant decrease was found in the percentage live spermatozoa when comparing the 500 µg/ml concentration to the control as well as the lower concentrations of $CdCl_2$ (P=0.001) (Figure 22).

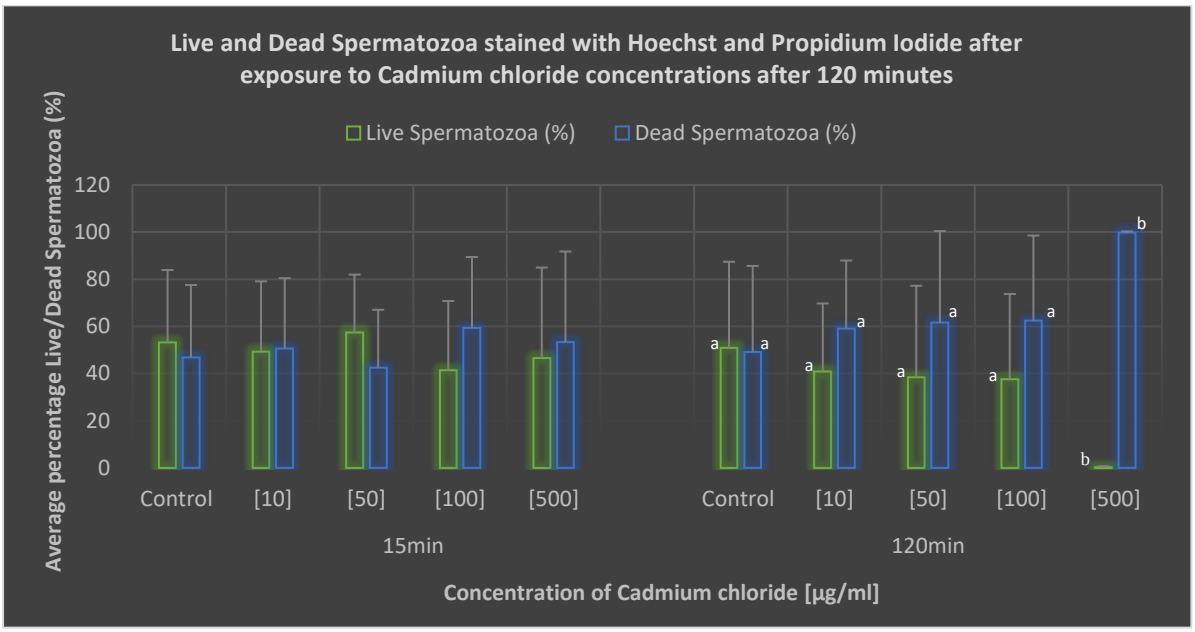


Figure 22: Average percentage live and dead vervet monkey spermatozoa that presented significant data at 120 minutes of exposure to Cadmium chloride concentrations. a and b = significantly different values at the same time point (P<0.05)

4.2.1.2.3 WST-1 assay

The vervet prepared suspensions were exposed to the following concentrations of $CuSO_4$: 10, 50, 100 and 250 µg/ml and $CdCl_2$: 10, 50, 100 and 500 µg/ml. These samples were treated with the WST-1 reagent and the absorbances were read after 60, 120 and 180 minutes of incubation (37 °C). The average absorbance readings are displayed in Table 20 and 21.

Table 20: Average absorbance readings of the vervet monkey prepared suspension (n=4) after exposure to $CuSO_4$ and treated with WST-1 for 60, 120 and 180 minutes

Time (min)	***$CuSO_4$ (µg/ml)***	***Average Absorbance Readings***
60	Control	0.048 ± 0.018
	[10]	0.009 ± 0.033
	[50]	-0.044 ± 0.044
	[100]	-0.033 ± 0.038
	[250]	-0.009 ± 0.025
120	Control	0.131 ± 0.033
	[10]	0.087 ± 0.042
	[50]	-0.024 ± 0.022
	[100]	-0.019 ± 0.023
	[250]	0.007 ± 0.010
180	Control	0.205 ± 0.034 [a]
	[10]	0.150 ± 0.062 [a]
	[50]	0.006 ± 0.010 [b]
	[100]	-0.004 ± 0.009 [b]
	[250]	0.008 ± 0.010 [b]

a and b = significantly different data relevant to the concentration of $CuSO_4$ (P<0.05)

Table 21: Average absorbance readings of the vervet monkey prepared suspension (n=3) after exposure to $CdCl_2$ and treated with WST-1 for 60, 120 and 180 minutes

Time (min)	*$CdCl_2$ (µg/ml)*	*Average Absorbance Readings*
60	Control	0.032 ± 0.027
	[10]	0.026 ± 0.016
	[50]	0.026 ± 0.031
	[100]	0.027 ± 0.029
	[500]	0.040 ± 0.010
120	Control	0.098 ± 0.020
	[10]	0.086 ± 0.007
	[50]	0.050 ± 0.057
	[100]	0.043 ± 0.061
	[500]	0.071 ± 0.013
180	Control	0.151 ± 0.021 [a]
	[10]	0.132 ± 0.018 [b]
	[50]	0.081 ± 0.062 [c]
	[100]	0.072 ± 0.062 [c]
	[500]	0.086 ± 0.014 [d]

a, b, c and d = significantly different data relevant to the concentration of $CdCl_2$ (P<0.05)

$CuSO_4$ treatment: The sperm average absorbance readings were not significant at the 60 and 120 minute time point. However, at 180 minutes a significant decrease was found in the average absorbance readings when comparing the 50 µg/ml, 100 µg/ml and 250 µg/ml concentrations to the control and 10 µg/ml concentration of $CuSO_4$ (P=0.031) (Figure 23). All three time points did, however, present the same trend where the absorbance decreased as the concentration of $CuSO_4$ increased.

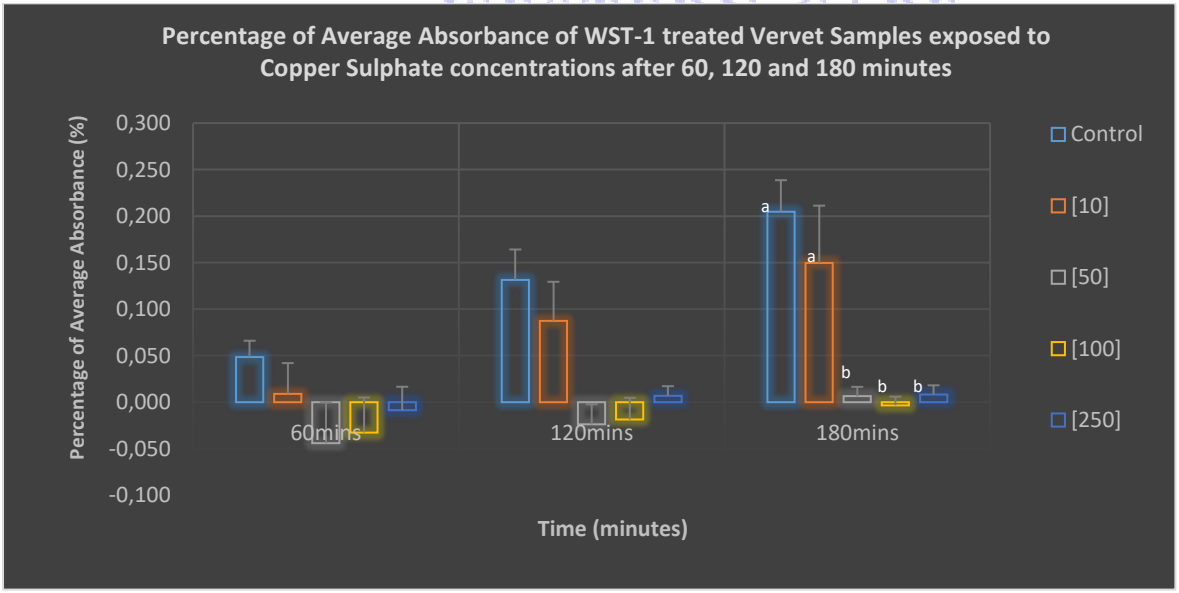


Figure 23: Average absorbance measurement for vervet monkey spermatozoa presenting significant data at 180 minutes of exposure to Copper sulphate concentrations. a and b = significantly different values at the same time point (P<0.05)

$CdCl_2$ treatment: The sperm average absorbance readings were not significant at the 60 and 120 minute time point. However, at 180 minutes a significant decrease was found in the average absorbance readings when comparing the 10 µg/ml, 50 µg/ml, 100 µg/ml and 500 µg/ml concentrations to the control (P=0.013) (Figure 24). All three time points did, however, present the same trend where the absorbance decreased until the 100 µg/ml concentration and slightly increased at the 500 µg/ml concentration of $CdCl_2$.

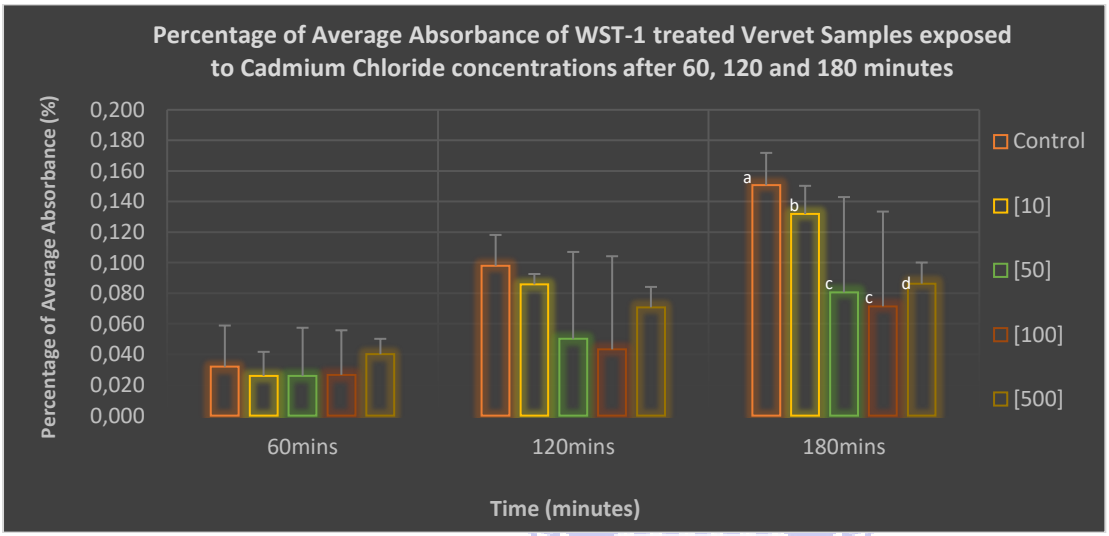


Figure 24: Average absorbance measurement for vervet monkey spermatozoa presenting significant data at 180 minutes of exposure to Cadmium chloride concentrations. a, b, c and d = significantly different values at the same time point (P<0.05)

4.2.1.3 Acrosome Intactness

The acrosome intactness of vervet monkey prepared samples was evaluated with the addition of the metals $CuSO_4$ (10 µg/ml, 50 µg/ml, 100 µg/ml and 250 µg/ml) and $CdCl_2$ (10 µg/ml, 50 µg/ml, 100 µg/ml and 500 µg/ml). The prepared samples were exposed to each metal and compared to the reagent after 15 and 120 minutes of incubation (37 °C). The average percentage intact and reacted acrosomes for $CuSO_4$ and $CdCl_2$ are displayed in Table 22 and 23.

Table 22: Average intact and reacted acrosome percentages of vervet monkey prepared samples (n=7) after exposure to $CuSO_4$ for 15 and 120 minutes

Time (min)	*$CuSO_4$ (µg/ml)*	Intact Acrosome (%)	*Reacted Acrosome (%)*
15	Control	26.15 ± 22.85	73.85 ± 22.85
	[10]	35.64 ± 24.81	64.36 ± 24.81
	[50]	21.09 ± 26.91	78.91 ± 26.91
	[100]	28.18 ± 33.29	71.82 ± 33.29
	[250]	24.43 ± 28.31	75.57 ± 28.31
120	Control	34.40 ± 20.38	65.57 ± 20.35
	[10]	34.23 ± 26.61	65.77 26.61
	[50]	27.75 ± 30.81	72.25 ± 30.81
	[100]	28.21 ± 24.67	71.79 ± 24.67
	[250]	25.04 ± 35.00	74.96 ± 35.00

Table 23: Average intact and reacted acrosome percentages of vervet monkey prepared samples (n=7) after exposure to $CdCl_2$ for 15 and 120 minutes

Time (min)	*$CdCl_2$ (µg/ml)*	Intact Acrosome (%)	*Reacted Acrosome (%)*
15	Control	43.52 ± 35.04	56.48 ± 35.04
	[10]	54.13 ± 40.79	45.87 ± 40.79
	[50]	52.14 ± 39.43	47.86 ± 39.43
	[100]	47.17 ± 36.50	52.83 ± 36.50
	[500]	48.75 ± 37.57	51.25 ± 37.57
120	Control	37.77 ± 36.08	62.23 ± 36.08
	[10]	47.45 ± 35.53	52.55 ± 35.53
	[50]	41.08 ± 33.13	58.92 ± 33.13
	[100]	30.93 ± 35.81	69.07 ± 35.81
	[500]	28.85 ± 23.15	71.15 ± 23.15

$CuSO_4$ treatment: The average percentage intact and reacted acrosomes at both 15 and 120 minutes were not significantly different between the control and four metal concentrations. It was also seen that the percentage intact acrosome values were low in comparison to the percentage reacted acrosomes at all concentrations of $CuSO_4$ and at both time points.

$CdCl_2$ treatment: The same effect was seen for the $CdCl_2$ treatment as was found for the $CuSO_4$ treatment, at both 15 and 120 minutes.

4.2.1.4 Hyperactivation

As previously mentioned the stimulant caffeine was used to induce hyperactivation. Caffeine was used at a concentration of 5 mM and 100 µg/ml of each metal, $CuSO_4$ and $CdCl_2$, in combination with it. A total of five samples were exposed to caffeine, $CuSO_4$ and $CdCl_2$, and a combination of the metal and caffeine. The motility of the samples we measured at 15, 20, 40, 60 and 90 minutes of incubation (37 °C). The average percentage hyperactivation for caffeine, $CuSO_4$, $CdCl_2$ and their combinations are displayed in Table 24 and 25.

Table 24: Average percentage sperm hyperactivation of the vervet monkey samples after exposure to $CuSO_4$ (100 µg/ml) and caffeine (5 mM) at 15, 20, 40, 60 and 90 minutes (n= 5).

Time (min)	***$CuSO_4$ and Caffeine (µg/ml) (mM)***	***Hyperactivation (%)***
15	Control	10.08 ± 10.58
	Caf [5]	11.99 ± 3.24
	$CuSO_4$ [100]	4.18 ± 3.64
	Caf [5]+ $CuSO_4$ [100]	16.81 ± 1.90
20	Control	7.57 ± 7.70
	Caf [5]	7.04 ± 7.14
	$CuSO_4$ [100]	3.35 ± 5.13
	Caf [5]+ $CuSO_4$ [100]	13.15 ± 12.38
40	Control	5.55 ± 6.26 [a]
	Caf [5]	6.70 ± 8.48 [a]
	$CuSO_4$ [100]	1.05 ± 1.38 [b]
	Caf [5]+ $CuSO_4$ [100]	5.55 ± 5.07 [c]
60	Control	3.79 ± 5.20
	Caf [5]	6.53 ± 10.09
	$CuSO_4$ [100]	1.65 ± 2.36
	Caf [5]+ $CuSO_4$ [100]	3.73 ± 7.79
90	Control	7.10 ± 8.73 [a]
	Caf [5]	6.39 ± 10.04 [a]
	$CuSO_4$ [100]	0.00 ± 0.00 [b]
	Caf [5]+ $CuSO_4$ [100]	0.80 ± 0.71 [c]

a, b and c = significantly different data relevant to the concentration of $CuSO_4$ and Caffeine ($P<0.05$)

Table 25: Average percentage sperm hyperactivation of the vervet monkey samples after exposure to $CdCl_2$ (100 µg/ml) and caffeine (5 mM) at 15, 20, 40, 60 and 90 minutes (n= 5).

Time (min)	*$CdCl_2$ and Caffeine (µg/ml) (mM)*	*Hyperactivation (%)*
15	Control	6.00 ± 1.59 [a]
	Caf [5]	14.08 ± 12.83 [b]
	$CdCl_2$ [100]	0.41 ± 0.83 [c]
	Caf [5]+ $CdCl_2$ [100]	12.81 ± 18.47 [d]
20	Control	7.67 ± 6.62 [a]
	Caf [5]	11.15 ± 16.30 [a]
	$CdCl_2$ [100]	0.75 ± 1.68 [b]
	Caf [5]+ $CdCl_2$ [100]	9.21 ± 15.65 [c]
40	Control	3.92 ± 4.96 [a]
	Caf [5]	6.80 ± 13.47 [a]
	$CdCl_2$ [100]	0.42 ± 0.93 [b]
	Caf [5]+ $CdCl_2$ [100]	7.21 ± 10.72 [c]
60	Control	4.88 ± 3.59 [a]
	Caf [5]	4.91 ± 10.58 [a]
	$CdCl_2$ [100]	0.30 ± 0.41 [b]
	Caf [5]+ $CdCl_2$ [100]	2.95 ± 5.96 [c]
90	Control	5.99 ± 8.47
	Caf [5]	7.96 ± 11.26
	$CdCl_2$ [100]	0.29 ± 0.41
	Caf [5]+ $CdCl_2$ [100]	9.55 ± 13.51

a and b = significantly different data relevant to the concentration of $CdCl_2$ and Caffeine (P<0.05)

$CuSO_4$ and Caffeine treatment: The percentage hyperactivation was measured over time for each treatment and it was found that only the treatment with 100 µg/ml $CuSO_4$ alone presented significant data at two time points (P=0.018). At 40 minutes, the percentage hyperactivation of 100 µg/ml $CuSO_4$ was significantly lower compared to the control (P=0.012), and at 90 minutes, the percentage hyperactivation of 100 µg/ml $CuSO_4$ (P<0.001) and Caf+$CuSO_4$ (P=0.013) was significantly lower compared to the control (Figure 25). A definite trend was, however, noted at each time point where the percentage hyperactivation was the lowest when spermatozoa were treated with 100 µg/ml $CuSO_4$ alone. Caffeine did not seem to increase the percentage hyperactivation compared to the control, but the combination of the caffeine and $CuSO_4$ kept the percentage hyperactivation values similar to the control, except at 90 minutes.

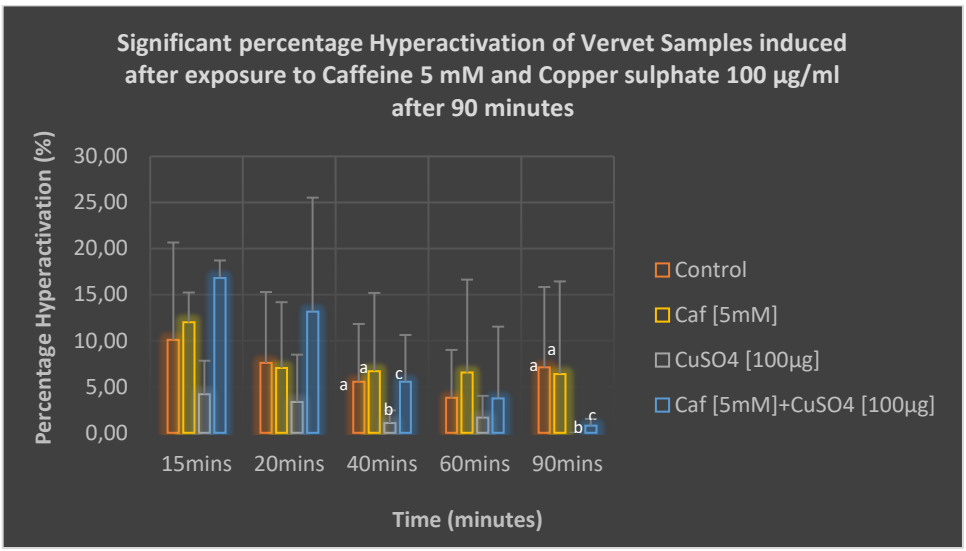


Figure 25: Average percentage sperm hyperactivation of the vervet monkey samples presenting significant data after exposure to $CuSO_4$ (100 µg/ml) and caffeine (5 mM) for 15- 90 minutes of incubation (37°C). a, b and c = significantly different values at the same time point ($P<0.05$).

$CdCl_2$ and Caffeine treatment: The percentage hyperactivation was measured over time for each treatment and for most time points, it was found that $CdCl_2$ alone or in combination with caffeine had significant data compared to the control samples presented ($P=0.025$). At 15 minutes, the percentage hyperactivation of caffeine ($P=0.006$) and the combination of Caf+$CdCl_2$ ($P=0.002$) was significantly higher compared to the control. The percentage hyperactivation of 100 µg/ml $CdCl_2$ alone was also significantly lower compared to the control at 20 ($P=0.021$), 40 ($P=0.007$) and 60 ($P=0.001$) minutes (Figure 26). A similar trend was noted for $CdCl_2$ treatment as with $CuSO_4$, where the percentage hyperactivation was lower when treated with the metal, caffeine maintained or increased percentage hyperactivation and the combination of caffeine and $CdCl_2$ kept the values similar and even higher than the control values at each time point except for the 90 minute time point.

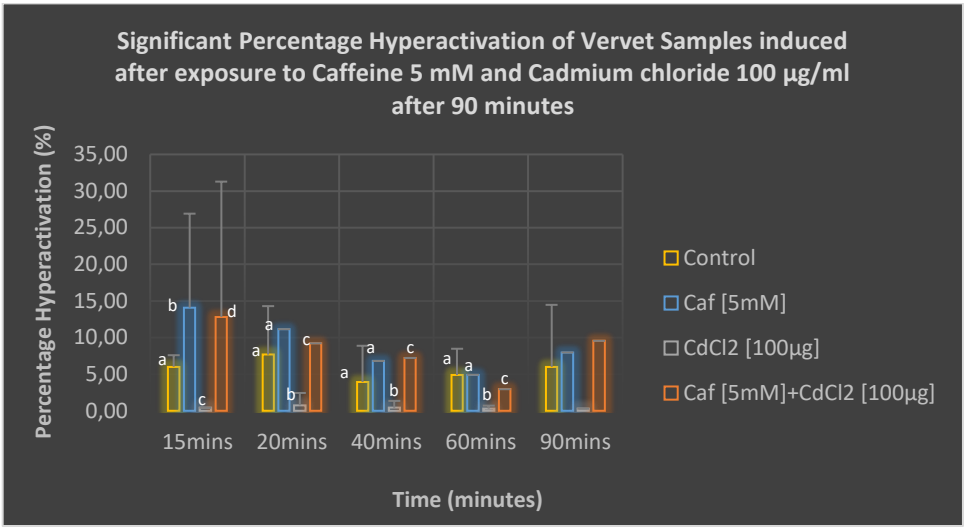


Figure 26: Average percentage sperm hyperactivation of the vervet monkey samples presenting significant data after exposure to $CdCl_2$ (100 µg/ml) and caffeine (5 mM) after 15-90 minutes of incubation (37 °C). a - d = significantly different values at the same time point ($P<0.05$).

4.2.2 Baboon

The prepared baboon samples were exposed to the metals $CuSO_4$ and $CdCl_2$, where after the following functional tests were performed: motility and longevity, vitality (E-N and H&P) and acrosome intactness.

4.2.2.1 Motility and longevity

A time-based study was performed to evaluate the motility parameters and longevity of the baboon sperm suspension. A total of four samples were exposed to each metal and motility was measured at 15, 45 and 75 minutes. The samples were exposed to the following concentrations of $CuSO_4$: 10, 50, 100 and 250 µg/ml (Table 26.1 and 26.2) and $CdCl_2$: 10, 50, 100 and 500 µg/ml (Table 27.1 and 27.2). Reason being for the lower total motility averages compared to the values reported in 4.1.2.2.1, may be due to the low total motility percentage for one of the four samples, which therefore resulted in a low total motility average and higher standard deviations compared to what was seen before.

$CuSO_4$ treatment: After 15 minutes of exposure to $CuSO_4$, three out of the thirteen motility parameters presented significantly lower values for the 50 µg/ml concentration when compared to the control and 10 µg/ml $CuSO_4$ concentrations. This decrease in the three kinematic parameters might be due to a sampling effect, since both the 100 µg/ml and 250 µg/ml concentrations had similar values to the control. After 45 minutes, one out of the thirteen parameters, straightness, decreased significantly after the 250 µg/ml concentration exposure. After 75 minutes, five out of the thirteen parameters presented significant lower values not only for the 250 µg/ml concentration, but for some parameters also for the 50 µg/ml and 100 µg/ml concentrations as well (Figure 27). A definite trend was seen, where most baboon sperm motility

parameters presented a decrease in values, especially after 75 minutes of exposure to the highest concentration of CuSO4, 250 µg/ml, compared to the control.

Table 26.1: Average measurements for baboon sperm motility parameters after exposure to copper sulphate (10-250 µg/ml) at 15, 45 and 75 minutes (n=4).

Time (mins)	*Conc. Of $CuSO_4$ (µg/ml)*	*Total Motility (%)*	*Progressive Motility (%)*	*Rapid Spermatozoa (%)*	*Medium Spermatozoa (%)*	*Slow Spermatozoa (%)*
15	Control	61.95 ± 13.14	35.98 ± 9.16	0.83 ± 0.86	13.38 ± 5.90	33.54 ± 9.33
	[10]	60.37 ± 18.75	29.34 ± 9.64	1.78 ± 2.51	11.22 ± 5.84	29.96 ± 7.52
	[50]	50.53 ± 32.91	22.68 ± 16.22	1.49 ± 2.79	8.29 ± 8.17	25.57 ± 18.01
	[100]	62.68 ± 17.21	29.74 ± 13.62	1.95 ± 2.69	11.20 ± 9.41	31.50 ± 4.97
	[250]	54.94 ± 14.92	26.63 ± 9.97	1.29 ± 1.77	9.00 ± 5.60	28.99 ± 9.12
45	Control	50.72 ± 12.50	16.39 ± 7.86	0.11 ± 0.22	3.45 ± 4.01	25.93 ± 3.52
	[10]	45.02 ± 26.63	13.04 ± 13.83	0.82 ± 1.63	3.96 ± 6.15	17.91 ± 10.21
	[50]	37.94 ± 29.16	10.08 ± 11.18	0.31 ± 0.62	2.01 ± 4.01	17.45 ± 12.93
	[100]	50.71 ± 15.48	14.38 ± 16.25	0.97 ± 1.94	3.64 ± 6.29	22.39 ± 7.39
	[250]	25.43 ± 20.85	9.45 ± 10.81	0.43 ± 0.87	2.91 ± 4.59	11.63 ± 11.02
75	Control	54.52 ± 13.89	15.09 ± 13.46	0.09 ± 0.16 [a]	3.25 ± 4.78	24.72 ± 11.62
	[10]	44.03 ± 27.94	12.92 ± 16.27	1.01 ± 1.76 [a]	3.85 ± 5.80	17.61 ± 11.45
	[50]	34.16 ± 29.16	9.86 ± 13.31	0.15 ± 0.26 [a]	2.74 ± 4.75	13.98 ± 12.91
	[100]	47.06 ± 9.28	12.20 ± 12.46	0.65 ± 1.13 [a]	3.50 ± 5.59	18.44 ± 8.57
	[250]	16.83 ± 15.58	4.37 ± 3.79	0.00 ± 0.00 [b]	0.72 ± 0.64	8.20 ± 7.63

Table 26.2: Average measurements for baboon sperm kinematic parameters after exposure to copper sulphate (10-250 µg/ml) at 15, 45 and 75 minutes (n=4).

Time (mins)	*Conc. of $CuSO_4$ (µg/ml)*	*VCL (µm/s)*	*VSL (µm/s)*	*VAP (µm/s)*	*LIN (%)*	*STR (%)*	*WOB (%)*	*ALH (µm)*	*BCF (Hz)*
15	Control	116.56 ± 18.75	88.07 ± 18.44	101.54 ± 21.38	75.21 ± 5.08 [a]	86.78 ± 1.23 [a]	86.68 ± 5.85	1.81 ± 0.15	15.42 ± 0.82 [a]
	[10]	110.46 ± 17.69	70.68 ± 21.12	83.40 ± 22.65	64.18 ± 16.23 [a]	84.21 ± 4.10 [a]	75.64 ± 16.15	2.26 ± 0.68	15.95 ± 3.45 [a]
	[50]	94.30 ± 35.28	59.82 ± 38.34	73.53 ± 42.22	57.35 ± 28.41 [b]	74.02 ± 19.82 [b]	72.33 ± 25.89	1.42 ± 0.99	11.20 ± 7.60 [b]
	[100]	106.22 ± 31.29	76.17 ± 26.41	90.14 ± 30.18	70.96 ± 4.43 [ab]	84.33 ± 1.57 [ab]	84.14 ± 5.16	1.86 ± 0.24	14.35 ± 0.72 [ab]
	[250]	106.36 ± 15.57	75.78 ± 17.84	89.13 ± 18.40	70.59 ± 9.22 [ab]	84.54 ± 4.69 [ab]	83.26 ± 7.26	1.82 ± 0.30	14.16 ± 0.49 [ab]
45	Control	80.56 ± 16.30	53.76 ± 10.81	63.03 ± 13.48	66.98 ± 6.05	85.51 ± 2.74 [a]	78.25 ± 5.13	1.74 ± 0.24	15.98 ± 1.57
	[10]	79.68 ± 31.92	46.14 ± 32.86	55.90 ± 35.20	54.83 ± 22.53	78.78 ± 12.12 [a]	67.30 ± 20.14	1.69 ± 0.60	14.73 ± 5.28
	[50]	63.98 ± 35.76	39.99 ± 33.59	46.86 ± 35.77	52.80 ± 25.14	78.38 ± 13.57 [a]	64.35 ± 23.46	1.09 ± 0.82	9.92 ± 7.41
	[100]	77.05 ± 30.98	49.01 ± 28.54	57.91 ± 30.69	60.84 ± 9.60	83.20 ± 4.27 [a]	72.85 ± 7.86	1.48 ± 0.59	13.72 ± 4.01
	[250]	60.84 ± 46.61	42.00 ± 32.34	48.30 ± 37.49	51.85 ± 34.69	65.41 ± 43.61 [b]	59.45 ± 39.76	1.18 ± 0.90	11.37 ± 7.95
75	Control	74.75 ± 22.90	47.60 ± 16.07	56.43 ± 20.17	63.36 ± 2.70 [a]	84.76 ± 1.70 [a]	74.80 ± 4.36 [a]	1.68 ± 0.19	16.86 ± 1.22 [a]
	[10]	81.58 ± 34.20	46.41 ± 37.15	56.01 ± 40.70	52.69 ± 25.06 [a]	78.96 ± 11.94 [a]	64.39 ± 24.04 [a]	1.86 ± 0.32	17.78 ± 6.20 [a]
	[50]	53.64 ± 53.20	35.14 ± 36.71	41.04 ± 42.75	42.60 ± 37.23 [b]	56.98 ± 49.35 [b]	49.83 ± 43.50 [b]	1.10 ± 1.01	10.20 ± 8.93 [b]
	[100]	77.73 ± 33.12	48.40 ± 28.03	58.19 ± 33.65	59.38 ± 11.37 [ab]	83.07 ± 0.72 [ab]	71.42 ± 13.31 [ab]	1.55 ± 0.53	12.23 ± 3.88 [c]
	[250]	50.25 ± 43.76	33.54 ± 29.87	38.79 ± 34.22	44.28 ± 38.70 [bc]	57.47 ± 49.82 [bc]	51.29 ± 44.60 [bc]	1.14 ± 0.99	11.34 ± 9.82 [c]

Conc-concentration, $CuSO_4$-copper sulphate, VCL-curvilinear velocity, VSL- straight-line velocity, VAP-average-path velocity, LIN-linearity, STR-straightness, WOB-wobble, ALH-amplitude of lateral head displacement and BCF-beat-cross frequency, a - c = significantly different data relevant to the concentration of $CuSO_4$ ($P<0.05$).

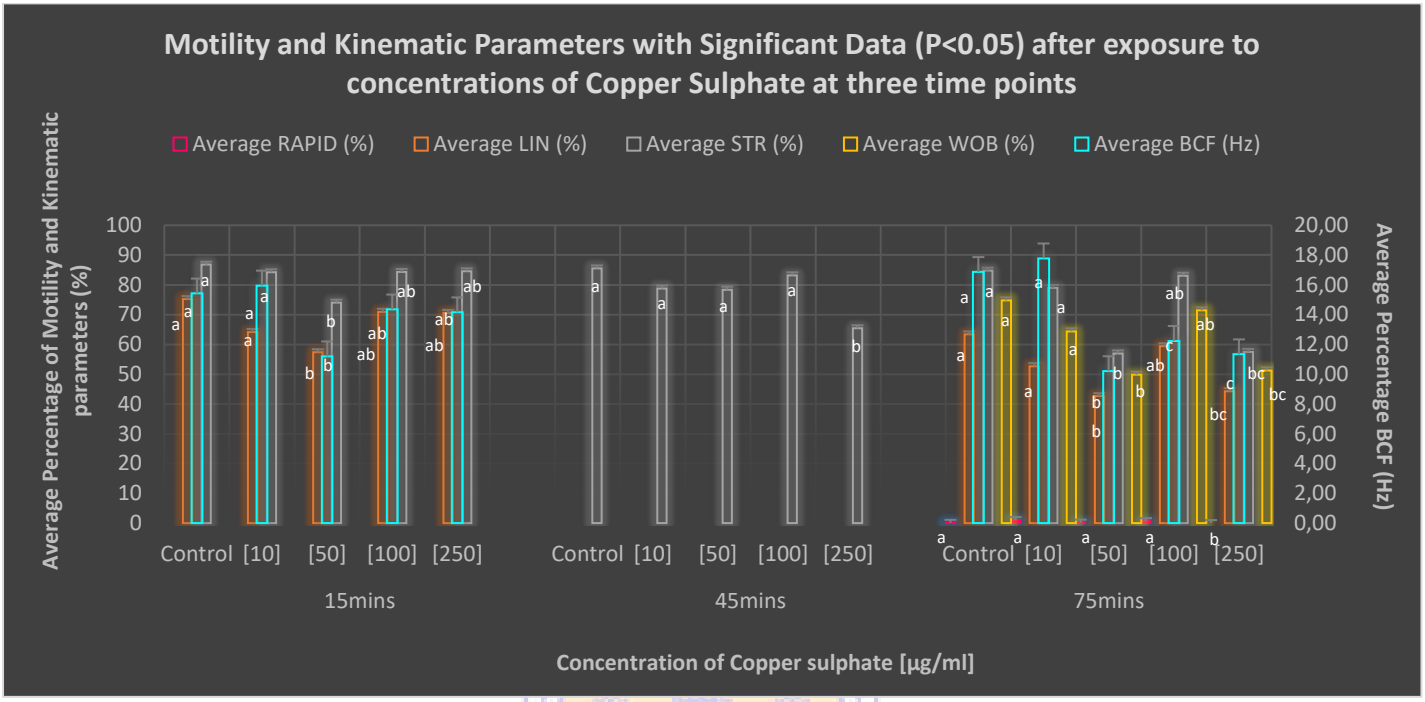


Figure 27: Average motility and kinematic parameters that presented significant data at 15, 45 and 75 minutes of exposure to Copper Sulphate (10-250 µg/ml). a - c = significantly different (P<0.05)

$CdCl_2$ treatment: After 15 minutes of exposure to $CdCl_2$, three out of thirteen sperm motility parameters presented significantly lower values not only for the 500 µg/ml concentration, but one parameter, LIN, also for the 10 µg/ml and 50 µg/ml concentration as well. This decrease in the three kinematic parameters for the two lowest metal concentrations might be due to a sampling effect, since the 100 µg/ml had similar values to the control. After 45 minutes, four out of the thirteen motility parameters decreased significantly after the 500 µg/ml concentration exposure. For percentage rapid spermatozoa, significant differences were also found for the 10 µg/ml, 50 µg/ml and 100 µg/ml concentrations, however, this may be due to a sampling effect as these values were higher than that of the control. After 75 minutes, eight of the thirteen motility parameters presented significant lower values not only for the 500 µg/ml concentration, but one parameter, rapid spermatozoa, also for the 100 µg/ml concentration (Figure 28 and 29). A trend was noted, where most baboon sperm motility parameters presented a decrease in values, especially after 75 minutes of exposure to the highest concentrations of $CdCl_2$, 100 µg/ml and 500 µg/ml, compared to the control.

Table 27.1: Average measurements for baboon sperm motility parameters after exposure to Cadmium chloride (10-500 µg/ml) at 15, 45 and 75 minutes (n=4).

Time (mins)	*Conc. of $CdCl_2$ (µg/ml)*	*Total Motility (%)*	*Progressive Motility (%)*	*Rapid Spermatozoa (%)*	*Medium Spermatozoa (%)*	*Slow Spermatozoa (%)*
15	Control	66.88 ± 34.88	46.11 ± 30.47	1.07 ± 1.96	18.26 ± 16.32	40.22 ± 19.00
	[10]	74.02 ± 36.83	51.66 ± 30.14	5.11 ± 6.35	25.33 ± 16.46	32.48 ± 21.58
	[50]	65.08 ± 43.81	47.70 ± 27.56	4.86 ± 7.91	19.22 ± 13.37	34.61 ± 20.48
	[100]	69.55 ± 36.04	45.30 ± 30.93	6.35 ± 8.94	16.96 ± 15.38	34.34 ± 22.64
	[500]	52.26 ± 39.17	24.80 ± 23.57	0.83 ± 0.95	10.05 ± 11.65	25.58 ± 21.73
45	Control	57.10 ± 34.14	26.15 ± 19.33	0.15 ± 0.18 [a]	7.17 ± 6.25	34.10 ± 22.53
	[10]	64.53 ± 29.44	36.79 ± 22.74	2.14 ± 1.74 [b]	8.76 ± 11.72	42.01 ± 23.46
	[50]	61.95 ± 33.13	35.87 ± 20.84	2.44 ± 2.82 [c]	11.67 ± 10.07	36.40 ± 24.07
	[100]	61.78 ± 27.10	31.84 ± 19.66	2.19 ± 2.45 [c]	11.38 ± 12.74	30.93 ± 18.77
	[500]	0.90 ± 1.08	0.00 ± 0.00	0.00 ± 0.00 [d]	0.00 ± 0.00	0.09 ± 0.18
75	Control	73.21 ± 16.45 [a]	32.19 ± 13.78	0.00 ± 0.00 [a]	6.68 ± 5.40 [a]	49.02 ± 16.60 [a]
	[10]	79.20 ± 7.32 [a]	34.09 ± 11.53	1.13 ± 1.67 [b]	6.56 ± 6.48 [a]	48.40 ± 8.93 [a]
	[50]	73.58 ± 7.40 [a]	29.06 ± 21.53	1.84 ± 2.72 [b]	8.40 ± 10.91 [b]	38.03 ± 6.85 [a]
	[100]	67.76 ± 8.30 [a]	22.74 ± 13.87	0.00 ± 0.00 [ac]	6.89 ± 8.04 [ab]	33.39 ± 8.93 [a]
	[500]	0.00 ± 0.00 [b]	0.00 ± 0.00	0.00 ± 0.00 [ac]	0.00 ± 0.00 [c]	0.00 ± 0.00 [b]

Table 27.2: Average measurements for baboon sperm kinematic parameters after exposure to Cadmium chloride (10-500 µg/ml) at 15, 45 and 75 minutes (n=4).

Time (mins)	*Conc. of $CdCl_2$ (µg/ml)*	*VCL (µm/s)*	*VSL (µm/s)*	*VAP (µm/s)*	*LIN (%)*	*STR (%)*	*WOB (%)*	*ALH (µm)*	*BCF (Hz)*
15	Control	125.47 ± 22.64	91.47 ± 15.29	108.71 ± 22.11	73.11 ± 3.89 [a]	84.60 ± 4.13 [a]	86.43 ± 2.59 [a]	2.01 ± 0.38	16.48 ± 2.92
	[10]	155.71 ± 34.36	95.45 ± 28.29	138.03 ± 36.94	62.04 ± 14.06 [b]	71.26 ± 18.53 [a]	87.97 ± 5.61 [a]	1.87 ± 1.08	11.78 ± 4.90
	[50]	137.41 ± 36.18	105.28 ± 29.69	121.27 ± 35.81	76.36 ± 6.28 [c]	87.02 ± 4.16 [a]	87.69 ± 4.10 [a]	1.81 ± 0.58	12.70 ± 3.79
	[100]	145.31 ± 45.38	113.72 ± 44.78	129.72 ± 48.40	76.77 ± 9.57 [ab]	87.11 ± 5.53 [a]	87.93 ± 6.34 [a]	1.85 ± 0.30	15.64 ± 3.02
	[500]	76.33 ± 53.98	50.87 ± 35.89	60.84 ± 44.04	50.01 ± 33.39 [d]	63.23 ± 42.29 [b]	59.40 ± 39.72 [b]	1.42 ± 0.97	11.69 ± 7.88
45	Control	101.48 ± 19.37	71.66 ± 21.87 [a]	85.58 ± 22.93	69.49 ± 8.87	83.15 ± 4.23 [a]	83.38 ± 7.10 [a]	1.82 ± 0.55	12.64 ± 3.71
	[10]	113.92 ± 28.02	81.13 ± 24.60 [a]	94.82 ± 29.41	70.50 ± 4.06	85.64 ± 1.70 [a]	82.34 ± 5.02 [a]	1.79 ± 0.29	15.17 ± 1.48
	[50]	124.39 ± 29.22	98.39 ± 31.25 [a]	111.94 ± 33.59	77.93 ± 7.53	87.52 ± 1.93 [a]	88.94 ± 6.68 [a]	1.79 ± 0.25	13.95 ± 1.86
	[100]	112.21 ± 29.68	85.12 ± 32.26 [a]	96.12 ± 33.22	74.14 ± 9.76	87.72 ± 4.43 [a]	84.32 ± 7.75 [a]	1.86 ± 0.31	15.22 ± 2.05
	[500]	22.59 ± 26.34	6.15 ± 8.19 [b]	9.05 ± 11.26	14.29 ± 20.01	32.70 ± 38.47 [b]	20.78 ± 27.10 [b]	0.00 ± 0.00	0.00 ± 0.00
75	Control	92.15 ± 15.45 [a]	63.41 ± 13.05 [a]	76.21 ± 15.88 [a]	68.53 ± 3.17 [a]	83.23 ± 0.72	82.35 ± 4.38	2.00 ± 0.22	13.75 ± 0.62
	[10]	93.02 ± 19.14 [a]	62.36 ± 17.30 [a]	75.08 ± 20.57 [a]	66.55 ± 6.95 [a]	83.00 ± 0.83	80.14 ± 7.69	1.87 ± 0.19	14.76 ± 1.29
	[50]	93.30 ± 36.77 [a]	63.65 ± 34.71 [a]	75.54 ± 39.38 [a]	65.82 ± 9.46 [a]	83.66 ± 2.11	78.58 ± 9.87	1.81 ± 0.11	14.96 ± 0.72
	[100]	81.72 ± 20.22 [a]	54.97 ± 19.92 [a]	64.77 ± 22.27 [a]	66.20 ± 10.83 [a]	84.46 ± 2.29	78.19 ± 10.87	1.84 ± 0.14	14.77 ± 1.36
	[500]	0.00 ± 0.00 [b]	0.00 ± 0.00 [b]	0.00 ± 0.00 [b]	0.00 ± 0.00 [b]	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Conc-concentration, CdCl2-cadmium chloride, VCL-curvilinear velocity, VSL-straight-line velocity, VAP-average-path velocity, LIN-linearity, STR-straightness, WOB-wobble, ALH-amplitude of lateral head displacement and BCF-beat-cross frequency, a – d = significantly different data relevant to the concentration of $CdCl_2$ ($P<0.05$).

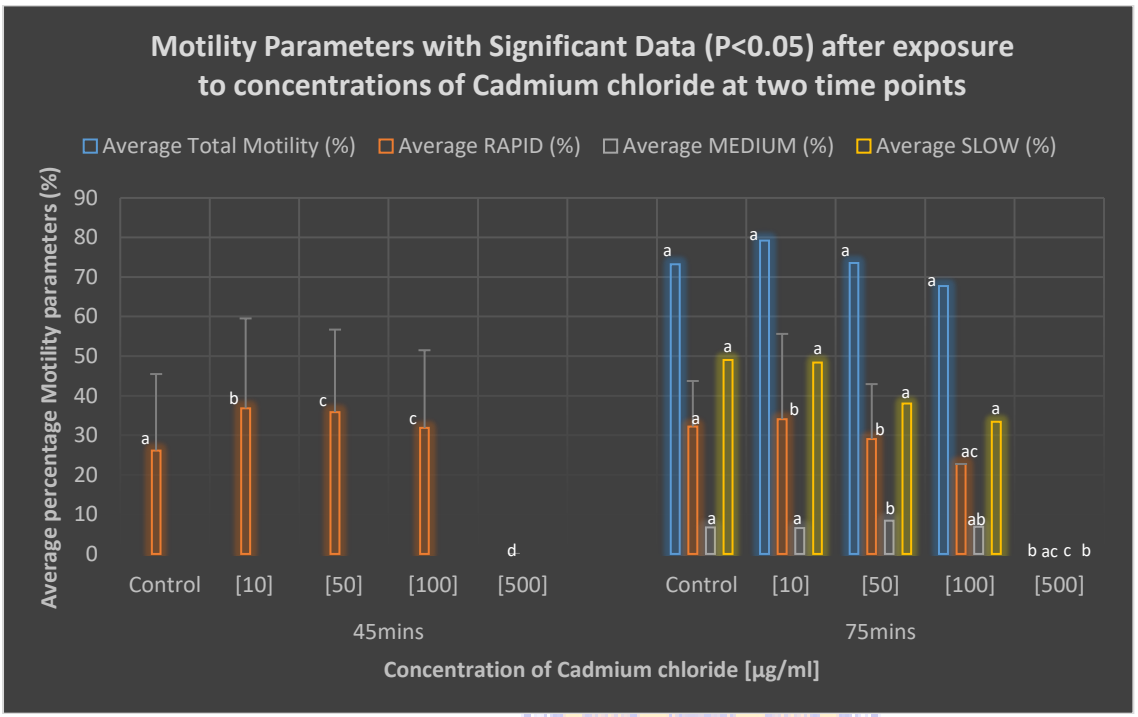


Figure 28: Average motility parameters that presented significant data at 45 and 75 minutes of exposure to Cadmium chloride (10-500 µg/ml). a - d = significantly different (P<0.05)

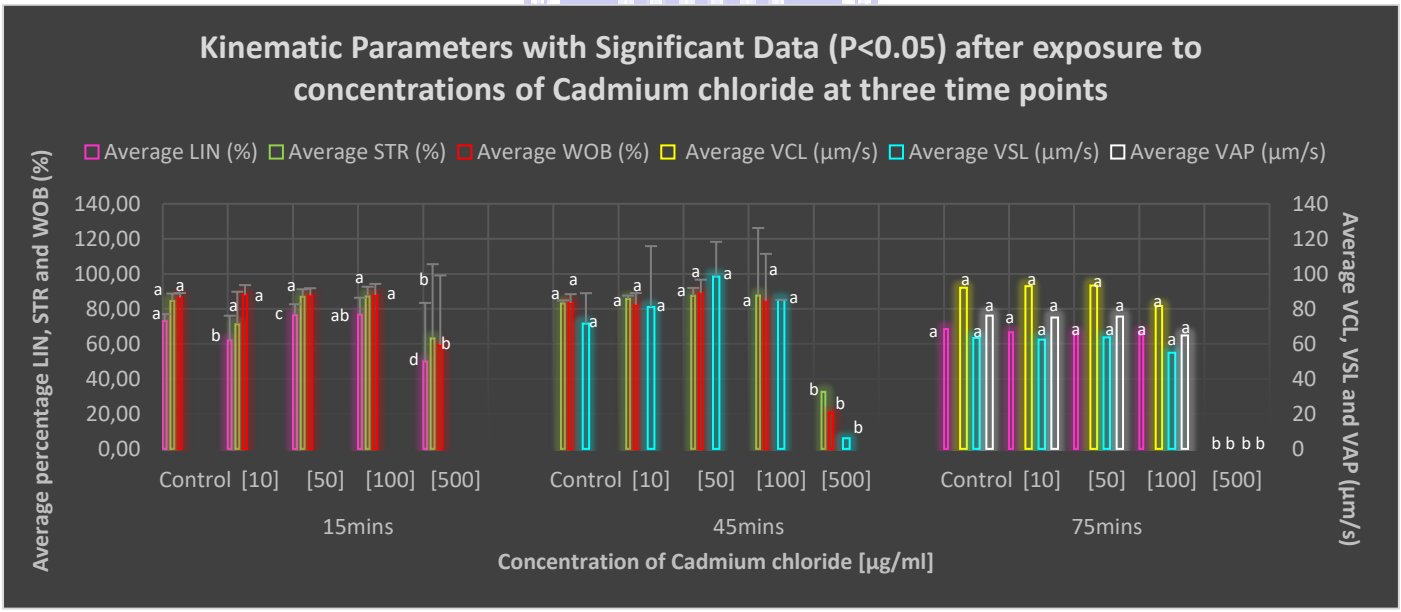


Figure 29: Average kinematic parameters that presented significant data at 15, 45 and 75 minutes of exposure to Cadmium chloride (10-500 µg/ml). a - d = significantly different (P<0.05)

4.2.2.2 Vitality

4.2.2.2.1 Eosin-Nigrosin (E-N)

The prepared baboon suspension was exposed to the E-N stain after incubation (37 °C). A total of three samples were exposed to each metal and vitality was measured at 15 and 90 minutes. The samples were exposed to the following concentrations of $CuSO_4$: 10, 50, 100 and 250 µg/ml (Table 28) and $CdCl_2$: 10, 50, 100 and 500 µg/ml (Table 29).

Table 28: Average vitality percentages of the baboon prepared suspensions (n=3) after exposure to the E-N staining kit at 15 and 90 minutes of $CuSO_4$ treatment.

Time (min)	*$CuSO_4$ (µg/ml)*	*Live Spermatozoa (%)*	*Dead Spermatozoa (%)*
15	Control	62.91 ± 11.37	37.09 ± 11.37
	[10]	46.75 ± 13.51	53.25 ± 13.51
	[50]	57.89 ± 8.84	42.11 ± 8.84
	[100]	34.27 ± 14.78	65.73 ± 14.78
	[250]	51.53 ± 18.39	48.47 ± 18.39
90	Control	51.53 ± 20.56	48.47 ± 20.56
	[10]	55.66 ± 15.13	44.34 ± 15.13
	[50]	40.44 ± 7.53	59.56 ± 7.53
	[100]	31.86 ± 9.74	68.14 ± 9.74
	[250]	55.73 ± 17.11	44.27 ± 17.11

Table 29: Average vitality percentages of the vervet prepared suspensions (n=3) after exposure to the E-N staining kit at 15 and 90 minutes of $CdCl_2$ treatment.

Time (min)	*$CdCl_2$ (µg/ml)*	*Live Spermatozoa (%)*	*Dead Spermatozoa (%)*
15	Control	44.97 ± 39.49 [a]	55.03 ± 39.49 [a]
	[10]	43.62 ± 10.25 [a]	56.38 ± 10.25 [a]
	[50]	39.64 ± 11.34 [a]	60.36 ± 11.34 [a]
	[100]	36.08 ± 15.28 [a]	63.92 ± 15.28 [a]
	[500]	12.50 ± 12.03 [b]	87.50 ± 12.03 [b]
90	Control	33.83 ± 31.27	66.17 ± 31.27
	[10]	27.08 ± 13.77	72.92 ± 13.77
	[50]	28.68 ± 25.09	71.32 ± 25.09
	[100]	19.90 ± 24.08	80.10 ± 24.08
	[500]	9.76 ± 9.10	90.24 ± 9.10

a and b = significantly different data relevant to the concentration of $CdCl_2$ ($P<0.05$)

$CuSO_4$ treatment: The data was found to be not significant for both live and dead spermatozoa at the two time points, as was seen for the vervet monkey. This either indicates that $CuSO_4$ had no effect on the vitality of the baboon sperm tested or the stain is unable to detect the effects of $CuSO_4$ on baboon sperm vitality.

$CdCl_2$ treatment: After 15 minutes of exposure to $CdCl_2$, a significant decrease (P=0.043) was seen for the percentage live spermatozoa for the 500 µg/ml $CdCl_2$. An opposite effect was noted for the percentage dead spermatozoa, with a significant increase (P=0.043) in values. However, after 90 minutes of exposure, the data was found to be not significant for both live and dead spermatozoa. The insignificance of this data was probably due to the large standard deviations recorded for all treatments, since a general trend for a decrease in the average percentage live spermatozoa was seen with an increase in the concentration of $CdCl_2$ (Figure 30). It thus seems that E-N staining could detect the effect of $CdCl_2$ on baboon sperm vitality.

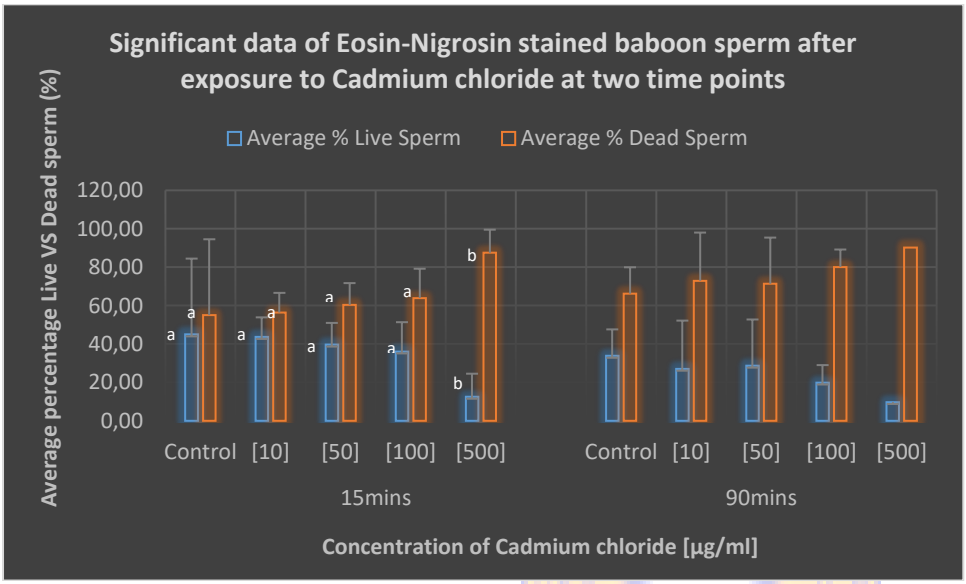


Figure 30: Average percentage live vs dead baboon spermatozoa that presented significant data at 15 and 90 minutes of exposure to Cadmium chloride (10-500 µg/ml). a and b = significantly different values at the same time point (P<0.05)

4.2.2.2.2 Hoechst and Propidium Iodide (H&P)

The baboon prepared suspensions were exposed to the H&P stains and allowed to incubate (37 °C). One sample was exposed to each metal and vitality was measured at 15 and 60-75minutes. The samples were exposed to the following concentrations of $CuSO_4$: 10, 50, 100 and 250 µg/ml and $CdCl_2$: 10, 50, 100 and 500 µg/ml.

Although only one sample was evaluated, an apparent effect was seen after 15, 60 and 75 minutes. After 15 minutes of exposure to $CuSO_4$, percentage live spermatozoa seemed to have decreased with increasing concentrations of $CuSO_4$ compared to the control. After 60 minutes of exposure to $CuSO_4$, the percentage live sperm presented a zero value for all concentrations of $CuSO_4$. After 15 minutes of exposure to $CdCl_2$, no apparent effect was seen with increasing concentrations of $CdCl_2$ compared to the control. After 75 minutes

of exposure to $CdCl_2$, the percentage live spermatozoa presented a zero value for the 500 µg/ml concentration. These results only indicate the effect of these metals on one sample, but a definite effect is seen after exposure to the highest concentrations of $CuSO_4$ and $CdCl_2$, causing a decrease in percentage live spermatozoa after 60 and 75 minutes of treatment.

4.2.2.3 Acrosome Intactness

The acrosome intactness of baboon prepared samples was evaluated with the addition of the metals $CuSO_4$ (10 µg/ml, 50 µg/ml, 100 µg/ml and 250 µg/ml) and $CdCl_2$ (10 µg/ml, 50 µg/ml, 100 µg/ml and 500 µg/ml). The prepared samples were exposed to each metal and compared to the reagent after 15 and 90 minutes of incubation (37 °C). The average percentage intact and reacted acrosomes for $CuSO_4$ and $CdCl_2$ are displayed in Table 32 and 33.

Table 32: Average intact and reacted acrosome percentages of baboon prepared samples (n=6) after exposure to $CuSO_4$ for 15 and 90 minutes

Time (min)	***$CuSO_4$ (µg/ml)***	**Intact Acrosome (%)**	***Reacted Acrosome (%)***
15	Control	74.27 ± 18.06 [a]	25.73 ± 18.06 [a]
	[10]	77.93 ± 11.16 [a]	22.07 ± 11.16 [a]
	[50]	60.21 ± 31.90 [a]	23.12 ± 16.61 [a]
	[100]	55.56 ± 31.17 [a]	44.44 ± 31.17 [a]
	[250]	19.16 ± 29.82 [b]	80.84 ± 29.82 [b]
90	Control	71.65 ± 13.23 [a]	28.35 ± 13.23 [a]
	[10]	75.02 ± 12.82 [a]	24.98 ± 12.82 [a]
	[50]	69.72 ± 17.46 [b]	30.28 ± 17.46 [b]
	[100]	45.35 ± 22.99 [c]	54.65 ± 22.99 [c]
	[250]	17.32 ± 26.97 [d]	82.68 ± 26.97 [d]

a - d = significantly different data relevant to the concentration of $CuSO_4$ ($P<0.05$)

Table 33: Average intact and reacted acrosome percentages of baboon prepared samples (n=5) after exposure to $CdCl_2$ for 15 and 90 minutes

Time (min)	***$CdCl_2$ (µg/ml)***	**Intact Acrosome (%)**	***Reacted Acrosome (%)***
15	Control	94.12 ± 7.23	5.88 ± 7.23
	[10]	91.05 ± 10.42	8.95 ± 10.42
	[50]	90.63 ± 12.52	9.37 ± 12.52
	[100]	92.60 ± 8.79	7.40 ± 8.79
	[500]	84.38 ± 7.52	15.62 ± 7.52
90	Control	91.51 ± 4.72	8.49 ± 4.72
	[10]	90.85 ± 8.88	9.15 ± 8.88
	[50]	90.22 ± 5.56	17.18 ± 20.21
	[100]	91.62 ± 7.40	8.38 ± 7.40
	[500]	85.21 ± 5.61	14.79 ± 5.61

$CuSO_4$ treatment: After 15 minutes of exposure, a significant decrease (P=0.005) was seen in average percentage intact acrosomes, with a concordant increase (P=0.001) in the average percentage reacted acrosomes, for the 250 µg/ml concentration compared to the control and other $CuSO_4$ concentrations. After 90 minutes of exposure, the same significant increase (P<0.001) and decrease (P<0.001) was seen as after 15 minutes, for average percentage intact and reacted acrosomes, with the addition of the 50 µg/ml and 100 µg/ml concentrations, compared to the control and 10 µg/ml $CuSO_4$ concentration (Figure 31).

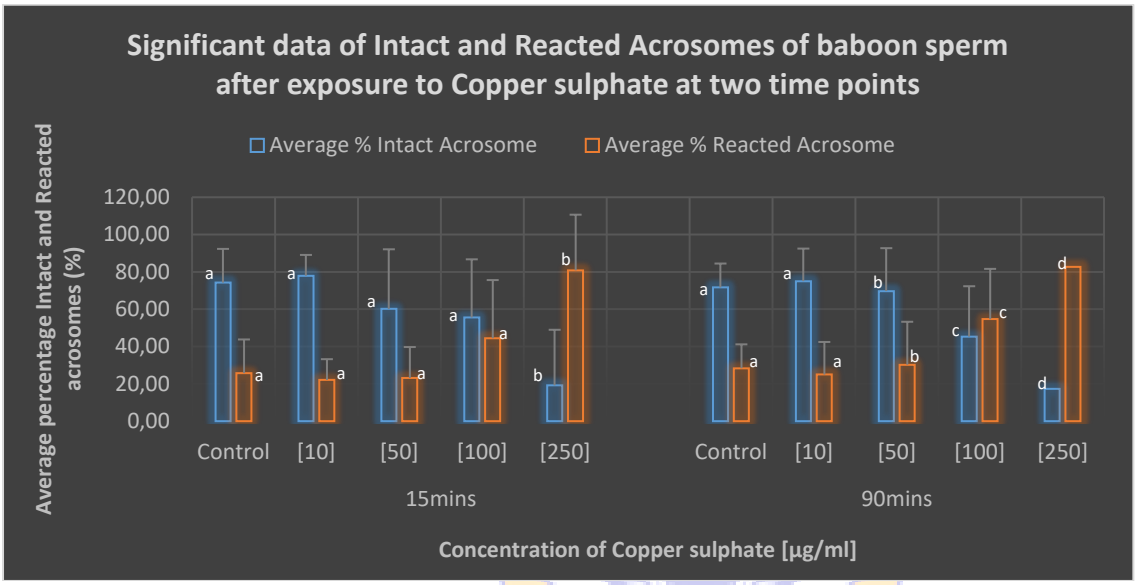


Figure 31: Average percentage intact and reacted acrosomes of baboon spermatozoa that presented significant data at 15 and 90 minutes of exposure to Copper sulphate (10-250 µg/ml). a - d = significantly different (P<0.05)

$CdCl_2$ treatment: After both 15 and 90 minutes of exposure, the average percentage intact acrosomes ranged between 84-94% for both the control and treatments and no effect was found for different $CdCl_2$ concentrations on baboon acrosome intactness.

4.2.3 Rhesus monkey

The prepared rhesus samples were exposed to the metals $CuSO_4$ and $CdCl_2$, where after the following functional tests were performed: motility and longevity, and hyperactivation.

4.2.3.1 Motility and longevity

A time-based study was performed to evaluate the motility parameters and longevity of the rhesus monkey sperm suspension. A total of three samples were exposed to each metal and motility was measured at 15, 60 and 120 minutes. The samples were exposed to the following concentrations of $CuSO_4$: 10, 50, 100 and 250 µg/ml (Table 34.1 and 34.2) and $CdCl_2$: 10, 50, 100 and 500 µg/ml (Table 35.1 and 35.2). Reason being

for the higher total motility averages compared to the value reported in 4.1.3.2.1, may be due to one sample out of the three that had a total motility of 90%, whereas the other two ranged between 30-50%. This is also resulted in higher standard deviations compared to what was seen before.

$CuSO_4$ treatment: After 15 minutes of exposure to $CuSO_4$, only one (BCF) out of the thirteen motility parameters presented significantly lower values for the 250 µg/ml concentration when compared to the control and other $CuSO_4$ concentrations. After 60 minutes, four out of the thirteen parameters, decreased significantly after the 250 µg/ml concentration exposure. After 120 minutes, nine out of the thirteen parameters presented significant lower values not only for the 250 µg/ml concentration, but for some parameters also for the 50 µg/ml and 100 µg/ml concentrations as well (Figure 32, 33 and 34). A definite trend was seen, where all the rhesus sperm motility parameters presented a decrease in values, especially after 120 minutes of exposure to the highest concentration of CuSO4, 250 µg/ml, compared to the control.

Table 34.1: Average measurements for rhesus monkey sperm motility parameters after exposure to Copper sulphate (10-250 µg/ml) at 15, 60 and 120 minutes (n=3).

Time ***(mins)***	***Conc. Of $CuSO_4$ (µg/ml)***	***Total Motility*** ***(%)***	***Progressive Motility*** ***(%)***	***Rapid Spermatozoa*** ***(%)***	***Medium Spermatozoa*** ***(%)***	***Slow Spermatozoa*** ***(%)***
15	Control	64.72 ± 26.00	51.14 ± 24.64	44.68 ± 25.92	9.87 ± 6.84	10.18 ± 1.45
	[10]	69.16 ± 23.94	58.15 ± 25.00	53.18 ± 27.74	8.33 ± 8.90	7.65 ± 5.09
	[50]	66.69 ± 23.93	54.20 ± 20.72	47.64 ± 23.30	9.18 ± 8.00	9.87 ± 2.13
	[100]	73.85 ± 22.12	59.09 ± 20.72	52.07 ± 24.64	10.63 ± 7.78	11.14 ± 4.59
	[250]	52.15 ± 43.93	41.26 ± 38.46	35.75 ± 37.57	8.25 ± 8.13	8.15 ± 4.67
60	Control	57.35 ± 24.58	33.01 ± 13.71	25.95 ± 11.37	12.02 ± 9.17	19.37 ± 10.99
	[10]	58.51 ± 23.63	34.65 ± 10.45	24.77 ± 9.47	13.86 ± 8.05	19.87 ± 12.22
	[50]	55.66 ± 26.45	30.44 ± 15.12	20.11 ± 11.51	14.30 ± 10.40	21.25 ± 12.88
	[100]	49.07 ± 36.85	23.14 ± 22.96	13.87 ± 15.68	12.65 ± 9.78	22.54 ± 13.41
	[250]	4.55 ± 5.06	1.01 ± 1.75	0.51 ± 0.87	0.71 ± 1.22	3.34 ± 3.19
120	Control	35.03 ± 37.50	11.58 ± 12.48 [a]	4.70 ± 5.17	11.02 ± 13.70 [a]	19.32 ± 19.13
	[10]	34.11 ± 29.58	9.55 ± 6.12 [a]	3.12 ± 1.66	9.24 ± 9.32 [a]	21.75 ± 20.33
	[50]	36.43 ± 34.80	10.62 ± 10.54 [a]	3.95 ± 3.69	11.86 ± 14.30 [a]	20.62 ± 17.69
	[100]	27.74 ± 30.96	4.05 ± 4.19 [b]	1.06 ± 1.28	5.01 ± 4.09 [b]	21.67 ± 26.07
	[250]	0.18 ± 0.32	0.18 ± 0.32 [c]	0.18 ± 0.32	0.00 ± 0.00 [c]	0.00 ± 0.00

Table 34.2: Average measurements for rhesus monkey sperm kinematic parameters after exposure to Copper sulphate (10-250 µg/ml) at 15, 60 and 120 minutes (n=3).

Time *(mins)*	*Conc. of $CuSO_4$ (µg/ml)*	*VCL (µm/s)*	*VSL (µm/s)*	*VAP (µm/s)*	*LIN (%)*	*STR (%)*	*WOB (%)*	*ALH (µm)*	*BCF (Hz)*
15	Control	176.69 ± 40.47	139.14 ± 41.15	154.49 ± 42.51	77.79 ± 6.29	89.63 ± 2.76	86.71 ± 4.91	2.57 ± 0.21	16.90 ± 1.18 [a]
	[10]	204.26 ± 59.85	167.38 ± 60.76	182.39 ± 59.82	80.56 ± 8.05	90.83 ± 4.29	88.56 ± 5.13	2.53 ± 0.42	17.19 ± 0.41 [a]
	[50]	189.00 ± 40.35	155.56 ± 43.00	171.77 ± 44.96	81.49 ± 6.29	90.34 ± 2.94	90.15 ± 5.16	2.16 ± 0.04	16.14 ± 1.33 [a]
	[100]	168.23 ± 41.03	126.06 ± 35.80	141.86 ± 40.63	74.39 ± 4.37	88.87 ± 1.30	83.69 ± 4.37	2.59 ± 0.31	17.92 ± 0.47 [a]
	[250]	149.88 ± 47.20	119.65 ± 38.51	134.07 ± 45.17	80.06 ± 10.76	89.49 ± 4.77	89.24 ± 7.68	2.12 ± 0.31	15.47 ± 2.92 [b]
60	Control	133.74 ± 50.35	99.09 ± 63.22	110.62 ± 61.66	70.07 ± 18.6	87.20 ± 6.73 [a]	79.82 ± 15.93 [a]	2.07 ± 0.68 [a]	18.48 ± 2.85 [a]
	[10]	129.99 ± 44.89	99.79 ± 54.07	108.87 ± 51.81	73.76 ± 14.22	89.94 ± 5.44 [a]	81.59 ± 10.81 [a]	2.17 ± 0.38 [a]	17.45 ± 1.60 [a]
	[50]	122.76 ± 43.02	92.45 ± 54.69	101.43 ± 51.71	71.50 ± 16.72	88.79 ± 7.02 [a]	79.93 ± 12.29 [a]	2.27 ± 0.43 [a]	17.50 ± 1.99 [a]
	[100]	93.63 ± 12.68	68.21 ± 17.47	78.95 ± 13.69	72.13 ± 11.53	85.48 ± 8.49 [a]	84.05 ± 5.43 [a]	2.01 ± 0.27 [a]	16.20 ± 0.42 [a]
	[250]	38.58 ± 44.15	19.80 ± 32.52	25.75 ± 38.80	24.42 ± 36.28	37.20 ± 41.19 [b]	34.95 ± 41.72 [b]	0.72 ± 1.25 [b]	4.68 ± 8.11 [b]
120	Control	85.03 ± 13.14	48.17 ± 22.66 [a]	56.36 ± 22.25 [a]	55.05 ± 17.13 [a]	83.97 ± 5.76 [a]	64.87 ± 15.50 [a]	1.98 ± 0.45 [a]	16.73 ± 5.70 [a]
	[10]	90.65 ± 31.00	55.72 ± 36.12 [a]	63.52 ± 35.12 [a]	57.66 ± 17.38 [a]	84.96 ± 7.63 [a]	67.11 ± 14.42 [a]	2.15 ± 0.23 [a]	16.65 ± 3.12 [a]
	[50]	85.51 ± 18.33	52.94 ± 35.03 [a]	60.11 ± 35.52 [b]	58.36 ± 25.70 [a]	84.62 ± 9.72 [a]	67.40 ± 21.46 [a]	2.26 ± 0.19 [a]	17.51 ± 3.66 [a]
	[100]	56.87 ± 15.69	25.38 ± 4.99 [b]	37.79 ± 2.32 [c]	45.40 ± 4.56 [a]	67.16 ± 12.86 [b]	70.01 ± 20.09 [a]	1.29 ± 1.18 [a]	11.29 ± 9.93 [a]
	[250]	14.13 ± 24.47	2.00 ± 3.47 [b]	3.93 ± 6.80 [d]	4.73 ± 8.19 [b]	17.02 ± 29.47 [b]	9.26 ± 16.04 [b]	0.00 ± 0.00 [b]	0.00 ± 0.00 [b]

Conc-concentration, $CuSO_4$-copper sulphate, VCL-curvilinear velocity, VSL- straight-line velocity, VAP-average-path velocity, LIN-linearity, STR-straightness, WOB-wobble, ALH-amplitude of lateral head displacement and BCF-beat-cross frequency, a - d = significantly different data relevant to the concentration of $CuSO_4$ (P<0.05).

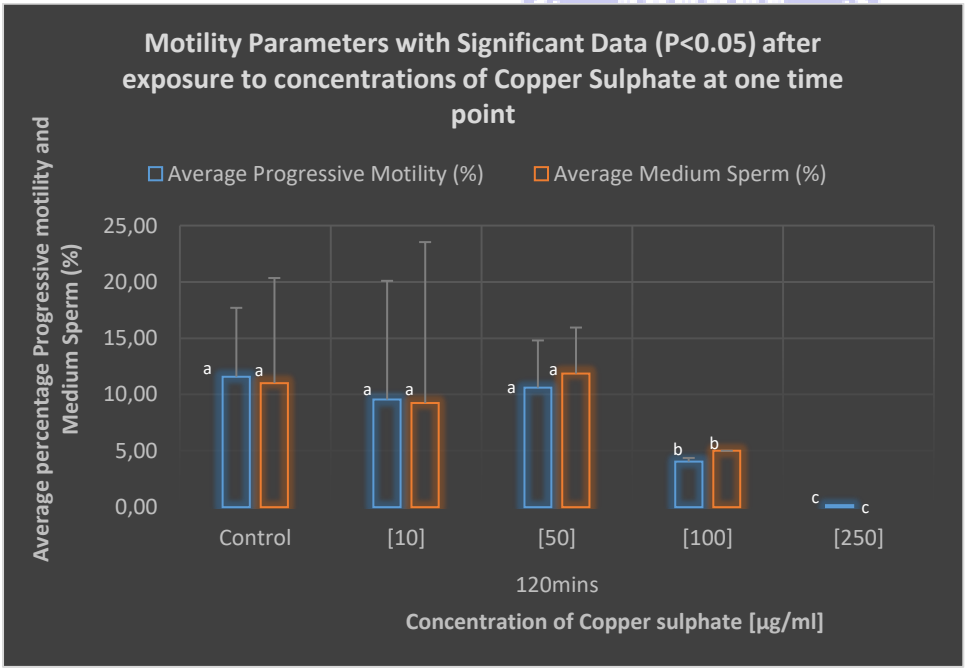


Figure 32: Average rhesus monkey motility parameters that presented significant data at 120 minutes of exposure to Copper Sulphate (10-250 µg/ml). a - c = significantly different values (P<0.05)

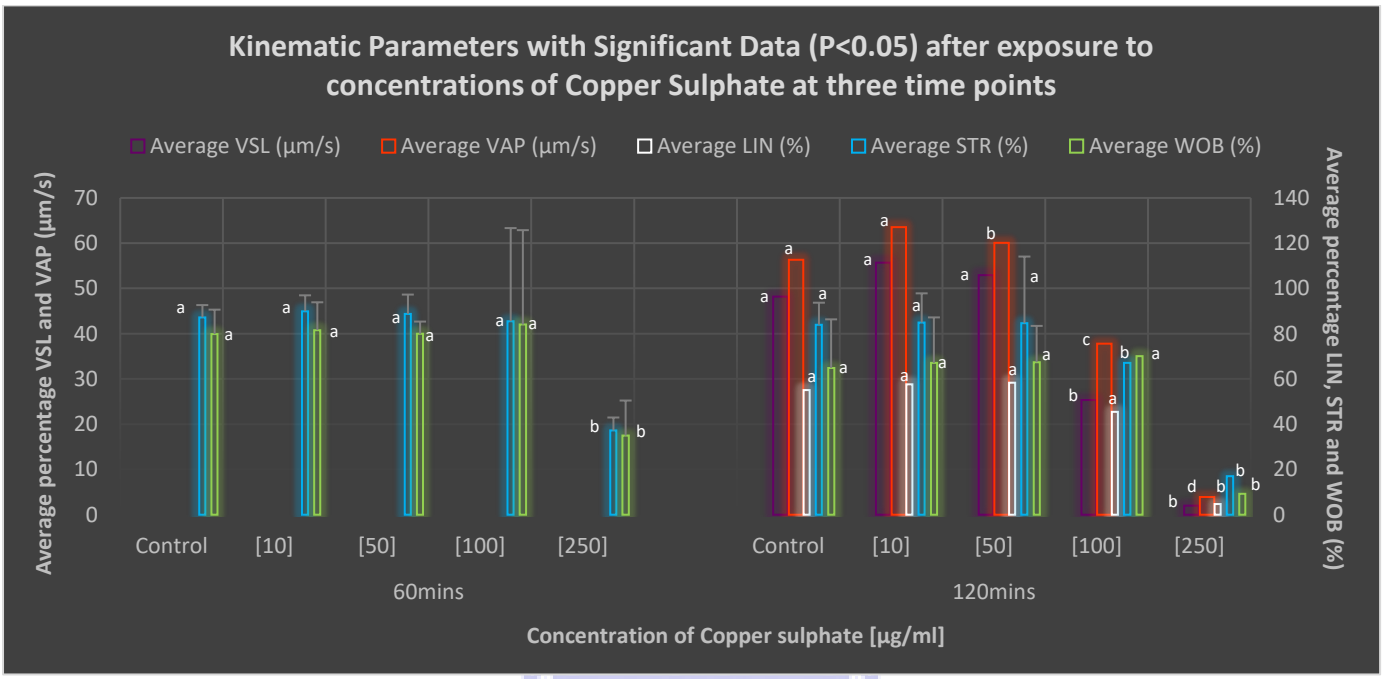


Figure 33: Average rhesus monkey kinematic parameters that presented significant data at 60 and 120 minutes of exposure to Copper Sulphate (10-250 µg/ml). a - d = significantly different values at the same time point (P<0.05)

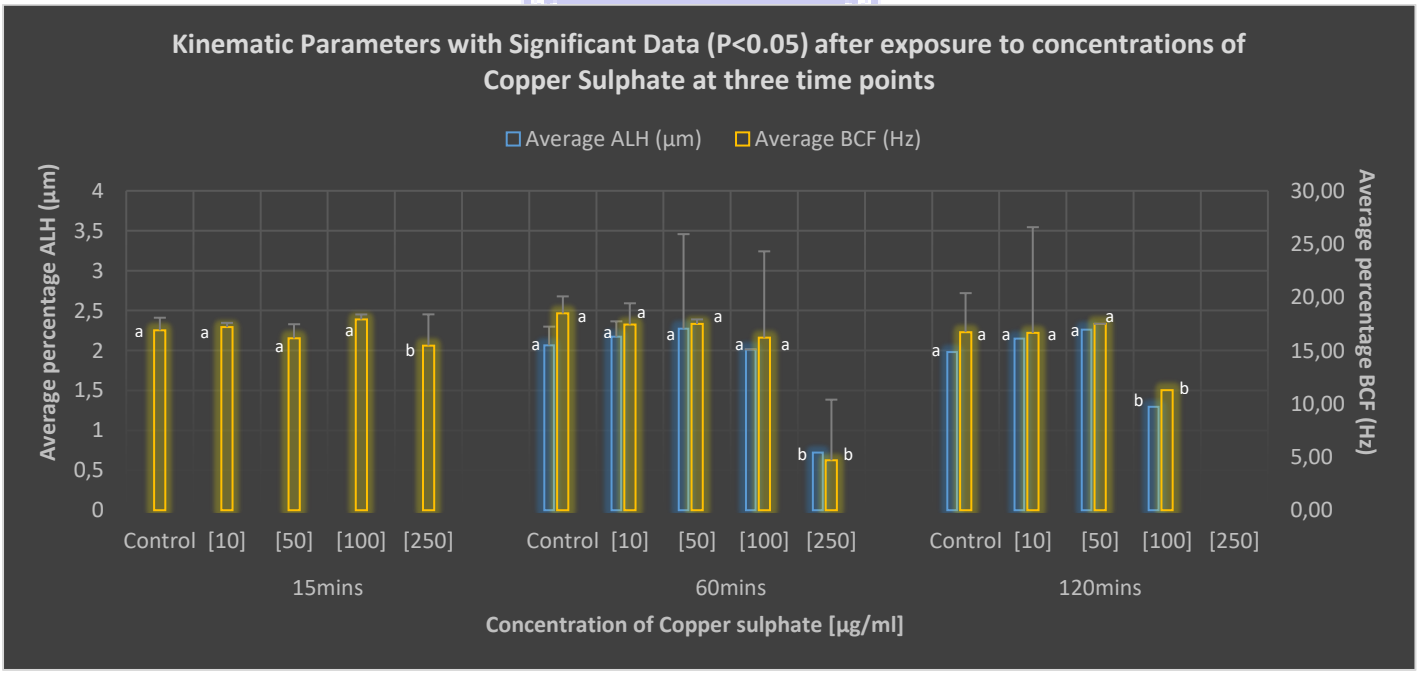


Figure 34: Average rhesus monkey kinematic parameters that presented significant data at 15, 60 and 120 minutes of exposure to Copper Sulphate (10-250 µg/ml). a and b = significantly different at each time point (P<0.05)

$CdCl_2$ treatment: After 15 minutes of exposure to $CdCl_2$, three out of thirteen sperm motility parameters presented significantly lower values for the 500 µg/ml concentration when compared to the control and other $CdCl_2$ concentrations. After 60 minutes, six out of the thirteen motility parameters presented significant lower values for the 500 µg/ml concentration. However, lower values for the control samples

might be due to a sampling effect, since the 10 µg/ml, 50 µg/ml and 100 µg/ml concentrations had higher values than the control. After 120 minutes, all except one motility parameter presented significantly lower values for the 500 µg/ml concentration. (Figure 35, 36 and 37). A trend was noted, where all the rhesus sperm motility parameters presented a decrease in values, especially after 120 minutes of exposure to the highest concentration of $CdCl_2$, 500 µg/ml, compared to the control.

Table 35.1: Average measurements for rhesus monkey sperm motility parameters after exposure to Cadmium chloride (10-500 µg/ml) at 15, 60 and 120 minutes (n=3).

Time	*Conc. of $CdCl_2$*	*Total Motility*	*Progressive Motility*	*Rapid Spermatozoa*	*Medium Spermatozoa*	*Slow Spermatozoa*
(mins)	*(µg/ml)*	*(%)*	*(%)*	*(%)*	*(%)*	*(%)*
15	Control	62.33 ± 26.27	47.88 ± 23.30	42.48 ± 24.70	9.02 ± 2.47 [a]	10.83 ± 3.06
	[10]	59.29 ± 31.35	46.01 ± 30.48	39.68 ± 30.76	10.83 ± 1.13 [a]	8.78 ± 3.58
	[50]	63.90 ± 32.66	50.43 ± 33.31	45.17 ± 34.89	8.41 ± 2.23 [a]	10.31 ± 2.78
	[100]	63.63 ± 30.00	45.36 ± 29.67	40.38 ± 30.70	8.61 ± 1.21 [a]	14.64 ± 4.23
	[500]	43.83 ± 38.44	24.61 ± 31.16	18.66 ± 27.81	8.07 ± 5.91 [b]	17.10 ± 7.18
60	Control	43.25 ± 45.46	27.48 ± 30.27	22.76 ± 25.42	7.20 ± 7.12	13.29 ± 13.11
	[10]	55.00 ± 26.83	31.96 ± 25.17	23.33 ± 20.88	13.83 ± 5.85	17.84 ± 2.00
	[50]	46.14 ± 37.55	30.95 ± 34.56	25.00 ± 31.61	9.01 ± 3.76	12.14 ± 2.63
	[100]	49.25 ± 36.79	30.46 ± 30.63	23.21 ± 29.03	12.38 ± 4.67	13.66 ± 3.58
	[500]	1.83 ± 1.58	0.09 ± 0.16	0.09 ± 0.16	0.09 ± 0.16	1.65 ± 1.45
120	Control	49.06 ± 45.88 [a]	27.93 ± 35.51 [a]	19.18 ± 25.53 [a]	12.11 ± 10.75 [a]	17.78 ± 9.61 [a]
	[10]	45.16 ± 47.53 [a]	29.33 ± 39.49 [a]	24.21 ± 33.05 [a]	8.11 ± 8.68 [a]	12.83 ± 5.80 [a]
	[50]	45.61 ± 54.72 [a]	28.58 ± 40.08 [a]	21.94 ± 31.03 [a]	8.88 ± 11.89 [a]	14.79 ± 11.80 [a]
	[100]	41.74 ± 56.56 [a]	18.59 ± 25.67 [a]	12.28 ± 17.06 [a]	10.76 ± 14.60 [a]	18.70 ± 24.90 [a]
	[500]	0.81 ± 1.15 [b]	0.00 ± 0.00 [b]	0.00 ± 0.00 [b]	0.00 ± 0.00 [b]	0.81 ± 1.15 [b]

Table 35.2: Average measurements for rhesus monkey sperm kinematic parameters after exposure to Cadmium chloride (10-500 µg/ml) at 15, 60 and 120 minutes (n=3).

Time	*Conc. of $CdCl_2$*	*VCL*	*VSL*	*VAP*	*LIN*	*STR*	*WOB*	*ALH*	*BCF*
(mins)	*(µg/ml)*	*(µm/s)*	*(µm/s)*	*(µm/s)*	*(%)*	*(%)*	*(%)*	*(µm)*	*(Hz)*
15	Control	166.46 ± 35.79	117.58 ± 14.08	137.76 ± 27.69	71.58 ± 6.70 [a]	86.24 ± 6.65	82.94 ± 1.66 [a]	2.53 ± 0.24	16.28 ± 1.04
	[10]	163.08 ± 59.12	115.21 ± 43.59	134.87 ± 54.96	70.32 ± 1.58 [a]	86.06 ± 2.67	81.78 ± 3.83 [a]	2.60 ± 0.29	16.03 ± 1.04
	[50]	172.98 ± 67.00	126.64 ± 48.34	146.25 ± 61.87	73.16 ± 4.07 [a]	87.63 ± 4.97	83.56 ± 4.06 [a]	2.56 ± 0.31	16.13 ± 1.10
	[100]	152.31 ± 55.11	109.12 ± 40.50	125.84 ± 49.90	71.40 ± 1.28 [a]	87.34 ± 3.29	81.83 ± 3.58 [a]	2.60 ± 0.33	16.31 ± 1.69
	[500]	103.61 ± 47.94	69.91 ± 37.56	82.27 ± 45.12	65.35 ± 11.37 [b]	84.83 ± 5.00	76.80 ± 10.39 [b]	2.16 ± 0.23	15.92 ± 0.48
60	Control	89.95 ± 78.89	64.99 ± 56.96 [a]	75.56 ± 66.95	48.18 ± 41.73 [a]	57.59 ± 49.97 [a]	55.83 ± 48.43 [a]	1.44 ± 1.24 [a]	11.44 ± 9.97 [a]
	[10]	117.88 ± 21.12	78.35 ± 20.54 [a]	92.05 ± 23.19	65.83 ± 5.55 [a]	84.96 ± 2.82 [b]	77.45 ± 5.35 [a]	2.41 ± 0.19 [a]	15.77 ± 0.95 [a]
	[50]	118.89 ± 45.37	76.95 ± 43.22 [a]	91.46 ± 48.02	61.94 ± 10.92 [a]	83.22 ± 3.08 [b]	74.23 ± 10.68 [a]	2.28 ± 0.12 [a]	17.15 ± 1.74 [a]
	[100]	117.26 ± 43.57	76.77 ± 46.76 [a]	91.49 ± 51.65	62.07 ± 15.52 [a]	82.62 ± 3.47 [b]	74.76 ± 16.01 [a]	2.18 ± 0.16 [a]	17.45 ± 3.90 [a]
	[500]	34.87 ± 30.30	4.21 ± 3.76 [b]	12.42 ± 10.81	8.11 ± 7.40 [b]	22.46 ± 19.68 [c]	23.86 ± 21.00 [b]	0.00 ± 0.00 [b]	0.00 ± 0.00 [b]
120	Control	99.00 ± 30.60 [a]	56.63 ± 39.58	67.89 ± 40.71 [a]	53.39 ± 23.42 [a]	80.38 ± 10.10 [a]	65.35 ± 20.92 [a]	1.91 ± 0.27 [a]	15.26 ± 4.14 [a]
	[10]	112.19 ± 51.93 [a]	71.01 ± 58.30	84.78 ± 57.80 [a]	57.42 ± 25.39 [a]	78.57 ± 15.20 [a]	71.28 ± 18.52 [a]	1.85 ± 0.49 [a]	11.55 ± 7.86 [a]
	[50]	98.27 ± 50.82 [a]	65.72 ± 54.49	75.65 ± 56.21 [a]	60.65 ± 24.08 [a]	83.05 ± 10.32 [a]	71.79 ± 20.07 [a]	1.17 ± 1.19 [a]	10.27 ± 10.64 [a]
	[100]	95.50 ± 8.43 [a]	42.17 ± 32.53	59.43 ± 23.24 [a]	42.82 ± 30.28 [a]	65.24 ± 29.22 [a]	61.40 ± 18.92 [a]	1.36 ± 1.13 [a]	10.84 ± 12.15 [a]
	[500]	23.93 ± 33.84 [b]	2.15 ± 3.04	8.03 ± 11.35 [b]	4.48 ± 6.34 [b]	13.37 ± 18.90 [b]	16.77 ± 23.72 [b]	0.00 ± 0.00 [b]	0.00 ± 0.00 [b]

Conc-concentration, CdCl2-cadmium chloride, VCL-curvilinear velocity, VSL-straight-line velocity, VAP-average-path velocity, LIN-linearity, STR-straightness, WOB-wobble, ALH-amplitude of lateral head displacement and BCF-beat-cross frequency, a – c = significantly different data relevant to the concentration of $CdCl_2$ ($P<0.05$).

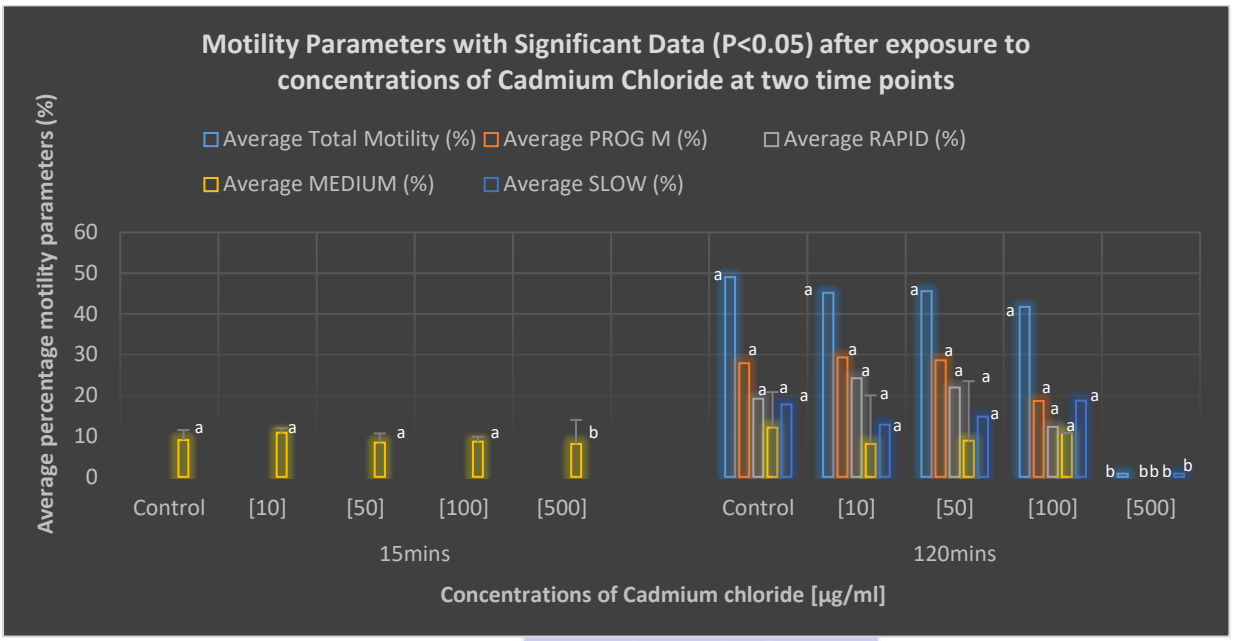


Figure 35: Average rhesus monkey motility parameters that presented significant data at 15 and 120 minutes of exposure to Cadmium chloride (10-500 µg/ml). a and b = significantly different at each time point (P<0.05)

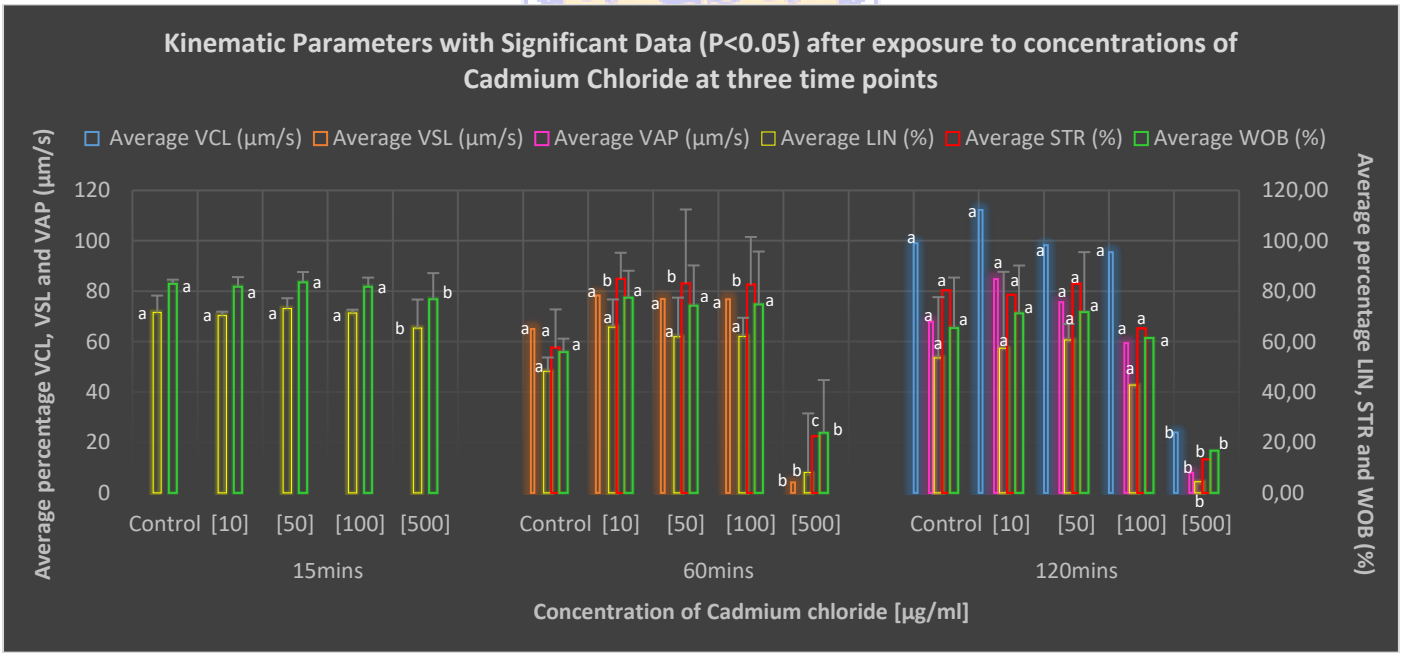


Figure 36: Average rhesus monkey kinematic parameters that presented significant data at 15, 60 and 120 minutes of exposure to Cadmium chloride (10-500 µg/ml). a - c = significantly different at each time point (P<0.05)

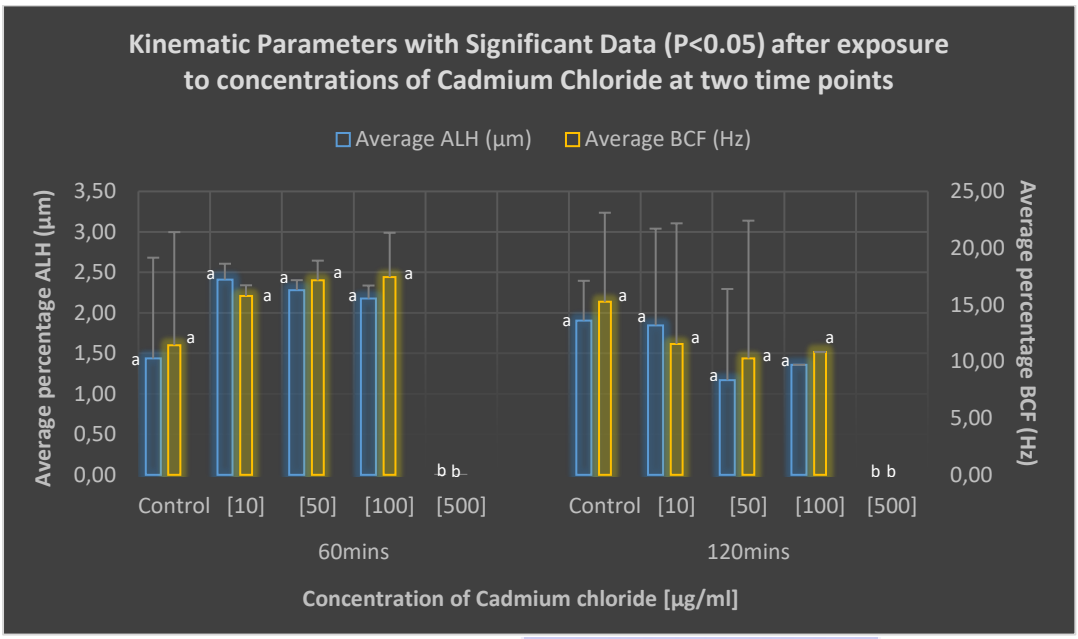


Figure 37: Average rhesus monkey kinematic parameters that presented significant data at 60 and 120 minutes of exposure to Cadmium chloride (10-500 µg/ml). a and b = significantly different at each time point (P<0.05)

4.2.3.2 Hyperactivation

As previously described the stimulant caffeine was used to induce hyperactivation. Caffeine was used at a concentration of 5 mM and 100 µg/ml of each metal in combination with it. One sample was exposed to caffeine, a metal and a combination of the two. Unfortunately, due to the rhesus monkey being a seasonal breeder, more semen samples could not be collected to increase the repeats of this experiment. The motility of the sample was measured at 10, 20, 35, 50, 65 and 80 minutes. The percentage hyperactivation are displayed in the Table 36 and 37.

Table 36: The percentage sperm hyperactivation of the rhesus monkey sample after exposure to $CuSO_4$ (100 µg/ml) and caffeine (5 mM) at 10, 20, 35, 50, 65 and 80 minutes incubation time (n= 1).

Time (min)	*$CuSO_4$ and Caffeine (µg/ml) (mM)*	*Hyperactivation (%)*
10	Control	63.47
	Caf [5]	70.41
	$CuSO_4$ [100]	71.86
	Caf [5]+ $CuSO_4$ [100]	71.14
20	Control	58.10
	Caf [5]	59.26
	$CuSO_4$ [100]	37.95
	Caf [5]+ $CuSO_4$ [100]	79.60
35	Control	59.11
	Caf [5]	68.80
	$CuSO_4$ [100]	35.64
	Caf [5]+ $CuSO_4$ [100]	64.09
50	Control	65.62
	Caf [5]	78.77
	$CuSO_4$ [100]	41.14
	Caf [5]+ $CuSO_4$ [100]	71.68
65	Control	67.65
	Caf [5]	81.70
	$CuSO_4$ [100]	28.57
	Caf [5]+ $CuSO_4$ [100]	74.44
80	Control	65.92
	Caf [5]	72.73
	$CuSO_4$ [100]	14.38
	Caf [5]+ $CuSO_4$ [100]	59.82

Table 37: The percentage sperm hyperactivation of the rhesus monkey sample after exposure to $CdCl_2$ (100 µg/ml) and caffeine (5 mM) at 10, 20, 35, 50, 65 and 80 minutes incubation time (n= 1).

Time (min)	*$CdCl_2$ and Caffeine (µg/ml) (mM)*	*Hyperactivation (%)*
10	Control	41.13
	Caf [5]	71.20
	$CdCl_2$ [100]	37.76
	Caf [5]+ $CdCl_2$ [100]	61.50
20	Control	36.31
	Caf [5]	94.48
	$CdCl_2$ [100]	29.07
	Caf [5]+ $CdCl_2$ [100]	74.14
35	Control	17.71
	Caf [5]	82.32
	$CdCl_2$ [100]	26.42
	Caf [5]+ $CdCl_2$ [100]	66.13
50	Control	16.26
	Caf [5]	66.48
	$CdCl_2$ [100]	14.97
	Caf [5]+ $CdCl_2$ [100]	53.95
65	Control	11.26
	Caf [5]	69.40
	$CdCl_2$ [100]	23.44
	Caf [5]+ $CdCl_2$ [100]	54.56
80	Control	31.14
	Caf [5]	76.72
	$CdCl_2$ [100]	8.98
	Caf [5]+ $CdCl_2$ [100]	49.27

After 80 minutes of exposure to $CuSO_4$, the percentage hyperactivation presented a lower value for the 100 µg/ml concentration and Caffeine maintained the percentage hyperactivation to values higher than the control. After 80 minutes of exposure to $CdCl_2$, the same effect was seen as with $CuSO_4$ exposure. However, due to the evaluation of only one sample for each metal, calculated averages could not be determined, statistics could not be performed, and therefore we cannot indicate the true effect of the metals on rhesus sperm hyperactivation. These results only indicate the effect of these metals on one sample, but a definite effect is seen after 80 minutes with the 100 µg/ml concentration of $CdCl_2$ and $CuSO_4$.

Chapter 5: Discussion and Conclusion

An estimated 50-80 million people world-wide are affected by infertility (Mati, 2004) and furthermore 48.5 million couples are unable to conceive (Mascarenhas et al., 2012). Infertility is present in both males and females and there are many factors that contribute to these high rates. According to Andrology Australia (2016), male infertility is diagnosed when, after testing both partners, reproductive problems are found in the male. It is estimate that one in twenty men has some kind of fertility problem often associated with low numbers of spermatozoa in his ejaculate. The causes of male infertility include: sperm production problems such as varicocele; blockage of sperm transport, for example through the absence of vas deferens; sexual problems such as erectile dysfunction; hormonal problems; or sperm antibodies through injury or infection in the epididymis (Andrology Australia, 2016).

Male infertility was the focus of this study, specifically the function of spermatozoa, as sperm dysfunction has been identified as a common cause of male infertility (Aitken et al., 1991). Sperm functional tests have been revealed to be a more suitable option to identify functionally deficient sperm (Lamb, 2010) than a standard semen analysis because it is not a test of fertility (Jequier, 2010).

Since sperm abnormalities are regarded as indicators for reduced fertility in both human and nonhuman primates, it's necessary to develop methods based on nonsubjective techniques for measuring individual sperm characteristics (Valle et al., 2012).

Non-human primates (NHPs) formed the research models for this study due to their phylogenetic closeness to humans (Bornman et al., 1988; Dancet et al., 2011). The focus species for this study were vervet monkey *(Chlorocebus aethiops)*, chacma baboon (*Papio ursinus*), and rhesus monkey (*Macaca mulatta).* These three primates were selected, as Old World Primates have been found to be more applicable human models than New World Primates (van der Horst, 2005). The Chacma baboon are closer to humans than macaque (Simerly et al., 2010) and have been found to be an appropriate research model for human reproduction (Dancet et al., 2011).

The aim of this study was to evaluate NHP spermatozoa using established sperm functional tests for humans, design novel techniques for sperm analysis in primates and standardize protocols for future studies on male infertility. It was ultimately our objective to assess NHP sperm function in order to use these species as primate models for future reproductive studies in order to explain high infertility rates in humans.

The functional tests employed for this study were sperm motility and longevity, vitality, DNA integrity, acrosome intactness and hyperactivation. The NHPs sperm functionality was first evaluated using these tests,

in order to optimise each test for the three primate species. Secondly, once all functional tests were performed, the tests were repeated with the addition of chemicals to the incubation media in order to verify the sensitivity of each functional test. The chemicals used were two heavy metals, namely copper sulphate ($CuSO_4$) and cadmium chloride ($CdCl_2$). As previously mentioned, high doses of $CdCl_2$ exposure causes rapid testicular edema, haemorrhage and necrosis (Puluputturi and Dayapulae, 2012) and $CuSO_4$ significantly reduces the motility of both moving sperm and resting sperm (Chen et al., 2011). Previous studies have shown that the metals decrease human sperm motility at concentrations of 250µg/ml $CuSO_4$ and 500µg/ml $CdCl_2$, after five hours of exposure (Israel, 2013; Prag, 2013). The metals were therefore used at these concentrations in order to produce an inhibitory affect on NHP sperm function. However, due to low quality and limited availability of semen samples, certain functional tests could not be completed for the baboon and rhesus monkey, respectively.

5.1 Optimization

As mentioned above, the NHPs sperm functionality was first evaluated using the selected tests usually employed to evaluate human semen samples, in order to optimise each test for the three primates to acquire species specific data. This baseline data were compared to results obtained from treatment tests and ultimately could be further applied in a clinical or experimental set-up.

5.1.1 Preparation method

This study required the appropriate preparation method to obtain a suitable sperm sample for functional testing for all three species of NHPs.

A study by Morell and Rodriguez-Martinez (2009) has demonstrated various separation and selection techniques to obtain improved sperm quality. Their separation technique included washing of semen and the selection technique included the methods of migration (swim-up), filtration (glass wool, membranes) and centrifugation on a colloid (density gradient, single layer) (Morell and Rodriguez-Martinez, 2009). Colloid centrifugation methods were found to offer the best possibility for selecting good quality spermatozoa and removing cellular debris and pathogens which may be present in seminal plasma (Morell and Rodriguez-Martinez, 2009). More specifically, density gradient centrifugation (DGC) separates motile, morphologically normal, chromatin-intact spermatozoa from the rest of the ejaculate (Morell, 2006). The silane-coated silica colloids have the advantage of being autoclavable and stable for long periods in salt solutions, thus permitting standardised ready-to-use formulations to be sold comercially, such as PureSperm (Morell and Rodriguez-Martinez, 2009).

A study by Hernandez-Lopez et al. (2005), compared the effects of two density gradient centrifugation substances, Percoll and PureSperm, on common marmoset semen and it was found that these substances are not toxic to marmoset sperm. As many NHPs develop a tight coagulum following ejaculation, spermatozoa are only easily recovered from the liquid phase. Samples are also prone to contamination from bacteria from the penis skin and/or hair surrounding the genitals. Therefore DGC using Percoll, PureSperm or other silica-based products, is the better choice to obtain good quality samples (Hernandez-Lopez et al., 2005). A second study by Sakkas et al. (2000), demonstrated the use of Percoll and PureSperm, as well as the Swim-up method to separate human spermatozoa with chromatin and nuclear DNA anomalies. It was found that the Swim-up technique was not efficient at isolating a population of spermatozoa with a low percentage of nuclear anomalies, whereas the DGC substances significantly reduced the percentage of spermatozoa with nuclear DNA damage (Sakkas et al., 2000).

These previous studies clearly indicate the benefit of using density gradient centrifugation, therefore the preparation methods compared in this study were PureSperm (PS) and Swim-up (SU). The PureSperm selected sperm preparations presented a significantly higher motility average and sperm concentration for the vervet monkeys compared to the Swim-up, and presented a similar trend for baboon and rhesus monkeys. It was therefore decided to use the PureSperm selection method for all further functional testing, unless stated otherwise.

5.1.2 Motility and Longevity

A time-based study was performed in order to evaluate motility and longevity, and motility parameters were measured every 15 minutes with the use of the CASA system. The motility parameters of the five vervet samples, however, presented no significant data when the different time points were compared. Although no significant data was seen, a definite trend was noted, with the average percentages being halved for almost all the parameters after 75 minutes. This insignificant data may be due to the high standard deviations arising from inconsistent samples as well as a change in sample size from five to four at 60 and 75 minutes due to low sample quality . Another reason for insignificance may be due to the short 15 minute interval period between time points of analysis and would therefore require longer periods of intervals between analysis in order to see an effect.

Only two motility parameters presented significant data when compared to the semen samples for the twelve baboon samples measured only after 15 minutes of incubation. Motility over time could, however, not be measured here. The two siginifcantly higher motility parameters were percentage Medium spermatozoa ($P=0.003$) and BCF ($P<0.001$) in PS samples compared to the semen. A higher percentage of

medium spermatozoa in PS would be expected as PS contains a higher percentage of motile spermatozoa compared to semen. The increased BCF in PS further proves the ability of the method to select good quality sperm for testing. According to a previous study, PS has been shown to significantly improve BCF compared to their original semen samples (Chen and Bongso, 1999). Additionally a trend of higher motility and kinematic parameters in PS compared to semen was noted for the baboon samples and was used as reference for subsequent testing.

Four motility parameters presented with significant data for the nine rhesus samples when compared to the semen samples. A time based study could also not be performed and therefore we were unable to evaluate longevity. The significant motility parameters in PS compared to semen were LIN (P=0.004), STR (P=0.001), WOB (P=0.016) and ALH (P=0.02). These parameters were seen to have low standard deviations for PS samples in comparison to the semen samples and other parameters. Again the PS preparations tend to have higher motility and kinematic parameters as seen for the vervet and baboon samples.

Reason being for the inability to perform time-based studies was due to time constraints and low quality samples for the baboon and rhesus samples. Therefore, this became an objective for validation testing in order to obtain better quality samples and measuring motility over time as longevity has been said to be an indicator of fertility (WHO, 1999). The vervet species, however, allowed for measurement over time, but presented no significant data and therefore it was decided to measure motility at less time points but with longer intervals in order to allow the treatments to take effect. Therefore validation testing included the use of time points 15, 60 and 120 minutes of incubation in order to see an effect and the continued use of PS for sperm preparation.

According to a previous study, vervet samples obtained by rectal electroejaculation had few or no motile spermatozoa, except for one that yielded approximately 0.2 X 10^6/ml with 28 % motility (Sparman et al., 2007). Similar results were also obtained for some vervet samples used in the current study and could therefore partly explain the insignificant data obtained after testing and analysis for vervet samples. However, the motility test in this instance was shown to be successful in measuring the motility parameters and the longevity of at least one species of the focus NHPs. According to the study by Seier et al. (1989), the motility percentage of vervet males with unknown fertility and males with known fetility was found to be 55.40 ± 18.40% and 43.64 ± 19.10%, respectively. These values are lower that what we obtained, with a total motility ranging from 50-80%. For baboon sperm motility, a study by Nyacheio et al. (2012) reported a motility percentage of 61.22-67.33%, which is similar to what we obtained from our semen samples (61.44 ± 18.76%) and an even higher percentage when prepared with PS (82.37 ± 13.60%). According to Lanzendorf

et al. (1990), the sperm motility of rhesus monkeys was reported to be 76.8-86.8%, however we obtained lower values for both semen (38.10 ± 27.46%) and PS (54.20 ± 26.17%) samples. The study by Maree and van der Horst (2012) found the motility percentage of the semen of these three species to be 57.4 ± 15.5% (vervet monkey), 73.4 ± 16.7% (chacma baboon) and 69.2 ± 10.2% (rhesus monkey). These findings were somewhat similar to what we obtained as the vervet monkey results fell into the range of 50-80%, the baboon results were in the 60% range and higher for PS, however our rhesus monkey results were lower than what was seen in this previous study. The study also obtained the following motility and kinematic parameters after Swim-up preparation with Ham's F10 medium (Invitrogen, Cape Town, South Africa). For vervet monkey, motility 94.7 ± 4.7%, VCL 328.8± 18.6 µm/s, VSL 293.0 ± 24.4 µm/s, VAP 315.4 ± 20.6 µm/s, LIN 89.0 ± 4.4%, STR 92.8 ± 3.0%, WOB 95.9 ± 1.8%, ALH 3.0 ± 0.2 µm and BCF 13.9 ± 1.2 Hz. For the baboon, motility 93.9 ± 2.9%, VCL 367.7 ± 45.5 µm/s, VSL 337.8 ± 40.2 µm/s, VAP 357.2 ± 44.2 µm/s, LIN 92.0 ± 3.6%, STR 94.7 ± 3.0%, WOB 97.2 ± 1.2%, ALH 3.4 ± 0.4 µm and BCF 15.4 ± 1.4 Hz, and for the rhesus monkey, motility 94.0 ± 5.5%, VCL 292.3 ± 19.2 µm/s, VSL 217.6 ± 34.3 µm/s, VAP 251.6 ± 27.9 µm/s, LIN 74.2 ± 9.3%, STR 86.1 ± 5.4%, WOB 85.9 ± 5.8%, ALH 4.0 ± 0.4 µm and BCF 17.3 ± 1.3 Hz (Maree and van der Horst, 2012). All these values for motility and kinematic parameters, for all three NHPs, are higher than what we obtained in the current study, except for LIN, STR, WOB and BCF, which presented lower values for the rhesus monkey. These higher values may be due to the difference in preparation of the spermatozoa in this previous study, as the semen samples were prepared with the Swim-up technique and we made use of the PureSperm method. Furthermore, sperm motion parameters of the rhesus monkey were evaluated as part of a study by Hung et al. (2009), however, no dramatic changes were noted and only after chemical activation was there a decrease (VAP, VSL, LIN, BCF and STR) and increase (VCL and ALH) in some parameters. The only similar effect was noted for ALH in PS samples being signifcantly higher than in the semen samples. These results therefore do indicate that we were successful in measuring the baseline motility parameters of our three NHPs.

5.1.3 Vitality

Vitality assessment involved the use of three methods, namely Eosin-Nigrosin staining, Hoechst and Propidium Iodide staining and WST-1 cytotoxicity assay. These three different techniques were employed in order to optimise which method would be most sensitive in determining vitality of the three primate species and apply it in the validation part of the study.

5.1.3.1 Eosin-Nigrosin (E-N)

Prepared samples were stained with E-N after a 15 minute and 75-90 minute (last time point reading) incubation time. The test was performed in order to evaluate the sensitivity of E-N to distinguish live spermatozoa, which remained white, from dead spermatozoa, which absorbed the eosin dye and stained pink. Vervet monkey samples were stained after incubation of 15 and 75-90 minutes and the percentage live sperm significantly decreased at the last time point. However, a discrepancy was noted at the first time point with the percentage total motility of the sample. The total motility was 78.30 ± 17.73% and the live sperm was 54.12 ± 17.52% at the 15 minute time point. The baboon and rhesus monkey samples could only be evaluated at one time point and therefore the ability of the test to distinguish between live and dead sperm could not be proven over time for these species. However, a similar discrepancy was noted for the baboon and rhesus monkey as found with the vervet samples, where the percentage total motility was higher than the percentage live spermatozoa at the 15 minute time point.

The discrepancy could be due to the delay in time, ranging from five to ten minutes, from when the motility was analysed up until when the E-N smears were prepared. Another possible reason for the low vitality and high motility percentage could be due to the E-N stain did not stain the spermatozoa accurately. However, for the vervet samples, the vitality test was still sensitive in determining live from dead sperm as well as indicating a short time frame during which E-N staining should be performed for sperm vitality testing. A previous study by Seier et al. (1989) indicated the E-N staining to be successful for testing vitality of vervet monkey samples and also indicating a maximum of 68.00 ± 14.90% live sperm with a 55.40 ± 18.40% total sperm motility. This therefore proves that the high vitality with a lower motility is not uncommon for this species. A previous study with the use of drill (African forest baboon) sperm, reported on good results for viability testing (Maya-Soriano et al., 2015) with the use of E-N and a study by Talwar et al. (1979) also indicated the successful use of the stain for viability testing of rhesus monkey sperm. Another study on rhesus monkey sperm indicated a percentage live spermatozoa of 59.6 ± 30.5 (Thomsen, 2013), which is close to the 51.38 ± 5.06% at the 15 minute time point obtained for rhesus monkeys in this study. In the instance of this project, the baboon and rhesus species would require more testing to evaluate more than one time point and with a larger sample size. The E-N staining technique was still employed for validation testing in order to further test the sensitivity of the technique for the three species and also to select good samples with better time management as previous studies have shown this technique to be successful.

5.1.3.2 Hoechst and Propidium Iodide (H&P)

Prepared samples were exposed to H&P and measured after 15 minutes and 45-60 minutes (last time point reading) of incubation. The test was performed in order to evaluate the sensitivity of it to distinguish live spermatozoa, which stained blue, from dead spermatozoa, which stained red. Vervet monkey samples were stained at 15 and 45-60 minutes; the data has shown a tendency to decrease at the last time point but this change was not significant. A similar discrepancy was noted for these samples as with the E-N stained samples. The live sperm was 49.58 ± 10.09% at the 15 minute time point, while the total motility was 61.35 ± 18.32%. The baboon and rhesus samples could only be evaluated at one time point and therefore the ability of the test to distinguish between live and dead sperm could not be proven over time for these species. However a similar discrepancy was noted for the baboon and rhesus monkey as with the vervet monkey samples, where the percentage total motility was higher than the percentage live spermatozoa at the 15 minute time point.

Reasons for the discrepancy could again be due to the time delay between motility analysis and preparation of the smears. Secondly, the H&P stains might not have accurately stained the sperm during the analysis. With regards to the baboon and rhesus samples, an added reason could be due to the low percentage total motility at the 15 minute time point seen for both species and as previously stated this low motility clearly indicates why the vitality of the samples were low.

A study by Mdhluli et al. (2004) indicated the successful use of Hoechst 33258 for vitality testing of vervet monkey spermatozoa and yielded 78.75 ± 5.64% live spermatozoa, while this study obtained a lower value of 49.58 ± 10.09% at the 15 minute time point. A previous study by Putkhao et al. (2013), incorporated a similar staining technique (Viability kit, Invitrogen) for rhesus monkeys, using the SYBR 14 dye, staining live spermatozoa green and Propidium Iodide, staining dead spermatozoa red. It indicated a much higher percentage live spermatozoa of 58.18 ± 1.74 while we obtained a percentage live spermatozoa of 38.56 ± 6.91. These previous studies further proves the reasons for the discrepancy in results and the test would require more testing and a larger sample size for all three species. As with E-N, the H&P staining technique was still employed for validation testing as these previous studies have shown the technique to be successful and to further test the sensitivity of the technique for the three species by selecting good samples with better time management.

5.1.3.3 WST-1 cytotoxicity assay

The WST-1 reagent was evaluated on three preparation methods, namely semen, PureSperm and Swim-up samples. Only the vervet monkey samples were used to evaluate this assay due to time constraints with the

other two species. The absorbances were read at 15 minute intervals until 45 minutes and the data presented a significant difference at the 15 minute time point, where semen presented a significantly higher absorbance value compared to Swim-up. However, Swim-up presented a significantly higher absorbance than PureSperm. As previously stated, the reason for these differences is due to the concentration of spermatozoa in the semen and Swim-up samples compared to the PureSperm. The semen and Swim-up had a higher concentration of spermatozoa and therefore more sperm cells to undergo the metabolic reaction when exposed to the reagent therefore producing the detectable formazan dye. According to Roche Diagnostics (2006), the reagent, tetrazolium salt, is cleaved by cellular enzymes, mitochondrial dehydrogenases, to formazan. The higher number of viable sperm cells in semen probably resulted in higher mitochondrial dehydrogenase activity which in turn increases the amount of formazan dye formed, which directly correlates to the number of metabolically active sperm cells (Roche Diagnostics, 2006). The dye is read by a spectrophotometer, resulting in the absorbance value indicating the formazan produced only by viable cells.

This assay proved to be a successful vitality test as it clearly indicated the relationship in the presence of live cells in the samples with the presence of the dye. A previous study by Aitken et al. (2003) indicated that the WST reagent is readily reduced by human spermatozoa. The PureSperm samples did present the lowest absorbance values compared to the semen and Swim-up, however, PureSperm was selected for validation tests, as it was the method of choice for preparation and all functional testing. An increase in absorbance values over time was also seen for all three methods, although this increase was not significant. As mentioned above, the reason for this increase could be due to the increased number of viable cells, increasing enzyme activity which increases the amount of dye formed over time.

5.1.4 DNA Integrity

The DeadEnd™ Fluorometric TUNEL System G3250 (Promega, USA) was employed in order to evaluate DNA integrity. The system is designed to detect and quantify apoptotic cells within a cell population. It measures nuclear DNA fragmentation, which is a hallmark of apoptosis (Promega Corporation, 2009) and thus, in terms of this project, it assesses sperm DNA integrity. The baboon and rhesus monkey samples were exposed to the system, however, the test proved unsuccessful for both species as fluorescence was not present and all samples revealed the same result as previously mentioned. There was no distinction between fragmented and non-fragmented DNA and therefore we no longer proceeded with this test and the evaluation of DNA integrity. This also provided reasoning for not attempting the test with the vervet monkey samples as well as further validation tests. According to a previous study by Mitchell et al. (2010), the conventional TUNEL

assay is not a sensitive method for analysing DNA fragmentation in human spermatozoa because the highly compacted nature of sperm chromatin impedes the ability of terminal transferase to access the sites of DNA cleavage. This provides reasoning for its failure when employed to evaluate the baboon and rhesus monkey samples. This assay would require modification and optimization for both human and primate sperm, which is further explained in the study by Mitchell et al. (2010), or alternatively, a different method for testing sperm DNA integrity should be selected. Furthermore, according to Agarwal et al. (2016), the TUNEL assay has its advantages in being sensitive, reliable, with minimal inter-observer variability and it can be performed on few spermatozoa, however, it lacks strict standardization, which makes comparison between laboratories more difficult, explaining the existence of many clinical thresholds (Feijo and Esteves, 2014).

Henkel (2016), stated that there are currently eight tests available for sperm DNA fragmentation analysis, namely sperm chromatin structure assay (SCSA), TUNEL assay, Acridine Orange (AO), aniline blue staining (AB), chromomycin A3 staining (CMA3), toluidine staining (TS), Comet assay and sperm chromatin dispersion test (SCD), with its improved version called Halosperm assay. These tests, besides TUNEL, should therefore be considered for use in future studies when testing DNA integrity in NHPs. Other studies successfully evaluating DNA integrity in baboons and rhesus monkey spermatozoa involved the use of AO test (Cseh et al., 2000) and the Comet assay (Li et al., 2007).

5.1.5 Acrosome Intactness

Acrosome intactness was measured through the use of reagents FITC-PSA and FITC-PNA. Samples were exposed to these reagents in order to determine acrosomal status of spermatozoa. Those spermatozoa with intact acrosomes fluoresced green and those without an acrosome would appear dark when analysed with a fluorescence microscope. The FITC-PNA reagent did not produce measurable results and it was decided to forego this reagent and employ the FITC-PSA for further testing. The reason for the failure and success of these two reagents respectively, could be due to their difference in binding specificity. PSA binds to α-methyl mannoside residues localized in the acrosome contents (Cross et al., 1986), while PNA binds to β-D-galactosyl residues localized on the outer acrosomal membrane (Mortimer et al., 1987). Therefore PSA labels the acrosome contents of suitably permeabilized spermatozoa while PNA labels glycoproteins of the outer acrosomal membrane in cells whose plasmalemma has been disrupted, and this might not have been the case with the primate spermatozoa at the time of evaluation (Mortimer et al., 1990). A study by Lybaert et al. (2009) has shown the successful use of FITC-PSA and FITC-PNA for acrosome reaction in mouse spermatozoa and another study by de Villiers (2006) has demonstrated the use of FITC-PNA for the evaluation of acrosomal status in vervet monkeys. Therefore it is recommended to undergo more testing in

order to distinguish whether or not FITC-PNA is suitable for other NHP spermatozoa. Furthermore, a study by Tollner et al. (1990) has demonstrated the successful labelling of cynomolgous monkey spermatozoa with the use of FITC-PSA as well as a study by Julius (1998), evaluating the induction of acrosome reaction in spermatozoa of Tana Mangabeys and De Brazza's monkeys.

Vervet monkey samples of Semen and PureSperm preparations were exposed to FITC-PSA after 15 and 45 minutes of incubation. However, the data was found to be insignificant for the comparison of the two preparation methods as well as the two time points. A tendency in percentage intact acrosomes to decrease was seen after 45 minutes with an increase in percentage reacted acrosomes for both Semen and PureSperm preparations. The acrosome intactness test was successful in distinguishing intact from reacted acrosomes, however, as previously stated, because of insignificant data, further testing is required and with a larger sample size. The baboon and rhesus monkey samples could only be evaluated at one time point and therefore the ability of the test to distinguish between intact and reacted acrosomes could not be proven over time for these species. The test was, however, still considered for use for validation testing.

5.1.6 Hyperactivation

Hyperactivation is important in human fertility as the impairment of it may be related to infertility. Spermatozoa of infertile patients have a diminished capability to become hyperactivated (Munire et al., 2004) and thus emphasise the importance of the evaluation of hyperactivation during sperm functional testing. Hyperactivation was induced via the Flush technique with the use of stimulants and assessed through the use of the CASA system. Preliminary experiments was performed with the two stimulants Caffeine and Procaine at concentrations ranging from 1 to 10 mM, however only caffeine was selected at a concentration of 5mM because, as found in a study by Hong et al. (1981), this concentration is optimal for stimulating sperm motility. Another study by Colas et al. (2010) found that caffeine significantly increased the proportion of capacitated ram spermatozoa and was a potent inductor of hyperactivation. Procaine, however, was discarded, as caffeine produced better results, and it has been shown to be an inhibitor of sperm motility due to its membrane stabilizing ability (Hong et al., 1981).

As mentioned in chapter 3, section 3.6.6, established rhesus monkey cut-off values were used in order to determine percentage hyperactivation for the 3 primate species. These cut-off values were: VCL≥130 µm/s, linearity ≤69% and ALH ≥7.5 µm (≥3.75 µm) (Baumber and Meyers, 2006).

Vervet monkey samples were exposed to caffeine for 5, 15 and 30 minutes. The caffeine significantly increased the percentage hyperactivation at each time point compared to the control, however, the results

was insignificant over time for both the control and caffeine samples. Baboon samples were exposed to caffeine from 5 to 60 minutes, resulting in a significantly higher percentage hyperactivation at the 15 and 30 minute time point compared to the control as well as an increase in hyperactivation over time. Rhesus samples were exposed to the stimulant from 15 to 60 minutes, and the percentage hyperactivation for the caffeine samples was significantly higher at the 45 and 60 minute time point compared to the control. However, there was no significant difference over time for both the control and caffeine rhesus samples. These results proved the stimulatory effect of caffeine to induce hyperactivation in spermatozoa of all three species according to VCL, LIN and ALH cut-off values (Baumber and Meyers, 2006). Hyperactivation was found to peak at 15 and 30 minutes for the baboon spermatozoa and at 45 and 60 minutes for the rhesus spermatozoa. It would be expected that the vervet monkey and baboon had many motile spermatozoa, however, one or two of the cut-off criteria for VCL, LIN and ALH may not have been met for these spermatozoa, as these criteria were specific for rhesus monkeys. Secondly, the cut-off values for the vervet monkey and baboon would be expected to be higher, than the standard rhesus monkey values applied here, if species specific values were first determined and applied for these two species. Time constraints in this study did, however, not allow for the establishment of more specific cut-off values for these NHPs.

According to previous studies by Chan et al. (1982), Glover et al. (1990) and Wolf et al. (1990), caffeine and cAMP have been reported to enhance nonhuman primate sperm motility. A study by Baumber and Meyers (2006), presented results, where caffeine did not cause a significant increase in hyperactivated motility of rhesus monkey spermatozoa but only when combined with cAMP was there a significant increase. Another study investigating hyperactivation in the cynomolgus monkey produced the same result (Mahony and Gwathmey, 1999). This could possibly explain why the data was insignificant over time for the tested samples in this study. However, a study by Nyachieo et al. (2010) demonstrated that baboon sperm motility was significantly higher when exposed to caffeine individually as well as combined with dbcAMP.

Their results are similar to hyperactivation results obtained in this study with the baboon caffeine samples being higher than the control, as well as being significant over time.

This Flush technique with the use of Caffeine at a concentration of 5mM was therefore found to be successful in stimulating and inducing hyperactivation in these three primate species and was employed for validation testing.

All sperm functional tests employed in this study, except for DNA integrity testing, was found to be successful in evaluating the spermatozoa of the three primate species. These techniques were then applied to the second part of the study, repeating the functional tests with an added chemical to cause inhibition of sperm function.

5.2 Validation

The second part of this study involved repeating all functional tests performed in order to verify their optimization and validate the sensitivity of these tests. Due to time constraints, low quality and limited availability of semen samples, certain tests could not be completed for baboon (WST-1 assay and hyperactivation) and rhesus monkey (vitality, acrosome reaction and WST-1), respectively.

Spermatozoa were prepared with the selected preparation method, PureSperm, and were exposed to chemicals copper sulphate ($CuSO_4$) and cadmium chloride ($CdCl_2$), shown to have an inhibitory effect on sperm function. This suspension was then used for all further functional testing. As previously stated, $CuSO_4$ has been seen to reduce sperm motility (Chen et al., 2011) and a study by Katayose et al. (2004) had found that higher concentrations of copper has significant adverse effects on sperm motility. $CdCl_2$ has also been shown to cause testicular edema (Puluputturi and Dayapulae, 2012) and studies have shown that low levels of cadmium can result in decreased semen quality and/or altered reproductive hormone levels (Wirth and Mijal, 2010). These heavy metals were seen to have an effect at concentrations in the range of 250 µg/ml and 500 µg/ml and were therefore used in this study in order to cause an inhibitory effect.

5.2.1 The effect of $CuSO_4$ and $CdCl_2$ on Motility and Longevity

A time-based study was performed, similar to the first part of the study, where vervet monkey samples were exposed to different concentrations of $CuSO_4$ and $CdCl_2$ at 15, 60 and 120 minutes. $CuSO_4$ significantly reduced various motility parameters all time points at the highest concentrations (100 µg/ml and 250 µg/ml) and a decrease in all parameters was seen after 120 minutes of exposure (Figure 17 and 18). The negative effect of CuSO4 was most prominently seen in progressive motility, rapid swimming spermatozoa, STR, WOB and BCF, which presented a common significant effect at two or all time points. After 15, 60 and 120 minutes of $CdCl_2$ treatment, two (15 min), eight (60 min) and nine (120 min) parameters were found to have significantly lower values for the 100 µg/ml and 500 µg/ml concentrations compared to the control and other $CdCl_2$ concentrations. After 120 minutes of exposure six parameters was also significantly lower compared to the control for the 50 µg/ml concentration (Figure 19 and 20). $CdCl_2$ was seen to significantly reduce

motility at the highest concentrations and a decrease in all parameters was seen after 120 minutes of exposure. The parameters which presented a common significant effect at two or all time points were progressive motility, rapid swimming sperm, STR, ALH and BCF. Both metals presented a significant negative effect at their highest concentrations in the same set of motility parameters (progressive motility, rapid swimming sperm, STR and BCF). These specific motility parameters should therefore be considered as a standard for evaluating the effect of $CuSO_4$ and $CdCl_2$, or even other metals, on vervet monkey sperm motility.

For the baboon samples, after 15, 45 and 75 minutes of $CuSO_4$ treatment, three (15 min), one (45 min) and five (75 min) parameters presented significantly lower values for the 250 µg/ml concentration compared to the control and other concentrations of $CuSO_4$ (Figure 27). However, some parameters presented a significant effect as well at 50 µg/ml and 100 µg/ml after 75 minutes. After 15, 45 and 75 minutes of $CdCl_2$ treatment, three (15 min), four (45 min) and eight (75 min) parameters presented significantly lower values for the 500 µg/ml concentration compared to the control and other concentrations of $CdCl_2$. After 45 minutes of exposure the percentage rapid swimming sperm also presented significance for all the $CdCl_2$ concentrations and was also significant for the control and 100 µg/ml concentration after 75 minutes (Figure 28 and 29). These results indicate that both metals significantly reduced motility at their highest concentrations and a trend was noted with a decrease in all parameters after 75 minutes of exposure. The common parameters that presented a significant effect to $CuSO_4$ at all three time points were LIN, STR and BCF, and to $CdCl_2$ at two or all three time points, were rapid swimming sperm, VSL, LIN, STR and WOB. The common parameters between the two metals were LIN and STR, therefore, they should be considered as a standard for evaluating the effect of $CuSO_4$ and $CdCl_2$, or even other metals, on baboon sperm motility.

For the rhesus monkey samples, after 15, 60 and 120 minutes of $CuSO_4$ treatment, one (15 min), four (60 min) and nine (120 min) parameters presented significantly lower values for the 250 µg/ml concentration compared to the control and other $CuSO_4$ concentrations. After 120 minutes of exposure five parameters were also significantly lower for the 100 µg/ml concentration and one parameter, VAP, for the 50 µg/ml concentration (Figure 32, 33 and 34). After 15, 60 and 120 minutes of $CdCl_2$ treatment, three (15 min), six (60 min) and twelve (120 min), parameters presented significantly lower values for the 500 µg/ml concentration compared to the control and other $CdCl_2$ concentrations. After 60 minutes of exposure one parameter, straightness, was also significantly lower for all the $CdCl_2$ concentrations compared to the control (Figure 35, 36 and 37). As for the baboon, these results indicate that both metals significantly reduced motility at their highest concentrations and a trend was noted with a decrease in all parameters after 120

minutes of exposure. The common parameters which presented a significant effect to $CuSO_4$ at two and all three time points were STR, WOB, ALH and BCF, and to $CdCl_2$ at two or all three time points, were medium swimming sperm, LIN, STR, WOB, ALH and BCF. The common parameters between the two metals were STR, WOB, ALH and BCF, therefore, they should be considered as a standard for evaluating the effect of $CuSO_4$ and $CdCl_2$, as well as other metals, on rhesus monkey sperm motility.

The last time point for each species presented the most parameters which were significant for both metals, except for the vervet samples, which had the most significant parameters at the 60 minute time point. This indicates that the metals had a more prominent inhibitory effect at the last time point and with the highest concentration of the metals.

According to a previous study, cadmium chloride has been found to reduce sperm concentration and motility in adult rats (Xu et al., 2001), and in human studies, it has been found that men with significantly higher blood cadmium levels had low sperm motility (Wirth and Mijal, 2010). With regards to copper, a study by Roblero et al. (1996) showed that 100 µg/ml copper ion (Cu2+) significantly affected human sperm motility. Other studies indicated that high concentrations of copper in the seminal plasma is correlated with reduced sperm motility and may render sperm immotile (Roblero et al., 1996; Wong et al., 2001; Eidi et al., 2010).

Previous studies by Roychoudhury et al. (2008) and Slanina et al. (2015) indicated motility parameters that were significantly affected from exposure to $CuSO_4$. The first study indicated a significant decrease in VAP, VCL and VSL at time points 0, 60 and 120 minutes, as well as STR, LIN, WOB, ALH and BCF were altered for rabbit sperm motility parameters (Roychoudhury et al., 2008). The study also indicated a non-significant decrease in ALH, which indirectly confirmed the decrease in progressive motility and BCF significantly decreased at the 0 and 60 minute time point (Roychoudhury et al., 2008). The second study indicated significantly lower values when analysing turkey spermatozoa for total motility and progressive motility after 30 minutes of exposure to $CuSO_4$ at concentrations ranging from 12.5 µg/ml to 50 µg/ml. A different study on turkey spermatozoa also presented a significant decrease in VCL, ALH and BCF (Slanina et al., 2015). In this current study the parameters which presented with significance for all three primate species across the three time points were progressive motility, rapid swimming spermatozoa, LIN, STR, WOB, ALH and BCF, which are similar to what was found in these previous studies and therefore confirms the negative effect of $CuSO_4$ on sperm motility and kinematics.

Copper is the metal cofactor for a variety of enzymes, including amine oxidase, copper-dependent superoxide dismutase, cytochrome oxidase and tyrosinase (Abdul-Rasheed, 2010). Copper not only promotes ROS formation, but can also bind directly to the free thiol groups of cysteine. In consequence, excess copper can lead to oxidation and crosslinks between proteins, thus inactivating enzymes or impairing structural proteins (Aydemir et al., 2006). Since spermatozoa contain high concentrations of polyunsaturated fatty acids and generate ROS, mainly superoxide anion and hydrogen peroxide, they are particularly susceptible to peroxidative damage (Wong et al., 2001), including lipid peroxidation. The enhanced production of free radicals reduces oxidative processes and glucose consumption, which reduces or abolishes sperm motility (Abdul-Rasheed, 2010) and damage to lipid membranes induced by ROS has been proposed as one of the major causes of human male infertility (Huang et al., 2000).

According to Adamkovicova et al. (2012, 2016), after Wister rats were exposed to $CdCl_2$, significant changes were seen in all sperm motion parameters. They reported a significant decrease in total motility, progressive motility, parameters reflecting velocity characteristics as well as STR, LIN, WOB, ALH and BCF. These findings confirmed the positive relationship between cadmium levels and asthenozoospermia, supporting the hypothesis that environmental exposure may contribute significantly to reduced sperm motility (Xu et al., 2001; Benoff et al., 2009). In this current study $CdCl_2$ was seen to significantly reduce progressive motility, rapid swimming spermatozoa, VSL, LIN, STR, WOB, ALH and BCF across the three time points and for all three species. These parameters were similar to what was found in these previous studies and therefore confirms the negative effect of $CdCl_2$ on sperm motility.

The decrease in sperm motility after exposure to $CdCl_2$ might be explained by cadmium's effects on microtubules and sperm mitochondrial function (Oliveira et al., 2009 (b)). A study by Kanous et al. (1993) found that cadmium inhibits microtubule sliding in bovine sperm, and microtubule sliding is in the base of flagellar motion (Oliveira et al., 2009 (b)). Oliveira et al. (2009 (b)) found that cadmium exposure affected sperm mitochondrial function and previous study by Au et al. (2001) also showed the deformation of mitochondrial cristae in sea urchin after direct exposure to cadmium. Since cadmium competes with calcium for calmodulin binding (Lindemann et a., 1991), and calcium calmodulin binding is important for sperm motility, calmodulin inhibition results in decrease in sperm motility (Schlingmann et al., 2007). As previously mentioned, the sperm membrane contains a characteristic high level of unsaturated fatty acids which makes spermatozoa susceptible to oxidative damage (Gravance et al., 2003). Low levels of ROS production by spermatozoa are necessary for physiological processes involving sperm capacitation and acrosome reaction.

However, excessive generation of ROS leads to reduced mitochondrial membrane potential and is associated with a decreasing energy availability, which may impede sperm motility (Wang et al., 2003; Tremellen, 2008).

As previously stated, in this study, a definite trend was noted, where all motility parameters presented a decrease at the last time point and some parameters were significantly reduced. This effect of the metals was seen in all three NHP species. Therefore, it is clear that $CusO_4$ and $CdCl_2$ reduced sperm motility in the three primate species and the motility tests employed were sensitive to this change.

5.2.2 The effect of $CuSO_4$ and $CdCl_2$ on Vitality

As previously stated, certain functional tests could not be completed due to the specified reasons, one of these tests being vitality for the rhesus monkey.

5.2.2.1 Eosin-Nigrosin (E-N) and heavy metal exposure

As in the first part of the study, prepared samples were exposed to concentrations of $CuSO_4$ and $CdCl_2$, after which they were stained with Eosin-Nigrosin after 15 minutes and at the last time point of exposure. The vervet monkey samples were found to be insignificant for both live and dead spermatozoa at the two time points, 15 and 120 minutes, for both metals. As previously stated, this either indicated that the metals had no effect on the vitality of the spermatozoa or the vitality test was unable to detect any change caused by the metal on the spermatozoa. Due to the sampling effect observed where the control had lower values than samples exposed to the metals, this could also be a reason for the results obtained.

The baboon samples proved insignificant for both live and dead spermatozoa at the two time points, 15 and 90 minutes, for the $CuSO_4$ concentrations, as seen for the vervet samples. However, exposure to $CdCl_2$ yielded different results. After 15 minutes a significant decrease was seen for the percentage live spermatozoa for the 500 µg/ml concentration, with a significant increase in values for the percentage dead spermatozoa. After 90 minutes of exposure, however, no significant difference between the control and exposed preparations were found for both live and dead spermatozoa. These results suggest that, as with the vervet monkey samples, $CuSO_4$ had no effect on the vitality of the baboon spermatozoa or the vitality test was unable to detect any change caused by the metal. The test did, however, detect the negative effect of $CdCl_2$ on sperm vitality, but only at the 15 minute time point, which may be due to the large standard deviations recorded at the 90 minute time point.

According to a previous study, significant negative correlations were found between seminal plasma copper concentrations and sperm vitality ($P<0.01$) (Eidi et al., 2010). Two studies by Babaei and Abshenas (2013)

and Tabassomi and Alavi-Shoushtari (2013) illustrated the successful use of the E-N stain to evaluate sperm vitality. Both studies indicated a significant decrease in percentage live spermatozoa after the consumption of copper by rats (Babaei and Abshenas, 2013) and a significant decrease in viability after exposure of water buffalo spermatozoa to 0.064 mg/l $CuSO_4$ (Tabassomi and Alavi-Shoushtari, 2013). This effect of $CuSO_4$ was however not evident in E-N stained spermatozoa in the current study. A study by Zhao et al. (2015), demonstrated the use of E-N staining for the evaluation of vitality in rat spermatozoa and found that cadmium significantly lowered sperm vitality ($P<0.01$) similar to the negative effect seen in our baboon samples.

Taking the results from previous studies into account, the use of the E-N stain to test the effect of the two heavy metals on primate spermatozoa was thus inconclusive in the current study. Since this vitality test was sensitive to the effect caused by one metal (CdCl2) in one primate species (baboon), it would require more testing in order to validate its sensitivity. The reason for this anomaly is not clear, and the possibility that the technique used is species specific can be ruled out due to previous studies on spermatozoa from drill (African forest baboon) (Maya-Soriano et al., 2015) rhesus monkey (Talwar et al., 1979) successfully employing E-N viability testing of primate spermatozoa.

5.2.2.2 Hoechst and Propidium Iodide (H&P) and heavy metal exposure

As in the first part of the study, prepared samples were exposed $CuSO_4$ and $CdCl_2$, after which they were stained with H&P after 15 minutes and the last time point of exposure. In the vervet monkey samples, no effect was seen on sperm vitality after 15 minutes of exposure to $CuSO_4$. However, after 120 minutes, a significant decrease in the percentage live spermatozoa was found for the 100 µg/ml and 250 µg/ml concentrations. Treatment with $CdCl_2$ proved the same result, with no effect on vitality at the 15 minute time point, but a significant decrease was seen after 120 minutes in the percentage live spermatozoa exposed to 500 µg/ml $CdCl_2$. For the baboon species, only one sample could be evaluated and therefore the ability of the test to detect the effect of the metals could not be proven. However, after 60 and 75 minutes of exposure to metals, the percentage live spermatozoa presented a 0 value for all the concentrations of $CuSO_4$ and at the 500 µg/ml concentration of $CdCl_2$. These provisional results suggest that the metals have a negative effect on baboon sperm vitality. However, in order to validate the sensitivity of test, more testing is required with a larger sample size. These results indicate that the metals did not have an immediate effect on sperm vitality but the vitality test was sensitive to the effects of the metals.

As stated with the Eosin-Nigrosin staining, previous studies have shown negative correlations between seminal plasma copper concentrations and sperm vitality (Eidi et al., 2010) and copper has been correlated

with the disturbance of semen parameters, one being sperm viability (Hamad et al., 2014). According to Pizent et al. (2012), several studies have found evidence that low-levels of cadmium affects semen quality. With regards to the vervet samples, an effect was only seen after the last time point for both metals, which indicates that a longer incubation period is required in order for the test to detect change in the sperm vitality. The provisional baboon results indicated that all $CuSO_4$ concentrations caused a decrease in vitality, while only the 500 µg/ml $CdCl_2$ had an effect, also at the last time point of testing. A previous study by Cardaci (2016), used the LIVE/DEAD assay (the SYBR® and Propidium iodide dyes) to measure sperm viability of the Drosophila after exposure to cadmium concentrations ranging from 10 µM to 500 µM $CdCl_2$. It was found that there was an increase in the number of dead sperm proportional to increasing amounts of $CdCl_2$ (Cardaci, 2016). The possible mechanism behind this effect may be explained by the work of Oliveira et al. (2009 (b)), which showed $CdCl_2$ exposure resulted in DNA fragmentation in rats. Therefore nuclear degradation is occurring in the $CdCl_2$ treated spermatozoa and this is supported by the high number of dead sperm (Cardaci, 2016). These previous findings therefore support the effects of copper and cadmium seen on sperm vitality.

5.2.2.3 WST-1 cytotoxicity assay and heavy metal exposure

As in the first part of the study, only vervet samples were tested, where samples were exposed to $CuSO_4$ and $CdCl_2$, after which they were treated with the WST-1 reagent. The absorbances were read at 60, 120 and 180 minutes of incubation (37 °C). The data presented a significant decrease in absorbance only after 180 minutes of exposure for both metals. This significance was seen at the 50 µg/ml, 100 µg/ml and 250 µg/ml concentrations $CuSO_4$ and all the $CdCl_2$ concentrations. A definite trend was seen with the $CuSO_4$ exposure, where the absorbance values decreased with an increase in $CuSO_4$ concentration for all three time points. After exposure to $CdCl_2$, the absorbance values decreased with an increase in $CdCl_2$ concentration except for the 500 µg/ml concentration where a slight increase was noted. Previous study by Aitken et al. (2003), indicated that human spermatozoa have the ability to reduce the WST-1 reagent and this significant effect was only seen after 180 minutes of incubation. This provides reasoning for the significant decrease in absorbance seen only after 180 minutes for this study. Another study by Ohtani et al. (2004), indicated that most tetrazolium (MTT, WST) assays have the ability to detect sperm toxicity caused by introduced chemical agents and WST-3 is thought to be the best for sperm analysis. Park et al. (2012) investigated the freezing and thawing damage caused to spermatozoa, and also demonstrated the successful use of WST-1 in order to evaluate sperm viability.

A study by Knazicka et al. (2012), evaluated the effect of copper ions on the viability of buffalo spermatozoa. The spermatozoa was exposed to concentrations of copper ranging from 3.9 µM/l to 1000 µM/l and viabilty

was measured by the MTT (metabolic activity) assay. Sperm viability was found to decrease significantly for all concentrations of copper and proved that this heavy metal, at high doses, are toxic to sperm motility and this subsequently disrupts the viability of spermatozoa (Knazicka et al., 2012). This result was also seen in the current study, especially for the vervet samples, where copper significantly decreased sperm motility and would therefore confirm the significant decrease in vitality seen via the WST-assay.

As mentioned in section 5.2.1, copper toxicity leads to ROS production followed by protein and lipid oxidation (Olivari et al., 2008). This was negatively correlated with sperm motility and viability (Tvdra et al., 2013). Once copper is taken up into a cell, the excess of it is reduced to cuprous ions that readily bind with sulfhydryl groups (Vlarengo et al., 1980), interfering with electron transport and inhibiting ATP production (Wimalasena et al., 2007). Copper also accumulates in the sperm mitochondria (Earnshaw et al., 1986), decreasing the mitochondrial membrane potential while causing ROS formation, leading to oxidative damage (Krumschnabel et al., 2005). In a study by Dawson et al. (1998), the effect of seminal plasma metals, one being cadmium, were compared to sperm viability and it was found that the metals levels were inversely correlated with the percentage of live sperm, (cadmium, $P<0.01$). The presence of these metals exerted a toxic effect on spermatozoa (Dawson et al., 1998). Previous studies also found that cigarette smoking has detrimental effects on sperm vitality, presenting negative correlations (Emad et al., 2012) and reduced sperm vitality (Chia et al., 1994). This cadmium induced toxicity effect is believed to be due to increased oxidative stress (Liu et al., 2010), the generation of ROS resulting in oxidative deterioration of lipids, proteins and DNA (Kaur and Sharma, 2015), which impairs sperm quality.

The current study's results and findings thus indicate that both metals had a negative effect on sperm vitality, with a decrease seen in absorbance values, as absorbance shows the direct proportion of viable cells therefore illustrating a decrease in live spermatozoa (Park et al., 2012). Therefore, this test was successful and sensitive to the change elicited by the metals on vervet sperm vitality. However, tests were performed on only one species, therefore more testing is required in order to further prove its sensitivity.

Out of the three vitality tests employed, the WST-1 assay was the most successful in evaluating sperm vitality and the effect of the metals, as the first tests proved insignificant results at some time points. The WST assay would therefore be best suited for further vitality and toxicity tests of other primate species.

5.2.3 The effect of $CuSO_4$ and $CdCl_2$ on Acrosome Intactness

As previously stated, certain functional tests could not be completed due to the specified reasons, one of these tests being acrosome intactness for the rhesus monkey.

As in the first part of the study, FITC-PSA reagent was employed to evaluate acrosome intactness. Samples were exposed to $CuSO_4$ and $CdCl_2$, after which they were exposed to the reagent. For the vervet monkey samples, however, the data was found to be not significant for percentage intact and reacted acrosomes, after 15 and 120 minutes, for both metals. The test did detect acrosomes that had reacted, as both metals presented lower values for the percentage intact acrosomes in comparison to the percentage reacted acrosomes for all concentrations of $CuSO_4$ and CdCl2, therefore distinguishing between intact and reacted, however this change was not significant. The baboon samples, however, presented a significant decrease in percentage intact and an increase in percentage reacted acrosomes for the 250 µg/ml $CuSO_4$ concentration after 15 minutes and additionally at the 50 µg/ml and 100 µg/ml concentration after 90 minutes of exposure. After $CdCl_2$ exposure, the results proved the same as the vervet samples and were not significant for percentage intact and reacted acrosomes, at both times. These results indicate that $CuSO_4$ had a negative effect on the intactness of baboon sperm acrosomes and this change was detected by the employed test, but not for vervet monkey spermatozoa. $CdCl_2$ presented no significant effect on both vervet monkey and baboon spermatozoa but the test did however detect a difference between intact and reacted acrosomes. Either the metals had no effect on acrosome intactness of the spermatozoa or the reagent did not accurately stain the acrosomal contents.

A study by Roblero et al. (1996) indicated that copper at a concentration of 100 µg/ml caused a significant decrease in human sperm viability after 5 hours of exposure. According to Aydemir et al. (2006), spermatozoa require a minimal level of ROS for normal capacitation and acrosome reaction. As mentioned, it has been shown that copper *in vitro* increased lipoprotein oxidation (Abuja and Albertini, 2001; Raveh et al., 2001). Therefore increased lipid peroxidation and altered membrane function can render sperm dysfunctional through impaired sperm functions, one being acrosome reaction reactivity (Cummins et al., 1994). A study by Roychoudhury et al. (2010) found that after copper exposure (3.57 to 4.85 µg $CuSO_4$/ml) there was a higher occurrence of rabbit spermatozoa with disordered membranes, which suggested alterations in anterior part of the head (acrosome). These findings therefore support the significant decrease in intact acrosomes seen for baboon samples after 90 minutes of $CuSO_4$ exposure, suggesting that the metal had an effect on the acrosomal membrane resulting in the release of acrosomal content. However, a study by Misro et al. (2008) found that a high release of copper drastically lowers human sperm motility and

viability but only marginally affected the acrosome status. This supports the insignificant results obtained when evaluating the vervet samples and possible species differences in the effect of this heavy metal on sperm acrosome intactness.

According to a study by Tielemans et al. (1999), cadmium exposure has not been associated with a significant reduction in human semen quality. This could explain why no effect was seen on acrosome intactness in both baboon and vervet species. However, several studies have found that infertile men with varicocele are typically oligozoospermic and the spermatozoa from these men had many defects, one of them being an acrosome reaction insufficiency (Rogers et al., 1985; Naftulin et al., 1991; Vigil et al., 1994; Benoff et al., 1995; El Mulla et al., 1995; Benoff, 1997; Benoff et al., 1997). This defect and others such as increased tapering head forms and reduced zona pellucida binding ability, results from an interaction between the observed increase in testicular cadmium and actin (Wang et al., 1996). Additionally, according to Oliveira et al. (2009 (b)), $CdCl_2$ (2 and 3mg) was found to significantly decrease the percentage of mice sperm with intact acrosomes. Arabi and Mohammadpour (2006) found that cadmium at a concentration of 750 µM/l significantly elevated malondialdehyde level/lipid peroxidation rate as well as indicated a negative effect of the metal on the membrane integrity of bull spermatozoa. Cadmium was observed to alter acrosomal membrane integrity and caused abnormal acrosome reaction (Arabi and Mohammadpour, 2006).

The incubation of cadmium with spermatozoa has been show to result in various and distinct morphological alterations of the sperm head membrane and especially of the acrosome region. This part of the spermatozoon is involved in the release of acrosomal enzymes and may therefore be highly sensitive to chemicals. In particular, cadmium, as well as other metals, has been shown to produce a large round hole in the acrosome sperm membrane (Castellini et al., 2009). As previously stated, low levels of ROS produced by spermatozoa are needed for physiological processes involving sperm capacitation and the acrosome reaction and increased cadmium levels results in the excessive generation of ROS (Wang et al., 2003; Tremellen, 2008) therefore impairing this physiological process. All these findings therefore suggest that an increase in cadmium levels could result in failure of acrosome reaction, however, this was not the aim of the present study.

With regards to this study and its findings, the acrosome intactness test was successful in distinguishing intact from reacted acrosomes, however, it was not significant and only sensitive to the change elicited by $CuSO_4$ and in one species. An increase in concentration of the metal resulted in a decrease in intact acrosomes, therefore causing more acrosomes to become reacted. However, due to this effect only being

evident in one species, more testing is required in order to further prove the effects of both metals on acrosome intactness and in more than one species.

5.2.4 The effect of $CuSO_4$ and $CdCl_2$ on Hyperactivation

As previously stated certain functional tests could not be completed due to the specified reasons, one of these tests being hyperactivation for the baboon.

As in the first part of the study, hyperactivation was evaluated through the use of the Flush technique. Samples were exposed to caffeine (5mM), $CuSO_4$ (100 µg/ml) and $CdCl_2$ (100 µg/ml), and a combination of a metal and caffeine. Vervet monkey samples were exposed and measured at 15, 20, 40, 60 and 90 minutes of incubation (37 °C) and the data was significant when treated with 100 µg/ml $CuSO_4$ over time. The data was also significant after 90 minutes of exposure to Caf (5mM) + $CuSO_4$ (100 µg/ml). After $CdCl_2$ treatment, the control samples presented significant data over time , after 15 minutes of exposure the data was significant for the caffeine and Caf (5mM) + $CdCl_2$ (100 µg/ml) samples and after 20, 40 and 60 minutes, the data was significant after treatment with 100 µg/ml $CdCl_2$. A trend was noted for both metals, where the percentage hyperactivation was lower when treated with the metals, caffeine maintained or increased percentage hyperactivation compared to the control and the combination of caffeine and the metal kept the values similar and even higher than the control values at each time point except after 60 ($CdCl_2$) and 90 ($CuSO_4$) minutes. For the rhesus monkey species, only one sample could be evaluated and therefore the ability of the test to detect the effect of the metals could not be proven. However, after 80 minutes of exposure to both metals, the percentage hyperactivation presented a lower value for the 100 µg/ml concentration of both metals and caffeine maintained the percentage hyperactivation to values higher than the control.

It is clear that both metals significantly lowered the percentage sperm hyperactivation for the tested primate species and as previously mentioned, the kinematic parameters of focus when determining hyperactivation are VCL, LIN and ALH (Baumber and Meyers, 2006). Therefore, according to the results obtained in 5.2.1, the parameters VCL, LIN, ALH, as well as STR was significantly and negatively affected by the highest concentrations of the these metals across the three primate species. $CuSO_4$ significantly reduced LIN, STR and ALH after 60 and 120 minutes of exposure to the 100 µg/ml and 500 µg/ml concentrations. $CdCl_2$ significantly reduced VCL, LIN, ALH and STR after 60, 75 and 120 minutes of exposure to the 100 µg/ml and 500 µg/ml concentrations. This significant effect on these kinematic parameters may therefore be related to the lowered hyperactivation levels seen here ranging from 15 to 90 minutes of exposure to the 100 µg/ml concentration of both metals.

According to Tabassomi and Alavi-Shoushtari (2013), the exposure of spermatozoa to higher copper concentrations (>0.064 mg/l) was detrimental to sperm parameters, one of them being progressive motility, where a significant decrease was seen. This could explain the significant effect caused by 100 µg/ml $CuSO_4$ to the vervet monkey samples as well as the effect seen after 80 minutes for the rhesus monkey sample. ROS are active participants in sperm capacitation, hyperactivation and sperm-oocyte fusion. Spermatozoa, however, lack cytoplasm, an important component containing antioxidants to counteract the damaging effects of high levels of ROS (Aitken et al., 1993(b); Tvdra et al., 2011). This therefore leaves sperm functional properties, in this case hyperactivation, vulnerable to the damaging effect caused by increased ROS production, which has previously been shown to result from excessive copper levels. However, a study by Bolanca et al. (2016), proved a different result, where high concentrations of copper were associated with increased human hyperactivated spermatozoa, indicated by low values of parameters LIN and STR. It was also seen that higher concentrations of copper, among other metals, negatively impact forward progression and sperm concentration (Bolanca et al., 2016). This was, however, not the case in the current study, as a definite decrease in percentage hyperactivation was seen when treated with $CuSO_4$.

A study by Hung et al. (2007) has shown that the exposure of rhesus monkey spermatozoa to environmental tobacco smoke (of which cadmium is a major component (Mukhopadhyay et al., 2010)) decreased sperm motility and its ability to undergo hyperactivation when exposed to cAMP and caffeine. Furthermore a study by Young et al. (1995) investigating rat spermatozoa, demonstrated that the percentage of motile cells mimicking hyperactivated motion was diminished with increased concentration (20-100 µg/ml) or increased exposure to cadmium. This proves the results found in the current study where the vervet samples were affected after 20, 40 and 60 minutes, as well as the lower values obtained for the rhesus sample after exposure to the 100 µg/ml $CdCl_2$ after 80 minutes.

The molecular events implicated in the initiation of capacitation include the removal of cholesterol from the sperm plasma membrane, ion fluxes resulting in alteration of sperm membrane potential and increased tyrosine phosphorylation of proteins involved in the induction of hyperactivation (Luconi et al., 1996; Osheroff et al., 1999). As previously explained, sperm samples treated with cadmium have shown structural and functional alterations of the sperm membrane due to peroxidation conditions. Lipid peroxidation impairs plasma membrane ion exchanges, which is necessary for maintenance of sperm movements (Rao et al., 1989). Copper and cadmium have both been shown to cause damage to the sperm membrane due to ROS and it would therefore affect the processes involved in the induction of hyperactivation by decreasing or impairing it.

The hyperactivation test was therefore shown to be successful as it was sensitive to the effect elicited by the metals as well as combined with caffeine. A decrease in hyperactivation was seen in particular for the vervet species which was proved by the significant data when treated with 100 µg/ml of $CdCl_2$ and $CuSO_4$. However, this effect was only proven for one species and therefore more testing would be required to further prove the sensitivity of this test for other primate species.

5.3 Conclusion

The current study involved an assessment of primate sperm function in order to establish the possibility of using these species as primate models for reproductive studies. We therefore hypothesised that by evaluating sperm functional tests and designing novel techniques for sperm analysis in primates, protocols will be standardized for use in future studies on male infertility. This would allow comparisons of human and NHP sperm function which may reveal or explain the high infertility rates in humans.

Sperm functional testing is seen as the second-tier level of sperm testing when the semen analysis (first-tier) reveals sperm abnormalities or if the couple is diagnosed with unexplained infertility (Vasan, 2011). The overall significance of sperm functional testing is that it complements the basic semen analysis, which at most only allows for the diagnosis of male infertility without providing evidence for an aetiological or physiopathological origin. Therefore there is a need to assess sperm functional competence in the extended evaluation of the infertile man (Oehninger et al., 2000). The functional tests investigated in this study were sperm motility and longevity, vitality, DNA integrity, acrosome intactness and hyperactivation. The first part of the study involved an optimization step, testing the competence of each functional test to efficiently evaluate primate sperm functional status. Most tests were found to be successful with the exception of one (Figure 38) and these tests were then further employed for validation testing. Furthermore, the vervet monkey delivered the most complete set of data and more significant results than the other two primate species investigated (Figure 39).

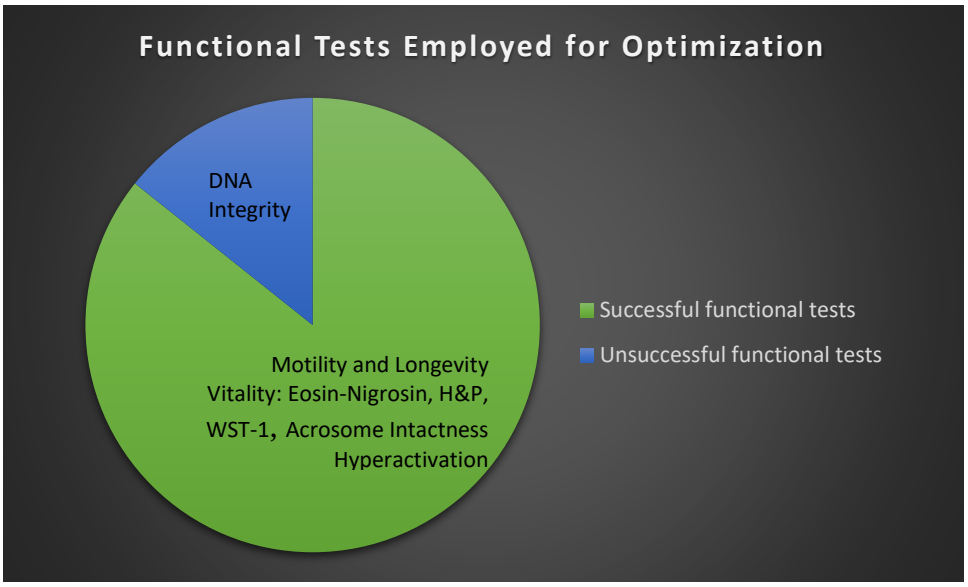


Figure 38: Functional tests that were successful in evaluation of sperm functional status during the optimization step

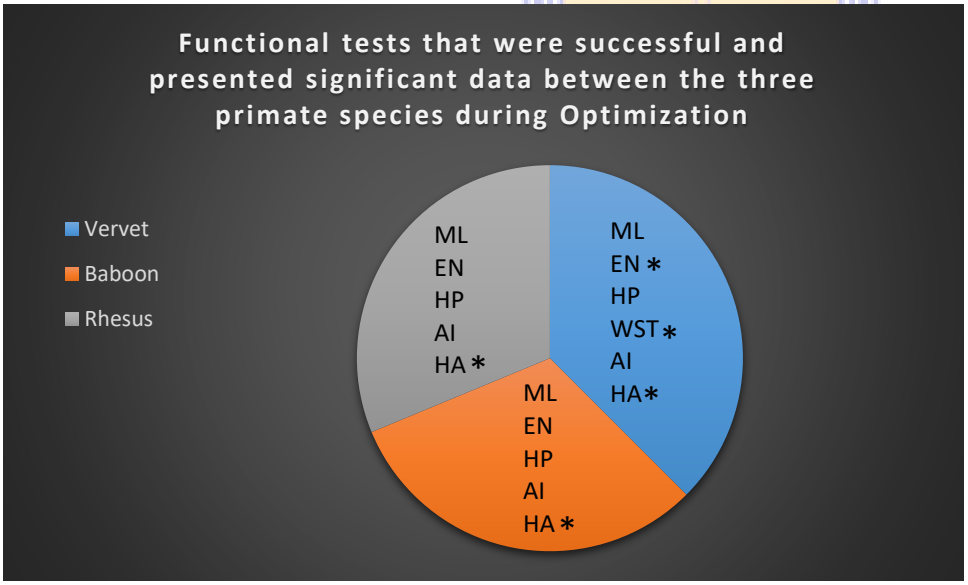


Figure 39: Functional tests performed that were successful for the three primate species as well as those that presented significant (*) results (P<0.05) during Optimization. ML – motility and longevity, EN – Eosin-Nigrosin stain, HP – Hoechst and Propidium Iodide stain, WST – WST- 1 cytotoxicity assay, AI – acrosome intactness, HA - hyperactivation

As previously explained, DNA integrity assessment using the TUNEL-assay in this study was not successful because no distinction could be made between fragmented and non-fragmentted DNA of certain species tested. It was therefore decided not continue with this sperm functional test in the remainder of the optimization part of the study as well as during validation testing. For future studies, it is recommended that DNA integrity should be re-evaluated in these three species with other methods in order to establish which method would best suit primates as well as human sperm. Others methods could include the Halosperm kit, Sperm chromatin structure assay (SCSA), Acridine orange test, Toluidine Blue, Aniline Blue, In situ nick translation assay, Comet assay and Sperm chromatin dispersion test (Schulte et al., 2010).

All functional tests were successfully performed when using samples from all three species, however, time constraints, low quality and low numbers of samples only allowed for either one sample or one time point to be evaluated, preventing analysis over time for baboon and rhesus monkey samples. The vervet monkey samples were therefore successfully used for all testing, providing results for analysis performed over time. However, this did not prevent the use of all three primate species for validation testing. All methods or techniques for functional testing were accepted and employed for use in the second part of the study.

The second part of the study involved the validation of all sperm functional tests, through the use of heavy metals to elicit a change or inhibitory action on sperm function in order to validate whether the tests were sensitive to this change. The chemicals copper sulphate and cadmium chloride were selected as they have been shown to negatively effect sperm function. Primate sperm was exposed to concentrations 10, 50, 100 and 250 µg/ml $CuSO_4$ and 10, 50, 100 and 500 µg/ml $CdCl_2$. All sperm functional parameters were found to be significantly decreased with the highest concentrations of the chemicals and if not significant, trends were seen of a reduction in results compared to controls. A significant decrease was seen in motility for all three species, vitality for vervet and baboon sperm, acrosome intactness for baboon sperm and hyperactivation for vervet spermatozoa (Figure 40).

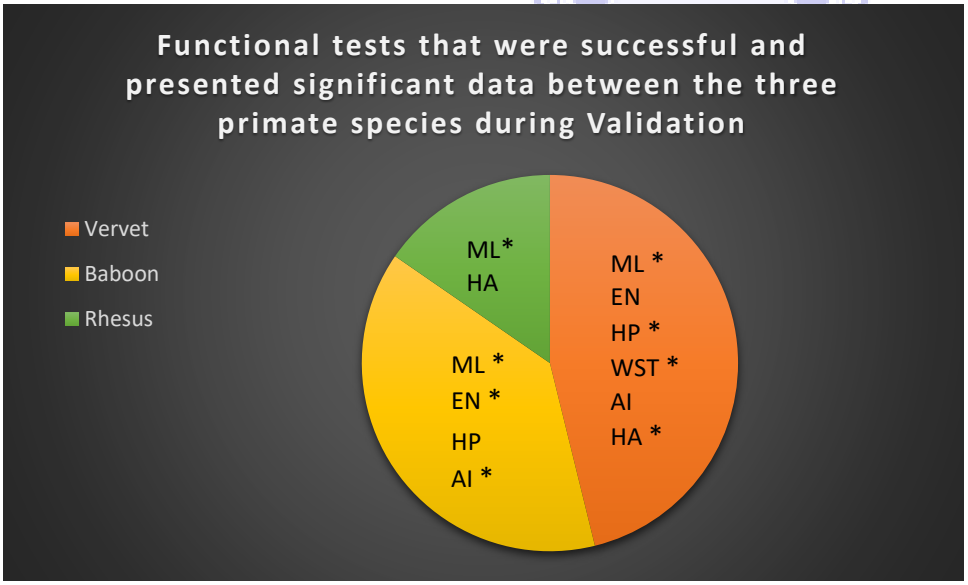


Figure 40: Functional tests performed that were successful for the three primate species as well as those that presented significant (*) results (P<0.05) during Validation. ML – motility and longevity, EN – Eosin-Nigrosin stain, HP – Hoechst and Propidium Iodide stain, WST – WST- 1 cytotoxicity assay, AI – acrosome intactness, HA - hyperactivation

For *motility*, both metals were found to significantly reduce motility for all three primates, at the highest concentration and a trend was noted with a decrease in all parameters after 120 minutes of exposure. These

significant reductions in motility parameters therefore prove the negative effect of these metals on sperm motility as well as the sensitivity of the motility test to the effect of these chemicals.

For *vitality,* the E-N proved no significant data for the vervet monkey spermatozoa when exposed to both metals and the same result was obtained for the baboon spermatozoa when exposed to $CuSO_4$. However, a significant negative effect was seen when baboon spermatozoa were exposed to $CdCl_2$. The H&P test did, however, detect a significant reduction in the percentage live spermatozoa for vervet monkey spermatozoa after exposure to the highest concentrations of both metals but this significant result was not seen for the baboon spermatozoa. The WST-1 assay proved to be the most successful out of the three vitality tests, as it detected a significant decrease in absorbances, which is directly proportional to viable spermatozoa, when exposed to all concentrations of $CuSO_4$ and 500 µg/ml $CdCl_2$, over time. These results prove the negative effect of these metals on sperm vitality and the sensitivity of these tests to distinguish live from dead spermatozoa after exposure to these chemicals.

For *acrosome intactness*, the test was successful in distinguishing between intact and reacted acrosomes, however, the data proved not significant for vervet spermatozoa after exposure to both metals and for baboon spermatozoa after exposure to concentrations of $CdCl_2$ but significant after exposure to $CuSO_4$.

For *hyperactivation*, both metals were found to cause a significant decrease in the percentage hyperactivation at the 100 µg/ml concentrations for the vervet spermatozoa. A significant effect was also seen when exposed to the combination of the metal and caffeine. These results indicate that the test was therefore successful and sensitive to the effect elicited by these chemicals.

These findings show that all tests employed were therefore successful in their abilities to determine sperm function and were sensitive to the inhibitory action of the chemicals $CuSO_4$ and $CdCl_2$.

As previously mentioned, metals may affect the male reproductive system directly, when they target specific reproductive organs, or indirectly, when they act on the neuroendocrine system (Pizent et al., 2012). Transition metals such as cadmium, chromium, nickel, vanadium and zinc constitute an important group of environmental factor that can disturb normal functioning of male reproductive system (Chandel and Jain, 2014). Increased levels of these metals in blood plasma or semen appear to be significantly and positively correlated with male infertility (Umeyama et al., 1986; Kumar et al., 2000). There is growing evidence that oxidative stress is implicated in the pathogenesis of male infertility (Aitken, 1994; Apostoli et al., 2007; Makker et al., 2009). Toxicological studies have shown that transition metals can accumulate in testes and/or

epididymis impairing their endocrine and reproductive functions (Pandey and Singh, 2002; Fossato da Silva et al., 2011; Liu et al., 2013). They can also adversely affect spermatogenesis and cause testicular necrosis through a direct effect on the testicular vasculature (Ragan and Mast, 1990; Aruldhas et al., 2005; Massanyi et al., 2007; Al-Attar, 2011). Several metals, including iron and cadmium, may increase ROS production, decrease glutathione and other antioxidant levels, enhance the lipid peroxidation of the cell membrane, cause apoptosis and contribute to the oxidative damage of DNA (Jones et al., 1979; Aitken et al., 1993 (b); Liang et al., 1999; Wellejus et al., 2000). This damage compromises paternal genomic contribution to the embryo (Tremellen, 2008) and increases the risk of infertility, miscarriage or serious disease in the offspring (Aitken and Koppers, 2011).

In order to further validate the sensitivity of these functional tests and the effect of these metals, further investigations should include a larger sample size from all three species as this was a limiting factor for many of the evaluations. Secondly, time periods of analysis should be lengthened in order to further investigate whether the metals need time in order to elicit a more prominent effect and also a lengthened period of study in order to allocate a sufficient amount of time for the investigation of more than one species. Thirdly, as there are many methods to evaluate one function, it should be considered to include more than one in order to establish which method works best for different species, contributing to the development of research primate models.

The many methods for evaluating sperm vitality could include, the use of the Eosin stain only, hypo-osmotic swelling test (WHO, 2010), Pentoxifylline (Khalili et al., 1999), MTT viability assay (Nasr-Esfahani et al., 2002; Byun et al., 2008) FluoVit vitality kit (Microptic, 2013) or the Vitaltest® (HalotechDNA, 2015). Methods for acrosome reaction include, the use of FITC-PNA (peanut-agglutinin) (Esteves et al., 2007), as it proved unsuccessful in this study and may require further investigation. Other methods include the use of the Coomassie brilliant blue (CBB) stain and acid phosphatase detection (Zhang et al., 2005), immunodetection techniques, differential interference contrast or phase contrast and flow cytometry (Pietrobon et al., 2001). For hyperactivation, together with the current Flush technique and SCA CASA system employed in this study, previous studies have illustrated the use of the Cellsoft CASA system with an equipped hyperactivation module (Cyro Resources, New York, NY) (Wang et al., 1991) to evaluate hyperactivation. A new method was developed in a study by Mazzilli et al. (2001), to evaluate sperm hyperactivation based on sperm head axis angle deviation through the use of the superimposed image analysis system (SIAS).

The evaluation of additional sperm functional tests and their sensitivity would contribute to the assessment of NHP sperm function and the development of suitable models for research studies. Future studies should

include the evaluation of more sperm functions or functional tests, such as, sperm morphology, location and viability of sperm mitochondria, cervical-mucus penetration, zona pellucida binding and acrosome reaction induction, since metals have been found to affect the male reproductive system.

Sperm morphology is a strong indicator of bodily and testicular health and morphology as a tool in the clinical diagnosis of a patient and also as a prognostic and predictive tool for the prediction of male fertility potential, makes its evaluation require further refinement (Menkveld et al., 2011). According to the WHO (2010), there are three recommended staining methods for sperm morphology analysis, the Papanicolaou, Shorr and Diff-Quik stain, whereafter the stained preparations are examined and classified as normal or abnormal. Additionally, van der Horst and Maree (2009), developed a new staining technique, the SpermBlue® staining process, to successfully evaluate sperm morphology of human and animal spermatozoa. A study by Kao et al. (2004), through the use of a new concurrent PCR method, found that mtDNA (mitochondrial DNA) content may be used for the assessment of the fertility and motility of human sperm and that depletion of mtDNA may play an important role in the pathophysiology of some types of male infertility and subfertility. The location and viability of the mitochondria can be assessed by staining the mitochondria with the fluorescent probe, MitoTracker® Red CM-H_2XRos (Invitrogen). The presence and intactness of individual proteins is determined by labelling it with specific probes in the mitochondrial electron transport chain. To label mitochondria, spermatozoa are simply incubated with the probes and can be treated with an aldehyde-based fixative for samples that need fixation. Semen is centrifuged and resuspended in prewarmed (37 °C) staining solution containing the MitoTracker®probe, whereafter they may be analyzed by fluorescence microscopy (Invitrogen, 2008).

Sperm-cervical mucus penetration test measures the ability of sperm in the semen to swim up into a column of cervical mucus or substitute (Ola et al., 2003). A study by Hull et al. (1984) found that spermatozoa that are unable to penetrate cervical mucus were also unable to fertilize a human oocyte *in vitro*. Zona pellucida binding assays have been used to predict fertility in humans (Kaskar et al., 1994) and have also been used to study how different sperm abnormalities influence fertility. Most assays use fresh or salt-stored oocytes collected from fresh ovaries but because ovaries are not easy to obtain on a regular basis, chilled and frozen-thawed ovaries have been tested, with varying results (Hermansson et al., 2007). The acrosome reaction induction test induces the acrosome reaction to evaluate if spermatozoa can actually undergo the acrosome reaction (can also be used as a positive control for treatment study). Sperm samples are treated with the Calcium Ionophore (A 23187) (Sigma, Cape Town, South Africa), followed by

an incubation in medium containing high bovine serum albumin to induce Acrosome reaction (Mukhopadhyay et al. 2008).

This study allowed for the evaluation of established sperm functional tests, where they were optimized for the indicated primate sperm and their sensitivity validated with the selected metals. Therefore, we achieved our first aim for assessing NHP sperm function and the evaluation of standard sperm functional tests, further proving that primates would be good models for reproductive studies. Secondly, although novel sperm functional tests were not developed per se, the standard tests were adjusted and modified in order to accommodate the species specific requirements of the semen and the quality of spermatozoa for each primate species. Therefore, more testing is required to further validate the sensitivity of these functional tests with larger sample sizes, more primate or mammalian species, longer incubation periods or time-based studies, as well as including other sperm functions for testing. We were able to inhibit sperm function, testing the sensitivity of each test, which was successful in detecting change and therefore reaching our second aim. The development of set protocols of each functional test for each of the studied three species is a necessity, taking into account the constraints and findings of this study, if they are to be considered for use as basic primate models in the future.

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