University of Pretoria

Faculty of Health Sciences

School of Medicine

Investigating alternative sperm preservation methods for assisted reproductive technologies

By

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Submitted in fulfilment of the requirements for the MSc Degree in Reproductive Biology, at the Faculty of Health Sciences, University of Pretoria

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To my mother, Christa Slabbert

“All that I am, or hope to be, I owe to my angel mother”

Abraham Lincoln

DECLARATION BY CANDIDATE

'I hereby declare that the thesis submitted for the degree MSc Reproductive Biology, at the Faculty of Health Sciences, University of Pretoria, is my own original work and have not previously been submitted to any other institution of higher education. I further declare that all sources cited or quoted are indicated and acknowledged by means of a comprehensive list of references.'

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Signature

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Faculty of Health Sciences Research Ethics Committee

15/05/2012

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SUMMARY

Introduction: Cryopreservation of human sperm is considered a routine practice in assisted reproduction laboratories. Semen samples are mainly cryopreserved as a back-up for procedures, donor sperm, and validation of samples from human immunodeficiency virus-positive patients. Human immunodeficiency virus semen samples generally result in a low yield of purified spermatozoa after decontamination. These samples need to be cryopreserved for later use. Unlike conventional cryopreservation, vitrification does not use harmful cryoprotectants, thereby potentially reducing sperm damage. Vitrification is not yet common practice for sperm cryopreservation in assisted reproduction. The aim of this study was to establish the feasibility of utilising vitrification as an alternative to current conventional cryopreservation of spermatozoa.

Methods: Semen samples were collected from human immunodeficiency virus-negative patients seeking diagnostic assistance from the unit. All samples were processed according to the unit's standard protocol. For Study 1A (n=10) washed samples were divided and cryopreserved using three different cryopreservation media, and two different freezing protocols. In Study 1B (n=15), washed samples were divided and preserved using cryoprotectant-free vitrification in 100 µl, 300 µl and 500 µl volumes. For Study 2 (n=35) washed samples were split and cryopreserved using cryoprotectant-free vitrification (utilizing the volume that resulted in the highest quality spermatozoa in Study 1B) and conventional slow freezing (using the medium and protocol that resulted in superior quality spermatozoa in Study 1A). Post thawing, motility and kinetic parameters (Studies 1 and 2), viability (Study 1), mitochondrial membrane potential (Study 2), and DNA fragmentation (Study 2) of the two groups were compared.

Results: Study 1A indicated that cryopreserving spermatozoa using Freezing Medium resulted in the highest quality spermatozoa with regards to motility and viability ($p<0.05$). Comparing the two preservation protocols, no conclusion could be reached on which protocol yielded superior results ($p>0.05$). The RBL freezing method is shorter, simpler and requires less equipment, and was therefore deemed the preferred method. Study 1B showed that the larger vitrification volumes (300 µl and 500 µl) yielded better spermatozoa in terms of motility and viability ($p<0.05$). No significant difference was observed with respect to the 300 µl and 500 µl vitrification volume groups. For practical reasons, 300 µl volumes will provide sufficient sperm for any procedure and, the intermediate volume ensures that more than one straw can be preserved. Study 2 found that cryoprotectant-free vitrification resulted in spermatozoa with significantly higher mitochondrial membrane potential and significantly lower apoptosis post thawing ($p<0.05$).

Discussion: Conventional cryopreservation methods may compromise various sperm parameters and final yield. In this study, cryopreservation and cryoprotectant-free vitrification had equivalent outcomes with respect to sperm motility. However, the latter method yielded superior results in terms of $\Delta\Psi$ and DNA sperm fragmentation. In conclusion, vitrification is an easy, rapid and more affordable technique that requires no special equipment. Using vitrification for purified sperm samples of patients could potentially result in a better post thaw quality for ART procedures.

Keywords: Human spermatozoa, conventional cryopreservation, cryoprotectant-free vitrification, motility, viability, mitochondrial membrane potential, DNA fragmentation

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LIST OF ABBREVIATIONS

$\triangle\psi m$:	Mitochondrial membrane potential
8-0HdG:	8-Hydroxy-2-deoxyguanosine
AI:	Artificial insemination
AIDS:	Acquired immunodeficiency syndrome
ALH:	Amplitude lateral head displacement
AR:	Acrosome reaction
ART:	Assisted reproductive technologies
ARV:	Antiretroviral
BMI:	Body mass index
BSA:	Bovine serum albumin
CASA:	Computer aided sperm analyzer
CBS™:	Cryo Bio System
CD4:	Cluster difference 4
CO_2:	Carbon dioxide
CP II:	CryoProtect II
CPA:	Cryoprotectant agent
CVM:	Cytomegalovirus
DGC:	Discontinuous density gradient centrifugation
DMSO:	Dimethyl sulfoxide

DNA:	Deoxyribonucleic acid
dUTP:	Deoxyuridine triphosphate
FITC:	Fluorescein isothiocyanate
GLS:	Generalized least squares
FM:	Freezing Medium
HAART:	Highly active antiretroviral therapy
HBV:	Hepatitis B
HCV:	Hepatitis C
HEPES:	Hydroxyethyl piperazineethanesulfonic acid
HIV-1:	Human immunodeficiency virus sub-type 1
HSA:	Human serum albumin
ICSI:	Intra cytoplasmic sperm injection
IS_M:	Irvine Scientific® freezing protocol
IUI:	Intrauterine insemination
IVF:	*In vitro* fertilization
LN_2:	Liquid nitrogen
L-NAME:	L-nitro-G-arginine methyl ester
MCICCP:	Carbonylcyanide m-chlorophenylhydrazone
$NaHCO_3^-$:	Sodium bicarbonate
NO:	Nitric oxide
NOS:	Nitric oxide synthase

O_2:	Oxygen
PBS:	Phosphate buffer saline
PCR:	Polymerase chain reaction
PETG:	Polyethylene terephthalate glycol
pH:	Potential of hydrogen
PI:	Propidium iodide
PUFA:	Polyunsaturated fatty acids
PVC:	Polyvinyl chloride
RBL:	Reproductive Biology Laboratory
RBL_M:	Reproductive Biology Laboratory standard freezing protocol
ROS:	Reactive oxygen species
RNA:	Ribonucleic acid
SBAH:	Steve Biko Academic Hospital
SF:	SpermFreeze
SNP:	Single nucleotide polymorphism
TdT:	Terminal deoxynucleotidy transferase
TUNEL:	Terminal deoxynucleotidyl transferase mediated dUTP nick end labelling assay
TYB:	Test yolk buffer
V_{100}:	100 µl vitrification volume
V_{300}:	300 µl vitrification volume

V_{500}: 500 µl vitrification volume

VAP: Average path velocity

VCL: Curvilinear velocity

VSL: Straight-line velocity

WBC: White blood cells

WHO: World Health Organization

WOB: Wobble

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CHAPTER 1

OVERVIEW OF STUDY

1.1 Motivation for study

Cryopreservation is used as a method of preserving different cells and tissues that includes male and female gametes and embryos.[1] With temperatures below sub-zero combined with the appropriate cryoprotective agents (CPA), the physiological and reproductive functions are preserved, making long-term storage possible, without the loss of viability.[2] Freezing of human spermatozoa are routinely applied in assisted reproductive technology (ART) laboratories.[3] Cryopreservation of male gametes offers the opportunity to pubertal boys and men to preserve their fertility, or to conserve gametes for impending ART treatments. Human semen cryobanking can be divided into two broad areas: semen banking for future use, and donor banking.[4] Cryopreservation 'success' is measured in terms of post thaw motility. Until now, cryopreservation has not provided complete protection, since the motility of the preserved sperm decreases to more or less 50% of their pre-freezing value.[5] An even greater decrease is observed in spermatozoa from infertile patients compared to fertile patients.[6] The methods of cryopreservation developed in the 1950s are still applied today in many species and therefore, it is safe to say, that modifications and improvement in the conventional methods are necessary.[2]

Vitrification of human spermatozoa are not yet routinely applied by infertility laboratories, as further investigations are needed in this area.[2] Vitrification preserves sperm by plunging the suspension directly into liquid nitrogen (LN_2). As a result, solidification of living cells without the formation of ice crystals is achieved during cooling.[7,8] The prescribed method for vitrification of oocytes, embryos and other tissues requires fast cooling rates and high CPA

concentrations,[9] but spermatozoa has a low tolerance level for these high concentrations. High concentrations of CPA are detrimental to spermatozoa because of the lethal osmotic effects and possible chemical alteration exerted.[10] A method without the use of conventional CPA and applying rapid freezing has been described for the vitrification of human spermatozoa.[7,10,11] This method entails replacing the conventional CPA with a protein and carbohydrate solution (1% Human serum albumin [HSA] and 0.25 M sucrose). A major disadvantage of cryoprotectant-free vitrification is that only minute volumes of spermatozoa (≤20 µl) can be vitrified at one time. Also, most of the proposed methods do not prevent direct contact with LN_2.[7,9,10] Four different aseptic vitrification techniques were suggested by Isachenko *et al.*[12,13] but only small volumes, ranging from 1 µl to 40 µl of sperm suspension could be vitrified. In 2011, the Isachenko group reported a novel aseptic cryoprotectant-free vitrification method for larger volumes of spermatozoa.[12]

In recent years there has been a global surge in the demand for ART in patients infected with blood-borne viral infections such as Human immunodeficiency virus (HIV), Hepatitis B (HBV) or Hepatitis C virus (HCV).[14] The Reproductive and Endocrine Unit at Steve Biko Academic Hospital (SBAH) is currently the unit with the longest standing history in semen decontamination experience. See Addendum B for the standard operating procedure (SOP) of the Reproductive and Endocrine Unit at SBAH (SOP nr: F1.15.1). Semen from HIV-1 positive males are washed using a discontinuous density gradient centrifugation (DGC) method together with a novel tube insert, to separate the sperm from the HIV-contaminated seminal plasma. The efficacy of the washing procedure must be verified and a small portion of the semen and the purified samples are sent to pathology laboratories for viral validation. Results are in general received after 48-72 h, depending on the transport distance. The remaining portion is cryopreserved until the viral validation outcome has been obtained. Cryopreserved samples are either maintained for future use or discarded. See Figure 1 for a summary of the decontamination protocol. Preliminary studies showed that sperm washing procedures together with the

ProInsert™ (Nidacon™ International, Mölndal, Sweden) results in a significantly lower yield of purified spermatozoa. The decrease in sperm yield together with the detrimental effects of cryopreservation reduces the sperm quality of the sample significantly, influencing the choice of the ART procedure (*in vitro* fertilization [IVF] or intra cytoplasmic sperm injection [ICSI]) to be performed.[15] Further investigations are necessary to develop alternative methods for sperm storage.

The study aimed to find alternative preservation methods that might be less detrimental and more optimal (without the negative effects of the conventional freezing methods with cryoprotectants). Finding quicker, simpler and more cost effective methods, will not only be of benefit to the Reproductive and Endocrine unit at SBAH, but could contribute to simpler and more accessible ART services.

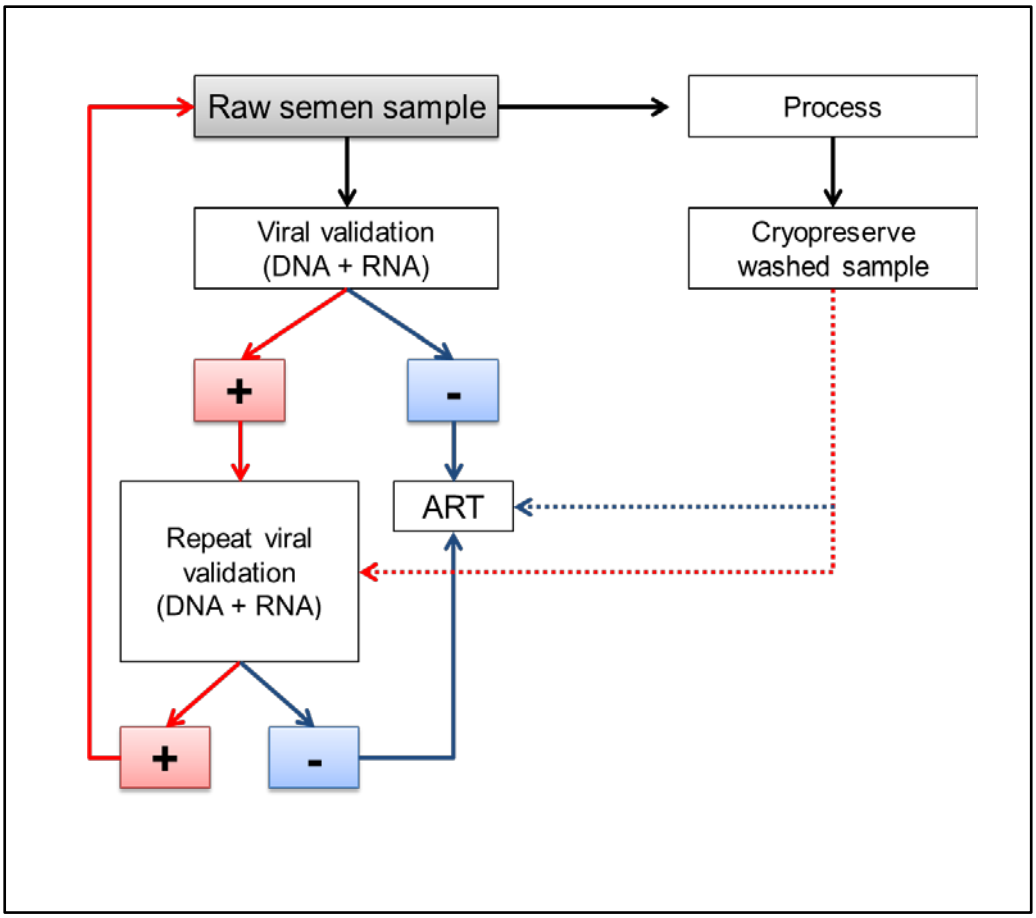

Figure 1: Line diagram explaining standard operating procedures for semen samples from HIV positive males at Steve Biko Academic Hospital

1.2 Research questions

1. Does the outcome of the quality of spermatozoa differ between the current commercial available cryopreservation methods?
2. Is it possible to vitrify larger volumes of spermatozoa than the standard volume?
3. Can cryoprotectant-free vitrification become the future norm for the preservation of spermatozoa in an assisted reproduction program?

1.3 Hypothesis

1.3.1 Study 1A

Ha: Cryopreserving human spermatozoa using the standard method employed by the SBAH, Reproductive Biology Laboratory (RBL) yields superior results when compared to other commercially available methods.

1.3.2 Study 1B

Ha: Vitrifying spermatozoa using larger volumes than the standardized norm, results in better sperm yield and quality post-thawing.

1.3.3 Study 2

Ha: Cryoprotectant-free vitrification of human spermatozoa yields equal or inferior results when compared to current conventional methods.

1.4 Aim

The aim of this study is to investigate alternative methods of sperm preservation for assisted reproductive technologies (ART).

1.5 Objectives

i) To determine the effect of commercially available cryopreservation methods on human spermatozoa post-thawing.

ii) To determine the implication of increasing the vitrification volumes of spermatozoa on the post thawing yield and quality.

iii) To determine if vitrification of spermatozoa will be a superior method to preserve sperm in comparison to current conventional methods.

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CHAPTER 2 LITERATURE REVIEW

2.1 Introduction

Preservation of human spermatozoa has been revolutionized over the past few decades. Although methods to cryopreserve spermatozoa has been improved and modified, there are still numerous opportunities and areas for improvement. Many detrimental effects are associated with cryopreservation (post-thaw) of human spermatozoa, the most common being a decrease in motility. Sperm motility is a strong predictor for fertilization *in vitro* and is therefore considered vital during ART.[16] Although the optimal cryopreservation protocol for human spermatozoa is yet to be defined[3], investigations into finding different methods to store sperm for longer periods of time, without the detrimental effects of freezing are urgently needed.

2.2 History of cryopreservation and vitrification

Storage and cryopreservation of sperm dating back to 1776, when Lazaro Spallanzani reported that sperm becomes immobile when cooled in snow. Nevertheless, it was not until 1866, when Montegazza suggested that men going to war, who were killed on the battle field, could produce a legal heir with his semen frozen and stored at home.[17] Montegazza found that after thawing semen stored over a long period of time at approximately -15°C, a small percentage remained motile. As a result, a sperm bank for cattle was suggested, and it was proposed that the same process could be followed for human semen. In 1949 Polge made a very significant progress, by discovering that the use of glycerol could provide protection to cells at very low temperatures. This is often cited as the defining moment in the establishment of cryopreservation.[18] The first babies after fertilization with cryopreserved

human sperm was born in 1953.[19] Success rates continued to increase by the implementation egg yolk as a cryoprotective medium, together with using gradual freezing techniques in pellets or straws in 1964.[6]

An alternative method of freezing is vitrification. Vitrification constitutes rapid cooling of water until it reaches a glassy state, without intracellular ice crystallization. Katkov *et al*.[20] described five methods to achieve vitrification:

- Equilibrium freezing allows to freeze out of the bulk of water with the use of CPA and storing at ultra-low temperature
- Lyophilisation using slow freezing to moderate low (-40°C) followed by secondary drying at 30°C (food and pharmaceutical industries)
- Ice free vitrification at fast cooling rates and high concentrations of CPA
- Ice free vitrification at fast cooling rates and in the absences of CPA
- High temperature vitrification by air/vacuum drying at temperatures above 0°C

The earliest experiments on vitrification were reported in 1930. However these were unsuccessful, because the fast cooling rates were unattainable at the time. After the discovery of cryoprotectants and the use of LN_2 it became possible to vitrify various types of cells, however, vitrification of human spermatozoa was unsuccessful. Vitrification of human spermatozoa became a reality only recently. The failed attempts to vitrify spermatozoa were possibly due to the high concentration of permeable CPA used during vitrification and the low tolerance of spermatozoa to these agents. Exposure to high concentrations of CPA can lead to toxic and osmotic shock and consequently would be lethal to spermatozoa.[20,21] The first successful vitrification of human spermatozoa without CPA was reported in 2002 by the Isachenko group.[10,22]

2.3 Application of cryopreservation

Cryopreservation of human spermatozoa is routinely applied by ART laboratories.[3] Human semen cryobanking can be divided into two distinct areas; autoconservation, and donor banking. Autoconservation is applicable to pre-vasectomy patients, fertility preservation before chemotherapy or radiotherapy, washed semen from men carrying infectious disease such as HIV-1, patients whose job description entails occupational risks, or for men who are unavailable on the day of the ART procedure. Donor banking is beneficial for couples who carry known hereditary or infectious diseases, or for single women wishing to conceive, or insemination of lesbian women. As of yet, there is no universally accepted method for preserving spermatozoa, as different methods yield relatively satisfactory results.[4] Figure 2 is a flow diagram summarizing the different applications of cryopreservation for human spermatozoa (adapted from Björndal, 2010[4]).

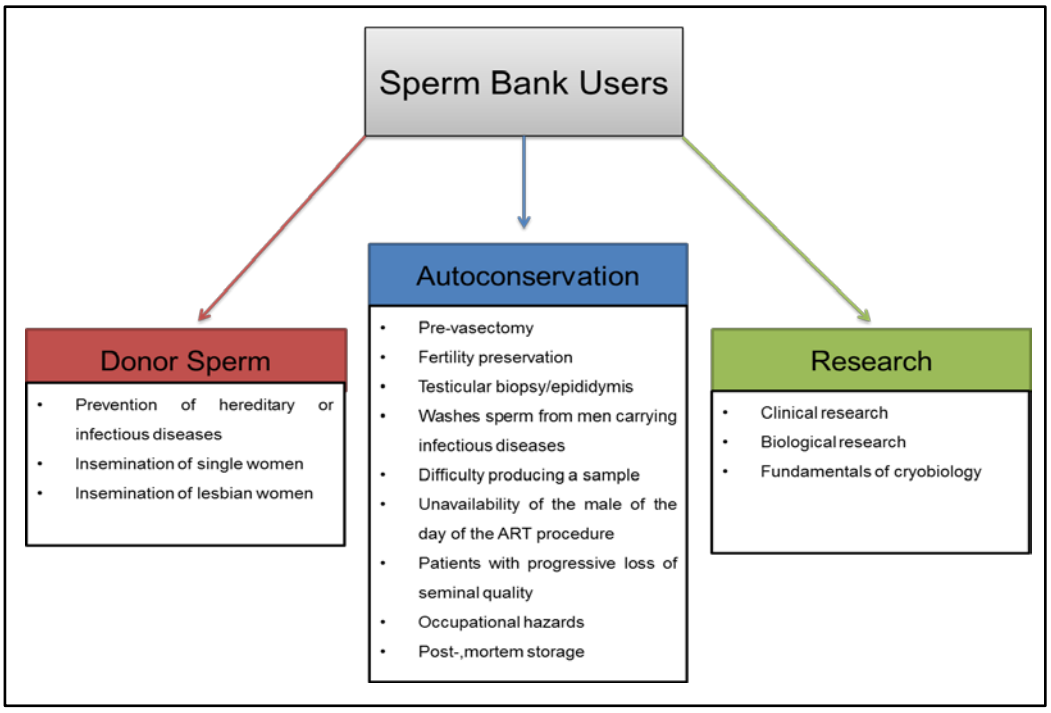


Figure 2: Flow diagram summarizing the various applications for cryopreservation of human spermatozoa[4]

2.4 Effects of conventional cryopreservation on human spermatozoa

Donnelly *et al.*[16] reported that post thawing, the average velocity of spermatozoa cryopreserved (static phase vapour for 15 min, after which the frozen vial was suspended into LN_2) was decreased by 45%.[16] An even greater decrease was observed in spermatozoa from infertile patients compared to that of fertile patients. Similar results were found by O'Connell *et al*.[23] when investigating the effects of cryopreservation on sperm morphology, motility and mitochondrial function. Motility parameters were assessed using computer aided semen analysis (CASA) and a highly significant reduction in all motility parameters after cryopreservation was observed. Menkveld[24] studied the effect of cryopreservation on the semen parameters of 64 cancer patients. The mean progressive motility (a) was reduced from 45.4±13.5% to 23.3±12.1%, post thawing. Cryopreservation was also implicated to decrease the number of spermatozoa with normal morphology.[16,23] Donnelly *et al*.[16] reported a 55% decrease in normal morphology in fertile donors and a decrease of 62.5% in infertile patients. Both Menkveld[24] and Donnelly *et al.*[16] noted a marked increase in the incidence of amorphous, megalo, and elongated heads. Interestingly, in the infertile group, there was an increase in midpiece abnormalities and a reduction in spermatozoa that had cytoplasmic droplets after freezing-thawing.

Thomson *et al.*[25] demonstrated, using TUNEL (Terminal deoxynucleotidyl transferase mediated dUTP Nick end labelling) assay, an increase in the percentage of sperm DNA (deoxyribonucleic acid) fragmentation after cryopreservation and thawing in patients suffering from fertility problems. Similar results were reported by Donnelly *et al.*[16] in a study to determine the DNA integrity (using the Comet Assay) and morphology of spermatozoa from fertile and infertile men. A significant decrease in sperm DNA integrity was observed in the infertile group post thawing. Surprisingly, no significant

difference in the DNA integrity of fresh and frozen-thawed semen from fertile men was observed.

2.5 Effects of cryoprotectant-free vitrification on spermatozoa

Data presented by Nawroth *et al*.[22] indicating that it is possible to vitrify human sperm in the complete absence of cryoprotectants in addition to the conventional programmable slow-freezing cryopreservation. The study group was divided into four categories: raw semen with and without cryoprotectants, and washed/processed spermatozoa with and without cryoprotectants. Programmable freezing with no cryoprotectants reduced sperm motility to 1.7% for raw and 0.2% for processed spermatozoa. After vitrification (with cryoprotectants), only 2% of spermatozoa remained motile. However, when the processed spermatozoa, frozen conventionally with cryoprotectants were compared to spermatozoa vitrified without cryoprotectants, 38% and 49% motile spermatozoa were observed, respectively. See table 1 for a summary of the results:

Table 1: Vitrification and cryopreservation of spermatozoa with and without cryoprotectants[22]

Method	Percentage motile
Programmable freezing *without* CPA*	0.2%
Programmable freezing *with* CPA*	38%
Vitrification without CPA*	49%
Vitrification with CPA*	2%

Isachenko *et al.*[7,11] found that regardless of whether spermatozoa were immersed directly into LN_2 or frozen in LN_2 vapour, the DNA integrity was found to be unaffected. To measure the 'fertilizing potential' of human spermatozoa, Isachenko *et al.* considered three variables:

1) Fertilization rate
2) Early cleavage of zygotes
3) Late development of embryos

During IVF procedures, equal fertilizing potential, regardless of the preservation method (cryoprotectant-free vitrification or frozen in vapour of LN_2) was observed.

Vitrification of human spermatozoa is a quick, straightforward technique that does not require any special cryobiological equipment.[7,11] In 2005, Isachenko *et al.*[9] described four different methodologies for cryoprotectant-free vitrification using:

i) Cryoloops
ii) Droplets
iii) Open pulled straws
iv) Open standard straws

The four methods are depicted in figure 3. The drawback of this alternative cryoprotectant-free vitrification of human spermatozoa was that only small volumes of spermatozoa (≤20 µl) could be vitrified at a time, and most of the proposed methods were open systems i.e. do not provide protection against direct contact with LN_2. These factors made this approach unsuitable for preservation of spermatozoa for ART.[8] The only closed aseptic technique was the open-pulled straw method. This method requires an open-pulled straw and a 0.5 ml insemination straw. The sperm suspension is drawn into the open-pulled straw, and placed in the 0.5 ml insemination straw. Both ends of the insemination straw are sealed, before being plunged into LN_2. A minor modification to this technique was made by the same researchers in 2007.[26] A

standard 0.25 ml insemination straw was cut at a 45˚ angle and the sperm suspension was pipetted onto the cut end of the straw. Thereafter the straw was inserted into the 0.5 ml insemination straw. Both ends were then sealed, and the straws were plunged into LN_2. The successful application of this vitrification technique was proven after the birth of two healthy babies.[27]

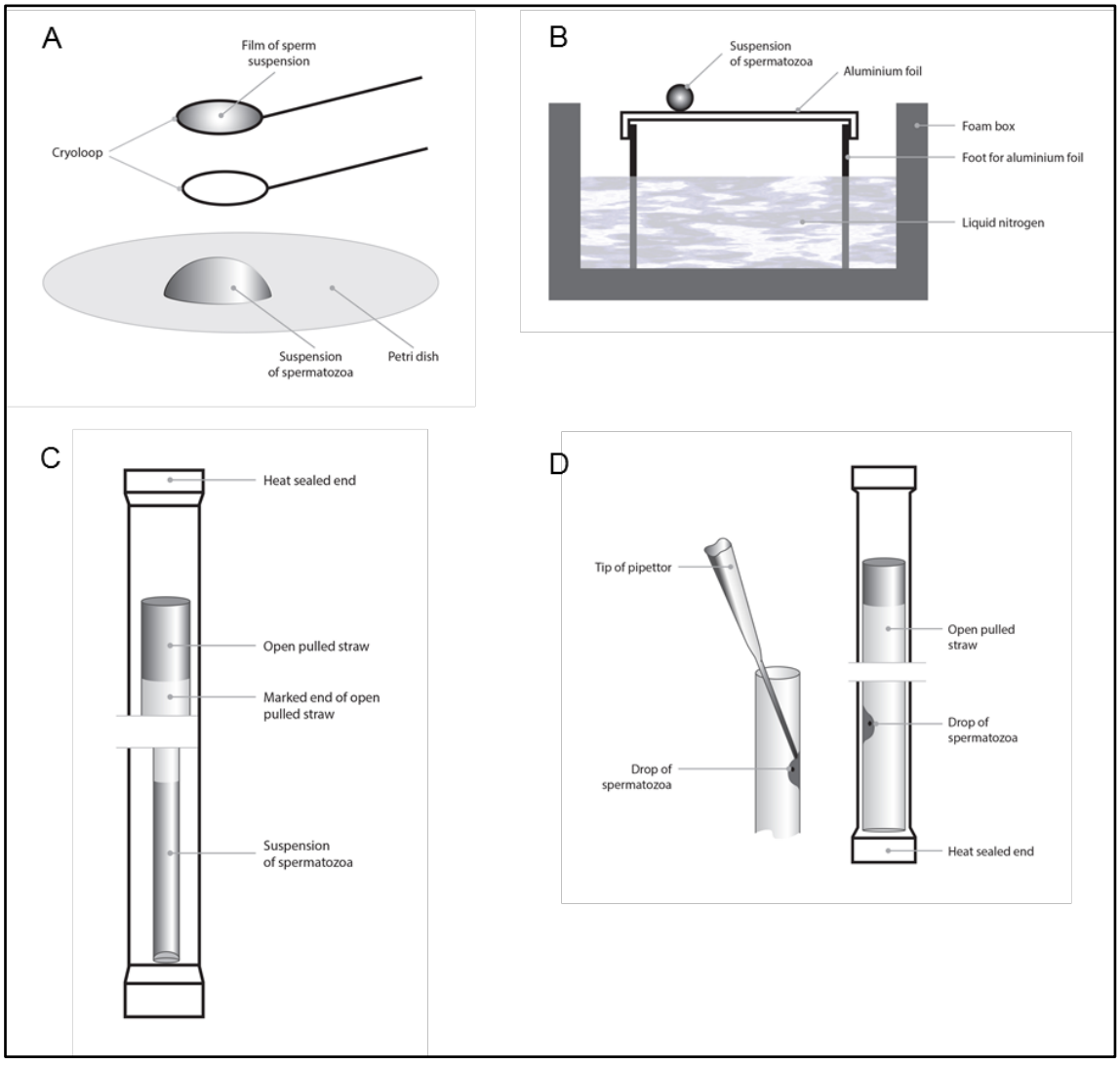


Figure 3: Line diagram, adapted from illustrations by Isachenko and colleagues demonstrating the different methodologies for vitrification of human spermatozoa[37] *(RBL picture library)*

A: Method for 'cryoloop' vitrification
B: Droplet vitrification
C: Straw in straw method
D: Open-pulled straw vitrification

2.6 Factors influencing the survival of human spermatozoa during and after preservation

The main objective of cryopreservation of cells is to maintain viability and functionality over periods of sub-zero temperature storage.[4] Various factors influence sperm survival after cryopreservation. In the following section, selected biological, biochemical and technical aspects influencing sperm survival will be discussed.

2.6.1 Biological aspects

2.6.1.1 Lifestyle

Abstinence from sexual activity or ejaculation before producing a semen sample alters the quality of the semen.[28] Short intervals between ejaculations reduce the sperm numbers, while extended abstinence periods reduce the motility. The World Health Organization (2010) recommends a minimum of two days and a maximum of seven days sexual abstinence.[29] De Jonge *et al.*[28] conducted a study to evaluate the influence of ejaculatory abstinence on semen parameters and DNA fragmentation. Sixteen men participated in the study, and each subject delivered a fresh semen sample after abstinence periods of one, three, five and eight days. Sperm numbers and semen volume increased with duration of the abstinence period. Abstinence did not influence pH, viability, morphology, total or grade A motility, or sperm DNA fragmentation. A short (24 hour) abstinence period negatively influenced chromatin quality. Marhsburn *et al*.[30] reported that an abstinence period of less than or equal to two days before an intrauterine insemination (IUI) procedure produces the highest pregnancy rates per cycle when compared to longer interval periods.

As tobacco use is accepted as a general health hazard, evidence shows that in both men and women, cigarette smoking influences reproductive health. Ramlau-Hansen *et al.*[31] investigated the association between smoking and semen characteristics in a group of healthy men. A 19% lower mean sperm concentration and a 29% lower total sperm count were observed in smokers. Kunzle *et al.*[32] found a significant decrease in sperm density, total sperm count, total number of motile sperm and citrate concentration (affecting pH). A conclusion was drawn that even though sperm variables in smokers remain within the normal range, men with borderline semen quality who wish to father children could considerably benefit to stop smoking. Surprisingly however, some studies show that male fertility and spermatogenesis in adult men are resistant to these deleterious effects.[33] Lopez-Teijon *et al*.[34] found no significant differences in semen parameters between smokers and non-smokers. Speculation can be drawn to how many packs of cigarettes the participants smoked per day, and for example the period the individuals were actively smoking. Sankako and colleagues confirmed in a study on rats that cigarette-smoke exposure harms semen and reproductive parameters, and that residual damages were still observed even after abstinence from cigarette-smoke exposure. Spontaneous recoveries in the semen and reproductive parameters were only demonstrated after sixty days abstinence from cigarette smoking.[35]

An increase in life expectancy along with an improvement in the socio-economic status results in higher maternal and paternal age, raising a concern of fertility maintenance during the aging process.[36] Cardona-Maya *et al*.[37] reported that semen volume, rapid progressive motility and total progressive motility, concentration and total sperm count are inversely related to age. A significant decline in volume, rapid and total progressive motility and sperm count exists among older men compared with younger men.[37] Similar results were found by Levitas *et al*.[36] Even though the exact mechanism responsible for these declining parameters in the ageing male is not fully understood, evidence suggests that age does affect semen quality leading to reduced reproductive capacity in older men.[36] Schmid and colleagues observed significantly higher

levels of zinc, copper and calcium in older patients (≥65 years), when compared to those of younger men (≤28 years). In the study, an association was found between higher sperm calcium and lower sperm motility, and an increased frequency in DNA fragmentation. Higher levels of sperm copper are associated with lower motility, and increased DNA fragmentation.[38]

Obesity causes significant disturbance in the hormone levels that can affect the reproductive system. Multiple hormonal changes associated with obesity cause alteration in sperm parameters and erectile dysfunction.[39] Hammoud *et al.*[39,40] investigated the effect of male obesity on sperm parameters and erectile dysfunction. The group found an increase in the incidence of oligozoospermia with an increase in body mass index (BMI). The prevalence of low progressively motile sperm was also higher with increased BMI. Even though the effect of obesity on male infertility seems to be minimal, with the increase in male obesity, it is predicted that the number of obese men with reduced fertility will also increase.[41] Hammiche *et al.*[42] showed that sperm concentration and total motile sperm count in men of subfertile couples are detrimentally affected by a high BMI and central adiposity. More studies are needed to gain insight into the underlying mechanisms and the true effects on the reproductive outcome.

Due to the increased demand for ART in patients and couples living with HIV, the question emerges as to what the effect of HIV and antiretroviral (ARV) treatment has on sperm and semen parameters.[43] Contradictory results have been reported regarding the association of HIV and ARV on semen characteristics. Krieger *et al*.[44] reported no difference whereas Crittenden *et al*.[45] only observed a decrease in the percentage of motile spermatozoa in men infected with HIV. However, several groups have reported a more significant effect of HIV on semen parameters when compared to controls.[46-48] Dondero *et al*.[46] reported a significant increase in cytoplasmic droplet forms and immature germ cells in HIV-positive men when compared to that of a control group. This

suggests that a stress condition exists after HIV infection, affecting spermatogenesis and the epididymal function. The effect of ARV treatment, duration of the infection, and HIV markers such as Cluster difference 4 (CD4) cell count and viral load on semen parameters has been reported.[49] CD4 count has been shown to affect semen volume[47,50-52], the concentration[46,47,50,53], motility[45,46,51,52,54] and morphology[47,50,53]. From the above evidence it is clear that semen parameters correlate positively with CD4 counts, which suggests that patients with chronic acquired immunodeficiency syndrome (AIDS) are less fertile than healthier HIV-1 infected individuals. Male patients infected with the HIV-1 virus have a higher tendency to have orchitis, hypogonadism, and leukospermia which could account for oligospermia and teratozoospermia.

2.6.1.2 Seminal quality

For ejaculated spermatozoa to be potentially functional, separation from the seminal plasma should take place quickly and efficiently. Prolonged exposure of spermatozoa to seminal plasma after ejaculation can cause irreversible decreases in sperm motility, viability and fertilizing capacity, since seminal plasma contains factors that inhibit sperm capacitation.[55] Capacitation is a complex of structural and functional alteration occurring in spermatozoa that enable them to undergo the acrosome reaction (AR) and fertilize the oocyte. However, this process is still not completely understood.[28] Sperm capacitation has been linked to sperm plasma membrane composition and fluidity modification, changes in intracellular ion concentrations and alterations in oxidative metabolism.[56] This is a vital process for both *in vitro* and *in vivo* fertilization to occur. Apart from mature spermatozoa, the seminal plasma may contain other types of cells such as epithelial cells, immature sperm cells and leukocytes.[29] Leukocytes have been shown to be one of the main causes of reactive oxygen species (ROS).[57] Physiological levels of ROS are necessary to preserve normal cell function, but when present in abundance are considered detrimental to the spermatozoa's function and survival.[58] Therefore it is vital for

the functionality and survival of spermatozoa to be separated from the seminal environment as soon as possible after liquefaction.

2.6.2 Biochemical aspects

2.6.2.1 Reactive oxygen species

Various biological mechanisms can lead to the production of ROS. All normal/living cells under aerobic conditions produce ROS. Reactive oxygen species (ROS) produced by mammalian spermatozoa are essential for sperm capacitation, AR, zona pellucida binding and oocyte fusion.[59] However, ROS are considered to be detrimental to sperm survival and function when present in supra physiological concentration. Oxidative stress occurs as a result of an imbalance between ROS production and a deficiency in the antioxidant strategies present in the male tract to protect spermatozoa from free radical attack.[60] Derivatives of ROS participate in cascades of chemical reactions that lead to free radicals that target all cellular components including lipids, proteins, nucleic acids and sugars.[58] High concentrations of polyunsaturated fatty acids (PUFA) are present in the plasma membranes of spermatozoa.[61] Reactive oxygen species (ROS) attack the lipids in the plasma membrane, leading to a cascade of chemical reactions called lipid peroxidation.[58] Due to a lack of vigorous cellular defence mechanisms against oxidative agents, human spermatozoa are extremely susceptible to peroxidative damage.[61,62] It has been hypothesized that oxidants interfere with normal sperm function via lipid peroxidation in the sperm plasma membrane which results in sperm malfunction.[61] Increased ROS levels have been shown to correlate with a decrease in sperm motility.[58] Factors protecting spermatozoa DNA from oxidative stress include the tight packaging of the DNA and the antioxidants in seminal plasma.[62] A by-product of DNA oxidation is 8-hydroxy-2-deoxyguanosine (8-OHdG). Strong correlations exist between DNA strand breaks and the 8-OHdG ions in spermatozoa.[60] A decrease in fertilization rates as well as poor embryo cleavage and quality has been reported in cases where

samples contain high prevalence of DNA damaged sperm.[63] Figure 4 is a diagrammatic representation (adapted from Cocuzza *et al.*[64]) of various factors influencing the production of ROS, and consequently the effect on spermatozoa.

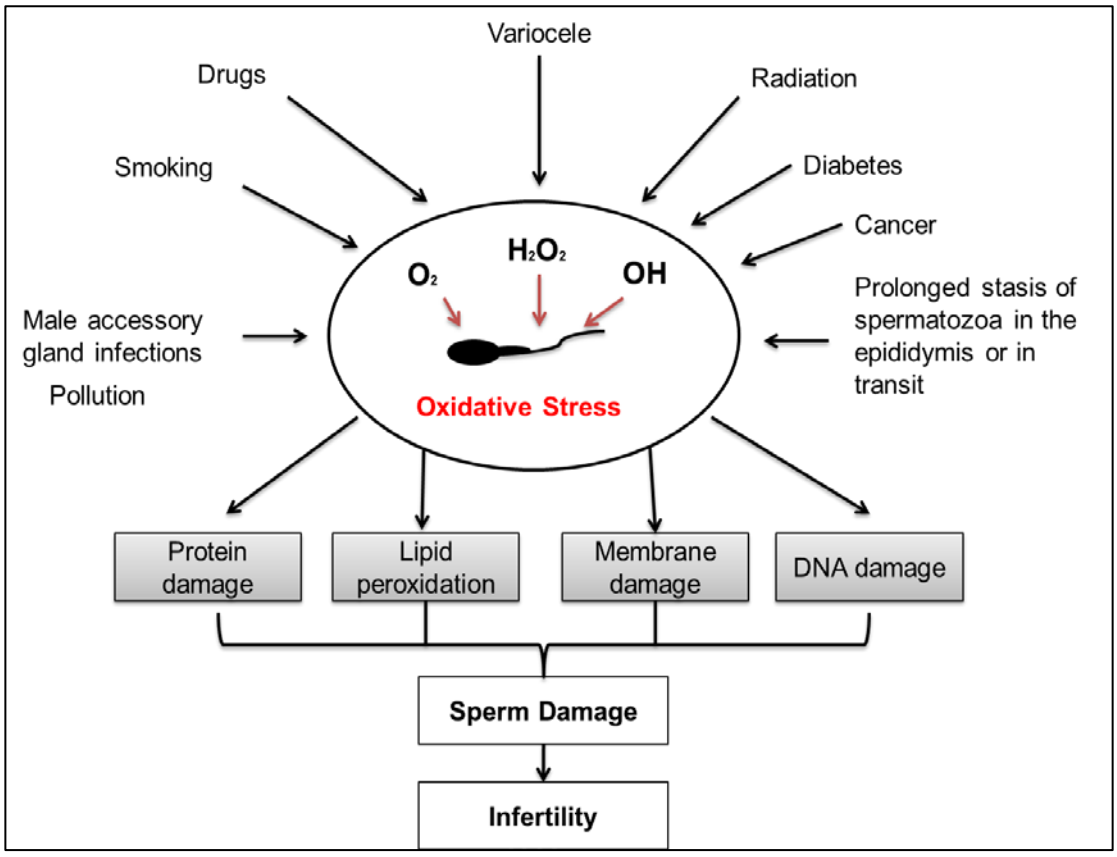


Figure 4: Factors influencing reactive oxygen species generation and the subsequent outcome for human spermatozoa

2.6.2.2 Nitric oxide (NO)

Nitric oxide (NO) is a diatomic, semi-stable free radical, produced from L-arginine via nitric oxide synthase (NOS).[65] In the male reproductive system NO is crucial for various physiological functions. Three forms of NOS exist, namely: 1) endothelial NOS (eNOS), 2) inducible NOS (iNOS), and 3) neuronal NOS (nNOS). Each isoform is vital for effective production of NO.[66-68] In spermatozoa, NO has a major effect on motility and plays an essential part in the AR, capacitation, zona pellucida binding, morphology, and viability.[66,69,70] *In vitro*, a high concentration of NO decreases sperm motility while increasing sperm peroxidation.[71,72] Wu *et al*.[73] investigated the effects of NO on human sperm motility, viability and fertilization. Results showed a significant decrease in sperm functions, sperm-zona binding and embryonic development after exposure to high levels of NO. Similar results were observed by Rosseli *et al.*[72] with regards to sperm motility and viability. When evaluating the direct effects of NO, that were chemically generated from single nucleotide polymorphism (SNP) on human spermatozoa, evidence suggested that NO, in a concentration dependent manner, induced toxicity as well as decreased forward progressive motility.[72] In contrast, physiological concentrations of NO increase sperm motility, decrease sperm lipid peroxidation and prolong viability.[71] Bahanzadeh *et al.*[74] evaluated the effects of the NOS inhibitor, L-nitro-G-arginine methyl ester (L-NAME), on epididymal sperm count, motility and morphology in varicocelized rats. Findings showed that L-NAME improved both the sperm count and morphology. Rosseli *et al.*[72] found that motility and viability were significantly maintained in human spermatozoa when treated with L-NAME. Figure 5 is a line diagram summarizing the up-and-down regulation of NO and the subsequent effect on sperm parameters (adapted from an illustration published by Doshi *et al.*[66]).

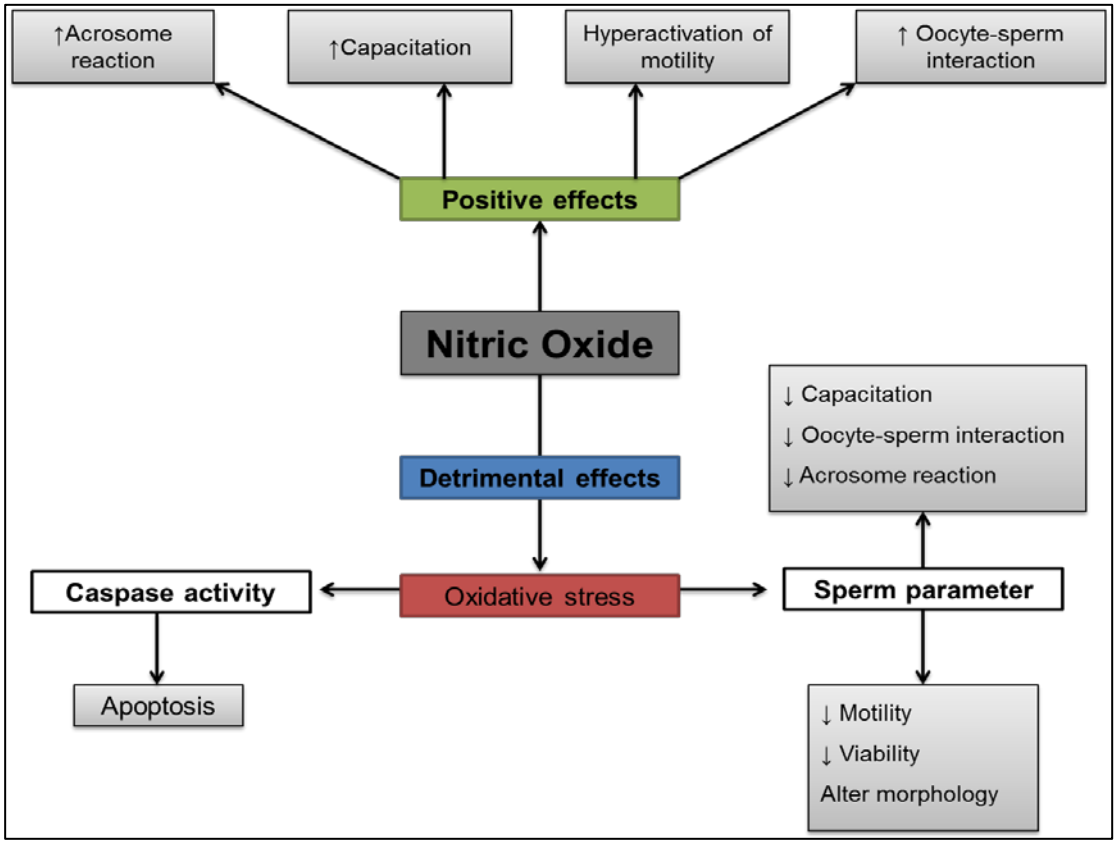


Figure 5: Model depicting the up and down regulation of nitric oxide and the effect on human spermatozoa[70]

2.6.3 Technical aspects

2.6.3.1 Temperature

To gain competence to fertilize an oocyte, the male gamete needs to undergo several metabolic and structural changes, known as capacitation. Sperm capacitation is a temperature-dependent phenomenon. Variations in the temperature could cause alterations in some events associated with capacitation.[56] Marin-Briggiler *et al*.[56] determined whether incubation at room temperature for 18 hrs. would allow human capacitation related events to occur. Results showed that incubation temperatures regulate cellular mechanisms involved in sperm capacitation. A temporary blockage of capacitation-related events was observed in spermatozoa incubated at room temperature (20˚C) for 18 hrs. Functional properties of these cells (non-capacitated and a low rate of spontaneous AR) can be retained when exposed to body temperature (37˚C).[56]

Human spermatozoa spend their entire lifespan inside the male and female genital tract, in a constant environment.[75] According to the WHO guidelines, semen samples should be handled at temperatures ranging from 20˚C to 40˚C to prevent a decrease in sperm motility.[29] Keppler *et al*.[76] analysed the motion or kinematic parameters and hyperactivation of sperm at different temperatures. Hyperactivation was triggered at temperatures above 40˚C. Hyperactivation is necessary for migrating spermatozoa to reach the site of fertilization. Human spermatozoa are severely sensitive to fluctuations in temperature. Temperatures lower than 4˚C and higher than 37˚C have proved to influence sperm functions such as motility negatively.[77] Sperm motility is a requirement for fertilization in humans.[78] Esfandiari *et al*.[75] investigated the effects of different temperatures on sperm, including ROS production, motility and other motion characteristics. Samples incubated at 37˚C had higher motility and other motion parameters, and lower ROS production, when compared to samples incubated at 4˚C and 25˚C.

Sperm DNA damage is also affected by temperature. Sperm DNA integrity is a significant component of fertility.[79] Jackson *et al.*[79] evaluated the effects of semen storage and separation techniques on sperm DNA fragmentation. A progressive decrease in DNA quality was observed when analysing samples incubated at 37˚C. Samples stored at room temperature for 24 hrs. had a significantly higher percentage of DNA fragmentation, with the largest increase in DNA damage occurring within the first 4 hrs. Therefore, diagnostic assessment of semen samples should be performed as soon as possible after production to minimize the levels of DNA damage.

2.6.3.2 Long-term incubation

High percentages of progressively motile spermatozoa are necessary for successful IVF.[80] Calamera *et al.*[81] investigated the effects of long term *in vitro* incubation on different functional sperm parameters. Incubation of human spermatozoa in sperm culture medium (up to 48 hrs.) supplemented with 3.5% HSA, led to a significant decrease in sperm motility and a deterioration of sperm motion parameters. Other sperm functions and structures such as DNA intactness or the ability to undergo the AR remained unaltered. Similar results were found by Zollner *et al.*[80] showing that the percentage of progressively motile spermatozoa decreased steadily with the time of incubation, with the greatest loss occurring in the first four hrs. of incubation (1.7%/hrs.). No difference was detected in the loss of motion after 24 and 48 hrs. of incubation.

2.6.3.3 Semen preparation

2.6.3.3.1 Techniques

Different types of processing methods exist to separate spermatozoa from seminal plasma for a variety of purposes such as diagnostic tests, therapeutic recovery for insemination and ART. Three processing methods are prescribed

by the WHO.[29] These include a direct swim-up, a simple washing technique, and discontinuous density gradient (DGC) methods. The nature of the semen sample largely determines the type of sperm preparation technique that will be best suited. The simple washing procedure is well-suited for semen samples of good quality. This procedure usually results in the highest yield of spermatozoa and is often used for preparing samples for IUI.[29] The conventional swim up method is easy to perform and cost effective.[29] This technique is performed by layering culture medium over a liquefied sample (see figure 6). This procedure results in a lower yield of spermatozoa, but sperm is selected for their motility and is useful where the percentage of motile spermatozoa in semen is low. The direct swim-up technique is frequently used for normozoospermic[(1)] samples.

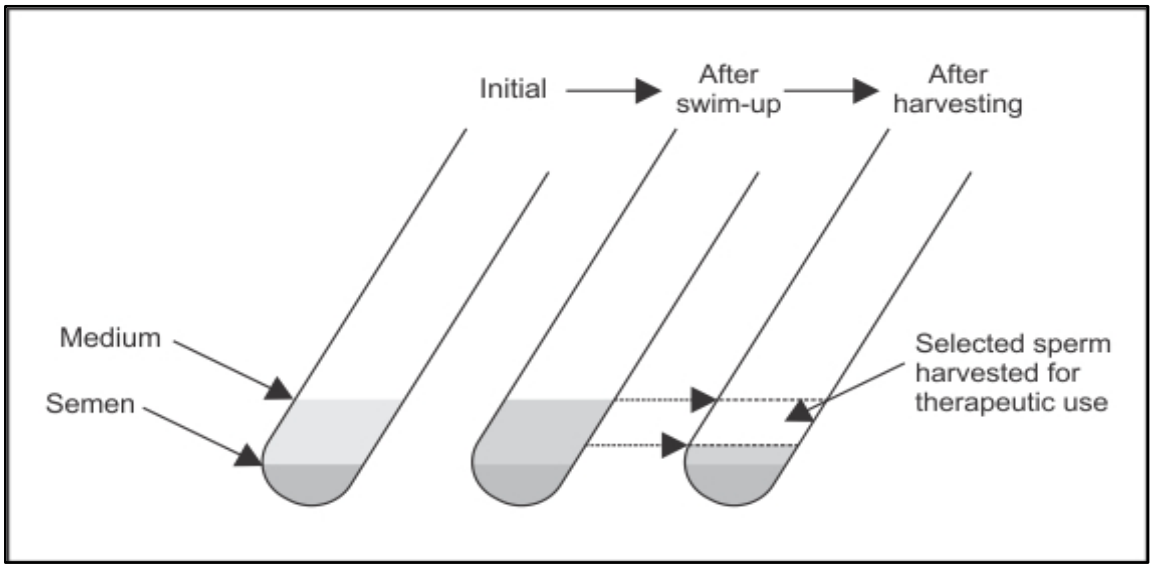


Figure 6: Line diagram explaining the 'direct swim up' method for semen preparation *(RBL picture library)*

[(1)] Concentration, progressively motile and morphological normal spermatozoa equal to or above the lower reference number[29]

Semen samples classified[29] as oligozoospermia[(2)], teratozoospermia[(3)], or asthenozoospermia[(4)] are recommended to be processed using DGC to optimize the end product for IVF/ICSI. This type of preparation technique usually yields a high percentage of motile spermatozoa, free of debris, contaminating leukocytes, non-germ cells and degenerating germ cells.[29] Furthermore this method is relatively expensive and requires the use of commercially prepared density gradients and sperm washing medium. Discontinuous gradient centrifugation methods entails layering seminal plasma over density gradients consisting of colloidal silica coated with silane and centrifugation. Cells are subsequently separated based on their density, and motile sperm actively migrates through the gradient material and form a soft pellet at the bottom of the tube. The layers are removed by pipetting, and the soft pellet is washed free of the density gradients in a clean tube containing a sperm washing medium.[29] Figure 7 is a line diagram illustrating the technique.

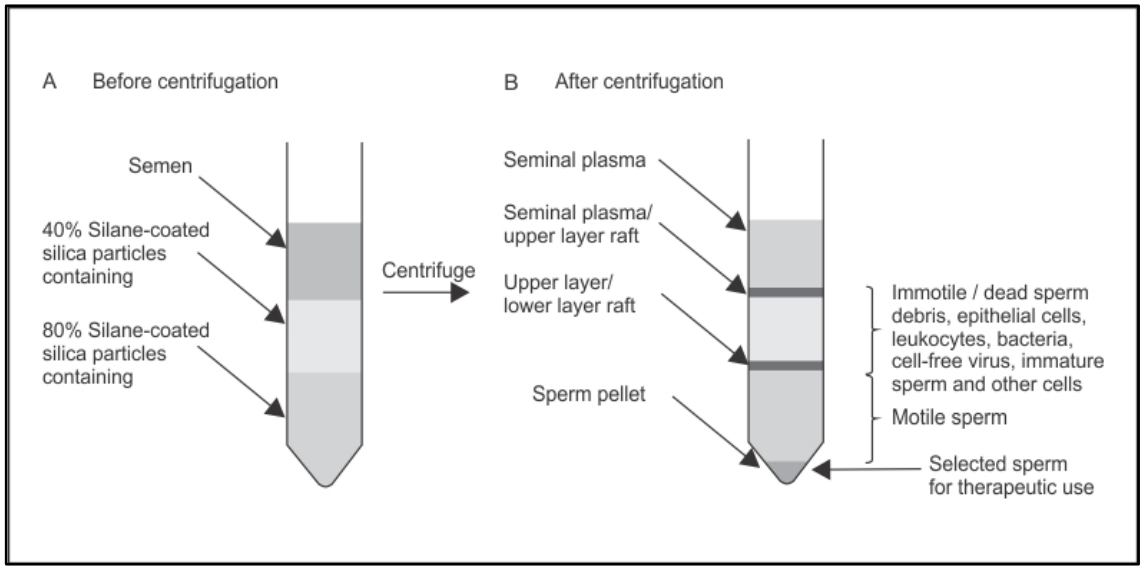


Figure 7: Discontinuous density gradient centrifugation method for therapeutic procedures *(RBL picture library)*

A) Loaded tube with gradients and semen
B) After centrifugation

(2) Concentration of spermatozoa below the lower reference level
(3) Percentage of morphological spermatozoa below the lower reference level
(4) Percentage of progressively motile (PR) below the lower reference level

During the use of highly active antiretroviral therapy (HAART), virus loads in serum and semen can be reduced. Semen washing techniques are used to prepare samples with undetectable levels of virus ribonucleic acid (RNA) for IUI, IVF or ICSI.[82] Several studies have been published showing little or no risk of transmission from mother to child.[83-86] The method to treat serodiscordant couples (where the male is positive) is to remove cell-free and cell-associated pathogens from sperm fractions, while maintaining optimal sperm quality.[87,88] Washing semen from HIV-infected individuals involves three steps. Liquefied semen is filtered through discontinuous buoyant density gradients. Thereafter, spermatozoa are washed to remove the excess seminal plasma and hyper-osmotic gradient media. The final step entails a modified swim-up method to recover highly motile spermatozoa free of leukocytes. Even though each step is highly efficient in reducing the viral content, the final aliquot of spermatozoa should nevertheless be tested to exclude any HIV RNA contamination. Viral validation is performed by using a modified polymerase chain reaction (PCR) or nucleic acid sequence based amplification.[84,88-90] At the Reproductive and Endocrine Unit of SBAH semen decontamination experiments were initiated to test the ability of a decontamination method involving a novel tube insert (ProInsert™) combined with density gradient centrifugation.

The studies focused on elimination HIV-1, HCV, cytomegalovirus (CVM) and white blood cells (WBC) from *in vitro* spiked semen samples.[91,92] Subsequently, validation studies on the semen decontamination procedure were performed to evaluate the efficacy of:

1) Removing bacteria and yeast from *in vitro* spiked semen samples; and
2) *In vivo* derived HIV-1 RNA and pro-viral DNA from semen of HIV-seropositive men.[15,93]

The decontamination procedure has proved to be effective in the removal of the bacteria and all HIV-1 pro-viral RNA and DNA from all neat semen.[87] The semen decontamination procedure involves processing semen samples using DGC (PureSperm™ 40 and 80%; Nidacon™ International Mölndal, Sweden),

making use of the novel polypropylene centrifugation tube insert (ProInsert™) without the additional swim-up step. Contamination of the sperm population is eliminated by using an elongated micropipette to aspirate the sperm pellet.[93]

2.6.3.3.2 Semen preparation media

A semen preparation medium that results in good post processing outcomes is an essential contributing factor in the treatment of infertility. An efficient sperm preparation medium is required to produce a high number of spermatozoa with normal morphology and large percentage of motile cells, while avoiding toxic effects.[94] A variety of gradient mediums for preparing spermatozoa for ART are available. The most widely used media for all methods of ART use to be Percoll®. However, replacement products have been introduced into the market. Most of these products contain silane-coated silica particles, are adjusted for osmolality, have very low toxicity, are non-irritant and are approved for human *in vivo* use.[95] Chiamchanya *et al*.[94] studied the effect of three semen preparation media, PureSperm® (Nidacon, Mölndal, Sweden), Sil-Select Plus™ (FertiPro, Beernem, Belgium) and SpermGrad™ (Vitrolife, Gothenburg, Sweden) on semen parameters, DNA damage and protamine deficiency. Results reveal that PureSperm® preparations yielded the best spermatozoa in terms of total motility, and progressive motile concentrations, as well as having the lowest percentage of protamine deficient sperm. The Sil-Select Plus™ preparation resulted in the lowest amount of DNA damage, but yielded a low percentage of motile spermatozoa. After processing with SpermGrad™ the highest percentage of DNA damaged spermatozoa were observed, but a higher percentage of morphological normal sperm when compared to the Sil-Select Plus™ preparation was found.

For ART procedures such as ICSI, IVF or IUI, simple sperm preparation techniques are used to separate spermatozoa from seminal plasma. For these procedures, the culture media should be a balanced salt solution supplemented

with proteins and contain a buffer appropriate for the environmental conditions in which the spermatozoa will be processed and stored.[29] A culture media should be capable of sustaining the active metabolism of spermatozoa *in vitro*.[55] The type of incubator used will determine with which buffer the culture media should be supplemented. Incubators containing atmospheric air, hydroxyethyl piperazineethanesulfonic acid (HEPES) or a similar buffer are advisable. Incubators containing 5% (v/v) carbon dioxide (CO_2), a media buffered with sodium bicarbonate ($NaHCO_3^-$) or a similar would be best suited. Buffering culture media to suite different incubator conditions ensures that the pH is compatible with sperm survival.[29] Although an acidic pH is detrimental to human spermatozoa, a high pH is well tolerated.[55] According to the WHO (2010) the lower reference limit for the pH of semen is ≥7.2.[29] Protein supplementation in culture media is required for optimal survival of human spermatozoa. Previously, bovine serum albumin (BSA) was used, but this is now recognized as carrying the risk of disease transmission, including prion disease (Creutzfeldt-Jakob disease, CJD).[82] The most commonly used supplement currently is HSA. Human serum albumin (HSA) is manufactured from blood obtained from hepatitis and HIV screened donors.[55] For ART procedures the purification of HSA is vital to ensure the protein supplement is free of any viral, bacterial and prion contamination.[29]

2.6.3.4 Cryopreservation procedure

2.6.3.4.1 Cryopreservation media

Permeating CPA most commonly used in sperm cryopreservation includes glycerol or egg yolk. Permeating cryoprotectants cross the plasma membrane of the cells being frozen.[17] Non-permeating cryoprotectants are macromolecules or sugars that increase the extracellular osmolality and aid in the dehydration of cells during slow freezing. The most commonly used non-permeating cryoprotectants are glucose and sucrose.[1,4]

CPA serves several functions by:

1) Enhancing osmotic pressure and pH;

2) Providing energy source to avoid the use of intracellular sperm phospholipid;

3) Preventing bacterial contamination by including an anti-biotic; and

4) Semen dilution while eliminating the harmful effect on survival produced by high dilution.

Nallella *et al.*[96] studied the ability of two cryopreservation protocols and three cryoprotectants to preserve sperm quality. The three cryoprotectants used were TYB (test yolk buffer), Sperm Freezing Medium (ORIGIO Medi-Cult, Malov, Denmark), and Enhance Sperm Freeze (VitroLife, Gothenburg, Sweden). Cryopreserving spermatozoa using TYB resulted in better post thaw sperm quality compared with Sperm Freezing Medium and Enhanced Sperm Freeze.[96] Cryoprotective media with egg yolk seem to achieve higher survival rates than media without egg yolk. However, as egg yolk is of animal origin, it contains a risk of introducing microbial agents or other unknown infectious diseases into the sample. Harmful effects of CPA include toxicity and osmosis. Cryoprotective agents (CPA) are chemical substances that in effect poisonous to the cells. As the metabolism of the cell slows down, the toxic effects are diminished, unless high concentrations are used at relatively high temperatures. The addition of CPA to a spermatozoa solution exerts osmotic stress on the cell owing to the increased osmolality of the medium.[4]

2.6.3.4.2 Addition/Removal of cryoprotective mediums

Human spermatozoa are extremely sensitive to shrinkage and swelling. To increase the cryo-survival rates, the slow addition and removal of CPA is a vital step. Rapid dilutions severely damage cryopreserved spermatozoa.[4] Most protocols involve a drop-wise addition of CPA with constant mixing over several min.[17] Human spermatozoa can swell to only 110% of their original volume, while shrinkage can take place to 75% of this value and still retain 90% of the original motility.[97] When CPA are added to cells, dehydration occurs, and the

cells shrink. Then, as the CPA enters the cell, the cell returns to its isotonic volume. When the CPA is removed by diluting the post-thaw specimen, water enters the cell quickly due to the osmotic gradient and the CPA leaves the cell more slowly, in this way the cell swells before equilibrium has been restored. Critical volume limits are exceeded during this process which results in permanent damage to the cell, presumably via the integrity of the cytoskeleton.[4,17,97] After dilution with CPA, semen should be packaged and cooled immediately. Exposure of the cells to cryoprotectants prior to freezing should be less than 10 min.[17]

2.6.3.4.3 Cooling rate

Human spermatozoa are generally considered to be relatively insensitive to cooling rates within the range of 1-25˚C/min.[17] Several methods have been utilized to achieve controlled cooling for human spermatozoa. Programmable freezing systems allow cells to be frozen gradually, providing more time for the cells to become dehydrated, thereby reducing damage caused by the formation of intracellular crystals.[4,17,98] Static phase vapour is a method by which stable temperature gradients are established in the vapour phase above a quantity of LN_2, and held within an insulated container. Straws are placed at a predetermined height above the LN_2 in the vapour phase before being plunged into the LN_2.[4] Disadvantages of this method include the rapid rate of temperature decreases, poor reproducibility of cooling rates, and the large variation observed between straws from the same semen sample.[4,98]

2.6.3.4.4 Packaging

Various packaging containers are available for human spermatozoa: [4]

- Glass ampoules: these have been discouraged for years based on their fragility;

- Plastic screw tops or 'Cryovials': made from polypropylene;
- Plastic straws or 'Pailettes': Originally made from polyvinyl chloride (PVC), but were replaced by straws made from polyethylene terephthalate glycol (PETG); and
- CBS™ (Cryo Bio Systems) high security straws manufactured from isometric resin.

The CBS™ has substantial advantages in terms of mechanical strength at cryogenic temperatures as well as impermeability to viruses. In Europe, straws are used mainly for preservation of human semen. In many laboratories, especially in the USA, plastic cryovials are used. Reasons for the use of cryovials in many laboratories are that these devices are easier to use, and larger volumes of the specimen can be stored.[17] A number of key facts should be taken into account when debating the use of vials vs. straws. To date, there have been no reliable prospective trials in which relative fertility of human spermatozoa frozen in straws was compared to those in cryovials However, it has been argued that there might be as much as a 6-to 8-fold higher post thaw fecundity for sperm frozen in straws. Physics shows that the larger radius of cryovials will impede heat transfer, resulting in uneven heat exchange throughout the sample. The contents of the cryovials will thaw more slowly and less uniformly when removed from cryo-storage when compared with that of straws. This poses a problem, since rapid thawing is required for optimal cryo-survival rates.[17]

2.6.3.4.5 Storage

Samples are stored at temperatures below -132˚C. Warming samples to above this temperature leads to recrystallization of frozen water molecules, which damages the cryopreserved cell. Currently there are four types of storage systems available: [99]

- Liquid phase nitrogen;
- Vapour phase nitrogen;
- Super cold air; and
- Mechanical freezers.

When deciding on the storage system that works best or will provide optimal results, various factors should be taken into account such as storage temperature, temperature fluctuations during audit, cross-contamination risk, safety, costs and storage space.[4,17,100] A temperature of -150˚C is generally considered to be the critical storage temperature for cells since this temperature provides a reasonably safe margin. In practice, storing cells in LN_2 (-196˚C) is convenient and reliable. However, storing cells in LN_2 does have disadvantages: LN_2 is expensive and the risk of microbial cross-contamination cannot be excluded. To prevent contamination from LN_2, it has been advised to store the cells in the gas phase. Vapour phase storage may reduce the hazard of contamination, but large temperature fluctuations may exist within the vapour phase. Another storing option is a mechanical (electrical) freezer. The most commonly used freezer for cryopreservation purposes are the upright, front opening freezers, reaching a cold point of -80˚C. Even though this front opening design is very convenient, these freezers are expensive and issues arise regarding temperature stability and variability. As cold air is significantly heavier than warm air, opening the freezer causes massive air exchanges and thereby affecting the temperature. Therefore, it is generally preferred to rather make use of LN_2 storage since the internal temperature variation is minimal and the environment is less frequently subjected to disturbance.[99]

2.6.3.4.6 Thawing

Thawing is the process where frozen water changes state-from solid to liquid. During the thawing process, biological activities of cells must be recovered.[101] Fast rewarming rates are required for optimal cell recovery.[99] Calamera *et al*.[102]

observed that to thaw samples at 40˚C for 3 min significantly increased sperm motility.

Presently, several thawing techniques exist: [101]

- Thawing at room temperature for 10 min and subsequently at 37˚C for 10 min;
- Thawing at 37˚C for 10 min; and
- Thawing at room temperature (24˚C) for 15 min.

The warming rate has an influence on the survival of living cells, but not as great impact as the cooling rate.[99] During slow rewarming, small intercellular crystals that might have formed during the freezing can grow and in this way influence cell recovery. After thawing, the cryoprotective additives should be washed out.[4]

2.7 Conclusion

Cryopreservation of human spermatozoa has transformed the field of ART. Preservation of male and female gametes has a wide variety of applications namely autoconservation and donor banking. From the above literature review, the following conclusions can be drawn:

- Cryopreservation has deleterious effects on human spermatozoa in terms of motility, viability, morphology, DNA integrity, etc.
- Aseptic cryoprotectant-free vitrification is possible, but not yet routinely applied by infertility laboratories as further investigations are necessary.
- Various biological, biochemical and technical factors influence the survival of human spermatozoa during and after preservation.

Cryopreservation techniques for mammalian spermatozoa have been available for more than 50 years. However, the solution to the deleterious effects of cryopreservation methods on human spermatozoa remains elusive. An optimal,

universally standardized and accepted method still needs to be determined. Figure 8 summarizes the biological, biochemical and technical factors influencing the functionality of spermatozoa. The diagram was adapted from Björndal *et al*.[4] This study focused on comparing different preservation methods on the post thawing yield of washed spermatozoa.

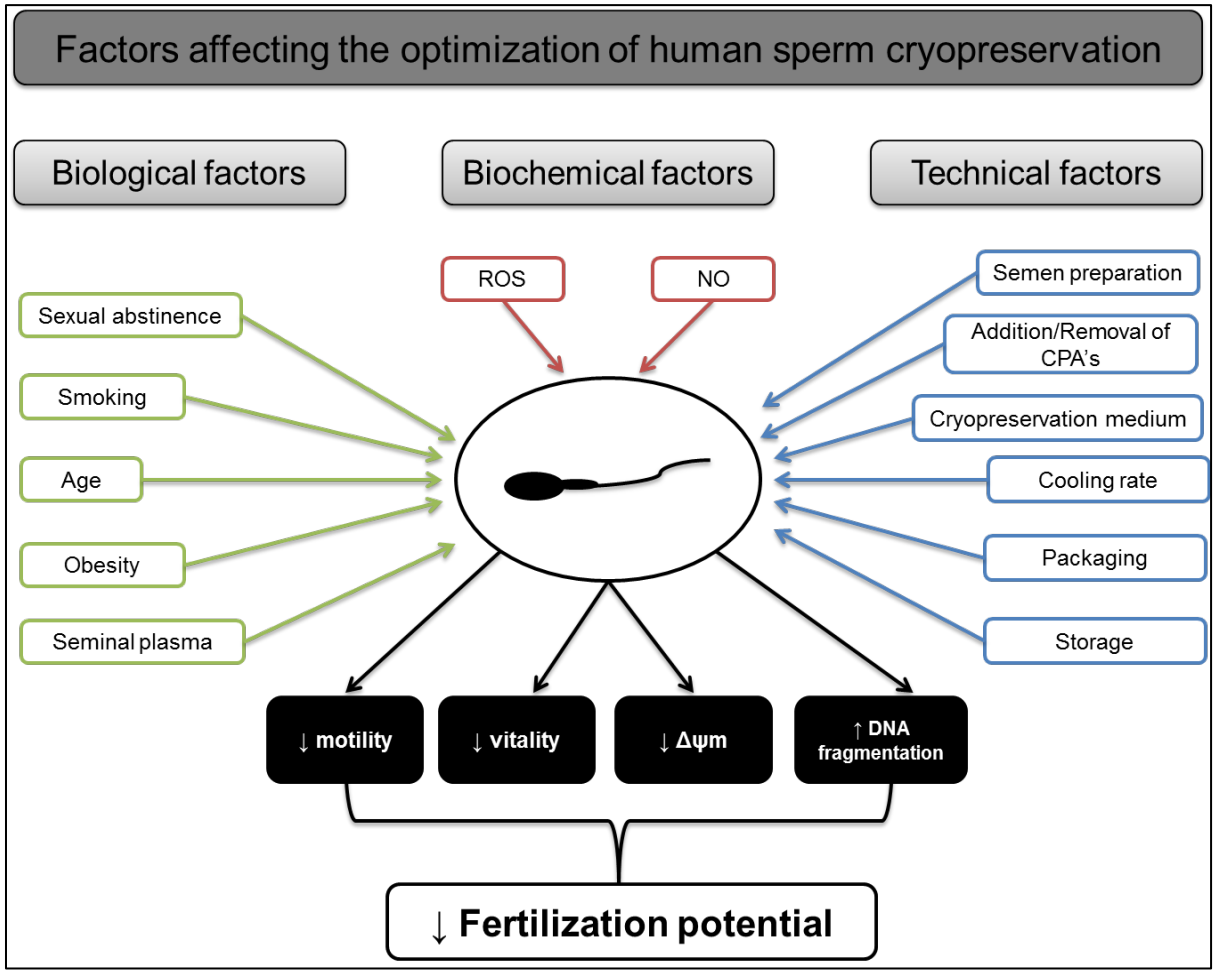


Figure 8: Summary of the biological, biochemical and technical factors influencing the functionality of human spermatozoa

ROS = reactive oxygen species, NO = nitric oxide, CPA = cryoprotective agents, $\Delta\psi m$ = mitochondrial membrane potential

CHAPTER 3 MATERIALS AND METHODS

3.1 Sampling

Semen samples from healthy patients, over the age of 18, seeking diagnostic assistance at the Reproductive Biology and Endocrine Unit, SBAH, were used. Each sample had a minimum concentration of 15 x 10^6 sperm/ml, total motility of at least 40% and a minimum volume of 1.5 ml. Samples were collected according to the SOP of RBL (see Addendum B: SOP nr: F1.18.1). Semen samples were left to liquefy for 30 min after which analyses were performed according to the guidelines of the WHO criteria (WHO, 2010).[29] The descriptive statistics of the patients' semen parameters are summarized in table 4 (see chapter 4, section 4.1). The number of semen samples included in this study was: Study 1A=10, Study 1B=15, Study 2=35.

3.2 Semen preparation

Each ejaculate was processed using the double DGC method. The density gradients were prepared by layering 2 ml of 40% PureSperm® (Nidacon, Mölndal, Sweden) over 2 ml of 80% PureSperm® (Nidacon, Mölndal, Sweden). The semen sample was then layered on top of the two gradient columns (1.5 ml) and centrifuged (centrifuge 5810R; Eppendorf®, Hamburg, Germany) for 20 min at 300 g. After centrifugation, the supernatant was removed and the pellet was resuspended in 5 ml PureSperm® wash (Nidacon, Mölndal, Sweden). The solution was centrifuged for 10 min at 500 g, the supernatant was discarded; the pellet was then resuspended, and subsequently divided into aliquots.[103]

3.3 Experimental set-up

3.3.1 Study 1: Quality control

3.3.1.1 Study 1A: Comparison between cryopreservation protocols used by well-established (leading) infertility units in South Africa

Study 1A had two main objectives: 1) to compare three different cryoprotective mediums from FertiPro (FertiPro, Beernem, Belgium), Irvine Scientific® (Irvine Scientific®, Santa Ana, USA) and Nidacon (Nidacon, Mölndal, Sweden), and 2) to compare two different preservation protocols. Table 2 summarizes the composition of the three cryopreservation media. A preliminary retrospective study revealed that the three mediums chosen for this study are used by some of the leading infertility units in South Africa. The medium and method that yielded the highest quality spermatozoa was subsequently used as a control in Study 2. See figure 10 for a summary of the experimental procedure.

Table 2: Composition of the cryoprotective mediums used in Study 1A: Product inserts

Product name	**Sperm CryoProtect™II**	**Freezing Medium**	**SpermFreeze**
Company	*Nidacon*	*Irvine Scientific®*	*FertiPro*
Buffer	HEPES	Tris-EDTA	HEPES
Protein	No Protein	Egg yolk protein	Human serum albumin (0.4%)
Antibiotic	No antibiotic	Gentamicin	No antibiotic
Glycerol	5%	12%	26.76%

3.3.1.1.1 Cryopreservation procedure

The washed sperm sample was divided into three aliquots. Each aliquot was cryopreserved with different cryopreservation media; 1) SpermFreeze (SF), 2) Freezing Medium (FM), 3) Sperm CryoProtect II (CP II). Cryopreservation mediums were added to the washed spermatozoa in 0.7:1, 1:1, and 1:3 ratios respectively (according to the manufacturer's recommendation). After the addition of the mediums, each aliquot was further subdivided into two different aliquots. The contents of each aliquot were aspirated into CBS™ high security straws (Cryo Bio System, Paris, France). Two different preservation protocols were used to freeze the straws: i) standard protocol (SOP nr: G3.2.1) of the RBL unit, and ii) Irvine Scientific® freezing protocol.

i) Standard protocol of the RBL unit: (RBL_M)

The spermatozoa and CPA solution were left at room temperature for 10 min where after the straws were placed in the vapour phase (1 cm from the liquid surface) of the LN_2 for 15 min. Subsequently, the straws were submerged into the LN_2 and stored in a LN_2 tank (Thermo Fisher Scientific, Johannesburg, South Africa) before post analyses (motility and viability) were performed. Figure 9 illustrates a floating device used (provided by Nidacon™ International) to ensure straws are suspended 1 cm from the LN_2 surface at all times. See SOP nr: G3.2.1 (Addendum B).

ii) Irvine Scientific® freezing protocol: (IS_M)

The spermatozoa and CPA solutions were left at room temperature for 10 min. After 10 min, the straws were placed in a glass beaker with tap water, and kept in a refrigerator (2-8°C) for 60 min. The straws were then placed in the vapour phase (1 cm from the liquid surface) of the LN_2 for 15 min, and subsequently submerged into the LN_2. Straws were stored in a LN_2 tank dedicated for research before post analyses (motility and viability) were performed.[104]

Figure 9: Floating device used to suspend straws in the nitrogen vapour (1 cm from the liquid nitrogen surface) *(RBL picture library)*

Thawing was initiated by placing the straws in tap water (23˚C) for 5 min. The end of the straw was cut open using sterile scissors. The contents were aspirated into a centrifuge tube containing 2 ml PureSperm® wash (37˚C). Tubes were centrifuged for 10 min at 500 g, to remove the cryopreservation medium. The supernatant was removed and the accumulated spermatozoa at the bottom of the tube were resuspended and post counts were performed (motility and viability).[104]

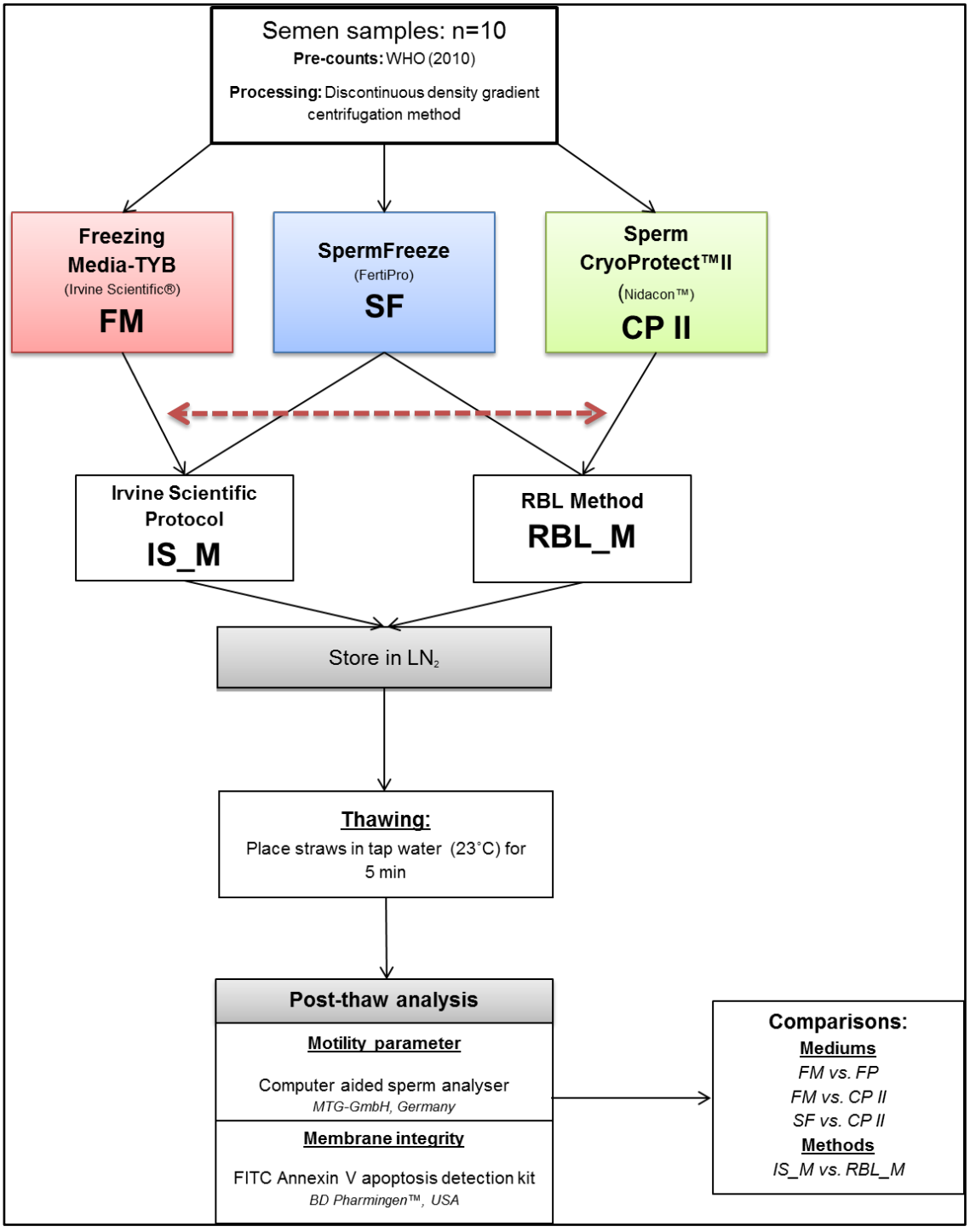


Figure 10: Flow diagram depicting the experimental procedure/setup to compare three commercially available cryopreservation media using two different preservation protocols

3.3.1.2 Study 1 B: Establishing a cryoprotectant-free vitrification protocol for larger volumes of spermatozoa

A method without the use of conventional CPA and applying rapid freezing has been described for the vitrification of human spermatozoa.[9,10,105] This method entails replacing the conventional CPA with a protein and carbohydrate solution (1% HSA and 0.25 M sucrose). In 2011, the Isachenko group reported a novel aseptic cryoprotectant-free vitrification method for larger volumes of spermatozoa.[13] Study 1 B used and adapted this method to determine an optimal vitrification volume, which will be suitable for IVF, ICSI and IUI procedures. The superior method was used as a control in Study 2. See figure 12.

3.3.1.2.1 Vitrification cooling procedure

The vitrification solution was prepared by dissolving HSA in double distilled water (1%) (Adcock Ingram, Johannesburg, South Africa) with the addition of sucrose powder (342.3g; Sigma Aldrich, St Louis, USA) to reach a 0.5 M concentration. The 0.5 M sucrose solution was added to the diluted spermatozoa immediately after processing (DGC) in a 1:1 ratio to reach a final concentration of 0.25 M. The remaining sucrose solution was frozen at -20˚C until further use. The diluted suspensions were maintained at room temperature for 5 min before the vitrification procedure commenced.[105]

For the vitrification procedure, 0.5 ml CBS™ straws were used. Before the straws were filled with the diluted sperm suspension, each straw was labelled with a line, 1.5 cm from the inner end of the cotton polyvinyl plug. This allows for a large enough air space inside the straw to prevent rupturing when immersed into LN_2. Straws were filled (using the modified syringe device) with volumes of 100 µl ($\mathbf{V_{100}}$), 300 µl ($\mathbf{V_{300}}$) and 500 µl ($\mathbf{V_{500}}$) of the sucrose and sperm solution respectively. Both ends of the straw were sealed hermetically using the flame of an alcohol burner and forceps and submerged horizontally

into the LN_2. Straws were stored in a LN_2 tank specifically designated for research.[105] Figure 11 is a simulated stepwise representation of the vitrification protocol followed in the study.

To thaw the vitrified samples, straws were immersed into a water bath (42˚C), and left for 20 sec. The outer surfaces of the straws were wiped dry with a paper towel to remove excess condensation, and sterilized using 70% alcohol. The heat sealed ends of the straws were cut open and the content was expelled into centrifuge tubes (BD Pharmingen™, Franklin Lakes, USA) containing 2 ml of PureSperm® wash (37°C). The tubes were centrifuged for 10 min at 500 g and the supernatant was removed. The accumulated sperm pellet was resuspended and post thaw analyses (motility and viability) were performed.[13]

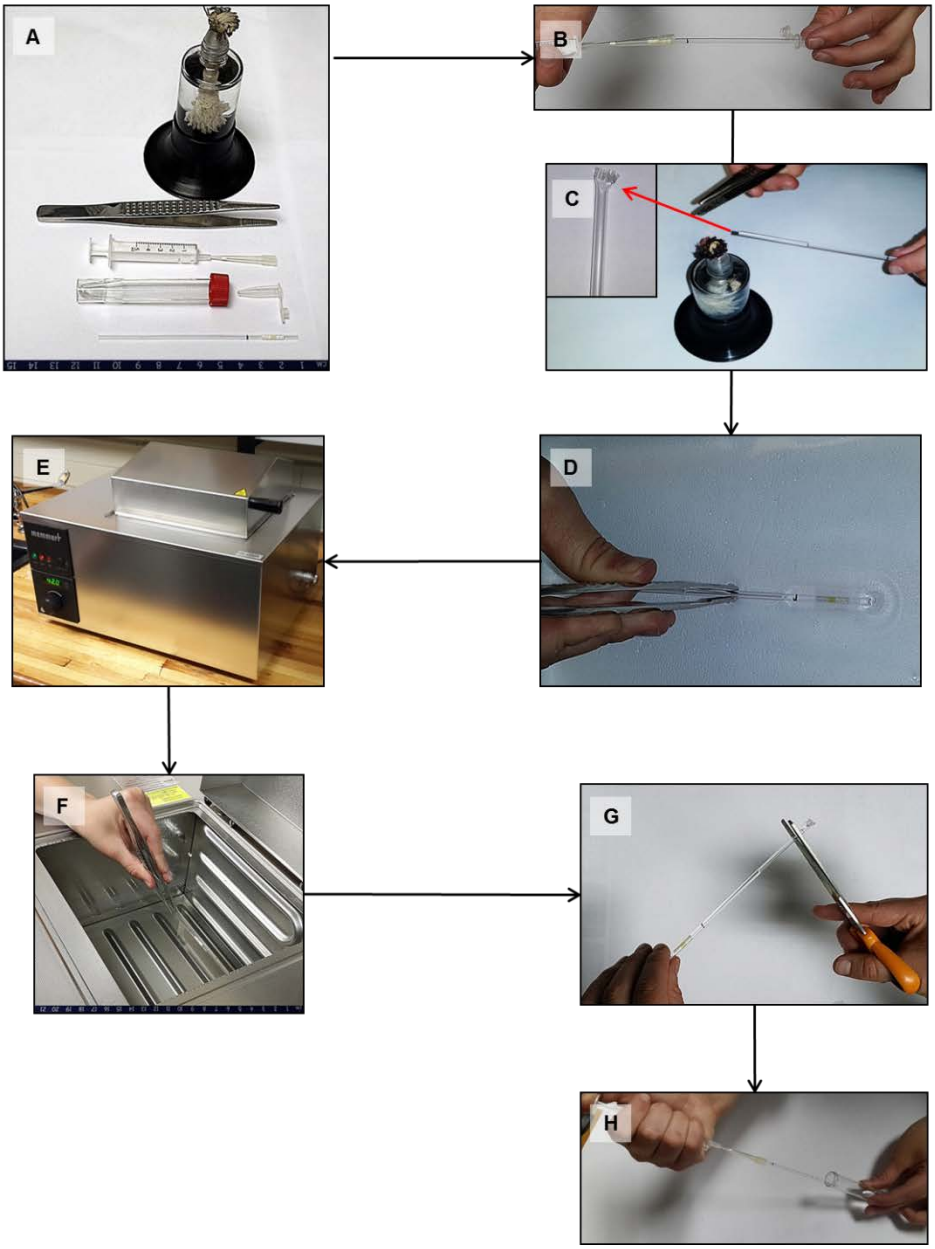


Figure 11: Step wise description of the vitrification procedure

A) Equipment needed for cryoprotectant free vitrification
B) Aspiration spermatozoa suspension into CBS™ straw
C) Flame sealing of straw
D) Cooling of straw
E) Water bath used for thawing set at 42˚C
F) Warming of straw
G) Cutting of straw using sterile scissors
H) Expelling spermatozoa suspension from straw

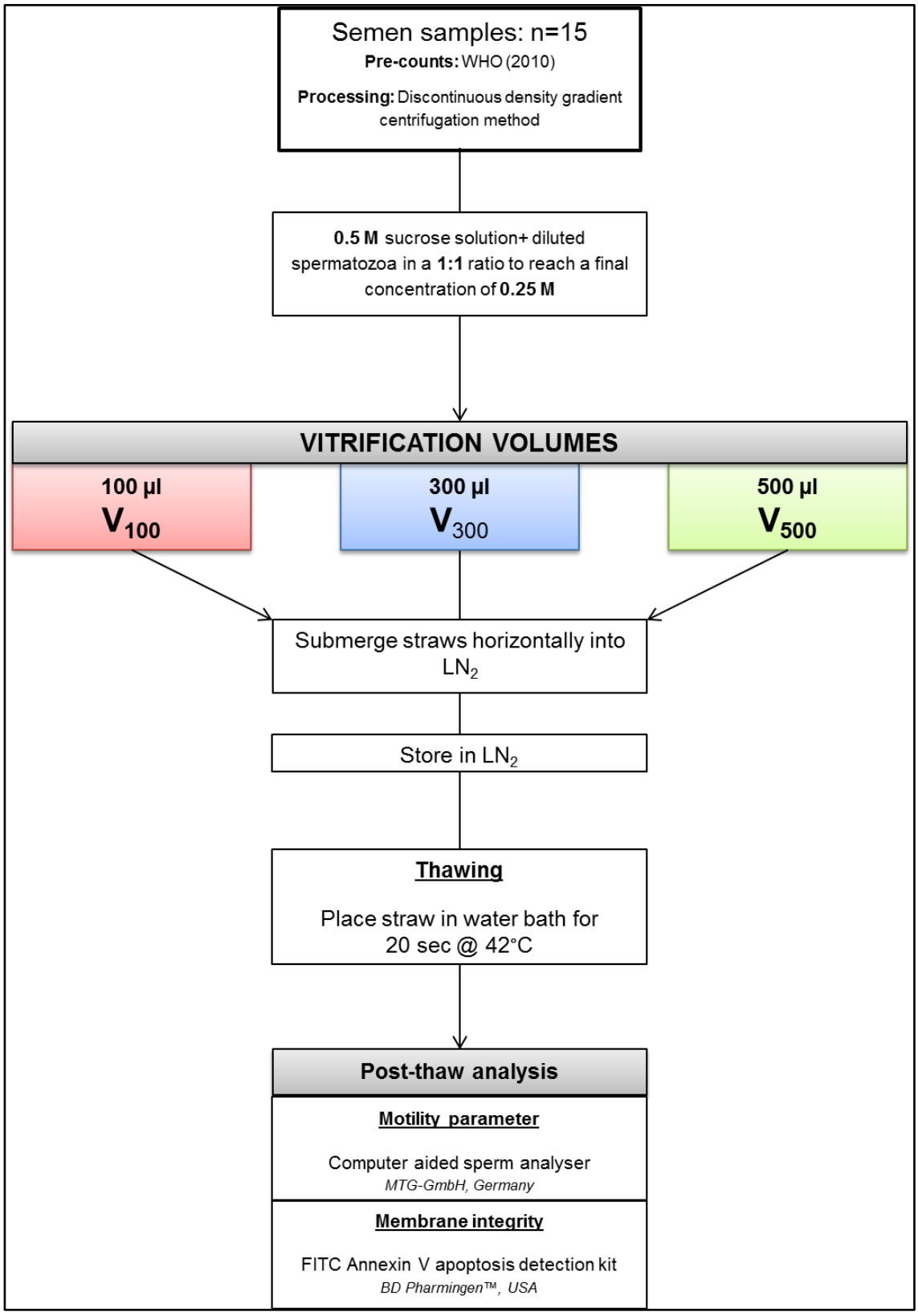


Figure 12: Flow diagram illustrating the study design and methodology for Study 1B

3.3.2 Study 2: Cryoprotectant–free vitrification vs. conventional cryopreservation methods for washed spermatozoa

The aim of Study 2 was to compare the two preservation methods from Study 1A and B that yielded the best quality spermatozoa. Study 1A compared three different cryoprotectant agents and two preservation methods with each other, and results indicated that cryopreserving spermatozoa using Freezing Medium, regardless of the freezing method, yielded superior quality spermatozoa. Results from Study 1B showed that spermatozoa vitrified in volumes of 300 µl or 500 µl yielded superior quality spermatozoa when compared to a smaller vitrification volume (100 µl). For any ART procedure only 1 x 10^6 sperm/ml is needed. The 300 µl volume proved to be optimal, providing more than sufficient sperm for any procedure and this is a small enough volume for more than one straw to be vitrified for the same sample to serve as a back-up. For this study, conventional cryopreservation, using Freezing Medium and the RBL protocol, was compared to cryoprotectant-free vitrification using 300 µl freezing volumes. Figure 14 is a flow diagram depicting the experimental procedure for Study 2.

3.3.2.1 Cryopreservation procedure

The washed sperm sample was divided into two aliquots. The first aliquot was frozen using the conventional cryopreservation method. Freezing Medium was added to the spermatozoa in a 1:1 ratio. After 10 min, the straws were placed horizontally in the vapour phase (1 cm from the liquid surface) for 15 min and subsequently submerged into the LN_2 and stored. See section 3.3.1.1 for a detailed description of the experimental procedure.

The cryopreserved straws were thawed in tap water (23˚C) for 5 min. After 5 min, the straws were sterilized using 70% alcohol, and the end of the straw was cut open using sterile scissors. The contents were expelled into a centrifuge

tube containing 2 ml PureSperm® wash (37˚C), and centrifuged for 10 min at 500 g. The pellet was resuspended and post counts were performed.

3.3.2.2 Vitrification procedure

A sucrose solution, serving as a non-permeable cryoprotectant was prepared beforehand and stored at -20˚C. See section 3.3.1.2. The sucrose solution was thawed at room temperature, incubated at 37˚C and added to the washed spermatozoa in a 1:1 ratio to reach a final concentration of 0.25 M. Straws were filled with 300 µl of the sperm-sucrose solution and sealed hermetically using the flame of an alcohol burner and forceps. The straws were submerged horizontally into the LN_2 and stored. The LN_2 tank used to store the cryopreserved straws are shown in figure 13.

For thawing, the straws were placed in a warm bath at 42˚C for 20 sec. The contents of the straws were expelled into centrifuge tubes containing PureSperm® wash (37˚C) and centrifuged for 10 min at 500 g. The supernatant was removed, the accumulated pellet was resuspended and post thaw analysis was performed. For a detailed description of the methodology see section 3.3.1.2.

Figure 13: Liquid nitrogen container used for storage of cryopreserved samples
(RBL picture library)

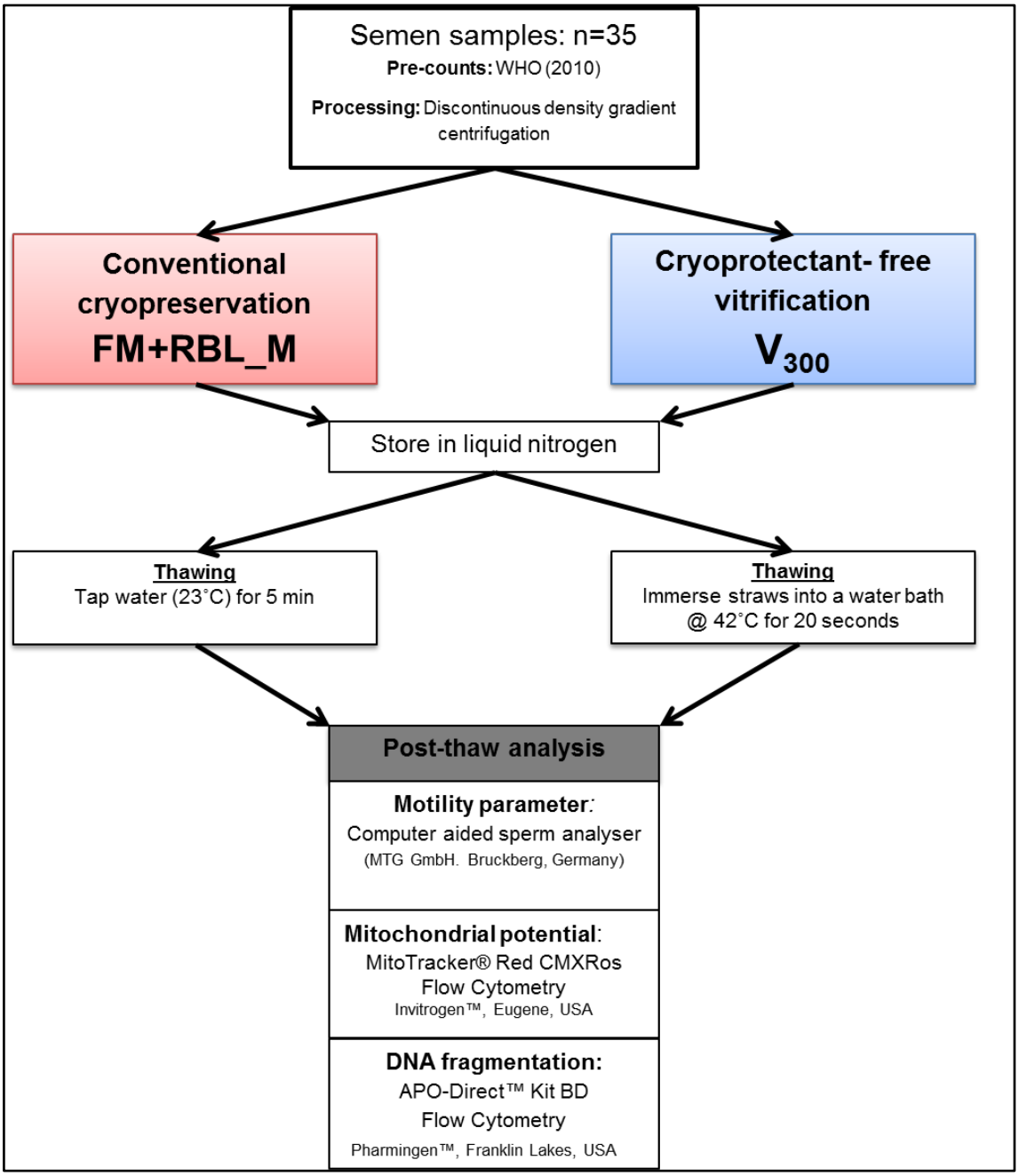


Figure 14: Flow diagram illustrating the experimental set-up for Study 2

3.4 Analysis

3.4.1 Study 1

3.4.1.1 Motility

Sperm motility was analysed microscopically prior to cryopreservation, as well as post thawing. The sperm motility was determined using computer aided sperm analysis (CASA; MTG-GmbH, Bruckberg, Germany) at 200 times magnification (Axioscope 40; Carl Zeiss, Oberkochen, Germany). The CASA classified the spermatozoa into four different motility categories:[29]

i) a (rapid progressive)
ii) b (slow progressive)
iii) c (non-progressive)
iv) d (Immotile)

The velocity parameters measured by the CASA system include (see figure 15):

i) VCL (Curvilinear velocity)
ii) VSL (Straight-line velocity)
iii) VAP (Average path velocity)
iv) WOB (Wobble)
v) ALH (Amplitude lateral head displacement)

Leja® counting chambers (20µm; Leja®, Nieuw-Vennep, Netherlands) were pre-warmed to 37˚C. For each analysis, 5 µl of the sperm solution was loaded into micro-chambers. For quality control purposes, 2 separate chambers were loaded with the same solution and video recordings of at least 200 cells[29]/ or 10 random representative fields per chamber was recorded for 30 sec.

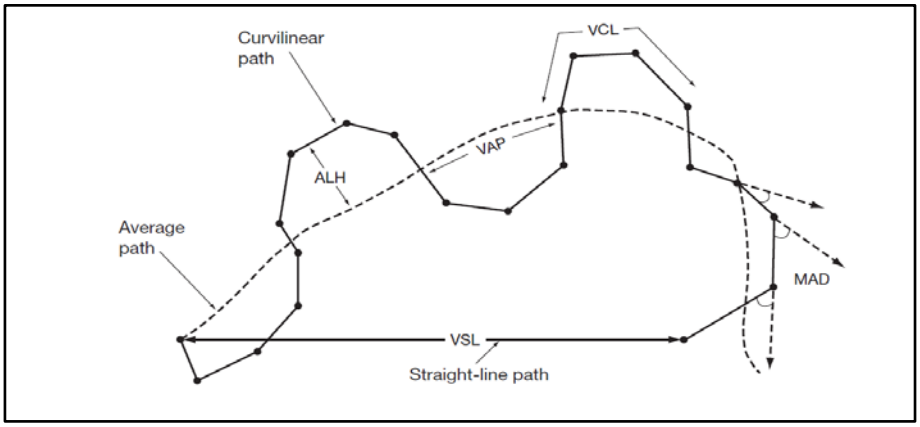


Figure 15: Velocity parameters measured by CASA systems[29]

3.4.1.2 Viability

Sperm viability was determined post thawing using a BD-Pharmingen™ FITC (Fluorescein isothiocyanate) Annexin V apoptosis detection kit (BD Pharmingen™, Franklin Lakes, USA). Staining was performed according to manufacturer's instruction. Thawed samples were washed twice (10 min at 500 g), first in 2 ml cold (4˚C) phosphate buffered saline (PBS; Sigma Aldrich, St Louise, USA), and secondly in 2 ml cold (4˚C) Annexin V binding buffer. Following the double wash, 100 µl of the sperm pellets were removed, 5 µl of Annexin V added, and incubated at room temperature (in the dark) for 15 min. Afterwards, 400 µl of binding buffer was added to the samples and flow cytometry evaluation (Beckman Coulter, Brea, USA) was performed within 20 min. Directly prior to the evaluation, 5 µl of propidium iodide (PI) was added to the samples.[106] Propidium iodide (PI) is detected in the FL3 and Annexin V in the FL1 channel of the flow cytometer. Figure 16 depicts a dot plot representing the flow cytometry result for one of the sperm samples included in this study.

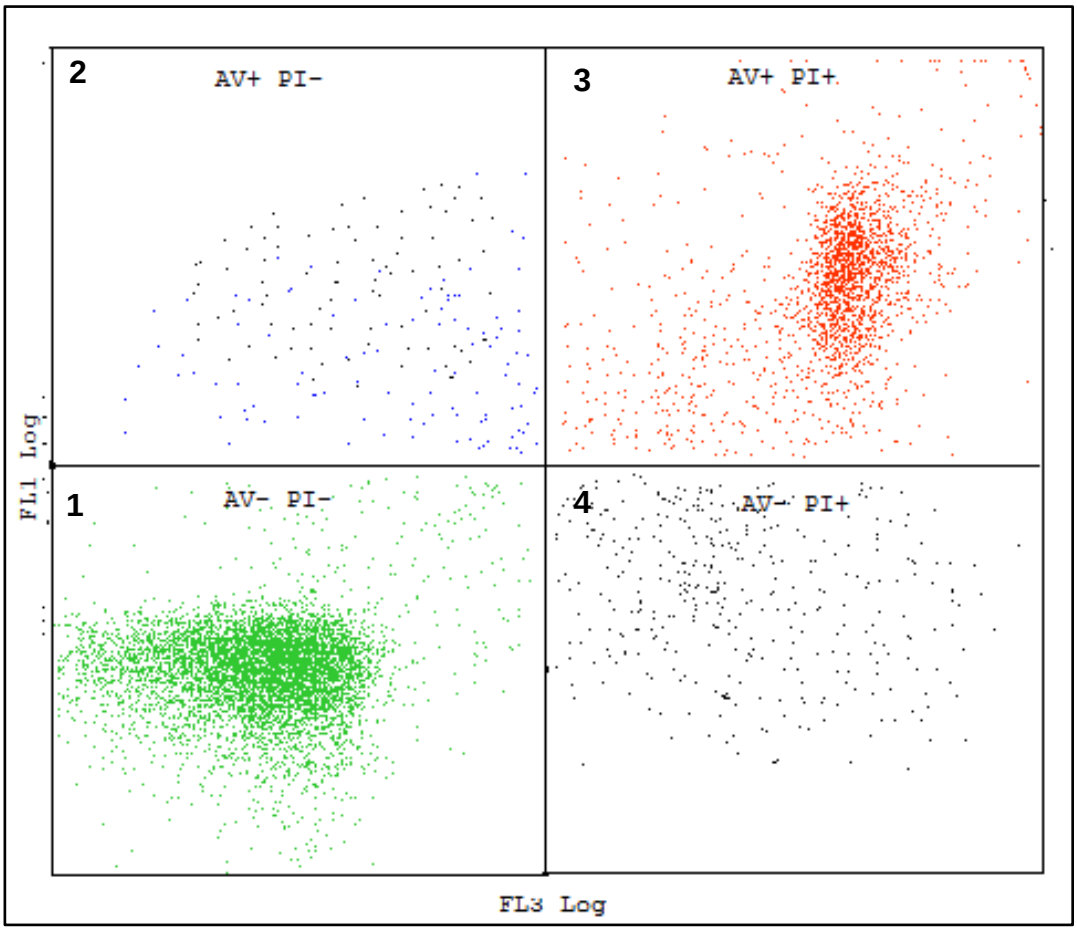


Figure 16: Dot plot illustrating the separation of spermatozoa into four quadrants based on viability status

1. **AV- PI-: Normal**
2. **AV+ PI-: Early apoptosis**
3. **AV+ PI+: Late apoptosis/early necrosis**
4. **AV- PI+: Necrosis**

3.4.2 Study 2

3.4.2.1 Motility

Sperm motility was analysed using a CASA system. See section 3.4.1.1. Figure 17 is a screen display of the MedeaLAB CASA program used to calculate the motility and velocity parameters.

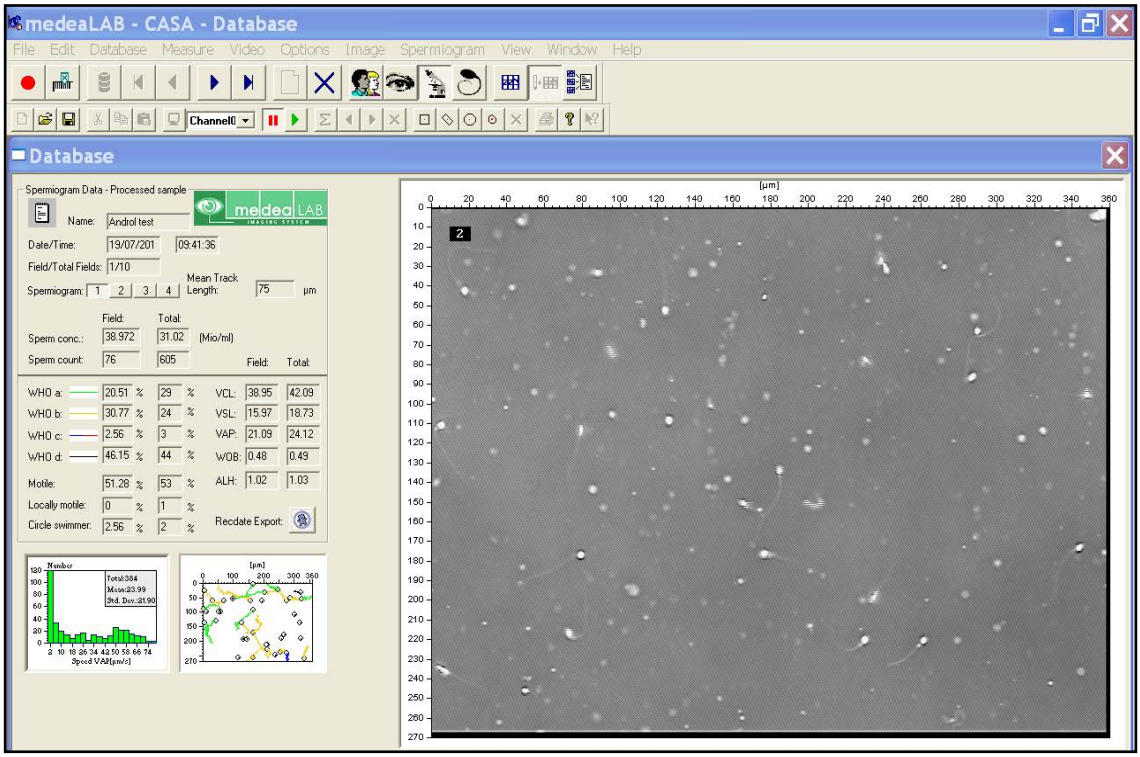


Figure 17: MTG-GmbH computer aided sperm analyser

3.4.2.2 Mitochondrial membrane potential ($\Delta\psi m$)

Mitochondrial membrane potential ($\Delta\psi m$) was determined using a method described by Marchetti *et al.*[107] with small alterations. The MitoTracker® Red CMXRos (Invitrogen™, Eugene, USA) probe was used. MitoTracker® Red CMXRos accumulates in the mitochondria of the cells depending on the $\Delta\psi m$, and the fluorescence fluorochromes reflects this potential. Mitochondrial membrane depolarisation is associated with a decrease in fluorescence.

A stock solution was prepared by diluting 50 µg MitoTracker® Red CMXRos in 100 µl dimethyl sulfoxide (DMSO). The solution was dispensed into smaller vials and stored at -20˚C until use. Thawed samples were washed in 2 ml PureSperm® wash for 10 min at 500 g, the supernatant was removed, and the sperm pellet was resuspended and diluted in PureSperm® wash to reach a final concentration of 5 x 10^6 sperm/ml. A volume of 2.6 µl of the MitoTracker® Red CMXRos stock solution was added to the spermatozoa, and subsequently left to incubate at 37˚C for 15 min. Thereafter, the solution was centrifuged for 10 min at 500 g and 100 µl of the sperm pellet was resuspended in 900 µl PureSperm® wash. The $\Delta\psi m$ was determined using flow cytometry. MitoTracker® Red CMXRos fluorescence was detected in the FL3 channel of the flow cytometer.[107]

3.4.2.3 DNA fragmentation

DNA fragmentation was analysed, using the APO-DIRECT kit. The APO-DIRECT™ assay (BD Pharmingen™, Franklin Lakes, USA) is a single step method for labelling DNA breaks with FITC-dUTP (Flourescein isothiocyanate deoxyuridine Triphosphate), followed by flow cytometry analysis.[108]

Thawed samples were washed in 2 ml PureSperm® wash for 10 min at 500 g. The supernatant was removed and the sperm pellet was resuspended in 1% (w/v) paraformaldehyde in PBS (pH 7.4) to adjust the concentration to 1-2 x 10^6 sperm/ml. The cell suspension was then incubated on ice for 30 min-60 min. The solution was centrifuged for 10 min at 500 g and the supernatant was discarded. The spermatozoa were washed (10 min at 500 g) twice in 5 ml PBS. After the double wash, the pellet was resuspended in the residual PBS by gently vortexing and the concentration was adjusted to 1 x 10^6 sperm/ml in 70% ice-cold ethanol. Spermatozoa were frozen at -20˚C for at least 24 hrs. before assessment.[108]

Before the staining step commenced, the frozen solutions were left at room temperature to thaw. Spermatozoa were resuspended by gently swirling the tubes and centrifuged for 10 min at 500 g. The supernatant was aspirated and the pellet was resuspended in 1 ml wash buffer. The spermatozoa were washed twice by centrifuging (10 min at 500 g) and the pellet was resuspended in 50 µl of the staining solution (10 µl reaction buffer, 0.75 µl TdT [terminal deoxynucleotidy transferase] enzyme, 8 µL FITC-dUTP and 32.25 µl distilled H_20/assay). Samples suspended in the staining solution were incubated for 60 min at 37˚C after which they were washed twice in 1 ml rinse buffer (10 min at 500 g). The supernatant was aspirated, and the pellet was resuspended in 0.5 ml of the PI/RNase staining buffer. The cell suspension was incubated for 30 min at room temperature in the dark, and subsequently, DNA fragmentation was then assessed using flow cytometry.[108] The FITC-labelled dUTP positive spermatozoa were measured in the FL1 channel of the flow cytometer.

3.5 Quality control

3.5.1 Computer aided sperm analysis

The CASA system was calibrated by the manufacturing company (MTG-medical technology Vertriebs-GmbH, Bruckberg, Germany, Version 5.4) and the motion parameters were verified manually and automated, i.e using a secondary CASA system:

i) Results from a semen sample were compared between the CASA used in this study (MTG-GmbH) and the Sperm Class Analyser® (SCA®, Barcelona, Spain). Due to limited access to the SCA® system, situated at an external department, only a single run was performed.

ii) Internal quality control was performed by Prof DR. Franken from Stellenbosch University (WHO Semenology expert). Results from the

manual reading by Prof DR. Franken were compared to the results obtained from the CASA and SCA® readings (table 3).

Table 3: Internal quality control of the computer aided sperm analysis system used for motility assessment of spermatozoa

Parameter	CASA	SCA®
	MTG-GmbH, Bruckberg, Germany	*SCA®, Barcelona, Spain*
VCL	42.09 µm/s	47.8 µm/s
VSL	18.73 µm/s	18.8 µm/s
VAP	24.12 µm/s	25 µm/s
WOB	0.49	0.522

VCL = curvilinear velocity, VSL = straight line velocity, VAP = average path velocity, WOB = wobble, ALH = amplitude of lateral head displacement

3.5.2 Flow cytometry

3.5.2.1 Annexin V/PI validation

Sperm viability (apoptosis and necrosis) was analysed using flow cytometry. The protocol was set up and validated by the inclusion of the following controls:

i) Apoptosis was induced by incubating sperm with hydrogen peroxide (H_20_2) (Sigma Aldrich, St Louise, USA) for 18 hrs.

ii) Annexin V-FITC binding to sperm was blocked by incubating sperm, according to the manufacturer's stipulations, with purified recombinant Annexin V (BD Pharmingen™, Franklin Lakes, USA).

iii) Necrosis was induced by incubating spermatozoa with 2 µl of Triton X (Sigma Aldrich, St Louise, USA) for 20 min. The controls are illustrated in Figure 18 A and B.[93]

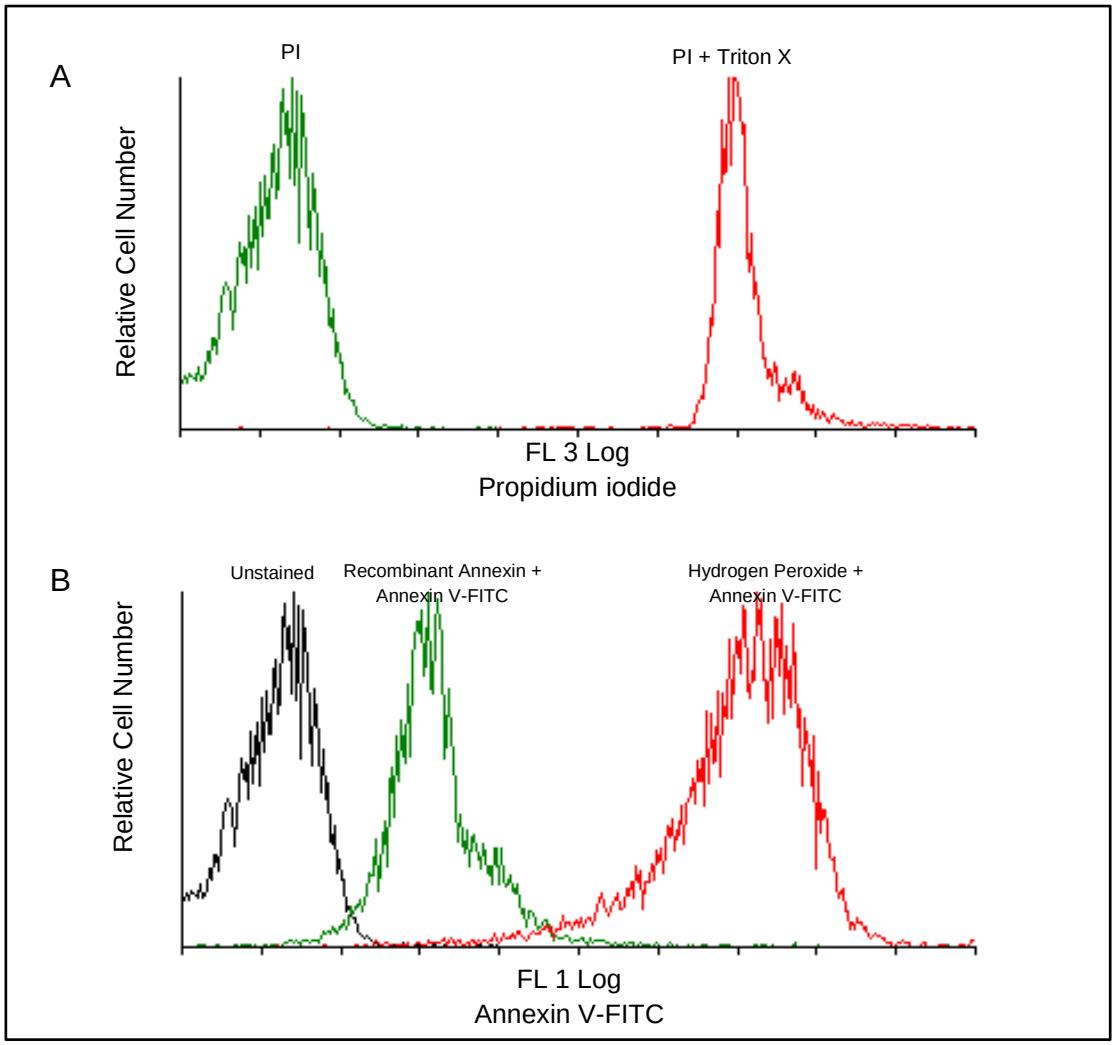


Figure 18: Flow cytometric histograms depicting overlay graphs of the controls included during the viability assay:

A) Triton-X to induce necrosis,
B) Hydrogen peroxide to induce apoptosis and recombinant Annexin to block the binding of Annexin V-FITC to the spermatozoa

3.5.2.2 MitoTracker® Red protocol set-up and validation

To serve as a positive control the $\Delta\psi m$ was abolished using m-Chlorophenylhydrazone (mCICCP; Sigma Aldrich, St Louise, USA). A stock solution of mCICCP was prepared by adding 204.6 mg of mCICCP to 1 ml of DMSO. The solution was stored in small tubes at -20˚C. Prior to experimentation the stock solution was thawed and 5 µl was added to 950 µl spermatozoa and incubated for 15 min, before staining with MitoTracker® Red CMXRos. For a negative control, aliquots containing spermatozoa were incubated with MitoTracker® Red CMXRos without any other additives.[93] See figure 19 for a graphical representation of the results.

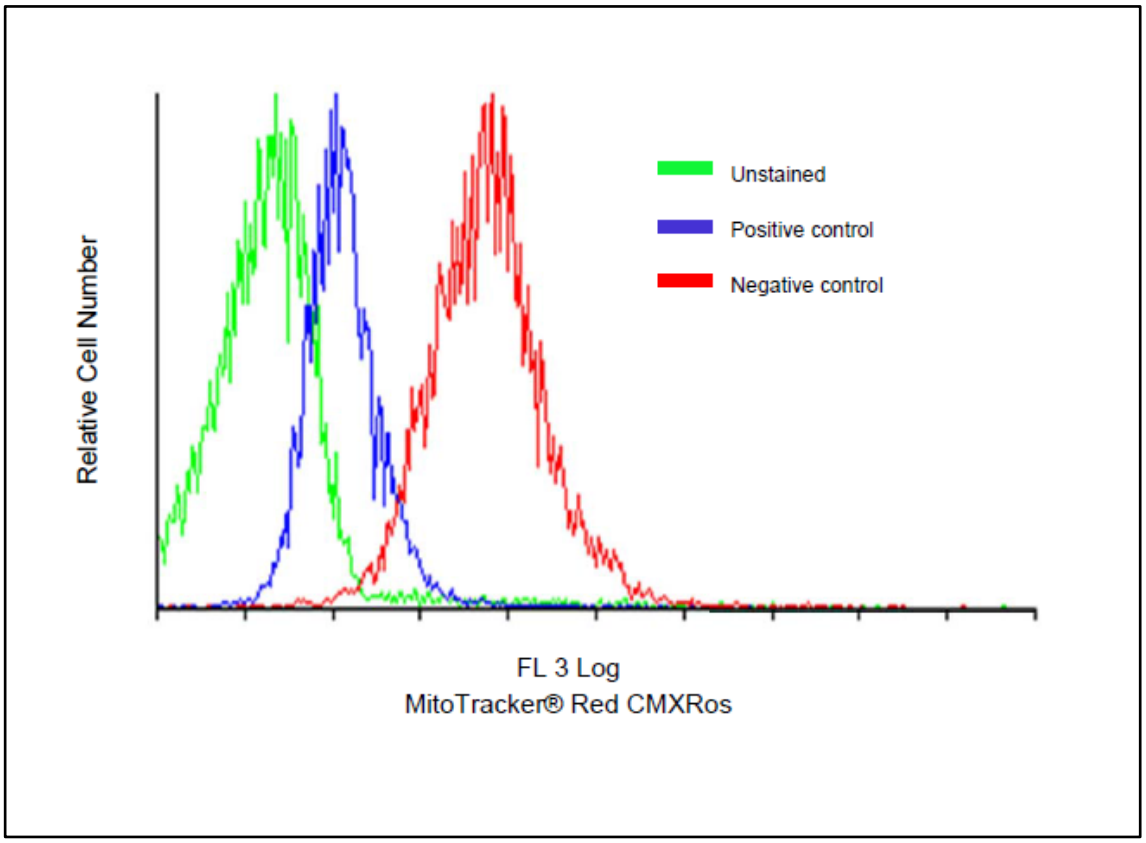


Figure 19: Cytofluorometric analysis of the mitochondrial membrane potential depicting a frequency histogram of unstained cells, positive control cells (mCICCP and stained with MitoTracker® Red CMXRos) and negative control cells (stained with MitoTracker® Red CMXRos)

3.5.2.3 APO-DIRECT™

The APO-DIRECT™ kit includes positive and negative control cells. The control cells were derived from a human lymphoma cell line that was already fixed. The positive and negative cells were stained according to the suggested protocol. The fluorescent stains included in the APO-DIRECT™ are PI and FITC-dUTP. Propidium iodide (PI) stains the total DNA and FITC-dUTP stains only the apoptotic cells. The FITC-labelled dUTP-positive cells were measured in the FL 1 channel and PI in the FL 3 channel of the flow cytometer (see figure 20).[108]

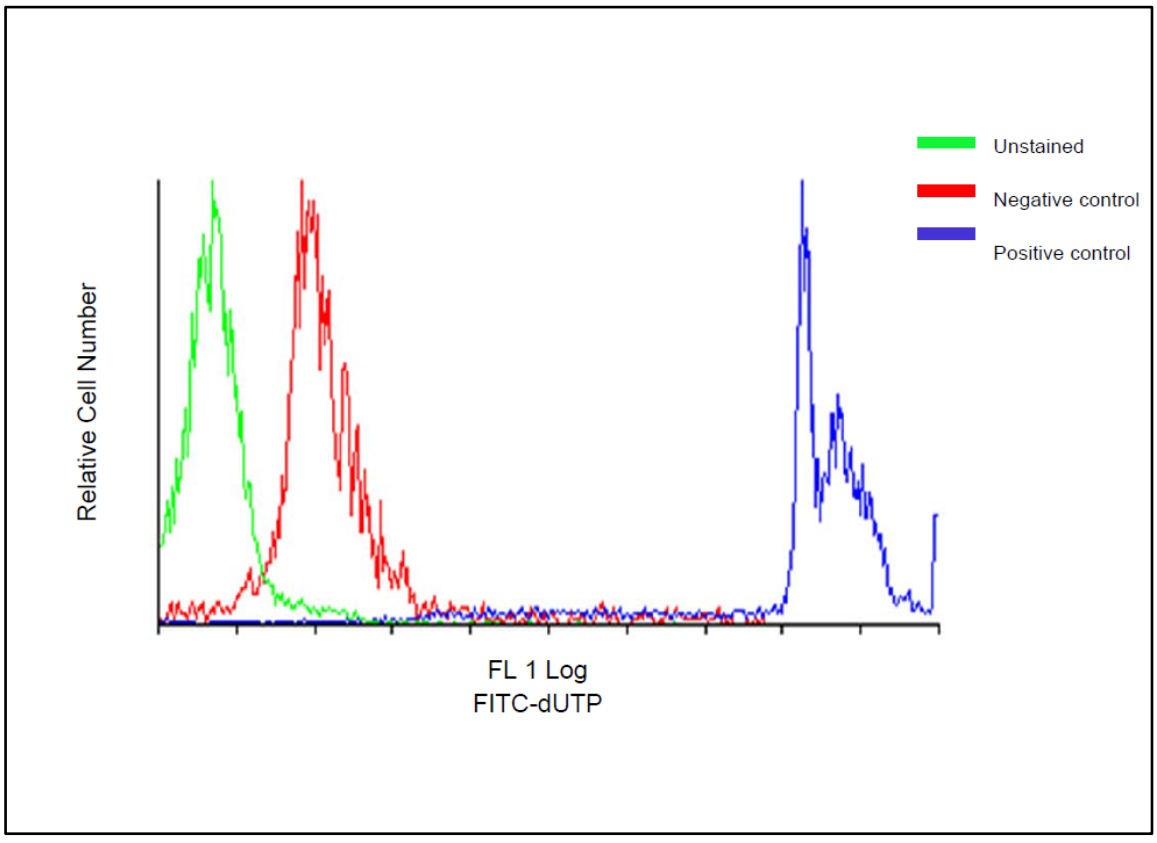


Figure 20: APO-DIRECT™ experimental set-up:
Flow cytometric histograms showing overlay plots of processed unstained spermatozoa (green), positive (blue) and negative control cell

3.6 Statistical analysis

Sperm parameters were compared in the 3 studies using a within subject two-factor study design.

i) Cryopreservation at three levels (Freezing Medium, Sperm Freeze, CryoProtect™ II)
ii) Freezing protocols at two levels (RBL protocol, Irvine Scientific® protocol)
iii) Vitrification volumes at three levels (100 µl, 300 µl, 500 µl)
iv) Preservation methods at two levels (Cryoprotectant-free vitrification, conventional cryopreservation

Since within subject data is not independent, the data analysis needs to account for the intra-subject variation. Therefore, a mixed model approach was adopted using random effects generalized least squares regression. The parametric data analysis assessed means (averages) following interpretation that the residuals were Gaussian distributed. Testing was performed at the 0.05 level of significance using Stata Release 11 statistical software.[109]

CHAPTER 4

RESULTS

4.1 Semen parameters of samples used

Table 4: Average microscopic sperm parameters: Volume, pH, concentration and progressive motility of sperm samples used in Studies 1 and 2

Parameter	Study 1A	Study 1B	Study 2
	Mean (Standard deviation)		
Volume (ml)	3.22 (1.515)	3.593 (1.067)	2.983 (1.071)
pH	7.41 (0.145)	7.44 (0.124)	7.407 (0.153)
Concentration (10^6/ml)	64.36 (14.779)	48.053 (20.067)	41.867 (17.805)
Progressive motility (%)	64.55 (8.241)	70.2667 (10.599)	74.467 (9.016)

4.2 Study 1A

4.2.1 Motility and velocity parameters of spermatozoa after cryopreservation using different mediums and freezing protocols

Graphical representation illustrating the total motility (a+b) and rapid progressive motility (a) of spermatozoa after cryopreservation using three different mediums are shown in figure 21 A. Spermatozoa cryopreserved using FM and SF had significantly higher percentages of total motility when compared to the CP II group ($p<0.05$). No significant difference was seen in the total motility of spermatozoa after cryopreservation using SF and FM ($p=0.957$). The

rapid progressive motility of spermatozoa in the FM group was significantly higher when compared to the SF ($p<0.05$) and CP II ($p<0.001$) groups. No significant difference was observed in the percentage of rapid progressive spermatozoa after cryopreservation using SF and CP II. See table 10 (Addendum A).

The velocity parameters of spermatozoa after cryopreservation using the three different mediums are illustrated in figure 21 B. No significant differences were observed after comparing the velocity parameters of spermatozoa cryopreserved using FM or SF. Cryopreserving spermatozoa using SF resulted in significantly higher VCL ($p<0.05$), and ALH ($p<0.05$) parameters compared to spermatozoa cryopreserved using CP II. Significantly higher VCL ($p<0.05$), VSL ($p<0.05$), VAP ($p<0.01$), WOB ($p<0.01$) and ALH ($p<0.01$) results were observed when comparing spermatozoa cryopreserved using FM to spermatozoa in the CP II group. Descriptive statistics are summarized in table 10 (see Addendum A).

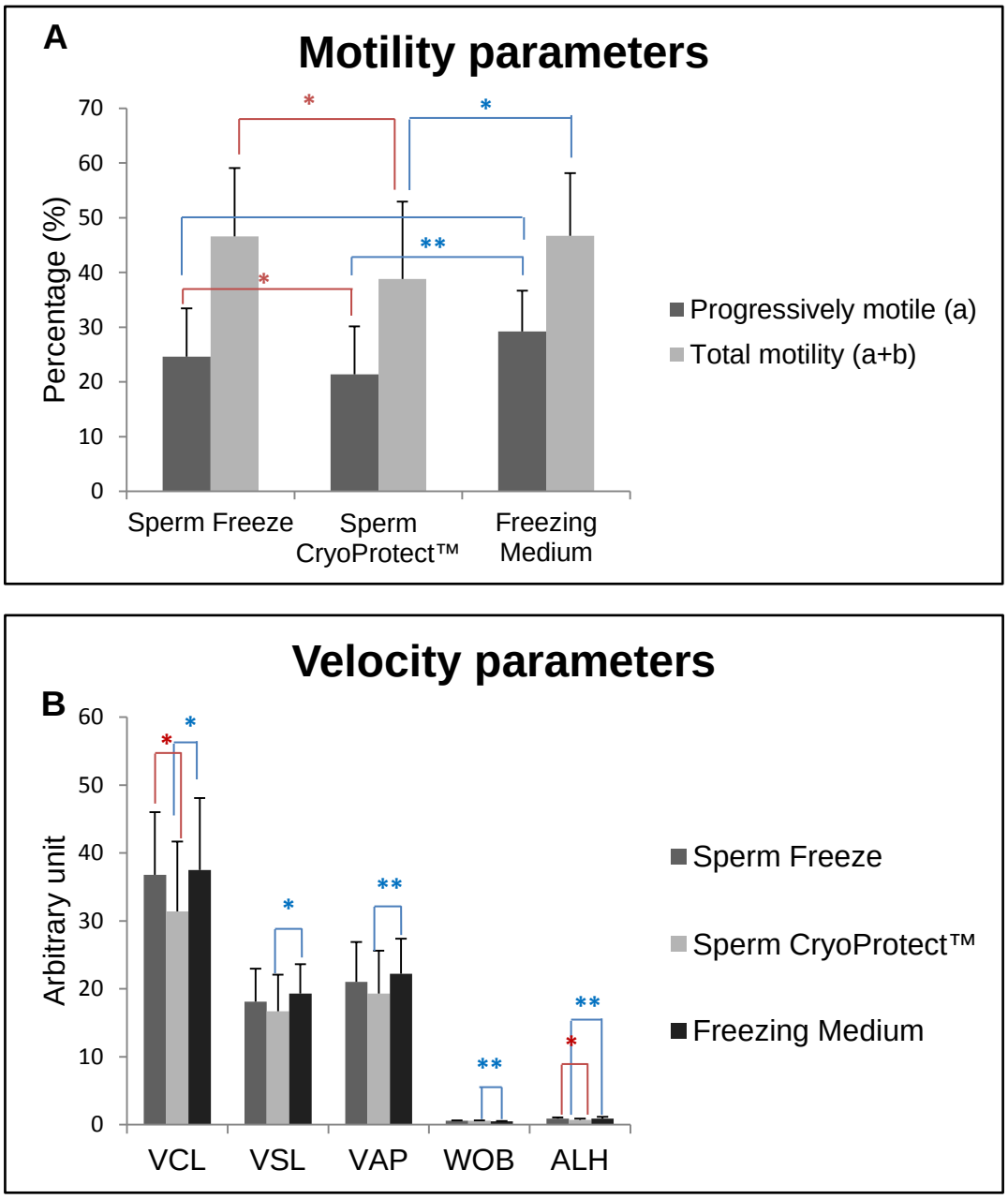


Figure 21: Bar graphs illustrating the A) progressively motile and total motility and B) velocity parameters after spermatozoa were cryopreserved using three commercially available cryoprotectants (*p<0.05, **p<0.001)

VCL = curvilinear velocity, VSL = straight line velocity, VAP = average path velocity, WOB = wobble, ALH = amplitude of lateral head displacement

4.2.2 Viability parameters of spermatozoa after cryopreservation using three different cryopreservation media

The viability parameters of spermatozoa cryopreserved using three different cryopreservation media, are depicted in figure 22. Cryopreserving spermatozoa using FM resulted in significantly higher percentages of normal spermatozoa (AV-[5]; PI-[6]) when compared to spermatozoa in the SF ($p<0.001$) and CP II ($p<0.001$) groups. Significantly lower percentages of late apoptotic/early necrotic (AV-; PI+[7]) spermatozoa were observed after cryopreservation using FM compared to spermatozoa in the SF ($p<0.001$) and CP II ($p<0.001$) groups. No significant differences were observed between the percentages of apoptotic (AV+[8]; PI-) and necrotic spermatozoa (AV-; PI+). Refer to table 11 (see Addendum A).

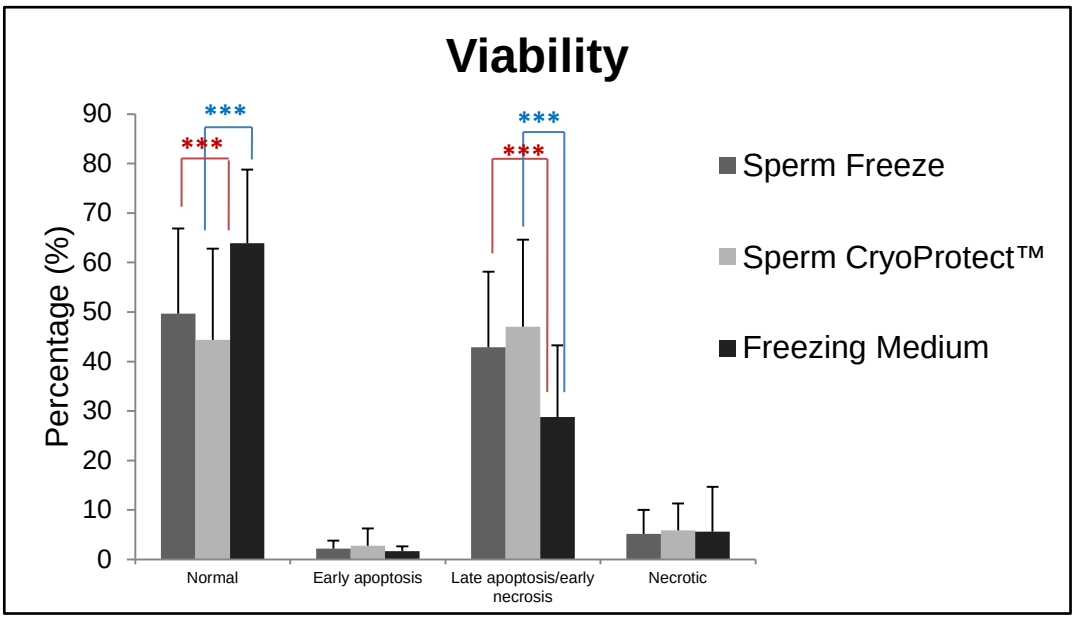


Figure 22: Assessment of apoptotic statuses of spermatozoa after cryopreservation using three commercially available cryoprotectants (*p<0.001)**

[5] AV[-]: Annexin V negative
[6] PI[-]: Propidium iodide negative
[7] PI[+]: Propidium iodide positive
[8] AV[+]: Annexin V positive

4.2.3 Motility and velocity parameters of spermatozoa cryopreserved utilizing two different freezing protocols

The results of the total motility, rapid progressive motility and velocity parameters of spermatozoa are depicted in table 5. No significant differences were observed in any of the sperm motion parameters post thawing.

4.2.4 Viability parameters of spermatozoa after preservation using two different freezing protocols

The descriptive statistics for the viability parameters of spermatozoa are summarized in table 6. No significant differences were found in the percentages of normal, apoptotic, late apoptotic/early necrotic or necrotic spermatozoa post thawing.

Table 5: Summary of the motility and velocity parameters of human spermatozoa cryopreserved using two different freezing protocols

Parameter	RBL Method	Irvine Scientific Method	p-value
	Mean (Standard deviation)		(95% CI)
Rapid Progressive (%)	24.8% (9.05)	25.3% (8.82)	0.784 (-2.768 ; 3.668)
Total Motility (%)	44.4% (14.02)	43.65% (12.34)	0.729 (-5.219 ; 3.653)
VCL (µm/s)	35.5 (10.60)	34.8 (10.01)	0.726 (-4.574 ; 3.185)
VSL (µm/s)	17.8 (4.78)	18.3 (5.12)	0.576 (-1.261 ; 2.270)
VAP (µm/s)	20.9 (5.51)	20.83 (6.23)	0.973 (-1.936 ; 1.870)
WOB (µm/s)	0.5 (0.059)	0.5 (0.042)	0.741 (-0.017 ; 0.024)
ALH (µm)	0.8 (0.24)	0.8 (0.20)	0.286 (-0.129 ; 0.038)

VCL = curvilinear velocity, VSL = straight line velocity, VAP = average path velocity, WOB = wobble, ALH = amplitude of lateral head displacement

Table 6: Viability results after cryopreserving spermatozoa utilizing two different freezing protocols

Parameter	RBL Method	Irvine Scientific Method	p-value
	Mean (Standard deviation)		(95% CI)
Normal (%) **(P^-A^-)**	50.44% (19.89)	54.79% (17.19)	0.141 (-1.445 ; 10.141)
Apoptotic (%) **(P^-A^+)**	2.03% (1.33)	2.4% (2.95)	0.533 (-0.704 ; 1.361)
Late Apoptotic/ Early Necrotic (%) **(P^+A^+)**	42.2% (18.17)	37.03% (16.57)	0.094 (-11.123 ; 0.878)
Necrotic (%) **(P^+A^-)**	5.3% (4.98)	5.8% (7.80)	0.692 (-1.939 ; 2.921)

4.3 Study 1B

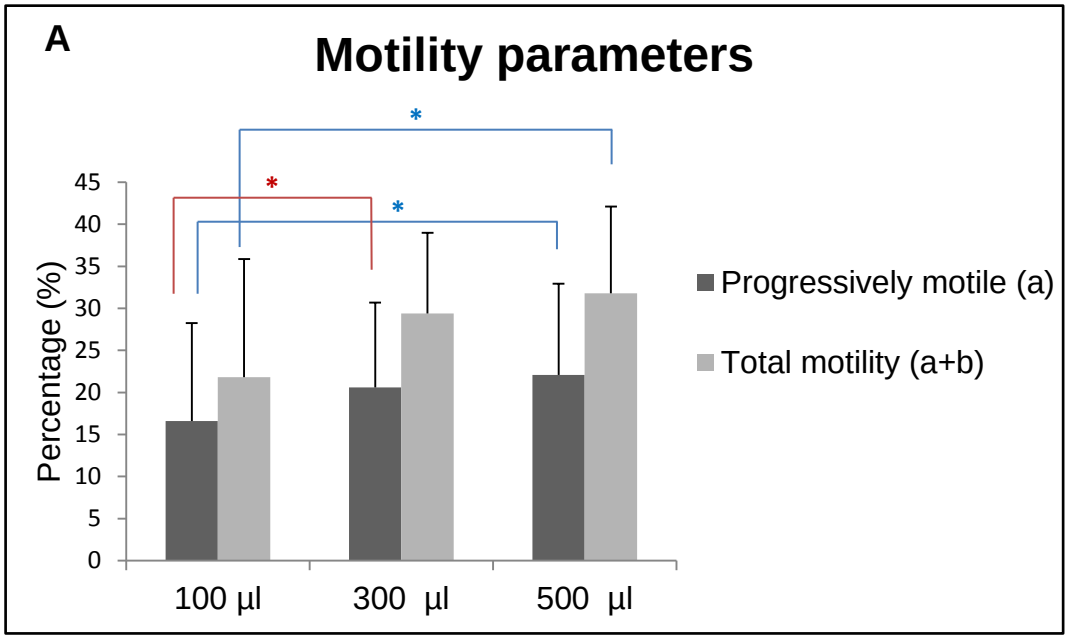
4.3.1 Sperm motility and kinetic parameters of spermatozoa post vitrification

The total motility (a+b) and rapid progressive motility (a) of spermatozoa are shown in figure 23 A. Vitrifying spermatozoa in V_{300} and V_{500} volumes resulted in significantly higher total (a+b) and rapid progressive motility (a) compared to spermatozoa vitrified V_{100} volumes ($p<0.05$). No significant differences were observed when comparing the motility of spermatozoa in the V_{300} and V_{500} volume groups.

The velocity parameters are depicted in figure 23 B. Spermatozoa vitrified in larger volumes (V_{300} and V_{500}) had significantly higher percentages of VCL, VSL, VAP, ALH, and WOB parameters when compared to spermatozoa in the V_{100} volume group ($p<0.05$). No significant differences were observed in the velocity parameters of spermatozoa vitrified in V_{300} and V_{500} volumes. See table 12 in Addendum A for descriptive statistics and p-values.

4.3.2 Viability parameters of spermatozoa after vitrification using different volumes

Refer to table 7 for the descriptive statistics of the viability parameters. No significant differences were observed in the percentage of normal (PI-; AV-), late apoptotic/early necrotic (PI+; AV+), apoptotic (PI-; AV+) or necrotic spermatozoa (PI+; AV-).



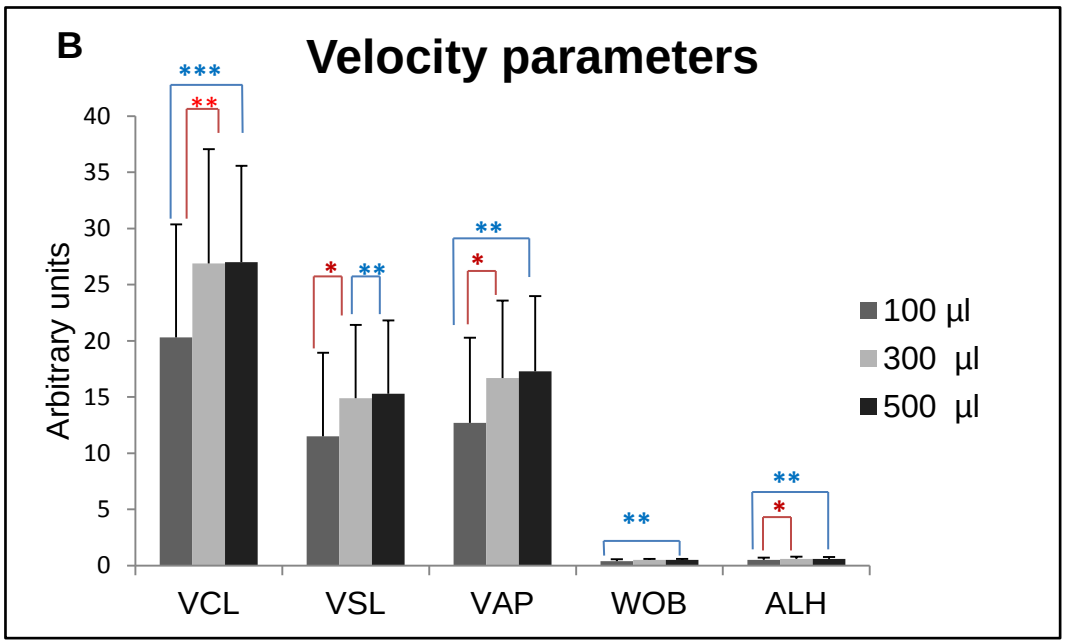


Figure 23: Motion and velocity parameters after vitrifying spermatozoa in 100, 300 and 500 µl volumes (*p<0.05, **p<0.01, ***p<0.001)

VCL = curvilinear velocity, VSL = straight line velocity, VAP = average path velocity, WOB = wobble, ALH = amplitude of lateral head displacement

Table 7: Apoptotic statuses of spermatozoa post vitrification in three different volumes

Parameter	100 µl	300 µl	500 µl	p-value (95% CI)		
	Mean (Standard deviation)			P^a	P^b	P^c
Normal (%) **(P^-A^-)**	28.4% (17.13)	23.7% (16.31)	25.9% (19.18)	0.635 (-13.192 ; 8.044)	0.695 (-8.495 ; 12.743)	0.39 (-15.316 ; 5.919)
Apoptotic (%) **(P^-A^+)**	13.5% (11.94)	8.8% (11.56)	8.7% (12.05)	0.261 (-13.089 ; 3.549)	0.975 (-17.729 ; -1.089)	0.274 (-12.959 ; 3.680)
Apoptotic/Early Necrotic (%) **(P^+A^+)**	47.3% (25.28)	62.8% (25.82)	61.3% (28.31)	0.11 (-3.167 ; 31.036)	0.855 (-18.694 ; 15.510)	0.075 (-1.575 ; 32.628)
Necrotic (%) **(P^+A^-)**	10.9% (14.25)	4.7% (3.07)	4.3% (3.46)	0.026 (-12.398 ; -0.784)	0.893 (-6.207 ; 5.407)	0.037 (-11.998 ; -0.384)

P^a → 500 µl vs. 100 µl
P^b → 500 µl vs. 300 µl
P^a → 300 µl vs. 100 µl

4.4 Study 2

4.4.1 Sperm motility and kinetic parameters

No significant differences were observed in the total motility (a+b), rapid progressive motility (a) or any of the kinetic parameters (VCL, VSL, VAP, WOB, and ALH) of spermatozoa after preservation using either cryoprotectant-free vitrification or conventional cryopreservation. See table 8.

4.4.2 Mitochondrial membrane potential

The percentages of Mitotracker® Red CMXRos staining in the two groups are illustrated in figure 24. Significantly higher $\Delta\psi m$ was observed in spermatozoa preserved using cryoprotectant-free vitrification ($p<0.001$). See table 13 in Addendum A for descriptive statistics and p-values.

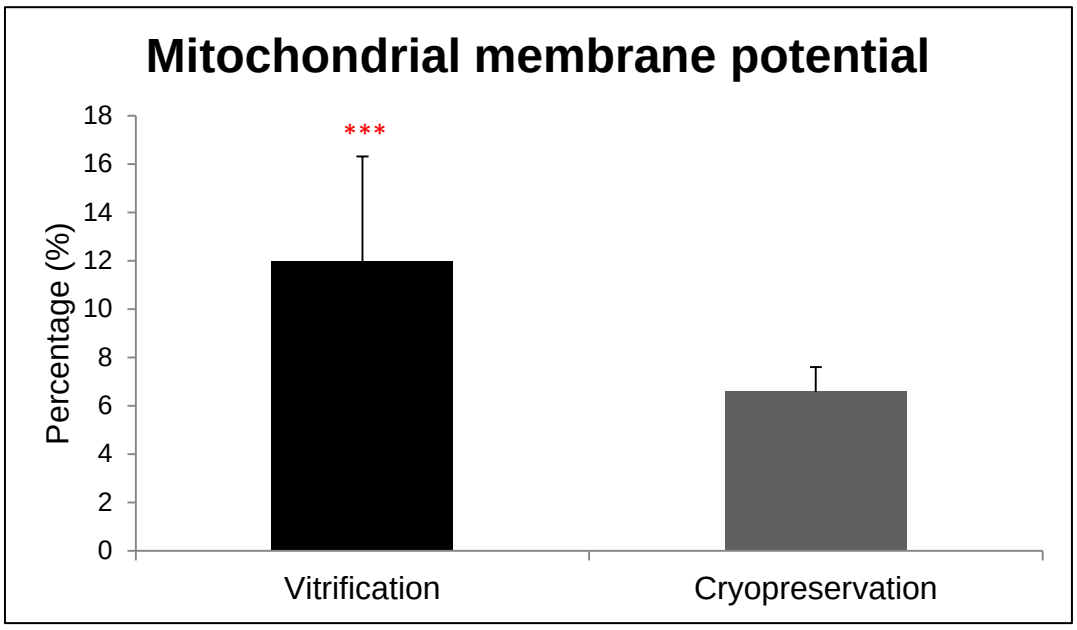


Figure 24: Percentage of Mitotracker® Red CMXRos staining after preservation of spermatozoa using cryoprotectant-free vitrification and conventional cryopreservation (*p<0.001)**

Table 8: Comparison of motility and velocity parameters of spermatozoa preserved using cryoprotectant-free vitrification and conventional cryopreservation

Parameter	Vitrification	Cryopreservation	p-value
	Mean (Standard deviation)		(95% CI)
Rapid Progressive (%)	11.520% (6.099)	12.540% (7.160)	0.423 (-1.477; 3.517)
Total Motility (%)	20.480% (7.399)	23.900% (12.583)	0.181 (-1.588; 8.428)
VCL (µm/s)	19.056 µm/s (6.469)	20.646 µm/s (7.854)	0.339 (-1.667; 4.848)
VSL (µm/s)	9.857 µm/s (3.698)	10.609 µm/s (4.516)	0.343 (-0.804; 2.309)
VAP (µm/s)	11.488 µm/s (4.075)	12.329 µm/s (5.027)	0.385 (-1.054; 2.735)
WOB (µm/s)	0.530 (0.072)	0.795 (1.421)	0.349 (-0.289; 0.819)
ALH (µm)	0.503 µm (0.118)	0.548 µm (0.140)	0.146 (-0.016; 0.104)

VCL = curvilinear velocity, VSL = straight line velocity, VAP = average path velocity, WOB = wobble, ALH = amplitude of lateral head displacement

4.4.3 DNA fragmentation

DNA fragmentation was measured using a TUNEL assay. Significantly lower percentages of apoptotic spermatozoa were observed after cryopreservation using cryoprotectant-free vitrification ($p<0.001$) (see figure 25). Descriptive statistics and p-values are summarized in table 14 (Addendum A).

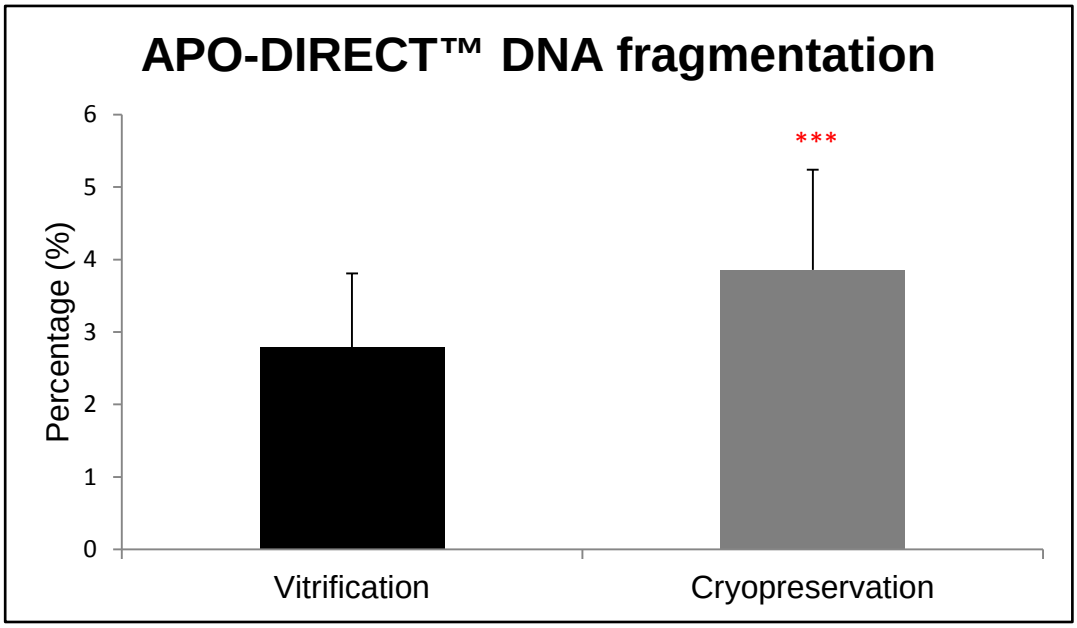


Figure 25: Percentage of DNA fragmented spermatozoa determined using a TUNNEL assay, after comparing cryoprotectant-free vitrification with conventional cryopreservation (*p<0.001)**

CHAPTER 5

DISCUSSION AND CONCLUSION

Vitrification of spermatozoa has vast potential, with opportunities for further investigations.[13] Cryo-damage is a result of the stressors induced by cryopreservation. The stressors include intracellular ice crystal formation, cellular dehydration, and osmotic injury during addition or removal of CPA (due to the raised solute levels within the cell), or alterations in membrane permeability.[110-112] Therefore, the success of the cryopreservation procedure is influenced by the rate of freezing and the cryopreservation media in which the cells are frozen. No universally accepted freezing protocol exists. Furthermore, a method for freezing and thawing of semen that optimize the recovery of motile spermatozoa has still not been firmly established. Various cryoprotectants are commercially available to protect sperm from the negative effects of the preservation procedure.[96] Spermatozoa however, have a low tolerance for cryoprotectants since these agents may possibly cause lethal osmotic effects and chemical changes in sperm.[10]

Vitrification without the use of conventional CPA and the application of rapid freezing has been described for human spermatozoa.[9-11] This method requires replacing the CPA with a protein and carbohydrate solution (1% HSA and 0.25 M sucrose). An advantage of vitrification is that no cryoprotectants are needed, thereby avoiding the lethal effects of cryoprotectant toxicity and possible osmotic damage to spermatozoa.[7,11] Studies 1 and 2 explored the potential and feasibility of substituting conventional cryopreservation procedures, with larger volume cryoprotectant-free vitrification.

The effect of cryopreservation on sperm morphology is well documented and has been shown to significantly decrease the percentage of normal sperm.[16,23] Preliminary retrospective studies on sperm morphology parameters of HIV-positive males at SBAH indicated no significant differences before and after cryopreservation (6.682±2.982 vs. 6.545±3.320, p=0.753). Since no differences were seen in the morphology, this study focussed on comparing the two methods with respect to sperm motility, sperm viability, $\Delta\psi m$, and DNA integrity.

The main objective of Study 1A was to determine which cryopreservation media and method yielded the best quality spermatozoa in terms of motility and viability. Three cryopreservation media included in this study is regularly used by South African ART laboratories were used. To ensure a sterility assurance level when using TYB (present in FM), the medium is membrane filtered and aseptically processed.[104] Superior results were obtained after spermatozoa were cryopreserved using FM. Significantly higher percentages of rapid progressive motile and normal spermatozoa were found. Cryopreserving with FM also resulted in significantly lower percentages of spermatozoa in the late apoptosis/early necrosis stage. Several studies revealed that preserving spermatozoa using a cryoprotective medium containing TYB, compared to other CPA, yielded better results in terms of motility and cryosurvival.[113,114] The mechanism behind TYB functioning is not entirely understood. The decrease in the deleterious effects on spermatozoa could possibly be due to an exchange of lipids between the sperm cell membranes and a phospholipid portion of the low-density protein of egg yolk. This low-density protein alters the cell membranes molecular composition while maintaining the fluidity.[113,115,116] For the second part of the experiment, two cryopreservation protocols were compared. No statistically significant differences were observed after cryopreserving spermatozoa using the different protocols. No definite conclusion could be drawn on which method resulted in better quality spermatozoa post freezing. As the RBL_M is quicker (±20 min vs. ±80 min), and no specialized equipment

such as a refrigerator is needed. Therefore, the RBL_M was selected as the preferred protocol.

Vitrification as a cryopreservation method has numerous advantages and benefits. Conventional cryopreservation methods take approximately 60 to 90 min to complete and in some instances even longer, whereas most vitrification protocols take only several min. Apart from the time saving aspect, vitrification has economic benefits when compared to conventional freezing methods. As no specialized equipment is necessary, and the cost of permeable cryoprotectants is eliminated. Two benefits are an absence of ice crystallization and minimization of osmotic injury. The prescribed method for vitrification of oocytes, embryos and other tissues requires rapid cooling rates and high CPA concentrations.[9] Spermatozoa are known to have low tolerance levels for high concentrations of cryoprotectants. Nevertheless, pioneers in the field of mammalian spermatozoa vitrification reported successful vitrification of human spermatozoa without the use of permeable cryoprotectants.[7,9-11] Various aseptic vitrification techniques were however investigated by Isachenko *et al.*[9] but only small volumes, ranging from 1 µl to 40 µl, of sperm suspension could be vitrified in these systems. In 2011, the Isachenko group reported a novel aseptic cryoprotectant-free vitrification method where spermatozoa are vitrified in larger volumes (up to 500 µl).[105] The larger-volume vitrification technique was proven a success after the birth of a healthy baby by means of IUI with vitrified spermatozoa.[117] This technique was utilized and adapted in Study 1B to establish an optimal vitrification volume.

The aim of Study 1B was to investigate the effects of larger vitrification volumes (100 µl, 300 µl, and 500 µl) on human spermatozoa. Results indicated that the two larger vitrification volumes (300 µl, and 500 µl), provide superior cryoprotective effects. When comparing the post thaw motility (total motility and rapid progressive motility) of spermatozoa vitrified in different volumes (100 µl, 300 µl, and 500 µl), significantly better results were obtained in the larger volume groups (300 µl, and 500 µl). No significant differences were

observed between the 300 µl and 500 µl vitrification groups. For practical implications and general convenience, spermatozoa vitrified in 300 µl volumes represented the better choice (see section 3.3.2).

Study 2 compared the outcomes from Studies 1A and B:

i) Conventional cryopreservation using Freezing Medium as cryoprotectant and using the SOP from RBL; and
ii) Larger volume cryoprotectant-free vitrification with 300 µl of the sperm/sucrose solution.

When comparing the kinetic parameters of the spermatozoa after cryopreservation and vitrification, no significant differences were observed in the in any of the measured parameters. Studies by Isachenko *et al.*[13] and Moskovtsev *et al.*[21] found significantly higher percentages of motile spermatozoa after cryoprotectant-free vitrification when compared to those yielded by conventional cryopreservation. Isachenko and co-workers also used FM as the CPA, but utilized the longer freezing method recommended by the manufacturer.[13] Originally the results from Moskovtsev and co-workers were reported after vitrifying smaller volumes, but comparable results were found when vitrifying sperm in 200 µl volumes.[21]

Spermatozoa preserved using the cryoprotectant-free vitrification technique had significantly higher $\Delta\psi m$, when compared to that of the conventional cryopreservation technique ($p<0.001$). Reductions in the $\Delta\psi m$ are often associated with a decrease in sperm motility.[118] However, results obtained from the current study do not necessarily explain this phenomenon, since no difference was observed in the sperm kinetic parameters between the two techniques. The decrease in the $\Delta\psi m$ could be considered as a feature of cell death where the identification of mitochondrial dysfunctions is an early marker of programmed cell death in somatic cells.[118,119] A reduction in the $\Delta\psi m$ defines an early stage of apoptosis preceding other manifestation processes such as DNA fragmentation and ROS production.[120,121] DNA strand breaks typically occur during the final stages of cell death. Results from the TUNEL

assay (APO-DIRECT™) confirmed this since significantly higher percentages of apoptotic spermatozoa were found after conventional cryopreservation ($p<0.001$). Conflicting results were reported by the Canadian group who found no significant difference between the DNA integrity of those spermatozoa vitrified or preserved by conventional methods.[21] Isachenko *et al.*[13] observed significantly higher rates of membrane integrity and percentages of spermatozoa with intact acrosomes after larger volume vitrification, compared to those using the conventional slow freezing method.

In conclusion, results from Study 1A indicated that cryopreserving spermatozoa with FM resulted in a post thaw harvest of quality spermatozoa, i.e. optimal sperm motility and viability. Comparing the two preservation protocols, no conclusion could be reached on which protocol yielded superior results. The freezing method from RBL is shorter, simpler and requires less equipment, and was therefore selected as the preferred method. Study 1B showed that vitrifying sperm, without permeable cryoprotectants, and at larger volumes is possible. The results indicated that the larger vitrification volumes (300 µl and 500 µl) yielded better spermatozoa in terms of motility and viability post thawing. No significant difference was observed with respect to the 300 µl and 500 µl vitrification volume groups. For practical reasons, 300 µl volumes will retain sufficient sperm for ART procedures and in addition, the intermediate volume ensures that more than one straw can be preserved and be available as a back-up. With a ±40% reduction in the total motility, vitrification is an alternative for the preservation of spermatozoa during ART. Results from Study 2 showed that the cryoprotectant-free vitrification method resulted in spermatozoa with significantly higher $\Delta\psi m$ and significantly lower apoptosis post thawing. Post thaw quality of spermatozoa found in this study is equivalent to results found in literature.

Intrauterine insemination (IUI) can be promoted as a first-line therapy in cases with at least 1 x 10^6/ml motile spermatozoa after washing for successful

fertilization. In circumstances with an inseminating motile count of lower than 1 x 10^6/ml, IUI can still be performed, provided the spermatozoa morphology score is ≥ 4%.[122] After thawing a single straw (300 µl) of spermatozoa preserved using conventional cryopreservation and cryoprotectant-free vitrification, the following results were obtained with respect to progressive and total motility (see table 9).

Table 9: Progressive and total motility post thawing

Parameter	Vitrification	Cryopreservation
	Mean (Standard deviation)	
Total motility (a+b)	3.04 x 10^6/ml (0.52)	3.67 x 10^6/ml (0.99)
Progressive motility (a)	1.71 x 10^6/ml (0.428)	1.92 x 10^6/ml (0.557)

From these results a conclusion can be reached that both these methods can be implemented for the storage of spermatozoa to be used in any future ART procedures. See figure 29 for a detailed summary of all the results obtained.

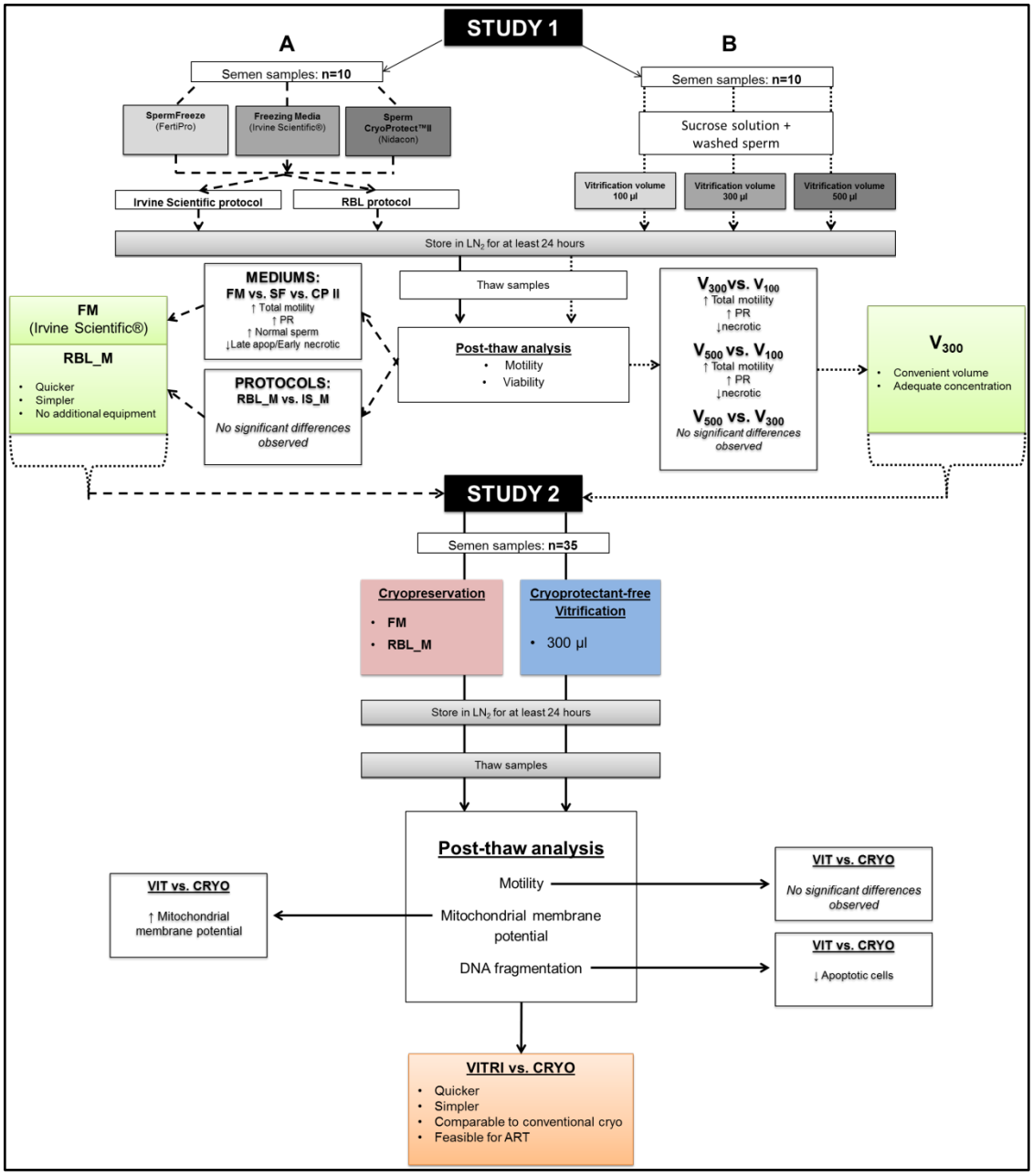


Figure 26: Summary of results for Studies 1 and 2

Vitrification of spermatozoa provides a simpler, faster, more cost-effective alternative to conventional cryopreservation methods. Initially, comparing the costs between cryoprotectant-free vitrification and conventional cryopreservation, the difference in cost for freezing 300 µl of sperm solution did not seem significant at all (R10.54 vs. R11.88 = 40% reduction)[9]. However, over time, a 40% reduction can significantly reduce the laboratory's freezing costs. Not only will this lead to a reduction in the cost of donor sperm, but it can contribute to the affordability and accessibility of ART treatments. Laboratories with limited resources and budget constraints can easily implement this technique. As cryoprotectant-free vitrification uses a sucrose solution, which can be prepared in the laboratory, no expensive cryopreservation media need to be purchased, there is no waiting period or transporting and delivering cost. The cost of the vitrification solution can easily be reduced even further, as a wide variety of less expensive HSA products is commercially available. In addition, this study illustrated how this technique can be simplified even further. Using sterile (ART approved) disposable items found in laboratories, a simple aspiration device can be created, therefore, there is no need to purchase an expensive aspirator. Furthermore, straws can be sealed using an alcohol flame burner and forceps, thereby, eliminating the need for electricity and the cost of heat-sealing devices.

Various suggestions can be made to enhance post thaw results:

- Supplementing cryopreservation media with antioxidants (vitamin E) has shown to significantly increase the post thaw motility, without affecting the viability or DNA fragmentation.[123]

- The induction of apoptosis can be reduced by optimizing the sperm preparation techniques used, the concentrations of cryoprotectants and the freezing/thawing protocols applied.[124,125]

[9] Quotes for preservation media obtained in August 2013

- Experiments should be conducted to determine the effects of different thawing rates and temperatures on the recovery rate of motile spermatozoa.

- Further investigation into the cryoprotective function of the sucrose solution at different concentration is suggested. A final sucrose solution of 0.25 M was used in this study. However, recently Zhu *et al.*[126] found that vitrifying spermatozoa in cryovials with a solution containing 0.20 M sucrose reduced cryoinjuries and maintained the important physiological functions.

- Investigating alternative cryopreservation storage and carrying devices such as cryovials can further reduce the cost for this technique, and might enhance post thaw quality.

- Determining the outcome of ART procedures using spermatozoa preserved using cryoprotectant-free vitrification.

Experimental results and theoretical analyses performed in this study prove the feasibility of implementing cryoprotectant-free vitrification into ART laboratories. In any clinical setting, it is of the upmost importance that any method used should be effective, reliable and safe. This cryoprotectant-free vitrification of human spermatozoa provides an attractive alternative to conventional freezing. Not only is a safe, contamination-free (no direct contact with LN_2) carrying device used, but this method requires no specialized or extra equipment. The outcome of comparing cryoprotectant-free vitrification with a conventional method has shown comparable results, if not superior to those obtained by conventional cryopreservation methods. Therefore, using vitrification for purified sperm samples could potentially result in a better post thaw quality for ART procedures. Whether cryopreservation is used for fertility preservation or as a back-up for ART procedures, the recovery rate of functional spermatozoa is imperative.

To conclude, this research project has highlighted and proved that alternative options for preservation of human spermatozoa do exist and would be feasible to implement in ART laboratories. Furthermore, new research opportunities were presented, and can subsequently result in a broader knowledge of cryopreservation for male gametes. Information derived from this study could possibly contribute to a better understanding of, or prompt new questions relating to the field of Cryobiology.

CHAPTER 6

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CHAPTER 7

ADDENDUMS

ADDENDUM A

DESCRIPTIVE STATISTICS FOR STUDY 1 AND 2

A.1 Study 1A: Comparison between cryopreservation protocols used by well-established (leading) infertility units in South Africa

The three mediums and two methods that were selected for this study are used by leading infertility units in South Africa. The medium and method that resulted in the highest quality spermatozoa was subsequently used as a control in Study 2.

Table 10: CASA parameters for spermatozoa after conventional cryopreservation using three different commercially available cryopreservation media

Parameter	Sperm Freeze	Sperm CryoProtect™	Freezing Medium	p-value (95% CI)		
	Mean (Standard deviation)			P^a	P^b	P^c
Rapid Progressive (%)	24.6% (8.86)	21.4% (8.77)	29.2% (7.48)	0.024 (0.609 ; 8.491)	0.0001 (3.859 ; 11.741)	0.106 (-7.191 ; 0.691)
Total Motility (%)	46.58% (12.54)	38.8% (14.19)	46.7 (11.47)	0.957 (-5.283 ; 5.584)	0.004 (2.466 ; 13.334)	0.005 (-13.184 ; -2.316)
VCL (µm/s)	36.8 (9.19)	31.4 (10.29)	37.5 (10.57)	0.719 (-3.878 ; 5.625)	0.012 (1.323 ; 10.827)	0.032 (-9.953 ; -0.450)
VSL (µm/s)	18.1 (4.86)	16.7 (5.39)	19.3 (4.34)	0.298 (-1.016 ; 3.311)	0.02 (-1.880 ; 2.446)	0.195 (-3.593 ; 0.733)
VAP (µm/s)	21.03 (5.87)	19.3 (6.30)	22.2 (5.18)	0.304 (-1.107 ; 3.553)	0.01 (0.575 ; 5.236)	0.157 (-4.013 ; 0.649)
WOB (µm/s)	0.6 (0.056)	0.6 (0.052)	0.5 (0.042)	0.304 (-1.107 ; 3.553)	0.01 (0.575 ; 5.236)	0.157 (-4.013 ; 0.649)
ALH (µm)	0.9 (0.19)	0.7 (0.20)	0.9 (0.25)	0.887 (-0.095 ; 0.111)	0.007 (0.039 ; 0.246)	0.04 (-0.238 ; -0.032)

P^a → Freezing Medium vs. Sperm Freeze

P^b → Freezing Medium vs. CryoProtect™

P^a → Sperm Freeze vs. CryoProtect™

Table 11: Summary of the viability parameters of human spermatozoa after conventional cryopreservation with three commercially available cryoprotectants

Parameter	Sperm Freeze	Sperm CryoProtect™	Freezing Medium	p-value (95% CI)		
	Mean (Standard deviation)			P^a	P^b	P^c
Normal (%) **(P^-A^-)**	49.7% (17.19)	44.4% (18.42)	63.9% (14.87)	0 (7.119 ; 21.309)	0 (12.417 ; 26.606)	0.143 (-12.392 ; 1.797)
Apoptotic (%) **(P^-A^+)**	2.2% (1.60)	2.8% (3.45)	1.7% (0.94)	0.47 (-1.731 ; 0.799)	0.0967 (-2.337 ; 0.193)	0.348 (0.659 ; 1.871)
Late Apoptotic/ Early Necrotic (%) **(P^+A^+)**	42.9% (15.27)	47% (17.60)	28.8 (14.45)	0 (-21.549 ; 6.741)	0 (-25.607 ; 10.798)	0.283 (-3.347 ;11.462)
Necrotic (%) **(P^+A^-)**	5.2% (4.8)	5.9% (5.41)	5.6% (9.11)	0.793 (-2.577 ; 3.375)	0.877 (-3.212 ; 2.741)	0.676 (-2.342 ; 3.611)

P^a → Freezing Medium vs. Sperm Freeze

P^b → Freezing Medium vs. CryoProtect™

P^a → Sperm Freeze vs. CryoProtect™

A.2 Study 1B: Establishing a cryoprotectant-free vitrification protocol for larger volumes of spermatozoa

Table 12: Motion and velocity parameters of human spermatozoa after vitrification utilizing three different volumes

Parameter	100 µl	300 µl	500 µl	p-value (95% CI)		
	Mean (Standard deviation)			P^a	P^b	P^c
Rapid Progressive	16.6 % (11.64)	20.6% (10.07)	22.1% (10.58)	0.03 (0.525 ; 10.475)	0.546 (-3.442 ; 6.509)	0.118 (-1.009 ; 8.942)
Total Motility	21.8% (14.08)	29.4% (9.6)	31.8% (10.3)	0.003 (3.537 ; 16.596)	0.471 (-4.130 ; 8.930)	0.021 (1.137 ; 14.197)
VCL	20.3 µm/s (10.07)	26.9 µm/s (10.16)	27 µm/s (8.57)	0.001 2.570 ; 10.705)	0.958 (-3.958 ; 4.177)	0.002 (2.461 ; 10.596)
VSL	11.5 µm/s (7.43)	14.9 µm/s (6.52)	15.3 µm/s (6.53)	0.772 (-2.471 ; 3.327)	0.01 (0.893 ; 6.691)	0.023 (0.466 ; 6.263)
VAP	12.7 µm/s (7.57)	16.7 µm/s (6.89)	17.3 µm/s (6.68)	0.002 (1.722 ; 7.404)	0.694 (-2.272 ; 3.411)	0.006 (1.152 ; 6.835)
WOB	0.4 (0.148)	0.5 (0.084)	0.5 (0.095)	0.009 (0.014 ; 0.099)	0.475 (-0.027 ; 0.059)	0.059 (-0.0016 ; 0.084)
ALH	0.5 µm (0.2)	0.6 µm (0.18)	0.6 µm (0.16)	0.002 (0.058 ; 0.253)	0.441 (-0.059 ; 0.136)	0.019 (0.019 ; 0.215)

P^a → 500 µL vs. 100 µL

P^b → 500 µL vs. 300 µL

P^a → 300 µL vs. 100 µL

VCL = curvilinear velocity, VSL = straight line velocity, VAP = average path velocity, WOB = wobble, ALH = amplitude of lateral head displacement

A.3 Study 2: Cryoprotectant–free vitrification vs. conventional cryopreservation methods for washed spermatozoa

Table 13: Comparison between percentages of Mitotracker® Red CMXRos fluorescence in spermatozoa after vitrification and cryopreservation

Parameter	Vitrification	Cryopreservation	p-value
	Mean (Standard deviation)		(95% CI)
CMXRos	11.988% (4.326)	6.581% (1.026)	<0.001 (-6.984; -3.830)

Table 14: Summary of the percentages of normal and DNA fragmented spermatozoa after cryopreservation utilizing two different methods

Parameter	Vitrification	Cryopreservation	p-value
	Mean (Standard deviation)		(95% CI)
NORMAL PI^+ $FITC\text{-}dUTP^-$	74.049% (3.839)	76.109% (5.924)	0.181 (-6.984; -3.830)
APOPTOTIC PI^+ $FITC\text{-}dUTP^+$	2.791% (1.017)	3.859% (1.381)	<0.001 (-1.588;8.428)

ADDENDUM B

STANDARD OPERATING PROCEDURES OF RBL

B.1 Semen decontamination procedures

RBL SOP: F1.15.1 (cover page)

Department	Department Obstetrics and Gynaecology Steve Biko Academic Hospital	Unit	Reproductive Biology Laboratory
SOP name	Decontamination procedures	SOP nr	F1.15.1
Category	Procedures	Author	J Roos
Approved by HOD		Approved date	21/11/2013

Standard Operating Procedure:

Decontamination procedures

Contents

Aim

The decontamination of an HIV$^+$ semen sample is essential for use in assisted reproduction. The complete removal of the HIV virus from an HIV$^+$ patients' semen sample, requires correct processing using the ProInsert™ together with PureSperm® density gradients. The process also removes debris and non-sperm cells such as white blood cells. The decontamination procedure requires basic semen analysis, viral validation in semen as well as sperm processing and cryopreservation. Viral validation results in the semen will determine whether the washed sperm will be sent away to confirm viral removal.

Materials

Product	Catalogue number	Product	Catalogue nr
Specimen jar	165A	Red cap conical test tubes	142-ASR-10
3ml Plastic Pasteur pipettes	BD Falcon 357575	CBS weighted ID rods	019021
Pipette tips	9401070, 94300110	High Security straw	010288
pH paper	9557	Yellow sterile filling nozzles	008656
Coverslips	GLAS2C29M2222SQ	Cryotubes	AE3772671100
Eppendorf tubes	P2TUB003C/000002	PureSperm®	PS100/PSW100
Tryphan blue	T8154	Spermfreeze	SPF
Hemacolour	1116610001	ProInsert™	NI-PI15-5
Supravital stain	VITAL	Entelan	1.07961.0500
Mixed anti-globulin reaction (MAR) test	SPM-G-S SpermMarIg	Immersion oil	1.04699.0500
Microscope slides		Goblets	006924
Canes		Ruler	
Liquid nitrogen		Gloves	
Wastebottle		Goggles	
Facemask			

B.2 Procedure for patient producing a semen sample

RBL SOP: F1.18.1 (cover page)

Department	Department Obstetrics and Gynaecology Steve Biko Academic Hospital	Unit	Reproductive Biology Laboratory
SOP name	Producing a semen sample	SOP nr	F1.18.1
Category	Procedures	Author	J Roos
Approved by HOD	C. Huyser	Approved date	12/01/2011

Standard Operating Procedure:

Guidelines for consulting embryologist: Procedure for patient producing a semen sample

Contents

Aim

The aim is to receive an optimum semen sample delivered according to World Health Organisation guidelines (WHO laboratory manual 5th edition, 2010). The consulting embryologist should use the following guidelines to explain the procedure of producing a semen sample to the patient.

Materials

- Liquid pink soap (Bio-scrub) (Dismed Pharma, Code number 1297)
- Sterile water (Intra-Pour, Reg.number 28/34/0137, Lot number 90DA039)
- Paper towel
- Specimen jar (Promex specimen jar, Cat. number 165A, Product code 162800, Lot number 259/04, Material master number 2606A)
- Polystyrene cup with lid
- Washing guidelines

Method

Self masturbation

- Discuss the guidelines on the production of a semen sample with the patient(s) using the washing guidelines as a reference
- Tell/Request/Ask the patient(s) to:
  - Urinate because it reduces the concentration of micro-organisms
  - Wash his hands and penis with the liquid pink soap (Bio-scrub) that is provided
  - Rinse/soap off his hands and penis with the bottled (sterile) water that is provided. Stress the importance of only using the sterile water and not tap water
  - Wipe his hands and penis dry with paper towels that are provided
  - Proceed to masturbate

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Department	Department Obstetrics and Gynaecology Steve Biko Academic Hospital	Unit	Reproductive Biology Laboratory
SOP name	Freezing and thawing semen samples	SOP nr	G3.2.1
Category	Cryopreservation	Author	M Stander
Approved by HOD		Approved date	13/11/2013

Standard Operating Procedure:

Freezing and thawing of semen samples

Contents

Aim

Semen samples are cryopreserved for later use during assisted reproductive procedures or for research purposes. Once cryopreserved, a semen sample can be stored indefinitely in Liquid Nitrogen (-196°C).

Materials

- Semen sample
- SpermFreeze™ (Fertipro; Harrilabs SPF) *(Fridge, Room 82296)*
- Freezing straws (IEPSA 10287) *(Cryopreservation cupboard, Room 82341)*
- Eppendorf tube
- PVC sealing powder (*Cryopreservation cupboard, Room 82341)*
- Clean container (e.g. Petri dish)
- Water
- Cryomarker (Nalgene Cryopen) (*Cryopreservation cupboard, Room 82341)*
- Syringe
- Pipette tip (50-200µl)
- Polystyrene container
- Liquid nitrogen
- Polystyrene floating device
- Scissors
- Red capped test tube (Greiner Bio-One, No. 164160)

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B.3 Freezing of semen samples

RBL SOP: G3.2.1 (cover page)

ADDENDUM C

REPORTING OF RESULTS

C.1 Draft article 1: Cryoprotectant-free vitrification: An alternative to conventional cryopreservation for human spermatozoa (first draft)

Submitted to Andrologia

LARGE VOLUME CRYOPROTECTANT-FREE VITRIFICATION: AN ALTERNATIVE TO CONVENTIONAL CRYOPRESERVATION FOR HUMAN SPERMATOZOA

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Keywords

Cryopreservation-DNA integrity-Human spermatozoa-Mitochondrial membrane potential- vitrification

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Summary

Vitrification is a simple and cost-effective method for the storage of human spermatozoa without the use of conventional cryoprotectants, by plunging the sperm suspension directly into liquid nitrogen. As a result, solidification of living cells without the formation of ice crystals is achieved during cooling. This study aimed to compare cryoprotectant-free vitrification to conventional cryopreservation protocols. Semen samples (n=35) were collected from patients seeking diagnostic assistance at the Reproductive and Endocrine Unit at Steve Biko Academic Hospital. Samples were processed using a discontinuous density gradient centrifugation method. Washed samples were split into two aliquots and cryopreserved either by means of cryoprotectant-free vitrification (sucrose + 1% albumin) or conventional slow freezing (Test-Yolk buffer). Post thawing, the sperm motion parameters, mitochondrial membrane potential ($\Delta\psi m$), and DNA fragmentation were compared between the two groups. No significant differences were observed in the sperm motility parameters ($p>0.05$). Significantly higher percentages of $\Delta\psi m$ (11.99%±4.326% vs. 6.58%±1.026%; $p<0.001$) and lower percentages of DNA fragmentation (2.79%±1.017% vs. 3.86%±1.38%; $p<0.01$) were observed when comparing cryoprotectant-free vitrification to conventional cryopreservation. Cryoprotectant-free vitrification appears to be a rapid and effective alternative to conventional methods resulting in good quality spermatozoa post thaw.

Introduction

Cryobiology is a fast evolving field, with promising applications in reproductive biology (Isachenko et al., 2012a). Temperatures below sub-zero together with appropriate cryoprotective agents (CPA) preserve the physiological and reproductive functions of cells, making long-term storage without the associated loss of viability possible. This cryopreservation method preserves the cells by plunging the suspension directly into liquid nitrogen. As a result, solidification of living cells without the formation of ice crystals is achieved during cooling (Isachenko *et al.,* 2004(a); Vutyavanich *et al.,* 2010). The described method for vitrification of oocytes, embryos and other tissues requires rapid cooling rates and high CPA concentrations (Isachenko *et al.,* 2005). Spermatozoa however, have low tolerance levels for high concentrations of cryoprotectants. These agents may cause possible lethal osmotic effects and chemical alterations in sperm (Isachenko *et al*., 2003). Vitrification of human spermatozoa is therefore not generally applied, and further investigations are needed in this area (Isachenko *et al.,* 2011).

Vitrification without the use of conventional CPA has been previously described for human spermatozoa (Isachenko *et al.,* 2003; Isachenko *et al.,* 2004(a); Isachenko *et al.,* 2004(b)). This method requires replacing the CPA with a protein and carbohydrate solution (1% human serum albumin (HSA) and 0.25 M sucrose). A major disadvantage of cryoprotectant-free vitrification is that only small volumes of spermatozoa (≤ 20 µl) can be vitrified. In addition, most of the proposed methods were described as open systems and do not prevent direct contact with liquid nitrogen (Isachenko *et al.,* 2003; Isachenko *et al.,* 2004(a); Isachenko *et al*., 2005). Different aseptic vitrification techniques were however investigated by Isachenko *et al.,* (2005), but only small volumes, ranging between 1 µl to 40 µl, of sperm suspension could be vitrified in these systems. In 2011, the Isachenko group reported a novel aseptic cryoprotectant-free vitrification method allowing the vitrification of larger volumes (up to 500 µl) of spermatozoa (Isachenko et al., 2012a).

This study aimed to compare the effects of conventional slow freezing vs. cryoprotectant-free vitrification with regards to motility parameters, mitochondrial membrane potential ($\Delta\psi m$), and DNA integrity of washed human spermatozoa.

Materials and methods

Institutional approval for this study was received from Steve Biko Academic Hospital (SBAH), and the Research Ethics Committee of the University of Pretoria (protocol number S48/2012). Informed consent was received from all participants.

Semen preparation

Semen samples (n=35) from healthy patients seeking diagnostic assistance from the Reproductive Biology and Endocrine Unit, SBAH were used. The inclusion criteria were a minimum concentration of 15 x 10^6 sperm/ml, total sperm motility of at least 40% and a minimum semen volume of 1.5 ml. The average values of the sample population included in the study were as follows: volume 2.98 ml (±1.071), concentration 41.87 x 10^6 sperm/ml (±17.81), total motility 74.47% (±9.02) and morphology 9.77% (±2.46). Semen analyses were performed according to the World Health Organization guidelines for the examination and processing of human semen (WHO, 2010). Each ejaculate was processed by means of a discontinuous gradient centrifugation method, according to the manufacturer's guidelines (Nidacon, 2010; Nidacon™ International, Mölndal, Sweden). After the final wash step, the supernatant was discarded; the pellet was resuspended, and split into two aliquots for: (i) conventional slow freezing, and (ii) cryoprotectant-free vitrification.

Conventional slow freezing

Freezing Medium-TYB (Test yolk buffer; Irvine Scientific®, Santa Ana, CA) was added to the washed spermatozoa in a 1:1 ratio. The sperm suspension was aspirated into 0.5 ml CBS™ straws (Cryo Bio System, Paris) and both ends

were sealed twice hermetically using an alcohol flame burner and forceps. The loaded straws were then kept at room temperature for 10 min. The manufacturer's protocol was adapted by excluding the refrigeration period. Subsequent to the room temperature incubation, straws were placed horizontally in the vapour phase for 15 min and submerged into liquid nitrogen. Straws were stored in a liquid nitrogen tank (Thermo Fisher Scientific, Johannesburg, South Africa) for at least 24 hrs.

For thawing, cryopreserved straws were immersed in water (23˚C) for 5 min (according to the manufacturer's instruction). Hereafter, the straws were sterilized using 70% alcohol, dried and the ends were cut open. The contents were expelled into a centrifuge tube (BD Pharmingen™, Franklin Lakes, NJ) containing 2 ml PureSperm® Wash (Nidacon™ International, Mölndal, Sweden) and centrifuged for 5 min at 300 g. The supernatant was removed; the pellet resupended and post sperm counts were performed.

Cryoprotectant-free vitrification for larger volumes

Results from a pilot study found no significant differences in the total motility [a+b: 29.4%±9.6 vs. 31.8%±10.3, p=0.546], rapid progressive motility [a: 20.6%±10.07 vs. 22.1%±10.58, p=0.546] or viability [23.7%±16.31 vs. 25.9%±19.18, p=0.695] of spermatozoa after vitrification in 300 µl and 500 µl volumes. For practical implications and general convenience, spermatozoa were vitrified in 300 µl volumes for the purpose of the current study. The vitrification solution was prepared by dissolving HSA in double distilled water (1%) (Adcock Ingram, Johannesburg, South Africa), with the addition of sucrose powder (342.3g; Sigma Aldrich, St Louis, MO) to reach a 0.5 M sucrose concentration. Immediately after processing, the sperm suspension was diluted in a 1:1 ratio with the vitrification solution to reach a final sucrose concentration of 0.25 M. Straws (0.5 ml, CBS™) were marked with an asterisk, 1.5 cm from the inner end of the cotton polyvinyl plug. This allows for a large enough air space to form inside the straw to prevent rupturing when immersed into liquid nitrogen. The vitrification and sperm solution (300 µl) were aspirated into the

straws. Both ends of the straws were sealed twice hermetically using an alcohol flame burner and forceps. Straws were then left at room temperature for 10 min and subsequently submerged horizontally directly into the liquid nitrogen (Isachenko *et al*., 2012a) and stored similarly to the conventional cryopreserved straws.

To thaw, vitrified straws were immersed into a water bath (42˚C) for 20 sec according to the protocol suggested by Isachenko *et al.,* 2012a. Thereafter, straws were processed as previously described for the conventional cryopreserved samples.

Sperm motility parameters

Motility parameters of washed spermatozoa were analysed both before freezing and post-thawing. The sperm motility parameters were analysed by computer aided sperm analysis (CASA) using a MTG-GmbH analyser (MTG-MedeaLAB, Bruckberg, Germany, Version 5.4) at 200 times magnification (Axioscope 40; Zeiss, SA). For each analysis, 5 µl of the sperm solution was loaded into Leja™ micro-chambers (20µm; Leja®, Nieuw-Vennep, Netherlands). For quality-control purposes, two separate chambers were loaded with the same spermatozoa solution, and video recordings of at least 200 cells/or 10 random fields per chamber were carried out for 30 sec.

Mitochondrial membrane potential (△ψm)

The △ψm analysis was determined by a technique adapted from Marchetti *et al.* (2002). MitoTracker® Red CMXRos (Invitrogen™, Eugene, USA) was used to analyse the △ψm. A stock solution of MitoTracker® Red CMXRos (1 mM) was added to the thawed spermatozoa at a final concentration of 150nM/10^6 and incubated (37˚C) for 15 min. Hereafter, spermatozoa were washed with 2 ml PureSperm® Wash (37˚C), the supernatant removed and the sperm re-suspended in 1 ml of this medium before flow cytometry analysis (Beckman

Coulter, Brea, CA). MitoTracker® Red CMXRos fluorescence was detected in FL3.

The $\Delta\psi m$ was abolished using m-Chlorophenylhydrazone (mCICCP; Sigma Aldrich, St Louis, MO) as a positive control. Spermatozoa were incubated with 50 µmol/l mCICCP for 15 min, before continuing with the MitoTracker® Red CMXRos staining. For a negative control, aliquots of unstained washed spermatozoa were analysed.

Detection of DNA fragmentation by the TUNEL assay

DNA fragmentation was analysed, using the APO-DIRECT™ kit (BD Pharmingen™, Franklin Lakes, USA) consisting of a single step method for labelling DNA breaks with FITC-dUTP (Flourescein isothiocyanate deoxyuridine Triphosphate), followed by flow cytometry analysis (APO-DIRECT™, 2012).

Thawed spermatozoa were fixed according to manufacturer's instruction and frozen at -20°C until analysis. For fixation, thawed spermatozoa were suspended in 1% (w/v) paraformaldehyde (Sigma Aldrich, Johannesburg, South Africa) in phosphate buffered saline (PBS, Sigma Aldrich, Johannesburg, South Africa) at a concentration of 2 x 10^6 spermatozoa/ml. The sperm suspension was incubated on ice for 30 min, whereafter spermatozoa were centrifuged and the supernatant discarded. The pellet was resuspended in the residual PBS by gentle vortexing. The sperm solution was adjusted to 1 x 10^6 spermatozoa/ml in 70% ice-cold alcohol.

Frozen fixed samples were thawed at room temperature and then resuspended by gently swirling the tubes where after it was centrifuged for 10 min at 500 g. The supernatant was aspirated and the pellet resuspended in 1 ml of the supplied wash buffer. Spermatozoa were washed twice by centrifuging (10min at 500 x g) and the pellet was re-suspended in 50µl of the staining solution (10 µl reaction buffer, 0.75 µl TdT Enzyme, 8µL FITC-dUTP and 32.25 µl distilled H_20/assay). Samples suspended in the staining solution were incubated for 60

min at 37˚C, where after the solution was washed twice in 1 ml of the supplied rinse buffer (10 min at 500 g). The supernatant was aspirated, and the pellet resuspended in 0.5 ml of the propidium iodide (PI)/RNase staining buffer. Thereafter, the cell suspension was incubated for 30 min at room temperature in the dark, after which DNA fragmentation was assessed using flow cytometry (Beckman Coulter, Brea, USA). FITC-labelled dUTP-positive spermatozoa were detected in the FL 1 channel. APO-DIRECT™ kits include positive and negative control cells which were stained according to the manufacturer's protocol. FITC-labelled dUTP-positive cells were measured in the FL 1 channel and PI in the FL 3 channel of the flow cytometer.

Statistical analysis

Data for the motility parameters, $\Delta\psi m$ and the DNA fragmentation are reported using descriptive statistics, mean and standard deviation. This study design employed random effects generalized least squares regression (GLS) i.e. a mixed model approach. Testing was done at the 0.05 level of significance using Stata Release 11 statistical software (StataCorp, 2009).

Results

No statistically significant differences were observed in the total motility, rapid progressive motility or velocity parameters of spermatozoa ($p>0.05$) post-thawing (see table 1). Significantly higher $\Delta\psi m$ (11.988% ± 4.326 vs. 6.581% ± 1.026, $p<0.001$) and lower percentages of DNA fragmentation (2.791% ± 1.017 vs. 3.859% ± 1.381, $p<0.01$) were found in spermatozoa cryopreserved by means of cryoprotectant-free vitrification compared to conventional cryopreservation (see figures 1 and 2).

Discussion

Cryopreservation is used as a method of storing different cell types and tissues, including male and female gametes as well as embryos. As in any emerging

technology, this method shows great potential, but also the need for further investigations and developments (Isachenko *et al*, 2012). An advantage of vitrification is that no cryoprotectants are needed, thus avoiding the lethal effects of cryoprotectant toxicity and osmotic damage specifically to spermatozoa, and is an alternative to conventional cryopreservation of spermatozoa (Isachenko *et al.,* 2004). Cryopreservation 'success' is measured in terms of post thaw motility. Until now, cryopreservation has not provided complete protection, since the motility of the preserved sperm decreases to more or less 50% of their pre-freezing value. An even greater decrease is observed in spermatozoa from infertile patients compared to fertile patients (Donnelly et al., 2001). Therefore, the semen parameters of the samples included in the study were all above the lower reference limits recommended by the WHO (2010). This study explored the potential and feasibility of substituting conventional cryopreservation procedures, with cryoprotectant-free vitrification. The effect of cryopreservation on sperm morphology is well documented (Donnelly *et al.,* 2001; O'Connell *et al.,* 2002) and has shown to significantly decrease the percentage of normal sperm. Since the morphological effects are well known, this study focussed on comparing these two methods with respect to sperm motility, $\Delta\psi m$, and DNA integrity. When the motility and velocity parameters of the spermatozoa were compared after conventional cryopreservation and vitrification, no statistically significant differences were observed. The percentages of rapid progressively motile (a) and total motile (a+b) spermatozoa after cryopreservation were 12.54% and 23.9% ($p>0.05$) respectively, compared to 11.52% and 20.48% ($p>0.05$) for vitrified spermatozoa. Studies by Isachenko *et al.* (2011) and Moskovtsev *et al.* (2012) reported significantly higher percentages of motile spermatozoa after cryoprotectant-free vitrification when compared to conventional cryopreservation. Moskovtsev *et al.,* (2012) initially found these results after vitrifying smaller volumes of spermatozoa, however, comparable results were documented when vitrifying spermatozoa in 200µl volumes. The larger-volume technique reported by the Isachenko group was successful after the birth of a

healthy baby by means of intra-uterine insemination with vitrified spermatozoa (Sanchez *et al.*, 2011).

Spermatozoa preserved by means of the cryoprotectant-free vitrification technique had significantly ($p<0.001$) higher $\Delta\psi m$, when compared to the conventional cryopreservation technique. The $\Delta\psi m$ results obtained in this study are not concurrent with results reported by Isachenko *et al*., 2012b (63%) and Isachenko *et al.,* 2008 (65%). Reductions in the $\Delta\psi m$ are often associated with a decrease in sperm motility (Thornberry *&* Lazebnik, 1998). The percentage of motile spermatozoa recovered post thawing was significantly lower than the results reported by Isachenko et al., 2012b and Isachenko et al., 2008. Motility results obtained from this current study do not necessarily explain this phenomenon, since no difference was observed in the sperm motility and velocity parameters between the two techniques. The decrease in the $\Delta\psi m$ could possibly be considered a feature of cell death where the identification of mitochondrial dysfunctions is early markers of programmed cell death in somatic cells (Green & Reed, 1998; Thornberry & Lazebnik, 1998). A reduction in the $\Delta\psi m$ defines an early stage of apoptosis preceding other manifestations processes such as DNA fragmentation and reactive oxygen species production (Kroemer *et al.,* 1997; Marchetti *et al.,* 2002). DNA strand breaks typically occur during the final stages of cell death. Results from the TUNEL assay (APO-DIRECT™) also confirmed this theory, since significantly ($p<0.01$) higher percentages of DNA fragmented spermatozoa were found after conventional cryopreservation. Isachenko et al., 2004 (a) also reported that DNA integrity were unaffected by vitrification, however, no significant effect was noticed in the conventional cryopreservation group either. These conflicting results can be due to different cryopreservation mediums used or freezing protocols utilized.

Results from this study indicated that, after thawing only one straw (300 µl) of spermatozoa preserved either by means of conventional cryopreservation or cryoprotectant-free vitrification, 1.92×10^6 (±0.557) and 1.71×10^6 (±0.428)

rapid progressively motile (a) spermatozoa were recovered respectively. Intra-uterine insemination (IUI) can be promoted as a first-line therapy in cases with at least 1 x 10^6 motile spermatozoa after washing for successful fertilization. In circumstances with an inseminating motile count of less than 1 x 10^6, IUI can still be performed, provided the morphology score is ≥ 4% (Ombelet *et al.,* 1997). Therefore these methods are comparable, and either can be implemented for the storage of spermatozoa to be used in any future ART procedures. Vitrification of spermatozoa provides a simpler, faster, more cost-effective alternative to conventional cryopreservation methods. Laboratories with limited resources and budget constraints can easily implement this technique as no specialized or extra freezing equipment is needed. In addition, this technique does not require any expensive cryopreservation mediums and can easily be performed in any laboratory.

Acknowledgements

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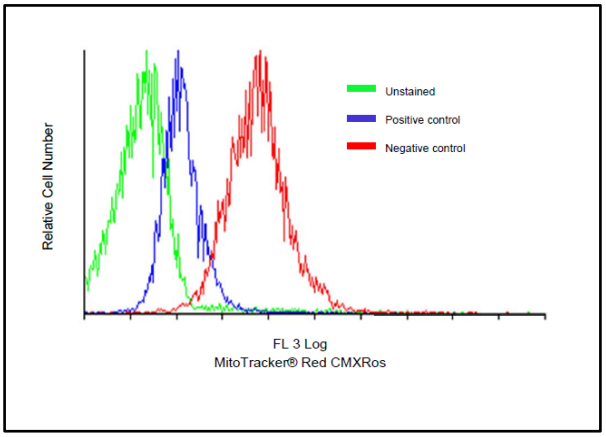
A



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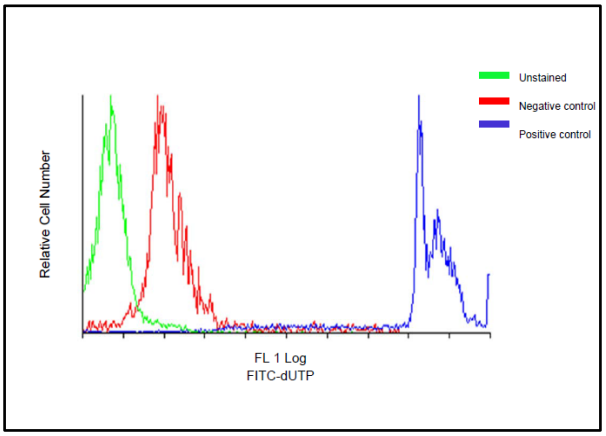

Figure 1: Controls used for the set-up and validation of the flow cytometry protocols:

A: Cytofluorometric analysis of the mitochondrial membrane potential depicting a frequency histogram of unstained cells, positive control cells (treated with mCICCP and stained with MitoTracker® Red CMXRos) and negative control cells (stained with MitoTracker® Red CMXRos)

B: APO-DIRECT™ experimental set-up: flow cytometric histograms showing overlay plots of processed unstained spermatozoa (green), positive (blue) and negative control cells (red)

Table 1: Summary of the motility and velocity parameters of spermatozoa post thawing

Parameter	Vitrification	Cryopreservation	p-value
	Mean (Standard deviation)		(95% CI)
Total Motility (%)	20.480% (7.399)	23.900% (12.583)	0.181 (-1.588; 8.428)
Rapid Progressive (%)	11.520% (6.099)	12.540% (7.160)	0.423 (-1.477; 3.517)
VCL (µm/s)	19.056 µm/s (6.469)	20.646 µm/s (7.854)	0.339 (-1.667; 4.848)
VSL (µm/s)	9.857 µm/s (3.698)	10.609 µm/s (4.516)	0.343 (-0.804; 2.309)
VAP (µm/s)	11.488 µm/s (4.075)	12.329 µm/s (5.027)	0.385 (-1.054; 2.735)
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ALH (µm)	0.503 µm (0.118)	0.548 µm (0.140)	0.146 (-0.016; 0.104)

VCL = curvilinear velocity, VSL = straight line velocity, VAP = average path velocity, WOB = wobble, ALH = amplitude of lateral head displacement

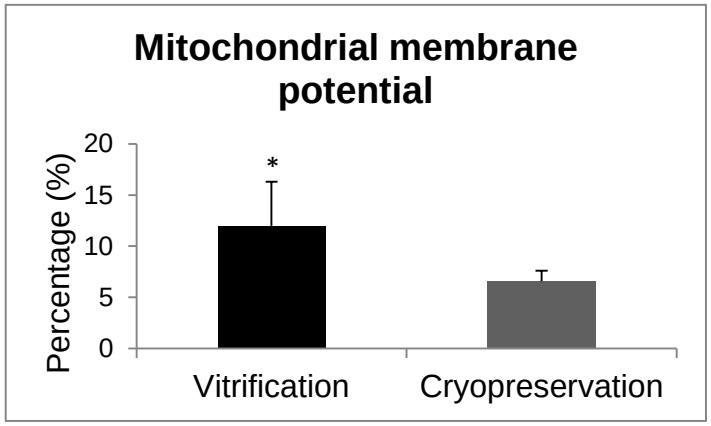


Figure 2: Percentage of Mitotracker Red CMXRos fluorescence after freezing of spermatozoa by means of cryoprotectant-free vitrification and conventional cryopreservation
(* = p<0.01)

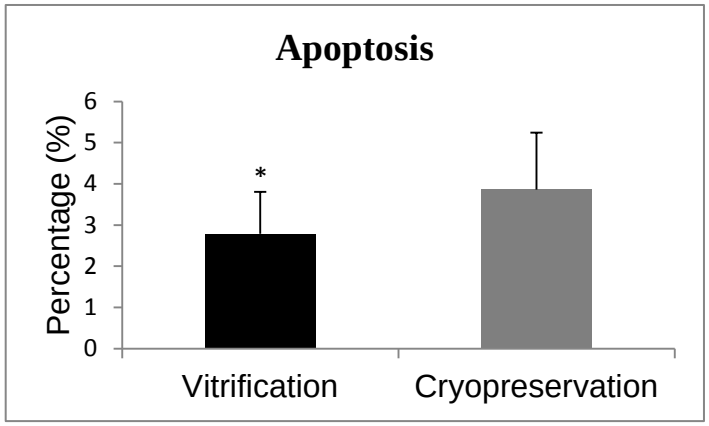


Figure 3: Bar graph representing the percentage of apoptotic spermatozoa measured by means of the TUNEL assay (*=p<0.01)

C.2 Oral presentation: The Annual Congress of the Division Biological Sciences: South African Academy of Science and Technology (October 2013)

Vitrifikasie van sperme: 'n alternatief vir konvensionele bevriesingsmetodes

Slabbert, M.[1] du Plessis, SS.[2] Huyser, C[1].

1) Departement Obstetrie en Ginekologie, Universiteit van Pretoria

2) Mediese Fisiologie, Departement Biomediese Wetenskappe, Stellenbosch Universiteit

1. INLEIDING

- Bevriesing van menslike spermatosoë word beskou as 'n roetine-praktyk in Geassisteerde Reproduktiewe Tegnologie (ART) laboratoriums
- Semen monsters word hoofsaaklik bevries om te berg vir ART prosedures, skenker sperme, en om gewasde monsters van menslike immuniteitsgebrekvirus (MIV)-positiewe pasiënte te behandel

INHOUDSOPGAWE

1. INLEIDING...Vervolg

- Semen monsters van MIV-patiënte lei oor die algemeen tot 'n lae opbrengs van gesuiwerde spermatosoë na semen prosessering, en verder word dan bevries word vir latere gebruik
- Vitrifikasie word tans nog nie beskou as algemene praktyk vir sperm bevriesing in ART laboratoriums nie
- In teenstelling met konvensionele bevriesingsmetodes gebruik vitrifikasie geen skadelike bevriesingsmiddele nie, wat 'n gevolglike vermindering van moontlikheid skade kan hê

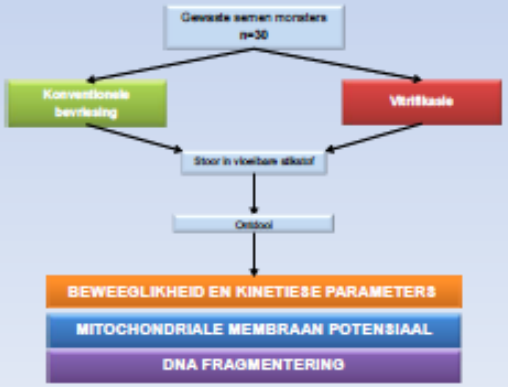
Isachenko E, et al. J Reprod Stem Biotechn. 2011; 2: 128-45; Isachenko E, et al. Hum Reprod. 2004;19(4):932-9

1. INLEIDING...Vervolg

- Gedurende die vitrifikasie proses word spermatosoa met bewarings middel behandel wat uit **sukrose, gedistilleerde water, en 1% HSA** bestaan en dan **direk** in vloeibare stikstof geplaas
- Die **vinnige** afkoeling tempo **limineer** die vorming van **glas kristalle** gedurende die vries proses

Vutyavanich T, *et al. Fertil Steril* 2010; 93(5) :1921-8;Isachenko V, *et al. RBM online.* 2005; 10(3) :350-4; Isachenko E, *et al. RBM online.* 2003; 6(2) :191-200; Isachenko V, *et al. Biol Reprod.* 2004; 71(4): 1167-73

3. METODES...vloeidiagram

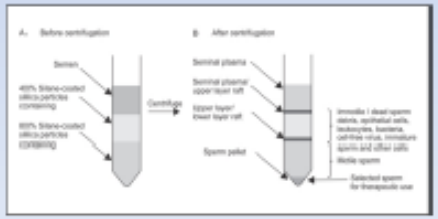


2. DOEL

Vitrifikasie met die huidige **konvensionele bevriesingsmetodes** van spermatosoë te vergelyk en te bevestig die voordeel en moontlike gebruik in ART laboratoriums

3. METODES...Prosessering

- Semen monsters **(n=30)** van MIV-negatiewe pasiënte is in die studie gebruik
- Alle monsters is geprosesseer volgens die eenheid se **standaard protokol**

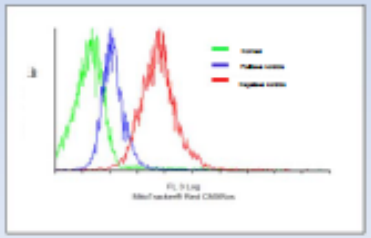


3. METODES...Bevriesings metode

3. METODES...Analises

MITOCHONDRIALE MEMBRAAN POTENSIAAL

- MitoTracker® Red CMXRos
- Vloeisitometrie (Beckman Coulter)

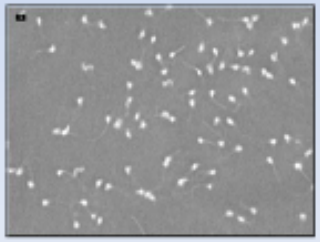


MitoTracker® red CMXRos [product insert].Eugene, USA: Invitrogen™; 2012

3. METODES...Analises

BEWEEGLIKHEID EN KINETIESE PARAMETERS

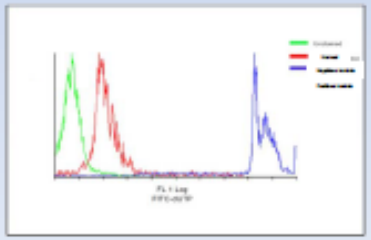
- MTG-GmbH CASA sisteem
- Leja™ mikroskoop plaatjies



3. METODES...Analises

DNA FRAGMENTERING

- APO-Direct™ Kit
- Vloeisitometrie (Beckman Coulter)



APO-DIRECT™ [product insert]. Franklin Lakes, USA: BD Pharmingen™; 2012

3. METODES

STATISTIESE ANALISES

- Om die veranderlikes van die spermatosoa parameters te analiseer, was beskrywende statistek (gemiddelde en standaard afwyking) gebruik
- Ontledings is uitgevoer deur die gebruik van GLS regressie, m.a.w. 'n gemengde model benadering
- Toetse is uitgevoer op die 0,06-peil van betekenis deur Stata Release 11 statistiese sagteware te gebruik

StataCorp LP. Statistical Software. 11th ed. USA: StataCorp Press; 2009

4. RESULTATE

MITOCHONDRIALE MEMBRAAN POTENSIAAL

Spermatosoë bevries deur middel van vitrifikasie het betekenisvolle hoër persentasies van ΔΨ in vergelyking met konvensionele bevriesing (11,99 % ± 4,326 % teen 6,58 % ± 1,026, p<0,001)

4. RESULTATE

BEWEEGLIKHEID EN KINETIESE PARAMETERS

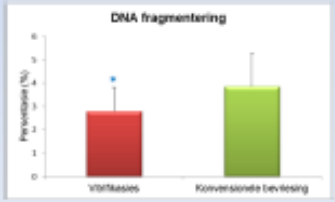
Geen betekenisvolle verskille is waargeneem nie (p>0.05)

Parameter	Vitrifikasie	Konventionele bevriesing	p-Waarde
	Gemiddelde (standaard afwyking)		(95% CI)
Vinnige Progressiewe Beweeglikheid (%)			
Totale Motiliteit (%)			
VCL (µm/s)			
VSL (µm/s)			
VAP (µm/s)			
WOB (µm/s)			
ALH (µm)			

4. RESULTATE

DNA FRAGMENTERING

Betekenisvolle laer persentasies van DNA fragmentering is in die vitrifikasie groep gevind (2,79 % ± 1,017 % teen 3,86 % ± 1,38 %, p<0.01)



5. BESPREKING

- Bevriesing metodes kan verskeie sperm parameters benadeel
- Die twee bevriesingsmetodes het vergelykbare resultate gehad met betrekking tot sperm beweeglikheid en kinetiese parameters
- Vitrifikasie het wel beter resultate opgelewer in terme van ΔΨ en DNA fragmentasie
- KOSTE: Deur gebruik te maak van vitrifikasie is daar n 40% afname in die totale bevriesings koste

DANKIE

5. BESPREKING

- Vitrifikasie is 'n maklike, vinnige en meer bekostigbare alternatiewe tegniek wat geen spesiale bevriesingstoerusting benodig nie
- Vitrifikasie vir gewaste sperm monsters kan lei tot selfs beter gehalte spermatosoë na ontdooiing vir ART prosedures

C.3 Poster presentation: Faculty of Health Sciences Faculty Day (August 2013)

SPERM VITRIFICATION:
An alternative to conventional cryopreservation

INTRODUCTION

- Cryopreservation of human sperm is considered a routine practice in assisted reproduction technology (ART) laboratories
- Semen samples are cryopreserved as back-up for ART procedures, donor programs, and to validate samples from HIV-positive patients
- Semen samples from HIV-seropositive patients generally result in a lower yield of purified spermatozoa after semen decontamination. These samples need to be cryopreserved for later use
- Vitrification is not yet common practice for sperm cryopreservation in ART laboratories.
- The study aimed:
  - i) To compared cryoprotectant free vitrification, described by Isachenko et al. (2012) to conventional cryopreservation methods
  - ii) Establish the feasibility of sperm vitrification, as opposed to current conventional cryopreservation for ART programs

MATERIALS AND METHODS

CRYOPROTECTANT-FREE VITRIFICATION

- Sucrose + 1% albumin (0.5M) was added to washed spermatozoa in 1:1 ratio
- CBS™ straws were filled with 300 µl of the solution and sealed on both ends using an alcohol flame burner
- Straws were submerged into the liquid nitrogen and stored
- To thaw the samples, straws were immersed into a water bath (42°C) for 20 sec

CONVENTIONAL SLOW FREEZING

- Freezing Media (Irvine Scientific®, Santa Ana, CA, USA) was added to the spermatozoa in a 1:1 ratio
- CBS™ straws were filled with 300 µl of the solution and sealed on both ends using an alcohol flame burner
- Straws were placed horizontally in the vapour phase for 15 min, submerged into liquid nitrogen and stored
- To thaw the samples, straws were placed in tap water (23°C) for 5 min

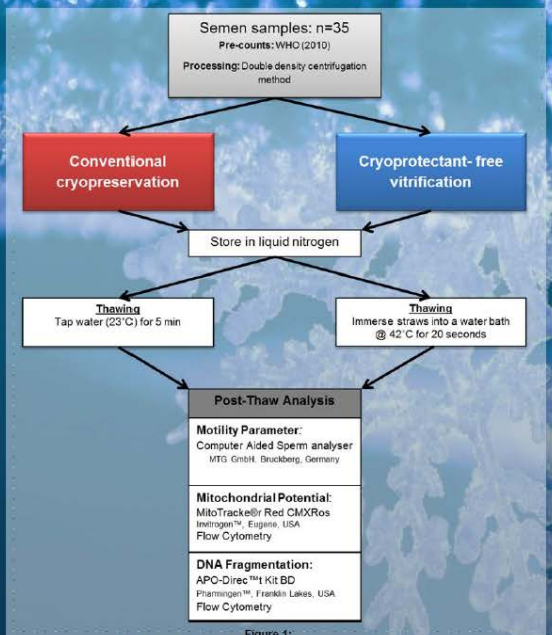


Figure 1:
Flow diagram illustrating the experimental set-up of the study

RESULTS

Table 1:
Summary of the motion and velocity parameters of spermatozoa post thawing

	Vitrification	Cryopreservation	
Parameter	Mean (Standard deviation)		P-Value (95% CI)
Rapid Progressive	11.520% (6.099)	12.540% (7.160)	0.423 (-1.477; 3.517)
Total Motility	20.480% (7.399)	23.900% (12.583)	0.181 (-1.588; 8.428)
VCL	19.056 µm/s (6.469)	20.646 µm/s (7.854)	0.339 (-1.667; 4.848)
VSL	9.857 µm/s (3.698)	10.609 µm/s (4.516)	0.343 (-0.804; 2.309)
VAP	11.488 µm/s (4.075)	12.329 µm/s (5.027)	0.385 (-1.054; 2.735)
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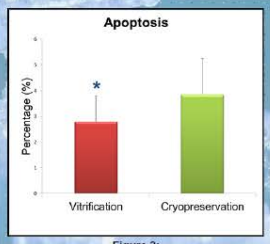

Figure 2:
Percentage of apoptotic (DNA fragmented) spermatozoa after comparing cryoprotectant-free vitrification with conventional cryopreservation ($P < 0.001$)

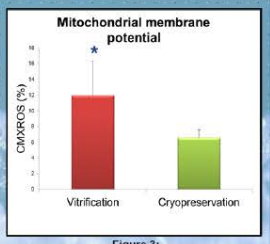

Figure 3:
Percentage mitochondrial membrane potential after freezing spermatozoa by means of cryoprotectant-free vitrification and conventional cryopreservation ($P < 0.001$)

DISCUSSION

- Conventional cryopreservation methods may compromise various sperm parameters and final yield
- In this study, cryopreservation and vitrification had equivalent outcomes with respect to sperm motility
- Vitrification yielded superior results in terms of mitochondrial membrane potential and DNA sperm fragmentation
- In conclusion, vitrification is easy to perform, less time consuming and an affordable method that does not require any special equipment
- The use of vitrification for purified sperm samples of HIV-positive patients could result in a better sperm quality for ART applicable ART procedures

ACKNOWLEDGEMENTS

- Prof P. Becker, Biostatistics Unit Medical Research Counsel (MRC) – statistics
- Reproductive & Endocrine Unit, Department Obstetrics & Gynaecology, University of Pretoria – research assistance
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