SEMEN DECONTAMINATION FOR THE ELIMINATION

OF SEMINAL PATHOGENS

by

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DEDICATION

This thesis is dedicated to:

Jesus Christ who has given me the strength to complete this journey

and

my wife, Vinita, for her continued support

DECLARATION BY CANDIDATE

"I hereby declare that the thesis submitted for the degree Philosophiae Doctor, at the University of Pretoria, is my own original work and has 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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STRUCTURE OF THE THESIS

SECTION A: LITERATURE REVIEW

Chapter 1 provides an overview of seminal constituents including seminal plasma, white blood cells, reactive oxygen species, bacteria, viruses and the potential negative impact thereof on the outcome of assisted reproductive treatment. **Chapter 2** is a presentation of semen processing procedures, and in **Chapter 3** assisted reproductive treatment for patients with potential seminal pathogens are addressed.

SECTION B: SEMEN PROCESSING WITH SUPPLEMENTS AND THE IMPACT THEREOF ON SPERM PARAMETERS

The addition of trypsin to upper density layers could improve the effectiveness of semen decontamination. The effect of semen processing with trypsin and trypsin inhibitor on sperm parameters was evaluated and results are described in **Chapters 4 and 5**, respectively.

SECTION C: EFFECTIVENESS OF SEMEN PROCESSING FOR THE ELIMINATION OF PATHOGENS

Semen processing by discontinuous density gradient centrifugation in combination with a centrifuge tube insert was evaluated. The method's effectiveness for the elimination of *in vitro* derived bacteria and white blood cells as well as *in vivo* derived human immunodeficiency virus from semen was evaluated; the results are presented in **Chapters 6, 7 and 8**, respectively.

SECTION D: CONCLUSIONS

Conclusions from the study are summarized in **Chapter 9**. Recommendations for the improvement of risk reduction procedures to be followed during assisted reproductive treatment for patients with seminal pathogens are given.

SECTION E: ANNEXURES

Annexure A consists of diagrammatic layouts of experiments performed during this research. **Annexure B** is a summary of the sperm parameters of participating donors. Published articles and presented posters originating from this work are given in **Annexure C**.

ETHICS CLEARANCE CERTIFICATE

Faculty of Health Sciences Research Ethics Committee

DATE: 8/10/2012

TO:

Mr. Jozef Fourie
Obstetrics and Gynaecology
Steve Biko Academic Hospital

Best Mr. Jozef Fourie

RE.: Protocol 37/2008 ~ Re-approval until December 2013.

PROTOCOL NO.	**37/2008**
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INVESTIGATOR	**Principal Investigator:** Mr. Jozef Fourie **Tel:** 012 354 2208/2067 **Fax:** 012 329 6258 **E-mail :** Jozef Fourie [jozef.fourie@up.ac.za]
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Herewith acknowledgement that the above '**Protocol 37/2008 ~ Re-approval until December 2013.'** document has been received by the Deputy Chair of the Faculty of Health Sciences Research Ethics.

It will be filed.

With regards

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STUDY OBJECTIVES

Effective semen decontamination prior to therapeutic assisted reproductive treatment is critical, specifically in countries such as South Africa with a high prevalence of seminal pathogens. The objectives of this study were therefore to determine the:

i) Impact of trypsin and trypsin inhibitor on sperm parameters obtained from HIV-1 sero-negative donors.

ii) Prevalence of various bacteria in the semen of males seeking assisted reproductive treatment at the Reproductive and Endocrine Unit at Steve Biko Academic Hospital.

iii) Effectiveness of discontinuous density gradient centrifugation in combination with a centrifuge tube insert for the removal of the following from semen:

   a. *in vitro* derived commonly found seminal bacteria,
   b. *in vitro* derived white blood cells and
   c. *in vivo* derived HIV-1.

SUMMARY

The presence of pathogens in semen can compromise the outcome of assisted reproductive treatment, together with the possibility of the female partner or offspring becoming infected. This is cause for concern, especially in South Africa with a high prevalence of HIV-1. Most of these infected individuals are in their reproductive years with the desire to have their own genetically related children. Therefore, assisted reproductive treatment with effective risk reduction procedures, such as semen processing for the elimination of these pathogens is crucial.

However, during sperm preparation by standard discontinuous density gradient centrifugation, the supernatant is aspirated to allow access to the purified sperm pellet. Pathogens from the upper layers can adhere to the inside surface of the test tube and flow down to re-infect the purified sperm sample. The use of a centrifuge tube insert may prevent the re-contamination of sperm samples after discontinuous density gradient centrifugation. Furthermore, seminal pathogens can bind specifically or non-specifically to spermatozoa, rendering semen decontamination procedures ineffective. Serine proteases, such as trypsin, have been demonstrated to effectively inactivate viruses and to break pathogen-sperm bonds. However, the addition of a protease to density gradient layers during semen processing could have a negative impact on sperm parameters. This research was therefore aimed towards the determination of:

i) The effect of semen processing with trypsin and trypsin inhibitor on sperm parameters.

ii) The prevalence of various bacteria in semen samples from men attending the Reproductive and Endocrine Unit at Steve Biko Academic Hospital.

iii) The effectiveness of semen processing by discontinuous density gradient centrifugation with a centrifuge tube insert, for the elimination of some of the most prevalent bacteria, white blood cells and *in vivo* derived HIV-1.

Evaluation of sperm parameters after semen processing indicated that trypsin and trypsin inhibitor did not have an impact on sperm mitochondrial membrane potential, vitality, motility and zona binding potential, or acrosin activity, respectively. Seminal bacteria were highly prevalent in patients wishing to participate in the Unit's assisted reproductive program, with 49.5% of semen samples presenting with positive bacterial cultures.

Semen processing by means of discontinuous density gradient centrifugation with the tube insert, eliminated significantly more *in vitro* derived (spiked) bacteria and white blood cells from semen compared to processing without the insert. Furthermore, the semen decontamination procedure was effective in removing HIV-1 RNA from 100% of samples and proviral DNA from 98.1% of semen samples from HIV-1 sero-positive patients.

The effectiveness of discontinuous density gradient centrifugation for the elimination of seminal pathogens could, therefore, be improved by the addition of trypsin to the upper density layer, without supplementing the bottom layer with trypsin inhibitor. Additionally, semen decontamination efficiency could also be improved by the prevention of re-contamination of processed sperm samples by the utilization of a tube insert during discontinuous density gradient centrifugation.

PUBLICATIONS

Published

1. Huyser, C., <u>Fourie, J</u>. 2010. SPERM ONLY PLEASE: Prevention of infections in an assisted reproduction laboratory in a developing country. *FVV in ObGyn*. Monograph. 97-106.

2. <u>Fourie, J</u>., Loskutoff, N., Huyser, C. 2012. Elimination of bacteria from human semen during sperm preparation using density gradient centrifugation with a novel tube insert. *Andrologia*. 44:513-517.

3. <u>Fourie, J</u>., Loskutoff, N., Huyser, C. 2012. Treatment of human sperm with serine protease during density gradient centrifugation. *J Assist Reprod Genet*. 29:1273-1279.

In preparation

1. <u>Fourie, J</u>., Loskutoff, N., Huyser, C. Improved removal of white blood cells from spiked human semen samples by density gradient centrifugation with the ProInsert™ as determined by flow cytometry. *J Assist Reprod Genet.*

2. Durandt, C., <u>Fourie, J</u>., Boyd, L., Huyser, C. Novel flow cytometric method for the determination of white blood cell contaminations in purified sperm samples. *Cytometry.* Collaboration with Pharmacology (University of Pretoria).

3. <u>Fourie, J</u>., Loskutoff, N., Huyser, C. Semen decontamination for HIV-1 sero-positive patients participating in a tertiary academic assisted reproduction program: A South African perspective. *J Assist Reprod Genet.*

Conference papers

1. Huyser, C., Loskutoff, N., <u>Fourie, J</u>. 2007. Developing and testing a semen decontamination procedure. Book of abstracts, p66. Presented at: International Federation of Fertility Societies (IFFS), XIX World Congress (April/May) Durban, RSA.

2. <u>Fourie, J</u>., Huyser, C., Loskutoff, N., Lindeque, K. 2008. Elimination of bacteria from spiked human semen samples. Book of abstracts, p37. Presented at: Medical Research Council's Research Day (16 and 17 October) Cape Town, RSA.

3. Huyser, C., <u>Fourie, J</u>. 2009. The implementation of ART procedures for HIV-positive patients in a South African context: A laboratory perspective. Presented

at: Third Alexandria Forum for Woman's Health and Development (18-19 March) Alexandria, Egypt.

4. <u>Fourie, J</u>., Loskutoff, N., Huyser, C. 2009. Evaluating a novel apparatus during density gradient centrifugation for the elimination of bacteria, HIV-1 RNA and proviral DNA from human semen. Book of abstracts, p280. Presented at: European Society for Human Reproduction and Embryology Campus (13-15 December) Genk, Belgium.

Poster presentations

1. <u>Fourie, J</u>., Loskutoff, N., Huyser, C. 2010. PureSperm: prevention of sperm pellet re-contamination by HIV-1 RNA, DNA and bacteria during density gradient centrifugation. Presented at Faculty Day: Faculty of Health Sciences, University of Pretoria.

2. <u>Fourie, J</u>., Loskutoff, N., Huyser, C. 2012. Treatment of human sperm with serine protease during density gradient centrifugation: Potential additive to reduce pathogenicity and to enhance ART outcome. Presented at Faculty Day: Faculty of Health Sciences, University of Pretoria.

LIST OF ABBREVIATIONS

$\Delta\psi_m$ -   Mitochondrial membrane potential

$\Delta\psi_m^{high}$ -   High mitochondrial membrane potential

ALH -   Amplitude of lateral head displacement

ART -   Assisted reproductive treatment

ATCC -   American type culture collection

ATP -   Adenosine triphosphate

AV -   Annexin V

b-DNA -   Branched DNA

CASA -   Computer aided semen analyses

CFU -   Colony forming units

DMSO -   Dimethyl sulfoxide

DNA -   Deoxyribonucleic acid

EBV -   Epstein-Barr virus

HBV -   Hepatitis B virus

HCV -   Hepatitis C virus

HDV -   Hepatitis D virus

HHV -   Human herpesvirus

HIV -   Human immunodeficiency virus

HPV -   Human papillomavirus

HSV -   Herpes simplex virus

HTLV -   Human T-lymphotrophic virus

HZA -   Hemizona assay

ICSI -	Intra-cytoplasmic sperm injection
IMC -	Insemination motile count
IUI -	Intra-uterine insemination
IVF -	*In vitro* fertilization
LIN -	Linearity
LLD -	Lowest limit of detection
MACS -	Magnetic-activated cell selection
mCLCCP -	Carbamoylcyanide m-chlorophenylhydrazone
NASBA -	Nucleic acid sequence based amplification
P -	Probability value
PBS -	Phosphate-buffered saline
PCR -	Polymerase chain reaction
PI -	Propidium iodide
PS -	Phosphatidylserine
r -	Recombinant
RBL -	Reproductive Biology Laboratory
RNA -	Ribonucleic acid
RT-PCR -	Reverse transcription polymerase chain reaction
SBAH -	Steve Biko Academic Hospital
SBTI -	Soybean trypsin inhibitor
SHV -	Semen hyperviscosity
STR -	Straightness
TPM -	Total progressive motility

VAP -   Average path velocity

VCL -   Curvilinear velocity

VSL -   Straight line velocity

WBCs -   White blood cells

WHO -   World Health Organization

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Sperm parameters determined for donors who participated in the study for the evaluation of:

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LITERATURE REVIEW

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SEMINAL CONSTITUENTS

CHAPTER 2

SEMEN PROCESSING PRIOR TO THERAPEUTIC ASSISTED REPRODUCTIVE TREATMENT

CHAPTER 3

ASSISTED REPRODUCTIVE TREATMENT FOR PATIENTS WITH SEMINAL PATHOGENS

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CHAPTER 1

SEMINAL CONSTITUENTS

1.1 INTRODUCTION

The initiation of life in an assisted reproductive laboratory carries with it an advanced responsibility to protect, and prevent any detriment to co-workers, patients and potential offspring during procedures. From an embryologist's viewpoint it could be said that the protection of potential human life should begin with the human gamete. The safeguarding of a semen sample could include: i) the proper handling of the sample depending on the specific application thereof and ii) the elimination of substances that could have a negative impact on spermatozoa, the embryo or the potential offspring. The aim of the present study was to validate current semen decontamination procedures in a human assisted reproduction program.

Semen consists of two fractions namely a fluid and a cellular fraction. The cell fraction comprises of mature spermatozoa, germ and non-sperm cells ("epithelial cells, CD4+ T lymphocytes, monocytes, polynuclear leukocytes and macrophages") (Bielanski, 2007). The liquid fraction, also known as seminal plasma, is a protein rich fluid (Southern, 2013) and includes various secretions from the accessory sex glands. All seminal constituents are potential vectors for the introduction of pathogens into the female reproductive tract during sexual intercourse, or intra-uterine insemination (IUI). These seminal pathogens may also be introduced into embryo culture systems whereby the outcome of assisted reproductive treatment (ART) may be compromised. Seminal constituents that are relevant to ART will be presented in this chapter.

1.2 SEMINAL CONSTITUENTS

1.2.1 Spermatozoa

Spermatozoa consist of a sperm head containing the paternal deoxyribonucleic acid (DNA), a midpiece containing mitochondria supplying energy for sperm movement, and a tail which provides motility (Hoogendijk, Kruger & Menkveld, 2007). The leading purpose of the haploid spermatozoon is to fertilize an oocyte where after a zygote is formed (Birkhead, 1999). Spermatozoa are produced from spermatogonia during spermatogenesis in the seminiferous tubules within the testes (Holstein, Schulze & Davidoff, 2003). The process consists of the following different stages:

i)   Spermatocytogenesis is a phase of an exponential increase in the number of spermatogonia by mitotic division. Followed by differentiation of the cloned spermoatogonia into primary spermatocytes (Johnson *et al*., 2000).

ii)   The primary spermatocytes then undergo two meiotic divisions yielding the haploid spermatids (Tanaka *et al*., 2003).

iii)   During spermiogenesis the round spermatids are transformed to testicular spermatids that are still attached to the Sertoli cells (Tachibana *et al*., 2005).

iv)   Excess cytoplasm is absorbed and the sperm are released from the Sertoli cells during the process of spermiation (Holstein, Schulze & Davidoff, 2003).

Spermatozoa are transported to the epidymis where the cells remain until ejaculation, after final maturation in the cauda epididymis (Hinrichsen & Blaquier, 1980). Various factors can impact on the quality of a sperm sample. The reference values for a normal sperm sample as described by the World Health Organization (WHO, 2010) are presented in Annexure B, page 223.

1.2.2 Seminal plasma

During ejaculation spermatozoa are suspended in seminal plasma consisting of secretions from the testes, epididymides and accessory sex glands, including the seminal vesicles, prostate and bulbourethral glands (Rossato *et al*., 2002). Apart from serving as a suspension medium, seminal plasma contains compounds that are required for sperm metabolism, motility and fertilization (Lay *et al*., 2001). In addition, seminal plasma also contains coagulation and liquefaction factors (Lay *et al*., 2001). Other compounds found in seminal plasma support sperm vitality, inhibit

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the uterine immune response against sperm, and prepare the endometrium for implantation of the embryo (Robertson, 2005).

Prior to fertilization sperm should undergo capacitation and hyperactivation where after binding to the zona pellucida and the acrosome reaction will follow (De Lamirande, Leclerc & Gagnon, 1997). Hyperactivation is however associated with poor progressive motility, capacitation should therefore not commence too long before the oocyte is reached. Decapacitation factors present in seminal plasma will prevent capacitation until sperm migrates out of seminal plasma and into the female reproductive tract (Begley & Quinn, 1982). However, according to Rogers *et al*. (1983), prolonged exposure of sperm to the decapacitation factors present in seminal plasma, could have a permanent negative impact on sperm fertilizing potential *in vitro*. The World Health Organization recommends that sperm should be isolated from seminal plasma within an hour after ejaculation (WHO, 2010). The inhibition of sperm capacitation by decapacitation factors present in seminal plasma could not be reversed by the processing of semen samples, or by incubation of the sperm in capacitating medium (Rogers *et al*., 1983). Levay *et al.* (1995) found that contaminating seminal plasma at a concentration of 50.0% in insemination medium decreased sperm-zona pellucida binding by 70.0%. Moreover, the presence of even low concentrations of seminal plasma in processed sperm samples may negatively impact, or even totally inhibit sperm fertilizing ability and should be prevented (Kanwar *et al*., 1979; Mortimer, 2000). Spermatozoa intended for use during ART must be processed and isolated from the seminal plasma as effectively as possible, directly after liquefaction (Mortimer & Mortimer, 1992; Mortimer, 2000).

1.2.3 White blood cells

White blood cells (WBCs), predominantly polymorphonuclear neutrophils, can be found in all sections of the male reproductive tract and is present in most human semen samples (El-Demiry *et al*., 1987; Englert *et al*., 2004; Yilmaz *et al*., 2005; WHO, 2010). Anderson *et al*. (1991) stated that the concentrations of WBCs in the semen samples from vasectomised patients are 85.0% to 90.0% less than the concentrations of WBCs found in semen samples from non-vasectomised patients. Keck and co-workers (1998) are of the opinion that the decreased WBC

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concentrations in the semen samples of vasectomised men, suggest that most seminal WBCs originate from the testes and the epididymides and not the accessory sex glands.

Seminal round cell counts consisting of WBCs, spermatids and epithelial cells (Johanisson *et al*., 2000) are determined during standard semen analyses. White blood cell validation requires the ability to distinguish between spermatids, lymphocytes, macrophages and the peroxidase-positive inflammatory polymorphonuclear neutrophils. Peroxidase positive cells change to a brownish colour after staining with ortho-toluidine, as described in the WHO (2010) guidelines, making these cells easily identifiable. The WHO classified seminal peroxidase-positive cell counts of more than 1×10^6 cells/ml as leukocytospermia. Leukocytospermia has been reported to be prevalent in 15.0% to 60.7% of semen samples from men attending infertility centres (Arata de Bellabarba *et al*., 2000; Lackner *et al*., 2006; Moretti *et al*., 2009).

Most researchers agree that leukocytospermia is correlated with male reproductive tract infections (Trum *et al*., 1998; Comhaire *et al.,* 1999; Arata de Bellabarba *et al*., 2000), but Barrat and co-workers (1990) were unable to demonstrate an association between the concentration of seminal peroxidase-positive cells and male genital tract infections. The authors recommended that seminal WBCs should be further differentiated by using monoclonal antibodies. Antibiotic treatment of patients presenting with leukocytospermia reduced bacterial counts, but not the number of seminal leukocytes (Krause *et al*., 2003). This, as well as the absence of raised leukocyte concentrations in the semen samples from men with known reproductive tract infections (Zinzendorf *et al.*, 2008), indicate that increased seminal leukocyte counts are not solely due to genital tract infections. A lower ejaculation frequency (Anderson & Politch, 1996) and other external environmental influences such as the consumption of alcohol and the smoking of cigarettes and marijuana, could also result in increased seminal leukocyte concentrations (Close, Roberts & Berger, 1990). Various factors could, therefore, contribute to the occurrence of leukocytes in semen samples and the presence thereof could impact negatively on sperm parameters and the outcome of ART.

Sperm progressive motility rates of patients with leukocytospermia can be significantly compromised (Yilmaz *et al*., 2005), whereby fertilization and pregnancy rates could be reduced (Donnely *et al*., 1998). Alvarez *et al*. (2002) reported a significant increase in DNA fragmentation of spermatozoa originating from leukocytospermic semen samples. Contaminating leukocytes have been reported to be a source of reactive oxygen species in processed sperm samples (Aitken, 1995; Fraczek *et al*., 2007), resulting in inhibited sperm function due to oxidative stress (Aitken *et al*., 1996). The effective removal of leukocytes from semen by processing reduced the effects of oxidative stress on sperm and resulted in increased rates of sperm-oocyte fusion (Aitken, 1995).

White blood cells are the predominant target cells of human immunodeficiency virus (HIV) and increases in the concentration of seminal WBCs in HIV-1 sero-positive patients correspond with increases in seminal HIV-1 load (Bujan *et al*., 2002). Englert *et al*. (2004) stated that the existence of leukocytes and macrophages in processed sperm samples from HIV-1 sero-positive male patients, could result in the transmission of the virus during ART. Therefore, sperm cells that are successfully isolated from seminal plasma and HIV-1-permissive leukocytes that are present in the semen samples from HIV-1 sero-positive patients, should be free of HIV-1 and safe to use during ART (Bujan *et al*., 2002; Nicopoullos *et al*., 2004). The effectiveness of discontinues density gradient centrifugation in combination with a centrifuge tube insert, for the elimination of *in vitro* added WBCs, was evaluated and the results are reported in Chapter 7.

1.2.4 Reactive Oxygen Species

"Reactive oxygen species, defined as including oxygen ions, free radicals and peroxides" (Tremellen, 2008), cause damage to sperm resulting in male infertility by oxidative stress (Hammadeh *et al*., 2006; Tremellen, 2008). Increased concentrations of reactive oxygen species in semen will result in peroxidation of the cell membrane phospholipids and ultimately over time, in cell death (De Lamirande & Gagnon 1995; Fraczek *et al*., 2007).

Fisher & Aitken (1997) reported that male germ cells in various stages of development can potentially produce reactive oxygen species. However, the production of reactive oxygen species is the highest by sperm cells that are immature, containing cytoplasmic droplets and presenting with abnormal head morphology (Gil-Guzman *et al*., 2001). Even though sperm cells are capable of generating reactive oxygen species, the contribution of sperm to seminal reactive oxygen species is low (Whittington & Ford, 1999). The principal source of reactive oxygen species in semen is leukocytes (Whittington & Ford, 1999; Henkel, 2005). Seminal bacteria, could also directly and indirectly through increased leukocyte concentrations, induce the production of reactive oxygen species (Fraczek *et al*., 2007).

When the level of seminal reactive oxygen species surpasses the seminal antioxidant activity, sperm and consequently male fertility, will be impacted negatively (Tremellen, 2008). Sperm motility is decreased within one hour of contact with reactive oxygen species by the depletion of intra-cellular adenosine triphosphate (De Lamirande & Gagnon, 1992). Reactive oxygen species are also known to induce DNA fragmentation and to decrease sperm fertilizing potential (Hammadeh *et al*., 2006). Sperm DNA fragmentation could have deleterious effects on the outcome of ART (Tesarik, Greco & Mendoza, 2004) and is also associated with early spontaneous abortion (Zini *et al*., 2008).

Antioxidant supplementation *in vivo* could have a therapeutic effect (Aitken, Jones & Robertson, 2012). Sperm cells are partially protected against the negative impact of reactive oxygen species, by the antioxidant factors present in human seminal plasma (Aitken, 1995; Adeel *et al*., 2012). However, spermatozoa are removed from the seminal plasma and isolated from the protective anti-oxidants during semen processing. It is therefore critical that the semen processing method employed for the removal of seminal plasma is effective in the removal of all reactive oxygen species and reactive oxygen species producing non-sperm and immature sperm cells.

1.2.5 Biomolecules

Various organic and inorganic compounds vital for several sperm functions are found in semen. The purpose of free amino acids present in seminal plasma is mainly

unidentified, however, a reduction in L-carnitine is correlated with poor sperm motility (Lenzi *et al*., 2004) and Tyrosine serves as a seminal antioxidant (Cervoni *et al*., 1992). Human seminal plasma proteins bind to sperm plasma membranes and play a role in sperm motility and sperm-oocyte binding (Vickram, Ramesh Pathy & Sridharan, 2012). Other proteins potentially found in seminal plasma after breaking of the sperm-testis barrier, are anti-sperm antibodies that could result in male infertility (Chamley & Clarke, 2007). The identification of thousands of proteins in seminal plasma by the utilization of proteomics could become a useful tool for the evaluation of male fertility in the future (Milardi *et al*., 2012).

Cholesterol is secreted by the prostate gland (Kravets *et al*., 2000) and is involved in the capacitation process, a prerequisite for successful fertilization (De Jonge, 2005), and also stabilizes sperm plasma membranes (Cross, 1998). Seminal trace elements include zinc and selenium acting as antioxidants (Sheweita, Tilmisany & Al-Sawaf, 2005; Atig *et al*., 2012). Zinc is a primary element involved during DNA transcription (Ebisch *et al*., 2007) and is correlated with sperm motility and concentration (Atig *et al*., 2012). Potassium and sodium are involved in the sperm acrosome reaction, a requirement for fertilization (Vickram, Ramesh Pathy & Sridharan, 2012).

1.2.6 Seminal bacteria

The prevalence of bacteriospermia amongst patients seeking ART ranges between 54.0% and 57.0% (Cottell *et al*., 2000; Gdoura *et al*., 2008; Kiessling *et al*., 2008). The occurrence of seminal bacteria could be due to urogenital tract infections, or due to contamination of ejaculated semen samples (Bielanski, 2007). During ART, the female reproductive tract's immunological defences are circumvented by the direct transfer of gametes or embryos into the uterus (Cottell *et al*., 1997; Krissi *et al*., 2004). Bacteria may therefore be inoculated into the uterus, or the *in vitro* culturing system, resulting in a potential negative impact on the outcome of ART due to poor quality embryos, or failed fertilization (Huyser *et al*., 1991; Cottell *et al*., 1997; Cottell *et al*., 2000; Kastrop *et al*., 2007).

Uterine infections post-IUI have been reported to be 0.01% (Broder, Sims & Rothman, 2007) and infections of embryo culture media, ranges between 0.4% and 6.7% (Cottell *et al*., 1996; Kastrop *et al*., 2007). Microbial infections of the culture system during intra-cytoplasmic sperm injection cycles appear to be limited, suggesting that most of the infections in embryo culture systems are due to bacteria being introduced into the culture system by contaminated sperm used for *in vitro* fertilization (IVF) (Kastrop *et al*., 2007). Seminal bacteria are inducers of oxidative stress by the generation of reactive oxygen species (Fraczek *et al.*, 2007) and producers of toxins during metabolic processes (Moretti *et al*., 2009). Incubation of bacteria with sperm *in vitro* may immobilize sperm cells by adhesion and/or agglutination, may cause apoptosis and can impair sperm-oocyte interaction (Keck *et al*., 1998; Villegas *et al*., 2005).

Patients with positive pathogenic micro-organism semen cultures should receive antibiotic treatment to clear the infection prior to ART. However, the treatment of patients with antibiotics will be unsuccessful in the removal of seminal skin contaminants. Huyser & co-workers (1991) reported that semen washing with an extra swim-up step, was superior in the elimination of seminal bacteria when compared to the treatment of patients with antibiotics. Effective semen processing procedures for the elimination of seminal bacteria prior to ART are therefore required, and are discussed in Chapter 3 and the effectiveness reported on in Chapter 6. Bacteria that are relevant to ART will be discussed in the following section, in the order of size and complexity as described by Elder & co-workers (2004).

1.2.6.1 Intra-cellular and non-culturable micro-organisms
The diagnosis of genital tract infections with bacteria from this group is problematic, because there is currently no *in vitro* method to isolate and identify these bacteria using standard microbiology culture techniques. Polymerase chain reaction analyses of semen samples even though costly is, therefore, the most viable method for detecting these bacteria in semen samples of men seeking ART (Hosseinzadeh, Eley & Pacey, 2004).

    (i) *Chlamydia trachomatis*, *Chlamydophila pneumoniae* and *Chlamydophila psittaci* are Gram-negative species from the group, *Chlamydiae*, that infects

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humans (Kuo *et al*., 1995). The subtypes L1-L3 and D-K of *C. trachomatis* are sexually transmitted and can cause sexually transmitted diseases (Keck *et al*., 1998; Elder, Baker & Ribes, 2004; Eley *et al*., 2005). The prevalence of the bacterium depends on the patient population and ranges between 4.9% in Europe (Hosseinzadeh *et al*., 2001) to 41.4% in North Africa (Gdoura *et al*., 2008). The main negative impact of infection with the bacterium is female infertility due to inflammation and blocking of the fallopian tubes (Eggert-Kruse *et al*., 1996; Keck *et al*., 1998). *Chlamydia trachomatis* causes sperm DNA damage and is accountable for 50.0% of non-gonococcal and most of gonococcal urethritis (Gallegos *et al*., 2008).

Chlamydia trachomatis can be found in seminal plasma as a metabolically inactive elementary body, or as an intra-cellular metabolically active replicating reticulate body (Hosseinzadeh *et al*., 2001). Elementary bodies attach to and will penetrate sperm whereby the bacterium will be transported into the female reproductive tract (Erbengi, 1993). Hosseinzadeh *et al*. (2001) suggested that sperm could be exposed to elementary bodies during ejaculation and sperm viability might only be affected during the hours after ejaculation due to the delayed formation of reticulate bodies. The negative impact of *C. trachomatis* on spermatozoa parameters may consequently be overlooked. Furthermore, due to the penetration of the bacterium into sperm, semen decontamination procedures will be ineffective for the removal of this pathogenic micro-organism prior to ART. Patients should thus be screened for the chlamydial infections and if needed treated prior to ART (Paavonen & Eggert-Kruse, 1999).

1.2.6.2 Cell wall deficient micro-organisms

Bacteria from the family *Mycoplasmataceae* (Kilic *et al.*, 2004) are cell-wall deficient and include the mycoplasmas and ureaplasmas that are naturally found in the male reproductive tract (Gdoura *et al*., 2007). These bacteria could, however, result in genitourinary tract infections (Gdoura *et al*., 2007). The most prevalent of the genital mycoplasmas are *M. hominis and U. urealyticum* which are found in 9.6% to 23.8% (Gdoura *et al*., 2008; Zinzendorf *et al.*, 2008) and 9.0% to 39.0% (Knox *et al*., 2003;

Zinzendorf *et al*., 2008; De Francesco *et al*., 2011) of semen samples from men attending infertility centres, respectively. *Ureaplasma parvum* and *M. genitalium* are less prevalent and is found in 3.0% and 5.0% of semen samples from men attending infertility units, respectively (Gdoura *et al*., 2008). *Mycoplasma hominis* and *U. urealyticum* will be presented in the following section:

(i) *Mycoplasma hominis* attaches to and penetrates sperm (Diaz-Garcia *et al*., 2006), resulting in the possible transport of the pathogen into the female reproductive tract, or through density layers during density gradient centrifugation. Colonization of the female reproductive tract with *M. homonis* is increased among sexually active women with multiple sexual partners, illustrating the sexual route of transmission (Gupta *et al*., 2009).

The bacterium has been demonstrated to impair the sperm acrosome reaction and to reduce sperm motility *in vitro*, possibly indirectly by bacterial metabolites, as well as directly by its attachment to spermatozoa (Kohn *et al*., 1998). The attachment of *M. hominis* to sperm has been reported to have a negative impact on sperm morphology by causing midpiece and tail alterations (Diaz-Garcia *et al*., 2006).

(ii) The variation in the prevalence of *U. urealyticum* could be explained by the diversity of the detection methods and due to the fastidious nature and complicated culture of the bacterium (Gdoura *et al*., 2007; Gdoura *et al*., 2008). Male patients with *U. urealyticum* infection in the reproductive tract could be asymptomatic carriers of the micro-organism (22.0%), or some of the most common symptoms as described by Zdrodowska-Stefanow and co-workers (2006) could be experienced: "dysuria (68.9%), hypogastric pain (42.2%) and reddening of the external meatus of the urethra and/or glans penis (26.7%)". *Ureaplasma urealyticum* attaches to and penetrates sperm where it replicates *in situ* (Nunez-Calonge *et al*., 1998) and where, due to the urealytic activity of the bacterium releases ammonium ions that is toxic to sperm (Abdulrazzak & Bakr, 2000).

In vitro incubation of sperm with *U. urealyticum* resulted in decreased sperm motility and damage to cell membranes (Nunez-Calonge *et al*., 1998). Colonization of the male reproductive tract with *U. urealyticum* has been reported to be correlated with reduced sperm concentration, increased seminal viscosity (Wang *et al*., 2006) and decreased sperm vitality (Rybar *et al*., 2012). Nevertheless, IVF rates do not seem to be influenced by *U. urealyticum* (Shalika *et al*., 1996; Kohn *et al*., 1998; Kanakas *et al*., 1999). However, reported higher abortion rates and reduced implantation rates in *U. urealyticum*-positive couples suggest that factors such as endometritis are also involved in the compromised fertility due to the bacterium (Shalika *et al*., 1996; Kanakas *et al*., 1999).

1.2.6.3 Gram-negative bacilli

Gram-negative bacilli that are relevant to ART include *Pseudomonas* spp., *Klebsiella pneumonia*, *Enterobacter cloacae* and *Escherichia coli*. *Escherichia coli* belonging to the family *Enterobacteriaceae* (Reddy *et al*., 2007; Tumbarello *et al*., 2007), is by far the most prevalent bacterium from this group, with an incidence of 7.0% to 20.0% in semen samples from men attending infertility units (Huyser *et al*., 1991; Sanocka-Maciejewska, Ciupinska & Kurpisz, 2005). *Escherichia coli* is known to colonize and infect the male reproductive system causing epididymo-orchitis and chronic prostatitis (Pellati *et al*., 2008). The bacterium attaches to sperm within minutes after contact, impacting negatively on sperm motility by causing sperm defects in the midpiece and tail (Diemer *et al*., 2000; Diemer *et al*., 2003). Furthermore, *E. coli* may inhibit the initiation of the acrosome reaction whereby sperm fertilizing potential and the outcome of ART could be compromised (Diemer *et al*., 2000; Diemer *et al*., 2003). The attachment of *E. coli* to sperm could result in the micro-organism being transported into the female reproductive tract resulting in cervical colonization and decreased pregnancy rates after ART (Fanchin *et al.*, 1998).

1.2.6.4 Gram-negative cocci

Neisseria gonorrhoea is a sexually transmitted pathogen belonging to the family *Neisseriaceae* (Fredlund *et al*., 2004). The bacterium predominantly colonizes the mucous membranes of the urogenital tract in humans (Rhoton-Vlasak, 2000).

Infection of male patients cause urethritis resulting in compromised male fertility (Pellati *et al*., 2008). The micro-organism attaches to sperm within fifteen minutes after contact in an *in vitro* culture environment (James-Holmquest *et al*., 1974). Immunoelectron microscopy indicated that after attachment the bacterium penetrates sperm where it replicates within vacuoles in the cytoplasm (Apicella *et al*., 1996). The bacterium could, therefore, be transported by sperm into the female reproductive tract (Gomez *et al*., 1979) and semen decontamination procedures would be unsuccessful to separate sperm from the pathogen. Infection in the female predominantly occurs at the endocervix (Rhoton-Vlasak, 2000; Pellati *et al*., 2008) and is mostly asymptomatic. Therefore, these patients may remain untreated for prolonged periods of time leading to complications such as gonorrhoeal disease (Rhoton-Vlasak, 2000). Early detection by selective screening of patient populations with a high probability of infection (Domes *et al*., 2012), followed by antibiotic treatment of infected patients are, therefore, critical (Tapsall, 2005).

1.2.6.5 Gram-positive cocci

The most prevalent seminal Gram-positive cocci among patients attending infertility centres are listed below and are from the *Staphylococcaceae* family (Dissemond, 2009):

(i) *Staphylococcus aureus* is found as part of the normal flora on the skin and in nasal passages, and is present in 7.0% (Sanocka-Maciejewska, Ciupinska & Kurpisz, 2005) to 8.0% (Shalika *et al*., 1996) of semen samples.

(ii) Coagulase-negative staphylococci include *Staphylococcus epidermidis*. This bacterium is found as part of the normal flora on the skin and it has been reported to be present in 15.0% (Cottell *et al*., 2000) to 49.0% of semen samples (Huyser *et al*., 1991).

(iii) *Enterococcus faecalis* is found as part of the normal flora in the gastrointestinal tract and has been reported to be present in 16.0% of semen samples from men attending an infertility unit (Huyser *et al*., 1991).

Most Gram-positive bacteria should be considered as normal flora in the male reproductive tract (Huyser *et al*., 1991; Purvis & Christiansen, 1993), that do not attach to sperm and processing of semen by discontinuous density gradient centrifugation should be effective to remove these bacteria from semen.

1.2.7 Seminal viruses

Semen is a major vector for the vertical and horizontal, or nosocomial transmission of sexually transmitted infections during intercourse and ART (Englert *et al*., 2004; Bezold *et al*., 2007). Healthcare workers assisting patients with ART should be aware of the prevalence of specific blood borne viruses in the geographical patient population that they assist. This will allow for a comprehensive and cost-effective screening program for the detection of a blood borne virus to be established. However, the identification of a male patient with a sexually transmitted viral infection should not result in the refusal of ART to the couple (Gilling-Smith *et al*., 2005). Depending on the specific virus that is identified, these patients should rather be referred for counselling, followed by the implementation of proven risk reduction procedures and ART (Huyser & Fourie, 2010). The effectiveness of semen decontamination procedures depend on the specific viral infection. Viruses most relevant to ART will be presented in the following section.

1.2.7.1 Double-stranded deoxyribonucleic acid (DNA) viruses

Double stranded DNA viruses include the family *Herpesviridae* (Chisholm & Lopez, 2011) [herpes simplex virus (HSV), Epstein-Barr virus (EBV), cytomegalovirus, human herpes viruses (HHV)], human papillomaviruses (HPV) and hepatitis B virus (HBV). Viruses belonging to this group cause lifelong latent infections that could result in reactivation of the viruses followed by disease (Decker *et al.,* 1996; Strickler *et al*., 2005; Sinclair & Sissons, 2006; Ikeda, 2013).

(i) Herpes simplex viruses (HSV) type 1 and 2 are transmissible by sexual contact and causes incurable, but treatable genital ulcer disease (Bezold *et al*., 2007; White *et al*. 2008). Herpes simplex virus type 1 was originally mostly associated with oral lesions and was predominantly contracted during childhood (Elder, Baker & Ribes, 2004). El Borai *et al*. (1998) stated that, due to an increase in the incidence of HSV-1, genital lesions caused by HSV type 1 and 2 are equally prevalent. The prevalence of seminal HSV among men attending infertility centres ranges between 3.7% to 49.5% as was determined in studies performed in North America (Bezold *et al*., 2007) and Greece (Kapranos *et al*., 2003), respectively. Infection of male patients with the virus has been reported to decrease sperm concentration and motility

(Kapranos *et al*., 2003; Bezold *et al*., 2007). Furthermore, HSV infection has been reported to increase the male to female transferral rate of HIV-1 (Butler *et al*., 2008), due to increased HIV-1 shedding in semen (Nagot *et al*., 2007). A meta-analysis by Wald and Link (2002) indicated that the risk of contracting HIV-1 is increased twofold among patients that are HSV-2 sero-positive. Kotronias and Kapranos (1998) demonstrated the attachment of HSV to sperm and Michou *et al*. (2012) reported the presence of HSV in 59.38% of processed sperm samples from men sero-positve for HSV.

(ii) Epstein-Barr virus (EBV) is primarily transferred by salivary contact during childhood and more than 90.0% of the global population become infected (Thompson & Kurzrock, 2004). Most individuals are lifelong asymptomatic carriers of the virus (Thompson & Kurzrock, 2004; Cohen, 2000), though primary infection during adolescence, or adulthood could result in infectious mononucleosis, also called glandular fever (Anagnostopoulos *et al*., 1995). Furthermore, EBV is associated with malignant lymphomas, namely Hodgkin's disease (Glaser *et al*., 1997), Burkitt's lymphoma and others (Thorley-Lawson & Gross, 2004). Epstein-Barr virus is present in 0.4% to 45.0% of semen samples from men attending infertility centres in North America and Greece, respectively, suggesting a sexual route of transfer (Bezold *et al*., 2007; Michou *et al*., 2012). Attachment of the virus to sperm has not been demonstrated. Nevertheless, the failure of semen processing procedures to remove the virus from 22.45% of semen samples (Michou *et al*., 2012), could be interpreted as a potential attachment of the virus to sperm.

(iii) The human herpesviruses (HHV) that are relevant to ART include HHV type 6, 7 and 8. Human herpesvirus type 6 and 7 are associated with neurological disorders (Ward *et al.,* 2005) and are present in 8.2% to 13.5% (Kaspersen, 2012; Michou *et al*., 2012) and 3.6% to 4.2% (Kaspersen, 2012; Michou *et al*., 2012) of semen samples, respectively. Kaspersen *et al*. (2012) demonstrated the attachment of HHV-6 to the sperm acrosome by confocal microscopy. The association of HHV-7 with sperm cells have not been reported. Human herpesvirus type 8 is less prevalent in semen than HHV-6 and 7 (Diamond *et*

al., 1997; Kaspersen, 2012) and is mostly found in oropharyngeal samples such as saliva (Pauk *et al*., 2000). The virus is associated with Kaposi's sarcoma (Glaser *et al*., 1997) and has been reported to penetrate sperm (Bagasra *et al*., 2005). The attachment to and the penetration of HHV into sperm cells will result in the failure of semen decontamination procedures to remove these viruses from sperm prior to ART.

(iv) Cytomegalovirus infections are generally asymptomatic and most adults (50.0% to 95.0%) test positive for cytomegalovirus antibodies (Bresson *et al*., 2003). Viral shedding into semen results in the virus being present in 8.7% to 43.0% (Bezold *et al*., 2007; Michou *et al*., 2012) of semen samples from men attending infertility centres in North America and Greece, respectively. Infection of the female partner during pregnancy, or activation of the virus from the latent state could be detrimental (Berry & Lal, 2012) by causing an array of clinical outcomes, from spontaneous or therapeutic abortions, intra-uterine death to multiple congenital abnormalities associated with cytomegalic inclusion disease, including blindness. Congenitally infected children can also present with deafness, dental or other developmental delays (Wong *et al*., 2000; Luck & Sharland, 2008). Pallier *et al*. (2002) performed electron microscopy after the *in vitro* incubation of cytomegalovirus with sperm. The researchers stated that 4.0% of sperm had viral particles in close proximity to the sperm membranes, suggesting the potential for virus-sperm attachment. Semen processing by discontinuous density gradient centrifugation followed by a wash step, has been ineffective in the removal of the virus from 89.4% of semen samples positive for the virus (Michou *et al*., 2012).

(v) Human papillomavirus (HPV) is a member of the *Papillomaviridae* family (De Villiers, 2013) with numerous subtypes of which HPV type 16, 18 and 33 are the predominant sexually transmitted subtypes (Dillner *et al*., 1996). The virus infects cutaneous and mucous squamous epithelial cells (Perino *et al*., 2011) and is associated with genital warts (Olatunbosun, Deneer & Pierson, 2001) as well as anogenital cancers (Zur Hausen, 2009).

The virus was found in 53.0% of semen samples from men with HPV infections and in 8.0% of semen samples from asymptomatic men, in a Canadian study (Olatunbosun, Deneer & Pierson, 2001). Flores-Sanchez *et al.* (2010) detected HPV-16, HPV-18, or both viruses in 29.6%, 16.1% and 14.1% of semen samples from men attending an infertility centre in France, respectively. Richter *et al.* (2013) reported that HPV-16 and/18 were detected in 19.5% of women attending public sector primary healthcare clinics in Gauteng Province, South Africa. Infection of the male partner is mostly asymptomatic however infection of the female partner, specifically with HPV-16 and 18, is a major cause of cervical cancer (Harper *et al.*, 2004; Nielson *et al.*, 2007). Human papillomavirus infection during pregnancy could result in spontaneous abortion (Hermonat *et al.*, 1997; Perino *et al.*, 2011), or vertical transmission of the virus to offspring (Cason & Mant, 2005) which could lead to recurrent respiratory papillomatosis (Rabah *et al.*, 2001). Even though HPV infection was not correlated with reduced pregnancy rates following ART (Perino *et al.*, 2011), the virus has been reported to cause male infertility by reducing sperm motility (Lai *et al.*, 1997; Garolla, 2013). Furthermore HPV has also been reported to be associated with the formation of antisperm antibodies that could potentially result in autoimmune infertility (Garolla, 2013). Infection with HPV during ART would therefore, be detrimental to pregnancy. Lai *et al.* (1997) and Foresta *et al.* (2010) reported that HPV penetrates sperm cells and semen processing has been reported to be ineffective in the removal of HPV from sperm (Foresta *et al.*, 2011).

(vi) Hepatitis B virus (HBV) is part of the *Hepadnaviridae* family (Hu *et al.*, 2000) and a major cause of hepatitis, cirrhosis and hepatocellular carcinoma (Perz *et al.*, 2006; Oger *et al.*, 2011). In countries with high HBV endemicity (such as South Africa), perinatal transmission is the major route of transmission, with horizontal transmission in childhood second (Lavanchy, 2004; Lok & McMahon, 2009). In areas with low HBV endemicity, sexual contact and needle sharing amongst high-risk adults are the predominant routes of transfer (Struve *et al.*, 1990). The chronicity of HBV infection is age dependant with 5.0%, 30.0% and 90.0% of adults, children and neonates developing chronic

hepatitis B, respectively (Hoofnagle *et al*., 2007). Hepatitis B virus vaccination has, therefore, been included in the South African Expanded Programme of Immunisation since 1995 (Spearman *et al*., 2013). The virus is present in 33.0% of semen samples from men with acute hepatitis (Hadchouel *et al*., 1985). Infection is correlated with reduced embryo implantation and pregnancy rates following ART (Pirwany *et al.,* 2004; Oger *et al*., 2011; Zhou *et al*., 2011). Male fertility could be compromised by HBV infection due to decreased sperm-zona binding and fertilization rates (Pirwany *et al*., 2004). Furthermore, infection of male patients is correlated with reduced sperm concentration, morphology, motility and viability (Lorusso *et al*., 2010; Zhou *et al*., 2011). The presence of HBV DNA within sperm cells has been demonstrated (Hadchouel *et al*., 1985; Huang *et al*., 2002), and semen processing procedures would, therefore, be ineffective in the removal of HBV from sperm prior to ART. The female partner from an HBV sero-discordant couple could be vaccinated against HBV, but the virus may still be transferred into the oocyte by infected sperm resulting in vertical transmission of the virus (Honeck *et al*., 2006).

1.2.7.2 Single-stranded ribonucleic acid (RNA) viruses

Viruses belonging to this group include the hepatitis C and D viruses (HCV and HDV).

(i) Hepatitis C virus (HCV) is part of the *Flaviviridae* family (Agnello *et al*., 1999). Most HCV infected individuals are chronically infected with an increased risk of chronic liver disease, cirrhosis and liver cancer (Alter, 2007). The virus is small (~10 000 nucleotides) with no reverse transcriptase activity and can, therefore, not be integrated into the DNA of host cells (Levy *et al*., 2000), allowing for treatment and curability of the virus (Seeff & Hoofnagle, 2002). The effect of HCV infection on sperm parameters is controversial. Garrido *et al*. (2005) reported that HCV infection had no negative impact on sperm parameters, while Moretti *et al*. (2008) demonstrated decreased sperm motility parameters as well as increased sperm apoptotic and necrotic statuses among HCV sero-positive patients. Even though HCV RNA is present in 20.0% to 38.0% of semen samples from HCV sero-positive patients (Leruez-

Ville *et al*., 2000; Bourlet *et al*., 2009), the sexual transmission rate of the virus is said to be low (Pasquier *et al*., 2000; Garrido *et al*., 2004). Rooney & Gilson (2006) reported that the probability of the sexual partner of a chronically infected individual to contract the virus is 0% to 3.0%. Most HCV sero-conversions are due to needle sharing between illegal drug users (Alter & Moyer, 1998; Kane *et al*., 1999; Alter, 2007). Therefore, due to the low probability of sexual transmission of the virus and the inability of the virus to integrate into the DNA of host cells, semen decontamination procedures should be effective in the purification of sperm samples from HCV sero-positive male patients. The effectiveness of semen processing for the elimination of HCV from semen is discussed in Chapter 3.

(ii) The Hepatitis D (HDV) virus contains an extremely small single stranded RNA genome (~1679 nucleotides) that lacks the ability to self-replicate (Taylor, 2006; Rizzetto, 2009). The virus is thus considered as defective, is not classified into a viral family (Howard, 2002) and belongs to its own separate genus (*Deltavirus*) (Hughes, Wedemeyer & Harrison, 2011). Hepatitis D virus is dependent on HBV for the synthesis of proteins relevant to assembly and propagation, therefore, individuals that are sero-positive for HDV will be co-infected with HBV (Taylor, 2006). Farci (2003) reported that 5.0% of HBV infected individuals, globally, are co-infected with HDV. Individuals super-infected with HDV in combination with HBV have a much greater risk of developing liver failure compared to patients solely infected with HBV (Taylor, 2006). The virus is present in bodily fluids and can be transferred via sexual intercourse (Elder, Baker & Ribes, 2004). Due to the presence of HBV and HDV within spermatozoa, semen decontamination procedures will be ineffective to remove the virus from sperm samples and ART for patients infected with HBV and HDV should not be considered. These patients should rather be counselled on preventing transmission of the viruses.

1.2.7.3 Retroviruses

Retroviruses relevant to ART include human immunodeficiency virus (HIV) type 1 and 2 as well as human T-lymphotrophic virus (HTLV) type 1 and 2.

(i) Human immunodeficiency virus (HIV) is the most relevant virus to ART. The virus can be transferred from mother to child (intra-uterine, intra-partum, or during breastfeeding), or by needle sharing between drug users (Bruneau *et al*., 1997). However, sexual intercourse is the major route of transmission of the virus and HIV has been detected in 6.6% to 25.0% of semen samples from men with blood viral loads below the lowest limit of detection (Lambert-Niclot *et al*., 2012; Politch *et al*., 2012). Two types of the virus namely HIV-1 and HIV-2 belong to the genus *Lentiviruses* in the family *Retroviridae* (King, Adams & Lefkowitz, 2012). These viruses have been documented causing lifelong, subclinical, asymptomatic infections and ultimately resulting in the degeneration of organs, cachexia and death (Narayan & Clements, 1989; Manavi, 2006). The occurrence of HIV-2 is, however, limited to mostly Western Africa, the virus is less infectious and infection result in less viral copies in plasma when compared to HIV-1 (Popper *et al*., 1999; McCutchan, 2006). Human immunodeficiency virus type 1 is categorized into three groups namely M, O and N, respectively. Group M is the major cause for the HIV-1 pandemic (Vidal *et al*., 2000; McCutchan, 2006) and is further divided into different subtypes of which subtype A, C, D, G, H and a circulating recombinant form (02_AG) are the most prevalent subtypes in Africa (Vidal *et al*., 2000; Bikandou *et al*., 2000; McCutchan, 2006; Hemelaar, 2012). The prevalence of HIV in South Africa has been reported to be 17.4% and 13.3% for female and male patients between the ages of 15 to 49, respectively (Statistics South Africa, 2013). These patients are within their reproductive years and many of them (29.0%) have the desire to have genetically related offspring (Myer, Morroni & Rebe, 2007). However, the potential of the virus to bind to and penetrate sperm is much debated (Baccetti *et al*., 1994; Quayle *et al*., 1998; Pudney *et al.,* 1999; Barboza *et al*., 2004). Furthermore, the sero-positivity of patients, specifically in patients with reduced CD4 counts, is correlated with reduced sperm motility, concentration and outcome of IUI (Nicopoullos *et al*., 2004). Treatment of HIV-1 sero-positive male patients

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with highly active antiretroviral therapy should, therefore, precede semen decontamination procedures and ART when assisting patients with reduced CD4 counts (<300 copies/ml). Risk reduction procedures during ART for HIV-1 sero-positive patients are described in Chapter 3 and the effectiveness of density gradient centrifugation with a centrifuge tube insert was evaluated and the results are reported in Chapter 8.

(ii) Human T-lymphotrophic virus (HTLV) has been classified in the genus *Deltaretrovirus* of the *Retroviridae* family (King, Adams & Lefkowitz, 2012). Two types of the virus exist namely HTLV-1 and HTLV-2, however, only HTLV-1 is associated with human diseases (Goncalves, 2010). Even though 90.0% of infected individuals remain asymptomatic lifelong carriers of the virus (Taylor *et al*., 1999; Goncalves *et al*., 2010), infection could result in adult T-cell leukemia, or neurodegenerative disease (Marriott & Semmes, 2005). The global prevalence of the virus is low, except for specific patient populations in the south-western parts of Japan with a viral prevalence of 27.0% (Mueller *et al*., 1996). The South African prevalence of the virus has been reported to be 0.01% (Du Plessis, Webber & Saayman, 1999). The major route of vertical transmission of the virus is by breastfeeding (Proietti *et al*., 2005). Genital secretions do contain HTLV-1 and the virus can, therefore, be transferred via sexual contact (Zunt *et al*., 2002). Epelboin (2011) stated that the guidelines for patient screening followed by decontamination procedures, as is performed for HIV sero-positive patients, should also be considered for HTLV-1 sero-positive patients. However, the impact of HTLV-1 on sperm parameters and if the virus attaches to sperm cells have not been reported. The effectiveness of semen processing for the removal of the virus from semen and the safety of ART for HTLV-1 sero-positive patients is, therefore, not known.

1.2.7.4 The association of viruses with spermatozoa

The association of viruses with sperm cells is summarized in Table 1.1.

Table 1.1: The association of viruses with sperm cells.

Virus	Association with sperm	Reference
Double-stranded DNA viruses		
• Herpes simplex virus	• Attaches to sperm	(Kotronias & Kapranos, 1998)
• Epstein-Barr virus	• Attachment not been demonstrated • Semen processing failed to remove the virus from 22.45% of samples	(Michou *et al*., 2012) "
• Human herpes virus	• Penetrates sperm	(Glaser *et al.*, 1997)
• Cytomegalovirus	• Virus potentially attached to sperm • Semen processing failed to remove the virus from 89.36% of samples	(Michou *et al*., 2012) "
• Human papillomavirus	• Penetrates sperm	(Lai *et al.*, 1997)
• Hepatitis B virus	• Penetrates sperm	(Hadchouel *et al*., 1985)

Table 1.1 (Continue): The association of viruses with sperm cells.

Virus	Association with sperm	Reference
Single-stranded RNA viruses		
• Hepatitis C virus	• Sperm are not target cells for the virus	(Briat *et al*., 2005)
	• Semen processing has been confirmed to be effective in removal of the virus	See Table 3.6 (page 88)
• Hepatitis D virus	• Hepatitis D virus is dependent on Hepatitis B virus (which has been confirmed to penetrate sperm)	(Taylor, 2006) (Huang *et al*., 2002)
Retroviruses		
• Human immunodeficiency virus	• The attachment and penetration of virus into sperm is debated	See Table 3.3 (page 83)
• Human T-cell leukaemia virus	• Association with sperm and effectiveness of semen processing for removal of the virus is not reported	

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CHAPTER 2

SEMEN PROCESSING PRIOR TO THERAPEUTIC ASSISTED REPRODUCTIVE TREATMENT

2.1 INTRODUCTION

The natural selection of progressively motile sperm, *in vivo*, occurs by the active migration of sperm from seminal plasma into the female reproductive tract after intercourse (Henkel & Schill, 2003; Suarez & Pacey, 2006). However, during assisted reproductive treatment (ART), semen processing should be performed to allow for the qualitative improvement of a sperm sample (Engel *et al*., 2001). Viable sperm populations with improved ratios of sperm with intact nuclear content should be isolated from seminal plasma (Larson *et al*., 1999; Jayaraman *et al*., 2012), immature sperm cells, non-sperm cells and seminal pathogens, prior to therapeutic use. Chapter 1 describes the impact of seminal constituents on sperm.

Various semen processing methods are currently utilized during the preparation of sperm for therapeutic use. According to Henkel & Schill (2003), the ideal sperm preparation technique should be aimed towards the following:

    i) Be economical, rapid and non-complicated.

    ii) Deliver a high yield of progressively motile spermatozoa.

    iii) Be non-detrimental to sperm.

    iv) Remove all non-sperm cells, pathogens, toxic metabolites and seminal compounds such as decapacitation factors.

    v) Allow processing of large semen volumes.

However, no single processing method entirely complies with all of the above mentioned requirements. Multiple semen processing techniques should, therefore, be available and the optimal method for a specific patient case should be selected, depending on the quality of the neat semen sample and the application of the processed sperm sample (Henkel & Schill, 2003; Jayaraman *et al*., 2012). Prepared sperm samples could be utilized for:

    i) Intra-uterine insemination (IUI), whereby the selected sperm fraction is inseminated directly into the uterus (Wainer *et al*., 2004).

    ii) *In vitro* fertilization encompasses the insemination of obtained oocytes outside the body (Huang *et al*., 2013).

iii) Intra-cytoplasmic sperm injection (ICSI) involves the injection of a single sperm into the ooplasma of an oocyte by means of micro-manipulation (Leruez-Ville *et al*., 2013).

iv) Intra-cytoplasmic morphologically selected sperm injection, using high magnification (>6,000 times) (Klement *et al*., 2013).

v) The sperm sample could be cryopreserved and stored for later use (Klaver *et al*., 2012).

The most relevant semen processing procedures that are currently available are presented in Section 2.2.1 to 2.2.3.2. These methods are however not specifically aimed towards the elimination of seminal pathogens. Semen decontamination, addressed in Section 2.2.4, has remained relatively unchanged since 1992 (Semprini and co-workers), whereby the authors employed discontinuous density gradient centrifugation (see Section 2.2.3.2) as the basis of sperm preparation during ART for patients with potential seminal pathogens.

2.2 SEMEN PROCESSING TECHNIQUES

2.2.1 Migration based methods

2.2.1.1 Swim-up method

Dilution of semen with medium followed by centrifugation and the re-suspension of the resultant sperm pellet in medium, as performed during sperm washing (Section 2.2.3.1), allows for the removal of seminal plasma. This method is, however, ineffective in the isolation of a motile sperm fraction from semen (Schuster *et al*., 2003). The selection of a highly motile sperm population (>90% motile) from the sperm pellet obtained by semen washing, can be achieved by allowing sperm to swim up into overlaying medium (Henkel & Schill, 2003; Schuster *et al*., 2003). Washed sperm samples are transferred to fresh test tubes and overlaid with medium (0.5 to 1 ml) followed by an incubation period of up to one hour at 37°C. After incubation, the upper layer containing the motile sperm fraction can be obtained by aspiration using a sterile pipette tip (Adiga & Kumar, 2001). The pelleting of spermatozoa with immature and non-sperm cells during the centrifugation step could, however, have a negative impact on sperm by the generation of reactive oxygen species (Aitken & Clarkson, 1988; Henkel & Schill, 2003). Neat semen samples presenting with increased numbers of non-sperm cells should, therefore, not be centrifuged prior to the swim-up step. The direct swim-up method (Figure 2.1), or the isolation of spermatozoa from semen by density gradient centrifugation (described in Section 2.2.3.2)

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prior to a swim-up step should be considered (Mortimer, 2000; World Health Organization, 2010).

The direct sperm swim-up method involves layering of liquefied semen beneath culture medium (or culture medium layered over the semen). During a subsequent incubation period ranging from 15 to 60 minutes at 37°C, depending on the application of the sperm cells, the motile sperm migrate from the semen into the culture medium, where after the progressively motile sperm fraction can be aspirated and utilized therapeutically. Sperm yield can be improved by the incubation of sperm at 37°C versus room temperature (Purvis & Egdetveit, 1993) and by increasing the surface area of the semen-medium interface by; the layering of several semen aliquots under media in multiple round bottom tubes, incubating samples at a 30° angle, or by using 4-well dishes (Purvis & Egdetveit, 1993; Adiga & Kumar, 2001; Henkel & Schill, 2003).

The selection of a protein supplemented medium for the sperm swim-up technique will depend on if spermatozoa are incubated under air, or within a CO_2-enriched atmosphere. A zwitterion-buffered (e.g. HEPES), or a bicarbonate-buffered medium should be used to maintain a physiological pH (6.8 to 7.2) under air, or under a CO_2-enriched atmosphere, respectively (Björndahl *et al*., 2010; World Health Organization, 2010). The swim-up method is simple to perform, cost-effective and no chemicals that could result in cytotoxicity are used (Soderlund & Lundin, 2000). The technique consistently produces sperm suspensions with superior velocity, acrosomal status, morphology and vitality when compared to density gradient centrifugation (Oehninger *et al*., 1990; Henkel & Schill, 2003; Ricci *et al*., 2009). However, due to low sperm yields obtained by the method, the swim-up procedure can generally only be utilized for the processing of semen samples with high sperm counts and motility (Chen *et al*., 1995; World Health Organization, 2010). Alternative devices utilized for the isolation of a highly motile sperm fraction (based on the principle of sperm migration) include the SEP-D kit (Gentis *et al*., 2012), a syringe method (Siam, 2012), the Zech-selector (Ebner *et al*., 2011) and a microfluidic sperm sorting channel (Schuster, 2003).
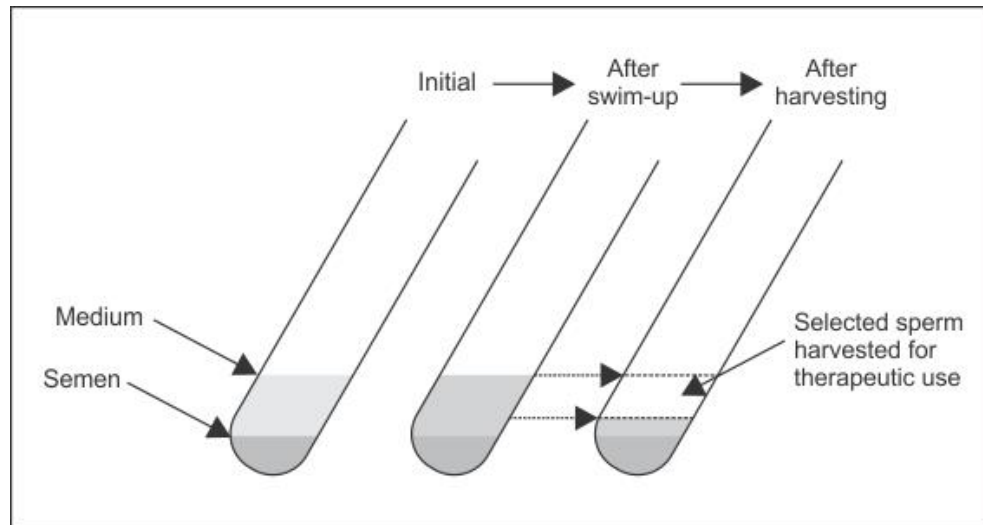
Figure 2.1: Direct swim-up of sperm from semen into medium (adapted from Björndahl *et al*., 2010).

2.2.1.2 Cumulus penetration method

Oocytes are surrounded by tightly packed granulosa cells known as the cumulus oophorus layer (Eppig, Wigglesworth & Pendola, 2002; Assou *et al*., 2006). Evidence suggests that sperm-cumulus contact is required for the optimum fertilization of bovine oocytes, by the induction of capacitation and the acrosome reaction (Fukui, 1990; Zhang *et al*., 1995; Tanghe *et al*., 2002). Hyaluron, a glycosaminoglycan, serves as the support structure on which the extracellular matrix of the cumulus-oocyte complex is assembled (Russell & Salustri, 2006). Mature, acrosome intact, viable sperm have the ability to bind to hyaluron via a hyaluron binding protein present on the sperm cell membrane (Ranganathan, Ganguly & Datta, 1994; Huszar *et al*., 2003). After binding to hyaluron, sperm will migrate through the cumulus layer in order to reach the oocyte and for fertilization to occur (Swain & Pool, 2008). De Jonge (2005), therefore, refers to the cumulus oophorus complex as a checkpoint where quality sperm with high fertilization potential are naturally selected and allowed access to the oocyte.

The capillary-cumulus model, illustrated in Figure 2.2, has been employed by various researchers. A column of medium (3 cm) is aspirated into a glass capillary (inner diameter – 0.7 mm) using a 1 ml syringe that is attached to the end of the glass capillary. Cumulus cells (one to two complexes) are then aspirated where after the open end of the capillary is

placed in a droplet containing semen/sperm. After an incubation period (depending on initial sperm concentration and application of prepared sperm), the sperm that migrated through the cumulus column can be obtained (Hong *et al.*, 2004). This method has been successfully utilised for the selection of higher percentages of sperm with normal morphology, increased zona-binding potential, improved motility parameters and chromatin packaging (Hong *et al.*, 2004; Rijsdijk & Franken, 2007; Franken & Bastiaan, 2009). Although the sperm yield is low, the capillary-cumulus penetration method is cost-effective and could be employed for the selection of quality sperm to be used for ICSI (Franken & Bastiaan, 2009).
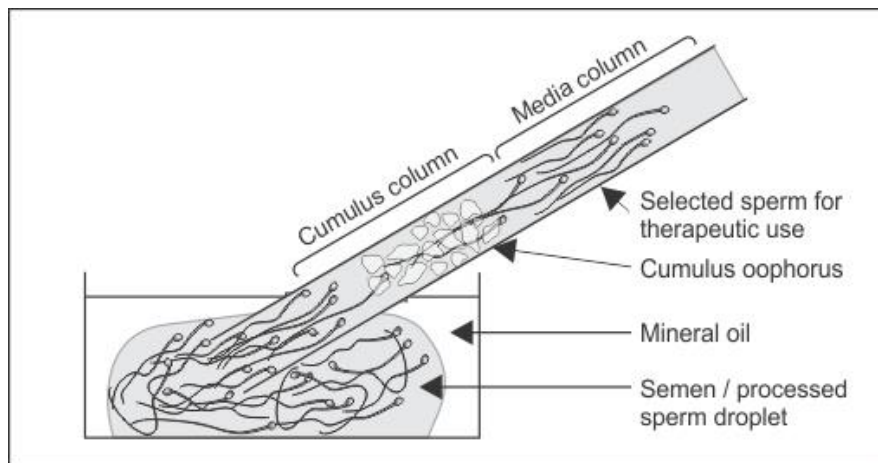
Figure 2.2: Cumulus penetration method of sperm selection (adapted from Franken & Bastiaan, 2009) (not drawn to scale).

2.2.2 Adherence based methods

2.2.2.1 Glass wool filtration

Glass wool filtration isolates motile sperm from other seminal constituents by i) the active migration of sperm as well as by, ii) the filtration of semen through densely packed glass wool fibres (Henkel & Schill, 2003). Non-vital spermatozoa are sticky and will adhere to the surface of the glass wool during filtration, whereby only viable sperm will be able to pass through a glass wool column (Foote, 2001; Björndahl *et al.*, 2010). The method as described by Jeyendran *et al.* (1986), with slight modifications as adapted by Engel *et al.* (2001), is illustrated in Figure 2.3 and is performed as follows: 15 mg of dispersed glass wool (microfiber code112, John Manville, USA) is packed to a depth of 6 mm in the body of a 1 ml, non-pyrogenic, sterile syringe. Any loose glass particles are then removed by

rinsing of the glass-wool column with medium two to three times. The glass-wool column is then suspended vertically where after diluted semen (400 µl semen and 800 µl medium) is layered on top of the column to be filtrated for 5 to 10 minutes at 37°C.

According to Sánchez *et al.* (1996) the success of glass wool filtration is directly related to the type of glass wool used. Potential risks of the technique such as damage to spermatozoa or the occurrence of glass wool fragments in the filtrate should, therefore, be prevented by using quality glass wool (Henkel & Schill, 2003). Nevertheless, glass-wool filtration is not generally utilized for the preparation of human semen for therapeutic use.
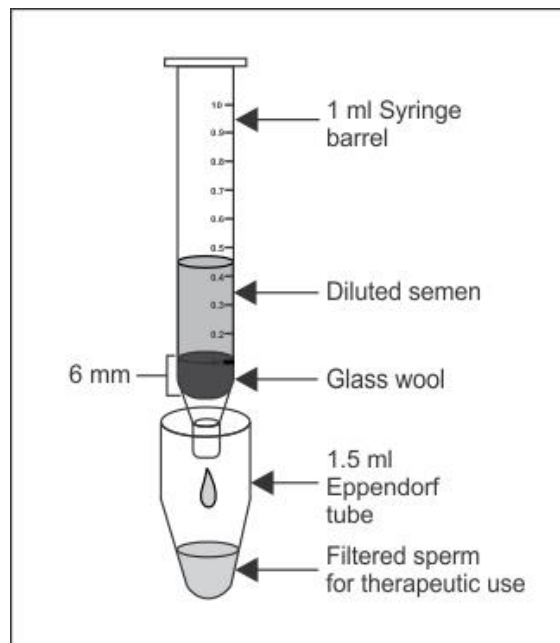
Figure 2.3: Glass wool filtration of sperm (adapted from Engel *et al.*, 2001).

2.2.2.2 Magnetic-activated cell selection (MACS)

Standard semen processing procedures are aimed towards the isolation of a viable, motile sperm fraction. The effectiveness of semen preparation methods could, however, be further improved by the selection of a sperm population that is also based on the apoptotic status of spermatozoa (Said *et al.*, 2008). Sperm apoptotic status is correlated with sperm deoxyribonucleic acid (DNA) fragmentation (Sakkas *et al.*, 1999) and 20.0% of sperm cells in the neat semen samples from sub-fertile men are apoptotic (de Vantéry Arrighi *et al.*, 2009). The DNA integrity of a selected sperm population and resultantly also the

outcome of ART could, therefore, be improved by the removal of apoptotic sperm (Sharma, Said & Agarwal, 2004).

Sperm apoptosis is associated with the externalization of phosphatidylserine (PS) to the outside surface of the cell membrane (Zhang *et al*., 2008). Superparamagnetic microbeads that are conjugated with Annexin V will specifically bind to PS on the outside surface of apoptotic cells (Glander & Schaller, 1999). Magnetic-activated cell selection, illustrated in Figure 2.4, can consequently be utilized for the removal of apoptotic cells from sperm samples (Said *et al*., 2008). Processed sperm cells are incubated (15 minutes at room temperature) with 100 µl Annexin V-conjugated microbeads per 10×10^{6} sperm cells. Superparamagnetic microbead-labelled sperm (apoptotic) are then separated from the non-labelled sperm by means of a magnetic separator (de Vantéry Arrighi *et al*., 2010). Magnetic-activated cell selection has been utilized to reduce the number of sperm cells with externalized PS by 70.0% (de Vantéry Arrighi *et al*., 2010). Furthermore, MACS has also been successfully employed to eliminate leukocytes from seminal fluid by utilizing anti-CD45-conjugated superparamagnetic microbeads (Krausz *et al*., 1992).

The disadvantage of MACS is, however, that the method can only be employed to remove magnetically labelled cells from semen. The method should, therefore, be utilized in combination with other semen processing procedures such as density gradient centrifugation (Tavalaee *et al*., 2012). Even though MACS is not widely employed yet, definite merits exist for this safe, rapid and efficient method of non-apoptotic sperm selection to be incorporated into ART programs (de Vantéry Arrighi *et al*., 2009; Gil, 2013).
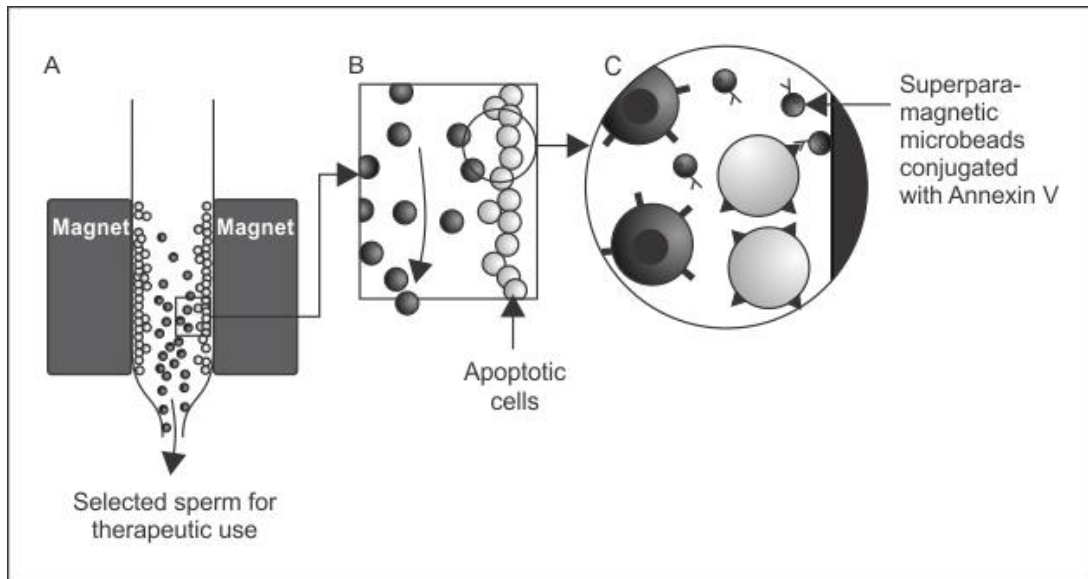
Figure 2.4: Magnetic-activated cell selection of non-apoptotic cells (adapted from Said *et al*., 2008).

2.2.3 Sedimentation based techniques

2.2.3.1 Simple washing of semen

The dilution of semen (5 to 10 times) with medium followed by centrifugation and obtaining of the sperm pellet is the simplest available method of semen processing. The time and speed of centrifugation could be adapted according to the application of the processed sperm sample. However, samples are generally centrifuged for 10 minutes, and 800 x g should not be exceeded (Mortimer, 2000). The sperm pellet formed during centrifugation can be obtained by aspiration of the supernatant whereby access to the processed sperm sample is allowed.

The disadvantage of the method is that all cellular seminal constituents, other than seminal plasma, are pelleted with the sperm cells and may continue to contaminate the processed sperm sample (Mortimer, 1994). Therefore, due to the negative impact of seminal constituents on sperm cells, this method of unselected sperm washing should be avoided. The method is generally employed for the preparation of sperm to be utilized for IUI (World Health Organization, 2010), or in combination with the swim-up method and density gradient centrifugation (Section 2.2.1.1 and 2.2.3.2, respectively), as well as for the processing of semen samples with low sperm concentrations prior to ICSI (De Vos *et al*., 1997).

2.2.3.2 Density Gradient Centrifugation

Density gradient centrifugation is currently the most widely utilized method of semen processing (Soderlund & Lundin, 2000). Two techniques are available, namely continuous (Gorus & Pipeleers, 1981) and discontinuous (Tomlinson *et al.*, 2001) density gradient centrifugation. Continuous gradients consist of medium with a gradual increase in density from the top to the bottom (Henkel & Schill, 2003). Discontinuous density gradients, on the other hand, display clear borders between the different density layers (Henkel & Schill, 2003) and is almost exclusively the method of choice in ART units (Björndahl *et al.*, 2010).

Discontinuous density gradient layers are prepared by layering media with different concentrations of silica particles on top of each other, preferably in a conical centrifuge tube (Mortimer, 1994). Originally, polyvinylpyrrolidone-coated silica particles (Percoll®) were added to media to obtain gradient layers with different densities (Lessley & Garner, 1983). However, due to the risks associated with potential endotoxins present in Percoll®, the product has never been approved by the U.S. Food and Drug Administration, and is no longer used during ART for humans (Andersen & Grinsted, 1997). The usage of silane-coated silica particles that is used in products such as PureSperm® (Nidacon International, Mölndal, Sweden), Pureception (SAGE In-Vitro Fertilization, Inc., Trumbull, CT, USA), IxaPrep® (MediCult, Copenhagen, Denmark), SilSelect® (FertiPro, Beernem, Belgium) and ISolate® (Irvine Scientific, Santa Ana, CA, USA), replaced the use of the polyvinylpyrrolidone-coated silica particles (Henkel & Schill, 2003). Mortimer (1994) reported that an optimum concentration of silica particles of 80% allows for a sufficient yield of quality spermatozoa. Density gradients, therefore, generally consist of a bottom and a top layer of 1,5 to 2 ml of 80% to 90% and 40% to 45% silica particles (Mortimer, 1994; Chen & Bongso, 1999; Soderlund & Lundin, 2000; Jayaraman *et al.*, 2012); with approximate densities of 1.06 and 1.10 g/ml, respectively (Björndahl *et al.*, 2010).

After liquefaction, semen aliquots of ≤1.5 ml (Chen & Bongso, 1999; Soderlund & Lundin, 2000; Li *et al.*, 2012) are layered on the prepared density gradients, where after the samples are centrifuged at 300 x g for 20 minutes (Mortimer, 2000). During centrifugation the sperm heads are aligned in the direction of the centrifugal force, allowing motile sperm with intact cell membranes and relative high densities >1.10 g/ml

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(Oshio *et al*., 1987), to actively migrate into the bottom density gradient layer (Andersen & Grinsted, 1997; Mortimer, 2000; Henkel & Schill, 2003). Immotile and/or immature spermatozoa have densities ranging between 1.06 to 1.09 g/ml (Oshio *et al*., 1987) and, therefore, most of these cells are trapped in the upper gradient layer. Motile sperm are consequently separated from seminal plasma, immotile sperm, leukocytes, and cellular debris. Post-centrifugation, the sperm that penetrated the bottom density gradient would have formed a sperm pellet that can be obtained by aspiration and discarding of the supernatant (Figure 2.5). After the density gradient centrifugation step, the isolated sperm should be washed free from the silane-coated silica particles as is described in Section 2.2.3.1.

Discontinuous density gradient centrifugation usually provides an uncontaminated fraction of highly motile spermatozoa with improved morphology, nuclear integrity (Tomlinson *et al.*, 2001; Jayaraman *et al*., 2012) and apoptotic status (Ricci *et al*., 2009). Furthermore, seminal leukocytes and reactive oxygen species are significantly reduced (Henkel & Schill, 2003; Yilmaz *et al*., 2005). Conversely, the layering of density gradients with suitable interphases requires skill and is time consuming because the suspension medium is the same, and thereby easily mixed (Henkel & Schill, 2003). Centrifugal force could have a potential negative impact on sperm DNA integrity due to the production of reactive oxygen species (Mortimer, 2000; Li *et al*., 2012). The interfaces between density gradients could become clogged with non-motile sperm, or other cells whereby the penetration of progressively motile sperm through the interface could be hampered (Björndahl *et al*., 2010). Furthermore, the method is costly when compared to other methods such as the swim-up method (Section 2.2.1.1).

Nevertheless, the rapidity of the method and superior sperm yield obtained (Chen & Bongso, 1999), qualifies density gradient centrifugation as the preferred method of sperm preparation, specifically when assisting patients with low sperm counts, or when sperm motility is compromised (Soderlund & Lundin, 2000). The buoyant density of cell-free seminal human immunodeficiency virus type 1 (HIV-1) is 1.042 g/ml (Kuji *et al*., 2008) and density gradient centrifugation has, therefore, been reported to be effective in the removal of the virus from semen (Gilling-Smith *et al*., 2006; Bujan *et al*., 2007; Savasi *et al*., 2007). However, the potential attachment (peptide binding) of pathogens to sperm

could result in pathogens being transported through the density gradients to contaminate the processed sperm samples. Furthermore, potential pathogens could attach to the inside surface of the test-tube and flow down to re-infect the purified sperm samples during aspiration of the supernatant (Loskutoff *et al*., 2005). Standard discontinuous density gradient centrifugation should, therefore, be adapted when assisting patients with potential seminal pathogens.
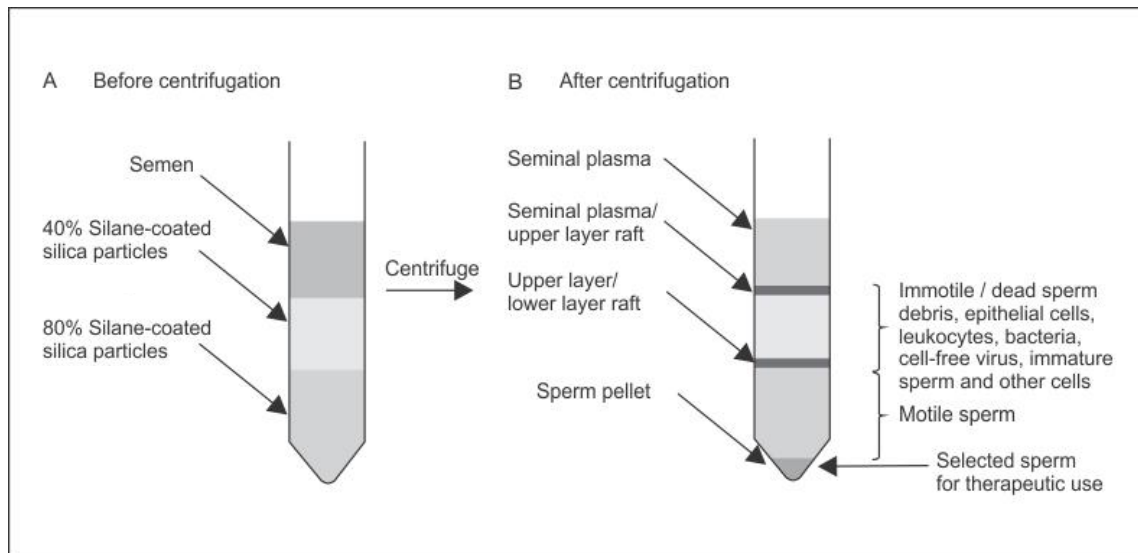
Figure 2.5: Discontinuous density gradient centrifugation (adapted from Björndahl *et al*., 2010).

2.2.4 Semen decontamination

The term semen decontamination has been suggested (Huyser & Fourie, 2010) to distinguish standard discontinuous density gradient centrifugation from improved techniques utilized for the elimination of seminal pathogens (Huyser & Boyd, 2012).

2.2.4.1 Density gradient centrifugation in combination with sperm swim-up

The processing of semen for the elimination of HIV-1 followed by IUI for the HIV-1 sero-negative female partner, was pioneered by Semprini and co-workers in 1992. The method employed by the researchers consisted of discontinuous density gradient centrifugation followed by washing of the sperm samples and an extra swim-up step (Figure 2.6). Since then, numerous ART units implemented discontinuous density gradient centrifugation in combination with sperm swim-up when providing ART to HIV-1 sero-positive male patients (Hanabusa *et al*., 2000; Bujan *et al*., 2004; Garrido *et al*., 2004; Kato *et al*., 2006).

However, the swim-up step is a time-consuming procedure and technique failure has been reported to occur in 7.7% of samples (Garrido *et al*., 2004). Improved methods of semen decontamination should therefore be considered when providing ART to patients with potential seminal pathogens.
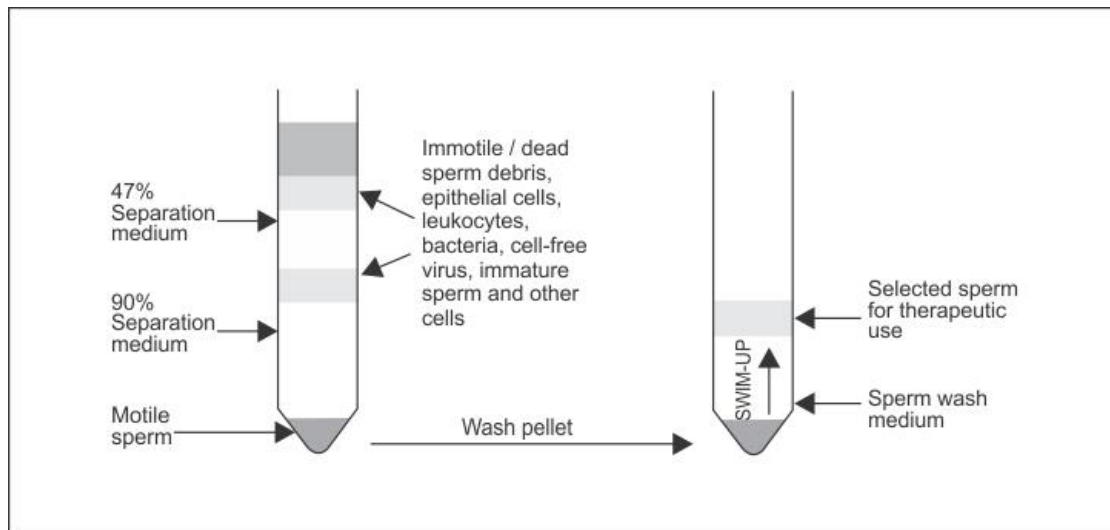
Figure 2.6: Semen decontamination by discontinuous density gradient centrifugation followed by a swim-up step (adapted from Politch *et al*., 2004).

2.2.4.2 Density gradient centrifugation with supplements

Pathogenic micro-organisms such as *Escherichia coli* (Sánchez *et al*., 1989; Diemer *et al*., 2000), *Ureaplasma urealyiticum* (Nunez-Calonge *et al*., 1998)*, Mycoplasma hominis* (Diaz-Garcia *et al*., 2006), *Neiseria gonorrhoeae* (James-Holmquest *et al*., 1974) and *Chlamydia trachomatis* (Erbengi, 1993) can attach to spermatozoa. Furthermore, enveloped viruses such as HIV could also potentially bind to sperm and result in horizontal and/or vertical transmission of the virus during ART (Fanibunda *et al*., 2008). Proteases such as trypsin could, therefore, be added to density gradients to break the pathogen-sperm bonds and to inactivate viruses (Loskutoff *et al*., 2005; Huyser *et al*., 2006).

Tang & Levy (1991) reported that trypsin treatments of 5 minutes at concentrations of 25 to 100 µg/ml were effective in the inactivation of HIV-1. Furthermore, Loskutoff *et al*. (2005) reported a significant reduction in the infectivity of HIV-1 ribonucleic acid after a brief (1 minute) exposure to trypsin at a concentration of 0.25%. Likewise Bielanski, Loewen & Hare (1988) successfully utilized trypsin at a concentration of 0.3% to inactivate bovine herpesvirus-1 during semen processing. Loskutoff and co-workers

(2005) were able to reduce the number of *in vitro* derived HIV-1 copies from an initial spiking concentration of 1 x 10^{7-8} copies/ml to below the lowest limit of detection in sperm samples, by utilizing discontinuous density gradient centrifugation with density layers supplemented with 0.25% trypsin. The addition of trypsin to upper density layers, therefore, appears to be beneficial for the inactivation of seminal pathogens during semen processing.

Nevertheless, trypsin could potentially have a negative impact on sperm parameters and the inhibition of the trypsin, by the addition of trypsin inhibitor to the bottom density layer, could be beneficial (Loskutoff *et al*., 2005). See Figure 2.7 for a schematic illustration of discontinuous density gradient centrifugation with upper and lower density layers that are supplemented with trypsin and trypsin inhibitor, respectively. Adding trypsin inhibitor could, however, inhibit the activity of spermatozoal acrosin, a protease involved in the fertilization process (Honda, Siruntawineti & Baba, 2002). Liu & Baker (1993) reported the total inhibition of sperm acrosin activity after a 6 hour incubation period of sperm with 2 mg/ml soybean trypsin inhibitor. Therefore, the effect of semen processing on sperm parameters with density gradient layers supplemented with either trypsin or trypsin inhibitor should be considered, prior to usage during ART. The effects of trypsin and trypsin inhibitor on sperm parameters were investigated and the findings are presented in Chapter 4 and Chapter 5, respectively.
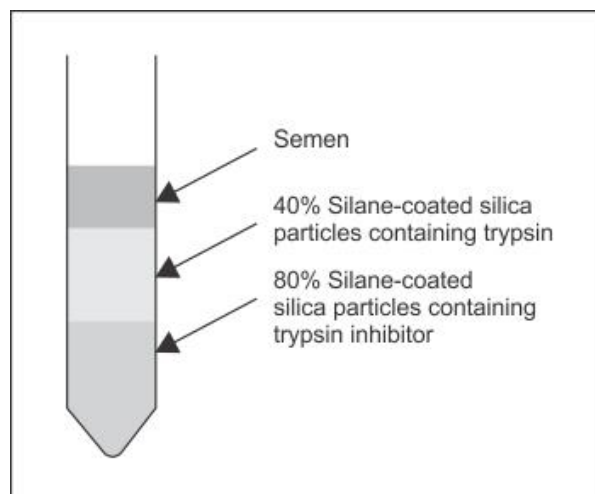
Figure 2.7: Semen decontamination by discontinuous density gradient centrifugation using upper and lower density layers supplemented with trypsin and trypsin inhibitor, respectively.

2.2.4.3 Density gradient centrifugation with a centrifuge tube insert

After standard density gradient centrifugation, the supernatant needs to be aspirated and discarded to allow access to the sperm pellet. However, Kaneko *et al.* (1986) suggested that re-contamination of the purified sperm pellet often occurs during aspiration of supernatant. Pathogens from the upper layers can adhere to the inside surface of the test tube and flow down to re-infect the sperm pellet. For this purpose, Kaneko and co-workers (1986) developed an inner column (Figure 2.8) to allow for the safe discarding of all impurities by removal of the inner column after density gradient centrifugation. In 2004, Politch *et al*. introduced a double tube gradient method (Figure 2.9) that prevented re-contamination of the purified sperm pellet similarly to the method as was employed by Kaneko and co-workers. Loskutoff *et al*. (2005) developed a simplified polypropylene tube insert that allowed access to the purified sperm pellet through the lumen of the insert by using an elongated pipette. This tube insert, now branded as the ProInsert™ (Nidacon International), was introduced to the market in 2013 (CE marking and FDA approval pending) and is illustrated in Figure 2.10. The effectiveness of discontinuous density gradient centrifugation in combination with the ProInsert™, without an extra swim-up step, for the elimination of micro-organisms, white blood cells and *in vivo* derived HIV-1 was investigated and the results are reported in Chapters 6, 7 and 8, respectively.
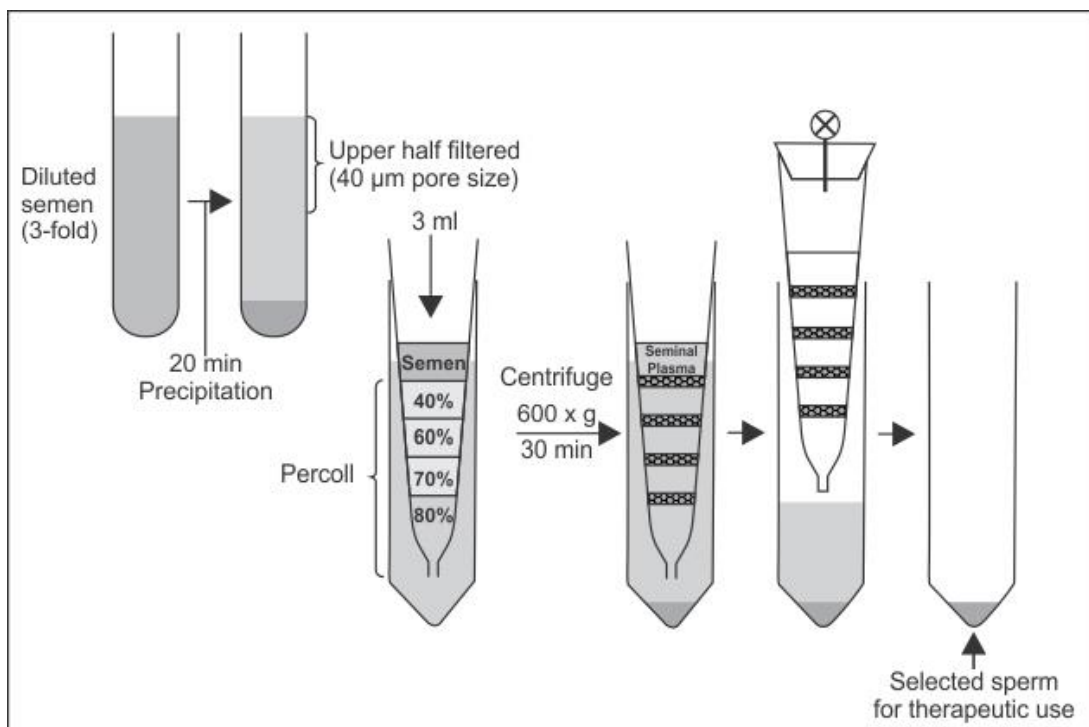
Figure 2.8: Semen decontamination using discontinuous density gradient centrifugation with an inner column (adapted from Kaneko *et al*., 1986).

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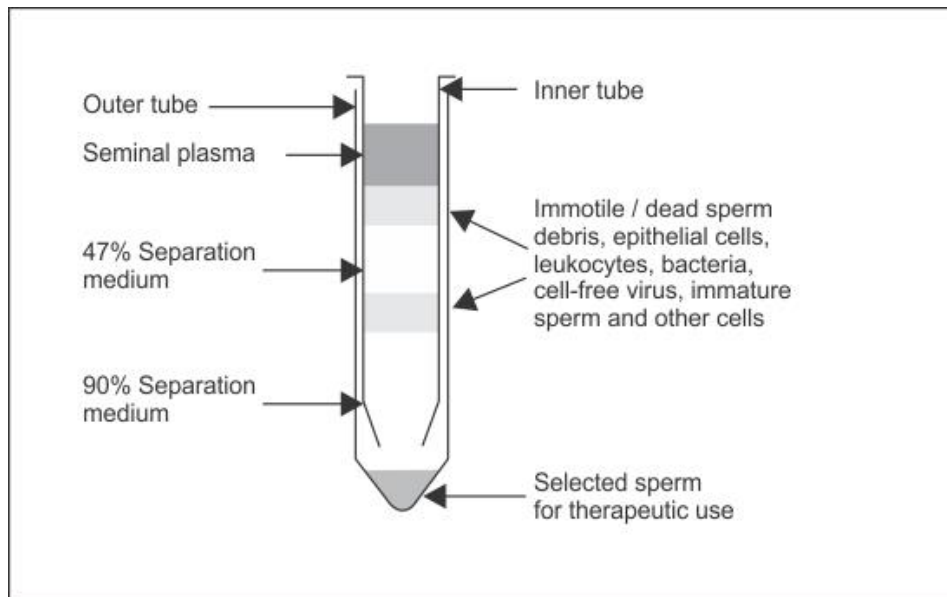
Figure 2.9: Semen decontamination using discontinuous density gradient centrifugation with an inner column (adapted from Politch *et al*., 2004).
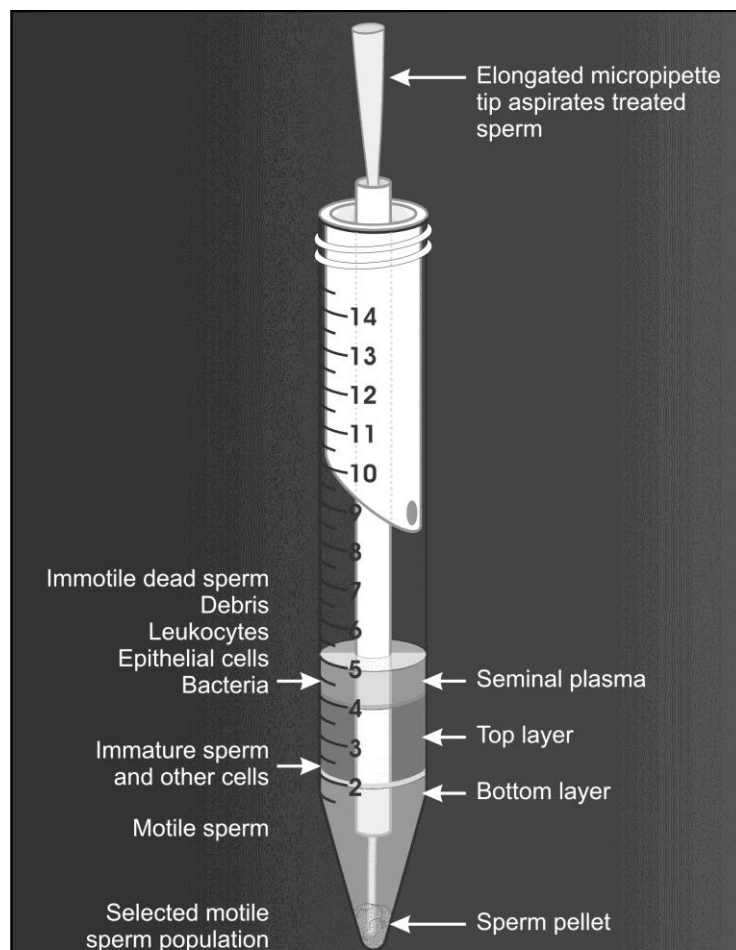
Figure 2.10: Semen decontamination using the ProInsert™.

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CHAPTER 3

ASSISTED REPRODUCTIVE TREATMENT FOR PATIENTS WITH SEMINAL PATHOGENS

3.1 INTRODUCTION

The general transformation towards a more accommodating approach to the provision of assisted reproductive treatment (ART) to human immunodeficiency virus type-1 (HIV-1) sero-positive patients, is in part due to the improved clinical care of infected patients, as well as by improvements made in risk reduction procedures during ART (Zutlevics, 2006). However, concerns regarding the treatment of patients with potential seminal pathogens with ART include: i) the probability of vertical and horizontal transmission of the specific pathogen, ii) the welfare of children with infected parents that may die due to the infection (Lyerly & Anderson, 2001) and iii) the possibility of nosocomial transfer of pathogens in an ART laboratory (Englert *et al.*, 2004).

During the remainder of this chapter it will become evident that effective risk reduction procedures for the treatment of patients with seminal pathogens are available and that the decision to withhold these methods from informed couples, is ethically and legally unsubstantiated (Lyerly & Anderson, 2001).

3.2 ASSISTED REPRODUCTIVE TREATMENT FOR PATIENTS WITH POTENTIAL SEMINAL MICRO-ORGANISMS

The prevalence, clinical impact and potential detrimental effect of specific seminal micro-organisms on ART are presented in Section 1.2.6. The presence of bacteria in an *in vitro* embryo culture system can compromise the outcome of ART by: i) a reduction in sperm motility (Nunez-Calonge *et al.*, 1998), ii) the induction of apoptosis/necrosis (Villegas *et al.*, 2005), or iii) causing degeneration of *in vitro* fertilized oocytes (Huyser *et al.*, 1991). During an embryo transfer, the introduction of pathogens such as *Mycoplasma genitalium*, into the uterus can lead to intra-uterine infections and female infertility (Kastrop *et al.*, 2007; Grzesko *et al.*, 2009). Infected sperm samples used for *in vitro* fertilization are a considerable cause (35.0%) of infected embryo culture systems (Kastrop *et al.*, 2007). Therefore, comprehensive standard operating procedures that are relevant to a specific unit's patient population and scope of practice must be developed and implemented, to

allow for the effective and safe provision of ART to patients with potential seminal micro-organisms. The following section presents practical guidelines that should be considered when assisting these patients with ART.

3.2.1 Aseptic delivery of semen samples

Effective washing prior to the delivery of a semen sample should reduce the infection of semen samples by skin contaminants. Krissi *et al.* (2004) reported that that the incidence of positive semen cultures was reduced from 94.2% to 32.7% by having men wash their hands before masturbation. Proper washing prior to the delivery of a semen sample is therefore recommended by the World Health Organization (WHO, 2010). Boucher *et al.* (1995) stated that the percentage of semen samples without bacteria can be increased by 36.5% by verbally discussing the washing guidelines with patients versus providing the guidelines in a written format. The guidelines for delivery of a semen sample as described by the WHO (2010) include the following:

i)   Patients should pass urine.

ii)   Antibacterial soap can be used to wash hands and penis.

iii)   Soap residue must be thoroughly rinsed away with water.

iv)   Hands and penis should be dried using a disposable towel.

v)   The semen sample should be delivered by masturbation after 2 to 7 days of abstinence into a sterile container that has been confirmed to be non-toxic to sperm cells.

Even though the number of semen samples with positive bacterial cultures can be decreased by effective washing prior to the delivery of semen samples, micro-organisms colonizing the urogenital tract will continue to contaminate semen samples.

3.2.2 Screening and antibiotic treatment of patients

The routine screening of all semen samples for micro-organisms and the sensitivity testing of seminal micro-organisms will result in the improved management of an ART cycle (Leterrier *et al.*, 2011). Depending on the antibiotic resistance profile of seminal pathogenic micro-organisms, patients should be treated with an appropriate antibiotic to clear up the specific genital tract infection prior to ART (Levy *et al*., 1999; Skau & Folstad, 2003; Damirayakhian, Jeyendran & Land, 2006).

However, non-culturable bacteria such as *Chlamydia trachomatis* may be overlooked during standard semen cultures and asymptomatic infections may remain untreated. Prophylactic broad-spectrum antibiotic treatment of asymptomatic male patients may result in the inoculation of the female partner's reproductive tract with antibiotic-resistant bacteria (Liversedge *et al.*, 1996; Rodin, Larone & Goldstein, 2003). Furthermore, the effective elimination of seminal bacteria by antibiotic treatment is not guaranteed (Weidner *et al.*, 1999; Vicari, 2000), and due to the presence of seminal bacteria being mostly due to contamination by skin flora (Kim & Goldstein, 1999; Krissi *et al.*, 2004), the treatment of these patients with antibiotics will be ineffective. Effective semen processing procedures for the elimination of seminal bacteria are, therefore, required.

3.2.3 Semen processing for the elimination of micro-organisms

Huyser *et al.* (1991) reported that semen processing is more effective than antibiotic treatment, by ridding 57.4% of semen samples free from microbial contaminants compared to 16.3% of samples after treatment with prescription antibiotics. However, depending on the processing method employed, 39.7% to 43.0% of sperm samples may remain positive for bacteria post-processing (Huyser *et al.*, 1991; Knox *et al.*, 2003). The outcomes of various processing methods for the elimination of *in vivo* derived bacteria from the semen samples of men seeking ART are presented in Table 3.1, page 77.

Density gradient centrifugation is the most widely used method of sperm preparation utilized for the elimination of seminal micro-organisms. Sterile pipette tips and tubes should be changed after the density gradient centrifugation step to avoid re-contamination of the purified sperm samples. Nicholson *et al.* (2000) reported that by using fresh pipette tips and test tubes for the washing step, the number of processed sperm samples that are free from bacteria can be increased by 44.0%. Nevertheless, technique failure often occurs and sexually transmitted pathogens such as *Ureaplasma parvum and Ureaplasma urealyticum*, have been found in processed sperm samples (Knox *et al.*, 2003).

3.2.4 Supplementation of semen processing and embryo culture media with antibiotics

The effectiveness of semen processing for the elimination of bacteria could be improved by the supplementation of semen processing media with antibiotics. The growth of seminal bacteria was successfully prevented by the treatment of semen samples with a

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broad spectrum of antibiotics; however the most commonly used in embryo culture (e.g. gentamycin, penicillin and streptomycin) were not effective for some of the most prevalent environmental bacteria found in semen, probably due to resistance after so many years of use (Huyser *et al.*, 2004). Regardless, these antibiotics (penicillin, streptomycin and gentamycin) are usually added to semen processing and embryo culture media (Magli *et al.*, 1996; Cottell *et al.*, 1997; Gardner & Lane, 2007; Kastrop *et al.*, 2007). Cottell & co-workers (1997) were able to eliminate bacteria from 95.0% of semen samples by processing semen in an antibiotic rich medium. This addition of antibiotics to culture media, however, may result in antibiotic resistant bacterial strains (Kastrop *et al.*, 2007), as well as decreased embryo cleavage rates (Magli *et al.*, 1996; Lemeire, Van Merris & Cortvrindt, 2007). The manufacturers of PureSperm® products (Nidacon International, Mölndal, Sweden) therefore decided against the supplementation of any of their semen processing media with antibiotics (personal communication with Prof. Paul Holmes, chief executive officer, Nidacon International).

Improved methods of semen processing for the elimination of seminal micro-organisms, without the use of antibiotics are therefore required. The effectiveness of discontinuous density gradient centrifugation in combination with the ProInsert™ (Nidacon International, see Section 2.2.4.3) for the elimination of *in vitro* derived micro-organisms was evaluated and results are reported in Chapter 6.

Table 3.1: Effectiveness of semen processing for the elimination of micro-organisms.

Processing method	Outcome	Reference
1 ml Semen washed in 2 ml of Ham's F-10 supplemented with 10% human serum and 0.05 g/L penicillin. Samples were centrifuged at 500 x g for 10 minutes - Sperm pellets were re-suspended in 2 ml Ham's F-10 and washed again - A **swim-up step** (30 min at 37°C) was performed using 1 ml of Ham's F-10	All bacteria were removed from 57.4% of semen samples with known contamination (N=102)	(Huyser *et al.*, 1991)
- Simple sperm wash – using Earle's balanced salt solution (EBSS) supplemented with pyruvic acid, 10% human serum, 100 IU/ml penicillin and streptomycin. Samples were centrifuged for 5 minutes at 500 x g where after sperm pellets were obtained followed by, **Swim-up** of sperm into 1.2 ml of supplemented EBSS (30 minutes at 37°C)	All bacteria were removed from 95.0% of processed semen samples (N=140)	(Cottell *et al.*, 1997)
Discontinuous density gradient centrifugation – 1ml layers of 90% and 45% PureSperm$^®$ (Nidacon International) followed by, - Two washing steps	All bacteria were removed from 58.8% of processed semen samples (N=68)	(Nicholson *et al.*, 2000)
1 ml 95% and 47.5% **density layers** (Puresperm$^®$) were prepared and overlaid with 1 ml semen - Samples were centrifuged (15 minutes at 300 x g) where after - Sperm pellets were washed twice by re-suspension in culture medium and centrifugation (5 minutes at 300 x g)	All bacteria were removed from 60.3% of semen samples with known positive *Ureaplasma* cultures (N=73)	(Knox *et al.*, 2003)
1 ml 80% and 40% **density layers** were prepared and overlaid with 1 ml semen - Samples were centrifuged (15 to 30 minutes at 300 to 400 x g) where after - Sperm pellets were washed twice by re-suspension in culture medium and centrifugation (4 to 10 minutes at 200 x g)	All bacteria were removed from 64.0% of processed semen samples (N=50)	(Abeysundara *et al.*, 2013)

3.3 ASSISTED REPRODUCTIVE TREATMENT FOR PATIENTS WITH BLOOD BORNE VIRUSES

Assisted reproductive treatment for patients infected with blood borne viruses serves a dual purpose:

i)   Treatment of potential male infertility caused by the infection (Keck *et al.*, 1998), and

ii) Risk reduction of vertical or horizontal transfer of viruses (Gilling-Smith *et al.*, 2006). The female partner from a HIV-1 sero-positive male stands a 0.1% to 0.2% chance to become infected with the virus per single act of unprotected sexual intercourse (Mastro & de Vincenzi, 1996). Therefore, HIV sero-discordant couples where the male partners are sero-positive are seeking ART to reduce the risk of HIV-1 transmission to the sero-negative female partners (Leruez-Ville *et al.*, 2002).

Providing semen decontamination procedures to couples where the male partner is sero-positive for viruses such as HIV-1 and hepatitis C virus (HCV) is, therefore, a crucial service. In the following section factors that should be considered prior to the establishing of an ART program for patients with seminal viruses will be presented.

3.3.1 Ethical considerations

The major objections against the treatment of HIV-1 sero-discordant couples with ART include concerns regarding the welfare of the child after untimely death of parent(s), as well as the potential transmission of the virus to the sero-negative partner, offspring, other patients, or health-care workers (Spriggs & Charles, 2003; Gilling-Smith *et al.*, 2005). These concerns are all ungrounded, the treatment of patients with blood borne viruses by ART can in fact reduce risk whereby detriment can be avoided (Spriggs & Charles, 2003).

The effective treatment of opportunistic infections due to HIV-1 and the use of improved anti-retroviral treatment, resulted in enhanced quality of life as well as increased life expectancies ($\geq$20 years from the time of diagnosis) (Nicopoullos *et al.*, 2004). Human immunodeficiency virus-acquired immune deficiency syndrome is, therefore, now considered as a manageable chronic condition (Kato *et al.*, 2006) and the refusal of ART to these patients is no longer acceptable (Nicopoullos *et al.*, 2004). On the contrary, the refusal of semen decontamination procedures to these patients could result in:

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i)   Childlessness due to patients having protected intercourse to reduce the possibility of transmission of the virus (Ohl *et al.*, 2003; Frodsham *et al.*, 2006). The negative implications of childlessness on the psychological wellbeing of patients could be detrimental, specifically in Africa due to cultural stigmas (Dyer, Lombard & Van der Spuy, 2009; Heys *et al.*, 2009).

ii)   Desperate couples could attempt to conceive by having unprotected intercourse whereby vertical or horizontal transmission of the infection could occur (Klein *et al.*, 2003; London, Orner & Myer, 2008; Matthews, 2013). The drive to have their own genetically related offspring is overpowering and 43.0% of female partners from HIV-1 sero-positive males, will even undergo insemination with stored sperm from their male partners in the event of untimely death caused by the infection (Klein *et al.*, 2003).

The refusal of ART to patients with HIV-1 would be discriminating in nature and would put patients at greater risk of contracting the virus (Spriggs & Charles, 2003). Lyerly & Anderson (2001) concluded: "It is neither ethically nor legally justifiable to categorically exclude individuals from infertility services on the basis of HIV infection."

3.3.2 Assisted reproductive treatment for patients infected with human immunodeficiency virus type 1 (HIV-1)

3.3.2.1 Patient health and antiretroviral therapy

Prior to commencing ART, HIV-1 sero-positive patients should be in good health and free from genital tract infections that could result in the increased shedding of HIV into semen (Fleming & Wasserheit, 1999; Winter *et al.*, 1999). The inclusion criteria regarding HIV serum load and CD4 counts from various ART units are summarized in Table 3.2, page 80. Patients excluded from an ART program based on these parameters should be referred to a virologist for antiviral therapy (Ohl *et al.*, 2003). Antiretroviral therapy is effective in the reduction of serum HIV-1 viral load and the increase of CD4 counts (Barth *et al.*, 2008; van Leeuwen *et al.*, 2008). The rate of viral shedding into semen can be reduced by 1.65 $\log_{10}$ units/ml by successful antiretroviral therapy within 6 months (Barroso *et al.*, 2000). However, the penetration of antiretroviral drugs into the male reproductive tract (Lowe *et al.*, 2004), could result in a negative impact on sperm parameters. Antiretroviral therapy is associated with decreased sperm progressive motility (Bujan *et al.*, 2007c; van Leeuwen *et al.*, 2008; Lambert-Niclot *et al.*, 2011). The negative impact of antiretroviral treatment on sperm progressive motility could be due to the inhibitory effect of nucleoside analogue

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reverse transcriptase inhibitors on mitochondrial deoxyribonucleic acid (DNA) replication (Pavili *et al.*, 2010). Furthermore, patients receiving antiviral therapy could still present with viral copies in their semen samples (Zhang *et al.*, 1998; Marcelin *et al.*, 2008; Nicopoullos *et al.*, 2010). Antiretroviral therapy should, therefore, be employed in combination with effective semen processing methods.

Table 3.2: Inclusion criteria for the acceptance of HIV-1 sero-positive male patients into assisted reproductive programs based on serum viral ribonucleic acid (RNA) load and CD4 count.

CD4 count/mm^3	Serum viral load	Reference
• >350	• <15,000 HIV-1 RNA copies/ml	(Sauer & Chang, 2002)
• >200 measured 2 times within 4 months prior to ART	• Stable no increase >0.5 log measure 2 times within 4 months prior to ART	(Ohl *et al.*, 2003)
• >350	• <3000 HIV-1 RNA copies/ml	(Bujan *et al.*, 2004)
• >200	• Stable blood viral load at least 4 months prior to ART	(Bujan *et al.*, 2007b)
• >200 measured 2 times within 4 months prior to ART	• Stable blood viral load at least 4 months prior to ART	(Savasi *et al.*, 2008)
• >250 over 6 month period prior to ART	• <50 000 HIV-1 RNA copies/ml over 6 month period prior to ART	(Sauer *et al.*, 2009)
• >200	• Stable blood viral load at least 4 months prior to ART	(Vitorino *et al*., 2011)

3.3.2.2 Semen processing for the elimination of HIV

The effectiveness of semen processing for the elimination of seminal pathogens is dependent on the specific method employed, as well as on the interaction of the virus with sperm cells. The attachment of HIV-1 to sperm and the penetration of the virus into the sperm cells are debated and reported findings are presented in Table 3.3, page 83. More evidence suggests that HIV-1 is able to bind to sperm than not. However, Quayle *et al.* (1998) stated that the reported attachment of HIV to sperm could be false in some cases, due to the subjectivity of methods such as electron microscopy. Furthermore, evaluated

samples may have been contaminated with virus due to ineffective semen processing (Quayle *et al.*, 1998). Nevertheless, the processing of semen samples from HIV-1 sero-positive patients, for the elimination of viral copies seems to be a viable option to assist sero-discordant couples (where the male is positive) to conceive.

However, even though a few thousand cycles (Table 3.4, page 84) have been performed during the last 15 years, cases of HIV-1 transferral after donor insemination have been reported (Wortley, Hammett & Fleming, 1998), illustrating that there certainly is risk involved. See Table 3.5, page 85 for a summary of the effectiveness of semen processing for the elimination of HIV-1 from semen at various ART units. The risk of HIV transmission can be minimized by the utilization of effective semen decontamination procedures followed by viral validation of processed sperm samples (Semprini & Fiore, 2004; Eke & Oragwu, 2011). When a positive result is obtained for an aliquot from a processed sperm sample, the remaining sample must be incinerated and the patient should provide a fresh semen sample to be decontaminated after two to three weeks (Garrido *et al.*, 2004a). However, from Table 3.4 it is clear that not all reproductive units, providing ART to HIV-1 sero-positive male patients, submit processed sperm samples for viral validation prior to therapeutic use. The provision of ART to couples where the male partner is HIV-1 sero-positive, is ethically justifiable and if stringent precautionary measures are taken, relatively safe. The effectiveness of discontinuous density gradient centrifugation in combination with a centrifuge tube insert, for the removal of *in vivo* derived HIV from semen samples of men seeking ART in South Africa, was evaluated and the results are presented in Chapter 8.

3.3.2.3 Viral validation of processed sperm samples

Therapeutic ART should not be performed unless the absence of HIV from the processed sperm sample is confirmed by reliable molecular methods of virus detection (Kato *et al.*, 2006; Garrido & Meseguer, 2006). Methods employed for the detection of HIV-1 in semen and sperm samples are based on the amplification of unique sequences of viral nucleic acids (Meseguer *et al.*, 2002). Available methods for the quantitative evaluation of HIV-1 viral load include reverse transcription polymerase chain reaction (RT-PCR), branched DNA (b-DNA), and nucleic acid sequence-based amplification (NASBA) (O'Shea *et al.*, 2000; Chan & McNally, 2008).

Reverse transcription PCR is the most commonly used molecular method to test for HIV-1 in semen samples. Variations of the method include, i) Multiplex PCR, allowing the simultaneous detection of two or more viruses (Canto *et al.*, 2006), ii) nested-PCR, where two successive amplification reactions can increase sensitivity and specificity (Garrido *et al.*, 2004a), and iii) real-time PCR, whereby the simultaneous amplification and detection of viral DNA result in reduced cost and time of the assay (Espy *et al.*, 2006; Pasquier *et al.*, 2006). Reverse transcription polymerase chain reaction is a sensitive method for the quantitative detection of HIV in blood plasma (Schutten *et al.*, 2000; Gibellini *et al.*, 2004). However, polymerase inhibitors found in seminal plasma may impede the chain reaction during analyses of semen samples (Dyer *et al.*, 1996), potentially resulting in false negatives, under-quantification or inconclusive results (Garrido *et al.*, 2004b). The effect of inhibitors can be reduced by the processing of semen samples (i.e. the removal of seminal plasma) as well as by the analyses of internal or amplification controls (Espy *et al.*, 2006; Garrido & Meseguer, 2006; Chan & McNally, 2008). Nested-PCR has a lower, lowest limit of detection (LLD), when compared to conventional RT-PCR. Garrido & Messeguer (2006) are of the opinion that the method can be utilized to detect a single viral copy/ml. False negatives obtained by RT-PCR could, therefore, be prevented by using nested-PCR (Meseguer *et al.*, 2002). Nested-PCR is, however, time consuming, costly (Dowell *et al.*, 2001) and not commercially available.

Nucleic acid sequence-based amplification may be less susceptible to inhibition and can be utilized reliably for the detection of seminal HIV-1 (Dyer *et al.*, 1996). However, false negatives have been obtained for African patients infected with non-B subtypes of HIV (O'Shea *et al.*, 2000). The b-DNA assay has been reported to be a specific, sensitive and reproducible method for the quantitative evaluation of HIV-1 RNA in low volumes (100 to 250 µl) of blood plasma, yielding results similar to quantitative PCR (O'Shea *et al.*, 2000; Pachl *et al.*, 1995; Cao *et al.*, 1995; Tsongalis, 2006). Nevertheless, the method is generally not used for the detection of seminal HIV-1. See Table 3.5 for the methods of viral validation utilized for the detection of HIV in processed sperm samples from sero-positive male patients at various ART units.

Table 3.3: Association of HIV-1 with sperm cells.

Method	Finding	Reference
• Electron microscopy • Fluorescence immunochemistry • *In situ* hybridization • PCR	• HIV-1 attach to and penetrates spermatozoa	(Baccetti *et al.*, 1994)
• Isolation of germ cells by an immunomagnetic bead technique • Detection of viral DNA by PCR	• No viral DNA was detected in germ cell populations	(Quayle *et al.*, 1997)
• Immunocytochemistry • *In situ* hybridization • Electron microscopy • *In situ* PCR • Electron microscopy	• No association of HIV-1 with sperm could be demonstrated	(Pudney *et al.*, 1999)
• Atomic force microscopy	• HIV-1 attaches to and merges with sperm cells	(Barboza *et al.*, 2004)
• Nested-PCR • *In situ* PCR	• HIV-1 DNA was found *in situ* in abnormal sperm cells	(Muciaccia *et al.*, 2007)
• Western blot analysis	• HIV-1 binds to mannose receptor on the sperm membrane	(Fanibunda *et al.*, 2008)
• Fluorescence *in situ* hybridization	• HIV-1 provirus was able to integrate into sperm chromosomes	(Wang *et al.*, 2011)

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Table 3.4: Summary of published outcomes for assisted reproductive treatment cycles including intra-uterine insemination (IUI), *in vitro* fertilization (IVF) and intra-cytoplasmic sperm injection (ICSI) (2004-2010), performed for sero-discordant couples where the male is positive.

ART procedure	Cycles	Couples	HIV-1 test of washed fraction		Clinical pregnancy rate	Reference
			DNA	RNA		
IUI	68	25	Yes	Yes	14.7%	(Manigart *et al.*, 2006)
	2840	853	Yes**	Yes**	15.1%	(Bujan *et al.*, 2007a)*
	2400	581	No	Yes	19.0%	(Savasi *et al.*, 2007)
	174	61	No	Yes	12.0%	(van Leeuwen *et al.*, 2009)
	429	151	No	Yes	13.5%	(Nicopoullos *et al.*, 2010)
Total	5911	1671				
IVF	107	76	Yes**	Yes**	29.0%	(Bujan *et al.*, 2007a*)
Total	107	76				
ICSI	73	73	Yes	Yes	46.0%	(Garrido *et al.*, 2004a)
	62	20	Yes	Yes	45.0%	(Manigart *et al.*, 2006)
	394	262	Yes**	Yes**	30.6%	(Bujan *et al.*, 2007a*)
	278	160	No	Yes	23.0%	(Savasi *et al.*, 2007)
	420	181	No	No	45.0%	(Sauer *et al.*, 2009)
Total	1227	696				
IVF/ICSI	38	27	Yes	Yes	60.6%	(Kashima *et al.*, 2009)
	44	28	Yes	Yes	18.2%	(Prisant *et al.*, 2010)
Total	82	55				
TOTAL	7327	2498				

*CREAThE – Centers for Reproductive Assistance Techniques to HIV couples in Europe
** Multicenter study between 8 centers; All centers except 1, performed HIV-1 DNA and RNA validation of sperm samples

Table 3.5: Effectiveness of semen processing for the elimination of HIV-1.

Seminal HIV-1 RNA load (copies/ml)	Processing method	Outcome	Reference
• 83.3% of samples ≥LLD • median 330 • range <50 – 30, 000	• Density gradient centrifugation - 56, 64, 72 and 80% • Percoll® gradients centrifuged at 400g for 30 minutes, followed by • Swim-up into 2 ml human tubal fluid medium at 37°C for 60 min	• HIV-1 RNA load <LLD in all (N=12) processed sperm samples (nested-PCR, in-house; LLD=50 copies/ml) • HIV-1 DNA not determined	(Hanabusa *et al.*, 2000)
• 40.7% of samples ≥LLD • Median 5,500 • Range <20 – 1, 000, 000	• Density gradient centrifugation – 45 and 90% • PureSperm® gradients centrifuged at 300 x g for 20 minutes, followed by • Two washing steps	• HIV-1 RNA detected in 6.4% of (N=125) processed sperm samples (COBAS® AMPLICOR monitor 1.5 (Roche SA, Neuilly sur Seine, France, RT-PCR, LLD=5 viral copies/10^6 spermatozoa) • HIV-1 DNA detected in 1.6% of the processed sperm samples (prototypic assay, COBAS® AMPLICOR Monitor 1.5, (Roche Molecular Systems), LLD=5 viral copies/10^6 spermatozoa)	(Leruez-Ville *et al.*, 2002)
___	• Semen samples were diluted 1:1 with medium and washed – 400 x g for 10 minutes • Pellet diluted with medium to original volume of semen sample • Density gradient centrifugation – 45,70 and 90% • PureSperm® gradients centrifuged at 300 x g for 20 minutes, followed by • Wash step, followed by • Swim-up step into 0.5 to 0.7 ml of medium for 45 minutes	• HIV-1 RNA detected in 7.7% of (N=26) and DNA in none of the processed sperm samples (N=26) (nested-PCR, in-house; LLD=1 copy/ml)	(Garrido *et al.*, 2004a)

Table 3.5 (Continue): Effectiveness of semen processing for the elimination of HIV-1.

Seminal HIV-1 RNA load (copies/ml)	Processing method	Outcome	Reference
• Added *in vitro* 1×10^8	• Density gradient centrifugation – 30, 45 and 90% with 0.25% trypsin added to 45% layer and 10µg/ml trypsin inhibitor added to 30 and 90% density layers • Isolate gradients centrifuged at 700 x g for 30 minutes	• HIV-1 RNA load = 227 and 228 viral copies/ml in duplicate processed sperm pellets, respectively (Bayer VERSANT® HIV-1 RNA 3.0, bDNA; LLD=75 copies/ml) • HIV-1 DNA not applicable	(Loskutoff *et al.*, 2005)
• HIV-1 RNA detected in 100% of semen samples	• Density gradient centrifugation – 45 and 90% • Percoll® gradients centrifuged at 110 x g for 30 minutes, followed by • Three washing steps	• HIV-1 RNA load <LLD in all (N=20) processed sperm samples (Applied Biosystems, Foster City, Ca, USA, Multiplex RT-PCR for tested for HIV and HCV, analytical sensitivity = 500 IU/ml) • HIV-1 DNA not determined	(Canto *et al.*, 2006)
___	• Density gradient centrifugation – 45 and 90% • Allgrad® gradients centrifuged at 560 x g for 22 minutes, followed by • Single washing step	• HIV-1 RNA load <LLD in all (N=13) processed sperm samples • HIV-1 DNA detected in 69.2% of sperm samples • Fermentas Life Sciences, Lithuania, RT-PCR; LLD not provided; and in-house nested-PCR LLD=1 viral copy/ml	(Cardona-Maya *et al.*, 2009)

3.3.3 Assisted reproductive treatment for patients infected with hepatitis C virus (HCV)

The transmission rate of HCV during sexual intercourse is low, even during frequent unprotected intercourse (Wyld *et al.*, 1997). However, the presence of HCV RNA in 12.5% (Bourlet *et al.*, 2002) to 20.0% (Bourlet *et al.*, 2009) of semen samples from chronically infected patients, is cause for caution when considering ART treatment for male patients infected with HCV (Halfon *et al.*, 2006). Sperm cells are not the target cells for HCV (Briat *et al.*, 2005) and semen decontamination procedures should, therefore, be effective in the isolation of sperm cells from HCV positive semen samples. However, the absence of HCV from processed sperm samples must first be confirmed by molecular assays, prior to the therapeutic use of processed sperm samples (Halfon *et al.*, 2006; Bourlet *et al.*, 2009).

3.3.3.1 Semen processing for the elimination of HCV

Results from various studies, summarized in Table 3.6, page 88, indicate that semen processing for the removal of HCV from semen is effective, and because the risk of viral transmission during ART is extremely low, HCV sero-positive male patients have been accepted into ART programs in France since 2000 (Cassuto *et al.*, 2002). Levy *et al.* (2002) and Bourlet *et al*., (2009) demonstrated successful ART for couples where the male is sero-positive, without sero-conversion of the female partner, or vertical transmission of the virus.

Table 3.6: Effectiveness of semen processing for the elimination of HCV.

Processing procedure	Outcome	Reference
• Density gradient centrifugation – 50, 70 and 90% • Percoll® gradients centrifuged followed by • Washing step	• HCV RNA not detected in any of the processed sperm samples from HCV positive semen samples (N=2) • AMPLICOR HCV amplification and detection kit, RT-PCR, Roche SA) (LLD=100 copies/ml)	(Levy *et al.*, 2000)
• Density gradient centrifugation 50, 70 and 90% followed by, • Washing step followed by • Swim-up into 1.1 ml of medium for 60 minutes at 37°C	• HCV RNA not detected in any of the processed sperm samples from HCV positive semen samples (N=4) • COBAS AMPLICOR HCV RT-PCR assay (Roche, Diagnostic systems, Meylan, France) (LLD=100 copies/ml)	(Pasquier *et al.*, 2000)
• Semen samples were diluted 1:1 with medium and washed – 400 x g for 10 minutes • Pellet diluted with medium to original volume of semen sample • Density gradient centrifugation – 45, 70 and 90% • PureSperm® gradients centrifuged at 300 x g for 20 minutes, followed by • Wash step, followed by Swim-up step into 0.5 to 0.7 ml of medium for 45 minutes	• HCV RNA detected in 24% of processed sperm samples (N=21) using in-house nested-PCR (LLD=1 viral copy/ml) • HCV RNA not detected in any of the processed sperm samples (N=21) AMPLICOR Monitor RT-PCR (LLD=600 IU/ml)	(Garrido *et al.*, 2004b)

Table 3.6 (Continue): Effectiveness of semen processing for the elimination of HCV.

• Density gradient centrifugation – 45 and 90% • PureSperm® gradients centrifuged at 300 x g for 20 minutes, followed by • Two washing steps	• HCV RNA not detected in any of the processed sperm samples from HCV positive semen samples (N=51) • HCV RNA amplification by COBAS AMPLICOR (Roche diagnostics) (LLD=50 IU/ml)	(Briat *et al.*, 2005)
• Density gradient centrifugation – 40 and 90% with no washing step	• HCV RNA not detected in any of the processed sperm samples from HCV positive semen samples (N=24) • COBAS AMPLICOR HCV Monitor assay (version 2), RT-PCR (LLD=10 copies/ml)	(Halfon *et al.*, 2006)
• Density gradient centrifugation – 50, 70 and 90% • PureSperm® gradients centrifuged followed by • Washing step	• HCV not detected in any of the processed sperm samples (N=153) from men sero-positive for HCV (N=86). • COBAS AMPLICOR HCV assay (version 2.0) RT-PCR (LLD=100 copies/ml)	(Bourlet *et al.*, 2009)
• Density gradient centrifugation – 40 and 80% • Pureception gradients centrifuged followed by • Washing step, followed by • Swim-up step into 1 ml of medium for 60 minutes	• HCV not detected in any of the processed sperm samples from men sero-positive for HCV and HIV-1 (N=16) • In-house nested-PCR (LLD=1 viral copy/ml)	(Savasi *et al.*, 2010)

3.4 ASSISTING AZOOSPERMIC PATIENTS

The retrieval of sperm from azoospermic patients by testicular biopsy and epididymal aspiration, will result in increased blood and white blood cell concentrations in sperm samples. This may result in an increased risk of viral transmission during ART (Garrido *et al.*, 2009). Bujan *et al.* (2007) suggested that this risk could be reduced by:

- Effective antiretroviral therapy followed by confirmation that blood and seminal viral loads are below the lowest limit of detection.
- Strive to prevent contamination of sperm samples with blood during the biopsy/sperm aspiration.
- Prepare sperm samples by discontinuous density gradient centrifugation, should sufficient motile sperm be retrieved. Alternatively, two washes could be performed when low sperm numbers are obtained.
- The confirmation of the absence of viral copies from washed sperm samples, followed by ICSI.

Successful ART cycles employing these guidelines have been performed without viral transmission (Manno *et al.*, 2003; Nicopoullos *et al.*, 2004; Garrido *et al.*, 2009; Leruez-Ville *et al.*, 2013).

3.5 SELECTION OF ASSISTED REPRODUCTIVE TECHNIQUE

Semen decontamination procedures follow intensive methods of processing and together with the cryopreservation of processed sperm samples, result in low insemination motile counts (IMC) of sperm available for therapeutic use (Ohl *et al.*, 2003; Kato *et al.*, 2006). Leruez-Ville *et al.* (2002), therefore, stated that IUI may not be effective in achieving a pregnancy and the use of IVF or ICSI may frequently be the only viable option. The specific ART procedure to follow will depend on the quality of the purified sperm sample, as well as on the female's aetiology. An IMC of $\geq 10 \times 10^6$ and normal sperm morphology of $\geq 5\%$ is positively correlated with pregnancy rate post-IUI (Nikbakht & Saharkhiz, 2011). Figure 3.1 illustrates suggested cut-off values that would qualify a sperm sample for a specific ART procedure, considering female aetiology.
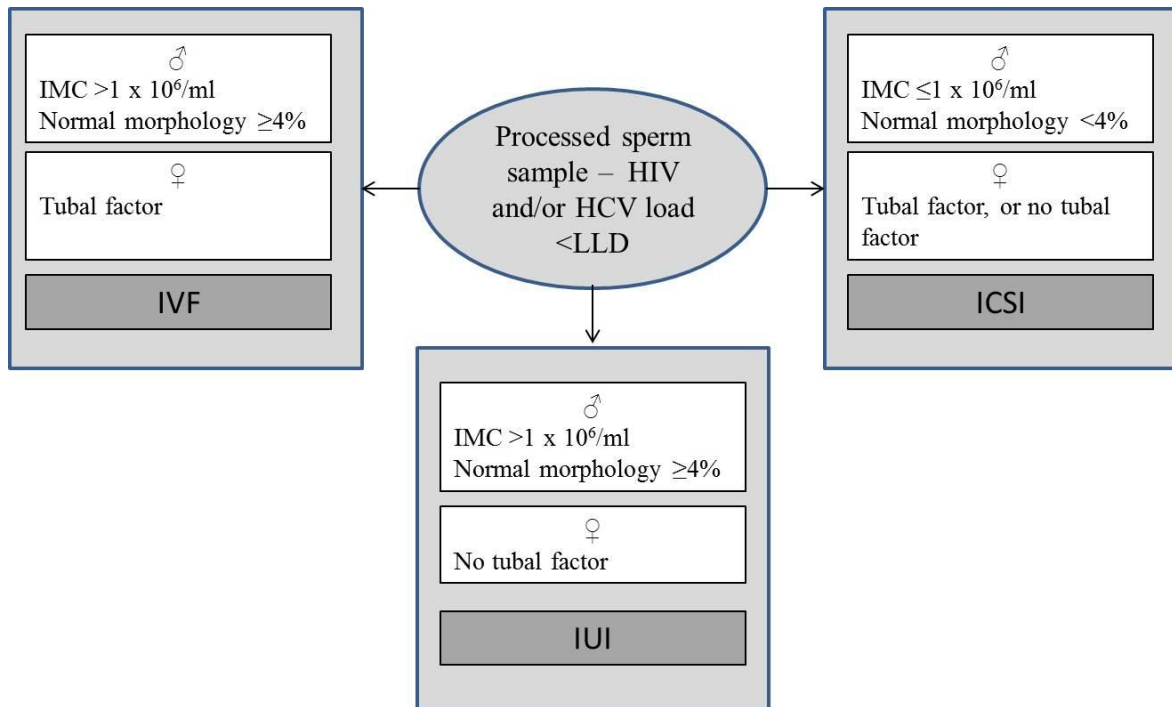
Figure 3.1: Assisted reproductive treatment procedure to follow depending on sperm parameters and female aetiology (adapted from Ombelet, 2003 & 2013; Huyser & Fourie, 2010).

Various factors will influence the choice of an ART procedure, with regards to male subfertility (Tournaye, 2012). According to Meseguer *et al.* (2002) the following should be considered when determining whether to make use of IUI or IVF/ICSI:

- When performing IUI, all available purified sperm should be inseminated to increase the probability for achieving a pregnancy. However, for IVF and ICSI, generally only a fraction of the purified sperm sample is required whereby the remaining sperm will be available for potential follow-up ART cycles.

- The IVF and ICSI procedures ensure that the female partner is exposed to minimal potentially infected sperm when compared to IUI.

- Pregnancy rates after IVF and ICSI is higher when compared to IUI.

The cost of an IVF or ICSI cycle is, however, considerably more than for IUI, and the choice of a specific ART procedure should, therefore, be considered holistically by the clinical and laboratory teams together with informed patients.

3.6 LABORATORY SAFETY

The stringent *in vitro* environment required for the effective culture of embryos, will suit the growth of bacteria and viruses equally well (Englert *et al.*, 2004). The estimated risk of contracting HIV-1 after percutaneous exposure to blood from HIV-1 sero-positive patients is 0.3% (Bell, 1997) and the estimated risk should be similar after percutaneous exposure to semen. Reported cases of nosocomial transfer of blood borne viruses during ART (Lesourd *et al.*, 2000) and the transferral of HIV and hepatitis B and C virus to healthcare workers (Gilling-Smith *et al.*, 2005) demonstrates the potential risk involved when providing ART to patients with potential seminal pathogens. The safety of health care workers, patients and potential offspring must be endeavoured without compromise, by adhering to strict written guidelines when assisting these patients.

According to the European Society for Human Reproduction and Embryology, all patients attending infertility units should be screened for sexually transmissible diseases prior to receiving ART (Magli *et al.*, 2008). Furthermore, post-test counselling must be available for patients with positive results for a chronic viral infection (Englert *et al.*, 2004; Sherr *et al.*, 2007). Englert *et al.* (2004), however, stated that patients should be given the right to refuse viral screening. Biological fluid from these patients should then be handled similarly as samples from patients that are known to be sero-positive for a sexually transmitted infection. In addition to general laboratory safety guidelines (Gilling-Smith & Almeida, 2003), guidelines specifically relevant to the handling of semen samples with potential pathogens (Huyser, 2013) are required and are presented in the following section:

- Staff members must be motivated towards a positive attitude during group discussions and extensively trained prior to the handling of potentially hazardous semen samples (Davidson & Gillies, 1993; Englert *et al.*, 2004).
- Health care workers must be vaccinated against vaccine preventable viruses such as hepatitis B virus (Weber, Rutala & Schaffner, 2010).

- Protective gear must be utilized including: gown/theatre clothes, shoe covers, hairnet, eye protection, gloves and masks (Gilling-Smith *et al.*, 2005; Weber, Rutala & Schaffner, 2010).

- Potentially hazardous samples must be handled in an access controlled, separate laboratory using dedicated equipment. Alternatively, high risk samples could be separated in time, by the batching of blood borne virus-positive patients together. After treating a group of patients with seminal pathogens the laboratory must be cleaned and disinfected before a new cycle with un-infected patients can commence (Gilling-Smith *et al.*, 2005).

- Preparation of semen samples should be performed in a Class II biosafety cabinet providing a sterile environment for the handling of semen samples. The safety of the operator and the prevention of contamination of the air in the laboratory, can be assured by a 100% recirculation of filtered air (Elder, Baker & Ribes, 2004; Englert *et al.*, 2004; Huyser & Fourie, 2010). Validation by certification of the biosafety cabinets should be performed annually (Huyser, 2013).

- No sharp objects and, where possible, only disposable items should be utilised (Gilling-Smith & Almeida, 2003).

- The risk of nosocomial transfer of pathogens in the humidified environment during embryo culture could be minimized, by utilizing incubators with separate compartments that can be dedicated to a specific patient (Gilling-Smith & Almeida, 2003).

- Work surfaces must be cleaned by wiping with distilled water post-handling of samples. Disinfecting agents such as Virkon (DuPont Chemical Solutions Enterprise, Wilmington, DE, USA) could be used to decontaminate working surfaces. However, due to the potential embryotoxic effect of these cleaning products, this should only be performed at the end of the working after all embryos are placed in incubators (Gilling-Smith *et al.*, 2005). Oosafe® (IC Products SA, Minusio, Switzerland) is a disinfectant effective against viruses that does not release volatile organic compounds and is therefore also safe to use in an ART laboratory. Heat resistant equipment such warm blocks could heat sterilized by autoclaving, or by oven sterilization at 180°C for three hours (Elder, Baker & Ribes, 2004).

- The embryo culture laboratory should be cleaned on a regular basis according to standard operating procedures, and cleaning equipment (such as mops and brooms) must be dedicated. See infection control in the IVF laboratory (Junk, 2009).

3.6.1 Precautions taken during the cryopreservation of processed sperm samples

The turn-around time for viral validation of processed sperm samples would typically be more than 48 hours in South Africa (Huyser & Fourie, 2010). The viability of processed sperm samples deteriorates during prolonged incubation *in vitro* and must, therefore, be cryopreserved while awaiting the viral validation results. Viruses, bacteria and fungi have been reported to have the ability to survive in liquid nitrogen (Letur-Konirsch *et al.*, 2003; Abou-Setta, 2004; Levy *et al.*, 2004). Hepatitis B and HIV DNA were found to remain stable during repeated cryopreservation and thawing cycles (8 times) (Krajden *et al.*, 1999). Cryopreservation straws may leak (Russell *et al.*, 1997) whereby contaminating pathogens could potentially be transferred between processed sperm samples during storage in liquid nitrogen.

Nosocomial infection with hepatitis B virus has been reported after transfer of the virus between cryopreserved bone marrow samples during storage (Tedder *et al.*, 1995). Micro-organisms, including potentially pathogenic, bacteria and fungi such *as Escherichia coli* and *Aspergillus* species have been detected in liquid nitrogen dewars (Fountain *et al.*, 1997; Bielanski *et al.*, 2003). Due to the ability to potentially store thousands of samples in a single storage dewar, contamination of the system could result in the widespread transfer of pathogens (Russell *et al.*, 1997). The following guidelines must, therefore, be followed when cryopreserving and storing sperm samples from patients with potential seminal pathogens:

- Samples should be cryopreserved using dedicated equipment and stored in separate liquid nitrogen dewars (Gilling-Smith *et al.*, 2005).
- Specialised cryopreservation straws that can be properly heat-sealed and that are manufactured from shatterproof ionomeric resin such as Cryo Bio System's high security straws (CBS[™], St-Ouen-sur-Iton, France) must be used (Gilling-Smith *et al.*, 2005; Bielanski, 2012).
- An episodic disinfection program of liquid nitrogen dewars could be considered (Bielanski, 2012).

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SECTION B

SEMEN PROCESSING WITH SUPPLEMENTS AND THE IMPACT THEREOF ON SPERM PARAMETERS

CHAPTER 4

THE EFFECT OF SEMEN PROCESSING WITH TRYPSIN ON SPERM PARAMETERS

CHAPTER 5

THE EFFECT OF SEMEN PROCESSING WITH TRYPSIN INHIBITOR ON SPERM PARAMETERS

CHAPTER 4

THE EFFECT OF SEMEN PROCESSING WITH TRYPSIN ON SPERM PARAMETERS

4.1 INTRODUCTION

Washing of sperm in an attempt to eradicate pathogens from semen is performed in various assisted reproduction laboratories (Bujan *et al.*, 2007; Sauer *et al.*, 2009; Nicopoullos *et al.*, 2011). However, pathogens may attach to sperm and be transported during density gradient centrifugation through the density layers (Fiore *et al.*, 2005; Gilling-Smith *et al.*, 2005). Trypsin has, therefore, been added to the upper of two density layers to inactivate and remove bound pathogens from sperm during density gradient centrifugation (see Section 2.2.4.2 for a discussion on semen processing by density gradient centrifugation with supplements). The addition of trypsin could enhance the effectiveness of semen processing procedures for the removal of pathogens, whereby risk reduction during assisted reproductive treatment (ART) can be improved. This has clearly been demonstrated by the effective removal of pathogens bound to the embryos of domestic livestock (Stringfellow & Givens, 2000). The addition of trypsin to density layers, therefore, promises to be a viable approach to improve the effectiveness of semen decontamination. Nevertheless, the effect of trypsin on human sperm parameters must be determined prior to the supplementation of density gradient layers for this procedure to be accepted by clinicians and regulatory officials.

This study was performed to evaluate the effect of trypsin on sperm-zonae binding potential, as well as on the following sperm parameters: vitality, mitochondrial membrane potential and motility.

4.2 EFFECT OF SEMEN PROCESSING WITH TRYPSIN ON SPERM-ZONAE INTERACTION

Binding of capacitated acrosome-intact spermatozoa to the ZP-3 and ZP-4 glycoproteins present on the zona pellucida is a prerequisite for fertilization (Gupta *et al.*, 2009). Trypsin (a proteolytic enzyme) could, however, negatively impact on sperm receptors, whereby sperm binding to the zona pellucida glycoproteins could be hampered. This part of the study was aimed towards the determination of the effect of trypsin on sperm-zonae binding potential.

The hemizona binding test (Arslan *et al*., 2006) is illustrated in Figure 4.1. This bio-assay is utilized to evaluate sperm-zonae binding potential, by physically counting the numbers of treated and untreated sperm that are bound to hemizonae from bisected, non-viable human oocytes (Franken, 1998). Unfertilized, non-vital oocytes remaining from an *in vitro* fertilization (IVF) program maintain their binding ability and can, therefore, be successfully utilized to perform this assay (Henkel *et al.*, 1999). The assay is, therefore, a cost-effective test that can be employed to predict the pregnancy outcome of intra-uterine insemination (IUI) procedures (Arslan *et al.*, 2006), as well as fertilization rates during IVF, with great sensitivity (Oehninger *et al.*, 1989).

4.2.1 METHODS

4.2.1.1 Experimental design

The experimental protocol is diagrammatically summarized and presented in Annexure A, Figure A.1 (see Section E, page 217).

4.2.1.2 Collection of semen

Semen samples (N=5) with parameters in the normal ranges as described by the World Health Organization (WHO, 2010), were received from donors (N=5). The donors were students enrolled at the University of Pretoria (19-26 years old) and were required to abstain for three days prior to delivery of the semen samples. Morphology evaluations were performed according to the Tygerberg criteria (Kruger, Menkveld & Stander, 1986). The semen parameters of these donors are summarized in Annexure B, Figure B1 (see Section E, page 224).

4.2.1.3 Semen processing

The semen samples were split and processed by means of discontinuous density gradient centrifugation, using PureSperm® Pro (Nidacon International, Mölndal, Sweden), with a recombinant, human sequence trypsin (rTrypsin) in the 40% density layer (the exact concentration is not provided due to intellectual property rights), versus standard PureSperm®, not supplemented with rTrypsin. Treated sperm samples were kept at 37°C until insemination of the hemizonae.

4.2.1.4 Source and preparation of human zonae pellucidae

Non-viable, unfertilized oocytes remaining from the Unit's IVF program were bisected by means of micromanipulation (Transferman; Eppendorf, Hamburg Germany) at 200 times magnification (Axiovert 200; Zeiss) using a 3.5 mm, 30 degree blade (BD Micro-Sharp™, Beckton, Dickinson and Company, Franklin Lakes, NJ, USA). Droplets of 50 µl PureSperm® Wash (Nidacon International) were prepared in culture dishes (Falcon 353004; Becton Dickenson Co.) and covered using FertiCult™ mineral oil (FertiPro; Beernem, Belgium). Hemizonae were placed in the micro-droplets and any attached sperm remaining from IVF were stripped by vigorous pipetting using 80 µm pipette (Flexipet®; Cook, Limerick, Ireland). Procedures were performed at room temperature (24°C) and oocytes were bisected on the day of experimentation, within 7 days after being aspirated from the ovaries.

4.2.1.5 Sperm-hemizonae interaction

Hemizonae (N=7 pairs per semen donor) were individually inseminated according to the standard operating procedure for IVF at the Reproductive Biology Laboratory with 1×10^5 progressively motile treated, versus untreated sperm. After an incubation period of 18 hours at 37°C, the hemizonae were transferred to freshly prepared PureSperm® Wash micro droplets. Loosely bound sperm were removed by pipetting 4 times using a 130 µm pipette tip (Flexipet®; Cook). The numbers of tightly bound sperm were counted double-blind by two evaluators at 400 times magnification (Axiovert 200; Zeiss).
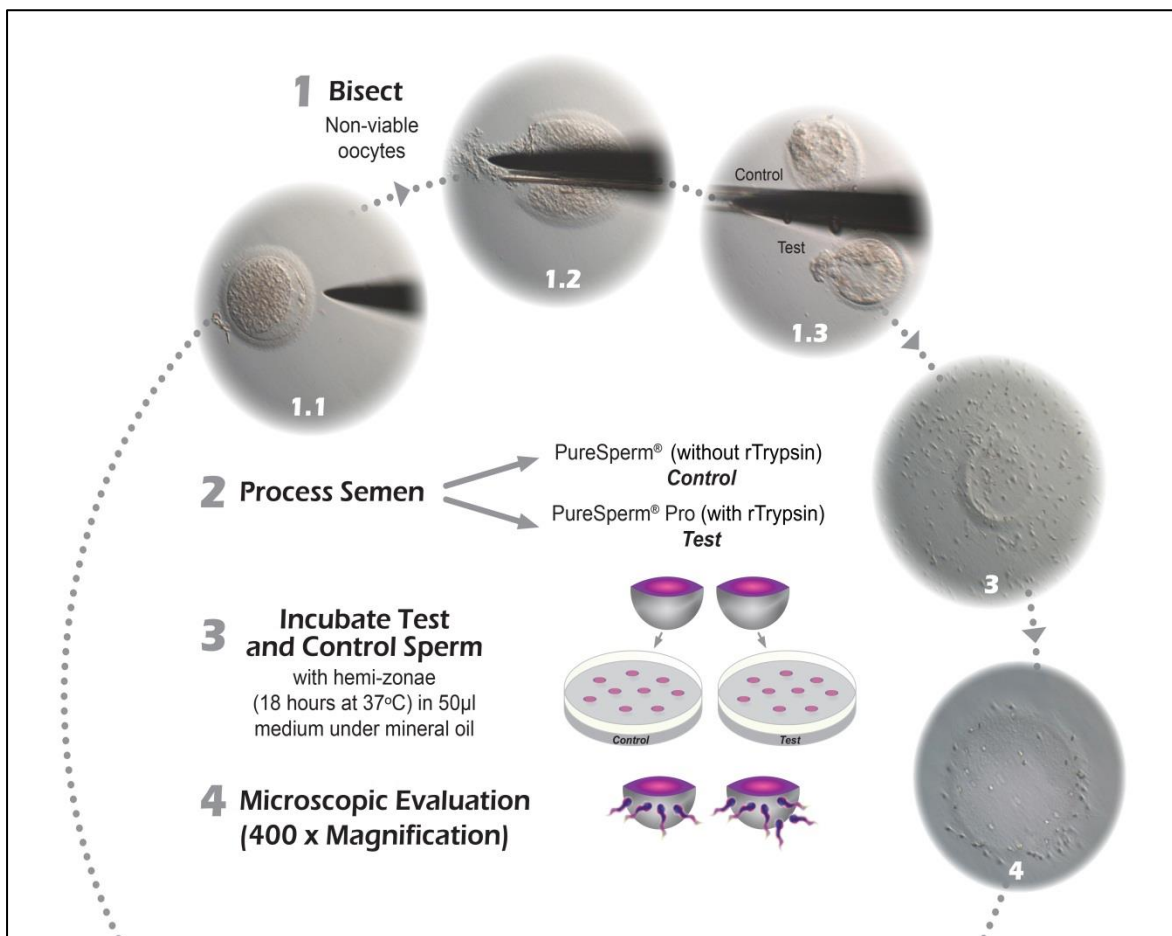
Figure 4.1: Schematic presentation of the hemizona assay.

4.2.1.6 Statistical analysis

The number of sperm bound to hemizonae after semen processing with PureSperm®, or PureSperm® Pro containing the rTrypsin were compared. Stata Statistical Software: Release 10 (StataCorp., 2007) was used to perform a random effects maximum likelihood regression, a mixed model analysis controlling for the dependence of data associated with specific semen donors.

4.2.2 RESULTS

Processing of semen with PureSperm® Pro (containing rTrypsin) did not cause a reduction in the number of sperm bound to the hemizonae when compared to processing with standard PureSperm® (without rTrypsin) (P=0.775). The geometric means (Ando *et al*., 2004) and 95% confidence intervals are summarized in Table 4.1. The numbers and log-transformed numbers of sperm bound to hemizonae post-processing with the PureSperm® products are given in Figure 4.2. Log-transformed numbers of bound sperm were analysed due to a skewed distribution of data when the absolute numbers of bound sperm were analysed.

Table 4.1: Summary of statistics indicating the geometric means of the numbers of sperm bound to the hemi-zonae post-semen processing with Puresperm® and PureSperm® Pro containing recombinant, human sequence trypsin.

Treatment	N	Geometric Mean	95% CI[*]
PureSperm®	23	19.57	14.46 ; 26.49
PureSperm® Pro	24	18.38	13.01; 25.97

[*]95% Confidence interval (CI)

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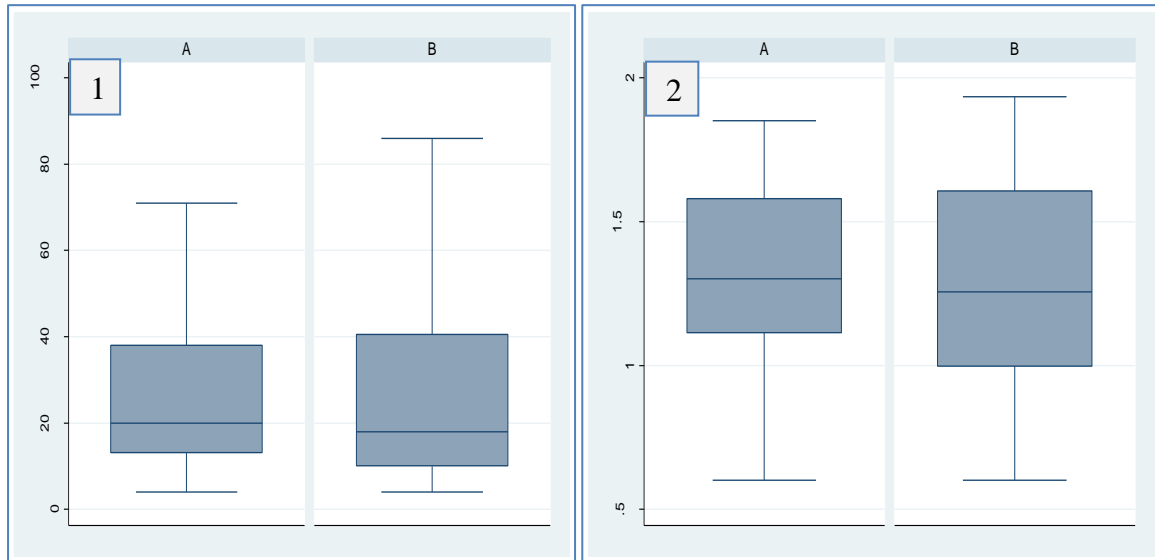
Figure 4.2: Box and whiskers plot indicating the (1) numbers and (2) log-transformed numbers of sperm (N=5 donors) bound to hemizonae (N=7 pairs of hemizonae/semen donor) post-processing with (A) PureSperm® compared to (B) PureSperm® Pro containing recombinant, human sequence trypsin.

4.3 THE EFFECT OF SEMEN PROCESSING WITH DENSITY LAYERS SUPPLEMENTED WITH TRYPSIN AT DIFFERENT CONCENTRATIONS ON SPERM VITALITY, MITOCHONDRIAL MEMBRANE POTENTIAL AND MOTILITY PARAMETERS

Silva, Solana & Castro (1999) reported a decrease in the total and progressive motility as well as vitality, of bovine sperm exposed to 0.3% trypsin for a period of 5 minutes. The negative impact of reduced motility parameters (Donnelly *et al.*, 1998) and vitality by a specific semen processing method, on sperm fertilization potential would render the method unsuitable for use in ART.

This experiment was aimed towards the determination of the effect of trypsin at different concentrations on the following human sperm parameters: vitality, mitochondrial membrane potential and motility.

4.3.1 METHODS

4.3.1.1 Experimental design

Semen samples were processed using discontinuous density gradient centrifugation containing trypsin at different concentrations in the upper density layer. Sperm parameters were evaluated post-processing. The experiment was replicated (N=9) and is diagrammatically illustrated in Annexure A, Figure A.2 (see Section E, page 218).

4.3.1.2 Collection of semen

The collection criteria and the method for delivery of the semen samples from donors (N=18) were similar as described in Section 4.2.1.2. Samples were pooled (N=2 samples/pool) and split prior to processing.

4.3.1.3 Semen processing

Prior to experimentation, the proteolytic activity of the recombinant, human sequence trypsin (rTrypsin) was tested and confirmed by the effective trypsinization (separation and dislodging) of tissue (HeLa) from the substrate according to standard operating procedures (Visagie, 2011). The rTrypsin is not commercially available and was supplied by (Nidacon International, Mölndal, Sweden), therefore the actual concentration is not available because of legal proprietary issues.

Discontinuous density layers (40 and 80%) were prepared by diluting PureSperm® 100% with PureSperm® Buffer (Nidacon International). The commercially available PureSperm® Pro is supplemented with 10 times diluted rTrypsin. Therefore, in this experiment, the rTrypsin was added to the 40% gradient at dilutions of 0, 2, 10 and 20 times. The prepared density layers were stored at 4°C and would be stable for up to 5 months after supplementation (Loskutoff, Morfeld & Crichton, 2003). However, fresh 40% gradient layers containing rTrypsin were prepared weekly. Bottom layers (80%) without any added supplements were used during experimentation.

The pooled semen samples were divided into 4 equal aliquots that yielded the test (N=3) and control (N=1) samples. The samples were processed by discontinuous density gradient centrifugation according to the product manufacturer's guidelines using the supplemented, or non-supplemented, density layers, respectively. Sperm parameters were evaluated 16 to 18 hours after processing.

4.3.1.4 Flow cytometric evaluations

Flow cytometry is viewed as an objective method to evaluate several sperm parameters simultaneously with high sensitivity and repeatability, for high numbers of sperm within a short period of time (Perticarari *et al*., 2007; Martinez-Pastor *et al.*, 2010). Depending on the purpose of the analysis, sperm are labelled with a specific fluorochrome, and individual sperm cells are subjected to a laser beam where after the scattered and emitted light from the sperm is recorded. The fluorescence intensity is measured, and the data is made available to the researcher for interpretation (Gillan, Evans & Maxwell, 2005; Martinez-Pastor *et al*., 2010). Evaluations for this part of the study were performed by using the FC500 flow cytometer (Beckman Coulter, Brea, CA, USA).

4.3.1.4.1 Flow cytometry controls

(i) Induction of apoptosis

Staurosporine induces apoptosis in sperm (Eley *et al.*, 2005; Mahfouz *et al.*, 2009), and was included as a positive control for the validation of the flow cytometry protocol.

The induction of apoptosis was performed as described by Mahfouz *et al.* (2009) with minor adjustments. Staurosporine (Sigma Chemical Co. St Louis, MO, USA) was diluted with dimethyl sulfoxide (DMSO) to a concentration of 1 mM, aliquots were stored as

stock solutions at -20°C. Prior to experimentation, the aliquots were thawed and incubated with sperm at a concentration of 20 μM for 16 hours at 37°C. The sperm samples were washed twice using PureSperm$^{®}$ Wash before the standard staining protocol for Annexin V-FITC was followed.

(ii) Induction of necrosis

Triton X is a surfactant that permeabilizes cell membranes (Jakop *et al.*, 2009). Therefore, enabling the membrane-impermeable nuclear propidium iodide (PI) stain to penetrate cell membranes, and serve as an appropriate positive control during the determination of sperm vitality.

Processed sperm samples were re-suspended to 1 ml with PureSperm$^{®}$ Wash, 2 μl of Triton X-100 (Sigma-Aldrich, St Louis, MO, USA) was added and left to incubate for 15 min prior to staining with PI (BD Biosciences, San Diego, CA, USA) (Falzone, Huyser & Franken, 2010).

(iii) Annexin blocking by recombinant Annexin V (AV)

A negative control for apoptosis was the saturation of AV-FITC binding sites by incubating sperm with purified recombinant AV. The recombinant AV is included as part of the apoptosis detection kit (BD Biosciences).

Sperm cells were washed twice with cold phosphate-buffered saline (PBS) (Sigma-Aldrich) and were then re-suspended in binding buffer (BD Biosciences) at a concentration of 1 x 10^{6} sperm/ml. An aliquot (100 μl) of the solution was transferred to a flow cytometry tube (BD Biosciences), and 10 μg of the purified recombinant AV was added. The samples were gently vortexed and incubated for 15 minutes, prior to staining with AV-FITC and PI.

(iv) Abolishment of mitochondrial membrane potential ($\Delta\psi_m$) by carbamoylcyanide m-chlorophenylhydrazone (mCLCCP)

The incubation of sperm with mCLCCP, an uncoupling agent, will induce the abolishment of $\Delta\psi_m$ (Marchetti *et al.*, 2002). As a positive control during the setup of the flow cytometry protocol for the detection of cells with high $\Delta\psi_m$ ($\Delta\psi_m^{high}$), processed sperm were incubated with 50 μmol/l mCLCCP (Marchetti *et al.*, 2002).

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Carbamoylcyanide m-chlorophenylhydrazone (Sigma-Aldrich) was diluted in DMSO (Sigma-Aldrich) to 1 mM/L (204.6 mg mCLCCP to 1 ml DMSO) and stored as a stock solution at -20°C. Prior to experimentation the stock solution was thawed and 50 µl was added to 5 x 10^{6} sperm cells suspended in 950 µl PureSperm$^{®}$ Wash, the cells were then incubated at 37°C for 15 minutes prior to staining with MitoTracker$^{®}$ (Marchetti *et al.*, 2002).

4.3.1.4.2 Sperm apoptotic and necrotic status

Apoptosis is characterized by the activation of caspases, followed by the externalization of phosphatidylserine (PS) from the inner plasma membrane to be exposed on the exterior of the membrane (Almeida, Sousa & Barros, 2009). Annexin V, a FITC-conjugated, cell membrane-impermeable phospholipid-binding protein, will exclusively bind to PS present on the outside surface of cell membranes (Martin *et al.*, 2005; Hoogendijk *et al.*, 2009). Flow cytometry using a 530 nm wavelength band pass filter can, therefore, be used to identify AV-FITC binding in apoptotic cells. Cell necrosis can be characterized by damaged cell membranes, and cell vitality can be determined by measuring the integrities of sperm membranes (WHO, 2010). Propidium iodide (PI), a membrane-impermeable nucleic acid stain, will penetrate the damaged membranes of dead sperm cells and stain nuclear material (Gillan, Evans & Maxwell, 2005). Necrotic sperm, stained with PI can be identified by examining the cells with light at a wave length of between 562 to 588 nm (Falzone, Huyser & Franken, 2010).

Double staining with AV-FITC and PI will allow for the identification of the following cell populations by means of flow cytometry: i) non-apoptotic cells, which exclude PI and are negative for AV-FITC; ii) apoptotic cells, which exclude PI but are positive for AV-FITC; and iii) secondary necrotic cells, which include PI and are positive for AV-FITC (Oosterhuis *et al.*, 2000).

4.3.1.4.3 Sperm apoptotic and necrotic status post-processing

To determine cell viability (apoptotic/necrotic status), BD Bioscience's AV-FITC apoptosis detection kit II containing AV-FITC and PI was used according to the product manufacturer's guidelines.

Aliquots (5×10^6 sperm) from the processed samples were washed twice; initially in 2 ml of cold PBS (Sigma-Aldrich) and thereafter, in 2 ml of cold AV binding buffer that was supplied as part of the apoptosis detection kit. Sperm pellets (100 µl) were obtained and 5 µl of AV-FITC and PI were added. The samples were vortexed briefly and incubated (24^oC) in the dark for 15 minutes, binding buffer (400 µl) was then added and flow cytometry evaluations were performed within 20 minutes after staining. A minimum of 1×10^4 sperm were analysed from each sample, and AV-FITC and PI stained sperm were detected in the FL1 and FL3 channels, respectively.

4.3.1.4.4 Sperm mitochondrial membrane potential post-processing

Mitochondria generate adenosine triphosphate (ATP) whereby sperm are supplied with energy needed for cell functions, including motility (Ramalho-Santos *et al.*, 2009). Mitochondrial membrane potential ($\Delta\psi_m$) is, thereby, a parameter that is used to evaluate mitochondrial functionality. Sperm with high $\Delta\psi_m$ ($\Delta\psi_m^{high}$) represent a population of sperm with a high number of motile sperm that are able to undergo the acrosome reaction and, therefore, have high fertilizing potential (Gallon *et al.,* 2006). Mitochondrial membrane potential is an essential parameter that should be evaluated to determine the effect of a specific treatment on the energy metabolism of the treated cells.

Mitotracker Red CMX Ros (Molecular Probes, Eugene, USA) (50 µg) was diluted in 100 µl DMSO (Sigma-Aldrich) to prepare a stock solution that was stored at -20^oC. Processed sperm samples (5×10^6 cells) were re-suspended in 1 ml PureSperm® Wash. Mitotracker (2.6 µl from the stock solution) was added to the sperm, and the suspensions were incubated at 37^oC for 15 minutes. The cells were washed, re-suspended in 1 ml PureSperm® Wash, and flow cytometry was performed to determine the percentages of cells with $\Delta\psi_m^{high}$ using the FL3 channel. A minimum of 1×10^4 cells were analysed per examination.

4.3.1.5 Computer-aided semen analysis of sperm motility parameters post-semen processing

Sufficient progressively motile sperm ($>10 \times 10^6$) are required for the successful fertilization of oocytes during IUI (Van Voorhis *et al.,* 2001). Computer-aided semen analysis (CASA) was introduced in the 1980s as a quick and objective method to

accurately evaluate sperm motility parameters, and to assist in the prediction of sperm fertilizing potential (Sidhu *et al.*, 1998; Jedrzejczak *et al.*, 2005).

The CASA system was calibrated by the manufacturing company (medeaLab CASA, Medical Technology Vertriebs GmbH, Altdorf, Germany), and quality control was performed by comparing results obtained by CASA to that obtained by a manual evaluation, performed by an experienced spermatologist (Prof. D. Franken, Department of Obstetrics and Gynaecology, University of Stellenbosch, WHO Semenology expert). Two-chamber, 20 µm deep, Leja® counting chambers (Leja Products, The Netherlands) were pre-warmed to 37°C. Duplicate sperm aliquots (5 µl) were loaded into both chambers, then at least 200 sperm and 10 microscope fields were evaluated per chamber (WHO, 2010) by means of CASA (MediaLAB, version 5.4 Altdorf, Germany) at 200 times magnification (Axioskop 40; Zeiss, Göttingen, Germany). Sperm were tracked and motility was evaluated during a live video recording. Representative tracked paths of sperm are illustrated in Figure 4.3.
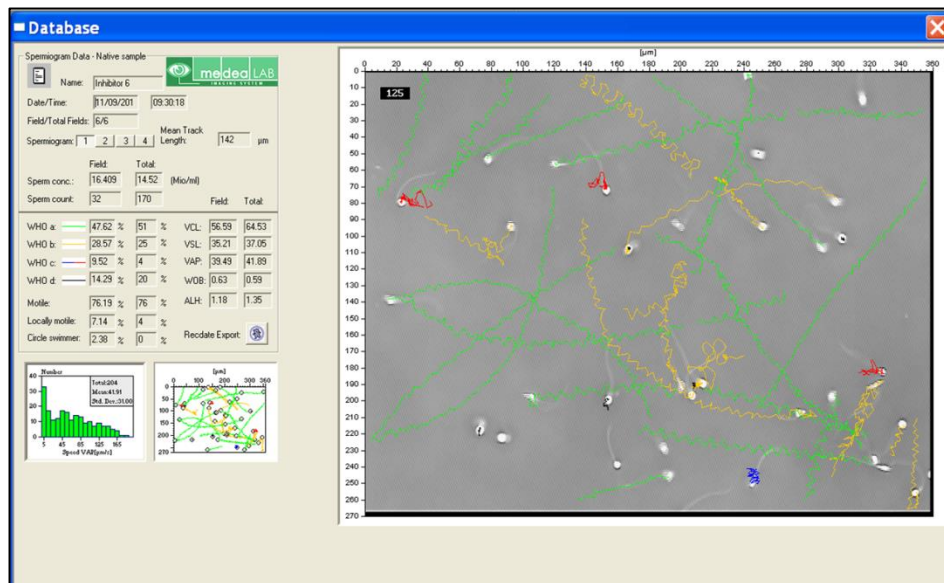
Figure 4.3: Sperm motility paths tracked during computer-aided semen analysis. Green and yellow paths represent sperm that were progressively motile. Blue and red paths were of non-progressively motile sperm and paths indicated in black were of immotile sperm.

4.3.1.6 Statistical analysis

Sperm parameters were examined post-processing using upper density layers containing rTrypsin diluted 20, 10, and 2 times of the concentration (legally protected intellectual property) as supplied by Nidacon International, and were compared to similar processing without rTrypsin. A random effects generalized least squares regression, a mixed model analysis, was employed to compare rTrypsin concentration to zero rTrypsin with respect to sperm parameters (StataCorp., 2007). Each comparison was performed at the Bonferroni adjusted level of significance of 0.017, i.e. 0.05/3, to preserve the 0.05 level of significance (Miller, 1981).

4.3.2 RESULTS

4.3.2.1 Activity of recombinant, human sequence trypsin (rTrypsin)

The rTrypsin used to supplement the PureSperm$^{®}$ upper gradient layer, was confirmed to be active, by the confirmation of enzymatic peptide bond breakage. The incubation of cultured HeLa cells with the rTrypsin resulted in the cells becoming round and dislodged from the bottom of the cell culture dish after two minute incubation at 37°C (Figure 4.4).
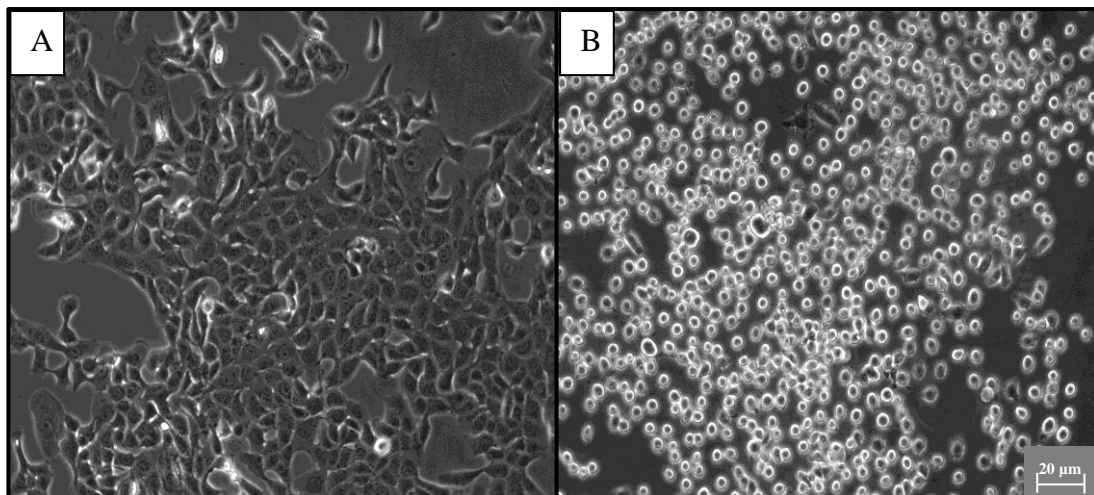
Figure 4.4: HeLa cells magnified 200 times (A) attached to the bottom of a tissue culture flask, and (B) after trypsinization by the addition of recombinant, human sequence trypsin.

4.3.2.2 Sperm apoptotic and necrotic status

Treatment of sperm with staurosporine (20 μM staurosporine for 16 hours at 37°C) increased the apoptotic status of the sperm population and served as a positive control during flow cytometry. Recombinant AV prevented binding of sperm with AV-FITC and served as an effective negative control during the setup of the flow cytometry protocol. Figure 4.5 illustrates the fluorescence intensities of untreated sperm and sperm treated with staurosporine and recombinant AV prior to staining with AV-FITC.
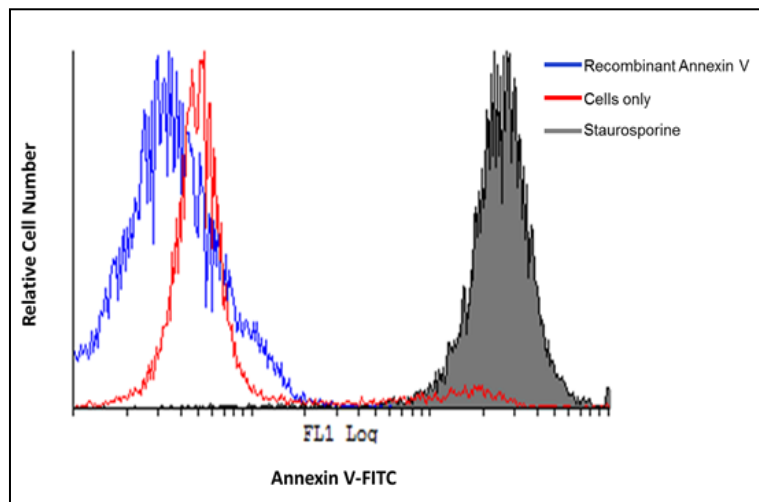
Figure 4.5: Flow cytometric histograms indicating the fluorescence intensities of untreated sperm (red) and sperm treated with staurosporine (grey). Recombinant Annexin V inhibited Annexin V-FITC binding to sperm (blue).

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Sperm necrosis was successfully induced by the incubation of 1 ml sperm (1×10^6 sperm/ml) with 2 µl Triton X for 15 minutes at $24^{o}C$. The necrotic statuses of treated and untreated sperm are illustrated in Figure 4.6.
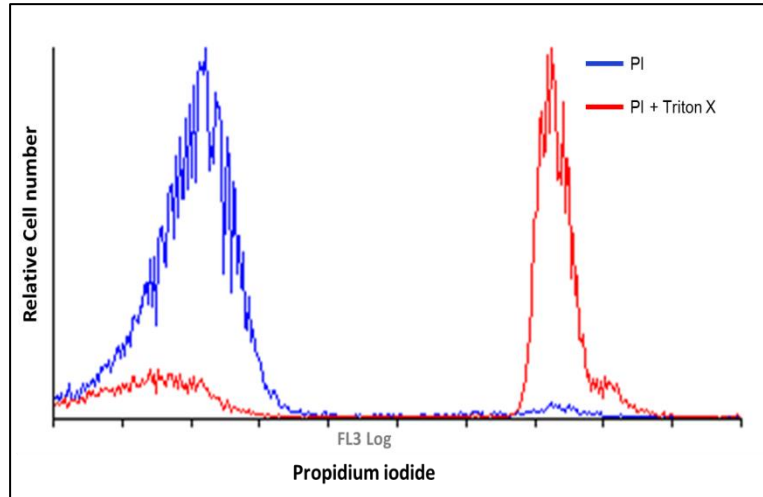
Figure 4.6: Flow cytometric histograms of untreated sperm (blue) and sperm treated with 2 µl/ml Triton X (red) prior to staining with propidium iodide.

Processing of semen using discontinuous density layers supplemented with rTrypsin diluted 20, 10, and 2 times the concentration as supplied by Nidacon International, did not have a negative impact on sperm apoptotic, or necrotic status when compared to processing with non-supplemented density layers. Treated versus non-treated sperm that were non-apoptotic and non-necrotic (Annexin V-PI-), apoptotic (Annexin V+PI-), necrotic (Annexin V-PI+), and apoptotic and necrotic (Annexin V+PI+), are diagrammatically illustrated in Figure 4.7 and the data is presented in Tables 4.2 and 4.3, respectively (page 128).
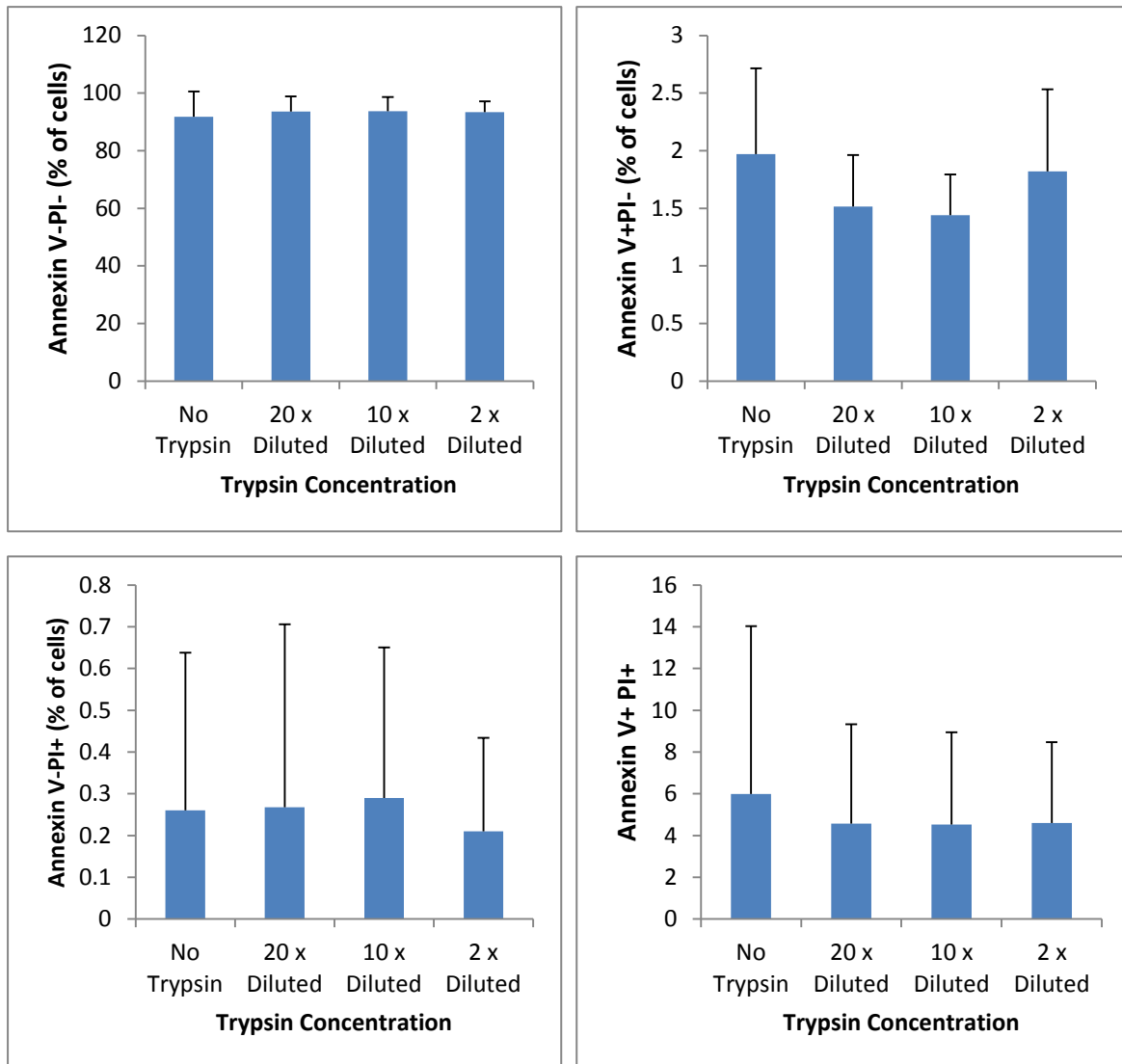
Figure 4.7: Percentages of sperm that were live (Annexin V-PI-), apoptotic (Annexin V+PI-), necrotic (Annexin V-PI+), and apoptotic and necrotic (Annexin V+PI+), after processing with and without recombinant, human sequence trypsin diluted 20, 10 and 2 times in discontinuous density layers.

Table 4.2: Apoptotic/necrotic statuses (AV-PI- & AV+PI-) of sperm treated with recombinant, human sequence trypsin compared to untreated sperm.

Trypsin concentration	N	(AV-PI-)				(AV+PI-)		
		Mean	SD	P-Value		Mean	SD	P-Value
No Trypsin	9	91.772	8.782	−		1.971	0.744	−
20 x Diluted	9	93.641	5.231	0.079		1.516	0.447	0.025
10 x Diluted	9	93.748	4.936	0.064		1.439	0.356	0.009
2 x Diluted	9	93.369	3.801	0.134		1.820	0.714	0.459

Table 4.3: Apoptotic/necrotic statuses (AV-PI+ & AV+PI+) of sperm treated with recombinant, human sequence trypsin compared to untreated sperm.

Trypsin concentration	N	(AV-PI+)				(AV+PI+)		
		Mean	SD	P-Value		Mean	SD	P-Value
No Trypsin	9	0.260	0.378	−		5.996	8.027	−
20 x Diluted	9	0.268	0.438	0.925		4.569	4.760	0.120
10 x Diluted	9	0.290	0.361	0.715		4.521	4.416	0.108
2 x Diluted	9	0.210	0.224	0.543		4.600	3.873	0.129

4.3.2.3 Sperm mitochondrial membrane potential ($\Delta\psi_m$)

Depolarization of sperm $\Delta\psi_m$ was successfully achieved by the incubation of sperm with 50 µmol/l mCLCCP for 15 minutes at 37°C prior to staining with MitoTracker[®]. The fluorescence intensities of MitoTracker[®] stained cells that were treated and non-treated with mCLCCP are indicated in Figure 4.8. The reduction in fluorescence intensity of the treated cells, indicated that mCLCCP served as an effective positive control during the setup of the flow cytometry protocol for the determination of $\Delta\psi_m^{high}$.

Sperm $\Delta\psi_m$ was not reduced by the addition of rTrypsin to the upper discontinuous density layer during density gradient centrifugation (P≥0.09). The percentages of cells with $\Delta\psi_m^{high}$ after processing without and with rTrypsin diluted 20, 10 and 2 times are diagrammatically illustrated in Figure 4.9 and are summarized in Table 4.4 (page 130).
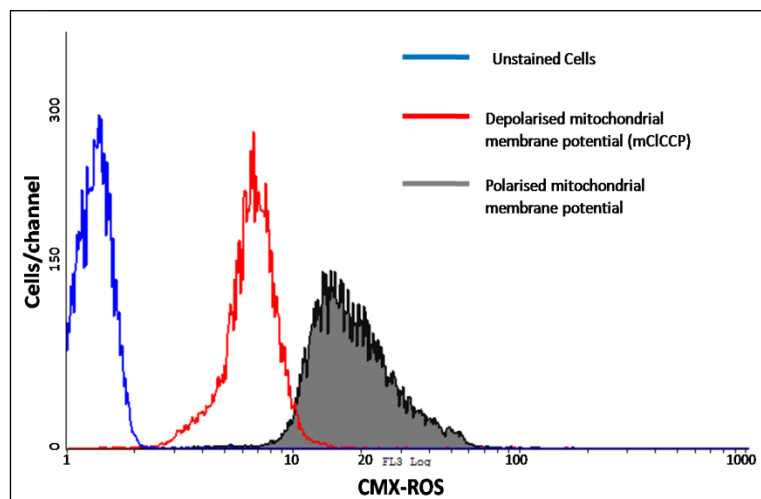
Figure 4.8: Histogram indicating the fluorescence intensities of processed sperm (blue), sperm stained with MitoTracker[®] Red CMX Ros (grey) and sperm treated with 50 µmol/l mCLCCP prior to staining with MitoTracker® Red CMX Ros (red).
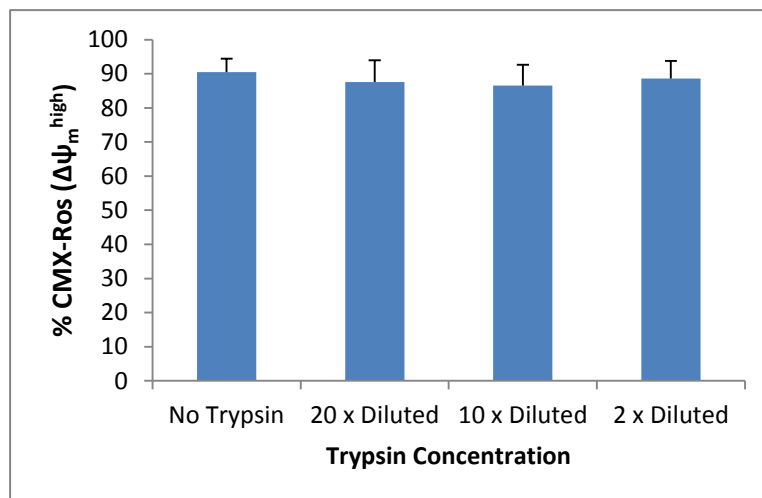
Figure 4.9: High mitochondrial membrane potentials ($\Delta\psi_m^{high}$) of untreated sperm and sperm treated with recombinant, human sequence trypsin after staining with CMX-Ros.

Table 4.4: High mitochondrial membrane potentials ($\Delta\psi_m^{high}$) of sperm treated with recombinant, human sequence trypsin compared to untreated sperm.

Trypsin concentration	N	Mean % ($\Delta\psi_m^{high}$)	SD	P-Value
No Trypsin	9	90.469	3.943	−
20 x Diluted	9	87.549	6.391	0.090
10 x Diluted	9	86.517	6.076	0.022
2 x Diluted	9	88.647	5.073	0.290

4.3.2.4 Sperm motility parameters

None of the measured sperm motility parameters including: progressive motility, average path velocity (VAP), straight-line velocity (VSL), curvilinear velocity (VCL), linearity (LIN=VSL/VCL), straightness (STR=VSL/VCL), or amplitude of lateral head displacement (ALH) were negatively impacted by sperm processing using rTrypsin. Motility parameters are diagrammatically illustrated in Figure 4.10 and are summuarized in Tables 4.5 to 4.8, respectively (page 132-133).
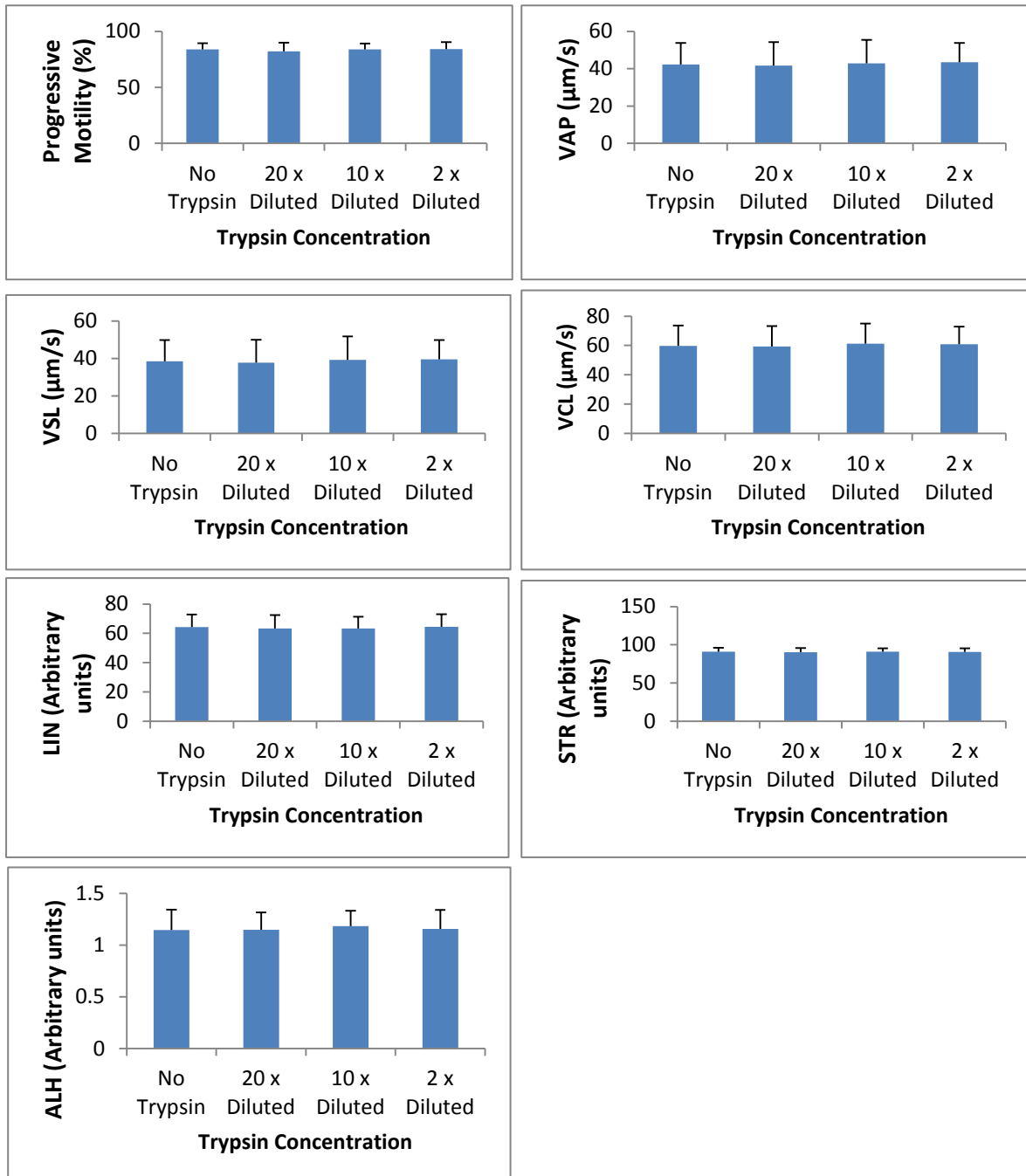
Figure 4.10: Sperm motility parameters of untreated sperm (without trypsin) and sperm treated with recombinant, human sequence trypsin.

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Table 4.5: Total progressive motilities (TPM) and average path velocities (VAP) of sperm treated with recombinant, human sequence trypsin compared to untreated sperm.

Trypsin concentration	N	(TPM - %)			(VAP - µm/s)		
		Mean	SD	P-Value	Mean	SD	P-Value
No Trypsin	9	83.889	5.476	−	42.202	11.52	−
20 x Diluted	9	82.222	7.567	0.281	41.657	12.598	0.703
10 x Diluted	9	84.000	5.220	0.943	42.938	12.465	0.607
2 x Diluted	9	84.222	6.305	0.829	43.408	10.419	0.399

Table 4.6: Straight line velocities (VSL) and curvilinear velocities (VCL) of sperm treated with recombinant, human sequence trypsin compared to untreated sperm.

Trypsin concentration	N	(VSL - µm/s)			(VCL - µm/s)		
		Mean	SD	P-Value	Mean	SD	P-Value
No Trypsin	9	38.474	11.261	−	59.731	13.882	−
20 x Diluted	9	37.793	12.224	0.666	59.225	13.982	0.713
10 x Diluted	9	39.252	12.438	0.621	61.212	13.748	0.281
2 x Diluted	9	39.459	10.367	0.532	60.904	12.010	0.281

Table 4.7: Linearity (LIN) and straightness (STR) of sperm treated with recombinant, human sequence trypsin compared to untreated sperm.

Trypsin concentration	N	(LIN - arbitrary units)				(STR - arbitrary units)		
		Mean	SD	P-Value		Mean	SD	P-Value
No Trypsin	9	64.254	9.057	–		90.878	5.393	–
20 x Diluted	9	63.243	9.887	0.545		90.246	5.696	0.506
10 x Diluted	9	63.389	8.394	0.605		90.914	4.756	0.970
2 x Diluted	9	64.503	9.088	0.881		90.510	4.996	0.698

Table 4.8: Amplitude of lateral head displacement (ALH) after treatment with recombinant, human sequence trypsin compared to untreated sperm.

Trypsin concentration	N	Mean (ALH - arbitrary units)	SD	P-Value
0 x Trypsin	9	1.144	0.199	–
20 x Diluted	9	1.147	0.168	0.910
10 x Diluted	9	1.183	0.148	0.113
2 x Diluted	9	1.157	0.182	0.618

4.4 DISCUSSION

Serine proteases such as trypsin are naturally occurring in semen, it is secreted by the auxiliary sex glands and is involved in liquefaction of semen samples (Paju *et al.*, 2000). However, the effect of the *in vitro* addition of trypsin during density gradient centrifugation, for the inactivation of viruses and to break the peptide pathogen-sperm bonds, must be determined prior to the supplementation of commercial density layers for use in human ART.

The results from this part of the study have been published as a peer-reviewed article (Fourie, Loskutoff & Huyser, 2012) and are presented in Annexure C. This study indicated that semen processing using discontinuous density layers supplemented with rTrypsin diluted 20, 10, and 2 times, did not have a negative impact on sperm apoptotic/necrotic status, $\Delta\psi_m$, or any of the measured motility parameters after a 16-18 hour incubation period. Furthermore, similar numbers of sperm were bound to hemizonae, post-processing using upper discontinuous gradient layers containing trypsin (PureSperm[®] Pro) when compared to the standard density upper layers (PureSperm[®] 40%) not containing rTrypsin, 19.75 vs. 18.38 sperm bound, respectively (P=0.775). The fertilization potential of sperm should, therefore, not be impacted negatively by the addition of rTrypsin to discontinuous density layers. This is in accordance with studies performed on bovine semen where treatment with trypsin had no negative impact on the outcome of ART (Mattson, Devlin & Loskutoff, 2008) for producing bovine embryos *in vitro*. On the contrary, trypsin treatment resulted in increased bovine sperm motility, (Figenschau & Bertheussen, 1999), fertilization rates (88% versus 63%; P<0.01) and the number of *in vivo* produced transferrable bovine embryos (Blevins, de la Rey & Loskutoff, 2008). Silva, Solana and Castro (1999), however, did report damage to bovine sperm cell membranes, as determined by the hypoosmotic swelling test, after treatment with trypsin at 0.3% for 5 minutes, but not at 0.25%.

The increased fertilization potential resulting from trypsin treatment could be as a result of the increased sperm motility. Trypsin is suggested to increase ATP generation by activating glyceraldehyde triphosphate dehydrogenase enzyme (Figenschau, Bertheussen, 1999) and in turn results in improved metabolization of the substrate leading to improved sperm motility. However, in this study, only non-significant (statistically) increases in the

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percentages of progressively motile sperm, VAP, VSL and VCL were observed post-rTrypsin treatment (10x and 2x diluted).

Semen hyperviscosity (SHV), is associated with male infertility, and is present among 26.2% of male partners of sub-fertile couples (Elia *et al.*, 2009). The prevalence of SHV could however be as high as 43.5% in a patient population with sexually transmitted infections such as *Ureaplasma urealyticum* (Zinzendorf *et al.*, 2008). Semen hyperviscosity can impact on various sperm functions, but most importantly it is suggested to negatively influence sperm motility parameters (TPM, VCL, VAP and ALH) (Elzanaty, Malm & Giwercman, 2004). Furthermore, the entrapment of sperm in the highly viscous seminal plasma may possibly result in decreased sperm yield during density gradient centrifugation (Elzanaty, Malm & Giwercman, 2004). Reduced concentrations of prostate-specific antigen, the main proteolytic enzyme found in seminal plasma, in hyperviscous semen samples suggests an association between SHV and proteolytic seminal enzymes (Elzanaty, Malm & Giwercman, 2004). This has also been demonstrated by the successful achievement of reduced viscosity and increased sperm motility after the *in vitro* treatment of viscous semen samples with α-chymotrypsin (Zavos & Zarmakoupis-Zavos, 1997), a serine protease related to trypsin. The differences between trypsin and chymotrypsin are in their substrate specificities, with trypsin being "more effective for breaking (peptide) bonds that are associated with cell to cell, cell to substrate, and cell to enveloped virus adhesions" when compared to chymotrypsin (Walsh, 1970). Trypsin treatment of viscous semen samples using 5 mg trypsin/ml has also been successful to decrease viscosity (Cohen & Aafjes, 1982).

The benefits of trypsin to semen and sperm include: improved liquefaction, reduced viscosity, the inactivation or detachment of peptide-bound enveloped viruses, increased ATP concentration and motility. These benefits together with the lack of a negative impact of trypsin on sperm parameters (at trypsin concentrations of not more than 0.25%), therefore, substantiates its addition to the upper discontinuous layers for the processing of semen by density gradient centrifugation. This would be especially important when providing ART to a patient population with a high incidence of sexually transmitted pathogens, or when sperm yield should be increased during the processing of semen samples with increased viscosity, or inferior motility.

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CHAPTER 5

THE EFFECT OF SEMEN PROCESSING WITH SOYBEAN TRYPSIN INHIBITOR ON SPERM PARAMETERS

5.1 INTRODUCTION

See Section 2.2.4.2 for a comprehensive discussion on semen processing by discontinuous density gradient centrifugation with supplements added to the upper (containing a lower concentration of silica particles) and lower (containing a higher concentration of silica particles) density layers. Treatment of sperm with trypsin at relatively high concentrations (0.3%) may have a potential negative impact on sperm membrane integrity (Silva, Solana & Castro, 1999) due to the proteolytic activity of the enzyme. However, the natural occurrence of trypsin inhibitors in human semen has been demonstrated (Suominen & Niemi, 1972). This suggests that the lower density gradient layers could be supplemented with trypsin inhibitor to prevent the possible negative impact of trypsin on sperm, when using upper density layers containing trypsin. However, trypsin inhibitor could inhibit other sperm acrosomal enzymes required during the fertilization process.

Following sperm-zona pellucida binding during the fertilization process, sperm undergo the acrosome reaction. During the acrosome reaction, acrosomal enzymes are released by exocytosis allowing sperm to penetrate the zona pellucida (Honda, Siruntawineti & Baba, 2002). The primary and most extensively studied acrosomal enzyme is the trypsin-like glycoproteinase, acrosin (Welker *et al.*, 1988; De Jonge *et al.*, 1993; Honda, Siruntawineti & Baba, 2002). High acrosin activity in sperm is correlated with high fertilization rates (De Jonge *et al.*, 1993). Furthermore, the failure of sperm to penetrate the zona pellucida after the inhibition of acrosin clearly demonstrates this trypsin-like enzyme's crucial role during the fertilization process (Liu & Baker, 1993).

The potential inhibition of acrosin by trypsin inhibitors will impact negatively on sperm fertilizing potential (Zaneveld *et al.*, 1971). Therefore, the impact of trypsin inhibitor on sperm parameters should be investigated. This study was aimed towards the determination of the effect of a trypsin inhibitor at different concentrations on sperm parameters.

5.2 METHODS

5.2.1 Experimental design

This experiment was replicated (N=9) and is diagrammatically illustrated in Annexure A, Figure A.3 (see Section E, page 219).

5.2.2 Collection of semen

The collection criteria and the method of delivery of semen samples (N=18) were similar as described in Section 4.2.1.2. After collection, the semen samples were pooled (N=2 samples per pool), and divided in equal aliquots yielding test and control samples that were individually processed.

5.2.3 Semen processing

Soybean trypsin inhibitor has been proven to not impact negatively on sperm motility parameters (Liu & Baker, 1993) and was, therefore, utilized during experimentation in this section of the study. Standard upper density gradient layers (PureSperm® 40%, Nidacon International, Mölndal, Sweden) were layered on top of lower (80%) layers containing soybean trypsin inhibitor at the following concentrations: 10 μg, 20 μg and 100 μg/ml soybean trypsin inhibitor, and as negative controls, semen samples were processed without soybean trypsin inhibitor in the lower layer. Lower density layers were prepared by diluting PureSperm® 100% (Nidacon International) with PureSperm® Buffer (Nidacon International) and soybean trypsin inhibitor (T6522, Sigma-Aldrich, St Louis, MO, USA).

Pooled semen samples were divided into 4 equal aliquots (three test and one control sample) and the samples were processed by means of discontinuous density gradient centrifugation using the prepared density layers according to the manufacturer's guidelines (Nidacon International).

5.2.4 Sperm acrosin proteolytic activity

The determination of the proteolytic activity of sperm acrosin provides an assessment of sperm fertilizing potential and should be performed when a comprehensive evaluation of sperm parameters is required (De Jonge *et al.*, 1993). A gelatin film substrate assay (gel test) according to the methods described by Welker *et al.* (1988) and Henkel *et al.* (1995), with minor adjustments, was utilized to determine the activity of acrosin present in sperm after subjecting samples to soybean trypsin inhibitor. The determination of the acrosin proteolytic activity using the gelatinolytic technique can be successfully utilized to predict fertilizing potential of sperm, a halo formation rate of <60%, or a mean halo diameter of <10 μm predicts poor fertilization rates (Henkel *et al.*, 1995).

5.2.4.1 Preparation of gel slides

Gelatin (Merck, Wadeville, Gauteng, South Africa) was dissolved in distilled water at 50^oC to a concentration of 5% (w/v). A 40 μl droplet of the gel suspension was placed in the centre of a microscope slide (25 x 75 mm) and spread by overlaying another slide on top of the first and then dragging the two slides apart. Slides were stored horizontally overnight at 4^oC. A relative humidity of 60% was maintained by keeping an open container with distilled water in the fridge containing the slides. Fixing of the slides was performed by dipping the slides in phosphate buffered saline (PBS) (Sigma-Aldrich, St Louis, MO, USA), containing 0.05% gluteraldehyde (EMD Bioscience Inc. an affiliate of Merck, Darmstadt, Germany) for two minutes, the slides were then rinsed twice in PBS for 15 seconds, and then rinsed in distilled water for 20 seconds. After fixing, the slides were stored vertically at 4^oC and at a relative humidity of 60% and could be used for up to 3 weeks after fixing (Henkel *et al.*, 1995).

5.2.4.2 Procedure of Gelatinolysis

Processed sperm samples were diluted using Earle's balanced salt solution (Life Technologies, Carlsbad, CA, USA) to a final concentration of 10×10^6 sperm/ml, and were equilibrated for 30 minutes at 37^oC. Smears were prepared by evenly coating the prepared slides with sperm (11.5 μl aliquots), using a cover slide. The slides were left to dry for 10 minutes at 24^oC and were then incubated in a moist chamber (95% relative humidity) at 39^oC for two hours. During this time the sperm cells underwent necrosis (Goodpasture,

Reddy & Zaneveld, 1981), resulting in the degeneration of the cell membranes and the leakage of acrosin onto the gel substrate.

Formed halos were observed at 400 x magnification and measured using a light microscope (Axiovert 200, Carl Zeiss, Göttingen, Germany) fitted with a digital camera (Axiocam, Carl Zeiss) utilizing Axiovision software (Version 4.8 June 2009, Carl Zeiss). Halo formation rates were determined by counting the number of halos per 100 sperm cells. Halo diameters were determined by measuring the halo diameters around 10 sperm cells with normal morphology according to the World Health Organization's criteria (WHO, 2010). Acrosin activity index was determined by multiplying the halo diameter by the halo formation rate (Henkel *et al*., 1995).

5.2.4.3 Inhibition of acrosin with soybean trypsin inhibitor

Liu & Baker (1993) demonstrated by means of the gel test, that soybean trypsin inhibitor, at a concentration of 2 mg/ml, will totally inhibit acrosin activity and, therefore, prevent halo formation during the gel test. The incubation of sperm with soybean trypsin inhibitor (2 mg/ml) for one hour at $37^{o}C$ was included in this study as a positive control.

5.2.5 Sperm apoptotic and necrotic status, mitochondrial membrane potential, and motility parameters post-trypsin inhibitor treatment

Sperm apoptotic and necrotic statuses, mitochondrial membrane potentials, and motility parameters of test and control sperm samples; as well as the control samples that were analysed during the initial development of the flow cytometry protocols, were performed as described in Section 4.3.1.4.1.

5.2.6 Statistical analysis

Sperm parameters were examined post-processing using lower density layers containing soybean trypsin inhibitor at concentrations of 10, 20 and 100 μg/ml, and were compared to similar processing without soybean trypsin inhibitor. A random effects generalized least squares regression, a mixed model analysis, was employed to compare trypsin inhibitor concentration to zero trypsin inhibitor with respect to sperm parameters (StataCorp., 2007). Each comparison was performed at the Bonferroni adjusted level of significance of 0.017, i.e. 0.05/3, to preserve the 0.05 level of significance (Miller, 1981).

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5.3 RESULTS

5.3.1 Activity of soybean trypsin inhibitor

The soybean trypsin inhibitor that was used to supplement density layers was confirmed to be active by the effective inhibition of halo formation after the incubation of sperm with soybean trypsin inhibitor (2 mg/ml for one hour at 37°C).   Halo formation around untreated sperm and sperm incubated with soybean trypsin inhibitor is illustrated in Figure 5.1.A and 5.1.B, respectively. No halos were observed around sperm that were incubated with soybean trypsin inhibitor (2 mg/ml).   The addition of 2 mg soybean trypsin inhibitor/ml to semen, therefore, served as an effective positive control, and the gel test could be considered as an accurate and objective method to test the acrosin activity of sperm.
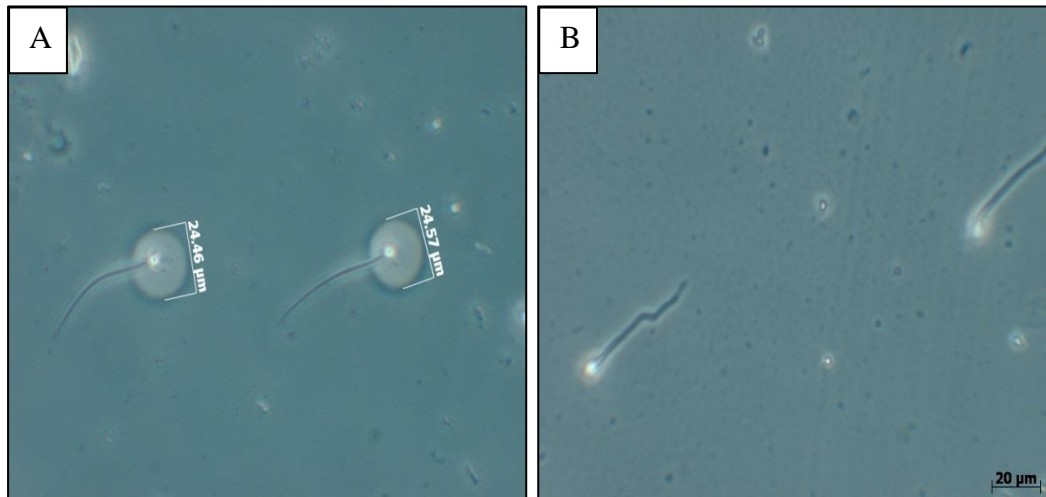
Figure 5.1: Halo formation on gelatin-coated microscope slides, indicating sperm acrosin activity of (A) untreated sperm and (B) the absence of halos around sperm incubated with soybean trypsin inhibitor (2 mg/ml semen) at 400 x magnification.

5.3.2 Sperm acrosin proteolytic activity

The mean halo diameter of control sperm was 25.35 µm [standard deviation (SD) = 2.39]. This is in accordance with halo diameters >19 µm of sperm with high fertilization potential (Henkel *et al.*, 1995). Evaluation of sperm using the gel test, indicated that halo diameter and the acrosin activity index were impacted negatively, by the processing of semen with soybean trypsin inhibitor (Figure 5.2). The processing of semen with the lower density layer containing 100 µg/ml resulted in a statistically significant decrease in halo diameter and acrosin activity index ($P<0.001$). However, halo formation rate was not impacted by semen processing with supplemented density layers. The data is given in Tables 5.1 and 5.2 (page 148).
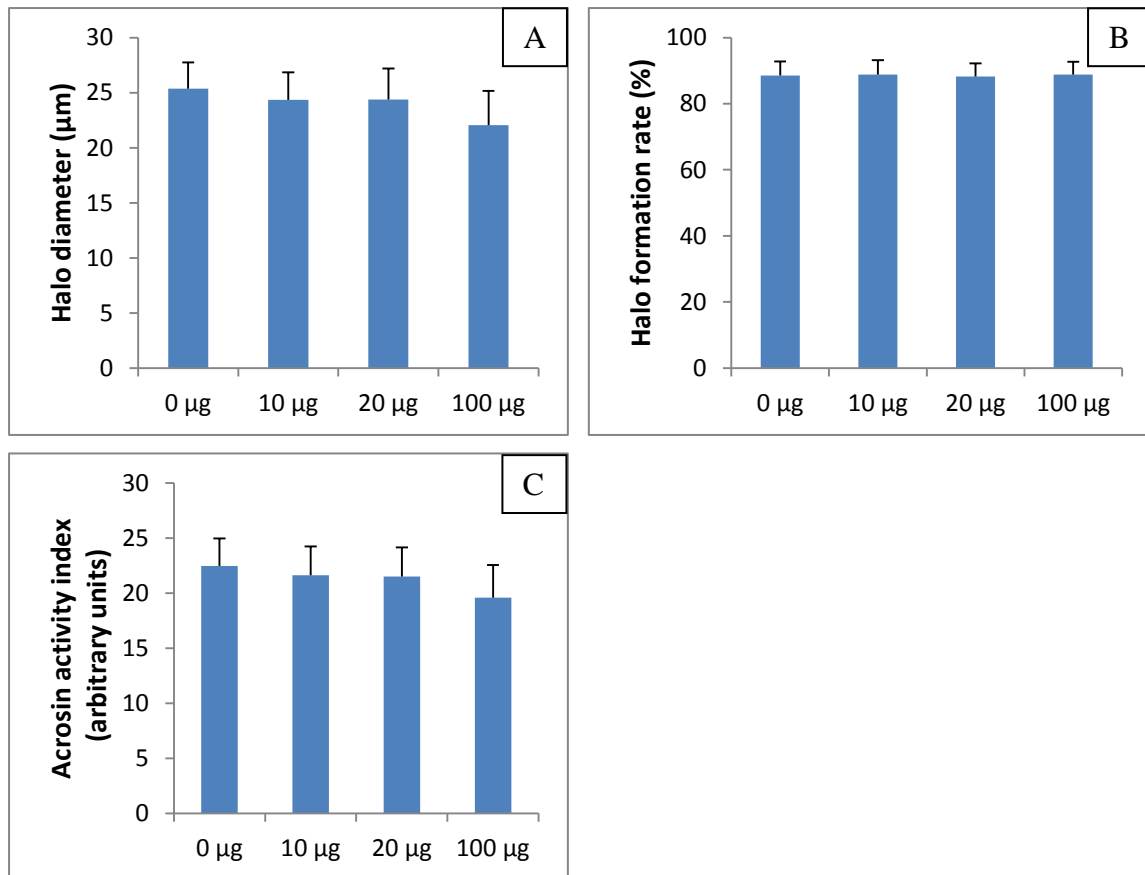
Figure 5.2: Bar charts indicating (A) halo diameters, (B) halo formation rates and (C) acrosin activity indexes of sperm processed by density gradient centrifugation with the lower density layers containing soybean trypsin inhibitor.

Table 5.1: Halo diameters and halo formation rates of sperm treated with soybean trypsin inhibitor compared to untreated sperm.

Trypsin concentration	N	(Halo size - µm)				(Halo formation rate - %)		
		Mean	SD	P-Value		Mean	SD	P-Value
0 µg	9	25.352	2.388	–		88.556	4.187	–
10 µg	9	24.354	2.483	0.038		88.778	4.410	0.411
20 µg	9	24.391	2.791	0.046		88.222	4.024	0.217
100 µg	9	22.066	3.084	<0.001		88.778	3.866	0.411

Table 5.2: Acrosin activity indexes for sperm processed with soybean trypsin inhibitor compared to untreated sperm.

Inhibitor concentration	N	Acrosin activity index (arbitrary units)	SD	P-Value
0 µg	9	22.457	2.501	–
10 µg	9	21.630	2.601	0.043
20 µg	9	21.508	2.639	0.020
100 µg	9	19.594	2.939	<0.001

5.3.3 Sperm apoptotic and necrotic status

Appropriate flow cytometry controls as described in Section 4.3.1.4.1 were included to verify that sperm apoptotic and necrotic statuses were evaluated correctly. The flow cytometry histograms in Figure 5.3 indicate that the treatment of sperm with staurosporine and recombinant Annexin V (AV) served as effective positive and negative controls for the detection of apoptotic sperm. Triton X was successfully utilized to permeabilize cell membranes whereby propidium iodide (PI) could penetrate cells and stain the nuclear material of necrotic cells, see Figure 5.4. The flow cytometry protocol that was utilized to determine sperm apoptotic and necrotic statuses in the following experiments could, therefore, be considered as accurate.
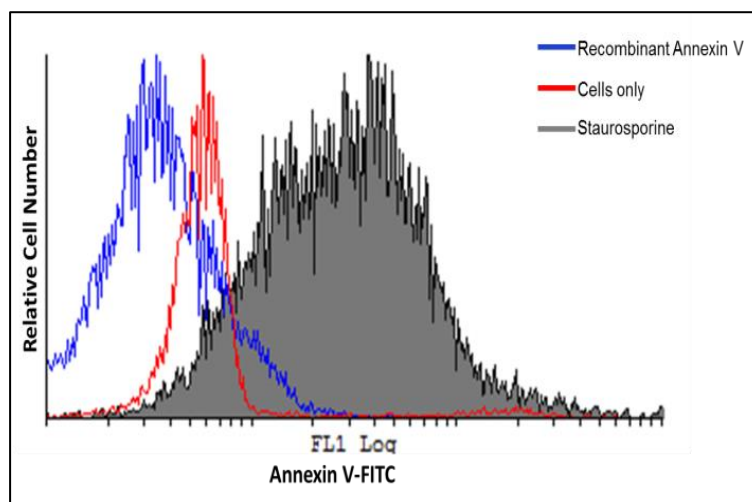
Figure 5.3: Flow cytometric histograms indicating the fluorescence intensities of untreated sperm (red) and sperm treated with staurosporine (grey). Recombinant Annexin V inhibited Annexin V-FITC binding to sperm (blue).
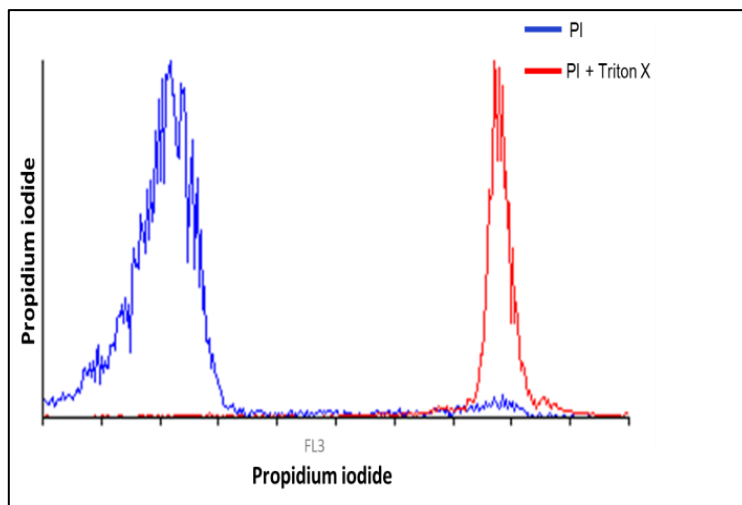
Figure 5.4: Flow cytometry histogram of: i) a non-treated, non-necrotic sperm population (blue); and ii) a necrotic sperm population that was treated with 2 μl/ml Triton X prior to staining with propidium iodide (red).

Processing of semen by means of discontinuous density gradeint centrifugation using lower density layers containing soybean trypsin inhibitor (10, 20 and 100 μg/ml), did not have a negative impact on sperm apoptotic, or necrotic status when compared to untreated sperm. The apoptotic and necrotic statuses of the processed sperm samples that were: i) non-apoptotic and non-necrotic (Annexin V-PI-), ii) apoptotic (Annexin V+PI-), iii) necrotic (Annexin V-PI+), and iv) apoptotic and necrotic (Annexin V+PI+), are diagrammatically illustrated in Figure 5.5 and results are summarized in Tables 5.3 and 5.4 (page 152), respectively.
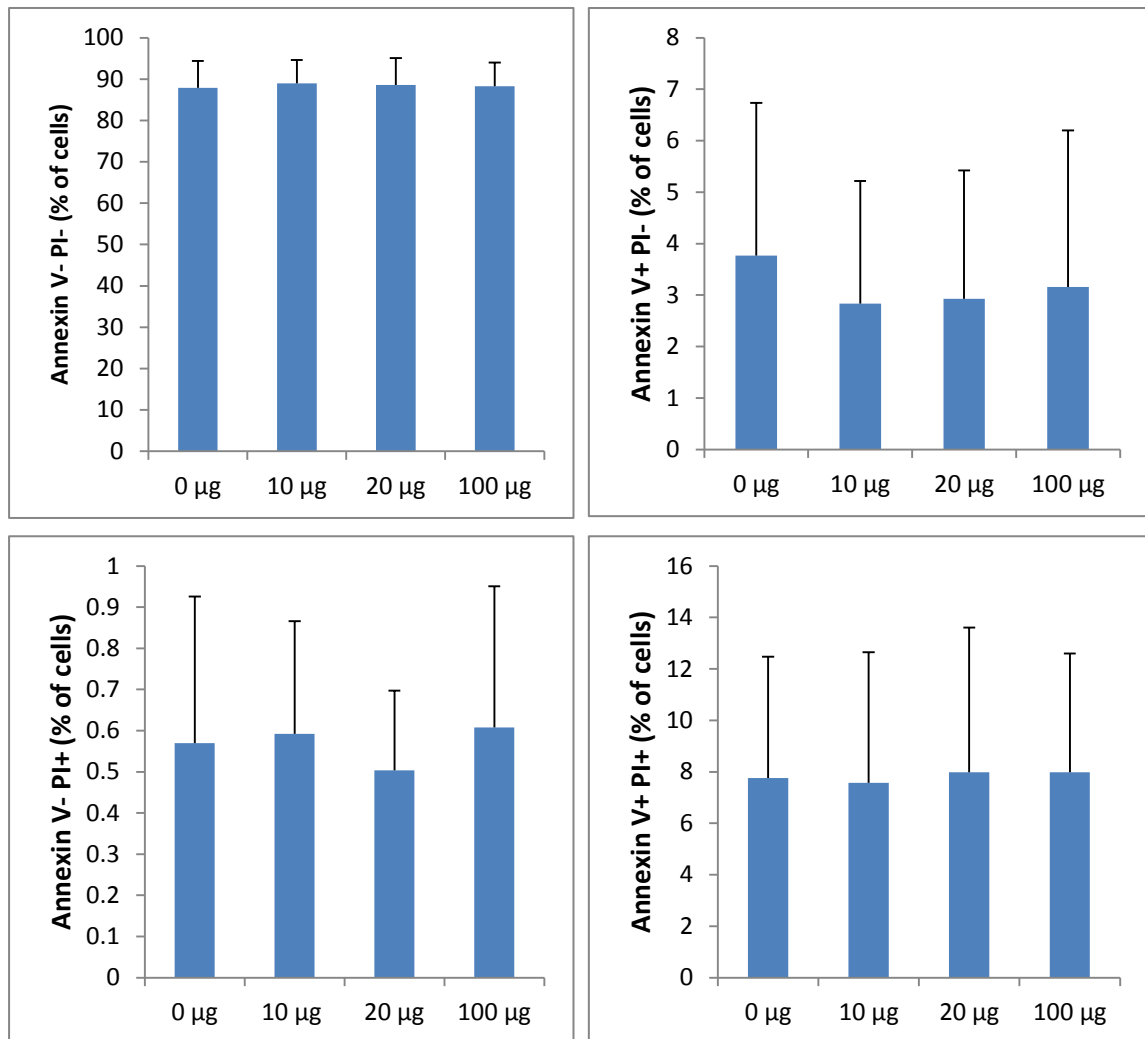
Figure 5.5: Percentages of live (Annexin V-PI-), apoptotic (Annexin V+PI-), necrotic (Annexin V-PI+), and apoptotic and necrotic (Annexin V+PI+) sperm, after processing with and without soybean trypsin inhibitor at different concentrations.

Table 5.3: Apoptotic and necrotic statuses (AV-PI- & AV+PI-) of sperm processed with soybean trypsin inhibitor compared to untreated sperm.

Inhibitor concentration	N	(AV-PI- %)				(AV+PI- %)		
		Mean	SD	P-Value		Mean	SD	P-Value
0 µg	9	87.908	6.454	–		3.764	2.970	–
10 µg	9	89.000	5.641	0.278		2.836	2.380	0.021
20 µg	9	88.582	6.536	0.503		2.923	2.493	0.038
100 µg	9	88.249	5.754	0.735		3.156	3.043	0.131

Table 5.4: Apoptotic and necrotic statuses (AV-PI+ & AV+PI+) of sperm processed with soybean trypsin inhibitor compared to untreated sperm.

Inhibitor concentration	N	(AV-PI+ %)				(AV+PI+ %)		
		Mean	SD	P-Value		Mean	SD	P-Value
0 µg	9	0.570	0.356	–		7.757	4.712	–
10 µg	9	0.592	0.273	0.762		7.571	5.078	0.807
20 µg	9	0.503	0.194	0.363		7.987	5.617	0.762
100 µg	9	0.608	0.343	0.606		7.986	4.607	0.764

5.3.4 Sperm mitochondrial membrane potential ($\Delta\psi_m$)

Treatment of sperm with carbamoylcyanide m-chlorophenylhydrazone abolished high mitochondrial membrane potential ($\Delta\psi_m^{high}$) as is illustrated in Figure 5.6. The protocol for the determination of sperm $\Delta\psi_m$ could, therefore, be considered as accurate.
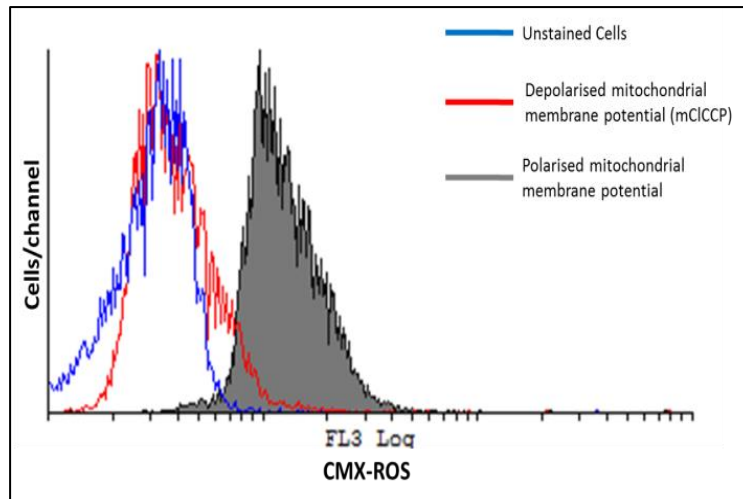
Figure 5.6: Histogram indicating the fluorescence intensities of unstained processed sperm (blue), sperm stained with MitoTracker® Red CMX Ros (grey) and sperm treated with 50 μmol/l mCLCCP prior to staining with MitoTracker® Red CMX Ros (red).

Sperm $\Delta\psi_m^{high}$ was not reduced by the addition of soybean trypsin inhibitor to the lower density layers utilized during semen processing by discontinuous density gradient centrifugation. The percentages of cells with $\Delta\psi_m^{high}$ after processing with soybean trypsin inhibitor at concetrations of 0, 10, 20 and 100 μg/ml are diagrammatically illustrated in Figure 5.7 and the data is given in Table 5.5 (page 154).

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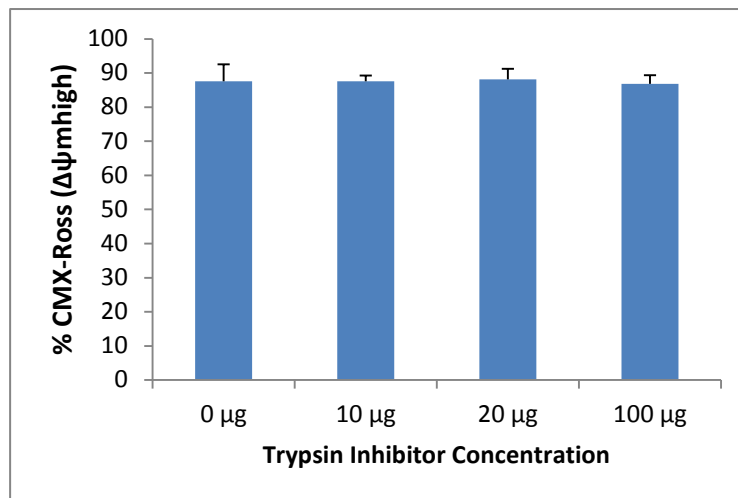
Figure 5.7: The percentages of sperm cells with high mitochondrial membrane potential post-processing with soybean trypsin inhibitor.

Table 5.5: High mitochondrial membrane potentials ($\Delta\psi_m^{high}$) of sperm treated with soybean trypsin inhibitor compared to untreated sperm.

Inhibitor concentration	N	Mean % ($\Delta\psi_m^{high}$)	SD	P-Value
0 µg	9	87.553	4.943	−
10 µg	9	87.570	1.678	0.991
20 µg	9	88.151	3.083	0.679
100 µg	9	86.791	2.581	0.598

5.3.5 Sperm motility parameters

Evaluation of the motility parameters of sperm samples that were processed by density gradient centrifugation with the lower density layers containing soybean trypsin inhibitor, indicated that sperm motility parameters were not impacted negatively by treatment with soybean trypsin inhibitor at concentrations of 10 and 20 µg/ml (Figure 5.8). However, the processing of semen samples with the lower density layers supplemented with 100 µg/ml resulted in significant decreases in total progressive motility (TPM), average path velocity (VAP), straight line velocity (VSL), curvilinear velocity (VCL) and amplitude of lateral head displacement (ALH) ($P \leq 0.002$). The straightness (STR) and linearity (LIN) of sperm motility were not affected by processing with trypsin inhibitor. Mean sperm motility parameters and statistical data are given in Tables 5.6 to 5.9 (page 157 and 158).
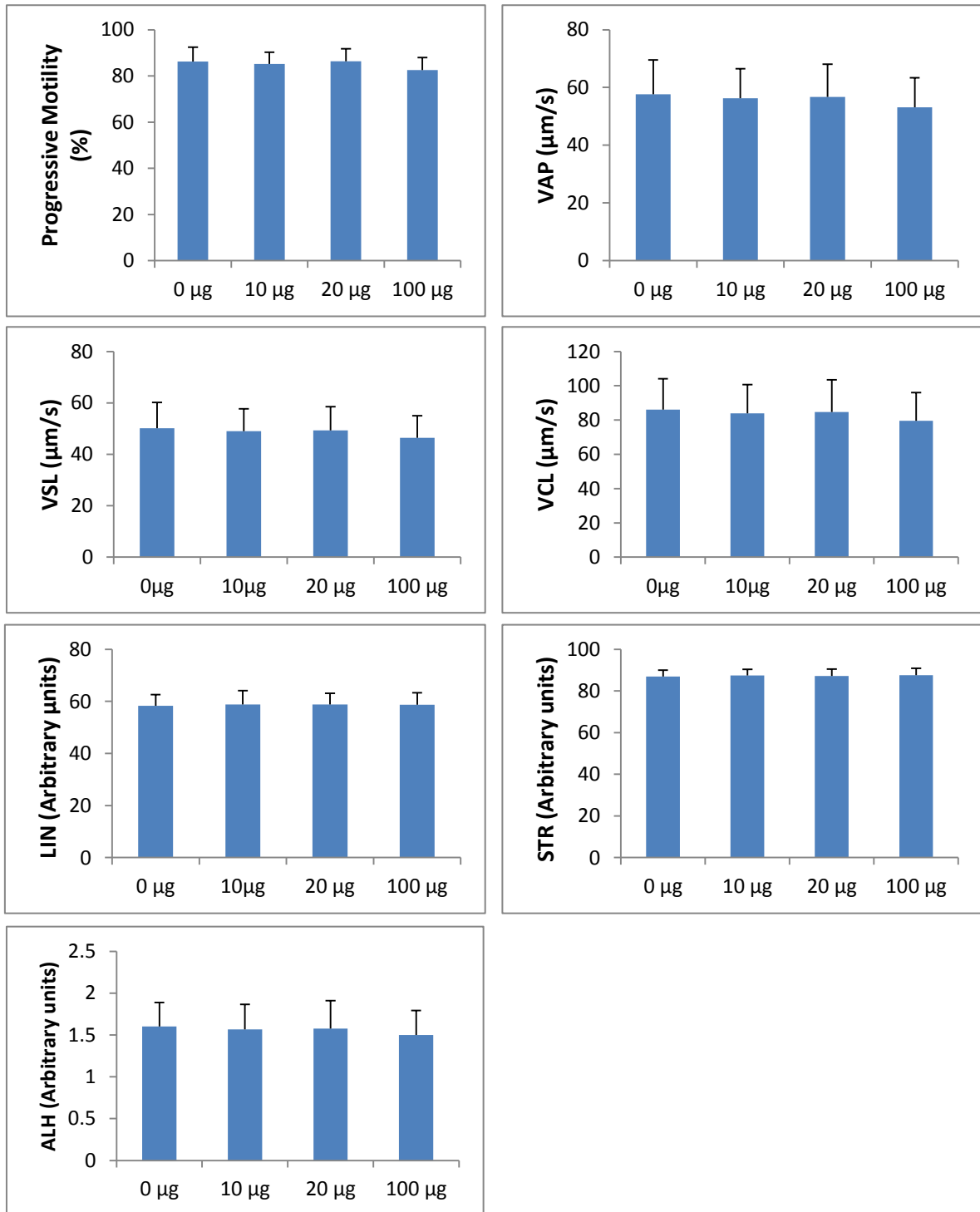
Figure 5.8: Sperm motility parameters of semen samples that were processed by density gradient centrifugation with soybean trypsin inhibitor.

Table 5.6: Total progressive motilities (TPM) and average path velocities (VAP) of sperm treated with soybean trypsin inhibitor compared to untreated sperm.

Inhibitor concentration	N	(TPM - %)				(VAP - μm/s)		
		Mean	SD	P-Value		Mean	SD	P-Value
0 μg	9	86.278	6.140	−		57.685	11.892	−
10 μg	9	85.139	5.089	0.226		56.236	10.293	0.165
20 μg	9	86.389	5.430	0.906		56.747	11.286	0.368
100 μg	9	82.556	5.364	<0.001		53.089	10.272	<0.001

Table 5.7: Straight line velocities (VSL) and curvilinear velocities (VCL) of sperm treated with soybean trypsin inhibitor compared to untreated sperm.

Inhibitor concentration	N	(VSL - μm/s)				(VCL - μm/s)		
		Mean	SD	P-Value		Mean	SD	P-Value
0 μg	9	50.091	10.090	−		86.104	18.040	−
10 μg	9	49.050	8.675	0.269		83.968	16.773	0.106
20 μg	9	49.330	9.161	0.419		84.628	18.802	0.264
100 μg	9	46.408	8.580	<0.001		79.536	16.493	<0.001

Table 5.8: Linearity (LIN) and straightness (STR) of sperm treated with soybean trypsin inhibitor compared to untreated sperm.

Trypsin inhibitor concentration	N	(LIN - arbitrary units)				(STR - arbitrary units)		
		Mean	SD	P-Value		Mean	SD	P-Value
0 µg	9	58.323	4.242	−		86.950	2.994	−
10 µg	9	58.808	5.327	0.603		87.363	2.955	1.15
20 µg	9	58.767	4.346	0.635		87.180	3.259	0.64
100 µg	9	58.655	4.670	0.722		87.585	3.228	1.77

Table 5.9: Amplitude of lateral head displacement (ALH) after treatment with soybean trypsin inhibitor compared to untreated sperm.

Trypsin inhibitor concentration	N	Mean (ALH - arbitrary units)	SD	P-Value
0 µg	9	1.602	0.284	−
10 µg	9	1.566	0.301	0.265
20 µg	9	1.576	0.334	0.410
100 µg	9	1.501	0.291	0.002

5.4 DISCUSSION

Acrosin is a sperm acrosomal serine protease. This protease plays a role during the fertilization process by the digestion of the zona pellucida, a glycoprotein layer surrounding the oocyte, allowing sperm to penetrate the oocyte (Goodpasture, Reddy & Zaneveld, 1981). Llanos *et al*. (1993) reported that the acrosome reaction was inhibited by the addition of trypsin inhibitor to human sperm. This suggests the relevance of a trypsin-like enzyme (potentially acrosin) in the sperm membrane fusion process (Llanos *et al.*, 1993). The significance of acrosin is further demonstrated by the association between low acrosin activity and low sperm fertilizing potential (Langlois *et al.*, 2005). An acrosin activity minimum of 25 μIU/10^6 sperm was, therefore, proposed by using a spectrophotometric assay, to obtain an expected fertilization rate of 50% (Kennedy *et al*., 1989; Langlois *et al.*, 2005). Henkel *et al*. (1995) also indicated that an acrosin activity index of <6 as determined by a gelatinolytic assay, is indicative of sub-fertility.

Trypsin inhibitors act by blocking the conversion of the inactive proacrosin to acrosin (Goodpasture, Reddy & Zaneveld, 1981). Liu & Baker (1993) demonstrated the potential negative impact of trypsin inhibitor on assisted reproductive treatment by preventing the penetration of human sperm through the zona pellucida, after one hour incubation of sperm with soybean trypsin inhibitor (2 mg/ml). The effect of trypsin inhibitors should, therefore, be determined prior to the supplementation of commercially available density layers. Loskutoff *et al*. (2005) reported that the supplementation of the lower density layer with 10 μg soybean trypsin inhibitor, was sufficient to inhibit the 0.25% trypsin that is required for effective semen decontamination procedures. Therefore, an in depth evaluation of the effect of soybean trypsin inhibitor on sperm parameters at the following soybean trypsin inhibitor concentrations: 0, 10, 20 and 100 μg/ml was performed in this study.

Results from the gel test indicated that halo diameters and acrosin activity indexes were significantly lower (P<0.001), for sperm samples that were processed with density layers containing 100 μg soybean trypsin inhibitor, compared to processing with the standard density layers (not containing soybean trypsin inhibitor). Halo formation rates were similar for all treatment groups, indicating that acrosin activity index was decreased due to the reduction in mean halo diameters by processing with soybean trypsin inhibitor. Sperm motility parameters (TPM, VAP, VSL, VCL and ALH) were also impacted negatively by

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processing with 100 µg soybean trypsin inhibitor (P≤0.002). Sperm apoptotic and necrotic statuses, as well as $\Delta\psi_m^{high}$ were similar for all the soybean trypsin inhibitor treatment groups.

Trypsin should be added to the upper density gradient layer at a concentration of not more than 0.25% (Silva, Solana & Castro, 1999). At this trypsin concentration, the trypsin inhibitor should be added at a concentration of 10 µg/ml to effectively inhibit the trypsin (Loskutoff *et al.*, 2005). In the present study, soybean trypsin inhibitor added to lower density layers at a concentration of 10 µg/ml did not have a negative impact on any of the measured human sperm parameters. However, due to the low concentrations of trypsin required to break virus-sperm bonds during sperm decontamination procedures, and due to the removal of sperm from the trypsin containing upper density layer during the washing step after density gradient centrifugation, the addition of trypsin does not impact negatively on the tested sperm parameters. Supplementation of the lower density layer with trypsin inhibitor to block the activity of trypsin on sperm is, therefore, unnecessary.

In conclusion, the addition of trypsin inhibitor to density layers would not be of benefit to semen decontamination procedures, and would increase the manufacturing cost and shorten the shelf life of the density layers. It is therefore recommended that lower density gradient layers used for discontinuous density gradient centrifugation, during semen decontamination procedures, do not require to be supplemented with trypsin inhibitor when the upper density layer is supplemented with trypsin.

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SECTION C

EFFECTIVENESS OF SEMEN PROCESSING FOR THE ELIMINATION OF PATHOGENS

CHAPTER 6

SEMEN PROCESSING FOR THE REMOVAL OF MICRO-ORGANISMS

CHAPTER 7

SEMEN PROCESSING FOR THE ELIMINATION OF WHITE BLOOD CELLS

CHAPTER 8

ELIMINATION OF *IN VIVO* DERIVED HIV-1 DNA AND RNA FROM SEMEN

BACKGROUND

During standard semen density gradient centrifugation processing methods, the supernatant is aspirated after centrifugation to allow access to a purified pellet consisting of motile sperm without seminal plasma and other cellular debris. Pathogens retained in the upper silica particle density layers of this procedure can adhere to the inside surface of the test tube and flow down to potentially re-infect the purified sperm pellet, should the semen contain pathogenic agents such as viruses (Politch *et al*., 2004; Loskutoff *et al*., 2005). Huyser & co-workers (2006) suggested that the re-infection of purified, human sperm samples could be prevented by using a tube insert during centrifugation. The following section of the study was performed to evaluate the effectiveness of semen processing by means of density gradient centrifugation using a novel tube insert for the elimination of seminal pathogens. The principle of using a tube insert is described and illustrated in Section 2.2.4.3.

CHAPTER 6

SEMEN PROCESSING FOR THE REMOVAL OF MICRO-ORGANISMS

6.1 INTRODUCTION

The presence of micro-organisms in semen can negatively impact on the outcome of assisted reproductive treatment (ART), and can result in infection of the female reproductive tract if exposed (Cottell *et al*., 2000; Gdoura *et al*., 2008; Kiessling *et al*., 2008). Micro-organisms include bacteria, fungi, archaea and protists. Viruses and prions are considered as non-living and are therefore not classified as micro-organisms (Biology Online, 2014). A detailed background on bacteriospermia is provided in Section 1.2.6. The purpose of this study was, therefore, to: i) determine the prevalence of bacteria in the semen of men seeking ART at the Reproductive and Endocrine Unit at Steve Biko Academic Hospital (SBAH), and ii) to evaluate the effectiveness of discontinuous density gradient centrifugation; together with a tube insert, for the elimination of prevalent bacteria and yeast from inoculated (spiked) human semen samples. This section of the overall study was published as a peer-reviewed scientific article (Fourie *et al*., 2012) and is presented in Annexure C.

6.2 METHODS

6.2.1 Determination of the prevalence of bacteria in semen

The prevalence of bacteria in semen samples (N=1,210) from men (N=1,038) participating in the ART program at SBAH were surveyed in 2007-2010. Patients were requested to sexually abstain for three days, according to the ART program's guidelines at the Reproductive Biology Laboratory.   Guidelines on how to deliver semen samples aseptically by means of masturbation (World Health Organization, 2010), were verbally described, according to Boucher *et al.* (1995), as well as given to the patients in a written format available in four indigenous languages: English, Afikaans, Sepedi and Zulu. Following liquefaction (37°C for 30 minutes), 200 µl semen aliquots were submitted to the National Health Laboratory's Microbiology Department for microscopy, culture and sensitivity evaluation according to the Unit's standard operating procedure (Working Group, Tshwane Academic Division, National Health Laboratory Service, Department of Microbiology, University of Pretoria, 2006).

6.2.2 Semen processing for the removal of bacteria and yeast from spiked semen samples

Experiments performed in this section of the study are illustrated in Annexure A, Figure A.4 (Section E, page 220). Semen from donors (N=5) were collected, pooled and stained (Gram's method) to ensure the absence of micro-organisms according to standard operating procedure (Working Group, Tshwane Academic Division, National Health Laboratory Service, Department of Microbiology, University of Pretoria 2006).   The pooled sperm concentration was adjusted to 40×10^{6} spermatozoa/ml by dilution with PureSperm$^{®}$ Wash (Nidacon International, Mölndal, Sweden).   Subsequently, 1 ml aliquots of the pooled semen samples were inoculated with bacteria or yeast commonly found in semen. *Escherichia coli* (ATCC 25922), *Enterobacter cloacae* (in-house strain), *Enterococcus faecalis* (ATCC 29212), Coagulase-negative staphylococci (in-house strain), *Staphylococcus aureus* (ATCC 25923) and *Candida albicans* (ATCC 90028), were individually added to the semen aliquots (in duplicate) at concentrations of 1×10^{3}, 10^{4}, 10^{5} and 10^{6} colony forming units (CFU)/ml.   The inoculated semen samples were processed using discontinuous density gradient centrifugation (PureSperm$^{®}$ - 40 and 80%, Nidacon International) with and without the use of a polypropylene tube insert (ProInsert™, Nidacon International), without an additional swim-up step. Bacteria and yeast quantifications were performed by inoculating Mac-Conkey and blood agar plates

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with 10 µl aliquots of the processed sperm samples. The numbers of CFU present were macroscopically counted following a 24 hour incubation period at 37°C. Non-spiked semen samples served as negative controls and unprocessed spiked semen samples were included as positive controls.

6.2.3 Statistical analysis

Stata Statistical Software: Release 10 (StataCorp., 2007) was used to perform a two factor analysis of variance. The main effects were: i) semen processing with, or without the ProInsert™ (Nidacon, International) and ii) micro-organism inoculation concentration (1 x 10^5 and 1 x 10^6 CFU/ml). The outcome variable was log CFU/ml of micro-organisms present in sperm samples after processing.

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6.3 RESULTS

6.3.1 Prevalence of bacteria in semen

The prevalence of gram positive and negative bacteria in semen of patients seeking ART at SBAH is provided in Table 6.1.

Table 6.1: Prevalence of bacteria in semen samples (N=1,210) of patients (N=1,038) participating in an assisted reproductive treatment programme at SBAH (2007-2010).

Organism	Gram	No.	(%)
No Growth		611	50%
Coagulase-negative staphylococci	+	280	23%
Escherichia coli	-	53	5%
Staphylococcus aureus	+	45	4%
α-Haemolytic streptococci	+	45	4%
Enterococcus spp.	+	39	3%
Enterococcus faecalis	+	38	3%
β-Haemolytic streptococci	+	16	2%
Klebsiella pneumoniae	-	17	1%
Enterococcus feacium	+	11	1%
Enterobacter cloacae	-	9	1%
Streptococcus spp.	+	9	1%
Staphylococcus spp.	+	12	1%
Ureaplasma spp.	Null	145	12%
Mycoplasma spp.	Null	57	5%

Pseudomonas spp., *Citrobacter* spp., *Haemophilus* spp., *Micrococcus* spp., *Neisseria* spp., *Bacillus* spp., *Acinetobacter* spp., *Enterococcus* spp. and *Aeromonas hydrophila* were present in <1% of semen samples.

6.3.2 The effectiveness of semen processing for the removal of bacteria and yeast from spiked semen samples

Sperm pellet retrieval using the ProInsert™ prevented re-contamination and removed significantly more micro-organisms (96.0%) from semen compared to processing without the insert [P<0.004 with respect to mean log(CFU)]. Figure 6.1 is a photo of blood agar plates indicating the numbers of *Staphylococcus aureus* CFU present after processing with (6.1 A) and without (6.1 B) the Proinsert™. Bacterial and yeast concentrations (CFU/ml) present after processing with and without the insert are illustrated in Table 6.2, page 169. Treated sperm pellets remained clear of micro-organisms below the spiking concentration of 1 x 10^5 CFU/ml. Figure 6.2 is a bar chart of the number of micro-organisms (CFU/ml) present after semen spiked with 1 x 10^6 CFU/ml were processed with and without the ProInsert™.
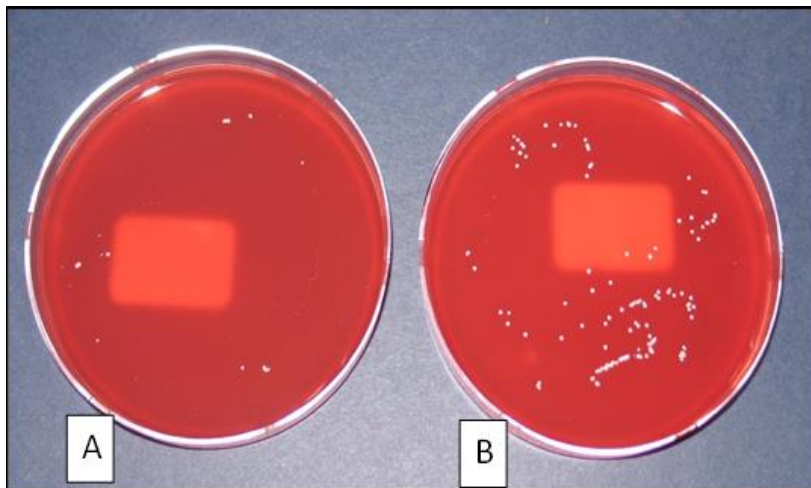
Figure 6.1: *Staphylococcus aureus* colonies after culture (24h) on blood agar plates. Semen samples were spiked with 1 x 10^6 colony forming units/ml. Processed (A) with the ProInsert™ and (B) without the insert.

Table 6.2: Number of bacterial colony forming units (CFU)/ml present in sperm samples post-processing with and without the ProInsert™.

Treatment		Bacteria (CFU/ml) present after processing					
Concentration of innoculated bacteria		*Entero-bacter cloacae*	*Entero-coccus faecalis*	*Escherichia coli*	*Cougulase negative staphylo-cocci*	*Staphylo-coccus aureus*	*Candida albicans*
		In-house	ATCC 29212	ATCC 25922	In-house	ATCC 25923	ATCC 90028
	Without Insert						
1 x 10³	1.1	100	0	100	100	0	0
	1.2	100	0	100	400	0	0
1 x 10⁴	2.1	100	4400	400	600	100	300
	2.2	1100	12100	600	1200	100	400
1 x 10⁵	3.1	4000	24800	23300	2200	2500	700
	3.2	7000	26800	25200	3700	5500	900
1 x 10⁶	4.1	25200	35000	49500	16000	15800	2200
	4.2	29600	36700	63600	23700	16700	1900
	With insert						
1 x 10³	1.1	0	0	0	0	0	0
	1.2	0	0	0	0	0	0
1 x 10⁴	2.1	0	0	0	0	0	0
	2.2	0	0	0	0	0	0
1 x 10⁵	3.1	0	200	100	100	400	0
	3.2	0	200	100	300	900	0
1 x 10⁶	4.1	400	6700	100	1200	1400	200
	4.2	700	9500	100	1400	1500	200
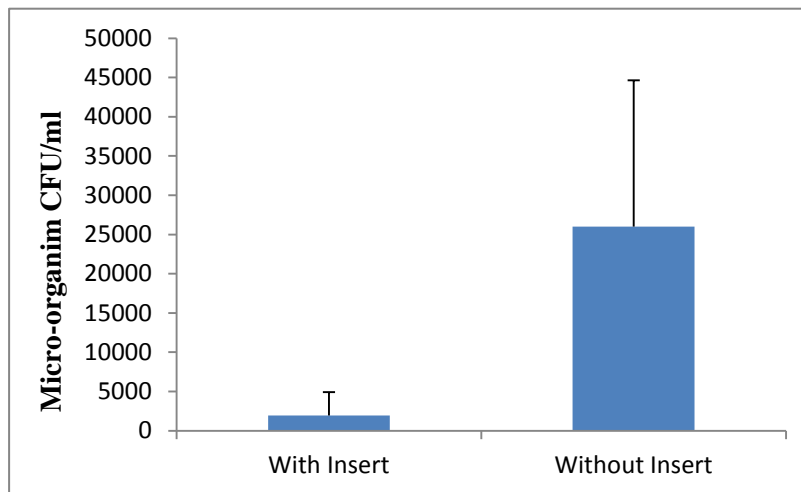
Figure 6.2 The numbers of micro-organisms (five bacterial strains and one yeast) present after semen spiked with 1 x 10^6 colony forming units (CFU)/ml were processed by means of discontinuous density gradient centrifugation, with and without the ProInsert™.

6.4 DISCUSSION

Bacteria were found to be present in 49.5% of semen samples from men seeking ART at SBAH.   This is in agreement with results from studies that reported the bacterial prevalence in unprocessed semen samples to be between 54.0% and 57.0% (Cottell *et al*., 2000; Gdoura *et al*., 2008; Kiessling *et al*., 2008).

The presence of bacteria in semen is mostly (94.2%) due to contamination (Krissi *et al*., 2004) by skin flora (Kim *et al*., 1999). Although patients are instructed to deliver semen samples to SBAH according to guidelines that are provided in a written format (available in 4 languages), the samples may still be contaminated inadvertently. The last line of defence against semen-derived bacterial contamination of the embryo culture system, therefore, is semen processing, by means of strict aseptic techniques and the proper changing of sterile pipette tips and tubes, between the density gradient centrifugation and washing procedures (Nicholson *et al*., 2000). The current study demonstrates that by utilizing the ProInsert™, re-infection of the purified sperm pellets post-density gradient centrifugation was prevented, and significantly (96.0%) more bacterial CFU were removed

from semen when compared to processing without the insert (P<0.004). Treated sperm pellets remained clear of bacteria below the spiking concentration of 1×10^5 CFU/ml.

In conclusion, the high prevalence of seminal pathogens warrants the need for improved semen processing procedures. In the present study, the novel ProInsert™ facilitated density gradient layering, retrieval of the treated sperm pellets without recontamination, and the effective removal of selected seminal pathogens. Used test tubes can be capped, whereby hazardous material is contained and can be appropriately disposed of providing a bio-secure method for the operator.   The ProInsert™ therefore allows for a cost-effective and user-friendly means to improve the effectiveness of discontinuous density gradient centrifugation to eradicate micro-organisms from semen.

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CHAPTER 7

SEMEN PROCESSING FOR THE ELIMINATION OF WHITE BLOOD CELLS

7.1 INTRODUCTION

White blood cells (WBCs) serve as target cells for viruses in semen and are responsible for the generation of reactive oxygen species. See Section 1.2.3 for a detailed description of the impact of leukocytospermia. The removal of WBCs from semen prior to the usage of sperm for assisted reproductive procedures is, therefore, critical. This section of the study was aimed towards the determination of the effectiveness of semen processing with the ProInsert™ for the elimination of WBCs from spiked human semen samples. In order to determine the presence of WBCs in processed sperm samples, a sensitive method for distinguishing accurately between sperm and WBCs, had to be developed.

7.2 METHODS

7.2.1 Experimental design

See Figure A.5 (Section E, page 221) for a diagrammatic illustration of the design of this experiment that was repeated 10 times.

7.2.2 Isolation of white blood cells

White blood cells were isolated from blood using the ACCUSPIN^{TM} System-HISTOPAQUE$^®$ (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer's guidelines.

Each replication of the experiment required one healthy donor of whom eight, 5 ml ethylenediaminetetraacetic acid (EDTA) supplemented vacutainer$^®$ tubes (BD Biosciences, San Diego, CA, USA) of whole blood were collected by a trained healthcare professional. The blood was decanted into the top chamber of an ACCUSPIN^{TM} System-HISTOPAQUE$^®$ tube. The samples were centrifuged at 1000 x g for 10 minutes at 24°C using a swing-out rotor (Eppendorf 5810R, Hamburg, Germany). Aspiration of the plasma layer provided access to the band of mononuclear cells, which formed during centrifugation. The cells were transferred to a clean test tube containing 5 ml phosphate buffered saline (Sigma-Aldrich) and washed by centrifugation at 500 x g for 10 minutes. After aspiration of the supernatant, two further washing steps were performed by re-

suspending the cells in 5 ml of PureSperm® Wash (Nidacon International, Mölndal, Sweden) and centrifugation at 500 x g for 10 minutes. The cells were suspended in 2 ml PureSperm® Wash and mononuclear cell concentrations were determined using a Neubauer hemocytometer (Hauser Scientific, Horsham, PA, USA) according to the World Health Organization's guidelines (WHO, 2010). The isolated WBCs were kept at room temperature (24°C) until the inoculation of semen samples.

7.2.3 Processing of semen to eliminate added white blood cells

Semen samples with parameters in the normal ranges, as described by the WHO (2010) were obtained from donors (N=10). The samples were spiked with WBCs at a concentration of 20×10^6 WBC/ml semen, and divided into test and control samples. Spiked samples were then processed by discontinuous density gradient centrifugation using PureSperm® 40 and 80 (Nidacon International) with, or without the use of the ProInsert™ (Nidacon International). Figure 7.1 represents images photographed at 1000 times magnification (Axiovert 200, Carl Zeiss, Göttingen, Germany) using a digital camera (Axiocam, Carl Zeiss) and Axiovision software (Version 4.8 June 2009, Carl Zeiss), of a (7.1A) raw spiked semen sample and (7.1B) a processed sperm sample after Papanicolaou staining as described the by the WHO (2010). Processed samples were analysed for the presence of WBCs using various flow cytometry (FC500, Beckman Coulter, Brea, CA, USA) methods.
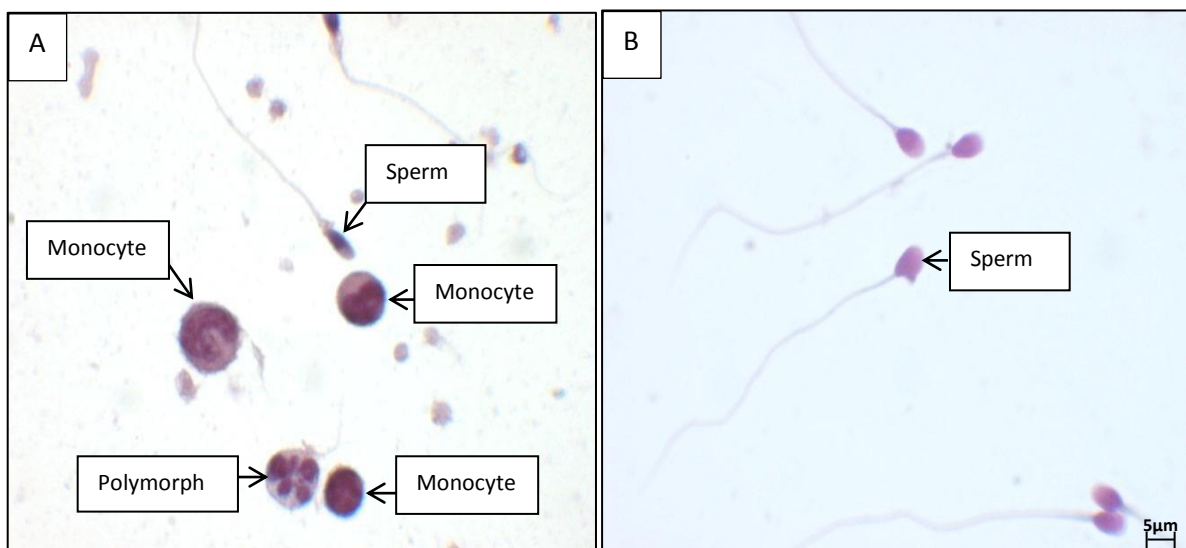
Figure 7.1: Photos taken of Papanicolaou stained: (A) raw semen spiked with WBCs $(20 \times 10^6/\text{ml})$ without density gradient centrifugation processing and (B) sperm after density gradient centrifugation processing of WBC-spiked semen sample (1000 times magnification).

7.2.4 Determination of white blood cell concentrations in processed sperm samples using CD45-FITC

Processed sperm samples were diluted to a final concentration of 1 x 10^6 sperm in 1 ml PureSperm® Wash (Nidacon International). The cells were stained by adding 20 µl of CD45-FITC (Becton Dickenson and Company, San Jose, CA, USA), a monoclonal antibody that will bind to the CD45 antigen present on the membrane surface of all leukocytes (Aitken, West & Buckingham, 1994), including those found in blood and semen (Ricci *et al*., 2000). After staining, the cells were left to incubate for 20 minutes at room temperature (24^oC) in the dark, prior to the evaluation of the samples by flow cytometry.

The initial development of the flow cytometry protocol was performed by analysing CD45-FITC-stained sperm and WBCs versus unstained cells. The accuracy of this protocol was then confirmed by analysing purified sperm cells that were spiked with WBCs at different known concentrations (no WBCs, 5 x 10^3 and 1 x 10^6 WBCs/ml). The fluorescence signal of 1 x 10^4 cells were detected in the FL1 channel.

7.2.5 Determination of white blood cell concentrations in processed sperm samples using propidium iodide (PI)

WBCs contain double the nuclear material of the haploid sperm cells. Therefore, after nuclear staining with PI (BD Biosciences), diploid WBCs should fluoresce at double the intensity of sperm when examined by flow cytometry. The FL3 flow cytometry channel was used to distinguish between WBCs and sperm during experimentation.

The cell membranes of WBCs and sperm needed to be permeabilized to allow for the diffusion of PI into the cells. This was achieved by adding 2 µl of Triton X-100 (Sigma-Aldrich St Louis, MO, USA) to 1 x 10^6 cells suspended in 1 ml of PureSperm® Wash (Nidacon International). Propidium iodide was added (20 µl/million cells) and the cell suspensions were left to incubate at room temperature (24^oC) in the dark for 20 minutes. Flow cytometry was performed by the evaluation of 1 x 10^4 cells in the FL3 channel. Identical concentrations of WBCs and sperm cells as was described in the previous section (7.2.4) were used to gauge the accuracy of this flow cytometry protocol for distinguishing between these cells.

7.2.6 Determination of white blood cell concentrations in processed sperm samples using CD45-FITC in combination with Vybrant$^®$ Dyecycle™ Ruby Stain

Sperm and WBCs present in processed samples were distinguished, by investigating:

(i)   The presence of CD45 antigens (Aitken, West & Buckingham, 1994) by staining with CD45-FITC (BD Biosciences, San Diego, CA, USA) (Ricci *et al*., 2000). However, due to the non-specific binding of CD45-FITC to sperm, as described in Section 7.3.1, cells were also differentiated between based on;

(ii)   The quantity of nuclear material (haploid sperm versus diploid WBCs), by staining with Vybrant$^®$ Dyecycle™ Ruby Stain (Life Technologies, Carlsbad, CA, USA).

Flow cytometry was performed after incubating 1×10^6 sperm suspended in 1 ml PureSperm$^®$ Wash (Nidacon International) with 20 µl CD45-FITC and 1 µl of the Ruby stain for 20 minutes in the dark. Samples were analysed for 2 minutes and CD45-FITC positive and Vybrant$^®$ Dyecycle™ Ruby stained cells were detected in the FL1 and FL6 channels after excitation at 488 nm and 638 nm wavelengths, respectively.

7.2.7 Statistical analysis

Semen processing with the ProInsert™ (Nidacon International) was compared to processing without the insert, with respect to the percentages of WBC's present in post-processed sperm samples. For this within semen sample study design, data analysis employed a random effects maximum likelihood regression, a mixed-model analysis controlling for the dependence of data associated with specific semen donors (StataCorp., 2007).

7.3 RESULTS

7.3.1 White blood cell concentrations in processed sperm samples using CD45-FITC

The WBCs that were isolated from whole blood and used in these experiments were successfully stained using CD45-FITC. Figure 7.2 is a histogram illustrating the increased fluorescence intensity of WBCs after staining with CF45-FITC. The number of CD45 positive cells increased in accordance with the increased WBC spiking concentration, as illustrated in Figure 7.3. However, CD45+ cells were detected in the population of purified sperm cells without added WBCs (Figure 7.3A), demonstrating the non-specific binding of CD45-FITC to sperm. Staining of cells solely with CD45-FITC would, therefore, be ineffective to accurately test for the presence of WBCs in processed sperm samples.
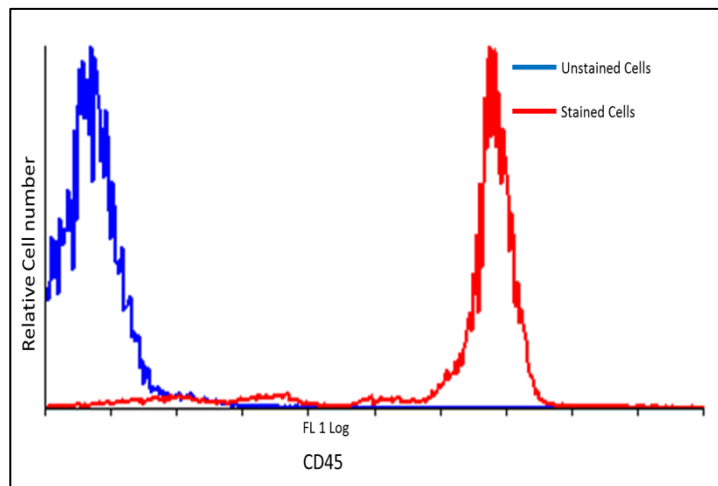
Figure 7.2: Histogram demonstrating the fluorescence intensities of WBCs without (blue) and with (red) the added CD45-FITC.
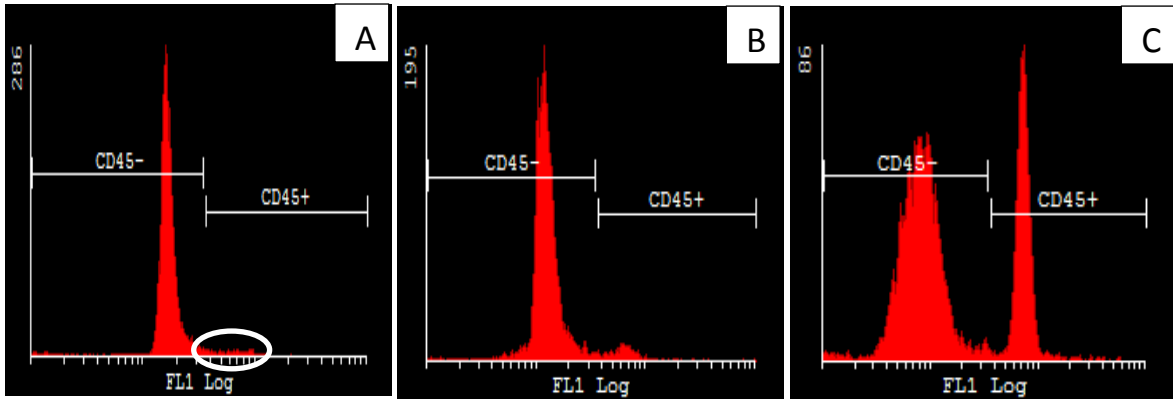
Figure 7.3: Histograms showing the numbers of CD45-FITC positive and negative cells after 1 x 10^6 sperm cells were spiked with (A) no WBCs, (B) 5 x 10^3 WBCs/ml and (C) 1 x 10^6 WBCs/ml.

7.3.2 White blood cell concentrations in processed sperm samples using propidium iodide (PI)

Nuclear DNA was successfully stained using PI (BD Biosciences), whereby haploid sperm and diploid WBC populations could be distinguished from each other in the FL3 channel. The numbers of diploid cells increased in accordance with the increased WBC spiking concentration (Figure 7.4). However, a low number of cells fluoresced with the same intensity as the diploid cells (Figure 7.4A) when un-spiked purified sperm samples were analysed. These cells that would be falsely interpreted as WBCs could be explained as diploid sperm cells (Weissenberg *et al*., 1998). This method would, therefore, not be an accurate and sensitive method to determine the presence of contaminating WBCs in processed sperm samples.
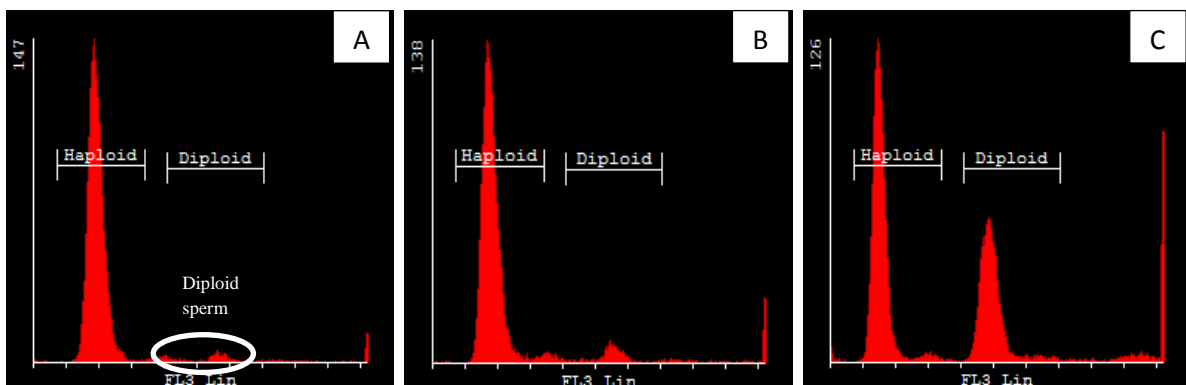
Figure 7.4: Histograms indicating the numbers of haploid and diploid cells stained with propidium iodide after 1 x 10^6 sperm were spiked with: (A) no WBCs, (B) 5 x 10^3 WBCs/ml and (C) 1 x 10^6 WBCs/ml.

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7.3.3 White blood cell concentrations in processed sperm samples using CD45-FITC in combination with Vybrant® Dyecycle™ Ruby Stain

This novel flow cytometry method was effectively utilized to distinguish between sperm and WBCs when purified sperm samples (1 x 10^6 sperm/ml), and sperm samples spiked with WBCs (5 x 10^3 and 1 x 10^6/ml), were analysed as controls during validation of the flow cytometry protocol. The number of WBCs detected by flow cytometry increased in accordance with increased WBC spiking concentrations. Furthermore, no false positives for WBCs were detected when purified un-spiked sperm samples were evaluated (Figure 7.5).
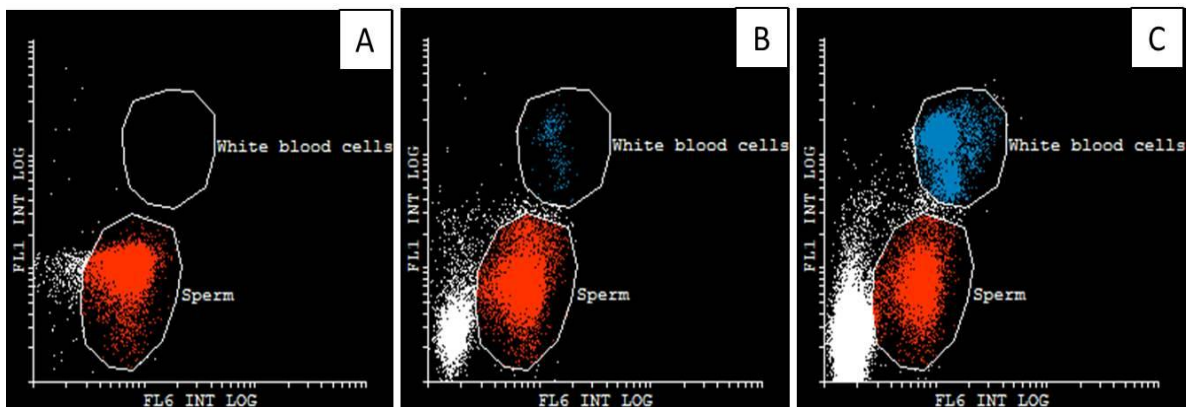
Figure 7.5: Dot plots demonstrating sperm and WBC populations for (A) purified sperm (1 x 10^6/ml) and sperm samples spiked with (B) 5 x 10^3/ml and (C) 1 x 10^6/ml WBCs that served as flow cytometry controls. The fluorescence intensities of CD45-FITC and Vybrant® Dyecycle™ Ruby stained cells are indicated on the Y and X axes, respectively.

7.3.4 Effectiveness of semen processing for the removal of white blood cells from spiked semen samples

Significantly more WBCs were removed from semen (spiked with WBCs 20 x 10^6/ml) by processing using discontinuous density gradient centrifugation in combination with the ProInsert™ when compared to standard processing without the insert (P=0.013). Figure 7.6 is a representative dot plot indicating the numbers of WBCs present in sperm samples after processing with and without the insert. The mean percentages of WBCs remaining in processed sperm samples were 0.2% and 0.8% for samples that were processed with and without the use of the insert, respectively (see Table 7.1, page 181).
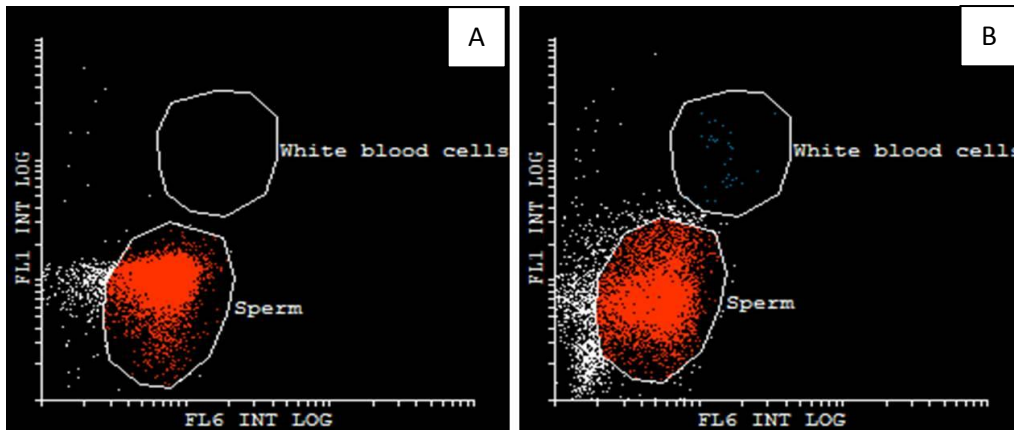
Figure 7.6: Representative dot plots indicating the numbers of white blood cells and sperm present in samples processed with (A) and without (B) the use of the ProInsert™ (Nidacon International). The fluorescence intensities of CD45-FITC and Vybrant® Dyecycle™ Ruby stained cells are indicated on the Y and X axes, respectively.

Table 7.1: Mean percentages of WBCs present in sperm samples processed by means of density gradient centrifugation with and without the ProInsert™.

Insert	Mean (%)	SD*	95% CI**
With	0.148	0.186	-
Without	0.844	0.918	-
Difference	**0.696**	**0.936**	**(0.145; 1.247)**

*Standard deviation (SD) and **95% confidence interval (CI)

7.4 DISCUSSION

Precise determination of the number of WBCs present in processed sperm samples was a prerequisite for this part of the study. CD45-FITC has been utilized previously by other researchers to determine the number of WBCs present in semen samples (Ricci *et al*., 2000). Nevertheless, in this study non-specific binding of CD45-FITC to sperm was shown. Staining of sperm and WBCs with PI allowed for the effective distinguishing between haploid and diploid cell populations. However, diploid sperm are present at a rate of 0.13% in the semen samples of fertile men (Kovács *et al*., 2008), and would falsely be identified as WBCs. Therefore these two staining methods would not be effective to accurately determine the number of contaminating WBCs in processed sperm samples.

A combination of CD45-FITC and a nuclear stain was investigated. Permeabilization of the cells using Triton X to allow for the entry of PI into cells would damage the CD45 antigens, and CD45-FITC would, therefore, not bind to WBCs. Consequently, the cell permeable nuclear stain Vybrant® Dyecycle™ Ruby Stain was used rather than the cell non-permeable PI stain. In the initial development of the appropriate protocol, the numbers of WBCs as detected by flow cytometry, correlated with the the known spiking concentrations (0, 5×10^3 and 1×10^6) in the control samples. Furthermore, no false positives for WBCs were detected by using this protocol. Accurate differentiation between sperm and WBCs was, therefore, possible. Hence, this novel staining method could be utilized to accurately compare semen processing using the ProInsert™ to processing without the insert, with respect to the number of WBCs present in processed sperm samples.

Preliminary experiments indicated that the lowest WBC spiking concentration that resulted in WBC contamination in processed sperm samples was 20×10^6/ml. This spiking concentration of 20 times the WHO's (2010) reference limit for leukocytospermia, was selected as the spiking concentration used in experiments. Similarly as reported after initial experimentation using CD45-FITC for the detection of white blood cells in sperm samples (Huyser *et al.*, 2006); in this study, processing of spiked semen samples by discontinuous density gradient centrifugation indicated that significantly more WBCs were removed from semen when the ProInsert™ was used compared to standard processing without the insert (P=0.013).

Efficient removal of WBCs from semen by using the ProInsert™ should also result in the improved removal of blood borne viruses that have target cells (e.g. leukocytes) other than sperm, whereby risk reduction during assisted reproductive treatment can be improved (Loskutoff *et al*., 2005). The usage of the ProInsert™ is, therefore, highly recommended, specifically when processing semen samples potentially contaminated with pathogens. However, due to low numbers of WBCs still present in processed sperm samples, even when the ProInsert™ was used, it is recommended that aliquots of all processed sperm samples from HIV-positive patients, destined for therapeutic use, be submitted for viral validation prior to use for assisted reproductive treatment.

7.5 REFERENCES

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CHAPTER 8

ELIMINATION OF *IN VIVO* DERIVED HIV-1 DNA AND RNA FROM SEMEN

8.1 INTRODUCTION

Infection with the human immunodeficiency virus (HIV) is no longer considered as life threatening to patients. Improvements made in multidrug highly active antiretroviral therapy has resulted in HIV-acquired immunodeficiency syndrome becoming a manageable, chronic disease (Lambert-Niclot *et al.*, 2012). However, the majority of infected individuals are in their reproductive years with the desire to have their own genetically related offspring (Sauer, 2005; Paiva *et al.*, 2007; Kanniappan, Jeyapaul & Kalyanwala, 2008), and it would be ethically incorrect to refuse assisted reproductive treatment (ART) to these patients (Englert *et al.*, 2001; Gilling-Smith, Smith & Semprini, 2001). This is of significance specifically in regions with a high HIV-1 prevalence such as in South Africa's Gauteng province, with an HIV-1 prevalence of 32.4% (Pillay *et al.*, 2008). A study investigating the sero-prevalence of HIV-1 among patients (N=303) seeking ART at the Reproductive Biology Laboratory (RBL) at Steve Biko Academic Hospital (SBAH), indicated that; 14.4% of couples were sero-discordant (7.1% male positive and 7.3% female positive), and 9.3% of couples were sero-concordant (Maharaj, 2011). A total of 16.4% (9.3% + 7.1%) of male patients seeking ART at SBAH are, therefore, HIV-1 sero-positive.

Shedding of HIV into the semen of infected patients occurs even when patients are receiving highly active antiretroviral therapy, resulting in HIV ribonucleic acid (RNA) and/or deoxyribonucleic acid (DNA) being present in 30.0% of semen samples from these patients (Politch *et al.*, 2012). Assisted reproductive procedures combined with semen decontamination techniques were described elsewhere (Section 2.2.4 and Chapter 3) and should be performed to reduce the risk of vertical and horizontal transmission of the virus (Huyser & Fourie, 2010). However, technique failure does occur, probably due to re-contamination of the processed sperm samples after density gradient centrifugation (Loskutoff *et al.*, 2005), resulting in the detection of HIV in processed sperm samples (Leruez-Ville *et al.*, 2002; Fiore *et al.*, 2005) (see Table 3.5, page 85). Risk reduction procedures performed during semen decontamination should be evaluated to ensure the effective and safe ART of HIV-1 sero-positive patients.

The effectiveness of semen processing by discontinuous density gradient centrifugation in combination with the ProInsert™ (Nidacon International, Mölndal, Sweden) for the removal of HIV-1 from semen samples of HIV-1 sero-positive patients, was evaluated in this section.

8.2 METHODS

8.2.1 Experimental design

See Figure A.6 (Section E, page 222) for a diagrammatic illustration of the procedural design.

8.2.2 Participants

Human immunodeficiency virus-1 sero-positive patients that participated in the semen decontamination program at RBL were required to provide the Unit with recent (<3 months old) blood viral validation results (HIV-1 DNA, RNA and CD4 count). Leukocytes expressing the CD4 receptor are the major target cells for HIV, and decreased CD4 counts are correlated with a supressed immune response (Lawn *et al*., 2006). Patients with CD4 counts of <300 cells/ml were, therefore, excluded from the program and were referred to a virologist for antiviral therapy. Patients were only entered into the ART program once the CD4 count increased to above 300 cells/ml. The semen decontamination procedure and potential risks involved in using purified sperm from HIV-1 positive men were discussed with patients. Patients were then required to sign an informed consent form that allowed the Unit to:

i)   Perform the semen decontamination procedure.
ii)   Submit a neat diagnostic semen sample and purified sperm sample for HIV-1 validation.
iii)   Cryopreserve and store purified sperm samples.
iv)   Utilize sperm samples for ART (if samples are confirmed to be HIV-1-free).

 A unique identification code was assigned to each participant in the program to ensure anonymity.

8.2.3 Collection of semen samples

Patients (N=95) that enrolled in the semen decontamination program were requested to provide two semen samples by masturbation per week (on Monday and Wednesday, or on Tuesday and Thursday), for one to two consecutive weeks, depending on the quality of the processed sperm samples. Most patients, therefore, had to provide the Unit with 4 semen samples. However, in cases where sperm yield was too low for a specific ART procedure, additional semen samples were required from the patient. On average, 4.24 semen samples were received from each patient. The first semen samples delivered each week served as diagnostic samples and samples delivered on the second visit of each week were processed for therapeutic use during ART. Samples were produced in a dedicated room, as described in Section 6.2.1. Patients were instructed to seal the semen containing sample cup using a ziplock bag that had to be placed in a polystyrene cup that was covered with a lid. Upon receiving semen samples from the patients, the samples were placed in a dedicated warming oven at 37°C and allowed to liquefy for 30 minutes.

8.2.4 Evaluation of neat semen samples

Precautionary measures as described in the standard operating procedure for the correct handling of hazardous biological material at RBL were employed (RBL, 2012). Procedures were performed by, or under the supervision of experienced scientists. Protective clothing that covered the operator's arms was worn. Furthermore, face masks, protective eye wear and double gloves were used when handling samples. All procedures were performed in a laboratory dedicated to the handling of semen samples from patients known to be HIV-1 sero-positive (Muciaccia *et al.*, 2007), in a Class II biological safety cabinet (Labotec, South Africa, Midrand, Gauteng). Standard macroscopic and microscopic semen analyses were performed on all samples according to the World Health Organization's guidelines on semen evaluation (WHO, 2010).

8.2.5 Viral validation of neat diagnostic semen samples

Semen aliquots were decanted into 2.5 ml Cryo Tubes® (Nalge Nunc International, USA, Rochester, NY) and were submitted for viral validation. Viral validation was performed by qualitative and quantitative polymerase chain reaction (PCR) for viral DNA and RNA, respectively. A minimum semen volume of 500 µl was required by the pathology laboratory for viral validation to ensure maximum sensitivity of the method (Pasquier *et al.*, 2006). Samples were transported to Lancet Laboratories (Richmond, Johannesburg, Gauteng, South Africa) on ice in a sealed polystyrene box at ± 4°C. Commercial validation assays were used to determine HIV-1 RNA viral load (COBAS® Ampliprep/COBAS® TaqMan® HIV-1 version 2, Roche Diagnostics, Indianapolis, USA) and to evaluate proviral DNA qualitatively (Amplicor HIV-1 DNA, version 1.5 Roche Diagnostics). With a lowest limit of detection (LLD) of 20 viral copies/ml semen, the COBAS® Taqman® test is a sensitive and commonly used method to determine seminal viral loads (Pasquier *et al.*, 2012).

Samples were treated according to the standard operating procedures of Lancet laboratories (personal communication Dr. J. Trusler, Lancet Laboratories). For RNA analyses, semen samples were diluted twofold by adding a lysis buffer and Proteinase K (COBAS® Amplirep/COBAS® Taqman® pre-extraction reagent). The samples were then incubated at 60°C for 60 minutes and centrifuged at 1800 x g for 10 minutes. Thereafter, negative human plasma was added to yield a 1:4 dilution of the semen samples, changing the LLD to less than 80 viral copies/ml. The addition of negative human plasma to semen samples has been reported to reduce the effect of PCR inhibitors (Bourlet *et al*., 2003; Pasquier *et al*., 2006). The diluted semen samples were then analysed for the presence of HIV-1 RNA, and if a validation error occurred, semen samples were diluted further with negative human plasma to yield a final dilution of 1:10 (LLD of less than 200 viral copies/ml).

For DNA analyses, semen samples were originally extracted on the MagnaPure 32 (Roche, Japan) using the TNA High Performance kit (Roche, Germany) as per the manufacturer's instructions. To overcome the sperm sample volume limitation, the Promega Maxwell system was utilised. Two hundred microliters of semen was incubated with 200 µl lysis buffer and 20 µl proteinase K at 56°C, and centrifuged for

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10 minutes at 200 x g. The lysed sample mixture was transferred to the Viral Maxwell extraction kit (Promega, UK) cartridges and total nucleic acid extracted on the Maxwell automated nucleic extraction platform (Promega, UK) and eluted in 100 µl Nuclease-free water. The elute was incubated with the Roche kit Internal Control at 100°C for 30 minutes and amplification and detection performed using the Amplicor HIV-1 DNA, version 1.5 Roche Diagnostics kit, as per the manufacturer's instructions. Samples that tested positive for HIV-1 or within the grey zone (indeterminate – optical density 0.2 to 0.8) were re-processed and tested in duplicate for confirmation.

Viral validation results were received using a secure email address from Lancet Laboratories within 72 to 96 hours after the samples were submitted.

8.2.6 Decontamination of semen samples

The weekly therapeutic semen samples, destined for semen decontamination, were evaluated as described in (Section 8.2.4). Semen decontamination was performed by processing semen samples by discontinuous density gradient centrifugation (PureSperm® - 40 and 80%, Nidacon International) with the use of a polypropylene tube insert (ProInsert™, Nidacon International), without an additional swim-up step. Processing of semen samples using the ProInsert™ is described in Section 2.2.4.3. Processed sperm samples were obtained (580 µl) and microscopic evaluations were performed on 10 µl aliquots. The remaining 570 µl of sperm samples were cryopreserved and stored to be used during ART after the samples were confirmed to be free of HIV-1 DNA and RNA.

8.2.7 Cryopreservation of purified sperm samples

Samples were cryopreserved using SpermFreeze™ (FertiPro, Beernem, Belgium) that was added drop-wise to the processed sperm while gently mixing (399 µl SpermFreeze™ to 570 µl sperm). The sperm suspensions were then aspirated into three 300 µl high security semen cryopreservation straws (Cryo Bio System, CBS™, St-Ouen-sur-Iton, France) using a micro-classic aspirator (Brand GMBH Co, Werthein, Germany). The high security ionomeric resin straws are equipped with sterile removable nozzles (CBS™) that prevent contamination of the outside surface of the straw during the loading process, with any pathogens that may potentially remain in the processed sperm samples. The straws were identified by inserting an identification rod

into the straws (CBS™).   These colour coded identification rods were labelled (Labxpert, Brady, Milwaukee, WI, USA) with the date of cryopreservation and the patient's unique identification code.   Both sides of the straws were then heat-sealed (CBS™) where after the straws were wiped with a tissue and 70% ethanol. See Figure 8.1 for a presentation of the equipment utilized during filling, identification and sealing of the high security straws.

Cryopreservation was performed by means of controlled rate cooling (Freeze Control CL-8800, Cryologic, Victoria, Australia). The cooling program was as follows:

     i)   Starting temperature 20°C.

     ii)   Cooling to -30°C at 6°C/minute.

     iii)   Cooling to -40°C at 5°C/minute.

     iv)   Non-controlled-rate cooling (free-fall) from -40°C.

When a temperature of -80°C was reached during the free-fall process, the straws were plunged into liquid nitrogen.   The cryopreserved straws were stored in a dedicated dewar (Thermo Electron Corporation, Bremen, Germany) that was locked for security purposes.
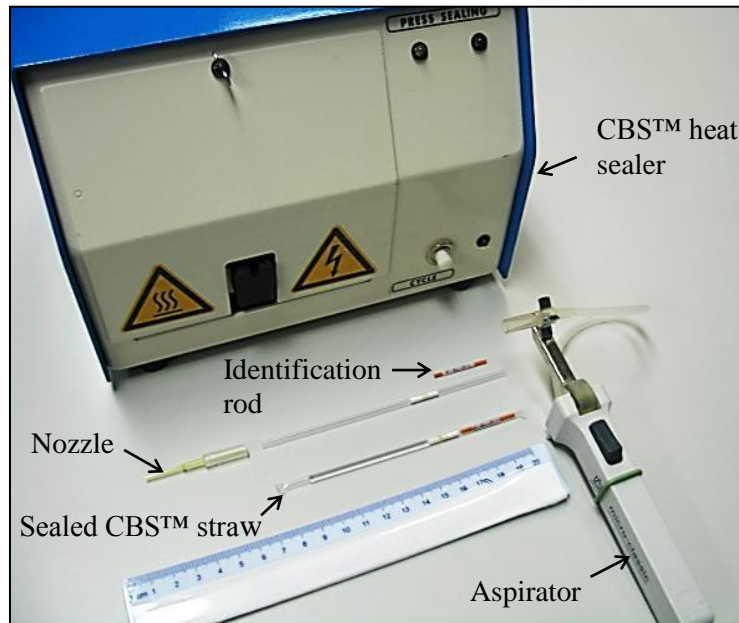
Figure 8.1: Equipment used during loading, identification and sealing of the high security straws.

8.2.8 Viral validation of processed sperm samples

Purified sperm samples were submitted for viral validation if the neat semen tested positive for HIV-1 RNA and/or DNA, or if the result was inconclusive due to a validation error. Samples were not submitted for viral validation if the diagnostic semen sample tested negative for viral DNA, or if the HIV-1 RNA load was below the LLD of the detection method. The purified sperm samples designated for HIV-1 DNA and RNA validation are diagrammatically illustrated in Figure 8.2, and the number of cryopreserved sperm straws that would remain for ART per week is illustrated in Table 8.1 (page 192).
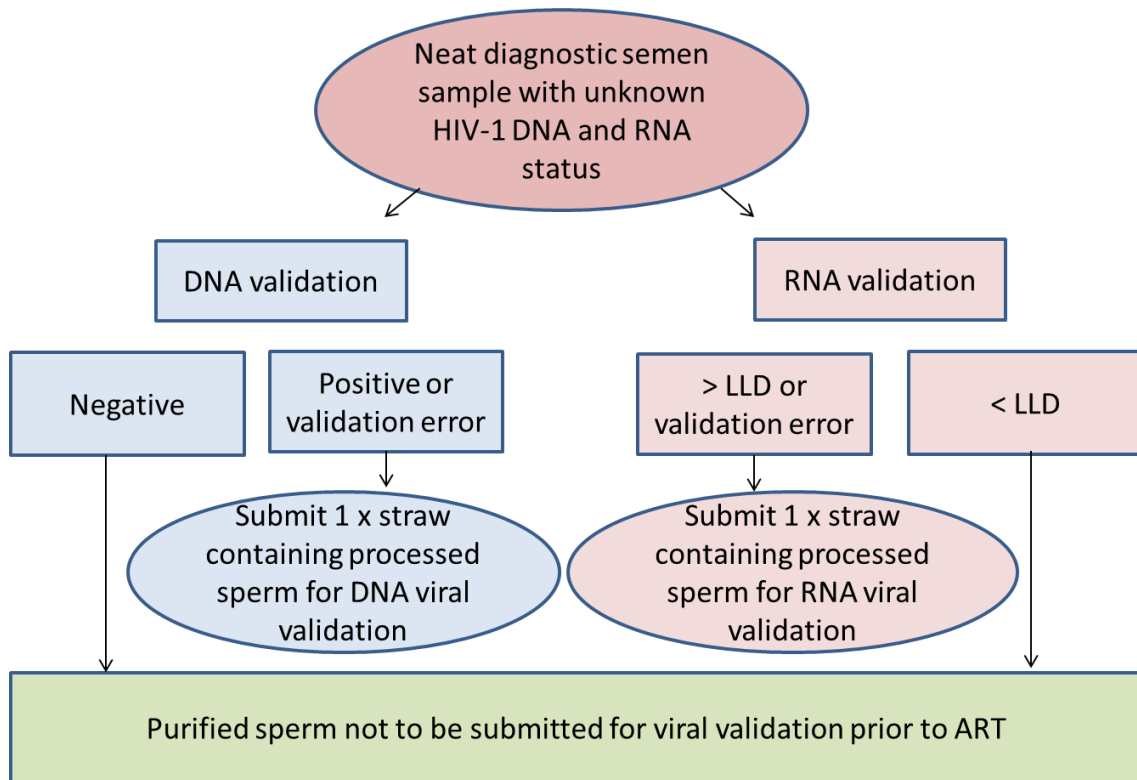
Figure 8.2: Processed sperm samples designated for HIV-1 DNA and RNA validation.

Table 8.1: The number of straws containing processed sperm remaining for assisted reproductive treatment depended on the viral validation results of the diagnostic neat semen sample.

HIV-1 DNA and RNA validation result of neat diagnostic semen sample		Number of straws containing processed sperm to be submitted for HIV-1 DNA and RNA validation		Number of straws containing purified sperm remaining for ART
DNA	RNA	DNA	RNA	
-	-	0	0	3
+	-	1	0	2
-	+	0	1	2
+	+	1	1	1

8.2.9 Statistical analysis

The RBL's patient database (Microsoft Excel 2010) was accessed to perform descriptive statistics on data from July 2007 to June 2012.

8.3 RESULTS

The blood plasma viral loads (BPVL) and seminal viral loads (SVL) of HIV-1 sero-positive patients (N=95) seeking ART at RBL at SBAH ranged between <LLD to 167,263 and <LLD to 1,443,350 copies/ml, respectively. The HIV-1 RNA status of blood and semen is presented in Table 8.2. Human immunodeficiency virus type 1 loads of <LLD were considered as negative and viral loads of ≥LLD were considered as positive. From all patients, 5.6% presented with undetectable BPVLs, of which 32.7%, delivered semen samples with SVL of ≥LLD. Furthermore, the disagreement between blood and seminal HIV-1 RNA status (+ and -) was a random event, i.e. 32.7% of patients had BPVL <LLD and SVL ≥LLD, while 37.0% of patients presented with BPVL ≥LLD and SVL <LLD (Table 8.2).

Table 8.2: The numbers and (%) of patients (N=95) and neat semen samples that tested positive and/or negative for HIV-1 RNA.

Blood HIV-1 RNA status	Semen HIV-1 RNA status		Total
	<LLD*	+	
<LLD*	33 (67.35)	16 (32.65)	49 (51.58)
+	17 (36.96)	29 (63.04)	46 (48.42)
Total	50 (52.63)	45 (47.37)	95 (100)

*   LLD=20-200 viral copies/ml

The positivity of neat semen samples for HIV-1 DNA and RNA is given in Table 8.3. Of all tested neat semen samples (N=186) from sero-positive patients, 53.8% tested positive for HIV-1 DNA, RNA or both (13.4, 11.3% and 29.0%, respectively). Results for 7.0% (N=13) of neat samples could not be obtained due to PCR validation errors. Validation errors for neat semen samples included: i) HIV-1 RNA errors (N=10), and ii) HIV-1 DNA and RNA errors (N=3). Processed sperm samples (N=103) were submitted

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for viral validation, results for 2% (N=2) of samples could not be obtained due to validation errors for DNA and RNA in both sperm samples. Furthermore, one submitted, processed sperm straw went missing at the pathology laboratory.

The semen decontamination procedure was effective in removing HIV-1 RNA and proviral DNA from 98.1% of semen samples of HIV-1 infected patients, as shown in Table 8.3. From a total of 103 processed sperm samples that were submitted for viral validation two samples tested positive for HIV-1 DNA and none for RNA.

Table 8.3: HIV-1 RNA and proviral DNA detection in semen (N=186) and purified sperm samples (N=103) from HIV-1 sero-positive men (N=95) participating in the assisted reproductive program at Steve Biko Academic Hospital.

	Neat Semen Samples (N=186)			Processed Sperm Samples (N=103)
Negative	**Positive**			**Negative**
DNA & RNA	**DNA**	**RNA**	**DNA & RNA**	**DNA & RNA**
46.24%	13.44%	11.29%	29.03%	98.06%
N=86	N=25	N=21	N=54	N=101

8.4 DISCUSSION

Human immunodeficiency virus may be transferred to 7.0% to 24.0% of females within the first couple of months after contraction of the virus by their male sexual partners (Pilcher *et al.*, 2004). Male patients receiving highly active antiretroviral therapy, and with blood viral loads below the LLD, may still present with 6.6% to 25.0% of semen samples positive for HIV-1 (Lambert-Niclot *et al.*, 2012; Politch *et al*., 2012). The current study indicated that 32.7% of patients with undetectable blood plasma HIV-1 RNA loads presented with HIV-1 RNA in their semen samples. Furthermore, 53.8% of semen samples from HIV-1 sero-positive male partners, of couples wanting to participate in the ART program at Steve Biko Academic Hospital, tested positive for HIV-1 DNA or RNA. The seminal viral load (SVL) ranged between <LLD to 1,443,350, with a mean of 22,780 copies/ml semen. Therefore, due to the potential high

HIV-1 SVL, the rate of male to female transferral is 1.9 times more than female to male transferral of the virus (European Study Group on Heterosexual Transmission of HIV, 1992). The use of condoms during intercourse is, therefore, recommended to discordant couples where the male is positive, resulting in these patients being unable to conceive their own genetically related offspring (Pasquier *et al.*, 2006).

Concrete evidence for an association between sperm and HIV-1 does not exist (Pudney *et al.*, 1999). Seminal HIV can be present as cell-free RNA, or in the form of proviral DNA, be associated with CD4 receptor-carrying lymphocytes and macrophages (Quayle *et al.*, 1997; Savasi *et al.*, 2013). The effective isolation of sperm from seminal plasma and white blood cells that serve as target cells for the virus, should then yield a population of sperm that is HIV-1 free and safe for use during ART. The promotion of semen washing procedures, consequently, results in more HIV-1 sero-discordant couples attending infertility units, rather than attempting to conceive by having unprotected intercourse (Lesage *et al.*, 2006).

Semen decontamination procedures employed at RBL consist of semen processing by discontinuous density gradient centrifugation in combination with the ProInsert™. From a total of 103 processed sperm samples that were submitted for viral validation, only two samples tested positive for HIV-1 DNA and none for RNA. The relatively low SVL (1,690 and 2,900 viral copies/ml, respectively) from patients with HIV-1 DNA in processed sperm samples suggests that SVL is not indicative of the effectiveness of semen decontamination procedures. The success rate for semen decontamination achieved at RBL was 98.1% for HIV-1 DNA and 100% for RNA. This is superior to results from other assisted reproductive units, where 6.4% to 69.2% of processed sperm samples, remained positive for HIV DNA and/or RNA (Leruez-Ville *et al.*, 2002; Meseguer *et al.*, 2002; Garrido *et al.*, 2004; Cardona-Maya *et al*., 2009). See Table 3.5 (Section A, page 85) for a summary of the HIV-1 RNA and DNA statuses of processed sperm samples from various ART units. The improved effectiveness of the decontamination method employed at RBL could be attributed to the secure method of obtaining the purified sperm pellet post-density gradient centrifugation through the ProInsert™ (Loskutoff *et al.*, 2005).

However, it must be noted that while this research was conducted the centrifugation tube insert underwent various design changes by the manufacturer (Nidacon International) and the final product was only introduced into the market in 2013. The changes in design allowed the operator to layer semen onto the density layers without contaminating the inner lumen of the insert. This was achieved by raising the lumen of the insert to above the level of where semen is added to the insert. See Figure 2.10 for an illustration of the ProInsert™. Therefore, the two processed sperm samples that tested positive for proviral DNA could have been contaminated post-processing when an initial prototype of the insert was tested, where the sterility of the inner lumen of the insert could have been compromised during loading of the semen.

Challenges that could be encountered when assisting HIV-1 sero-discordant couples with semen decontamination procedures include the following:

i)   Researchers agree that the complete removal of HIV-1 from semen cannot be guaranteed (Savasi *et al.*, 2013). Furthermore, the PCR method utilised for viral validation's LLD was 20 to 200 viral copies/ml, whereby false negative results are possible (Pasquier *et al.*, 2006). Patients must, therefore, be counselled that semen decontamination is merely a risk reduction method and that risk cannot be totally eliminated (Gilling-Smith, 2005). Additionally patients must sign an informed consent form wherein the potential consequences of technique failure are acknowledged.

ii)   The presence of seminal PCR inhibitors results in the inhibition of HIV-1 RNA amplification whereby viral validation errors are obtained (Lesage *et al.*, 2006). Patients should be informed that in the event of PCR errors, viral validation should be repeated, whereby less sperm will be available for ART.

iii)   One straw containing processed sperm was lost at the pathology laboratory. Effective communication between the assisted reproductive laboratory, courier company and the pathology laboratory is critical to ensure that all parties are aware of the value of these specimens to the patients, and that samples must be handled with extreme care. Several meetings were held and continue to be scheduled between RBL and representatives from Lancet laboratories.

iv)   Viral validation of neat semen and processed sperm samples is costly. According to a quotation obtained from Lancet Laboratories the current

(May 2013) cost of HIV-1 DNA and RNA validation is R928 (78€) and R967 (81€), respectively. This study demonstrated that seminal viral load (ranges from <LLD to 1,443,350 copies/ml semen) was no indication of the effectiveness of the semen decontamination. It is, therefore, recommended that only purified sperm samples, and not neat semen samples should be submitted for viral validation.

v)   To ensure that sufficient purified sperm cells were available for ART, most patients were requested to provide semen samples (N=4) during two consecutive weeks, where after sperm had to be cryopreserved and stored while awaiting viral validation results. This was inconvenient and costly to patients. Same day viral validation (Lesage *et al.*, 2006) of purified sperm samples would require only one semen sample on the day of the assisted reproductive procedure. Purified sperm could be stored at room temperature until an aliquot of the sample is confirmed to be virus free. Hereby purified sperm samples would not be compromised during the cryopreservation process. Same day viral validation is however not readily available, specifically not in developing countries such as South Africa.

vi)   Viral validation of processed sperm samples could be impossible in some African countries due to a lack of pathology services. Under these circumstances, risk reduction procedures should be maximised to ensure the safe usage of purified, untested sperm for ART. Semen decontamination procedures should be followed by single sperm washing and intra-cytoplasmic sperm injection (Mencaglia *et al.*, 2005). This approach has been reported to be the safest method for HIV-1 sero-discordant couples to conceive (Garrido *et al.*, 2004). Savasi *et al*. (2013) suggest that the intra-cytoplasmic sperm injection technique, is the only assisted reproductive procedure that should be applied to HIV-1 sero-discordant couples where the male is positive.

In conclusion, HIV-1 sero-positive male patients with undetectable blood plasma viral loads should be counselled regarding the potential presence of virus in their semen samples. These patients should be dissuaded from having unprotected intercourse in an attempt to conceive. Assisted reproductive treatment in combination with semen decontamination by discontinuous density gradient

centrifugation and the ProInsert™ is the safest reported option for these patients to conceive. Furthermore, processed sperm samples should only be utilized for ART once the samples have been confirmed to be free from virus by PCR testing, if these tests are available.

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SECTION D

CONCLUSIONS

CHAPTER 9

CONCLUSIONS AND RECOMMENDATIONS

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The refusal of assisted reproductive treatment (ART) to patients that are sero-positive for HIV-1 is ethically and legally unwarrantable (Lyerly & Anderson, 2001). However, although a few thousand cycles (Table 3.4, page 84) have been performed globally, the risks involved are demonstrated by the transmission of HIV-1 after donor insemination (Wortley, Hammett & Fleming, 1998). The safety of patients, health care workers, and potential offspring must, therefore, be considered during the endeavour to accomplish a pregnancy by ART. Even though HIV-1 sero-positive male patients may present with undetectable blood plasma viral loads, in this study HIV-1 was detected in 32.7% of semen samples from these patients. Consequently patients must be counselled and strongly dissuaded from attempting to conceive by having unprotected intercourse. Instead, effective semen processing procedures for the elimination of seminal pathogens must be employed prior to therapeutic ART. The effectiveness of semen processing as a risk reduction method was investigated and the following conclusions and recommendations were made:

Semen decontamination

- Numerous semen processing procedures are currently available; however, most of these methods are not aimed towards the elimination of seminal pathogens. Huyser & Fourie (2010) suggested the term semen decontamination to specifically refer to processing procedures that are aimed towards the removal of seminal pathogens. The effectiveness of semen decontamination procedures is not guaranteed (Leruez-Ville *et al*., 2002; Knox *et al*., 2003; Nicopoullos *et al*., 2010), and will partly depend on the specific seminal pathogen. Pathogens that adhere to, or penetrate sperm might not be effectively eliminated from sperm samples during processing. The association of relevant viruses with sperm is presented in Table 1.1 (page 22) and the following bacteria have been reported to attach to sperm: *Escherichia coli* (Sánchez *et al*., 1989; Diemer *et al*., 2000)*, Ureaplasma urealyiticum* (Nunez-Calonge *et al*., 1998)*, Mycoplasma hominis* (Diaz-Garcia *et al*., 2006)*, Neiseria gonorrhoeae* (James-Holmquest *et al*., 1974), *Chlamydia trachomatis* (Erbengi, 1993)*.

- Trypsin can be added to semen processing media to inactivate viruses and to break pathogen-sperm bonds (Loskutoff *et al.*, 2005).

The effect of trypsin and trypsin inhibitor on human sperm parameters

- The effect of semen processing with density layers supplemented with trypsin was evaluated. The study indicated that semen processing with upper density layers supplemented with trypsin, did not have a negative impact on any of the evaluated sperm parameters. Trypsin should however, not be added to density layers at concentrations higher than 0.25% (Silva *et al.*, 1999).

- Bottom density layers would have to be supplemented with trypsin inhibitor at a concentration of 10 µg/ml, to inhibit trypsin at a concentration of 0.25% (Loskutoff *et al.*, 2005). An experiment performed to determine the effect of semen processing with lower density layers supplemented trypsin inhibitor (10 µg/ml) indicated no detrimental impact on any of the tested sperm parameters. Nevertheless, the absence of a negative impact of semen processing with trypsin on sperm parameters indicates that bottom density layers should not be supplemented with trypsin inhibitor.

The effectiveness of semen decontamination using a tube insert

- Sperm samples processed by density gradient centrifugation may be re-infected with pathogens during aspiration of the supernatant (Kaneko *et al.*, 1986; Loskutoff *et al.*, 2005). The ProInsert™ (Nidacon International, Mölndal, Sweden) has been developed to provide safe access to purified sperm pellets without bringing sperm into contact with the potentially contaminated upper density layers (Loskutoff *et al.*, 2005). The effectiveness of discontinuous density gradient centrifugation in combination with the ProInsert™ for the removal of bacteria, white blood cells and *in vivo* derived human immunodeficiency virus from semen was evaluated.

  - Seminal bacteria could have a negative impact on the outcome of ART and may result in infection of the female reproductive tract (Cottell *et al.*, 2000; Gdoura *et al.*, 2008; Kiessling *et al.*, 2008). Discontinuous density gradient centrifugation in combination with the ProInsert™ was effectively utilized, in this study, to eliminate significantly (96.0%) more *in vitro* derived bacteria from semen when compared to processing without

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the insert (P<0.004). All bacteria were removed from semen samples below a bacterial inoculation concentration of 1×10^5 colony forming units/ml.

- The removal of white blood cells from semen by effective semen processing is critical to prevent the transfer of viruses (Bujan *et al*., 2002; Englert *et al*., 2004) as well as to limit the production of reactive oxygen species (Whittington & Ford, 1999; Henkel, 2005). In this study novel flow cytometric analyses of processed sperm samples were utilized to demonstrate that significantly more (P=0.013) *in vitro* derived white blood cells were removed from semen by processing with the ProInsert™ compared to processing without the insert.

- The documented failure rate of semen processing for the elimination of *in vivo* derived HIV-1 ranges between 6.4% to 69.2% (Leruez-Ville *et al*., 2002; Garrido *et al*., 2004; Cardona-Maya *et al*., 2009). During the current study, discontinuous density gradient centrifugation in combination with the ProInsert™ was successfully utilized to eliminate:

  - HIV-1 RNA from 100% and
  - DNA from 98.1%

of semen samples (N=103). Seminal HIV-1 load was not an indication of the effectiveness of semen decontamination and a semen sample containing 1,443,350 viral copies/ml was successfully purified.

The effectiveness of discontinuous density gradient centrifugation for the elimination of seminal pathogens could therefore be improved by utilization of the ProInsert™. Furthermore, the ProInsert™ facilitated the layering of density layers and test tubes could be capped after use, allowing for the containment and effective disposal of hazardous material (Fourie, Loskutoff & Huyser, 2012). Utilization of the ProInsert™ during ART, specifically when assisting patient populations with a high probability of having seminal pathogens, should be seriously considered.

Viral validation of processed sperm samples

- The reported failure of semen processing for the elimination of HIV-1 supports the argument that semen decontamination procedures reduce, but do not eliminate risk (Gilling-Smith *et al*., 2005). Processed sperm samples must therefore be submitted for viral validation prior to therapeutic use. However, patients should be informed about the sensitivity of molecular testing and the potential for obtaining false negative results.

Selection of ART procedure

- The specific ART procedure to follow after successful semen decontamination should be agreed upon by the clinical and laboratory teams, as well as by the patients. The benefits of the intra-cytoplasmic sperm injection for patients with potential seminal pathogens include the following:

  - Less purified sperm are required for therapeutic use when compared to *in vitro* fertilization and intra-uterine insemination. More sperm will resultantly be available during potential follow up ART cycles.

  - Each oocyte is exposed to a single sperm cell whereby the risk of exposure to HIV is limited.

  - After semen decontamination, a single sperm can be washed further by pipetting in fresh media using the sperm injection needle prior to intra-cytoplasmic sperm injection.

  - Pregnancy rates per cycle are increased following intra-cytoplasmic sperm injection when compared to intra-uterine insemination (28.5% versus 12.6%) (Nyboe Andersen *et al*., 2009).

  Therefore, although thousands of intra-uterine inseminations have been performed globally without HIV transferral (see Table 3.4), intra-cytoplasmic sperm injection is the ART method with the lowest risk for patients with potential seminal pathogens (Savasi *et al*., 2013).

Standard operative procedures during ART for patients with potential seminal pathogens

- The provision of ART services to HIV-1 sero-positive male patients should be regulated. National establishments must ensure that consensus is reached and that

these guidelines comply with international standards and are adhered to (Magli *et al*., 2008; Practice Committee of American Society for Reproductive, Medicine, 2013). The following should be considered when preparing such guidelines:

- Thorough training of staff designated to the handling of potentially hazardous samples.

- Written safety guidelines for the handling of hazardous material must be in place.

- Having separate, dedicated laboratories and equipment for patients with blood-borne viruses. Alternatively, the treatment of HIV-1 sero-negative and positive patients could be divided in time (Gilling-Smith *et al*., 2005).

- Screening of patients for prevalent infections prior to ART.

- Providing of counselling to patients testing positive for blood-borne viruses and referral to a virologist for treatment.

- Comprehensive written informed consent must be obtained from patients prior to ART.

- Adhering to strict inclusion and exclusion (CD4 count and serum viral load) criteria for patients (See Table 3.2, page 80).

- The utilization of proven semen decontamination procedures.

- Storage of cryopreserved processed sperm samples by using high security straws in dedicated liquid nitrogen dewars.

- The viral validation of processed sperm samples by molecular methods with comprehensive quality control analyses in place.

Conclusion

Potential harm to human life during ART can be reduced by the utilization of proven semen decontamination procedures. The effectiveness of semen decontamination could be improved by the supplementation of upper density layers with trypsin, without having a negative impact on sperm parameters. Furthermore, significantly more seminal pathogens can be removed from semen by the utilization of the ProInsert™ during discontinuous density gradient centrifugation. It is therefore highly recommendable that the ProInsert™ be utilized for the processing of all semen samples potentially containing pathogens. However, the reported failure rate of 1.9% HIV-1 DNA in processed sperm samples, suggests that processed sperm samples from HIV-1 sero-positive patients must

be submitted for viral validation prior to therapeutic use. Even though (as discussed previously) the reported failures may have resulted from technical error, because of a temporary design change of the ProInsert™ that increased the probability of inadvertent contamination. Still, technical error is, and will always be, a concern, so viral validation should continue to insure the decontamination treated sperm can be used therapeutically.

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SECTION E

ANNEXURES

ANNEXURE A

DIAGRAMMATIC EXPERIMENTAL LAYOUTS

ANNEUXRE B

SEMEN PARAMETERS OF DONORS

ANNEUXRE C

PEER-REVIEWED PUBLISHED ARTICLES AND PRESENTED POSTERS

ANNEXURE A

DIAGRAMMATIC EXPERIMENTAL LAYOUTS

Diagrammatic illustrations of experiments performed during the research are presented in this section.

i)   **Evaluation of the effect of semen processing with**:

- PureSperm® Pro supplemented with recombinant, human sequence trypsin (rTrypsin), compared to standard PureSperm® (not supplemented with rTrypsin), on sperm-zona pellucida binding (Figure A.1).

- Density gradient layers supplemented with trypsin at different concentrations on sperm: vitality, mitochondrial membrane potential and motility parameters (Figure A.2).

- Density gradient layers supplemented trypsin inhibitor on sperm parameters (Figure A.3).

ii)   **Effectiveness of density gradient centrifugation in combination with the ProInsert™ for the removal of:**

- *In vitro* derived micro-organisms (Figure A.4).

- White blood cells from spiked semen samples (Figure A.5).

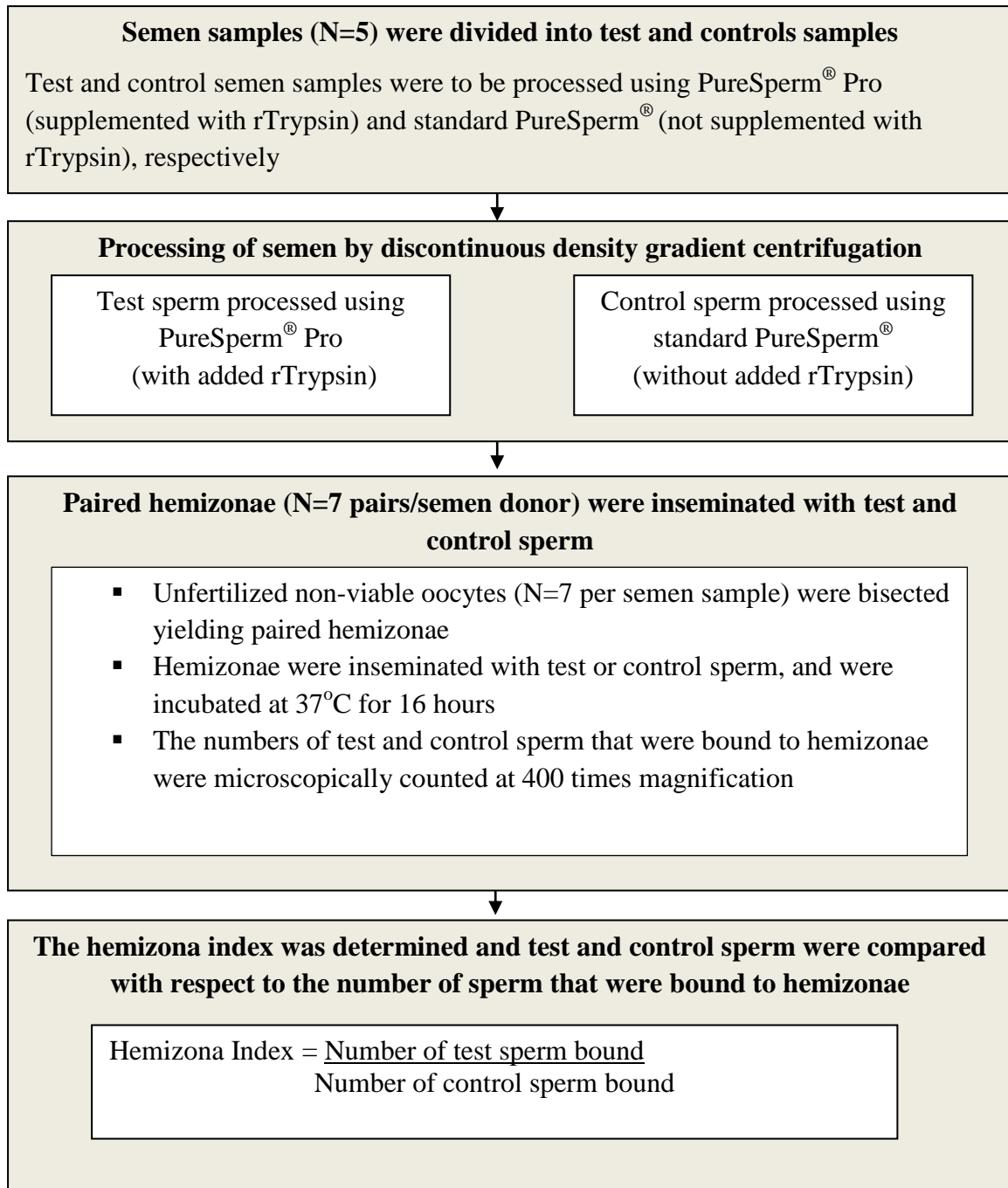- *In vivo* derived HIV-1 from semen samples (Figure A.6).

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Semen samples (N=5) were divided into test and controls samples

Test and control semen samples were to be processed using PureSperm® Pro (supplemented with rTrypsin) and standard PureSperm® (not supplemented with rTrypsin), respectively

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Processing of semen by discontinuous density gradient centrifugation

| Test sperm processed using PureSperm® Pro (with added rTrypsin) | Control sperm processed using standard PureSperm® (without added rTrypsin) |

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Paired hemizonae (N=7 pairs/semen donor) were inseminated with test and control sperm

- Unfertilized non-viable oocytes (N=7 per semen sample) were bisected yielding paired hemizonae
- Hemizonae were inseminated with test or control sperm, and were incubated at 37°C for 16 hours
- The numbers of test and control sperm that were bound to hemizonae were microscopically counted at 400 times magnification

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The hemizona index was determined and test and control sperm were compared with respect to the number of sperm that were bound to hemizonae

$$\text{Hemizona Index} = \frac{\text{Number of test sperm bound}}{\text{Number of control sperm bound}}$$

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Figure A.1: Diagrammatic experimental layout of the evaluation of the effect of semen processing, with density gradient layers supplemented recombinant, human sequence trypsin (rTrypsin), on sperm-zonae binding potential.
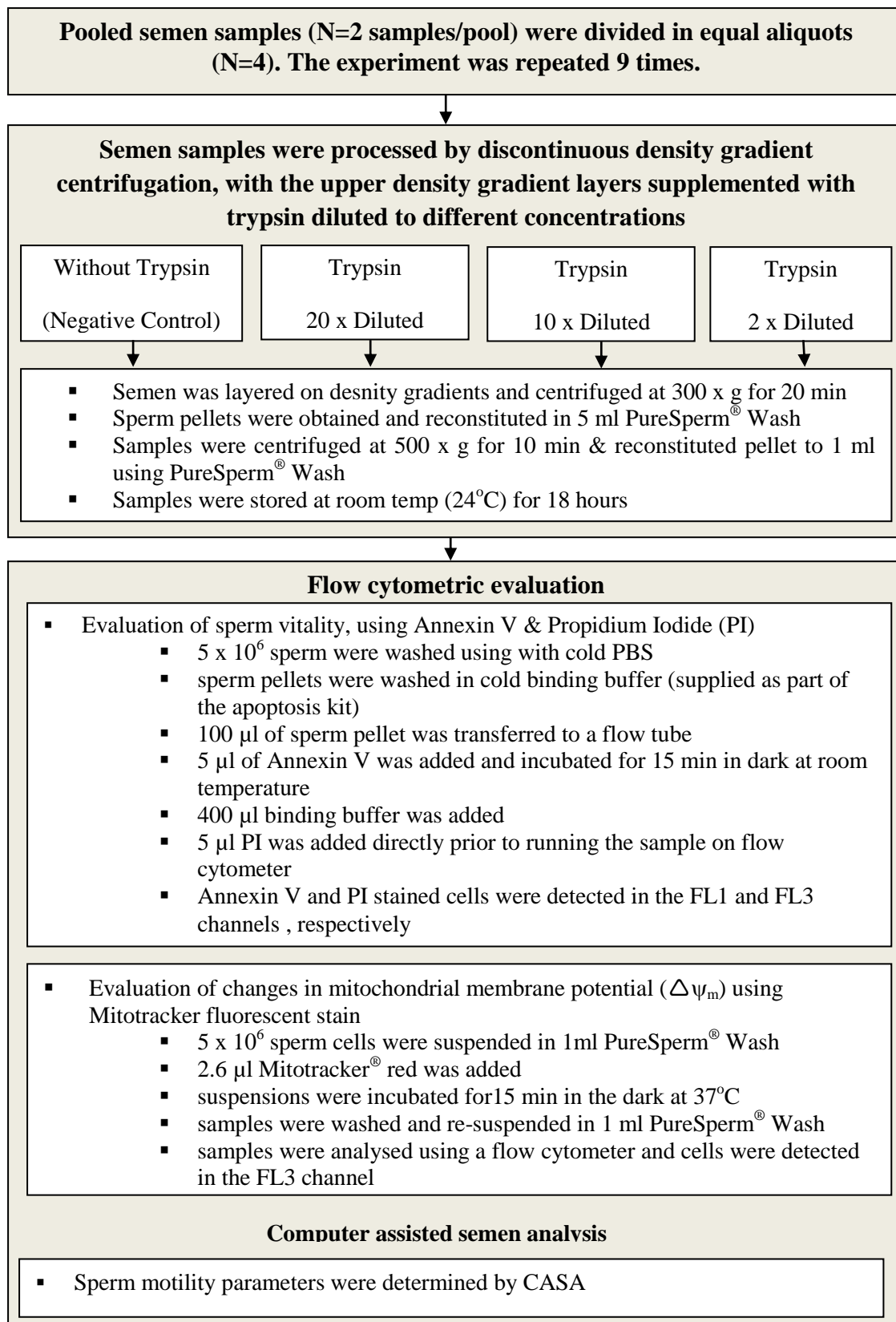
Pooled semen samples (N=2 samples/pool) were divided in equal aliquots (N=4). The experiment was repeated 9 times.

Semen samples were processed by discontinuous density gradient centrifugation, with the upper density gradient layers supplemented with trypsin diluted to different concentrations

Without Trypsin (Negative Control)	Trypsin 20 x Diluted	Trypsin 10 x Diluted	Trypsin 2 x Diluted

- Semen was layered on desnity gradients and centrifuged at 300 x g for 20 min
- Sperm pellets were obtained and reconstituted in 5 ml PureSperm® Wash
- Samples were centrifuged at 500 x g for 10 min & reconstituted pellet to 1 ml using PureSperm® Wash
- Samples were stored at room temp (24°C) for 18 hours

Flow cytometric evaluation

- Evaluation of sperm vitality, using Annexin V & Propidium Iodide (PI)
  - 5×10^6 sperm were washed using with cold PBS
  - sperm pellets were washed in cold binding buffer (supplied as part of the apoptosis kit)
  - 100 µl of sperm pellet was transferred to a flow tube
  - 5 µl of Annexin V was added and incubated for 15 min in dark at room temperature
  - 400 µl binding buffer was added
  - 5 µl PI was added directly prior to running the sample on flow cytometer
  - Annexin V and PI stained cells were detected in the FL1 and FL3 channels , respectively

- Evaluation of changes in mitochondrial membrane potential ($\Delta\psi_m$) using Mitotracker fluorescent stain
  - 5×10^6 sperm cells were suspended in 1ml PureSperm® Wash
  - 2.6 µl Mitotracker® red was added
  - suspensions were incubated for15 min in the dark at 37°C
  - samples were washed and re-suspended in 1 ml PureSperm® Wash
  - samples were analysed using a flow cytometer and cells were detected in the FL3 channel

Computer assisted semen analysis

- Sperm motility parameters were determined by CASA

Figure A.2: Diagrammatic experimental layout of the evaluation of the effect of rTrypsin at different concentrations on sperm vitality, mitochondrial membrane potential and motility parameters.
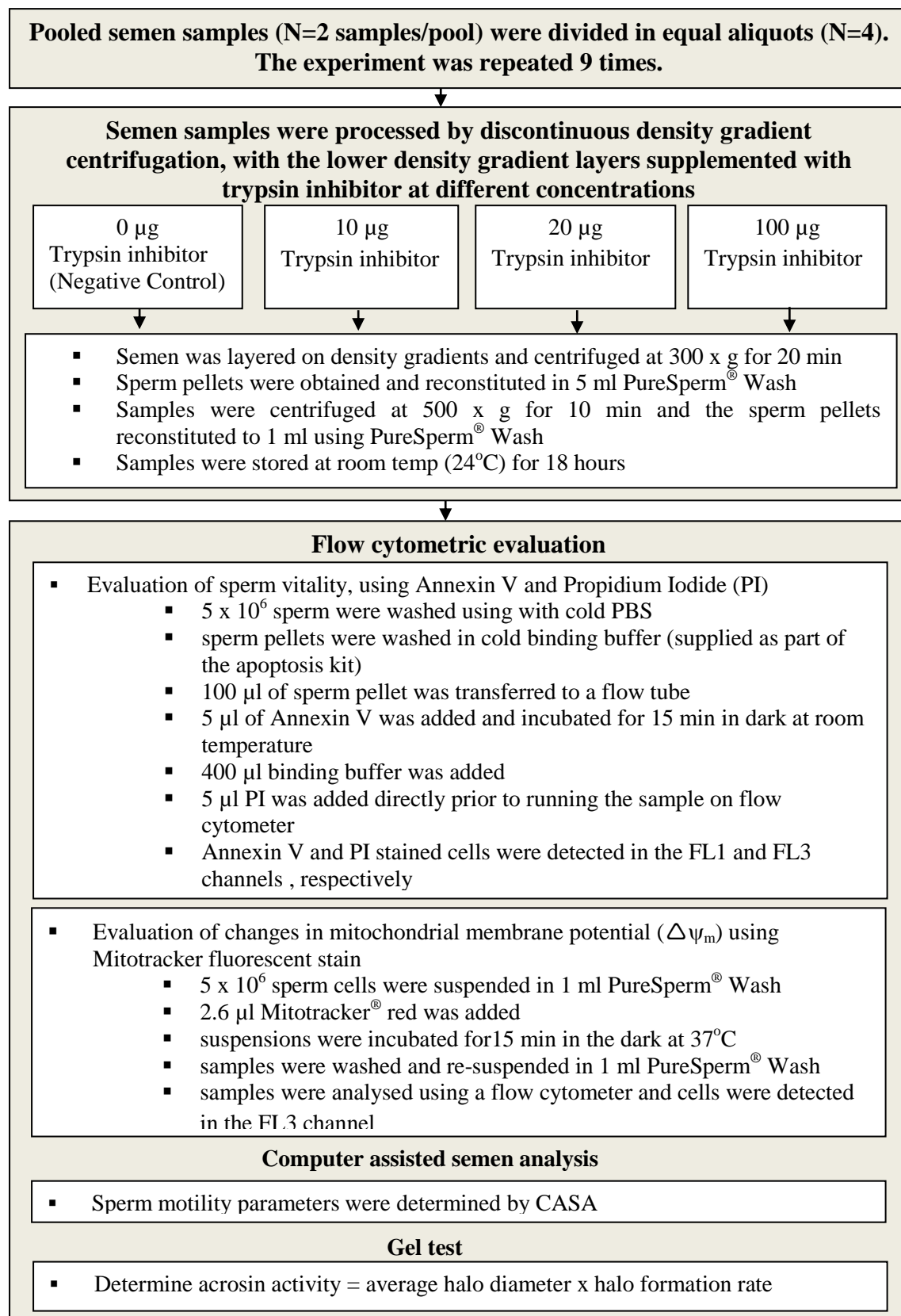
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> **Pooled semen samples (N=2 samples/pool) were divided in equal aliquots (N=4). The experiment was repeated 9 times.**

> **Semen samples were processed by discontinuous density gradient centrifugation, with the lower density gradient layers supplemented with trypsin inhibitor at different concentrations**

0 µg Trypsin inhibitor (Negative Control)	10 µg Trypsin inhibitor	20 µg Trypsin inhibitor	100 µg Trypsin inhibitor

- Semen was layered on density gradients and centrifuged at 300 x g for 20 min
- Sperm pellets were obtained and reconstituted in 5 ml PureSperm® Wash
- Samples were centrifuged at 500 x g for 10 min and the sperm pellets reconstituted to 1 ml using PureSperm® Wash
- Samples were stored at room temp (24°C) for 18 hours

Flow cytometric evaluation

- Evaluation of sperm vitality, using Annexin V and Propidium Iodide (PI)
  - 5×10^6 sperm were washed using with cold PBS
  - sperm pellets were washed in cold binding buffer (supplied as part of the apoptosis kit)
  - 100 µl of sperm pellet was transferred to a flow tube
  - 5 µl of Annexin V was added and incubated for 15 min in dark at room temperature
  - 400 µl binding buffer was added
  - 5 µl PI was added directly prior to running the sample on flow cytometer
  - Annexin V and PI stained cells were detected in the FL1 and FL3 channels , respectively

- Evaluation of changes in mitochondrial membrane potential ($\triangle\psi_m$) using Mitotracker fluorescent stain
  - 5×10^6 sperm cells were suspended in 1 ml PureSperm® Wash
  - 2.6 µl Mitotracker® red was added
  - suspensions were incubated for15 min in the dark at 37°C
  - samples were washed and re-suspended in 1 ml PureSperm® Wash
  - samples were analysed using a flow cytometer and cells were detected in the FL3 channel

Computer assisted semen analysis

- Sperm motility parameters were determined by CASA

Gel test

- Determine acrosin activity = average halo diameter x halo formation rate

Figure A.3: Diagrammatic experimental layout of the evaluation of the effect of semen processing with trypsin inhibitor on sperm parameters.

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| Semen samples were pooled (N=5 samples/pool). The experiment was performed in duplicate | Bacterial isolates were obtained from the Department of Microbiology NHLS |

Adjustment of sperm concentrations

- Sperm concentrations were adjusted to 40×10^6/ml using PureSperm® Wash
- Pooled semen samples were divided into 5 aliquots that were to be spiked with micro-organisms and processed

Semen aliquots were spiked with the following micro-organisms and incubated together for 30 minutes at 37°C

Micro-organism spiking concentrations: 1×10^3; 1×10^4; 1×10^5; 1×10^6; 1×10^7 colony forming units/ml semen

- *Enterobacter cloacae* (In-house)
- *Enterococcus faecalis* (ATCC 29212)
- *Escherichia coli* (ATCC 25922)
- Coagulase-negative staphylococcus (In-house)
- *Staphylococcus aureus* (ATCC 25923)
- *Candida albicans* (ATCC 90028)

Spiked semen samples were divided in test and control samples that were processed by discontinuous density gradient centrifugation with and without the insert

| With ProInsert™ (Test) | Without ProInsert™ (Control) |

Processed sperm samples were submitted for quantitative micro-organism evaluation

- Processed sperm samples were cultured on blood agar plates
- Non-spiked sperm samples served as negative controls
- Spiked non-processed sperm samples were included as positive controls

Figure A.4: Diagrammatic experimental layout of the evaluation of the effectiveness of semen processing with the ProInsert™ for the elimination of *in vitro* derived micro-organisms.

White blood cells were isolated from whole blood	Semen samples (N=9) were obtained from donors (N=9).
ACCUSPIN™ System-HISTOPAQUE®	

Semen samples were spiked with white blood cells

- 20×10^{6} WBCs/ml semen

- Spiked semen samples were split to yield a test and control sample

Spiked semen samples were divided in test and control samples that were processed by discontinuous density gradient centrifugation with and without the insert

With ProInsert™ (Test)	Without ProInsert™ Control)

Flow cytometric evaluation to determine number of white blood cells in processed sperm samples

- Samples were stained using CD45 FITC and Vybrant® Dyecycle™ Ruby Stain
- CD45 FITC and Ruby stained cells were detected in the FL 1 and FL6 flow cytometry channels, respectively
- Processing with and without the ProInsert™ were compared with respect to the number of white blood cells present in processed sperm samples

Figure A.5: Diagrammatic experimental layout of the evaluation of the effectiveness of semen processing with the ProInsert™ for the elimination of white blood cells from spiked semen samples.

Consultation with HIV-1 sero-positive patients

- HIV-1 sero-positive patients (N=95) wanting to participate in semen decontamination and assisted reproductive program
- Consultation with patients to determine health status (blood HIV-1 RNA viral load & CD4 count) → CD4 counts must be >300 cells per ml to participate

Week 1

Day 1 (Monday/Tuesday) Evaluation of diagnostic semen sample

- Macroscopic evaluation
- Microscopic evaluation
- External HIV-1 DNA and RNA validation of semen samples at Lancet laboratories
  - Using the COBAS® Ampliprep/ COBAS® TaqMan® platform

Day 2 (Wednesday/Thursday) Decontaminate therapeutic semen sample

- Process semen by density gradient centrifugation using the ProInsert™

Cryopreservation of purified sperm sample

- Cryopreserve sample using SpermFreeze™
- Store cryopreserved sperm samples in CBS™ straws (N=3/sample)

Viral validation of purified therapeutic sperm samples depended on viral status of diagnostic semen sample

- Diagnostic semen sample positive for HIV-1 DNA or validation error→ submit 1 x purified sperm straw for viral validation
- Diagnostic semen sample positive for HIV-1 RNA or validation error→ submit 1 x purified sperm straw for viral validation
- Diagnostic semen sample negative for HIV-1DNA and RNA→ No straw to be submitted for viral validation

Week 2 - Repeat procedures from Week 1

Assisted reproduction using purified sperm was performed if:
- Therapeutic semen sample tested negative for HIV-1 DNA and RNA
- An aliquot of the purified sperm tested negative for HIV-1 DNA and RNA

Figure A.6: Diagrammatic experimental layout of the evaluation of the effectiveness of semen processing with the ProInsert™ for the elimination of *in vivo* derived HIV-1 from semen samples.

SEMEN PARAMETERS OF DONORS

Summaries of sperm parameters of donors used in the following studies.

Evaluation of the:

- Effect of semen processing with PureSperm$^{®}$ Pro, supplemented with recombinant, human sequence trypsin (rTrypsin), compared to standard PureSperm$^{®}$ (not supplemented with rTrypsin), on sperm-zona pellucida binding (Figure B.1).

- Effect of semen processing with density gradient layers supplemented with trypsin at different concentrations on sperm: vitality, mitochondrial membrane potential and motility parameters (Figure B.2).

- Effect of semen processing with density gradient layers supplemented trypsin inhibitor on sperm parameters (Figure B.3).

- Effectiveness of density gradient centrifugation in combination with the ProInsert™ for the removal of white blood cells from spiked semen samples (Figure B.4).

In all figures, the World Health Organization's (2010)* reference values for normal sperm parameters are indicated with dotted lines.

*World Health Organization. 2010. Laboratory manual for the examination and processing of human semen. Cambridge, UK: Cambridge University Press, 5th Ed.
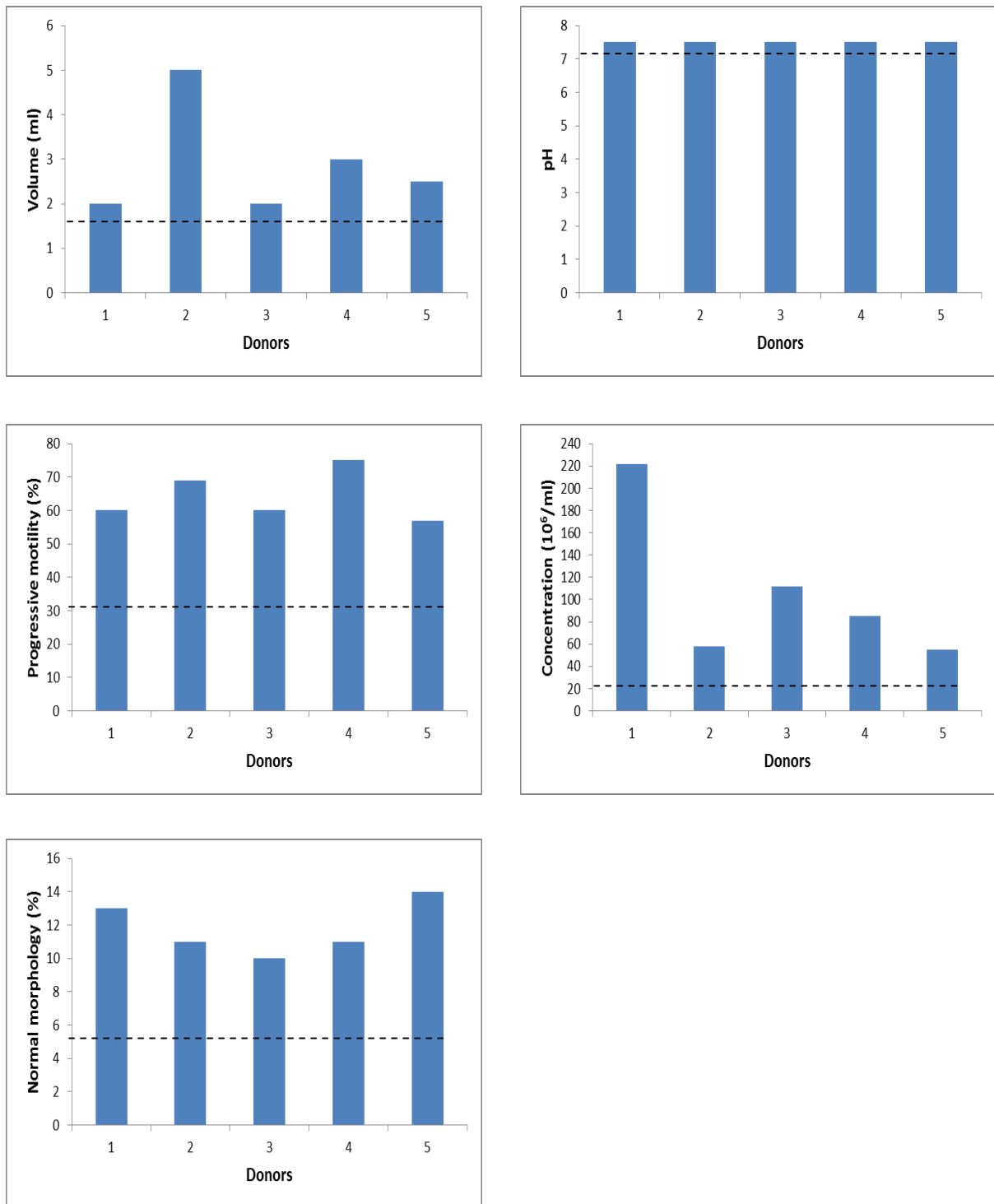
Figure B.1: Sperm parameters determined for donors who participated in the experiment for the evaluation of the effect of semen processing with PureSperm® Pro (supplemented with rTrypsin), compared to standard PureSperm® (not supplemented with rTrypsin) on sperm-zonae pellucidae binding.

~ 224 ~
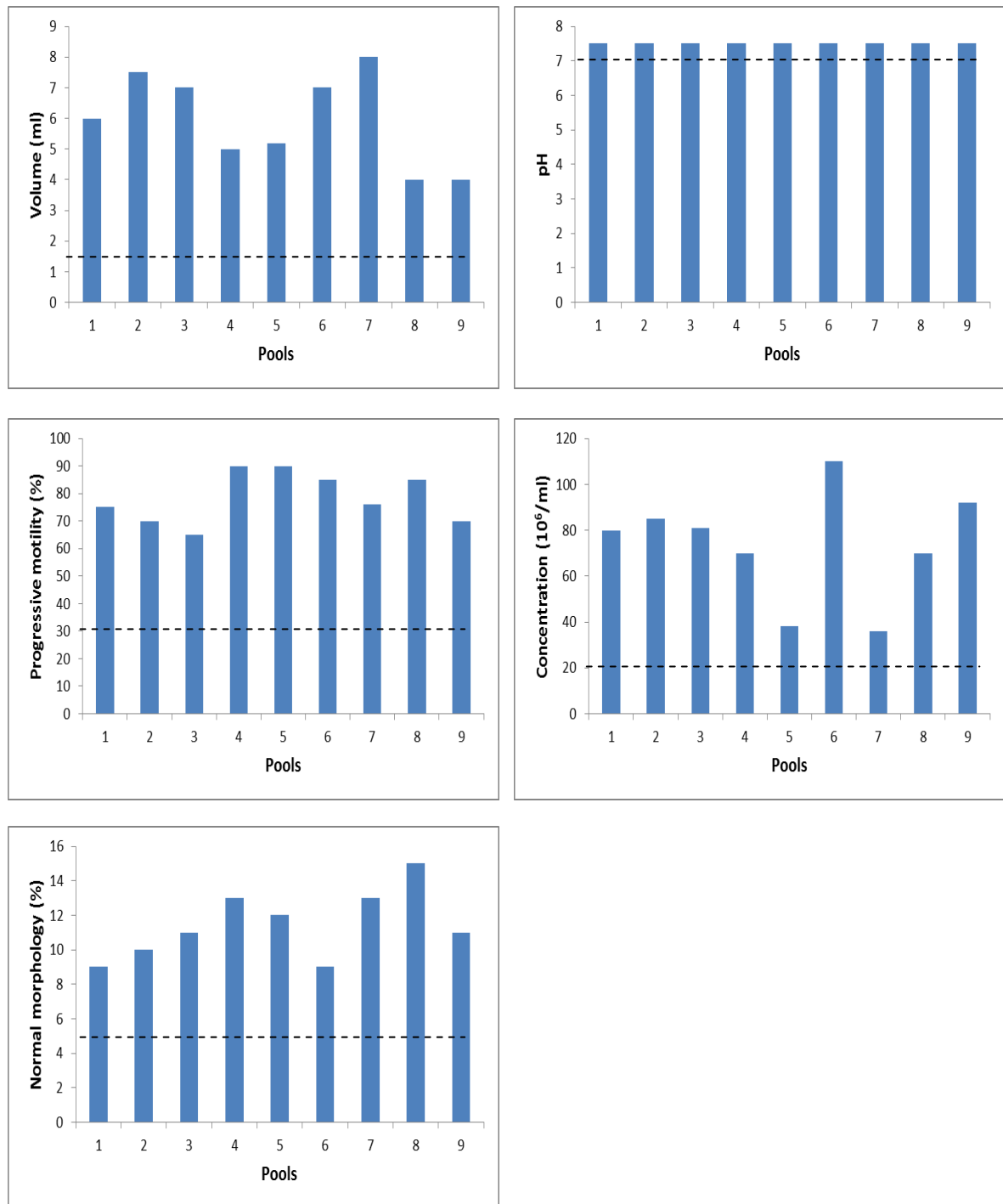
Figure B.2: Sperm parameters determined for donors that participated in the study for the evaluation of the effect of rTrypsin on sperm parameters (N=9 pools and 2 donors/pool).
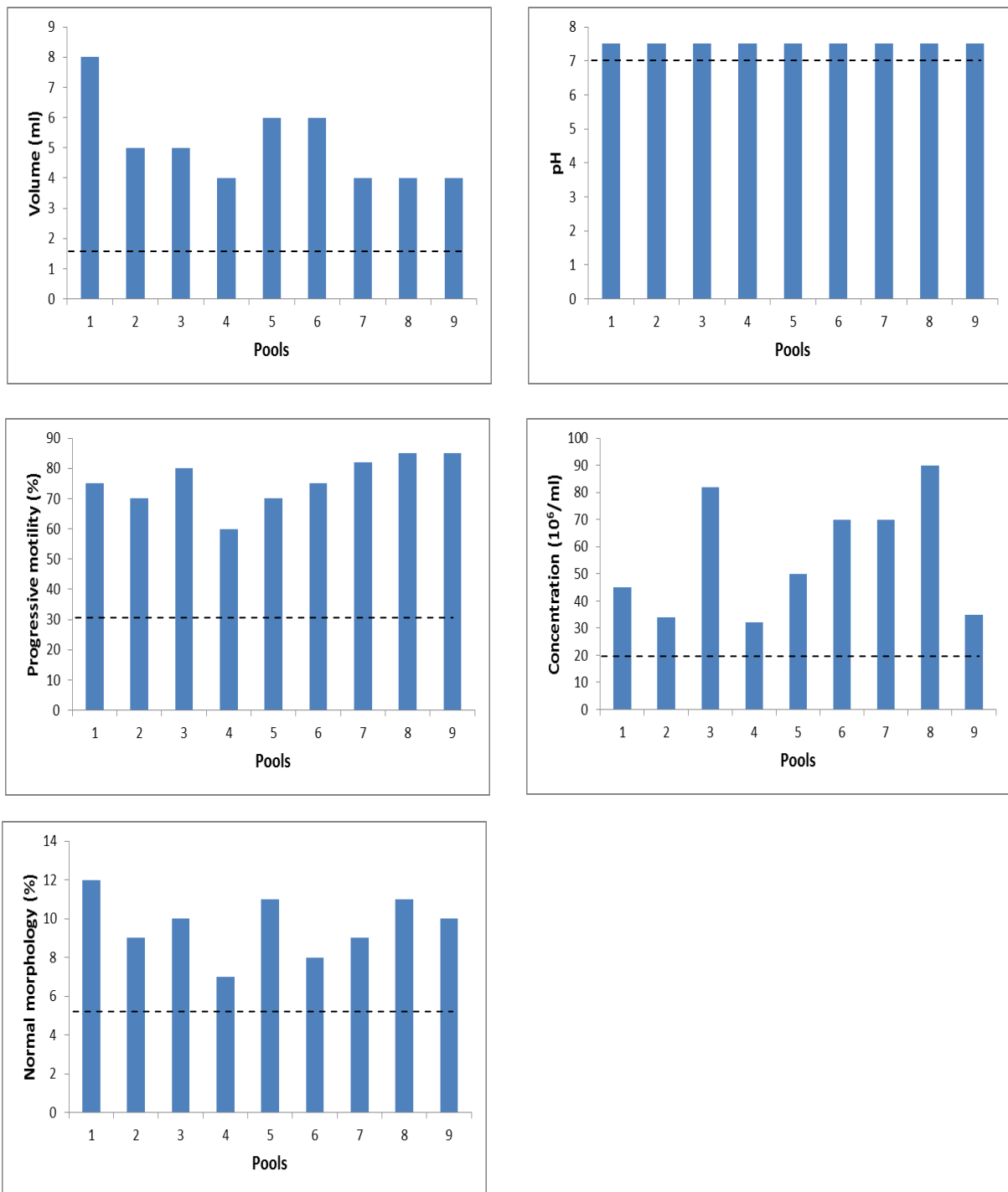
Figure B.3: Sperm parameters determined for donors that participated in the study for the evaluation of the effect of trypsin inhibitor on sperm parameters (N=9 pools and 2 donors/pool).
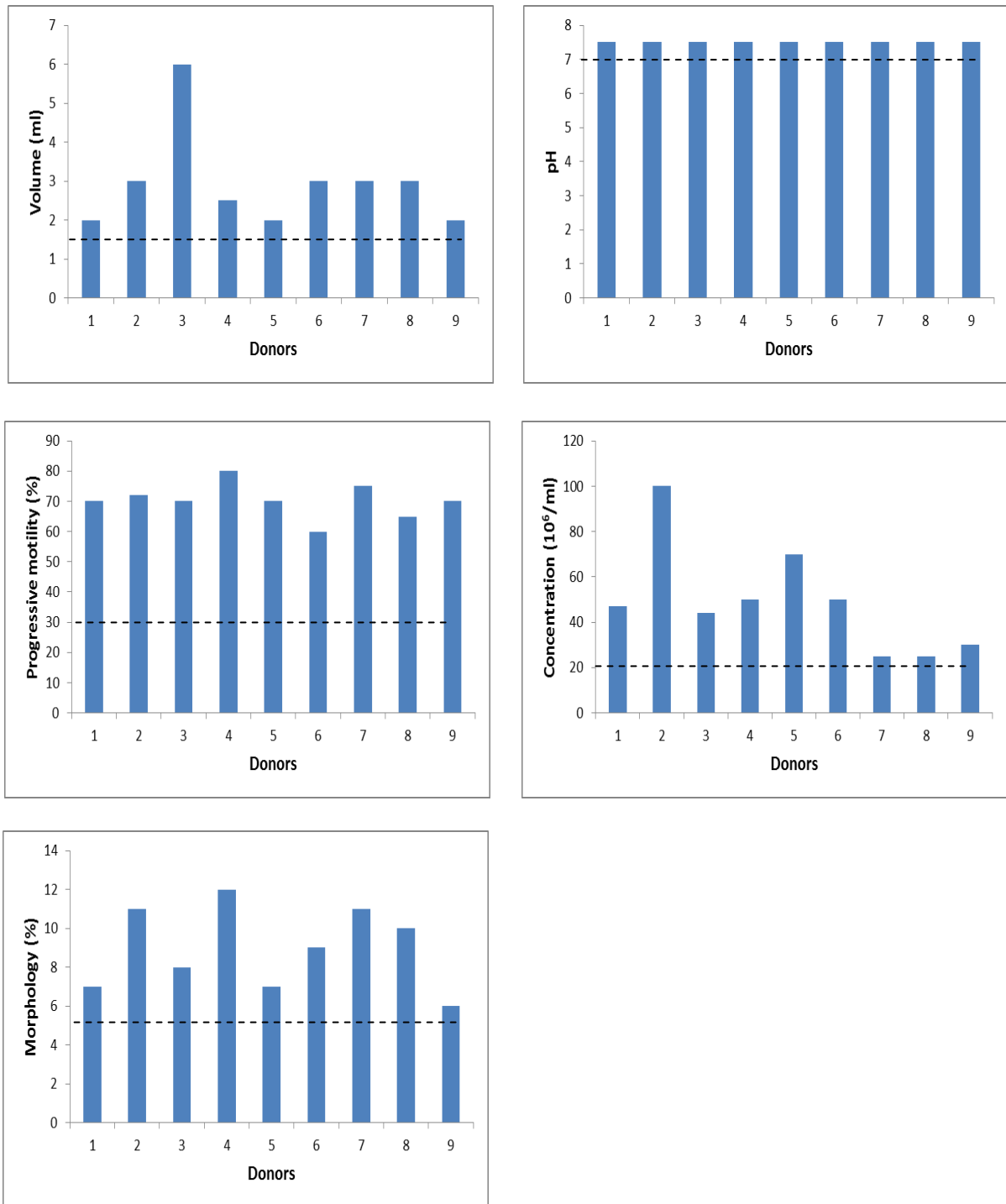
Figure B.4: Sperm parameters determined for donors who participated in the study for the evaluation of the effectiveness of semen processing for the removal of white blood cells from semen samples (N=9 pools and 2 donors/pool).

ANNEXURE C

PEER-REVIEWED PUBLISHED ARTICLES AND PRESENTED POSTERS

Articles that were published in international, peer-reviewed journals and posters that were presented are listed in this section.

Articles:

- Fourie, J., Loskutoff, N., Huyser, C. 2012. Treatment of human sperm with serine protease during density gradient centrifugation. *J Assist Reprod Genet*. 29, 1273-1279.

- Fourie, J.M., Loskutoff, N., Huyser, C. 2012. Elimination of bacteria from human semen during sperm preparation using density gradient centrifugation with a novel tube insert. *Andrologia*. 44, 513-517.

Permission for the inclusion of the articles in this thesis was received from Plenum Press (see Page 229) and John Wiley and Sons publishers (see page 237), respectively.

Poster presentations:

- Fourie, J., Loskutoff, N., Huyser, C. 2010. PureSperm: prevention of sperm pellet re-contamination by HIV-1 RNA, DNA and bacteria during density gradient centrifugation. Presented at Faculty Day: Faculty of Health Sciences, University of Pretoria – 18 August 2010.

- Fourie, J., Loskutoff, N., Huyser, C. 2012. Treatment of human sperm with serine protease during density gradient centrifugation: Potential additive to reduce pathogenicity and to enhance ART outcome. Presented at Faculty Day: Faculty of Health Sciences, University of Pretoria – 28 August 2012.

Journal of assisted reproduction and genetics Billing Status:

Order detail ID: 63833744 Article Title: Treatment of human sperm with serine protease during density gradient centrifugation. Author(s): Fourie, J. ; Loskutoff, N. ; Huyser, C.DOI: 10.1007/S10815-012-9851-6Date: Nov 01, 2012ISSN: 1573-7330 Publication Type: e-Journal. Volume: 29 Issue: 11 Start page: 1273 Publisher: Plenum Publishers (Journals) Author/Editor: ALPHA, Scientists in Reproductive Medicine.

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Title of your thesis / dissertation	Semen decontamination for the elimination of seminal pathogens
Expected completion date	Oct 2013

TECHNOLOGICAL INNOVATIONS

Treatment of human sperm with serine protease during density gradient centrifugation

J. Fourie · N. Loskutoff · C. Huyser

Abstract

Purpose Seminal pathogens can bind specifically or non-specifically to spermatozoa, rendering semen decontamination procedures ineffective, whereby vertical or horizontal transmission of the infection could occur. Serine proteases have been demonstrated to effectively inactivate viruses and to break pathogen-sperm bonds. However, the addition of a protease to density gradient layers during semen processing could negatively impact on sperm parameters. This study investigated the effect of the addition of a recombinant, human-sequence protease (rhProtease) on sperm parameters during density gradient centrifugation.

Methods (i) Pooled semen samples ($n=9$) were split and processed by density gradient centrifugation, with the top density layers supplemented, or non-supplemented with rhProtease at three different concentrations (diluted 2, 10 and 20 times). Sperm parameters were then analysed by flow cytometry and computer-assisted semen analyses. (ii) Semen samples ($n=5$) were split and similarly processed using PureSperm® Pro, with rhProtease in the 40 % density gradient layer, or standard PureSperm® not supplemented with rhProtease (Nidacon, International) respectively. The Hemizona assay was then utilized to compare sperm-zona binding post processing.

Results Evaluation of sperm parameters indicated that rhProtease did not, at any of the tested concentrations, have an impact on (i) mitochondrial membrane potential, vitality, motility, or (ii) zona binding potential.

Conclusion We report that the addition of rhProtease to density gradients is a non-detrimental approach that could improve the effectiveness of semen processing for the elimination of seminal pathogens, and benefit assisted reproduction outcome.

Keywords Density gradient centrifugation · Semen processing · Serine protease · Sperm parameters

Capsule The addition of rhProtease to density gradient layers did not impact negatively on: sperm apoptotic or necrotic status, mitochondrial membrane potential, or motility.

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Introduction

In an attempt to purify sperm and to eradicate pathogens, semen processing is performed by various assisted reproductive laboratories [1–3]. However, the binding of viruses such as human immunodeficiency virus–1 (HIV-1) [4, 5] and other pathogenic micro-organisms, including: *Escherichia coli* [6, 7], *Ureaplasma urealyticum* [8], *Mycoplasma hominis* [9], *Neiseria gonorrhoeae* [10] and *Chlamydia trachomatis* [11], to the spermatozoa, may result in the transport of these pathogens through the gradient layers during density gradient centrifugation (DGC). Processed sperm pellets, therefore, may remain positive for pathogens. The adherence of viruses to sperm membranes may contribute to the reported failure rate of 10 % during semen processing for the elimination of HIV and hepatitis [12]. Consequently, techniques to inactivate pathogens and to break the virus-sperm bonds are, therefore, investigated.

Serine proteases such as chymotrypsin and trypsin are natural constituents of semen, that are secreted by the auxiliary seminal glands and play a role in semen liquefaction

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[13]. Tang et al. (1991) reported that HIV is susceptible to inactivation by trypsin at low concentrations [4]. Therefore, the effectiveness of DGC for the removal of viruses from semen could be improved by the addition of trypsin to density gradient layers [14]. In addition, serine protease has also been reported to remove bound proteins that may be responsible for sperm-antibody formation, thereby increasing fertilizing potential [15]. However, the effect of trypsin on human sperm parameters must first be considered. Binding of capacitated, acrosome-intact spermatozoa to the ZP3 and ZP4 glycoproteins of the zona-pellucida is a prerequisite for fertilization [16]. Trypsin could have a negative impact on sperm receptors, whereby sperm binding to the zona pellucida glycoproteins could potentially be compromised. Silva, Solana & Castro (1999), reported a decrease in the total and progressive motility, as well as vitality of bovine sperm treated for a period of 5 min with trypsin at a concentration 0.3 % [17].

Any potential negative effects of reduced motility or vitality on sperm fertilizability [18] will cause any semen processing method to be undesirable. The current study, therefore, investigated the effect of semen processing by DGC, supplemented with a recombinant, human-sequence serine protease (rhProtease), on human sperm parameters.

Materials and methods

Institutional approval for the study was received from Steve Biko Academic Hospital and the Medical Research Council's Ethics Committee, University of Pretoria (protocol number 37/08). Informed written consent was received from all participants.

Processing of semen using density gradients supplemented with rhProtease

Prior to experimentation, the proteolytic activity of rhProtease (Nidacon International, Mölndal, Sweden) was tested and confirmed by the effective trypsinization of HeLa cells [19]. Density gradients (40 & 80 %) were prepared by diluting PureSperm®-100 % with PureSperm® Buffer (Nidacon, International). rhProtease was added to the 40 % gradient at dilutions of 0, 2, 10 and 20 times. Semen samples with parameters in the normal ranges, as described by the World Health Organization (WHO) [20], were pooled ($n=2$ samples/pool) and divided into test ($n=3$ samples) and control ($n=1$) samples. The samples were processed by DGC according to the product manufacturer's guidelines using the supplemented, or non-supplemented, density layers, respectively. Sperm parameters were evaluated 16 to 18 h after processing and the experiment was repeated nine times.

Flow cytometry controls

Sperm vitality (apoptosis & necrosis) and mitochondrial membrane potential were determined by means of flow cytometry (FC500, Beckman Coulter, Brea, CA, USA). Protocols were set up and validated by the inclusion of the following controls: (i) sperm apoptosis was induced by incubation with 1 μM staurosporine (Sigma-Aldrich, St Louis, MO, USA) for 18 h at 37 °C [21]; (ii) Annexin V-FITC binding to sperm was blocked by incubating sperm, according to the manufacturer's guidelines, with purified recombinant Annexin V (BD Biosciences, San Diego, CA, USA); (iii) necrosis was induced by incubating 1×10^6 sperm (1 ml) with 2 μl of Triton X (Sigma-Aldrich); and (iv) mitochondrial membrane potential ($\Delta\psi_m^{high}$) was abolished by incubating sperm with 50 μmol/l carbamoylcyanide m-chlorophenylhydrazone (mCLCCP, Sigma-Aldrich) for 15 min at 37 °C [22].

Vitality

Sperm vitality post-processing was determined using a BD Pharmingen™ FITC Annexin V apoptosis detection kit (BD Biosciences). Aliquots (5×10^6 sperm) from the processed samples were washed twice; first in 2 ml of cold phosphate-buffered saline (Sigma-Aldrich) and, secondly, in 2 ml of cold Annexin V binding buffer. Sperm pellets (100 μl) were obtained and 5 μl of Annexin V-FITC and propidium iodide (PI) were added. The samples were vortexed and incubated (24 °C) in the dark for 15 min, binding buffer (400 μl) was then added and flow cytometry evaluations were performed within 20 min after staining. Annexin V-FITC and PI stained sperm were detected in the FL1 and FL3 channels, respectively.

Mitochondrial membrane potential

Mitotracker Red CMX Ros (Molecular Probes, Eugene, USA) (50 μg) was diluted in 100 μl DMSO (Sigma-Aldrich) to prepare a stock solution that was stored at −20 °C. Processed sperm samples (5×10^6 cells) were re-suspended in 1 ml Pure-Sperm® Wash (Nidacon, International). Mitotracker (2.6 μl from the stock solution) was added to the sperm, and the suspensions were incubated at 37 °C for 15 min. The cells were washed, re-suspended in 1 ml PureSperm® Wash, and flow cytometry was performed to determine the percentages of cells with high mitochondrial membrane potential ($\Delta\psi_m^{high}$) using the FL3 channel.

Computer assisted semen analyses (CASA)

Two-chamber, 20 μm deep, Leja® counting chambers (Leja Products, The Netherlands) were pre-warmed to 37 °C.

Duplicate sperm aliquots (5 μl) were loaded into both chambers, then at least 200 sperm and 10 microscope fields were evaluated per chamber [20] by means of CASA (MediaLAB, version 5.4 Altdorf, Germany) at 200 times magnification (Axioskop 40; Zeiss, Göttingen, Germany).

Sperm-zona interaction

The hemizona assay was utilized to evaluate sperm-zona binding potential [23]. Semen samples ($n=5$) with parameters in the normal ranges, as described by the WHO [20], were received from donors ($n=5$). Samples were split and processed by means of DGC, using PureSperm® Pro (Nidacon, International), with rhProtease in the 40 % density gradient layer, or standard PureSperm® not supplemented with rhProtease (Nidacon, International). Prepared sperm samples were kept at room temperature (37 °C) until insemination of the hemizonae.

Non-viable, unfertilized oocytes remaining from the Unit's in vitro fertilization (IVF) program were bisected by means of micromanipulation (Transferman; Eppendorf, Hamburg Germany) at 200 times magnification (Axiovert 200; Zeiss) using a 3.5 mm, 30 degree blade (BD Micro-Sharp™, Beckton, Dickinson and Company, Franklin Lakes, NJ, USA). Droplets of 50 μl PureSperm® Wash (Nidacon, International) were prepared in culture dishes (Falcon 353004; Becton Dickenson Co.) and covered using FertiCult™ mineral oil (FertiPro, Beemem, Belgium). Hemizonae were placed in the micro-droplets and any attached sperm remaining from IVF were stripped by vigorous pipetting using 80 μm pipette (Flexipet®; Cook, Limerick, Ireland). Procedures were performed at room temperature (24 °C) and oocytes were bisected on the day of use.

Hemizonae ($n=7$ pairs per semen donor) were individually inseminated with 1×10^4 progressively motile test or control sperm. After an incubation period of 16 h at 37 °C, the hemizonae were transferred to freshly prepared micro droplets. Loosely bound sperm were removed by pipetting 4 times using a 130 μm pipette tip (Flexipet®; Cook). The numbers of tightly bound sperm were double-blind counted by two evaluators at 400 times magnification (Axiovert 200; Zeiss).

Statistical analysis

Sperm parameters were examined after semen processing, using top density gradient layers containing rhProtease diluted 20, 10, and 2 times, and were compared to similar processing without rhProtease. Stata Statistical Software: Release 10 [24] was used to perform a mixed model analysis, and to preserve the 0.05 level of significance, each test was done at the Bonferroni adjusted level of significance of 0.017, i.e. 0.05/3 [25].

Stata Statistical Software was also used to compare the number of sperm bound to hemizonae post processing using PureSperm® and PureSperm® Pro. This comparison was performed using a mixed-model approach under the maximum likelihood option. This method controls for the dependence of data associated with specific semen donors.

Results

Activity of rhProtease

The rhProtease used to supplement the PureSperm® density gradient layers, was confirmed to be active by trypsinization. Incubation of cultured HeLa cells with the rhProtease resulted in the cells becoming round and dislodged from the bottom of the cell culture dish after 2 min incubation at 37 °C.

Flow cytometric controls

The incubation of sperm with staurosporine and recombinant Annexin was effective for the induction of apoptosis and blocking of Annexin V-FITC binding, respectively. Fluorescence intensities of Annexin V-FITC stained sperm, after treatment with staurosporine and recombinant Annexin, are illustrated in Fig. 1a. Figure 1b illustrates the necrotic status of sperm after treatment with Triton X, and Fig. 1c illustrates the abolishment of $\Delta\psi_m^{high}$ by treatment of sperm with mCLCCP.

Vitality, mitochondrial membrane potential and motility parameters

Processing of semen samples by means of DGC with the top density layer containing rhProtease, diluted 2, 10 and 20 times, did not have a negative impact on apoptotic and necrotic status, mitochondrial membrane potential (Table 1), or any of the motility parameters of sperm including: progressive motility, average path velocity, curvilinear velocity and straight line velocity (Table 2).

Sperm-oocyte interaction in the hemizona assay

Processing of semen using PureSperm® Pro with rhProtease did not cause a reduction in the number of sperm bound to the hemizonae when compared to processing with standard PureSperm® ($P=0.732$), not supplemented with rhProtease. The results are summarized in Table 3.

Discussion

Treatments of sperm for 5 min at concentrations of 25–100 μg trypsin/ml has been found previously to effectively

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Fig. 1 Fluorescence intensity of treated sperm, included as controls, during set up of flow cytometry protocols; (a) Staurosporine to induce apoptosis, and purified recombinant annexin to block sperm annexin V-FITC binding; (b) Triton X induced necrosis; (c) mitochondrial membrane potential was abolished by treatment with mCLCCP
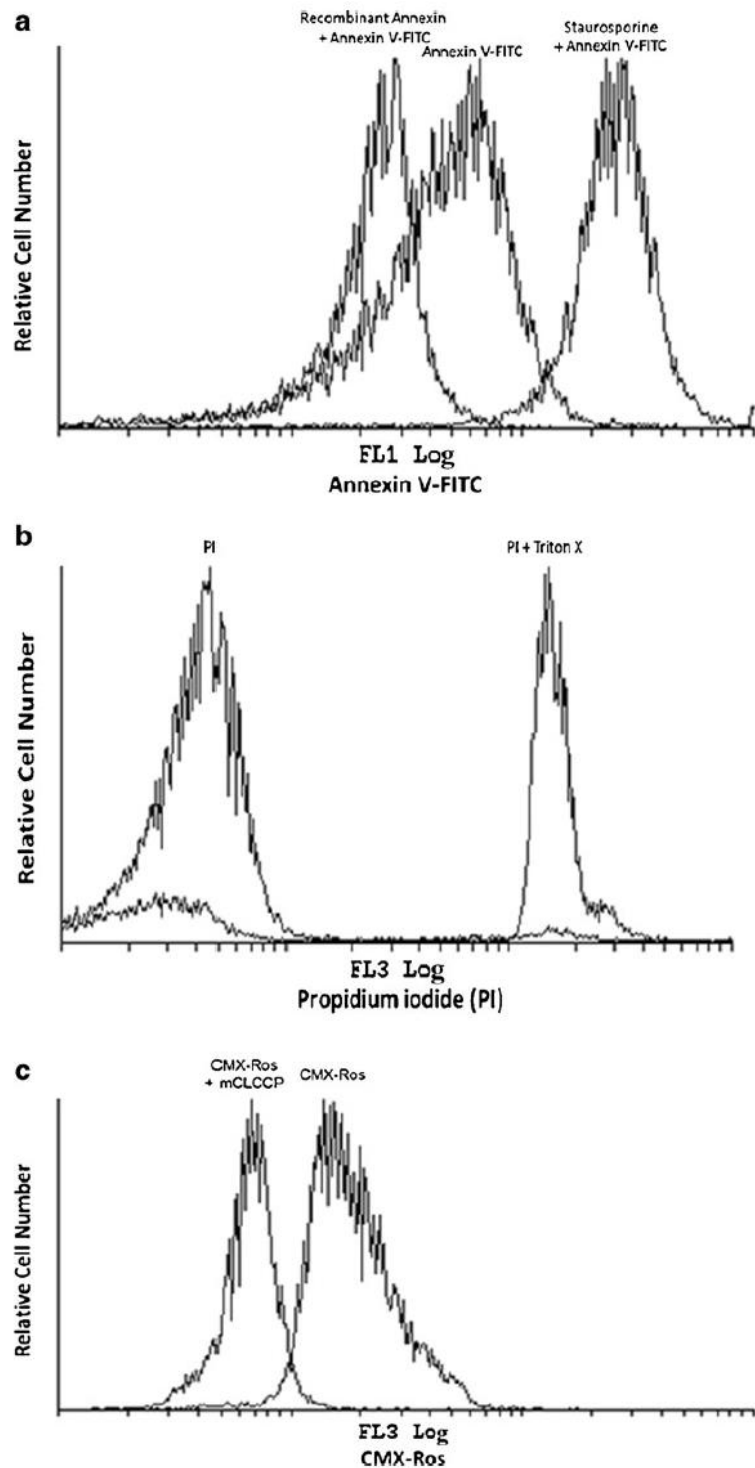
inactivate HIV-1, depending on the number of viral copies present [4]. Loskutoff et al. (2005) reported a significant reduction in the infectivity of HIV-1 RNA after a brief (1 min) exposure to 0.25 % trypsin and the effective removal of HIV-1 and HCV RNA from spiked human semen by processing semen with density gradients supplemented with

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Table 1 Apoptotic/necrotic status (AV-PI-) of sperm treated with rhProtease compared to untreated sperm

rhProtease concentration	(AV-PI-)			MMP		
	Mean	SD	P-Value	Mean	SD	P-Value
0× Trypsin	91.772	8.782		90.469	3.943	
20× Diluted	93.641	5.231	0.079	87.549	6.391	0.090
10× Diluted	93.748	4.936	0.064	86.517	6.076	0.022
2× Diluted	93.369	3.801	0.134	88.647	5.073	0.290

Table 3 Numbers of sperm bound to the hemizonae post-processing with standard Puresperm® compared to PureSperm® Pro

Treatment	Mean number bound	SE	95 % CI
PureSperm (-rhProtease)	26.26	6.60	13.33; 39.19
PureSperm Pro (+rhProtease)	28.51	6.60	15.58; 41.44

Standard error (SE) & 95 % confidence interval (CI)

trypsin at the same concentration [14]. Furthermore, trypsin treatment at a concentration of 0.3 % and 0.25 % has successfully been utilized to inactivate bovine herpesvirus-1 during the washing of bovine semen [26].

Another application may be in cases of semen hyperviscosity, where the progressive motility of sperm is decreased [27], potentially resulting in decreased sperm yield during semen processing. Hyper-viscous semen samples could therefore be treated to reduce viscosity [28]. This is of importance especially in patient populations with high incidences of sexually transmitted infections such as HIV [29] and *Ureaplasma urealyticum* [30, 31], with increased prevalence of seminal hyper-viscosity. The addition of trypsin to semen has been proven to effectively reduce seminal viscosity [32, 33], whereby sperm yield during processing could also be improved.

The ability of serine proteases to inactivate and remove viruses attached to sperm [14] and the potential to prevent sperm-antibody formation [15], together with improved sperm yield during processing, demonstrates the advantages for use as a supplement during semen processing. The addition of rhProtease to density layers in combination with the usage of a ProInsert™ (Nidacon, International), could therefore be beneficial, specifically when assisting patients with potentially infected semen samples [34]. However, the effect rhProtease has on sperm parameters should be considered prior to its inclusion into DGC protocols. Therefore, we evaluated sperm parameters post-processing to determine whether rhProtease could have any detrimental effects.

This study indicated that semen processing with the top density gradient layer supplemented with rhProtease, diluted

0, 2, 10 and 20 times, did not impact negatively on: sperm apoptotic status, necrotic status, mitochondrial membrane potential, or motility. Sperm-zona binding potential was similar for sperm processed with the rhProtease contained in PureSperm® Pro, when compared to the standard PureSperm® (Nidacon, International) without rhProtease. The low concentrations of rhProtease added to the density gradient layers, together with the washing step after exposure to the enzyme and silane-coated silica particles, could contribute to the lack of a negative impact of rhProtease on sperm parameters. These results are in agreement with that of other researchers. Mattson (2008) reported that the treatment of bovine sperm with trypsin had no negative impact on sperm parameters or in vitro embryo production [35]. In another study, fertilization rates and the number of in vivo produced transferrable bovine embryos were improved by the processing of semen with density gradients supplemented with trypsin [36]. Loskutoff et al. (2005) reported that DGC of semen with trypsin added to density layers had no detrimental effects on sperm motility or viability [14]. Furthermore, trypsin treatment of human sperm, prior to swim up, resulted in increased motility and adenosine triphosphate (ATP) concentration [37]. Westhoff and Kamp (1997) suggested that the increased ATP concentration could be attributed to the activation of the glycolytic enzyme glyceraldehyde triphosphate dehydrogenase (GADPH) by trypsin [38], explaining the improved motility.

In conclusion, the addition of rhProtease to the top layer during density gradient centrifugation is non-detrimental to human sperm. The usage of rhProtease improves (i) sperm yield of hyper-viscous semen samples, (ii) the effectiveness of semen processing for the elimination of seminal pathogens

Table 2 Progressive motility of sperm treated with rhProtease compared to untreated sperm

rhProtease concentration	Progressive motility			Average path velocity			Straight line velocity			Curvilinear velocity		
	Mean	SD	P-Value	Mean	SD	P-Value	Mean	SD	P-Value	Mean	SD	P-Value
0× Trypsin	83.889	5.476		42.202	11.520		38.474	11.261		59.731	13.882	
20× Diluted	82.222	7.567	0.281	41.657	12.598	0.703	37.793	12.224	0.666	59.225	13.982	0.713
10× Diluted	84.000	5.220	0.943	42.938	12.465	0.607	39.252	12.438	0.621	61.212	13.748	0.281
2× Diluted	84.222	6.305	0.829	43.408	10.419	0.399	39.459	10.367	0.532	60.904	12.010	0.281

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and, potentially, (iii) the prevention of sperm-antibody formation, all of which could enhance the outcome assisted reproduction.

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ORIGINAL ARTICLE

Elimination of bacteria from human semen during sperm preparation using density gradient centrifugation with a novel tube insert

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Summary

The occurrence of bacteria in sperm samples intended for *in vitro* fertilisation can compromise the outcome of assisted reproductive techniques. Effective semen processing procedures should therefore be implemented to remove bacteria from semen. Unfortunately, technique failure does occur whereby bacteria can be found in processed sperm preparations. To improve the effectiveness of semen processing, a novel centrifuge tube insert was developed to facilitate the layering of density gradients and semen, and to prohibit the re-infection of purified sperm pellets. The purpose of this study was to: (i) determine the prevalence and type of bacteria present in semen of patients participating in the Unit's assisted reproduction program and (ii) evaluate the effectiveness of density gradient centrifugation with the novel tube insert, for the elimination of bacteria and yeast from spiked human semen samples. A survey in 2007–2010 indicated that 50% of semen samples were found to have positive bacterial cultures. Semen processing by means of density gradient centrifugation with the novel tube insert eliminated significantly more *in vitro* derived (spiked) bacteria and yeast from semen compared to processing without the insert ($P < 0.004$). Therefore, it is highly recommended that the centrifuge tube insert, ProInsert™, be incorporated into assisted reproductive programs.

Introduction

Bacteriospermia, the occurrence of bacteria in semen (Keck *et al.*, 1998), is present among 54–57% of patients attending infertility centres (Cottell *et al.*, 2000; Gdoura *et al.*, 2008; Kiessling *et al.*, 2008) with the presence of bacteria in semen attributed to systemic and local reproductive tract infections, or to contamination post-ejaculation (Bielanski, 2007). During assisted reproductive techniques (ART) including intrauterine insemination (IUI), *in vitro* fertilisation and intracytoplasmic sperm injection (Krissi *et al.*, 2004), the natural immunological defence mechanisms present in the female reproductive tract are bypassed (Cottell *et al.*, 1997). Consequently, bacteria may be introduced into the upper genital tract, or the embryo culture system, potentially leading to a compromised outcome of ART and, or infection of the female genital tract (Huyser *et al.*, 1991; Cottell *et al.*, 1997, 2000; Kastrop *et al.*, 2007). The frequency of micro-organism infections post-IUI is approximately 0.01% (Broder *et al.*, 2007) and infections of *in vitro* embryo culture systems range between 0.35% and 0.68% (Cottell *et al.*, 1996; Kastrop *et al.*, 2007).

Patients with semen cultures positive for bacteria should undergo antibiotic treatment prior to ART. However, antibiotic treatment will be ineffective against skin contaminants present in semen. Therefore, antibiotics (penicillin, streptomycin and gentamycin) are usually added to semen processing and embryo culture media (Magli *et al.*, 1996; Cottell *et al.*, 1997; Gardner & Lane,

2007; Kastrop *et al.*, 2007). This addition of antibiotics to culture media, however, may result in antibiotic resistant bacterial strains (Kastrop *et al.*, 2007), as well as a decreased embryo cleavage rate (Magli *et al.*, 1996; Lemeire *et al.*, 2007). Semen washing, with an extra swim-up step, has been reported to be more effective in decreasing the incidence of potential pathogens in sperm samples compared to antimicrobial therapy by prescription antibiotics (Huyser *et al.*, 1991).

Semen processing procedures that are effective in the elimination of bacteria from semen are required. Depending on the processing method employed, 5–43% of sperm samples will remain positive for bacteria post-processing (Huyser *et al.*, 1991; Cottell *et al.*, 1997; Knox *et al.*, 2003). Procedural failure could be attributed to the contamination of the sperm pellets post-processing. A medical grade polypropylene, centrifuge tube insert (Proinsert™; Nidacon International, Mölndal, Sweden), has been developed to avoid inadvertent contamination without further washing (Loskutoff *et al.*, 2005).

The purpose of this study was to: (i) determine the prevalence of bacteria in semen of men attending an infertility centre at Steve Biko Academic Hospital (SBAH) and (ii) evaluate the effectiveness of density gradient centrifugation (DGC) using a centrifuge tube insert for the removal of prevalent bacteria and yeast from spiked human semen samples.

Materials and methods

Institutional approval for the study was received from SBAH and the Medical Research Council's Ethics Committee, University of Pretoria (protocol number 37/08).

Prevalence of bacteria in semen

The prevalence of bacteria in semen samples ($n = 1210$) from men ($n = 1038$) participating in the ART program at SBAH were surveyed in 2007–2010.

Patients were requested to sexually abstain for 3 days. Guidelines to deliver semen samples for diagnostic evaluation (World Health Organisation, 2010) were verbally discussed with patients (Boucher *et al.*, 1995). These guidelines were also available in a written format in four national indigenous languages.

Following liquefaction (37 °C for 30 min), 200 μl semen aliquots were submitted to the National Health Laboratory's Microbiology Department for microscopy, culture and sensitivity evaluation according to the Unit's standard operating procedures (SOP) (Working Group, Tshwane Academic Division, National Health Laboratory Service, Department of Microbiology, University of Pretoria, 2006).

Semen processing for the elimination of bacteria and yeast from spiked semen samples

Semen from donors ($n = 5$) were collected, pooled and gram-stained to ensure the absence of micro-organisms according to SOP. The pooled sperm concentration was adjusted to 40×10^6 spermatozoa per ml by dilution with PureSperm Wash (PSW-100; Nidacon International). Subsequently, 1 ml aliquots of the pooled semen sample were inoculated with bacteria or yeast commonly found in semen. *Escherichia coli* (ATCC 25922), *Enterobacter cloacae* (in-house strain), *Enterococcus faecalis* (ATCC 29212), Coagulase-negative staphylococci (in-house strain), *Staphylococcus aureus* (ATCC 25923) and *Candida albicans* (ATCC 90028) were individually added to the semen aliquots (in duplicate) at concentrations of 1×10^3, 10^4, 10^5 and 10^6 colony forming units per ml (CFU per ml). The inoculated semen samples were processed using DGC (PureSperm® 40 and 80%; Nidacon International) with and without the use of the polypropylene centrifuge tube insert (ProInsert™; Nidacon International) (Fig. 1), without an additional swim-up step. Bacteria and yeast quantifications were performed by inoculating Mac-Conkey and blood agar plates with 10 μl aliquots of the processed sperm samples. The numbers of colony forming units present were macroscopically counted following a 24 h incubation period at 37 °C. Nonspiked semen samples served as negative controls, and unprocessed spiked semen samples were included as positive controls.

Statistical analysis

STATA Statistical Software: Release 10 (StataCorp., 2007) was used to perform a two factor analysis of variance (ANOVA) to compare the numbers of bacteria and yeast colony forming units present in sperm samples post-processing, either with or without the insert, and at two spiking concentrations [log (1×10^5 and 1×10^6) CFU per ml].

Results

Prevalence of bacteria in semen

The prevalence of bacteria in semen is indicated in Table 1.

Semen processing for the elimination of bacteria and yeast from spiked semen samples

Sperm pellet retrieval using the novel ProInsert™ eliminated recontamination and removed significantly more micro-organisms (96%) from semen compared to processing without the insert [$P < 0.004$ with respect to mean log (CFU)]. Treated sperm pellets remained clear of
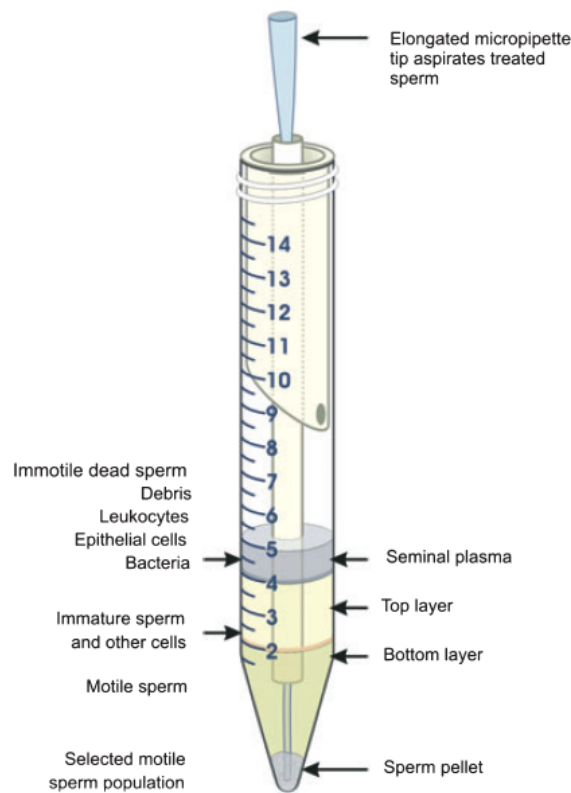
Fig. 1 Centrifuge tube containing medical grade polypropylene insert (ProInsert™; Nidacon International, Mölndal, Sweden).

Table 1 Prevalence of bacteria in semen samples ($n = 1210$) of patients participating in an ART programme at SBAH surveyed in 2007–2010

Organism	Gram	No.	(%)
No growth		611	50
Coagulase-negative staphylococci	+	280	23
Escherichia coli	–	53	5
Staphylococcus aureus	+	45	4
α-Haemolytic streptococci	+	45	4
Enterococcus spp.	+	39	3
Enterococcus faecalis	+	38	3
β-Haemolytic streptococci	+	16	2
Klebsiella pneumoniae	–	17	1
Enterococcus feacium	+	11	1
Enterobacter cloacae	–	9	1
Streptococcus spp.	+	9	1
Staphylococcus spp.	+	12	1
Ureaplasma spp.	Null	145	12
Mycoplasma spp.	Null	57	5

Pseudomonas spp., *Citrobacter* spp., *Haemophilus* spp., *Micrococcus* spp., *Neisseria* spp., *Bacillus* spp., *Acinetobacter* spp., *Enterococcus* spp. and *Aeromonas hydrophila* were present in <1% of semen samples.

micro-organisms below the spiking concentration of 1×10^5 cfu per ml. Bacterial and yeast concentrations (CFU per ml) present after processing with and without the insert are illustrated in Table 2.

Discussion

Presence of bacteria in semen

Bacteria were present in 50% of semen samples from men seeking ART at SBAH during the period 2007–2010. This is in agreement with results from studies that reported bacterial prevalence in neat semen samples to be between 54% and 57% (Cottell *et al.*, 2000; Gdoura *et al.*, 2008; Kiessling *et al.*, 2008). The presence of bacteria in an *in vitro* embryo culture system can compromise the outcome of assisted reproductive procedures, by impacting directly on sperm quality by a reduction in motility (Nunez-Calonge *et al.*, 1998), by the induction of apoptosis/necrosis (Villegas *et al.*, 2005) or by causing degeneration of *in vitro* fertilised oocytes (Huyser *et al.*, 1991). During embryo transfer, the introduction of pathogens, such as *Mycoplasma genitalium*, into the uterus can lead to intrauterine infections that may lead to infertility (Kastrop *et al.*, 2007; Grzesko *et al.*, 2009). Infected sperm samples used for *in vitro* fertilisation are a considerable cause (35%) of infected embryo culture systems (Kastrop *et al.*, 2007). Therefore, the sterile delivery and effective preparation of sperm samples intended for use in ART should be a priority.

Urination and proper washing prior to collection of a semen sample is recommended by the World Health Organisation (WHO, 2010). Appropriate washing will significantly reduce bacterial infection of semen samples (Krissi *et al.*, 2004); nevertheless, micro-organisms resident within the male genital tract will continue to contaminate ejaculates (Woolley *et al.*, 1992). Semen samples from patients enrolling in an ART program should be examined for the presence of micro-organisms and those patients presenting with reproductive tract infections should undergo antibiotic treatment prior to ART. However, micro-organisms such as *Mycoplasma genitalium* cannot be cultured on substrates generally used for the detection of mycoplasmas. Patients with asymptomatic undetected infections will therefore be overlooked and the infection will remain untreated (Grzesko *et al.*, 2009). Due to the fact that bacterial presence in semen is mostly attributable to contamination by skin flora (Kim & Goldstein, 1999; Krissi *et al.*, 2004), treatment of these patients with antibiotics will be ineffective (Huyser *et al.*, 1991). Therefore, the importance of strictly adhering to the prescribed washing guidelines to deliver a semen sample must be stressed to patients (Boucher *et al.*, 1995). Sufficient washing prior to the delivery of a semen sample will reduce the presence of outer skin

Bacteria in human semen and removal thereof

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Table 2 Number of bacterial colony forming units per ml present in sperm samples post-DGC processing with and without the novel tube insert[a] (duplicate observations per spiking concentration)

Treatment		Micro-organisms (CFU per ml) present after processing					
		Enterobacter cloacae	Enterococcus faecalis	Escherichia coli	Coagulase-negative staphylococci	Staphylococcus aureus	Candida albicans
Spiking concentration (CFU per ml)	Without	In-house	ATCC 29212	ATCC 25922	In-house	ATCC 25923	ATCC 90028
	Insert						
1×10^3	1.1	100	0	100	100	0	0
	1.2	100	0	100	400	0	0
1×10^4	2.1	100	4400	400	600	100	300
	2.2	1100	12100	600	1200	100	400
1×10^5	3.1	4000	24800	23300	2200	2500	700
	3.2	7000	26800	25200	3700	5500	900
1×10^6	4.1	25200	35000	49500	16000	15800	2200
	4.2	29600	36700	63600	23700	16700	1900
	With insert						
1×10^3	1.1	0	0	0	0	0	0
	1.2	0	0	0	0	0	0
1×10^4	2.1	0	0	0	0	0	0
	2.2	0	0	0	0	0	0
1×10^5	3.1	0	200	100	100	400	0
	3.2	0	200	100	300	900	0
1×10^6	4.1	400	6700	100	1200	1400	200
	4.2	700	9500	100	1400	1500	200

[a]Processing with the insert significantly ($P < 0.004$) reduced the micro-organisms with respect to mean log (cfu) for all six micro-organisms.

contaminants, but will not eliminate bacteria, as microorganisms may also be present in the anterior urethra (Kohn *et al.*, 1998; Damirayakhian *et al.*, 2006).

The last line of defence against seminal-derived bacterial contamination of the embryo culture system is semen processing, utilising strict aseptic techniques and proper changing of sterile pipette tips and tubes between the DGC and washing procedures (Nicholson *et al.*, 2000). Unfortunately, technical error often occurs. Bacterial contaminants, as well as sexual transmitted pathogens such as *Ureaplasma parvum* and *Ureaplasma urealyticum*, have been found in processed sperm samples (Huyser *et al.*, 1991; Cottell *et al.*, 1997; Knox *et al.*, 2003). During standard processing methods, the supernatant is aspirated to allow access to the purified sperm pellet. Pathogens from the upper layers can adhere to the inside surface of the test tube and flow down to re-infect the purified sperm. Details of the method have been previously described (Loskutoff *et al.*, 2005). The current study demonstrated that, by utilising the ProInsert™, re-infection of the purified sperm pellets post-DGC was and significantly (96%) more bacterial colony forming units were removed from semen when compared to processing without the insert ($P < 0.004$), all without a further swim-up step. Treated sperm pellets remained clear of bacteria below the spiking concentration of 1×10^5 CFU per ml. Similar semen processing methods using the ProInsert™ has also proved to be effective in the removal of human immunodeficiency virus subtype 1 (HIV-1) and hepatitis C virus from *in vitro* spiked semen (Loskutoff *et al.*, 2005).

In conclusion, the high prevalence of seminal pathogens warrants the need for improved semen processing procedures. In the present study, the novel ProInsert™ device facilitated discontinuous density gradient layering, retrieval of the treated sperm pellet without recontamination, and effective removal of selected seminal pathogens. Used test tubes containing the insert after semen processing can be capped, and the potential hazardous material contained within the test tube can be appropriately disposed of. The ProInsert™, therefore, allows for a cost-effective and user-friendly means to improve the effectiveness of DGC to eliminate pathogens from semen. The results of this report reflects the comments by Anderson & Politch (2003) that more attention be given to develop improved semen processing methods, particularly in developing countries.

Acknowledgements

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