Analyses of spermatozoa surface proteins using different separation techniques

by

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DECLARATION

By submitting this thesis, I declare that the entirety of the work contained therein is my own, original work, that I am the owner of the copyright thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining my qualification

Kay Arlene Fortuin :_____

Date signed :_____

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Hereby I'd like to give all Praise and Glory to God all Mighty who's Grace was always sufficient and who's love prevailed in all times. His Mercy and Blessings carried me through shallow waters and lifted me above all struggle. I stand firm in Him and will for all the days of my life.

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DEDICATION

For my Mother, Jacqueline Jennifer Fortuin.

Romans 8:18

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KEY WORDS

- Male infertility
- Human sperm membrane proteins
- Sperm separation techniques: wash, swim-up, Percoll density centrifugation
- Detergents: cetyl-trimethyl-ammonium bromide, saponin, sodium dodecyl sulphate, Tween-20
- Sodium-dodecyl sulphate polyacrylamide gel electrophoresis

ABSTRACT

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Passage of spermatozoa through the female reproductive tract is essential for the regulation of fertilization, ensuring that healthy sperm reach the oocyte. Previous studies were devoted to morphological selection of sperm cells by the cervical mucus. However, research prove that the loss of integrity of the sperm plasma membrane is associated with infertile men, irrespective of their normal semen parameters. This indicates that the sperm plasma membrane plays an important role in fertilization. Further studies indicated that sperm surface proteins assist penetration through the female reproductive tract and would therefore provide useful insight in understanding other factors associated with male infertility. The aim of this project was to determine if there are any differences between sperm surface proteins of fertile donor samples in relation to infertile patient samples using different separation techniques and different detergents.

Three different sperm separation techniques were employed, including wash, swim-up (SU) and Percoll density gradient centrifugation (DGC).Parallel to this, the deoxy-ribose nucleic acid (DNA) fragmentation of these cells were analysed for comparison of the extent of DNA damage induced due to different separation techniques used. This provided evidence that the best separation technique is the DGC as it minimises the amount of DNA fragmentation caused.

Four different detergents were used in the process of extracting the membrane proteins from spermatozoa, namely sodium dodecyl sulphate (SDS),saponin,cetyl-trimethyl-

ammonium bromide (CTAB), and TWEEN-20. The membrane proteins were then separated on a12% SDS poly-acrylamide gel electrophoresis (PAGE), and analysed by Coomassie blue and silver staining techniques as well as densitometry. Due to the different chemical nature of the detergents that extracted different surface proteins, CTAB (cationic) and SDS (anionic) extracted the most because of its strong solubilising abilities as non-ionic detergents. Common proteins that were extracted in donor samples included; 115, 92.5, 89, 61, 55.5, 51.5, 47, 44.5, 43, 38.5, 34 and 28 kDa proteins. In patients, commonly occurring proteins included; 92.5, 74.5, 70, 60.5, 51.5, 50, 44.5, 43, 36, 29.5, and 25.5 kDa proteins.

Marked differences were found between membrane proteins extracted from donor samples in comparison to patient samples. Identification of these proteins was done using the SwissProt database and a literature search. Mostly non-genomic progesterone receptors were identified; others included oestrogen receptor, a phosphotyrosyl protein, P34H, equatorial segment protein, mannose lectin receptor, human guanylylcyclase receptor, epididymal protease inhibitor receptor, PH30 and estradiol binding protein.

The function of the membrane surface proteins identified in this study plays a vital role in fertilization. A few of these functions include sperm attachment and binding to the oocyte as well as penetration thereof. Others play a role in signalling events such as capacitation, hyperactivation and acrosome reaction. The absence of these proteins in patient sperm possibly accounts for the functional inability to successfully achieve fertilization suggesting that this provides molecular insight to reasons for infertility amongst men. In addition to this, proteins presented by patient samples that were absent in healthy donors

may too account for their infertility status. Estradiol binding protein and PH30 are two proteins presented only in patient samples. Their function plays a role in the inhibition of the acrosome reaction and sperm-egg fusion, respectively.

In conclusion, these differences in protein expression between fertile donors and patients may form the molecular basis of infertility amongst men and indicates possibilities for novel proteonomic approaches to improve andrological diagnosis in future.

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CHAPTER 1

INTRODUCTION

1.1. Transport through the female reproductive tract

Upon ejaculation at copulation, millions of sperm cells are deposited in the upper region of the female vagina by the male (Figure 1; Williams *et al.*, 1993). Of this, only 10% will enter the cervix; 1% the uterus and 0.1% the fallopian tube. Eventually, one spermatozoon will fertilize the oocyte (Williams *et al.*, 1993). In essence, sperm movement through the female reproductive tract ensures the selection of spermatozoa for motile and morphologically normal sperm cells to succeed (Suarez and Pacey, 2006). The vast decrease in the number of sperm progressing to the upper regions of the female reproductive tract suggests that this is a very stringent process of sperm selection (Henkel, 2012).

In the human, spermatozoa encounter the following natural barriers by the female reproductive tract during sperm movement. The process of fertilization include the following (Henkel, 2012):

1.1.1. Cervix

1.1.2. Uterus

1.1.3. Oviduct

1.1.4. Oocyte

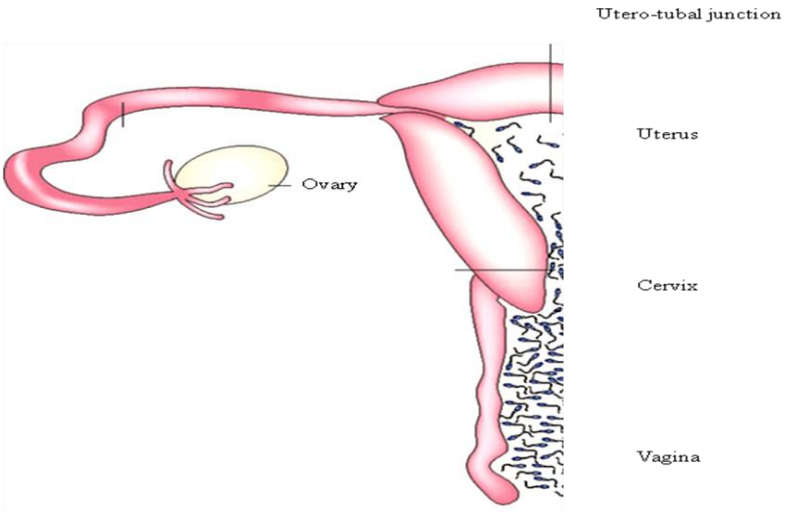


Figure 1: Diagram indicating the progression of human spermatozoa through the female reproductive tract (Modified from Kaupp *et al.* (2008).

1.1.1. The cervix and its cervical mucus

The lining of the cervical canal of the cervix in the human female reproductive tract is a thin layer of mucus known as the cervical mucus (Lagow *et al.*, 1972), containing mucus, soluble proteins, ions, simple sugars, immunoglobulins and enzymes (Katz *et al.*, 1997). The cervix and its secretions are the first barrier spermatozoa are required to cross in the female reproductive tract after ejaculation (Sobrero & MacLeod, 1962; Mortimer, 1983). This is achieved when spermatozoa swim out of their seminal plasma into the vaginal environment. Uterine and vaginal contractions aid the colonization process (Overstreet & Katz, 1977). However, the process by which the spermatozoa migrate through the mucus molecules is dependent on and facilitated by flagellar movements and seminal enzymes present on the sperm-mucus interface

(Overstreet *et al.*, 1980), suggesting that the cervical mucus filters sperm on basis of motility rather than morphology (Hanson and Overstreet, 1981). However, this could be argued, as morphologically abnormal sperm have lower motility (Overstreet *et al.*, 1980). In mammals it is also evident that the cervical mucus decreases the number of morphologically abnormal sperm cells (Perry *et al.*, 1996). Previous studies further support the concept that cervical mucus has the ability to store sperm for many days before ovulation (Perry *et al.*, 1996). Interactions between spermatozoa and the fluids in the female reproductive tract are essential for functioning and survival of these cells (Barratt& Cooke, 1991). This effect is not well known and the molecular basis is poorly understood (Chakroun Feki *et al.*, 2004).

Physical properties of the cervical mucus in humans account for its functions, few of these functions include:

1.1.1.1. Filtering out abnormal spermatozoa in humans

The physical and biochemical properties of the cervical mucus have the ability to select sperm by a filtering process (Hanson and Overstreet, 1981). It nurtures and biochemically supports sperm cells (Katz, 1991), thereby serving as a reservoir for storing and filtering spermatozoa. From here, sperm cells are released at a constant rate into the uterus (Freundl *et al.*, 1988). This suggests that the cervix serves as reservoir site (Overstreet and Katz, 1977) to filter out morphologically and functionally abnormal cells (Freundl *et al.*, 1988; Katz, 1991). Therefore, sustenance and colonization in the cervix and its mucus requires highly motile spermatozoa (Mattner, 1963).

Surface interactions of the sperms' membrane with the mucus molecules are suggested to take place (Katz *et al.,* 1989). Since it is well known that cervical mucus has the ability to exclude DNA damaged sperm, the large amount of excluded spermatozoa cannot only be based on their motility and morphology (Bianchi *et al.,* 2004).

1.1.1.2. Capacitation

Capacitation is a molecular event (Flesch *et al.,* 2001) whereby the sperms'membrane proteins and lipids undergo various biochemical changes (Florman and Ducibella, 2006; Gadella and Visconti, 2006). This is inclusive of an extensive amount of organization and rearrangement of the entire membrane (Flesch *et al*., 2001). Molecules on the plasma membrane integrate, adhere to or get removed from the sperms surface (Ickowicz *et al*., 2012), and surface receptors are activated or unmasked (Fabro *et al.,* 2002; Bahat *et al*., 2003). These surface receptors require exposure as they bind to structures in the female reproductive tract (Bedford, 1983).The process initiates downstream signalling cascades, achieved by the changes in ion fluxes in the plasma membrane (Cross, 1998). This is accounted for in response to the chemical stimuli from the oocyte (Fabro *et al.,* 2002; Bahat *et al.,* 2003) and is essential as it prepares the sperm cell for binding to the oocyte and successful fertilization thereof (Yanagimachi, 1988; De Jonge, 2005; Ickowicz *et al*., 2012).

1.1.2. Uterus

Just before ovulation, contraction intensity of the smooth muscle cells lining the uterus (Lyons *et al.,* 1991) stimulated by seminal components (Crane and Martin, 1991) increases (Lyons *et al.*, 1991), thereby, assisting the transportation of spermatozoa through the vestment by expelling spermatozoa from the cervix into the uterus (Suarez and Pacey, 2006). Sperm are rapidly moved through the uterus. This is a proposed method to enhance the cells survival from immune attack by the vagina or the cervix (Suarez and Pacey, 2006), as both, leukocytes and phagocytes may attack both normal and abnormal spermatozoa. They are primarily aimed at eliminating defective sperm cells, as these cells loose most of its defense mechanisms afforded by components in its seminal plasma (Suarez and Oliphant, 1982).

1.1.3. Oviduct

The oviduct is divided into three compartments, each with their own physiological function (Rath *et al.*, 2008). In order, these compartments are;

- Isthmus with the uterotubal junction (UTJ)
- Ampulla
- Infundibulum

In many mammalian species, the UTJ serves as an anatomical, mucosal and physiological barrier to spermatozoa in their passage through the female reproductive tract (Suarez and Pacey, 2006). However, in the human, no significant function could

be found (Suarez and Pacey, 2006; Henkel, 2012). It is a distinct site that connects the uterus with the fallopian tube (Hafez and Black, 1969), and is characterized by very narrow lumen with mucosal folds (Hook and Hafez, 1968) with a thick muscular layer that extends from the uterine wall (Hafez and Black, 1969). It possesses prominent cilia (Hook and Hafez, 1968), suggesting that these mucosal folds are to entrap sperm cells (Suarez, 2007). Furthermore, Dukelow and Riegle (1972) suggest that the UTJ specifically restricts immotile and damaged sperm from entering the oviduct by impairing the transportation of abnormal sperm cells (Saacke, 1982).

At this point, the UTJ also appears to differentiate between poor and high-quality DNA in spermatozoa (Hourcade *et al.*, 2010) and preserves the functions of good quality spermatozoa, thus enhancing their viability and motility (Hunter and Wilmut, 1984). Yet, the molecular mechanism by which the UTJ can discriminate between the integrity of the DNA is poorly understood (Henkel, 2012). As stipulated by Burkitt (2012), an assumption can be made that all sperm cells reaching this point are motile and capable of reaching the site of fertilization. Progression through the mammalian UTJ is then influenced by molecules present on the sperms' surface (Holt, 2009).

A study performed on murine sperm cells suggests that there are epitopes present on the sperms surface to allow for interaction with the UTJ for penetration (Krege *et al.*, 1995).This assumption is supported by studies with sperm from knock-out mice that lack a chaperone protein, calmegin, making it impossible to transverse through the UTJ (Holt, 2009). Other studies by Cho *et al.*(1998), Ikawa *et al.*(2001) and Nishimura *et al.*(2004), using germ cells from knock-out mice also prove that fertilin-B and ADAM1a proteins are important for assisting the movement of sperm through

the UTJ, suggesting that sperm has some unknown interaction with the UTJ (Holt, 2009). Sperm cells possessing these surface molecules could naturally occur during spermatogenesis, thus producing a population of sperm already rendered incapable of passing the UTJ (Holt, 2009).

The fallopian tube contains soft tissue, yet is also a very complex structure (Burkitt *et al.*, 2012) rich in nutrients which assist in supporting the life span of the sperm (Suarez, 2008). The complexity of the tubes' mucosal surface regulates the passing of sperm to the oocyte (Zamboni, 1972). This is achieved by slowing down sperm movement towards the ovary as the surface creates obstruction for spermatozoa (Suarez and Pacey, 2006). This is required to prevent introducing two sperm nuclei into the oocyte as mentioned by Ducibella (1996), which could result in unusual development.

Once the sperm is in the fallopian tube, it will attach itself to the epithelium, or swim off in another direction (Burkitt *et al.*, 2012). Attachment prevents polyspermic fertilization by allowing only a few sperm cells to be released to the oocyte at a time (Suarez and Pacey, 2006), and by housing only few sperm cells in the fallopian tube (Ducibella, 1996). Sperm encounter many complex barriers up until they reach the fallopian tube, where capacitation and fertilization occurs (Suarez and Pacey, 2006). A safe haven is then established for sperm by the fallopian tube. As described by Rodriguez-Martinez *et al.* (1990), spermatozoa will not be under attack by the female's defense systems in this region, as in the vagina, cervix and uterus.

In the oviduct, only certain sperm undergo capacitation (Eisenbach, 1999). The process is not understood, but could possibly be due to the involvement of sperm membrane receptors (Holt and van Look, 2004). *In vitro* studies indicate that if spermatozoa are already capacitated once they reach the oviduct, they cannot cross the UTJ (Shalgi *et al*. 1992). Furthermore, animal studies showed that capacitation will reduce the binding ability to the epithelium due to the loss of surface proteins (Lefebvre and Suarez, 1996). Together with the male germ cells being hyperactivated, a condition which is characterized by vigorous flagellar movement and a large lateral head displacement causing a whiplash movement required for spermatozoa to detach itself and progress through the lumen towards the oocyte (Jansen, 1980; Ho and Suarez, 2001; Holt, 2009). Hyperactivation also occurs in the fallopian tube, whereby the flagellar beats are increased, which is essential for the sperm to overcome forces exerted by the epithelium (Ho and Suarez, 2001).

After the UTJ, sperm reach the isthmus (Beck and Boots, 1972), which in many species serves as a functional sperm reservoir (Pacey *et al.,* 1995), which is retaining and storing sperm cells (Hunter, 1975) and only releasing them once ovulation has occurred (Baillie *et al.,* 1997). Upon ovulation, with the assistance of sperm capacitation and hyperactivation, spermatozoa are gradually released from the reservoir for fertilization (Morales *et al.*, 1988).

To date, there is no substantial research to verify mechanisms on how sperm cells bind to the epithelium of the isthmus (Henkel, 2012). Some animal studies suggest that sperm binding occurs through acidic heparin-binding proteins, which derive from the bovine seminal vesicle that coat the sperm head (Manjunath and Sairam, 1987),

and bind to annexin receptors containing fructose (Ignotz *et al*., 2007). Homologues of these bovine seminal vesicles were found in both mice and human (Lefebvre *et al*., 2007). Recent studies carried out on the bull revealed oxidation and reduction of sulphhydryl groups of the sperms' surface proteins (Gualtieri *et al*., 2009), which modulated adhesion of sperm to the oviductal epithelium as well as capacitation (Gualtieri *et al*., 2009).

The isthmus restricts sperm from movement. This owes to the downstream fluid and constrained space of the narrow lumen in the Fallopian tube (Katz *et al.,* 1989). In turn, a situation whereby the sperm has to increase its amount of energy for higher motility is created (Katz *et al.,* 1975). Once the sperm increases its flagellar beats, it inhibits motions away from the epithelium of the UTJ and retains sperm to the surfaces (Katz *et al.,* 1989). As a result, poorly motile sperm will remain trapped in this area and eventually be eliminated from the epithelium (Hunter, 1996). The mechanisms by which these cells are detached from the epithelium are by the activation of capacitation and hyperactivation under the influence of ovulation (DeMott and Suarez, 1992; Ho and Suarez, 2001). Hence, spermatozoa that are incapable of this process are drastically reduced in numbers at this site (Hunter, 1996).

1.1.4. Oocyte interaction

Oocytes are surrounded by s cumulus cells, which, in terms of their microstructure, are similar to that of the cervical mucus (Yuldin *et al.,* 1988). However, their

viscoelastic properties are different (Katz *et al.*, 1989). The cumulus cells are inflexible, and enzymes present in the sperm acrosome are required to penetrate them (Katz *et al.*, 1989). Both, the cumulus cells as well as the cervical mucus microstructure molecules have electrochemical affinities for molecules present on the surface of the sperm cell, creating an affinity of spermatozoa to these epithelial cells and thus creating more resistance for sperm cells to penetrate through them (Katz *et al.*, 1989).

Sperm transport to the cumulus is mediated by chemoattractants from the follicular fluid, cumulus cells and the oocyte itself (Sun *et al.*, 2005). Only capacitated sperm are responsive to this process (Cohen-Dayag *et al.*, 1995; Fabro *et al.*, 2002; Giojalas *et al.*, 2004). Progesterone is responsible for creating the chemical attractant concentration gradient that guides the gamete towards the egg in mammalian species (Eisenbach and Giojalas, 2006). This steroid (Teves *et al.*, 2006; Guidobaldi *et al.*, 2008) is the only physiological chemoattractant (Guidobaldi *et al.*, 2008) secreted by the cumulus cells (Teves *et al.*, 2006; Guidobaldi *et al.*, 2008)

1.2. Surface properties of spermatozoa

Successful fertilization in mammals incorporates the union of two gametes and involves a flow of cell to cell and cell to matrix interactions (Lefevre *et al*., 1997). For the sperm to successfully fertilize an oocyte, sperm are required to be mature (Abu *et al*., 2011). Spermatozoa face a stringent selection process in the female reproductive tract (Holt and Van Look, 2004), whereby sperm competition and sperm selection occurs (Holt *et al*., 2010). As described by Holt (2010), these mechanisms are complex and consider various sperm factors including the following:

- The ability of the sperm to undergo the acrosome reaction (Abu et al., 2011)
- Motility (Holt, 2010).
- Sperm count and morphology (Ombelet et al., 1998).
- Morphological problems (Gomendio and Roldan, 1991)
- Zona binding (Abu et al., 2011)
- Ability of the sperm to interact with the female reproductive tract (Holt, 2010)

These factors are of importance, because, as previously mentioned, the cervix would select sperm on basis of their motility and morphology (Perry *et al*., 1996). The oviduct excludes immotile and damaged cells (Dukelow and Riegle, 1972). There are reports stipulating that sperm motility and mid-piece volume are one of the crucial determinants of the sperms fertilization ability (Gomendio & Roldan 1991; Anderson & Dixson, 2002). Failure of any of the sperm cells' systems, such as its motility, or damage to the DNA, will result in inability to fertilize the oocyte (Holt and Van Look, 2004), because the female reproductive tract is capable of selecting male traits that

would increase the rate of fertilization success (Holt *et al*., 2010). Many researchers support this hypothesis by concluding that the female reproductive tract selects male gametes by natural selection (Holt *et al*., 2010).

1.2.1. The acrosome

The acrosome is a Golgi-derived organelle covering the anterior two thirds of the sperm nucleus (Chemes and Sedo, 2012). It is of importance as it assists in sperm penetration and oocyte activation (Chemes and Sedo, 2012). Spermatozoa with no acrosome or deformed flagella are regarded incompetent to fertilize and egg (Holt and Van Look, 2004), therefore rendering the patient infertile (Chemes and Sedo, 2012). A common feature of severe teratozoospermia is acrosomal hypoplasia, which is characterized by a small detached acrosome with diminished contents and this result in a change in chromatin condensation (Chemes and Sedo, 2012). Microscopically, these cells appear big and irregular in shape and can be caused by; abnormally compacted chromatin without a membrane (Chemes and Rawe, 2003). Because these sperm cells have no acrosome, its nucleus appears round (Chemes *et al*., 1987). Insufficient chromatin condensation worsens their potential to fertilize the egg (Nistal *et al*., 1978). This condition is a result of abnormal development of Golgi proacrosomic vesicles that fails to attach and spread over the spermatid nucleus as described by Chemes and Sedo (2012).

Upon acrosome reaction, hyaluronidase is released from the acrosome (Shams-Borhan and Harrison, 1981). Hyaluronidase is an acrosomal enzyme detected in various

species (Stambaugh and Buckley, 1970; Brown, 1975; Morton, 1976) possessing different forms (Harrison, 1988). The enzyme is involved in penetrating the cumulus oophorus for fertilization (Cummins and Yanagimachi, 1986; Yudin *et al.,* 1988; Lin *et al.,* 1994). Spermatozoa that have not undergone the process of capacitation are rendered incapable of the acrosome reaction (Austin, 1951).

1.2.2. Motility

If the sperm flagellum is defective, sperm motility is also compromised (Chemes and Sedo, 2012) and relevant sperm are then disqualified from the fertilization process (Holt and Van Look, 2004). Motility is important for the movement of the male germ cell through the female reproductive tract and also for the penetration of the oocyte (Holt and van Look, 2004; Malo *et al.,* 2006). Sperm with good motility have been proven to show less sperm head abnormalities (Cho *et al.,* 2003)

1.2.3. Morphology

Anomalies to the sperm head and neck are classified by different morphologies of the sperm cell (Chemes and Sedo, 2012). This is one of the most predictive semen parameters for pregnancy (Bonde *et al*., 1998; Guzick *et al*., 2001). Due to the difficulty in classifying the variations of the human sperm head into categories, information on sperm head morphology is scarce (Utsuno *et al*., 2013). These authors concluded that spermatozoa with abnormal sperm heads and large nuclear vacuoles

shows a higher percentage of having DNA fragmentation. Evaluating the sperm head, acrosome, mid-piece and tail has shown associations with the DNA integrity of the cell (Garolla *et al*., 2008; Oliveira *et al*., 2010; Braga *et al*., 2011; Wilding *et al*., 2011).

1.2.4. Zona binding

The second last barrier sperm are required to penetrate is the zona pellucida (ZP), which is generally as thick as the head of the sperm cell (Katz *et al.,* 1989). Binding of sperm cells to the ZP is only achieved once they have been capacitated (Yanagimachi, 1994). At this point, the female reproductive tract analyses the morphology (van den Bergh *et al.,* 2009) and DNA quality of the sperm in some not identified way once again (Ye *et al.,* 2006). Suggestions are made that the final selections could test the cells ability to induce the acrosome reaction (Nijs *et al.,* 2010) as well as the presence of excessive cytoplasmic retentions (Paes Almeida Ferreira de Braga *et al.,* 2009).

1.3. Chemical features relating to DNA quality of spermatozoa

1.3.1. Protamines

Eighty five percent of the sperms' DNA is complexed to protamines (Churikov *et al.,* 2004; Rousseaux *et al.,* 2005) and during spermiogenesis, the chromatin of the sperm cell undergoes an extensive amount of compaction, whereby the histones are replaced

by specific nuclear proteins related to the testis, as well as transitional proteins and protamines (Aoki and Carrell, 2003; Dadoune 2003). Any abnormality in the expression of the nuclear proteins changes the structure of chromatin, creating the possibility for male infertility (Carrell and Liu, 2001; Olivia, 2006). Epigenetic changes could result in certain paternal genes being expressed when the embryo is being developed (Hammoud *et al.,* 2009). It may also cause an increase in DNA fragmentation (Aoki *et al.,* 2005b; Aoki *et al.,* 2006), reducing the DNA integrity of the sperm cell (Tarozzi *et al.,* 2009; Tavalaee *et al.,* 2009), and decreasing the ability of the sperm to penetrate the oocyte. Overall, this causes a decrease in the fertilizating ability of spermatozoa (Carrell and Liu, 2001; Aoki *et al.,* 2005a; Aoki *et al.,* 2006).

DNA damage is one of the factors that affect the sperm cells of infertile men, thereby contributing to male infertility, particularly idiopathic infertility (Barratt *et al.,* 2010). Thus, DNA integrity seems to have great impact on the fertility status of male germ cells (Madrid-Bury *et al.,* 2005; Morrell *et al.,* 2007). Therefore, if the sperms' membrane is damaged, it will not progress through the female reproductive tract (Holt, 2009).

1.3.2. Reactive oxygen species (ROS)

Reactive oxygen species are highly reactive derivatives of oxygen with half-life times in the nano- to milli-second range. Many of these molecules are free radicals, which are characterized by the unpaired electrons in the outer shell (Lopes *et al.,* 2010). ROS are formed in the mitochondria of any aerobic cell during oxidative phosphorylation whilst adenosine-triphosphate (ATP) is produced (Fujii *et al.,* 2005; Halliwell, 2006). During these processes, the electrons leak from the mitochondrial electron transport chain, forming most of the ROS (Fujii *et al.,* 2005; Halliwell, 2006). Hence, ROS are produced when oxygen is used as a substrate in the reactions involving oxygenase and electron transfers (Chandra *et al.,* 2009).

A strict balance needs to be established between the amount of ROS present and the anti-oxidation defenses (Harvey, 2007). This is essential for the regulation of physiological functioning of cells including the interactions of sperm with the oocyte (Harvey, 2007; Lopes *et al.,* 2010). Oxidation, in moderation, is required for the formation of disulfide bonds in the sperm nucleus (Premkumar and Agarwal, 2012). However, oxidative stress can cause damage to the spermatozoa (Premkumar and Agarwal, 2012). On the other hand, lack of oxidants may too (Lewis *et al.,* 1997) lead to oxidative damage of the cell (Aitken *et al.,* 1995a). Due to their high reactivity, radicals have the ability to modify purine and pyrimidine bases in the DNA, causing damage in the DNA are therefore referred to as the most toxic inflictor of oxidative damage (Agarwal, 2004).

1.4. Analysis of DNA quality by the female reproductive tract

In the human spermatozoa, DNA fragmentation as a diagnostic paramter has only become of interest since the early 2000's (Evenson and Wixon, 2006). From numerous studies it has been deduced that patients with ejaculates exhibiting a percentage of more than 30% spermatozoa with DNA damage have increased failure in conception (Evenson and Wixon, 2006). Many aspects account for the causes of DNA damage in sperm cells (Henkel *et al.,* 2003), amongst them are:

- Apoptosis, which is a process of programmed cell death (Wyllie, 1980). This mechanism is biochemically characterized by the activation of endonucleases which cause breakages in the DNA (Gandini *et al.*, 2000).
- Excessive ROS production, leading to oxidative stress (Duran *et al.,* 2002) that causes DNA fragmentation to the cell (Lopes *et al.,* 1998; Duru *et al.,* 2000).
- Tension applied during spermatogenesis onto the chromatin while condensation thereof. Therefore, topoisomerase attempts to release the tension, but causes strand breaks in the process (Sakkas *et al*., 1999; Marcon and Boissonneault, 2004).
- Chronic or acute infections in the male reproductive tract lead to the increase of leukocytes which in turn causes an increase generation of ROS (Aitken and West, 1990), resulting in oxidative damage (Aitken and De Iuliis, 2010) to the DNA (Alvarez *et al.*, 2002).

In conclusion to the causes of DNA damage in sperm cells, it is evident that apoptosis and ROS are amongst the leading causes thereof. During the apoptotic process, the

cells' are killed and eliminated altogether (Wyllie, 1980; Gorczyca *et al.,* 1993). This is indicated by the translocation of phosphatidyl serine (PS) from the inner to the outer leaflet of the plasma membrane as an early event (Vermes *et al.,* 2005) demonstrating the sperm cells' inappropriateness for fertilization.

With regard to excessive ROS production, it should be taken into consideration that the membrane function and motility are of importance at each level of the progression of spermatozoa through the female reproductive tract. This process includes molecular interaction, adherence to the epithelia and oocyte vestments as well as penetration thereof (Overstreet *et al.,* 1980; Katz, 1989; Ho and Suarez, 2001; Holt and van Look, 2004; Guaitieri *et al.,* 2009). Furthermore, DNA damage caused by free radicals during oxidative stress induces lipid peroxidation of lipids the plasma membrane, which in turn leads to disruption in the plasma membrane, and impairs motility (Aitken, 1995a). Therefore, impairment of motility results in the inability to progress through the female reproductive tract (Holt and van Look, 2004)

1.5. Biochemical rationale: protein interactions and sperm functioning

Many sperm surface proteins are associated with sperm function, of which some could be important to diagnose certain cases of infertility amongst men (Liu *et al.,* 1996; Naz and Leshie, 1999). Studying sperm proteins allows for insight into the molecular aspects of reproduction and could assist with identifying different causes of male infertility in humans (Marinez-Heredia *et al.,* 2006). In the testis, the sperm cell undergoes meiotic and mitotic differentiation, whereby these cells gain various

differentiated proteins (Schroter *et al.,* 1999). Besides spermatozoa having to achieve successful fertilization, it is also required to withstand the immune defenses presented by the female reproductive tract and present itself in a manner that will differentiate itself from the poor quality spermatozoa (Schroter *et al.,* 1999).

Gametes interaction, is mediated by cell surface proteins (Vacquier, 1998). A series of studies was carried out on fertilization and sperm membrane proteins, yet, these processes are still poorly understood (Rajeev and Reddy, 2004). Many proteins on the sperm cells' surface have been identified (Wolf *et al.,* 1992; Diekman *et al.,* 2000). However, their clinical importance is still vague (Wolf *et al.,* 1992; Diekman *et al.,* 2000). A number of researchers are stating that epigenetic changes are responsible for certain pathologies (Lima *et al.,* 2010), indicating a possibility that this could be involved in male infertility (Jenkins and Carrell, 2011).

Many infertile men have sperm abnormalities, such as low sperm count or immotile spermatozoa, which can easily be detected. However, there is a group of men that have normal semen characteristics, but are unable to fertilize the oocyte *in vitro* (Jeremias and Witkin, 1996). Routine semen analysis gives minimal information regarding sperm chromatin defects or surface proteins that could be abnormal (Jeremias and Witkin, 1996). Since these membrane surface molecules are required to stabilize the plasma membrane and assist in interactions between spermatozoa, the female reproductive tract and the oocyte, molecular approaches are required to identify these patients with idiopathic infertility and analyze their problems (Jeremias and Witkin, 1996).

Integrins are described as a family of surface receptors that assist with cell to cell interactions and cell to extracellular environment interaction (Hynes, 1987; Hemler, 1990). Many integrins are recognized as ligand proteins which contain the Arg-gly-asp sequence (Fusi *et al.,* 1992). Integrins are proteins that consist of non-covalently linked α-and β-subunits with different proteins (Jeremias and Witkin, 1996) due to its amino acid tripeptide recognition sequence (arginine-glycine-aspartic acid) (RGD) (Albelda and Buck, 1990). This RGD tripeptide has shown involvement in adhesion and penetration of human sperm cells to the zona-free hamster eggs (Fusi *et al.,* 1992a; Henkel *et al.,* 2012), and there is sufficient evidence that supports the idea of integrins playing a role in assisting sperm adherence to the oolemma (Fusi *et al.,* 1992). β_1-integrins are found on the surface of spermatozoa, showing a relationship between thier expression of cell adhesion molecules and its ability to fertilize the egg *in vitro* (Klentzeris *et al.,* 1995). Sub-families of these integrins are $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, and $\alpha_6\beta_1$, which show a higher expression in spermatozoa from patients suffering from tubal or idiopathic infertility than patients suffering from male factor infertility (Jermias and Witkin, 1996).

These proteins include fibronectin, vitronectin and laminin (Fusi *et al.,* 1992). Vitronectin is localized on the equatorial region of the sperms head (Jeremias and Witkin, 1996), and fibronectin all over the surface (Anapliotou *et al.,* 1995; Fusi and Bronson, 1992; Fusi *et al.,* 1992a). Both proteins are correlated with the sperms cell's ability to undergo acrosome reaction (Fusi *et al.,* 1992a).With the inclusion of laminin, these three proteins assist in spermatozoa adhering to the epithelium of the endometrium and fallopian tubes (Jeremias and Witkin, 1996). Additionally, these

proteins have the ability to encourage attachment of the sperm to ligands on the oocyte (Fusi *et al.*, 1992a; Anapliotou *et al.*, 1995) for penetration through the oocyte (Jeremias and Witkin, 1996).

These ligand proteins are required to assist the sperm cell to successfully progress through the female reproductive tract (Jeremias and Witkin, 1996). This assumption is supported by Rath (2008) who suggested that the molecular connection between the uterine epithelial cells and sperm cells may be similar to the lectin interactions. Further research proved that spermatozoa that are not attached to epithelial cells of the uterus had damaged plasma membranes (Rodriguez-Martinez *et al.*, 1990) indicating that an intact outer membrane is required for the male germ cell to attach to the uterine wall (Taylor *et al.*, 2008).

Since motility of these sperm cells is impaired in the fallopian tubes due to the viscous fluid present (Overstreet *et al.*, 1980), their function is mediated by receptor-ligand interactions (Krege *et al.*, 1995; Ikawa *et al.*, 1997; Cho *et al.*, 1998). In mice, it was found that regardless of good morphology and motility, the absence of certain surface proteins inhibits passage through the UTJ (Krege *et al.*, 1995; Ikawa *et al.*, 1997; Cho *et al.*, 1998). These findings then further support the notion that the passage of sperm cells through the UTJ depends on direct contact between the sperms' membrane and the epithelium (Rath *et al.*, 2008).

1.6. Aim

The aim of this study was to establish differences in surface proteins present on the spermatozoa's membrane in humans using dissimilar sperm separation techniques to identify differences in fertile and infertile men at a molecular level.

In this regard, sperm surface proteins from fertile donors and infertile patients were isolated and analyzed by means of SDS-polyacrylamide gel electrophoresis. These results were then to be related with functional parameters such a motility and sperm DNA fragmentation using different separation techniques.

CHAPTER TWO

MATERIALS AND METHODS

2.1. Sample collection

For all procedures, ethical clearance was approved by the University of the Western Cape and Tygerberg Hospital, Stellenbosch University.

A total 20 semen samples was collected in sterile plastic beakers from donors (n=10) and patients (n=10) attending the Reproductive Biology Unit at Tygerberg Hospital (Tygerberg, Cape Town, SA) after a period of three day abstinence to sexual intercourse, with informed consent.

2.2. Sperm concentration and motility

Sperm concentration and motility were determined using the Sperm Class Analyzer (SCA) (Microptic S.L., Barcelona, Spain) version 4.1.0.1. This analyzer is capable of measuring and assessing sperm motility, concentration and morphology, having a higher precision and quantitative rate than manual methods (WHO, 2010). The minimum requirements for the semen samples used were least 50% forward progression and total sperm concentration of less than 100M/ml. This was done to ensure that an optimal concentration of protein was available for SDS-PAGE.

To avoid particles of dust being detected by the analyzer, the slides (Lasec, Cape Town, SA) were washed and rinsed thoroughly with distilled water, then dried with towelling paper. Thereafter, 5 µl semen were pipetted in the centre of the slide, covered with a 22 mm x 22 mm coverslip (Chance Propper LTD, Warley, England) and placed onto the stage of the microscope, which had been heated to 37ºC. The slide was left on the stage for 1-2 minutes to avoid flow of the sample. During this time, the SCA programme was set to analyse human sperm motility, providing data on total motility in percentage (%) and concentration of spermatozoa in million cells per millilitre (X 10^6/ml). Using a negative phase lens, with the green filter, the sample was then analysed at 10X magnification. A minimum number of 200 spermatozoa were captured in four different fields. All patient samples used never had less than 50 million cells per millilitre. However, their motility ranged between 7% - 50% for their total percentage of motile sperm.

2.3. Media

In this study, Human Tubule Fluid Medium, according to Quinn *et al*. (1985). This medium mimics the environment created by the epithelial cells in the fallopian tube (Tay *et al*. (1997). Constituents of this medium included the following, which was prepared in 1L distilled water:

- Sodium chloride (Saarchem, Gauteng, SA): 5.931 g
- Potassium chloride (Kimix, Cape Town, SA): 0.35 g
- Calcium chloride dehydrate (Saarchem, Gauteng, SA): 0.301g

- Magnesium sulfate heptahydrate (Kimix): 0.050 g
- Potassium dihydrogen phosphate (Saarchem): 0.050 g
- Phenol red (Sigma Aldrich, Steinheim, Germany): 0.005 g
- Sodium bicarbonate (Kimix): 2.1 g
- Glucose (anhydrous) (Saarchem): 0.5 g
- Sodium pyruvate (Biochemical Int LTD, England, UK): 0.036 g
- Sodium lactate (60% syrup; Sigma Aldrich): 3.9982 ml
- Hepes (Sigma Aldrich): 5.206 g

The osmolarity of this medium was adjusted using to 280 mOsmol/kg with distilled water using an osmometer (Wescor, Logan, USA). Then, aliquots of 50 ml were stored at -20°C until use. Before working with the medium, 50 ml tubes were defrosted in an incubator (Lasec) and supplemented with bovine serum albumin (BSA) (Sigma Aldrich) to yield a 1% (HTF-BSA)

2.4. Sperm separation techniques

Since there is no separation technique that serves as the ideal technique for the isolation of spermatozoa (Henkel and Schill, 2003), three different sperm separation techniques, namely wash swim-up and density gradient centrifugation, were used to separate spermatozoa from the seminal plasma. These methods have different efficiencies and depending on the quality of the ejaculate (Henkel and Schill, 2003) the recovery rate, motility, morphology and amount of DNA damage will vary (Byrd *et al.*, 1994).

2.4.1. Wash

This technique was the first sperm separation technique available, whereby the semen sample is first diluted with culture medium, centrifuged, the supernatant discarded and subsequently the pellet is resuspended (Edwards *et al.,* 1969). From this very basic method, Mahadevan and Baker (1984) developed the swim-up method, pathing the way for the development of more sophisticated separation techniques to isolate and increase the number of motile and, most importantly, functional sperm from an ejaculate (Henkel and Schill, 2003).

Washing a semen sample serves purpose to yield the largest amount of sperm in the ejaculate (WHO, 2010) giving the pellet is rich with motile and a highly concentrated number of spermatozoa (Boomsma *et al*., 2004). For the washing, the semen sample was diluted 1: with 0.1 M phosphate buffered Saline (PBS) (Oxoid, Basingstoke, Hampshire, England), pH 7.4, and centrifuged (Hermle Z160M centrifuge, Labortechnik, Wehingen, Germany) at 300xg for 10 minutes at room temperature. The supernatant was discarded, and the pellet used for sperm membrane protein extraction.

2.4.2. Swim-up (SU)

For the isolation of progressively motile spermatozoa from the ejaculate the swim-up technique was developed (Mahadevan and Baker, 1984). This technique allows

spermatozoa to swim out of a pellet and into the overlaying culture medium, thus allowing for extraction of the most motile spermatozoa (WHO, 2010). However, the method results a lower yield of spermatozoa than the washing technique (WHO, 2010).The swim-up (Zini *et al.,* 2000; Younglai *et al.,* 2001) and density gradient centrifugation (Larson *et al.,* 1999; Sakkas *et al.,* 2000) technique can to an extent, reduce DNA-fragmented sperm, but not remove them all (Utsuno *et al.,* 2013)

Aliquots of 200 µl were washed in 1ml HTF-BSA in a conical 15 ml test tube, followed by discarding the supernatant and gently overlaying the resulting pellet with 500 µl HTF-BSA, then gently placing the sample at a 45° angle for 1 hour in a Series 2000 incubator (Lasec) at 37°C. During this incubation, spermatozoa are allowed to swim up into the medium. After the incubation, the supernatent was carefully aspirated and washed twice in PBS (Oxoid), as descibed above. Thereafter, sperm from the pellet were used for membrane protein extraction.

2.4.3. Discontinuous Density Gradient Centrifugation (DGC)

Density gradient centrifugation using Percoll creates a density gradient that separates sperm with high motility from bacteria and the seminal plasma in the ejaculate (Ziebe and Anderson, 1993). The principle behind this technique is to layer the semen, which has a lower density, over the higher density medium (Henkel and Schill, 2003). By employing centrifugation, spermatozoa will move towards the bottom of the test tube; however, highly motile sperm will be able to penetrate faster through the boundaries of the gradient created by the density medium opposed to poorly motile sperm

(Henkel and Schill, 2003). A soft pellet containing highly motile sperm yields as a result (Henkel and Schill, 2003).

Since the advent of separating sperm into fractions as described by Gorus and Pipeleers (1981), separation using continuous gradient centrifugation has widely been applied in medically assisted reproduction procedures. Thus, density gradient centrifugation is a very popular technique when processing normal semen samples (Chen and Bongso, 1999) since it selects sperm with not only high motility, but also those with good fertilizing ability (Elglert *et al.,* 1992).

In this study, a discontinuous density gradient was used. A 100% Percoll (Amersham Pharmacia Biotech AB, Uppsala, Sweden) stock solution was aseptically prepared in a 1.5M NaCl solution, of which a 80% and 40% gradient was then prepared in HTF-BSA. The technique was performed by pipetting 400 µl of 80% Percoll solution (1.10 g/ml) into a 1.5ml Eppendorf vial. This layer was then carefully overlaid with 400 µl 40% Percoll solution (1.04 g/ml) in HTF-BSA. Finally, 400 µl liquefied semen were overlaid. The Eppendorf vial was then carefully placed in a centrifuge (Labortechnik) and centrifuged at 600xg for 20 minutes at room temperature. A soft pellet was yielded and aspirated using a Pasteur pipette. The spermatozoa were then washed with PBS and used for membrane protein extraction.

2.5. Removal of plasma membrane proteins

2.5.1. Preparation of samples

To analyse membrane proteins, these protein have to be extracted from the sample using detergents (Matthews *et al.*, 2006). In this study, the following detergents were prepared as a 1% solution in 0.02M Tris -HCl (Sigma Aldrich) buffer (pH 8.0):

- Tween-20 (Sigma Aldrich)
- Cetyl trimethylammonium bromide (CTAB) (Sigma Aldrich)
- Sodium dodecyl-sulphate (SDS) (Sigma Aldrich)
- Saponin (Sigma Aldrich)

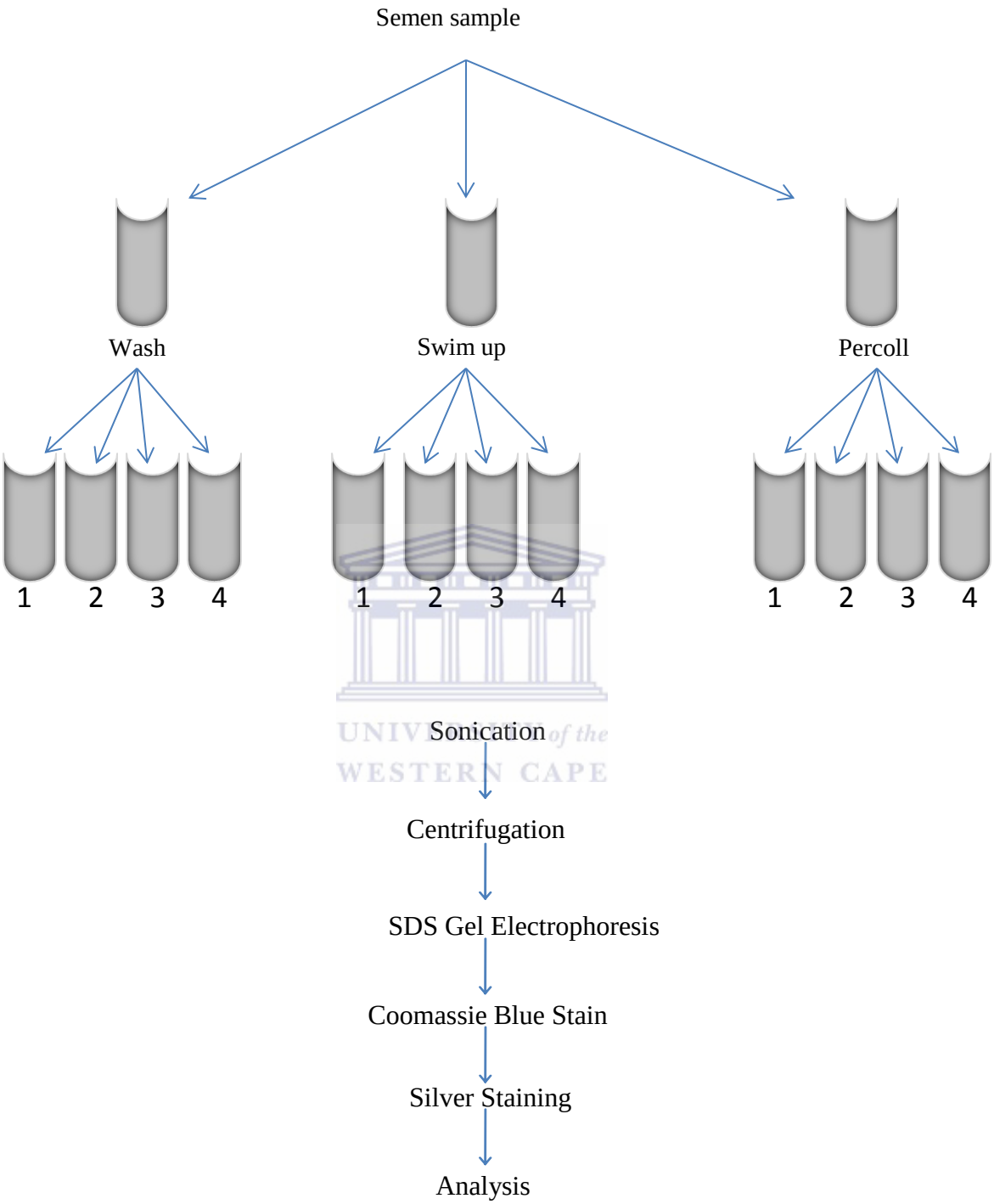
Protease inhibitors were added before working, these included 1mM solutions in 0.1% dimethyl sulphoxide (DMSO) (Sigma Aldrich):

- Benzidine (Sigma Aldrich)
- Phenylmethanesulfonyl fluoride (PMSF) (Sigma Aldrich)

2.5.2. Study design

Figure 2 shows a flow diagram representing the layout of the experimental study. As depicted, each sample underwent three different separation techniques (Wash; Swim-up (SU); Percoll density gradient centrifugation (DGC)). Furthermore, for each technique, the sample was treated with four detergents, possessing different chemical properties (1: CTAB; 2: Saponin; 3: SDS; 4: Tween 20). The proteins were then

separated on SDS-PAGE according to their molecular weights, then analysed by Coomassie and Silver staining techniques.



2.5.3. Extraction of plasma membrane proteins

Plasma membrane proteins were extracted by a protocol followed according to Rajeev and Reddy (2004). Briefly, the sperm pellets obtained after employing the three different separation techniques were sonicated using a Qsonica XL-2000 Series (Lasec) with ultrasound for 8 bursts, 15 seconds each. After sonication, the samples were centrifuged at 5000xg for 10 minutes at 4^{o}C. The supernatants were aspirated and diluted 1:1 with Laemmli's loading buffer. This mixture was heated on a heating block at 95°C for 15 minutes before loading it onto SDS polyacrylamide gel electrophoresis (SDS-PAGE) (Biorad, Hercules, California, USA).

2.6. Determination of protein concentration

Protein concentrations in the samples were determined using the Bradford assay kit (Biorad) and 96-well plates. The final protein concentration used for electrophoresis was adjusted to 100 µg/ml. Briefly, samples were diluted with lysis reagent, consisting of a 1M NaOH (Saarchem) in 0.1% SDS (Sigma Aldrich) solution. The absorbance was determined using a LT 4000 spectrophotometer (Lasec) at a wavelength of 630nm. The standard curve (Figure 3) used was a 0mg/ml; 1.25 mg/ml; 2.5 mg/ml; 0.5 mg/ml and 10 mg/ml of Bovine Serum Albumin (BSA) (Sigma Aldrich), respectively.

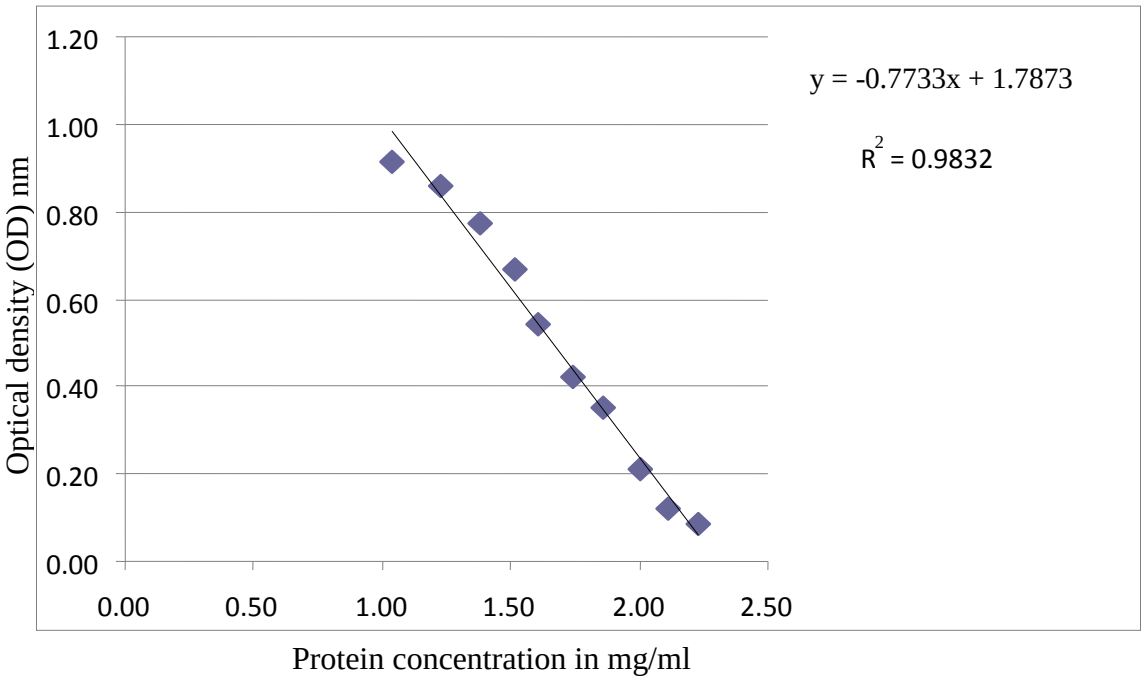


Figure 3: Standard curve for the determination of the protein concentration using bovine serum albumin (BSA). Optical density was measured at 630 nm.

2.7. Gel electrophoresis

2.7.1. Laemmli's loading buffer

Laemmli's loading buffer for the electrophoresis was prepared, for usage before loading the samples onto the gel. This buffer was prepared according to Laemmli (1970), consisting of the following:

- 20% glycerol (Sigma Aldrich)

- 4% SDS (Sigma Aldrich)
- 10% β-mercaptoethanol (Sigma Aldrich)
- 0.01% Bromophenol blue (Bio-Rad, Hercules, USA)

2.7.2. Electrophoresis

During electrophoresis, an electrical field is created in gels that separate protein molecules according to their size and charge (Bonner and Hargreaves, 2011). An estimation of the molecular weight of the proteins that have been separated can be made by comparing its electrophoretic migration to a protein standard of known molecular weights (Bonner and Hargreaves, 2011).

In this study, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the protein molecules. SDS gels were prepared using the Fluka-SDS Gel Preparation Kit (Sigma Aldrich). The gels were prepared according to Laemmli (1970), whereby a 12% separation gel and a 4% stacking gel were prepared. The volumes loaded per well included 20 µl sample and 7 µl molecular weight marker (Optima Scientific), ranging from 10-kDa to 170 kDa.

Initially, the gel ran at 200V for 15 minutes, followed by 150V for 45 minutes on a mini-PROTEAN Tetra cell (Biorad). Thereafter, the gel was placed in Coomassie brilliant blue stain and left overnight on a low speed orbital shaker (Labnet, Woodbridge, USA).

2.8. Coomassie brilliant blue staining

Coomassie stain is a commonly employed technique used to visualise and quantify proteins (Wilson, 1983). The stain was originally produced as an acid wool dye by Imperial Chemical Industries (Wilson, 1983). Later, Fazekas de St. Groth and colleagues (1963) provided results that showed proportional values of protein concentration after gel electrophoresis. Improvements were made on this technique and today it is widely used to visualise proteins in gels (Luo *et al.,* 2006).

The principle behind the technique proposes that the dye forms a strong non-covalent bond with the proteins in the gel, creating a negatively charged ionic form of the dye, which is a combination of van der Waals forces and electrostatic interactions (Diezel *et al.,* 1972). Therefore, it is essential to de-stain the gel in order to visualise the protein bands (Diezel *et al.,* 1972).

2.8.1 Staining procedure

After electrophoresis, the gels were gently removed from in between the two glass plates and placed into 200 ml 0.2% Coomassie dye solution: 50% ethanol (Polychem Suppliers, Cape Town, SA), 7.5% glacial acetic acid (Saarchem Merck), and then left overnight on an orbit LS gel rocker at 50 RPM (Labnet) for destaining the following day.

2.8.2 De-staining procedure

The de-staining solution consisted of 40% ethanol (Polychem Suppliers, Cape Town, SA) and 10% glacial acetic acid (Saarchem Merck):

Four hundred ml ethanol (B&M Scientific) in addition with 100ml glacial acetic acid (Saarchem Merck) were topped with 600 ml distilled water (dH_2O) to 1L.

Gels were placed in 600ml de-stain solution on the gel rocker (Labnet) for 1-3 hours. The de-stain solution was changed after each 30 minute interval until the gel appeared transparent.

2.9. Silver staining

Silver staining is a procedure that is about 100 times more sensitive than Coomassie staining. It detects protein concentrations in the very low nanogram range (Chevallet *et al.,* 2006), whereby Coomassie detects protein concentration only between 10 ng and 20 µg per band (Luo *et al.,* 2006). Here, silver ions are allowed to bind to proteins, then are reduced for visualisation of silver metal bands on the gel (Chevallet *et al.,* 2006). The protocol was performed according to Blum *et al.* (1986).

The following chemicals were prepared for the staining procedure:

- Sensitizing solution: 0.02% sodium thiosulphate (Riedel-deHaen, Hannover, Germany) solution

This was prepared by dissolving 0.2 g sodium thiosulphate in 1000 ml distilled water.

- Silver nitrate (Sigma Aldrich): 0.2% solution

Silver nitrate can be re-used and stored in a dark glass bottle at 4ºC; to prepare, 0.1 g silver nitrate was dissolved in 500 ml distilled water.

- Developing solution (freshly prepared): 3% Na_2CO_3 (Sigma Aldrich), 0.025% formaldehyde (Kimix), 10 mg/L sodium thiosulphate

From the stock solution of sodium thiosulphate (Riedel-deHaen), 25 ml were used in addition to 15 g Na_2CO_3 and 125 µl 40% formaldehyde topped to 500 ml with distilled water for the developing solution.

- Stop solution: tris-acetic acid

The stop solution was prepared by adding 50 g tris base (Sigma Aldrich) to 25 ml glacial acetic acid (Saarchem Merck) and topping it to 1000 ml with distilled water.

2.9.1. Procedure

After the Coomassie de-staining, the gels were rinsed in distilled water for 10 minutes and then pre-treated for 1 minute in 150 ml sodium thiosulphate solution. The gels were rinsed thrice in 150 ml distilled water, each for 20 seconds. Impregnation of the gels occurred by incubating them for 20 minutes in 150ml silver nitrate solution at 4ºC. To remove excess silver nitrate, the gels were rinsed with 150 ml distilled water for 7 seconds.

Gels were then soaked in 150 ml developing solution until bands appear; the reaction was stopped by soaking the gels in 150 ml tris acetic acid solution for 25 minutes and stored in distilled water for further analyses.

2.10. Analysis of SDS gels

Gels were analysed using the Visionworks LS software programme at the University of the Western Cape, Department of Biotechnology. The system calculated the approximate size (kDa) of the expressed protein bands in relation to the molecular weight marker (Optima Scientific, Cape Town) (Fig 4), as well as the are density of protein bands, thus providing an indication of the protein concentration (Figure 5).

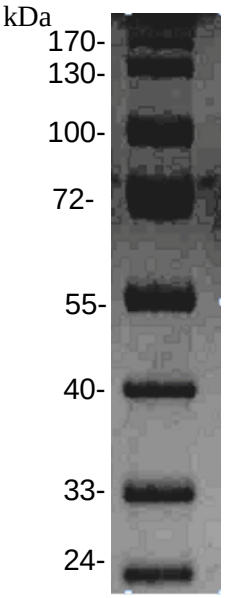


Figure 4: Molecular weight marker ranging from 24-170 kDa

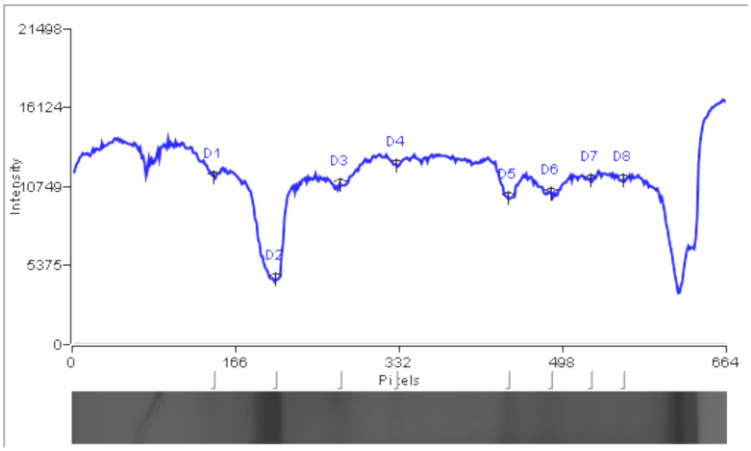


Figure 5: Inversely proportional graph showing the intensities of bands on one well of the gel (donor sample separated by DGC using the detergent Saponin). This was used to measure the area densities of particular bands of interest in kg/m^3.

2.11. Statistical analysis

Normal distribution was tested using Kolmogorov-Smirnov test, considering a P-value of less than 0.05 ($P< 0.05$) as statistically significant. For statistical evaluation of the protein band density after SDS-PAGE and DNA fragmentation in relation to the different sperm separation techniques independent t-tests were performed. Due to the small sample size, statistical sampling was employed in order to estimate sample sizes that will give significant results for. MedCalc® statistical software (version 12.3.0; Mariakerke, Belgium) was used for all statistical tests and analysis of data.

2.12. Identification of membrane surface proteins

Two approaches were employed to identify commonly occurring surface membrane proteins of donor and patient samples. One approach included a SwissProt database search using the identified molecular weights of the extracted proteins as basis. Alternatively, a literature search in the following articles was done:

1. Ahmad and Naz, 1995
2. Aitken *et al.,* 1995b
3. Baldi *et al.,* 1998
4. Benoff *et al.,* 1993
5. Benoff, 1998
6. Buddhikot *et al.,* 1999
7. Falkenstein *et al.,* 1999
8. Lambard *et al.,* 2004)
9. Le'gare *et al.,* 1999
10. Luconi *et al.,*1998
11. Luconi *et al.,* 1999
12. Luconi *et al.,* 2002
13. Naz *et al.,* 1991
14. Naz, 1999
15. Rochwerger *et al.,* 1992
16. Sabeur *et al.,* 1996
17. Sabeur *et al.,* 1996b
18. Saunders *et al.,* 2001
19. Schroter *et al.,* 1999
20. Wang *et al.,* 2005
21. Wolkowicz et al, 2003
22. Yang *et al.,* 2009

Both methods ensured accurate nomenclature of membrane proteins of interest.

2.13. Terminal deoxynucleotide transferase mediated dUTP nick-end labelling (TUNEL)-assay

After sperm separation, aliquots of the samples were analysed for DNA damage using the TUNEL assay kit (Promega: Apoptosis detection system, Madison, USA), containing equilibration buffer, nucleotide mix, TdT enzyme and 20X SSC solution. The purpose of this technique is to assess DNA strand breaks in the spermatozoa

(Hughes *et al.*, 1996; Lopes *et al.*, 1998). Smears were prepared from the pellet of each separation technique and stained as follows for the detection of DNA fragmented spermatozoa.

Each slide was fixed by immersing them in freshly prepared 4% methanol free formaldehyde (Kimix) in PBS (Oxoid) (pH 7.4) for 25 minutes at 4ºC. Thereafter, slides were washed in fresh PBS (Oxoid) for 5 minutes at room temperature, followed by permeabilizing the cells for 5 minutes in a 0.2% Triton X-100 (Sigma Aldrich, Steinheim, Germany) in PBS (Oxoid). A repetition of washing the cells in fresh PBS (Oxoid) for 5 minutes occurred twice. Any excess liquid on the surface of the slide was removed by gently tapping the slide on paper towel. The cells were then equilibrated with a 100 µl equilibration buffer (Promega), provided with the kit, for 10 minutes. While the cells were equilibrating, the nucleotide mix (Promega) was thawed on ice; the nucleotide mix (Promega) was needed to prepare the TdT incubation buffer. This incubation buffer was calculated and prepared according to the number of slides to be stained. The buffer included equilibration buffer (Promega) (45 µl per reaction), nucleotide mix (Promega) (5µl per reaction) and TdT enzyme (Promega) (1µl per reaction). Twenty micro-litres equilibration buffer (Promega) were added to each slide and covered with plastic coverslips provided with the kit, to prevent the cells from drying out. A humidifying chamber was made, and the cells were placed inside then covered with aluminium foil, and incubated (Lasec) for 60 minutes at 37ºC. After the hour, 40ml 2x SSC (Promega) were prepared from a 20x SSC (Promega) stock solution. The coverslips were removed and slides immersed in the solution for 15 minutes. Thereafter, the sample was washed in fresh PBS (Oxoid)

for 5 minutes at room temperature This was repeated thrice and 100 sperm cells were then analysed immediately with a fluorescent microscope at 40x magnification (Zeiss, Oberkochen, Germany). Fragmented sperm cells were distinguished from non-fragmented ones by fluorescing green (Figure 6).

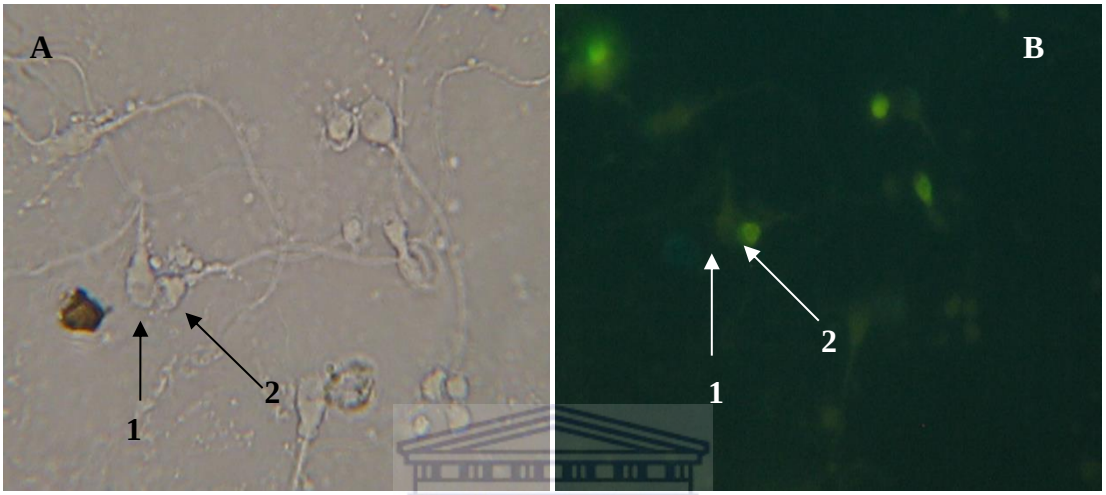


Figure 6: Illustration of DNA fragmentation detection with the TUNEL- assay. A: visualization of sperm cells under normal light microscopy, 1 and 2 indicate two sperm cells. B: visualization of sperm cells under fluorescent light, 1 indicating a sperm cell with no DNA fragmentation; 2 indicating a DNA fragmented sperm cell. (x1000 magnification) (Figure modified from: Mupfiga, 2009)

CHAPTER THREE

RESULTS

3.1. Comparison of donor and patient protein extraction using different separation techniques

Three different separation techniques were used to separate the seminal plasma from the spermatozoa. These included the wash, swim-up (SU) and Percoll density gradient centrifugation (DGC). This was done to evaluate which method possibly gives the best selection of sperm cells based on their type of proteins extracted. Both,healthy donors and infertile samples were exposed to these techniques.

The three separation techniques, extracted a total of 49 different sperm membrane surface proteins from donor samples. The molecular weights ranged from 21-175.6 kDa (Table 1). In contrast, patient samples extracted a total of 61 proteins with molecular weights ranging from 14-180.4 kDa (Table 2). Amongst donor samples, 12 common protein bands were observed ranging between 28-115 kDa, whereas amongst the patients 11 protein bands with molecular weights between 25-126 kDa frequently appeared (Table 3). Theses two experimental groups shared 7 commonly occuring proteins with molecular weights ranging from 36-93 kDa.

3.1.1 Membrane proteins extracted from donor samples

In donor samples, separation by the wash method (Figure 7A) extracted a total number of 49 sperm surface proteins, of which the molecular masses ranged between 25-175.6 kDa (Table 1). The SU only separated 19 sperm surface proteins (Figure 7B) with proteins of molecular weights ranging between 26-157.7 kDa (Table 1). Similarly to the washing technique, DGC extracted a total of 41 sperm membrane proteins (Figure 7C), with molecular masses ranging between 21-163 kDa (Table 1). From donor samples a total of 6 commonly occuring proteins that were not present in the patient group were extracted (Table 4). For all 3 techniques employed, a prominent protein band was visualized at an approximate molecular mass of 71 kDa. The 71 kDa band appears darker and thicker when separated with SU (Figure 7B) or DGC (Figure 7C) opposed to separation using the wash method (Figure 7A).

Table 1: Summary of all proteins extracted from donors sperm. The numbers shown in this table represent molecular weights (kDa). (C: CTAB; SA: Saponin; SD: SDS; T: Tween 20)

Wash				Swim up				Percoll			
C	SA	SD	T	C	SA	SD	T	C	SA	SD	T
		175.6									
										163.1	
	158.5		157.8	157.7				158.5			
	142.1	141.9						141.3			141
		131.4									
										126.5	
123								123.1		122.6	
122		121.8									
		115.4		115.3				114.8	114.5	113.5	114.8
			109.4						111.5		108.8
		102.9				106.2					107
								99.5	99.5		
								98.9			
								97.4		97.4	
96			95							95.9	
93			93.6						93.5	94.4	
	92.1	92.1									91
	89.6	88.7		88.8				89.1	89.1		89.3
			83.3								
80	79.6	80.8	81.1			78.9					
		75.9		75.9				75.9	73.9		75.9
		74.4									
		72						72.8		71.8	
71			71					71.3			
70	70.2							70.2		69.4	70.3
					67.4		68.4		68.4	68.8	
									67.1		67.1
			64.9					64			66.3
	59.5	61.5		61.6	60.5		61.1	61.7	61	62	61.7
	58.4										59
57.3	57	57.4						57			
		55.3			55.4	56.1	56.0	54	55	56	
		53.2									53
		51.8					51.2	52	52	51	
50		50.7			50.5			50		50	
49	48.4	48									
46		47.4			46.8		47.4	46		46.8	47
45	44.3	44	44.8						44.2		45
		42				43.8				43	43
40		40									40
38	38.5	38							39	39	39

37	36.8	36	36.2		36.4		36.6	36			
		35						35			
34	34.6	34	34		34.1		34.6		34	34	34
	32.5				31.6		31.7				
						29.3					
28					27	27	28				
		25				26.3					
								21			

Table 2: Summary of all proteins extracted from patient sperm. The numbers shown in this table represent the molecular weights in kDa.

Wash				SU				DGC			
C	SA	SD	T	C	SA	SD	T	C	SA	SD	T
			180.4								
		175.6									
158	158	158	158		126	142	134	148	114	148	
151	151	151		151							
141	141	141	141						96		
135	135	135	135								
		131.4									
				126		126	126	126		126	
120			122								
115	115	115	115			115					
										110	
105	105	105	105	105	105						
	103	103									
		98.2		98	98	98	98	94	84		
93	93	93	93	93				92			93
			91					91		91	
		86.6			87	88	89.4				
				84.3	83	83		81	81	81	81
79	79	79	79				79	77.8			
	75	76		74	74	74	75	74		74	74
72	72	72	72				70.44	71.6	71		
70	69.8		70		70				69	69	70
65.7				67	67.4	68.9		68	67	68	66
						66	66	66	65	65	
					63		63			63	
				62		62	62				62
	60.3	61.51		61	61			60	61		60
				58				59	59	59	59
					57			57			
				56	56	56	56		56		56
55	55	55	55					55			
54		53.7	54							54	54
51	51	51				52	51		52.6	52	50
50	50		50	50					51.5	50	
48				47.6				49	49	49	
46	46	46	46			46		46			46
				44	44	45			45		44
43	43	43	43	43	43	43	43	43	42	43	
					38	37.2	40	39		39	40
35	35	35		35	36	35	36.4	36	36	36	36
					34		34	35	34		34

				32.2		32	33	32	32	33	
29	29	29	29	29.3	30	30	30	30	30	30	30
								28	28	28	28
				27	27	27	27				27
		25		25.8		26	26	25	25	25	
									24		
									22		
									19		19
14	15.7	14	15.4								

Table 3: List of commonly occurring membrane surface proteins in spermatozoa from fertile donors and infertile patients. The identification was made from the SwissProt database and literature search.

kDa and identification of protein		kDa and identification of protein	
115	Phosphotyrosyl Protein	**92.5**	Progesterone Receptor
92.5	Non-genomic Progesterone Receptor	**74.5**	Phosphoprotein
89	Surface receptor	**70**	Human guanylyl cyclase receptor
61	Mannose lectin	**60.5**	Oestrogen Receptor β
55.5	Steroid binding domain of the progesterone genomic receptor	**51.5**	Major progesterone band
		50	Oestrogen Receptor β
51.5	Major non-genomic progesterone band	**44.5**	Progesterone Receptor
47	Minor progesterone band	**43**	Surface protein
44.5	Progesterone Receptor 43 kDa protein	**29.5**	Estradiol-binding protein
38.5	Equatorial Segment Protein	**25.5**	PH-30
34	P34H		
28	Progesterone Receptor		

Table4: Molecular weights of surface proteins extracted from donor sperm and not present in patient samples similarly to the proteins extracted from the patient samples that were not present in the donor samples.

Donors (kDa)	Patients (kDa)
115	74.5
89	70
55.5	29.5
44.5	25.5
38.5	
28	

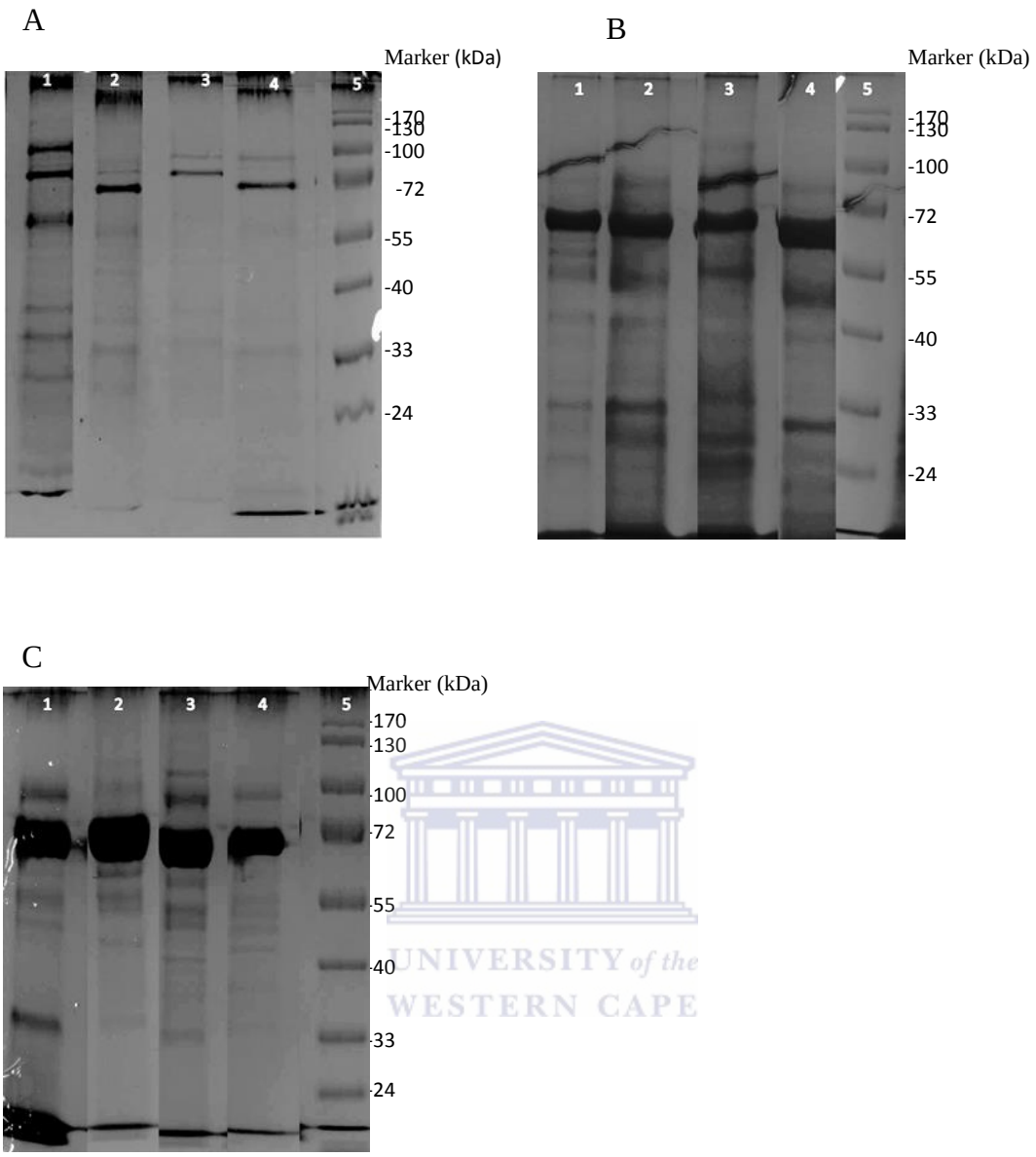


Figure 7: Donor sample membrane proteins separated on 12.5% poly-acrylamide gel (PAGE) after employing the washing technique (A), SU (B) and DGC (C).

Lane 1: CTAB; lane 2: Saponin; lane 3: SDS; lane 4: Tween 20; lane 5: molecular weight marker.

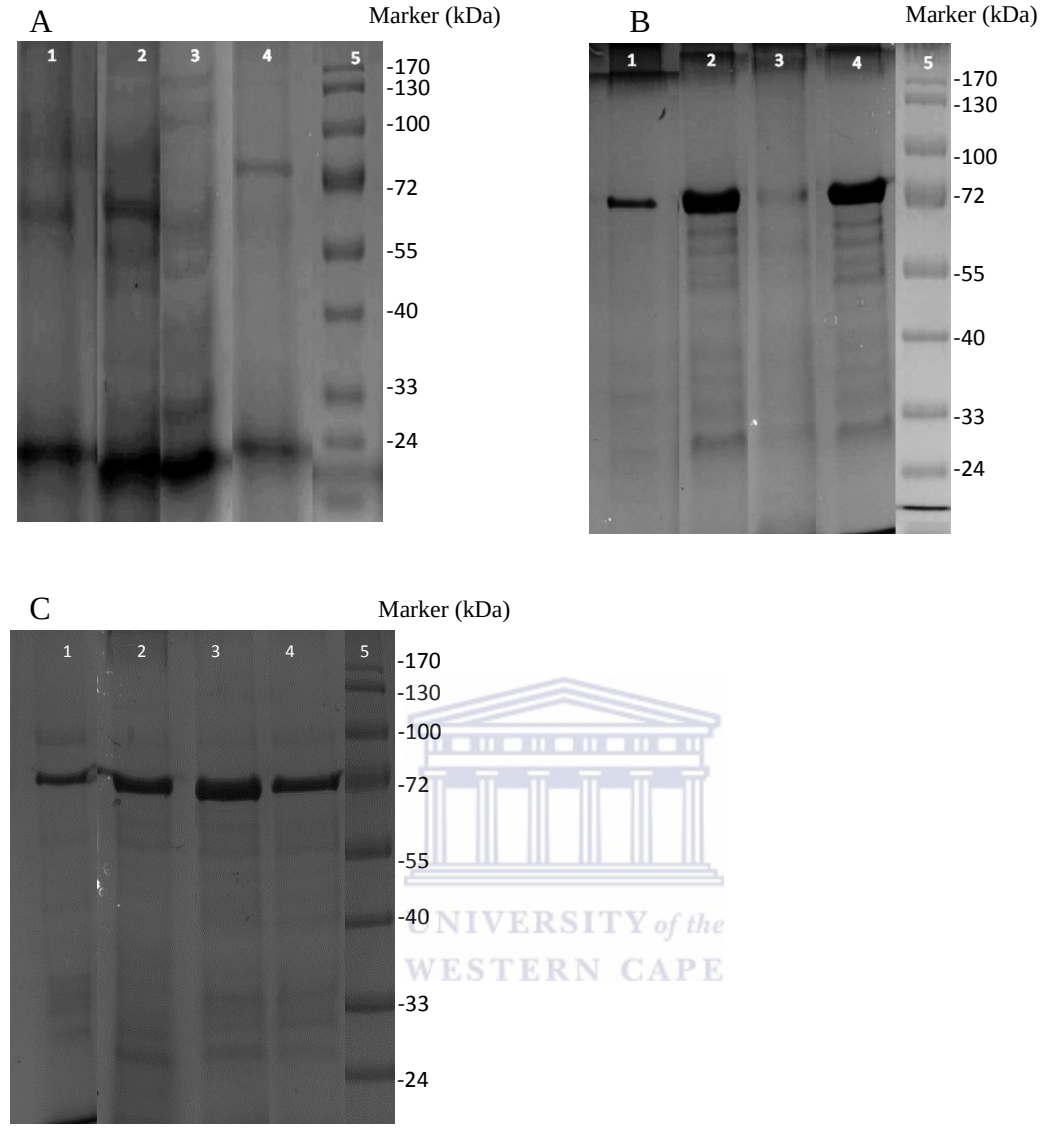


Figure 8: Membrane proteins separated on 12.5% PAGE from patient samples after employing the wash technique (A), SU (B), DGC (C).

Lane 1: CTAB; lane 2: Saponin; lane 3: SDS; lane 4: Tween 20; lane 5: molecular weight marker.

3.1.2. Membrane proteins extracted from patient samples

Membrane surface proteins of patient samples separated by means of the washing method extracted 34 proteins (Figure 8A) ranging from 14-180.4 kDa in molecular weight (Table 2). The common approximate 71 kDa protein band was present. However, it appeared extremely faint (Figure 8A). The swim-up technique (Figure 8B) employed on patients extracted 3 more protein bands than the wash method, with a total number of 37 proteins. Their molecular masses ranged between 26-126 kDa (Table 2). With this method, the 71 kDa band was, dependent on the patients, sometimes absent, or detected as an approximate 74 kDa protein (Table 2). The most membrane proteins were detected in patient samples separated using DGC. This method extracted 43 proteins in total (Figure 8C) of which the molecular masses ranged between 19-148 kDa (Table 2). The patient samples extracted 5 commonly occuring proteins that were not present in donor samples (Table 4). For this separation technique, the 71 kDa protein band appeared markedly darker (Figure 8C), but not as dark and thick as in donor samples (Figure 7).

3.2. Detergents with different properties

For each of the 3 separation techniques, 4 detergents were used for extraction of membrane proteins namely, CTAB (cationic detergent), Saponin (non-ionic detergent), SDS (anionic detergent) and Tween 20 (non-ionic detergent). These detergents were of different chemical nature, therefore extracting different types of surface proteins, depending on their properties.

3.2.1. Donors

A total of 49 proteins among the different detergents were extracted when the wash method in donors. From these, SDS extracted the most proteins (n=28) and TWEEN 20 the least (n=11) (Figure 9A; Table 5). For the SU technique, amongst a total of 19 proteins, CTAB extracted the least (n=5), and Saponin and Tween 20 the most (n=9) (Figure 9B; Table 6). In DGC separated sperm, CTAB extracted the most proteins (n=22) of the total of 41 compared to Saponin which extracted the least (n=14) (Figure 9C; Table 7).

A common pattern of proteins extracted in a certain range of molecular masses was detected amongst the different detergents. For instance, most of the proteins extracted with CTAB and SDS were between 37-120 kDa, whereas Saponin and Tween 20 aggregated in a slightly lower region of between 32-100 kDa (Table 11)

3.2.2. Patients

In patients, in sperm separated from seminal plasma by washing led to the extraction of a total of 34 proteins, of which CTAB extracted the most (n=22), and Tween 20 the least (n=19) (Figure 9A; Table 8). After SU, CTAB and SDS extracted the most proteins (n=21) and Saponin the least (n=19) of a total of 37 proteins (Figure 9B; Table 9). Forty three different proteins were extracted after DGC was utilized, CTAB extracting the most (n=27) and Tween 20 the least (n=20) (Figure 9C; Table 10).

Similar to donor groups, the molecular masses of proteins extracted by the patient samples exhibited approximately the same pattern. CTAB and SDS extracted proteins

of slightly higher molecular weight ranging between 37-120 kDa. Saponin and Tween 20 extracted proteins in a lower range of 32-100 kDa (Table 11).

Table 5: Summary of all proteins extracted from donors using the washing technique.

The numbers in the table represent the molecular weight of the proteins in kDa.

Wash			
CTAB	SAPONIN	SDS	TWEEN 20
		175.6	
	158.5		157.8
	142.1	142	
		131.4	
123			
122		121.8	
		115.4	
			109.4
		102.9	
96			95
93			93.5
	92.1	92.1	
	89.6	88.7	
			83.3
80	79.6	80.8	81.1
		75.9	
		74.4	
		72	
71			71
70	70.2		
			64.9
	59.5	61.5	
	58.4		
57.3	57	57.4	
		55.3	
		53.2	
		51.8	
50		50.7	
49	48.4	48	
46		47.4	
45	44.3	44	44.8
		42	
40		40	
38	38.5	38	
37	36.8	36	36.2
		35	
34	34.6	34	34
	32.5		
28			
		25	

Table 6: Summary of all proteins extracted from donors using the swim-up technique.

The numbers in the table represent the molecular weight of the proteins in kDa.

Swim up			
CTAB	SAPONIN	SDS	TWEEN 20
157.7			
115.3			
		106.2	
88.8			
		78.9	
75.9			
	67.4		68.4
61.6	60.5		61.1
	55.4	56.1	56.0
			51.2
	50.5		
	46.8		47.4
		43.8	
	36.4		36.6
	34.1		34.6
	31.6		31.7
		29.3	
	27	27	27
		26.3	

Table 7: Summary of all proteins extracted from donors using DGC technique. The numbers in the table represent the molecular weight of the proteins in kDa.

Percoll			
CTAB	SAPONIN	SDS	TWEEN 20
		163.1	
158.5			
141.3			141
		126.5	
123.1		122.6	
114.8	114.5	113.5	114.8
	111.5		108.8
			107
99.5	99.5		
98.9			
97.4		97.4	
		95.9	
	93.5	94.4	
			91
89.1	89.1		89.3
75.9	73.9		75.9
72.8		71.8	
71.3			
70.2		69.4	70.3
	68.4	68.8	
	67.1		67.1
64			66.3
61.7	61	62	61.7
			59
57			
54	55	56	
			53
52	52	51	
50		50	
46		46.8	47
	44.2		45
		43	43
			40
	39	39	39
36			
35			
	34	34	34
21			

Table 8: Summary of all proteins extracted from patients using the washing technique. The numbers in the table represent the molecular weight of the proteins in kDa.

Wash			
CTAB	SAPONIN	SDS	TWEEN 20
			180.4
		175.6	
158	158	158	158
151	151	151	
141	141	141	141
135	135	135	135
		131.4	
			122
120			
115	115	115	115
105	105	105	105
	103	103	
		98.2	
93	93	93	93
			91
		86.6	
79	79	79	79
	75	76	
72	72	72	72
70	69.8		70
65.7			
	60.3	61.5	
55	55	55	55
54		53.7	54
51	51	51	
50	50		50
48			
46	46	46	46
43	43	43	43
35	35	35	
29	29	29	29
		25	
14	15.71	14	15.4

Table 9: Summary of all proteins extracted from patients using the SU technique. The numbers in the table represent the molecular weight of the proteins in kDa.

SU			
CTAB	SAPONIN	SDS	TWEEN 20
	126	142	134
151			
126		126	126
		115	
105	105		
98	98	98	98
93			
	87	88	89.4
84.3	83	83	
			79
74	74	74	75
			70.4
	70		
67	67.4	68.9	
		66	66
	63		63
62		62	62
61	61		
58			
	57		
56	56	56	56
50		52	51
47.6			
		46	
44	44	45	
43	43	43	43
			40
	38	37.23	
35	36	35	36.4
	34		34
32.2		32	33
29.3	29.7	30	30
27	27	27	27
25.8		26	26

Table 10: Summary of all proteins extracted from patients using DCG technique. The numbers in the table represent the molecular weight of the proteins in kDa.

DGC			
CTAB	SAPONIN	SDS	TWEEN 20
148	114	148	
	96		
126		126	
		110	
94	84		
92			93
91		91	
81	81	81	81
77.8			
74		74	74
71.6	71		
	69	69	70
68	67	68	66
66	65	65	
		63	
			62
60	61		60
59	59	59	59
57			
	56		56
55			
		54	54
	52.6	52	50
	51.5	50	
49	49	49	
46			46
	45		44
43	42	43	
			40
39		39	
36	36	36	36
35	34		34
32	32	33	
30	30	30	30
28	28	28	28
			27
25	25	25	
	24		
	22		
	19		19

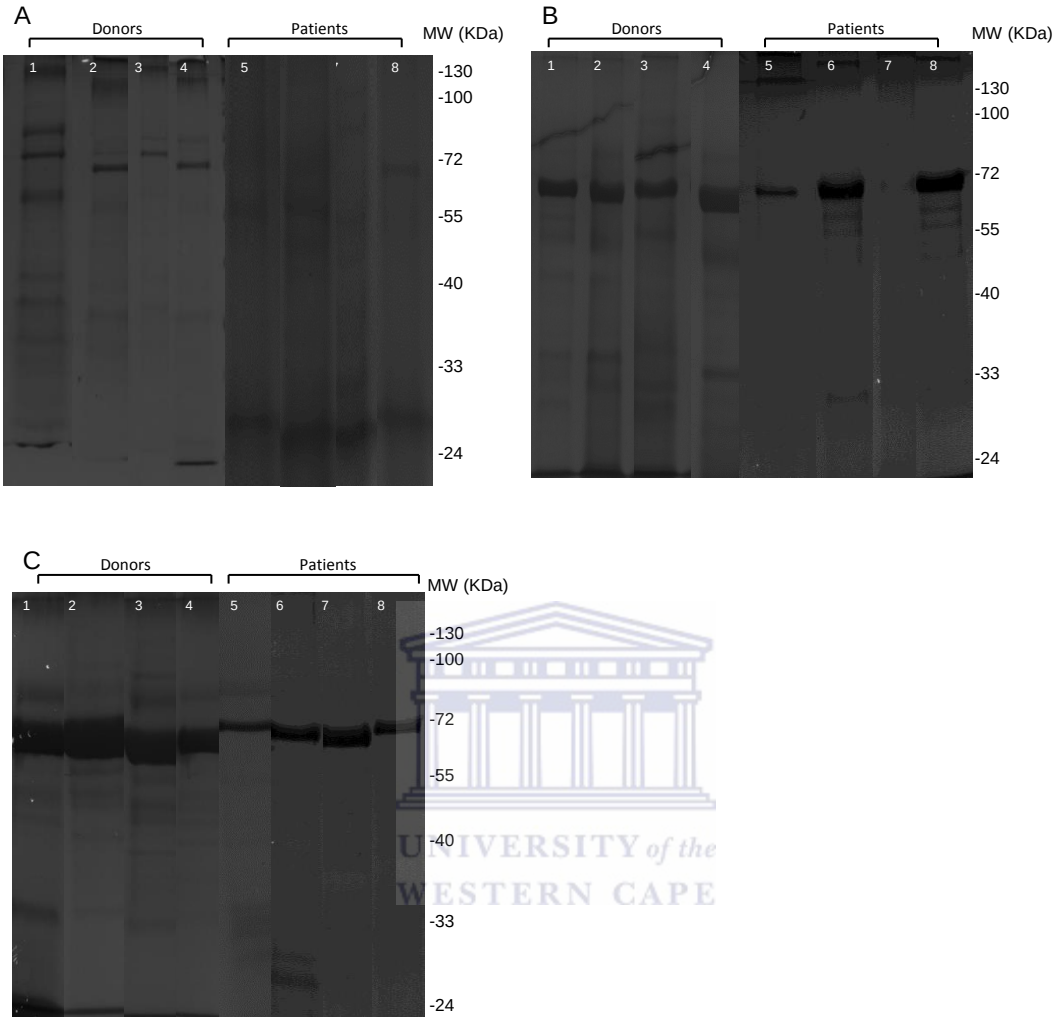


Figure 9: SDS-PAGE of membrane proteins after separation donor and patient samples. (A): wash technique, (B): SU, (C): DGC.

Lanes 1-4; donor membrane proteins extracted with CTAB, Saponin, SDS and Tween 20, respectively. Lanes 5-8: patients' membrane proteins extracted with CTAB, Saponin, SDS and Tween 20, respectively.

Table 11: Range in which the respective detergents, CTAB (cationic) and SDS (anionic) opposed to Saponin and Tween 20 (non-ionic), extracted proteins of both donor and patient samples.

CTAB and SDS (kDa)	Saponin and TWEEN-20 (kDa)
37	32
38	33
39	34
40	35
42	36
43	37
44	38
45	39
46	40
47	42
48	43
49	44
50	45
51	46
52	47
53	48
54	49
55	50
56	51
57	52
58	53
59	54
60	55
61	56
62	57
63	58
64	59
65	60
66	61
67	62
68	63
69	65
70	66
71	67
72	68
74	69
75	70
76	71
77	72
79	74
80	75
81	76
82	79
83	80
84	81
86.6	82
88.8	83
91	84
92	89
93	90
94	91

95	92
96	93
97	94
98.9	95
99.5	96
103	98
105	100
106	
110	
113	
114	
115	
120	

3.3. Similarities and differences in proteins extracted from donors and patients for different techniques

Donor and patient samples were compared in relation to the different separation techniques employed (wash, SU and DGC). In both, donors and patients, the wash and DGC separation extracted the most amounts of common sharing proteins from the sample (Table 12 and 13).

For the donor group, 9 common protein bands were extracted from the plasma membranes after the samples were separated by both washing and DGC technique, opposed to SU that extracted 3 commonly occurring protein bands (Table 12).

For patients, after washing the sperm cells, 17 protein bands were detected. After separation with SU: 13 and separation with DGC: 16 (Table 13).

Table 12: Summary of donors samples sharing common proteins for the wash, SU and DGC. The numbers are representative of the molecular mass of the proteins in kDa.

Wash	SU	DGC
158	61.5	115
92	56	89
80	27	75.5
57		61.5
48		55.5
45		52
38		47
36		39
34		34

Table 13: Summary of patient samples sharing common proteins for the wash, SU and DGC. The numbers are representative of the molecular mass of the proteins in kDa.

Wash	SU	DGC
158	126	81
151	98	74
141	88	70
135	74	67.5
115	68	65.5
105	62	60.5
93	56	59
79	44	51.5
72	35.5	49
55	33	43
51	29.5	36
50	27	34
46	26	33
43		30
35		28
29		25
15		

3.4. The 71 kDa protein

A prominent band was present in both patient and donor samples (Figure 10). The average molecular mass (kDa) of the protein band across gels (n=6) of 48 different lanes was calculated as an approximate 71 kDa (excluding outliers). Considering that visual differences in its protein concentration of its bands were observed, the average area density of the protein band was determined in 38 donors and 36 patients, respectively. Significant differences for the band density were established for this 71 kDa protein band (P=0.0094) as in donor samples this protein was thrice more dense than in patient samples (Figure 11).

Figure 10: Visual differences between the 71 kDa protein band occurring in donors (A) and patients (B).

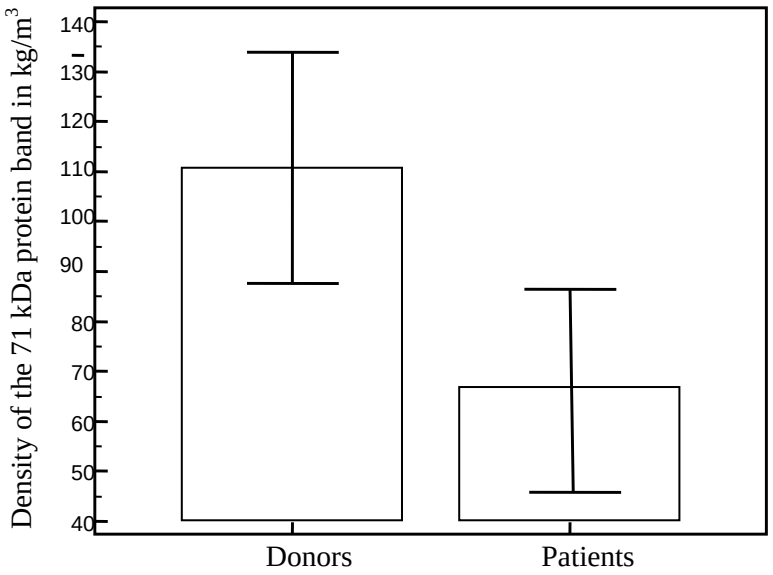


Figure 11: Comparison of band density of the 71 kDa protein band of donors and patients. On average, the band density in donor samples is about thrice of that appearing in patient samples (P= 0.0094).

3.5. DNA fragmentation

DNA fragmentation of sperm cells was tested in sperm samples from donors and patients after sperm separations using the wash and DGC techniques (Fig 12). Although no significance differences (P=0.701) were seen when comparing donor sperm DGC and patient sperm DGC using the sample size of n=5, statistical sampling revealed that if the sample size were increased to n=13 significant differences would be seen. Similarly, by analyzing the data from the sample n=5 of the DGC for donor of patient samples, no significance was seen with DGC method in patients and washing method in donors. However, it would be significant if the sample size were to be increased to n=9 (sampling test). Significant differences were observed between

DGC of donor samples and washing the donor samples (P= 0.001) as well as between washing the donor samples and washing patient samples (P< 0.001).

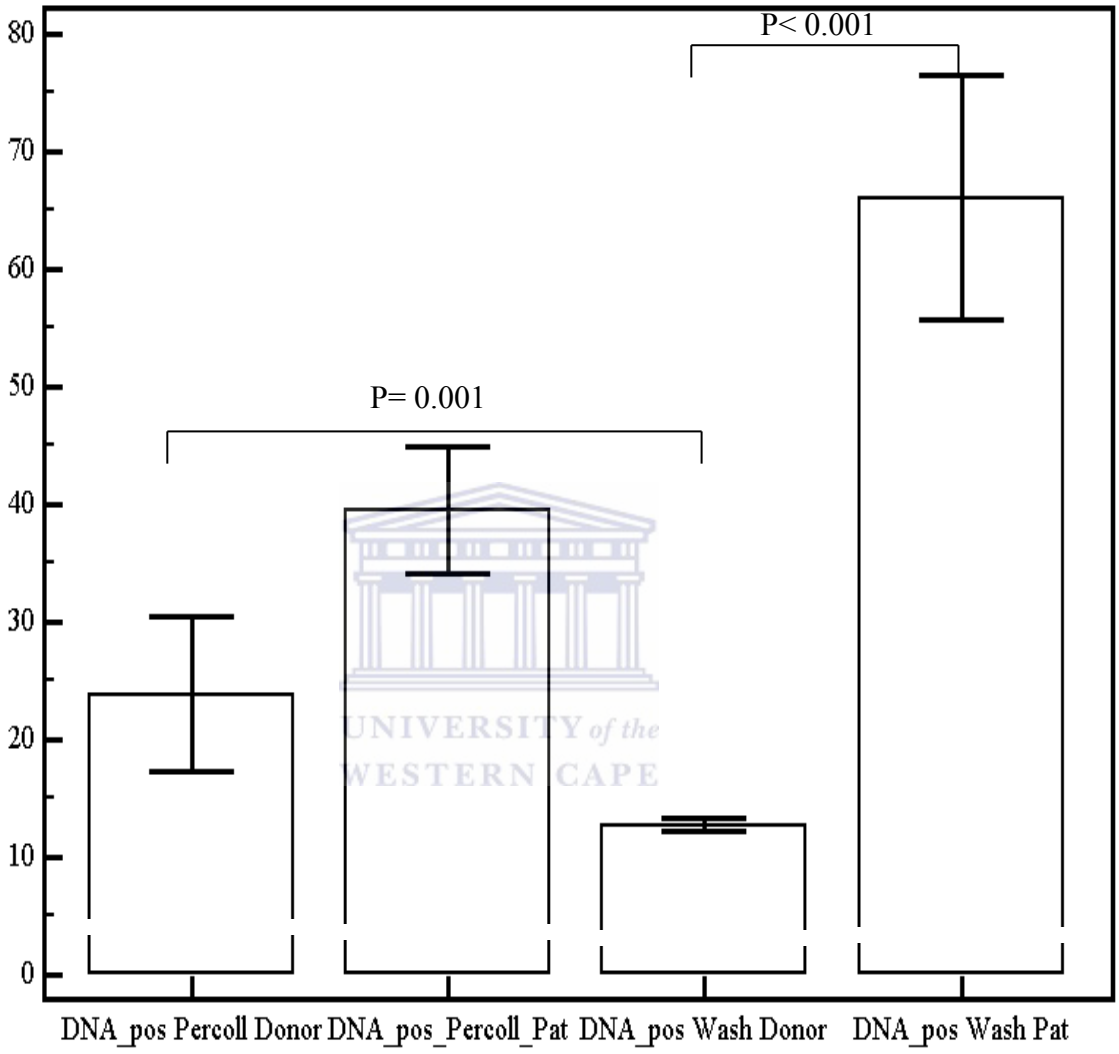


Figure 12: Comparison of the total the percentages of DNA-fragmented spermatozoa in donors and patients after washing and DGC.

CHAPTER FOUR

DISCUSSION

4.1 Plasma membrane

In the human testis, the sperm cell undergoes meiotic and mitotic differentiation, whereby these cells gain many different proteins (Schroter *et al.,* 1999). Besides spermatoza having to achieve successful fertilization, male germ cell are also required to withstand the immune defenses presented by the female reproductive tract. Therefore, they present in a manner that will differentiate those spermatozoa that are able to fertilize oocytes from spermatozoa with poor quality (Schroter *et al.,* 1999).

A total number of 6198 human sperm proteins have been reported (Amaral *et al.,* 2013). Since spermatozoa need to interact with the female reproductive tract and the oocyte efficiently (Flesch and Gadella, 2000), these proteins are associated with cellular functioning of the sperm cell (Amaral *et al.,* 2013; Ashrafzadeh *et al.,* 2013) and play an important role in the fertilization process (O'Rand *et al.,* 1979). The function of these proteins is of such importance that the loss of their integrity, even with normal semen parameters, is associated with male infertility (Wassarman, 1990).

As pointed out by Pixton (2004), many underlying causes of male infertility are poorly understood. However, the information on these causes of sperm dysfunction in men that suffer from an unknown cause of infertility is inadequate (Mackenna *et al.,*

1995). Commonly, a rise in information regarding defective zona binding accounts for poor fertilization rates or no fertilization occurring in assisted reproduction (Esterhuizen *et al.,* 2001; Liu *et al.,* 2001; Bastiaan *et al.,* 2003; Liu and Baker, 2003), suggesting that the plasma membrane is of utmost importance in the process of fertilization (Wassarman, 1990).

As the sperm cell progresses through the female reproductive tract, the most suitable sperm for fertilization is selected (Henkel, 2012). From the cervix, the number of sperm cells is reduced due to the elimination of cells with poor morphology and motility (Pretorius *et al.,* 1984). As these cells progress to the uterus, all non-functional sperm are eliminated on bases of their motility and ability to undergo capacitation and acrosome reaction (Henkel, 2012). At the isthmus, capacitation is induced, hyperactivation occurs (DeMott and Suarez. 1992; Ho and Suarez, 2001) and spermatozoa are required to be responsive with good DNA integrity (Henkel, 2012). Once the sperm cells eventually reach the cumulus and the zona pellucida, they undergo hyperactivation and their DNA integrity is assessed (Henkel, 2012). Apparently, this process seems to go along with the cells ability to bind to the zona pellucida, (Menkveld *et al.*, 1991; Liu and Baker, 1992).

Certain causes of male infertility may now be diagnosed with specific membrane proteins being identified in humans (Liu *et al.,* 1996; Naz and Leshie, 1999). For the sperm to attach to and penetrate the oocyte, specific ligands and receptors are required (Jeremias and Witkin, 1996). If the sperm cell is unable to bind to the zona pellucida, it may cause poor fertilization or complete fertilization failure, especially in assisted reproduction methods (Oehninger *et al.,* 1997; Liu and Baker, 2000, 2003;

Esterhuizen *et al.,* 2001; Liu *et al.,* 2001; Bastiaan *et al.*, 2003).Therefore, a detailed molecular approach is needed to identify these abnormalities as well as their underlying causes (Jeremias and Witkin, 1996).

4.1.1. Classification of membrane proteins

Membrane proteins can be broadly classified as integral or peripheral (Brewis and Gadella, 2010). Certain transmembrane glycoproteins are made in the testis. These proteins, which are usually hormone receptor proteins, such as progesterone and oestrogen receptors, have extracellular portions that are glycosylated and protrude to the outer cell surface (Schröter *et al.,* 1999). Another group of proteins are produced once the cycle of spermatogenesis is complete. Therefore, this production of proteins occurs outside the testis in other areas of the male reproductive tract (Schröter *et al.,* 1999). The last type of proteins is those that seem to undergo secondary integrations into the plasma membrane of the spermatozoa (Schröter *et al.,* 1999).

4.1.2. Functions of commonly extracted membrane proteins

4.1.2.1. Non-genomic progesterone receptors

Most of the proteins extracted in this study were non-genomic progesterone receptors (Table 14 and 15) (92-93 kDa; 51-52 kDa; 46-48 kDa; 45 kDa; 28 kDa).

Table 14: Common proteins extracted from donor sperm plasma membranes and their associated function

kDa	Name of protein	Function
115	Phosphotyrosyl Protein(116 kDa)	• Human sperm activation (Aitken *et al.*, 1995b)
92-93	Non-genomic Progesterone Receptor (90-95 kDa) (Luconi *et al.*, 2002)	• Stimulates capacitation • Hyperactivates sperm • Induces the acrosome reaction (Baldi *et al.*, 1998)
89	89 kDa surface receptor (Ahmad and Naz, 1995)	• Autophosphorylation for a signal transduction (Ahmad and Naz, 1995)
61	Mannose lectin (Benoff, 1998)	• Expressed after capacitation (Benoff *et al.*, 1993)
55.5	Steroid binding domain of the Progesterone genomic receptor (52-57 kDa) (Sabeur *et al.*, 1996; Luconi *et al.*,1998)	• Inhibits calcium influx (Sabeur *et al.*, 1996; Luconi *et al.*,1998)
51.5	Major non-genomic progesterone band (Sabeur *et al.*, 1996)	• Initiates the acrosome reaction (Sabeur *et al.*, 1996)
47	Minor progesterone band (46-48kDa) (Sabeur *et al.*, 1996)	• Initiates the acrosome reaction (Sabeur *et al.*, 1996)
44.5	Progesterone Receptor (45 kDa) (Luconi *et al.*, 2002)	• Stimulates capacitation • Hyperactivates the sperms motility • Induces the acrosome reaction (Baldi *et al.*, 1998)
43	43kDa protein (Naz, 1999)	• Autophosphorylation (Naz, 1999)
38.5	Equatorial Segment Protein (38 kDa) (Wolkowicz et al, 2003)	• Binding sperm to the egg and fusion thereof (Wolkowicz et al, 2003)
34	P34H	• Sperm-zona pellucida binding (Le'gare*et al.*, 1999).
28	Progesterone Receptor	• Induces acrosome reaction (Falkenstein *et al.*, 1999; Buddhikot *et al.*, 1999)

Table 15: Common proteins extracted from the plasma membrane of patient sperm and their associated functions

kDa	Name of protein	Function
92.5	Progesterone Receptor (90-95 kDa) (Luconi *et al.*, 2002)	• Stimulates capacitation • Hyperactivates the sperms motility • Induces the acrosome reaction (Baldi *et al.*, 1998)
74.5	Phosphoprotein (75kDa) (Naz *et al.*, 1991)	• Involved in capacitation (Naz *et al.*, 1991)
70	Human guanylyl cyclase receptor (hGC) (Yang *et al.*, 2009)	• Involved in capacitation (Yang *et al.*, 2009)
60.5	Oestrogen Receptor β (Saunders *et al.*, 2001)	• Possible residues of spermatogenesis (Lambard *et al.*, 2004)
51.5	Major progesterone band (Sabeur *et al.*, 1996)	• Initiates the acrosome reaction (Sabeur *et al.*, 1996)
50	Oestrogen Receptor β (Saunders *et al.*, 2001)	• Possible residues of spermatogenesis (Lambard *et al.*, , 2004)
44.5	Progesterone Receptor (45 kDa) (Luconi *et al.*, 2002)	• Stimulates capacitation • Hyperactivates the sperms motility • Induces the acrosome reaction (Baldi *et al.*, 1998)
43	43kDa protein (Naz, 1999)	• Autophosphorylation (Naz, 1999)
36	Epididymal protease inhibitor receptor (Wang *et al.*, 2005)	• Formation of protein complexes (Wang *et al.*, 2005)
29.5	Estradiol-binding protein (29 kDa) (Luconi *et al.*, 1999)	• Inhibits acrosome reaction (Luconi *et al.*, 1999)
25.5	Weak secondary PH-30 band (26 kDa) (Schroter *et al.*, 1999)	• Sperm-egg binding • May also inhibit the union of the sperm to the egg (Rochwerger *et al.*, 1992)

In addition, the steroid binding domain of this non-genomic progesterone receptor (55-56 kDa) was identified. The differences and similarities between fertile donor and infertile patient samples are apparent in this study.

Progesterone is a steroid secreted by the cumulus cells surrounding the oocyte (Sabeur *et al.,* 1996) and found at high concentrations in follicular fluid (Lobo *et al.,* 1985; Frederick *et al.,* 1991; Anderson, 1993). The effects of progesterone on mammalian sperm cells are of physiological importance for fertilization to occur (Luconi *et al.,* 2002) and exhibits rapid effects on sperm cells upon interaction with their surface receptors (McEwen, 1991; Nemere and Norman, 1991; Wehling, 1997).

Sabeur *et al.* (1996) suggested that human sperm have two non-genomic progesterone receptors, a major and minor (Sabeur *et al.,* 1996). Both major (51-52 kDa) and minor (47 kDa) progesterone bands were identified in this study as identified in a study by Sabeur *et al.* (1996) (Table 14 and 15). Other progesterone receptors were too identified in this study. These included non-genomic progesterone receptors of 92-93 kDa, 55-56 kDa, 47 kDa and 28 kDa in molecular mass (Table 14 and 15).

Progesterone receptors are of significant importance as they stimulate capacitation, hyperactivate sperm cells and induces the acrosome reaction (Baldi *et al.,* 1998) via its surface receptors (Sabeur *et al.,* 1996) located on the plasma membrane of viable sperm heads (Blackmore and Lattazio, 1991; Tesarik *et al.,* 1992). Two of the progesterone receptors, 28 and 47 kDa, were missing in patient samples but present in donors.

Both these receptors play a role in the initiation and induction of the acrosome reaction, respectively (Sabeur *et al.,* 1996; Falkenstein *et al.,* 1999; Buddhikot *et al.,* 1999) (Table 14 and 15), and the lack of these receptors in patients may therefore be an indication of these patients' infertility.

Another noticeable difference between donor and patient samples in this study was the absence of the steroid binding domain of the progesterone genomic receptor (55-56 kDa) in patients. The functional importance of this domain is to inhibit the calcium influx to the cell (Sabeur *et al.,* 1996; Luconi *et al.,* 1998). Only a millimolar concentration of calcium influx is required for capacitation in man (Stock and Fraser, 1989; DasGupta *et al.,* 1993). Therefore, this needs to be modulated to regulate capacitation and the acrosome reaction (Naz and Rajesh, 2004). Without this receptor being present in patient samples, this suggests that capacitation, in patients may be compromised.

4.1.2.2. Oestrogen receptors

Aside from progesterone, oestrogen was found in high quantities in human follicular fluid (Lobo *et al.,* 1985; Frederick *et al.,* 1991; Anderson, 1993). Similarly to progesterone, it also possesses two hormone receptors, oestrogen receptor α (ERα) (Green *et al.,* 1986) and β (ERβ) (Kuiper *et al.,* 1996). In this present study, ERβ was identified as commonly occurring protein extracted from patient samples (50 kDa; 60.5 kDa). ERβ was shown to be found on the membrane of spermatocytes,

spermatogonia and developing spermatids, and not in mature spermatozoa (Luconi et al., 1999; Saunders *et al.,* 2001).

Luconi *et al.* (1999) suggest that these receptors function to increase internal calcium concentrations as well as stimulate tyrosine phosphorylation of proteins (Luconi *et al*., 1999). As a result of the tyrosine phosphorylation pathway, the calcium influx due to the stimuli of progesterone is prohibited (Luconi *et al*., 1999). With the inability of progesterone to stimulate its receptors come further problems in the fertilization process. Amongst them are the inability to undergo the acrosome reaction, hyperactivation and capacitation.

For mammalian sperm to acquire their ability to progressively move through the female reproductive tract and interact with the oocyte, epididymal maturation is required (Bedford *et al.,* 1983). During the period of sperm cells passing through the tracts of the male reproductive system, sperm surface properties are significantly modified (Ross *et al.,* 1990; Le'gare *et al.,* 1999). These distributional changes result in biochemical alterations of the membrane (Le'gare *et al.,* 1999), possibly suggesting that cells possessing these receptors are not mature, and can therefore not fertilize an oocyte.

Taking all these aspects of the oestrogen receptor into consideration, one can conclude that the expression of this ERβ receptor present in patient samples could be another factor explaining their infertility. Therefore, patients could possibly be identified with this proteomic approach. It could also be said that these cells

possessing this surface membrane protein are immature, also aiding to its inability of successful fertilization.

4.1.2.3. Protein phosphorylation receptors

Membrane surface proteins extracted from donor and patient samples both commonly expressed sperm surface proteins required to undergo protein phosphorylation (Table 14 and 15). In this study, these surface membrane proteins included phosphotyrosol protein (115 kDa); mannose lectin receptor; phosphoprotein (74.5 kDa); human guanylyl cylase receptor (70 kDa), and an un-named 43 kDa surface membrane protein.

Protein phosphorylation is an important biological event required to regulate the functioning of many receptors (Hunter and Cooper, 1985; Yarden and Ullrich, 1988) by the post-translational modification of proteins (Naz and Rajesh, 2004) in sperm (Visconti and Kopf, 1998). The activation of this process is mediated through ligand-receptor interaction, which in turn induces a signal transduction pathway (Hunter and Cooper, 1985; Cadena and Gill, 1992). The mechanism involved in this process to regulate protein activity is the addition or removal of phosphate groups from serine, threonine or tyrosine residues of proteins as described by Naz and Rajesh (2004). During this process of removal or addition of these phosphate groups, allosteric modifications are induced that result in conformational changes of the proteins which in turn either activate or inactivate them (Naz and Rajesh, 2004).

The entire process is under control of cyclic adenosine monophosphate (cAMP), which is a second messenger present in all cell types (Naz and Rajesh, 2004). This cAMP dependant pathway activates protein kinase A that in turn regulates protein tyrosine phosphorylation (Naz and Rajesh, 2004). As an end result, the cell gains the ability to undergo capacitation, acrosome reaction (Naz *et al.*, 1991; 1993), hyperactivation as well as binding to, penetration of and fusion of the spermatozoa with the oocyte (Naz and Rajesh, 2004). This is supported by a study, using mice, by Visconti *et al.* (1995) that proved that compounds required for tyrosine phosphorylation to occur are too needed for the process of capacitation.

4.1.2.4. Receptors required for the fusion of the sperm to the egg

Before the sperm crosses the oolema there is receptor-ligand interaction that occurs (Fusi *et al*., 1992) for the completion of fertilization (Primakoff and Myles,2002). The interaction of surface proteins of the sperm cell and the oocyte is of importance for the assistance of adhesion and fusion of these two cells (Primakoff and Myles,2002).

Equatorial Segment Protein (38.5 kDa) and P34H (34 kDa) (Table 14) were two of the commonly occurring receptor proteins that have only been extracted in donor samples. As these proteins are known to mediate binding of the sperm to the oocyte (Wolkowicz et al, 2003), they are essential for the attachment of the sperm to the zona pellucida and require recognition and interaction between complementary molecules present on both gametes as described by O'Rand (1988) and Wassarman (1988). Once the sperm cell reaches and binds to the zona pellucida they receive a signal to undergo

the acrosome reaction which is needed to penetrate the zona (Primakoff and Myles,2002).

With both equatorial segment protein and P34H being absent in patient samples as shown in this study, this suggests an incompetent condition of these cells to adhere and bind to the zona pellucida, and possibly the inability to induce acrosome reaction too. With these defects it is evident that a sperm cell will be incapable of fertilizing the egg and is therefore rendered infertile.

4.2. Optimal methods for membrane protein extraction

4.2.1. Donors

Amongst the semen samples from healthy donors, most proteins were extracted from the plasma membrane after either washing the sample or separation by DGC. In this context, it must be realized that washing the samples did not only provide many sperm of from which most proteins could be extracted, but also include proteins from debris or leukocytes. Therefore, this sperm separation technique seems to be rather inappropriate, not only from the diagnostic proteomic view, but also from the point of the sperm cells' functionality. Many reports pointed the detrimental effect and the problems involved on the fertilizing potential out (Miller *et al.*, 1996). More gentle methods such as DGC would be better, and could possibly be the preferred technique of choice, as it selects sperm with good motility patterns (Jaroudi *et al.*, 1993).

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4.2.2. Patients

The best separation technique for patient samples yielding spermatozoa of which the most protein extraction could occur was DGC. These cells were obtained from infertile patients' samples with poor quality sperm cells. Therefore, density gradient medium enriches these types of cells (Amersham Bioscience, 2001). The properties of Percoll are of great importance as these cells have poor motility and other unknown defects, rendering them infertile and Percolls medium creates an environment similar to physiological conditions (Amersham Bioscience, 2001). In addition to this, DGC also rendered less sperm with fragmented DNA (Figure 12)

Washing and performing the SU on patient samples rendered less sperm cells for protein extraction although equal amounts of protein were loaded per well per gel. Since these cells are already of poor quality, exposing them to high speed centrifugation creates potential for the generation of increased levels of ROS that damages the sperm cells membrane (Aitken and Clarkson, 1988), with the membrane being damaged. As a result, sperm surface proteins would also be damaged. With employing the wash technique on patient samples the amount of DNA fragmentation was remarkably high (Figure 12).

4.3. Classification of detergents for extracting membrane proteins

The type of detergents being used for the dissolution of proteins plays a significant role in the approach to characterizing proteins and their functions (Garavito and

Ferguson-Miller, 2001). The detergents used assist in isolating and solubilizing membrane proteins (Garavito and Ferguson-Miller, 2001) due to their chemical nature. In addition, the preference of the detergent has an impact on specific proteins (Garavito and Ferguson-Miller, 2001). CTAB as cationic and SDS as anionic detergent extracted the most proteins with a slightly higher molecular mass than Saponin and Tween-20 as non-ionic detergents. The variation in the amount of proteins extracted is due to the properties of the used detergents.

Detergents of an ionic nature, such as CTAB and SDS have very strong solubilizing abilities (Macfarlane, 1983; Seddon *et al.,* 2004), which is known to increase renaturation yields. This is made possible because CTAB and SDS inhibit the formation of intermolecular bonds that could lead to aggregation (De Bernardez Clark, 2001).

Contrary to CTAB and SDS, non-ionic detergents, such as Saponin and Tween 20 are of a milder class of detergents (Reisinger and Eichacker, 2006) that destabilize the sperm membrane (Jakop *et al.,* 2009). These classes of detergents are less likely to denature the protein, hence it does not separate protein-protein bonds (Sigma-Aldrich, 2008)

4.4. 71 kDa protein

There were marked differences in one protein that were expressed denser in donors than the patient samples. This protein was approximately 71 kDa in molecular weight, and presented in both patient and donor groups. However, the area densities in donors

were thrice as higher than in patients, suggesting that this protein is expressed in higher quantities in healthy than infertile men. This protein is known as the heat shock-related protein 8 isoform 1, as similarly detected in a study performed by Martinez-Heredia *et al.* (2006). It belongs to the heat shock protein 70 family, functioning in the process of protein folding assisting with the binding to polypeptides to facilitate correct folding (Martinez-Heredia *et al.,* 2006). The low expression of this protein amongst patient samples could be due to a lack of expression during the early stages of spermatogenesis as described by Rajeev and Reddy (2004). The marked differences in expression of this surface membrane protein between donor and patient samples could possibly be a molecular marker of incorrect protein folding within the sperm cell. This then further contributes to the possibility of infertility.

4.5. Molecular approaches in Andrology

Many infertile men have sperm abnormalities that are easily detected. However, there are subgroups of men who have normal semen characteristics, but are unable to fertilize the oocyte *in vitro* (Jeremias and Witkin, 1996). Routine semen analysis gives minimal information regarding chromatin defects or its surface protein molecules that could be abnormal in the sperm cell (Jeremias and Witkin, 1996), making these techniques insufficient in determining the fertility status of a man (Milardi *et al.*, 2012). Therefore, novel molecular approaches are developed to detect and analyze these problems (Sutovsky, 2012), as it may provide insight to allow identification of molecular markers of the sperm proteome for male infertility (Milardi *et al.,* 2012)

Events that occur during the fertilization process are mediated through the interactions of the sperm plasma membrane and the female reproductive tract (Darszon *et al.*, 2012). Therefore, genomic and proteomic approaches deliver insight into the process of sperm maturation (Dacheux *et al.*, 2012), which assists with detail to the functional abilities of spermatozoa during the process of fertilization (Ferrer *et al.*, 2012). Signaling pathways involved in the process of fertilization such as capacitation and other events that occur in the female reproductive tract for the preparation of the spermatozoa for fertilization are too being studied (Signorelli *et al.*, 2012).

Advances have been made regarding the interaction between the sperm and the oocyte (Gupta *et al.*, 2012). This including the identification of the sperm receptors as well as proteins present on the eggs surface (Gupta *et al.*, 2012). Upon identifying, quantifying and characterizing surface proteins on sperm cells, this provides insight into the cellular functions (Brewis and Gadella, 2010). Therefore, proteins present on the plasma membrane of the sperm surface would assist in the molecular interaction occurring at early fertilization events (Brewis and Gadella, 2010). Thus, providing better insight to the requirements needed to be met by the spermatozoa for successful fertilization in its journey through the female reproductive tract. This is of importance, especially for assisted reproductive techniques and for the diagnosis of idiopathic infertility amongst men.

4.6. Conclusion

In conclusion, the advent of molecular approaches to characterize and diagnose male infertility may be the possible solution running parallel with routine examinations. This proteomic approach could assist with the explanation as to why patients are rendered infertile without obvious cause by identifying surface membrane proteins that are required in the process of fertilization. It is evident in this study that certain surface proteins are absent from patient samples, yet present on donors, or present on patient samples surface membrane and absent on donors'. The function of these proteins is closely related to their ability to fertilize the oocyte, which renders them fertile or infertile. Therefore, the infertility status of men may go beyond what meets the eye.

CHAPTER FIVE

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