CHAPTER 1

General Introduction

Adapted from:

1) Said TM, Agarwal A, Zborowski M, Grunewald S, Glander H-J, Paasch U. Utility of magnetic cell separation as a molecular sperm preparation technique. *J Androl*, 2008, 29 (2), 134-142.

2) Said TM, Paasch U, Glander H-J, Agarwal A. Role of caspases in male infertility. *Hum Reprod Update,* 2004, 10 (1), 39-51.

3) Agarwal A, Said TM. Role of sperm chromatin abnormalities and DNA damage in male infertility. *Hum Reprod Update,* 2003, 9 (4), 331-345.

4) Agarwal A, Said TM. Oxidative stress, DNA damage and apoptosis in male infertility – A clinical approach. *BJU International*, 2005, 95 (4), 503-507.

I. Introduction

Assisted reproductive techniques (ART) have become the treatment of choice in many cases of infertility; however the current success rates for these procedures remain suboptimal [1]. Programmed cell death (apoptosis) is a mode of cellular death based on a genetic mechanism that induces a series of cellular, morphological and biochemical alterations leading the cell to suicide [2]. There is likelihood that some sperm selected for ART will display features of apoptosis despite their normal appearance, which may be partially responsible for the low blastocyst development rates, pregnancy rates and recurrent pregnancy loss seen with ART [3-5]. The negative effects of sperm apoptosis, specifically DNA fragmentation appear at the time of activation of the paternal genome [6].

Various sperm preparation techniques are currently used as main components of ART procedures [7]. These techniques appear to deliver comparable results and have a common objective, which is the selection of a sufficient number of viable motile sperm capable of fertilizing the oocyte(s) [8]. Current routine sperm preparation techniques depend on a sedimentation or migration approach to separate spermatozoa based on their motility or density. These preparation techniques are directly dependant on routine sperm parameters, mainly motility and morphology. Nevertheless, molecular events such as sperm apoptosis are overlooked along the course of routine ART, which may negatively impact the final outcomes.

Numerous reports link the presence of apoptosis markers in human sperm with the failure of *in-vivo* and *in-vitro* fertilization [4,5,9-11]. One of the early specific apoptotic events reported in human spermatozoa is the externalization of the phospholipid phosphatidylserine (PS) from its normal location in the inner leaflet of the sperm plasma membrane to the outer surface [12,13]. On the other hand, sperm DNA fragmentation is a late apoptotic feature that has been identified in ejaculated spermatozoa [14-18]. The development of new protocols for sperm selection based on the presence of these markers and apoptosis-like manifestations should be considered to improve ART success rates.

II. Apoptosis in Human Spermatozoa

II.a. Apoptosis phenotype

The inclusion of non-apoptotic spermatozoa is one of the pre-requisites for achieving successful fertilization. Although apoptosis is an on-going physiological phenomenon that maintains the number of germ cells within the supportive capacity of the Sertoli cells [19], deregulated apoptosis has been associated with the presence of abnormal spermatozoa in semen in terms of counts, motility and morphology [20-22]. As opposed to somatic and testicular germ cells, the significance of apoptosis phenotype in ejaculated spermatozoa remains controversial [23]. Although ejaculated spermatozoa display several apoptosis-like characteristics as in somatic cells, such apoptosis-

related features may not indicate the presence of apoptosis death functions [17]. The failure to eliminate these abnormal spermatozoa during spermatogenesis--also termed as “abortive apoptosis”--may be the reason for their presence in semen [20,21].

II.b. Externalization of phosphatidylserine

In spermatozoa with an intact plasma membrane, the phospholipid PS is located on the inner leaflet of the plasma membrane only. PS has a high and selective affinity for annexin V, a 35-36 kD phospholipid binding protein [24]. Annexin V cannot pass through an intact plasma membrane; therefore, annexin V binding to spermatozoa characterizes a disturbed integrity of the membrane. The binding is due to translocation of PS from the inner to the outer leaflet of plasma membrane resulting in externalization of PS (EPS) on the external surface [25]. This translocation of PS is one of the earliest detectable features of cells undergoing the terminal steps of apoptosis [12,26]. EPS negatively correlates with the sperm motility [27]. Reduced integrity of spermatozoal membrane is more frequently seen in spermatozoa from infertile men, and this reduced integrity contributes to childlessness despite normal routine sperm parameters [28,29].

II.c. Mitochondrial dysfunction

In humans, spermatozoal mitochondria are susceptible to apoptotic stimuli due to their compartmentalization within the midpiece region [30]. Intact mitochondrial membrane potential (MMP) is determined to be essential for the spermatozoal motility [31]. The disruption of MMP is considered a key marker for the apoptosis signaling cascade and is noted in human spermatozoa following cryopreservation and thawing [32]. A strong correlation could be found between MMP and levels of DNA fragmentation in human spermatozoa [33]. In support of the implication of apoptosis in male infertility, EPS, mitochondrial dysfunction, and nuclear DNA damage were detected in significantly higher levels in infertile men and those with varicoceles [11,34,35].

II.d. Caspase activation

Caspases are a family of cysteine proteases that have a documented role in apoptosis. Individual family members are referred to in order of their publication, therefore, interleukin-1β-converting enzyme (ICE), the first family member acquired the name caspase-1 (CP-1). Caspases were implicated in apoptosis with the discovery that cell death abnormal-3 (CED-3) is related to mammalian ICE or CP-1 [36,37]. Samples from infertile patients are characterized by high numbers of cells with activated caspases, especially in cytoplasmic residues, with a strong correlation to EPS [38]. The presence of precursors and activated forms of CP-8 and CP-9 in conjunction with CP-3 in human spermatozoa has also been confirmed [39].

Caspase activation is believed to be a well-defined point of no return for apoptosis progression, and a number of apoptotic events downstream of caspase activation have been characterized among which DNA fragmentation stands as a relatively late apoptotic event [40]. Evidence supports that within the cellular component of the testicular tissue, caspases play a central role in the apoptotic process that leads to DNA fragmentation of Sertoli cells [41]. Activated CP-3 induces activation of caspase-activated deoxyribonuclease (CAD; also called DNA fragmentation factor-40 or caspase-activated nuclease), which is integrally involved in degrading DNA. Therefore, CP-3 executes the final disassembly of the cell by generating DNA strand breaks [42].

Caspase mediated apoptosis was reported in sperm in response to cryopreservation and exposure to Chlamydia trachomatis [43,44]. Sperm DNA fragmentation was found to be prevalent in fractions of sperm with positive immunostaining for CP-3, suggesting a relation between them [18]. Further, a significant positive correlation was seen between CP-3 in the sperm midpiece and DNA fragmentation in low motility semen specimens, suggesting that caspase-dependent apoptotic mechanisms could originate in the cytoplasmic droplet or within mitochondria and function in the nucleus [45], Figure 1. In addition, increased CP-3 activity seems to be correlated with low sperm morphology and motility [46].

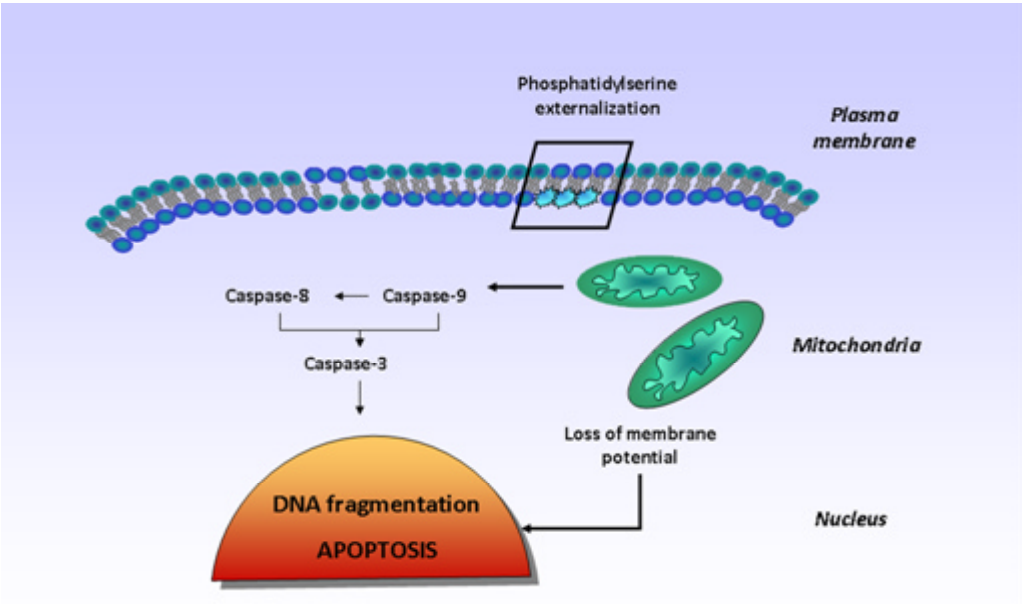

Figure 1. *Apoptosis cascade in human spermatozoa. Early, PS is translocated from the inner to the outer leaflet of the plasma membrane. Initiator caspases (CP-8 and CP-9) activate effector CP-3 and together with loss of mitochondrial membrane potential lead to DNA fragmentation.*

III. Pathogenesis of Sperm DNA Damage

III.a. Origin of sperm DNA damage

Environmental stress, gene mutations and chromosomal abnormalities can disturb the highly refined biochemical events that occur during spermatogenesis. This can ultimately lead to an abnormal chromatin structure that is incompatible with fertility [47]. Sperm nuclear chromatin abnormalities/DNA damage could occur at the time of, or be the result of, DNA packing at spermiogenesis [48]. Or, alternatively, it could be the result of free-radical induced damage [49] or a consequence of apoptosis [14]. However, the exact mechanism(s) by which chromatin abnormalities/DNA damage arises in human spermatozoa is not precisely understood.

III.b. Oxidative stress

In recent years, researchers have become more concerned about the generation of reactive oxygen species (ROS) in the male reproductive tract because at high levels, ROS is potentially toxic to sperm quality and function [50]. Many reports have indicated that high levels of ROS are detected in semen samples of 25% to 40% of infertile men [51]. Although the mechanism(s) by which human spermatozoa generate ROS remain to be clarified, there appears to be a significant role for the enzymen ß - nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [52]. The cytoplasmic enzyme glucose-6-phosphate dehydrogenase (G6PD) controls the rate of glucose flux and intracellular availability of NADPH through the hexose monophosphate shunt. This in turn is used as a source of electrons by spermatozoa to fuel the generation of ROS via NADPH oxidase [53].

Two factors protect the sperm DNA from oxidative insult: the characteristic tight packaging of the DNA and the antioxidants present in seminal plasma [54]. However, oxidative stress may develop as a result of an imbalance between ROS generation and scavenging activities [55]. In general, deoxyribonucleic acid bases and phosphodiester backbones are very susceptible to peroxidation. In addition, spermatozoa are particularly susceptible to OS-induced damage because their plasma membranes contain large quantities of polyunsaturated fatty acids and their cytoplasm contains low concentrations of scavenging enzymes [56].

Strong evidence suggests that high levels of ROS mediate the occurrence of high frequencies of single- and double-strand DNA breaks commonly observed in the spermatozoa of infertile men [57-59]. Studies in which the sperm was exposed to artificially produced ROS resulted in a significant increase in DNA damage in the form of modification of all bases, production of base-free sites, deletions, frame shifts, DNA cross-links and chromosomal re-arrangements [60-62]. Furthermore, ROS play an important role in mediating apoptosis by inducing cytochrome *c* and caspases 9 and 3, which in turn results in a high frequency of single and double stranded DNA strand breaks [63].

III.c. Sperm morphological attributes

Teratozoospermia occurs as a result of defective spermatogenesis and is characterized by an abundance of spermatozoa carrying surplus residual cytoplasm. The retention of residual cytoplasm promotes spermatozoa to generate endogenous ROS via mechanisms that may be mediated by the cytosolic enzyme G6PD [64]. Therefore, patients presenting with teratozoospermia are at greater risk of developing pathogenic levels of ROS, and subsequently apoptosis and sperm DNA damage (Figure 2).

In general, ROS production is highest in immature spermatozoa from males with abnormal semen parameters and appears to be associated with high levels of DNA damage [65]. However, immature spermatozoa with cytoplasmic retention are not the only abnormal male germ cells that are associated with high levels of DNA damage and ROS production. Spermatozoa with abnormal head morphology, midpiece defects and tail defects also display the same characteristics. Production of ROS positively correlates with the sperm deformity index (SDI), which is calculated by dividing the total number of deformities observed by the number of sperm evaluated [66]. Similarly, spermatozoa with large nuclear vacuoles were shown to present with higher levels of DNA fragmentation [67].

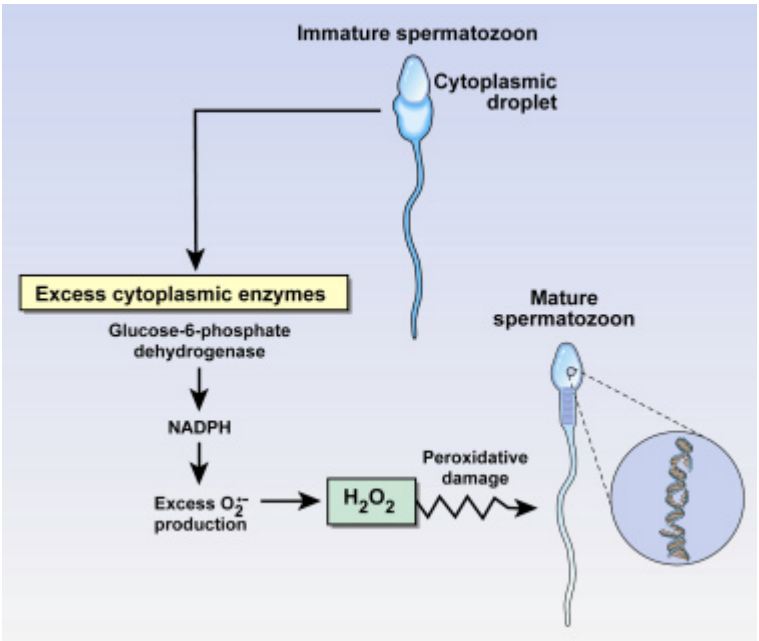

Figure 2. *Mechanism for the link between oxidative stress and sperm DNA damage. Immature spermatozoa characterized with cytoplasmic droplets have deranged redox metabolic activity and greater ability to produce ROS. In turn, oxidative stress leads to damage of the sperm DNA damage. (From: Agarwal and Said.* BJU International*, 2005, 95, 503-507)*

IV. Magnetic Cell Separation: Principles and Techniques

Techniques for cell isolation play a major role in a variety of biological and biomedical applications including disease management. Magnetic cell separation offers the advantages of simplicity of operation, low cost and specificity and sensitivity afforded by use of immunospecific reagents. The outstanding examples of the magnetic cell separation application include the isolation of rare progenitor cells from the human umbilical cord blood and the use of mobilized peripheral blood as a substitute for bone marrow transplantation in patients that underwent irradiation and chemotherapy [68,69].

Typically, magnetic cell separation employs the use of magnetic particles conjugated to proteins or antibodies to tag cells of interest. To label and to separate cells, many types of magnetic microbeads and nanobeads have been developed. The addition of iron oxide compounds to polystyrene spheres or to a sugar based skeleton is a typical approach to bead production [70].

An early type of magnetic separator was developed for use in combination with micrometer-sized Dynabead magnetic particles (Invitrogen, Carlsbad, CA) [71]. Dynabeads were the first magnetic particles used for clinical cell separation applications, in particular, bone marrow purging of gliomas prior to transplantation [72]. Due to their relatively large size (from 1 μm to 4.5 μm or) the preferred applications of Dynabeads is negative cell selection, where the cells targeted by the magnetic label are discarded, and only the unlabeled cells are used [73].

Multiple additional techniques were also introduced as solutions for isolation of a desired cell from a heterogeneous mixture of cells. These techniques include magnetic cell capture from suspensions directly in the field of view of a microscope [74] and a specialized system based on bioferrofluids, that is, submicron magnetic particles conjugated to targeting antibodies (Invitrogen, Carlsbad, CA) [75,76]. Recently, the FDA approved the use of CellSearch™ system for monitoring metastatic breast cancer (Veridex LLC, Warren, NJ).

In comparison to micrometer-sized magnetic particles, the use of colloidal magnetic particles as labeling reagents offers advantages in forming stable suspensions and fast reaction kinetics, similar to immunofluorescence labels. The small size of the particles, in the range of tens to a hundred nanometers, comes with the cost of low magnetic moment, requiring high magnetic fields and gradients, and therefore the use of specialized, magnetic affinity-type columns, typically obtained by soft steel alloy inserts inside the cell suspension container (Figure 3). This concept has been implemented in a particularly successful design, the Magnetic Activated Cell Sorting (MACS) Microbeads and Columns™, developed and commercialized by Miltenyi Biotec GmbH, Bergisch Gladbach, Germany [77].

As the colloidal magnetic particles are extremely small (<100 nm), the use of a high-gradient magnetic field is required to retain the labeled cells, approaching 1 tesla (1 tesla = 10,000 gauss) and the local gradients of up to 1,000 tesla per meter [78,79]. The MiniMACS™ column

(Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) is specifically designed to generate this strong magnetic field while maintaining optimal cell viability and function. By using a MACS column with a biocompatible coating of ferromagnetic solid support placed in a powerful magnet, the magnetic force is sufficient to retain the target cells labeled with minimum microbeads [80]. MACS microbeads are used to magnetically label the target cell population. They are not visible with light microscopy, biodegradable, and gentle on cells [77]. The column schematic is shown in Figure 3.

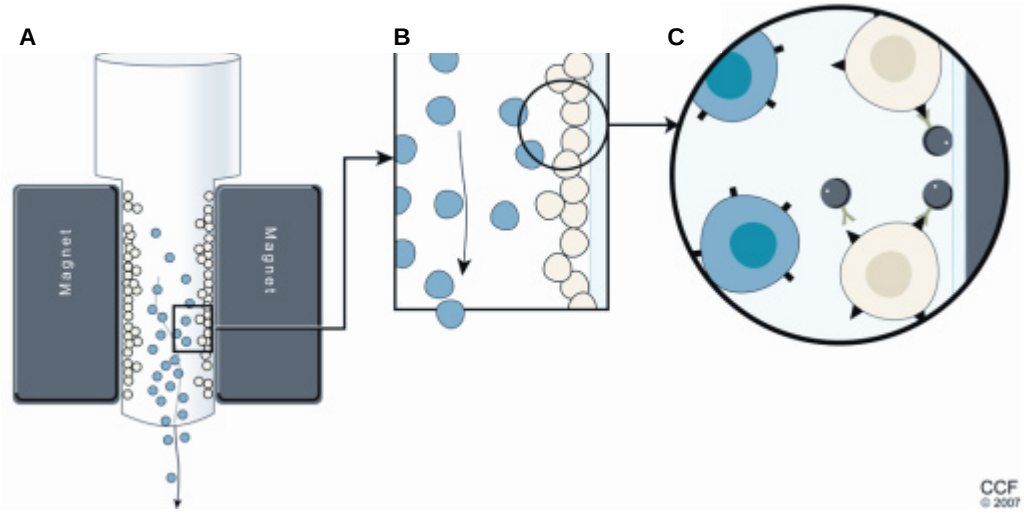

Figure 3: *Schematic diagram of MiniMACS™ magnetic cell separation column. A) The solid support consists of closely packed, sub-millimeter spheres made of soft steel alloy. An external magnetic field magnetizes the solid support, which attracts magnetically labeled cells from a mixture applied to the column. B) A thin slice across the solid support shows interstitial spaces available for flow of the cell mixture. C) The un-labeled cells flow freely through the column, while the magnetically labeled cells are retained inside the column and could be recovered by removal of the column from the magnetic field, followed by elution. (From: Said et al. J Androl, 2008, 29, 134-142).*

V. Role of Magnetic Cell Separation in Male Reproduction

Paramagnetic microbeads coupled with the use of specific antibodies can effectively separate cells. Therefore, the successful application of MACS relies mainly on the selection of specific antibody and an appropriate labeling strategy. Based on the antibody used, there are multiple applications for the MACS technology in the field of male reproduction. Initially, magnetic beads were used to select and characterize acrosome-reacted sperm as well as to evaluate its ability to penetrate zona-free hamster oocytes [81,82]. However, since the acrosome reaction of the

fertilizing spermatozoon must be exactly synchronized with its penetration through the egg vestments [83], the selection of an already acrosome-reacted sperm would be of little value in the context of *in-vitro* human fertilization.

MACS combined with anti-CD45 microbeads has been used repeatedly with great success to eliminate leukocytes from the seminal fluid [84,85]. MACS could be also used to isolate antibody-labeled from antibody-free spermatozoa in males with autoimmune infertility. These patients could benefit from this protocol as it avoids potential damage to fragile sperm and does not seem to induce any modification of semen qualities. Subsequently, the concentration of sperm after MACS separation might be useful for insemination in infertile couples with autoimmune male infertility [86-88].

MACS has been employed to facilitate the analysis of distinctive homogeneous spermatogenic cell populations by overcoming the heterogeneity of somatic and germ cells within the testicular tissue [89-91]. Immunoglobulin G was applied against the c - kit receptor protein for the immuno-magnetic isolation of spermatogonia. The use of the c-kit receptor as a marker for cell sorting of immature germ cells is supported by multiple studies that document its presence on the outer membrane of spermatogonia [92-94].

In a recent study, MACS did not eliminate all tumor cells from murine testicular cell suspensions [95]. EL-4 tumor cells were labeled using R - phycoerythrin (PE) conjugated rat anti-CD49f monoclonal antibody. Following MACS with anti-PE microbeads, the number of spermatogonial stem cells ($CD49f^+$) increased significantly from 3.94% to 40.46%, while the number of EL-4 cells decreased from 10.35% to 3.54%. However, spermatogenesis was observed in only a limited number of testes that received purified cell suspensions transplantation and malignancy developed in one out of 20 mice. The lack of absolute MACS efficiency in this study may be attributed to the non-specificity of the antibodies used. Similarly, MACS did not prove to be an effective sex selection method for the isolation of Y-bearing sperm since the H-Y antigen used as a surface marker is considerably expressed among X-chromosome-bearing sperm as well [96].

Annexin V which has high affinity for PS but lacks the ability to pass through an intact sperm membrane could be used as a marker in conjunction with MACS to label apoptotic cells [28]. Indeed, paramagnetic microbeads conjugated with annexin V has been used effectively to label spermatozoa with externalized PS [97]. MACS separation of a sperm population yields 2 fractions: annexin-negative (intact membranes, non-apoptotic) and annexin-positive (externalized PS, apoptotic) [98].

Aims and Contents of the Thesis

Despite recent advances in ART such as assisted hatching and preimplantation genetic screening, the current pregnancy and live-birth rates remain to be improved in order to alleviate the socio-economic burden of failed cycles. The increase in ART applications associated with suboptimal success rates has mandated the development of an ideal sperm preparation technique that could be used in these applications. While the role of spermatozoa in ART failure has been overlooked, strong evidence suggests that proper sperm selection will result in improved success rates. As an example, sperm with excessive nuclear histones are prone to premature chromosomal condensation, which has been identified as one of the prevalent causes of fertilization failure in ART [99]. Recent review of literature describes that DNA damage in the male germline is associated with poor fertilization rates following *in vitro* fertilization, defective preimplantation embryonic development, and high rates of miscarriage and morbidity in the offspring, including childhood cancer [100]. Sperm aneuploidies have been also suggested to have an impact on the outcome of intracytoplasmic sperm injection [101].

In this thesis, our aims were to: 1) examine the role of sperm morphological attributes, specifically cytoplasmic retention droplets in the occurrence of ROS-mediated DNA damage; 2) test the hypothesis of using the magnetic cell separation technology as a sperm preparation method; 3) examine the extent of improvement in sperm parameters following MACS application; and 4) identify the protocol limitations and which ART procedures would benefit the most from its application.

Immature, morphologically abnormal spermatozoa are sometimes characterized by the presence of cytoplasmic droplets in the midpiece, which are rich in G6PD, which increases the intracellular availability of NADPH and subsequently acts to increase ROS production. In **Chapter 2**, we investigate the impact of sperm cytoplasmic droplets on DNA damage and examine the role played by NADPH and ROS in mediating such damage. In **Chapter 3**, we further elucidate the relationship between sperm morphology and ROS-mediated DNA damage by employing the SDI, a quantitative index that encompasses all abnormalities in sperm morphological attributes.

The hypothesis of using MACS with annexin V microbeads as a sperm preparation technique is based on its ability to remove apoptotic sperm; nevertheless there are other components in the ejaculate such as leukocytes, debris, plasma, etc. that should be removed as well. Therefore, there is a need to complement MACS with other routine sperm preparation techniques. In **Chapter 4**, we have optimized MACS by examining its combination with either double density

gradient centrifugation or by one-step sperm wash. We also examined the quality of spermatozoa selected using the combined approach in terms of motility, viability and apoptosis markers. **In Chapter 5,** we characterized the morphology of spermatozoa selected using our MACS protocol. Detailed morphological evaluations are provided in the form of SDI scores, percentages of sperm with acrosomal defects, midpiece defects, cytoplasmic droplet and tail defects. The data generated should allow us to evaluate the advantages of integrating MACS as a part of sperm preparation protocols.

Our novel suggested protocol for sperm preparation is a 2 step procedure that combines a routine sperm preparation technique with MACS. Subjecting semen samples to multiple manipulations could result in substantial sperm loss, which may prevent the resulting sample from being successfully used during the course of ART. Therefore in **Chapter 6**, we have undertaken the evaluation of the extent of cell loss and sperm recovery rates following the procedure.

Despite recent methodological advances, the detrimental effects of cryopreservation on human spermatozoa are still markedly noted. Apoptosis plays a role in the decrease of sperm quality after cryopreservation as evidenced by the impairment of the membrane integrity, including the externalization of PS. In **Chapter 7**, we assessed the effects of integrating MACS in cryopreservation–thawing protocols on sperm motility and cryosurvival rates.

Chapter 8 is dedicated to evaluating the fertilization potential of non-apoptotic sperm separated by MACS. We used animal models to assess if MACS could be of benefit in identifying a sperm population of higher fertilization potential that can be used to enhance ART outcomes. In our research, we used the zona-free hamster penetration assay and the sperm chromatin decondensation assay following hamster oocyte intracytoplasmic injection. These assays serve as models for *in-vitro* fertilization and intracytoplasmic sperm injection, respectively. In addition, we evaluated the extent of DNA fragmentation in MACS separated spermatozoa and its correlation with fertilization potential. Collectively, these findings should enable us to identify which ART procedure would benefit the most from MACS application.

In order to substantiate the impact of apoptosis on failed fertilization, the relationship between the sperm's ability to penetrate oocyte and the expression of apoptosis markers was investigated in **Chapter 9**. Samples with low sperm penetration capacity were compared with those with high sperm penetration capacity in terms of EPS, disrupted MMP and activated CP-3. Finally, the findings of this thesis are discussed and summarized in **Chapter 10**.

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