CHAPTER 10

General Discussion, Future Directions and Summary

Sperm Morphology, Oxidative Stress and DNA Damage

There is substantial evidence from a number of interventional and observational studies that oxidative stress (OS) is a major causative factor in the increased level of DNA damage seen in spermatozoa [1-4]. It has been found that reactive oxygen species (ROS) production is highest in immature spermatozoa with abnormal head morphology and cytoplasmic retention [5,6]. It has been also suggested that there is an increased β-nicotinamide adenine dinucleotide phosphate (NADPH) production via glucose-6-phosphate dehydrogenase (G6PD) in the immature spermatozoa with cytoplasmic retention and this NADPH causes high ROS production via NADPH oxidase activity [5].

In view of this evidence, the use of NADPH for ROS generation appears to be the most similar to the actual form of OS resulting from spermatozoa *in vivo*. NADPH has been shown to induce significantly high levels of lipid peroxidation, loss of motility, and DNA damage in mature motile spermatozoa from healthy donors when incubated for 24 hours [7]. Though there have been several reports on NADPH inducing ROS generation by spermatozoa of healthy donors [7-9], the levels of induced ROS production via NADPH by the immature and mature spermatozoa of infertile patients remained to be examined. In our study, we documented that higher level of sperm DNA damage was seen in immature sperm fractions of patients screened for infertility. The higher DNA damage was associated with high ROS generated in response to NADPH. This finding is consistent with another report that documents higher ROS levels in immature sperm fractions in infertile men [10]. This finding provides insight into the pathogenesis of infertility since subfertile men are expected to have a larger proportion of immature and morphologically abnormal spermatozoa [11].

Clinical Implications of ROS Mediated Sperm DNA Damage

The results regarding the occurrence of DNA damage in response to NADPH elucidate the pathogenesis of sperm DNA damage and its relation with OS encountered in cases of teratozoospermia, specifically those characterized by the presence of cytoplasmic retention droplets. A potential clinical application would be the selection of the optimum sperm preparation protocol during the course of assisted reproductive techniques (ART). Samples characterized by the presence of cytoplasmic retention droplets should be prepared using double density gradient centrifugation, which has the ability to efficiently separate those immature, morphologically abnormal spermatozoa [6]. A technique such as swim-up, which allows prolonged contact between mature and immature spermatozoa after pelleting, should be avoided. Such prolonged contact will lead to mature spermatozoa being exposed to high levels of ROS generated by the immature sperm and subsequently suffer from DNA fragmentation.

Whereas the impact of sperm DNA fragmentation on fertilization rates remains controversial, there is a wider agreement concerning its negative effects on embryo development and abortion rates following in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) [12-15]. Although low levels of sperm DNA damage can be repaired by the oocyte, apoptosis and embryo fragmentation can occur if the damage is extensive [16]. Decreased embryo cleavage rates and embryo quality have been reported in sperm samples containing a high frequency of damaged DNA [17].

Routine and Molecular Sperm Preparation Techniques

Currently, routine sperm preparation techniques used during ART are dependant on sperm properties such as motility and morphology. Although the most normal appearing and motile spermatozoa are selected during ART, there is always a chance that sperm containing varying degrees of DNA damage may be used. In support of the impact of paternal DNA status on the ability to achieve a live birth, a study evaluated the different sperm aneuploidies in couples with unexplained recurrent pregnancy loss. The sperm aneuploidy rate in chromosomes X, Y, 13, 18 and 21 was reported to be significantly higher in couples with recurrent pregnancy loss compared to the general population and fertile controls (2.77 ± 0.22 vs. 1.48 ± 0.12 and 1.19 ± 0.11). In addition, it was also reported that the percentage of aneuploid sperm correlates with the percentage of apoptotic sperm [12]. Therefore, the miscarriage rate seen following ART possibly reflects the fact that genomically compromised spermatozoa are sometimes used and lead to irreparable DNA damage in the embryo. In support, failed ART cycles rates were attributed, at least in part, to the inclusion of apoptotic, DNA damaged sperm as a result of absent *in vivo* sperm selection barriers [13,14]. Therefore, there is a need to improve routine sperm preparation protocols by including techniques that enable the selection of non-apoptotic, DNA intact spermatozoa. Such an approach represents the evolution of preparation techniques, which expands beyond routine sperm parameters such as motility and morphology to include the sperm molecular characteristics.

A new electrophoretic system has been recently described for the rapid isolation of populations of spermatozoa exhibiting high levels of DNA integrity [18]. The first human pregnancy has been reported in a couple suffering from long-term infertility associated with extensive sperm DNA damage following the application of this electrophoretic system [19]. Despite the undisputed benefits of this technique, the complexity of the separation apparatus used may be a limiting factor against its widespread use in Andrology Laboratories, specifically those with limited resources.

In our study, we have used one of the early features of apoptosis, which is the externalization of phosphatidylserine (PS) residues normally present on the inner leaflet of the sperm plasma

membrane to design a new sperm preparation strategy. Colloidal paramagnetic microbeads (~50 nm in diameter) conjugated with annexin V binding to PS was used to separate dead and apoptotic spermatozoa by Magnetic Activated Cell Sorting (MACS). Cells with externalized PS would bind to these microbeads, while non-apoptotic cells with intact membranes would not bind and subsequently could be used during ART. Annexin V MACS is a simple, inexpensive, non-technically challenging procedure that takes less than 30 minutes to perform and therefore could be easily introduced in the clinical practice.

Standardization of MACS as a Sperm Preparation Technique

In our present research, we have standardized MACS as a preparation technique that yields motile, viable, morphologically normal spermatozoa, which display higher cryosurvival rates as well as oocyte penetration capacity [20-25]. The protocol combines 2 different readily available, inexpensive techniques aiming at improving the results of ART. First, double density gradient centrifugation is used to remove seminal plasma and other extraneous constituents of the seminal fluid leaving only mature viable spermatozoa [6]. Second, spermatozoa displaying the apoptosis surface marker (externalized PS) are immuno-labeled and removed using MACS. Thus, we hypothesized that combining the advantages of both density gradient centrifugation and advanced molecular sperm preparation techniques would yield spermatozoa with superior quality and function.

The labeling strategy employed in our protocol is based on considering PS externalization in human sperm as an apoptotic manifestation. However, alterations in the sperm phospholipid bilayer were also reported to be a part of the normal sperm physiology. During sperm capacitation, protein kinases have been identified as mediators for signaling pathways that lead to externalization of PS and phosphatidylethanolamine [26-28]. Nevertheless, annexin V binding was not seen in human spermatozoa undergoing capacitation, which indicates that the previously suggested association between PS externalization and capacitation may have been due to species-specific differences in sperm capacitation as well as different methodologies used for capacitation induction [29]. In support of the association of PS externalization and annexin V binding with apoptosis, sperm motility, morphology and viability were significantly reduced in the annexin-positive fraction [20-22,25]. The strong correlation between PS externalization, caspase-3 activation and mitochondrial changes typical of apoptosis provides further support for this observation [24,30-32].

Potential MACS Application

In line with the above-mentioned findings, we found that the application of density gradient centrifugation in combination with MACS yields an annexin V-negative sperm population that

demonstrates a significantly lower incidence of DNA fragmentation [23]. This finding is supported by the fact that DNA damage is indeed a late apoptotic event. However, it is of importance to note that density gradient centrifugation by itself was reported to select spermatozoa with 50% lesser DNA damage [33]. Nevertheless in our study, the combined protocol using MACS was compared to controls prepared using only density gradient centrifugation. The results showed a further decrease in percentage of DNA damaged sperm. Spermatozoa prepared by MACS after density gradient centrifugation had 30% lesser rate of DNA damage compared to those prepared by only density gradient centrifugation, which clearly indicates the beneficial effect of adding annexin V MACS to isolate non-apoptotic sperm.

In our study, we found that annexin V MACS if performed prior to cryopreservation yields a sperm population with higher motility and cryosurvival rates following thawing. The higher cryosurvival in MACS separated sperm may be a direct result of excluding those that display manifestations of apoptosis. In support, non-apoptotic sperm separated by MACS show significantly higher levels of intact mitochondria and lower caspase activation following cryopreservation–thawing compared to sperm that were not separated [24,30,32]. A recent report documented that spermatozoa with abnormal morphology are more susceptible to DNA damage during cryopreservation [34]. Since MACS separates sperm with better morphological features and DNA integrity, tolerance to cryodamage should be expected.

Our results showing that MACS separation enhances the sperm-oocyte penetration potential indicate its potential benefit during ART procedures such as intrauterine insemination (IUI) or IVF. On the other hand, the value of using MACS prior to ICSI requires further investigations. Similarly, other published reports document the association of apoptosis and DNA damage with the fertilization rates following IUI and IVF but not with ICSI [35-37]. The discrepancy may be due to the technical nature of ICSI that plays an important role in minimizing the impact of sperm preparation methods.

During ICSI, all biological and genetic selection is bypassed as the oocyte membrane is mechanically pierced. Although ICSI has been linked in a number of reports to an increased incidence of perinatal hazards in the offspring conceived with this technique no significant differences were found between children born after ICSI and IVF in this regards [38]. During ICSI, one motile and as far as possible, a morphologically normal sperm is selected for injection. Therefore, the benefits of enriching a cellular population with larger percentage of DNA intact sperm will be diminished. On the other hand during IUI and IVF, a significantly larger number of spermatozoa are used, whether placed inside the uterine cavity or in direct contact with the oocyte. A sperm population with higher percentage DNA integrity will have juxtapositionally higher chances of a DNA intact sperm penetrating the oocyte.

Advantages and Limitations of MACS

The transfer of our new approach to clinical Andrology laboratories may lead to significant improvement in selected ART success rates and subsequently reduce the physical, psychological as well as financial burdens for patients. Authors have reported that the percentage of blastocyst development was 50% higher in men with lesser than 20% damaged sperm in their ejaculates [13]. Similarly, another study has reported that men with >30% DNA damaged sperm have lower blastocyst development rates and ongoing pregnancy rates [39]. Therefore, the elimination of DNA damaged sperm using sperm separation techniques could result in significantly higher blastocyst development rates and limited miscarriages.

Despite the obvious added benefits of MACS, the technique may not be suitable for the preparation of sperm samples with limited counts. The use of density gradient centrifugation in addition to MACS will result in some degree of sperm loss that may not be acceptable in samples with severe oligozoospermia.

FUTURE DIRECTIONS

The application of MACS in combination with density gradient centrifugation allows for sperm selection based on sperm apoptosis in addition to routine parameters such as motility and morphology. Data included in this thesis serve as a basis for future projects that would evaluate the safety of magnetic cell sorting in the context of human reproduction. Although annexin V sperm, which is not bound to magnetic beads, constitute the actual population that will be used during ART, it is critical to evaluate if any bead-bound sperm will be concomitantly included as well. In addition, previous research indicate that routine sperm preparation techniques such as density gradient centrifugation and swim up may indirectly influence the sex of the offspring [40,41]. Therefore, the additional use of MACS should be also assessed to identify if the ratio of X- to Y-bearing sperm will be inadvertently impacted.

The potential clinical application of MACS as a sperm preparation technique warrants the validation of the current findings in large cohorts of infertile men undergoing IUI and IVF. Although MACS could also be used to isolate spermatozoa with compromised genomic integrity, the value of integrating MACS in sperm preparation prior to ICSI requires further investigation in a clinical ART program. To date, we have relied on animal models to evaluate MACS in sperm preparation. Although solid conclusions could be reached using this approach, the limitations are obvious. It has been shown that the integrity of the paternal genome impacts only later stages of embryo development and not early stages of fertilization [42]. Therefore, the expected arrest of human male pronucleus formation following hamster oocyte ICSI, does not offer definitive answer whether it is beneficial to select DNA intact sperm using MACS before

ICSI. The added benefits of MACS may also differ between patients according to the etiology of infertility and the type of sperm defects present.

The continuing development of magnetic cell separation techniques offers opportunities for improved sperm cell sorting. A particularly interesting non-bead approach relies on cell magnetophoretic mobility. Magnetophoretic mobility is analogous to the electrophoretic and sedimentation mobilities encountered in electrical and sedimentation separations, respectively [43]. The magnitude of the mobility of a magnetically tagged cell distinguishes these cells from unlabeled ones and allows an effective cell sorting in continuously flowing cell suspensions [44,45]. The advantage of this method lies in being non-bead based. Therefore, there should be no concerns regarding magnetic beads being accidentally introduced in the oocyte. This approach may lead to further improvement in the quality of the sorted sperm cells by selection of spermatozoa with higher fertilization potential. This could be achieved by selecting spermatozoa that display motion kinetics which characterize hyperactivated sperm undergoing capacitation.

SUMMARY

In the context of male infertility, seminal reactive oxygen species (ROS), sperm DNA damage and apoptosis are inter-linked to constitute a unified pathogenic molecular mechanism. The impact of these factors on male infertility, their clinical significance and management options have always been a subject of controversy. The specific aims of our research 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 magnetic activated cell sorting (MACS) application; and 4) identify the protocol limitations and which ART procedures would benefit the most from its application.

In **Chapter 2**, we explored the role played by β-nicotinamide adenine dinucleotide phosphate (NADPH), which is present in sperm cytoplasmic droplets, in the pathogenesis of DNA damage. Semen samples from 28 men undergoing infertility evaluation were separated into mature and immature sperm fractions using double density gradient centrifugation. Both fractions were incubated with 5 mM NADPH and evaluated for ROS and DNA damage levels using chemiluminescence and deoxynucleotidyl transferase-mediated fluorescein-dUTP nick end labeling (TUNEL) assay, respectively. Higher levels of ROS were seen in immature spermatozoa incubated with NADPH. Higher ROS levels were also noted in spermatozoa rich in cytoplasmic residues regardless of NADPH exposure. The increase in ROS showed significant positive correlation with sperm DNA damage. These results collectively documented the role of NAPDH in ROS-mediated sperm DNA damage.

After establishing the implication of sperm cytoplasmic droplets in ROS production and its correlation with DNA damage, the subsequent experiment presented in **Chapter 3** described our efforts to evaluate the impact of other abnormal sperm morphological attributes. We used the Sperm Deformity Index (SDI) as a novel quantitative expression of sperm morphology, which is calculated by dividing the total number of deformities observed by the number of sperm evaluated. Semen samples from men undergoing infertility screening (n = 7) were compared to healthy donors (n = 6) in terms of SDI, ROS levels and DNA damage.

SDI showed a significant positive correlation with the percentage increase in sperm DNA damage in samples treated with NADPH and controls. Such increase appeared to be more evident in patients with semen samples containing high incidence of morphologically abnormal spermatozoa as evaluated by the SDI. Therefore, we concluded that SDI is an index for sperm

morphology that could be used to identify potential infertile males with abnormal prevalence of OS-induced DNA damage.

In **Chapter 4,** we conducted an experiment to evaluate the advantage of integrating MACS with routine sperm preparation technique and to identify how the protocol could be optimized to maximize its benefit. Semen specimens collected from 15 healthy donors were prepared by either density gradient centrifugation or by one-step sperm wash technique separately and in combination with annexin V MACS. The sperm yield was evaluated following each approach as regards motility, viability, and morphology as well as markers of apoptosis: active caspase-3, mitochondrial membrane potential and externalization of phosphatidylserine (PS). Our results showed that the combination of double density gradient centrifugation and annexin V MACS was superior to all other preparation methods combined or separate in providing spermatozoa with highest quality in terms of motility, viability and apoptosis markers (caspase-3, mitochondrial membrane potential and externalized PS). On the other hand, no differences in sperm morphology were observed between any of the evaluated sperm fractions. These results indicated that the combination of density gradient centrifugation and annexin V MACS is a better approach for sperm preparation.

The equivocal results regarding sperm morphology following annexin V MACS preparation was thoroughly investigated in a follow up experiment as described in **Chapter 5**. Sperm morphology using the strict Tygerberg criteria and the SDI scores were assessed following annexin V MACS conducted on semen samples from 50 healthy donors. Non-apoptotic spermatozoa presented with significantly higher proportions of sperm with normal morphology and significantly lower SDI scores as well as lower percentages of sperm with acrosomal defects, midpiece defects, cytoplasmic droplet and tail defects. A significant correlation was found between the sperm morphology attributes studied and the expressed apoptosis markers: caspase-3 activation and mitochondrial membrane potential. Therefore, it appears that annexin V MACS can be also used to prepare a sperm population with superior morphological profile.

Thereafter, we have undertaken in **Chapter 6** the evaluation of sperm recovery following the use of MACS combined with density gradient centrifugation as a sperm preparation technique. Our aim was to ensure that the combined approach will not result in significant sperm loss, thus rendering the samples unsuitable in a clinical setting. Semen samples collected from healthy donors (n = 19) were divided into 2 portions: the first was prepared by density gradient centrifugation and MACS, while the second was prepared by only density gradient centrifugation. Compared to the sperm count after density gradient centrifugation, the average

number of cells lost during the combined approach was limited to only 15%. The number of total motile sperm showed a significantly positive correlation with the concentrations of spermatozoa and volumes of beads used. These results indicated that our suggested protocol results in high sperm yield and minimal cell loss.

In **Chapter 7**, we investigated if non-apoptotic spermatozoa prepared by a combination of density gradient centrifugation and annexin V MACS display increased tolerance to cryopreservation-thawing. Semen samples were collected from 10 healthy donors and were subjected to cryopreservation-thawing following preparation with density gradient centrifugation and annexin V MACS. Control aliquots consisted of sperm prepared by only density gradient centrifugation and sperm prepared with annexin V MACS conducted after thawing. Non-apoptotic sperm separated by MACS prior to cryopreservation had significantly higher motility following cryopreservation–thawing than sperm not separated by MACS. Similarly, non-apoptotic spermatozoa had higher cryosurvival rate than sperm cryopreserved without prior separation by MACS. Therefore, data indicate that separating a distinctive population of non-apoptotic spermatozoa with intact membranes could optimize cryopreservation–thawing outcome.

Animal models were used as described in **Chapter 8** to evaluate the fertilization potential as the ultimate function of non apoptotic sperm separated by annexin V MACS. The zona free hamster oocyte sperm penetration assay (SPA) was used to assess the sperm-oocyte penetration capacity in samples from 16 healthy donors. Non-apoptotic sperm exhibited significantly higher oocyte penetration potential compared to annexin-positive sperm and controls not separated by MACS. In support of the correlation between sperm apoptosis and fertilization, the sperm-oocyte penetrating capacity showed a significant positive correlation with motility, mitochondrial membrane integrity, as well as a significant negative correlation with the percentage of active caspase-3 and binding to externalized PS. Therefore, we were able to conclude that MACS may be of potential benefit during ART procedures such as intrauterine insemination (IUI) or *in vitro* fertilization (IVF).

In **Chapter 8**, we have also evaluated the fertilization potential of spermatozoa prepared by MACS following intracytoplasmic sperm injection (ICSI) using an animal model. Non-apoptotic sperm from 19 healthy donors had sperm chromatin decondensation (SCD) values following hamster oocyte-ICSI that were comparable to controls not prepared by MACS. On the other hand, results of the TUNEL assay showed that, annexin V-negative sperm following MACS had the lowest incidence of DNA fragmentation. We found a weak correlation between apoptosis

markers (caspase-3 activation, mitochondrial membrane potential and DNA damage) with the results of SCD, which may indicate a weak impact of apoptosis on early fertilization stages. Therefore, it appears that although MACS separates spermatozoa with higher DNA integrity, this may not be translated into higher fertilization rates following ICSI.

Finally in **Chapter 9**, we thoroughly investigated the correlation of apoptosis markers and the SPA in order to substantiate our previous findings. Semen samples from 76 healthy donors were prepared by density gradient centrifugation and annexin V MACS. Thereafter, apoptosis markers were evaluated and the SPA was conducted. Samples were grouped according to SPA results into normal (>20%) and abnormal (<20%). Semen samples with abnormal SPA (n = 22) showed significantly higher amounts of spermatozoa with externalized PS, disrupted mitochondrial membrane potential and activated caspase-3 compared to samples with normal SPA (n = 54). All three apoptosis markers displayed a significant negative correlation with the percentage of penetrated oocytes as well as with the numbers of sperm per penetrated oocyte. These results provided further support for the negative impact of apoptosis on *in vivo* and *in vitro* fertilization. Consistently, the beneficial effect of annexin V MACS is shown.

Based on the results of our findings, we conclude that: 1) a rise in sperm DNA damage should be expected in conjunction with immature spermatozoa rich in cytoplasmic droplets due to NADPH-mediated increase in ROS; 2) MACS could be considered as a flexible, inexpensive, fast and simple cell sorting system for the separation of large numbers of non-apoptotic spermatozoa; 3) the combination of MACS with double density gradient centrifugation yields a sperm population characterized by higher motility, viability and morphology. Moreover, spermatozoa prepared by MACS display reduced DNA fragmentation. In this context, MACS may be considered a unique molecular preparation technique that complements conventional sperm preparation protocols; and 4) the separation of a non-apoptotic fraction results in a sperm population that displays higher tolerance to cryopreservation. Most importantly, it results in a sperm population with higher fertilization potential as documented by higher oocyte penetration capacity. Thus, the inclusion of an additional step of sperm preparation involving the isolation of apoptotic sperm using MACS may significantly enhance the outcome of some ART procedures such as IUI or IVF where the sperm DNA integrity is expected to play a significant role in determining success rates. On the other hand, the benefits of MACS usage prior to ICSI were not evident in our current research. The limitation of our animal model together with the limited impact of sperm DNA on early fertilization stages could have led to such lack of evidence.

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