

## Magnetic activated cell sorting (MACS): Utility in assisted reproduction\*

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Assisted reproductive techniques (ART) have now been extensively incorporated in the management of infertile couples. But even after rapid methodological and technological advances the success rates of these procedures have been below expectations. This has led to development of many sperm preparation protocols to obtain an ideal semen sample for artificial reproduction. Sperm apoptosis has been heavily linked to failures in reproductive techniques. One of the earliest changes shown by apoptotic spermatozoa is externalization of phosphatidyl serine. Magnetic activated cell sorting (MACS) is a novel sperm preparation technique that separates apoptotic and non-apoptotic spermatozoa based on the expression of phosphatidylserine. This has led to the incorporation of MACS as a sperm preparation technique. The review highlights the principle and mechanism of this novel technique and enumerates its advantages as a sperm preparation technique. Its utility in ART as an efficient tool for sperm recovery and its application in cryopreservation of semen samples is also explained.

Assisted reproduction techniques (ART) have become the treatment of choice in many cases of male and female factor infertility. However the success rate of these techniques still remains suboptimal. One of the factors determining successful assisted reproduction is the quality of sperm<sup>1</sup>. The increase in ART applications along with their suboptimal success rates has mandated the development of an ideal sperm preparation technique that could compliment these applications. The current pregnancy and live birth rates achieved with ART techniques need to be improved in order to lessen the emotional and socio-economic burden of a failed ART procedure<sup>2</sup>.

Conventional semen analysis includes evaluation of sperm concentration, motility, morphology and viability. However it does not provide any information about programmed cell death (apoptosis) which may play a role in low fertilization and implantation rates seen with assisted reproduction techniques. Numerous sperm preparation techniques have been used to maximize the outcome of ART techniques. These preparation techniques utilize motile spermatozoa that are capable of fertilizing the female oocyte<sup>3</sup>. Current sperm preparation techniques

are based on sedimentation or migration approach and separate spermatozoa on the basis of their motility and density. Sperm preparation by double density gradient centrifugation has shown great potential in sperm preparation for assisted reproduction<sup>4</sup>. In addition, the one-step washing technique is also considered a good alternative for processing cryopreserved samples<sup>5</sup>. This group showed that washing cryopreserved-thawed samples by one-step addition of diluting medium does not appear to compromise the samples compared to with stepwise addition of medium. However these techniques overlook molecular events such as apoptosis which play an important role in ART failures. Recently, Ainsworth *et al*<sup>6</sup> described a new sperm preparation technique based on electrophoretic separation of spermatozoa exhibiting high levels of DNA integrity. This technique shows promise and the first human pregnancy has been reported in a couple with long term infertility associated with extensive sperm DNA damage. Although promising, the complex apparatus and procedure involved in this technique is a limiting factor in its widespread usage.

Apoptosis or programmed cell death is a physiological process of critical importance in the human system. In the male reproductive system it controls the overproduction of spermatozoa, thus assisting the nursing and supportive capacity of Sertoli cells. Apoptosis proceeds in two main phases. First is a commitment phase which is followed by an

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execution phase. This is characterized by a series of stereotypic changes that include cell shrinkage, plasma membrane disruption, phosphatidylserine externalization and chromatin condensation and fragmentation<sup>7-9</sup>. However dysregulated apoptosis has been implicated in the pathogenesis of male infertility. It may also be responsible for the failure of ART despite the use of morphologically normal and motile spermatozoa. The failure of elimination of apoptotic spermatozoa during spermatogenesis (abortive apoptosis) may be the reason for their presence in semen<sup>10,11</sup>. Ejaculated human spermatozoa have been shown to display characteristics typical of apoptosis such as caspase activation, decreased mitochondrial membrane potential (MMP) and plasma membrane translocation of phosphatidylserine (PS)<sup>12-15</sup>. This may be one of the reasons for the current low pregnancy and birth rates with assisted reproductive techniques.

### Evaluation of apoptotic markers

#### Mitochondrial membrane potential

A number of critical events in apoptosis originate in mitochondria<sup>16-19</sup>. Mitochondrial involvement in apoptosis revolves around various mechanisms including activation of caspases, disruption of electron transport chain leading to changes in oxidative phosphorylation and ATP synthesis<sup>20</sup>, loss of mitochondrial membrane potential and involvement of pro and anti-apoptotic family of proteins. A lipophilic cationic dye (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine chloride) is used to detect intact transmembrane potential of mitochondria in spermatozoa using the Mitosensor kit. Sperm with intact mitochondria excite red fluorescence due to the formation of dye aggregates while the sperm with disrupted potential fluoresce green due to dye monomers.

#### Caspase 3

Caspases are cysteine specific aspartate dependent proteases that are the key effectors of apoptosis, although some are involved in activation of cytokines, rather than cell death. Ekert *et al*<sup>21</sup>, used a fluorescent labeled inhibitor of caspase (FLICA) which is cell permeable, non-cytotoxic and covalently binds to active caspase-3 and showed a significant negative correlation between percentage of spermatozoa with activated caspase-3 and sperm motility and viability ( $P < 0.0001$ ).

#### Externalization of phosphatidyl serine

During early phases of disturbed membrane function, membrane phospholipid asymmetry occurs prior to disturbed integrity of the plasma membrane<sup>22</sup>. In normal, viable spermatozoa with intact plasma membrane, phosphatidyl serine (PS), a negatively charged molecule, is located on the inner leaflet of the plasma membrane<sup>23</sup>. Translocation of PS occurs from the inner leaflet to the outer leaflet of plasma membrane thus exposing PS on the external surface<sup>22</sup>. This externalization of PS is one of the earliest features of apoptotic cells<sup>9</sup>. This has been studied using a monoclonal mouse anti-human phosphatidylserine antibody<sup>24</sup>.

#### DNA fragmentation

Sperm DNA integrity is of critical importance for transmission of genetic material to offspring. DNA fragmentation is one of the hallmarks of apoptosis. It has been proposed that DNA fragmentation may be due to flaws in endogenous endonuclease activity resulting in DNA nicks<sup>25-27</sup>. Sperm and DNA strand breaks have been evaluated using several techniques like terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick end labeling (TUNEL), Sperm chromatin structure assay (SCSA) acridine orange staining (AOT) amongst others<sup>28</sup>. TUNEL can simultaneously detect single and double strand breaks, however it cannot be employed in routine clinical use due to a lack of useful thresholds and the standardized forms of this assay have yet not been validated. The SCSA is a flow cytometric study technique which reveals the DNA fragmentation index (% DFI). This is a simple and less time consuming method for the analysis of DNA damage in human spermatozoa. The chances of live birth decreases when DNA fragmentation index (DFI)  $\geq 30\%$  (refs. 29-31).

#### Magnetic activated cell sorting

Magnetic activated cell sorting or MACS is a sperm preparation technique for ART that has been used recently. MACS has been shown to yield motile, viable, morphologically normal spermatozoa that displays significant cryopreservation tolerance and higher fertilization potential<sup>32-36</sup>. It allows separation of apoptotic spermatozoa which may be one of the causes of ART failure even in patients with otherwise normal sperm parameters.

*Principle*—MACS technology uses annexin V-conjugated superparamagnetic microbeads (50 nm) to separate nonapoptotic spermatozoa from those with

deteriorated plasma membranes and externalization of PS. These microbeads are composed of biodegradable matrix, and it is therefore not necessary to remove them from spermatozoa after the separation process. MACS microbeads do not alter structure, function, or activity status of labeled spermatozoa and are not known to interfere with subsequent experiments.

As mentioned earlier, externalization of PS is an early process in apoptosis. Depending on  $Ca^{2+}$ , PS has a high affinity for annexin V, which is 35-36 kDa phospholipid binding protein showing highly selective binding to PS. Annexin-V does not have the ability to pass the intact sperm membrane, so the annexin-V binding to spermatozoa characterizes disturbed integrity of the sperm membrane<sup>12</sup>. Thus annexin enables the identification of cells with altered membrane integrity at an earlier stage than staining with supravital stains<sup>22</sup>. Based on annexin binding and subsequent magnetic separation 2 fractions are obtained: annexin-negative (unlabeled- intact membrane; non-apoptotic) and annexin positive (labeled- altered membrane; apoptotic).

**MACS technique**—MACS involves the incubation of spermatozoa with Annexin-V-conjugated microbeads for 15 min at room temperature; 100  $\mu$ L of microbeads are used for every 10 million separated cells. The sperm-microbead suspension is loaded into a separation column containing iron spheres which is fitted in a magnet (MiniMACS). A magnetic field of 0.5 T (Tesla) can be applied between the poles of the magnet and up to 1.5T within the iron spheres of the column (Fig. 1). The cells labeled with microbeads are retained on the MACS column while the unlabeled cells pass through. Thus, with MACS technology both labeled and unlabeled cell fractions can be easily isolated with high purity. The labeled cells are the apoptotic cells that constitute the annexin-V positive fraction while the unlabeled cells are the non-apoptotic spermatozoa which comprise the annexin-V negative fraction. The column is removed from the separator and the retained cells are eluted using the annexin-V binding buffer as the enriched, positively selected cell fraction. The entire procedure of selection and separation takes less than 30 min, and the cells can immediately be used for further experiments.

**Advantage of MACS**—MACS acts on the sperm molecular level as opposed to routine sperm preparation techniques that rely only on sperm density and motility. This way MACS is a unique preparation

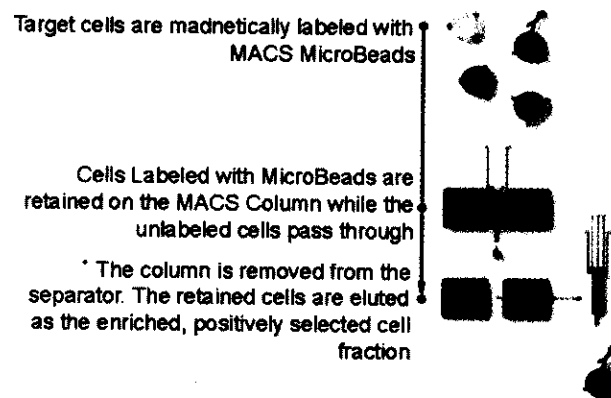


Fig. 1—The principle of MACS method depends on the labeling of the cell-surface marker with a specific antibody combined with the use of MACS microbeads which are used to magnetically label the target cell population. By using a MACS column containing iron balls with a coated cell friendly matrix placed in a permanent magnet, the target cells labeled with a minimum of microbeads will be retained. On rinsing the column with a buffer, all the unlabelled (annexin-negative) cells will be washed out and on removing the column from the magnet; the labeled (annexin-positive) fraction can be obtained.

method that complements the conventional sperm preparation protocols. MACS separation has showed an increase in percentage of motile sperm<sup>24</sup>. It has been used to optimize the cryopreservation-thawing outcome and enhance cryosurvival rates following cryopreservation. It has enhanced the percentage of spermatozoa with intact mitochondria and mitochondrial cryosurvival rates following cryopreservation. By separating the apoptotic spermatozoa it has also improved the success rates of assisted reproduction techniques. MACS provide optimal purity and recovery with reliable and consistent results. Technically, it is a convenient procedure with an easy to use system applicable in every lab. It provides rapid results due to short incubation time and bead detachment after the procedure is not necessary.

### MACS and cryopreservation

Cryopreservation of semen specimens has become an integral part of assisted reproduction protocols, especially in patients with malignancies prior to undergoing chemotherapy or radiotherapy and also people undergoing voluntary sterilization.

Cryopreservation has gained significance with the use of intracytoplasmic sperm injection (ICSI) in which non-motile but viable can still be successfully used. However cryopreservation shows deleterious effects on the spermatozoa membrane. This leads to a

decrease in sperm viability, motility and cryosurvival rates<sup>37</sup>. Apoptosis has been postulated to contribute to this decrease in sperm quality after cryopreservation<sup>38</sup>. These findings indicate a need for a novel sperm preparation technique that can separate apoptotic spermatozoa before cryopreservation and thus improve the post thaw motility and cryosurvival rates. Said *et al.*<sup>24</sup> have demonstrated an enhancement in sperm motility and cryosurvival rates following cryopreservation using MACS-annexin-V conjugated microbeads. Furthermore, it was demonstrated that the annexin negative (ANMB negative) sperm have significantly higher motility than the raw samples whereas the ANMB positive sperm show significantly lower motility (Table 1)<sup>24</sup>. In addition, highest cryosurvival rates were seen in the ANMB- negative spermatozoa that were separated prior to freezing compared to the fraction that was cryopreserved without MACS (Table 1).

#### Utility of MACS in sperm preparation protocols

The selection of vital, viable, non apoptotic spermatozoa is the backbone of any assisted reproductive technique. It is essential to obtain optimal conception rates and reduce failures in ART. MACS is one technique that can remove apoptotic spermatozoa but it is limited in its ability to remove other components of semen like debris, leucocytes, seminal plasma etc. This points towards the need to combine MACS with other sperm preparation techniques so that they complement each other and provide spermatozoa with the highest quality in terms of motility, viability and apoptotic markers (caspase 3, MMP)<sup>24</sup>. Furthermore it has been shown that the combination of density gradient centrifugation (DGC) and annexin-V magnetic cell sorting is superior to all other sperm preparation techniques in terms of providing motile, viable and non apoptotic

spermatozoa. Also it has been shown that the annexin-negative sperm fraction prepared by MACS following density gradient had significantly higher motility values than the spermatozoa prepared by density gradient only ( $P = 0.02$ ) and the annexin-negative spermatozoa prepared by MACS following one-step wash ( $P < 0.0001$ ). Sperm viability was also shown to be significantly higher in annexin negative sperm fraction prepared by MACS following density gradient compared to spermatozoa prepared with density gradient only ( $P = 0.01$ ). On the other hand, sperm viability was comparable in fractions prepared by density only and annexin-negative spermatozoa prepared by MACS following one-step wash (Table 2). The ANMB-positive fractions obtained following DGC+MACS or following one-step wash had the lowest motility and viability values as compared with ANMB-negative spermatozoa and density gradient preparations (Table 2).

Sperm deformity index (SDI) is based on the total number of defects/total sperm counted has been shown to be a more powerful predictor of male fertility and of *in vitro* fertilization outcome compared with the assessment of the proportion of sperm with normal morphology<sup>39</sup>. In terms of effect of MACS on morphology, Aziz *et al.*<sup>32</sup> have shown that SDI and percentage of sperm with acrosomal defects, midpiece defects, tail defects and cytoplasmic droplets were significantly lower on combining MACS with double density gradient techniques. Also a significant correlation between sperm morphology attributes studied and the expression of apoptotic markers such as caspase-3 activation and MMP that reflects the mitochondrial membrane integrity has been shown. The strongest association was seen between SDI scores and caspase-3 activation and MMP.

#### Effect of MACS on sperm recovery

Even after development of various sperm preparation techniques the recovery rate of functional spermatozoa remain unsatisfactory<sup>3</sup>. Methodologies and newer sperm preparation techniques are required to decrease the rate of congenital abnormalities<sup>40</sup>. Said *et al.*<sup>36</sup> have modified the protocol that combines annexin V-MACS with the density gradient centrifugation (DGC). Compared with the sperm count after only DGC, though the average number of cells decreased by 15.2±19.1% following MACS but the sperm recovery rates following DGC+MACS were comparable to those after only DGC (73.8±12.1% vs. 66.7±19.1%). The higher sperm

Table 1—Motility and cryosurvival rates before and after separation by magnetic activated cell sorting (MACS)<sup>24</sup>  
[Values are mean ± SD]

Parameter	Motility (%)	Cryosurvival rate (%)
Unprocessed (Raw)	64.5 ± 6.43	30.29 ± 16.06
ANMB-negative	76 ± 15.06 <sup>a</sup>	76.6 ± 59.75
ANMB-positive	41 ± 29.61 <sup>a</sup>	12.7 ± 31.19

<sup>a</sup> $P < 0.05$  was considered statistically significant compared with unprocessed semen

ANMB = Fraction separated by annexin-V magnetic beads

Table 2—Sperm parameters assessed by different sperm preparation techniques<sup>35</sup>  
[Values are mean ± SE]

Preparation technique	Sperm fraction	Motility (%)	Viability (%)	Morphology (%)	
				WHO	Kruger's criteria
DGC	Mature sperm pellet	74.37 ± 12.32 <sup>b,c</sup>	75.53 ± 10.77 <sup>b</sup>	21.33 ± 10.02	8.14 ± 3.23
DGC + MACS	Annexin-negative	82.52 ± 9.18 <sup>a,c</sup>	82.91 ± 6.73 <sup>a,c</sup>	21.36 ± 8.91	8.38 ± 3.48
DGC + MACS	Annexin-positive	10.65 ± 9.65 <sup>a,b,c</sup>	30.22 ± 9.67 <sup>a,b,c</sup>	20.71 ± 7.14	6.79 ± 2.81
HTF wash + MACS	Annexin-negative	49.94 ± 19.59 <sup>a,b</sup>	71.58 ± 11.78 <sup>b</sup>	21.92 ± 8.73	7.75 ± 3.11
HTF wash + MACS	Annexin-positive	7.15 ± 9.02 <sup>a,b,c</sup>	22.67 ± 11.64 <sup>a,b,c</sup>	18 ± 6.93	5.42 ± 2.50

DGC = Density gradient centrifugation; MACS = Magnetic activated cell sorting; HTF = Human tubal fluid.  
<sup>a</sup>P < 0.05 was considered significant using paired t-test when compared with DGC; <sup>b</sup> with DGC+MACS; and <sup>c</sup> with HTF wash+MACS

recovery rate obtained in this protocol advocates its use as a sperm preparation technique prior to assisted reproduction.

#### MACS and apoptotic markers

The annexin-V negative fraction showed the lowest percentage of caspases-3 activation as well as highest mitochondrial membrane integrity. The converse of this is also true, that annexin-V positive fraction demonstrated the highest expression levels of these apoptotic markers. MACS coupled with annexin V microbeads has been repeatedly shown to efficiently isolate apoptotic spermatozoa<sup>33</sup>. Paasch *et al.*<sup>14</sup> demonstrated that MACS separation results in depletion of spermatozoa with activated caspases.

#### MACS and enhanced sperm fertilization potential in ART

The increase in the number of ART techniques and the associated low success rates emphasizes on the need to develop an ideal sperm preparation technique. DGC technique is now a standard sperm preparation technique as it provides spermatozoa with lesser DNA damage and higher MMP, but the ability to separate apoptotic spermatozoa is lacking<sup>41,42</sup>. On the other hand it works at a molecular level to provide spermatozoa devoid of any dead and apoptotic forms. The two techniques complement each other and provide the highest quality spermatozoa for assisted reproductive procedures. It has been highlighted above that use of MACS resulted in higher quality sperm in terms of motility, viability and morphology. Despite this, the fertilization potential remains the ultimate sperm function. The zona free hamster oocyte penetration assay was used to assess the sperm-oocyte penetration capacity. Non-apoptotic sperm

exhibited significantly higher oocyte penetration potential compared to annexin-positive sperm and controls not separated by MACS. To support the correlation between sperm apoptosis and fertilization, the sperm-oocyte penetrating capacity showed a significant positive correlation with motility ( $r=0.07$ ), mitochondrial membrane integrity ( $r = 0.07$ ), as well as a significant negative correlation with the percentage of active caspases 3 ( $r = -0.6$ ) and binding to externalized PS ( $r=-0.5$ )<sup>36</sup>.

Using the sperm penetration assay Said *et al.*<sup>36</sup> showed that the percentage of oocytes penetrated by annexin-negative spermatozoa was significantly higher than annexin-positive group and the control group<sup>36</sup>. Also the sperm capacitation index was higher in annexin negative group as compared to annexin-positive and control groups (Table 3).

Said *et al.*<sup>36</sup> evaluated the fertilization potential of spermatozoa prepared by MACS following intracytoplasmic sperm injection (ICSI) using an animal model. Non-apoptotic sperm from 18 fertile donors were used and sperm chromatin decondensation (SCD) values following ICSI were comparable to controls that were not prepared by MACS. They showed a weak negative 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<sup>36</sup>.

#### Future trends

The application of MACS allows selection and separation of apoptotic spermatozoa in addition to routine parameters like motility and morphology. Evidence suggests that it is an efficient tool that can be applied to clinical setting in patients undergoing

Table 3—Impact of magnetic cell separation on sperm fertilization potential of various fractions using sperm penetration assay<sup>36</sup>  
[Values are mean  $\pm$  SD]

	SPA (n = 16)	
	Oocytes penetrated (%)	Sperm capacitation index
Annexin - Negative (Aliquot A)	44.5 $\pm$ 12.6	1.8 $\pm$ 0.3
Annexin - Positive (Aliquot B)	20.8 $\pm$ 5.3	1.3 $\pm$ 0.4
Control (Aliquot C)	33.8 $\pm$ 6.9	1.5 $\pm$ 0.6
<i>P</i> values <sup>a</sup>		
A vs. B	<0.001	<0.001
A vs. C	0.001	0.04
B vs. C	<0.001	0.01

SPA = Sperm penetration assay

<sup>a</sup> *P* < 0.05 was considered significant using one-way analysis of variance with repeated measures

assisted reproductive procedures and reduce the psychosocial and financial burden of a failed ART procedure. MACS could be implemented in cryopreservation-thawing process in order to optimize cryosurvival outcomes. MACS can also be considered during sperm preparation techniques prior to intrauterine insemination or *in vitro* fertilization. MACS could also be used to isolate spermatozoa with compromised genetic integrity. Nevertheless, the value of integrating MACS in sperm preparation prior to ICSI requires further investigation in a clinical ART program.

### Conclusions

Apoptosis has a detrimental effect on the male fertility. MACS has been developed as a flexible, easy and rapid cell separation technique that has the ability to separate spermatozoa based on molecular characteristics of the sperm. In addition it is a relatively low-cost technique that can have wide applicability in andrology laboratories. MACS advocates the use of this protocol as sperm preparation technique prior to assisted reproduction. The higher sperm recovery rate obtained following the combination of density gradient separation with MACS may better preserve sperm motility and improve cryosurvival rates following cryopreservation. The deleterious effects of apoptosis on sperm-oocyte penetration are significantly reduced following the separation of non-apoptotic spermatozoa using MACS. Also the contribution of MACS in enhanced efficiency of assisted

reproduction undermines its role in sperm preparation techniques. The improved results with the incorporation of MACS in assisted reproduction techniques may lead to reduced economic and emotional burden on patients under therapy.

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