CHAPTER 6

Evaluation of Sperm Recovery following Annexin V MACS Separation

Said TM, Agarwal A, Grunewald S, Rasch M, Glander H-J, Paasch U
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ABSTRACT

Magnetic-Activated Cell Sorting (MACS) using paramagnetic annexin V-conjugated microbeads eliminates spermatozoa with externalized phosphatidylserine, which is considered one of the features of apoptosis. Our objective was to evaluate the sperm recovery following the use of MACS as a sperm preparation technique. Mature spermatozoa were separated and divided into 2 fractions. The first was prepared by density gradient centrifugation (DGC) and MACS, while the second was prepared by only DGC. Following MACS, the percentage of cells collected in the annexin-negative fraction was significantly higher than the annexin-positive fraction and the sperm recovery rate was 73.8 ± 12.1%. In conclusion, the integration of MACS with DGC can be considered as an effective sperm preparation technique that does not lead to significant cell loss. Separating a distinctive population of non-apoptotic spermatozoa with intact membranes may optimize the outcome of assisted reproduction.

Key words: Annexin; recovery rate; Magnetic-Activated Cell Sorting; apoptosis; sperm preparation

INTRODUCTION

Assisted reproductive techniques (ART) have become the treatment of choice in many cases of male and female infertility. Successful fertilization requires a sperm plasma membrane with normal integrity and function (Flesch *et al*., 2000). A spermatozoal membrane with impaired integrity is known to occur more frequently in infertile men and contributes to infertility despite the presence of normal routine sperm parameters (Duru *et al*., 2001, Glander *et al*., 1999, Sugkraroek *et al*., 1991). This may be one of the reasons behind the current suboptimal clinical pregnancy and live birth rates following ART.

The plasma membrane is one of the key structures in sperm of infertile men displaying apoptotic features (Glander *et al*., 1999). Early phases of disturbed membrane functions are associated with asymmetry of the membrane phospholipids and changes in lipid composition (Schiller *et al*., 2000). The phospholipid phosphatidylserine (PS), which is normally present on the inner leaflet of the plasma membrane becomes externalized to the outer leaflet (Vermes *et al*., 1995). The externalization of PS is currently accepted as a membrane marker for early apoptosis (Martin *et al*., 1995). Annexin V is characterized by high affinity to PS and does not have the ability to pass the intact sperm membrane. Therefore, annexin V binding to spermatozoa characterizes disturbed integrity of the sperm membrane (Glander *et al*., 1999).

Colloidal super-paramagnetic microbeads (~50 nm in diameter) conjugated with annexin V have been shown to separate the dead and apoptotic spermatozoa by Magnetic-Activated Cell Sorting (MACS). Cells exposing PS bound to these microbeads (annexin-positive) are enriched to high extent within a column containing iron balls when placed in a very strong magnetic field. Cells with intact membranes will remain unlabelled (annexin-negative), and shall pass freely through the column (Miltenyi *et al*., 1990, von Schonfeldt *et al*., 1999).

The binding of paramagnetic annexin V microbeads (ANMB) during MACS is an effective method to eliminate spermatozoa at early apoptotic stages from fresh and cryopreserved samples (Grunewald *et al*., 2001). ANMB-negative spermatozoa have been characterized as non-apoptotic with the lowest amount of caspase activation, disruption of mitochondrial membrane potential and DNA fragmentation (Paasch *et al*., 2004, Said *et al*., 2005a). Furthermore, these cells display higher fertilization rates when used for animal model IVF and ICSI (Said *et al*., 2006). The combination of MACS with density gradient centrifugation (DGC) in a single sperm preparation protocol results in spermatozoa with superior quality (Said *et al*., 2005a). Nevertheless, cell loss and sperm recovery rates during and after the procedure remain to be evaluated. The objective of our study was to evaluate the sperm cell recovery rate after MACS separation in order to assess the future potential of this method for the preparation of compromised samples.

MATERIALS AND METHODS

Sample preparation

Following approval of the Institution Review Board, semen samples were collected from 19 healthy donors with semen parameters exceeding World Health Organization reference ranges for the normal fertile population (1999). The liquefied semen samples were prepared by double density gradient centrifugation (PureCeption®, SAGE BioPHARMA, Bedminster, NJ). In brief, samples were loaded onto a 40% and 80% discontinuous gradient and centrifuged at 1600 rpm for 20 minutes at room temperature. The resulting 80% pellet representing the mature fraction was washed by centrifugation for additional 7 minutes and re-suspended in human tubal fluid media (HTF, Irvine Scientific, Santa Ana, CA). The sperm cell suspension was divided into 2 aliquots: the first was further separated into annexin-negative (non-apoptotic) and annexin-positive (apoptotic) fractions using MACS, while the second was left unseparated as a control. Sperm recovery rates were calculated following density gradient centrifugation compared to raw semen samples and following MACS compared to post-density gradient centrifugation samples using the following formula:

$$\frac{\text{Number of motile spermatozoa after the separation}}{\text{Number of motile spermatozoa before the separation.}} \times 100$$

Isolation of spermatozoa with deteriorated membranes by MACS

The sperm suspensions were divided into 2 sperm fractions by passage through a magnetic field (OctoMACS; Miltenyi Biotec, Bergisch Gladbach, Germany) based on the binding of paramagnetic annexin V microbeads to PS present on the surface of spermatozoa (Paasch *et al*., 2003a). Briefly, the washed spermatozoa were incubated with 100 µL ANMB at room temperature for 15 minutes, and placed on top of the separation column containing iron balls (Figure 1). The ANMB–labeled apoptotic spermatozoa (ANMB-positive) were retained in the separation column, which was placed in a magnet, whereas spermatozoa with intact membranes passed through (ANMB-negative). The power of the magnetic field was measured as 0.5 Tesla between the poles of the magnet and up to 1.5 Tesla within the iron globes of the column. After removing the column from the magnetic field, the retained fraction was eluted using an annexin-binding buffer (Margolis *et al*., 1983).

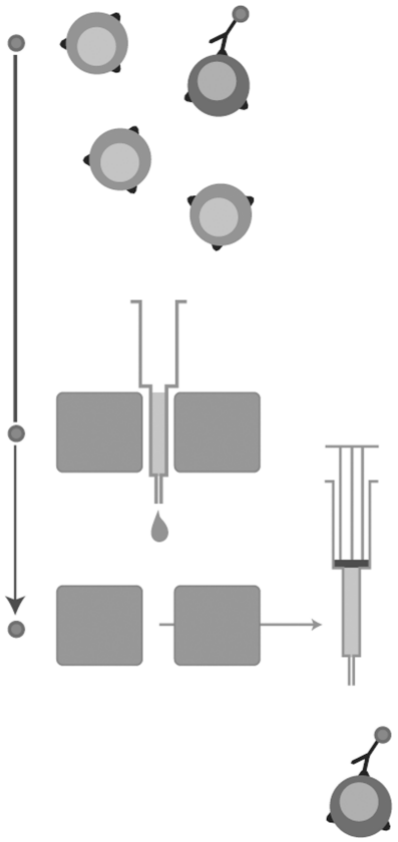

Figure 1. *The principle of the MACS method depends on labeling the cell surface marker with a specific antibody combined with the use of MACS microbeads, which are super-paramagnetic particles that are coupled to highly specific monoclonal antibodies, used to magnetically label the target cell population. 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. As the column is rinsed with buffer, all the unlabeled (annexin negative) cells will be washed out thoroughly. By removing the column from the magnet, the labeled fraction (annexin positive) can be obtained.*

Statistical analysis

Student's paired *t*-test was used to calculate the difference between samples. Hypothesis testing was two – tailed, and P values $0<0.05$ were considered statistically significant. All values are given as mean ± standard deviation (SD). Calculations were performed with Statistica 6.0 software (StatSoft; Tulsa, OK).

RESULTS

Sperm count and motility in samples post-DGC and following the MACS separation are given in Table 1. Following DGC + MACS, the percentage of cells collected in the annexin-negative fraction was significantly higher than the annexin-positive fraction ($p<0.0001$). Compared to the sperm count after DGC, the average number of cells decreased by 15.2 ± 19.1% following MACS. The amount of beads was adjusted according to the sperm count as outlined by the manufacturer (100 µL for each 10 X 10^6 cells subjected to separation). The mean volume of beads used was 718.4 ± 321.5 µL. The number of total motile sperm following DGC + MACS showed a significantly positive correlation with the number of spermatozoa subjected to separation and volume of microbeads used ($r=0.81$, $p<0.0001$; $r=0.76$, $p<0.0001$, respectively). The sperm recovery rates following DGC + MACS were comparable to those following only DGC (73.8 ± 12.1% vs. 66.7 ± 19.1%).

Parameter	Separated sperm (DGC only)	DGC + MACS	
		Annexin-negative	Annexin-positive
Total sperm count (X 10^6)	74 ± 32	61 ± 32	3.3 ± 3.0
Motility (%)	73 ± 10	74 ± 12	5.9 ± 4.3

Table 1. *Sperm concentration and motility in samples post-DGC only and in samples post-DGC + MACS Values are expressed as mean ± standard deviation. DGC = density gradient centrifugation; MACS = magnetic activated cell sorting.*

DISCUSSION

Unorthodox methodologies have been recently accepted as superior techniques for sperm preparation in order to decrease the rate of congenital abnormalities (Schulman *et al*., 2005). Despite various advances in sperm preparation methodology, the recovery rate of functional spermatozoa remains unsatisfactory (Henkel *et al*., 2003). In our study, the recovery rate of spermatozoa after MACS separation has been tested. Paramagnetic microbeads coupled with specific antibodies are considered an effective tool for cell separation (McCloskey *et al*., 2000). Based on the antibody used, leukocytes may be extracted from ejaculates or immature germ cell population may be separated from testicular tissue (Ochsendorf *et al*., 1997, van der Wee *et al*., 2001). The beads may also be used for immunomagnetic separation of membrane-intact and non-apoptotic spermatozoa (Glander *et al*., 2002, Grunewald *et al*., 2001, Paasch *et al*., 2003b, Paasch *et al*., 2005). The paramagnetic annexin V-conjugated microbeads are able to

eliminate spermatozoa with externalized PS (apoptotic cells) and disintegrated plasma membranes from cryopreserved semen samples (Grunewald *et al.*, 2001, Paasch *et al.*, 2005).

In the current study, we have assessed the sperm recovery following a preparation protocol that combines annexin V-MACS with density gradient centrifugation. The number of spermatozoa separated as ANMB-negative (unlabeled with intact membranes) was higher than the number separated as ANMB-positive (labeled apoptotic spermatozoa). This was primarily due to the strict inclusion of semen samples from donor population. The number of sperm retrieved correlated significantly with the original number of cells subjected to separation as well as the number of beads used. Therefore, we anticipate that the sperm retrieval can be maximized by increasing the numbers of separated cells and beads used.

The factors that could alter sperm recovery may be technical such as the concentration of beads used or factors that are related to the sample quality. Since the samples will be subjected to density gradient centrifugation prior to MACS, abnormalities such as high semen viscosity or low motile sperm count may lead to a significant decrease in recovery rates. MACS is based on the separation of apoptotic sperm, therefore samples with high incidence of deregulated apoptosis will be characterized by increased pool of ANMB-positive sperm. Such samples are expected in cases with idiopathic infertility, low sperm motility, high incidence of morphological abnormalities and abnormal reactive oxygen species production (Said *et al.*, 2004). These cases would benefit the most from our novel sperm preparation protocol.

DGC is currently established as a sperm preparation technique prior to ART (Chen *et al.*, 1999) and has been standardized to complement MACS (Said *et al.*, 2005a). Therefore, the main objective of our study was to evaluate sperm recovery following DGC + MACS in order to assess its feasibility as a sperm preparation technique. In our present study, the estimated average number of lost cells (15%) was considerably higher compared to our previous work (1%) (Grunewald *et al.*, 2001). The difference between these 2 estimations could be attributed to the different preparation protocols employed in both studies. In the previous study, cell loss was estimated following glass wool filtration and MACS, while in the present study DGC and MACS were used.

In general, MACS is a feasible and safe method that may be used to provide a high quality sperm fraction (Glander *et al.*, 2002, Grunewald *et al.*, 2001, Paasch *et al.*, 2003b, Paasch *et al.*, 2005). The high sperm recovery rate following DGC + MACS advocates the use of this protocol as sperm preparation technique prior to assisted reproduction. Separating a distinctive population of non-apoptotic spermatozoa with intact membranes and subjecting it to IVF or ICSI is a step further in optimizing the outcome of assisted reproduction. Nevertheless, future experiments using animal models that evaluate embryo livability and genetic integrity would be still needed before the technique could be applied to human cases.

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