CHAPTER 7

Effects of Magnetic-Activated Cell Sorting on Sperm Motility and Cryosurvival Rates

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ABSTRACT

Objective: Superparamagnetic annexin V-conjugated microbeads can separate spermatozoa with externalized phosphatidylserine, which is considered one of the early features of late apoptosis. Our objective was to evaluate the effect of magnetic-activated cell sorting in cryopreservation-thawing protocols on sperm motility and cryosurvival rate.

Design: Prospective-controlled study.

Setting: Andrology department at a University based medical institution.

Patients: Ten healthy volunteer sperm donors.

Interventions: Sperm populations were separated using annexin-V magnetic-activated cell sorting before and after the cryopreservation-thawing process.

Main outcome measures: Sperm motility and cryosurvival rate.

Results: Annexin-negative sperm separated by magnetic-activated cell sorting had significantly higher motility following cryopreservation-thawing than sperm that were not separated. Similarly, annexin-negative spermatozoa also had higher cryosurvival rate than sperm cryopreserved without magnetic-activated cell sorting and sperm that were annexin-positive.

Conclusions: The separation of a distinctive population of non-apoptotic spermatozoa with intact membranes may optimize the cryopreservation-thawing outcome. Magnetic-activated cell sorting using annexin-V microbeads enhances sperm motility and cryosurvival rates following cryopreservation.

Key words: annexin; cryosurvival rate; magnetic-activated cell sorting; mitochondrial membrane potential; sperm

INTRODUCTION

Cryopreservation of human semen is the most commonly accepted method of preserving male reproductive capacity. Cryopreserved spermatozoa may be used in assisted reproductive techniques (ART) (1), especially in cases where the patient elects to undergo vasectomy for contraception or, most importantly**,** when a patient is diagnosed with cancer and the treatment may render him infertile (2). The indications for sperm cryobanking have been greatly expanded by recent breakthroughs in ART, in which immotile but viable sperm can be used successfully for oocyte fertilization through intracytoplasmic sperm injection (ICSI).

Despite recent methodological advances, cryopreservation exerts detrimental effects on spermatozoa that lead to significant decreases in sperm viability and motility and ultimately in cryosurvival rates (CSR) (3). The fertility potential of cryopreserved mammalian spermatozoa is lower than that of fresh sperm. The reduction arises from both a lower post-thaw viability and sublethal dysfunction in a proportion of the surviving subpopulation (4). Programmed cell death (apoptosis) most likely contributes to the decrease in sperm quality after cryopreservation (5).

The sperm plasma membrane is one of the key structures affected by cryopreservation that displays apoptotic features (6). Early phases of disturbed membrane functions are associated with asymmetry of the membrane phospholipids. The phospholipid phosphatidylserine (PS), which is normally present on the inner leaflet of the plasma membrane, becomes externalized to the outer leaflet (7). The externalization of PS is a known early marker for apoptosis (8). Because annexin-V has a high affinity for PS, it cannot pass through an intact sperm membrane. Therefore, when annexin-V binds to spermatozoa, it signifies that the integrity of the membrane has been disturbed (9).

Colloidal super-paramagnetic microbeads (~50 nm in diameter) conjugated with annexin-V may be used to separate dead and apoptotic spermatozoa by magnetic-activated cell sorting (MACS). Cells with PS that has externalized to the outer leaflet will bind to these microbeads. When placed into a column containing iron balls and passed through a strong magnetic field, those cells remain in the separation column. On the other hand, cells with intact membranes remain unlabelled and pass freely through the column (10, 11).

The process of cryopreservation increases the amount of apoptotic spermatozoa, which in turn decreases the success rates of ARTs. The binding of superparamagnetic annexin-V microbeads (ANMB) can effectively eliminate spermatozoa in early apoptotic stages from cryopreserved samples (12). Therefore, ANMB-negative spermatozoa may have higher survival potential after cryopreservation. The objective of our study was to determine if the inclusion of MACS in cryopreservation-thawing protocols improves sperm motility and the CSR.

MATERIALS AND METHODS

Sample preparation

This study was approved by our Institution Review Board. Semen samples were collected from 10 healthy donors, and semen parameters exceeded the World Health Organization (WHO, 1999) (13) reference ranges for the normal fertile population. To separate the predominantly mature spermatozoa, the liquefied semen was loaded onto a 55% and 80% discontinuous SupraSperm gradient (MediCult, Jyllinge, Denmark) and centrifuged at 500*g* for 20 minutes. The resulting 80% pellet (mature spermatozoa) was aspirated and re-suspended in human tubal fluid media (HTF, Irvine Scientific, Santa Ana, CA).

The sperm cell suspension was divided into 2 separate fractions. The first was subjected to MACS followed by cryopreservation and thawing whereas the second was cryopreserved-thawed first and then subjected to MACS. Sperm motility was assessed in all fractions at each step of the experiment. The different steps of our experiment design are illustrated in Figure 1.

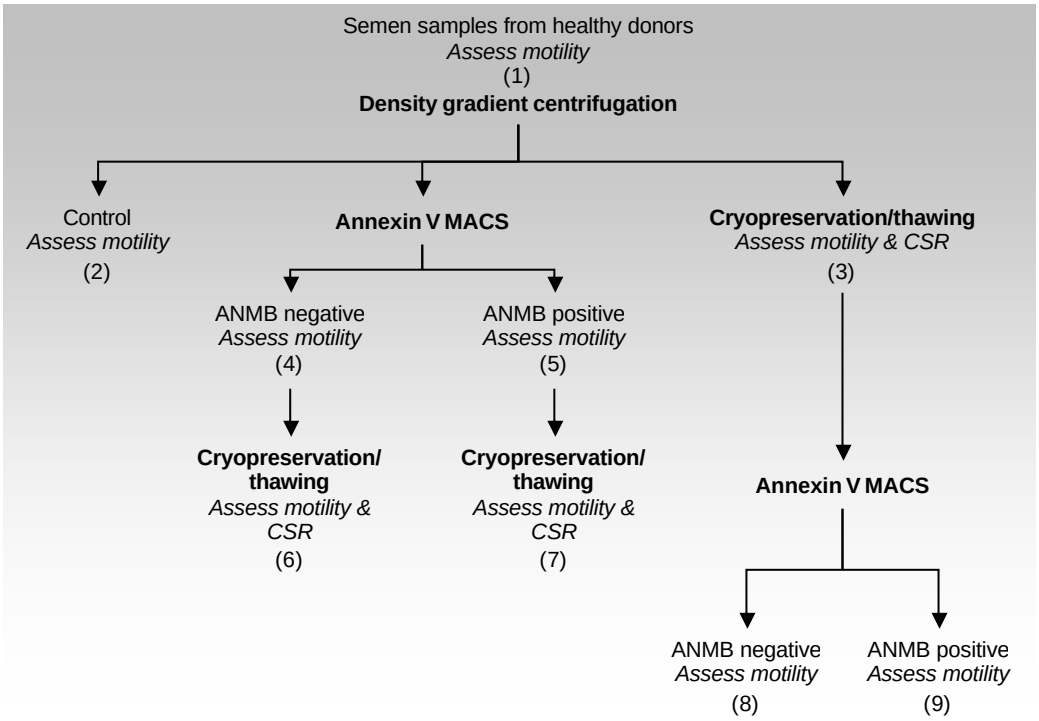

Figure 1. *Flow diagram of overall experiment design: spermatozoa from same sample were subjected to cryopreservation-thawing before and after MACS. Number in parenthesis represents the aliquot number. MACS = magnetic activated cell separation; ANMB = annexin-V magnetic microbead; CSR = cryosurvival rate.*

Isolation of spermatozoa with deteriorated membranes by MACS

The sperm suspensions were passed through a magnetic field (MiniMACS; Miltenyi Biotec, Bergisch Gladbach, Germany), and the spermatozoa were classified as either ANMB-positive or ANMB-negative based on the binding of the microbeads to their outer surface (14).

Briefly, the washed spermatozoa were incubated with 100 µL ANMB at room temperature for 15 minutes, placed on top of the separation column containing iron balls, which was placed in a magnet. The apoptotic spermatozoa were retained in the separation column and labeled as ANMB-positive whereas the spermatozoa with intact membranes passed through the column and were labeled 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 the column was removed from the magnetic field, the retained fraction was eluted using an annexin-binding buffer (15). The number of cells collected in each column exceeded 1 X 10^6/mL.

Cryopreservation – thawing protocol

All specimens were cryopreserved using TEST-Yolk Buffer (TYB, 20% egg yolk and 12% glycerol, Irvine Scientific, Santa Ana, CA), (16). TYB was added to the sperm samples at room temperature. An aliquot of the freezing medium equal to 25% of sperm sample volume was added to the specimen and gently mixed for 5 minutes using a Hema-Tek aliquot mixer (Miles Scientific, Elkhart, IN). This was repeated to give a final 1:1 (v/v) ratio of freezing medium to the sperm samples. Cryovials (1.5 mL, Corning, Pittsburg, PA) containing the specimens were placed in the freezer at -20°C for 8 minutes and thereafter in liquid nitrogen vapor at -80°C for 2 hours. The vials were finally transferred to liquid nitrogen tanks at -196°C. Twenty-four hours after the samples were frozen**,** a vial was removed and thawed by incubating it at 37°C for 20 minutes. Spermatozoa were washed and re-suspended in HTF media immediately after thawing, and sperm motility was re-assessed.

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 ± SD. All calculations were performed with Statistical 6.0 software (StatSoft; Tulsa, OK).

RESULTS

Raw semen samples collected from the donors had a sperm concentration of 89.5 ± 22.3 X 10^6/mL and percentage motility of 64.5 ± 6.43. The percentage motility of the different aliquots

are listed in Table 1. After MACS separation pre-freeze, the ANMB-negative sperm had significantly higher motility values (76 ± 15.06, P = 0.03) than the raw samples (64.5 ± 6.43) whereas the ANMB-positive sperm had significantly lower values (41 ± 29.61, P < 0.0001). The ANMB-negative sperm consistently had higher motility than the ANMB-positive sperm (P = 0.006).

The cryopreservation-thawing process significantly decreased the motility of the spermatozoa that were not subjected to MACS separation compared with the pre-freeze control (20 ± 12.02 vs. 62.5 ± 20.45, P = 0.0001). Similarly, the cryopreservation-thawing process decreased sperm motility in the ANMB-negative spermatozoa (38.5 ± 11.31, P = 0.01) and in the ANMB-positive spermatozoa (4 ± 7, P < 0.0001). However, the ANMB-negative sperm were the least affected by the cryopreservation-thawing process. The ANMB-negative sperm had higher motility following cryopreservation-thawing than the ANMB-positive sperm (P < 0.0001) and the sperm that were not separated by MACS (P = 0.003).

The use of TYB did not lead to any decrease in sperm motility. Motility values obtained after density gradient centrifugation were comparable to those obtained when TYB was added prior to cryopreservation in the samples that were not separated by MACS (62.5 ± 20.45 vs. 68.00 ± 10.33). When MACS was performed on the samples after the cryopreservation-thawing process, sperm motility was severely affected. Motility in the cryopreserved-thawed spermatozoa pre-MACS was significantly higher than that in the post-MACS ANMB-positive cells (20.00 ± 12.02 vs. 0.00 ± 0.00, P = 0.0005) and that of the ANMB-negative cells (20.00 ± 12.02 vs. 2.58 ± 0.81, P = 0.0005).

The percentage of sperm that survived the cryopreservation process (percentage CSR) was calculated using the following formula: 100 X post-thaw total motile sperm/pre-freeze total motile sperm. The CSR was highest in the ANMB-negative spermatozoa that were separated prior to freezing. This sperm aliquot had a significantly higher CSR than the sperm cryopreserved without MACS (76.6 ± 59.75 vs. 30.29 ± 16.06, P = 0.04) and the sperm that were ANMB-positive (76.6 ± 59.75 vs. 12.7 ± 31.19, P = 0.04).

Aliquot Number	Aliquot content (n=10)	MACS timing	CPT	Motility (%)
1	Raw semen	N/P	No	64.5 ± 6.43
2	Control (post density gradient)	N/P	No	62.5 ± 20.45
3	Control (post density gradient)	N/P	Yes	20 ± 12.02[b,c]
4	ANMB-negative	Pre-freeze	No	76 ± 15.06[a]
5	ANMB-positive	Pre-freeze	No	41 ± 29.61[a]
6	ANMB-negative	Pre-freeze	Yes	38.5 ± 11.31[b]
7	ANMB-positive	Pre-freeze	Yes	4 ± 7[b,c]
8	ANMB-negative	Post-thaw	Yes	2 ± 2.58[b,c]
9	ANMB-positive	Post-thaw	Yes	0.00 ± 0.00[b,c]

Table 1. *Motility values obtained from spermatozoa separated and non separated by MACS. N/P = MACS was not performed; ANMB = fraction separated by annexin-V magnetic beads; CPT = cryopreservation-thawing. Values presented as mean ± standard deviation; P <0.05 considered significant compared to: a) raw semen, b) non-cryopreserved control, and c) ANMB-negative spermatozoa separated by MACS and cryopreserved.*

DISCUSSION

Despite various advances in cryopreservation methodology, the recovery rate of functional post-thaw spermatozoa remains unsatisfactory. Specifically, sperm motility significantly decreases after freezing (17). In our study, the cryopreservation-thawing process significantly decreased the percentage of motile spermatozoa. Sperm motility may have decreased because of a change in temperature and the formation and dissolution of ice in the extracellular environment (4). On the other hand, TYB may be excluded as a potentially damaging factor since sperm motility was maintained after it was added.

The cryopreservation-thawing process induces many changes in mammalian spermatozoa (18, 19). The lipid components of cell membranes tend to reorganize after being subjected to cooling, which may decrease the stability of the lipid bilayer (20, 21). In turn, sperm membrane integrity becomes impaired as PS translocates from the inner to the outer leaflet of the sperm plasma membrane (6, 22, 23), which is considered one of the early signs of apoptosis (7, 8). Annexin-V is a 35-36 KDa phospholipid-binding protein that specifically binds to PS (6, 8, 9) and therefore can be used to detect early deleterious changes in the sperm plasma membrane that follow cryopreservation (24).

Superparamagnetic microbeads coupled with the use of specific antibodies can effectively separate cells. Based on the antibody used, leukocytes may be extracted from ejaculates or an immature germ cell population may be separated from testicular tissue (25, 26). The beads may also be used for immunomagnetic separation of membrane-intact and non-apoptotic spermatozoa (9, 12, 14, 27). The superparamagnetic annexin V-conjugated microbeads are able to eliminate spermatozoa with externalized PS (apoptotic cells) and disintegrated plasma membranes from cryopreserved semen samples (12, 27).

In the current study, we assessed the integration of MACS coupled with annexin V-conjugated microbeads in our cryopreservation protocol. The procedure delivers 2 sperm fractions: ANMB-positive (labeled apoptotic spermatozoa) and ANMB-negative (unlabeled with intact membranes). Prior to cryopreservation, ANMB-negative spermatozoa separated by MACS had the highest motility compared with the ANMB-positive and non-separated spermatozoa. Following cryopreservation and thawing, ANMB-negative spermatozoa still had the highest motility and CSR compared with the other fractions. Therefore, it appears that the elimination of spermatozoa with early apoptotic changes positively affects motility and CSR after cryopreservation.

Sperm cryopreservation and thawing is associated with increased reactive oxygen species (ROS) production and decreased antioxidant levels (28, 29). In our earlier study, we described that ROS levels have a positive correlation with the extent of apoptotic sperm (30). Therefore, the higher cryosurvival rates in ANMB-negative sperm following cryopreservation-thawing could be related, at least in part, to the exclusion of the ROS producing ANMB-positive sperm. In support, superoxide dismutase and catalase that act as selective scavengers for ROS, were able to improve sperm recovery after cryopreservation-thawing (31).

In general, MACS is a feasible and safe method that may be used to produce a high-quality sperm fraction (9, 12, 14, 27). Although the separation columns and their magnetic field do not exert any detectable effect on the spermatozoa (12), sperm motility decreased dramatically in our current study when MACS was performed on cryopreserved-thawed samples. Because the deleterious effect of MACS was manifested only in the cryopreserved-thawed samples and not

in the fresh samples, we therefore believe that this procedure can be integrated in cryopreservation protocols provided it is conducted on pre-freeze specimens.

MACS, if used, should be performed before cryopreservation. In our present study, sperm motility deteriorated when MACS was performed after cryopreservation, possibly because the cryopreservation procedures (eg, centrifugation re-suspension) made the cells vulnerable to damage. Moreover, ANMB-negative spermatozoa may still display a very early phase of PS translocation. In that case, only a limited number of beads would bind to the sperm—too few to be retained in the column.

Cryopreservation of human sperm is a fundamental tool for the preservation of male fertility. However, the process of cryopreservation impairs sperm fertility. Separating a distinctive population of non-apoptotic spermatozoa with intact membranes before subjecting it to the cryopreservation-thawing process may optimize the outcomes. Our findings suggest that MACS coupled with ANMB not only can be used to perform this separation but that it enhances sperm motility and CSR following cryopreservation.

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