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Magnetic-activated cell sorting before cryopreservation preserves mitochondrial integrity in human spermatozoa

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Abstract

Superparamagnetic annexin-V conjugated microbeads are able to eliminate spermatozoa with externalized phosphatidylserine, a membrane feature of apoptotic cells as well as spermatozoa with deteriorated plasma membrane. Our objective was to evaluate the effects of annexin-V Magnetic-Activated Cell Sorting (MACS) in cryopreservation–thawing protocols and on integrity of sperm mitochondrial transmembrane potential and mitochondrial integrity survival rate (MSR). Mature spermatozoa of 10 healthy donors were prepared by density gradient centrifugation and divided into 2 aliquots afterwards. The first one was subjected to annexin-V MACS followed by cryopreservation and thawing, while the second was cryopreserved–thawed without MACS to serve as control. Annexin-negative sperm separated by MACS showed significantly higher levels of intact mitochondria following cryopreservation–thawing ($45.4 \pm 8.6\%$) compared to sperm that were not separated ($15.8 \pm 4.6\%$, $p < 0.01$). Separating a distinctive population of non-apoptotic spermatozoa with intact membranes may optimize cryopreservation–thawing outcome. MACS using annexin-V microbeads enhances the percentage of spermatozoa with intact transmembrane mitochondrial potential and mitochondrial integrity survival rates following cryopreservation.

Introduction

Cryopreservation of human spermatozoa is a widespread method applied for fertility preservation in patients undergoing cancer therapy or those who are planning permanent contraceptive measures such as vasectomy (Sibert et al. 1999). In addition it can become necessary during treatment cycles of assisted fertilization techniques. Despite many advances in the field of cryobiology, cryopreserved spermatozoa still have decreased fertility potential compared to those present in fresh semen samples (Donnelly et al. 2001; Anger et al. 2003). Apoptosis, the programmed cell death contributes to this decrease in sperm function (Glander and Schaller 1999; Agarwal and Allamaneni 2004; Paasch et al. 2004b).

Early manifestations of apoptosis including damage of the sperm membrane with externalization of the phospholipid phosphatidylserine (EPS)

to the outer surface (Glander and Schaller 1999) and disruption of the mitochondrial membrane potential (MMP) were observed in human spermatozoa following cryopreservation and thawing (O'Connell et al. 2002). As human spermatozoa are preferentially susceptible to mitochondria-mediated apoptosis the disruption of the MMP is a key point in apoptosis signaling transduction (Grunewald et al. 2005). Furthermore, intact MMP is essential for the spermatozoal motility (Evenson et al. 1982).

Based on the high and selective affinity of the 35–36 kDa phospholipid binding protein annexin-V to phosphatidylserine (Vermes et al. 1995), apoptotic spermatozoa and spermatozoa with deteriorated plasma membranes can be sub-populated by superparamagnetic annexin-V microbeads (ANMB) in ANMB-positive and -negative fractions in a magnetic cell sorter (MACS), (Paasch et al. 2003).

Annexin-V MACS was previously proved to separate spermatozoa with activated caspases – key initiator and executor enzymes of apoptosis – preferentially in the ANMB-positive fraction while ANMB-negative sperm were characterized by lowest activation of the apoptosis signaling cascade (Paasch et al. 2004a). The MACS system has no detrimental effects on sperm motility, viability and morphology (Grunewald et al. 2001).

The aim of our study was to evaluate the benefit of inclusion of annexin-V MACS in a standard cryopreservation protocol.

Material and methods

Sample preparation

Semen samples were collected from 10 healthy donors with semen parameters exceeding reference ranges for the normal fertile population (WHO 1999). In order to separate 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 min. 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, while the second was cryopreserved–thawed without a separation step. Assessment of the integrity of MMP was performed for all fractions at each step of the experiment. Different steps entailed in our experiment design are illustrated in Figure 1.

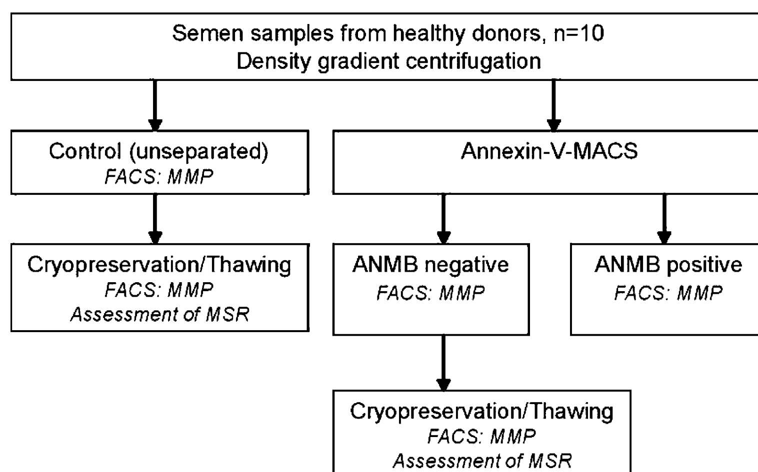

Figure 1. Flow diagram of overall experiment design: spermatozoa from same sample were subjected to cryopreservation–thawing either separated by Annexin-V MACS previously or not. MACS = magnetic activated cell separation; ANMB = annexin-V magnetic microbead; MMP = mitochondrial membrane potential; MSR = mitochondrial integrity survival rate.

Separation of spermatozoa with deteriorated membranes by MACS

The sperm suspensions were divided into 2 sperm fractions by passage through a magnetic field (MiniMACS; Miltenyi Biotec, Bergisch Gladbach, Germany) based on the binding of ANMB to phosphatidylserine. Phosphatidylserine can be present on the surface of spermatozoa due to externalization during apoptosis. Alternatively the ANMB bind to phosphatidylserine on the inner leaflet of deteriorated and therefore permeable plasma membranes. Briefly, the washed spermatozoa were incubated with 100 μl ANMB at room temperature for 15 min, and placed on top of the separation column containing iron balls. The ANMB–labeled spermatozoa (ANMB-positive) were retained in the separation column, which was placed in a magnet, whereas non-apoptotic 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). The number of cells collected in each column exceeded 1×10^6/ml.

Cryopreservation–thawing protocol

All specimens were cryopreserved using Test-Yolk Buffer (TYB, 20% egg yolk and 12% glycerol, Irvine Scientific, Santa Ana, CA). TYB was added to sperm samples as a cryoprotectant 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 min 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 and sperm samples. Cryovials (1.5 ml, Corning, Pittsburg, PA) containing the specimens were placed in the freezer at −20 °C for 8 min and thereafter in liquid nitrogen vapor at −80 °C for 2 h. 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 min. Spermatozoa were washed and re-suspended in HTF media immediately after thawing and mitochondrial transmembrane potential was re-assessed.

Integrity of mitochondrial membrane potential (MMP)

A lipophilic cationic dye (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl carbocyanine chloride) was used to detect intact transmembrane potential of mitochondria in spermatozoa (ApoAlert Mitosensor® Kit, Clontech, CA, USA). Spermatozoa with intact mitochondria excite an intense red fluorescence due to forming of the dye aggregates. The monomer dye fluoresces green indicating disrupted mitochondrial membrane potential. Mitosensor was used according to the instructions of the manufacturer. Briefly, all aliquots were incubated at 37 °C in dark for 20 min in 1 μg of the lipophilic cation diluted in 1 ml PBS.

Negative controls were processed identically for each fraction, except that the stain was replaced with PBS.

Flow cytometry analyses

All flow cytometry analyses where performed by the flow cytometer FACS Calibur (Becton Dickinson, USA). In each assay 10,000 cells where examined at a flow rate of <100 cells/s. Both fluorescences can be measured by FACS. Green emission is detected in fluorescence channel 1 (FL1) and red emission is detected in fluorescence channel 2 (FL2).

The percentage of intact mitochondrial membrane potential and the mean fluorescence was calculated by a specific software (EXP32ADC, Coulter). The superparamagnetic microbeads with the size of about 50 nm in diameter are too small to be detected by optical methods and to change the scatter properties of spermatozoa in the flow cytometer (Jackaman et al. 1977). As a positive control the binding of FITC-conjugated *Pisum sativum* agglutinin to the acrosome was employed for setting the bitmap on dot plot (Glander et al. 1996). The intracellular localization of the fluorescence signals was investigated by fluorescence microscopy (Jenamed, Carl Zeiss, Germany).

Statistical analysis

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

Results

Following standard semen preparation including density gradient centrifugation 77.7 ± 12.9% of spermatozoa collected from healthy donors had intact mitochondria. Values of human spermatozoa containing intact mitochondria obtained from different aliquots in our experiment are illustrated in Table 1.

Fluorescence microscopy revealed fluorescence signals of intact MMP (red fluorescence) in the midpiece region only. The optical examination served as an internal quality control of the Mitosensor® assay and proved the FACS results (Figure 2).

In non-cryopreserved semen samples of healthy donors the inclusion of annexin-V MACS led to a slight, but not significant increase in MMP-intact sperm within the ANMB-negative fraction. ANMB-positive sperm had a significantly lower percentage of intact mitochondria ($p < 0.01$). Consistently, spermatozoa with intact structure of the outer cell membrane (ANMB-negative) had significantly higher values of intact mitochondria transmembrane potential compared to those with externalized phosphatidylserine (ANMB-positive, $p < 0.01$).

Cryopreservation–thawing led to a significant decrease of MMP-intact spermatozoa ($p < 0.01$). Compared to the unseparated as well as to the ANMB-positive aliquot, the ANMB-negative fraction had a significantly lower percentage of spermatozoa with disrupted MMP following cryopreservation–thawing ($p < 0.01$). The mitochondrial integrity survival rates (MSR) of the different sperm subpopulations were calculated as ratio of the percentage of spermatozoa with intact MMP of the cryopreserved and the neat aliquot:

MMP-intact spermatozoa [%] after cryopreservation divided by MMP-intact spermatozoa [%] neat.

MACS separation previous to cryopreservation improved the cryopreservation survival rate within the ANMB-negative fraction significantly. MSR was increased about 36.1 ± 18.9% ($p < 0.01$, Table 2).

Discussion

Cryopreservation of human sperm is an essential tool for the preservation of male fertility. However, despite continuous methodological optimization, the process of cryopreservation and thawing leads to an activation of apoptosis signal transduction in a certain amount of the cryopreserved spermatozoa probably contributing to the reduction of the fertilizing capacity (Sakkas et al. 1998; Paasch et al. 2004b; Peris et al. 2004). In human spermatozoa mitochondria are preferentially susceptible to apoptotic stimuli due to their compartimentation within the midpiece region (Grunewald et al. 2004). The disruption of the MMP is a crucial point and was chosen as a key

Table 1. Values of intact mitochondria obtained from spermatozoa separated and non separated by annexin-V magnetic activated cell sorting (MACS).

Aliquot (n = 10)	MACS timing	CPT	Spermatozoa with intact MMP %
Control (post density gradient)	N/A	No	77.7 ± 12.9
Control (post thawing)	N/A	Yes	15.8 ± 4.6#
ANMB-negative	Pre-freeze	No	81.8 ± 12.8*
ANMB-positive	Pre-freeze	No	13.5 ± 7.0
ANMB-negative (post thawing)	Pre-freeze	Yes	45.4 ± 8.6

ANMB = fraction separated by annexin-V magnetic beads; CPT = cryopreservation-thawed; MMP = mitochondrial membrane potential. N/A = not available, no MACS separation. Values presented as mean ± standard deviation; Wilcoxon test for paired samples, $p < 0.05$ considered significant compared to: #non-cryopreserved control, and *ANMB-negative spermatozoa separated by MACS and cryopreserved.

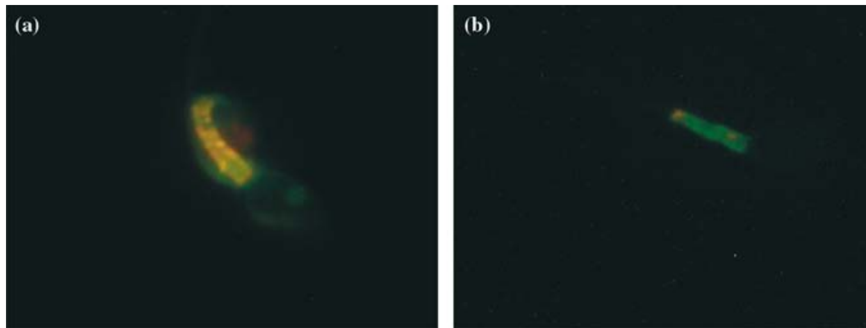

Figure 2. Mitochondrial membrane potential (MMP) in human spermatozoa: (a) Intact mitochondria of ANMB-negative sperm excite a red fluorescence in the midpiece region due to intra-mitochondrial aggregation of the lipophilic cations (Mitosensor® assay). (b) In contrast, mitochondria of ANMB-positive sperm fail to aggregate the lipophilic cations and display a green fluorescence.

Table 2. Mitochondrial integrity survival rates (MSR) calculated as ratio of MMP–intact spermatozoa [%] before and after cryopreservation.

Aliquots ($n = 10$)	MSR
Control (unseparated)	0.21 ± 0.07
ANMB-negative	0.57 ± 0.14
p (Control vs. ANMB-negative)	< 0.001

ANMB = fraction separated by annexin-V magnetic beads; CSR = Cryopreservation survival rates. Values presented as mean ± standard deviation; Wilcoxon test for paired samples, $p < 0.05$ considered significant.

marker of the activation of apoptosis signaling cascade.

In our study, the process of cryopreservation–thawing significantly decreased the percentage of spermatozoa with intact mitochondrial membrane potential. This goes with the previously observed plasma membrane changes: the spermatozoal cell membrane displays certain reordering of their lipid components, leading to a loss of stability of the lipid bilayer (Schiller et al. 2000). In turn, sperm membrane integrity becomes impaired with translocation of phosphatidylserine from the inner to the outer leaflet of the sperm plasma membrane (Glander and Schaller 1999; Hinkovska-Galcheva et al. 1989). This translocation is considered one of the early signs of terminal phase of apoptosis (Martin et al. 1995). The specific binding of annexin-V to phosphatidylserine can be used for detection (Shen et al. 2002) and magnetic separation of spermatozoa with disturbed plasma membrane (Paasch et al. 2003). In the current study, we have assessed the integration of annexin-V MACS in a standard cryopreservation protocol. The procedure delivers 2 sperm fractions: ANMB-positive (spermatozoa with labeled phosphatidylserine) and ANMB-negative (unlabeled with intact membranes) passed spermatozoa. Prior to cryopreservation, annexin-V MACS enriched significantly mitochondria-intact spermatozoa within the ANMB-negative fraction, while ANMB-positive spermatozoa presented predominantly with disrupted MMP. The mitochondrial damage due to cryopreservation and thawing was lowest in the ANMB-negative subpopulation. In turn, cryosurvival rates were superior compared to unseparated or ANMB-positive spermatozoa. Therefore, it appears that the elimination of spermatozoa with early apoptotic changes has a positive effect on preservation of MMP and cryosurvival rates following cryopreservation.

In general, MACS is a feasible and safe method that may be used to provide a high quality sperm fraction. The separation columns and their magnetic field do not exert any detectable effect on the spermatozoa (Grunewald et al. 2001).

Despite various advances in cryopreservation methodology, the recovery rate of functional post-thaw spermatozoa remains still unsatisfactory (Donnelly et al. 2001). Separating a distinctive population of membrane and mitochondria intact spermatozoa and subjecting it to cryopreservation–thawing may optimize the outcome of the process. Our findings are of importance in advocating the use MACS coupled with annexin-V microbeads to enhance the percentage of spermatozoa with intact mitochondria and mitochondrial cryosurvival rates following cryopreservation.

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