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Cryopreservation and Thawing Is Associated with Varying Extent of Activation of Apoptotic Machinery in Subsets of Ejaculated Human Spermatozoa[1]

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ABSTRACT

We investigated the impact of cryopreservation and thawing on levels of caspases-3, -8, and -9 activity, intact mitochondrial membrane potential ($\Delta\psi_m$), and DNA fragmentation in human spermatozoa. Eleven pools of cryopreserved and eight pools of fresh semen samples were examined. Mature and immature fractions were separated on a two-layer density gradient (47% and 90%) and further subdivided based on the externalization of phosphatidylserine and its binding to annexin V-labeled superparamagnetic microbeads (ANMB). Levels of activated caspases were assessed using fluorescein-labeled inhibitors of caspases (FLICA), $\Delta\psi_m$ using a lipophilic cationic dye, and DNA fragmentation by the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay. Cryopreservation was significantly associated with activation of caspases-3, -8, and -9, as well as disruption of the mitochondrial membrane potential but no significant changes were observed in DNA fragmentation. In mature sperm, caspase activation was only detected in the ANMB$^+$ fraction, whereas in immature sperm, both ANMB$^+$ and ANMB$^-$ fractions showed activated caspase levels. In ANMB$^+$ immature sperm, apoptosis seemed to be triggered by a surface ligand-receptor mechanism as well as by disruption of mitochondria, whereas in ANMB$^-$ immature sperm, apoptosis was induced by activation of caspase-9 following loss of intact $\Delta\psi_m$. These results demonstrate that selection of annexin V-negative mature spermatozoa might be of clinical relevance for fertility preservation, as this sperm fraction shows no activated apoptosis during the cryopreservation process.

apoptosis, caspases, cryopreservation, human spermatozoa, magnetic cell depletion, male reproductive tract, signal transducers, sperm, spermatogenesis

INTRODUCTION

During cryopreservation, spermatozoa are exposed to physical and chemical stress [1–3], changes in lipid composition of sperm plasma membrane [1, 2], reduced head size [3], and externalization of phosphatidylserine residues [4]. Factors contributing to these changes include temperature, maturity of the cell [5], cryoprotectant used, and rate of cooling [4, 6, 7].

Ejaculated human spermatozoa show evidence of DNA fragmentation [8–16], plasma membrane translocation of phosphatidyl serine [4, 17–21], and recently caspase activation has also been demonstrated [22–25]. These characteristics are typical of programmed cell death in somatic cells [26–29]. In somatic cells, apoptosis is regulated at three levels: plasma membrane (presence of Fas receptors), nucleus (presence of p53 inducing upregulation of Bax gene and downregulation of Bcl-2 expression), and cytoplasm (activation of Bax and release of cytochrome c and caspase 3 cascade in the cytosol) [30–32]. Features characteristic of apoptosis in human ejaculated spermatozoa include ultrastructural observations of abnormalities of the chromatin, mitochondria, the nuclear envelope, the plasma membrane, presence of cytoplasmic vacuoles and apoptotic bodies [33], presence of DNA fragmentation and phosphatidyl serine externalization. The presence of somatic-cell apoptotic markers in ejaculated spermatozoa may be as a result of the remnants of the immature sperm exhibiting a cytoplasmic droplet that is abnormally released by the seminiferous tubules. When present in abundance, immature spermatozoa could be responsible for infertility and may exhibit expression of markers such as creatine kinase, HSPA2, Bcl-XL, and caspase 3 [34]. Also, somatic-cell apoptosis could be due to abortive testicular apoptosis [12, 13] or may be reflective of mechanisms related to endocrinopathies, varicoceles, and inflammation/infection [29]. Also, because spermatozoa are terminally differentiated cells, like neutrophils, they may exhibit a defined ex vivo lifetime that could be inherent to mature spermatozoa or could be related to anoikis, programmed cell death when cells are not anchored to an extracellular matrix. However, spermatozoa may also have many structural and functional characteristics not typical of somatic cells. This makes it likely that, should apoptosis occur, mechanisms and indicators of sperm apoptosis could also be unique. Poor chromatin packaging and/or damaged DNA have been implicated in the failure of sperm decondensation after intracytoplasmic sperm injection (ICSI), resulting in fertilization failure [35, 36].

Recently, cryopreservation-induced caspase activity, activation of caspase-9 along with disruption of the $\Delta\psi_m$, and the release of mitochondria-associated regulating proteins have been demonstrated in cryopreserved spermatozoa [6, 21, 37]. Therefore, our aim was to further characterize the impact of cryopreservation and thawing on apoptosis-related signaling in sperm. The rationale for studying this type

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of signal transduction in fresh and cryopreserved spermatozoa was to differentiate the extent of performance of apoptosis identified by externalization of the phosphatidylserine and to study how immature and mature spermatozoa respond to cryopreservation. It is critical to clarify if the important apoptosis-specific caspases are associated with the signaling cascade and if they are in synchrony with other known changes both at the DNA and membrane levels. The experiment used a factorial, repeated-measure design examining apoptosis in fresh (unfrozen) and cryopreserved (frozen) samples. Both immature and mature spermatozoa were separated into annexin V-labeled superparamagnetic microbeads (ANMB) $ANMB^+$ and $ANMB^-$ spermatozoa. We measured the levels of activated caspase-3, -8, and -9, intact mitochondrial membrane potential, and DNA strand breaks in fresh and frozen and immature and mature spermatozoa.

MATERIALS AND METHODS

Sample Collection and Preparation of Semen Pools

Following institutional approval from The Cleveland Clinic Foundation and informed consent from 30 healthy donors, a total of 84 ejaculates were used to create 8 pools of fresh and 11 pools of cryopreserved semen. These numbers were based on our earlier pilot studies and sample-size calculations. We used pooled semen samples instead of single ejaculates to ensure the availability of adequate numbers of cells to carry out the various assays following two serial separations, one on density gradient and the other following passage through a magnetic field by magnetic cell sorting (MACS) technique. In addition, due to the factorial nature of the experiment as well as the multiple parameters being measured, the design necessitated the pooling of semen samples from individual donors over time.

Semen samples were collected by masturbation into sterile containers. Following liquefaction, an aliquot was examined for sperm concentration, motility, and morphology according to the World Health Organization standard guidelines [38]. Each ejaculate fulfilled the following criteria: sperm concentration $>20 \times 10^6$/ml and >50% motility. Each donor contributed an average of three samples, and the average volume of each pooled sample was 4.5 ml. The average total number of spermatozoa in each pool was $734.6 \pm 272.3 \times 10^6$.

Experimental Design

This design necessitated an analytical technique to account for the correlated nature of the samples. The experiment used a factorial, repeated-measures design model for this analysis and comprised 84 samples. The protocol involved 40 aliquots to be processed for each pool and 5–9 washing and resuspension steps per aliquot.

Density gradient centrifugation was performed and immature and mature spermatozoa were separated into ANMB-positive and ANMB-negative ($ANMB^+$ and $ANMB^-$) fractions [6, 21]. In addition, aliquots were used for measuring caspase-3, -8, and -9, mitochondrial membrane potential, and DNA damage.

We first assessed both two- and three-way interactions among the effects: immature vs. mature spermatozoa, $ANMB^+$ and $ANMB^-$ and cryopreserved vs. fresh sample on each continuous variable of apoptosis. An interaction was kept in the model when $P < 0.10$. If neither the two- or three-way interactions were significant, our primary analysis directly interpreted the main effects by collapsing over levels and adjusting for other main effects. If an interaction between variables was significant at the $P < 0.10$ level, then our primary analysis was to examine the effect of each factor within levels of the other interacting factor or factors. For subgroup comparisons within three-way interactions, a Bonferroni correction was used to give a significance criterion of 0.0125 (cryopreservation assessed four times): within mature/immature and $ANMB^+$ and $ANMB^-$ and 0.025 when assessing the cryopreservation effect within levels of one factor. We used repeated measures analysis of variance (RMA) model assuming a common correlation (compound symmetry structure) among measurements within the same pool.

Within-factor analyses were done using either RMA as explained above or with two-sample *t*-tests. Mature and immature control samples (without superparamagnetic separation) were compared with *t*-tests on each outcome. Correlations among continuous variables were assessed using Spearman correlation analysis and 95% confidence intervals. Values are given as mean ± SD (SD) or mean t ± standard error of the mean (SEM) when reporting results from the RMA. The significance level for each hypothesis was 0.05. These analyses were done using SAS version 8.2 (SAS Institute Inc., Cary, NC). The details of each assay are described below.

Cryopreservation of Semen Samples

For cryopreservation, semen samples were diluted dropwise with an equivalent volume of freezing medium TEST (TES (N-tris[hydroxymethyl] methyl-2-aminoethanesulfonic acid) and Tris yolk buffer) containing 12% (v/v) glycerol (Irvine Scientific, Santa Ana, CA). The samples were placed into cryovials and frozen at −20°C for 8 min and in liquid nitrogen vapor at −100°C for 2 h. Finally, the tubes were plunged into liquid nitrogen at −196.5°C for long-term storage. Vials were thawed by placing them in an incubator at 37°C for 30 min.

Isolation of Mature and Immature Sperm Populations

The liquefied semen was loaded onto a 47% and 90% discontinuous gradient using Isolate (Irvine Scientific) and centrifuged at $500 \times g$ for 20 min at room temperature. The resulting interface between the 47% and 90% layer (fraction 1, immature spermatozoa) and the 90% pellet (fraction 2, mature spermatozoa) was aspirated and transferred into separate test tubes [39]. The pellet from both fractions was resuspended in Biggers-Whitten-Whittingham medium (BWW; Irvine Scientific) and centrifuged at $500 \times g$ for 7 min. Both fractions were examined for sperm concentration and motility. An aliquot was separated from both fractions to serve as the control, and the rest was subjected to superparamagnetic bead separation (see below).

Separation of Spermatozoa with Externalized Phosphatidylserine

The mature and immature sperm suspensions obtained after density gradient separation were further divided into two subfractions. This was achieved by passage through a magnetic field using magnetic cell sorting (MACS) technique (MiniMACS; Miltenyi Biotec, Bergisch-Gladbach, Germany, and Auburn, CA) based on the binding of superparamagnetic microbeads (ANMB) to phosphatidylserine (PS) present on the surface of spermatozoa. The translocation of phosphatidylserine on the external surface of apoptotic spermatozoa has been used previously for a highly specific and sensitive separation using the superparamagnetic ANMB [21]. The MACS step was done using unseparated mature and immature sperm suspensions as controls.

The washed spermatozoa were incubated with 100 μl ANMB at room temperature for 15 min. The ANMB-labeled spermatozoa were retained in the separation column ($ANMB^+$), which was placed in the magnetic field, while the nonlabeled spermatozoa ($ANMB^-$) passed through. After removing the column from the magnetic field, the retained fraction was eluted.

Detection of Activated and Inhibited Caspases

Activated caspase-3, -8, and -9 were detected in living spermatozoa using the carboxyfluorescein-labeled fluromethyl ketone (FMK)-peptide-inhibiting substrate of caspases (CaspaTag Intergen Company, Purchase, NY) according to the manufacturer's instructions. These cell-permeable and noncytotoxic caspase inhibitors bind covalently to active caspases [39]. The kits used were caspase-3, CaspaTag Caspase-3 (DEVD) activity kit as the FAM-DEVD-FMK inhibitor. This is a carboxyfluorescein analogue of benzyloxycarbonyl-aspartylglutamylvalylaspartic acid fluoromethyl ketone. This is a potential inhibitor of caspase-3 and caspase-3-like caspases. Caspase-8, CaspaTag Caspase-8 (LETD) activity kit has the FAM-LETD-FMK inhibitor. This is a carboxyfluorescein analogue of benzyloxycarbonyl-leucylglutamylthreonylaspartic acid fluoromethyl ketone (zLETD-FMK) that is a potential inhibitor of caspase-8. Caspase-9, CaspaTag Caspase-9 (LEHD) activity kit has the FAM-LEHD-FMK inhibitor. This is a carboxyfluorescein analogue of benzyloxycarbonyl-leucylglutamylhistidylaspartic acid fluoromethyl ketone (zLEHD-FMK) that is a potential inhibitor of caspase-9. These inhibitors are cell permeable and noncytotoxic.

The detection of activated caspases by application of FLICA was performed with flow cytometry. In brief, a 150-fold stock solution of the inhibitor was prepared in dimethyl sulfoxide (DMSO). It was further diluted in phosphate buffered saline (PBS) to make a 30-fold working so-

TABLE 1. Measurement of activated caspases, intact mitochondrial membrane potential ($\Delta\psi_m$), and DNA fragmentation (TUNEL$^+$) in fresh and cryopreserved spermatozoa after adjusting for effects due to ANMB$^+$/ANMB$^-$ and mature/immature subfractions.[a]

Parameter	Caspase-3	Caspase-8	Caspase-9	$\Delta\psi_m$	TUNEL$^+$
Fresh (%)	39.6 ± 3.7	41.3 ± 3.6	41.3 ± 3.9	49.6 ± 3.9	33.8 ± 4.1
Cryopreserved (%)	55.4 ± 3.2	54.6 ± 3.1	57.4 ± 3.3	37.6 ± 3.5	40.2 ± 3.6
Difference (%)	15.8 ± 4.9[a]	13.4 ± 4.8[a]	16.2 ± 5.2[a]	−12.0 ± 5.2[b]	6.4 ± 5.4

[a] Values from repeated measures analysis model are expressed as least squares mean ± SEM.
[b] $P < 0.05$ was significant.

lution. The prepared aliquots of all fractions were incubated at 37°C for 20 min with 10 μl of the working solution, washed with the rinse buffer provided with the kit, and analyzed by flow cytometry. Human neutrophils (5×10^6 cells) treated with 1 mM cycloheximide for 6 h were used as positive controls for induction of apoptosis [22]. The negative controls were processed identically for each fraction, except that the stain was replaced with 10 μl PBS.

To ensure measurement of true caspase activity, we added a pan-caspase inhibitor (Ac-DEVD-CHO, Alexis, Germany) to the spermatozoa during artificial capacitation (30% BSA in BWW, 5% CO_2 over 3 h). The inhibitor was replaced with an equal volume of Z-FA-FMK (Alexis). Activation of caspase-3, -9, and -8 and mitochondrial transmembrane potential were measured. In addition, the percentage of cells with hyperactivation and linear motility was assessed by computer-assisted sperm-motion analyzer. The process of capacitation was monitored by chlortetracycline assay and protein phosphorylation was assessed by Western blotting.

Detection of Mitochochondrial Membrane Potential

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 vital 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 for 20 min in 1 μg of the lipophilic cation diluted in 1 ml PBS.

In brief, sperm conjugated with fluorescein isothiocynate (FITC) and rhodamine isothicynate (RITC) conjugated *Pisum sativum* were used as control settings after induction of acrosome reaction (with 30% albumin prepared in BWW medium [vol/vol] at 37°C and 5% CO_2 incubation) for fluorescence detection within the FHL1 and FHL2 channel. Whole ejaculate sperm cells were used to set the forward and side scatter for the FACS device for each run. Human neutrophils (5×10^6 cells) treated with 1 mM cyclohexamide for 6 h were used as positive controls for apoptosis [40]. Negative controls were processed identically for each fraction except that the stain was replaced with 10 μl PBS. In addition, the lipophilic cation used is a bicolor probe, which includes both the positive and negative dye. The percentage of spermatozoa having intact mitochondria is indicated by $\Delta\psi_m$. In addition, the lipophilic cation used (Mitosensor) is a bicolor probe, which includes both the positive and negative dyes.

Evaluation of DNA Fragmentation in Spermatozoa

The proportion of cells with DNA fragmentation was measured in the same fractions using the TUNEL assay and flow cytometry Kit for Apoptosis, (APO-DIRECT; Chemicon, USA). The assay was used according to the instructions of the manufacturer. Briefly, the sperm pellets were resuspended in 1% (w/v) paraformaldehyde in PBS at a concentration of 1–2 $\times$ 10^6/ml after the superparamagnetic bead separation procedure. Once the pellets were placed on ice for 30–60 min, ice-cold 70% (v/v) ethanol was added.

Somatic cells were used without (negative control) and after induction of apoptosis (positive control) for each run according to the manufacturer's instructions. Two washings were done to elute the ethanol before incubating the cells at 37°C for 60 min with staining solution (TdT reaction buffer, TdT enzyme, fluorescein-dUTP, distilled H_2O). The samples were rinsed to stop incubation and then resuspended in 0.5 ml of propidium iodide/Rnase A solution. The cells were incubated in the dark for 30 min at room temperature. Flow cytometry analysis of DNA fragmentation was performed within 3 h. The negative controls were processed identically for each fraction except that the TdT enzyme was replaced with an equal volume of PBS.

Flow Cytometric Analysis of Activated Caspases, Trans*-Membrane Mitochondrial Potential and DNA Fragmentation*

The extent of activated caspase-8, -9, and -3, $\Delta\psi_m$, and DNA fragmentation was evaluated by flow cytometry analyses. All the fluorescence signals of labeled spermatozoa were analyzed by the flow cytometer FACscan (Becton Dickinson). A minimum of 10 000 spermatozoa was examined for each assay at a flow rate of <100 cells/sec. The sperm population was gated using 90-degree and forward-angle light scatter to exclude debris and aggregates. The excitation wavelength was 488 nm supplied by an argon laser at 15 mW. Green fluorescence (480–530 nm) was measured in the FHL1 channel and red fluorescence (580–630 nm) in the FHL-2 channel. The percentage of positive cells and the mean fluorescence was calculated on a 1023-channel scale by software Expo32ADC (Coulter, Germany).

RESULTS

Cryopreservation was significantly associated with activation of caspases-8, -9, and -3, as well as disruption of the mitochondrial membrane potential, but no significant changes were observed for DNA fragmentation (Table 1, Figs. 1 and 3).

This activation pattern of caspases was also found within the mature and immature fraction of spermatozoa obtained by density gradient centrifugation (Table 2). The proportion of mature and immature cells having intact $\Delta\psi_m$ was lower in the cryopreserved pools than in the fresh pools but did not reach the level of significance in both subfractions.

TABLE 2. Measurement of activated caspases, intact mitochondrial membrane potential, and DNA fragmentation (TUNEL$^+$) in fresh and cryopreserved spermatozoa after density gradient centrifugation in control samples (no superparamagnetic separation).[a]

	Mature fraction		Immature fraction	
Parameter	Fresh	Cryopreserved	Fresh	Cryopreserved
Caspase-3 (%)	20.3 ± 8.6	38.1 ± 13.3[b]	38.6 ± 11.7	65.7 ± 10.1[b]
Caspase-8 (%)	19.7 ± 8.1	37.1 ± 12.3[b]	37.0 ± 10.9	66.9 ± 9.4[b]
Caspase-9 (%)	22.7 ± 9.0	38.3 ± 10.6[b]	40.6 ± 11.6	63.0 ± 7.5[b]
$\Delta\psi_m$ (%)	58.3 ± 24.8	50.7 ± 23.7	53.1 ± 12.0	37.4 ± 15.9
TUNEL$^+$ (%)	26.0 ± 11.3	36.1 ± 17.5	32.4 ± 11.3	45.6 ± 18.5

[a] Values are expressed as mean ± SD.
[b] $P < 0.025$ (Bonferroni correction) was significant by Student *t*-test.

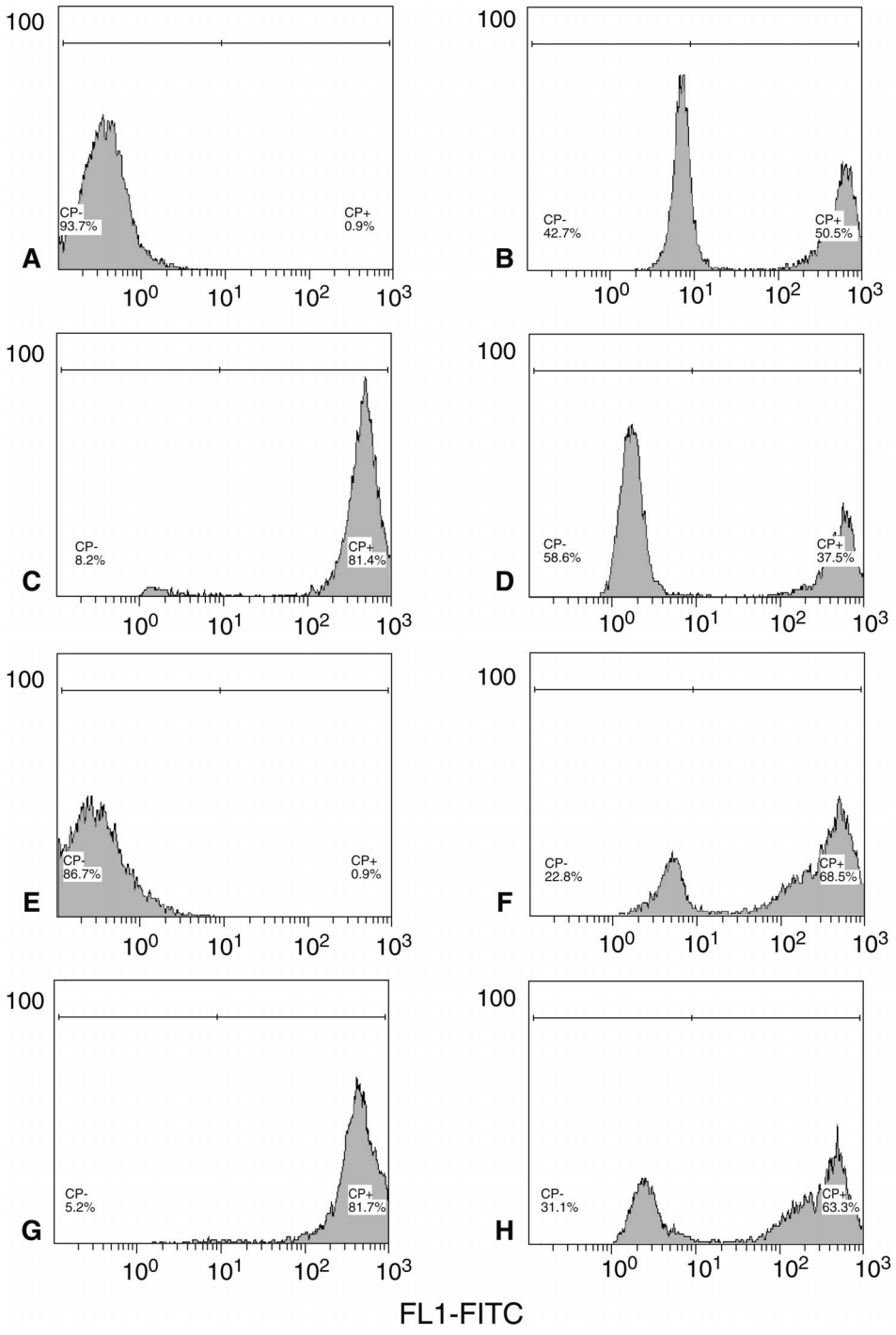

FIG. 1. The X-axis of each histogram depicts the intensity of fluorescence in the green spectrum FITC channel of the cytometer (FL1 channel) on a logarithmic scale. The Y-axis depicts the frequency in terms of the number of cells. The two clearly demarcated cell populations are the caspase-negative (left peak) and caspase-positive (right peak) cells. **A**) Mature negative control, (**B**) mature control, (**C**) mature annexin-labeled superparamagnetic beads (ANMB)$^+$, (**D**) mature (ANMB)$^-$, (**E**) immature negative control, (**F**) immature control, (**G**) immature (ANMB)$^+$, (**H**) immature (ANMB)$^-$.

There were also no significant differences among the fresh and cryopreserved spermatozoa in terms of the proportion of cells having apoptotic DNA fragmentation (TUNEL$^+$, Table 2).

In Tables 1 and 2, the possible interactions have not been considered. Results showing the interactions among cryopreserved/fresh, ANMB$^-$/ANMB$^+$, and mature/immature fractions for samples undergoing magnetic cell separation are reported in Tables 3 and 4. The three-way interactions were significant for activated caspase-3 and -9 and the two-way interactions were significant for activated caspase-8 (cryopreserved/fresh vs. ANMB$^-$/ANMB$^+$) and activated caspase-3 and -9 (mature/immature vs. ANMB$^-$/ANMB$^+$). None of the interactions were significant for $\Delta\psi_m$ or TUNEL$^+$.

All interactions were significant for degree and not direction. This implies that it is meaningful to report the main effects without interactions (Table 3) in each case. Because the within-factor analyses were also interesting, we therefore reported the data in both ways. A significant two-way interaction was seen in the mean levels of activated caspase-8 between fresh vs. cryopreserved and ANMB$^+$ vs. ANMB$^-$ effects ($P = 0.098$; Table 4). Cryopreserved fractions showed significantly higher levels of ANMB$^+$ subfraction compared with ANMB$^-$ subfraction adjusting for mature and immature fractions in the RMA model.

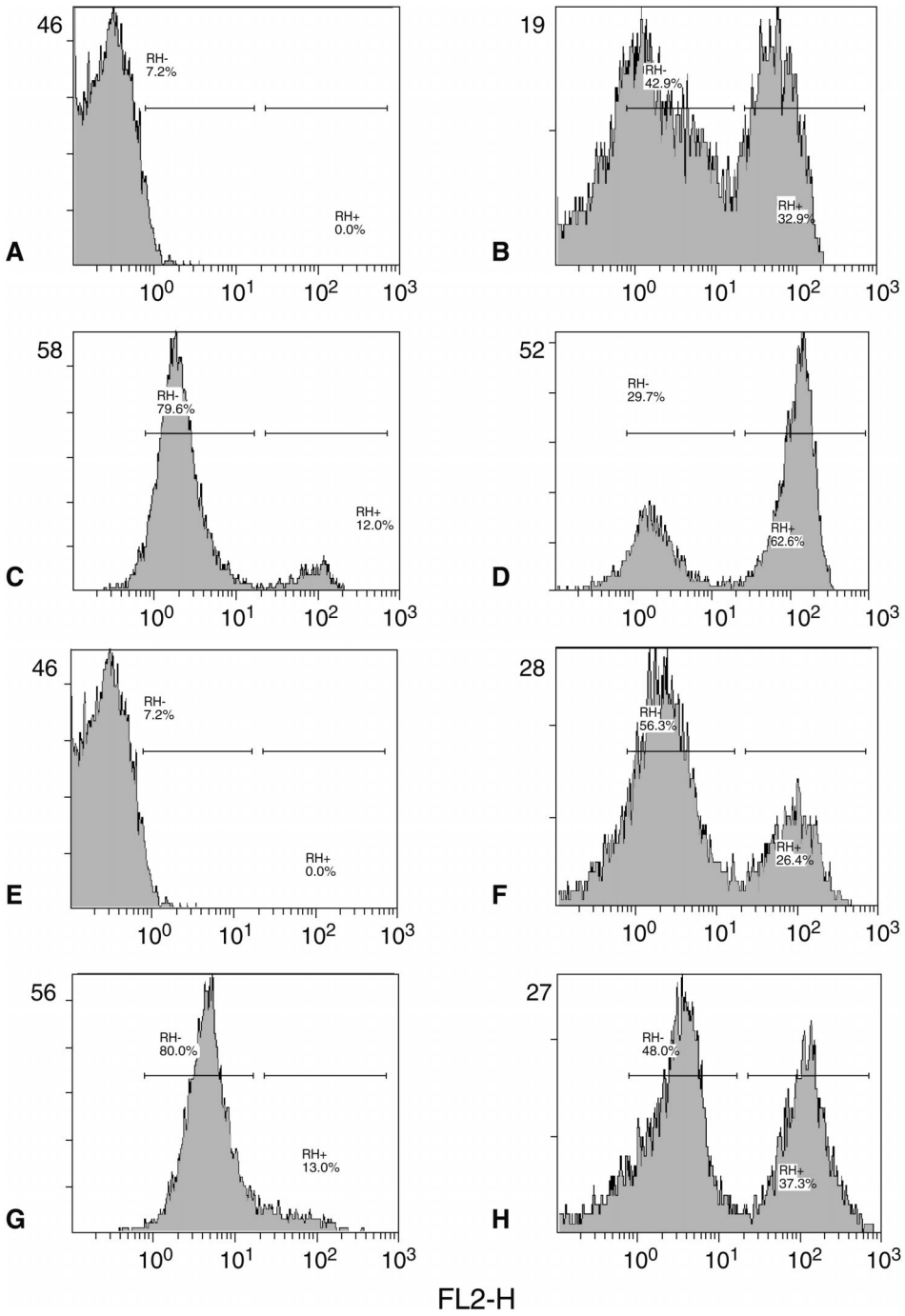

FIG. 2. Sample histograms of spermatozoa in the various subfractions stained with the lipophilic cationic dye for the intact mitochondrial potential ($\Delta\psi_m$) along with the negative controls. The X-axis of each histogram depicts the intensity of fluorescence in the red spectrum (rhodamine channel of the cytometere FHL2 channel) on a logarithmic scale. The Y-axis depicts the frequency in terms of the number of cells. The two clearly demarcated cell populations are the cells with disrupted $\Delta\psi_m$ (left peak) and the cells with intact $\Delta\psi_m$ (right peak). **A**) Mature negative control, (**B**) mature control, (**C**) mature ANMB$^+$, (**D**) mature ANMB$^-$, (**E**) immature negative control, (**F**) immature control, (**G**) immature ANMB$^+$, (**H**) immature ANMB$^-$.

The levels of apoptotic markers in mature and immature spermatozoa after the paramagnetic separation are shown in Tables 5 and 6. Mature annexin V$^-$ sperm were free of significant caspase activation, disruption of the *trans*-membrane mitochondrial potential, and DNA fragmentation after cryopreservation. This subpopulation shows the lowest activation level of all apoptotic markers in comparison with all other fractions in fresh samples and after freezing-thawing.

For ANMB$^+$ subfractions of mature spermatozoa, only caspase-3 levels were significantly higher in cryopreserved pools compared with fresh samples after correcting for multiple comparisons, whereas, to a nonsignificant amount also, the other caspases were activated. No significant differences were seen in the mature ANMB$^+$ subfractions of the fresh and cryopreserved pools in terms of the proportion with intact $\Delta\psi_m$ or apoptotic DNA fragmentation (TUNEL$^+$).

In immature sperm, both ANMB$^+$ and ANMB$^-$ fractions showed activated caspase levels, which further increased after cryopreservation. Compared with the fresh pools, significantly lower proportions of cells with intact $\Delta\psi_m$ were seen in the immature ANMB$^-$ fraction of the cryopreserved pools. No significant differences were seen among the fresh and cryopreserved pools in terms of the proportion of cells having apoptotic DNA fragmentation (Table 6).

Both the mature and the immature ANMB$^+$ subfractions had significantly higher levels of activated caspases in the cryopreserved pools compared with fresh pools (0.05 significance criterion), the immature ANMB$^-$ subfraction of

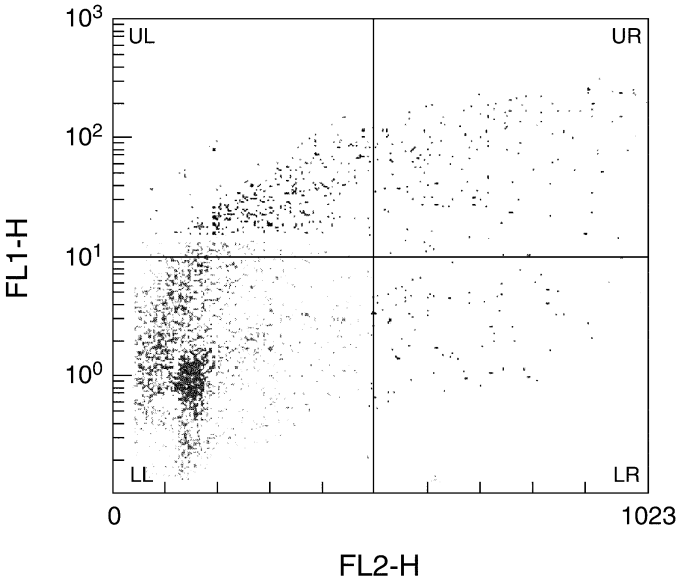

FIG. 3. Sample cytogram of sperm sample stained for the TUNEL assay. The X-axis depicts the intensity of fluorescence in the red spectrum (FHL2 channel) on a linear scale. The Y-axis depicts the intensity of fluorescence in the green spectrum (FITC channel of the cytometer; FL1 channel) on a logarithmic scale. The gate setting divides the cells in four populations: upper left (UL), early apoptotic cells; upper right (UR), late apoptotic cells; Lower left (LL), vital cells; lower right (LR), necrotic cells. Cells in the UL and UR regions combined together give the proportion with DNA fragmentation.

the cryopreserved pools also had significantly higher levels of activated caspase-3 and -9 compared with the fresh pools. No difference was found in the levels of activated caspase-8.

Even though the differences in caspase activation within the various subgroups were small, they were close to the level of significance. Additional samples were examined to study the biological impact of caspase activation on the physiological role such as sperm function. Caspase inhibition resulted in a significant reduction of both capacitated and phosphorylated sperm. The percentage of hyperactivated sperm decreased from 14.0% ± 8.0% to 6.1% ± 4.4%. Capacitation performed in the presence of caspase inhibitor resulted in a decrease in caspase activation (19.5% ± 16.9% vs. 12.2% ± 7.2%; $P < 0.05$). Finally, inhibition of caspase activation resulted in a significant enhancement in the intact membrane potential (70.9% ± 16.0% vs. 88.8% ± 7.3% $P < 0.05$).

DISCUSSION

The results obtained in our study indicate for the first time that cryopreservation and thawing triggers caspases and disruption of $\Delta\psi_m$ to varying extents, thereby providing evidence in favor of our study hypothesis. Freezing showed a significant overall activation of caspases with a decrease in mitochondrial membrane potential. Although this decrease in mitochondrial membrane potential was only 12% ($P < 0.05$) in fresh and frozen ejaculates without separation into immature and mature spermatozoa (Table 1), it is an important finding. This decrease was also observed in fractions separated by density gradient. Both mature and immature fractions had significant activation of caspases followed by a decreased number of sperm with intact mitochondrial membrane potential after cryopreservation.

Although the activation of caspases in response to cryopreservation and thawing was fairly uniform, the mechanism of activation differed between annexin V^+ and annexin V^- fractions in both mature and immature sperm fractions. We found that all cryopreserved $ANMB^+$ samples showed higher levels of caspase-3, -8, and -9 activity (Tables 5 and 6), similar to our earlier reports [24].

We found significant interactions between the effects of fresh vs. cryopreserved, mature vs. immature, and $ANMB^+$ vs. $ANMB^-$ fractions on the levels of activated caspase (Tables 3 and 4). These interactions strongly suggest that cryopreservation and thawing triggers activated caspase activity in spermatozoa by a mechanism that may be linked to the translocation of phosphatidylserine to the surface of the cell. It also suggests that this mechanism may be different for mature and immature spermatozoa. Selection of annexin V^- mature sperm after freezing might be of clinical relevance because there were no statistical increases either in caspase activity or in disruption of $\Delta\psi_m$ after freezing and thawing. Also, this subfraction displayed the least amount of DNA damage. This finding is supported by our earlier study, where we did not find any activation of caspase-9 after freezing of mature spermatozoa following separation on a density gradient, whereas spermatozoa with activated caspase-8 and -3 could be removed after thawing by passage through MACS (Grunewald et al., unpublished data). The importance of caspase activation due to external triggers, e.g., cryopreservation, is reflected by the additional experiments that confirm multiple effects on spermatozoa subjected to capacitation. A negative influence of caspase activation was also evident in various molecular checkpoints, e.g., phosphorylation, hyperactivation, and integrity of the mitochondrial membrane potential.

TUNEL, although popular, cannot by itself distinguish between apoptosis and necrosis [8, 41–43]; therefore, it is

TABLE 3. Interactions among cryopreserved/fresh, mature/immature, and $ANMB^+/ANMB^-$ subfractions after superparamagnetic cell separation.[a]

Parameter	3-way *P*-values[b]	2-way *P*-values: Cryopreserved/fresh vs. Mature/immature	Cryopreserved/fresh vs. $ANMB^+/ANMB^-$	Mature/immature vs. $ANMB^+/ANMB^-$
Caspase-3 (%)	0.03[c]	0.53	0.26	0.06[c]
Caspase-8 (%)	0.44	0.59	0.098[c]	0.01[c]
Caspase-9 (%)	0.02[c]	0.60	0.54	0.04[c]
$\Delta\psi_m$ (%)	0.51	0.98	0.47	0.46
$TUNEL^+$ (%)	0.97	0.95	0.52	0.76

[a] Repeated measures analysis model adjusting for cryopreserved/fresh, mature/immature, and $ANMB^+/ANMB^-$ accounting for correlation within pool.
[b] Interactions with $P < 0.10$ were significant.
[c] Cryopreserved/fresh, mature/immature, and $ANMB^+/ANMB^-$.

TABLE 4. Two-way interactions between impact of cryopreservation and $ANMB^+ANMB^-$ on activated caspase-8 while adjusting for immature/mature subfractions (interaction: $P = 0.098$).[a]

Subfraction	Cryopreserved	Fresh	Difference[b]
$ANMB^+$ (%)	78.0 ± 3.5	60.2 ± 4.1	17.8 ± 5.5[c]
$ANMB^-$ (%)	31.3 ± 3.5	22.3 ± 4.1	9 ± 4.5
Difference[b] (%)	46.7 ± 3.5[c]	37.9 ± 4.1[c]	

[a] Repeated measures analysis model with adjusted mean ± SEM and differences in means ± SEM reported.
[b] Difference of 2 rows or columns means ± SEM.
[c] $P < 0.0125$ significant (Bonferroni correction).

TABLE 5. Measurement of activated caspases, intact mitochondrial membrane potential, and DNA fragmentation (TUNEL$^+$) in mature fresh and cryopreserved spermatozoa after superparamagnetic separation.[a]

	Mature ANMB$^+$		Mature ANMB$^-$	
Parameter	Fresh	Cryopreserved	Fresh	Cryopreserved
Caspase-3 (%)	51.0 ± 18.5	75.7 ± 13.2[b]	9.4 ± 4.9	19.2 ± 15.0
Caspase-8 (%)	54.3 ± 17.5	72.6 ± 12.6	11.5 ± 5.9	17.0 ± 9.6
Caspase-9 (%)	56.5 ± 22.7	77.5 ± 10.8	10.4 ± 5.6	19.5 ± 12.7
$\Delta\psi_m$ (%)	31.7 ± 23.5	20.0 ± 12.0	75.8 ± 15.5	63.7 ± 29.3
TUNEL$^+$ (%)	45.3 ± 9.0	53.3 ± 12.4	19.3 ± 14.5	24.4 ± 16.0

[a] Values are expressed as mean ± SD.
[b] $P \leq 0.0125$ (Bonferroni correction) significant by Student *t*-test.

important to complement annexin-V/propidium iodide staining with other markers of DNA damage [44]. The lack of significant differences among the fresh and cryopreserved spermatozoa in terms of the proportion of cells having apoptotic DNA fragmentation (Table 2) can be explained as also the mature ANMB$^+$ subfractions of the fresh and cryopreserved pools in terms of the proportion of apoptotic DNA fragmentation as evident by TUNEL assay by our recent publication [45]. We demonstrated that, although there was a strong correlation between apoptosis and sperm DNA damage in the patient group in whole ejaculate, the total amount of DNA damage in the immature and mature spermatozoa cannot be explained by apoptosis alone. Apoptosis can explain only a fraction of such DNA damage [12]. DNA damage can also occur due to oxidative stress, which was not examined in the present study [46–49].

As a first step in our study, our intent was to identify important members of the apoptotic machinery. Inhibiting caspase activity before cryopreservation of sperm did not enhance the postthaw motility [50]. However, we were able to prevent caspase activation and downstream signaling in ejaculated spermatozoa by inhibiting caspases (unpublished results). In other studies, although addition of staurosporine or peptide caspase inhibitors did not accelerate or delay cell death, staining was observed with an anti-caspase-3 antibody using indirect immunofluorescence [51]. Additional studies are needed to further assist in our understanding of the signaling pathways that may be present in a highly specialized cell such as spermatozoa.

Mature annexin V$^+$ fraction showed a higher degree of caspase activation, disruption of $\Delta\psi_m$, and TUNEL$^+$ compared with the annexin V$^-$ fraction following cryopreservation. Receptor-mediated apoptosis signaling and the role of mitochondria in amplifying death signals may not be operational in this subfraction because no significant disruption in the $\Delta\psi_m$ was seen, in contrast with the significant amounts of caspase-3 activation (Table 5). We therefore speculate that, in the annexin V$^+$ mature spermatozoa, freezing and thawing triggers the final executor caspase-3 directly by some mitochondria-dependent but caspase-8-independent mechanism similar to that observed in somatic cells [52]. These differences also suggest that annexin V$^+$ sperm correspond to a group of cells that may have a higher degree of dysfunctional mitochondria or that these mitochondria may have already undergone disruption of the mitochondrial membrane potential.

The annexin V$^-$ immature sperm in our study were found to have activated caspases-9 and -3 (Table 6). Caspase-8 was also seen to be activated, although to a lesser degree. This activation might be attributed to the ability of caspases to activate each other during terminal stages of apoptosis [53]. After annexin V sorting, the immature ANMB$^-$ sperm fraction showed a significant decrease of mitochondrial membrane potential between fresh and cryopreserved aliquots (Table 6).

Cytochrome c released due to mitochondrial damage does not activate procaspase-8 but is known to primarily activate procaspase-9 [52, 54–57] and downstream assembling of the apoptosome in response to Bcl-2 family proapoptotic signals, free radicals, and other cellular stress [58]. Consequently, the reaction pattern of annexin V$^-$ immature sperm following cryopreservation is similar to cells undergoing Type-II (mitochondrial) apoptosis. Recent studies suggest that caspase-8 is not always activated early in the context of Fas (CD95) signaling [57, 59–61]. This has led to the suggestion of Type I [27, 62–65] and Type II apoptosis [66–68]. Fas receptors have been detected on the surface of intratesticular germ cells [64] as well as in subsets of ejaculated spermatozoa [6, 12, 13]. However, their functional impact during spermatogenesis is controversial. Men with abnormal semen parameters have higher levels of Fas expression in their ejaculated spermatozoa, indicating that an abortive apoptosis has taken place [12, 13]. Several investigations have proposed a different role for the Fas/FasLigand other than mediating apoptosis [18, 69]. In a recent study, we demonstrated that a subpopulation of spermatozoa might have a different frequency of Fas distribution following cryopreservation [21]. Presence of Fas on ejaculated spermatozoa correlate strongly both with decreased sperm concentration and with abnormal morphol-

TABLE 6. Measurement of activated caspases, intact mitochondrial membrane potential, and DNA fragmentation (TUNEL$^+$) in the immature fresh and cryopreserved spermatozoa after superparamagnetic separation.[a]

	Immature ANMB$^+$		Immature ANMB$^-$	
Parameter	Fresh	Cryopreserved	Fresh	Cryopreserved
Caspase-3 (%)	71.1 ± 13.8	83.2 ± 8.9	26.9 ± 8.7	43.5 ± 15.7[b]
Caspase-8 (%)	66.1 ± 16.4	83.4 ± 11.1	32.9 ± 20.5	45.5 ± 15.0
Caspase-9 (%)	73.2 ± 13.6	87.0 ± 8.2	25.0 ± 9.8	45.7 ± 15.7[b]
$\Delta\psi_m$ (%)	23.4 ± 12.4	16.4 ± 10.6	67.6 ± 8.3	50.4 ± 14.0[b]
TUNEL$^+$ (%)	47.6 ± 11.9	55.3 ± 17.8	23.1 ± 15.9	27.8 ± 13.2

[a] Values are expressed as mean ± SD.
[b] $P \leq 0.0125$ (Bonferroni correction) significant by Student *t*-test.

ogy. Cryopreservation also led to conflicting results in Fas distribution depending on the method used, i.e., native vs. cryopreserved samples: donors ($P > 0.05$) and patients ($P < 0.01$). A 72% increase in Fas staining was seen following MACS separation irrespective of whether or not the samples were cryopreserved. We have demonstrated earlier the presence of Fas by immunohistochemistry in testicular tissue of infertility patients [6]. We, therefore, hypothesized that immature annexin V-positive sperm might also be capable of undergoing Type I apoptosis. Activation of Fas receptor leads to annexin V flip-flop and activation of caspases and may also influence mitochondrial membrane potential [6], and caspase inhibitors hamper activation of Fas receptor. However, a recent study failed to demonstrate substantial amounts of Fas receptors on the ejaculated spermatozoa of normozoospermic and nonnormozoospermic men and does not support the abortive apoptosis theory [70]. Further research is needed to clarify the role of Fas in spermatogenesis and in ejaculated sperm.

Cryopreservation-related changes at the membrane of mature spermatozoa indicate that the cells that are truly positive for the phosphatidylserine transition are in the process of undergoing apoptosis, i.e., they are already dying, and thus one would expect the caspases to be activated. In contrast, annexin V^+ immature sperm had significantly activated caspases-8 and -9 and the highest levels of activation of caspase-3, although this did not reach statistical significance. One explanation for the lack of significant caspase-3 activation may be attributed to the fact that the increase of activation was less in comparison with the fraction of mature annexin V^+ sperm (12.1% vs. 24.7%).

Our studies lend further support to the hypothesis of abortive apoptosis because cryopreservation of annexin V^+ spermatozoa resulted in activation of caspase-8 in these subfractions [11, 13]. Activation of apoptosis is likely by an unknown mitochondrial-independent mechanism because it cannot account for caspase-3 activation in the absence of a change in mitochondrial membrane potential [51]. The activation of caspases was accompanied by a loss of mitochondrial transmembrane potential, although it did not reach the level of significance. Technical, biological, and other unknown factors may explain this. The more plausible explanation would be that immature annexin V^+ sperm are not only abnormal in terms of morphological appearance but also in terms of functional maturity, i.e., mitochondrial membrane integrity. Therefore, these sperm have smaller absolute values to start with compared with the annexin V^- fraction. Our results thus demonstrate that, in annexin V^+ immature sperm, the majority of the sperm may be apoptotic as measured by caspase activation either due to a surface receptor mechanism or mitochondrial injury, whereas annexin V^- immature sperm may be apoptotic largely due to mitochondrial injury. Activation of caspase-8, -9, and -3 in concert with disruption of $\Delta\psi_m$ in immature annexin V^+ fractions does not immediately affect the levels of DNA fragmentation. The inability of the postthaw apoptotic machinery to damage sperm DNA may be due to its tightly packed nature. Another reason might be the short postthaw incubation period, whereby activated enzymes may not have had sufficient time to produce DNA strand breaks. These differences might also be related to the different techniques employed [8, 18, 19, 69].

Although we did not examine the fertilizing ability of apoptotic spermatozoa, other studies have demonstrated that the presence of spermatozoa with damaged DNA is indicative of apoptosis [9, 41, 71]. Additional research is needed to clearly establish the role of apoptosis in DNA damage and assisted reproductive techniques outcome. Several studies report significant correlation between sperm DNA damage and fertilization as well as pregnancy rates following both conventional in vitro fertilization and ICSI [14, 48, 72–74].

Our study design was limited by the fact that we used pooled semen samples instead of single ejaculates. The primary reason was to ensure the availability of an adequate number of cells to carry out the various assays following two serial separations. The protocol involved about 40 aliquots to be processed for each pool and about 5–9 washing and resuspension steps per aliquot. To ensure variability among the pools, the donor samples were randomly assigned to each pool. The effects studied represent effects on a pool of participants, and so within-subject comparisons and therefore within-subject conclusions were not possible. However, the results are more applicable on a cellular level, describing some of the characteristics of the ejaculated human spermatozoon.

In conclusion, cryopreservation and thawing induces caspase activation in mature annexin V^+ in ejaculated human spermatozoa as well as in immature annexin V^+ and annexin V^- sperm. It is also associated with disruption of the $\Delta\psi_m$ in the immature annexin V^- fraction. Cells showing this phenomenon can be separated by the annexin V superparamagnetic separation technique. Immature annexin V^+ spermatozoa exhibit Type-I and Type-II apoptosis, whereas mature annexin V^- spermatozoa are unlikely to be apoptotic. Cells without susceptibility to apoptosis activation can thus be separated by MACS, which may prove to be of clinical relevance.

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