Blackwell Publishing

Asian J Androl 2005; 7 (1): 61–69
DOI: 10.1111/j.1745-7262.2005.00009.x

AJA

·*Original Article*·

Immunomagnetic removal of cryo-damaged human spermatozoa

Uwe Paasch, Sonja Grunewald, Katja Wuendrich, Torsten Jope, Hans-Jurgen Glander

Division of Andrology, Department of Dermatology, University of Leipzig, Leipzig D-04103, Germany

Abstract

Aim: To estimate the dissipation of mitochondrial transmembrane potential (mTMP, $\Delta\psi_m$) and activation of sperm caspases (aCP) as signs of apoptosis in human spermatozoa during cryopreservation and to evaluate the efficiency of immunomagnetic cell separation (MACS) of these spermatozoa via annexin V-binding. **Methods:** The mTMP and aCP in fresh and cryopreserved spermatozoa were detected by fluorescence microscopy and by Western blots. The sperm suspensions were divided into two sperm fractions (with intact and deteriorated membranes) by magnetic cell separation (MiniMACS, Miltenyi Biotec, Bergisch Gladbach, Germany) in dependence on their binding to superparamagnetic annexin V-microbeads (AN-MB). **Results:** The cryopreservation decreased the portion of spermatozoa with intact mTMP from 80.1 % ± 7.2 % to 53.5 % ± 13.1 % and increased the spermatozoa with activated pan-caspases (aCP) from 21.8 % ± 2.6 % to 47.7 % ± 5.8 % (n = 10; mean ± SEM; $P < 0.01$). The activation of caspases 1, 3, 8, and 9 in the cryopreserved spermatozoa was confirmed by Western blots (n = 22). MACS reduced significantly the percentage of cryopreserved spermatozoa with dissipated mTMP to 8.1 ± 3.9 ($P < 0.01$) and also those with aCP to 9.3 % ± 2.2 %. Western blot analyses confirmed the increase of the activated caspase3, 9, and 8 in the AN-MB-positive fraction ($P < 0.05$) compared with the AN-MB-negative fraction. The MACS separation effect was confirmed by anti-annexin V-antibodies. There was no significant influence of the separation column and the magnetic field on the sperm functions. **Conclusion:** The cryopreservation impaired the mTMP and enhanced the activation status of caspases in human spermatozoa. The immunomagnetic sperm separation via binding of AN-MB could deplete low quality spermatozoa from cryopreserved semen samples. *(Asian J Androl 2005 Mar; 7: 61–69)*

Keywords: spermatozoa; cryopreservation; immunomagnetic separation; plasma membrane; annexin V; apoptosis; caspases; mitochondria

1 Introduction

Cryopreservation of semen specimens has become increasingly necessary for patients with malignancies before radiation or chemotherapy and is administered in order to perform assisted reproductive procedures later. The programmed cell death (PCD, the apoptosis) very likely contributes to the decrease of sperm vitality after cryopreservation. Cryopreservation and thawing are associated with impairment of mitochondrial transmembrane potential (mTMP), activation of caspases, and impairment of the membrane integrity, including the translocation of phosphatidylserine from the inner to the outer leaflet of the sperm plasma-membrane [1–3] as parts of the apoptosis machinery.

Correspondence to: Hans-Jurgen Glander, Professor of Andrology, Division of Andrology, Department of Dermatology, University of Leipzig, Stephanstrasse 11, D-04103 Leipzig, Germany.
Tel: +49-341-971-8640, Fax: +49-341-971-8649
E–mail: glah@medizin.uni-leipzig.de
Received 2004-06-15 Accepted 2004-11-18

Caspases (cytosolic cysteine containing aspartate specific proteases, CP) are transducers within the different pathways of apoptosis signaling network. Caspases cleave their target proteins at the amino acid aspartate if they become proteolytically activated in a cascade. From a functional point of view, caspases act either as initiators (CP 8, 9, and 10) or as effectors (CP 3, 6, and 7) in apoptosis [4]. Caspase 8 was identified to be the most important initiator enzyme of the caspase cascade. Caspase 9 interacts together with many other regulators and transductors in apoptosis via mitochondria. Caspase 1 transduces inflammation signals and links these processes with apoptosis. All these initiator caspases are activators of downstream caspases. Caspase 3, the most important among them, executes the final disassembly of the cell by cleaving a variety of cell structure proteins and generation of DNA strand breaks [4].

The externalization of the phospholipid phosphatidylserine (EPS) from the inner to the outer leaflet of the plasma membrane is an early feature of the terminal phase of apoptosis [5] and can be monitored by annexin V-binding. Depending on Ca^{2+}, annexin V has a high affinity for phosphatidylserine (PS) in humans. Annexin V is a 35–36 kD phospholipid binding protein that cannot pass the intact plasma membrane. PS is accessible for annexin V on the external surface of plasma membrane of apoptotic spermatozoa as well as on the permeable membrane of dead spermatozoa [1, 2]. Colloidal superparamagnetic microbeads conjugated with annexin V (Miltenyi Biotec, Bergisch Gladbach, Germany) bind to the dead and apoptotic spermatozoa and retain them within an external magnetic field provided by separation columns (magnetic cell separation, MACS). This method may also be promising for patients who have already been exposed to chemotherapy or radiation with negative effects on spermatozoa before cryopreservation. In this way it may be possible to eliminate such damaged spermatozoa prior to intracytoplasmic sperm injection. For an optimal conception rate it is desirable to mark and to eliminate apoptotic and dead spermatozoa with deteriorated membrane structures.

Therefore, we investigated the effect of cryopreservation on the mitochondrial transmembrane potential (mTMP) and the caspase activation (aCP) in association with membrane integrity of human spermatozoa. There were three objectives of our study: (i) to monitor the mTMP and the activation of caspases in fresh and cryopreserved spermatozoa by fluorescence microscopy and Western blot; (ii) to supervise the separation effects of MACS in relation to mTMP, activation of caspases, and computer assisted sperm motion analysis; and (iii) to evaluate the effects of separation column on sperm functions (i.e. motility, acrosomal status, and eosin-supravital staining).

2 Materials and methods

2.1 Semen samples

Following institutional approval, the semen samples were derived from 14 healthy donors. Written informed consent was obtained. Semen samples were collected by masturbation into sterile, plastic Petri dishes, and were investigated according to the standard guidelines of the WHO [6]. The ejaculates were used for further experiments only if the following requirements were fulfilled: (a) volume of the ejaculate > 2 mL, and had a pH value > 7.2, and < 1×10^6 leukocytes per mL ejaculate; (b) the sperm concentration was greater than 20 million·mL^{-1}; (c) more than 15 % of the spermatozoa showed a normal morphology (strict criteria); and (d) more than 50 % appeared progressively motile. Highly viscous ejaculates were excluded.

2.2 Freezing technique before detection of mitochondrial transmembrane potential (mTMP, $\Delta\psi_m$) and of pancaspases activation

The semen samples were cryostored with freezing medium TEST {TES [N-tris(hydroxymethyl) methyl-2-aminoethanesulfonic acid] and Tris} yolk buffer (TYB), resulting in the best vitality parameters of spermatozoa after cryostorage as previously demonstrated [1]. For cryopreservation the semen samples were diluted dropwise with an equivalent volume of freezing medium TEST yolk buffer containing 12 % (v/v) glycerol (Irvine Sci., Catalogue No. 9971, Santa Ana, California, USA). The samples were placed into Cryo Tubes 2.0 mL (Faust Laborbedarf AG, Schaffhausen, Switzerland) and frozen with the system Nicool LM 10 (Air liquide, Wiesbaden, Germany). A slow cooling rate at level 2 decreasing the temperature from +24 ˚C to +5 ˚C within 15 min, was followed by incubation for 15 min at level 10 for a decrease to –70 ˚C. Finally, the tubes were plunged into liquid nitrogen and stored at –196 ˚C. The thawing was performed by incubation in a 37 ˚C water bath for 5 min.

2.3 Freezing technique before blotting

After determination of sperm concentration the samples were divided into three aliquots. The first aliquot remained untreated (control). The other two aliquots were placed into special Nunc Cryo Tubes (Nalge Nunc International, Roskilde, Denmark) and gently mixed with glycerol to get a final concentration of 7 % or 14 %, respectively. The diluted semen samples were frozen with the system Nicool LM 10 (Compagnie Francaise de Produits Oxygenes) according to a standard protocol. Finally, the tubes were plunged into liquid nitrogen for storage at –196 ˚C. The samples were thawed by an incubation at 37 ˚C for 5 min.

2.4 Detection of mitochondrial transmembrane potential (mTMP, $\Delta\psi_m$)

The integrity of the mitochondrial transmembrane potential (mTMP, $\Delta\psi_m$) was monitored by lipophilic cations (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine chloride, MitoLight™, Chemicon, Temecula, CA, USA). Spermatozoa with intact $\Delta\psi_m$ excite an intense fluorescence in the red range due to forming multimers of aggregates. Green fluorescence of their monomers indicates disrupted mTMP (Figure 1). The results were analyzed by fluorescence microscopy (Jenamed Co., Jena, Germany). No toxic influence of the method on the motility parameters was found ($P > 0.05$).

2.5 Detection of active caspases (cytosolic aspartate specific proteases) in vital spermatozoa

The semen samples were filtered through glass wool to remove the gelatinous masses, diluted with human tubal fluid (HTF) [7] and washed twice with HTF (400 ×*g*, 5 min). The supernatants were discarded and the pellets were used for further experiments. Active pan-caspases were detected in living spermatozoa through the use of the carboxy-fluorescein labelled caspase inhibitor FAM-VAD-FMK (carboxyfluorescein, FAM, derivate of benzyloxycarbonyl valylalanyl aspartic acid fluoromethyl ketone, zVAD-FMK). This cell-permeable and non-cytotoxic caspase inhibitor binds covalently to active caspases 1–9. The fluorogenic substrate becomes fluorescent upon cleavage by the caspases. The detection of activated caspases by the inhibitor was performed according to the instruction manual of the manufacturers of the fluorescein pan-caspase (VAD) activity kit (CaspaTag™, S7300, Intergen Co., Oxford, England) with controls.

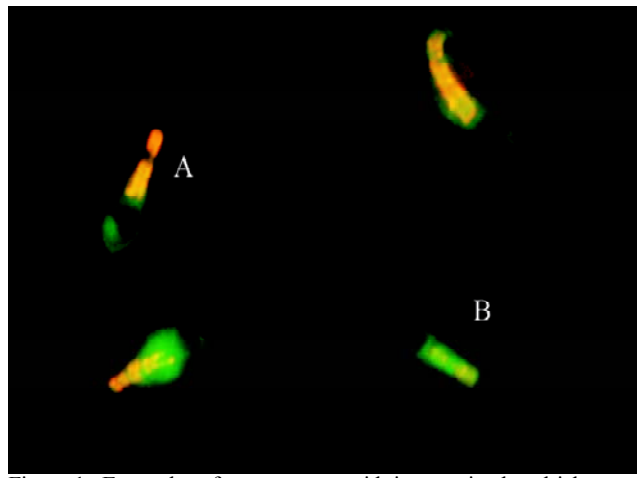

Figure 1. Examples of spermatozoa with intact mitochondrial transmembrane potential, mTMP, $\Delta\psi_m$ (A, red fluorescence in the mid-piece) and dissipated mitochondrial transmembrane potential (B, green fluorescence in the mid-piece).

2.6 Western blotting and densitometric evaluation of caspases

After filtration through glass wool the semen samples ($n = 22$) were diluted with 0.2 mol·L^{-1} Soerensenbuffer, pH 7.4 (90 mL 0.2 mol·L^{-1} $Na_2HPO_4 \cdot 2H_2O$, 10 mL 0.2 mol·L^{-1} $NaH_2PO_4 \cdot H_2O$, 100 mL 16 % NaCl, aqua dest. ad 2 L) and washed twice with 0.2 mol·L^{-1} Soerensenbuffer, pH 7.4 (700 ×*g*, 7 min). After discarding the supernatants the pellets were dissolved in 1.5 volumes of SDS-sample-buffer, pH 6.7 (50 mmol·L^{-1} Tris-HCl, 2 % sodium dodecyl sulfate, SDS, freshly added 1mmol·L^{-1} Na_3VO_4, 2 % mercaptoethanol). Nucleic acids were degraded and viscosity was reduced by 2 % benzonase (Merck, Bad Soden, Germany). The sperm lysates with 14 % (v/v) solution of bromophenol blue (Merck, Darmstadt, Germany) were heated for 5 min at 95 °C (Sample Thermostat, Gebrüder Liebisch, Bielefeld, Germany). The protein concentration of the samples was determined using amido black [8]. For separation of proteins (15 %) SDS-PAGE was carried out under reducing conditions on a vertical slab gel apparatus (Mini Protean 3 cell, Bio-Rad Laboratories GmbH, Munich, Germany). The method applied corresponded to Laemmli [9]. Each lane was loaded with heated sperm lysate (95 °C, 5 min), containing 50 µg proteins. Recombinant proteins of caspases (Chemicon, Hofheim, Germany) were used as positive controls and prestained BenchMarks™ (Life Technologies, Karlsruhe, Germany)

for the evaluation of the molecular weights. Electrophoresis was performed at 100 V for 10 min followed by 200 V for 1 hour (Power Pac 300, Bio-Rad, Munich, Germany). The protein transfer to nitrocellulose membranes (0.2 µm pore size) followed the method of Kyhse-Andersen [10], who used a discontinuous buffer system in a semidry transfer system (Pegasus, Phase, Luebeck, Germany) at 0.8 mA/cm^2 for 70 min (Protran, Schleicher & Schuell, Germany). Membranes were blocked with 2 % bovine serum albumin (BSA)-Tris-Triton, pH 7.5 for 1 hour (50 mmol·L^{-1} Tris, 150 mmol·L^{-1} NaCl, 0.2 % Triton-X-100) followed by an incubation with the primary antibody rabbit anti human caspase 1 (1:40, Oncogene, Boston, USA), anti human caspase 3 (1:100, A3537, Dako, Hamburg, Germany), anti-human caspase 8 (1:1000, AF 832, R&D Systems, Wiesbaden, Germany) and anti-human caspase 9 (1:500, AB 16970, Chemicon, Hofheim,Germany) in 2 % BSA-solution with 0.03 % NaN_3. Blots were washed with 0.5 % BSA-Tris-Triton-solution six times within 30 min and incubated for 1 hour with peroxidase-conjugated secondary antibody (1:10000, cat.-No: 112-036-003, Dianova, Hamburg, Germany). Non-immune rabbit serum at the same dilution ratio instead of the first antibody was used as negative control. All incubations were performed at 4 °C. Relative differences between protein amounts were examined by Luminol-H_2O_2-detection according to Faulkner and Fridovich [11]. Bound secondary antibodies were visualized by X-ray films (Konica, Type 3A4, BW Plus Roentgen, Kamp-Crutfort). Analysis of the fluorescence signals was performed by the ChemilmageTM 4400-System (Alpha Innotech Corporation, San Leandro, Canada). The sum of all pixel data of each protein signal was examined after background correction using AlphaEase software (Biozym Diagnostik, Oldendorf, Germany). Fluorescent signals of unfrozen spermatozoa were each set as 100 %. Each signal was measured three times and the average was then calculated. The protein signals of each Western blot were independently evaluated by determination with a fixed camera adjustment.

2.7 Depletion of apoptotic spermatozoa by magnetic cell separation (MACS)

The sperm suspensions were divided into two sperm fractions by the passage through a magnetic field (MiniMACS) depending on the binding of superparamagnetic annexin V- microbeads (AN-MB) to phosphatidylserine (PS) of spermatozoa.

The washed spermatozoa were incubated with 100 µL AN-MB at room temperature (25 °C) for 15 minutes. The AN-MB-labeled spermatozoa were retained in the separation column, which was placed in a strong permanent magnet, while the non-labeled spermatozoa pass through. After removing the column from the magnetic field, the retained fraction was eluted. There was no significant influence of the separation column and the magnetic field on the sperm functions of semen samples (% spermatozoa before *vs.* after passage; $P > 0.05$), such as progressively motile spermatozoa 32.5 % ± 4.7 % *vs.* 31.5 % ± 4.9 %, spermatozoa with intact acrosome 16.7 % ± 2.0 % *vs.* 15.4 % ± 1.9 %, spermatozoa stainable by eosin 29.2 % ± 6.4 % *vs.* 29.6 % ± 6.7 %. The passage through the column led to a sperm loss of 0.8 % ± 1.2 % (mean ± SEM).

2.8 Evaluation of the separation effect of annexin V-microbeads and immunomagnetic cell separation technique

The separation effect of the MACS system was confirmed by anti annexin V-antibodies conjugated with fluorescein isothiocyanate, FITC (BMS147FI, Bender MedSystems, Vienna, Austria) in flow cytometric analysis [12]. The acrosomal status of the spermatozoa by fluorescence microscopy was evaluated by pisum sativum agglutinin conjugated with FITC.

2.9 Computer-aided sperm motion analysis (CASA)

The Stroemberg-Mika cell motion analyser (Version 4.4, Mika Medical GmbH, Rosenheim, Germany) was applied for determination of sperm motility. From the determined parameters the velocity curve linear (VCL) (µm·s^{-1}), velocity average path (VAP) (µm·s^{-1}), and velocity straight line (VSL) (µm·s^{-1}) were considered in our experiments. The parameters were set as follows: number of frames, 32; minimum area of sperm head, 25 pixels; maximum area, 300 pixels; immotile cells VCL, <5 µm·s^{-1}; non-progressive motile cells VCL, <10 µm·s^{-1}; tail-detection, 5 pixels; and tail-size, 15 pixels. The correct identification of cells as spermatozoa is given by the tail detection system.

2.10 Statistical analysis

Data analyses were performed by non-parametric tests (Mann–Whitney U-test, Friedmann-test, Spearman's test) as appropriate for data type and distribution (investigated with the Shapiro–Wilk-W-test or Kolmogorov–Smirnov-

test). Calculations were performed with the statistical computer program STATISTICA 6.0 Edition for Windows (StatSoft, Inc., Tulsa, OK, USA). $P < 0.05$ were considered as statistically significant.

3 Results

3.1 Motility, detection of mitochondrial transmembrane potential (mTMP, $\Delta\psi_m$) and active pan-caspases, aCP (cytosolic aspartate specific proteases) in vital spermatozoa before and after cryopreservation

The cryopreservation decreased the portion of spermatozoa with intact mTMP from 80.1 % ± 7.2 % in fresh semen samples to 53.5 % ± 13.1 % ($P < 0.01$; Table 1) and increased significantly the percentage of spermatozoa with active caspases (aCP) to 47.7 % ± 5.8 % from 21.8 % ± 2.6 % before cryostorage ($P < 0.01$; Table 2). The motility parameters of the spermatozoa decreased significantly after cryopreservation: WHO motility A+B before *vs.* after cryopreservation: 59.6 % ± 3.7 % *vs*.16.2 % ± 2.9 % ($P < 0.01$); and averages of speeds: VSL 29.4 ± 2.6 *vs*. 21.6 ± 2.2 and VAP 39.5 ± 3.1 *vs*. 30.6 ± 2.0 (μm·s^{-1}, mean ± SEM, $P < 0.05$).

3.2 Western blotting of caspase 1, 3, 8, and 9 in spermatozoa before and after cryostorage

3.2.1 Caspase 1

The 10 kDa- and the 20 kDa-subunits of activated caspase 1 were detectable by Western blot-analysis in spermatozoa before and after cryopreservation. The cryopreservation significantly enhanced the percentage of the 20 kDa caspase 1 subunit. This effect was intensified by an increase in glycerol concentration from 7 % to 14 % ($P < 0.05$; Figure 2A). The small 10 kDa-subunit did not signifi cantly correlate with the percentage of the large 20 kDa-subunit, nor did it significantly change after cryopreservation.

3.2.2 Caspase 3

Immunoblot-signals of 55 kDa, 32 kDa, 17 kDa, and 12 kDa were found for caspase 3 in the ejaculated spermatozoa (Figure 2B). The 32 kDa-band represented the

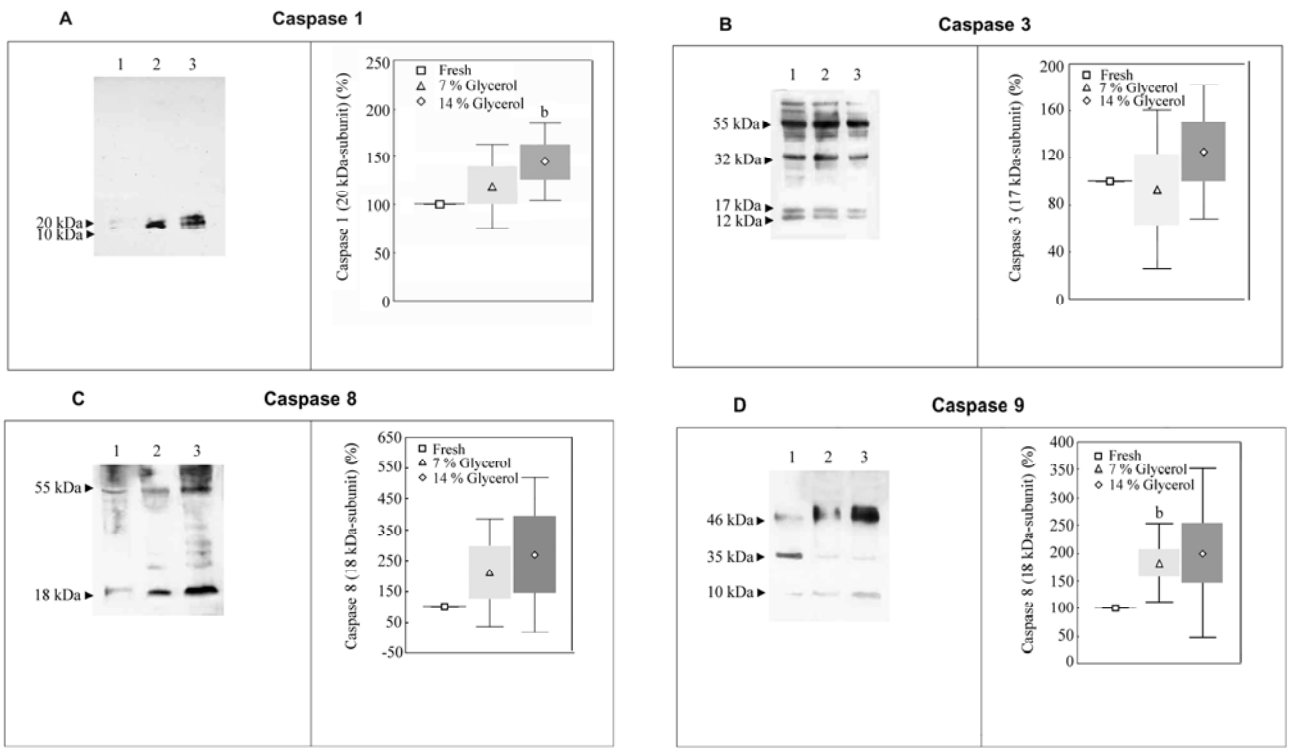

Figure 2. Examples of Western blots of caspase 1 (A), 3 (B), 8 (C), and 9 (D) in cryopreserved spermatozoa compared to control. Western-blot lane 1: control, lane 2: spermatozoa cryopreserved with 7 % glycerol, lane 3: spermatozoa cryopreserved with 14 % glycerol. Relative amount of activated caspase proteins in spermatozoa of healthy donors ($n = 22$). □ control, Δ cryopreservation with 7 % glycerol, ◊cryopreservation with 14 % glycerol. $^bP < 0.05$, compared with control.

Table 1. Percentage of fresh and cryopreserved spermatozoa with intact mitochondrial transmembrane potential (mTMP, $\Delta\psi_m$) before and after immunomagnetic cell separation, MACS. Wilcoxon test, n=10, values with identical superscripts are significantly different (mean ± SD, P< 0.01).

	mTMP (%) (Before MACS)	mTMP (%) (After MACS)	
		AN-MB-negative	AN-MB-positive
Fresh spermatozoa	80.1 ± 7.2[a,e]	90.6 ± 8.2[e,c]	6.8 ± 4.5[c]
Cryopreserved spermatozoa	53.5 ± 13.1[a,f]	88.0 ± 6.6[f,d]	8.1 ± 3.9[d]

precursor of caspase 3 while the two smaller bands of 17 kDa and 12 kDa indicated the active subunits. The detected signal at 55 kDa may represent the known dimer of caspase 3. With increasing concentration of glycerol a non-significant decrease of the relative amount of caspase 3 precursor (32 kDa) in the spermatozoa was detected to 88 % ± 31 % at 7 % glycerol and to 59 % ± 29 % at 14 % glycerol (control 100 %, $P > 0.05$). The decrease of zymogene was associated with an increase of the 17 kDa-subunit to 125 % ± 56 %, but not of the 12 kDa-subunit ($P > 0.05$). The percentage of the 55 kDa-dimer remained almost unchanged ($P > 0.05$).

3.2.3 Caspase 8

The precursor of caspase 8 (55 kDa) and the activated enzyme (18 kDa) could be detected in all semen samples examined (Figure 2C). The cryopreservation non-significantly raised the activated caspase 8 (18 kDa subunit) both with 7 % and 14 % glycerol to 212 % and 270 % of the control, respectively ($P > 0.05$).

3.2.4 Caspase 9

The spermatozoa showed the 46 kDa caspase 9 precursor, the active enzyme (10 kDa) and a 35 kDa-signal (Figure 2D). No significant alterations of caspase 9 precursor in spermatozoa was detected after cryopreservation ($P > 0.05$) independently on the concentration of glycerol. The 35 kDa signal might have been detected because of a non-specific reaction of caspase 9 antibody with an unknown 35 kDa protein, since the applied antibody was not known to be interactive with the 35 kDa protein. However, the cryopreservation led to a significant increase of the activated caspase 9 (10 kDa subunit) in the spermatozoa ($P < 0.05$). Activation was enhanced from 183 % to 200 % by the increase in glycerol concentration from 7 % to 14 % ($P > 0.05$). The 35 kDa-signal was not significantly influenced by the cryopreservation process ($P > 0.05$).

3.3 Magnetic cell separation (MACS)

The AN-MB-negative, non-apoptotic, fresh spermatozoa showed a significantly higher percentage of spermatozoa with intact mTMP $\Delta\psi_m$ than AN-MB-positive ones: 90.6 % ± 8.2 % *vs.* 6.8 % ± 4.5 % ($P < 0.01$), and in cryopreserved spermatozoa the percentages amounted to 88.0 ± 6.6 % *vs.* 8.1 ± 3.9 % ($P < 0.01$; Table 1). Thus, after cryopreservation the number of sperm with intact mTMP $\Delta\psi_m$ was significantly decreased in line with impairment of plasma membrane. The overall motility correlated positive but not significant with the amount of spermatozoa with intact mTMP ($P > 0.05$).

The MACS separation technique also resulted in a significant depletion ($P < 0.001$) of spermatozoa with activated pan-caspases (aCP) in the AN-MB-negative

Table 2. Activated pan-caspases (aCP) in fresh and cryopreserved spermatozoa before and after immunomagnetic cell separation, MACS. [f]Differences between fresh and cryopreserved semen samples are significant $P < 0.01$, compared with the fresh spermatozoa; AN-MB-negative: spermatozoa without ability to bind magnetic annexin V-microbeads; AN-MB-positive: spermatozoa with bound magnetic annexin V-microbeads, mean ± SEM; n = 10 paired semen samples. MACS decreased significantly ([a,b]$P < 0.001$) the percentage of fresh and cryopreserved spermatozoa with aCP.

Sperm characteristics	Fresh spermatozoa	Cryopreserved spermatozoa
AN-MB-positive (%)	14.4 ± 2.5	49.0 ± 8.1[f]
AN-MB-negative (%)	85.7 ± 2.5	51.0 ± 8.1[f]
Pan-aCP before MACS (%)	21.8 ± 2.6[a]	47.7 ± 5.8[f, b]
Pan-aCP in AN-MB-positive (%)	97.7 ± 1.0	89.1 ± 2.3[f]
Pan-aCP in AN-MB-negative(%)	9.2 ± 1.4[a]	9.3 ± 2.2 [b]

Table 3. Activated caspases 1, 3, 8, and 9 after immunomagnetic cell separation (MACS). AN-MB-negative: spermatozoa without ability to bind magnetic annexin V-microbeads was set at 100 %; AN-MB-positive: spermatozoa with bound magnetic annexin V-microbeads; mean ± SEM; $n = 10$, $^bP<0.05$, significant to AN-MB-negative sperm which were set as 100 %.

Caspases-subunits	AN-MB-positive spermatozoa (%)
Caspase 1 (20 kDa-subunit)	70 ± 151
Caspase 3 (12 kDa-subunit)	135 ± 41
Caspase 8 (18 kDa-subunit)	478 ± 87[b]
Caspase 9 (10 kDa-subunit)	128 ± 10

fractions of cryopreserved spermatozoa to 9.3 % ± 2.2 % and in the AN-MB-negative spermatozoa of the fresh semen group to 9.2 % ± 1.4 %. In an incubation period over 5 hours the spermatozoa that have passed the magnetic separation column did not show any significantly different motility and vitality to a control assay. The percentages of aCP in the AN-MB-negative fraction did not significantly differ between fresh and cryopreserved sperm ($P > 0.05$; Table 2). There was a significantly positive correlation between the overall percentage of spermatozoa with aCP and those binding annexin V-microbeads in the fresh as well as in the cryopreserved semen sample group ($r = 0.97$, $r = 0.99$; $P < 0.001$), whereby cryopreservation increased both parameters.

3.3.1 Western blotting of caspase 1,3,8, and 9 in the annexin V-positive- and annexin V-negative sperm fraction

The separation effect of MACS on activation status of caspases in spermatozoa was also evaluated on protein level by Western blot technique. It was found that the caspases 3, 8, and 9 showed a tendency to higher activation in the annexin V-positive spermatozoa compared with the annexin V-negative fraction, which was set as 100 % (Table 3). However, the differences were significant ($P < 0.05$) in caspase 8 only. The activation of caspases was associated with a decline of the enzyme-precursors except the caspase1-precursor (Figure 3).

4 Discussion

Cryopreservation of human sperm is a fundamental tool for the preservation of male fertility [13]. After cooling changes of sperm proteins [14] and phase transitions of the lipids take place in the membrane of spermatozoa. This reordering of membrane components may lead to a loss of stability of the lipid bilayer and exposure of phosphatidyl serine (PS) on the sperm surface, which occurs in nucleated cells during early phases of late apoptosis [5]. Cryoprotectants, in addition to their cryoprotective properties, may be a trigger of the programmed cell death, which is associated with dissipation of mTMP, $\Delta\psi_m$, impairment of the membrane integrity, including the externalization of PS of plasma membrane [1, 2] and aCP [12]. Therefore, measuring the mitochondrial transmembrane potential and caspase activation status might be of interest for the evaluation of cytotoxic potential of cryoprotectives. Recent studies suggested that the machinery of programmed cell death (PCD, apoptosis) including activation of caspases exists in human spermatozoa [1, 2, 12, 15]. Mitochondria are addressed as outstanding organelles in mediation of apoptosis as well as in maintaining a sufficient motility to assure fertilization[3]. The effects we found suggest an apoptosis-like influence on sperm mitochondria by cryopreservation, especially by glycerol.

The caspases activate DNAse, which generates DNA strand breaks [4] resulting in decreased male fertility [15]. Therefore, the amount of human spermatozoa with activated caspases is of clinical interest to minimize their usage in assisted fertilization techniques. The coexistence of activated caspases with externalization of PS or disinte-

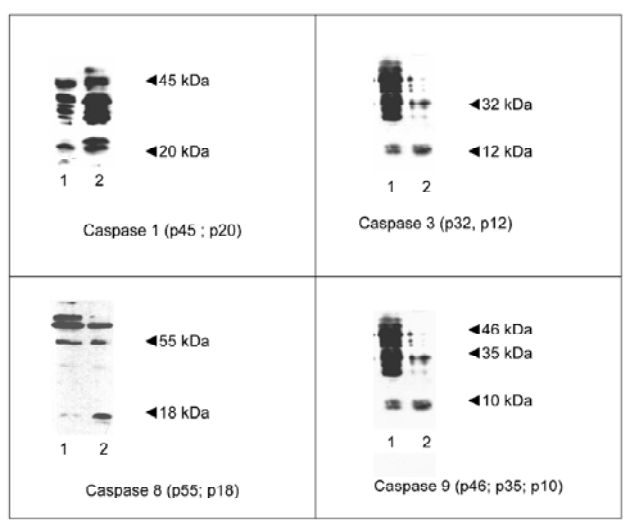

Figure 3. Examples of Western-blots of caspase 1, 3, 8 and 9 after magnetic cell separation (MACS). Western-blot lane 1: spermatozoa without ability to bind magnetic annexin V-microbeads (AN-MB-negative spermatozoa); Western blot lane 2: spermatozoa with bound magnetic annexin V-microbeads (AN-MB-positive spermatozoa)

grated membranes allowed the MACS of such spermatozoa from cryopreserved semen samples because of the specific binding between PS and superparamagnetic annexin-V-conjugated microbeads (AN-MB). The MACS-method is commonly applied in hematology to separate blood stem cells with magnetic immunobeads binding to the surface of the cells [16]. However, it cannot be excluded that the AN-MB-negative fraction contains spermatozoa in a very early phase of PS translocation, binding a few beads only, that are too little to be retained in the column. A deleterious influence of the separation column and the magnetic field on the spermatozoa was not observed.

The differences of activation status of caspases between both sperm fractions after MACS were in context with the results of the computer-assisted sperm motion analysis. Surprisingly, within the AN-MB-positive fraction of the cryopreserved spermatozoa a lower percentage of spermatozoa with activated caspases was revealed compared with fresh spermatozoa. Possibly, TYB mediates an inhibition of the caspases cascade because that phenomenon disappears if cryopreservation is performed without TYB (see blotting experiments). Depending on their function, three groups of caspases could be differentiated: the initiator caspases 2, 8, 9, 10; the effector caspases 3, 6, 7; and caspases 1, 4, 5, 11, 13 involved in inflammation [4, 17]. We examined CP 1, 3, 8, and 9 as caspase 1 is involved in TNF-receptor mediated cell death, caspase 3 executes the final disassembling of the cell by cleaving of a variety of cell structure proteins, caspase 8 is the most important initiator enzyme of the caspase cascade, and caspase 9 triggers the mitochondrial (intrinsic) apoptosis [3, 4, 17].

Standardized cryopreservation resulted in activation of important subtypes of caspases to a varying extent, depending on degree of maturation [18]. Interestingly, the activation status of caspase 8, which is known to mediate membrane receptor apoptosis, showed an association to the impaired integrity of plasma membrane. The activation of caspase 9 after cryopreservation might be explained by the detected loss of mitochondrial transmembrane potential with release of cytochrome c into the cytoplasm and subsequent activation of caspase 9 [3, 4, 17]. Several mechanisms of activation of caspases after cryopreservation may play a role: (i) Cryopreservation of spermatozoa leads to various structural and functional alterations of sperm membranes [1, 2, 15], which may lead to a loss of stability of the lipid bilayer [19] and a sublethal cryodamage of spermatozoa. The impairment of integrity of sperm membrane might initiate the caspase cascade [17]; (ii) Cryoprotectants (e.g. glycerol) in themselves contribute to activation of caspases via direct toxic effects on mitochondria, since cytotoxic stress involves mitochondrial perturbations, followed by DNA fragmentation [3, 15]. Glycerol is assumed to protect cells during cryopreservation but it is not an indifferent substance [13]. Increasing, glycerol concentration elevated the loss of fluidity of sperm plasma membranes and decreased the fertilizing capacity of spermatozoa. A higher activation of caspases was detected in spermatozoa that had been cryopreserved with 14 % glycerol in comparison with 7 % glycerol. With an increase of glycerol concentration, the toxic effect of glycerol may predominate the desired cell protection. Thus, mechanisms associated with apoptotic processes deserve attention in cryopreserved spermatozoa in order to conserve vital sperm functions after thawing. The superparamagnetic annexin V-conjugated microbeads worked as an effective tool to eliminate cryopreserved spermatozoa with impaired membrane structures, which also very likely contain disintegrated DNA. Whether MACS really decrease the ratio of spermatozoa with degenerated DNA and reduced fertilizing capacity shall be examined in the future. Furthermore, the question shall be examined if the separation of immature sperm prior the cryopreservation and the supplementation of the cryopreservation media with antioxidants could improve the recovery of motile sperm with intact DNA following cryopreservation. There are reports indicating that damage during cryopreservation is partly caused by a burst in oxygen radical production mainly by immature sperm [20].

MACS may not only be used for providing a high quality sperm fraction. This technique may also be applied for the evaluation of semen sample quality by detecting the ratio of sperm count between AN-MB-negative and AN-MB-positive fraction. The known feasibility and safety of MACS enrichment procedure in patients with autologous transplantation of peripheral blood stem cells [16] was confirmed by andrological examination methods.

Taken together, the cryopreservation decreases the percentage of spermatozoa with intact mitochondrial transmembrane potential and enhances the activation status of caspases in human spermatozoa. The immunomagnetic sperm separation via binding of annexin V

microbeads is a useful method to enrich vital and non-apoptotic spermatozoa from cryopreserved semen samples.

Acknowledgment

Supported by grants from the German Research Council (DFG, GL 199/4-1).

References

1 Glander HJ, Schaller J. Binding of annexin V to plasma membranes of human spermatozoa: a rapid assay for detection of membrane changes after cryostorage. Mol Human Reprod 1999; 5: 109–15.
2 Duru NK, Morshedi M, Schuffner A, Oehninger S. Cryopreservation-thawing of fractionated human spermatozoa and plasma membrane translocation of phosphatidylserine. Fertil Steril 2001; 75: 263–8.
3 O'Connell M, McClure N, Lewis SE. The effects of cryopreservation on sperm morphology, motility and mitochondrial function. Hum Reprod 2002; 17: 704–9.
4 Earnshaw WC, Martins LM, Kaufmann SH. Mammalian caspases: structure, activation, substrates, and functions during apoptosis. Annu Rev Biochem 1999; 68: 383–424.
5 Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. A novel assay for apoptosis: flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescence labelled annexin V. J Immunol Methods 1995; 184: 39–51.
6 World Health Organization. WHO Laboratory Manual for Examination of Human Semen and Sperm-Cervical Mucus-Interaction, 4th ed. Cambridge: Cambridge University Press, 1999.
7 Quinn P, Kerin JF, Warnes GM. Improved pregnancy rate in -human *in vitro* fertilization with the use of a medium based on the composition of human tubal fluid. Fertil Steril 1985; 44: 493–8.
8 Henkel AW, Bieger SC. Quantification of proteins dissolved in an electrophoresis sample buffer. Anal Biochem 1994; 223: 329–31.
9 Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227: 680–5.
10 Kyhse-Andersen J. Electroblotting of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. J Biochem Biophys Methods 1984; 10: 203–9.
11 Faulkner K, Fridovich I. Luminol and lucigenin as detectors for O_2^{-}. Free Radic Biol Med 1993; 15: 447–51.
12 Paasch U, Grunewald S, Fitzl G, Glander HJ. Deterioration of plasma membrane is associated with activated caspases in human spermatozoa. J Androl 2003; 24: 246–52.
13 McGonagle LS, Goldstein M, Feldschuh J, Foote RH. The influence of cryoprotective media and processing procedures on motility and migration of frozen-thawed human sperm. Asian J Androl 2002; 4: 137–41.
14 Cao WL, Wang YX, Xiang ZQ, Li Z. Cryopreservation-induced decrease in heat-shock protein 90 in human spermatozoa and its mechanism. Asian J Androl 2003; 5: 43–6.
15 Donnelly ET, Steele EK, McClure N, Lewis SE. Assessment of DNA integrity and morphology of ejaculated spermatozoa from fertile and infertile men before and after cryopreservation. Hum Reprod 2001; 16: 1191–9.
16 Despres D, Flohr T, Uppenkamp M, Baldus M, Hoffmann M, Huber C, *et al*. CD34+ cell enrichment for autologous peripheral blood stem cell transplantation by use of the CliniMACs device. J Hematother Stem Cell Res 2000; 9: 557–64.
17 Cohen GM. Caspases: the executioners of apoptosis. Biochem J 1997; 326: 1–16.
18 Weng SL, Taylor SL, Morshedi M, Schuffner A, Duran EH, Beebe S, *et al*. Caspase activity and apoptotic markers in ejaculated human sperm. Mol Hum Reprod 2002; 8: 984–91.
19 Glander HJ, Schiller J, Suss R, Paasch U, Grunewald S, Arnhold J. Deterioration of spermatozoal plasma membrane is associated with an increase of sperm lyso-phosphatidylcholines. Andrologia 2002; 34: 360–6.
20 Park NC, Park HJ, Lee KM, Shin DG. Free radical scavenger effect of rebamipide in sperm processing and cryopreservation. Asian J Androl 2003; 5: 195–201.