

Activation pattern of caspases in human spermatozoa

Uwe Paasch, M.D., Ph.D.,^a Sonja Grunewald, M.S.,^a Ashok Agarwal, Ph.D.,^b and Hans-Jürgen Glandera, M.D., Ph.D.^a

University of Leipzig, Leipzig, Germany, and The Cleveland Clinic Foundation, Cleveland, Ohio

Objective: We investigated the activation of caspases 8, 9, 1, and 3 in human ejaculated spermatozoa to study main pathways of apoptosis. Potential functional impact of this phenomenon and possible activation mechanisms were examined by [1] subjecting cells to freezing and thawing, and [2] testing the dependence of caspase activity on membrane integrity.

Design: Experimental study.

Setting: Andrology Center Leipzig (European Academy of Andrology).

Patient(s): Ten healthy volunteers, 40 semen samples.

Intervention(s): Sperm populations were separated by annexin V magnetic activated cell sorting (MACS) according to externalized phosphatidylserine.

Main Outcome Measure(s): Active caspases 8, 9, 1, and 3 were examined in human ejaculated spermatozoa before and after cryopreservation.

Result(s): The active caspases were localized in the postacrosomal region (caspases 8, 1, and 3) or in the midpiece (caspase 9). Cryopreservation led to a significant increase in the number of cells showing activation of all caspases. The MACS separation resulted in a significant depletion of sperm positive for activated caspases within the externalized phosphatidylserine–negative fraction.

Conclusion(s): Caspases 8, 9, 1, and 3, representing the main pathways of apoptosis, are present in human spermatozoa and can become activated, particularly after freezing and thawing, and are associated with changes in the outer cell membrane. Spermatozoa showing these changes and caspase activation can be efficiently removed from semen samples by the annexin V MACS technique. (*Fertil Steril*® 2004;81(Suppl 1): 802–9. ©2004 by American Society for Reproductive Medicine.)

Key Words: Caspases, externalization of phosphatidylserine, apoptosis, human spermatozoa, immunomagnetic cell depletion

The term *apoptosis* defines programmed cell death, which is molecularly and morphologically distinct from necrosis (1). Recent models of apoptosis include receptor-mediated pathways and intrinsic triggered apoptosis, as well as cytotoxic or stress-induced forms (2).

Up to now it has been unclear whether apoptosis in ejaculated spermatozoa takes place in a similar way as in somatic cells or whether spermatozoa, which are thought to have a transcriptionally inactive nucleus, undergo abortive forms of this process (3, 4). Acute apoptotic changes due to withdrawal of FSH occurring in germ cells might be independent from classic pathways of programmed cell death (5). Furthermore, the functional impact of programmed cell death in human sperm is poorly understood.

Several studies investigated CD95 as a marker of receptor-mediated apoptosis (6), regulator proteins, such as bax and bcl-x (7), disturbances of plasma membrane integrity (8), and DNA strand breaks (9, 10) as indicators of programmed cell death in human spermatozoa.

The connections between these features of programmed cell death are caspases (cytosolic cysteine-containing aspartate-specific proteases). These enzymes are found to be major transducers and effectors within the different pathways of apoptosis-signaling network in somatic cells. They comprise a family of highly specific proteases, which contain the amino acid cysteine in their active sites. After proteolytic activation in a cascade (11, 12) their targets are cleaved after the amino acid aspartate (13).

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Reprint requests: Uwe
Paasch, M.D. Ph.D.,
Department of
Dermatology/Andrology
Unit, University of Leipzig,
Leipzig, Germany (FAX:
49-341-9718649; E-mail:
paau@medizin.uni-
leipzig.de).

^a Department of
Dermatology/Andrology
Unit, University of Leipzig.

^b Center for Advanced
Research in Human
Reproduction and Infertility,
Urological Institute, The
Cleveland Clinic
Foundation.

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From a functional point of view, caspases involved in apoptosis act either as initiators (caspases 8, 9, and 10) or as effectors (caspases 3, 6, and 7) (14–16). Caspase 8 was identified as the most important initiator enzyme of the caspase cascade triggered by activation of death receptors (e.g., CD95 or tumor necrosis factor receptor) via adapter molecules (e.g., Fas associated protein with death domain (FADD) and forming the death-inducing signaling complex (17). Caspase 9 interacts with many other regulators and transducers (e.g., cytochrome c released from disintegrated mitochondria) in intrinsic apoptosis. Caspase 1 transduces inflammation signals and links these processes with the programmed cell death. In addition, caspase 1 can be activated by p53 through direct transcription (18). All these initiator caspases are activators of downstream caspases. Caspase 3 (19), the most important among them, executes the final disassembly of the cell by cleaving a variety of cell structure proteins and generating DNA strand breaks (20).

Externalization of the phospholipid phosphatidylserine from the inner to the outer leaflet of the plasma membrane is an early feature of the terminal phase of apoptosis (21). Magnetic activated cell sorting (MACS) using the high and selective affinity of superparamagnetic annexin V microbeads for externalized phosphatidylserine (EPS) can be used to subpopulate spermatozoa. Annexin V is a 35- to 36-kd phospholipid-binding protein that cannot pass the intact plasma membrane (22). However, EPS of dead spermatozoa can also bind annexin V.

Therefore, we investigated the amount and localization of activated initiator caspases 8 and 9 in conjunction with their shared effector, caspase 3, in human spermatozoa. Caspase 1 was studied because it mediates alternative pathways of apoptosis via p53 and inflammation signals (18, 19). In addition, the functional impact of caspases in human spermatozoa was examined by testing the dependence of caspase activity on membrane integrity and their response to cryopreservation.

MATERIALS AND METHODS

The experimental design of our study is shown in Figure 1.

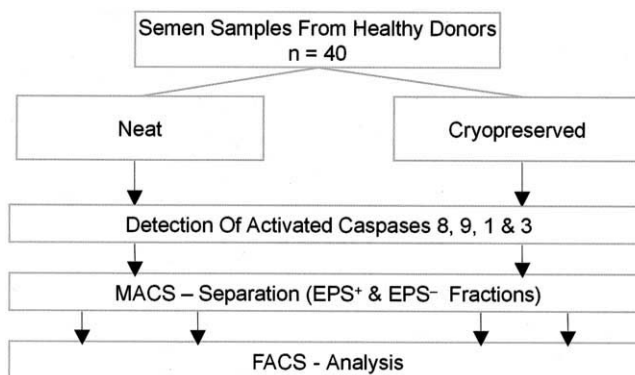
Selection Criteria of the Semen Samples

Semen samples (n = 40) were provided by 10 healthy volunteers (donors) at our department of andrology in accordance with ethical guidelines. Volunteers provided written informed consent. Samples were taken after sexual abstinence of 2 to 3 days.

Semen samples were collected by masturbation into sterile plastic cups. Specimens were examined for routine semen characteristics, such as sperm concentration and motility according to the World Health Organization (WHO) guidelines (23). The donor specimens had the following criteria: sperm concentration $>20 \times 10^6/\text{mL}$, $>50\%$ appearing progressively motile (WHO a+b), $>15\%$ of the spermatozoa

FIGURE 1

Experimental design of the study.



Paasch. Activation pattern of caspases in human spermatozoa. *Fertil Steril* 2004.

with a normal morphological shape, and leukocyte concentration $<1 \times 10^6/\text{mL}$.

Sperm Preparation

The semen samples used in this study were aliquoted into two portions. One aliquot was investigated immediately in neat condition and the other after cryopreservation. The aliquots were diluted 1:1 in 0.9% NaCl and filtered through glass wool to remove the gelatinous masses (24). Thereafter they were washed twice ($400 \times g$, 5 minutes) with annexin V binding buffer (Miltenyi Biotec; Bergisch-Gladbach, Germany). The supernatants were discarded, and the pellets were used for further experiments.

Freezing Technique

The semen samples were cryostored with freezing medium TES and Tris yolk buffer, which produces the best vitality parameters of spermatozoa after cryostorage, as previously demonstrated (8). For cryopreservation the semen samples were diluted dropwise with an equivalent volume of freezing medium TES and Tris yolk buffer containing 12% (vol/vol) glycerol (Irvine Scientific; Santa Ana, CA; catalogue no. 9971).

The samples were placed into 2.0-mL cryotubes (Faust Laborbedarf; Schaffhausen, Switzerland) and frozen (system Nicool LM 10; Air Liquide, Wiesbaden, Germany). A slow cooling rate (level 2) decreased the temperature from room temperature to 5°C within 15 minutes and was followed by 15 minutes incubation at level 10 for a decrease to -70°C (25). Finally, the tubes were plunged into liquid nitrogen and stored at -196.5°C . Vials were thawed by being placed in a 37°C water bath for 5 minutes.

Detection and Evaluation of Active Caspases in Vital Spermatozoa

Active caspases 8, 9, 1, and 3 were detected in living spermatozoa through the use of the carboxyfluorescein-la-

TABLE 1

Selective detection of active caspases with specific carboxyfluorescein-labeled caspase inhibitors.

Caspases	Abbreviation	Inhibiting peptide sequence
1	FAM-YVAD-FMK	Benzyloxycarbonyl-tyrosylvalylalanylaspargate
8	FAM-LETD-FMK	Benzyloxycarbonyl-leucylglutamylthreonylaspargate
9	FAM-LEHD-FMK	Benzyloxycarbonyl-leucylglutamylhistidylaspargate
3	FAM-DEVD-FMK	Benzyloxycarbonyl-valylglutamylisoleucylaspargate

Inhibitor: carboxyfluorescein (FAM)-peptide-fluoromethylketone (FMK).

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beled caspase inhibitors (Table 1). These cell-permeable and noncytotoxic caspase inhibitors bind covalently to active caspases (26). The fluorogenic substrate becomes fluorescent upon cleavage by the caspases (27). The detection of activated caspases by the inhibitor was performed according to the instruction manual of the manufacturers of the fluorescein caspase activity kit (CaspTag, S730x; Intergen, Oxford, United Kingdom), with appropriate controls.

A 150-fold stock solution of the inhibitor was prepared in dimethyl sulfoxide. It was further diluted in phosphate-buffered saline to make a 30-fold working solution. All the prepared aliquots of all fractions were incubated at 37°C for 20 minutes with 10 μ L of the working solution and washed with the rinse buffer provided with the kit. The negative controls were processed identically for each fraction, except that the stain was replaced with 10 μ L of phosphate-buffered saline.

Flow cytometric analysis of the samples evaluated the extent of activation of caspases 8, 9, 1 and 3. The spermatozoa parted by MACS were adjusted to a minimum concentration of 1.0×10^6 spermatozoa mL^{-1} . The fluorescence signals of labeled spermatozoa were analyzed by flow cytometer (Coulter Epics XL; Beckman Coulter, Germany). A minimum of 10,000 spermatozoa were examined for each assay at a flow rate of <100 cells/s. The sperm population was gated with 90° and forward-angle light scatter to exclude debris and aggregates. Green (fluorescein isothiocyanate conjugate-derived) fluorescence was measured with a 488-nm band pass filter.

The percentage of positive cells and the mean fluorescence was calculated by special software (Expo32ADC; Coulter) on a 1023-channel scale. The superparamagnetic microbeads of approximately 50 nm diameter are too small to be detected by optical methods and to change the scatter properties of spermatozoa in the flow cytometer (28). As a positive control, the binding of fluorescein isothiocyanate conjugate-conjugated pisum sativum agglutinin to the acrosome was used for setting the bitmap on dot plot (8, 29).

The intracellular localization of active caspases in vital spermatozoa was investigated by fluorescence microscopy (Jenamed; Carl Zeiss, Jena, Germany).

Depletion of Spermatozoa With Deteriorated Membranes by MACS

The sperm suspensions were divided into two parts by the passage through a magnetic field (MiniMACS; Miltenyi Biotec) based on the binding of superparamagnetic annexin V microbeads to EPS present on the surface of spermatozoa (30). 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 iron globes of the column.

The washed spermatozoa were incubated with 100 μ L annexin V microbeads at room temperature for 15 minutes. The annexin V microbead-labeled apoptotic spermatozoa (EPS⁺) were retained in the separation column, which was placed in the magnet; the nonlabeled spermatozoa (EPS⁻) passed through. After the column was removed from the magnetic field, the retained fraction was eluted.

Statistical Analysis

Evaluation of differences and correlations data was performed by nonparametric tests (Mann Whitney *U* test, Wilcoxon rank-sum test, Spearman test), as appropriate for data type and distribution (investigated by Shapiro-Wilk test). Student's *t*-test for paired sample was used to compare the odds ratios. All calculations were performed with Statistica 6.0 software (StatSoft; Tulsa, OK). *P* values <.05 were considered statistically significant. All values are given as mean \pm SD.

RESULTS

Activity of Caspases 8, 9, 1, and 3

All of the investigated caspases could be detected within neat and cryopreserved semen samples. The active enzymes were localized predominantly in the postacrosomal region (caspases 8, 1, and 3). In contrast, the mitochondrial initiator caspase was located only in the midpiece (caspase 9, Fig. 2). The second finding serves as an internal quality control for the specificity of enzyme detection.

Cryopreservation led to a significantly increased percentage of sperm showing activation of all types of enzymes ($15.7\% \pm 6.0\%$ vs. $40.9\% \pm 9.5\%$, neat vs. cryopreserved;

FIGURE 2

Donor sperm out of the EPS⁺ fraction having activated caspase 1 in the postacrosomal region.



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$P < .01$, Table 2). The amount of EPS⁺ cells increased from $12.1\% \pm 6.2\%$ to $40.6\% \pm 11.2\%$.

All caspases in our study were activated in human ejaculated spermatozoa (neat, nonseparated semen samples), and their presence was ranked as follows: caspase 3⁺ > 8⁺ > 9⁺ > 1⁺ ($P > .05$). Cryopreservation did not affect this ranking. Differences within this group became partly significant owing to a small increase in caspase 1 activation (caspase 3⁺ > 1⁺ and caspase 8⁺ > 1⁺; $P < .001$). The greatest increase was found in sperm positive for caspase 3 ($32.6\% \pm 6.7\%$), followed by caspase 8⁺ sperm ($30.5\% \pm 6.7\%$), caspase 9⁺ sperm ($22.2\% \pm 11.7\%$), and caspase 1⁺ sperm ($15.5\% \pm 6.1\%$, Table 2).

Impact of Annexin V Magnetic Separation

The MACS separation according to EPS resulted in a significant enrichment ($P < .01$) of sperm free of all types of activated caspases within the EPS⁻ fraction. Conversely, cells having activated caspases were enriched into the EPS⁺ fraction ($P < .01$; EPS⁻ vs. EPS⁺; $P < .01$; Fig. 3). The separation effect was more distinct for caspases 8 and 9 than for caspases 1 and 3.

As mentioned above, the EPS⁻ fraction out of neat and cryopreserved semen showed low caspase levels, but between the different types of caspases subtle distinctions could be seen. Compared with the other types of caspases, there were slightly greater amounts of EPS⁻ sperm having caspase 3 activation (significant for caspase 3 vs. caspase 9 [$P = .037$; neat], Table 3).

In contrast, caspase 9 was most often activated in EPS⁺ sperm (neat: significant compared with caspases 1 and 3; $P < .01$; cryopreserved: compared with all other caspases, $P < .01$). Interestingly, lesser amounts of sperm were found to be caspase 1⁺ (caspase 1 vs. caspases 3, 8, and 9; $P < .01$) in that fraction.

Cryopreservation did not influence the percentage of caspases 8⁺, 9⁺, 1⁺, and 3⁺ sperm within the EPS⁻ fraction (EPS⁻ neat vs. cryopreserved: $P > .05$).

However, the amount of cryopreserved EPS⁺ sperm having activated caspases 8, 9, 1, and 3 was significantly increased compared with the neat ejaculates (caspases 8⁺, 9⁺, 1⁺, and 3⁺ in EPS⁺ neat vs. cryopreserved: $P < .01$). This indicates sufficient separation capacity of the MACS system in terms of enrichment of EPS⁻ and caspases⁻ cells.

TABLE 2

Percentage of spermatozoa having caspases activated within the whole sample and the annexin V MACS-generated subpopulations in neat and cryopreserved donor samples.

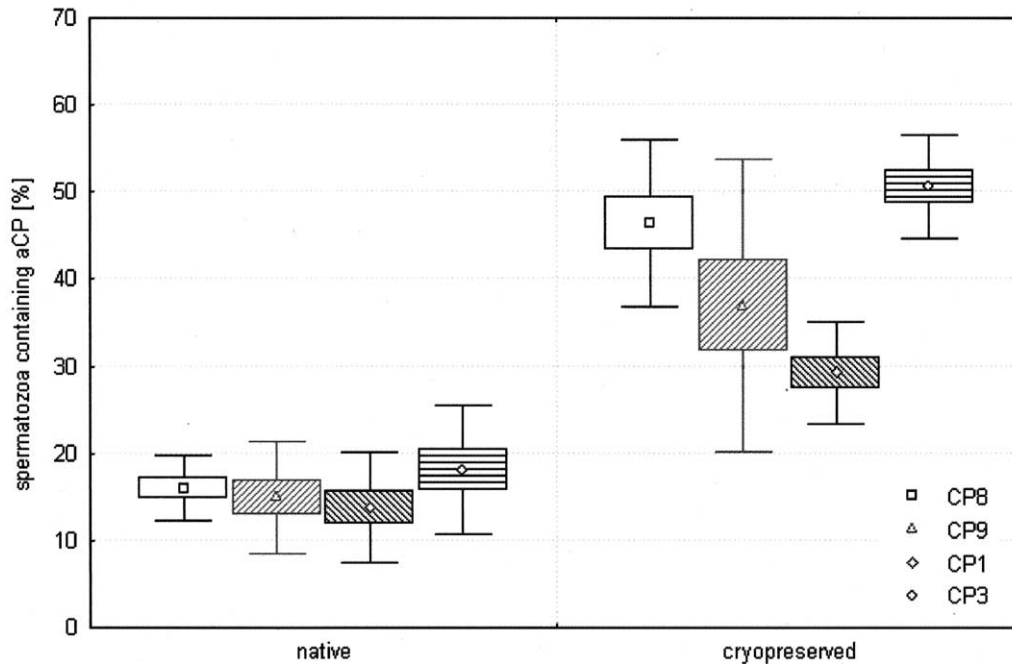
Caspases	EPS ⁺ (%)	CP ⁺ total (%)	CP ⁺ in EPS ⁻ (%)	CP ⁺ in EPS ⁺ (%)
8				
Neat	10.7 ± 4.3	16.0 ± 3.8	10.3 ± 2.2	65.3 ± 6.9
Cryopreserved	49.2 ± 11.6	46.5 ± 9.6	9.5 ± 2.9	83.5 ± 5.4
9				
Neat	12.3 ± 10.2	14.9 ± 6.5	7.4 ± 3.3	68.3 ± 5.6
Cryopreserved	32.3 ± 19.2	36.9 ± 16.8	10.8 ± 5.9	92.4 ± 2.0
1				
Neat	12.2 ± 5.1	13.8 ± 6.3	9.3 ± 6.1	48.1 ± 5.8
Cryopreserved	31.5 ± 2.5	29.3 ± 5.8	8.9 ± 5.3	73.6 ± 7.8
3				
Neat	13.1 ± 5.1	18.1 ± 7.5	13.2 ± 6.7	50.8 ± 9.9
Cryopreserved	49.7 ± 11.5	50.7 ± 5.9	14.3 ± 6.2	86.7 ± 4.3

Note: Values are expressed as mean ± SD. CP = caspase; EPS = externalized phosphatidylserine.

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FIGURE 3

Percentage of caspases (CP) 8⁺, 9⁺, 1⁺, and 3⁺ sperm within EPS⁺ and EPS⁻ subpopulations of neat (native) and cryopreserved samples. Box: mean ± SE; whisker: mean ± SD.



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Relationship Between Membrane Damage and Caspase Activation

The MACS separation resulted in a concomitant depletion and enrichment of cells bearing activated caspases. Cryopreservation led to an increase of cells with EPS and those with activated caspases. The relationship between membrane damage and activation of caspases was investigated with the odds ratio (OR) based on log₂ equation:

$$OR = (\text{right positive} \times \text{right negative}) / (\text{false positive} \times \text{false negative})$$

$$\log_2 OR = \ln(OR) / \ln(2)$$

Establishing log₂ transforms the OR into a normally distributed random variable. An increase of the log₂OR of 1 results in a doubling of the OR. The log₂OR then represents the chance of a sperm with active caspases compared with sperm without active caspases to be also annexin V⁺. Dif-

TABLE 3

Comparison of caspase 8, 9, 1, and 3 activation in nonseparated and subpopulated spermatozoa in neat and cryopreserved semen samples, demonstrated by P values (Wilcoxon test).

Compared CP	Nonseparated		EPS ⁻		EPS ⁺	
	Neat	Cryopreserved	Neat	Cryopreserved	Neat	Cryopreserved
8 vs. 9	ns	ns	ns	ns	ns	.005
8 vs. 1	ns	.001	ns	ns	.005	.022
8 vs. 3	ns	ns	ns	.028	.005	ns
9 vs. 1	ns	ns	ns	ns	.005	.005
9 vs. 3	ns	ns	.037	ns	.007	.013
1 vs. 3	ns	.001	ns	ns	ns	.005

Note: The different caspase types are activated to a similar amount in neat semen; differences were found after cryopreservation and particularly within the EPS⁺ subpopulation. P<.05 was significant by Wilcoxon test. CP = caspase; EPS = externalized phosphatidylserine; ns = not significant.

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TABLE 4

The \log_2 OR of CP 8, 9, 1, and 3 displays the factor of the opportunity of CP⁺ sperm in comparison to CP⁻ sperm to be also annexin V⁺.

Caspases	\log_2 OR neat cells	\log_2 OR cryopreserved cells	$\Delta \log_2$ OR	<i>P</i>
8	4.1 ± 0.6	5.7 ± 0.7	1.6	.0003
9	4.9 ± 0.7	7.0 ± 1.4	2.1	.0019
1	3.6 ± 1.5	5.2 ± 1.1	1.7	.037
3	2.9 ± 0.5	5.5 ± 0.8	2.6	.00002

Note: Values are expressed as mean ± SD. *P* < .05 was significant by *t*-test for paired samples. CP = caspase.

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ferences between the \log_2 OR of the neat and the cryopreserved semen samples were tested with Student's *t*-test for paired samples (Table 4).

The interrelationship between the activation of a caspase subtype and the binding of annexin V on disturbed membranes was visible in all samples investigated, either neat or cryopreserved. The closest connection was found for caspase

9 (rank order of association: caspase 9 > 8 > 1 > 3; caspases 8, 1, and 3 vs. caspase 9: *P* < .05) in neat and cryopreserved semen samples. Caspase 3 showed the weakest relationship: caspases 8 and 9 vs. caspase 3: *P* < .01).

Because of cryopreservation the association of activation of caspases 8, 9, 1, and 3 with EPS increased significantly (rank order: caspase 9 > 8 > 3 > 1) in spermatozoa (Fig. 4). This indicates a close correlation of membrane damage and activation of initiator caspases of the receptor-mediated and especially the mitochondria-derived pathway. In contrast, caspase 1⁻-mediated alternative pathways and the effector caspase 3 seem to be either less vulnerable to membrane damage or are not instantly activated.

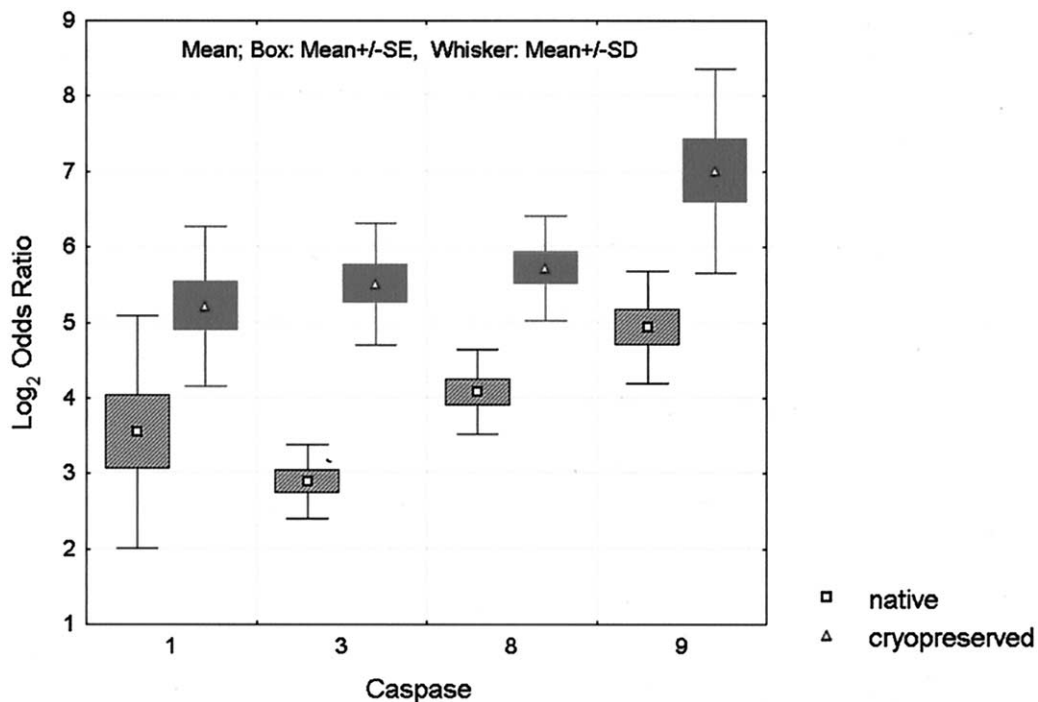
DISCUSSION

In this study we investigated the presence and activation of caspases 8, 9, 1, and 3 in human ejaculated spermatozoa.

Consistent with the special structure of sperm, the active caspases were observed predominantly in the postacrosomal region (caspases 8, 1, and 3). In addition, mitochondria-associated caspase 9 was particularly localized in the mid-piece.

FIGURE 4

\log_2 OR of caspases 8⁺, 9⁺, 1⁺, and 3⁺ neat (native) and cryopreserved sperm. Student's *t*-test for paired samples: ***P* < .01, **P* < .05 vs. cryopreserved samples.



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The different proteases were found to be activated to a similar degree in neat spermatozoa. This might be because of the concomitant autoactivation between the members of this enzyme family. The percentage of spermatozoa containing active caspases was not significantly different for the caspase subtypes and ranked as caspase 3⁺ > 8⁺ > 9⁺ > 1⁺. This might reflect the function of caspase 3 as main shared effector caspase, whereas the remaining caspases represent parts of different apoptotic pathways.

After cryopreservation and thawing an increased amount of spermatozoa with activated caspases was seen. The percentages of spermatozoa containing active caspases 8 and 3 were especially increased, whereas caspase 1 was less activated, and caspase 9 was intermediate. This correlates with higher rates of EPS, a sign of disturbed membrane integrity and poor classic sperm parameters (8). The lesser increase in caspase 1 activity might be explained by its role in linking inflammatory signals to the apoptosis network, which is not related to cryopreservation.

To clarify how caspases are activated by cryopreservation even under optimized cryoprotection (31), the dependency from membrane integrity was investigated by annexin V MACS.

Principally, the MACS separation according to EPS resulted in a significant depletion of sperm having activated caspases 8, 9, 1, or 3 within the EPS⁻ fraction and a simultaneous enrichment of sperm bearing active caspases 8, 9, 1, or 3 into the EPS⁺ fraction.

The relationship between caspase activation and externalization of phosphatidylserine was found to be present by calculation of OR of logarithmic-transformed data (log₂OR). The closest association of caspase activation and membrane damage was detected for caspase 9. This effect could be attributed to the sperm's specialized structure, in which the mitochondria are located in the midpiece close to the outer membrane. On the other hand, the presence of active caspase 3 in sperm is less accompanied by loss of membrane asymmetry (EPS⁺ sperm) compared with the other caspases. Because the protease is the shared effector enzyme of multiple apoptosis pathways, an activation triggered independently from membrane integrity is very likely.

Cryopreservation and thawing increased the association between caspase activation and EPS. The percentage of caspases 8⁺, 9⁺, 1⁺, and 3⁺ sperm was significantly elevated within the EPS⁺ subpopulation, but not influenced within the EPS⁻ fraction. This indicates that cryopreservation-induced caspase activation is due to membrane changes during freezing and thawing.

The types of caspases found indicate that apoptosis might not only act via the CD95–death-inducing signaling complex–caspase 8 pathway in human sperm. The presence of caspase 9 emphasizes the important role of mitochondria in apoptosis signaling. Although caspase 1 is less active in neat

and cryopreserved samples, the cytokine-processing caspase displays alternative pathways of apoptosis (18, 19). These findings are supported by the prominence of activated caspase 3, acting as a shared effector caspase of multiple pathways.

The mere presence of an active caspase does not seem to be the crucial lethal factor, because overall survival time of a single spermatozoon could not be prolonged by inhibitors of caspases (32).

However, a wide spectrum of cell cytoskeletal proteins and membrane components are targets of caspase 3 (33). Therefore, its role in decreased fertilization capacity is presumed.

Further studies are needed to evaluate the status of caspase activation in subpopulations of spermatozoa, because its relationship with DNA damage is well known (34). The impact of activation of caspases in terms of DNA fragmentation should be investigated to clarify its origin.

The clinical significance of our findings is emphasized by the known correlation of high levels of DNA fragmentation and poor fertilization rates after IVF (35) and intracytoplasmic sperm injection (36). It needs to be tested whether caspase activation correlates in the same manner. If this hypothesis is found to be true, annexin V–based MACS separation of sperm might be introduced as a new tool to enrich spermatozoa with a higher capacity to fertilize eggs, leading to an overall increase in success rates in assisted reproduction.

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