Cell and Tissue Banking (2006) 7:81–90
DOI 10.1007/s10561-005-0276-7

Activation of caspases in human spermatozoa during cryopreservation – an immunoblot study

Katja Wündrich[1], Uwe Paasch[1], Monika Leicht[2] and Hans-Jürgen Glander[1,*]
*[1]Department of Dermatology/Andrology Unit; [2]Department of Physiology, University of Leipzig, Stephanstrasse 11, D-04103 Leipzig, Germany; *Author for correspondence (e-mail: hans-juergen.glander@medizin.uni-leipzig.de)*

Received 17 November 2004; accepted in revised form 29 December 2004

Key words: Caspases, Cryopreservation, Glycerol, Human spermatozoa, Western blot

Abstract

Cellular stress to ejaculated spermatozoa such as cryopreservation is known to induce caspase-derived, apoptotic signaling. Therefore, the proenzymes and active forms of caspases 1, 3, 8 and 9 were examined by western blot technique in unfrozen and frozen human spermatozoa of infertility patients and of healthy donors. Twenty-two semen samples derived from healthy donors and 26 semen samples of unselected infertility patients were divided into 3 parts, two of them were cryostored at −196 °C with 7% or 14% (v/v, final concentration) of glycerol. The caspases were detected by immunoblots with polyclonal rabbit-anti-caspases-antibodies after 15% sodium dodecyl sulfate-polyacrylgel electrophoresis (SDS-PAGE) under reducing conditions. For evaluation of the differences between amounts of caspase protein the luminol/H_2O_2 method was applied. A significant increase of activated caspase-1 in donors, of caspase-8 in patients and caspase-9 in patients and donors after cryopreservation were found, whereas, the application of 14% glycerol resulted in higher amounts of activated caspase than did 7% glycerol. Possibly, glycerol may also contribute to activation of caspases via direct toxic effects to mitochondria during cryopreservation of spermatozoa. This finding strongly supports an hypothesis of a higher mitochondria-derived apoptosis-sensitivity of spermatozoa in patients than in healthy donors during cryopreservation. Inactive caspase-3 was reduced subsequent to cryopreservation in patients ($p < 0.05$) and non-significant in donors ($p > 0.05$). Active caspase-3 was detectable in all samples but without significant differences between the three assays. It is concluded that mechanisms associated with apoptotic processes deserve attention in cryopreservation of spermatozoa in order to conserve vital sperm functions after thawing.

Introduction

Cryopreservation of semen specimens has become increasingly necessary for patients with malignancies before radiation or chemotherapy and is administrated in order to perform assisted reproductive procedures later. To ensure post-thaw salvage-rate of healthy, motile sperm, the cryostorage demands the usage of cryoprotective agents to prevent cell damage. Glycerol is one of the oldest most widely used, and it is able to penetrate human sperm membranes (Gao et al. 1992). However, it has ambivalent effects on the spermatozoa. Glycerol not only protects the spermatozoa against cryoinjury due to dehydration and intracellular ice-formation (Lovelock and Polge 1954) it also impairs the cell integrity (Buhr et al. 2001), motility (Paasch and Glander 1997) and

fertilizing capacity of spermatozoa (Jeyendran et al. 1985).

Even though its ability to prevent cryoinjury is highly variable from species to species, as well as among individuals within a species (Agca and Critser 2002). While this is a generally-accepted fact, the biochemical mechanism by means of which it occurs is as yet unclear, as glycerol has been proven to reduce motility at final concentrations higher than 7–14% (Critser et al. 1988; Paasch and Glander 1997). In addition, apoptotic changes at the membrane were detected using 10% glycerol (Glander and Schaller 1999).

The programmed cell death (PCD, the apoptosis) very likely contributes to the decrease of sperm vitality after cryopreservation (Anzar et al. 2002; Grunewald et al. 2001). Cryo-preservation and thawing are associated with impairment of the membrane integrity, including the translocation of phosphatidyl serine from the inner to the outer leaflet of the sperm plasma-membrane (Glander and Schaller 1999; Duru et al. 2001; Schuffner et al. 2001), post-thaw damage to the mitochondrial membrane (O'Connell et al. 2002; Paasch et al. 2003a) and activation of caspases (Grunewald et al. 2001). All of these processes are widely accepted as keystone events of apoptosis. The impact of cryoprotectants on levels of activated caspases in spermatozoa has not been elucidated fully (Glander and Schaller 1999; Grunewald et al. 2001). The family of aspartic acid-directed cysteine proteases called caspases (cysteinyl aspartate-specific proteinases) represents the prime effectors of apoptosis (Nicholson and Thornberry 1997). They are expressed as inactive proenzymes and are processed into active forms by cleavage in cells that undergo apoptosis. Activation of caspases may be induced by self-proteolysis (auto-activation) in a proteolytic cascade, that is due to cross-talk with other caspases, or by regulator proteins. Up to now 14 caspases are described. They comprise initiator caspases, e.g. caspase-8 and caspase-9, which recognise induction signals of apoptosis, and effector caspase-6, -7 and -3. The latter one is the major execution protease (Bratton et al. 2000). Caspase-1, belongs to the inflammation-associated mediators of immune response to microbial pathogens that culminate in cytokine production (Creagh et al. 2003). An activation of caspases in spermatozoa was already shown by fluorescence cytochemical investigations (Paasch et al. 2003a).

At first, spermatozoa were thought to have a death-programme that may not depend on caspases (Weil et al. 1998). However, their presence and functional competence could be demonstrated later (Paasch et al. 2001) and were confirmed by others (Weng et al. 2002; Wang et al. 2003). Moreover, caspase-1 was found in residual bodies of spermatogenesis (Blanco-Rodriguez and Martinez-Garcia 1999) and in mature spermatozoa (Paasch et al. 2002). Cryopreservation as such is known to activate caspases in cryopreserved human spermatozoa with a significant correlation to deterioration at the membrane (Grunewald et al. 2001; Paasch et al. 2003a). However, the activation pattern differs according to the sub-type of caspases and the degree of spermatozoal maturation (Weng et al. 2002; Wang et al. 2003; Paasch et al. 2003, 2003b).

The aim of our study was to further characterize the impact of glycerol based cryoprotection on sperm apoptosis. Therefore pro-enzymes and active forms of caspases-1, -3, -8 and -9 were detected and quantified by western blot technique before and after freezing of human spermatozoa of infertility patients and of healthy donors performed with 7% or 14% (v/v, final concentration) of glycerol.

Materials and methods

Semen samples

Following institutional approval, 48 semen samples were used. Of these 22 were derived from 14 healthy donors, and the remaining 26 were derived from patients who attended our infertility center. 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 (World Health Organization 1999). The ejaculates of the donors 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 $< 1 \times 10^6$ leukocytes per ml ejaculate; (b) the sperm concentration was greater than 20 million/ml; and (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. The ejaculates of patients showed spermiogram parameters as follow: sperm

concentration 62.8 ± 14.7 million/ml, $11.5 \pm 1.5\%$ of spermatozoa showed a normal morphology and $21.9 \pm 3.2\%$ were rapidly progressively motile. The corresponding values in the semen samples of donors amounted to 123.5 ± 32.0 million/ml, $21.7 \pm 3.8\%$ with a normal morphology and $59.6 \pm 5.7\%$ were rapidly progressively motile.

Freezing technique

After determination of sperm concentration the samples were divided into 3 aliquots. The first aliquot remained untreated (control). The other two aliquots were placed into special Nunc Cryo Tubes (Nalge Nunc International, 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 (Grischenko et al. 2003). Finally, the tubes were plunged into liquid nitrogen for storage at −196 °C. The samples were thawed by an incubation at room temperature for 5 min.

Preparation of sperm lysates

The semen samples were filtered trough glass wool to remove the gelatinous masses, diluted with 0.2 M Soerensenbuffer, pH 7.4 (90 ml 0.2 M $Na_2HPO_4 \cdot 2H_2O$, 10 ml 0.2 M $NaH_2PO_4 \cdot H_2O$, 100 ml 16% NaCl, aqua dest. ad 2 l) and washed twice with 0.2 M Soerensenbuffer, pH 7.4 ($700 \times g$, 7 min). After discarding the supernatants the pellets were dissolved in 1.5 volumes of SDS–sample–buffer, pH 6.7 (50 mM Tris–HCl, 2% SDS, freshly added 1 mM 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).

Western blotting of caspases

The protein concentration of the samples was determined using amido black (Henkel and Bieger 1994). 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 (1970). 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 h (Power Pac 300, Bio-Rad, Munich, Germany). The protein transfer to nitrocellulose membranes (0.2 μm pore size) followed the method of Kyhse-Andersen (1984), who used a discontinuous buffer system in a semidry transfer system (Pegasus, Phase, Luebeck, Germany) at 0.8 mA/cm2 for 70 min (Protran, Schleicher and Schuell, Germany). Membranes were blocked with 2% BSA–Tris–Triton, pH 7.5 for 1 h (50 mM Tris, 150 mM 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 6 times within 30 min and incubated for 1 h 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 (1993). Bound secondary antibodies were visualized by X-ray films (Konica, Type 3A4, BW Plus Roentgen, Kamp-Crutfort).

Densitometric evaluation

Analysis of the fluorescence signals was performed by the Chemilmage™ 4400-System (Alpha

Innotech Corporation, San Leandro, Canada). The sum of all pixel data of each protein signal was examined after background correction using software AlphaEase (Biozym Diagnostik, Oldendorf, Germany). Fluorescent signals of unfrozen spermatozoa of donors and patients were each set as 100%. Each signal was measured 3 times and the average was then calculated. The protein signals of each western blot were independently evaluated by determination with a fixed camera adjustment.

Statistical analysis

Data analyses were performed by non-parametric Friedmann-test as appropriate for data type and distribution. Normal distribution was proved by Shapiro–Wilk-*W*-test using the computer program STATISTICA 99′ Edition from StatSoft, Inc. (Tulsa, OK 74104, USA). The p values < 0.05 were considered as statistically significant.

Results

Western blotting of caspase-1, -3, -8, and -9 of healthy donors and infertility patients

Caspase-1

The 10 kDa- and the 20 kDa-subunits of activated caspase-1 were detectable by western blot-analysis in donors and patients before and after cryopreservation. The cryopreservation significantly enhanced the percentage of the 20 kDa caspase-1 subunit in spermatozoa of donors. This effect was intensified by an increase in glycerol concentration from 7% to 14% (Figure 1). A similar trend was noted in patients, but because of the higher standard deviation, was without significance. The small, 10 kDa-subunit did not significantly correlate with the percentage of the large, 20 kDa-subunit, nor did it significantly change after cryopreservation in donors and in patients. However, it showed an increase along with the concentration of glycerol used in patients. In all assays of cryopreservation, the spermatozoa of patients showed a higher activation of caspase-1 than could be observed in the spermatozoa of donors.

Caspase-3

Immunoblot-signals of 55 kDa, 32 kDa, 17 kDa, and 12 kDa were found for caspase-3 in the ejaculated spermatozoa of donors and patients (Figure 1). The 32 kDa-band represented the 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 (Cain et al. 1999). With increasing concentration of glycerol a significant decrease of the relative amount of caspase-3 precursor (32 kDa) in spermatozoa of patients was detected: $88 \pm 31\%$ at 7% glycerol and $77 \pm 27\%$ at 14% glycerol ($p < 0.05$), in contrast to that of donors, who did not show significant alterations ($p > 0.05$), although there was also slight a decrease. The decrease of zymogene was associated with an increase of the 17 kDa-subunit but not of the 12 kDa-subunit ($p > 0.05$). The percentage of the 55 kDa-dimer remained almost unchanged ($p > 0.05$).

Caspase-8

The precursor of caspase-8 (55 kDa) and the activated enzyme (18 kDa) could be detected in all the donors and patients examined (Figure 1). The cryopreservation significantly raised the activated caspase-8 (18 kDa) in patients ($p < 0.01$) and in donors ($p > 0.05$). The increase in glycerol to 14% led to a further increase of the detectable amount of active caspase-8 ($p < 0.05$), compared to an increase of 7% in glycerol with cryostorage in patients. In contrast, healthy donors showed a non-significant increase ($p > 0.05$) in the activation of caspase-8 (18 kDa subunit) after cryopreservation both with 7% and 14% glycerol.

Caspase-9

Donors and patients both showed the 46-kDa caspase-9 precursor, the active enzyme (10 kDa) and a 35 kDa-signal (Figure 1). In healthy donors as well as in infertility patients, no significant alteration 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 spermatozoa of both infertility patients and donors ($p < 0.05$). Activation was enhanced by the increase in glycerol concentration from 7% to 14% ($p < 0.05$), and was more pronounced in the spermatozoa of patients than in that of donors. The 35 kDa-signal was not significantly influenced by the cryopreservation process ($p > 0.05$).

Discussion

Cryopreservation of human sperm is a fundamental tool for the preservation of male fertility in a variety of circumstances. In view of recent reports (Glander and Schaller 1999; Grunewald et al. 2001), we hypothesized that cryoprotectants, in addition to their cryoprotective properties, may be a trigger of the programmed cell death. In this study, the impact of glycerol on caspase activity in spermatozoa has been demonstrated, thereby providing strong evidence in favor of the hypothesis.

Ejaculated spermatozoa derived from healthy donors as well as from infertility patients were tested for precursors and activated caspases-1, -2, -8, and -9 both before and after cryostorage with 7% or 14% glycerol. We found a significant increase of activated caspase-1 in donors, caspase-8 in patients, and caspase-9 in both patients and donors after cryopreservation. Caspase-3, the terminal effector caspase, showed an overall decrease of the zymogen and an overall increase in the 17 kDa active enzyme ($p < 0.05$). In addition, the

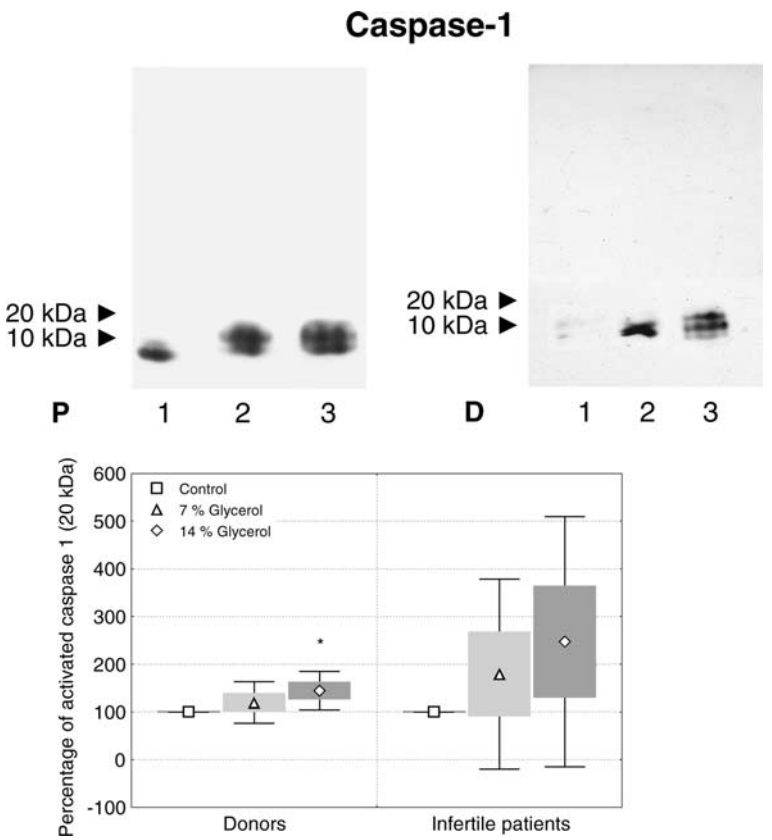

Figure 1. Examples of western-blots of caspase-1, -3, -8, and -9 in cryopreserved spermatozoa compared to control. Western-blot lane 1: control; lane 2: spermatozoa cryopreserved with 7% glycerol; lane 3: spermatozoa cryopreserved with 14% glycerol. Relative amount of activated caspase proteins in spermatozoa of healthy donors (D, $n = 22$) and of infertile patients (P, $n = 26$). □ control, △ cryopreservation 7% glycerol, ◇ cryopreservation 14% glycerol. *significant differences compared to control, $p < 0.05$.

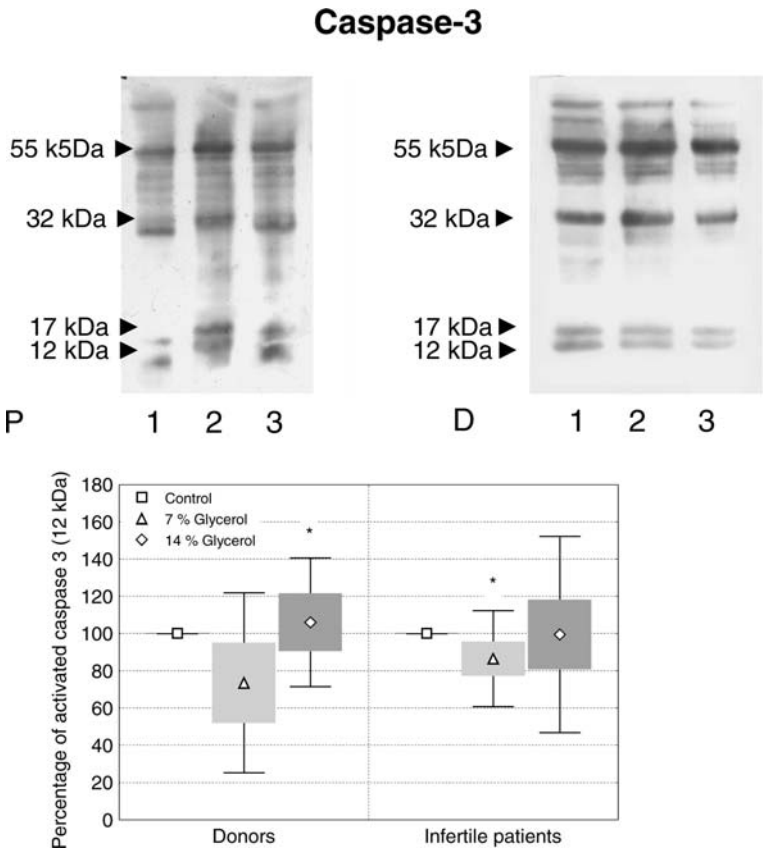

Figure 1. (Continued)

application of 14% glycerol resulted in higher amounts of activated caspase than did 7% glycerol. In contrast, the amount of detectable 12 kDa sub-unit did not increase in patients.

Recent studies suggested that the machinery of programmed cell death (PCD, apoptosis) exists in human spermatozoa (Paasch et al. 2003a; Duru et al. 2001; Glander and Schaller 1999; Donelly et al. 2001). The activation of caspases plays a fundamental role in apoptosis. Caspases are a family of highly specific proteases. After proteolytic activation in a cascade (Thornberry and Lazebnik 1998; Wolf and Green 1999) their targets are cleaved after the amino acid aspartate (Nicholson and Thornberry 1997). Depending on their function, three groups of caspases could be differentiated: initiator caspases-2, -8, -9, -10, effector, or downstream, caspases-3, -6, -7, and caspases-1, -4, -5, -11, -13 involved in inflammation (Faleiro and Lazebnik 2000). Initiator and effector caspases have been identified in human spermatozoa in close correlation to their maturation status (Paasch et al. 2003b). Standardized cryopreservation resulted in activation of important subtypes of caspases to a varying extent (Paasch et al. 2003b), also depending on degree of maturation (Weng et al. 2002; Wang et al. 2003; Paasch et al. 2003).

Caspase-1, also known as interleukin 1β-converting enzyme, is the first described member of the family of cysteine proteases and involved in TNF-receptor mediated cell death (Green 1998; Creagh et al. 2003). Caspase-1 influences the release of cytochrome c from mitochondria by depolarization of the mitochondrial membrane and amplifies the death signal (Susin et al. 1997). Both subunits of activated caspase-1 (10 kDa and 20 kDa) showed a significant increase after cryopreservation in both donors and in infertile patients. Since this enzyme also mediates signals of inflammation, its contribution to signaling in spermatozoa remains to be elucidated.

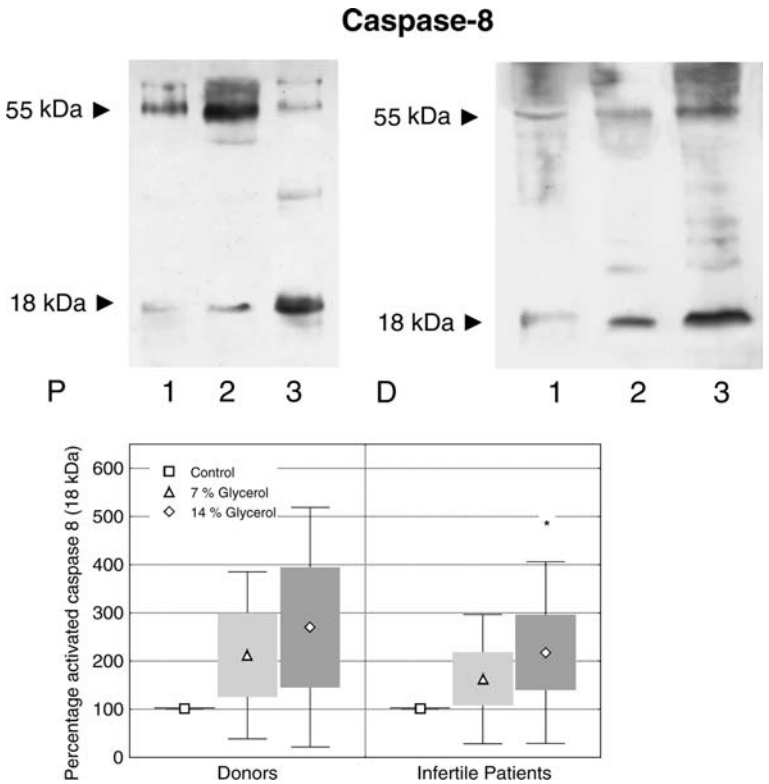

Figure 1. (Continued)

Caspase 3 executes the final disassembling of the cell by cleaving of a variety of cell structure proteins (Faleiro et al. 1997) and can be activated by all other upstream caspases (Tewari et al. 1995). The designated dimer of caspase-3 (55 kDa) has been detected in all samples at constant level. It only showed a small increase in the cryopreserved spermatozoa of patients at a level of 14% glycerol. With an increase in the glycerol concentration, the precursor of caspase-3 (32 kDa) decreased significantly after cryopreservation. This effect was stronger in donors than in patients. The decrease in the precursor was accompanied by an expected increase in the 17 kDa- but not in 12 kDa-subunits ($p > 0.05$). Significant consumption of caspase-3 precursor in patients without accompanying increase in the 12 kDa active subunits may reflect the inability of sperm to express those enzymes. However the other active subunit (17 kDa) showed a non significant increase in both, patients and donors.

Caspase 8 is described as the most important initiator caspase for the death receptor pathway via CD95 (type I apoptosis), which triggers DISC, the death inducing signalling complex (Kischkel et al. 1995). In comparison with the control group, the amount of caspase-8 precursor (55 kDa) did not significantly decrease ($p > 0.05$) in the cryopreserved spermatozoa of both patients and donors. However, an increase in the 18 kDa-subunit of caspase-8 in cryopreserved spermatozoa was detected with increasing glycerol concentrations in both groups. This effect was significant in patients ($p < 0.05$) but not in donors. The presence of the death-receptor protein CD 95 at the surface of ejaculated sperm has been interpreted as abortive apoptosis during spermatogenesis (Sakkas et al. 1999; Sakkas et al. 2002). This hypothesis is supported by the increased amount of caspase-8 and receptor-protein CD95 after cryopreservation in patients only (Grunewald et al. 2001). Activation of caspase-8 in donors could therefore be interpreted as a result of cross-talks, once apoptosis has been fully activated.

Recent data on caspase activation of ejaculated spermatozoa also indicate a predominant role of

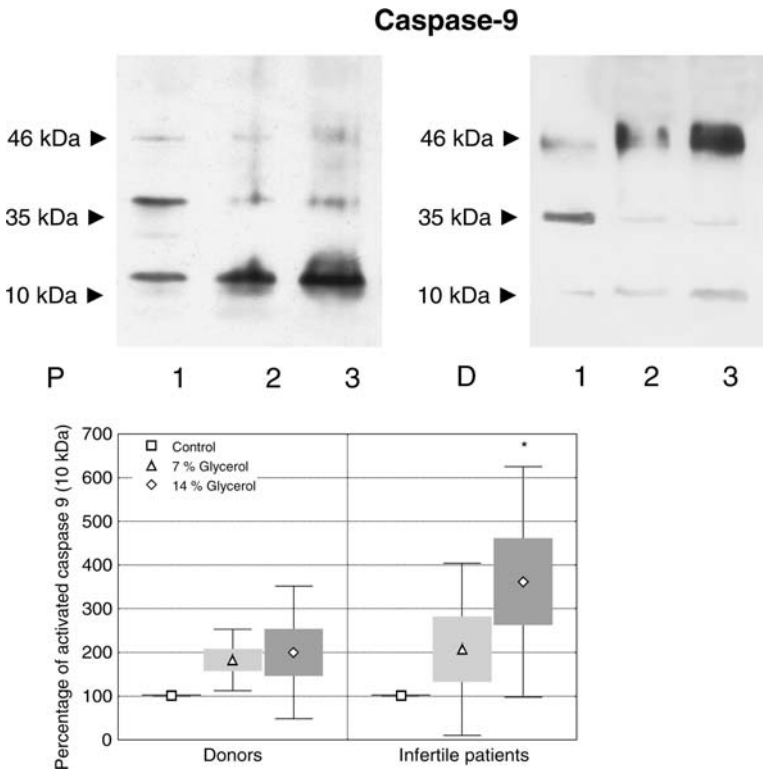

Figure 1. (Continued)

mitochondrial (type II) mediated apoptotic signaling in sub-populations after cryopreservation (Paasch et al. 2003; Paasch et al. 2003b). Caspase-9 triggers intrinsic apoptosis in close co-operation with regulators and transducers. Our results indicate no significant difference in caspase-9 precursor (46 kDa) in cryopreserved spermatozoa of donors and patients, compared to control group ($p > 0.05$). After cryopreservation the active enzyme showed a significant glycerol-dependent increase, which was more pronounced in patients than in donors. This strongly supports the existence of a higher sensitivity to mitochondria derived apoptosis in spermatozoa of patients due to cryopreservation.

The impact of cryopreservation on activation of caspases-1, -3, -8, and -9 in the spermatozoa of donors or infertile patients respectively has already been demonstrated by other methods (Grunewald et al. 2001; Paasch et al. 2003). Several mechanisms may be identified: (i) Cryopreservation of spermatozoa leads to various structural and functional alterations of sperm membranes (Glander and Schaller 1999; Donelly et al. 2001; Duru et al. 2001; Anzar et al. 2002), which may lead to a loss of stability of the lipid bilayer (Glander et al. 2002) and a sublethal cryodamage of spermatozoa (Alvarez and Storey 1993). The impairment of integrity of sperm membrane might initiate the caspase cascade (Bratton et al. 2000). (ii) Cryoprotectants in themselves clearly contribute to activation of caspases via direct toxic effects on mitochondria, since cytotoxic stress involves mitochondrial perturbations, followed by DNA fragmentation (Fulda et al. 1999). The higher level in activation of caspases-1 and -9 in cryopreserved spermatozoa of patients may indicate that these cells have a lower cryotolerance and a higher susceptibility to caspase activation than does the spermatozoa of donors.

Glycerol is assumed to protect cells during cryopreservation but it is not an indifferent substance. Increasing glycerol concentration elevated the loss of fluidity of sperm plasma membranes (Buhr et al. 2001) and decreased the fertilizing capacity of spermatozoa (Jeyendran

et al. 1985). A higher activation of caspases, the enzymes for apoptosis machinery was detected in spermatozoa cryopreserved 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. In summary, mechanisms associated with apoptotic processes deserve attention in cryopreserved spermatozoa in order to conserve vital sperm functions after thawing.

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