

Cryopreservation-Thawing of Fractionated Human Spermatozoa Is Associated With Membrane Phosphatidylserine Externalization and Not DNA Fragmentation

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ABSTRACT: The objective of these studies was to evaluate the effect of cryopreservation-thawing of human spermatozoa on DNA fragmentation and membrane integrity. This was a prospective, controlled cohort study, performed at a university-based infertility center. Ejaculates were examined from 5 donors and 16 men undergoing infertility evaluation. Purified sperm populations were prepared by gradient centrifugation, cryopreserved using a manual method and TEST-yolk buffer and glycerol (TYB-G), followed by quick-thaw. Annexin V binding was used for assessing membrane translocation of phosphatidylserine, and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) was utilized for the evaluation of DNA fragmentation. The results were as follows: the percentage of live cells with intact membranes (annexin V⁻, live) was significantly reduced after cryopreservation-thawing. On the other hand, the percentages of live cells with phosphatidylserine translo-

cation (annexin V⁺, live) and of necrotic (dead) cells increased significantly after thawing. TUNEL revealed percentages of cells with DNA fragmentation in the prefreeze and postthaw samples that were not significantly different. In a further attempt to examine differences in response to various cryoprotection protocols, experiments were carried out using no cryoprotection, glycerol alone, or TYB-G. Samples frozen with TYB-G demonstrated significantly higher percentages of live cells without phosphatidylserine translocation than the other conditions. We concluded that cryopreservation-thawing of human sperm from patients and donors was associated with membrane change, as revealed by membrane translocation of phosphatidylserine, while having no major impact on DNA fragmentation.

Key words: Phosphatidylserine translocation, TUNEL, annexin V binding, cryodamage.

J Androl 2001;22:646-651

Evidence has been presented that membrane-stress phase transitions encountered by the plasma membrane during freezing-thawing make a cell more prone to fracture, and to a lesser extent lipid peroxidation contributes to sublethal cryodamage in human spermatozoa (Alvarez and Storey, 1992, 1993). Lipid diffusion through the plasma membrane is significantly compromised in frozen-thawed human sperm compared with fresh sperm (James et al, 1999). The membrane phospholipid content decreases after cryopreservation, with loss of phosphatidylcholine and phosphatidylethanolamine being the most pronounced (Alvarez and Storey, 1992). During cryopreservation-thawing, phospholipids that are normally located on the inner layer of plasma membrane have been reported to move to the outer layer; frozen-thawed ram

spermatozoa depicted translocation of phosphatidylglycerol to the outer layer (Hinkovska-Galcheva et al, 1989).

It has been demonstrated that phosphatidylserine is externalized in cells with membrane disturbance (Vermees et al, 1995). Plasma membrane phosphatidylserine externalization is widely accepted as an early marker of programmed cell death (Martin et al, 1995). Using annexin V (which can selectively bind translocated phosphatidylserine) and the vital dye propidium iodide, Glander and Schaller (1999) reported on the effects of cryopreservation-thawing on human spermatozoa and suggested that this assay could be used to detect membrane integrity of frozen-thawed spermatozoa.

Sperm cryopreservation may also affect DNA/nuclear protein interactions. Although cryopreservation had no effect on the chromosomal content of human spermatozoa (Martin et al, 1991), others have reported on the effects of freezing leading to changes of sperm chromatin condensation using acridine orange, Feulgen-DNA cytophotometric analysis, and aniline blue staining (Royere et al, 1988, 1991; Hammadeh et al, 1999). Prelytic DNA frag-

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Received for publication October 16, 2000; accepted for publication January 25, 2001.

mentation is commonly accepted as a marker of programmed cell death. Various assays, including the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL), have been used to evaluate DNA integrity of human spermatozoa (Manicardi et al, 1995; Sun et al, 1997; Aitken et al, 1998; Lopes et al, 1998; Hughes et al, 1999).

In the present study, we aimed to evaluate the effects of cryopreservation-thawing on membrane and DNA integrity of human spermatozoa. We used the annexin V binding assay for assessing plasma membrane externalization of phosphatidylserine and the TUNEL assay for evaluation of DNA integrity.

Materials and Methods

Patients' Inclusion Criteria and Study Design

These studies were performed under approval of the Institutional Review Board at Eastern Virginia Medical School. Sixteen ejaculates from men undergoing evaluation for infertility at our andrology laboratory (study group) and 5 ejaculates from fertile men participating in our artificial insemination donor (AID) program (control group) were examined independently. Semen specimens were collected after a 2- to 4-day sexual abstinence period. All patients suffered from primary infertility more than a 1-year duration, had a normal physical examination, and were nonsmokers. All men had a negative antisperm antibody test (assessed by mixed antiglobulin reaction), and less than 0.5×10^6 leukocytes/mL of semen (peroxidase staining; World Health Organization, 1999). In order to provide a sufficient number of cells for all tests, subjects with a concentration of motile spermatozoa $>20 \times 10^6$ /mL in the original sample were included in the study.

After semen liquefaction for 30 minutes at room temperature, a basic semen analysis (concentration, motility, and morphology) was performed. This was immediately followed by separation of the motile sperm fraction with Percoll density-gradient separation. Prefreeze evaluation of motion parameters, annexin V binding, and TUNEL assays were performed in the fractions with high sperm motility (90% layer fractions).

In the first experiments, the purified motile sperm populations of 10 samples from patients (study group) and 5 samples from donors (control group) were cryopreserved using the manual method with TEST-egg yolk buffer and glycerol (TYB-G; Irvine Scientific, Santa Ana, Calif) as the extender-cryoprotectant medium. Postthaw analysis of motion parameters, annexin V binding, and TUNEL assays were repeated after a minimum of 24 hours of cryostorage and within 30 minutes of postthaw.

In additional semen samples from 6 patients, following separation of the motile sperm fraction, 1 aliquot of sperm was incubated with TYB-G or glycerol (G) alone at room temperature for 20 minutes in order to evaluate whether egg yolk or G had any effect on plasma membrane phosphatidylserine externalization and DNA fragmentation (this aliquot was not cryopreserved). Finally, in order to assess the effects of different cryoprotectant protocols, the remaining sperm suspensions from

those samples were divided into 3 aliquots and frozen using 1) no cryoprotectant, 2) a drop-wise dilution with G alone at a final concentration of 6%, or 3) a drop-wise dilution with TYB-G. In all these samples, postthaw analyses of motion parameters, annexin V binding, and TUNEL assays were repeated after a minimum of 24 hours of cryostorage and within 30 minutes of postthaw.

Sperm Preparation

Sperm concentration and motion parameters (progressive motility, velocity, and linearity) were objectively evaluated using the HTM-IVOS semen analyzer (Hamilton Thorne Research, Beverly, Mass) with fixed parameter settings (Oehninger et al, 1990). Sperm concentration and motility readings were manually monitored and corrections were made as appropriate. Sperm concentration and motility were assessed according to World Health Organization (1999) criteria, and sperm morphology was examined according to strict criteria after Diff-Quik staining (American Scientific Products, McGraw, Ill; Kruger et al, 1988).

The sperm fractions with high motility were isolated using discontinuous Percoll (Pharmacia, Uppsala, Sweden) gradient separation (90% and 40% layers). Up to 2 mL of semen was carefully placed on Percoll layers, centrifuged at $380 \times g$ for 20 minutes, and the pellet of the 90% layer was carefully washed with human tubal fluid (HTF; Irvine Scientific) supplemented with 0.3% human serum albumin (HSA; Irvine Scientific) at $380 \times g$ for 10 minutes. The supernatant was discarded and the pellet was resuspended in 0.5 mL of HTF supplemented with 0.3% HSA.

Detection of Membrane Phosphatidylserine Translocation

Annexin V is a calcium-dependent, high-affinity phosphatidylserine-binding protein. Here, we used annexin V Cy3.18 (Ann VCy3; Sigma Chemical Co, St Louis, Mo) for detection of phosphatidylserine externalization with simultaneous assessment of cell viability. In order to differentiate between live cells with and without phosphatidylserine translocation and necrotic cells, we used 6-carboxyfluorescein diacetate (6-CFDA) in combination with Ann VCy3. The nonfluorescent 6-CFDA enters the cell and is converted to the fluorescent compound, 6-carboxyfluorescein (6-CF). This conversion is a function of the esterases that are present only in living cells. Thus, no fluorescence can be observed in necrotic (dead) cells.

By fluorescence microscopy, 6-CF is observed as green fluorescence and Ann VCy3 as red. Three patterns of fluorescence are observed: 1) live, normal cells that stain only with 6-CF (green); 2) live cells with translocation of membrane phosphatidylserine that stain with both 6-CF (green) and Ann VCy3 (red); and 3) necrotic or dead cells that stain only with Ann VCy3 (red).

Sperm suspensions from the high-motility fractions were washed twice with phosphate-buffered saline (PBS; Irvine Scientific) and adjusted to 1×10^6 /mL. A 50- μ L aliquot of sperm suspension was placed on a poly-L-lysine coated slide and stained with 6-CFDA/Ann VCy3 solution in the presence of calcium. After incubation in the dark for 10 minutes, the slide was covered with a 24 \times 50 mm coverslip and immediately read

Comparison of annexin V binding and TUNEL results before and after cryopreservation-thawing: study and control groups

	Patients (study group) (n = 10)			Donors (control group) (n = 5)		
	Prefreeze	Postthaw	P	Prefreeze	Postthaw	P
Annexin V assay						
Normal cells (%)	85 ± 4	59 ± 2	<.0001	73 ± 5	61 ± 3	.0005
Annexin V ⁺ live cells (%)	11 ± 3	24 ± 2	.002	21 ± 4	30 ± 3	.001
Necrotic cells (%)	3 ± 1	15 ± 2	<.0004	6 ± 1	8 ± 1	.007
TUNEL (%)	13 ± 2*	14 ± 2	0.4	5 ± 1*	5 ± 1	0.6

* $P = .01$.

blindly by 2 observers using a fluorescence microscope. At least 100 spermatozoa were counted per slide. Our laboratory has reported an intraobserver variability of <6% and an interobserver variability of <3% for the technique (Barroso et al, 2000).

Detection of DNA Fragmentation

DNA cleavage may yield double-stranded, low-molecular-weight DNA fragments as well as single-strand breaks in high-molecular-weight DNA. Such DNA strand breaks can be detected by TUNEL. Here, we used the In Situ Cell Death Detection kit (Boehringer-Mannheim, Indianapolis, Ind).

Briefly, the purified motile sperm fractions were washed twice in PBS supplemented with 0.3% HSA, and the concentration was adjusted to 20×10^6 cells/mL. One hundred microliters of sperm suspension was fixed with 100 μ L of 4% paraformaldehyde for 30 minutes at room temperature, and washed in PBS supplemented with 0.3% HSA. Cells were permeabilized using 0.1% Triton X-100 in 0.1% sodium citrate for 2 minutes at room temperature, and washed twice in PBS supplemented with 0.3% HSA. Then, spermatozoa were incubated with the TUNEL reaction mixture (calf thymus TdT plus dUTP label) in the dark at 37°C for 1 hour, and washed twice in PBS supplemented with 0.3% HSA. The final pellet was transferred to a final volume of 250 μ L in PBS. Fifteen microliters of sperm suspension was put on a slide, mixed with 5 μ L of antifade medium, covered with a coverslip, and analyzed immediately using epifluorescent microscopy.

Each cell was assigned as having DNA fragmentation (intense green fluorescence) or was considered normal (no fluorescence). For each test, 1 aliquot of fixed and permeabilized spermatozoa were incubated either with only dUTP label solution (negative controls) or using only DNase I, 1 mg/mL, for 10 minutes at room temperature (positive controls). At least 100 cells were analyzed by 2 investigators in duplicate slides and the results were averaged. Our laboratory has reported an intraobserver variability of <8% and an interobserver variability of <7% for the technique (Barroso et al, 2000).

Sperm Cryopreservation

The techniques for freezing-thawing have been previously described (Morshedi 1996; Srisombut et al, 1998). Briefly, sperm suspensions were mixed slowly with freezing medium containing TYB-G in a drop-wise fashion until a 2:1 volume ratio of sperm suspension to freezing medium was attained in 10 minutes. The mixture was transferred into 2-mL cryovials at a vol-

ume of 0.4 mL/vial, refrigerated for 1 hour, and cooled at 5 cm over the liquid nitrogen for 20 minutes. Then, vials were plunged into liquid nitrogen at -196°C . The specimens were thawed in a water bath at 40°C for 3 minutes after 24 hours of cryostorage.

Statistical Analysis

Parametric and nonparametric paired and unpaired *t*-tests, Spearman rank correlations, and analysis of variance (ANOVA) were used as appropriate. Data are presented as means \pm standard errors of the mean. The level of significance was set at $P < .05$.

Results

The mean basic semen parameters of all patients ($n = 16$) included in the study were as follows: concentration, 76 ± 14 ($\times 10^6/\text{mL}$); motility, $70\% \pm 6\%$; concentration motile, 49 ± 13 ($\times 10^6/\text{mL}$); curvilinear velocity, 42 ± 2 ($\mu\text{m/s}$); linearity, $62\% \pm 2\%$; and normal morphology, $8\% \pm 2\%$. The mean basic semen parameters of donors ($n = 5$) included in the study were as follows: concentration, 116 ± 21 ($\times 10^6/\text{mL}$); motility, $74\% \pm 4\%$; concentration motile, 88 ± 18 ($\times 10^6/\text{mL}$); curvilinear velocity, 66 ± 6 ($\mu\text{m/s}$); linearity, $62\% \pm 2\%$; and normal morphology, $18\% \pm 5\%$.

The Table presents annexin V binding and TUNEL results of the cohorts studied comparing prefreeze versus postthaw using the standard TYB-G protocol. The percentage of live cells with intact membranes (Ann V⁻, live) was significantly reduced after cryopreservation-thawing in the study and control groups ($P < .0001$ and $P = .0005$, respectively). The percentage of live cells with phosphatidylserine externalization (Ann V⁺, live) increased significantly in the postthaw samples of patients ($P = .002$) and controls ($P = .001$). There was also a significant increase in the percentage of necrotic (dead) cells in postthaw samples ($P < .0004$ and $P = .007$, respectively, for the patient and the control groups).

TUNEL results (Table) revealed no significant differences in the percentages of cells with DNA fragmentation in the prefreeze and postthaw samples of both patient and donor groups. Of note, the percentage of TUNEL-positive

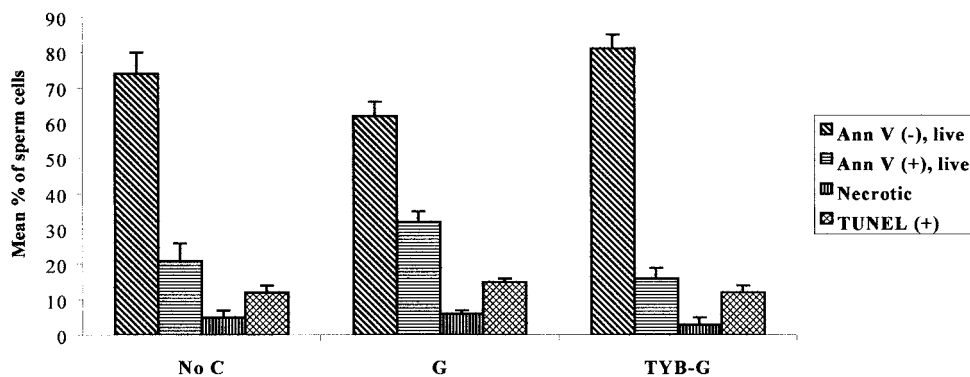


Figure 1. Prefreeze results using no cryoprotectant (No C), glycerol (G) alone, and TYB-G on plasma membrane (annexin V binding) and DNA integrity (TUNEL). Separated fractions of highly motile sperm were analyzed. No significant difference was observed between the groups, considering all parameters analyzed.

cells was significantly higher in the prefreeze motile sperm fractions of patients than donors ($P = .01$).

In both groups, postthaw progressive motility and velocity were significantly lower than prefreeze values (data not shown). There was no significant correlation between phosphatidylserine translocation or TUNEL results (prefreeze or postthaw) and motility or loss of progressive motility following cryopreservation thawing.

Comparisons of annexin V binding and TUNEL assays following addition of TYB-G to the fresh samples ($n = 6$ patients) and after the various cryopreservation-thawing protocols tested are shown in Figures 1 and 2, respectively. The results of addition of G and TYB-G to the fresh samples did not differ from those of samples analyzed without cryoprotectant (No C; Figure 1). However, after cryopreservation-thawing, all protocols resulted in an increase of the percentage of cells with phosphatidyl-

serine translocation and of necrotic cells, while reducing the percentage of live, normal cells ($P < .01$ for all comparisons). The group with No C was the only one showing a significant increase in TUNEL-positive cells before and after freezing-thawing ($P < .05$).

Figure 2 shows that the postthaw percentage of annexin V⁻ normal cells was significantly higher in TYB-G than in the No C and G groups ($P < .0001$). In addition, the postthaw percentage of annexin V⁺, necrotic (dead) cells was significantly lower in the TYB-G group than in the No C ($P = .001$) and G ($P = .007$) groups. No significant differences were observed in the postthaw percentages of annexin V⁺, live cells and TUNEL-positive cells between the groups. However, we observed a trend toward more DNA fragmentation in the cryopreservation protocol without cryoprotectant compared with the G and TYB-G groups ($P = .07$).

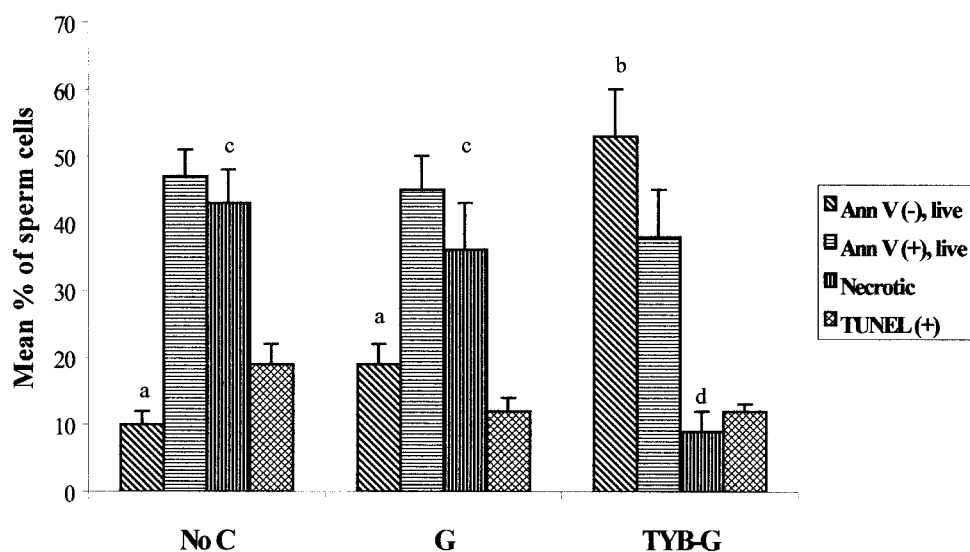


Figure 2. Effect of cryopreservation-thawing (postthaw values) using no cryoprotectant (No C), glycerol (G) alone, and TYB-G on plasma membrane (annexin V binding) and DNA integrity (TUNEL). Separated fractions of highly motile sperm were analyzed. Values with different superscripts indicate statistically significant differences.

The results of progressive motility were as follows: prefreeze, $81\% \pm 5\%$; prefreeze with TYB-G, $75\% \pm 7\%$; postthaw without cryoprotectant, $0.4\% \pm 0.1\%$; postthaw with G alone, $12\% \pm 5\%$; and postthaw with TYB-G, $26\% \pm 4\%$ (overall comparison using ANOVA, $P < .001$). Velocity parameters were also significantly reduced (data not shown).

Discussion

In the present study, we primarily evaluated the effects of cryopreservation-thawing (using a standard protocol of manual quick-freezing with TYB-G and quick thawing) on both membrane and DNA integrity of fractionated, motile fractions of human spermatozoa. The clinical preparation of cryopreserved semen using intrauterine insemination-ready processed samples by washing-centrifugation or gradient separation has recently been advocated (Sharma and Agarwal, 1996; Larson et al, 1997). Our results indicated that cryopreservation-thawing altered plasma membrane symmetry and was associated with translocation of phosphatidylserine, while DNA integrity was maintained.

It has been reported that annexin V binding combined with propidium iodide staining is more sensitive in detecting a deterioration of membrane functions than the vital stain propidium iodide alone (Glander and Schaller, 1999). Propidium iodide is normally a membrane-impermeable dye. However, dead cells lose their resistance to the influx of propidium, resulting in intracellular staining. In our study, we used annexin V in combination with 6-CFDA; this compound is converted to 6-CF by esterases in the living cells, which allows for a clear distinction under epifluorescent microscopy of live, normal cells; live cells with altered membranes (here depicting phosphatidylserine translocation); and necrotic (dead) cells. We observed that annexin V stained the entire spermatozoon (head, midpiece, and flagellum) in more than 90% of the annexin V⁺, live cells on a slide (data not shown). We postulate that phosphatidylserine is therefore translocated to the outer leaflet at different domains of the cell membrane.

Our results confirmed and extended previous studies in relation to 2 aspects. First, we showed that the percentage of annexin V⁺, live cells increased significantly following freezing-thawing as compared with prefreeze values (Duru et al, 2001). These cells are alive, but they have lost membrane integrity as demonstrated by translocation of phosphatidylserine. In addition, the cryopreservation-thawing process also induced necrosis. Therefore, freezing-thawing significantly reduced the percentage of normal cells. Second, in a further attempt to show any effect of the freezing medium on readings of annexin V binding,

and whether differences existed among various cryoprotection protocols, we tested samples using no cyoprotection, G alone, and TYB-G in both fresh and postthaw conditions. The presence of egg yolk or G did not influence the results of annexin V binding in the fresh samples. In addition, and as expected, experiments resulted in significantly higher percentages of live cells with intact membranes (annexin V⁻, live) and progressive motility after freezing-thawing with TYB-G. However, this protocol did not prevent phosphatidylserine externalization, compared with the G-alone and no cryoprotectant groups.

The present results also extended our preliminary observations that in fractionated spermatozoa of another cohort of patients and donors, cryopreservation-thawing resulted in membrane alteration with a perturbed transbilayer asymmetry, as evidenced by externalization of phosphatidylserine (Duru et al, 2001). Whether this is also true for samples from infertile men with more severe degrees of oligoasthenoteratozoospermia needs to be evaluated.

It has been suggested that an aminophospholipid translocase specifically mediates a rapid adenosine triphosphate-dependent translocation of aminophospholipids from the exoplasmic to the cytoplasmic leaflet (Muller et al, 1999). In cryopreserved ram spermatozoa, the sequestering of endogenous phosphatidylserine to the cytoplasmic leaflet is maintained in intact cells, but not in impaired cells. Furthermore, in this system, postthaw activity of the putative aminophospholipid translocase was significantly reduced in intact cells (Muller et al, 1999). If the activity of this putative enzyme is inhibited in cryopreserved human spermatozoa, an increased externalization of phosphatidylserine is to be expected.

The present experiments indicated no significant effect on DNA fragmentation (as measured by TUNEL) after freezing-thawing using the standard TYB-G protocol. Moreover, there were no variations in DNA fragmentation percentages between G-alone and TYB-G treatments. Although the cohort of patients studied herein did not differ from the donor group in terms of the basic sperm parameters (except morphology), this group of men had significantly higher basal levels of DNA fragmentation than controls. Our studies focused on examination of samples readily following thawing. More studies are needed to address delayed (ie, time-dependent) effects on chromatin-DNA integrity.

In conclusion, cryopreservation-thawing of motile sperm from patients and donors using standard methodology (manual freezing with TYB-G) was associated with plasma membrane translocation of phosphatidylserine, an expression of membrane alteration, and perhaps a damage. Conversely, freezing with TYB-G had no obvious effect on DNA fragmentation as assessed by TUNEL.

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