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Comparative Evaluation of Fresh and Washed Human Sperm Cryopreserved in Vapor and Liquid Phases of Liquid Nitrogen

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ABSTRACT: Infectious organisms such as hepatitis B were recently shown to survive in liquid nitrogen. To prevent cross-contamination of semen samples via liquid nitrogen, studies were undertaken to evaluate human sperm survival in the vapor phase of liquid nitrogen at −189°C. The study was conducted in 2 separate experiments. In the first experiment, a total of 30 unwashed, fresh semen samples (15 normozoospermic and 15 oligozoospermic) were evaluated for motility, vitality, and morphology after freeze–thaw survival in vaporous (−189°C) and liquid nitrogen (−196°C; control) phases. Similar evaluations were carried out in a second experiment on 27 samples (15 normozoospermic and 12 oligozoospermic) that were previously washed by the swim-up method. Motile sperm recovery rates were significantly different between liquid and vapor phases (unwashed, normozoospermic: 42.76% ± 3.23% vs 45.52% ± 4.44%, $P < .05$; washed, normozoospermic: 34.44% ± 4.41% vs 37.58% ± 3.90%, $P < .05$; unwashed, oligozoospermic: 16.53% ± 3.34% vs 18.25% ± 4.36%, $P < .05$; washed, oligozoospermic: 10.32% ± 2.54% vs 12.25% ± 2.81%, $P < .05$). Recovery rates for motility were much higher for unwashed samples compared with washed semen samples. In all experiments the recovery of normal and live forms showed no significant differences between the vapor and liquid nitrogen storage phases ($P > .05$). The results demonstrate that both washed and unwashed human sperm survive satisfactorily with good recovery in the vapor of liquid nitrogen and can be recommended for future storage in medically assisted conception programs.

Key words: Nitrogen vapor, fertilizability, cryopreservation.

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Sperm cryopreservation has been an important tool in assisted reproductive technology (ART). There have been many comprehensive reports on the cryopreservation of sperm in fertile and infertile men (Glander et al, 1989; Mossad et al, 1994; Tomlinson and Barratt, 1999). Furthermore, cryosurvival of washed and unwashed human sperm in normal and subnormal semen samples in the liquid phase of liquid nitrogen has also been reported (Keel and Webster, 1989; Hammadeh et al, 1999).

The need to screen donors for viruses such as human immunodeficiency virus (HIV) 1 and 2, hepatitis B and C viruses (HBV, HBC), and human T-cell leukemia virus (HTLV-I and -II) when tissues or cells are to be cryopreserved was emphasized by Tedder et al (1995). They observed leakage of cryopreservation bags in which bone marrow samples were stored, which thus led to contamination of liquid nitrogen dewars with the hepatitis B virus and subsequent transmission to patients after transplantation. Following the report of cross-contamination of human cells within liquid nitrogen storage vessels, McLaughlin (1999) recommended guidelines to ensure the safety of cryopreservation of donor gametes. It was recommended that straws and ampules containing gametes should be sealed and free from external contamination, ejaculates from unscreened individuals should not be stored in the same liquid nitrogen vessel as semen from donors who have been screened and found negative for contaminants, and storage of the material in cryopreservation dewars in a way that does not allow the liquid nitrogen to come in direct contact with the stored material. This can be achieved by storage in a system that does not require immersion of the material in the liquid phase, although concern has been expressed that this may compromise cell viability. Routine planned decontamination of cryopreservation dewars and ancillary equipment also help prevent the buildup of contamination.

With this background, the objective of this study was to evaluate the post-thaw recovery of viably motile and morphologically normal sperm in washed and unwashed normozoospermic and oligozoospermic semen samples after cryopreservation in the vapor phase of liquid nitrogen.

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Materials and Methods

Experiment 1: Evaluation of Storage of Whole Fresh Unwashed Semen Samples in Liquid Nitrogen Vapor

Semen—Fresh semen samples were obtained from adult men aged 25–35 years who were enrolled in a semen analysis pro-

tocol at the andrology clinic of the National University Hospital, Singapore. Fifteen normozoospermic and 15 oligozoospermic samples ($\leq 20 \times 10^6$ sperm/mL) were collected by masturbation in sterile containers after 2 to 3 days of sexual abstinence. Fresh samples were assessed for motility, vitality, and morphology using guidelines of the World Health Organization (WHO; 1999). Motile counts were calculated using a Makler chamber and vitality and morphology were assessed with eosin-nigrosin staining of smears and the hypo-osmotic swelling test (HOST).

Freezing—To 1 mL of the liquefied normozoospermic and oligozoospermic semen samples, 0.7 mL of Sperm Freeze solution (FertiPro, Brussels, Belgium) was added drop by drop while being gently mixed at room temperature. Sperm Freeze is a ready-to-use, chemically defined freezing medium containing HEPES, 15% glycerol, and 0.4% human serum albumin (negative for HIV and hepatitis B). The mixture was equilibrated for 10 minutes at room temperature and then equally divided into 2 aliquots. Each 0.85-mL aliquot was transferred to labeled, 1.8-mL plastic cryotubes (Nunclon, Roskilde, Denmark). The cryotubes were labeled ''V'' and ''L'' for vapor and liquid nitrogen phases, respectively. The L-phase cryotubes served as controls.

The V and L cryotubes were capped tightly and stored in a refrigerator at 4°C for 1 hour. In the meantime, the freezing dewar (height 66 cm; diameter 25 cm; MVE, Paris, France) was filled with liquid nitrogen up to a height of 15 cm. After 1 hour at 4°C the cryotubes were placed in a wire basket and lowered into the freezing dewar to a level of 37.5 cm above the liquid nitrogen (−53°C as measured with a thermocouple). The lid of the freezing dewar was closed tightly to prevent any leakage of liquid nitrogen vapor. After 15 minutes at −53°C the cryotubes were lowered to 27.5 cm (−109°C) above the liquid nitrogen, and after 15 minutes at this level (−109°C) the cryotubes were very quickly clipped to aluminum canes (MVE). The V cryotubes were clipped to the upper part of each cane and the L cryotubes of samples from the same patient were clipped to the lower part of each cane so that the V tubes were exposed to the vapor and the L tubes were immersed in the liquid nitrogen (−196°C). The canes were then quickly inserted into canisters and the canisters were lowered into storage dewars (MVE) containing liquid nitrogen. The time to attach the cryotubes to canes, place them in canisters, and then lower the canisters into storage dewars was less than 15 seconds. The liquid nitrogen level in the storage dewar was maintained at 15 cm and the V cryotubes were always at 15 cm above the liquid nitrogen (−189°C as measured by a thermocouple). All samples were stored 3 days before thaw and analysis.

Thawing—Cryotubes were taken out of the storage dewar and kept on a laboratory bench at room temperature (25°C) for 30 to 45 minutes. The thawed samples were assessed for sperm motility, vitality, and morphology according to WHO (1999) guidelines.

Experiment 2: Evaluation of Storage of Washed Semen Samples in Liquid Nitrogen Vapor Phase

Semen—For this experiment semen samples were obtained from adult men aged 26–34 years who were undergoing semen analysis at the andrology clinic of the National University Hospital, Singapore. Fifteen normozoospermic and 12 oligozoospermic samples collected by masturbation in sterile containers after 2 to 3 days of sexual abstinence were used for this experiment. Prewash and prefreeze analysis for motility, vitality, and morphology were carried out according to WHO (1999) protocols, similar to those in experiment 1.

Washing of Semen Samples—All semen samples were washed using the conventional swim-up method. Briefly, 1 mL of liquefied semen was mixed with 1 mL of in vitro fertilization culture medium (Ferticult-IVF, FertiPro) in 5-mL sterile plastic tubes. The suspension was then centrifuged at $500 \times g$ for 10 minutes. The supernatant was decanted and the sperm pellet was resuspended in 0.25 mL of the same medium. One-milliliter aliquots of fresh Ferticult-IVF medium were transferred to 10-mL plastic tubes, and the sperm suspension was slowly introduced under the medium in each tube. The tubes were loosely capped and incubated at 37°C in 5% CO_2 in air for 1 hour. The upper layer containing motile sperm just above the sperm-medium interface was separated and transferred to sterile 5-mL tubes for analysis and freezing.

Freezing, Storage, and Thawing—Each washed sample was assessed for sperm motility, morphology, and vitality (eosin-nigrosin smear and HOST) using WHO (1999) protocols. Each sample was divided into 2 aliquots labeled V and L, mixed with Sperm Freeze (FertiPro) solution, transferred to cryotubes, and frozen exactly according to the method used in experiment 1. Final storage in vapor (−189°C) and liquid nitrogen for 3 days was carried out, followed by thawing and analysis similar to that in experiment 1.

Evaluation and Calculation of Thawed Semen Parameters

Sperm Motility—Motile sperm were calculated according to WHO (1999) guidelines. Approximately 10 μL of thawed semen was placed in the center of the well of a Makler chamber (Sefi Instruments, Haifa, Israel) and covered with a coverglass. The chamber remained at 37°C for 5 minutes in order for the sperm to spread out and homogenize. The chamber was then placed on a warm stage of an upright microscope and the number of motile sperm (ie, evidence of movement) in 10 horizontal squares was quickly counted at 200× magnification. A mean of 3 such counts were taken and the results were expressed as $\times 10^6$/mL. The recovery rate of motile sperm following freezing and thawing was calculated as follows:

$$\text{motile recovery rate (\%)} = \frac{\text{post-thaw motile count} \times 10^6/\text{ml}}{\text{fresh motile count} \times 10^6/\text{ml}} \times 100$$

Sperm Vitality Measurement Using Eosin-Nigrosin—One part of the thawed semen sample was mixed with 3 parts of 1% aqueous eosin for 30 seconds on a precleaned, grease-free glass slide. Then, 3 parts of a 10% aqueous nigrosin solution was mixed with the eosin-semen mixture. Smears were evenly spread out onto fresh slides using the eosin-nigrosin-smear mixture. A total of 200 sperm were counted at 400× magnification for live : dead ratios and morphology (head and tail abnormalities). Live sperm stained white, dead sperm stained pink. The recovery rate of vital and morphologically normal sperm following freezing and thawing was calculated as follows:

Table 1. *Sperm recovery after cryopreservation of fresh unwashed semen samples in liquid and vapor phases of liquid nitrogen.*

	Recovery (Mean ± SD %)			
	Normozoospermic		Oligozoospermic	
Sperm parameters	Liquid	Vapor	Liquid	Vapor
Motility	42.76 ± 3.23*	45.52 ± 4.44*	16.53 ± 3.34‖	18.25 ± 4.36‖
Vitality				
Eosin-nigrosin	44.98 ± 3.69†	44.37 ± 3.61†	31.18 ± 3.19¶	31.61 ± 3.47¶
HOST	57.23 ± 8.5‡	57.57 ± 9.07‡	44.55 ± 6.24#	45.75 ± 6#
Morphology	74.95 ± 6.45§	73.15 ± 5.16§	52.89 ± 6.3**	51.48 ± 6.31**

Paired Student's *t* test: * $p < .05$; † $p > .05$; ‡ $p > .05$; § $p > .05$; ‖ $p < .05$; ¶ $p > .05$; # $p > .05$; ** $p > .05$.

$$\text{vital recovery rate (\%)} = \frac{\text{percentage of thawed white sperm}}{\text{percentage of fresh white sperm}} \times 100$$

$$\text{normal forms recovery rate (\%)} = \frac{\text{percentage of thawed normal sperm}}{\text{percentage of fresh normal sperm}} \times 100$$

Sperm Vitality Measurement Using HOST—A hypo-osmotic swelling solution (0.735 g sodium citrate and 1.351 g fructose in 100 mL of distilled water) was prepared and warmed to 37°C. To 1 mL of the warm HOST solution, 0.1 mL of the fresh or thawed sample was added, and the mixture was incubated at 37°C for 1 hour. After incubation, a drop of the well-mixed sample was placed onto a glass slide, covered with a coverslip, and curling of sperm tails was observed under phase contrast optics at a magnification of 400×. A total of 200 spermatozoa were counted and assessed for curled and noncurled tails. The recovery of live sperm after freezing and thawing was calculated as follows:

$$\text{vital recovery rate (\%)} = \frac{\text{percentage of thawed sperm with curled tails}}{\text{percentage of fresh sperm with curled tails}} \times 100$$

Statistical Analysis

Statistically significant differences in recovery rates of motility, vitality, and morphology among sperm cryopreserved in vaporous and liquid nitrogen were calculated using the paired Student's *t* test. A *P* value $<.05$ was considered significant.

Results

Evaluation of Storage of Whole Fresh, Unwashed Semen Samples in the Liquid Nitrogen Vapor Phase

Table 1 summarizes the percentage recovery of normozoospermic and oligozoospermic sperm following liquid and vapor nitrogen cryopreservation. The mean fresh prefreeze motility and vitality percentages (using HOST) for normozoospermic samples were 62.41% ± 4.80% and 75.10% ± 4.60%, respectively; and for oligozoospermic samples they were 55.33% ± 3.80% and 72.3% ± 2.8%, respectively. For normozoospermic samples, the mean ± SD percentage in post-thaw recovery rate of motile sperm in the vapor phase (−189°C) was significantly greater than recovery in the liquid phase at −196°C (45.52% ± 4.44% vs 42.76% ± 3.23%; $P < .05$). Recovery of live (vital) sperm was not significantly different between the liquid and vapor phases of liquid nitrogen (eosin-nigrosin: 44.98% ± 3.69% vs 44.37% ± 3.61%, $P > .05$; HOST: 57.23% ± 8.50% vs 57.57% ± 9.07%, $P > .05$). The percentages of normal forms surviving in both liquid and vapor phases were also not significantly different (74.95% ± 6.45% vs 73.15% ± 5.16%; $P > .05$).

For oligozoospermic samples the motile, live, and morphologically normal recovery percentages were much lower than they were for normozoospermic samples (Table 1). For oligozoospermic samples as well, the percentage of motile recovery was significantly higher in the vapor phase (−189°C) when compared with the liquid phase (18.25% ± 4.36% vs 16.53% ± 3.34%; $P < .05$). For oligozoospermic samples, the percentage recoveries of live (vital) and normal forms were also not significantly different between the 2 phases (Table 1).

Evaluation of Storage of Washed Semen Samples in Liquid Nitrogen Vapor

Table 2 summarizes the influence of liquid and vapor nitrogen cryopreservation on normozoospermic and oligozoospermic sperm samples previously washed by the conventional swim-up method. The values for recovery of motile and vital washed sperm were much lower than corresponding values for unwashed fresh sperm for both normozoospermia and oligozoospermia groups (Tables 1 and 2). The percentage recovery of motile sperm was significantly higher for washed normozoospermic samples frozen in the vapor phase compared with washed normozoospermic samples frozen in the liquid phase (37.58% ± 3.90% vs 34.44% ± 4.41%; $P < .05$). The percentage recovery of live and normal sperm in washed normozoospermic samples frozen in the vapor phase was not significantly different from the values of washed samples frozen in the liquid phase (Table 2).

The motile, live, and morphologically normal sperm

Table 2. *Sperm recovery after cryopreservation of washed semen samples in liquid and vapor phases of liquid nitrogen*

	Recovery (Mean ± SD %)			
	Normozoospermic		Oligozoospermic	
Sperm parameters	Liquid	Vapor	Liquid	Vapor
Motility	34.44 ± 4.41*	37.58 ± 3.90*	10.32 ± 2.54‖	12.25 ± 2.81‖
Vitality				
Eosin-nigrosin	39.28 ± 3.71†	39.54 ± 3.78†	27.68 ± 2.61¶	27.92 ± 2.7¶
HOST	49.49 ± 8.84‡	49.86 ± 8.89‡	38.67 ± 5.7#	38.91 ± 5.60#
Morphology	78.65 ± 6.82§	77.80 ± 7.27§	49.06 ± 6.33**	48.20 ± 7.41**

Paired Student's *t* test: * $p < .05$; † $p > .05$; ‡ $p > .05$; § $p > .05$; ‖ $p < .05$; ¶ $p > .05$; # $p > .05$; ** $p > .05$.

recovery rates of washed, frozen normozoospermic samples were much higher than corresponding values for oligozoospermic samples in both liquid and vapor phases (Table 2). The percentage recovery of motile sperm in the vapor was significantly higher than it was for the liquid phase for oligozoospermic washed samples. The freeze–thawed, recovered live, and normal washed oligozoospermic sperm samples followed similar trends as the unwashed samples for both phases of liquid nitrogen in which there were no significant differences between liquid and vapor phases (Table 2).

Discussion

With most fresh unwashed human sperm freezing protocols (rapid manual or slow programmed machine methods), the motile recovery rates for normozoospermic samples are between 40%–50% using glycerol as a cryoprotectant. The results of the present study compare well with such recovery rates for unwashed normozoospermic samples frozen both in liquid and vapor phases (42.76% ± 3.23% to 45.52% ± 4.44%). Motile recovery rates were much better in the vapor (−189°C) phase compared with liquid nitrogen (−196°C) for washed and unwashed samples. In addition, the recovery rates of motile sperm in frozen–thawed normozoospermic specimens were much higher compared with the oligozoospermic specimens, suggesting that normozoospermic samples freeze and store better in vapor than oligozoospermic samples do. It is also apparent that oligozoospermic samples freeze and store better in vapor than in liquid. The improvement in cryopreservation observed by freezing to −189°C could be the result of the difference in freezing method. Specifically, the sample that was stored in the vapor phase was cooled from −109°C to −189°C in vapor and the sample stored in liquid nitrogen was cooled from −109°C to −196°C in liquid. Given that heat transfer in a liquid is more efficient than in a vapor, the cooling rates are likely different between these samples, which may explain the difference in motility recovered.

The percentage of live sperm in post-thaw oligozoospermic unwashed samples was much lower than it was in unwashed normozoospermic samples. Also, recovery rates of sperm vitality between the vapor and liquid phases using the WHO eosin-nigrosin smear and HOST had no significant differences. It thus appears that temperatures of −189°C to −196°C did not have a drastic effect on live sperm forms, and as such, vapor freezing of human sperm will not be detrimental to vital sperm forms. Furthermore, recovery of live sperm forms using HOST showed higher values than the eosin-nigrosin smear for both oligozoospermic and normozoospermic samples. Because the principle of HOST is to detect damage to the integrity of head and tail membranes, unlike the eosin-nigrosin smear, which detects only the integrity of sperm head membranes, higher values for HOST over eosin-nigrosin are expected. However, vapor-phase temperatures of −189°C do not appear to have greater damaging effects on the sperm membranes compared with the liquid phase. Selective permeability of water molecules to reach osmotic equilibrium under exposure to hypo-osmotic conditions reflects the functional integrity of sperm tail membranes, thus resulting in straightening or curling of sperm tails. It appears that the sperm tail membranes are much better preserved in the freeze–thaw procedure; hence it is possible to recover adequate frozen-thawed live sperm for intracytoplasmic sperm injection using HOST in ART programs in both normal and subnormal semen samples frozen and thawed in a vapor phase (Chan et al, 1992; Ahmadi and Ng, 1997; Sallam et al, 2001). The changes in vitality and HOST results in this study may also be due to the addition and removal of hypertonic concentrations of glycerol. Addition of glycerol to human sperm before cooling and their removal after warming has shown to cause osmotic stress to sperm membranes, thus making them leaky (Gao et al, 1993, 1995).

The percentage of morphologically normal sperm was much less in post-thaw oligozoospermic, unwashed semen samples compared with that of normozoospermic unwashed semen. No significant differences were observed, however, between vapor and liquid phases. The sperm

from men with oligozoospermia produce more reactive oxygen species (ROS) than do sperm from men with normal sperm counts (Aitken et al, 1989; Iwasaki and Gagnon, 1992). Furthermore, ROS production occurs during the freeze-thaw procedure (Mazzilli et al, 1995; Wang et al, 1997). These factors may also contribute to reduced sperm survival, motility, and aberrant morphology in oligozoospermic semen compared with normozoospermic semen samples.

Recent studies on the effect of storage temperature on sperm cryopreservation by Trummer et al (1998) showed greater decreases in sperm motility recovery among specimens maintained at −70°C than among those maintained at −196°C (47% vs 39%). However, no differences in post-thaw sperm morphology were detected among sperm cryopreserved at −70°C vs −196°C. The results of the present study were, interestingly, contrary in that sperm motility recovery values were higher in vapor at −189°C, although they were consistent in terms of recovery of normal forms. Lesser fluctuations in temperature between the vapor phase at −189°C and liquid phase at −196°C because of narrow intervals compared with −70°C and −196°C may be the reason for the contrary results of post-thaw motility recovery.

Although the motile recovery counts of washed semen samples were much lower than they were for unwashed samples, the trends of significance between liquid and vapor phases were similar for washed and unwashed samples. This may be attributable to the protective nature of seminal plasma when unwashed fresh semen samples are frozen and stored (Mortimer, 1990). Seminal plasma contains high levels of energy substrates such as fructose, which play an important role in sperm motility by providing energy through mitochondria located around the mid-piece. The absence of these substrates in washed, frozen–thawed samples perhaps resulted in lower motility recovery values compared with unwashed samples.

In addition, vital sperm recovery was reduced in cryopreserved, washed sperm samples compared with unwashed samples. This may also be due to the protective nature of seminal plasma. Metal ions such as zinc, which are found in seminal plasma, contribute to maintaining the integrity of sperm membranes such as the plasma and acrosomal membranes.

Good correlations between oocyte penetration and hypo-osmotic swelling, between percentages of motile and swollen spermatozoa, and between percentages of swollen spermatozoa and spermatozoa that did not stain with eosin were reported previously by Jeyendran et al (1992). Very little relationship was reported between the percentage of swollen spermatozoa and the percentage of normal sperm present in the ejaculate. HOST and eosin-nigrosin smears appear to be good complementary tests for evaluating sperm vitality after freezing and storage of washed and unwashed, and normal and subnormal semen samples for artificial insemination and ART programs. The results of this study show that freezing of human semen in nitrogen vapor is as good as freezing in liquid nitrogen and is therefore a safer and better substitute for storage of male gametes in sperm banks in the light of the fears of cross-contamination with infectious microorganisms such as HIV and hepatitis B, which can thrive in liquid nitrogen.

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