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Cryopreservation of Human Semen

Comparison of Cryopreservatives, Sources of Variability, and Prediction of Post-thaw Survival

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ABSTRACT: Human semen was cryopreserved using Human Sperm Preservation Medium, TEST-Yolk buffer, or glycerol alone. Sperm characteristics for each specimen were measured before and after freezing to determine which cryopreservative resulted in better cryosurvival and recovery of motile sperm. Sperm frozen in Human Sperm Preservation Medium had a significantly better recovery of all semen parameters (motility, velocity, and recovery) than either TEST-Yolk or glycerol alone. Statistical analyses also were done to examine the variability between and within donor semen specimens. Differences between donors, between specimens, and measurements within donors all contributed to variability of sperm characteristics. Specimen-to-specimen variability for a given donor represented 12% to 47% of the total variability, whereas processing and measurement variability represented 12% to 41%. Donors also varied in the ability of their sperm to tolerate freezing. There was a relationship between motile count after dilution with cryopreservative and post-thaw motile count. This relationship allows the prediction of poor-thaw survival before freezing a specimen.

Key words: Post-thaw sperm, donor insemination, infertility.
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Cryopreservation of human semen is known to result in diminished motility (Taylor et al, 1982; Aitken et al, 1983; Hammitt et al, 1989; see review, Centola, 1989), various forms of structural damage (Alexander, 1977; Serafini et al, 1986) including acrosome loss (Centola et al, 1990a), as well as diminished in vitro penetration of zona-free hamster oocytes (Cohen et al, 1981; Critser et al, 1987). Furthermore, artificial insemination with frozen–thawed donor semen has resulted in reduced pregnancy rates. Pregnancy rates have been reported as low as 5% (Richter et al, 1984) to 43% (Wong et al, 1989) to 61% (Steinberger and Smith, 1973). A dual consecutive (2-day) insemination with frozen donor semen resulted in a success rate of 40% in our patient population (Centola et al, 1990b).

Optimum cryosurvival is dependent not only on freezing methodologies, but also on the cryopreservative used. Cryopreservatives include glycerol alone (Jeyendran et al, 1984), buffers containing egg yolk (Hammitt et al, 1988), and even milk (Prins and Weidel, 1986). Prins and coworkers (1986) compared eight different cryopreservatives, with sperm frozen in yolk buffer demonstrating the highest post-thaw survival. Mahadevan and Trounson (1983) developed a modified Tyrode's medium containing 7.5% glycerol, referred to as Human Sperm Preservation Medium (HSPM). Comparison of sperm frozen in HSPM with egg yolk-citrate-glycerol medium showed no significant difference in post-thaw motility or viability between the two cryopreservatives. However, the pregnancy rate was higher for semen frozen in HSPM than in the egg yolk-citrate medium, although the values were not significantly different ($P = 0.67$) (Mahadevan and Trounson, 1983).

Post-thaw sperm motility has been unpredictable based on prefreeze values. Harrison and Sheppard (1980) suggested that cryosurvival is low when initial motility is low, and that an initial sperm density of greater than 40 million/mL and an initial motility of 60% is necessary for adequate cryosurvival. Others have corroborated this concept (Keel and Karow, 1980), and still others have suggested that post-thaw motility is not related to prefreeze motility (Behrman and Sawada, 1966; Amelar and Dubin, 1980).

The objectives of the current study were to compare the effects of three cryopreservatives (HSPM, TEST-yolk, and glycerol) on post-thaw sperm motility and concentration; to determine the variability in motility and count (fresh and post-thaw) between and within a group of known fertile donors; and to determine if cryosurvival could be predicted from sperm motion parameters before freezing.

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Materials and Methods

Semen Specimens

Semen specimens were acquired from known fertile donors participating in the therapeutic donor insemination program. The specimens were collected by masturbation and delivered within a half-hour to the laboratory, where they were maintained on a 37°C warming block until complete liquefaction. Each specimen was assessed by computer-assisted semen analysis with a Hamilton-Thorn analyzer (HTM-2030; Hamilton-Thorn Research, Danvers, MA) using standard set-up parameters (Centola et al, 1990a).

Semen Cryopreservation

Human Sperm Preservation Medium (HSPM)—Human Sperm Preservation Medium was prepared according to the method of Mahadevan and Trounson (1983) and stored frozen until use. Vials of HSPM were thawed and maintained at 37°C. Warmed (37°C) HSPM was added in 0.05- to 0.1-mL droplets to the fresh semen on a mechanical rotator until an equal volume of HSPM had been added (1:1 dilution HSPM:semen). This took approximately 4 to 5 minutes. After thorough mixing, a 5.5-μL aliquot of the specimen was removed with an Eppendorf pipettor for computer-assisted semen analysis. The specimen then was placed into sterile cryovials (1.8-mL vials; Corning Vials, Corning, NY) at a volume of 1.0 mL semen-HSPM per vial.

TEST-Yolk Buffer—Commercially available TEST-Yolk buffer was used (freezing medium; Irvine Scientific, Irvine, CA). TEST-Yolk was warmed to 37°C, then gradually added to semen until a 1:1 dilution was achieved (Mahadevan and Trounson, 1983; Prins and Weidel, 1986). The specimen was added to cryovials as described for HSPM.

Glycerol Alone—Glycerol at 37°C to a final concentration of 7.5% vol/vol was added dropwise to the specimen with thorough mixing on a mechanical rotator using standard laboratory procedures (Sherman, 1954, 1963; Mahadevan and Trounson, 1983). The final specimen then was added to cryovials as above, using a volume of 0.5 mL per vial. A previous pilot study in this laboratory (unpublished) demonstrated that there was no significant difference in post-thaw sperm parameters when vials contained a volume of 0.5 mL versus 1.0 mL. We thus adhered to our standard protocol of using a volume of 0.5 mL when using glycerol as the cryopreservative because the dilution factor was less (0.075:1). When using HSPM or TEST-yolk preservatives, the volume per vial was 1.0 mL, because the specimen was diluted more (1:1 dilution).

All vials were loaded onto metal freezing canes. Our routine laboratory methodology uses slow manual cooling in liquid nitrogen (LN_2) vapor and storage in the liquid phase (Centola et al, 1990a). After a 48-hour minimum storage in the liquid phase, selected vials were removed, thawed at room temperature for 5 minutes, then equilibrated at 37°C for approximately 10 minutes, and once again assessed by computer-assisted semen analysis.

Statistical Analyses

Sperm characteristics were measured in the fresh ejaculate, in the specimen after dilution with cryopreservative but before freezing, and also after freezing and thawing, for each specimen. Cryopreservatives were compared in terms of post-thaw/fresh ratios of total sperm count, motile count, and progressive velocity; and in terms of post-thaw/fresh differences in percent rapid and percent motile. Ratios were used for comparisons of total count, motile count, and velocity because the distributions of the ratios were less skewed and had more stable variability than distributions of differences.

Comparisons were made using a mixed-model analysis of variance with preservative as a fixed effect and donor as a random effect. Mean values and their standard errors were corrected for donor effects.

Variability of sperm characteristics was partitioned into components due to variation between donors, between specimens for a given donor, and between measurements of a given specimen. We used an analysis very similar to one described by Huechel et al (1983). For each sperm characteristic, X, the k-th measurement of the j-th specimen from the i-th donor was modeled as:

$$X_{ijk} = X + S_i + T_{ij} + \epsilon_{ijk},$$

where X is an overall mean, S_i is a donor-specific mean, T_{ij} is a specimen-specific mean, and ϵ_{ijk} is the variation of measurements around the specimen-specific mean. Each of the components were assumed to be independent, so the variance of X was the sum of the variances of S, T, and ϵ. These variances were estimated by the REML option of the VARCOMP procedure in SAS (SAS Institute, 1985).

The probability of low (<10 million/mL) post-thaw motile counts was estimated from normal distributions that depended on the motile count after dilution with cryopreservative. We chose 10 million motile sperm post-thaw as a cutoff, because at 10 million motile sperm/vial, a maximum of two vials is needed per dose (dose equals >20 million motile sperm) for artificial insemination. Both the mean and standard deviation of the normal distributions for post-thaw motile count were proportional to the diluted motile count. Proportionality factors for the mean were estimated by a weighted analysis of covariance with diluted count as covariate, and including a donor-by-diluted count interaction. Intercept and donor main effect terms were excluded because a diluted count of zero should imply a post-thaw count of zero. Observations were weighted by 1/diluted count^2 because of the proportionality of standard deviation to diluted count.

Results

Comparison of Cryopreservatives

Table 1 shows means and standard deviations of each sperm characteristic for each cryopreservative. Data were compiled from 125 specimens from 34 donors (1 to 11 per donor, median = 2). Some specimens were tested with more than one cryopreservative. Table 2 shows the recovery of these characteristics after freezing and thawing. As described in the previous section, recovery was measured as percent of prefreeze values for total count, motile count, and progressive velocity, and as change from prefreeze values for percent rapid and percent motile. Human Sperm Preservation Medium had significantly better recovery ($P <$ 0.05) of all characteristics than either TEST-Yolk or glyc-

Table 1. *Means (± standard deviation) of sperm characteristics before and after freezing with three cryopreservatives*

	TEST-yolk (n = 19)	Glycerol (n = 45)*	HSPM (n = 80)
Count (×10⁶/ml)			
Fresh	99.8 (53.3)	101.8 (42.8)	102.3 (38.8)
Thawed	32.3 (16.5)	34.8 (19.1)	45.9 (26.6)
Motile count (×10⁶/ml)			
Fresh	81.9 (52.0)	86.4 (42.4)	82.0 (38.8)
Thawed	11.8 (12.3)	14.9 (13.3)	22.5 (16.3)
Percent rapid			
Fresh	44.1 (13.7)	45.0 (16.6)	36.0 (19.8)
Thawed	6.8 (8.5)	6.5 (7.4)	18.1 (13.4)
Percent total motility			
Fresh	76.5 (12.4)	82.0 (10.6)	78.3 (13.4)
Thawed	31.4 (18.1)	37.6 (18.5)	47.4 (14.8)
Progressive velocity (μ/sec)			
Fresh	34.8 (4.2)	34.2 (6.1)	31.5 (6.5)
Thawed	22.6 (6.1)	21.2 (5.6)	29.4 (8.1)

HSPM-human sperm preservation medium.

* Thawed values for count and motile count in the glycerol group have been adjusted to be comparable to the 1:1 dilution ratio used with TEST-yolk and HSPM. The actual dilution ratio with glycerol was 0.075:1.

erol. For two characteristics, post-thaw values with HSPM were not significantly different from prefreeze values. The ratio of post-thaw count to fresh count was 0.457, not significantly different from the value of 0.5 due to dilution alone. The ratio of post-thaw to fresh progressive velocity was 0.967, not significantly different from 1. Other characteristics did show deterioration after thawing, but not as much as with the other cryopreservatives. The only significant difference between TEST-Yolk and glycerol was that the former had better recovery of progressive velocity.

Variability

Variability of sperm characteristics was due in part to differences between donors and in part to differences between specimens and measurements within donors. Data for 330 specimens from 22 donors were used to estimate variance components for donors. Table 3 shows that donor-to-donor variability represented less than half of the total variability for each characteristic. Most of the variation was due to within donor factors such as variation between specimens for a given donor and variation in processing and measurement of a given specimen (such as mixing, sampling, and counting).

To study within-donor sources of variation further, 28 specimens from 15 donors (1 to 3 per donor, median = 2) were examined at each step of the HSPM freezing protocol: fresh specimen, specimen after dilution with HSPM before freezing, the first and last aliquotted vials before freezing, and a post-thaw test vial. Measurements of two vials from each specimen before freezing permitted processing and measurement variability to be estimated. Table 4 shows the results of these measurements. Specimen-to-specimen variability for a given donor represented 12% to 47% of the total variability in diluted prefreeze values. Processing and measurement variability of a given specimen represented 12% to 41%. Although the numbers of specimens and donors are small, it appears that both components of variability (specimen-to-specimen, and processing and measurement) make comparable contributions to variability. Note, however, that because the vials were set after dilution, any variability in the dilution process was included in the specimen component of variability.

Predicting Low Post-thaw Motile Count

The objective of this analysis was to determine whether prefreeze sperm characteristics could be used to predict low (<10 million/mL) post-thaw motile counts. The data consisted of sperm characteristics for 230 ejaculates from 20 donors (1 to 3 per donor, median = 11.5). Each characteristic was measured three times: fresh sample, sample after dilution with HSPM, and post-thaw. Graphs of post-thaw motile count versus each of the prefreeze measurements showed that the closest association was with motile count after dilution with HSPM (Fig 1). Both the mean and the standard deviation of post-thaw count were approximately proportional to the count of the diluted specimen.

Analysis of covariance showed that the slope of the re-

Table 2. *Recovery of sperm characteristics following freezing and thawing with three cryopreservatives**

	Cryopreservatives		
Characteristic	TEST-yolk (n = 19)	Glycerol (n = 45)	HSPM (n = 80)
Count (thawed/fresh)	0.282 (0.053)	0.328 (0.032)	0.457 (0.028)
Motile count (thawed/fresh)	0.110 (0.040)	0.111 (0.024)	0.261 (0.021)
Percent rapid (thawed − fresh)	−37.5 (2.8)	−39.8 (1.7)	−19.4 (1.5)
Percent total motile (thawed − fresh)	−41.3 (3.2)	−46.3 (2.0)	−33.5 (1.7)
Progressive velocity (thawed/fresh)	0.738 (0.049)	0.623 (0.030)	0.967 (0.026)

HSPM-human sperm preservation medium.

* Table entries are presented as the mean ± SEM. Means and standard errors are corrected for differences among donors. Table values for count and motile count in the glycerol group have been adjusted to be comparable to the 1:1 dilution ratio used with TEST-Yolk and HSPM. The actual dilution ratio in the glycerol group was 0.075:1.0.

Table 3. *Between-donor and within-donor components of variance (±SEM)**

Characteristic	Between	Within	Between/Total
Count ($\times 10^6$/ml)			
Fresh	747.5 (296.8)	1605.0 (129.8)	0.32
Diluted	378.0 (158.5)	1326.3 (107.3)	0.22
After thawing	418.0 (181.6)	724.5 (59.0)	0.37
Motile count ($\times 10^6$/ml)			
Fresh	522.6 (210.3)	1339.3 (130.0)	0.28
Diluted	364.7 (155.2)	1036.9 (100.7)	0.26
After thawing	139.3 (60.4)	344.3 (33.6)	0.29
Percent rapid			
Fresh	169.3 (71.6)	344.2 (33.7)	0.33
Diluted	134.9 (58.6)	247.1 (24.2)	0.35
After thawing	37.5 (19.0)	175.3 (17.1)	0.18
Percent total motile			
Fresh	67.0 (24.7)	66.3 (5.4)	0.50
Diluted	97.4 (39.2)	127.5 (10.4)	0.43
After thawing	53.4 (23.2)	191.2 (15.4)	0.22
Progressive velocity (μ/sec)			
Fresh	11.0 (4.3)	14.7 (1.4)	0.43
Diluted	19.3 (7.5)	24.4 (2.4)	0.44
After thawing	2.2 (1.6)	28.8 (2.8)	0.07

* Results are based on 330 specimens from 22 donors (1 to 40 per donor; median = 14.5) for count and percent motile, and on a subset consisting of 230 specimens from 20 donors (1 to 30 per donor; median = 11.5) for other parameters. Dilution is 1:1 with HSPM.

lationship varied significantly from donor to donor (P = 0.014). Since the slope is the proportion of diluted count recovered after thawing, this suggests that donors vary in the ability of their sperm to tolerate freezing. The average recovery proportion was 0.52. This is consistent with the proportion 0.246 for HSPM in Table 2, because the latter refers to fresh count, before 1:1 dilution with HSPM. The standard deviation of the proportion across donors was estimated as 0.12.

The combination of variation in slope between donors with variability around the mean for a given donor leads to an estimate of the standard deviation of post-thaw motile count as 0.26 × diluted motile count. We calculated the probability that post-thaw motile count would be inadequate (<10 million/mL) using a normal distribution with a mean of 0.52 × diluted motile count and a standard deviation of 0.26 × diluted motile count. Probabilities are shown in Table 5. The probability is 47% for a diluted count of 20 million/mL, and declines to 11% for a diluted count of 50 million/mL.

Discussion

We compared three commonly used cryopreservatives: human sperm preservation medium (HSPM, Mahadevan and Trounson, 1983), TEST-Yolk buffer, and 7.5% glycerol. In conjunction with this analysis, we also examined the interdonor and intradonor variability of sperm motility and concentration after cryopreservation with HSPM.

There was a consistent and significant loss of motile sperm concentration after cryopreservation using HSPM, as well as with TEST-Yolk and glycerol (Tables 1 and 2). There are numerous reports of the decrease in motility after freezing and thawing, but no published reports of loss in number (or concentration) of sperm after thawing. Under ideal circumstances, if no loss of cells occurred during the freezing and thawing process, the ratio of thawed to fresh sperm (Table 2) for count and motile count would be 0.5, taking in to account the dilution with the cryopreservative. With the HSPM cryopreservative, the ratio drops to 0.46, which is not a very significant loss. For the other two cryopreservatives, there is a greater decrease in the ratio. This could not be attributed to dilution errors. Although this has not been reported in the literature, others have indicated similar results (J. Critser, personal communication). Fragmentation or disintegration of spermatozoa into unrecogniz-

Table 4. *Proportions of total variability in sperm characteristics attributable to between-donor between-specimen, and processing and measurement variation*

Characteristic (after dilution with HSPM, before freezing)	Between donor	Between specimen	Processing and measurement
Count ($\times 10^6$)	0.25	0.47	0.28
Motile count ($\times 10^6$/ml)	0.31	0.28	0.41
Percent rapid	0.66	0.22	0.12
Percent total motility	0.49	0.12	0.39
Progressive velocity (μ/sec)	0.60	0.28	0.12

HSPM-human sperm preservation medium.

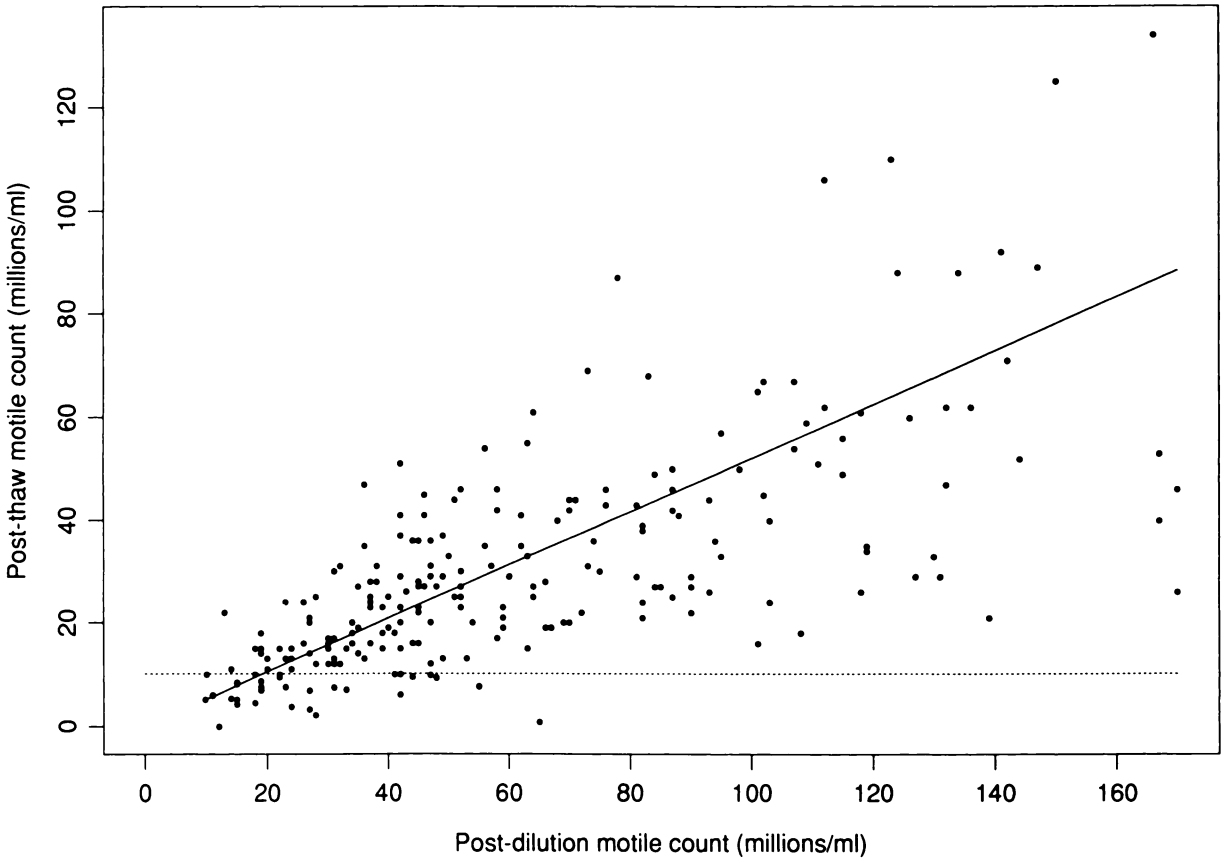

FIG. 1. Relationship between motile count after thawing and motile count after dilution for 230 samples from 20 donors (1 to 30 specimens per donor; median = 11.5). The solid line shows the average relationship for all donors; there were significant differences in slope between donors (see text). The dotted line at 10 million/ml indicates our cutoff for the minimum acceptable motile count after thawing.

able cell fragments after thawing may account for this apparent loss of numbers, because cell debris and fragments unrecognizable as intact cells by the computer-assisted semen analysis system are often seen on examination of the post-thaw specimen. Among the three cryopreservatives examined, HSPM yielded the best recovery of sperm concentration and motion parameters (Table 2). Recovery with TEST-Yolk buffer was not significantly different from that with glycerol, except that the former had better recovery of progressive velocity. These data suggest that HSPM is a superior cryopreservative based on post-thaw recovery of motile sperm, confirming the earlier data of Mahadevan and Trounson (1983).

Differences between donors, between specimens, and between measurements within donors all contributed to the variability of sperm characteristics (Tables 3 and 4). This variability was not surprising, because there is normally a considerable fluctuation in sperm count that is often seasonal (WHO, 1987). In our population of donors, donor-to-donor variability contributed less than half of the total variability for each characteristic. The primary sources of variation were factors affecting the sperm parameters for a given donor and specimen (within-donor variability). We also found significant differences between donors in the tolerance of their sperm to freezing. On average, post-thaw motile count was 52% of the prefreeze HSPM-diluted motile count. Proportions for individual donors varied around this average, however, with a standard deviation of 12 percentage points. It is also important to consider variability associated with measurements and processing techniques, which may be of similar magnitude to intradonor variability.

Table 5. *Estimated probability that motile count after thawing will be less than 10 million per milliliter**

HSPM-diluted motile count (million/ml)	Probability
20	0.47
30	0.24
40	0.15
50	0.11

HSPM-human sperm preservation medium.

* Probabilities were calculated from a normal distribution with mean = 0.52 × diluted motile count and standard deviation = 0.26 × diluted motile count.

A fairly strong relationship exists between motile count after dilution with HSPM but before freezing and post-thaw motile count (Fig 1). We were able to use this relationship to estimate the probability that a particular specimen will have an inadequate post-thaw motile count. We chose a cut-off for post-thaw motile count of 10 million motile sperm/mL. The standard dose for insemination is >20 million motile sperm; thus, a maximum of two vials (each with 1 mL of semen-cryopreservative) would equal a dose for insemination. The probability of inadequate post-thaw count is high when diluted prefreeze count is less than 20 million/mL, but drops sharply at higher prefreeze counts (Table 5). It is interesting to note that Harrison and Sheppard (1980) suggested that an initial sperm density of over 40 million/mL and an initial motility of 60% was necessary for successful cryopreservation.

Based on our data, it may be appropriate to assess sperm concentration and motility after dilution with the cryopreservative rather than in the fresh sample. Specimens with a diluted count of <20 million/mL are likely to have a post-thaw motile count of <10 million/mL. A decision of whether to proceed with the processing can then be made. Using this scheme would not only save the technician time, but also liquid nitrogen usage, because both the freezing and post-thaw analysis would be eliminated for those donor specimens that did not fall within the acceptable range.

These conclusions are based on results in our population of known fertile donors. They must be validated in other patient populations before prefreezing screening criteria can be adapted. In addition, criteria should be relaxed for patients with cancer or those undergoing vasectomy for whom cryopreservation may be the only chance for maintaining biologic fertility. These patients can be appropriately counseled as to the probability of adequate post-thaw sperm survival based on initial examination of the ejaculate. Furthermore, the initial results of cryopreservation and probability analysis might induce development of a better freezing protocol specifically tailored to an individual's specimens. They also might be counseled to consider assisted techniques such as in vitro fertilization or microinjection.

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