

Cryopreservation of human spermatozoa: comparison of two cryopreservation methods and three cryoprotectants

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Objective: To evaluate the ability of two cryopreservation methods and three cryoprotectants to preserve sperm quality.

Design: A prospective clinical study.

Setting: Male infertility clinic at a tertiary healthcare center.

Patient(s): Twenty infertile men and 10 healthy donors.

Intervention(s): In the first experiment, semen was cryopreserved by either the Irvine Scientific method (IS) or the Cleveland Clinic Foundation (CCF) method. In the second experiment, semen was cryopreserved by the IS method and one of three cryoprotectants: TES and Tris yolk buffer, Sperm Freezing Medium, or Enhance Sperm Freeze.

Main Outcome Measure(s): Postthaw sperm motility, cryosurvival, and kinematics.

Result(s): Percentages of postthaw sperm motility and cryosurvival were higher in the IS cryopreservation method compared with in the CCF method (15.94 ± 9.19 vs. 12.07 ± 7.31 and 47.42 ± 17.44 vs. 35.76 ± 17.56). However, the CCF method resulted in significantly better sperm kinematics. Postthaw motility in the donors and patients was highest in the samples frozen in TES and Tris yolk buffer medium.

Conclusion(s): The IS method was associated with more flash freezing compared with the CCF method and resulted in better preservation of sperm motility and a higher cryosurvival rate. TES and Tris yolk buffer was most effective at protecting sperm from the negative effects of the cryopreservation process. This may be due to the presence of egg yolk along with glycerol. (Fertil Steril® 2004;82:913–8. ©2004 by American Society for Reproductive Medicine.)

Key Words: Human spermatozoa, cryopreservation, cryoprotectants, TEST-yolk buffer, Sperm Freezing Medium, Enhance Sperm Freeze, cryopreservation methods

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Sperm cryopreservation is routinely performed in assisted reproduction centers and andrology laboratories. During the cryopreservation process, osmotic effects of freezing and thawing lower the fertilizing capacity of the spermatozoa by damaging cell membranes (1–3) and severely impairing sperm motility (4, 5), creating morphology alterations such as coiled tails, and causing damage to acrosome and structural and functional integrity (6, 7).

The technical aspects of freezing and thawing sperm and preparing cryopreservation media have been refined over the years (8–11). Various methods of cryopreservation have been evaluated for their effects on sperm motility (5, 12, 13). However, there is no standard

freezing and thawing method, and the methods for freezing and thawing semen that optimize motility recovery have not been firmly established. In addition, the optimum rate of temperature drop during freezing remains controversial (5, 8, 12). The flash-freezing technique in which the sample is plunged directly into liquid nitrogen produces sperm recovery rates that are comparable to those seen with computer-controlled, slow-staged freezing (13).

In addition, a variety of cryoprotectants are available to protect sperm from the negative effects of the cryopreservation process. Many andrology laboratories use TES and Tris yolk buffer (TYB; Irvine Scientific, Santa Ana, CA), but some of the other media commonly used

are the following: Sperm Freezing Medium (Medi-Cult, Copenhagen, Denmark) and Enhance Sperm Freeze (Conception Technologies, San Diego, CA). Although all of these cryoprotectants preserve sperm quality to a certain extent, they have never been compared to determine which one is most effective.

The main purpose of our current study was to determine the cryopreservation method and cryoprotectant associated with the best postthaw sperm quality. Specifically, we compared two rapid-freezing cryopreservation methods and three commercially available cryoprotectants for their effects on a number of variables, including sperm motility and sperm motion characteristics.

MATERIALS AND METHODS

The Cleveland Clinic Foundation Institutional Review Board approved this study. Semen samples were obtained from 20 subfertile patients and 10 healthy donors 2 to 3 days after sexual abstinence. Basic semen analysis was performed according to 1999 World Health Organization (WHO) guidelines (14).

Semen Analysis

After liquefaction, 5 μL of semen was loaded on a counting chamber (MicroCell, Conception Technologies, La Jolla, CA). Total sperm count ($\times 10^6/\text{mL}$) and percentage motility were measured manually and sperm motion kinetics were assessed with a computer assisted semen analyzer (CASA, IVOS, 10.7s; Hamilton Thorne Research, Beverly, MA). Sperm morphology (percentage normal) was assessed according to both 1999 WHO guidelines (14) and Kruger's strict criteria (15).

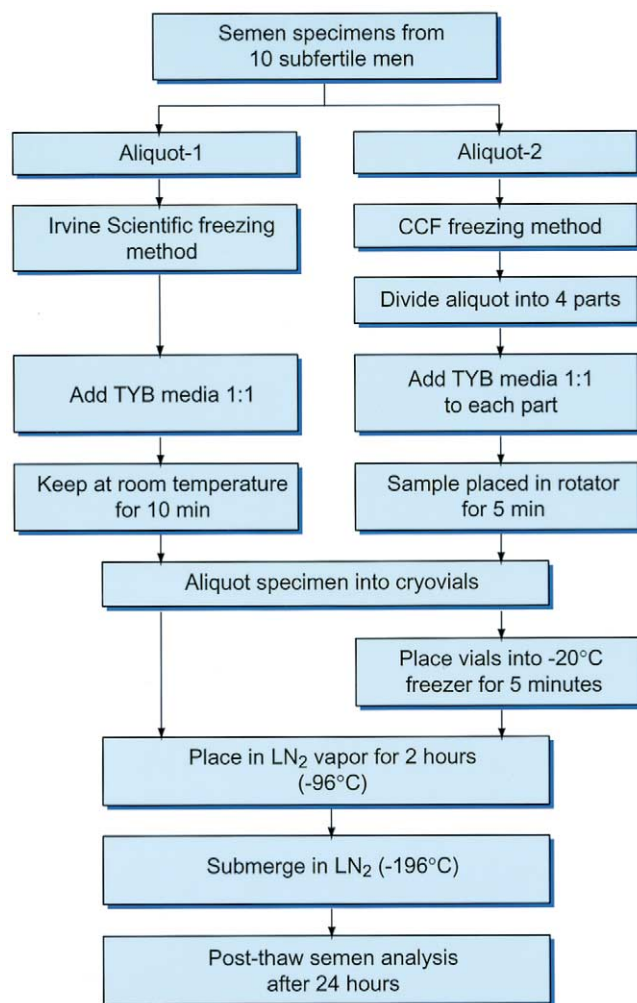
Comparison of Two Cryopreservation Methods

After initial semen analysis, samples from 10 patients were divided into two equal aliquots. Each aliquot was cryopreserved by two freezing methods—the Cleveland Clinic Foundation (CCF) method and the Irvine Scientific (IS) method—and by using TYB as the freezing medium (Fig. 1).

In the CCF method, a 5-mL vial of freezing medium (TYB, Irvine Scientific) was thawed. An aliquot equal to 25% of the original semen sample was added to an equal volume of the freezing media. This process was repeated 4 times to give a final ratio of 1:1 (vol/vol) of freezing medium to ejaculate. The aliquots were placed in cryovials at -20°C for 8 minutes and then in nitrogen vapors at -96°C for 2 hours. Finally, the aliquots were immersed in liquid nitrogen at -196°C until analysis (16). In the IS method, the entire volume of freezing medium (TYB) was added at one time, 1:1 (vol/vol) of freezing medium to ejaculate. The aliquots were placed in cryovials, followed by being placed in nitro-

FIGURE 1

Flow diagram of experiment 1 (comparison of two cryopreservation methods).



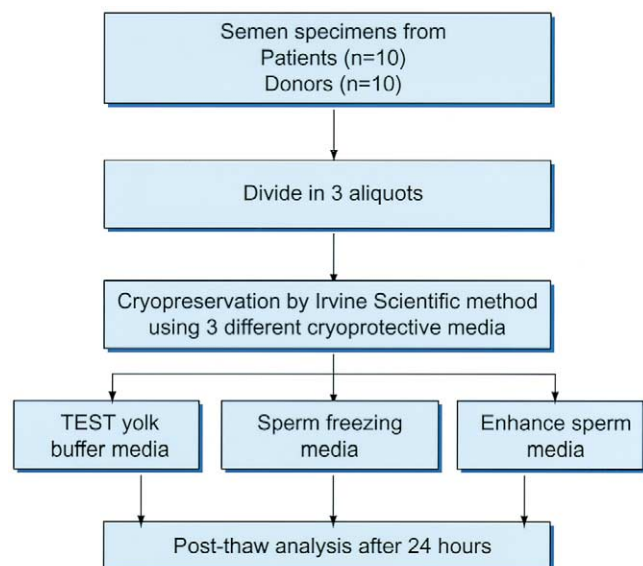
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gen vapors at -96°C for 2 hours, and then samples were immersed in liquid nitrogen at -196°C .

The frozen aliquots from both methods were thawed in an identical manner. The samples were kept at room temperature for 5 minutes and then in an incubator at 37°C for 20 minutes. Semen analysis was performed with the freezing media intact. Postthaw sperm concentration ($\times 10^6/\text{mL}$), motility (percentage) and total motile sperm count ($\times 10^6$), sperm motion characteristics, and morphology (percentage normal; WHO and Kruger's criteria) were evaluated. The preeeze and postthaw sperm motion kinetics measured by CASA included curvilinear velocity (micrometers per second), straight-line velocity (micrometers per second), average path velocity (micrometers per second), linearity (percentage), and amplitude of lateral head displacement

FIGURE 2

Flow diagram of experiment 2 (comparison of three cryoprotectants).



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(micrometers). Percentage cryosurvival (the proportion of preefreeze motile sperm that remained motile immediately after thaw) and longevity (the percentage motile sperm at 0, 60, 120, and 180 minutes) were assessed after thawing.

Comparison Among Three Cryoprotectants

Semen samples from 10 subfertile patients and 10 healthy donors each were divided into three aliquots (Fig. 2). Each aliquot was cryopreserved by using one of three different commercially available cryoprotectants: TYB (Irvine Scientific), Sperm Freezing Medium (Medi-Cult), and Enhance Sperm Freeze (Conception Technologies). All three aliquots were then cryopreserved by the IS freezing method. The samples were thawed, and the post-thaw total motile sperm count, percentage motility, and morphology (WHO and Kruger's) were evaluated. Motility was analyzed at 0, 60, 120, and 180 minutes after thawing.

Statistical Analysis

Paired Student's *t* test was used to compare the two cryopreservation methods and three different cryoprotectants. Results are given as mean and standard deviation. A *P* value of $<.05$ was considered statistically significant. Statistical software Graph Pad InStat version 3.00 (Graph Pad Software, Inc., San Diego, CA) was used.

TABLE 1

Semen characteristics in postthaw samples cryopreserved by the Cleveland Clinic Foundation (CCF) method and the Irvine Scientific (IS) method.

Variable	CCF method (mean \pm SD)	IS method (mean \pm SD)	<i>P</i> *
Count ($\times 10^6$ /mL)	43.06 \pm 32.66	41.66 \pm 33.91	.54
Motility (%)	12.07 \pm 7.31	15.94 \pm 9.19	.006
TMS ($\times 10^6$)	5.64 \pm 6.61	5.87 \pm 5.94	.65
WHO morphology (%)	21.77 \pm 9.47	20.90 \pm 9.29	.50
VCL (μ m/s)	22.46 \pm 6.77	18.59 \pm 6.98	.01
VSL (μ m/s)	9.82 \pm 3.76	7.78 \pm 3.61	.02
VAP (μ m/s)	14.76 \pm 4.93	11.84 \pm 4.64	.04
Linearity (%)	46.36 \pm 7.79	44.57 \pm 7.75	.57
ALH (μ m)	1.62 \pm 0.41	1.4 \pm 0.41	.05
Postthaw longevity (%)			
0 min	12.07 \pm 7.32	15.94 \pm 9.19	.006
60 min	9.76 \pm 6.51	7.73 \pm 4.99	.77
120 min	7.82 \pm 5.24	7.68 \pm 6.73	.95
180 min	6.75 \pm 4.07	7.73 \pm 0.38	.38
% Cryosurvival	35.76 \pm 17.56	47.42 \pm 17.44	.008

Note: TMS = total motility sperm count; WHO = World Health Organization; VCL = curvilinear velocity; VSL = straight-line velocity; VAP = average path velocity; ALH = amplitude of lateral sperm head displacement.

* *P* < .05 was significant by paired Student's *t* test.

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RESULTS

Comparison of Two Cryopreservation Methods

In the patient samples that were cryopreserved by the CCF and IS methods, the percentage motility was significantly lower in the postthaw samples than in the preefreeze samples (preefreeze motility: 32.59% \pm 13.81%; postthaw motility for IS method: 15.94% \pm 9.19%, *P* < .01; postthaw motility for CCF method: 12.07% \pm 7.31%, *P* < .001). The semen characteristics of the postthaw samples cryopreserved by the CCF and IS methods are illustrated in Table 1. The postthaw percentage of motile sperm and percentage cryosurvival were significantly higher in samples processed by the IS method (Table 1). On the other hand, the CCF method resulted in a significantly higher postthaw curvilinear velocity, straight-line velocity, and average path velocity (Table 1).

In the patients, the percentage of sperm with normal morphology (WHO criteria) was significantly lower in the postthaw samples than in the preefreeze samples with both cryopreservation methods (preefreeze: 34.10% \pm 10.75%; postthaw for IS method: 20.88% \pm 9.29%, *P* < .0006; postthaw for CCF method: 21.77% \pm 9.47%, *P* < .002). However, the percentage difference in postthaw normal sperm morphology between the two methods was not statistically significant (Table 1). Postthaw sperm longevity at 60, 120,

TABLE 2

Postthaw motility, cryosurvival rate, and morphology of semen samples cryopreserved in three different cryoprotectants.

Variable	TYB	Sperm Freezing Medium	Enhance Sperm Freeze	<i>P</i> ^a	<i>P</i> ^b	<i>P</i> ^c
Donors (n = 10)						
Postthaw motility (%)	26.75 ± 10.13	19.37 ± 6.41	17.59 ± 5.95	.04	.04	.31
Cryosurvival (%)	47.92 ± 15.68	36.58 ± 13.95	32.27 ± 11.68	.03	.025	.25
WHO morphology (%)	27.3 ± 7.81	29.5 ± 6.58	27.0 ± 7.22	.17	.79	.02
Kruger's morphology (%)	7.7 ± 3.12	9.00 ± 1.94	8.2 ± 2.09	.05	.34	.02
Patients (n = 10)						
Postthaw motility (%)	23.70 ± 9.67	21.16 ± 7.35	16.04 ± 6.60	.05	.006	.004
Cryosurvival (%)	38.30 ± 11.83	34.75 ± 10.68	26.16 ± 8.75	.082	.003	.003
WHO morphology (%)	24.80 ± 7.61	24.4 ± 6.05	25.4 ± 7.07	.78	.60	.40
Kruger's morphology (%)	8.00 ± 2.35	7.70 ± 2.45	7.90 ± 2.18	.49	.67	.64

Note: All values are mean ± SD. *P* < .05 was considered significant by paired Student's *t* test. TYB = TES and Tris Yolk Buffer; WHO = World Health Organization.

^aTYB vs. Sperm Freezing Medium.

^bTYB vs. Enhance Sperm Freeze.

^cSperm Freezing Medium vs. Enhance Sperm Freeze.

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and 180 minutes was not significantly different between the two methods (Table 1).

Comparison of the Three Cryoprotectants

In the donor samples, postthaw motility immediately after thawing was significantly higher in the aliquots frozen with TYB cryoprotectant than in those frozen with Sperm Freezing Medium (*P* = .04) and Enhance Sperm Freeze (*P* = .04; Table 2). The cryosurvival rate was higher in the donor samples frozen in TYB than in those frozen in Sperm Freezing Medium (*P* = .03) and Enhance Sperm Freeze (*P* = .025).

In the patient group, postthaw sperm motility was significantly different among the three cryoprotectant groups. Postthaw motility for the aliquots frozen with TYB cryoprotectant was higher than that in the aliquots frozen in Sperm Freezing Medium (*P* = .05) and Enhance Sperm Freeze (*P* = .006; Table 2). The cryosurvival rate was lowest in the patient samples that were frozen in Enhance Sperm Freeze compared with in Sperm Freezing Medium (*P* = .003) and TYB (*P* = .003). The cryosurvival rates in the patient samples cryopreserved in either TYB or Sperm Freezing Medium were not significantly different (Table 2).

In both the donors and patients, the samples cryopreserved in TYB had the highest sperm longevity at all four time measurements (0, 60, 120, and 180 minutes), compared with the case of the aliquots that were frozen in Sperm Freezing Medium and Enhance Sperm Freeze media (Table 3). Similarly, in donors and patients, postthaw normal morphology according to the WHO and Kruger's strict criteria was significantly lower than the prefreeze values for all three cryoprotectants (Table 4). No significant difference was found in postthaw morphology (WHO and Kruger's) among the three cryoprotectants in either the patients or donors (Table 2).

DISCUSSION

In the present study, we compared the effect of two cryopreservation methods (IS and CCF methods) on sperm motility, kinematics, survival, and morphology in a population of infertile men. The study results revealed a significant postthaw decrease in sperm motility and percentage normal morphology compared with the case of the prefreeze samples, irrespective of the cryopreservation method used. Whereas the IS method for semen cryopreservation provided superior postthaw sperm motility and cryosurvival compared with the CCF method (16), the latter method resulted in significantly better sperm kinematics. Both of these methods involve rapid freezing. However, freezing may occur more quickly with the IS method.

The advantages of the fast-freezing and slow-staged cooling methods have long been debated. Studies have reported results in favor of both the fast-freezing method (5) and the slow-staged cooling method (12, 17, 18). A recent study showed that there was no difference in sperm quality preservation when semen samples were frozen by fast-freezing technique or by slow, controlled freezing method, either in liquid nitrogen or vapor-phase nitrogen (19). Computerized slow-staged freezing was reported to limit cryoinjury to low-quality spermatozoa (12). A study by Verheyen et al. (5) focused on the interaction between the cooling temperature and the thawing method. According to their study, the freeze-thaw process was most successful when sperm were thawed at 37°C after rapid vapor freezing and at 22°C after the slower, computer-controlled freezing. Such interaction between cooling and thawing rates was confirmed by another study, which highlighted the role of water movement rather than ice crystal formation as the cause in cryoinjury (9).

TABLE 3

Postthaw longevity of samples cryopreserved in three different cryoprotectants.

Minutes	TYB	Sperm Freezing Medium	Enhance Sperm Freeze	<i>P</i> ^a	<i>P</i> ^b	<i>P</i> ^c
Donors (n = 10)						
0	26.75 ± 10.13	19.37 ± 6.41	17.59 ± 5.95	.04	.04	.31
60	11.07 ± 6.84	6.52 ± 5.34	5.97 ± 4.98	.021	.003	.734
120	7.92 ± 7.59	3.78 ± 4.60	3.05 ± 3.96	.013	.006	.104
180	5.22 ± 5.91	2.52 ± 3.74	2.76 ± 4.28	.109	.093	.731
Patients (n = 10)						
0	23.70 ± 9.67	21.16 ± 7.35	16.04 ± 6.60	.05	.006	.004
60	7.88 ± 4.23	5.84 ± 3.84	6.14 ± 3.88	.034	.051	.731
120	6.65 ± 6.67	5.21 ± 5.31	4.10 ± 4.00	.101	.049	.17
180	5.01 ± 5.33	3.38 ± 3.52	2.49 ± 3.45	.06	.005	.116

Note: Longevity was assessed by the percentage of motile sperm at different time intervals. All values are mean ± SD. *P* < .05 was considered significant by paired Student's *t* test. TYB = TES and Tris Yolk Buffer.

^aTYB vs. Sperm Freezing Medium.

^bTYB vs. Enhance Sperm Freeze.

^cSperm Freezing Medium vs. Enhance Sperm Freeze.

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The second part of our study investigated sperm motility, cryosurvival, and morphology by the same freezing method (IS method) but by employing three different but commonly used cryoprotectants in donors and subfertile men. The samples that were frozen with TYB had significantly higher postthaw total motility and cryosurvival rates than did the sperm frozen with Sperm Freezing Medium and Enhance Sperm Freeze.

Lucena and Obando (20) showed that cell survival is <15% when human spermatozoa are subjected to the freeze–thaw process without the addition of a cryoprotectant.

Therefore, use of a cryoprotective agent is indispensable in preventing injury to human spermatozoa during the cryopreservation process. A good cryoprotectant readily permeates cells and is relatively nontoxic in concentrations of ≥1 M (21). Cryoprotectants work by lowering the concentration of electrolytes during freezing and therefore decrease the extent of osmotic shrinkage at a given temperature. The extent of protection depends primarily on the molar ratio of the cryoprotective agent to endogenous solutes inside and outside the cells. The general protective mechanism of action is colligative.

TABLE 4

Prefreeze and postthaw morphology of semen samples cryopreserved in three different cryoprotectants.

Variable	Prefreeze normal morphology (%)	Cryoprotectant	Postthaw normal morphology (%)	<i>P</i> ^a
Donors (n = 10)				
WHO	37.6 ± 8.52	TYB	27.3 ± 7.81	.0001
		Sperm Freezing Medium	29.5 ± 6.58	.0006
		Enhance Sperm Freeze	27.0 ± 7.22	.0001
Kruger's	11.2 ± 2.89	TYB	7.7 ± 3.12	.0004
		Sperm Freezing Medium	9.0 ± 1.94	.001
		Enhance Sperm Freeze	8.2 ± 2.09	.0001
Patients (n = 10)				
WHO	35.7 ± 8.97	TYB	24.8 ± 7.61	.0001
		Sperm Freezing Medium	24.4 ± 6.05	.0007
		Enhance Sperm Freeze	25.4 ± 7.07	.0008
Kruger's	11.2 ± 3.52	TYB	8.0 ± 2.35	.0001
		Sperm Freezing Medium	7.7 ± 2.45	.0004
		Enhance Sperm Freeze	7.9 ± 2.18	.0002

Note: All values are mean ± SD. *P* < .05 was significant by paired Student's *t* test. TYB = TES and Tris Yolk Buffer; WHO = World Health Organization.

^aPrefreeze vs. postthaw morphology.

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Previous studies from our laboratory found that semen parameters deteriorated more significantly when they were cryopreserved in glycerol alone as compared with in a combination of glycerol and TYB (14). Our present results demonstrated that TYB preserves sperm functions better than Sperm Freezing Medium and Enhance Sperm Freeze. Other studies have also shown that TYB results in better recovery of motile sperm (17, 22–24). However, the limitation of our study was small sample size. Further large studies can be conducted to conclusively prove the effectiveness of TYB over other media by comparing effectiveness by using sperm function tests.

The mechanism by which TYB functions is unclear. TES and Tris yolk buffer for sperm cryopreservation is composed of egg yolk; a compound of lipoproteins, phospholipids, cholesterol; and various other, less abundant components in combination with Tris ([hydroxymethyl] aminomethane) buffers. Egg yolk may help reduce the deleterious effects on membrane structures that occur during rapid cooling (25, 26), possibly because of an exchange of lipids between spermatozoa cell membranes and a phospholipid portion of the low-density protein of egg yolk that alters cell membrane molecular composition and maintains its fluidity (27). This action may also increase the stability of the membrane enzyme system, acrosin–proacrosin.

In summary, our study results have demonstrated that the IS method for semen cryopreservation provides superior postthaw sperm motility and cryosurvival compared with the case of the CCF method. However, the CCF method resulted in significantly better sperm kinematics. Our study also demonstrates that use of TYB cryoprotectant results in better preservation of postthaw sperm quality compared with the case of Sperm Freezing Medium and Enhance Sperm Freeze.

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