DETRIMENTAL EFFECTS OF CRYOPRESERVATION ON THE STRUCTURAL AND FUNCTIONAL INTEGRITY OF THE SPERM MEMBRANE

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Many centers have been disappointed with the pregnancy rate following the insemination of cryopreserved–thawed sperm, despite the maintenance of an adequate motile density. The possibility exists that damage to the sperm membrane might occur despite preservation of other semen parameters. Simple measurements of structural integrity (viability) and functional integrity (hypoosmotic swelling test) were performed on thawed specimens. In each instance, both the viability and HOS scores were less than the critical 50% level. Specimens from three different commercial centers had very poor HOS and viability scores from two of the centers, and, though the scores were generally ≥50% from the third center, this was achieved by eliminating 11 of 12 donors. Reducing the glycerol concentration from 12 to 7% and switching from Nunc vials to plastic embryo straws did not improve the poor sperm membrane tests. The possibility exists that if modification of the cryopreservation technique leads to improved HOS and viability scores, perhaps improved pregnancy results will be realized.

Key Words: Cryopreservation; HOS testing; Viability test.

INTRODUCTION

Cryopreservation and thawing have been found to cause damage to the plasma membrane and acrosome of human spermatozoa, as evidenced by significant ultrastructural changes demonstrated by electron microscopy [11, 17]. Several studies have found significantly reduced fertility potential of frozen/thawed sperm, compared to fresh sperm following intracervical insemination [10, 14, 15]. Electron microscopic (EM) evaluation, though important from a research perspective, would not be practical for clinical evaluation of specimens. However, if membrane damage is almost always followed by impairment to motile density (MD), then sophisticated EM studies would not be necessary. The majority of commercial centers are concerned only about whether the thawed specimen has an adequate motile density.

Previous data, however, have found a dichotomy between MD and sperm membrane damage [4], as evidenced by low scores on two very practical tests of membrane damage: sperm viability and the hypoosmotic swelling test (HOS) [4, 9]. These preliminary data demonstrated

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that in 7 men with normal MD of $\geq 10 \times 10^6$/mL all 7 had HOS scores and viability less than the clinically critical 50% level with precryopreservation, HOS, and viability mean scores of 68.5 and 70, respectively, but post-thaw values of 32.8 and 33.7% [5].

The purpose of the study presented herein was to determine if a larger series would confirm the initial preliminary data. Furthermore, commercial sperm banks that do not evaluate membrane tests, and one claiming to do so, would be tested by both viability and HOS tests post-thaw. Finally, modifications of the cryopreservation technique would be tried to see if improved sperm membrane tests might be achieved.

MATERIALS/METHODS

Viability Testing. The specimen is placed into a Forma-Scientific incubator, set at 37 °C for 30 min (or longer, depending on liquefaction time). Using a standard autopipet, a 7-lambda (1 lambda = 1/1000 mL) aliquot is removed from the specimen and placed on slide. An equal 7-lambda drop of 0.5 g eosin stain is mixed with the semen on the slide. After coverslip is placed on top, the slide is placed under a phase microscope at 400 × power. Using the standard exclusion method, 100 individual cells are counted under the scope. The cells are divided into one of two groups: those demonstrating a red color (showing that the cell is dead and the membrane had deteriorated), and those demonstrating a normal white color (showing a membrane that is structurally intact). Normal levels range from 60% viable to 100% viable. Fifty percent to 60% is a gray-zone region.

Hypoosmotic Swelling Test. A 100-lambda aliquot of semen is mixed with a 1-mL (1000-lambda) portion of HOS reagent. The reagent is comprised of water, sodium citrate, and fructose. The solution of semen/HOS reagent is then placed in a heat block incubator, at 37 °C, for 30 min. After the incubation period, the solution is removed, and a 7-lambda portion of it is taken and placed on a slide with a coverslip. One hundred individual cells are next counted and separated into one of three groups: open, restricted, and unaffected. Both open and restricted cells are considered normal in this test. They indicate sperm membranes that have the ability to actively transport water across their membrane; thus, their tails demonstrate an acute swelling. Those that are unaffected are considered abnormal and demonstrate damage to the functional integrity of the membrane. Normal scores range from 60% showing HOS swelling changes to 100%. Fifty to 59% are considered gray-zone scores.

Sperm Count. Both the sperm count and the motility percentage are used by the majority of centers offering cryopreservation of human sperm to judge the post-cryopreservation effectiveness of their ejaculates. By multiplying the count by the motility, a motile density score is calculated (normal scores are $\geq 10 \times 10^6$). Although a portion of this study was designed to demonstrate the inaccuracy of judging by motile density, the motility and count were used not to judge a specimen but to verify that the specimens met with standard post-freezing evaluations, and then to evaluate the specimens with the HOS and viability tests. Thus, only specimens demonstrating over 10×10^6/mL motile sperm were included. A 7-lambda aliquot of semen was removed and counted in Makler chamber and percent motility was also determined.

Freezing and Thawing. The ejaculate was then placed in a 20-mL conical tube and mixed with an equal proportion of test yolk buffer (TYB) as the cryoprotectant. TYB contains sodium citrate, fructose, penicillin-G, and the active ingredient, glycerol (which comprises 12% of the whole solution). The semen and TYB were pipetted together, slowly (to reduce bubble formation), for 4 min. After this time, the conical tube was capped and placed in a 500-mL water bath at 37 °C, then placed into a BioCool Freezer set at 10 °C. The specimen in the water bath remained in the BioCool for 60 min to reduce the

specimen temperature (on average) to 12 °C. The conical tubes were removed after the appropriate time period from the BioCool and quickly placed under a sterile hood. The contents of the tubes were then pipetted into 1-mL Nunc vials. The Nunc vials were then placed into liquid nitrogen vapors (− 100 °C, 2 in. from the actual liquid), for 90 min. At the end of this period, the Nunc vials were separated into canes, then plunged into the liquid nitrogen, at which time specimen was cryopreserved.

The Nunc vials were removed from the liquid nitrogen for thawing and placed into a 37 °C incubator for 5 min. Following thawing, repeat viability, HOS, sperm count, and motility tests were evaluated.

Corroborating Initial Preliminary Data. An additional 6 specimens with pre-cryopreservation semen specimens with MDs over 15×10^6/mL and viability and HOS scores over 60% were cryopreserved and tested for these same parameters post-thaw.

Evaluation of Semen from Commercial Sources. The commercial sources, excluding center number 1, did not perform baseline or initial viability or HOS tests on their specimens. Thus, there were no pre-freeze data to compare post-thaw results. In all eight commercial samples, the specimens were shipped to the laboratory via DryShipper. Each source had individual thawing procedures, which were followed exactly as prescribed. After thawing, the HOS, viability, count, and motility tests were evaluated on the specimen.

Cryopreservation of Semen in Embryo Freezing Straws. The freezing procedure in this portion resembles that of standard freezing technique. The one variation is that in place of the 1-mL Nunc vials in which to store the semen, 0.5-mL embryo freezing straws were substituted. These straws are 6-in. plastic tubes. All of the timing, temperatures, and cryoprotectant/semen ratios remained constant.

Cryopreservation of Semen Following Cryoprotectant Glycerol Reduction from 12 to 7%. This procedure is the same as the standard freezing method, but the glycerol is reduced to 7%. Each specimen was evaluated by HOS, viability, count, and motility. The variation in the cryoprotectant was as follows: The cryoprotectant was reduced to 7% via refrigeration media. Refrigeration media is TYB with the exception of glycerol. Thus, after reducing the glycerol concentration to 7%, all of the standard freezing methods were followed.

RESULTS

The pre-freeze and post-thaw HOS and viability scores in 6 semen samples are seen in Table 1. All 6 specimens had a post-thaw MD of $\geq 10 \times 10^6$/mL, but all also had HOS and viability scores < 50%. Changing to embryo straws rather than Nunc vials did not improve post-thaw results, as seen in Table 2 where the specimens were equally split into two aliquots: One was frozen in a Nunc vial and the other in a plastic embryo straw. Comparison of freezing in TYB with 12% glycerol to TYB reduced to 7% glycerol did not improve viability and HOS scores (Table 3). Once again all were <50%.

The post-thaw viability tests and HOS scores are seen in Table 4. Only commercial center 1 (CC1) showed viability scores above 50% in all three specimens tested, and an HOS test above 50% in two of three specimens. All five specimens from the other two centers had both HOS scores and viability tests <50%. Commercial center 1 was the only center of the three that evaluated viability (but did not test HOS). They stated that to produce a viability score over 50% it was necessary to eliminate 11 of 12 donors.

TABLE 1 Freezing Data: Viability and HOS Scores of Donor Semen

	Viability (%)		HOS Test (%)	
Donor Number	Before Freezing	After Thawing	Before Freezing	After Thawing
1	71	38	67	35
2	58	26	58	26
3	61	24	61	24
4	71	30	69	30
5	65	37	62	37
6	83	42	82	40
7	82	39	81	38
8	92	48	85	45
9	88	35	87	33
10	92	42	90	37
11	96	38	94	37
12	83	28	78	24
13	77	42	74	40
Mean	78	36	76	34
±1 SD	12.3	7.1	11.7	6.5

Note. Patients 1-7 were previously reported [6].

DISCUSSION

The data presented herein corroborate previous preliminary results, suggesting that despite maintaining an adequate motile density most sperm specimens demonstrate sperm membrane damage [6]. This may explain why some centers report lower pregnancy rates following insemination of cryopreserved-thawed sperm than fresh sperm [1]. In fact, this may explain why so few pregnancies have been achieved following the insemination of cryopreserved-thawed sperm from either cancer patients or post-vasectomy patients [2, 7, 13]. Recently some pregnancies from cryopreserved sperm from patients with Hodgkin's disease before treatment have been recorded using IVF-ET [16].

TABLE 2 Freezing Data: Results of Cryopreservation of Semen in Embryo Straws

	HOS Scores (%)		
		Post-thaw	
Donor Number	Pre-freeze	Nunc Vial	Straw
1	77	42	24
2	86	46	22
3	96	39	30
Mean	86	42	25
±1 SD	9.5	3.5	4.1

TABLE 3 Hypoosmotic Swelling Test Scores Following Cryopreservation and Thawing of Semen Following Reduction in Glycerol from 12 to 7%

	HOS Scores (%)		
		Post-thawing	
Donor Number	Pre-freeze	12% Glycerol (Control)	Glycerol Reduction (7%)
1	92	42	35
2	88	38	35
3	83	28	24
Mean	88	36	31
±1 SD	4.5	7.2	6.3

The fact that two of three commercial centers demonstrated very poor HOS and viability scores and the third one needed to eliminate over 90% of prospective donors to acquire specimens with >50% viability indicates that the low scores obtained by our center were not uniquely related to a technical problem. Nevertheless, the fact that some centers report a success rate of therapeutic donor insemination (TDI) with frozen/thawed sperm similar to the use of fresh sperm suggests that possibly some slight difference in technique might ameliorate membrane damage [12, 8].

It is not certain that merely maintaining adequate motile density and an HOS and viability score ≥50% will preserve the fertility potential as well as fresh sperm. However, since very few pregnancies have been recorded when the latter two tests are <50% even with the other semen parameters quite normal [4], it makes sense to at least measure these parameters and, if poor, consider switching to another technique that maintains normal values.

Nevertheless, freezing might cause reduced fertility in other ways. Recently, some data have been presented demonstrating that freezing of seminal plasma significantly reduces its

TABLE 4 Viability and HOS Scores Following Thawing of Cryopreserved Commercial Sources

Specimen Number/Center	Post-thaw (%)	
	Viability	HOS
CC1-1	72	65
CC1-2	62	52
CC1-3	51	47
CC2-1	17	13
CC2-2	24	20
CC3-1	28	24
CC3-2	39	31
CC3-3	19	17
Mean	39	34
±1 SD	21	19

motility-enhancing capability [3]. At present, there is no definite technique that produces good post-thaw HOS and viability scores, and this is the goal of future research. Presently we are increasing the speed of cryopreservation, and the preliminary results are encouraging. We are also investigating changing the thawing procedure.

REFERENCES

1. Beck WW Jr (1978): Artificial insemination and preservation of semen. Urol Clinics North Am 5:593-605
2. Bracken RB, Smith RD (1980): Is semen cryopreservation helpful in testicular cancer? Urology 15:581-583
3. Check DJ, Check JH, Bollendorf A (1991): Fresh versus frozen seminal plasma for enhancing sperm motility in asthenozoospermic males. Arch Androl 26:79-81
4. Check JH, Epstein R, Nowroozi K, Shanis BS, Wu CH, Bollendorf A (1989): The hypo-osmotic swelling test as a useful adjunct to the semen analysis to predict fertility potential. Fertil Steril 52:159-161
5. Check JH, Nowroozi K, Wu CH, Bollendorf A (1988): Correlation of semen analysis and hypo-osmotic swelling test with subsequent pregnancies. Arch Androl 20:257-260
6. Check ML, Check JH (1991): Poor hypo-osmotic swelling test results from cryopreserved sperm despite preservation of sperm motility. Arch Androl 26:37-41
7. Friedman S, Broder S (1981): Homologous artificial insemination after long-term semen cryopreservation. Fertil Steril 35:321-324
8. Gillett WR, Cameron MC, Maekay-Duff M, Seddon RJ (1986): Pregnancy rates with artificial insemination by donor: the influence of the cryopreservation method and coexistent infertility factors. New Zealand Med J 891-893
9. Jeyendran RS, Van der Ven HH, Perez-Palaez M, Carbo BG, Zaneveld LJD (1984): Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. J Reprod Fertil 70:219-227
10. Lecton J, Selwood T, Trounson A, Wood C (1980): Artificial donor insemination: frozen versus fresh semen. Aust New Zealand J Obstet Gynaecol 20:205-207
11. Mahadervan MM, Trounson AO (1984): Relationship of fine structure of sperm head to fertility of frozen human semen. Fertil Steril 41:257-293
12. Richter MA, Haning RV Jr, Shapiro SS (1984): Artificial donor insemination: fresh versus frozen semen: the patient as her own control. Fertil Steril 41:277-280
13. Sanger WG, Armitage JO, Schmidt MA (1980): Feasibility of semen cryopreservation in patients with malignant disease. JAMA 244:789-790
14. Sherman JK (1973): Synopsis of the use of frozen human sperm since 1964: State of the art of human semen banking. Fertil Steril 24:397-412
15. Silva PD, Meisch J, Schauberger CW (1989): Intrauterine insemination of cryopreserved donor semen. Fertil Steril 52:243-245
16. Tournaye H, Camus M, Bollen N, Wisanto A, Van Steirteghem, Devroey P (1991): In vitro fertilization techniques with frozen-thawed sperm: a method for preserving the progenitive potential of Hodgkin patients. Fertil Steril 55:443-445
17. Wooley DM, Richardson DW (1978): Ultrastructural injury to human spermatozoa after freezing and thawing. J Reprod Fertil 53:389-394