ANDROLOGY

Optimal Utilization of Cryopreserved Human Semen for Assisted Reproduction: Recovery and Maintenance of Sperm Motility and Viability

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Purpose: *Our purpose was to evaluate sperm motility and viability and the maintenance of these parameters in already cryopreserved semen samples following repeated freezing/thawing cycles.*

Methods: *Human spermatozoa were subjected to five cycles of cryopreservation/thawing. Recovery of sperm motility and viability and the proportion of viable nonmotile sperm were determined up to 6 hr after thaw.*

Results: *Sperm motilities (prefreeze motility, 70.1%; n = 9 samples) after each of five freeze/thaw cycles were 24.4, 8.0, 3.5, 1.5, and 1.8%. The recovery of sperm viability was higher than that of motility after each cycle: 39.1, 25.3, 22.6, 17.8, and 16.5%. Recoveries of motility and viability were improved if the thawed samples were left in the original cryopreservation medium prior to refreezing vs. if a washing/resuspension step was included. The recovery of sperm motility in the first thawing cycle was indicative of the expected motile sperm recovery in the second thawing cycle.*

Conclusions: *Cryopreserved semen that is intended to be reused in future assisted reproduction treatments should be thawed only once and aliquoted in the original freezing medium before refreezing. The recovery of sperm motility and viability in the second thawing cycle, thus the applicability of the sample in conventional in vitro fertilization or intracytoplasmic sperm injection may be anticipated in >90% of the samples. In view of intracytoplasmic sperm injection it is important that sperm viability is maintained better than motility; after the first, second, and third thawing cycles the ratios of motile:nonmotile viable sperm were 1:1, 1:4, and 1:7, respectively.*

KEY WORDS: sperm cryopreservation; viability; motility; banking; freeze/thaw cycles.

INTRODUCTION

Successful freezing and thawing of human sperm have been performed for over four decades (1). Freezing protocols have undergone several changes in an attempt to minimize the detrimental effects of the cryopreservation on sperm motility and function. It is generally believed that multiple factors may contribute to the cryodamage of sperm cells, causing a decline in motility (2–4). In cryopreserved/thawed semen samples the recovery of sperm motility is between 25 and 50% of that of the prefreezing value, and there are substantial man-to-man variations (5–7). In studies with a single freezing/thawing cycle, there were differing opinions about whether, in addition to a lower recovery of motility, thawed sperm also show an abbreviated retention of motility and viability. For instance, some investigators found a diminished maintenance of sperm motility and viability (8), while others did not find a difference in long-term retention of sperm motility between fresh and cryopreserved/thawed samples in sperm pellets recovered from Percoll centrifugation (reviewed in Ref. 9). In a third laboratory, the authors

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did report differences in the rate of sperm motility decline in postthaw samples vs. fresh semen along with diminished acrosomal integrity and sperm penetration rates of zona-free hamster oocytes in the thawed samples (6,10).

The key issue for fertilization potential is the recovery of sperm motility in the thawed sample. To optimize the motile sperm yield, several investigators have studied the effects of various cryoprotectants, freezing and thawing protocols, postthaw processing, and sperm motility patterns (3–5,7,10,11–16). Traditionally, motility has also been used to estimate sperm viability and to predict fertility. However, a recently developed staining technique, which is not detrimental to sperm motility, has facilitated the examination of sperm viability independently from motility (17). This has allowed us to estimate another important sperm parameter: sperm viability in the motile and nonmotile sperm fractions.

The introduction of intracytoplasmic sperm injection (ICSI), which allows the efficient utilization of a small number of sperm for the treatment of severe male-factor infertility, initiated a considerable interest in the possible refreezing and later use of semen in men who have only a limited number of vials or low concentrations of cryopreserved sperm available. Such situations could arise, for example, after banking of sperm in men with major medical illnesses, prior to chemo- or radiation therapy, or in men who, for reasons such as vasectomy or impotency, are unable to produce spermatozoa. It has also become increasingly clear that our new approach, in which independent aliquots of the thawed sample are simultaneously evaluated for changes in sperm motility and viability, is likely to be useful for ICSI application because it provides an estimate of the proportion of viable nonmotile sperm that can potentially be utilized in this assisted reproduction modality.

To address the question of efficient use and further refreezing of cryopreserved human sperm we have examined four aspects: (a) the effects of repeated freeze/thaw cycles on the viability and motility of human spermatozoa; (b) the effects of washing and resuspension of the thawed sperm in fresh cryomedium vs. retention of the thawed samples in the original medium prior to refreezing; (c) the time-related maintenance of sperm motility and viability after two thaw cycles; and (d) the predictive value of the original and first thaw cycle motilities for the recovery of motility after the second thaw.

MATERIALS AND METHODS

Samples

Normospermic semen samples studied (>20 million sperm/ml, >40% sperm motility) originated in the Sperm Cryopreservation Program of the Sperm Physiology Laboratory, Department of Ob/Gyn, Yale University School of Medicine. The samples were obtained by masturbation after an abstinence period of 48 hr. The specimens were collected into sterile polyethylene cups and were allowed to liquefy for 30 min at room temperature. An initial semen analysis was performed on a small aliquot of each sample before freezing. The prefreezing sperm concentrations were within the range of 35–143 million/ml and the motility ranged from 51 to 86%. The samples used were cryopreserved for at least 1 year prior to these experiments. All studies were approved by the Human Investigation Committee of Yale School of Medicine.

Cryopreservation and Thawing of Spermatozoa

After the initial semen analysis, the specimens were diluted 1:1 (v/v) with a medium of 35% egg yolk, 12.5% glycerol, and 1.5% sodium citrate (6.25% glycerol final concentration) prepared freshly each week in our laboratory. The sperm samples and the medium were combined then equilibrated at 37°C for 10 min and were placed in 1.8-ml plastic cryovials. After approximately 10 min, precooling in liquid nitrogen vapor by placing the holding rack on the top shelf of the freezer, the samples were then plunged into liquid nitrogen (−196°C). During the repeated cryopreservations, the thawed specimens were left in their original cryopreservation medium and simply refrozen by the same procedure. In experiment 2, designed to compare two refreezing methods, half of the samples were washed with 3 vol of human tubal fluid (HTF; Irvine Scientific, Irvine, CA) at 400*g* for 10 min. Each washed pellet was then resuspended in HTF, and glycerol–egg yolk cryopreservation medium at a 1:1 ratio was added prior to refreezing. Refrozen specimens were stored in liquid nitrogen for at least 24 hr before each subsequent thaw. Specimens were removed from liquid nitrogen and placed directly into a 37°C water bath for 5 min. The thawed semen samples were then placed on the bench top and were kept at room temperature.

Assessment of Sperm Motility

A computer-assisted semen analysis (CASA) instrument (IVOS Version 10.6; Hamilton-Thorne Research,

Beverly, MA) was used to determine sperm motility. Samples were placed in a 10-μm Makler chamber and five to seven microscopic fields, originating in two drops of semen, were evaluated at 36.5°C. The CASA parameters were as follows: 30 frames per sec acquired at 60 Hz; minimum cell size, 2.0 pixels; nonmotile sperm head size cutoff, 3.0 pixels; and low VAP and low VSL cutoff: 5.0 μm/sec.

Evaluation of Sperm Viability

Viability was tested by the live/dead fluorescent vital stain (Molecular Probes Inc., Eugene, OR), which consists of a membrane-permeant nucleic acid stain (SYBR 14) and the conventional dead-cell stain, propidium iodide. The SYBR 14 is supplied as a 1 m*M* solution in DMSO, and the propidium iodide as a 2.4 m*M* aqeuous solution in water. The reagents are stored at −20°C protected from light. The methods used were modified from the original description (17). After warming the reagents to room temperature just prior to use, a 1:50 aqueous dilution of SYBR 14 was made and was added to each specimen at a 1:20-to-1:50 ratio. This concentration is higher than the recommended 1:200 ratio, but the higher concentration gives better results because the residual egg-yolk medium on the sperm surface apparently interferes with dye binding. The samples were incubated at 36°C for 15 min. Propidium iodide solution was then added to each specimen (1:200) to achieve a final concentration of 12 μ*M*. After incubating for 5 min more, a drop of each specimen was placed on a glass slide and the slides were evaluated at ×400 magnification under a fluorescent microscope (Olympus Optical Ltd., Tokyo). Under these conditions, the live sperm with intact membranes fluoresce green, while the dead cells with damaged membranes fluoresce red. The percents viable motile and viable nonmotile sperm were recorded after evaluating at least 200 cells from each of two slides for each sample. The motile fraction of the viable sperm population, that is, the proportion of motile green vs. total green sperm, was also recorded. We also noted occasional motile red sperm, which indicate a still motile but decaying or apoptopic sperm. However, the incidence of these is very rare and does not merit further discussion.

EXPERIMENTAL DESIGN

Experiment 1: Effect of Repeated Freezing and Thawing on Sperm Motility and Viability (Nine Subjects). These experiments were designed to determine the extent of decline in motility and viability after repeated freeze/thaw cycles. Motility and viability measurements were carried out 30 minutes after each thaw.

Experiment 2: Effect of Sperm Washing Between Repeated Freeze/Thaw Cycles (13 Subjects). These experiments addressed the effects of sperm washing and use of fresh cryomedium on sperm motility and viability between the repeated cryopreservation/thaw cycles. Each specimen was divided into two aliquots after the first thaw. One aliquot was left in the original cryopreservation medium during the subsequent freeze/thaw cycles. The other aliquot was washed, resuspended in cryopreservation medium and refrozen after each of the three freeze/thaw cycles. Motility and viability were evaluated within 30 min after thaw.

Experiment 3: Time Related Changes in Sperm Motility and Viability in the Postthaw Period (11 Subjects). In these experiments we examined the changes in motility and viability in two freezing/thawing cycles. The thawed sperm samples were incubated at 37°C for up to 6 hr. One half of each sample was used for evaluation after the first thawing cycle, whereas the other half of each sample was immediately refrozen and used after the second thaw. In the first cycle, motility and viability were evaluated immediately after thaw and at 1, 2, 3, and 6 hr after thaw. Samples after the second freeze/thaw cycle were evaluated at 0 time and 1, 2, and 3 hr postthaw.

Statistical Analysis

Data from the experiments were subjected to statistical analysis using the paired *t* test in comparing the sperm aliquots subjected to the subsequent freezing cycles and in comparing the two preparation/freezing conditions of experiment 2. To adjust for the multiple comparisons, a *P* value of <0.01 was considered statistically significant. All data are expressed as mean ± Standard error of the mean (SE).

RESULTS

Experiment 1: Effects of Repeated Freezing and Thawing on the Motility and Viability of Human Spermatozoa. In the first set of experiments (prefreeze motility, 70.1 ± 5.2%; prefreeze viability was not determined; n = 9 samples), we evaluated sperm motility and viability during five repeated freeze/thaw cycles. The mean sperm motility after the first thaw was 24.4 ± 2.9% (thus, 34.8% of the prefreeze motility was recovered). The mean motilities in the second, third, fourth, and fifth cycles were 8.0 ± 2.0, 3.5 ± 1.3, 1.5 ± 0.6,

and 1.8 ± 1.4%, respectively. The respective mean viability values in the postthaw samples of the five cycles were 39.1 ± 3.6, 25.3 ± 2.4, 22.6 ± 3.3, 17.8 ± 3.6, and 16.5 ± 5.0% (level of significance is $P <$ 0.001 between the first and the second thaw cycles in both motility and viability, no differences were found in either parameters in subsequent cycles). The maintenance of viability was higher than the maintenance of motility after each of the thawing cycles (Fig. 1). However, there was a substantial diversity in the recovery of motility and viability among the samples. For instance, the recovery of motility after the second thawing cycle was 34.8%, but the range among the nine samples was from 18 to 64%.

Experiment 2: Effects of Sperm Washing Between Freeze/Thaw Cycles on Sperm Motility and Viability. One aliquot of each cryopreserved/thawed sample (original motility, 77.2 ± 2.7%; n = 13) was left in the original cryopreservation medium, while the other aliquot was washed prior to refreezing. The mean motility values in the sperm samples kept in the original medium vs. the washed samples (Fig. 2) were significantly higher after the second (27.2 ± 3.9 vs. 10.9 ± 1.7%; $P < 0.001$) and third (7.4 ± 1.2 vs. 0.7 ± 0.1%; $P < 0.0001$) thaw cycles, but not after the fourth, when the recovered sperm motility was very low (2.7 ± 1.0 vs. 0.2 ± 0.1%). The mean sperm viabilities in the sample pairs showed a comparable decline after each of the respective freeze/thaw cycles: 50.3 ± 3.4 vs. 36.1 ± 2.5, 24.9 ± 2.4 vs. 8.9 ± 1.3, and 19.7 ± 3.2 vs. 6.8 ± 0.9% ($P < 0.005$ in all comparisons) (Fig. 3).

Experiment 3: Motility and Viability During the Postthaw Period. We observed sperm motilities and viabilities and the incidences of motile sperm in the viable sperm fraction during a period of 6 hr after the first thaw and for 3 hr after the second thaw in 11 samples (prefreeze motility, 76.7 ± 3.1%; n = 11). *After the first thaw* the mean incidences of viable sperm at 0, 1, 2, 3, and 6 hr were 56.3 ± 4.4, 50.8 ± 3.4, 42.8 ± 4.2, 43.2 ± 2.7, and 33.8 ± 4.6%, respectively (Fig. 4a). Whereas the avarage viability showed a moderate time-related decline, the proportion of motile sperm in the viable sperm population increased in the first hour (at 0 hr, 37.6 ± 3.1%; at 1 hr, 58.1 ± 4.3%; at 2 hr, 39.8 ± 4.3%; at 3 hr, 34.9 ± 1.9%; and at 6 hr, 34 ± 3.7%), and the increase in the first hour was statistically significant ($P < 0.001$) (Fig. 4b). *After the second thaw* the mean sperm viabilities at 0 time, 1 hr, 2 hr, and 3 hr were 28.3 ± 3.2, 28.7 ± 3.6, 23.4 ± 4.6, and 23.4 ± 3.6%, respectively (Fig. 5a). The sperm motilities in the viable sperm population at the same points were 18.5 ± 1.0, 24.9 ± 1.9, 22.6 ± 3.2, and 13.9 ± 1.0%. The increase in motility in the first hour after thaw was again statistically significant ($P < 0.001$) (Fig. 5b). Despite a gradual decline in sperm viability, sperm motility in the viable sperm population increased in the first 60 min after both the first (24.4 ± 3.5 to 30.0 ± 3.2%) and the second (5.7 ± 1.2 to 7.6 ± 1.4%) thaws, although significance was not reached at $P < 0.01$, the level we chose in these studies.

Motility Recovery After the Second Thaw. We evaluated 24 samples after the first and second thaws to determine whether the sperm motility in the first thaw

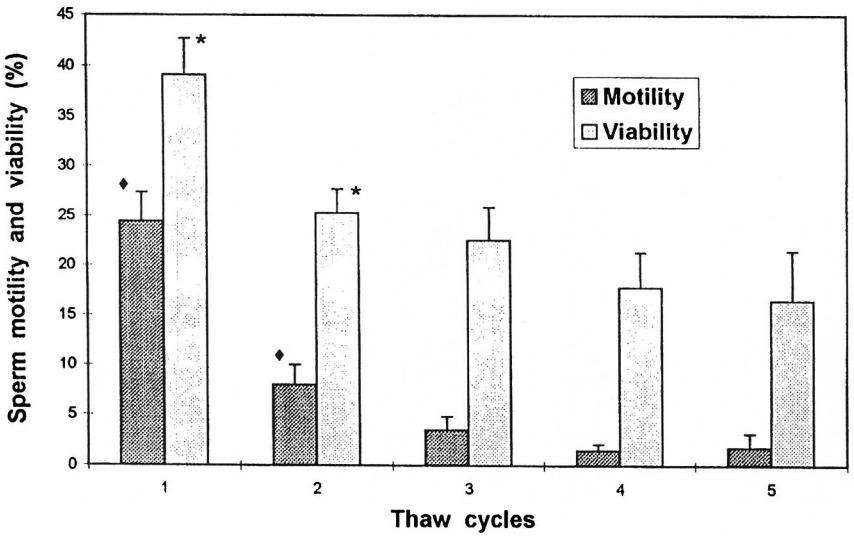

Fig. 1. Sperm motility and viability after five repeated freeze/thaw cycles. Bars designated with * and ♦ are different ($P < 0.001$).

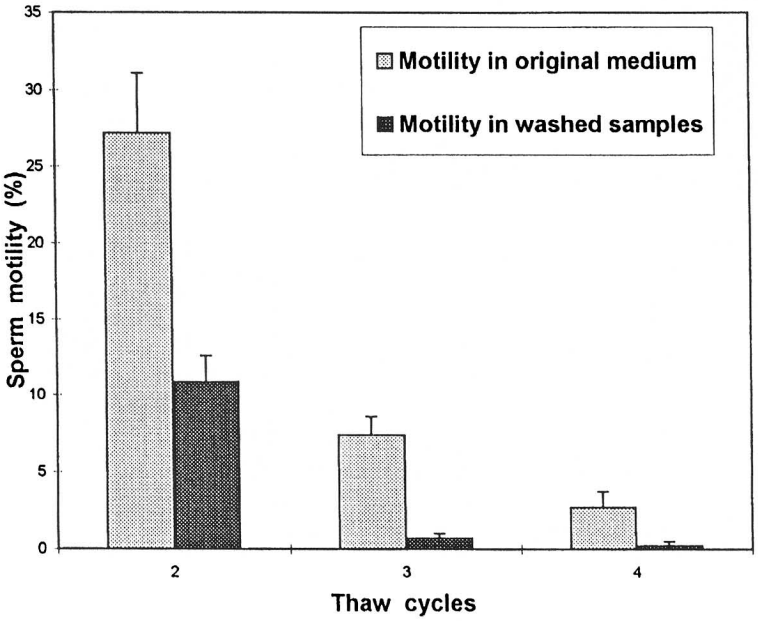

Fig. 2. Recovery of sperm motility after the three freeze/thaw cycles. The sperm samples were kept in the original cryomedium or they were washed and resuspended in new cryomedium prior to refreezing.

compared with the prefreeze motility would give an indication of the expected motility recovery after the second thaw. In 15 samples (62%) the motility in the second thaw was between 25 and 50% of that of the first thaw. The motility recovery in seven other samples (29%) was between 10 and 25%. Thus, in 90% of samples after the second thaw >10% of sperm motility was recovered compared with after the first thaw. In three-quarters of the samples the motility recovery was 15%, and in about two-thirds of the samples, it was at least 25% of that after the first thaw (Fig. 6). These data indicate that the motile sperm concentration and

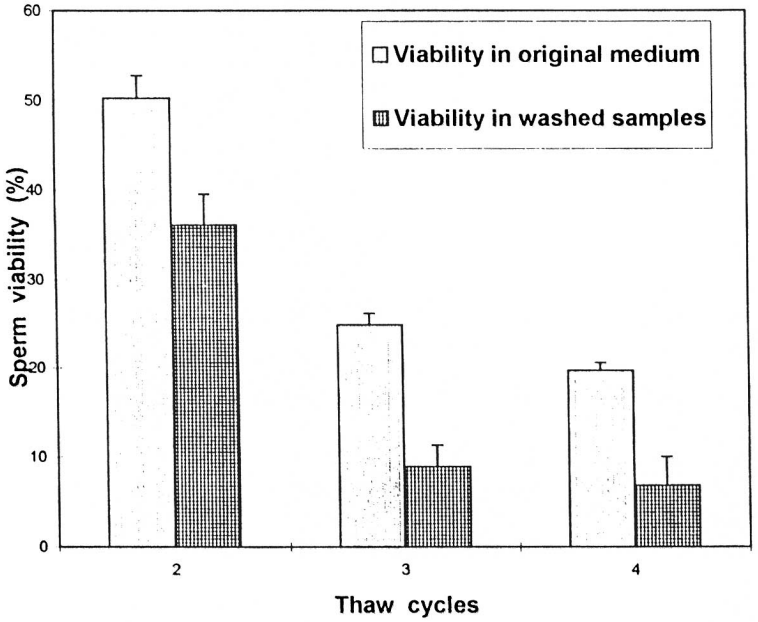

Fig. 3. Sperm viabilities after the three freeze/thaw cycles. The sperm samples were treated as in the legend to Fig. 2.

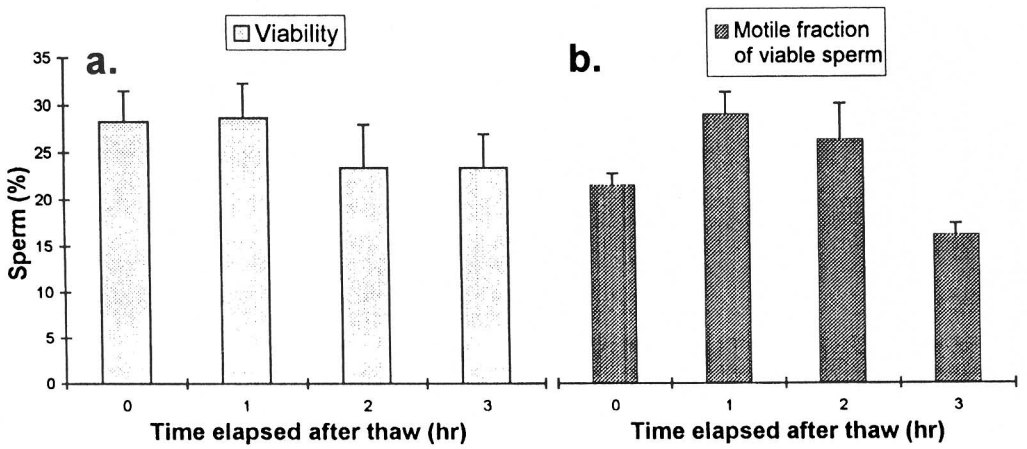

Fig. 4. Data for the first thaw: sperm viability and sperm motility in the viable sperm fraction during a period of 6 hr postthaw.

the motility recovery after the first thaw would allow the predetermination of whether in vitro fertilization (IVF) or ICSI would be the appropriate choice of assisted reproduction modality after the second thawing cycle. Whereas the data in Fig. 6 provide a threshold estimate as to whether there will be sufficient motile sperm to plan conventional IVF or ICSI, there was no significant relationship by correlation analysis between the motile sperm recoveries in the first and second cycles, due to the wide range of sample-to-sample variations in recoveries.

Incidence of Viable Motile and Viable Nonmotile Sperm After the Thaw Cycles. Because of the importance of the nonmotile but viable sperm fraction for ICSI, we examined the proportion of viable motile vs. viable nonmotile sperm in 24 samples within the first hour after the first and second thaws and in 6 samples within the first hour after the third thaw. After the first thaw the mean motility and viability were 25.9 ± 2.6 and 52.9 ± 2.7%. This indicates that only 47.8 ± 3.6% of the viable spermatozoa were motile. After the second and third thaws the motility and viability were even more disproportionate: 6.6 ± 0.9 vs. 26.4 ± 2.0 and 2.7 ± 1.0 vs. 19.7 ± 3.1%, respectively. Thus, in the thawed samples after the first, second, and third thaw cycles, for each motile sperm there was an increasing proportion of viable nonmotile sperm (Table I).

DISCUSSION

The present work was designed to determine the optimal methods for refreezing, for future assisted reproduction cycles, already cryopreserved semen samples of men with diminished current sperm produc-

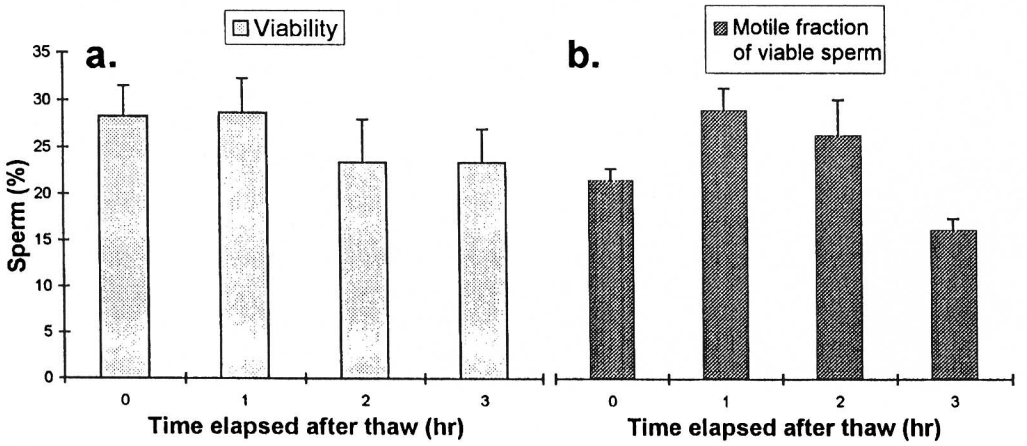

Fig. 5. Data for the second thaw: sperm viability and sperm motility in the viable sperm fraction during a period of 3 hr postthaw.

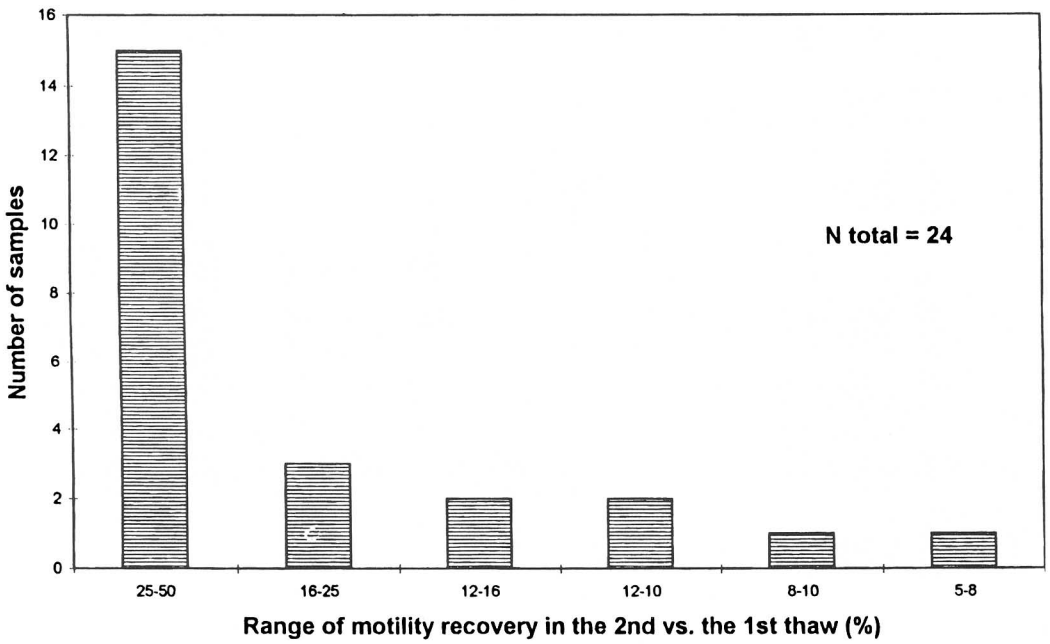

Fig. 6. Ranges of motility recovery after the second thaw compared with the first thaw in 24 samples.

tion. We found that the unused portion of previously cryopreserved semen can be refrozen and thawed with a predictable motile sperm yield. However, after the second thaw, the success of further freeze/thaw cycles is unpredictable and did result in very low sperm motility recovery. Sperm motility after the third thaw ranged between only 8 and 0.2%. When only a limited number of vials or vials with low motile sperm concentrations are available, the samples should be subdivided into smaller aliquots after the first thaw to limit the freeze/thaw cycle exposure of samples.

The problem of optimal utilization of cryopreserved sperm of presently azoospermic men is important. In a recent report, addressing thawing at 37°C or at room temperature, the higher temperature option provided significantly higher motile sperm yields upon two cycles of refreezing (18). The reported rates of motile sperm recovery are comparable to our present results: recoveries after the first, second, and third freezing/thawing cycles were 47, 64, and 38%, compared to our 35, 33, and 44%, respectively. The mean prefreeze motilities were also comparable: 70.2 vs. 73.2%.

Table I. Incidence of Viable Motile and Viable Nonmotile Sperm After Three Thawing Cycles (Mean ± SE)

	Viable motile (%)	Total viable (%)	Ratio, motile/nonmotile
1st thaw (N = 24)	25.9 ± 2.6	52.9 ± 2.7	1:1
2nd thaw (N = 24)	6.6 ± 0.9	26.4 ± 2.0	1:3
3rd thaw (N = 6)	2.7 ± 1.0	19.7 ± 3.1	1:6.3

In the course of our experiments, we also explored whether sperm should be left in the original cryopreservation medium or refrozen in fresh cryopreservation medium. The data from experiment 2 indicate that the centrifugation and resuspension process was more damaging to sperm than leaving the thawed samples in the original semen–medium mixture. In addition to the mechanical stress of centrifugation, the removal of the seminal fluid with its antioxidant capacity is a likely contributory factor to the decline of sperm motility after the washing step (19). The washing step resulted in significant declines in both the motility and the viability parameters. We recommend leaving the unused portion of the thawed samples in the original cryopreservation medium before refreezing, to avoid the detrimental effects of sperm washing.

Because of the fact that cryopreserved sperm is used in assisted reproduction, for either IVF or ICSI, depending on the level of motile sperm concentration, we wished to determine the rate of postthaw sperm motility recovery and the elapsed time to peak motility. Sperm motility increased significantly in the first hour, and this increase was maintained during the second hour. However, the motility subsequently declined. We

found this pattern occuring after both the first and the second thaws. Sperm viability showed a gradual decline during the postthaw period. Because in the selection of sperm for ICSI, sperm motility facilitates the recognition of viability, the observation of this maximum sperm motility window, between 30 and 90 min postthaw, will be useful for the timing of IVF or ICSI procedures.

Comparisons of sperm motility and viability during the repeated freeze/thaw cycles indicated that motility declines substantially in each cycle, but sperm viability is less affected. This observation is relevant for at least two areas of sperm physiology. First, it suggests that, due to the stress of freezing and thawing, in addition to sperm membrane cryodamage, the integrity of the energy generating or utilization system coupled to sperm motility is also diminished. Second, the better preservation of sperm viability than sperm motility is a clinically important finding. When minimal or no motile sperm were available, there were still high proportions of viable nonmotile sperm in the thawed samples that could be used for ICSI (Table I).

With respect to the prediction of motility recovery in the second thawing cycle, we observed that 90% of the samples had greater than 10% motility recovery compared with that in the first thaw. As Fig. 6 indicates, 75% had over 15% recovery and in 63% of the samples the motility recovery was between 25 and 50% after the second thaw compared to the first thaw. Thus, based on the total motile sperm in the initial sample and the recovery of motility after the first thawing cycle, the applicability of IVF or ICSI may be anticipated. It is of note that in these normal cryopreserved samples banked for various reasons a better predictive power may be achievable with respect to recovery of sperm motility and viability than in severely oligospermic and asthenospermic men. This is because the majority of sperm in the cryopreserved samples are mature sperm arising from normospermic specimens in which there is a single common damage factor: cryopreservation that causes diminished motility and viability. In contrast, in many subfertile men with nonobstructive oligospermia, the sperm are immature due to incomplete spermiogenetic development (20,21). In these sperm, in addition to the retained cytoplasm, there is also a deficiency of spermiogenetic membrane remodeling, which together cause an increase in the rate of intrinsic sperm lipid peroxidation along with the consequential membrane damage and diminished viability (22,23).

In summary, we have examined the utilization of cryopreserved/thawed sperm samples. It has became clear that in the second thawing cycle it is possible reliably to recover a substantial percentage of motile sperm, but from the third cycle on, the recovery of motility diminishes. For this reason, we suggest aliquoting the samples after the first thaw prior to refreezing for future utilization. The aliquots should be kept in the original semen–cryopreservative mixture because washing and resuspension of sperm in fresh medium causes a severe loss of motility. After thawing, most samples reached their maximal motility following an approximately 60-min incubation at 37°C. The overall loss of motility is more severe than that of viability. Thus, for each motile sperm there are additional viable nonmotile sperm in the thawed samples. Finally, in 90% of the samples the motility recovery in the first thawing cycle is predictive of the recovery of motility in the second thawing cycle.

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REFERENCES

1. Sherman JK: Freezing and freeze-drying of human spermatozoa. Fertil Steril 1954;5:357–371
2. McGann LE: Differing actions of penetrating and nonpenetrating cryoprotective agents. Cryobiology 1978;15:382–386
3. Serres C, Jouannet P, Czyglic F, David G: Effects of freezing on spermatozoa motility. *In* Human Artificial Insemination and Semen Preservation, G David, WS Price (eds). New York, Plenum Press, 1980, pp 147–161
4. Henry MA, Noiles EE, Gao D, Mazur P, Critser JK: Cryopreservation of human spermatozoa. IV. The effects of cooling rate and warming rate on the maintenance of motility, plasma membrane integrity, and mitochondrial function. Fertil Steril 1993;60(5):911–918
5. Keel BA, Webster BW, Roberts DK: Effects of cryopreservation on the motility characteristics of human spermatozoa. J Reprod Fertil 1987;81:213–220
6. Critser JK, Arneson BW, Baker DV, Huse-Benda AR, Ball GD: Cryopreservation of human spermatozoa. II. Postthaw chronology of motility and of zona-free hamster ova penetration. Fertil Steril 1987;47:980–984
7. Kolon TF, Philips KA, Buch JP: Custom cryopreservation of human semen. Fertil Steril 1992;58:1020–1023
8. Keel BA, Webster BW: Semen analysis data from fresh and cryopreserved donor ejaculates: Comparison of cryoprotectants and pregnancy rates. Fertil Steril 1987;52:100–106
9. La Lanou D, Gastard D, Guivarch A, Laurent MC, Poulain P: Strategies in frozen donor semen procreation. Hum Reprod 1995;10:1765–1774
10. Critser JK, Huse-Benda AR, Aaker DV, Arneson BW, Ball GD: Cryopreservation of human spermatozoa: 1. Effects of

holding procedure and seeding motility, fertilizability and acrosome reaction. Fertil Steril 1987;47:656–663
11. Richter A, Haning V, Shapiro SS: Artificial donor insemination. Fresh versus frozen semen: The patient as her own control. Fertil Steril 1984;41:277–281
12. Hammond MG, Jordan S, Sloan CS: Factors affecting pregnancy rates in a donor insemination program using frozen semen. Am J Obstet Gynecol 1986;155:480–484
13. Mahadevan M, Trounson A: Effect of cryoprotective media and dilution methods on the preservation of human spermatozoa. Andrologia 1983;15:355–360
14. Critser JK, Huse-Benda AR, Aaker DV, Arneson BW, Ball GD: Cryopreservation of human spermatozoa. III. The effect of cryoprotectants on motility. Fertil Steril 1988;50:314–320
15. Verheyen G, Pletincx I, Van Steirteghem A: Effect of freezing method, thawing temperature and post-thaw dilution/washing on motility (CASA) and morphology characteristics of high-quality human sperm. Hum Reprod 1993;8(10):1678–1684
16. Davis RO, Drobnis EZ, Overstreet JW: Application of multivariate cluster discriminate function, and stepwise regression analyses to variable selection and predictive modeling of sperm cryosurvival. Fertil Steril 1995;63:1051–1057
17. Garner DL, Johnson LA: Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. Biol Reprod 1995;53(2):276–284
18. Leffler KS, Walters CA: A comparison of time, temperature, and refreezing variables on frozen sperm motility recovery. Fertil Steril 1996;65:272–274
19. Aitken RJ, Krausz CS, Buckingham D: Relationship between biochemical markers for residual sperm cytoplasm, reactive oxygen species generation, and the presence of leukocytes and precursor germ cells in human sperm suspensions. Mol Reprod Dev 1994;39:268–279
20. Huszar G, Corrales M, Vigue L: Correlation between sperm creatine phosphokinase activity and sperm concentrations in normospermic and oligospermic men. Gamete Res 1988;19:67–75
21. Huszar G, Vigue L: Incomplete development of human spermatozoa is associated with increased creatine phosphokinase concentration and abnormal head morphology. Mol Reprod Dev 1993;34:292–298
22. Huszar G, Sbracia M, Vigue L, Miller D, Shur B: Sperm plasma membrane remodeling during spermiogenetic maturation in men: Relationship among plasma membrane beta-1,4-galactosyltransferase, cytoplasmic creatine phosphokinase, and creatine phosphokinase isoform ratios. Biol Reprod 1997; 56:1020–1024
23. Huszar G, Vigue L: Correlation between the rate of lipid peroxidation and cellular maturity as measured by creatine kinase activity in human spermatozoa. J Androl 1994;15:71–77