Improvements in Qualitative Characteristics of Cryopreserved Human Spermatozoa Following Recovery via the SpermPrep™II Filtration Method

PANAYIOTIS M. ZAVOS, NIKOLAOS SOFIKITIS*, TOSHIKO TODA† and IKUO MIYAGAWA*

*Department of Animal Sciences, University of Kentucky, Lexington, KY 40546 and Andrology Institute of Lexington, Central Baptist Hospital, Lexington, KY, USA, and *Department of Urology and †Department of Obstetrics and Gynecology, Tottori University School of Medicine, Yonago, 683*

ZAVOS, P.M., SOFIKITIS, N., TODA, T. and MIYAGAWA, I. *Improvements in Qualitative Characteristics of Cryopreserved Human Spermatozoa Following Recovery via the Use of the SpermPrep™II Filtration Method.* Tohoku J. Exp. Med., 1991, 165 (4), 283-290 — Motile, morphologically normal human spermatozoa can be separated from semen via a disposable SpermPrep™ filtration method. This method was employed with great success using frozen-thawed spermatozoa. The present study examined the qualitative characteristics of filtered spermatozoa via a new SpermPrep™II filtration method in conjunction with the short-term freeze preservation of these spermatozoa. The isolation procedure yielded populations of spermatozoa with very high percentage motility and progressive motility grade (0-4) and which were free of seminal debris. For 20 semen samples the prefreeze values of percentage of motility and grade for the fresh and post-filtered were 58.6% and 3.1 vs 84.1% and 3.6, respectively. The sperm freeze preservation procedure involved dilution of the spermatozoa in Test-Yolk buffer (TYB) with 7% glycerol (v/v) and freezing and thawing in a conventional manner. Post-thaw percent motility, and survival % were substantially higher ($p < 0.05$) for the filtered fraction than for the parent semen. The filtered fractions yielded post-thaw mean values of motility percent and grade of 68.9 and 3.4 vs 47.6 and 3.0 for the parent semen, respectively. Survival following incubation at 37°C for 3 hours yielded respective values of 75.9% and 53.2% ($p < 0.05$). It was concluded that the major factor in improving post-thaw quality recovery overall was the filtration via the SpermPrep™II as applied in this study. This technique could have significant clinical applications in the use of frozen-thawed specimens for non-coital reproduction purposes. — semen preparation; SpermPrep™II;

Received November 6, 1991; revision accepted for publication November 18, 1991.

Address for correspondence: Panayiotis Zavos, Ed.S., Ph.D., Professor, Dept. of Animal Sciences, 607 Garrigus Bldg. University of Kentucky, Lexington, KY 40546, USA. Presented at the ESHRE-IVF 7th Annual Meeting, Paris, France, June 28 to July 3, 1991 (Award Paper)

frozen-thawed semen

Today, spermatozoa used for either intrauterine insemination (IUI) or in vitro fertilization insemination (IVF-AI) could yield higher rates of conception if these spermatozoa are selected on the basis of their motility, progressive motility and morphological characteristics (McClure et al. 1989; Free et al. 1991). Improvements in rates of conception could be realized if frozen-thawed spermatozoa are selected on the basis of similar characteristics. Such selection of spermatozoa could be properly applied at the time of IUI or other forms of assisted reproductive techniques because the seminal plasma, cryoprotective agents, and other background materials and debris should be removed from the spermatozoa before these procedures are performed.

A number of manipulative techniques for fresh semen are currently available to remove the undesirable spermatozoa, debris, undesirable bioactive substances such as "decapacitation factors", spasmogens that can cause smooth muscle contractions following IUI and other constituents including lytic enzymes and microorganisms and, of course, to increase sperm quality. These various techniques have been used to separate motile sperm from semen, and each has both desirable and undesirable attributes. The ideal separation technique would: 1) be rapid, simple, and inexpensive; 2) recover all or most motile sperm in the specimen; 3) result in no damage or physiologic alteration of the separated sperm; 4) remove dead sperm and other cells, including microorganisms; 5) remove toxic and bioactive substances; 6) process large volumes of semen; and 7) allow the final volume of the sperm suspension to be controlled. Depending on the application, these criteria differ in their importance. Also the relatively lengthy time period required to perform these procedures is of particular importance since the life expectancy of post-thaw spermatozoa is limited (Friberg and Gemzell 1977; Critzer et al. 1987; Binor et al. 1980; Keel and Karow 1980).

Recently a new technique has been introduced which encompasses most of the above attributes including a high degree of sperm recovery and is also quite fast and reproducible (Zavos and Centola 1990a, 1991; Zavos 1991). Because of these advantages, the SpermPrep™ technique could have significant effects, in the manner that either fresh or frozen-thawed specimens are prepared and improved prior to their use in artificial (noncoital) reproduction procedures. Additional improvements could be realized if the fresh spermatozoa are selected on the basis of their qualitative characteristics prior to being cryopreserved. The present study was undertaken to study the qualitative characteristics of fresh filtered spermatozoa via a new SpermPrep™II method in conjunction with the short-term freeze preservation of these spermatozoa.

MATERIALS AND METHODS

Ejaculates were collected from 20 men who were referred to our andrological laboratory

for male infertility workup. Ejaculates were collected with exactly 4 days of abstinence each time. All patients collected their own ejaculates using the seminal collection device (SCD) at intercourse (Zavos 1985; Zavos 1987; Zavos and Goodpasture 1989).

Semen evaluation. After semen samples were produced and completely liquefied (within 15–30 min), each specimen was evaluated according to standard procedures recommended by the World Health Organization (WHO) with a phase-contrast microscope (World Health Organization 1987). Semen measures included volume, sperm count per milliliter, percentage sperm motility, grade of sperm motility (Zavos and Cohen 1980), sperm morphologic features and presence or absence of debris (Cohen et al. 1985). All seminal parameters were evaluated by the same technician.

Semen preparation. After semen evaluation, each sample was divided into various aliquots and treated as follows: 1) fresh raw specimen; 2) diluted 1:1 (by volume) with Test Yolk Buffer (TYB); 3) filtered via SpermPrepTMII in TYB; 4) filtered in TYB and added glycerol (7% v/v); 5) same as in treatment 4 and frozen-thawed; and 6) as in treatment 1, diluted 1:1 by volume in TYB with 7% glycerol (v/v) and frozen-thawed. The TYB medium was supplemented with adequate levels of antibiotics, a pH balance of 7.2–7.4, and a balanced osmotic pressure of 320–325 mOsm (using D-glucose). Data for the various treatments applied were compared to each other using one way analysis of variance followed by either the Student's *t*-test or the paired Student's *t*-test where appropriate.

SpermPrepTMII filtration procedure. The SpermPrepTMII was used very similarly as previously described for the SpermPrepTM technology (Zavos and Centola 1990a, b; Zavos 1991) with some simplified modifications and according to the manufacturer's specifications (ZBL, Inc., P.O. Box 23777, Lexington, Ky 40523, USA). SpermPrepTMII comes with an extension funnel which was attached to the column during the preparation steps. It should be emphasized also, that proper standard laboratory techniques were employed in our laboratory throughout the filtration process. Those techniques included complete sterility and maintenance of all semen diluents, the SpermPrepTMII filter and all other materials within a temperature range of 30–35°C.

1. Hydration. The SpermPrepTMII, prior to its use, was hydrated by placing 6.0 mL of Test Yolk buffer (TYB) in the barrel of the column. Using a Pasteur pipette, the top disc was then removed to allow proper mixing of the TYB with the beads. Similarly, using the Pasteur pipette, the beads were gently mixed with the TYB to form a suspension and assure that all air bubbles were removed from the bottom of the filter supported by the bottom disc. Care was taken not to disturb the bottom disc.

2. Sedimentation. After suspension, the beads were allowed to settle for 10 min to the bottom of the filter and to undergo complete hydration. As the sedimentation was completed, the bottom closure was removed and 4–5 mL of the TYB was allowed to run through and was discarded. This step normally enabled the removal of any small air bubbles from the filter line and any possible fragments or other impurities that may be present within the filter. The filter was capped again via the bottom closure and all the filtrate was discarded. The capped filter was placed into the 15 mL test tube and held until the already mixed and properly liquefied specimen containing the spermatozoa was ready for filtration. More TYB was added to the filter at this time to bring the level to 5–6 mL.

3. Filtration. Filtration was begun by placing the well mixed, liquefied semen, diluted via TYB, into the filter. As it may be noted, no pre-filtration sperm wash was necessary when the SpermPrepTMII is used when compared to the SpermPrepTMI (Zavos and Centola 1990a). The liquefied seminal aliquot to be filtered contained $118.6 \pm 30.6 \times 10^6$ well mixed spermatozoa. Proper liquefaction and mixing of the seminal specimen is of utmost critical importance to the success of this procedure, since non-liquefied or high viscosity specimens could interfere negatively with proper filtration. Also, as the seminal fraction was placed in the filter, it was gently mixed with the existing TYB medium already in the filter to form a uniform suspension. This prevented all the seminal fraction from settling in bulk, directly onto the upper surface of the filter beads and possibly clotting the

filter. Our experience also showed that improper mixing at this time would interfere with proper filtration. Simultaneously as the seminal aliquot was added and mixed, the bottom closure was removed to begin the filtration. Filtration was continued for 10 min and periodically more TYB was added to the filter to maintain the medium at its original level (8–10 mL level). This step maintained a uniform hydrostatic pressure on the filter during the total filtration time. Approximately 0.5–1.0 mL of filtrate was recovered during each minute of filtration. At the end of filtration the filter was closed via the closure cap, the filter was removed from the test tube, the filtrate was centrifuged, resuspended in 1.0 mL TYB and assessed as previously described.

4. Cryopreservation and incubation. The specimens were cryopreserved using our routine laboratory methodology by being placed in 0.5 mL French straws and frozen in liquid nitrogen vapor for 5 hr and then placed directly into liquid nitrogen and stored until thawed. The thawed specimens were incubated for 3 hr in a 37°C waterbath and readings of percentage motility and grade of progressive motility were taken every 30 min for the total duration of the incubation period.

RESULTS

The mean seminal characteristics of the 20 specimens prior to application of the SpermPrep™II selection procedure are shown in Table 1. All specimens were normospermic according to WHO standards. Application of the SpermPrep™II filtration method resulted in Recovery of significantly greater ($p < 0.01$) quality of spermatozoa than the fresh non-filtered specimens (Table 2). The mean of $61.3 \pm 12.2 \times 10^6$ total spermatozoa obtained with the SpermPrep™II filter represents a mean recovery of 51.7% of all spermatozoa applied to the filter during the period of filtration. The SpermPrep™II column therefore yielded a recovery of spermatozoa of adequate qualitative and quantitative characteristics that could be used for IUI purposes or cryopreserved and used further for IUI at a later date.

The percent motile sperm due to SpermPrep™II filtration improved by a mean of 25 percentages, the grade of motility improved by a mean of 0.2 points (scale 0–4), the percent of morphologically normal spermatozoa increased by a mean of approximately 24%, and the debris score decreased by a mean of 2.5 points (scale 0–4). The time required to harvest the spermatozoa through SpermPrep™II filtration was 10 min in all cases (filtration time only). This

TABLE 1. *Fresh seminal ejaculate characteristics of specimens used (means $\pm$ S.E.)^a*

Parameters assessed	Values ($n = 20$)
Volume (ml)	3.8 ± 0.6
Concentration (total)	318.6 ± 31.1
Motility (%)	58.6 ± 5.9
Grade (0–4)	3.1 ± 0.3
Morphology (%N)	64.3 ± 6.1
Debris (0–4)	2.9 ± 0.5

^aNormospermic specimens according to WHO standards.

TABLE 2. *Changes in sperm parameters of specimens (n=20) after processing via the SpermPrepII™ filtration method (means $\pm$ S.E)*

Parameters assessed	Fresh	Post filtered ^{a,b}
Motility (%)	58.6 $\pm$ 5.9	83.1 $\pm$ 9.1
Grade (0-4)	3.1 $\pm$ 0.3	3.6 $\pm$ 0.2
Morphology (%N)	64.3 $\pm$ 6.1	87.8 $\pm$ 12.1
Debris (0-4)	2.9 $\pm$ 0.5	0.4 $\pm$ 0.1

^aPrefiler count=118.6 $\pm$ 30.7; postfilter count: 61.3 $\pm$ 12.2 ($\times 10^6$ sperm).

^bRecovery percent: 51.7 spermatozoa.

Significant improvements noted in all parameters assessed ($p < 0.05$).

TABLE 3. *Sperm percent motility among various treatments assessed at 30 min intervals over 3 hr incubation period (means)*

Treatments	Incubation time in minutes (n=20)						
	0	30	60	90	120	150	180
1. Fresh	58.6	56.2	53.1	49.3	44.5	41.0	36.1
2. Fresh (TYB)	63.1	64.0	61.8	57.2	55.1	53.0	50.1
3. Filtered (TYB)	84.1	83.2	82.9	80.0	78.1	75.1	74.0
4. Filtered (TYB) ^a	83.1	82.0	80.0	76.1	73.2	71.3	69.0
5. Filtered (F/T) ^b	68.9	66.1	63.2	61.0	58.1	55.0	52.3
6. Fresh (F/T) ^b	47.6	42.3	38.1	33.2	30.1	28.1	25.3

With 7% glycerol.

^bFrozen-Thawed; incubations performed after being thawed 24 hr later.

TABLE 4. *Sperm grade of progressive motility among treatments assessed at 30 min intervals over 3 hr incubation period (means)*

Treatments	Incubation time in minutes (n=20)						
	0	30	60	90	12	15	180
1. Fresh	3.1	3.0	3.0	2.9	2.8	2.6	2.4
2. Fresh (TYB)	3.2	3.3	3.2	3.1	3.0	3.0	2.9
3. Filtered (TYB)	3.6	3.6	3.5	3.5	3.4	3.3	3.2
4. Filtered (TYB) ^a	3.6	3.5	3.5	3.4	3.2	3.2	3.0
5. Filtered (F/T) ^b	3.4	3.2	3.1	3.1	3.0	2.9	2.8
6. Fresh (F/T) ^b	3.0	3.0	2.9	2.6	2.5	2.4	2.2

^aWith 7% glycerol.

^bFrozen-Thawed.

mode of filtration was quite fast allowing direct filtration of the fresh diluted specimen requiring no further preparation such as sperm wash or other time consuming procedures.

The cryopreservation process had deleterious effects on the spermatozoa in both the filtered and the fresh aliquots ($p < 0.01$). However, those deleterious effects were more severe in the fresh cryopreserved than the filtered aliquots. These negative effects were magnified even further after the 3 hr incubation process was completed. The filtered fractions yielded post-thaw mean values of motility percent and grade of motility of 68.9 and 3.4 vs. 47.6 and 3.0 for the parent semen, respectively. Survival following incubation at 37°C for 3 hr yielded respective values of 75.9% and 53.2% ($p < 0.01$; Table 3). Similar patterns were noted in the grade of progressive motility measurements which yielded respective values of 82.3% and 73.3% ($p < 0.05$; Table 4).

DISCUSSION

The objective of the current study was to study the qualitative characteristics of fresh filtered spermatozoa via a new SpermPrepTMII method in conjunction with the short-term freeze preservation of these spermatozoa. As known, spermatozoa can now be stored in liquid nitrogen at subzero temperatures (as low as -196°C) and survive with relatively high fertility after thawing. However, many of the contained spermatozoa are killed or rendered immotile by freezing and thawing. The majority of the results have indicated that approximately 30% to 70 of spermatozoa that were motile prior to freezing regained their motility after thawing (Beck and Silverstien 1975). Hence, for optimum fertility and to overcome the shortcomings of frozen-thawed human semen, larger numbers of spermatozoa are used for frozen than for nonfrozen (fresh) semen at the time of performance of artificial insemination.

Also, to overcome these shortcomings, attempts have been made recently to separate the post-thaw motile from the non-motile spermatozoa so that the selected motile spermatozoa only, can be used for either intrauterine insemination (IUI) or other forms of assisted reproduction (Centola and Zavos 1990; Zavos 1991; Zavos and Centola 1991). With the advent of in vitro fertilization and other artificial reproductive techniques, it is considered important to use the best spermatozoa available from a semen sample. Indeed, recent evidence with fresh spermatozoa has suggested that spermatozoa separated via various methods have a greater fertilizing capacity than spermatozoa in the whole ejaculate (Forster et al. 1983; Tanphaichitr et al. 1988; Katayama et al. 1989). The retrieved spermatozoa normally are selected on the basis of motility, progressive motility and morphological characteristics. As known, motility and progressive motility are essential qualities for the spermatozoa to penetrate the various investments of the oocyte during in vitro insemination. However, although sperm motility characteristics are of considerable importance in evaluating and properly predicting the

fertility potential of an ejaculate or specimen, its use as a diagnostic tool by itself is sometimes inaccurate and, in some instances, exaggerated. For example, when using motility alone, neither the outcome of a human in vitro fertilization (Van der Ven et al. 1986) nor the zona-free hamster oocyte penetration test (Aitken et al. 1982) can be predicted with any degree of reliability. Also, improving the percent motility alone does not cause a significant improvement in conception rates after intrauterine insemination (Aitken et al. 1982; Hoing et al. 1986) or in the ability of the spermatozoa to penetrate cervical mucus (Santos and Asch 1985).

The data presented in this study indicated that the SpermPrep™II filtration method yielded spermatozoa of significantly higher qualitative characteristics both fresh and post-thaw state when compared to those not filtered ($p < 0.05$). Although the cryopreservation process had deleterious effects on both the fresh and the SpermPrep™II filtered spermatozoa ($p < 0.05$), these effects were more severe in the fresh than in the filtered cells. Furthermore, the deleterious effects were magnified even further after the 3 hr incubation period was completed yielding survival rates of 75.9% and 53.2% for filtered and fresh spermatozoa, respectively ($p < 0.05$).

In conclusion, the clinically and statistically significant improvements with the SpermPrep™II method over the non-processed seminal specimens in the yield of high quality spermatozoa, together with the freezability of the recovered spermatozoa, demonstrates that this new technique could be the method of choice for selecting motile, morphologically normal spermatozoa from either normospermic patients or from patients with spermatogenic dysfunctions for use as fresh or frozen-thawed in the various assisted reproductive techniques including IUI.

References

- 1) Aitken, R.J., Best, F.S.M., Richardson, D.W., Djahanbakhch, O. & Lees, M.M. (1982) The correlates of fertilizing capacity in normal fertile men. *Fertil. Steril.*, **38**, 68-76.
- 2) Beck, W.W. & Silverstein, I. (1975) Variable motility recovery of spermatozoa following freeze preservation. *Fertil. Steril.*, **26**, 863-869.
- 3) Binor, Z., Sokoloski, J.E. & Wolf, D.P. (1980) Penetration of the zona-free hamster egg by human sperm. *Fertil. Steril.*, **33**, 321-327.
- 4) Centola, G.M. & Zavos, P.M. (1990) Qualitative/quantitative improvements in post-thaw human semen using SpermPrep™. *J. Assisted Reproductive Technology-Andrology*, **2**, 335-339.
- 5) Cohen, J., Edwards, R., Fehilly, C., Fishel, S., Hewitt, J., Purdy, J., Rowland, G., Steptoe, P. & Webster, J. (1985) In-vitro fertilization: A treatment for male infertility. *Fertil. Steril.*, **43**, 422-432.
- 6) Critzer, J.K., Arneson, B.W., Aaker, D.V., Huse-Benda, A.R. & Ball, G.D. (1987) Cryopreservation of human spermatozoa. II. Postthaw chronology of motility and of zona-free hamster ova penetration. *Fertil. Steril.*, **47**, 980-984.
- 7) Forster, M.S., Smith, W.D., Lee, W.I., Berger, R.E., Karp, L.E. & Stenchever, M.A. (1983) Selection of human spermatozoa according to their relative motility and their interaction with zona-free hamster eggs. *Fertil. Steril.*, **40**, 655-660.
- 8) Free, D., Stutts, L., Merryman, D., Stringfellow, E., Horseman, V. & Honea, K. (1991) A comparison of donor semen processing techniques for use in intrauterine insemina-

- tions and their corresponding pregnancy rates. *Fertil. Steril.*, (Abstr. Suppl.) 591.
- 9) Friberg, J. & Gemzell, C. (1977) Sperm-freezing and donor insemination. *Int. J. Fertil.*, **22**, 148-154.
 - 10) Hoing, L.M., Devroey, P. & Van Steirteghem, A.C. (1986) Treatment of infertility because of oligoasthenoteratospermia by transcervical intrauterine insemination of motile spermatozoa. *Fertil. Steril.*, **45**, 388-391.
 - 11) Katayama, K.P., Stehlik, E. & Jeyendran, R.S. (1989) In vitro fertilization outcome: glass wool-filtered sperm versus swim-up sperm. *Fertil. Steril.*, **52**, 670-672.
 - 12) Keel, B.A. & Karow, A.M. (1980) Motility characteristics of human sperm, nonfrozen and cryopreserved. *Arch. Androl.*, **4**, 205-212.
 - 13) McClure, D.R., Nunes, L. & Tom, R. (1989) Semen manipulations improved sperm recovery and function with a two-layer Percoll gradients. *Fertil. Steril.*, **51**, 874-877.
 - 14) Santos, R. & Asch, R.H. (1985) In vitro penetration of human sperm into bovine cervical mucus-effect of semen washing and swim-up. Proceedings of the 41st Annual Meeting of the American Fertility Society, Chicago, IL. Sept. 28-Oct. 2.
 - 15) Tanphaichitr, N., Millette, C.F., Agulnick, A. & Fitzgerald, L.M. (1988) Egg-penetration ability and structural properties of human sperm prepared by Percoll-gradient centrifugation. *Gamete Res.*, **20**, 67-73.
 - 16) Van der Ven H.H., Jeyendran, R.S., Al-Hasani, S., Perez-Pelaez, M., Dietrich, K. & Zaneveld, L.J.D. (1986) Correlation between human sperm swelling in hypoosmotic medium (HOS) test and in vitro fertilization. *J. Andrology*, **7**, 190-196.
 - 17) World Health Organization (1987) WHO Laboratory Manual for the Examination of Human Semen and Semen-Cervical Mucus Interaction. Cambridge University Press, Cambridge, pp. 1-13.
 - 18) Zavos, P.M. (1985) Characteristics of human ejaculates collected via masturbation and a new seminal fluid collection device. *Fertil. Steril.*, **43**, 491-492.
 - 19) Zavos, P.M. (1988) Utilisation clinique d'un dispositif de recueil du sperme (SCD); parametres seminaux et ameliorations d'ejaculats recueillis par rapport sexual avec le SCD et par masturbation, chez des patients presentant divers dysfonctionnements spermatogenes et ejaculatoires. *Contracept. Fertil. Sex.*, **16**, 475-481.
 - 20) Zavos, P.M. (1991) Selection de spermatozoides viables a partir d'echantillons de spermes humains cogeles-decongeles: Comparaison de la methode du "swim-up" et d'une nouvelle methode de filtration: le SpermPrep™ Contracept. *Fertil. Sex.*, **19**, 293-297.
 - 21) Zavos, P.M. & Cohen, M.R. (1980) The pH of the cervical mucus and the postcoital test. *Fertil. Steril.*, **34**, 234-238.
 - 22) Zavos, P.M. & Goodpasture, J.C. (1989) Clinical improvements of specific seminal deficiencies via intercourse with a seminal collection device versus masturbation. *Fertil. Steril.*, **51**, 190-193.
 - 23) Zavos, P.M. & Centola, G.M. (1990a) Qualitative and quantitative improvements in human spermatozoa recovered via the swim-up and a new semen filtration method. *Infertility*, **13**, 25-34.
 - 24) Zavos, P.M. & Centola, G.M. (1990b) Ameliorations seminales des spermatozoides utilises pour la fecondation artificielle: Comparaison entre la methode de "nage ascendant" et une nouvelle methode de filtration du sperme sur colonne. *Contracept. Fertil. Sex.*, **18**, 943-948.
 - 25) Zavos, P.M. & Centola, G.M. (1991) Selection of sperm from oligozoospermic men for ARTA: Comparisons between swim-up and the SpermPrep™ filtration. *J. Assisted Reproduc. Technol. Androl.*, **1**, 338-345.
-