ELSEVIER

PREPARATION AND RECOVERY OF FROZEN-THAWED BOVINE SPERMATOZOA VIA VARIOUS SPERM SELECTION TECHNIQUES EMPLOYED IN ASSISTED REPRODUCTIVE TECHNOLOGIES

J.R. Correa and P.M. Zavos

Department of Animal Sciences, University of Kentucky, Lexington, KY 40546 USA

Received for publication: *December 12, 1995*
Accepted: *June 14, 1996*

ABSTRACT

A number of semen manipulative techniques are currently available to remove the undesirable spermatozoa, debris and other factors and to increase sperm quality. The use of motility stimulants such as caffeine or others could optimize the recovery and quality of frozen-thawed spermatozoa processed by a variety of sperm selection techniques. Frozen-thawed specimens from 5 bulls were slowly diluted and washed with Ham's F-10 medium containing 3% BSA (w/v) and 0 or 2mM caffeine. Aliquots containing approximately 50 x10^6 total sperm cells were used for conventional sperm wash, swim-up, Percoll density gradient centrifugation (80, 70, 55 and 40% Percoll gradients) and Sephadex (SpermPrep™I) filtration. Quantitative and qualitative characteristics of selected spermatozoa included: total sperm (x10^6), percentage and grade (0 to 4) of motility, percentage of spermatozoa with coiled tails and response to the hypoosmotic swelling (HOS) test (percentage of swollen spermatozoa). When compared to washed specimens, fewer spermatozoa were recovered via the swim-up, Percoll and SpermPrep™I filtration methods. Quantitative and qualitative characteristics of these spermatozoa improved further after processing with Ham's F-10 containing 2 mM caffeine, followed by selection via the various techniques. Enhancement of sperm motility, in conjunction with the most appropriate sperm selection technique, represents an efficient method for the recovery of spermatozoa with improved qualitative characteristics.

Key words: sperm selection, sperm quality, motility stimulation, caffeine

INTRODUCTION

Selection of spermatozoa should be properly applied at the time of AI, IVF or other forms of assisted reproductive technologies (ART) to remove the seminal plasma, cryoprotective agents, and other background materials (22,28). With the advent of IVF and other forms of ART, it is considered important to use the best spermatozoa available from a frozen-thawed semen specimens (28). A number of semen manipulative techniques are currently available to remove the undesirable spermatozoa, debris and other factors and to increase sperm qualitative characteristics. These various

Acknowledgments

The authors wish to express their appreciation to the American Breeders Service for providing frozen semen specimens for this research effort. This study was presented at the 21th Annual Meeting of the American Society of Andrology, Minneapolis, Minnesota, April 25-29, 1996.

© 1996 by Elsevier Science Inc.
0093-691X/96/$15.00
PII S0093-691X(96)00293-X

techniques include sperm wash, swim-up, swim-down or sedimentation type methods, Ficoll centrifugation, Percoll density gradient centrifugation, glass-wool and Sephadex filtration (6-8,11,13,15,16,20,22,26,28-35). Several methods of separating motile from non-motile spermatozoa have been recently evaluated using cryopreserved human and equine spermatozoa (8,23,29-34). In general, sperm selection via Sephadex filtration methods have been reported to be more efficient in yielding higher numbers of spermatozoa with improved viability (7). Frozen-thawed spermatozoa are often transiently immotile or have reduced progressive motility. Treatment of frozen-thawed spermatozoa with motility stimulants has resulted in the activation of previously immotile spermatozoa (9,25). Sperm motility stimulants commonly used include natural or synthetic derivatives of metylxanthines (phosphodiesterase inhibitors) such as caffeine and pentoxifylline (9,10,12,13,25). Inhibition of phosphodiesterase activity results in elevated levels of intracellular cAMP and enhancement of sperm motility (10,17). Thus, stimulation of sperm motility may increase the yield and qualitative characteristics of spermatozoa recovered via a variety of sperm selection techniques (9,10,12,25,27).

The objective of this study was to assess the quantitative and qualitative characteristics of frozen-thawed bovine spermatozoa, in the presence or absence of caffeine, recovered via a number of sperm selection techniques.

MATERIALS AND METHODS

Frozen-thawed Semen Preparation

Frozen-thawed semen from 5 bulls was used in this study (ABS Global, Inc., Madison, WI, USA). Frozen specimens were thawed at 37°C for 10 sec and transferred to a water bath at 21°C for 1 min to complete thawing (5). Post-thawed semen specimens (from each bull) were pooled and mixed to obtain a final sperm count of approximately 50 $\times 10^6$ after centrifugation-reconstitution. The semen specimens were split into 2 aliquots and diluted 1:1 with Ham's F-10 medium (ZBL, Inc., Lexington, KY, USA) containing 3% BSA (w/v) and 0 (control media) or 2mM caffeine (final concentration; experimental media). These media were used for dilution, resuspension and selection of spermatozoa. Sperm specimens were diluted and centrifuged, using a conical centrifuge tube, at 300 x g for 5 min and the supernatant was discarded. Sperm pellets were reconstituted with control or experimental media (sperm wash) to yield approximately 100 $\times 10^6$ sperm/ml. The reconstituted sperm specimens were then aliquoted into 0.5- ml portions. Two aliquots (reconstituted with control or experimental media) were used for each selection technique. Sperm washed aliquots were further processed via swim-up, Percoll density gradient centrifugation, and Sephadex (SpermPrep™I) filtration. A separate aliquot was maintained as an absolute control (thawed without further processing). Spermatozoa recovered after final processing were assessed for sperm concentration ($\times 10^6$ sperm/ml), for the percentage and grade of motility (0 to 4), for the percentage of spermatozoa with coiled tails (4), and response to hypoosmotic swelling (HOS) test considering the percentage of swollen spermatozoa (3,14). The HOS test was performed by adding 50.0-μl of the sperm specimen to 0.5-ml of a 100 mOsm/l HOS diluent (3,4). Spermatozoa were exposed to the HOS test to determine the proportion of spermatozoa with intact and biochemically active membranes (14). Sperm specimens were assessed for sperm concentration immediately after recovery via the various techniques employed and washed (as previously described) for final processing and sperm quality assessment. The Makler counting

chamber was employed to assess the sperm concentration and motility characteristics (18). Grade of motility was assessed on a scale of 0 to 4 (33). Also, the total functional sperm fraction (TFSF) was calculated (30). The TFSF is the product of the motility (%) by swollen spermatozoa (%) by total sperm count ($x10^6$).

Swim-up Procedure

The swim-up procedure was performed by gently over layering 1.0-ml of control or experimental media over the sperm specimen. The culture tube was held at a 45° angle for 1 h (37°C) to allow the maximal number of motile spermatozoa to migrate into the overlayered medium. At the end of incubation, 0.8-ml of the over layered medium was carefully removed without disturbing the sperm specimen-Ham's F-10 interface and processed for final evaluation.

Percoll Density Gradient Centrifugation

A modified discontinuous 4 layer technique was used for the Percoll separation method (2). An isotonic 90% (v/v) Percoll stock solution was prepared by adding 10X BSA-free control or experimental media to 100% Percoll (9 Percoll:1 BSA-free Ham's F-10). The BSA-free Ham's F-10 media had 10 times (10X) the solute concentration, and therefore the osmolarity, of the original Ham's F-10 (325 mOsm/l) in order to obtain an isotonic medium upon dilution with 100% Percoll. The Percoll stock solution was diluted further to obtain 80, 70, 55 and 40% (v/v) Percoll solutions. Percoll solutions (0.5-ml aliquots) were layered in increasing concentrations, starting with 80% at the bottom. The sperm specimen was layered onto the top of the upper Percoll layer (40%), and was centrifuged for 15 min at 300 x g. The 80% fraction containing the sperm pellet was collected (after removing the upper layers) with a disposable Pasteur pipette and washed once more. The recovered spermatozoa were resuspended in 0.5 mL of medium and processed for final evaluation.

SpermPrep™ Filtration Method

The SpermPrep™I filtration method (ZBL, Inc.) was used as previously described (28,29). The mechanism by which the SpermPrep™I filtration method separates spermatozoa is via gravity flow, following exclusion chromatography principles (30). Dead or immotile spermatozoa are retarded by the filter matrix while motile spermatozoa pass or penetrate the pores between the hydrated beads (differential filtration) and end up in the filtrate first (31). The beads used are made out of a physiologically inert polysaccharide derivative which at hydration and subsequent swelling and enlargement (3 times the original size) develop rough ridges on their surface which further aid the entrapment and removal of dead or immotile spermatozoa. Before use, the SpermPrep™I (Figure 1) was hydrated by placing 4.0-ml of control or experimental media in the barrel of the column. The filter matrix (Sephadex beads) was gently mixed with the media by forming a suspension and ensuring that air bubbles were removed from the bottom of the filter. The beads were allowed to settle for 10 min to the bottom (sedimentation) and to undergo complete hydration. When the sedimentation was completed, the bottom closure was removed and 2.0 to 3.0-ml of the medium was allowed to run through. This step normally enabled the removal of any small bubbles and debris from the filter line. The filter was capped again with the bottom closure and the filtrate was discarded. The capped filter was placed into a 15.0-ml conical centrifuge tube and held (37°C) until the sperm specimen was ready

for filtration. The sperm specimen was placed in the filter and it was gently mixed with the Ham's F-10 medium to form a uniform suspension and to prevent all the sperm from setting directly onto the filter and possibly clotting the filter. As the sperm specimen was added and mixed, the bottom closure was removed and filtration began. Filtration was continued for 5 min, and more medium was periodically added to the filter to maintain the level of medium at its original level. This step maintained a uniform hydrostatic pressure on the filter during the total filtration time. At the end of filtration, the filter was closed with the bottom closure and removed from the centrifuge tube. The filtrate was collected in the centrifuge tube and processed further for final evaluation.

Statistical Analysis

The results were reported as means ± SD. Statistical analysis of the data was performed by ANOVA. Significance between treatment means were determined by the Least Significant Difference method (24).

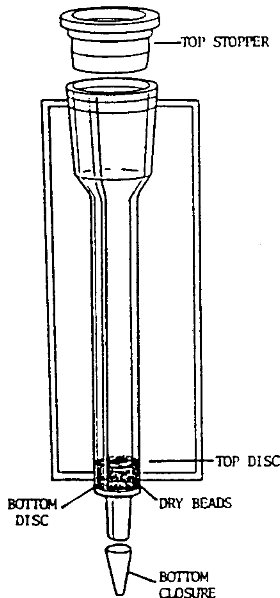

Figure 1. Diagrammatic illustration of the SpermPrep™I filter column and its components.

RESULTS

The sperm characteristics of specimens processed via various sperm selection techniques are shown in Table 1. Processing of frozen-thawed bovine spermatozoa via the various techniques employed in this study resulted in the harvesting of spermatozoa with improved qualitative characteristics. Compared to washed specimens fewer sperm were recovered when swim-up, Percoll and SpermPrep™I filtration methods were employed. Spermatozoa recovered via the swim-up and Percoll methods were quantitatively and qualitatively similar to each other. Higher numbers of motile spermatozoa responsive to the HOS test were recovered via the SpermPrep™I filtration method ($P<0.05$). In general, treatment of specimens with Ham's F-10 media containing 2 mM caffeine resulted in higher percentage of sperm motility, which subsequently resulted in the recovery of higher numbers of motile spermatozoa reactive to the HOS test (TFSF), regardless of the selection technique employed.

Table 1. Qualitative and quantitative characteristics of frozen-thawed bovine spermatozoa recovered via various selection techniques (means ± SD)

	Sperm characteristics assessed				
Method employed	Motility (%)	Grade (0 to 4)	HOS[a] (%)	Total sperm[b] (x10^6)	TFSF[c] (x10^6)
Control, unprocessed	70.0±1.1	3.4±0.1	39.0±1.4	50.0±0.0	13.7±1.1
Sperm wash, 2mM caffeine	75.0±1.8*	3.4±0.1	41.0±1.5	50.0±0.0	15.4±1.8*
Sperm wash, 0mM caffeine	73.0±2.5	3.4±0.1	39.0±1.1	50.0±0.0	14.2±2.5
Swim-up, 2mM caffeine	80.0±1.2*	3.5±0.1	50.0±1.9*	10.0±2.4*	4.0±2.1*
Swim-up, 0mM caffeine	78.0±2.0	3.5±0.1	45.0±3.8	7.0±1.6	2.5±1.4
Percoll, 2mM caffeine	78.0±2.0*	3.4±0.1	46.0±2.2*	10.5±2.3*	3.7±2.1*
Percoll, 0mM caffeine	74.0±2.1	3.4±0.1	41.0±1.9	8.5±1.8	2.3±3.2
SpermPrep™I, 2mM caffeine	93.0±2.0*	3.7±0.1*	65.0±3.0*	27.0±1.1*	16.3±2.0*
SpermPrep™I, 0mM caffeine	90.0±1.0	3.5±0.1	56.0±2.3	23.5±2.0	12.0±2.2

[a]HOS = Hypoosmotic swelling test (percentage of swollen spermatozoa).
[b]Total sperm = Numbers of spermatozoa recovered after processing (x10^6).
[c]TFSF = Total functional sperm fraction (motility (%) by swollen spermatozoa (%) by total sperm (x10^6)).
*Significant differences between specimens reconstituted and recovered with media containing 0 vs 2 mM caffeine ($P<0.05$).

DISCUSSION

The objective of this study was to compare the quantitative and qualitative characteristics of frozen-thawed bovine spermatozoa recovered via various sperm preparation techniques after treatment of spermatozoa with Ham's F-10 medium containing 0 or 2 mM caffeine to stimulate sperm motility. Our results indicate that spermatozoa separated via a variety of sperm selection methods were qualitatively superior to control and washed specimens. However, some signs of processing-related stress such as head-to-head agglutination and altered swimming patterns were observed in spermatozoa recovered via swim-up and Percoll selection methods (19,21). This stress to the spermatozoa was probably related to the relatively lengthy time period required for sperm recovery when employing these techniques. The time interval required for sperm selection is of importance because the cryopreservation and thawing process reduce the viability and longevity of these spermatozoa both in vivo and in vitro (1,8,28). The overall quantitative and qualitative characteristics of these spermatozoa improved further when 2 mM caffeine was incorporated into the Ham's F-10 medium. For all specimens, the percentage of spermatozoa with intact membranes (HOS test) was lower than the percentage of motile sperm. It is possible that some motile spermatozoa had damaged membranes caused by the freezing and thawing procedures (3).

Additional improvements in sperm qualitative characteristics are possible after a period of incubation with media containing caffeine following sperm selection (17). Severe reductions in sperm motility, as seen in frozen-thawed spermatozoa are considered to contribute to low fertilization rates (28). Various studies tend to support the clinical role of methylxanthines in enhancing fertilization (17,27). Also, caffeine can be removed from the fertilization media, after a period of stimulation prior to AI or IVF, if harmful side effects such as delayed or abnormal embryonic development are suspected (25).

In summary, employment of various sperm selection techniques was found to be a necessary step in enhancing the quality of the recovered spermatozoa. The use of caffeine, as applied in this study, further enhanced the quantitative and qualitative profiles of the recovered spermatozoa. Enhancement of sperm motility, in conjunction with the most appropriate sperm selection technique, represents an efficient method for the recovery of spermatozoa with improved qualitative characteristics. Further studies will be conducted to assess the fertilizing ability of spermatozoa selected via the techniques employed in this study.

REFERENCES

1. Beck WW, Silverstein I. Variable motility recovery of spermatozoa following freeze preservation. Fertil Steril 1975;26:863-867.
2. Choudhry TM, Berger T, Dally M. In vitro fertility evaluation of cryopreserved ram semen and its correlation with relative in vitro fertility. Theriogenology 1995;43:1195-1200.
3. Correa JR, Zavos PM. The hypoosmotic swelling test: its employment as an assay to evaluate the functional integrity of the frozen-thawed bovine sperm membrane. Theriogenology 1994;42:351-360.

4. Correa JR, Zavos PM. Frozen-thawed bovine spermatozoa diluted via slow or rapid dilution method: measurements on the occurrence of osmotic shock and sperm viability. Theriogenology 1995;44:963-971.
5. Correa JR, Rodriguez MC, Patterson DJ, Zavos PM. Thawing and processing of cryopreserved bovine spermatozoa at various temperatures and their subsequent effects on sperm viability, osmotic shock and sperm membrane functional integrity. Theriogenology 1996;46:413-420.
6. Dmowski WP, Gaynor L, Rao R, Lawrence M, Scommegna A. Use of albumin gradients for X and Y sperm separation and clinical experience with male sex preselection. Fertil Steril 1979;31:52-57.
7. Dravland JE, Morimer D. A simple discontinuous Percoll gradient procedure for washing human spermatozoa. IRCS Med Sci 1985;13:16-21.
8. Drobnis EZ, Zhong CQ, Overstreet JW. Separation of cryopreserved human semen using sephadex columns, washing, or percoll gradients. J Androl 1991;12:201-208.
9. Fattouh ESM, Abdou MSS. Effect of caffeine on the post-thaw motility of buffalo spermatozoa. Theriogenology 1991;36:149-154.
10. Garbers DL, Lust WD, First NL, Lardy HA. Effect of phosphodiesterase inhibitors and cyclic nucleotides on sperm respiration and motility. Biochemistry 1971;10:1825-1831.
11. Graham EF, Graham JK. The effect of whole ejaculate filtration on the morphology and the fertility of bovine semen. J Dairy Sci 1990;73:91-97.
12. Hammitt DG, Bedia E, Rogers PR, Syrop CH, Donovan JF, Williamson RA. Comparison of motility stimulants for cryopreserved human semen. Fertil Steril 1989;52:495-502.
13. Harris SJ, Milligen MP, Denniss KJ. Improved separation of motile sperm in asthenospermia and its application to artificial insemination homologous (AIH). Fertil Steril 1981;36:219- 221.
14. Jeyendran RS, Van der Ven HH, Perez-Pelaez M, Crabo BG, Zaneveld LJD. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. J Reprod Fertil 1984;70:219-225.
15. Kaneko S, Morikawi C, Sato H. Development of multiple exposure photography method for analysis of sperm motility and preparation of washed sperm with ficoll density gradient. Jpn J Fertil Steril 1980;25:491-493.
16. Katayama KP, Stehlik E, Jeyendran RS. In vitro fertilization outcome: glass wool-filtered sperm vs swim-up sperm. Fertil Steril 1989;52:670-672.
17. Lambert H, Steinleitner A, Eisermann J, Serpa N, Cantor B. Enhanced gamete interaction in the sperm penetration assay after coincubation with pentoxifylline and human follicular fluid. Fertil Steril 1992;58:1205-1208.
18. Makler A. The improved ten-micrometer chamber for rapid sperm count and motility evaluation. Fertil Steril 1980;33:337-338.
19. Makler A, Jakobi P. Effects of shaking and centrifugation on human sperm motility. Arch Androl 1981;7:21-26.
20. McClure DR, Nules L, Tom R. Semen manipulations improved sperm recovery and function with a two-layer Percoll gradient. Fertil Steril 1989;51:874-877.
21. Nevo AC, Mohan R. Migration of motile spermatozoa into sperm-free medium and the 'dilution effect'. J Reprod Fertil 1969;18:379-381.
22. Rusell DL, Rogers BJ. Improvement in the quality and fertilization potential of a human sperm population using the rise technique. J Androl 1987;8:25-33.

23. Rutz EM, Grove RJ, Zavos PM. Recovery of equine sperm through SpermPrep™ filtration method: comparisons. J Ass Reprod Technol Androl 1991;2:140-141.
24. SAS User's Guide: Statistics, SAS Institute, Inc., Cary, NC, 1989.
25. Sikka SC, Hellstrom WJG, Naz RK. Pentoxifylline: Role in management of male infertility/mechanisms of action. Mol Androl 1993;5:169-177.
26. Tanphaichitr N, Millette CF, Agulnick A, Fitzgerald LM. Egg-penetration ability and structural properties of human sperm prepared by Percoll gradient centrifugation. Gam Res 1988;20:67-73.
27. Tesarik J, Mendoza C, Carreras A. Effects of phosphodiesterase inhibitors caffeine and pentoxifylline on spontaneous and stimulus-induced acrosome reactions. Fertil Steril 1992; 58:1185-1190.
28. Zavos PM. Preparation of human frozen-thawed seminal specimens using the SpermPrep filtration method: improvements over the conventional swim-up method. Fertil Steril 1992; 57:1326-1330.
29. Zavos PM, Centola GM. Qualitative and quantitative improvements in human spermatozoa recovered via the swim-up and a new semen filtration method. Infertility 1990;13:25-34.
30. Zavos PM, Centola GM. Selection of sperm from oligozoospermic men for ARTA: comparisons between swim-up vs SpermPrep™ filtration. J Ass Reprod Technol Androl 1991;1:338-345.
31. Zavos PM, Centola GM. Methods of semen preparation for intrauterine insemination and subsequent pregnancy rates. Tohoku J Exp Med 1992;168:583-590.
32. Zavos PM, Grove RJ, Rutz EM. Qualitative improvements of sperm recovered through SpermPrep™ filter. J Ass Reprod Technol Androl 1991;2:206-207.
33. Zavos PM, Correa JR, Zarmakoupis PN. Improvements and short-term viability of mouse epididymal spermatozoa recovered through the SpermPrep™ filtration method. Theriogenology 1994;42:1035-1042.
34. Zavos PM, Sofikitis N, Toda T, Miyagawa I. Improvements in qualitative characteristics of cryopreserved human spermatozoa following recovery via SpermPrep™I filtration method. Tohoku J Exp Med 1991;163:283-290.
35. Zavos PM, Correa JR, Sofikitis N, Kofinas GD, Zarmakoupis PN. A method of short-term cryostorage and selection of viable sperm for use in the various assisted reproductive techniques. Tohoku J Exp Med 1995;176:75-81.