Middle East Fertility Society Journal
Vol 3, No I, 1998

Occurrence of osmotic shock in human spermatozoa: its effects on the quaiitative measurements of frozen-thawed spermatozoa

Panayota N. Zarmakoupis-Zavos, M.D.[*‡§]
Pavlos Aslanis, M.D.[§]
Panayiotis M. Zavos, Ed.S., Ph.D.[*‡§]
Juan R. Correa, Ph.D.[‡§]
Spyros Antypas, M.D.[”]

Andrology Institute of Lexington and Kentucky Centerfor Reproductive Medicine, Lexington, Kentucky; and Greek-American Andrology Institute of Athens and Children's Hospital "Ayia Sofia", Athens. Greece

Middle East Fertility Society Journal
Copyright © Middle East Fertility Society
Vol. 3, No I, 1998

Occurrence of osmotic shock in human spermatozoa: its effects on the quaiitative measurements of frozen-thawed spermatozoa

Panayota N. Zarmakoupis-Zavos, M.D.*‡§
Pavlos Aslanis, M.D.§
Panayiotis M. Zavos, Ed.S., Ph.D.*†‡§
Juan R. Correa, Ph.D.‡§
Spyros Antypas, M.D.¶

Andrology Institute of Lexington and Kentucky Centerfor Reproductive Medicine, Lexington, Kentucky, and Greek-American Andrology Institute of Athens and Children's Hospital "Ayia Sofia", Athens. Greece

ABSTRACT

Objective: Mammalian spermatozoa swell under hypotonic conditions. The exposure of spermatozoa to isotonic conditions after exposure to hypertonic conditions during cryopreservation results in the occurrence of osmotic shock, which is characterized by coiling of the distal end of the sperm tail. The objective of this study was to assess the effects of osmotic shock (% sperm coiling) on human cryopreserved spermatozoa following thawing and subsequent dilution in two different media.

Design: Twenty ejaculates were collected, split and frozen in TEST-Yolk buffer (TYB) containing a final concentration of 8% or 12% glycerol (v/v). Specimens were thawed after a 10 day cryostorage period and diluted 1: 1 (v/v) via a fast or slow dilution method (mode) using TYB or Ham's F-10 media. Specimens were washed and assessed for qualitative characteristics and for the occurrence of osmotic shock.

Setting: Andrology Institute of Lexington, Inc., Lexington, Kentucky.

Patients: Twenty male subjects were instructed to produce a semen specimen each via the use of a semen collection device at intercourse.

Main Outcome Measure(s): Evaluation of osmotic shock occurrence as a function of glycerol level in the freezing media, dilution rate and media used for sperm washing procedures.

Result(s): The slow dilution method yielded improved qualitative characteristics than the fast dilution method, regardless of glycerol level or media used for sperm wash. The occurrence of osmotic shock was higher as the level of glycerol increased (from 8 to 12%) in specimens diluted at a fast mode and washed with Ham's F-10.

Conclusion(s): It is believed that the observations made in this study are of great clinical significance to all involved with freezing and handling cryopreserved spermatozoa. Abrupt dilution of glycerol levels in cryopreserved sperm for IVF, or in vivo by the female reproductive tract fluids during artificial insemination (Al) could explain in part, the consistently lower fertility rates obtained when cryopreserved spermatozoa are used as compared to fresh ones.

Key words: spermatozoa, freezing, glycerol, dilution, osmotic shock

Current success in the cryopreservation of mammalian spermatozoa is attributable in large part to the fortuitous observation made by Polge in 1949 that glycerol carries unique cryoprotective properties (1). As of today, glycerol is the most commonly used cryoprotective agent for spermatozoa and other cells or tissues. Glycerol-treated spermatdzoa can be frozen and cryostored in liquid nitrogen at subzero temperatures (-196°C). However, many spermatozoa are killed, rendered immotile or physiologically altered by the freezing-thawing procedures (2). Evidence is available that glycerol may have an adverse effect

*Kentucky Center for Reproductive Medicine, Lexington, Kentucky.
*Reprint requests: Panayiotis Zavos, Andrology Institute of Lexington, P.O. Box 23777, Lexington, Kentucky 40523 (Fax:606-2786906).
‡Andrology Institute of Lexington, Lexington, Kentucky.
&reek-American Andrology Institute of Athens, Athens, Greece.
¶Department of Pediatric Surgery Children's Hospital "Ayia Sofia", Athens, Greece.

on post-thaw sperm quality and fertilization potential of human spermatozoa and spermatozoa from various domestic species (3-14). Spermatozoa cryopreserved in the presence of glycerol have been shown to yield significantly lower post-thaw sperm motility and the outcome of the sperm penetration assay (SPA), and to induce acrosomal ultrastructural changes when compared to fresh ones (7-10, 15, 16). Structural and biochemical changes in glycerol-treated spermatozoa and other cell types include increased leakage of intracellular enzymes and osmotic-related changes on the cellular membrane (3, 17- 19).

The exposure of spermatozoa to hypotonic solutions and the subsequent changes are termed osmotic shock. Swelling of spermatozoa and other cells may occur in other ways than by decreasing the osmotic pressure of the surrounding fluid. Isosmotic solutions of non-polar solutes, such as glycerol, can cause sperm swelling due to their ability to permeate the cell membrane, causing water to enter and to balance the internal and external osmotic pressures. The reports of cells being injured by the addition or removal of glycerol are usually attributed to osmotic shock rather than to chemical toxicity (17). In the case of frozen-thawed spermatozoa, osmotic shock refers to the cellular changes that occur during the exposure of spermatozoa to isotonic conditions after a period of hypertonic exposure induced by the addition of glycerol (5, 10-12, 14). This type of injury to frozen-thawed spermatozoa is characterized by coiling and swelling of the distal end of the sperm tail (5, 1 O-l 2, 14). The osmotic shock phenomenon can be used as a measurement of sperm behavior to osmotic conditions. The osmotic shock phenomenon may occur when the extracellular glycerol concentration in frozen-thawed semen is reduced abruptly during the performance of various in vitro sperm manipulations such as dilution and preparation of sperm for the various assisted reproductive technologies (ART), and also in vivo by the female reproductive tract fluids during the performance of artificial insemination (AI) (5, 6, 8, 9, 10-12, 14). Since the occurrence of osmotic shock involve structural changes on the sperm membrane, its functional status must be evaluated. The hypoosmotic swelling (HOS; 20) test evaluates whether an intact membrane is biochemically active. During the HOS test, the biochemically active spermatozoa, when exposed to hypoosmotic stress due to the influx of water, will undergo swelling and subsequently increase in volume to establish an equilibrium between the intracellular fluid compartment of the spermatozoon and the extracellular environment (20).

The objective of this study was to assess the effect of the speed of dilution (dilution mode) and glycerol removal during sperm washing procedures on frozen-thawed human sperm viability and occurrence of osmotic shock.

MATERIALS AND METHODS

Semen Collection and Assessment

Twenty subjects were instructed produce a semen specimen each after 4 days of sexual abstinence via the use of a semen collection device (Male Factor Pak™; MFP™; ZDL, Inc., Lexington, KY, USA) at intercourse. The MFP™ consists of a nonspermicidal condom made of polyurethane (2 1, 22). Specimens were allowed to liquefy for 15 to 30 minutes and assessed for volume (ml), count ($x10^6$), percentage and grade of motility (0 to 4), morphology (% normal), and for the sperm membrane functional integrity as measured by the HOS test. Assessment of seminal characteristics was performed according to the World Health Organization (WHO; 23) guidelines using a phase-contrast microscope at a magnification of 400x. Sperm motility assays were conducted under blind conditions. The technique used to estimate progressive motility (grade) was similar to the one described by Zavos et al (24). Sperm progressive motility was graded as follows: Grade 1, oscillating movement but stationary; Grade 2, slow movement with no fixed direction; Grade 3, slow progressive movement; and Grade 4, fast progressive movement. The percentage of spermatozoa with coiled tails was established as previously described (5, 10- 14). Application of the HOS test for frozen-thawed human spermatozoa was performed as described by Jeyendran et al

Table 1. Semen characteristics (n=20) of fresh specimens collecteat intercourse (means±SD)

Volume (ml)	Semen characteristics assessed				
	Total Count (x106)	Motility (%)	Grade (0 to 4)	Morphology (% normal)	HOS* (%)
3.8±1.1	374.3±29.3	65.8±5.1		61.8±7.2	73.4±3.1

*Hypoosmotic swelling (HOS) test (percentage of swollen spermatozoa)

(20). The HOS test was performed by adding and mixing 50 µl of the differently diluted spermatozoa to 0.5 ml of the 150 mOsm/l HOS diluent. The sperm mixture was then incubated for 1 h in a water bath at 37°C followed by assessment of sperm swelling patterns (20). Semen specimens were prepared for freezing and cryopreservation at -196°C following the initial assessment.

Freezing Procedure

Semen specimens were split into 3 aliquots (Aliquots 1 to 3). Aliquot 1 (fresh) was used without further processing. Aliquots 2 and 3 were prepared for freezing as previously described **(2, 25).** The medium used for freezing consisted of nonthermoprecipitated TYB (NT-TYB; ZDL, Inc.) containing 16 or 24% glycerol (v/v). Aliquot 2 was diluted 1:1 (v/v) with NT-TYB containing 16% glycerol to yield a final concentration of 8% glycerol. Aliquot 3 was diluted 1:1 (v/v) with NT-TYB containing 24% glycerol to yield a final concentration of 12% glycerol. Specimens were cryostored for 10 days at -196°C, followed by thawing at 37°C in a water bath for 2 minutes.

Dilutions of Frozen-Thawed Human Spermatozoa

The media used for dilution consisted of modified Ham's F-l 0 (SpermPrep™media; ZDL, Inc.) containing 3% (w/v) bovine serum albumin, and NT-TYB. Both media contained 0% glycerol. Each aliquot (2 and 3) was split into four equal volumes (0.5 ml) and diluted with either Ham's F-10 or TYB at a rapid or slow mode. Rapid dilution consisted of adding an equal volume of dilution media in a single step. Following rapid dilution, the semen specimen was mixed. Slow dilution consisted of adding the same volume of dilution media at a rate of 0.1 ml/min (5 min) via a drop-wise method (1 O-14) using a tuberculin syringe (22G needle). The semen specimens were allowed to equilibrate for 1 to 2 min following completion of the dilution and were centrifuged for 8 min at 400 x g (1 O-14). The supernatant was discarded and the sperm pellets were resuspended to 1 ml,

Table 2. Qualitative characteristics of cryopreserved spermatozoa frozen in nonthermoprecipitated TYB containing 8 or 12% (v/v) glycerol, followed by thawing (means&SD)

Treatments	Semen characteristics assessed (n=20)			
	Motility (%)	Grade (0 to 4)	Morphology (% normal)	HOS' %
8% glycerol	51.1±7.6[a]	3.3±0.3[a]	58.2±6.3[a]	61.3±45.6[a]
12% glycerol	48.2±8.4[a]	3.1±0.4[a]	57.1±6.8[a]	58.0±5.3[a]

● Hypoosmotic swelling (HOS) test (percentage of swollen spermatozoa)
"No significant differences were noted between the two treatments (P>0.05)

Table 3. Qualitative characteristics of cryopreserved spermatozoa frozen in nonthermoprecipitated TYB media containing 8% (v/v) glycerol, followed by rapid or slow dilution in two different media (means±SD)

Treatments	Semen characteristics assessed (n=20)				
	Motility %	Grade (0 to 4)	Morphology (% normal)	HOS* %	Osmotic shock† (% coiling)
Ham's F- 10, rapid dilution	43.4±3.1[a]	2.5±0.2[a]	41 .2±3.5[a]	45.7±4.1[a]	22.4±1.6[a]
Ham's F- 10, slow dilution	48.7±3.4[ab]	2.8±0.2[ab]	46.7±3.2[a]	49.4±3.5[a]	20.3±1.5[a]
TYB, rapid dilution	45. 1±3.2[a]	2.8±0.2[ab]	45.8±3.2[a]	55. 1±3.6[b]	17.6±1.6[b]
TYB, slow dilution	50.2±3.8[b]	3.2±0.3[b]	55. 1±3.4[b]	60.3±3.3[b]	12.1±1.2[c]

'Hypoosmotic swelling (HOS) test (percentage of swollen spermatozoa)
[abc]Significant differences noted among different treatments (P<0.05)

with the corresponding media used for dilution, and allowed to equilibrate for 5 min in a water bath (37°C) before assessment as previously described.

Statistical Analysis

The obtained values were expressed as means±SD. Analysis of Variance methods were used for statistical analysis of the variables and sample means studied. The Least Significant Difference method was used to determine significance among the various means per treatment (26). The statistical model employed included the effects of semen donor, semen status (fresh vs frozen) semen characteristics, and dilution method.

RESULTS

The results obtained in this study are summarized in Tables 1 to 4. Semen specimens were considered normospermic according to the WHO guidelines (Table 1). Sperm qualitative characteristics decreased after freezing and thawing as compared to fresh specimens. Those changes were more noticeable (P>0.05) in specimens cryopreserved using NT-TYB media containing 12% glycerol. Percentage of sperm motility and maintenance of the sperm membrane functional integrity were the sperm characteristics most affected by the freezing-thawing procedure (Table 2). No differences (P>0.05) were noted however, in sperm qualitative characteristics at post-thaw, if no further dilution of the samples was performed. Sperm characteristics were significantly reduced (P<0.05) in semen specimens frozen in NT-TYB containing 8% glycerol and diluted rapidly or slowly with Ham's F-10, or slowly with NT-TYB (Table 3). The maintenance of the sperm membrane functional integrity was higher, and the occurrence of osmotic shock was lower, in frozen-thawed specimens diluted with NT-TYB than Ham's F-10, regardless of the dilution mode (P<0.05).

Sperm characteristics were significantly reduced (P<0.05) in specimens frozen with NT-TYB containing 12% glycerol than those frozen with NT-TYB containing 8% glycerol, regardless of dilution rate (Tables 3 and 4).Significant improvements in all characteristics, except percentage of motility, were noted in frozen-thawed specimens (i 2% glycerol) diluted slowly with NT-TYB as compared to specimens diluted rapidly with NT-TYB, and slowly or rapidly with Ham's F-l 0 (Table 4).

DISCUSSION

The main objectives of this study were to assess the effects of the dilution method (dilution mode and glycerol removal) on frozen-thawed human spermatozoa viability, occurrence of osmotic shock and sperm membrane functional status. The results obtained in this study confirm similar observations by Zavos (5), which described the occurrence of osmotic shock in spermatozoa frozen and thawed in the presence of glycerol, followed by dilution. Various studies with spermatozoa from domestic species have also

demonstrated the occurrence of osmotic shock in spermatozoa frozen and thawed in the presence of glycerol. Fiser and Fairfull (6) reported that post-thaw survival of ram spermatozoa can be influenced by the glycerolization method prior to freezing but that post-thaw sperm motility and acrosomal integrity can be maintained even after a rapid decrease in glycerol concentration (6). Vazquez and Graham have noted that immediate decrease in osmotic pressure when frozen-thawed boar sperm were placed into an environment without the cryophylactic agent glycerol damaged the cells and caused reduction in motility (27). Jeyendran et al. (8,9) reported that glycerol-treated cryopreserved human spermatozoa possessed adequate post-thaw progressive. motility, but that incubation in glycerol-free medium caused a severe reduction in motility and low sperm penetration during the SPA. The authors concluded that human spermatozoa develop a dependency to glycerol and that the removal of glycerol from the incubation medium causes a reduction in motility, ability of the spermatozoa to become capacitated and subsequent fusion with oocytes. Glycerol seems to be contraceptive for rooster sperm (28, 29) and if glycerol is removed, fertility is recovered as assessed by AI, every 3 to 4 d rather than 7 to 14 d as with fresh sperm. Slow removal of glycerol is essential to reduce or overcome such contraceptive effects.

Post-thaw injury to spermatozoa and other viable cells due to the abrupt removal of glycerol is usually attributed to the occurrence of osmotic shock (17, 30-34). The osmotic shock phenomenon caused by the exposure of frozen-thawed spermatozoa to isotonic conditions after a period of hypertonic exposure, is characterized by increased coiling of the sperm tail which results in loss of progressive motility (5, 10-14). For this reason, spermatozoa should be diluted slowly to allow gradual osmotic adjustment to take place between the intra- and extracellular compartments, thus preventing or minimizing the osmotic shock phenomenon from occurring (5, 10- 14). The results obtained in this study indicate that frozen-thawed human spermatozoa should be diluted slowly during in vitro sperm manipulation procedures. The results also suggest a possible effect of chemical injury when diluting and washing the spermatozoa with media chemically different to the one employed for cryopreservation, i.e. NT-TYB vs Ham's F-10. In general, superior percentage and grade of motility, reduced percentage of spermatozoa with coiled tails, and greater ability of spermatozoa to react to the HOS test were obtained when post-thaw spermatozoa were diluted slowly using NT-TYB. Adequate numbers of progressively motile spermatozoa with biochemically active membranes are required for successful fertilization (20). It is believed that the observations made in this study are of great clinical significance to all involved with freezing and handling cryopreserved spermatozoa. Abrupt dilution of glycerol levels in cryopreserved sperm for IVF or other ART procedures, or

Table 4. Qualitative characteristics of cryopreserved spermatozoa frozen in nonthermoprecipitated TYB media containing 12% (v/v) glycerol, followed by rapid or slow dilution in two different media (mean&D)

Treatments	Semen characteristics assessed (n=20)				
	Motility (%)	Grade (0 to 4)	Morphology (% normal)	HOS' (%)	Osmotic shock† (% coiling)
Ham's F-l 0, rapid dilution	36.2 ± 3.3^{a}	2.0 ± 0.2^{a}	37.2 ± 3.3^{a}	39.1 ± 4.0^{a}	31.2 ± 1.8^{a}
Ham's F-IO. slow dilution	41.1 ± 3.1^{b}	2.2 ± 0.2^{ab}	39.1 ± 3.1^{a}	41.2 ± 4.0^{ab}	26.1 ± 1.9^{bc}
TYB, rapid dilution	40.3 ± 3.2^{ab}	2.3 ± 0.2^{ab}	41.3 ± 3.3^{ab}	43.7 ± 3.9^{ab}	30.2 ± 2.0^{a}
TYB, slow dilution	43.5 ± 3.0^{b}	2.5 ± 3.0^{b}	45.6 ± 3.0^{b}	45.8 ± 3.9^{b}	24.3 ± 1.8^{c}

'Hypoosmotic swelling (HOS) test (percentage of swollen spermatozoa)
^{abc}Significant differences noted among the various treatments ($P<0.05$)

in vivo by the female reproductive tract fluids during the performance of AI could explain, in part, the consistently lower fertility rates obtained when cryopreserved spermatozoa are used as compared to the fresh ones (2, 5). Furthermore, similar deficiencies could explain the consistently poorer performance of cryopreserved spermatozoa in penetrating the cervical mucus (35-37).

REFERENCES

1. Polge C, Smith AV, Parkes AV. Revival of spermatozoa after vitrification and dehydration at low temperatures. Nature 1949; 164:666.
2. Zavos PM. Principles of cryopreservation of human spermatozoa: state-of-the-art. Infertility 199 1; 13 :239-46.
3. Bower RE, Crabo BG, Pace MM, Graham EF. Effect of dilution and glycerol removal on the release of glutamic oxaloacetic transaminase (GOT) from boar spermatozoa. J Anim Sci 1973;36:319-24.
4. Wilmut I, Polge C. The low temperature preservation of boar spermatozoa. I. The motility and morphology of boar spermatozoa frozen and thawed in the presence of permeating protective agents. Cryobiology 1977; 14:471-8.
5. Zavos PM. Opisthosmotic shock of frozen-thawed human spermatozoa. Infertility 1983;5:247-55.
6. Fiser PS, Fairfull RW. The effect of glycerol concentration and cooling velocity on cryosurvival of ram spermatozoa frozen in straws, Cryobiology 1984;21:542-51.
7. Jeyendran RS, Van Der Ven HH, Kennedy W, Perez-Pelaez M, Zaneveld LJD. Comparison of glycerol and a zwitterion buffer system as cryopiotective media for human spermatozoa: effect of motility, penetration of zona-free hamster oocytes and proacrosin/acrosin. J Androl 1984;5:1-7.
8. Jeyendran RS, Van Der Ven HH, Perez-Pelaez M, Zaneveld LJD. Effect of glycerol and cryopreservation on oocyte penetration by human spermatozoa. Andrologia 1985;17:241-8.
9. Jeyendran RS, Van Der Ven HH, Perez-Pelaez M, Zaneveld LJD. Non-beneficial effects of glycerol on the oocyte penetrating capacity of cryopreserved and incubated human spermatozoa. Cryobiology 1985;22:434-7.
10. Correa JR, Zavos PM. Frozen-thawed bovine spermatozoa diluted by slow or rapid dilution method: measurements on occurrence of osmotic shock and sperm viability. Theriogenology 1995;44:963-71.
11. 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;46:413-20.
12. Correa JR, Zavos PM. Preparation and recovery of frozen-thawed bovine spermatozoa via various sperm selection techniques employed in assisted reproductive technologies. Theriogenology 1996;46:1225-32.
13. Zavos PM, Correa JR, Zarmakoupis-Zavos PN. Measurements of the sperm motility index via the sperm quality analyzer and its relationship to other sperm qualitative parameters. Theriogenology 1996;46:421-7.
14. Correa JR, Heersche, Jr. G, Zavos PM. Sperm membrane functional integrity and response of frozen-thawed bovine spermatozoa during the hypoosmotic swelling test incubation at varying temperatures. Theriogenology 1997;47:715-21.
15. Mahadevan MM, Trounson AO. Relationship of fine structure of sperm head to fertility of frozen human semen. Fertil Steril 1984;41:287-293.
16. Joyce C, Jeyendran RS, Zaneveld LJD. Release, extraction and stability of hyaluronidase associated with human spermatozoa. J Androl 1985;6: 152-6 1.
17. Frim J, Mazur P. Interactions of cooling rate, warming rate, glycerol concentration, and dilution' procedure on the viability of frozen-thawed human granulocytes. Cryobiology 1983;20:657-76.
18. Penninckx F, Cheng N, Kerremans R, Van Damme B, De Loecker W. The effects of different concentrations of glycerol and dimethylsulfoxide on the metabolic activities of kidney slices. Cryobiology 1983;20:51-60.
19. Penninckx F, Poelmans S, Kerremans R, De Loecker W. Erythrocyte swelling after rapid dilution of cryoprotectants and its prevention. Cryobiology 1984;21 :25-32.
20. 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;70:219-25.
21. Zavos PM, Goodpasture JC. Clinical improvements of specific seminal deficiencies via intercourse with a seminal collection device versus masturbation. Fertil Steril 1989;51:190-3.
22. Zavos PM, Kofinas GD, Sotikitis NV et al. Differences in seminal parameters in specimens collected via intercourse and incomplete intercourse (coitus interruptus). Fertil Steril1994;6 1:1174-6.
23. World Health Organization. WHO Laboratory Manual for the Examination of Human Semen and Semen-Cervical Mucus Interaction. 3rd edition, Cambridge University Press, Cambridge, 1992.
24. Zavos PM, Correa JR, Zarmakoupis PN. Improvements and short-term viability of mouse epididymal spermatozoa recovered through the SpermPrep™ filtration method. Theriogenology 1994;42:1035-42.
25. Zavos PM, Sofikitis N, Toda T, Miyagawa I. Improvements in qualitative characteristics of cryopreserved human spermatozoa following recovery via the SpermPrep™ II filtration method. Tohoku J Exp Med 1991;163:283-90.
26. SAS User's Guide: Statistics. SAS Institute Inc., Cary, NC, USA, 1989.

27. Vazquez IA, Graham EF. Effect of dialysis and glycerol on extender for freezing boar spermatozoa. 9th Int Congr Anim Reprod AI, Madrid, Spain, 1980;3:404.
28. Lake PE. The history and future of the cryopreservation ofavian germ plasm. Poult Sci 1986;65:1-15.
29. Hammerstedt RI-I, Graham JK. Cryopreservation of poultry sperm: The enigma of glycerol. Cryobiology 1992;29:26-38.
30. Mazur P, Leibo SP, Miller RH. Permeability of the bovine red cell to glycerol in hyperosmotic solutions at various temperatures. J Membr Biol 1974; 15: 107- 136.
31. Whittingham DG. Survival of rat embryos after freezing and thawing. J Reprod Fertil 1975;43:575-8.
32. Thorpe PE, Knight SC, Farrant J. Optimal conditions for the preservation of mouse lymph node cells in liquid nitrogen using cooling rate techniques. Cryobiology 1976;13:126-33.
33. Law P. The tolerance of human platelets to osmotic stress. Exp Hematol 1983;11:351-7.
34. Schneider V, Mazur P. Osmotic consequences of cryoprotectant permeability and its relation to the survival of frozen-thawed embryos. Theriogenology 1984;21:68-79.
35. Zavos PM, Cohen MR. Bovine mucus penetration test: an assay for fresh and cryopreserved human spermatozoa. Fertil Steril 1980;34:175-176.
36. Zavos PM, CohMR. The pH of the cervical mucus and the postcoital test. Fertil Steril 1980;34:234-8.
37. Correa JR, Pace MM, Zavos PM. Relationships between frozen-thawed sperm characteristics assessed via the routine semen analysis, sperm functional tests and fertility of bulls in an artificial insemination program. Theriogenology 1997;48:721-31.

Received on September 6, 1997; revised and accepted on December 6, 1997