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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 (AI) 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 spermatozoa 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

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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, 10-12, 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 (21, 22). Specimens were allowed to liquefy for 15 to 30 minutes and assessed for volume (ml), count ($\times 10^6$), 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 collected intercourse (means±SD)

Volume (ml)	Semen characteristics assessed				
	Total Count (x10 ⁶)	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-10 (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)

^aNo 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-10 (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 cryoprotective 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-10, 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-10, 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).

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