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Evaluation of Techniques for the Cryopreservation of Washed Spermatozoa: Comparisons between Ham's F-10 and TEST-Yolk Media

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ZAVOS, P.M., CORREA, J.R. and ZARMAKOUPIS-ZAVOS, P.N. *Evaluation of Techniques for the Cryopreservation of Washed Spermatozoa: Comparisons between Ham's F-10 and TEST-Yolk Media.* Tohoku J. Exp. Med., 1998, **184** (4), 277-284 —— The objective of this study was to develop new techniques for the cryopreservation of washed spermatozoa. Two media (Ham's F-10 and nonthermoprecipitated TEST-yolk buffer [NT-TYB]) containing 7% (v/v) glycerol were compared to semen cryopreservation by adding glycerol directly to the semen. Twenty four men collected a semen specimen each after 4 days of sexual abstinence via the use of a semen collection device at intercourse. Specimens were assessed for volume (ml), count ($\times 10^6$), percentage and grade of motility, morphology (% normal) and acrosomal status (% intact acrosomes). Each ejaculate was split into 3 aliquots (Aliquots 1 to 3) and processed for freezing. Aliquot 1 was prepared for cryopreservation by adding glycerol (7% [v/v] final concentration) directly via a dropwise mode. Aliquot 2 and 3 were diluted 1 : 1 (v/v) with Ham's F-10 and NT-TYB, respectively. Aliquots 2 and 3 were then centrifuged ($400 \times g$ for 10 minutes) and resuspended into the corresponding media containing 7% (v/v) glycerol to complete the sperm wash procedure. All aliquots were frozen in 0.5 ml french straws. Sperm specimens were frozen in liquid nitrogen (LN_2) vapor from $+23^{\circ}C$ to $-68^{\circ}C$ at a slow rate ($2.3^{\circ}C$/minute), after which the specimens were plunged directly into LN_2 and stored for 30 days. The quality of the spermatozoa were monitored throughout each step of the overall procedure by measuring the motility characteristics of the spermatozoa. Straws corresponding to each aliquot were thawed in a water bath at $37^{\circ}C$ for 2 minutes, followed by assessment of sperm motility and acrosomal status. The percentage of motility after thawing was $31.6 \pm 5.6\%$, $32.8 \pm 1.8\%$ and $37.3 \pm 1.9\%$ in Aliquots 1 to 3, respectively. Similarly, the grade of motility was 2.4 ± 0.2, 2.6 ± 0.1 and 3.0 ± 0.1 in Aliquots 1 to 3, respectively. The acrosomal status (% intact acrosomes) in Aliquots 1 to 3 was 41.2 ± 2.6, 43.1 ± 3.6 and 51.6 ± 4.5, respectively. The results

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suggest that the characteristics of spermatozoa washed and frozen in NT-TYB (Aliquot 3) were improved over those spermatozoa prepared via direct addition of glycerol to the semen (Aliquot 1) or by using Ham's F-10 (Aliquot 2). The most significant reduction noted during freezing was in the loss of acrosomal integrity. The results obtained in this study point out that washed spermatozoa can be cryopreserved with some success and that the recovered spermatozoa could be used for intrauterine insemination in an artificial insemination program using husband's or donor sperm, or for the various assisted reproductive technology procedures. It is the opinion of the authors that the information generated in this study is of importance for those scientists and clinicians involved in the handling and manipulation of cryopreserved spermatozoa. ———— spermatozoa; sperm wash; freezing; Ham's F-10; TEST-yolk buffer

Current success in the cryopreservation of mammalian sperm is attributable in large part to the fortuitous observation made by Polge et al. (1949) that glycerol carries unique cryoprotective properties. The use of glycerol in conjunction with a liquid nitrogen (LN_2) vapor freezing technique provides the technology that supports human semen banking (Sherman 1963, Zavos 1991). Various media have been employed for cryopreservation of human semen (Zavos 1991). Those media are based upon the use of glycerol as the principal (permeable) cryoprotectant in the presence or absence of extenders that include sodium citrate and Tes-Tris (TEST) buffered solutions, which often contain nonpermeable macromolecules such as egg yolk (EY) or albumin (Zavos 1991; Zavos et al. 1995). Since the initial introduction and application of TEST-yolk buffer (TYB) in human semenology (Zavos et al. 1980), the EY containing buffer has been extensively used as a sperm diluting media and as an extracellular cryoprotective agent in semen cryopreservation due to the unique properties of EY (Graham et al. 1972; Zavos et al. 1980, 1995; Jaskey and Cohen 1981; Bolanos et al. 1983; Kofinas and Zavos 1992a, b; Allan et al. 1997). Employment of EY in semen cryopreservation and preparation for use in assisted reproductive technologies (ART) has been shown to yield high recovery rates of spermatozoa with adequate motility and fertilizing capacity (Graham et al. 1972; Zavos et al. 1980, 1994b; Falk et al. 1990; Kofinas and Zavos, 1992a, b; Paulson et al. 1992; Gamzu et al. 1994; Hensleigh et al. 1996). Ham's F-10 is a complex and versatile media, which can be used for sperm wash and incubation, and as fertilization media for a variety of ART procedures (Zavos and Centola 1992; Correa and Zavos 1996). Modification of Ham's F-10 by adding albumin and HEPES buffer allows for the manipulation of spermatozoa without the need of an environment containing CO_2 such as when performing various in-vitro gamete manipulations during in-vitro fertilization (IVF).

In the human, semen may be extended using a variety of glycerolated media, usually added to the semen at a 1 : 1 (v/v) ratio (Sherman 1963; Zavos 1991). Direct addition of glycerol has been proved to be the most effective method to

minimize semen dilution inherent in the use of extenders (Zavos 1991). Spermatozoa cryopreserved in this manner must be washed several times in order to remove the seminal plasma and glycerol prior to their use for artificial insemination (AI) or in the various ART. Since the quality and viability of frozen-thawed spermatozoa is reduced by the cryopreservation process, the specimens must be prepared for AI or ART, as fast as possible, to avoid compromising the fertilizing potential of the spermatozoa (Zavos 1992). Other methods involve the extension of semen with cryopreservation media, followed by freezing. However, this methodology may not be an option in semen specimens with various quantitative and/or qualitative deficiencies. Performance of sperm wash procedures prior to cryopreservatios could prove to be useful in preventing those difficulties encountered when freezing diluted or nondiluted semen for AI or ART procedures. The objective of this study was to develop new techniques for the cryopreservation of washed spermatozoa. Two resuspension media (Ham's -10 and nonthermoprecipitated TYB [NT-TYB]) were compared to the conventional method of semen cryopreservation.

Material and Methods

Semen collection and assessment

Twenty four subjects were instructed to collect a semen specimen each after 4 days of sexual abstinence via the use of a semen collection device at intercourse (Zavos 1985a, b; Zavos and Goodpasture 1989; Zavos et al. 1994a). The semen collection device consisted of a nonspermicidal condom made of polyurethane (Male Factor Pak™; ZDL, Inc., Lexington, KY, USA). Semen specimens were assessed after proper liquefaction (30 minutes) according to procedures recommended by the World Health Organization (WHO 1992) using a phase-contrast microscope. Semen characteristics assessed included volume (ml), sperm count ($\times 10^6$ spermatozoa), percentage and grade of motility (0 to 4), morphologic features (% normal) and for acrosomal status (% intact acrosomes). All seminal parameters were evaluated by the same technician under blind conditions. Specimens were prepared for freezing and cryopreservation following initial assessment.

Experimental design

A diagrammatic representation of the experimental procedures followed are depicted in Fig. 1. Each semen specimen was split into 3 aliquots (Aliquots 1 to 3). The media used for semen dilution was Ham's F-10 supplemented with 3% (w/v) bovine serum albumin and NT-TYB. The same media was used for resuspension and cryopreservation in the presence of 7% (v/v) glycerol. Aliquot 1 was prepared for cryopreservation by adding glycerol (7% [v/v] final concentration) directly via a dropwise mode (Sherman 1963; Correa and Zavos 1995). Aliquot 2 and 3 were diluted 1 : 1 (v/v) with Ham's F-10 and NT-TYB, respec-

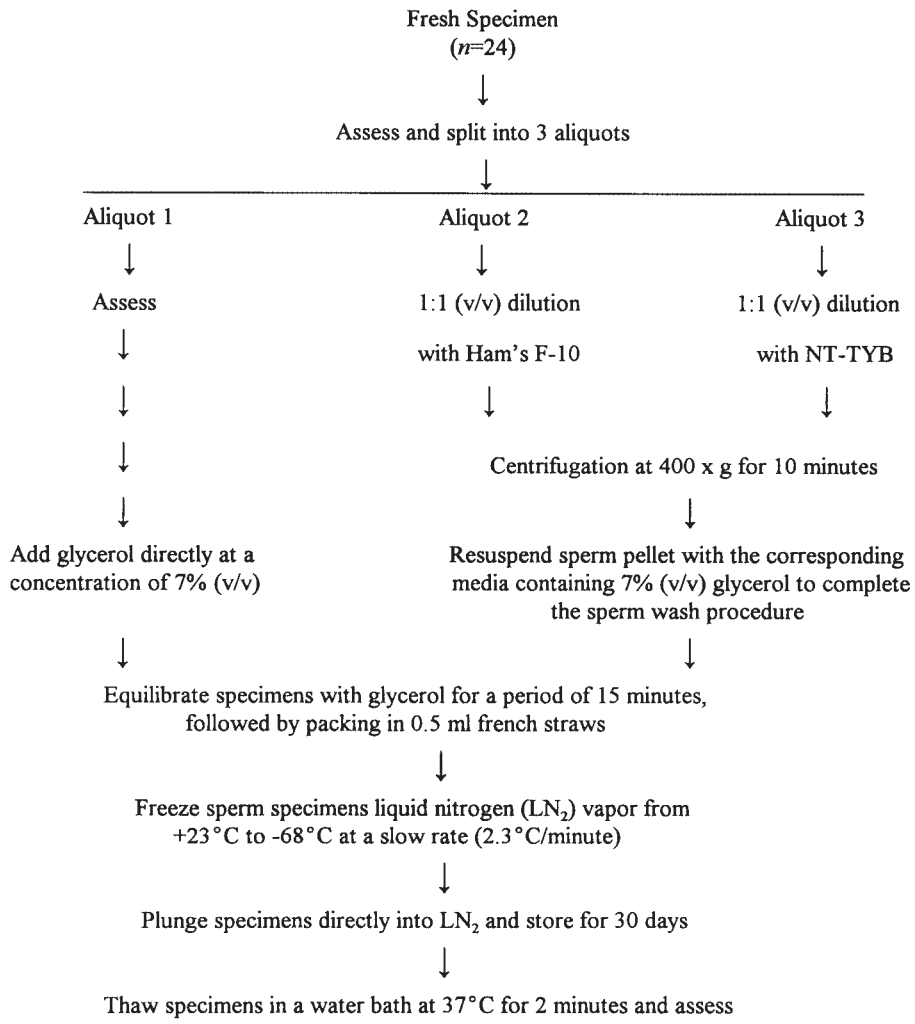

Fig. 1. Diagrammatic representation of the experimental procedures followed.

tively. Aliquots 2 and 3 were then centrifuged (400 × g for 10 minutes) and the sperm pellet was resuspended with the corresponding media to complete the sperm wash procedure. Specimens were equilibrated with glycerol for a period of 15 minutes, followed by packing in 0.5 ml french straws. Sperm specimens were frozen in LN_2 vapor from $+23°C$ to $-68°C$ at a slow rate (2.3°C/minute), after which the specimens were plunged directly into LN_2 and stored for 30 days. The quality of the spermatozoa was monitored throughout each step of the cryopreservation procedure by measuring the motility characteristics of the spermatozoa. Straws corresponding to each aliquot were thawed in a water bath at 37°C for 2 minutes, followed by assessment of the percentage of sperm motility and grade, and for the acrosomal status (% intact acrosomes), as previously described. The results were reported as means $\pm$ S.D. The data was analyzed by analysis of variance (ANOVA) procedures using the SAS Statistical Package (SAS Institute Inc. 1989). A level of $p<0.05$ was considered statistically significant. The statistical model included the effects of semen donor, media and cryopreservation method employed, and sperm characteristics assessed.

Results

The results obtained in this experiment are summarized in Tables 1 and 2. Fresh semen specimens were considered normospermic according to WHO standards. Sperm characteristics did not vary significantly during the preparation steps prior to freezing ($p > 0.05$). Significant differences were noted in the percentage of motility and intact acrosomes between fresh and frozen-thawed specimens, regardless of the cryopreservation method employed ($p < 0.05$). The progressive motility was also significantly decreased in specimens cryopreserved via direct addition of glycerol to the semen or sperm washed with Ham's F-10 as compared to fresh specimens ($p < 0.05$). The sperm characteristics most affected by the cryopreservation process was injury to the acrosome membrane as compared to fresh specimens ($p < 0.05$). The sperm characteristics of specimens washed and cryopreserved using NT-TYB were significantly superior than those of specimens cryopreserved via direct addition of glycerol or washed with Ham's F-10 ($p < 0.05$). The sperm characteristics of specimens washed and cryopreserved using Ham's F-10 tended to be higher, but not significantly different, than in specimens cryopreserved via direct addition of glycerol ($p > 0.05$).

Table 1. *Characteristics of semen specimens ($n = 24$) collected via the use of a semen collection device at intercourse (means ± S.D.)*

Volume (ml)	Count ($\times 10^6$)	Motility (%)	Grade (0 to 4)	Morphology (% normal)	Intact acrosomes (%)
3.1±0.3	214.8±38.2	55.8±2.4	3.1±0.1	59.6±5.2	77.3±7.8

Table 2. *Qualitative characteristics of spermatozoa ($n = 24$) cryostored via direct addition of glycerol or washed and frozen with Ham's F-10 or nonthermoprecipitated TYB (means ± S.D.)*

Cryopreservation method	Sperm characteristics assessed		
	Motility (%)	Grade (0 to 4)	Intact acrosomes (%)
Semen, 7% glycerol[a]	31.6±5.6	2.4±0.2	41.2±2.6
Ham's F-10, 7% glycerol[b]	32.8±1.8	2.6±0.1	43.1±3.6
NT-TYB, 7% glycerol[b]	37.3±1.9	3.0±0.1	51.6±4.5

[a] Aliquot 1 was prepared for cryopreservation by adding glycerol at a final concentration of 7% (v/v) directly via a dropwise mode.

[b] Aliquots 2 and 3 were cryopreserved with Ham's F-10 and NT-TYB containing 7% (v/v), respectively.

Discussion

The objective of this study was to develop new techniques for the cryopreservation of washed spermatozoa. Two resuspension media (Ham's F-10 and NT-TYB) were compared to the conventional method of semen cryopreservation, which consists of direct addition of glycerol to the semen. It was believed that washing of the spermatozoa before freezing could prove to be useful in preventing those difficulties encountered when freezing diluted or nondiluted semen for intrauterine insemination (IUI) or ART procedures. The results obtained in this study indicate that the cryopreservation procedure significantly reduced the sperm qualitative characteristics, regardless of the employed cryopreservation procedure. However, sperm characteristics were less affected in specimens washed and cryopreserved using NT-TYB.

The use of cryopreserved spermatozoa provides the advantage of arranging patients and performance of IUI and various ART procedures such as IVF, intracytoplasmic sperm injection (ICSI) and others (Zavos and Centola 1992; Zavos et al. 1995, 1997a, b). It has been documented that the treatment of human spermatozoa with TYB can enhance their ability to penetrate zona-free hamster oocytes (ZFHO) and bind to the zona pellucida as measured by the sperm penetration assay (SPA) or hemizona (HZA) assays (Bolanos et al. 1983; Falk et al. 1990; Lanzendorf et al. 1992; Paulson et al. 1992; Gamzu et al. 1994). The fertilization potential of spermatozoa incubated and cryostored in TYB can also be enhanced by selection of spermatozoa via various sperm preparation techniques employed in ART after the cryostorage period (Zavos et al. 1995; Correa and Zavos 1996; Correa et al. 1997). Preincubation of spermatozoa in TYB have been shown to increase the percentage of human oocytes fertilized via IVF procedures (Bolanos et al. 1983; Katayama et al. 1989a, b; Falk et al. 1990; Gamzu et al. 1994). It seems that a higher proportion of the sperm population undergo capacitation during TYB incubation, which results in synchronization of the acrosome reaction following sperm washing and preparation for use in the various ART procedures (Falk et al. 1990; Kofinas and Zavos 1992a; Gamzu et al. 1994). The increased percentage of acrosome-reacted spermatozoa that is seen after incubation in TYB may indicate that a larger percentage of the sperm population acquired the ability to penetrate the ovum and account for the higher rates of fertilization observed by others (Bolanos et al. 1983; Falk et al. 1990; Kofinas and Zavos 1992b; Gamzu et al. 1994).

The results obtained in this study point out that washed spermatozoa can be cryopreserved with some success and that the recovered spermatozoa could be used further for IUI in an AI program using husband's or donor sperm, or for the various ART procedures. It is the opinion of the authors that the information generated in this study is of significant clinical importance for those scientists and clinicians involved in the handling and manipulation of cryopreserved spermat-

ozoa and their employment in various clinical procedures.

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