

# EFFECT OF CENTRIFUGE SPEED, REFRIGERATION MEDIUM, AND SPERM WASHING MEDIUM ON CRYOPRESERVED SPERM QUALITY AFTER THAWING

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Cryopreservation and subsequent thawing of semen for assisted reproductive procedures decreases sperm motility; motility further reduces when the cryoprotectant medium is removed because of the osmotic shock and centrifugation done to prepare the sperm. To compare motility and thus pregnancy rates, this study examined the effects of adding an additional refrigeration medium and three different centrifugation speeds for sperm preparation. Semen samples from 16 healthy normal volunteers were obtained by masturbation after 48 h of abstinence. Sperm motility and other motion characteristics were analyzed with a computer-assisted semen analyzer before freezing, after thawing, and after centrifugation. Each sample was divided into 6 aliquots and frozen using the liquid nitrogen vapor method. After thawing, human tubal fluid (HTF) with 5% human serum albumin was added to 3 aliquots, and refrigeration medium (identical to freezing medium without glycerol) was added to the remaining 3 tubes for each specimen. The tubes containing the two media were then washed by centrifugation at 100g, 300g, and 500g for 10 min. Aliquots with refrigeration medium did not significantly differ from those with HTF for percent motility, curvilinear velocity, straight-line velocity, and amplitude of lateral head displacement at any centrifugation speed. Motile sperm count was significantly greater only at 100g and 300g for refrigeration medium ( $p = .02$  and  $.01$ ) and HTF ( $p = 0.001$ ); at 300g, average path velocity in refrigeration medium aliquots ( $p = .01$ ) and linearity in HTF ( $p = .01$ ) were greater. The results indicated that the reduction in motility and other motion characteristics probably cannot be overcome by changing factors such as the sperm preparation medium or centrifugation speed. More effective cryopreservation techniques or preparation methods that eliminate centrifugation need to be developed.

**Keywords** centrifugation, cryopreservation, motility, refrigeration medium, sperm

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Cryopreserving human spermatozoa is important and useful in treating subfertility. However, semen quality is lower in cryopreserved sperm compared with the unfrozen ejaculate. Many investigators have described reductions in both the proportion of motile spermatozoa and other motion characteristics immediately after thawing [3, 4, 6, 9, 12, 13, 21]. The proportion of motile spermatozoa decreases significantly after the semen is diluted with cryomedium. A further decrease occurs immediately on thawing and is accompanied by a decrease in the velocity and amplitude of lateral head displacement [14]. Sperm-processing methods such as the swim-up technique and gradient separation (Percoll) that are commonly used to process cryopreserved semen specimens for intrauterine insemination and in vitro fertilization (IVF) use centrifugation steps that can lead to low recovery of motile sperm, particularly from specimens with poor semen characteristics before cryopreservation [15].

The aim of this study was to determine (1) if the use of a buffer similar to the freezing medium but without glycerol could help reduce the damage to the sperm as compared to the regular buffer, and (2) the effect of centrifugation speed on the sperm motion characteristics.

## MATERIALS AND METHODS

*Chemicals.* TEST-yolk buffer-freezing medium, refrigeration medium, and modified human tubal fluid (HTF) with 5% human serum albumin were purchased from Irvine Scientific (Santa Ana, CA, USA).

*Assessment of Semen Variables.* Normal healthy volunteers ( $n = 16$ ) meeting the WHO guidelines for normal semen characteristics [23] provided the semen specimens by masturbation after 48 h of abstinence. Liquefied semen samples were analyzed on a computer-assisted motion analyzer (CASA; Cell-Trak, Model VP 110, Santa Rosa, CA, USA) before freezing and after thawing. For each measurement, a 5- $\mu$ L aliquot was loaded on a counting chamber (MicroCell, Conception Technologies, La Jolla, CA, USA) and analyzed for percent motility, curvilinear velocity (total distance traveled by a given sperm divided by the total time elapsed), straight-line velocity (straight-line distance from the beginning of the sperm track divided by time), average path velocity, linearity (departure of sperm track from a straight line), and amplitude of lateral head displacement (mean width of sperm head oscillation).

*Sperm Cryopreservation Procedure.* Each specimen after the initial CASA analysis was divided into 6 aliquots. TEST-yolk buffer with glycerol (freezing medium) was used for cryopreservation. Freezing medium equal to 25% of the original specimen volume was added to each aliquot. The specimen was gently mixed for 5 min in an aliquot mixer (Hema-Tek, Miles, Elkhart, IN, USA). This procedure was repeated three more times to give a final 1:1 (v/v) ratio of freezing medium to sperm specimens. Cryovials containing the aliquots were frozen at  $-20^{\circ}\text{C}$  for 8 min and thereafter in liquid nitrogen vapor at  $-100^{\circ}\text{C}$  for 2 h. The vials were then placed in liquid nitrogen at  $-196^{\circ}\text{C}$  for long-term storage.

*Post-thaw Semen Analysis.* For analysis, cryovials were thawed at room temperature for 5 min and then placed in a  $37^{\circ}\text{C}$  incubator for 20 min. Three of the 6 aliquots were prepared by removing the cryomedium and then washing with HTF to examine the effect on sperm motion characteristics. The 3 remaining aliquots were prepared by adding refrigeration

medium. Refrigeration medium is identical to the TEST-yolk freezing medium except for 12% glycerol. Refrigeration medium is used to maintain semen samples at temperatures of 2 to 5°C for periods greater than 96 h. The refrigeration medium was added in a stepwise manner as in freezing protocol so that a final 1:1 ratio (v/v) of refrigeration medium to sperm specimen was obtained. The tubes containing the 2 media were then washed at centrifugation 100g, 300g, and 500g for 10 min. The supernatant was removed after centrifugation and the pellet resuspended in 100 µL HTF. Sperm motility and other motion characteristics were analyzed by CASA, and the number of motile sperm was also determined manually.

*Assessment of Viability.* Viability was evaluated by eosin–nigrosin staining after cryopreservation. An aliquot from each sample was mixed with an equal volume of 0.05% eosin and twice the volume of nigrosin to improve the contrast. After 2 min, smears were prepared and air dried. A total of 100 spermatozoa in duplicate were evaluated at ×400 under a light microscope. The dead sperm appear pink; sperm without the dye were counted as viable.

*Statistical Analysis.* Repeated measures analysis of variance was used to assess the effect of media and centrifugation speed using the SAS statistical software package (SAS Institute, 1992). A *p* value of ≤.01 was considered as statistically significant. All data are shown as median and interquartile range. Semen samples were studied individually to avoid any possible interaction from pooling the samples.

## RESULTS

Table 1 summarizes the results for both refrigeration medium and human tubal fluid and the three centrifugation speeds. Percent motility did not differ between the two media at any of the three speeds. However, the motile sperm count was significantly higher at 100g and 300g when the refrigeration medium was used. The motile sperm count also was significantly higher at 300g with HTF medium. Other motion characteristics did not significantly differ except for average path velocity at 300g with refrigeration medium and percent linearity with HTF at 300g.

## DISCUSSION

Although human spermatozoa are described as cold-shock resistant compared to that of domesticated animals, large decreases in motility and viability are common effects of freezing [13]. Thawing also affects survival of cryopreserved spermatozoa. Although, motility is not directly related to fertilizing capacity [7], it is generally accepted to be an important indicator of cryopreserved sperm quality [6]. The availability of fewer motile sperm in intrauterine insemination is thought to be the cause of the lower fecundity rates compared to intrauterine insemination performed with fresh spermatozoa. Pregnancy rates are comparable when using fresh or frozen spermatozoa for intrauterine insemination or IVF when the cervix is bypassed [10, 20, 22]. To prepare cryopreserved spermatozoa for assisted reproductive procedures, the cryoprotectant must be removed using sperm preparation techniques such as the swim-up procedure or colloidal (Percoll) gradient separation. In both techniques, removing the cryoprotectant causes osmotic shock, which, in addition to the deleterious effects of freezing and thawing,

**Table 1.** Effect of centrifugation with refrigeration or human tubal fluid medium on sperm motion characteristics

Characteristics	Washing medium	Centrifugation speed			<i>p</i> value	
		(rpm)	Q1	Median		Q2
Motility (%)	REF	100	13.0	21.5	28.5	.37
		300	11.5	18.9	23.9	.79
		500	14.8	18.5	27.5	.58
	HTF	100	11.7	17.0	28.4	.05
		300	10.0	17.1	25.1	.12
		500	9.5	15.1	27.0	.16
MSC (10 <sup>6</sup> )	REF	100	4.2	7.2	9.0	.02*
		300	4.9	8.2	13.1	.01*
		500	5.0	9.8	12.2	.07
	HTF	100	4.0	7.5	10.6	.17
		300	5.4	7.7	10.3	.001*
		500	3.7	8.8	13.9	.13
VAP (μ/s)	REF	100	22.5	28.6	31.0	.32
		300	20.8	26.6	29.8	.01*
		500	22.7	24.8	27.4	.06
	HTF	100	24.3	26.5	28.4	.33
		300	21.8	24.7	28.2	.44
		500	22.9	26.5	27.5	.36
VCL (μ/s)	REF	100	38.7	44.2	46.7	.57
		300	33.3	41.3	48.6	.27
		500	38.1	40.6	48.6	.58
	HTF	100	41.4	45.5	49.7	.36
		300	42.3	48.6	58.0	.78
		500	40.9	46.2	49.1	.76
VSL (μ)	REF	100	11.1	15.2	16.8	.56
		300	11.0	14.4	16.2	.13
		500	10.5	13.3	15.2	.58
	HTF	100	11.9	14.0	15.3	.17
		300	11.5	13.8	15.3	.21
		500	10.2	14.7	15.3	.37
Linearity (%)	REF	100	28.2	33.6	35.6	.38
		300	27.7	30.8	35.7	.67
		500	28.1	32.0	33.7	.76
	HTF	100	27.0	29.8	33.3	.99
		300	23.8	29.0	32.3	.01*
		500	24.2	31.0	33.7	.50
ALH (μ)	REF	100	1.7	1.8	2.1	.26
		300	1.7	1.8	2.3	.71
		500	1.7	1.9	2.1	.36
	HTF	100	1.8	2.0	2.1	.92
		300	2.1	2.2	2.3	.50
		500	1.8	1.9	2.1	.55

*Note.* \**p* < .05 was significant. REF, refrigeration medium; HTF, human tubal fluid medium; MSC, motile sperm count; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity; LIN, linearity; ALH, amplitude of lateral head displacement.

further decreases sperm motility. In our study, freezing and thawing reduced the number of motile sperm by about 50%, a finding similar to that reported by other investigators [5, 18, 19]. The resulting percentage motility is in turn further reduced by dilution, and centrifugation [8, 9, 21].

During centrifugation, it is essential that forces greater than 100g not be used [11]. The harmful effects of washing and centrifugation have been attributed to the generation of reactive oxygen species, which can irreversibly damage the spermatozoa and impair their fertilizing ability [1, 2]. Centrifugation per se may mechanically damage spermatozoa, and the damage can be sublethal [16, 17]. This damage may be severe for cryopreserved sperm that have already been injured during freezing. Washing also results in sublethal damage to cryopreserved human spermatozoa [8]. Alternative methods for washing spermatozoa without centrifugation have been suggested [14], such as the swim-up technique or Sperm Prep columns. However, the Sperm Prep column does not concentrate the motile sperm, and this can be a problem with oligozoo- or asthenozoospermic ejaculates. Low-speed centrifugation and reduced osmotic shock in our study had no beneficial effect on sperm survival or motion characteristics, except the number of motile sperm and average path velocity were greater at a speed of 300g.

In conclusion, neither low centrifugation speeds nor adding only refrigeration medium after removing cryomedium is sufficient to reduce osmotic shock. Sperm preparation methods that avoid centrifugation may be more promising.

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