Human Reproduction vol.15 no.10 pp.2173–2179, 2000

Improvement in motion characteristics and acrosome status in cryopreserved human spermatozoa by swim-up processing before freezing*

S.C.Esteves[1,2], R.K.Sharma[1], A.J.Thomas Jr[1] and A.Agarwal[1,3]

[1]Andrology Research & Clinical Laboratories, Department of Urology, The Cleveland Clinic Foundation, Cleveland OH 44195, USA and [2]Andrology & Human Reproduction Clinic (ANDROFERT), Rua Tiradentes, 289/61, Campinas, São Paulo, Brazil 13023–190

[3]To whom correspondence should be addressed at: Andrology Research & Clinical Laboratories, Department of Urology, The Cleveland Clinic Foundation, 9500 Euclid Avenue, A19.1, Cleveland, Ohio 44195, USA. E-mail: agarwaa@ccf.org

The purpose of this study was to examine if selecting a sperm population with improved motion characteristics before freezing reduces the deleterious effects of cryopreservation. Semen specimens from 15 normal donors were divided into two equal aliquots. The first aliquot received no treatment (control), and the second was processed by swim-up from a washed sperm preparation to select a sperm population with better motility and motion characteristics (swim-up). Both aliquots were cryopreserved by the liquid nitrogen vapour method. Percentage motility and motion characteristics were evaluated by computer-assisted semen analysis. Acrosome integrity as well as spontaneous and calcium ionophore-induced acrosome reactions before freezing and after thawing were assessed by fluorescein isothiocyanate conjugated peanut agglutinin combined with a supra vital dye (Hoechst-33258). Swim-up processing enabled selection of a sperm population with better motion characteristics, percentage motility and viability before freezing ($P < 0.001$), but with no difference in percentage of acrosome-intact spermatozoa ($P = 0.63$). After thawing, the swim-up specimens exhibited faster velocity and progression than untreated specimens ($P < 0.001$). They also had higher percentages of spermatozoa with intact acrosomes and spermatozoa able to undergo acrosome reaction in response to calcium ionophore ($P < 0.05$). Selecting a highly motile sperm population before freezing enhances overall post-thaw spermatozoa quality.

Key words: acrosome/cryopreservation/human spermatozoa/sperm processing/swim-up

Introduction

Cryopreservation impairs sperm motility (Critser *et al.*, 1987), viability (Alvarez and Storey, 1993), penetration into cervical mucus, penetration of zona-free hamster eggs (Critser *et al.*, 1987), acrosome structure (McLaughlin *et al.*, 1992), and activity of acrosome protease and acrosin (Mack and Zaneveld, 1987). The fertilizing ability of human spermatozoa is reduced after cryopreservation, which can be explained partially by a reduction in the percentage of normal intact acrosomes and in total acrosin activity (Mack and Zaneveld, 1987). Efforts to improve the fertilizing ability of cryopreserved spermatozoa will be useful for clinical applications.

Under normal physiological conditions, ejaculated spermatozoa must be washed free from seminal plasma in order to undergo capacitation, which is necessary before they can undergo acrosome reaction (AR) and penetrate the zona pellucida (Zaneveld *et al.*, 1991; Henkel *et al.*, 1993; Wang *et al.*, 1993). Seminal plasma is removed as the spermatozoa traverse the cervical mucus and capacitation occurs as they are transported across the cervix, uterus and Fallopian tubes.

Prolonged exposure to seminal plasma results in marked decline in both motility and viability. The presence of seminal plasma in an IVF system inhibits the AR, which is a critical component of the fertilizing ability of human spermatozoa (Liu and Baker, 1988; Polanski and Lamb, 1988; Henkel *et al.*, 1993; Calvo *et al.*, 1994; Parinaud *et al.*, 1995). In addition, ejaculated spermatozoa are damaged by reactive oxygen species emanating from seminal leukocytes and damaged spermatozoa (Aitken and Clarkson, 1988). It is essential, therefore, that in assisted reproduction procedures such as intrauterine insemination (IUI) and IVF, ejaculated spermatozoa are separated from the seminal environment as soon as possible.

The swim-up procedure accomplishes this, and enables the selection of populations of highly motile spermatozoa for assisted reproduction procedures. It has been used by many laboratories as the main sperm washing technique (Berger *et al.*, 1985; Brandeis and Manuel, 1993). It seems reasonable to speculate, therefore, that removing the seminal plasma and selecting highly motile spermatozoa by the swim-up technique, before freezing, may reduce the loss of viability, motility and acrosome integrity during cryopreservation.

The purpose of this study was to determine if using swim-up to select a sperm population with better motion characteristics before freezing can minimize the deleterious effects of cryopreservation, as examined by sperm motion and functional measures.

Materials and methods

Chemicals

TEST yolk-buffer freezing medium, sperm-washing medium [modified Biggers-Whitten-Whittingam (BWW)] and Dulbecco's phos-

*Presented, in part, at the 93rd American Urological Association Annual Meeting (abstract # 785), San Diego, CA, USA.

© European Society of Human Reproduction and Embryology

phate-buffered saline (PBS) were purchased from Irvine Scientific (Santa Ana, CA, USA). Fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA), calcium ionophore A23187, and bis-benzimide (Hoechst-33258) were obtained from Sigma Chemical Co. (St Louis, MO, USA).

A stock solution of FITC-PNA was prepared by dissolving 2 mg of FITC-PNA in 1 ml PBS, and it was stored in 600 µl aliquots at –20°C. Hoechst-33258 was prepared by dissolving 1 mg of H33258 in 1 ml PBS, and it was stored in 10 µl aliquots at –20°C.

Semen collection, sperm processing and cryopreservation procedures

Sperm specimens from 15 healthy volunteers were obtained by masturbation into sterile specimen cups. All subjects were asked to observe 2–3 days of sexual abstinence before donating. Semen samples had normal volume, count and motility according to the World Health Organization criteria (WHO, 1999). After the initial semen analysis, each semen specimen was divided into two equal aliquots. The first aliquot received no treatment (control). The second aliquot was processed by the swim-up method from a washed sperm preparation (indirect method) to give a highly motile sperm population (Russell and Rogers, 1987). It should be noted that the centrifugation speed and duration were reduced as far as possible to minimize the potential damage caused by this process. Briefly, the liquefied semen sample was diluted with an equal volume of BWW. After centrifugation at 300 *g* for 10 min, the supernatant was removed and the pellet resuspended in 2 ml of BWW. A second centrifugation was followed by resuspension to a final volume of 600 µl of BWW supplemented with 0.3% bovine serum albumin (BSA) factor V (Irvine Scientific). This fraction was divided in three equal aliquots of 200 µl. Each aliquot was underlayered beneath 800 µl of BWW supplemented with 0.3% BSA in 15 ml centrifuge tubes. The tubes were loosely capped and placed in a 37°C incubator, under 5% CO_2 in air, at a 45° angle for 1 h. During this period, motile spermatozoa migrated from the underlayered sperm suspension to the upper layer. The top 600–700 µl of the supernatant containing the actively motile spermatozoa was removed with extreme care. Control and swim-up specimens were analysed by computer-assisted semen analysis using a computer-assisted semen analyser (CASA, Motion Analysis, Cell-Trak, model VP110, Santa Rosa, CA, USA). Sperm motion characteristics such as percent motility, curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), linearity (LIN), and amplitude of lateral head displacement (ALH) were measured. A small aliquot (0.1 ml) was removed from control and swim-up specimens for assessment of acrosome status.

Both aliquots were then cryopreserved by the liquid nitrogen vapour method. TEST-yolk buffer with glycerol was used as a freezing agent for cryopreservation (Esteves *et al.*, 1998). In brief, a 5 ml vial of the freezing agent was thawed by incubation at 37°C. An aliquot of the medium equal to 25% of the original specimen volume was then added to the specimen. The specimen was gently mixed for 5 min using the Hema-Tek aliquot mixer (Miles, Elkhart, IN, USA). This process was repeated until the ratio of freezing medium to ejaculate was 1:1. Cryovials were placed at –20°C for 8 min and submerged in liquid nitrogen vapour (N_2) at –79°C for 2 h. Vials were then immersed in liquid N_2 at –196°C for long-term storage. After at least 48 h in liquid nitrogen, the vials were incubated at 37°C for 20 min, washed twice in BWW to remove the cryomedium, and reconstituted in 0.5 ml BWW medium with 0.3% BSA (Agarwal *et al.*, 1995). A small aliquot (0.1 ml) was removed from each specimen for measurement of percentage motility, motion characteristics and frequency of spontaneous AR assessment after thawing.

Calibration set-up of the sperm motion analyser

The CASA calibration set-up was as follows: 2-well, 20 µm, duration of data capture (frames): 15 (control) and 30 (swim-up); minimum motile speed (µm/s): 600 (control) and 800 (swim-up); distance scale factor (µm/pixel): 0.9457; centroid cell size minimum (pixels): 2; centroid cell size maximum (pixels): 8; number of cells to find per well: 200; minimum number of fields per sample: 3. A high degree of correlation was seen between the CASA and both manual sperm counts (r^2 = 1, slope = 1) and motility (r^2 = 0.97, slope = 0.97), thereby establishing the accuracy of our CASA measurements. The reproducibility of the analyser results was determined by using a calibration videotape recording. The baseline values for the raw specimens were: sperm count was 38.3–42.5$\times10^6$/ml (30 frame/s) and motility: 61.6–75.0% (30 frame/s); the washed specimens had baseline values of 33.1–34.7$\times10^6$/ml (60 frame/s) for sperm count and 78.1–91.5% (60 frame/s) for motility. For these computations, results $>$2 SD were rejected.

Sperm capacitation and ionophore challenge

After thawing, a highly motile sperm population from each specimen was isolated by the swim-up method as described previously. The spermatozoa were then capacitated for 3 h by incubation in a BWW medium with 3% BSA at 37°C, under 5% CO_2 in air. Each capacitated sample was divided into two aliquots: one was challenged with 2.5 µmol/l calcium ionophore A23187 solution, and the other was treated with diluted dimethylsulphoxide (DMSO) alone to serve as a control. Both aliquots were then incubated at 37°C for 1 h under the same conditions.

Assessment of viability, acrosome status and ionophore-induced AR

Spermatozoa with intact acrosomes were counted before freezing and after thawing. Calcium ionophore-induced AR was measured only after cryopreservation.

Acrosome status was assessed with FITC-PNA, in conjunction with a nuclear stain, bis-benzimide as a viability test (Cross *et al.*, 1986; Mortimer *et al.*, 1990; Aitken *et al.*, 1993). Viability and acrosome status were assessed simultaneously by mixing 100 µl of sperm specimen with 100 µl of 2 µg/ml Hoechst-33258 solution and incubating for 10 min in the dark. Spermatozoa were then washed in PBS solution by centrifugation at 300 *g* for 5 min to remove excess stain, and the pellet was resuspended in 100 µl of BWW. A 20 µl aliquot of this solution was smeared on a microscope slide and allowed to dry. Air-dried slides were immersed in ice-cold methanol for 30 s to permeabilize the sperm membranes and allowed to air dry. The fixed smears were immersed in a 40 µg/ml FITC-PNA solution, incubated at room temperature for 20 min in a foil Coplin jar (Alleigance Healthcare Corp., McGaw Park, IL, USA), and washed gently in PBS to remove the excess label. Scoring was completed within 48 h of staining.

A Leitz Orthoplan fluorescence microscope (Leitz, Wetzlar, Germany) equipped with a Ploemopak epi-illumination module and a mercury ultraviolet source was used to examine the slides at ×1000 magnification in the presence of an anti-quenching agent (Cargille immersion oil, type DF, Fisher Scientific, Pittsburgh, PA, USA) to minimize the loss of fluorescence. Filter cube I.2 was used for FITC-PNA, which fluoresces apple-green, and cube A.2 for Hoechst-33258, which fluoresces a bright medium blue. Hoechst-33258 stains the nuclei of damaged cells (dead spermatozoa), which show a bright blue-white fluorescence, and is excluded from viable cells (live spermatozoa), which show a pale blue fluorescence. The same spermatozoa were examined for FITC-PNA labelling and for Hoechst-33258 staining by interchanging the two filters.

Categorization of staining patterns

Acrosome staining on FITC-PNA labelling was classified as follows. In an intact acrosome, the acrosomal region of the sperm head exhibited a uniform apple-green fluorescence. In a reacted acrosome, only the equatorial segment of the acrosome was stained.

Viability staining on Hoechst-33258 was classified as follows. In viable spermatozoa, the sperm head showed a pale-blue fluorescence, and in dead spermatozoa, the sperm head showed a bright blue-white fluorescence. A total of 200 spermatozoa per sample was scored. The frequency of spermatozoa with intact and reacted acrosomes was based on viable cells only.

Reproducibility of the acrosome scores

To evaluate the inter-observer reproducibility of the acrosome scores, in a blind study one slide from each donor ($n = 15$) was evaluated by two observers. To determine the intra-observer reproducibility of the acrosome scores, the same slides were re-evaluated by one observer.

Statistical analysis

The Student's paired *t*-test was used to analyse statistical differences in percentage motility, motion characteristics, viability and frequency of acrosome-intact spermatozoa in control and swim-up groups before and after cryopreservation, since the data were normally distributed as assessed by the Kolgomorov–Smirnov test for homogeneity and normality. Pearson's correlation coefficient was used to assess inter-observer and intra-observer reproducibility for acrosome scores. An alpha level of 0.05 or less was considered statistically significant and all tests were two-tailed. Statistical analysis was performed using the SAS statistical software package (Cary, NC, USA).

Results

Pre-freeze and post-thaw sperm characteristics in control and swim-up specimens are compared in Table I. Before freezing, the swim-up group had significantly higher percentage motility and velocity (VCL, VSL, and VAP). Viability scores in processed sperm specimens (swim-up) were significantly higher than in controls. After thawing, VCL, VSL, VAP, and ALH values were significantly higher in the swim-up group (Table I). On the other hand, percentage motility, LIN, and sperm viability were comparable between swim-up and control specimens (Table I). Before freezing, the total number of motile spermatozoa in control and swim-up specimens averaged 28.1 $\pm$ 15.3$\times10^6$ (range: 8.6–56.0) and 10.7 $\pm$ 6.7$\times10^6$ (range: 2.5–28.6; $P < 0.001$). After thaw, the concentration of motile spermatozoa was 9.0 $\pm$ 12.7$\times10^6$ (range: 1.7–53.0) in control specimens, and 2.2 $\pm$ 1.9$\times10^6$ (range: 0.4–7.5) in swim-up specimens (not significantly different).

The percentages of viable spermatozoa with intact acrosomes in control and swim-up groups before and after cryopreservation are shown in Table I. The percentages were not different before freezing, although individual variability was observed (Figure 1a). The freeze–thaw process significantly decreased percentages of spermatozoa with intact acrosomes in both swim-up and control specimens. However, the percentage of spermatozoa that lost acrosomal contents during cryopreservation, assessed by subtracting the percentage of viable-intact acrosome spermatozoa post-thaw from the percentage pre-freezing, was lower in the swim-up group, although individual variability was also observed (Figure 1b).

The effects of calcium ionophore on the acrosome status of viable cryopreserved spermatozoa in control and swim-up groups are shown in Table II. Acrosome reaction in response to ionophore challenge was significantly increased in both control and swim-up groups, as shown by the loss in acrosome integrity after ionophore challenge. However, AR in response to ionophore challenge was higher in specimens processed by swim-up before freezing. In the swim-up group, AR to ionophore challenge (ARIC) scores, calculated by subtracting the frequency of acrosome intact spermatozoa after ionophore challenge from the baseline values before, were >10% in all but two specimens (86.7%), whose scores were >5% but <10% in one, and <5% in the other (Figure 2). In the control group, ARIC scores were >10% in nine out of 15 specimens (60%). In the remaining six specimens, two had ARIC scores between 5 and 10%, and four had scores <5%. When an individual specimen was compared to its own control, pre-freeze swim-up processing was found to improve the ARIC scores in 12 out of 15 specimens. In two individuals, ARIC scores remained practically unchanged, while in another, the ARIC score was lower in the swim-up specimen. Interestingly, pre-freeze swim-up processing improved ARIC scores to >10% in both control specimens with scores between 5 and 10%, and in three out of four control specimens with ARIC scores <5% (Figure 2).

The mean difference in the measured percentage of spermatozoa with intact acrosomes between the first and second observer was not significant (1.3 $\pm$ 9.5%), and the average coefficient of variation was 6.5%. The intraclass correlation coefficient (ICC) between the observers was 0.81 [95% confidence interval (CI), 0.62–0.91]. The coefficient of variation was 1.6% for the duplicate evaluation of the same 15 slides by a single observer. The ICC between two readings by the same observer was 0.98 (95% CI, 0.95–0.99).

Discussion

Even with modern cryopreservation protocols for human semen, cryosurvival rates remain poor because of factors such as membrane damage and sub-lethal damage. Freezing and thawing causes a 25–75% reduction in sperm motility (Keel and Black, 1980; Critser *et al.*, 1987). Final motility depends largely on the initial quality of the sample, the cryoprotectant, or the method of freezing. Some of the effects on motility appear to be secondary to a loss in membrane integrity. Cryopreservation directly damages sperm membrane, resulting in loss of membrane permeability and cell death (Keel and Black, 1980; Cross *et al.*, 1986; Russell and Rogers, 1987; Mortimer *et al.*, 1990; Aitken *et al.*, 1993; Agarwal *et al.*, 1995; Parinaud *et al.*, 1995; Esteves *et al.*, 1996). The sub-lethal damage results from the combined effects of cell dehydration/re-hydration, membrane lipid phase transition, alteration in the enzyme activity or energy metabolism, and activation of lipid peroxidation cascade with generation of reactive oxygen species (Barthelemy *et al.*, 1990; McLaughlin *et al.*, 1992). For these reasons, conception rates with frozen–thawed spermatozoa are inferior to those obtained with freshly ejaculated specimens (Peterson *et al.*, 1988).

Table I. Effects of pre-freeze sperm processing of spermatozoa by swim-up on percentage motility, motion parameters, viability, and acrosome integrity in 15 normozoospermic men before and after cryopreservation

Sperm parameters	Control group (mean ± SD)	Swim-up (mean ± SD)	Percentage change[a] (mean ± SD)	*P*-value
Total sperm count ($\times 10^6$)				
Pre-freeze	49.4 ± 23.9	12.1 ± 6.9	–	<0.001
Post-thaw	24.4 ± 18.7	6.6 ± 5.7	–	0.001
Total motile spermatozoa ($\times 10^6$)				
Pre-freeze	28.1 ± 15.3	10.7 ± 6.7	–	0.003
Post-thaw	9.0 ± 12.7	2.2 ± 1.9	–	0.06
Percentage motility				
Pre-freeze	56.1 ± 13.2	85.2 ± 10.6	+60.8 ± 49.3	<0.001
Post-thaw	28.0 ± 7.1	30.1 ± 7.1	+12.5 ± 33.6	NS
Percentage change[a]	–48.1 ± 14.5	–63.9 ± 10.1		<0.001
Straight-line velocity (VSL) (μm/s)				
Pre-freeze	20.0 ± 4.9	38.7 ± 7.3	+112.1 ± 98.1	<0.001
Post-thaw	6.6 ± 2.3	12.4 ± 5.6	+89.11 ± 58.7	<0.001
Percentage change[a]	–65.5 ± 11.5	–67.7 ± 11.7		NS
Curvilinear velocity (VCL) (μm/s)				
Pre-freeze	45.0 ± 9.4	87.6 ± 12.2	+108.9 ± 88.9	<0.001
Post-thaw	22.7 ± 6.1	33.2 ± 7.7	+51.9 ± 34.9	<0.001
Percentage change[a]	–47.5 ± 16.7	–61.25 ± 10.7		0.005
Linearity (LIN)				
Pre-freeze	43.2 ± 5.5	43.9 ± 6.7	+3.0 ± 19.2	NS
Post-thaw	31.8 ± 7.1	33.7 ± 7.7	+7.6 ± 21.1	NS
Percentage change[a]	–25.51 ± 17.5	–22.5 ± 16.6		NS
Amplitude of lateral head displacement (ALH) (μm)				
Pre-freeze	2.7 ± 0.5	2.5 ± 0.4	–1.6 ± 35.3	NS
Post-thaw	1.2 ± 0.4	1.5 ± 0.5	+30.4 ± 25.2	<0.001
Percentage change[a]	–53.9 ± 23.3	–38.95 ± 21.6		0.006
Average path velocity (VAP) (μm/s)				
Pre-freeze	28.8 ± 6.0	56.7 ± 8.1	+111.0 ± 88.5	<0.001
Post-thaw	11.5 ± 3.7	18.5 ± 5.9	+67.6 ± 45.8	<0.001
Percentage change[a]	–58.5 ± 15.3	–66.7 ± 10.7		0.05
Sperm viability (%)				
Pre-freeze	89.4 ± 3.1	93.1 ± 4.3	+4.1 ± 3.7	0.007
Post-thaw	35.1 ± 5.1	36.9 ± 5.1	+6.1 ± 14.3	NS
Percentage change[a]	–60.7 ± 5.8	–60.3 ± 5.2		NS
Acrosome integrity (%)				
Pre-freeze	89.3 ± 5.9	88.2 ± 7.7	–1.1 ± 8.1	NS
Post-thaw	66.5 ± 9.2	72.1 ± 7.2	10.1 ± 17.5	0.04
Percentage change[a]	–25.5 ± 9.6	–17.9 ± 9.7		0.03

[a]Calculated as mean of individual changes. $P < 0.05$ was considered significant.
NS = not significant.

Intact ejaculated spermatozoa may be damaged by oxygen radicals emanating from seminal leukocytes and damaged spermatozoa (Aitken and Clarkson, 1988), so that the stress caused by the cryopreservation procedure itself may add to damage inflicted already by free oxygen radicals. It is not known if the processes of freezing and thawing damage all spermatozoa equally, or if there is selective damage to a more susceptible population such as those suffering lipid peroxidation. If damage is selective, removing intact spermatozoa immediately from potentially damaging leukocytes and damaged spermatozoa may improve sperm survival after cryopreservation.

The swim-up procedure eliminates the seminal plasma and immotile and dead spermatozoa along with exfoliated epithelial cells, cellular debris, leukocytes and amorphous material. Spermatozoa selected by the swim-up method in an experimental model perform better in the sperm penetration assay (SPA) than untreated specimens (Russell and Rogers, 1987).

In our study, all ejaculates had normal pre-freeze sperm characteristics. Cryopreservation shifted these characteristics in both control and swim-up specimens into the subnormal or sub-optimal range, as showed by decreased sperm motility and viability. However, specimens processed by swim-up from a washed sperm preparation before freezing had better post-thaw motion characteristics (VSL, VCL, VAP, and ALH). Spermatozoa separated by the SpermPrep method (Zavos *et al.*, 1991) had higher percentages of motile cells and the subjective motility ratings after the freeze–thaw process were better than the same characteristics in spermatozoa from the same ejaculates frozen in semen, even though cells still suffered damage. It was found (Kobayashi *et al.*, 1991) that post-thaw motility was higher in samples concentrated by Percoll centrifugation before freezing. Another study (Pérez-Sánchez *et al.*, 1994) demonstrated that spermatozoa selected by the swim-up technique, post-thaw, contained more morphologically normal forms, fewer totally degenerated spermatozoa with damaged head and tail membranes, and more progressively motile spermatozoa with greater velocities and amplitudes of lateral head displacement than did untreated frozen–thawed semen. However, the proportion of damaged viable cells was

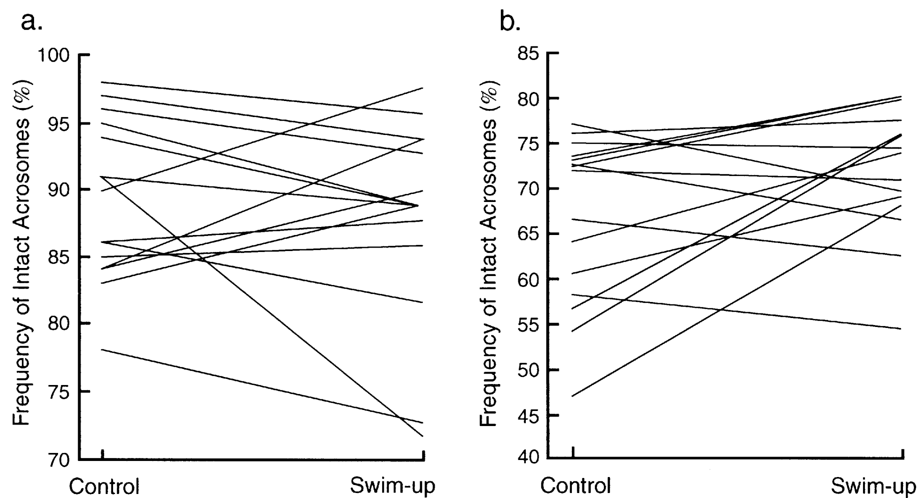

Figure 1. Effect of swim-up on acrosome integrity before freezing and after thawing. (**a**) The effect of sperm processing by swim-up on acrosome integrity before freezing was not uniform among the 15 donors. The frequency of intact acrosomes increased in the swim-up processed aliquots in six individuals, and the opposite occurred in the remaining nine. (**b**) The effect of pre-freeze sperm processing by swim-up on acrosome integrity after thawing also was not uniform. The frequency of intact acrosomes increased in nine individuals and decreased in six.

Table II. Effects of pre-freeze sperm processing by swim-up on post-thaw calcium-ionophore induced acrosome reaction in 15 normozoospermic men

Frequency of live spermatozoa with intact acrosome (%)	Control group (mean ± SD)	Swim-up group (mean ± SD)	Percentage change[a] (mean ± SD)	P-value[b]
Before ionophore challenge	66.5 ± 9.2	72.1 ± 7.2	10.1 ± 17.5	0.04
After ionophore challenge	55.1 ± 9.9	52.1 ± 10.6	–3.6 ± 21.6	NS
Percentage change[a]	–17.1 ± 11.2	–27.6 ± 13.2	–	0.003
P-value[c]	< 0.001	< 0.001		–

[a]Calculated as mean of individual changes.
[b]$P < 0.05$ was considered significant between control and swim-up groups.
[c]$P < 0.05$ was considered significant between the frequency of live spermatozoa with intact acrosome before and after ionophore challenge.
NS = not significant.

identical in both groups. Specimens prepared by Percoll separation techniques before freezing retained motility longer than did controls, although comparison with pre-freeze values revealed that selected and unselected spermatozoa lost viability from the freeze-thaw process to the same extent (Sharma and Agarwal, 1996).

In our study, although swim-up separation before freezing resulted in a lower total and motile sperm count compared to the control specimens, the results of motile sperm count after thawing were comparable between the control and swim-up specimens. The optimum concentration of total motile spermatozoa that can result in successful fertilization and pregnancy in IUI varies from 1 to 5×10^6. However, pregnancies have been achieved with as few as 0.1×10^6 motile spermatozoa (Berg *et al.*, 1997). Greater sperm concentration does not result in a corresponding increase in fecundity rates (Ombelet *et al.*, 1995).

Our results, as well as those obtained previously (Pérez-Sánchez *et al.*, 1994; Sharma and Agarwal, 1996), show that any improvements in sperm motion characteristics after selection by a swim-up or Percoll procedure do not seem to be a consequence of the selected spermatozoa being more resistant to the freeze–thaw process. First, the selected spermatozoa had higher absolute changes in some sperm characteristics after cryopreservation because they had higher initial values for motility, rather than better resistance to cryo-injury. Second, both sperm preparations (control and selected) had significant loss of viability after thawing, and the proportion of viable cells damaged was identical in both groups. The post-thaw swim-up group was not included in our study, since there are reports of its use in the literature (Graczykowski and Siegel, 1991). These authors reported low recovery rates after swim-up from cryopreserved specimens, as well as decreased motion characteristics. In a previous study, we also compared the effectiveness of swim-up to recover motile spermatozoa in fresh and cryopreserved specimens. Our results indicated that swim-up provides similar yields of spermatozoa from cryopreserved specimens (recovery rate: 13%; range: 9.7–29.4) compared to fresh ones (22%; range: 11–43; not significant) in normozoospermic individuals, although sperm motion characteristics and acrosome status were significantly lower in the former (Esteves *et al.*, 1999). In addition, cryopreserved

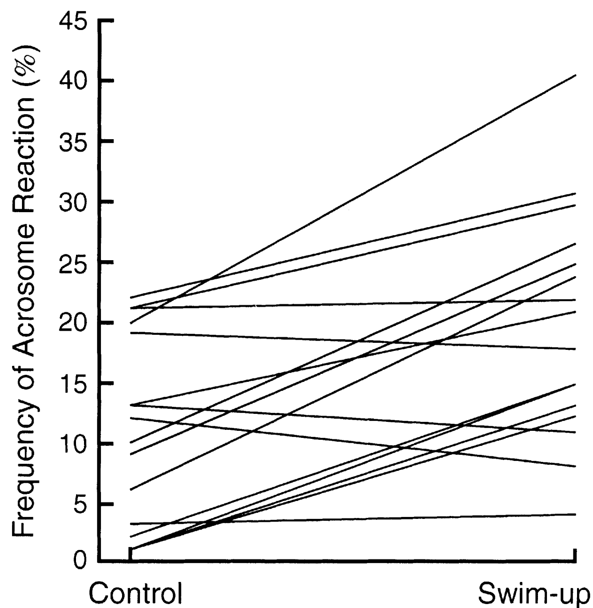

Figure 2. Effects of pre-freeze sperm processing by swim-up on post-thaw acrosome reaction to ionophore challenge (ARIC) (see text).

spermatozoa are known to be more fragile than fresh spermatozoa. Swim-up washing after thawing takes >1.5 h (thawing takes 20 min, removal of cryomedia takes ~20 min, and swim-up takes another 60 min) compared with <30 min in our protocol.

On the other hand, we found that specimens processed by swim-up from a washed sperm preparation before freezing had higher percentages of intact acrosomes after thawing. This effect cannot be explained by higher initial values for acrosome integrity, because pre-freeze acrosome integrity was similar in both sperm preparations. Along the same lines, the percentage of cryopreserved spermatozoa with intact acrosomes that were able to undergo AR in response to calcium ionophore was significantly higher in swim-up specimens, and these findings are relative changes rather than absolute changes. To our knowledge, this is the first study to report a beneficial effect of pre-freeze swim-up processing on post-thaw acrosome status. The reason for this is unclear, but we speculate that the highly motile sperm population selected by swim-up may not be affected by oxygen radicals emanating from damaged leukocytes and from other spermatozoa susceptible to or damaged by lipid peroxidation. To support this hypothesis, a recent study from our laboratory has demonstrated that ejaculates frozen with antioxidant-supplemented cryoprotectant had improved ability to respond to calcium ionophore post-thaw (Esteves *et al.*, 1998). In a study using transmission electron microscopy, it was demonstrated (Barthelemy *et al.*, 1990) that the deleterious effects of cryopreservation on sperm acrosomes were less evident if spermatozoa had been frozen without seminal plasma. These investigators postulated that the buffering effects of semen vary among individuals, and washing spermatozoa eliminates the important individual variable of seminal plasma.

Only spermatozoa with normal intact acrosomes can undergo AR, which is essential for the spermatozoa to penetrate the zona pellucida and to fuse with the oolemma. Also, the AR must occur at the correct time for the spermatozoa to be able to penetrate the zona pellucida (ZP). The correct timing of AR is ensured by physiological AR inducers in the cumulus oophorus and in the ZP (Tesarik *et al.*, 1992). A normal sperm sample should contain a high proportion of spermatozoa with intact acrosomes capable of undergoing AR after incubation in capacitating conditions. Therefore, the difference between the percentage of intact acrosomes after ionophore treatment and the baseline value is important (Takahashi *et al.*, 1992). There is now sufficient evidence that specialized sperm function tests, such as the AR, are better predictors of the fertility potential than traditional semen characteristics (Takahashi *et al.*, 1992; Henkel *et al.*, 1993; Calvo *et al.*, 1994; Yovich *et al.*, 1994). Using an AR to ionophore challenge (ARIC) cut-off at 10%, the sensitivity of the test in predicting subfertility was 54% with a positive predictive value of 64% and specificity of 85% (Cummins *et al.*, 1991). Patients with ARIC scores above 10% have consistently better fertilization rates than those with lower scores (Henkel *et al.*, 1993; Calvo *et al.*, 1994). In our study, ARIC scores were >10% in 86.7% of the cryopreserved swim-up specimens, and 60% of controls. When a specimen was compared to its own control, swim-up processing improved the ARIC scores in 83% (5/6) of the subjects whose unprocessed specimen scores were <10%. It should be noted, however, that in a previous study (Esteves *et al.*, 1998), the variation in the AR in fresh and cryopreserved specimens from the same individuals was analysed in two different germinative cycles (median interval 5 months; range 5–8 months). It was concluded that acrosome results differ significantly in the same individual at different time intervals.

In conclusion, the swim-up method using a washed sperm preparation before freezing offers the possibility of selecting spermatozoa with better post-thaw motility, acrosome integrity, and ability to undergo AR. These effects may be relevant to cancer patients as well as to donor spermatozoa for use in artificial donor insemination. Although our data were obtained from individuals with normal sperm characteristics, the results are not likely to be substantially different in semen cryopreserved for other reasons, such as before cancer treatments; cancer patients' specimens freeze as effectively as those from normal men (Agarwal *et al.*, 1995). Cancer patients generally have poor semen quality before cryopreservation. However, it has also been shown that 12–60% of men with cancer, depending on the type of disease, present with normal or slightly abnormal sperm parameters at the time of sperm banking (Agarwal *et al.*, 1995; Hallak *et al.*, 1999). Because the yield of spermatozoa obtained by the swim-up procedure is low, oligozoospermic specimens may not be suitable, and swim-up procedures may have to be performed in multiple tubes to maximize the number of motile spermatozoa from a given sample. In addition, the possibility of providing multiple ejaculates can be considered, particularly in cancer patients, who usually have poor pre-freeze semen characteristics.

Whether swim-up processing before freezing also improves fertilization and pregnancy outcomes with assisted reproductive procedures such as IUI or conventional IVF warrants further investigation.

Acknowledgements

Jar-Chi Lee assisted with the statistical analysis of results and Robin Verdi provided secretarial help. This study was supported by the Cleveland Clinic Foundation Research Program Committee (grant no. 5490), Cleveland, Ohio, USA. S.E.E. received financial support from The Cleveland Clinic International Center, Cleveland, Ohio, and FAPESP (Bolsa de Mestrado, Fundação de Amparo à Pesquisa do Estado de São Paulo, Brasil). This study was presented as a Thesis at the University of Campinas School of Medicine (UNICAMP, Brazil) to obtain the title of Master in Surgery.

References

Agarwal, A., Tolentino, M.V. Jr, Sidhu, R.S. *et al.* (1995) Effect of cryopreservation on semen quality in patients with testicular cancer. *Urology*, **46**, 382–389.

Aitken, R.J. and Clarkson, J.S. (1988) Significance of reactive oxygen species in defining the efficacy of sperm preparation techniques. *J. Androl.*, **9**, 367–376.

Aitken, R.J., Buckingham, D.W. and Fang, H.G. (1993) Analysis of the responses of human spermatozoa to A23187 employing a novel technique for assessing the acrosome reaction. *J. Androl.*, **14**, 132–141.

Alvarez, J.G. and Storey, B.T. (1993) Evidence that membrane stress contributes more than lipid peroxidation to sublethal cryodamage in cryopreserved human sperm: glycerols and other polyols as sole cryoprotectant. *J. Androl.*, **14**, 199–208.

Barthelemy, C., Royere, D., Hammahah, S. *et al.* (1990) Ultrastructural changes in membranes and acrosome of human sperm during cryopreservation. *Arch. Androl.*, **25**, 29–40.

Berg, U., Brucker, C. and Berg, F.D. (1997) Effect of motile sperm count after swim-up on outcome of intrauterine insemination. *Fertil. Steril.*, **67**, 747–750.

Berger, T., Marrs, R.P. and Moyler, D.L. (1985) Comparison of techniques for selection of motile spermatozoa. *Fertil. Steril.*, **43**, 268–273.

Brandeis, V.T. and Manuel, M.T. (1993) Effects of four methods of sperm preparation on the motile concentration, morphology, and acrosome status of recovered sperm from normal semen samples. *J. Assist. Reprod. Genet.*, **10**, 409–416.

Calvo, L., Dennison-Lagos, L., Banks, S.M. *et al.* (1994) Acrosome reaction inducibility predicts fertilization success at in-vitro fertilization. *Hum. Reprod.*, **9**, 1880–1886.

Critser, J.K., Arneson, B.W., Aaker, D.V. *et al.* (1987) Cryopreservation of human spermatozoa. II. Post-thaw chronology of motility and zona-free hamster ova penetration. *Fertil. Steril.*, **47**, 980.

Cross, N.L., Morales, P., Overstreet, J.W. *et al.* (1986) Two simple methods for detecting acrosome-reacted human sperm. *Gamete Res.*, **15**, 213–226.

Cummins, J.M., Pember, S.M., Jequier, A.M. *et al.* (1991) A test of the human sperm acrosome reaction following ionophore challenge. Relationship to fertility and other seminal parameters. *J. Androl.*, **12**, 98–103.

Esteves, S.C., Sharma, R.K., Thomas, A.J. Jr *et al.* (1996) Suitability of the hypoosmotic swelling test to assess viability of cryopreserved sperm. *Fertil. Steril.*, **66**, 798–802.

Esteves, S.C., Sharma, R.K., Thomas, A.J. Jr *et al.* (1998) Cryopreservation of human spermatozoa with pentoxifylline improves the post-thaw agonist-induced acrosome reaction. *Hum. Reprod.*, **13**, 3384–3389.

Esteves, S.C., Sharma, R.K., Thomas, A.J. Jr *et al.* (1999) Effectiveness of swim-up technique to recover functionally intact spermatozoa for assisted reproduction. *Fertil. Steril.*, **72**, 94.

Graczykowski, J.W. and Siegel, M.S. (1991) Motile sperm recovery from fresh and frozen–thawed ejaculates using a swim-up procedure. *Fertil. Steril.*, **55**, 841–843.

Hallak, J., Kolettis, P.N., Sekhon, V.S. *et al.* (1999) Cryopreservation of sperm from patients with leukemia. Is it worth the effort? *Cancer*, **85**, 1973–1978.

Henkel, R., Muller, C., Miska, W. *et al.* (1993) Determination of the acrosome reaction in human spermatozoa is predictive of fertilization *in vitro*. *Hum. Reprod.*, **8**, 2128–2132.

Keel, B.A. and Black, J.B. (1980) Reduced motility longevity in thawed human spermatozoa. *Arch. Androl.*, **4**, 213.

Kobayashi, T., Kaneko, S., Hara, I. *et al.* (1991) Concentrating human sperm before cryopreservation. *Andrologia*, **23**, 25–28.

Liu, D.Y. and Baker, H.W.G. (1988) The proportion of human sperm with poor morphology but normal intact acrosomes detected with *Pisum sativum* agglutinin correlates with fertilization *in vitro*. *Fertil. Steril.*, **50**, 288–293.

Mack, S.R. and Zaneveld, L.J. (1987) Acrosomal enzymes and ultrastructure of unfrozen and cryotreated human spermatozoa. *Gamete Res.*, **18**, 375–383.

McLaughlin, E.A., Ford, W.C. and Hull, M.G. (1992) Motility characteristics and membrane integrity of cryopreserved human spermatozoa. *J. Reprod. Fertil.*, **95**, 527–534.

Mortimer, D., Curtis, E.F. and Camenzind, A.R. (1990) Combined use of fluorescent peanut agglutinin lectin and Hoechst 33258 to monitor the acrosomal status and vitality of human spermatozoa. *Hum. Reprod.*, **5**, 99–103.

Ombelet, W., Puttemans, P. and Bosmans, E. (1995) Intrauterine insemination: a first-step procedure in the algorithm of male subfertility treatment. *Hum. Reprod.*, **10**, 90.

Parinaud, J., Vieitez, G., Moutaffian, H. *et al.* (1995) Variations in spontaneous and induced acrosome reaction: correlation with semen parameters and in-vitro fertilization results. *Hum. Reprod.*, **10**, 2085–2089.

Pérez-Sánchez, F., Cooper, T.G., Yeung, C.H. *et al.* (1994) Improvement in quality of cryopreserved human spermatozoa by swim-up before freezing. *Int. J. Androl.*, **17**, 115–120.

Peterson, E.P., Alexander, N.J. and Moghissi, K.S. (1988) A.I.D. and AIDS – too close for comfort. *Fertil. Steril.*, **49**, 209–210.

Polanski, F.F. and Lamb, E.J. (1988) Do the results of semen analysis predict future fertility? A survival analysis study. *Fertil. Steril.*, **49**, 1059–1065.

Russell, L.D. and Rogers, B.J. (1987) Improvement in the quality and fertilization potential of a human sperm population using the rise technique. *J. Androl.*, **8**, 25–33.

Sharma, R.K. and Agarwal, A. (1996) Sperm quality improvement in cryopreserved human semen. *J. Urol.*, **156**, 1008–1012.

Takahashi, K., Wetzels, A.M.M., Goverde, H.J.M. *et al.* (1992) The kinetics of the acrosome reaction of human spermatozoa and its correlation with *in vitro* fertilization. *Fertil. Steril.*, **57**, 889–894.

Tesarik, J., Mendoza, C. and Carreras, A. (1992) Effects of phosphodiesterase inhibitors caffeine and pentoxifylline on spontaneous and stimulus-induced acrosome reactions in human sperm. *Fertil. Steril.*, **58**, 1185–1190.

Wang, C., Lee, G.S., Leung, A. *et al.* (1993) Human sperm hyperactivation and acrosome reaction and their relationships to human *in vitro* fertilization. *Fertil. Steril.*, **59**, 1221–1227.

World Health Organization (1999) *WHO Laboratory Manual for the Examination of Human Semen and Semen–Cervical Mucus Interaction*, 4th edn. Cambridge University Press, Cambridge, UK.

Yovich, J.M., Edirisinghe, W.R. and Yovich, J.L. (1994) Use of acrosome reaction to ionophore challenge test in managing patients in an assisted reproduction program: a prospective, double-bind, randomized controlled study. *Fertil. Steril.*, **61**, 902–910.

Zaneveld, L.J., De Jonge, C.J., Anderson, R.A. *et al.* (1991) Human sperm capacitation and the acrosome reaction. *Hum. Reprod.*, **6**, 1265–1274.

Zavos, P.M., Sofikitis, N., Toda, T. *et al.* (1991) Improvements in qualitative characteristics of cryopreserved human spermatozoa following recovery via SpermPrep II filtration method. *Tohoku J. Exp. Med.*, **165**, 283–290.

Received on February 21, 2000; accepted on June 19, 2000