

Comparison of various methods of processing human cryopreserved–thawed semen samples

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We compared the efficacy of various methods of processing cryopreserved–thawed samples for the recovery of functionally adequate spermatozoa as assessed by the response to the sperm stress test (SST), an index of temperature activated sperm membrane lipid peroxidation, and immediate and delayed changes in sperm viability and motion parameters. Donor semen samples ($n = 28$) were cryopreserved–thawed and divided into six equal parts, one part was used as control and the remaining parts were used to compare five methods of sperm processing as follows: direct Percoll gradient processing, washing by one-step or stepwise addition of the washing medium followed by Percoll processing, and washing by one-step or stepwise addition of the washing medium. Additional samples ($n = 10$) were evaluated for the immediate and delayed (6 h at 37°C) impact of one-step and stepwise washing (without Percoll separation). Compared with wash-only methods, samples processed using Percoll had a significantly higher SST score ($P = 0.001$), motility, rapid spermatozoa ($>50 \mu\text{m/s}$), curvilinear velocity and motility index ($P < 0.001$). Comparing various Percoll methods, direct Percoll processing resulted in the highest number of motile spermatozoa recovered ($P < 0.00001$) and a higher SST score based on curvilinear velocity ($P = 0.001$). Stepwise washing gave a significantly higher number of motile spermatozoa ($P < 0.001$) but with a significantly lower SST score based on the concentration of motile spermatozoa ($P = 0.001$), motility ($P = 0.001$) and motility index ($P = 0.01$). Sperm viability and motion parameters after 6 h of incubation showed no difference between one-step and stepwise washing. In conclusion, compared with wash-only methods, Percoll processed samples resulted in the recovery of spermatozoa with superior quality as assessed by SST and motion analysis. One-step washing of the samples gave an overall comparable recovery compared to the samples prepared stepwise. Having significantly higher SST scores, similar viability and the maintenance of motility, one-step

washing may be a better method of processing thawed samples than the stepwise washing.

Key words: cryopreservation/gradient separation/human spermatozoa/sperm stress test/sperm washing

Introduction

Advances in assisted reproduction have significantly increased the use of cryopreserved semen samples. In addition to cryopreserved husband's and donor's ejaculated spermatozoa, requests for cryopreservation of epididymal and testicular spermatozoa have increased dramatically. Recovery of the optimal number of functionally intact spermatozoa from thawed samples has always been a goal of semen cryopreservation programmes. Sperm cryopreservation is known to result in loss of motility and viability which often varies between samples (Beck and Silversten, 1975; Critser *et al.*, 1987; Keel *et al.*, 1987; Centola *et al.*, 1992) and perhaps between the various methods of cryopreservation. In the preparative phase of cryopreservation, cells initially shrink when they are exposed to a hypertonic cryoprotectant; they then return back to near normal volume as the cryoprotectant penetrates. During the freezing phase, cells shrink again as water leaves the cell due to the freezing of extracellular water. Cells must go through the same process when they are thawed (Hammerstedt *et al.*, 1990). Gao *et al.* (1995) found that human spermatozoa can swell to 1.1 times and shrink to 0.75 times their iso-osmotic cell volume while maintaining their normal function. Post-cryopreservation sample osmolality could reach 700–900 mOsmol/kg or higher. Therefore, post-freezing methods of semen processing must address methods of bringing down the osmolality to ~285 mOsmol/kg with minimal effects on sperm motility and viability (Morshedi, 1996).

Methods for preparing cryopreserved samples have varied from a simple thaw with no special preparation technique for intracervical insemination (ICI), a simple wash with or without swim-up, to gradient separation (Percoll or ISolate) of the motile fraction for intrauterine insemination (IUI) or other assisted reproductive techniques. Theoretically, to process a cryopreserved–thawed sample, extreme care must be taken to add the washing medium gradually in order to avoid sudden exposure of sperm cells to a low (though normal) osmolality environment. Osmolar shock to already compromised spermatozoa may cause motility, viability and other functional impairments. However, related reports in the literature are contradictory. Using cryopreserved mouse spermatozoa, Nakagata and Takashima (1992) found no

difference in motility between one-step and stepwise washed samples but a significant decline in the fertilizing ability of spermatozoa washed by the one-step addition of the washing medium. On the other hand, Songsasen *et al.* (1997) reported that mouse cryopreserved–thawed samples prepared by one-step washing resulted in higher blastocyst formation (33%), compared with 17% for the samples processed by stepwise washing. They found no change in the viability of cryopreserved spermatozoa washed by stepwise or one-step addition of the diluting medium. Larson *et al.* (1997) found that, compared with one-step washing, Percoll processing of thawed semen samples resulted in a significantly higher percentage motility, slightly higher percentage normal morphology but significantly lower overall motile sperm recovery. They also reported that Percoll processing of semen samples prior to cryopreservation resulted in samples with superior sperm motion parameters. Ord (1993) recommended the gradual addition of the diluting medium to the thawed samples prior to loading on top of the Percoll layers.

Nevertheless, the question remains of whether a single method of sperm preparation can be found to be suitable for all types of cryopreserved–thawed semen samples. Moreover, it is not clear whether the routine methods of evaluating the quality of sample post-preparation are the most adequate and informative. This confusion is often compounded by the fact that, except in the in-vitro fertilization (IVF) cases, sample evaluation is performed immediately post-processing. In this case, we frequently remain with the question of whether or not there will be differences between methods of sample preparation with regard to the maintenance of sperm vital functions several hours post-insemination.

Alvarez and Storey (1985) reported that the rate of lipid peroxidation in rabbit and mouse spermatozoa is increased in a high temperature environment. Alvarez *et al.* (1996) also found that the rate of motility loss of human spermatozoa was correlated with the rate of endogenous lipid peroxidation at a temperature of 24–40°C. Incubation of spermatozoa in 40°C to activate lipid peroxidation (sperm stress test, SST) has been used to predict IVF outcome (Alvarez *et al.*, 1996).

The purpose of this study was to use the SST to evaluate the quality of different sperm preparation methods for cryopreserved–thawed human semen samples. The sperm preparation methods that were compared in this study included: stepwise washing, by gradual addition of the washing medium to the thawed semen; one-step washing, by the addition of the medium at once; Percoll processing, by direct addition of the thawed sample on top of Percoll gradients; stepwise washing followed by overlaying of the washed sample on top of the Percoll gradients and one-step washing of the sample followed by overlaying it on top of the Percoll gradients. We postulated that the changes in sperm motion parameters after each processing method and after the exposure to SST conditions could be used as simple indicators for the selection of an efficacious method for preparing cryopreserved semen samples. The delayed effects of the change in osmotic environment on sperm

viability and motion parameters due to the slow or quick addition of the washing medium were also investigated.

Materials and methods

Semen samples

These studies were performed upon approval of the Institutional Review Board of Eastern Virginia Medical School, USA. Cryopreserved semen samples ($n = 38$) from six fertile donors who had fathered a child within 1 year were studied. The donors were asked to collect samples by masturbation into sterile specimen cups following 48–72 h of abstinence. Following complete liquefaction at room temperature, all fresh samples were analysed for sperm concentration and motion parameters by computer-assisted semen analysis (CASA; Cell Soft, Cryoresources, NY, USA). Sperm concentration, percentage motility, mean curvilinear and straightline velocities (VCL, VSL), linearity ($\text{LIN} = \text{VSL}/\text{VCL} \times 10$) and the motility index ($\text{VCL} \times \text{percentage motility}$) were determined. Setting parameters and the definition of measured sperm motion parameters for the CASA have been published elsewhere (Oehninger *et al.*, 1990). All semen samples had a sperm concentration of $\geq 60 \times 10^6/\text{ml}$, motility of $\geq 60\%$, $\geq 10\%$ normal morphology by strict criteria (Kruger *et al.*, 1987), $\leq 1 \times 10^6/\text{ml}$ round cells, and had been screened to exclude urogenital tract infections and anti-sperm antibodies.

Cryopreservation–thawing methods

After initial semen analysis, each sample was slowly and gently mixed with the freezing medium containing TES-Tris-citrate, 12% glycerol and 20% egg yolk (Irvine Scientific; Santa Ana, CA, USA) (Morshedi *et al.*, 1995). The volume ratio of semen to the freezing medium was 1:1. The mixture was divided into aliquots in 2 ml cryo vials and refrigerated at 1–8°C for 30 min to allow equilibration. The vials were then transferred onto a freezing tray and placed 5 cm above the liquid nitrogen for 30 min before they were placed on an aluminium cane and plunged into liquid nitrogen. After a minimum of 180 days of storage, the samples were thawed at 42°C for 3 min (quick thaw) and evaluated for basic sperm motion parameters using CASA.

Sperm preparation methods

To obtain an adequate amount of thawed samples for all procedures, a minimum of three vials (~3 ml) from each ejaculate were thawed, pooled and divided into six equal parts and processed as follows: (i) stepwise washing (SW), one part of the sample (i.e. 0.5 ml) was gradually diluted with three times the volume of Ham's F-10 (Gibco; Grand Island, NY, USA) containing 0.3% human serum albumin (HSA, Irvine Scientific) at 100 μl increments and mixed gently each time a portion of the medium was added. The sample was then divided into aliquots in 2–3 tubes and centrifuged at 370 g for 10 min. The supernatants were aspirated with a pipette and the pellets were resuspended in 0.5 ml of Ham's F-10 and reanalysed for sperm motion parameters. (ii) One-step washing (OW), one part of the sample was diluted at once (one-step) by addition of Ham's F-10 and 0.3% HSA as in SW. The sample was then centrifuged, resuspended and analysed as in SW. In both washing methods (SW and OW) the final result was a ratio of 1:3 of the cryopreserved semen:diluent. (iii) Percoll processing (P), discontinuous Percoll gradients (Sigma, St Louis, MO, USA) were prepared by overlaying 0.5 ml of 90% isotonic Percoll with 0.5 ml of 40% isotonic Percoll in Ham's F-10 with 0.3% HSA. One part of the sample was directly added on top of



Figure 1. Study design. SST = sperm stress test.

the Percoll layers and centrifuged at 300 *g* for 20 min. Spermatozoa from the 90% layer were retrieved, washed once with Ham's F-10 and 0.3% HSA, resuspended and analysed as in SW. (iv) Stepwise washing followed by overlaying on Percoll (SW/P), one part of the sample was diluted with the washing medium as in SW and the resulting mixture was centrifuged as in SW. The resulting resuspended pellet was added on top of 40:90% Percoll gradients and processed as in the Percoll processing method (P). (v) One-step washing followed by overlaying on Percoll (OW/P), one part of the sample was diluted and processed as in OW. The resulting resuspended pellet was added on top of 40:90% Percoll gradients and processed as in the Percoll processing method (P). (vi) Unprocessed control (C), one part of the sample was kept unprocessed at room temperature.

Sperm stress test (SST)

Aliquots of 100 μ l from each sperm preparation method and the control were transferred into 2 ml cryotubes and incubated in shaking water bath at 40° C for 4 h (Alvarez *et al.*, 1996). After incubation, the sperm samples were allowed to cool down to room temperature for 5 min and the motion parameters were reanalysed. The stress test scores were calculated by the ratio of the post-incubation over pre-incubation of percentage motilities, concentration motile spermatozoa, percentage rapid spermatozoa (50 μ m/s), VCL, VSL, mean linearity and the motility index.

Experimental design

Experiment 1

After thawing, 28 semen samples from the six selected donors were analysed for sperm concentration and motion parameters as described (post-thaw analysis). Each sample was then divided into six equal parts and processed as described previously followed by determination of sperm motion parameters and the SST (post-processing) (Figure 1). For all post-thawing dilution steps of

experiment 1 and 2, each sample was divided into equal portions and sperm concentration and motion parameters were determined to be <5% different from each other. If the measurements were found to be dissimilar, the portions were mixed and divided again followed by a second measurement, or a third, if necessary.

Experiment 2

In addition, assessment of the delayed effects of stepwise and one-step washing (without Percoll processing) was performed using 10 cryopreserved semen samples from the same group of donors. Post-thawing sperm concentration and motion parameters were recorded; sperm viability was also determined prior to processing of each sample. Eosin dye (Sigma, St Louis, USA) at a concentration of 0.5% in phosphate-buffered saline, pH 7.4, was used to assess the viability by counting at least 200 non-motile spermatozoa. One of the portions was diluted (washed) in a stepwise fashion and analysed exactly the same way as in SW. Sperm viability was also determined following the washing. The other portion was washed and analysed as in OW followed by an assessment of the sperm viability. Both portions were then incubated at 37°C in 5% CO₂ in air for up to 6 h. Sperm concentration, motion parameters and viability were determined for both fractions post-incubation (Figure 1).

Statistical analysis

Comparisons between the motion parameters and stress test scores for the different sperm preparation methods were performed by analysis of variance. Comparisons between each sperm preparation method were conducted by a paired Student's *t*-test. Linear regression analyses were used for testing the correlation between concentration of motile spermatozoa and stress test scores in each sperm preparation method. All analyses were performed using StatMost statistical software for Windows. The results are presented as mean \pm SE. *P* < 0.05 was considered to be statistically significant.

Table I. Motion parameters of cryopreserved–thawed semen after different sperm preparation methods

| Preparation methods | Motion parameters | | | | | | |
|---------------------|-----------------------------|--|-----------------------------|-----------------------------|------------------------------|-----------------------------|-----------------------------|
| | Motility (%) | Conc. motile ($\times 10^6/\text{ml}$) | VCL ($\mu\text{m/s}$) | VSL ($\mu\text{m/s}$) | LIN | Rapid sperm (%) | Motility index |
| C | 52.2 \pm 2.9 ^a | 61.5 \pm 6.6 ^a | 41.5 \pm 1.2 ^a | 25.2 \pm 0.9 ^a | 6.04 \pm 0.12 ^a | 35.4 \pm 2.4 ^a | 21.7 \pm 1.4 ^a |
| SW | 41.9 \pm 3.4 ^b | 42.5 \pm 6.7 ^b | 29.8 \pm 1.1 ^b | 15.6 \pm 1.1 ^b | 5.07 \pm 0.18 ^b | 20.9 \pm 1.9 ^b | 12.6 \pm 1.2 ^b |
| OW | 39.6 \pm 3.8 ^b | 37.1 \pm 6.2 ^c | 32.7 \pm 1.2 ^c | 17.9 \pm 1.1 ^b | 5.35 \pm 0.17 ^b | 24.7 \pm 1.9 ^b | 12.9 \pm 1.3 ^b |
| P | 60.2 \pm 3.7 ^c | 30.7 \pm 5.2 ^c | 45.1 \pm 1.3 ^a | 28.7 \pm 1.4 ^c | 6.28 \pm 0.18 ^a | 42.2 \pm 2.3 ^a | 27.5 \pm 2.1 ^a |
| SW/P | 56.5 \pm 4.0 ^c | 25.6 \pm 4.2 ^d | 47.0 \pm 1.6 ^a | 29.9 \pm 1.8 ^c | 6.26 \pm 0.22 ^a | 44.2 \pm 2.9 ^a | 27.3 \pm 2.5 ^a |
| OW/P | 53.7 \pm 4.3 ^c | 21.5 \pm 2.8 ^d | 46.7 \pm 1.2 ^a | 28.9 \pm 1.3 ^c | 6.13 \pm 0.15 ^a | 43.8 \pm 2.4 ^a | 25.6 \pm 2.4 ^a |

a,b,c,d Statistically significant difference between values with different superscript ($P < 0.05$).

Rapid sperm = % of spermatozoa with velocity $\geq 50 \mu\text{m/s}$.

MI (motility index) = motility \times VCL/100; VCL = curvilinear velocity; VSL = straight line velocity; LIN = linearity; C = unprocessed control; SW = stepwise washing; OW = one-step washing; P = Percoll processing; SW/P = stepwise washing followed by overlaying on Percoll; OW/P = one-step washing followed by overlaying on Percoll.

Results

Experiment 1

Sperm motion parameters after sperm preparation

The sperm motion parameters after various sperm preparation methods are summarized in Table I. There were no differences in percentage motility among the samples directly processed with Percoll (P) or washed and then processed with Percoll (SW/P, OW/P). Although the percentage motility of the Percoll processed samples was significantly higher ($P < 0.001$) than the washed only samples, there were no differences observed among P, SW/P and the OW/P, and between the motility of the samples washed in one-step (OW) or in a stepwise fashion (SW).

The concentration of motile spermatozoa in SW was significantly higher ($P < 0.001$) than in the Percoll processed and SW samples. There were no differences in concentration of motile spermatozoa between P and OW, and between SW/P and OW/P.

Compared with wash only methods, P, SW/P, OW/P and C samples had a significantly higher VCL ($P < 0.001$). There were no differences in VCL among C, P, SW/P, and OW/P. The VCL of the OW samples was significantly better than that of SW at $P < 0.001$.

The VSL of P, SW/P and OW/P were significantly ($P < 0.001$) higher than those of SW or OW. However, there were no differences in VSL among P, SW/P and OW/P, and between SW and OW.

The mean linearity of the samples processed via the P, SW/P and OW/P was significantly higher than those of SW or OW ($P < 0.05$). However, there were no differences in LIN among P, SW/P and OW/P, and between SW and SW.

The percentages of rapid spermatozoa were significantly higher ($P < 0.001$) in P, SW/P and OW/P than in SW or OW. However, there were no differences in rapid spermatozoa among C, P, SW/P and OW/P, and between SW and OW.

The MI were significantly higher ($P < 0.001$) in C, P, SW/P and OW/P than in SW or OW. However, there were no differences among C, P, SW/P and OW/P, and between SW and OW.

The SST scores for each motion parameter from the different sperm preparation methods are summarized in

Table II. The SST scores calculated based on the percentage motility were significantly higher ($P = 0.001$) in samples processed via the P, SW/P and OW/P, than the OW, SW and the C. However, the SST scores for the OW samples were significantly higher ($P = 0.001$) than those of SW or the C. There were no differences in SST scores among P, SW/P and OW/P, and between the C and SW.

The SST scores based on the concentration of motile spermatozoa were significantly higher in P, OW/P, SW/P and OW than in SW or C. There were no differences in SST scores among P, SW/P, OW/P and OW, and between C and SW. The SST scores calculated based on VCL were similar among P, SW and OW but significantly higher than those of SW/P and OW/P, all at $P = 0.0007$.

The SST scores calculated based on VSL were similar among P, SW/P, OW/P, SW and the OW but significantly higher ($P = 0.0006$) than those of C. Similarly, the SST scores calculated based on LIN were similar among P, SW/P, OW/P, SW and the OW but significantly higher ($P = 0.00002$) than those of C.

There were no differences in SST scores calculated based on the percentages of rapid spermatozoa among all sperm preparation methods. The SST scores calculated based on the MI were similar among P, SW/P, OW/P and OW but significantly better ($P = 0.01$) than those of SW or C. The concentration of motile spermatozoa in each vial from various preparation methods did not influence the calculated SST scores.

Experiment 2

In the second phase of the study, the samples processed via the SW gave a significantly higher ($P < 0.03$) number of motile spermatozoa than those processed via the OW. However, the two methods gave similar results for sperm viability and sperm motion characteristics after washing. Furthermore, following the 6 h incubation period at body temperature, viability and motility characteristics were similar for both methods.

Discussion

Cryopreservation–thawing and subsequent processing result in a significant decline in the number of motile and viable

Table II. Stress score* of each motion parameter after different sperm preparation methods

| Preparation methods | Motion parameters | | | | | | |
|---------------------|------------------------------|--|--------------------------------|--------------------------------|--------------------------------|------------------------------|------------------------------|
| | Motility (%) | Conc. motile ($\times 10^6/\text{ml}$) | VCL ($\mu\text{m/s}$) | VSL ($\mu\text{m/s}$) | LIN | Rapid sperm (%) | Motility index |
| C | 0.34 \pm 0.03 ^a | 0.22 \pm 0.02 ^a | 0.85 \pm 0.06 ^{a,b} | 0.67 \pm 0.05 ^a | 0.79 \pm 0.03 ^a | 0.95 \pm 0.36 ^a | 0.29 \pm 0.03 ^a |
| SW | 0.27 \pm 0.05 ^a | 0.22 \pm 0.04 ^a | 1.03 \pm 0.07 ^a | 0.83 \pm 0.08 ^{a,b} | 0.81 \pm 0.05 ^{a,b} | 0.96 \pm 0.17 ^a | 0.30 \pm 0.06 ^a |
| OW | 0.61 \pm 0.07 ^b | 0.52 \pm 0.06 ^b | 0.95 \pm 0.04 ^a | 0.94 \pm 0.08 ^{a,b} | 0.91 \pm 0.04 ^{a,b} | 0.91 \pm 0.10 ^a | 0.59 \pm 0.08 ^b |
| P | 0.77 \pm 0.03 ^c | 0.63 \pm 0.05 ^b | 0.96 \pm 0.03 ^a | 0.87 \pm 0.04 ^b | 0.89 \pm 0.04 ^b | 0.91 \pm 0.07 ^a | 0.73 \pm 0.03 ^b |
| SW/P | 0.76 \pm 0.04 ^c | 0.61 \pm 0.06 ^b | 0.85 \pm 0.04 ^b | 0.80 \pm 0.06 ^b | 0.92 \pm 0.03 ^b | 0.85 \pm 0.13 ^a | 0.67 \pm 0.06 ^b |
| OW/P | 0.77 \pm 0.05 ^c | 0.56 \pm 0.05 ^b | 0.86 \pm 0.03 ^b | 0.82 \pm 0.05 ^b | 0.93 \pm 0.05 ^b | 0.74 \pm 0.07 ^a | 0.68 \pm 0.06 ^b |

*Ratio of post-incubation motion parameter and the same parameter pre-incubation.

^{a,b,c}Statistically significant difference between values with different superscript ($P < 0.05$).

Rapid sperm = % of spermatozoa with velocity $\geq 50 \mu\text{m/s}$.

MI (motility index) = motility \times VCL/100; VCL = curvilinear velocity; VSL = straight line velocity; LIN = linearity; C = unprocessed control; SW = stepwise washing; OW = one-step washing; P = Percoll processing; SW/P = stepwise washing followed by overlaying on Percoll; OW/P = one-step washing followed by overlaying on Percoll.

spermatozoa. There is evidence that cryopreservation may enhance lipid peroxidation mediated by the loss of a peroxidative enzyme, superoxide dismutase, during the freezing–thawing process (Alvarez *et al.*, 1987). It has been demonstrated that the exposure of human spermatozoa to different reactive oxygen species (ROS) results in a marked decrease in various sperm functions (Aitken and Clarkson, 1988; Aitken *et al.*, 1989; Alvarez and Storey, 1992; Agarwal *et al.*, 1994; Oehninger *et al.*, 1995). Consequently, during cryopreservation and thawing spermatozoa may acquire increased susceptibility to the peroxidative damage. Aitken and Clarkson (1988) evaluated the outcome of different sperm preparation protocols by measurement of ROS generation and the capacity for sperm–oocyte fusion. They found that a subpopulation of spermatozoa characterized by poor motility and impaired fertilizing capacity which is responsible for the generation of high amount of ROS could be isolated on the low density portion of Percoll gradients. This subpopulation appeared to respond to mechanical stimulus by increased ROS production which can impair the functional capacity of normal spermatozoa in the same suspension.

Although the cryopreservation phase needs careful observation for the optimal recovery of functionally normal spermatozoa, the post-thaw processing of semen samples also requires a similar scrutiny. Re-enforcing this is the report by Gilmore *et al.* (1997) indicating that the addition of the cryoprotecting agent causes much less damage to the spermatozoa than removal of the agent (i.e. processing of the sample).

The methods of preparing samples post-cryopreservation could significantly influence the sperm recovery rate and may also add additional stress to the already compromised spermatozoa. Semen samples can also be prepared prior to cryopreservation to have them ready for IUI (Larson *et al.*, 1997). With this method, not only is a sample with superior quality obtained but also the need to wash samples post-thaw for IUI is eliminated. Nevertheless, processing semen samples post-cryopreservation is routinely performed for many assisted reproductive techniques. The ideal sperm preparation technique should be fairly rapid, simple, inexpensive,

and should enable the user to recover the highest number of functionally and structurally normal spermatozoa. This description of an ideal method is often more hope than reality. Routinely, we employ the preparative techniques based on some scientific notions but also often based on what satisfies our immediate needs for the particular settings available. However, we still remain with the basic question of which method is the most suitable for processing cryopreserved semen samples. Should a simple wash of the sample, a swim-up or a gradient separation such as Percoll or ISolate be used? Should the sample be directly transferred on the gradient layers or be diluted/washed prior to the transfer? What criteria should be used to assess the quality of the processed samples prior to insemination?

A cryopreserved–thawed sample has an osmolality that can reach as high as 700–900 mOsmol/kg. To process the sample, a buffer based medium with an osmolality of ~ 285 mOsmol is often used. Scientifically, addition of the medium should be carried out with care and on a gradual basis to avoid the osmotic shock to already compromised spermatozoa. However, there have been several published data of adequate recovery from samples processed by direct transfer onto the gradient layers (Ord, 1993; Larson *et al.*, 1997), or by one-step addition of the diluting medium, disregarding the osmolality factor (Larson *et al.*, 1997; Songsasen *et al.*, 1997). Should the osmolality change be considered as a factor which may compromise the functionality of recovered spermatozoa? Can osmolality shock be used to eliminate a population of already compromised spermatozoa and recover the most adequately functional spermatozoa?

In this study, we compared some of the most common procedures used to process cryopreserved–thawed samples. The study showed that there is a significant loss of motile spermatozoa associated with all of the methods evaluated. Considering the fact that there are peroxidative and reactive elements in the semen which may negatively influence sperm function, preparing cryopreserved samples for IUI readiness may be a better alternative. Studies are underway in our laboratory to answer this question. How these two methods of sperm preparation affect pregnancy outcome also requires further study.

With the outcome parameters measured in this study, the issue of osmolality shock appeared to be less consequential. Although the samples processed by the direct transfer on to Percoll gradients (normal osmolality) had a significantly higher percentage motility, they were comparable in other aspects to those which were washed first and then transferred. In addition, the washing step did not appear to significantly influence any of the parameters measured. Samples washed by the stepwise addition of diluting medium had a significantly higher number of motile spermatozoa. However, the percentage of rapidly motile spermatozoa and other motion parameters measured were similar to those of the samples processed by one-step washing. Combined with the measurement of the degree of lipid peroxidation (SST) and the delayed outcome evaluations (see below), samples processed by one-step addition of the washing medium showed better outcome scores.

Since the loss of motility is associated with the degree of lipid peroxidation activated by high temperature (Alvarez and Storey, 1985; Alvarez *et al.*, 1987, 1996), we used the SST to evaluate which sperm preparation method would offer optimal results for the processing of cryopreserved-thawed semen samples. Compared to SW and OW, Percoll processing, SW/P, and OW/P procedures resulted in the recovery of a lower concentration of motile spermatozoa (which may be due to loss during the preparation process) but of superior quality in other motility parameters. The SST scores based on percentage motility, concentration of motile spermatozoa and the motility index for these procedures were comparable with each other and similar to those of the samples processed via OW. However, the SST scores based on percentage motility and the number of motile spermatozoa for SW samples were significantly lower than those of OW. In no instance were the SST scores of the samples processed as SW higher than those of the OW. It appears therefore, that the separation of the motile fraction to obtain an optimal number of functionally adequate spermatozoa (with the parameters measured) is beneficial. Moreover, the consequential effects of osmolality shock on the response of motile spermatozoa under stress conditions may not be significant.

One of the main questions in the evaluation of the processing methods is how sperm functions are maintained hours post-manipulation. Most outcome measures are carried out immediately or shortly after samples are processed with a particular method. We decided to determine if samples processed via a stepwise or a one-step addition of the washing medium scored differently 6 h after incubation at 37°C (body temperature). Although the samples processed by stepwise addition of the washing medium scored better in regard to the total number of motile spermatozoa, the difference disappeared after the incubation. Evaluation of the immotile fraction of spermatozoa with the viability test indicated no difference between the two methods both immediately prior to and after 6 h of incubation. The results indicate that the samples processed by the two methods give comparable results for the time period studied. Recalling the fact that the one-step processed samples scored better

with the stress test, it appears that this mode of processing may be superior to the stepwise washing. At ambient temperature, others have shown that sperm functions can be adequately preserved in TES/Tris egg yolk buffers supplemented with citrate and fructose (Aitken *et al.*, 1996). Modifications of sperm capacitation conditions in order to affect the plasma membrane cholesterol content have also been suggested to result in an enhancement of sperm fertilizing capacity (Benoff *et al.*, 1996).

In conclusion, gradient separation of the motile sperm fraction by the direct transfer of cryopreserved-thawed samples may be adequate for the types of samples evaluated. Washing cryopreserved-thawed samples by a one-step addition of diluting medium does not appear to compromise the samples compared with stepwise addition of the medium.

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