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Hypo-osmotic Swelling Test and Sperm Motility after Swim-up and Two-layer Percoll Gradient Preparation of Frozen-Thawed Sperm

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ABSTRACT

Objective To evaluate the percentage of motility and hypo-osmotic swelling test (HOST) for frozen-thawed sperm, compare between sperm preparation by washing and swim-up and two-layer Percoll gradient techniques.

Design Experimental study.

Setting Department of Obstetrics and Gynaecology, Faculty of Medicine, Siriraj Hospital, Mahidol University.

Subjects and methods Semen samples from 35 men were cryopreserved by the liquid nitrogen vapor method for six months. Then the frozen-thawed samples were divided into two aliquots, which were separated by washing and swim-up, and two-layer Percoll gradient techniques. The percentage of sperm motility and HOST were evaluated before and after sperm separation by both techniques.

Results Both sperm separation techniques resulted in a significant increase in sperm motility. However, washing and swim-up separated far better sperm motility than two-layer Percoll gradient (80.7 ± 13.1 versus 41.7 ± 19.7 ; $p < 0.001$). The HOST results after washing and swim-up also showed significantly higher percentage than two-layer Percoll gradient techniques (37.4 ± 22.4 versus 19.9 ± 15.7 ; $p < 0.001$), and the percentage of swollen sperm tail after the latter

separation technique was lower than initial post-thaw sperm (19.9 ± 15.7 versus 21.4 ± 6.7 ; $p < 0.05$).

Conclusion Preparation of cryopreserved sperm by washing and swim-up procedure improves sperm motility and sperm tail hypo-osmotic swelling test better than that preparation by two-layer Percoll gradient procedure. However, the HOST do not provide significant information in evaluating the viability of cryopreserved sperm.

Key words : hypo-osmotic swelling test, sperm motility, cryopreservation, swim-up technique, Percoll gradient

Artificial insemination with donor semen has been used in the treatment of male factor infertility for a long time. More recently, concerns regarding the transmission of human immunodeficiency virus (HIV) in semen and evidence of such transmission[1] have made it mandatory to use cryopreserved semen for therapeutic donor insemination. Cryopreservation is associated with reduced sperm survival, motility, and fecundity.[2-4] Recent studies, however, show that cryopreservation does not significantly impair the conception rate[5] and that frozen-thawed spermatozoa can be substituted for fresh spermatozoa for successful in vitro fertilization and embryo transfer (IVF-ET).[6]

During the freezing and thawing cycles, stored human spermatozoa may be subject to structural and functional damage. Cryopreserved sperm often have lower motility percentage and velocity,[7] reduced longevity in the female genital tract,[8] and reduced ability to penetrate zona-free hamster eggs in vitro.[9] Efficient recovery of motile and cryosurvival of spermatozoa is important after cryopreservation because higher numbers of such sperm may increase fecundability. A variety of methods have been used for the preparation of fresh and frozen-thawed sperm in order to select motile sperm fractions, free from seminal plasma, for intrauterine insemination (IUI) and IVF-ET.[6,10,11] In general, cryopreserved sperm are more fragile than nonfrozen sperm, separation techniques must be evaluated for their ability to preserve sperm viability and function. The ability of the human sperm tail to swell in the presence of a hypo-osmotic solution has recently been advocated as an indicator of the membrane integrity of normal functional spermatozoa.[12,13]

The aim of this study is to evaluate the percentage of motility and hypo-osmotic swelling test (HOST) for frozen-thawed human spermatozoa, compare between two most often used sperm preparation techniques : a washing and swim-up, and two-layer Percoll gradient techniques.

Materials and Methods

Semen was obtained from 35 men who attending our infertility clinic during April to August 1995. All samples had normal sperm concentration, motility, and morphology according to the World Health Organization criteria.[14] Semen was allowed to liquefy at room temperature and then was diluted 1 : 1 (vol/vol) with a citrate-egg yolk-glycerol cryopreservative diluent. The diluted samples were drawn into 0.25 ml straws. The straws were placed in the ultracold refrigerator at-0° C for 1 hour, then quickly transferred into the liquid nitrogen container.[15] After cryostorage for six months, semen was thawed at room temperature until ice was no longer visible. The thawed samples were examined for sperm motility and HOST, then divided into two aliquots, which

were separated by washing and swim-up, and two-layer Percoll gradient techniques. After sperm separation, percentage of motility and HOST were evaluated again on spermatozoa prepared by both techniques.

Hypo-osmotic swelling test : the test was performed with 0.1 ml of thawed semen mixed with 1 ml of hypo-osmotic, mixed solution of fructose and sodium citrate.[12] Following incubation at 37° C for 30 min, the spermatozoa were examined under phase-contrast microscopy for the percentage of swollen tails.

Washing and swim-up : 1 ml of thawed semen was resuspended in 3 ml of human tubal fluid (HTF) medium supplemented with 10% human serum albumin. After centrifugation for 10 min at 300 xg, the supernatant was removed and the pellet was overlayered with 0.5 ml of HTF medium. The sample incubated at 37° C for 60 min, then the supernatant was aspirated for further analysis.

Two-layer Percoll gradient : a layer of 2 ml of 40% Percoll (Sigma ; St. Louis, MO, USA) was layered over 2 ml of 80% Percoll in a 15-ml centrifuge tube. One milliliter of thawed semen was layered over this gradient, and then centrifuged for 20 min at 500xg. The semen and 40% layers were pipetted off and discarded. The 80% layer was washed twice with 3 ml of HTF medium followed by centrifugation for 10 min at 250xg. The final pellet was resuspended in 0.5 ml of HTF medium, and sperm motility and HOST was evaluated.

Statistical analysis was performed by using the Mann-Whitney U-test, and analysis of variance (ANOVA) when appropriate.

Table 1. Semen characteristics before and after freezing*

Semen characteristics	Before freezing	After freezing
Spem concentration (x 10^6/ml)	81.0 ± 36.6	64.1 ± 30.0
Motility (%)	65.0 ± 10.3	28.7 ± 13.0
Motile concentration (x 10^6/ml)	52.7 ± 25.6	18.4 ± 20.7
Hypo-osmotic swelling test (%)	64.1 ± 11.6	21.4 ± 6.7

*Values are means ± standard deviation

Table 2. Recovery of motile sperm and hypo-osmotic swelling test following separation of cryopreserved semen*

	Motility (%)	HOST (%)
Thawed semen before wash	28.7 ± 13.0	21.4 ± 6.7
Washing and swim-up	80.7 ± 13.1**	37.4 ± 22.4**
Two-layer Percoll gradient	41.7 ± 19.7	19.9 ± 15.7

*Values are means ± standard deviation

**Significantly different from two-layer Percoll gradient ; $p < 0.001$

Results

Table 1 shows semen characteristics before and after freezing of 35 samples from the male partners with the mean age of 33 years (range 26-42). The post-thaw survival rate of the spermatozoa was 44.2%.

Recovery of motile sperm and the result of HOST following sperm separation by washing and swim-up, and two-layer Percoll gradient techniques are shown in Table 2. In comparison with the sperm motility before processing (28.7 ± 13.0), both separation techniques resulted in a significant increase in sperm motility ($p < 0.001$). However, washing and swim-up separated far better sperm motility than two-layer Percoll gradient (80.7 ± 13.1 versus 41.7 ± 19.7 ; $p < 0.001$). The HOST results showed that the percentage of swollen spermatozoa after washing and swim-up was significantly higher than initial thawed semen ($p < 0.01$) and after two-layer Percoll gradient ($p < 0.001$). Anyhow, the HOST result after two-layer Percoll gradient was lower than initial thawed semen (19.9 ± 15.7 versus 21.4 ± 6.7 ; $p < 0.05$).

Discussion

The fertilizing capacity of frozen-thawed sperm is generally found to be lower than that of fresh sperm in human insemination.[16] Although a variety of sperm isolation techniques have been used successfully with fresh sperm, little comparative data exists on their effectiveness with cryopreserved sperm. Byrd and co-workers[11] has demonstrated that Percoll density gradient separation results in more motile and morphologically normal sperm than simple washing and Sephadex columns techniques, but lower pregnancy rate after IUI. In this study, the motility and HOST of post-thaw sperm after washing and swim-up showed significantly higher percentage than that separated by two-layer Percoll gradient technique. This is in contrary to fresh semen that two-layer Percoll gradient separated sperm with higher percentage of motility and HOST.[17-19] The poor sperm motility after two-layer Percoll gradient separation may, in part, due to the method of washing of cryoprotective media before layering over the 40% Percoll layer. Post-thaw motility of sperm is an important parameter linked to favorable outcome after artificial insemination.[20] The present study demonstrated that after cryopreservation there was significant decreases in the percentage of sperm motility, and markedly improved by washing and swim-up preparation. The hypo-osmotic swelling test of frozen-thawed sperm was also significantly lower when compared to that of fresh sperm. This may be due to osmotic effects and intracellular ice formation during the process of cryopreservation that leads to cellular damage to the sperm. The washing and swim-up procedure seemed slightly to improve the percentage of sperm tail swelling, but no improvement at all after separation by two-layer Percoll gradient. The present data showed that the hypo-osmotic swelling test could not provide predictive information regarding the ability of the sperm after the cryopreservation process. This is consistent with the results of previous studies.[21-23]

In conclusion, preparation of cryopreserved sperm by washing and swim-up procedure improves sperm motility and sperm tail hypo-osmotic swelling test better than that preparation by two-layer Percoll gradient procedure. However, the HOST do not provide significant information in evaluating the viability of cryopreserved sperm. The result attributes warrant consideration of the washing and swim-up method for preparation of cryopreserved sperm.

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