

## EFFECT OF AN INTERMEDIATE HOLD WITH VAPOR FREEZING ON SUBSEQUENT HYPOOSMOTIC SWELLING IN THAWED SPERM

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Cryopreservation and thawing of sperm exerts an adverse effect on functional integrity of the sperm membrane as measured by the hypoosmotic swelling (HOS) test. Supercooling using the liquid nitrogen (LN<sub>2</sub>) vapor technique may damage membranes by favoring ice crystal formation. Slight slowing of the cooling process may allow escape of intracellular fluid. This study was conducted to evaluate modification of the LN<sub>2</sub> vapor technique by employing an intermediate hold in a freezer (to slow down the rate of cooling) on HOS scores on specimens thawed 1 month after freezing. Semen samples were obtained from male partners of infertile couples with a requirement of a baseline HOS score <70% but ≥60%. The HOS test was performed on the unprepared semen sample prior to freezing and immediately post-thaw 1 month later on the aliquot frozen with LN<sub>2</sub> vapors only vs. the equal fraction subjected to an intermediate hold. The mean initial HOS score was 68.5% and was 47% in thawed specimens that had been cryopreserved with and without an intermediate hold. There were no differences in the percentage of specimens exhibiting a >50% HOS score following vapor freeze (70%) or vapor freeze with a hold (74%). Thus, these data do not demonstrate any advantage of slowing the vapor freezing process by utilizing an intermediate hold.

**Keywords** hypoosmotic swelling test, vapor freezing, ice crystals, sperm, HOS

The hypoosmotic swelling (HOS) test is used to evaluate the functional integrity of the sperm membrane [5]. When a male consistently scores <50% when tested for HOS changes, pregnancies rarely ensue in vivo [2] or in vitro [1]. In previous studies cryopreservation and thawing also adversely effected the HOS score [3, 4]. One study of semen specimens taken from patients with Hodgkin's disease or testicular tumors prior to treatment found that the specimen of patients whose pre-freeze values were within the normal range for HOS scores (a mean of 62 ± 14%) showed reduction to a subnormal mean of 33 ± 13% following freezing with liquid nitrogen (LN<sub>2</sub>) vapors, but was not as low using a computerized slow-staged freezing process (39 ± 10%) [8]. Another study using fertile donors and vapor freezing was com-

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pared to a technique using a commercial semiprogrammable freezer. Although this study found significantly lower mean HOS scores with the former ( $64 \pm 3\%$ ) compared to the latter ( $68 \pm 3\%$ ) [7], the clinical significance of this difference is not apparent. Also, commercial rate controlled freezers are expensive and thus not available to all centers.

The study was conducted in an attempt to improve the HOS scores above vapor freezing by holding the sperm first in a  $-20^{\circ}\text{C}$  freezer prior to placing the specimen into  $\text{LN}_2$  vapors.

## MATERIALS AND METHODS

The study included patients presenting to the Cooper Institute for Reproductive Hormonal Disorders for infertility; 27 males with baseline semen specimens having an HOS score of  $\geq 60\%$  but  $< 70\%$  were included. However, the HOS score on the day of testing was the one recorded for the study so the men were not eliminated if the HOS score was  $\geq 70\%$  or  $< 60\%$  on the test day. The 27 semen samples were divided into two equal aliquots. Semen samples were diluted in a 1:1 ratio with test yolk buffer with glycerol, which was gradually added over a 10-min interval while being mixed on a rotator. Aliquot A was placed into  $\text{LN}_2$  vapors in 1.5-mL screw top vials for 30 min and then plunged into  $\text{LN}_2$ . Aliquot B was first placed into a  $-20^{\circ}\text{C}$  freezer for 20 min, then  $\text{LN}_2$  vapors for 10 min, and finally plunged into  $\text{LN}_2$ .

The HOS test was performed as described by Jeyendran et al. [5]. The initial prediluted specimen was tested. Repeat HOS testing was performed on the two thawed aliquots 30 days later (thawing occurred in a  $37^{\circ}\text{C}$  water bath for 5 min).

Student *t* tests were used to compare the mean HOS scores of the two specimens and chi-square analysis was used to compare the frequency of HOS score  $\geq 50\%$  post-thaw. A *p* value of  $< .05$  was considered significant.

## RESULTS

The initial mean HOS scores on the pre-freeze samples were  $69 \pm 16\%$ . The result on the post-thaw aliquot A (vapor- $\text{LN}_2$ ) was  $47 \pm 11\%$  compared to  $47 \pm 12\%$  for aliquot B (freezer-vapor- $\text{LN}_2$ ) ( $p = 1.0$ ). The HOS scores were  $\geq 50\%$  in 19 of 27 (70%) specimens frozen first in vapors vs. 20 of 27 (74%) using the freezer before vapors (chi-square,  $p = \text{NS}$ ).

## DISCUSSION

There are some data suggesting that cellular damage to the sperm membrane may occur by intracellular ice formation if the cooling rate is too fast by not allowing sufficient time for enough intracellular water to diffuse extracellularly [6, 7]. However, too slow of a cooling rate may cause sperm damage by "solution effects." The theory is that excessive intracellular water diffuses out of the cells osmotically and then freezes. This produces very high extracellular and intracellular solution concentrations resulting in extreme cell shrinkage [6].

One study of donor quality sperm compared vapor freezing to a commercial semi-programmable freezer and the results showed good preservation of HOS scores (65% in the former and 68% in the latter) [7]. The results of another study comparing vapor freezing to computerized slow-staged freezing of semen from men with testicular tumors or Hodgkin's disease prior to therapy showed that the latter preserved the HOS score better than the former in both men with apparently normal semen (39% vs. 33%) and men with abnormal semen (29% vs. 24%), but both were considerably lower than the initial pre-freeze specimens of 62% and 45% [8].

We purposely selected men whose HOS scores were not initially below 50% because these sperm are probably substantially subfertile prior to freezing. An attempt was made to select males whose HOS scores were closer to the "normal semen" group in the study by Ragni et al. [8]. The mean HOS scores on the preselected group was 64%, but was 69% on the day of freezing. Though slowing the cooling process first using the freezer prior to LN<sub>2</sub> vapors did not improve the HOS scores, both techniques resulted in post-thaw HOS score >50% in ≥70% of the samples (even though the mean HOS score was <50%). There have not been studies as yet to determine if an HOS score <50% subsequent to freeze/thawing has the same clinical significance as a poor HOS score on a fresh ejaculate.

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