EFFECT OF SHORTENED EXPOSURE TIME TO THE CRITICAL PERIOD FOR ICE CRYSTAL FORMATION ON SUBSEQUENT POST-THAW SEMEN PARAMETERS FROM CRYOPRESERVED SPERM

M. L. CHECK
D. J. CHECK

Cooper Center for Reproductive Endocrinology, Marlton, New Jersey, USA

J. H. CHECK

The University of Medicine and Dentistry of New Jersey,
Robert Wood Johnson Medical School at Camden, Cooper Hospital
and University Medical Center, Camden, New Jersey, USA

R. LONG
M. PRESS

Cooper Center for Reproductive Endocrinology, Marlton, New Jersey, USA

Cryopreservation of human sperm using present methods leads to a reduced fertility potential of the specimen. In many instances this prevents the successful fertilization of the female partner from the frozen–thawed specimens of males whose semen has been cryopreserved prior to surgery, chemo-therapy, or even vasectomy. Furthermore, even though some donor specimens can be successfully used for achieving pregnancies, one needs to place the sperm intrauterine to approach the same pregnancy rates as those of fresh intracervical insemination. The main mechanism considered for sperm damage by cryopreservation is ice crystal formation. The most critical time for forming ice crystals is from 0 to −10°C. In the present study the effect of a modified rapid cryopreservation technique with reduction of exposure time to the 0 to −10°C temperature range was compared to standard freezing procedures on subsequent semen parameters. Though no significant differences were found on post-thaw motile densities or hypoosmotic swelling test scores, a new, equally effective, but more rapid technique for cryopreservation is reported.

Keywords: ice crystal formation, cryopreservation, semen, hypoosmotic swelling test, sperm, thaw

There have been studies suggesting very low pregnancy results following insemination of cryopreserved thawed semen specimens in the female partners of men whose sperm was frozen prior to treatment of the male partners, therapies that could destroy the intratesticular

Address correspondence to Jerome H. Check, 7447 Old York Road, Melrose Park, PA 19126.

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sperm population (e.g., testicular surgery, chemotherapy, radiation therapy for cancer or prior to vasectomy). In one study, only one pregnancy resulted in 21 couples inseminated with sperm from such men [6]. Even when using "donor quality" sperm, most centers report a reduced monthly pregnancy rate following intracervical insemination with frozen sperm in the 5–12% range [2, 11] compared to the higher rates reported for fresh donor insemination of 9 to 27% [1, 7, 8, 9]. For example, the study by Smith et al. [11] demonstrated a monthly fecundity rate of 20% fresh vs 8% frozen; Brown et al. [2] found 27% fresh vs 10% frozen; and Richter et al. [9] found 19% fresh vs 5% frozen.

Though reduced motile densities (MD) are usually found as compared to baseline levels, generally the thawed specimens have adequate MDs that would not necessarily explain the lowered success. A possible explanation for the decreased fertility potential of the cryopreserved sperm may be related to damage to the functional integrity of the sperm membrane as evidenced by decreased hypoosmotic swelling (HOS) test scores, even in specimens where MDs $\geq 10 \times 10^6$/mL were maintained [5].

In commercial semen banks, there is a marked variation in mean HOS test scores with one center's mean score at 57.7% (subfertile level <50%), but with mean levels for two other centers at 19% and 23.3% [4]. No apparent significant variation in method of freezing could be determined for the better center, except their selection of men with better quality sperm. It was suggested that their low HOS test scores may be secondary to ice crystal damage to the membrane. It was thought that most of the ice crystal formation occurs between 0 and –10°C.

The present study evaluated the effect of a more rapid freezing technique which limits exposure of the sperm to the theoretical critical temperature range of 0 to –10°C.

MATERIALS AND METHODS

Evaluation of the Semen (Pre-Freeze). The specimen was allowed to liquify at 37°C for 30 min. Three aliquots were removed from the specimen, including two seven lambda portions, one to measure viability and the other to evaluate the count and motility of the specimen. The remaining aliquot was 100 lambda, mixed on a 1:10 ratio with HOS reagent, and then evaluated 0.5 h later. The specimen was also tested for volume and sperm motility, which was graded on an A, B, C scale. The specimens were then split into two separate but equal portions and prepared for freezing.

Standard Freezing Methods. One 5-mL vial of test yolk buffer (TYB) was allowed to thaw at 37°C, until it reached that temperature. It was then slowly mixed on a 1:1 ratio with the specimen in 15-mL conical vial over a period of 5 min. The specimen was then pipetted into 1-mL Nunc vials, which were labeled and marked for storage. The Nunc vials were then placed in 37°C water in a 500-mL beaker and placed into a 0°C environment for 90 min. At the end of this stage, the specimens were removed, and placed in a "wheel," a device that suspended the vials approximately 1 in. above the liquid nitrogen (LN_2) surface, and were left suspended there for 60 min. After this, the specimens were placed in metal canes for storage, utilizing a cardboard outer sleeve for protection, then plunged into the LN_2. Thawing was performed by simply holding the specimen in the hand for 5 min.

Rapid Freezing Techniques. The TYB was allowed to liquify and was mixed with the specimen in the same manner as described in the standard freezing process. After labeling, the Nunc vials were then placed in a 60-mL isopropyl alcohol bath (in a 100-mL beaker), then placed in a freezer set at –20°C for 22 min. The specimen was then removed from the alcohol bath and placed in a "wheel," again suspending the vials 1 in. above the LN_2 surface. At the end of this, the specimens were placed

in canes, covered, then plunged into the LN_2. Thawing was achieved by removing the specimens from the LN_2, holding it in the hand for 30 s, then placing it in a 40°C water bath for 3 min. After thawing, postcryopreservation MDs and HOS tests were performed on all specimens in the same manner as previously described.

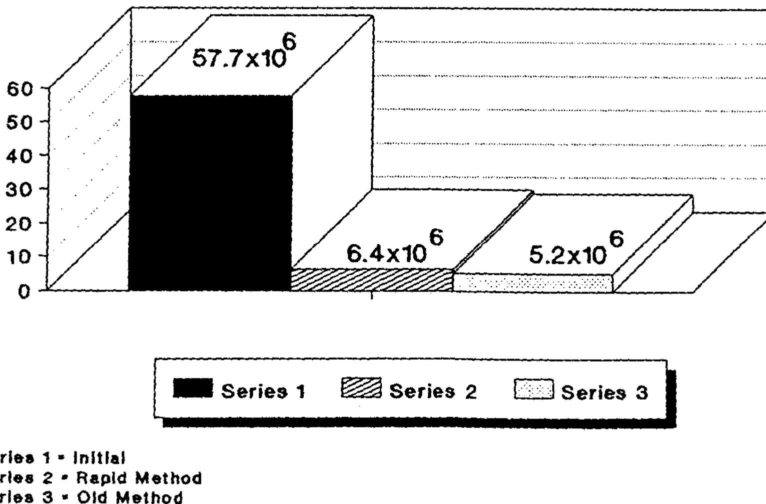
Statistical Analysis. Comparison to determine a statistically significant difference between samples with rapid and standard cryopreservation technique was performed using the paired t test, with a .05 level of significance.

RESULTS

The initial precryopreservation mean MD was 57.7×10^6 (Fig. 1). After post-thaw testing, the mean MD fell to a level of 5.2×10^6 with the standard cryopreservation method, and 6.4×10^6 with the rapid freezing technique. The precryopreservation HOS test score was 69.5% compared to 36% with the standard technique versus 35% with rapid freezing (p = NS) (Fig. 2). Following thawing 27 of 56 (48.2%) males with the rapid procedure vs 25 of 56 (44.6%) with standard freezing demonstrated HOS test scores >50% (p = NS).

DISCUSSION

In one study, timed intrauterine insemination (IUI) was performed using cryopreserved donor sperm; 20 pregnancies resulted from 82 inseminations (24% per attempt), with 19 of 35 (55%) women conceiving [10]. In a group of patients with poor successes with intracervical insemination (ICI) of frozen–thawed sperm (only 3.3% success), IUI using cryopreserved sperm (but apparently from another bank) resulted in a 37% success per attempt. The question is whether IUI can overcome the defect induced by freezing (possibly the sperm loses longevity of fertilization potential, e.g., early induction of acrosome reaction), or has the commercial



Series 1 - Initial
Series 2 - Rapid Method
Series 3 - Old Method

FIGURE 1 Comparison of motile densities following cryopreservation with rapid vs. standard methods.

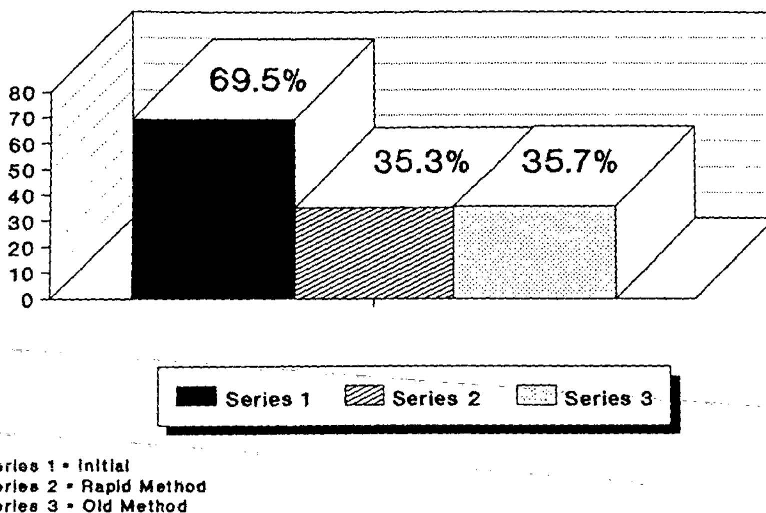


FIGURE 2 Comparison of hypoosmotic swelling tests following cryopreservation with rapid vs. standard methods.

center used by Silva (guaranteeing 24 × 10^6 total motile sperm) found an improved method of freezing.

The mean MDs of either the standard or rapid freezing methods were below the level of 10 × 10^6/mL considered normal by WHO. However, the means were in the 5 to <10 × 10^6/mL range and we recently demonstrated that an 81% 6-month pregnancy rate could be achieved with intercourse alone if a female factor was identified and corrected, even if the MDs were between 5 and <10 × 10^6/mL [3]. The MDs reported in our study are probably falsely low since they were evaluated immediately after thawing. Subsequently, we found that if the specimen is first incubated at 37°C for 10 min prior to evaluation, the MDs are usually increased from 70 to 100%. Rapid freezing did not improve the semen parameters of MD or HOS test scores. However, a distinct advantage of the new technique is that it saves 1.5 h of time. Both techniques allowed close to 50% of the semen tested to maintain an HOS score greater than 50%.

There is a high rate of decreased HOS test scores of cryopreserved sperm, but not a high rate of poor fertilization in an in vitro fertilization (IVF) program. It is possible that techniques such as IVF or IUI, not requiring longevity of fertilization potential, can overcome the fertility problem associated with decreased functional integrity of the sperm membrane. However, poor HOS scores following freezing may not have the same significance as poor HOS scores on fresh sperm, since we recently found that, of various semen parameters evaluated, HOS < 50% was the best predictor of poor IVF outcome. Some preliminary data in our IVF lab have suggested that despite no reduction in fertilization or cleavage rate with sperm with low HOS scores, pregnancy rates are considerably reduced.

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