

Rapid Cryopreservation technique using test-yolk buffer to the standard procedure / semen parameters

ML Check, JH Check, DJ Check, M Press, R Long

The University of Medicine / Dentistry of New Jersey, Robert Wood Johnson Medical School at Camden, Cooper Hospital/University Medical Center, Department of Obstetrics/Gynecology Division of Reproductive Endocrinology / Infertility, Camden, NJ, USA

abstract

Though many pregnancies have been established with frozen-thawed sperm, it is generally believed that the pregnancy rate is reduced compared to fresh sperm. One study found a 25% pregnancy rate with intracervical placement of fresh, vs. only 1% with frozen sperm, vs 11% with intrauterine placement of frozen-thawed sperm. Previously, we found a high rate of subnormal hypo-osmotic swelling test (HOS) scores (< 50%) following freeze-thawing using a standard technique (TEST-Yolk Buffer, Irvine Scientific). It is possible that sperm membrane damage may occur by ice crystal formation, especially during the critical 0° to -10°C stage. It was suggested that reducing the length of time of exposure of the sperm during the initial cool down time might lessen ice crystal formations. Thus, the standard procedure was modified such that although the same buffers and cryoprotectants were employed differences with the new method include:

- a. adding TEST-Yolk Buffer(TYB) all at once with the new vs a drop at a time with the old
- b. placing the specimen in a 70% isopropyl alcohol bath in a 20°C freezer for only 21 minutes with the new vs. a water bath in a 4°C refrigerator for 90 minutes with the old
- c. a reduced time in the nitrogen vapors with only 12 minutes exposure with new vs. 1 h with the old.

The initial samples (n=56) were divided into 2 aliquots and they were both frozen with new and old techniques. Baseline pre-freeze count, motility, and HOS test were performed on each of the 56 specimens. The same semen parameters were then repeated on thawed specimens 3-4 weeks later.

Statistical comparison between samples with old and new techniques were made using paired t-test with a 0.05 level of significance. The mean initial motile density ($\times 10^6/\text{ml}$) was 58 which reduced to 5.2 with old versus 6.4 with new (p=NS). The initial mean HOS score was 70% compared to 36% with old and 35% with new (p=NS). Following thawing there were 27 of 56(48%) maintaining the HOS above 50% vs 25 of 56 (47%) with the old(p=NS). Thus, the new procedure did not provide any beneficial change in sperm quality. However, since the length of time of freezing is considerably reduced (by approximately 1.5 h), the new procedure may be preferable.

Introduction

There have been studies suggesting very poor pregnancy results following insemination of cryopreserved thawed semen specimens in the female partners of men whose sperm was frozen prior to male therapies that could destroy the intratesticular sperm population (eg testicular surgery or chemo or radiation therapy for cancer) or prior to vasectomy. In one study, there was only 1 pregnancy in 21 couples inseminated with sperm from such men (Friedman / Broder, 1981).

Even using "donor quality" sperm, most centers report a reduced monthly pregnancy rate with frozen sperm in the 5-12% range (Smith et al, 1981; Brown et al, 1988) compared to the higher rates reported for fresh donor sperm of 9 to 27% (Richter et al, 1984; Bordson et al, 1986; Glezerman, 1981; Kossoy et al, 1988). For example, the study by Smith et al demonstrated a monthly fecundity rate of 20% fresh vs 8% frozen; Brown et al - 27% fresh vs 10% frozen; and Richter - 19% fresh vs 5% frozen.

Though reduced motile densities (MD) are usually found as compared to baseline levels, generally the thawed specimens have adequate MD's 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 hypo-osmotic swelling test (HOS) scores even in specimens where MD's $\geq 10 \times 10^6$ /ml were maintained (ML Check / JH Check 1991).

In commercial semen banks, there is a marked variation in mean HOS scores with one center's mean score at 57.7% (subfertile level $< 50\%$ Check et al, 1989) but with mean levels for 2 other centers at 19% and 23.3% (ML Check et al, 1991). 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 scores may be secondary to ice crystal damage to the membrane. It was thought that most of the ice crystal formation occurs between 0 to -10°C .

The present study was conducted to evaluate the new speeded-up freezing technique, especially during 0 to -10° cooling stage; and the possible improvement of HOS scores and perhaps sperm motility.

Hypothesis

It has been assumed that the majority of damage suffered by the sperm membrane damage is caused by ice crystal formation, especially during the critical 0 to -10°C stage. By reducing the length of time of exposure of the sperm during initial cool down, we hypothesized that this step may lessen ice crystal damage. This may be manifested by improvement in hypo-osmotic swelling test scores.

Materials / Methods

1. **Evaluation of the semen (Pre-Freeze):** The specimen was allowed to liquify at 37°C for 30 minutes. Three aliquots of semen were removed from the specimen; two seven lambda portions, one which measured viability, the other 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 the sperm motility was graded on an A, B, C scale. The specimens were then split into two separate, but equal portions, and prepared for freezing.

2. **Standard Freezing Method:** One 5ml 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 a 15ml conical vial over a period of 5 minutes. The specimen was then pipetted into 1ml Nunc vials, which were labeled and marked for storage. The Nunc vials were then placed into a 37°C water bath (in a 500ml beaker) and placed into a 0°C environment for 90 minutes. At the end of this stage, the specimens were removed, and placed into a "wheel", a device which suspended the vials above the LN₂ surface. The specimens were approximately 1 inch above the LN₂ surface, and suspended there for 60 minutes. After this, the specimens are placed into metal canes for storage, utilizing a cardboard outer sleeve for protection, then plunged into the LN₂. Thawing was performed by simply holding the specimen within the hand for 5 minutes.

3. **Rapid Freezing Technique:** 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 into a 60ml isopropyl alcohol bath (within a 100 ml beaker), then placed into a freezer set at -20°C for 22 minutes. The specimen was then removed from the alcohol bath, and placed into a "wheel", suspending the vials 1 inch above the LN₂ surface. At the end of this, the specimens were placed into canes, covered, then plunged into the LN₂. Thawing was achieved by removing the specimens from the LN₂, simply holding it in the hand for 30 seconds, then placing it into a 40°C water bath for three minutes. After thawing, post-cryopreservation motile densities and HOS tests were performed on all specimens in the same manner as previously described.

Results

The initial pre-cryopreservation mean motile density was 57.7×10^6 (Figure 1). After post thaw testing, the mean motile density fell to a level of 5.2×10^6 with the old cryopreservation method, and 6.4×10^6 with the rapid freezing technique. The pre-cryopreservation HOS score was 69.5% (Figure 2). There were no differences in post-thaw HOS scores between standard and rapid cryopreservation methods. The mean scores dropped with both techniques below the clinically significant level of 50%, but still 48.2% and 44.6% of specimens frozen with rapid and standard techniques surpassed the critical 50% level.

Discussion

In one study, timed IUI was performed using cryopreserved donor sperm. 20 pregnancies resulted from 82 inseminations (24% per attempt) with 19 of 35 (55%) women conceiving (Silva, 1989). 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 eg early induction of acrosome reaction) or has the commercial center used by Silva (quarantining 24×10^6 total motile sperm) found and improved method of freezing.

The mean motile densities 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 $< 10 \times 10^6$ /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 MD's were between 5 to $< 10 \times 10^6$ ml (Check et al, 1991). The motile densities 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 minutes prior to evaluation, the MD's are usually increased from 70-100%. Rapid freezing did not improve the semen parameters of motile density of HOS scores. However, a distinct advantage of the new technique is that it saves 1 1/2 hours of time. We were pleased to see that both techniques allowed close to 50% of the semen tested to maintain an HOS score over 50%.

There is a high rate of decreased HOS scores of cryopreserved sperm, but not a high rate of fertilization in an 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 HOS $< 50\%$ was the best predictor of poor IVF outcome of various semen parameters evaluated. It is hoped that we can evaluate the HOS scores on specimens from Silva's commercial source, to compare not just percentage motility, but quality (%A, B, and C sperm).

If high MD's and HOS scores apparent high pregnancy rates are confirmed using Silva's commercial source, then it becomes important to determine if the improved specimens are related to superior selection of donors, or from the use of a superior cryopreservation technique. If their technique is indeed better than others, those methods could be applied to patients needing to cryopreserve their sperm prior to treatments which cause sperm damage for future use.

Conclusion

The use of a rapid freezing process did not produce any significant improvement in motile densities or hypo-osmotic swelling test scores compared to standard procedure. The procedure, however, was shortened by 1.5 h without sacrificing quality. Continued research to find ways to lessen sperm damage by freezing is needed in order to improve the fertility

potential. It is believed that as part of the assessment of any new procedure, an HOS test should be performed.

References

- Bordson BL, Ricci E, Dickey RP, Dunaway H, Taylor SN, Curole DN(1986). Comparison of fecundability with fresh and frozen semen in therapeutic donor insemination. *Fertil Steril* 46:466-469.
- Brown CA, Boone WR, Shapiro SS(1988). Improved cryopreserved semen fecundability in an alternating fresh-frozen artificial insemination program. *Fertil Steril* 50:825-827
- Check ML, Check JH, Long R(1991). Detrimental effects of cryopreservation on the structural and functional integrity of the sperm membrane. *Arch Androl* 27:155-160
- Check ML, Check JH(1991). Poor hypo-osmotic swelling test results from cryopreserved sperm despite preservation of sperm motility. *Arch Androl* 26:37-41
- Friedman S, Broder S(1981). Homologous artificial insemination after long-term semen cryopreservation. *Fertil Steril* 35:321-324.
- Glezerman M(1981). Two hundred and seventy cases of artificial donor insemination: Management and results. *Fertile Steril* 35:180-187.
- Kosoy LR, Hill GA, Herbert CM, Brodie BL, Dalglish CS, Dupont WD, Wentz AC(1988). Therapeutic donor insemination: the impact of insemination timing with the aid of a urinary luteinizing hormone immunoassay. *Fertil Steril* 49:1026-1029.
- Richter MA, Haning RV Jr, Shapiro SS(1984). Artificial donor insemination: fresh versus frozen semen: the patient as her own control. *Fertil Steril* 41:277-280.
- Smith KD, Rodriguez-Rigau LJ, Steinberger E(1981). The influence of ovulatory dysfunction and timing of insemination donor (AID) with fresh or cryopreserved semen. *Fertil Steril* 36:496-502.