Clinical freeze preservation of whole human testicular tissue: the practicality of pre-freeze in vitro culture (IVC) to optimize post-thaw sperm motility

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Abstract
The clinical effectiveness of intracytoplasmic sperm injection (ICSI) has enabled testicular sperm processing and freezing to be a fertility preservation option for men. Our ART Lab has strived to simplify the pre-freeze and post-thaw processing procedures needed by Embryologists to isolate viable testicular sperm, while optimizing fertilization and pregnancy outcomes using in vitro culture (IVC) of testicular tissue at 30°C.

Objective: Effective and practical testicular tissue freeze preservation.

Methodology: Over a 2-year period, 40 adult men (24 to 62 years old; 84% vasovasostomy cases) were scheduled to have testicular tissue frozen at the California Cryobank. A retrospective analysis was performed to demonstrate the efficacy of our whole tissue freeze preservation/IVC methodology for cryobanking purposes. We assessed sperm motility patterns for up to 1 week and compared pre-freeze and post-thaw total and progressive sperm motility patterns, contrasting differences by ANOVA (p<0.05).

Results: Total and progressive motility of fresh sperm was elevated by +48 hours IVC, increasing to a peak mean of 52.1% at 96 hours IVC and progressive motility was sustained at 1 week IVC at levels similar to +48 hours. The delayed cryopreservation of testicular tissue after +48 hours IVC, yielded overt motility post-thaw in all cases following a brief equilibration period of the dispersed tubular contents at 37°C.

Conclusion: Pre-freeze IVC and whole biopsy freeze preservation of testicular tissue is a highly effective approach, insuring optimal post-thaw outcomes for future intracytoplasmic sperm injection use.
Labs to enhance motility [13,16], reducing the time and labor needed to find viable sperm. However, there is still no guarantee that a sufficient number of motile sperm will be isolated in a timely manner to inject all the mature oocytes under optimum conditions.

Considering the importance of having viable sperm available for ICSI post-oocyte retrieval, methods aimed to enhance post-thaw motility are critical, if we are to reliably isolate the best quality, viable sperm. One such practice adopted by many laboratories involves the short-term chemical exposure of testicular sperm to Pentoxifylline (30 min incubation) to activate and promote progressive motility [24,25]. Alternatively, no chemical treatment is necessary if a lab adopts an effective IVC strategy to enhance the maturation and motility of testicular sperm prior to freezing [5]. Actively motile testis sperm are less susceptible to cryodamage and complete motility impairment, thus insuring a simpler, less time-consuming process of isolating normal, motile sperm. In addition, the time and labor of pre-freeze and post-thaw processing is reduced by simply processing whole tissue in small motile sperm. In addition, the time and labor of pre-freeze and post-thaw processing is reduced by simply processing whole tissue in small

By +3hr post-TESE at 37°C, a baseline examination of specimen quality was performed on at least 100 sperm to quantitate the presence of motility (classified as the 0hr evaluation). Testicular cell suspensions were analyzed for the Total % motility and categorized by a motility index of Type: I= twitching, II= undulating motion of the flagellum, III= slow progressive motion and IV= rapid movement. Sperm longevity was evaluated daily for up to 1 wk (+168 hours post-TBx). Motility results were assessed daily until overt progressive motility was attained and the incubating TBx pieces were cryopreserved.

**Testicular cryopreservation and thawing**

Whole TBx pieces (approximately 2x2x1mm cubes) were generally cryopreserved at +48 to +96 hours post-TBx, upon confirming the presence of Type III and IV motile sperm (>10%). The TBx pieces resided in the cryovials for IVC in the heated Styrofoam box until freezing. Each vial was diluted in 5 steps (1:1; 100µl/1-2 min step) using Sperm Maintenance Medium (28% glycerol; Irvine Scientific) as a cryoprotectant. Note, an elevated level of cryoprotectant was used to permeate the tissue mass. Five patient vials were placed onto a labeled cane for long-term freeze preservation, while the remaining [sixth] vial was secured on a separate cane for standardized post-thaw testing. The canes containing cryovials were held upright in a tube rack and placed into a refrigerator for pre-freeze cooling. The 14% glycerol solution (1.0 ml final volume) was allowed to permeate the tissue for 3-18hr at 5°C before direct LN2 vapor freezing, and subsequently transfer to long-term LN2, vapor freezing, and finally liquid cryostorage.

Thawing of TEST vials was performed at ambient temperature for 1 min to allow for outgassing of LN2 vapors, then rapidly warmed in a 37°C water bath for an additional 5 minute. The TBx tissue was removed by pipette aspiration and placed directly into a 0.5M sucrose solution at room temperature for elution of glycerol from cellular tissue. After 5 minutes, the TBx tubules were rehydrated in and rinsed twice into fresh Sperm Wash Medium for 5 minute intervals to dilute out the residual extracellular glycerol. The equilibrated tissue was then moved to fresh medium droplets under oil, shread by needles and the cellular contents allowed to warm at 37°C for 1-3 hours. Upon equilibration of the dispersed cells, post-thaw assessment of motility types was again performed and re-evaluated for up to 1 week, under IVC incubation conditions (30°C±0.1°C). The TEST thaw results were recorded on to the Patient's final report, providing valuable information for future ICSI application, as needed.

**Study design and statistics**

Over a 24 month interval between 2014-2015, 40 adult men (24 to 62 years old) were scheduled to have testicular tissue frozen for possible future ICSI use. A retrospective analysis was performed to demonstrate the efficacy of our whole tissue freeze preservation/IVC methodology for cryobanking purposes. We assessed sperm motility patterns for up to 1 week and compared pre-freeze and post-thaw total and progressive sperm motility patterns, contrasting differences by ANOVA and T-tests (p<0.05).

**Results**

Testicular tissue freeze preservation services were provided to men scheduled for either a Vasovasostomy surgery (n=32, 84%) or to assist NOA/Anejaculatory Cancer patients (n=8). Only two of the latter NOA patients failed to yield sperm, thus 95% of the clients had tissue cryopreserved. Total and progressive motility significantly elevated by +48 hours IVC (46.8% and 12.4%) compared to 26.4% and 5.2% at +3 hours and 32.5% and 5% at +24 hours, respectively. Total motility
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Discussion

Testicular freeze-preservation of vasovasotomy patients is essentially an insurance policy to prevent a possible second surgery in case the vasectomy reversal is ineffective and future IVF treatment is required. Whether a TBx involves long-term freeze preservation or short-term ICSI cycle use to mitigate scheduling conflicts and treatment issues, it is important to maintain the active vitality (i.e., motility) of testicular sperm. Weakly motile sperm tend to be susceptible to cryostress, contrary to strong, progressively motile sperm. We have observed the latter phenomenon in this and prior studies [5], observing a downward shift in the initial % post-thaw motility in each movement category. The IVC outcomes of this study are consistent with prior observations [5,8,9]. The more overt the movement of sperm, the easier the task at hand is for the Embryologist diligently assessing the cellular milieu to isolate viable sperm for injection. The timely injection of mature oocytes with motile sperm optimizes potential embryo development and pregnancy outcomes [5,7,11,18]. Overall, excellent conservation of sperm viability was achieved in this study with minimal pre-freeze processing by cryopreserving whole testicular tissue.

Although testicular processing and cryopreservation has been clinically successful for more than two decades [12,17,18], many Embryologist still dread handling and assessing testicular sperm due to its potential labor intensiveness. The IVC of testicular tissue and cryopreservation of whole tissue tubular masses offers an efficacious cryopreservation procedure aimed to optimize pre-freeze and post-thaw motility. It is important to maintain the active vitality (i.e., motility) of testicular spermatozoa.

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peakd at +96 hours (52.1%), while progression elevated (p<0.05) to 20.3% and 24.5% by +96 and 120 hours, respectively, and remained high at +168 hours (20%). Mean pre-freeze total and progressive sperm motilities declined (p<0.05) from 44.4%±2.0%SE and 13.3% to 32.1%±2.2 and 6.7%, respectively, post-thaw. The reduced motility recovery rate of both total (73%) of progressive (50%) movements, are generally lessened by additional overnight IVC considering the median pre-freeze IVC interval was +72 hours. The motility patterns of pre-freeze / equilibrated (3hr, 37°C), post-thaw samples was: I=18.7%/17%; II=12.8%/8.4%; 9.6%/5.1%; and IV=3.7%/1.6%, respectively.
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