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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.

Introduction

Early efforts to diagnostically evaluate testicular tissue and isolate sperm for IVF purposes revealed the possibility of finding motile sperm [1], and successfully fertilizing oocytes by intracytoplasmic sperm injection (ICSI) which generated embryos capable of achieving a pregnancy [2]. Typically, testicular sperm is not motile post-biopsy. As it matures *in vitro*, however, sperm twitching, undulation and forward motility progressively occur [3-5]. The amount and pattern of initial motility may vary between individuals, and predictability is more prevalent in obstructive azoospermia patients. Initially, 5-10% twitching may be observed in a fresh specimen (0-3 hours), with the infrequent occurrence of more active sperm. It has been shown that in vitro culture (IVC) of testicular tissue does promote motility onset and progression [6,7], with an intermediate incubation temperature of 30-32°C optimizing motility formation and longevity [5,8,9]. Although testicular sperm motility is not required to achieve fertilization by fresh sperm post-ICSI [10], it is generally considered preferential for increasing pregnancy potential [11]. The search for motile testis sperm can test the patience of many Embryologists, both in terms of effort, time and eye strain, unless efforts are made to enhance motility. The latter situation is particularly challenging when attempting to select viable cryopreserved sperm post-thaw [8,12].

The delayed use of fresh testicular sperm after +24 hours post-biopsy [13,14] or longer [5] has proven effective in helping coordinate surgical procedures (i.e., anticipating the oocyte retrieval date) and optimizing sperm selection for ICSI. Alternatively, frozen-thawed testicular sperm has also been effectively used for over two decades [5,15,16], resulting in live birth rates similar to fresh testis sperm [5,17,18]. A variety of testicular processing methodologies have evolved to isolate individual sperm from abundant cellular debris [19], including enzymatic digestion [20] and fine mincing [21]/shredding [5]. Most IVF Labs worldwide have adopted a policy of freezing freshly isolated testicular sperm 3 to 24 hours post-biopsy in suspension [22,23] or as whole tubules [12]. One problem with the latter practices is that these fresh testicular sperm possess low levels of twitching to non-progressively motile sperm, which are vulnerable to cryostress. In turn, post-thaw motility is typically extremely low to apparently non-existent (i.e., barely twitching), requiring patience and willingness of Embryologists to extensively search the cellular/sperm suspension at 400X magnification to isolate viable sperm for ICSI. A routine policy of 24 hour incubation post-thaw has been implemented by some IVF

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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 2-6h 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 tubular masses (approximately $2mm^3$), without repetitive sperm washing procedures. Considering the importance of the centrosome of human sperm [26], it is possible that testicular sperm embedded in tissue during freezing are afforded greater protection [5]. In combination with extended IVC (+24-96hr), an efficient testicular biopsy (TBx) cryopreservation protocol can be implemented to eliminate the need to synchronize surgical procedures, while minimizing the time expended to identify, isolate and effectively inject motile sperm into oocytes. The purpose of this study was to document the practicality and overall effectiveness of a testicular tissue freeze preservation practice that incorporates pre-freeze IVC with whole tissue cryopreservation to insure reliable post-thaw motility for future ease of ICSI sperm selection.

Materials and methods

Testicular biopsy (TBx) processing and assessments

Surgically recovered testicular tissue was transported to the California Cryobank in HEPES-buffered HTF medium + 5% HSA (Sperm Wash Medium, Irvine Scientific, Santa Ana, CA) under insulated ambient conditions. Details regarding the processing of TBx samples has been previously detailed [5]. In brief, each TBx sample is typically dissected under stereomicroscopy into 7 smaller tubular clumps of whole tissue using fine needles (27ga-1cc syringes). One TBx piece (i.e., "TEST" sample) was thoroughly dissected (i.e., shred) in one or more 100µl droplets under light oil in a 100mm sterile dish (Falcon 1029) to disperse the cellular contents of tubules. The large dish was placed on a 37°C surface to equilibrate, warm and evaluate after 1-3 hours (i.e., baseline 0hr assessment), before being placed into a Styrofoam box (maintained on a 37-42°C surface) to create an internal incubation temperature of 30°C (acceptable range 29-31°C). The remaining TBx tubular masses were placed into labeled cryovials containing 0.5 ml Sperm Wash Medium containing gentimicin. The vials were securely closed and placed into the Styrofoam box for IVC for subsequent freezing once sufficient progressive motility (>10%) was confirmed upon evaluation of the pre-freeze fresh TEST sample.

The dispersed cellular contents of the TEST sample were subjectively assessed. The degree of spermatogenesis was gauged from high power field (hpf; 400x) determinations and described as: normal = >5 sperm/hpf, moderate = 3-5 sperm /hpf, fair = 1-2 sperm/hpf and low = <1/hpf. Poor spermatogenic activity associated with NOA patients was potentially further defined by 1 sperm/X number of hpf's or simply the total number of sperm found, if any. These poor NOA cases are not the subject of discussion in this paper.

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 LN_2 vapor freezing, and subsequently transfer to long-term LN_2 vapor and finally liquid cryostorage.

Thawing of TEST vials was performed at ambient temperature for 1 min to allow for outgassing of LN_2 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, shred 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±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

peaked 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.

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 milleu 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 approach to managing TBx cases, by simplifying procedural steps and eliminating exhaustive searching for twitching sperm post-thaw. Whole testicular tissue freezing following IVC promoting motility enhancement has proven be a highly effective treatment that reliably sustains post-thaw motility. The availability of progressively motile sperm simplifies valuable Embryology time needed to identify viable sperm for ICSI. Overall, the testicular cryopreservation strategy applied in this study alleviates the need for additional surgeries through the efficient freeze preservation of progressively motile testis sperm.

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