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ORIGINAL ARTICLE

Sperm Biology

Predictors of sperm recovery after cryopreservation in testicular cancer

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Our objective was to identify predictors of improved postthaw semen quality in men with testicular cancer banking sperm for fertility preservation. We reviewed 173 individual semen samples provided by 67 men with testicular germ cell tumor (TGCT) who cryopreserved sperm before gonadotoxic treatment between 1994 and 2010 at our tertiary university medical center. Our main outcomes measures were independent predictors for the greater postthaw total motile count (TMC) in men with TGCT. Men with NSGCT were more likely to be younger ($P < 0.01$) and had high cancer stage (II or III, $P < 0.01$) compared with men with seminoma. In our multiple regression model, NSGCT histology, use of density gradient purification, and fresh TMC > median fresh TMC each had increased odds of a postthaw TMC greater than median postthaw TMC. Interestingly, age, advanced cancer stage (II or III), rapid freezing protocol, and motility enhancer did not show increased odds of improved postthaw TMC in our models. In conclusion, men with TGCT or poor fresh TMC should consider preserving additional vials (at least 15 vials) before oncologic treatment. Density gradient purification should be routinely used to optimize postthaw TMC in men with TGCT. Larger, randomized studies evaluating cancer stage and various cryopreservation techniques are needed to assist in counseling men with TGCT regarding fertility preservation and optimizing cryosurvival.

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INTRODUCTION

Testicular cancer is one of the most common cancers among men 15–44 years old with approximately 8400 cases diagnosed in the United States in 2010 alone.[1] Advances in early diagnosis and treatment have made the disease one of the most curable cancers.[1] With excellent long-term survival and great cure rates for testicular germ cell tumors (TGCT), the impact of cancer therapies on fertility are important quality of life issues for these young men and their partners.[2] Specifically, systemic cytotoxic therapies have a known detrimental impact on dividing germ cells. Many treated men become oligozoospermic or azoospermic and <50% of men reported successful conception without assisted technologies after treatment of TGCT with surgery and/or chemotherapy.[2,3] Therefore, the American Society of Clinical Oncology (ASCO) advocates sperm cryopreservation as an effective method of fertility preservation in young men with cancer.[4] Unfortunately, freezing and thawing of cryopreserved sperm samples has a negative impact on sperm quality and may impact successful assisted reproduction. Men with testicular cancer specifically have worse sperm quality compared to procreative controls and men with other common cancers.[5,6] Men with TGCT have a sperm survival rate of only 44.8% and the lowest odds of having a postthaw TMC above 5 million compared to controls and other cancers.[5]

Therefore, optimizing postcryopreservation sperm recovery through various techniques including freezing methods, motility enhancers, and media is very important and has implications for the level of reproductive interventions needed and associated costs.[7] Unfortunately, there is very little evidence for predictors of optimal postthaw semen parameters among men with testicular cancer. Many studies have found conflicting evidence for the association of TGCT histology (seminoma *vs* nonseminomatous germ cell tumor (NSCGT)) with sperm quality.[5,8–10] In addition, there is limited evidence for the impact of rapid freezing protocols, motility enhancers, and density gradient purification for improvement in postthaw sperm quality for men with testicular cancer.[5,6,11]

In this study, we characterized fresh and thawed cryopreserved semen quality among men with testicular cancer by histological type (seminoma *vs* NSGCT) and identified potential predictors of improved postthaw semen quality in these men. We hypothesize that the histological type of TGCT and use of rapid freezing, motility enhancer, and density gradient purification impact postthaw semen quality.

MATERIALS AND METHODS

We completed a retrospective evaluation of a prospectively maintained database for men undergoing cryopreservation between 1994 and 2010 at The University of Washington Male Fertility Laboratory, following Institutional Review Board approval. These analyses included men who were diagnosed with TGCT and underwent orchiectomy and were undergoing cryopreservation prior to further oncologic treatment.

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Although only one ejaculate was obtained per visit, participants have provided multiple samples for cryopreservation.

Each sample was processed individually by utilizing different protocols based on fresh semen parameter to reduce inter-sample variability and optimize postthaw semen quality.[12] Fresh semen was measured for volume, pH, viscosity, liquefaction, and concentration using a Neubauer phase-contrast hemocytometer. Computerized (Hamilton Thorne IVOS) and manual motility measures were also made. We evaluated semen smears by strict (Tygerberg) morphology, and a differential count of leukocytes and immature germ cells was performed. Semen samples with motility below 25% were evaluated for sperm viability using Trypan Blue. Specimens with normal motility (≥50%, with ≥ 25% progressively motile sperm) and low numbers (<1000 mm^{-3}) of round cells (leukocytes + immature germ cells) were cryopreserved directly without sperm purification. A density gradient method was employed prior to cryopreservation when >1000 round cells per cubic mm were present in semen.

In semen samples with poor motility (<50% motile or <25% progressively motile), spermatozoa were treated with a motility stimulant. The semen sample was diluted and incubated with an equal volume of human tubal fluid (HTF) (Human Tubal Fluid, InVitroCare, Frederick MD, USA) or Ham's F10 (GIBCO), containing 0.5% SSS (Synthetic Serum Supplement, Irvine Scientific©, Santa Ana, CA, USA) and pentoxifylline (7.2 mmol l^{-1} final concentration, Sigma© Chemical Co., St. Louis, MO, USA) with or without 2-deoxyadenosine (2 mmol l^{-1} final concentration, Sigma© Chemical Co.) for 10 min at 37°C. Sperm motility characteristics were re-evaluated within 5 min after treatment. Following treatment, diluted semen was either cryopreserved directly or purified by density gradient.

Density gradient purification was performed using isotonic Percoll (Pharmacia© Uppsala, Sweden; prior to 1997) or PureSperm© (Nidacon, Mölndal, Sweden) suspension columns in 15-ml conical centrifuge tubes. Each column consisted of a discontinuous gradient of 0.75 ml 80% and 1 ml 40% gradient solution diluted in HTF or Ham's F10 medium. Semen, or diluted semen, was carefully layered above the 40% layer, and tubes were centrifuged for 15 min at 244 *g*. The pellet in the 80% layer was centrifugally washed twice in medium for 7 min at 244 *g* to obtain purified sperm for cryopreservation.

Freezing medium (Irvine Scientific©, Santa Ana, CA, USA) was slowly added to the treated or untreated semen or purified sperm preparation according to manufacturer's directions. Either a fast or slow freeze protocol was used. Fast freeze consisted of rapid freezing in liquid nitrogen vapors for 20–30 min, followed by immersion in liquid nitrogen. Slow freezing was accomplished by placing the sample in 37°C water that was allowed to reach 5°C over a time period of 2.5 h. The sample was then placed in vapor for 20–30 min and subsequently plunged in liquid nitrogen. A small portion of the sample was frozen and thawed a minimum of 2 days later, to analyze the motility and recovery of the sperm postcryopreservation ("postthaw").

For each patient, age, testicular cancer diagnosis, histological type, cancer stage, and number of visits and preserved vials were recorded. For each semen sample, total motile count and percentage motility were calculated from semen analysis just prior to freezing ("fresh") and postthaw. The use of rapid or slow freezing protocols, motility enhancers (pentoxifylline [PX], 2- deoxyadenosine [DPA]), special media (HTF, Ham's F10) and density gradient purification was also recorded for each sample.

Variables were summarized by count (%), mean (standard deviation) or median (inter-quartile range [IQR]) by cancer histology (seminoma and NSGCT). Since most subjects had multiple sperm samples (median = 3, IQR = 2–5), we used generalized estimating equation (GEE) with an exchangeable or unstructured covariance matrix for all analyses to account for correlation within subjects. Cryopreservation characteristics were compared with histology using uni-variate GEE regression to evaluate differences by histology type. A multiple GEE regression model was used to predict odds of postthaw TMC > median postthaw TMC from age, fresh TMC (dichotomized at the median), cancer stage (I, II, III), histology, freeze protocol (rapid *vs* slow), motility enhancer and density gradient. All analyses were conducted in R-statistics v. 3.0.3 using two-sided tests evaluated with a 0.05 significance level.

RESULTS

Of 103 testis cancer patients who cryopreserved sperm, 67 men who had data on either fresh TMC, fresh percentage motility, postthaw TMC, or postthaw percentage motility were included in the analysis. A total of 173 samples from these 67 men were analyzed. Twenty men had seminoma and 47 had NSGCT. Sperm cryopreservation characteristics are shown in **Table 1** (patient-level data) and **Table 2** (visit level data). Men with NSGCT were younger when compared with those with seminoma (26 years *vs* 31 years, $P < 0.01$). Additionally, a larger proportion of samples obtained from men with NSGCT were those of stage II or III disease compared to sample obtained from men with seminoma (51% *vs* 15%, $P < 0.01$). There were no statistically significant differences in fresh total motile count (TMC), postthaw TMC, or postthaw percentage motility for the sperm samples obtained from men with seminoma versus NSGCT (**Table 2**).

Table 3 shows results from GEE regression models for predictors of high postthaw TMC (defined as postthaw TMC > median

Table 1: Sperm cryopreservation characteristics by tumor histology (seminoma *vs* NSGCT), patient level data*

	Seminoma (n=20)	*NSGCT (n=47)*	*P*[§]
Mean baseline age in years (s.d.)	30.6 (5.8)	26.4 (5.1)	<0.01
Stages II and III versus I, *n* (%)	3 (15)	24 (51)	<0.01
Median number of visits (IQR)	2 (1.8, 4.2)	2 (2, 3)	NA

*Mean, median, frequency as specified; [§]*t*-test comparing baseline age between groups, Chi-squared test comparing cancer stage. NSGCT: nonseminoma germ cell tumor; s.d.: standard deviation; IQR: inter-quartile range; NA: not available

Table 2: Sperm cryopreservation characteristics by tumor histology (seminoma *vs* NSGCT), visit level data*

	Seminoma (n=58)	*NSGCT (n=115)*	*P*[ψ]
Median number vials (IQR)	4 (2, 6)	3 (2, 5)	0.83
Rapid freeze (%)	37 (64)	76 (66)	0.49
Density gradient, yes (%)	27 (47)	46 (40)	0.55
Motility enhancer, yes (%)**	23 (55)	34 (42)	0.95
HTF, yes (%)	24 (41)	35 (30)	0.90
Ham's F10, yes (%)	18 (31)	44 (38)	0.89
Median fresh TMC (IQR)	26.2 (8.7, 88.5)	33.6 (11, 83.2)	0.57
Mean % fresh motility (s.d.)	47.3 (16.8)	50.6 (20.4)	0.59
Median postthaw TMC	2.6 (1, 7.2)	5.8 (0.9, 19.2)	0.95
Median postthaw ΔTMC	20.3 (6.6, 50)	20.6 (10.2, 57.3)	0.34
Median postthaw % motility	25 (10.5, 38.5)	28 (14, 45)	0.73
Mean postthaw Δ% motility (s.d.)	21.1 (15.4)	21.6 (18.6)	0.80

*Mean, median, frequency as specified; **Pentoxifylline and 2-deoxyadenosine; [ψ]GEE regression model results predicting NSGCT versus seminoma. Missing values, *n*: median number of vials=3; Density gradient=1; Motility enhancer=50; Fresh TMC=3; Fresh % motility=3; Postthaw TMC=45; Postthaw ΔTMC=45; Postthaw % motility=13; Postthaw Δ% motility=16. NSGCT: nonseminoma germ cell tumor; HTF: human tubal fluid; TMC: total motile count; IQR: inter-quartile range; s.d.: standard deviation; GEE: generalized estimating equation

Table 3: Unadjusted and aORs (OR, aOR, respectively) of high postthaw TMC for histology, age, fresh TMC, stage, rapid freeze, motility enhancer, and density gradient purified

Predictor	*OR (95% CI)*	*P*	*aOR (95% CI)*	*P*[ψ]
NSGCT versus seminoma	2.51 (0.93–6.78)	0.07	4.25 (1.06–17)	0.04
Age (years)	0.99 (0.92–1.07)	0.82	1.03 (0.9–1.18)	0.68
High fresh TMC[§]	16.11 (6.29–41.26)	<0.01	24.68 (7.95–76.59)	<0.01
Stage II/III versus I	1.24 (0.49–3.15)	0.66	0.68 (0.23–2.06)	0.50
Rapid versus slow freeze	2.58 (0.98–6.79)	0.05	1.24 (0.34–4.48)	0.75
Motility enhancer	0.71 (0.31–1.63)	0.41	0.65 (0.22–1.88)	0.43
Density gradient	7.38 (2.93–18.54)	<0.01	8.15 (2.51–26.45)	<0.01

[ψ]GEE regression model results predicting postthaw TMC > median (postthaw TMC). There were 109 observations on 49 subjects (15 seminoma). The aORs correspond to a model that included all predictors; [§]Fresh TMC was also dichotomized at the median. NSGCT: nonseminoma germ cell tumor; TMC: total motile count; OR: odds ratio; aOR: adjusted odds ratio; CI: confidence interval; GEE: generalized estimating equation

postthaw TMC). In univariate comparisons with postthaw TMC, high fresh TMC and use of a density gradient were associated with higher postthaw TMC, and histology and rapid versus slow freeze approached statistical significance. In the multiple regression model that included all predictors in **Table 3**, high fresh TMC and use of a density gradient remained statistically significant, and NSGCT histology also achieved statistical significance, where having NSGCT versus seminoma resulted in higher postthaw TMC (OR = 4.25, 95% CI: 1.06–17.0, $P = 0.04$). Age, stage II or III disease, rapid versus slow freezing protocol and use of motility enhancer did not achieve statistical significance in the multiple regression model.

DISCUSSION

Fertility preservation through sperm cryopreservation is an important aspect of pretreatment oncologic management, especially in young men, given the negative impact of specific treatments on semen quality.[4,7] Cryopreservation is particularly important for fertility preservation in TGCT where semen quality is poor compared with other cancer diagnoses and controls.[5,6] TGCT was associated with poorest likelihood of postthaw TMC >5 million among cancer diagnoses and had significantly lower odds of successful intrauterine insemination (IUI) with TMC >5 million.[5] Therefore, in addition to possibly preserving additional vials in patients with TGCT, identifying optimal cryopreservation procedures and predictors of postthaw semen quality are important. In our study, we found several factors associated with improved postthaw TMC in preserved specimens among men with TGCT. NSGCT histology, use of density gradient purification, and greater fresh TMC were all associated with greater postthaw TMC. Interestingly, in this same model, age, advanced cancer stage (II or III), rapid freezing protocol, and motility enhancer were not associated with changes in postthaw TMC.

There are inconsistent findings in the literature regarding TGCT histology (seminoma *vs* NSGCT) and postcryopreservation semen quality. Botchan *et al*.[8] and Fraietta *et al*.[13] found increased sperm concentration, TMC, and percentage motility in men with seminoma compared to NSGCT. However, Hansen *et al*.[9] found decreased TMC in men with seminoma histology. Similarly, previous studies have found conflicting evidence for an association between cancer stage and semen parameters.[14,15] Hallak *et al*.[14] determined that the effects of cryopreservation were no affected by cancer stage while Agarwal *et al*.[15] found that postthaw semen quality declines with higher stage. In the present study, we found that NSGCT histology had increased odds of greater postthaw TMC (OR: 4.3) when compared to seminoma. We did not find an association between cancer stage and improved postthaw TMC in our study. The suspected relationship between TGCT histology and cryosurvival is incompletely understood and may be related to testicular development, Sertoli cell function, or gene and protein expression.[16,17]

TGCT histology would have a limited role, clinically as a predictor of postthaw semen quality since most experts recommend counseling regarding sperm cryopreservation prior to any oncologic treatment including orchiectomy.[4,7] Unfortunately, compliance with this recommendation among clinicians is poor. Although most clinicians agree that sperm banking should be offered before any treatment, only 25%–39% of oncologists actually counseled young male cancer patients about fertility preservation and sperm banking.[18,19] As a result, many testicular cancer patients may not be counseled and appropriately referred for sperm banking until following orchiectomy, yet before gonadotoxic therapies. At large tertiary referral centers such as our institution, many testicular cancer patients undergo orchiectomy at referring institutions prior to sperm banking and subsequent oncologic treatment. With information regarding TGCT histology in these cases, patients with seminoma may be requested to provide additional vials for preservation compared to patients with NSGCT.

There is also very limited research on the effect of various cryopreservation techniques including rapid freezing, use of motility enhancers and density gradient purification on postthaw semen quality in patients diagnosed with TGCT. Rapid freezing protocols are commonly used for sperm cryopreservation and have shown to provide better postthaw motility and cyrosurvival compared to slow freezing in nononcologic controls.[20] Additionally, vitrification, an ultra-rapid freezing method, may offer improved results compared to rapid freezing protocols, although it is not widely available and was not evaluated in our study.[21] In healthy, nononcologic controls, motility enhancers such as PX, an inhibitor of cyclic adenosine monophosphate (cAMP) phosphodiesterases, and DOA, an adenosine analogue, have shown a variable improvement in sperm motility.[22–25] Density gradient purification or centrifugation has also been shown to improve cryosurvival specifically in oligozoospermic, nononcologic controls.[26,27] There is only a single study evaluating artificial motility enhancers, PX and DOA, in testicular cancer patients.[11] There are no previous studies evaluating the use of various freezing protocols or density gradient purification on cryosurvival in testicular cancer. In our study, rapid versus slow freezing and motility enhancers were not associated with the postthaw TMC in TGCT, in contrast to previous literature. However, we did find that use of a density gradient significantly improved the odds of postthaw TMC (OR: 8.2) in men with testicular cancer.

There are several important considerations from our study. Men with oligospermia (TMC <5–10 million) and TGCT (regardless of seminoma of nonseminoma histology) may consider preserving additional vials before oncologic treatment. Additionally, density gradient purification may improve postthaw TMC in men with TGCT, and should be utilized in these men during sperm cryopreservation. Clinically, postthaw TMC and cryosurvival has important implications for assisted reproductive technologies in couples desiring pregnancy. Several studies have found that a postthaw TMC >5–10 million is predictive of successful IUI.[28–30] Therefore, we believe that men with TGCT should cryopreserve a minimum of 15 vials before oncologic treatment. Each vial yields approximately a TMC of 1 million. Preserving 15 vials would offer a couple desiring fertility, two attempts at IUI and would ensure viable sperm for IVF if both IUI attempts failed. Optimizing postthaw TMC and cryosurvival through density

gradient purification may forgo the need for IVF and offer significant cost benefit for couples.[7]

There are several limitations that effect interpretation of our results. This is a single institution retrospective study evaluating several cryopreservation protocols without randomization. Our sample size was limited with fewer patients with seminoma and Grades II and III disease. Finally, we did not assess molecular measures of cryosurvival such as DNA fragmentation index and histone/protamine ratio, which may confound improvements seen with various cryopreservation techniques.[31] Larger, randomized studies assessing the impact of various cryopreservation techniques are needed to optimize postthaw semen quality in men undergoing cryopreservation for testicular cancer. These studies must confirm the lack of an effect of cancer grade, rapid freezing and motility enhancers on sperm recovery.

CONCLUSIONS

We found several factors associated with improved postthaw TMC in preserved specimens among men with TGCT: NSGCT histology, greater fresh TMC, and use of density gradient purification. Men with testicular cancer with seminoma histology or lower fresh TMC (<25–30 million) should consider preserving additional vials before oncologic treatment. Density gradient purification should be considered to optimize postthaw TMC in men with TGCT. Larger, randomized studies evaluating cancer stage and various cryopreservation techniques are needed to confirm our findings and assist in counseling men with TGCT regarding fertility preservation and optimizing cryosurvival.

AUTHOR CONTRIBUTIONS

JMH, DPP, APP, CZ, TJW contributed in the study conception and design. JMH, CV, NAL performed the acquisition of data. JMH, DPP, APP, CZ performed the analysis and interpretation of data. JMH, DPP, CV drafted the manuscript. JMH, DPP, CV, NAL, APP, CZ, CHM, TJW provided critical revision and all authors provided final approval.

COMPETING INTERESTS

All authors declare no competing interests.

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