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Testicular Tissue Cryopreservation in Prepubertal Male Children: An Analysis of Parental Decision-Making

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Abstract

Background—Infertility is an unfortunate treatment-related consequence for some pediatric malignancies as well as some non-malignant conditions treated with stem cell transplant. Unlike pubertal males, prepubertal males cannot produce semen for cryopreservation. This manuscript reports on the acceptability and safety of a multi-institutional protocol for offering testicular tissue cryopreservation to families of prepubertal male children at highest risk for infertility. Data on decision influences, decision-making control, and emotional state when considering this option are described.

Procedure—Prepubertal males facing gonadotoxic therapy were offered testicular cryopreservation. Post-biopsy, patients were followed for acute side effects. In addition, parents and patients were asked to complete questionnaires, whether or not they chose to cryopreserve tissue.

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Results—Seventy-four prepubertal male children were approached. Fifty-seven families (77%) consented to the testicular biopsy; 48 of 57 underwent the procedure. There was one post-operative side effect. Parents who agreed to testicular cryopreservation and those that did not felt in control of this decision. Parents who consented to the biopsy and refusers were not deterred by the experimental nature of the protocol. An important decision-making influence was the risk of the biopsy.

Conclusion—Biopsy and cryopreservation of testicular tissue from prepubertal male children was performed successfully and safely at three institutions. Parents faced with this option at diagnosis can make an informed decision and weigh carefully the risks and benefits. Although asked to make a decision soon after they were given a difficult diagnosis, parents uniformly felt in control of this decision.

Keywords

decision-making; fertility preservation; prepubertal; testicular cryopreservation

INTRODUCTION

Multifaceted treatment regimens for pediatric cancer have led to significant increases in overall survival rates and an increase in pediatric cancer survivors. Estimates indicate that approximately one in 640 young adults in the USA will be a survivor of childhood cancer [1]. Cure, however, often comes at a cost and unfortunately, a consequence of the treatments currently used to treat pediatric cancers (as well as some non-malignant diagnoses) is infertility [2–4]. Pubertal males can preserve fertility by semen cryopreservation. However, for male children who have not gone through puberty, preserving fertility is a challenge. One possible approach to address this issue is the use of cryopreserved testicular tissue [5].

The prepubertal testicle contains spermatogonial stem cells (SSCs) that under the appropriate conditions will eventually generate mature sperm. In rodent models, spermatogonial transplantation has resulted in restored spermatogenesis, and mice have actually reproduced *in vivo*. Researchers have demonstrated that microinjecting a crude suspension of germ cells into immunocompromised mice rendered sterile can restore spermatogenesis and fertility [6,7]. The success of this research in animal models has created an exciting potential opportunity for translation into human clinical practice, particularly for patients who are facing gonadotoxic therapy that will leave them infertile. In an ideal situation, prepubertal male children could freeze testicular tissue at diagnosis prior to any gonadotoxic therapy. When ready to start a family, this cryopreserved tissue could be thawed and the stored germ cells reimplanted into the patient's own testes [8]. Alternatively, cryopreserved testicular germ cells could be matured and expanded *in vitro* to allow fertilization with intracytoplasmic sperm injection [9].

Presently, clinical applications of testicular cryopreservation and SSC culturing in humans are purely experimental. There are several key issues that require further inquiry before testicular cryopreservation procedures can be successfully translated to human clinical practice. One challenge is that germ cells yield a low number of SSCs, as 10^4 germ cells may contain only two stem cells [8]. Therefore, if the use of cryopreserved tissue from a

testicular biopsy is to be successful, methods are needed to increase the number of SSCs available to be subsequently matured *in vitro* or autotransplanted. A critical parameter is to develop appropriate culture conditions for human SSC viability and expansion, based in part on the animal modeling of SSC biology in rodents and non-human primates. In addition, identifying techniques for preventing malignant contamination from reimplanted tissue is imperative. Developing and refining this culture procedure is at the cornerstone of successfully translating this scientific advancement to clinical practice.

The present study explored our experience with testicular tissue cryopreservation at three major medical centers. We examined the acceptability and safety of this innovative fertility preservation technique not only for prepubertal male children with malignant diagnoses but also those with non-malignant conditions facing significant gonadotoxic therapy. Additionally, using data from questionnaires, we explore the decision-making influences, perceived level of personal decision-making control and mood states of those who are offered this fertility preservation opportunity at diagnosis.

METHODS

Utilizing the expertise of an interdisciplinary team, including clinicians, researchers, and andrologists, a research protocol was developed for testicular tissue acquisition, distribution, and storage. After full committee review, the Institutional Review Boards (IRB) at The Children's Hospital of Philadelphia (CHOP), Seattle Children's Hospital and Memorial Sloan Kettering Cancer Center approved the study.

Parents of prepubertal male children with diagnoses at highest risk for treatment-related gonadal damage including high risk neuroblastoma, rhabdomyosarcoma, osteosarcoma, Ewing sarcoma or sarcoma not otherwise specified (NOS) were offered the opportunity for testicular biopsy and cryopreservation. In cases when the final pathology was not known, those with small round blue cells on frozen section of their tumor in the operating room were eligible. Patients preparing for stem cell transplant for treatment of hematological disorders such as aplastic anemia or certain immunodeficiencies were also included. Finally, pubertal males with one of the study eligible diagnoses who attempted to sperm bank, but failed, or were medically unable to bank, were also offered participation in this study. Patients with a coagulopathy, cryptorchidism, or testicular involvement of their tumor were excluded.

The consent process emphasized that the use of cryopreserved testicular tissue in humans to restore fertility is experimental and whether or not the tissue would be clinically useful to their son in the future was not known. Given the limited developmental ability of young male children to understand the concept of fertility, a waiver of assent was approved by the IRB for subjects 12 years or younger.

Once consent was obtained, a testicular biopsy was performed by an urologist during a procedure when the patient was already under general anesthesia for their clinical care, that is, central line placement, bone marrow aspirates/biopsies or primary tumor biopsy. The volume of testis tissue biopsied varied depending on the age of the child and the testis size.

On average it was approximately 80mm^3. A separate operative procedure for the testicular biopsy alone was not permitted per this protocol and the biopsy always occurred before any cancer therapy was initiated.

Half of the biopsy was frozen for the subject's potential future use at the Hospital of the University of Pennsylvania's Penn Fertility Care. Collaborating sites identified local labs for storage. The remainder was used for research to isolate SSCs and determine optimal culturing conditions. Patients were followed for any adverse outcomes, including excessive pain, bleeding, or infection, both intra-operatively and post operatively for a period of 1 week. Two to four weeks later, parents (both those who agreed to the biopsy and those that refused the procedure) were asked to complete a set of questionnaires on factors influencing their decision, on how much control they felt they had over the decision, and on what their emotional state of mind was at the time of the decision.

A study specific questionnaire was administered to parents and patients. This questionnaire was designed to characterize the demographics of the cohort and to explore a series of factors that may have influenced a family's decision to cryopreserve or not. In the original iteration of this questionnaire, factors that may or may not have been endorsed as decision-making influences included: religion, ethics, finances, risks of biopsy procedure, and experimental nature of tissue cryopreservation. In 2011, we expanded the number of factors that may be considered when making the decision to cryopreserve and we added two other instruments to the questionnaire battery, the Decision-Making Control Instrument (DMCI) and the Profile of Mood States (POMS). Prior empirical work supports the reliability and validity of both measures [10,11]. The DMCI is a nine-item self-report measure of strength of control in decision-making. With this questionnaire we aimed to measure the perceived voluntary nature of parents making decisions for their seriously ill children [10]. The POMS is a 30-item self-report measure of mood states. There are six identifiable mood factors, including tension-anxiety, depression-dejection, anger-hostility, fatigue-inertia, vigor-activity, and confusion-bewilderment. These six subscales yield a global estimate of affective state referred to as the Total Mood Disturbance (TMD) score [11]. Our aim with the POMS was to determine whether there was a difference in mood states between parents who chose to cryopreserve from those that did not.

Feasibility and safety of testicular biopsy were summarized using descriptive statistics. Demographics were compared between those who chose to cryopreserve testicular tissue and those that did not, using *t* test for continuous variable such as age and Fisher's exact test for categorical variable such as race. The factors influencing decision-making were also compared between accepters and refusers using Fisher's exact test. Total score and subscales of DMCI and POMS were compared between accepters and refusers using Wilcoxon test.

RESULTS

Beginning in January 2008 at CHOP and then subsequently in April 2012 at Seattle and April 2013 at Memorial, 74 eligible male children were approached for this experimental protocol; 8 of these were from our collaborating sites. Fifty-seven families consented to the testicular biopsy (77%). Forty-eight of 57 actually underwent testicular biopsy during a

surgical procedure that they were already having for their oncology care. Nine patients had either a benign histology or a frozen biopsy not consistent with a study eligible malignancy and therefore never had a testicular biopsy performed. Of the 48 patients who had a biopsy, 7 have died from their disease.

The basic demographics of study subjects by accepters/refusers is shown in Table I. There were no significant differences in age of patient, race, religion, or education level of those parents who chose this procedure for their son and those who refused. Of the subjects who had the procedure, the most common diagnosis was neuroblastoma (18.8%, Table I) and those patients facing stem cell transplant (25%, Table I) for hematologic disease such as aplastic anemia or immunodeficiencies.

Testicular biopsies were successfully obtained on all 48 male children with no intra-operative adverse events. There was one postoperative scrotal cellulitis in a 17-month-old who was being transplanted for an immunodeficiency. He was treated with intravenous antibiotics and the cellulitis resolved. There were no other post-operative infections, and no bleeding issues. Post-biopsy there were no reports of excessive pain documented.

Study questionnaires provided informative data on beliefs, factors influencing the decision to cryopreserve or not, decision-making control, and the emotional state of the parent/patient at the time the decision was made. We collected questionnaire data on 66 parents and/or patients, including subjects that agreed to the biopsy and those that refused. Table II contains the percentage of parents who affirmed a factor was considered very much, somewhat or not at all. Refusers felt more overwhelmed at the time of the decision than those who chose to move forward with the testicular biopsy ($P = 0.0221$). Moreover, refusers were more likely to weigh the risks of the testicular biopsy procedure than those that consented to the procedure ($P = 0.0070$). Both acceptors and refusers were not deterred by the experimental nature of the protocol. Neither groups' decision was influenced significantly by religion, ethics, or finances.

Those that agreed to the testicular biopsy were more likely than refusers to endorse the beliefs that the science of reproductive medicine will advance to a point where tissue will be useful and that the science will be refined in the future so that their son can use the tissue to attempt pregnancy ($P = 0.0022$). Accepters were also more likely to endorse that fertility is important and worth trying to preserve, even though there are no guarantees ($P < 0.0001$). Those that agreed to the testicular biopsy were also more often influenced by a desire for genetic preservation for their son ($P = 0.0047$) and a hope to mitigate the future psychological trauma that may be caused if their son is rendered infertile by his therapy ($P = 0.0186$). Lastly, 100% of the families who chose to have the procedure felt that, in retrospect, they had made the right decision.

Several items on the study-designed questionnaire provide a measure of how families prioritize fertility preservation within the context of the cancer itself, the treatment timeline and overall risks. One item asked if, hypothetically, they would be willing to delay treatment in order to obtain testicular tissue for freezing. While the majority of parents answered that they would not be willing to delay treatment (62.3%), a third (32.1%) would delay cancer

directed therapy within reason in order to pursue this option; an additional 5.7% of those surveyed were not sure. Another question explored if families would be willing to go through a separate operative procedure and exposure to anesthesia solely for the testicular biopsy. A larger percentage of parents endorse being willing to do this than to delay therapy (50.9% vs. 32.1%) but 41.5% answered no to allowing an additional trip to the operating room just for the testicular biopsy, and 7.6% were not sure if they would consider this option for their son.

Results from the DMCI demonstrated that parents who agreed to testicular tissue cryopreservation and those that did not both felt in control of their decision, and endorsed that it was made by them without the inappropriate influence of others. There was no difference detected using the DMCI total score ($P = 0.5439$) or the subscores between those choosing cryopreservation and those who did not. Moreover, data from the POMS demonstrated that neither group differed in their emotional state at the time of their decision, as there was no significant difference in the total mood disorder index ($P = 0.2153$).

As part of the informed consent process, parents, and when appropriate, the patients needed to decide the disposition of the stored clinical tissue if the patient should die at any point in the future. Ninety-four percent of the time the decision was made to release the clinical specimen to research in this circumstance. In 6% of cases, parents chose to have the specimen discarded.

DISCUSSION

The aims of this multi-institutional collaboration are twofold: to provide the human tissue that is needed to refine the methods for growing and expanding SSCs, and to enable the collection and storage of testicular tissue that may be used by individual patients in the future to restore fertility. Because this study requires a surgical procedure, parental desire and acceptability of SSC collection via testicular biopsy was an important consideration. Our data indicate that participation in research that will allow for the translation of current animal experiments on SSC collection and transplantation into clinical care is highly desired by parents of prepubertal male children facing gonadotoxic treatments. The high acceptance rate (77%) of this multi-institutional research option affirms that families are willing to undergo the testicular biopsy procedure for tissue cryopreservation, even when there are no guarantees that the science will exist in the future to allow their sons to use this banked tissue to achieve pregnancy. Moreover, institutions of varying size and composition can successfully participate.

A potential barrier to the overall success of this research is that a family is being asked to make a critical decision during an already stressful time about an additional surgical procedure that is experimental in nature. An important part of the consent process for this protocol is communicating that it is unclear if banked tissue would be useful in the future, and that the science does not currently exist to use this tissue in humans to achieve pregnancy. The questionnaire data demonstrate that, although the time at diagnosis is very stressful, families want to be presented with experimental fertility options, and are able to make thoughtful decisions about fertility preservation.

Offering experimental fertility preservation options to families facing a new life threatening illness can also create ethical questions regarding the voluntary nature of consent. Are patients and families with no other choice for fertility preservation unduly influenced by a theoretical idea, a false hope, which may not ever become a viable clinical option? Does the intense emotional burden of having a child diagnosed with cancer compromise a parent's ability to make an informed and voluntary decision for their sons? DMCI data from this cohort demonstrates that for both those who chose the biopsy and those who refused, there is a feeling of ownership for the decision and that the choice was active, informed, and in the control of the parent and their son. Researchers studying the bioethics of fertility preservation in prepubertal male children uniformly agree that in order to provide appropriate protections for the vulnerable population involved in this research, the proposal to harvest and store tissue must be conducted under the oversight of an IRB [12–14]. Ethicists conclude that as long as certain ethical cautions are observed in how the tissue is collected and used, there is nothing in medical ethics that makes it impermissible to bank testicular tissue in the hopes of offering affected male children an option that may 1 day help them have biologically related children as adults [12–14].

Because the use of testicular tissue to achieve human pregnancy is still experimental, the patient's clinical care, in terms of timely initiation of therapy and overall safety, is of paramount concern. Every effort is made to incorporate the biopsy as seamlessly as possible into the child's clinical course/planned surgical interventions and to not delay moving forward with treatment. In addition, the study requires that the testicular biopsy be done at the time of another clinical procedure in the operating room, not as a stand-alone procedure, thus minimizing the risk of multiple anesthesia exposures. This approach seems to offer the greatest protection to human research subjects. However, families may be more flexible in regard to timing and risk than one would expect, emphasizing the idea that fertility preservation is a priority. Our data suggest that many families would be willing to go through a separate operative procedure and exposure to anesthesia solely for the testicular biopsy. This appears more acceptable than the idea of delaying their child's treatment, although some would also consider this.

In those that accepted the testicular biopsy option, there was a significant level of hopefulness that the reproductive science will exist in the future so that stored tissue can be used to attempt pregnancy. The pace of scientific advances to date supports this belief and provides concrete evidence that this level of parental hopefulness is not misguided. Clear progress has been made by the laboratory scientists associated with our protocol as well as by other investigators in the field in terms of SSC biology, isolation, and culture condition optimization [15–20].

A fundamental hurdle for SSC transplantation is the small amount of SSCs that can be obtained from a testicular biopsy of a small male child [19]. However, rodent model research has demonstrated that SSCs can be expanded under proper culture conditions, and then transplanted into infertile recipients who resume spermatogenesis and create healthy progeny [6,7]. Moreover, Brinster et al., 2012 demonstrated that long-term cryopreservation (>14 years) of testis cells from mouse, rat, rabbit, and baboon safeguards SSC viability, and that these cells can colonize the seminiferous tubules of recipient testes [15,19]. This is an

encouraging finding, as testicular tissue taken from prepubertal male children will potentially remain frozen for several decades before potential use. Successful SCC transplantation, restoration of spermatogenesis, and SCC viability after thawing has now been documented in a variety of animal models [21–24].

SCC transplantation in non-human primate models have been attempted but initial studies did not evaluate the presence or function of donor sperm after transplantation [25,26]. Hermann et al. have now demonstrated that autologous and allogeneic transplantation of SCCs in infertile rhesus macaques can produce functional sperm and that these sperm are able to fertilize rhesus oocytes via ICSI [16]. The significance of this work cannot be underestimated, as procedures for testicular transplantation of SCCs in mammals with the most physiological similarities to humans will ultimately be what transforms this science to human clinical use. These advances are a clear highlight of testicular tissue and SCCs fertility preservation research over the past 5 years.

Research techniques for enriching and expanding the number of SSCs in culture prior to transplantation are also crucial to successful translation to human use. Several independent research teams have successfully performed *in vitro* expansion of SCCs in rodents and rabbits using varied combinations of hormones and growth factors, with glial-cell derived neurotrophic factor (GDNF) being recognized as a key substance in SCC self renewal [15,20]. There also has been reported progress on culturing human SSCs from the testes of adult men with prostate cancer [17]. This study is particularly encouraging, as the tissue being used to culture SSCs is from human subjects. This work remains preliminary, however, and a robust bioassay is necessary to assess whether cultured human-derived SSCs actually have spermatogenic potential.

Alternative strategies to autotransplantation have also been developed which merit consideration. Sato et al., 2011, demonstrated the *in vitro* production of functional sperm cells from neonatal mouse testis tissue, which were then subsequently used to produce healthy, fertile offspring using microinsemination [18]. Subsequently they were also able to use neonatal testis tissue that had been cryopreserved and thawed and showed complete spermatogenesis *in vitro* [18]. This method of *in vitro* generation of spermatozoa offers promise for the future use of testicular tissue from prepubertal male children. This method bypasses the need for surgical SSC transplantation or autografting of testicular tissue and even more importantly, bypasses the concern regarding transplanting potentially malignant cells back into a male now cured of his disease [27].

The present study demonstrates that offering testicular tissue cryopreservation to those facing gonadotoxic treatment is acceptable to families, can be done safely and, can be replicated at other institutions of varying size. Parents and patients are able to make a self-determined and informed decision about this option, despite the intensity of the time surrounding diagnosis and the stress associated with the initiation of curative therapy. The experimental nature of utilizing frozen testicular tissue to restore fertility does not appear to influence the decision-making process but the risk of the testicular biopsy itself is considered, especially by those that ultimately refused the option for their son. Thus, testicular biopsy and tissue cryopreservation holds promise for this cohort of patients. While

significant progress has been made, continued clinical and basic science research is required to determine whether the tissue banking efforts of this protocol will be ultimately successful for creating live births for these patient populations in the future.

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TABLE I

Parent/Subject Characteristics by Accepters and Refusers

	Accepters	Refusers	*P*-value
	n = 57	n = 17	
Subject age, mean ± standard deviation (range) years	6.7 ± 4.3 (0.2–14.5)	7.0 ± 4.5 (0.8–15)	0.8034
Race, n (%)			0.6006
White	45 (78.9%)	12 (70.6%)	
Black	7 (12.3%)	2 (11.8%)	
Asian	2 (3.5%)	1 (5.9%)	
Other	3 (5.3%)	2 (11.8%)	
Religion, n (%)			
Protestant	15 (26.8%)	5 (31.3%)	0.3209
Catholic	22 (39.3%)	7 (43.8%)	
Jewish	5 (8.9%)	0	
Muslim	2 (3.6%)	1 (6.3%)	
Other	8 (14.3%)	0	
None	4 (7.1%)	3 (18.8%)	
Education level of parent consenting[a], n (%)			0.7724
Some HS	6 (11.5%)	2 (18.2%)	
Graduated high school	7 (13.5%)	1 (9.1%)	
Some college	13 (25.0%)	1 (9.1%)	
Graduated college	17 (32.7%)	4 (36.4%)	
Graduate school	9 (17.3%)	3 (27.3%)	
Diagnoses of biopsied subjects, n (%)			
Neuroblastoma	9 (18.8%)		
Rhabdomyosarcoma	7 (14.6%)		
Osteosarcoma	5 (10.4%)		
Ewing Sarcoma	8 (16.7%)		
Sarcoma NOS	7 (14.6%)		
Hematologic disease	6 (12.5%)		
Immunodeficiencies	6 (12.5%)		

[a]These data not available for five families who agreed to biopsy but did not complete questionnaire and six families who refused biopsy and did not complete questionnaire.

TABLE II

Decision Influences (Accepters and Refusers)

Factors	Yes, very much (%)	Yes, somewhat (%)	No, not considered (%)	Differences between accepters and refusers
Religious beliefs	6	21	73	0.6044
Ethical issues	4.6	15.4	80	0.7104
Financial considerations	6.2	18.5	75.4	0.2143
Too overwhelmed by diagnosis	5	37.5	57.5	0.0221
Accepters	0	38.2	61.8	
Refusers	33.3	33.3	33.3	
Limited time to decide	12	41	47	0.8016
Risk of testicular biopsy	18.2	54.5	27.3	0.0070
Accepters	12.7	61.8	25.5	
Refusers	45.5	18.2	36.4	
Experimental nature of freezing procedure	18.2	40.9	40.9	0.9355
Health of frozen tissue when thawed	10.6	50	39.4	0.9446
Hopeful that science will advance	82.5	12.5	5	0.0022
Accepters	91.2	8.8	0	
Refusers	33.3	33.3	33.3	
Worth opportunity, even though no guarantees	79.5	10.3	10.3	<0.0001
Accepters	90.9	9.1	0	
Refusers	16.7	16.7	66.7	
Desire to preserve son’s option of having genetically related children	73	19.2	7.7	0.0047
Accepters	85.7	14.3	0	
Refusers	20	40	40	
Desire to prevent son’s psychological distress over infertility	48	48	4	0.0186
Accepters	60	40	0	
Refusers	0	80	20	