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Histology of Testicular Biopsies Obtained for an Experimental Fertility Preservation Protocol in Boys with Cancer

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Abstract

Purpose—Cryopreservation of testicular tissue with subsequent re-implantation after therapy has fertility preservation potential for pre-pubertal boys with childhood cancer. We present the histology and the feasibility of testicular tissue procurement for this novel approach.

Patients and Methods—We performed a prospective cohort study of boys at significant risk for treatment-associated gonadotoxicity who were eligible for an experimental research protocol between 2008 and 2011. Open testicular biopsy was performed while they were anesthetized for another treatment related procedure. Half of the specimen was reserved for cryopreservation, while the other half was used for research purposes. Semi-thin sections of the biopsy specimens were evaluated for histological features and compared to age-adjusted reference values.

Results—Of the 34 boys who underwent biopsy between March 2008 and October 2011, 29 had solid tumors and 5 underwent hematopoietic stem cell transplantation for benign disease. Of these, 27 had adequate tissue for histologic analysis. The median age of the boys was 8.7 years (IQR=2.2 – 11.5 years). All children had either normal (81.5%) or increased (18.5%) numbers of normal

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germ cells per tubules for their age. However, 18.5% (5/27) of boys had no evidence of Adult dark (Ad) spermatogonia, and 56% (9/16) of boys had no evidence of primary spermatocytes on biopsy, that would be expected for their age norms. These findings are suggestive of abnormal germ cell maturation.

Conclusion—The preliminary histologic findings of abnormal spermatogenesis maturation in testes of pre-pubertal boys with cancer warrants further investigation.

Keywords

fertility preservation; childhood cancer; testicular biopsy; spermatogenesis

Introduction

Advances in therapy for children with cancer during the past several decades have resulted in dramatic improvements in long-term survival. Unfortunately, anti-cancer therapies often induce adverse effects in normal tissues and may cause significant long-term sequelae. For instance, exposure to high cumulative doses of cyclophosphamide often results in azospermia and decreased fertility potential in males¹. Radiation exposure, including total body irradiation conditioning for hematopoietic stem cell transplantation (HSCT), can also impair spermatogenesis². Given the success of modern anti-cancer therapies, greater focus has been placed upon longer-term quality of life and survivorship issues, such as fertility preservation in childhood cancer patients.

For post-pubertal adolescents and young men, sperm banking is a viable option and is available at most cancer centers. However, successful collection of a mature sperm specimen is not possible for pre-pubertal and peri-pubertal boys. Alternative techniques for fertility preservation for these younger patients are under active investigation in the research setting, but are not currently available clinically³.

Our multi-disciplinary group at the Children's Hospital of Philadelphia (CHOP) is investigating the feasibility of an experimental protocol that involves cryopreservation of testicular tissue specimens with the possibility for subsequent re-implantation of viable germ cells after completion of gonadotoxic therapy in selected patients⁴. While this technique has the potential to preserve fertility for pre-pubescent boys, several questions remain prior to clinical implementation. To address these issues, our group has previously demonstrated successful xenogeneic transplantation of human spermatogonial stem cells (SSC) into the seminiferous tubules of immunodeficient mice⁵. We have also reported the safety of the testicular biopsy procedure and the informed consent and participation willingness by parents for this experimental protocol^{4,6}.

In the current study, we report the preliminary histologic findings of testicular biopsies obtained from pre-pubertal males prior to the initiation of gonadotoxic therapy. Our *a priori* hypothesis was that these specimens will demonstrate normal histology for age.

Materials & Methods

Study Design and Population

We performed a prospective cohort study of all pre-pubertal males and peri-pubertal males at significant risk of treatment-associated gonadotoxicity who were unable to bank sperm and underwent open testicular biopsy between 2008 and 2011. Our protocol and the subject recruitment process have been previously described in detail⁶.

Briefly, all subjects had been diagnosed with solid tumors (*e.g.*, sarcoma, neuroblastoma) or were to undergo HSCT for non-malignant diseases (*e.g.*, aplastic anemia, immunodeficiency). Patients with hematologic malignancies were excluded from this study due to the need to start immediate anti-cancer treatment and the risk of testicular involvement with leukemia. Patients with coagulopathy, cryptorchidism, or known testicular involvement with cancer were also excluded.

Testis biopsy protocol

An open testicular biopsy was performed while patients were under anesthesia for another treatment-related operation, such as central line placement or bone marrow biopsy. Per protocol, testicular biopsies were never a primary procedure so as to minimize the risk of anesthesia exposure. All testicular biopsies occurred prior to initiation of anti-cancer treatment or HSCT conditioning. One-half of each biopsy specimen was cryopreserved at the Penn Fertility Care of the Hospital of the University of Pennsylvania for potential clinical use by the patient at a later date as previously described⁶.

The other half of the testicular biopsy was further divided into two specimens. The larger specimen was reserved for isolation of SSCs for research purposes. The remaining tissue was submitted for routine histopathologic analysis at CHOP, and these data are reported in this study. After fixation in 2% glutaraldehyde, the specimens were embedded in bisphenol A diglycidyl ether epoxy resin and then sectioned every 0.4 microns. The semi-thin sections were then stained with toluidine blue. Histological analysis was performed using light microscopy at 600x total magnification (Figure 1). At least 50 tubular cross sections of the specimen were examined.

Statistical Analysis

All data were prospectively collected. The primary outcome variable was total number of germ cells per tubule. The secondary outcome variables were numbers of gonocytes, adult dark (Ad) spermatogonia, and primary spermatocytes. Also reported was presence of Leydig cells, testicular fibrosis, tubular atrophy, microlithiasis, and carcinoma *in situ*. The primary predictor variable was clinical diagnosis. Other predictor variables included patient age and race/ethnicity.

A standardized template was used to reduce any variability between reporting pathologists. The total number of germ cells per tubule was reported as an ordinal variable: reduced, mildly reduced, normal, or increased based upon an age-adjusted reference value chart⁷. This chart had been created based on testicular biopsies from 94 normally descended testes⁷.

The remaining histological variables were dichotomous: present, absent, or unable to be determined. Using the reference chart and biopsy results of the contralateral testis in patients with unilateral undescended testis, we estimated that 95% of our cohort should have a normal appearing testicular biopsy for their age. Given our sample size of 27 patients, we estimated a power of 86% to detect a 20% difference between our cohort and the age-adjusted references⁷.

Statistical analyses were performed with Stata 13.0 (StataCorp LP, College Station, Texas, USA) using median values and interquartile range (IQR), for continuous variables. Proportions were used for categorical variables. Our research protocol was approved by the CHOP Institutional Research Board. Informed consent was obtained from the parents or legal guardians per the Declaration of Helsinki.

Results

Thirty-four male children and adolescents were enrolled and underwent open testicular biopsy. While all patients had adequate testicular tissue for viable cryopreservation, 7 specimens had insufficient tissue for histopathologic analysis and were thus excluded. Of the 27 specimens available for analysis, the median age of the patients was 8.7 years (IQR=2.2 – 11.5 years). Twenty-three (85.2%) of the 27 boys were non-Hispanic Caucasians.

As shown in Table 1, cancer diagnoses included neuroblastoma (n = 6), Ewing sarcoma (n = 5), osteosarcoma (n = 3), rhabdomyosarcoma (n = 2), synovial sarcoma (n = 2), pleuropulmonary blastoma (n = 1), rhabdoid tumor (n = 1), and chondrosarcoma (n = 1). An additional child initially diagnosed with osteosarcoma underwent testicular biopsy per protocol at time of tumor biopsy, but was subsequently found to have a benign aneurysmal bone cyst instead of cancer. His data are included in Table 2.

Five children with non-malignant diseases awaiting HSCT underwent testicular biopsies for this study (Table 2). Histological analysis of the 27 specimens revealed that 22 children (81.5%) had normal germ cells per tubules for age, while the remaining 5 children (18.5%) had increased germ cell counts. All children with increased numbers of germ cells per tubule had cancer diagnoses. Of the boys older than 6 months for whom Ad spermatogonia would be expected for their age, 5/26 (19%) boys had no evidence of Ad spermatogonia. Primary spermatocytes are usually seen in children older than six years; however, 56% (9/16) of children who were older than 6 years had no evidence of primary spermatocytes on biopsy.

The presence of fetal gonocytes beyond 6 months of age is another indicator of abnormal maturation⁸. Fetal gonocytes were not seen in any testicular specimens. Leydig cells were present in specimens from 24 children (89%), absent in one (3.7%), and could not be determined in two specimens (7.4%). The patient with absent Leydig cells was a 10 year old with Ewing sarcoma, who also had histologic evidence of testicular fibrosis and atrophy. The 9-year-old patient with embryonal rhabdomyosarcoma also had evidence of microlithiasis of unclear clinical significance. No testicular biopsy specimen had metastatic cancer involvement or intra-tubular germ cell neoplasia.

Discussion

Our long-term goal is to re-transplant cryopreserved SSC back into the seminiferous tubules of the patients following completion of gonadotoxic anticancer therapy. Although others and we have made progress towards achieving this goal, further work is required to assess the clinical potential of this experimental technique⁹. In particular, determination of whether the germ cells harvested from pre-pubertal patients with cancer have normal age-expected histology requires further assessment.

In our histologic analyses of pre- and peri-pubertal boys about to undergo gonadotoxic anticancer therapy, we did not detect a difference in the number germ cell per tubules for age in any specimen compared to reference values. However, we also noted an age-adjusted maturation delay of spermatogenesis. Nearly 20% of specimens from patients greater than 6 months of age lacked Ad spermatogonia that would be expected for age norms. Similarly, 56.3% of biopsies had no evidence of primary spermatocytes on biopsy when it would be expected for their age^{8, 10}.

The clinical significance of the lack of Ad spermatogonia and primary spermatocytes observed in these patients remains uncertain. However, in boys with history of cryptorchidism, the lack of Ad spermatogonia on testis biopsy has been correlated with subsequent abnormal semen analyses¹¹. The reduced fertility in cryptorchid boys has been hypothesized to be attributable to maturation arrest, more specifically from failed transformation of gonocytes into Ad spermatogonia⁸. However, the pathologic data from cryptorchid patients to those of our study patients may not be directly comparable and must be interpreted with caution¹¹.

Although gonadotoxic therapy is a well-recognized cause of reduced fertility, there may be other underlying causes for impaired spermatogenesis in the setting of malignancy. The possible non-treatment related mechanisms are poorly understood but are likely multifactorial¹². Proposed theories for impaired spermatogenesis in malignant diseases are pre-existing defects in germ cells, local and systemic tumor effects, and alterations of the endocrine and immune systems¹². Another hypothesis is that impaired spermatogenesis results from increased pro-inflammatory cytokines related to the child's stress response to the disease state^{13, 14}.

If boys with serious childhood diseases truly have delayed maturation in spermatogenesis, future re-implantation of testicular tissue may not result in successful clinical fertility. However, *ex vivo* maturation of spermatogenesis with subsequent intracytoplasmic sperm injection may overcome this and also circumvent the need for more invasive surgical re-implantation of cryopreserved testicular tissue³.

While our pilot study does not evaluate the clinical potential of this experimental fertility-preserving technique, obvious concerns exist regarding the re-implantation of tissue obtained from children with cancer prior to treatment. In our study, no patient had microscopic evidence of testicular involvement with primary tumor, minimizing the potential risk of inadvertent transplantation of viably cryopreserved cancer cells.

Another concern about this experimental protocol is the risk of overtreating patients by adding an additional surgical procedure of possibly limited or no benefit to the patient. Not every cancer treatment results in gonadotoxicity, although it remains somewhat difficult to predict *a priori* which patients will experience reduced fertility due to anti-cancer or pre-HSCT therapy. We limited study enrollment to children receiving therapies associated with the greatest risk of gonadotoxicity, but cannot be certain that every patient included in this study will ultimately experience reduced fertility related to cancer treatment.

Our study has several limitations. It is a relatively small and heterogeneous cohort with respect to age and underlying diagnosis. We could not control for Tanner stage or other clinical parameters of puberty, as this was not prospectively recorded. Initial histologic analysis was not centrally performed, and the reviewing pathologists were not blinded to the patients' clinical history, which may introduce expectation bias. It was also not possible to compare biopsy specimens from our study patients to normal controls, as it would place healthy patients at unnecessary risk from anesthesia and invasive procedures. However, we compared our cohort to reference values based upon 94 children with normally descended testes undergoing biopsy for various reasons⁷. These data were obtained from children with scrotal/inguinal pathology and 33 children at autopsy for unreported causes of death. Since these reference values were not obtained from truly "healthy" controls, the delayed maturation observed in our cohort may represent an even greater deviation from normal spermatogenesis.

Conclusion

Cryopreservation of testicular tissue with subsequent surgical re-implantation is a novel experimental approach that may allow future fertility for children following chemotherapy or HSCT. Preliminary testicular histologic analyses demonstrate normal to increased levels of germ cells; however, an age-adjusted delayed maturation of spermatogenesis was observed in many of these biopsy specimens. Further investigation regarding the impact of these preliminary histologic findings is warranted with the goal of improving future fertility for children treated with gonadotoxic therapies.

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Key of Definitions for Abbreviations

Ad	adult dark
CHOP	Children's Hospital of Philadelphia
HSCT	hematopoietic stem cell transplantation (HSCT)
IQR	interquartile range (IQR)
SSCs	spermatogonial stem cells

Figure 1.

Microscopy pictures of two pre-pubertal testicular biopsy specimens.

A demonstrates age-expected maturation to primary spermatocytes. (Arrow indicates primary spermatocytes)

B shows a specimen with maturation arrest at Ad spermatogonia and Adult pale spermatogonia stages, which is inappropriate for patient age.

Table 1

Histologic findings for children with cancer

Age	Diagnosis	Total germ cell per tubules	Presence of Ad spermatogonia	Presence of primary spermatocytes
4 months	Pleuropulmonary blastoma	Normal	Yes	No
6 months	Ewing sarcoma	Normal	Yes	No
7 months	Neuroblastoma	Normal	Yes	No
1.3 years	Rhabdomyosarcoma	Increased	Yes	No
2.2 years	Neuroblastoma	Normal	Yes	No
2.3 years	Neuroblastoma	Increased	Yes	No
2.8 years	Neuroblastoma	Increased	No	No
3.8 years	Neuroblastoma	Normal	Yes	No
4.3 years	Rhabdoid tumor	Increased	Yes	Yes
6.9 years	Neuroblastoma	Normal	No	No
8.8 years	Rhabdomyosarcoma	Normal	Yes	Yes
9.8 years	Synovial sarcoma	Normal	Yes	No
9.9 years	Ewing sarcoma	Normal	Yes	No
9.9 years	Chondrosarcoma	Increased	Yes	No
10.3 years	Ewing sarcoma	Normal	No	No
10.7 years	Ewing sarcoma	Normal	Yes	Yes
11.5 years	Osteosarcoma	Normal	Yes	No
12.5 years	Ewing sarcoma	Normal	Yes	No
12.7 years	Osteosarcoma	Normal	Yes	Yes
13.1 years	Osteosarcoma	Normal	Yes	Yes
17.8 years	Synovial sarcoma	Normal	No	Yes

Table 2

Histologic findings for children with non-cancer diagnoses

Age	Diagnosis	Total germ cell per tubules	Presence of Ad spermatogonia	Presence of primary spermatocytes
11 months	Chronic granulomatous disease	Normal	Yes	No
5.4 years	Aplastic anemia	Normal	No	No
8.7 years	Interferon-gamma immunodeficiency	Normal	Yes	No
11.9 years	Aplastic anemia	Normal	Yes	Yes
12.8 years	Aplastic anemia	Normal	Yes	Yes
12.4 years	Aneurysmal bone cyst*	Normal	Yes	No

* Pre-biopsy diagnosis was osteosarcoma, but the lesion was subsequently found to be benign.