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ORIGINAL ARTICLE ***Andrology***

Testicular tissue cryopreservation: 8 years of experience from a coordinated network of academic centers

H. Valli-Pulaski[1,5], K.A. Peters[5], K. Gassei[5], S.R. Steimer[5], M. Sukhwani[5], B.P. Hermann[1,5,15], L. Dwomor[1], S. David[3], A.P. Fayomi[3], S.K. Munyoki[4], T. Chu[1,5], R. Chaudhry[2], G.M. Cannon[2], P.J. Fox[2], T.M. Jaffe[2], J.S. Sanfilippo[1], M.N. Menke[1,5], E. Lunenfeld[6,8], M. Abofoul-Azab[8], L.S. Sender[9], J. Messina[9], L.M. Klimpel[9], Y. Gosiengfiao[10], E.E. Rowell[10], M.H. Hsieh[11], C.F. Granberg[12], P.P. Reddy[13], J.I. Sandlow[14], M. Huleihel[6,7], and K.E. Orwig[1,3,4,5,*]

[1]Department of Obstetrics, Gynecology & Reproductive Sciences, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, USA [2]Department of Urology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, USA [3]Department of Molecular Genetics and Developmental Biology Graduate Program, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, USA [4]Integrative Systems Biology Graduate Program, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, USA [5]Magee-Womens Research Institute, Pittsburgh, PA 15213, USA [6]The Center of Advanced Research and Education in Reproduction (CARER) , Ben-Gurion University of the Negev, Beer-Sheva, Israel [7]The Shraga Segal Department of Microbiology, Immunology and Genetics, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer-Sheva, Israel [8]Depratment of Obstetrics and Gynecology, Soroka Medical Center and Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer-Sheva, Israel [9]Hyundai Cancer Institute, CHOC Children's Hospital, Orange, CA 92868, USA [10]Ann & Robert H. Lurie Children's Hospital of Chicago, Chicago, IL 60611, USA [11]Sheik Zayed Institute for Pediatric Surgical Innovation at Children's National Health System, Washington, DC 20010, USA [12]Department of Urology, Mayo Clinic, Rochester, MN 55902, USA [13]Division of Pediatric Urology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH 45229, USA [14]Department of Urology, Medical College of Wisconsin, Milwaukee, WI 53226, USA [15]Present address: Department of Biology, The University of Texas at San Antonio, San Antonio, TX 78249, USA

*Correspondence address. University of Pittsburgh School of Medicine, Magee-Womens Research Institute, 204 Craft Avenue, Pittsburgh, PA 15213, USA. Tel: 412-641-2460; E-mail: orwigke@upmc.edu

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STUDY QUESTION: Is it feasible to disseminate testicular tissue cryopreservation with a standardized protocol through a coordinated network of centers and provide centralized processing/freezing for centers that do not have those capabilities?

SUMMARY ANSWER: Centralized processing and freezing of testicular tissue from multiple sites is feasible and accelerates recruitment, providing the statistical power to make inferences that may inform fertility preservation practice.

WHAT IS KNOWN ALREADY: Several centers in the USA and abroad are preserving testicular biopsies for patients who cannot preserve sperm in anticipation that cell- or tissue-based therapies can be used in the future to generate sperm and offspring.

STUDY DESIGN, SIZE, DURATION: Testicular tissue samples from 189 patients were cryopreserved between January 2011 and November 2018. Medical diagnosis, previous chemotherapy exposure, tissue weight, and presence of germ cells were recorded.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Human testicular tissue samples were obtained from patients undergoing treatments likely to cause infertility. Twenty five percent of the patient's tissue was donated to research and 75% was stored for patient's future use. The tissue was weighed, and research tissue was fixed for histological analysis with Periodic acid-Schiff hematoxylin staining and/or immunofluorescence staining for DEAD-box helicase 4, and/or undifferentiated embryonic cell transcription factor 1.

MAIN RESULTS AND THE ROLE OF CHANCE: The average age of fertility preservation patients was 7.9 (SD = 5) years and ranged from 5 months to 34 years. The average amount of tissue collected was 411.3 (SD = 837.3) mg and ranged from 14.4 mg—6880.2 mg. Malignancies ($n = 118$) were the most common indication for testicular tissue freezing, followed by blood disorders ($n = 45$) and other conditions ($n = 26$). Thirty nine percent ($n = 74$) of patients had initiated their chemotherapy prior to undergoing testicular biopsy. Of the 189 patients recruited to date, 137 have been analyzed for the presence of germ cells and germ cells were confirmed in 132.

LIMITATIONS, REASONS FOR CAUTION: This is a descriptive study of testicular tissues obtained from patients who were at risk of infertility. The function of spermatogonia in those biopsies could not be tested by transplantation due limited sample size.

WIDER IMPLICATIONS OF THE FINDINGS: Patients and/or guardians are willing to pursue an experimental fertility preservation procedure when no alternatives are available. Our coordinated network of centers found that many patients request fertility preservation after initiating gonadotoxic therapies. This study demonstrates that undifferentiated stem and progenitor spermatogonia may be recovered from the testicular tissues of patients who are in the early stages of their treatment and have not yet received an ablative dose of therapy. The function of those spermatogonia was not tested.

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Key words: Spermatogonial stem cells / fertility preservation / testis / testicular tissue cryopreservation / spermatogenesis

Introduction

Chemotherapy and radiation treatments for cancer and other conditions can be gonadotoxic and cause permanent infertility (Meistrich, 2009, Green *et al*., 2010, Lambertini *et al*., 2016). This is an important human health concern because cancer survivors and other infertile patients report that their fertility status impacts their emotional well-being, relationships, finances, and general health (Schover, 2009, Bak *et al*., 2012, Wu *et al*., 2013, Ellis *et al*., 2016). Therefore, the American Society of Clinical Oncology and the American Society for Reproductive Medicine recommend that patients be educated about the reproductive side effects of their treatment and about options to preserve their fertility (Martinez and International Society for Fertility Preservation–ESHRE–ASRM Expert Working Group, 2017, Oktay *et al*., 2018).

Cryopreservation of sperm is standard of care and the best option for adult men and adolescent boys to preserve their fertility. Unfortunately, there are no options to preserve the fertility of prepubertal boys who are not yet making sperm. We estimate that each year in the United States, more than 2000 boys will receive gonadotoxic treatments for cancer or other conditions (e.g. myeloablative conditioning prior to bone marrow transplantation) that will put them at significant risk for infertility (Valli *et al*., 2014a). Several cell- and tissue-based methods have emerged from the research pipeline during the past 2 decades to address the reproductive needs of this growing patient population (Gassei *et al*., 2017, Del Vento *et al*., 2018, Medrano *et al*., 2018). These promising technologies have prompted centers around the world, including our coordinated network of centers in the USA and Israel, to cryopreserve testicular biopsies for prepubertal boys with anticipation that new reproductive technologies will be available to them in the future (Bahadur *et al*., 2000, Keros *et al*., 2007, Sadri-Ardekani *et al*., 2009, Ginsberg, 2011, Sadri-Ardekani *et al*., 2011, Wyns *et al*., 2011, Goossens *et al*., 2013, Picton *et al*., 2015, Pietzak Iii *et al*., 2015, Onofre *et al*., 2016, Ho *et al*., 2017, Poganitsch-Korhonen *et al*., 2017, Uijldert *et al*., 2017, Heckmann *et al*., 2018, Stukenborg *et al*., 2018,).

Counseling of prepubertal patients and their families is complicated by the sensitive subject matter and the lack of standard of care options for preserving fertility of patients who are not producing sperm. The provision of fertility preservation services for young patients is further challenged by the fact that children's hospitals are not typically equipped with the infrastructure to offer fertility care. Nonetheless, adult survivors of childhood cancer desire to have children (Nieman *et al*., 2007, Schover, 2005, Ellis *et al*., 2016). A multidisciplinary approach increases the acceptance of fertility preservation procedures in the prepubertal and adolescence populations and their families. Furthermore, family support and hope for future parenthood have a positive effect on the decision-making process, whereas pressure from oncologists to not delay treatment has a negative effect on perception of fertility preservation (Wyns *et al*., 2015).

In this study, we established a coordinated network of centers that employed a standardized protocol to provide experimental testicular tissue cryopreservation services to patients at risk of infertility due to their medical treatments. While each center performed testicular biopsies on site, most centers utilized a centralized testicular tissue processing facility at the University of Pittsburgh. This mechanism enhanced access to fertility preservation care at children's hospitals. We report our collective experiences freezing testicular tissues for 189 patients, all of whom have donated a portion of their tissue to research. Combining the recruiting power of several institutions allowed us to characterize the patient population requesting fertility preservation care and draw conclusions of statistical significance that may help to inform fertility preservation practice. We examined patient testicular tissues to determine the impacts of age, diagnosis, and previous chemotherapy exposure on the population of undifferentiated spermatogonia. Our results indicate that the number of undifferentiated spermatogonia per seminiferous tubule increase with age and that it is possible to recover undifferentiated spermatogonia from the testes of patients who are already in the early stages of their chemotherapy treatments.

Materials and Methods

Study approval

Patients' testicular samples were obtained through the Fertility Preservation Program of the University of Pittsburgh Medical Center and seven coordinated recruitment sites at the Ann & Robert H. Lurie Children's Hospital of Chicago (IL), Cincinnati Children's Hospital Medical Center (OH), Children's National Medical Center (Washington, DC), Children's Hospital of Orange County (CA), Medical College of Wisconsin (Milwaukee, WI), Mayo Clinic (Rochester, MN), and Ben Gurion University (Be'er Sheva, Israel). Human subjects research for this coordinated network of centers was reviewed and approved by the University of Pittsburgh Institutional Review Board (PRO13040487) and registered with clinicaltrials.gov (NCT02972801). Each center protocol was also approved by their respective Institutional Review Board.

Patient recruitment and eligibility

Most families were informed about the study by their hematology/oncology team. Some families found the information on the internet and contacted the center directly to inquire about the study. Most centers have a dedicated fertility preservation navigator, who counsels the families about the procedure. At other centers the urologist who is performing the biopsy does the counseling. Each participating institution is responsible for counseling and consenting at their center. Some centers cover costs from philanthropic/departmental/institutional funds, while others pass costs to patients or insurance. The decision to participate was made exclusively by patients and/or their guardians. For patients under the age of 18, both parents/guardians were required to sign the consent form (when reasonably available) and it was the guardians' decision whether the under 18-year-old patient signed an assent form. All families were counseled about the risk and benefits of the study, including surgical complications. Written informed consent was obtained from all participants prior to inclusion in the study. All adverse events were reported to the coordinating center in Pittsburgh.

Eligibility criteria are described in Table 1. All centers have the same eligibility criteria. All patients and/or guardians were informed of the eligibility criteria and how they qualify. The information about drug and dose was either provided by the treating physician or retrieved from patient's medical record. Alkylating chemotherapy exposure was calculated using cyclophosphamide equivalent dose (CED) calculator (Green *et al.*, 2014; https://fertilitypreservationpittsburgh.org/fertility-resources/fertility-risk-calculator/).

Table 1 Inclusion and exclusion criteria for testicular tissue cryopreservation.

Inclusion criteria	Exclusion criteria
Males of any age.	Diagnosed with psychological, psychiatric, or other conditions which prevent giving fully informed consent.
Scheduled to undergo surgery, chemotherapy, drug treatment, and/or radiation with significant risk of causing infertility. Significant risk: • Cyclophosphamide equivalent dose >4 g/m^2 • Total body irradiation (TBI) • Testicular radiation >2 5 Gy • Cranial radiation >40 Gy • Cisplatin 500 mg/m2	Diagnosed with an underlying medical condition that significantly increases their risk of complications from anesthesia and surgery.
Have a medical condition or malignancy that requires removal of all or part of one or both testicles.	Cyclophosphamide equivalent dose >7.5g/m^2
Have two testicles if undergoing elective removal of all or part of a testicle for fertility preservation only.	

Tissue removal, transportation, and cryopreservation

The method of tissue removal was either a unilateral orchidectomy (available only to patients with two testes) or a testicular biopsy. The decision between orchiectomy and testicular biopsy was made by the patient and/or guardians or dictated by the patient's medical condition. Most patients or families opted for testicular tissue biopsies; eight patients/families opted for orchiectomy. In seven of those eight cases, orchiectomy was medically indicated; one case was elective, and the decision was made by the parents. Seventy five percent of the tissue was frozen for patient's future use and 25% was de-identified and designated for research. For patients who underwent biopsy, unilateral open testicular biopsy was performed and about 20% of the volume of the testis was removed. Patient tissues were processed and frozen at three centers—University of Pittsburgh (USA), Mayo Clinic (USA), and Ben Gurion University (Israel). The other recruitment sites sent their tissue to Pittsburgh for processing. The tissue was transported in Quinn's Advantage Blastocyst Medium (Origio, Denmark) on ice using express courier.

Patients' samples were either frozen as cell suspension ($n = 9$) or intact tissue pieces ($n = 180$) using a slow freezing (SF) protocol. Freezing intact pieces of testicular tissue is the preferred approach of our centers because it preserves the option for tissue- or cell-based therapies in the future. Cell suspensions were made using a two-step enzymatic digestion that was described previously (Hermann *et al.*, 2007). SF of intact pieces of testicular tissues were performed using methods that were previously described by others (Keros *et al.*, 2007). All centers used the same freezing protocol.

Staining of testicular tissue sections

Human testicular tissue fragments were fixed with 4% paraformaldehyde overnight, paraffin-embedded, and sectioned (5 μm). The slides were stained as previously reported in Valli *et al.* (2014b). Briefly, antigen retrieval was performed with sodium citrate buffer. Tissues were blocked in buffer containing 3% bovine serum albumin and 5% normal serum from the host species of the secondary antibody and sections were stained for 90 minutes at room temperature with primary antibodies ((mouse anti-undifferentiated embryonic cell transcription factor 1 (UTF1), 1:500, MAB4337, Millipore, USA; and rabbit anti-DEAD-box helicase 4 (DDX4), 1:200, ab13840, Abcam, USA)). Isotype-matched normal IgG was used as negative control. Primary

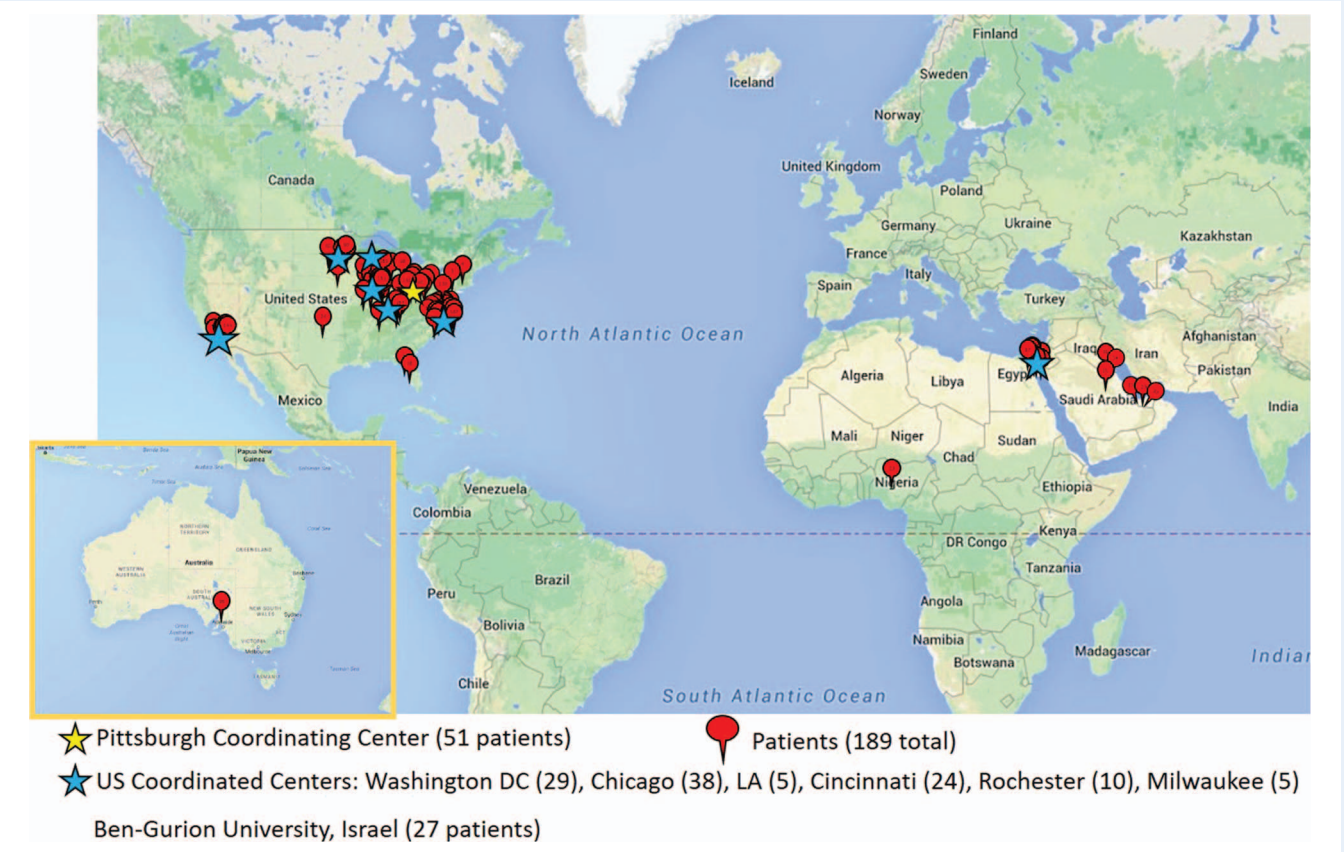

Figure 1 Testicular tissue cryopreservation from a coordinated network of academic and medical centers. Patients travel from around the world to access experimental testicular tissue freezing services offered by our coordinated network of medical centers. The Pittsburgh coordinating center is indicated by the yellow star and coordinated recruitment sites are indicated by the blue stars. Participating centers include the University of Pittsburgh Medical Center (PA), Ann & Robert H. Lurie Children's Hospital of Chicago (IL), Cincinnati Children's Hospital Medical Center (OH), Children's National Medical Center (Washington, DC), Children's Hospital of Orange County (CA), Medical College of Wisconsin (Milwaukee, WI), Mayo Clinic (Rochester, MN), and Ben Gurion University, Be'er Sheva, Israel. Participating patients' places of residence are indicated by red balloons.

antibody was detected using AlexaFluor-488 or AlexaFluor-568 conjugated secondary antibody (1:200, Invitrogen, USA). The slides were mounted with VectaShield mounting medium containing DAPI (Vector Laboratories, USA) for detection of nuclei. Sections were observed with a Nikon Eclipse 90i fluorescence microscope and images captured with NIS-Elements software (Nikon, Tokyo, Japan).

For Periodic acid-Schiff (PAS) and hematoxylin staining, the slides were stained according to manufacture instructions (Sigma-Aldrich, USA). A_{dark} spermatogonia were identified as relatively small, spherical, or slightly ovoid cells on the basement membrane of seminiferous tubules, having dark, dense chromatin in their uniformly stained nuclei. A_{pale} spermatogonia were identified as relatively larger, oval, or almost round cells on the basement membrane of the seminiferous tubules, having pale, elongated nuclei with coarser or more granular chromatin (Clermont and Antar, 1973, Clermont and Leblond, 1959).

The number of spermatogonia per cross section was determined by PAS hematoxylin staining and/or UTF1 immunostaining and/or DDX4 immunostaining. At least 40 seminiferous tubule cross sections were counted, except in five cases where insufficient tissue was available. In those cases, 9 to 33 cross sections were counted. Spermatogonia per seminiferous tubule counts were made in every 4th cross section, with 5 µm between each section (20 µm between counted sections). Typically, four of more non-adjacent cross sections were counted per sample.

Statistics

ANOVA and two-sample *t*-tests were used to determine the effect of age, diagnosis, and chemotherapy exposure on the number of UTF1- and DDX4-positive cells per testis cross section. Groups were considered different if *P*-values were <0.05.

Results

Testicular tissue cryopreservation patient population

Testicular tissues from 189 patients have been collected and cryopreserved between January 2011 and November 2018 (Fig. 1). The average age of patients was 7.9 years (SD = 5.0 years) and

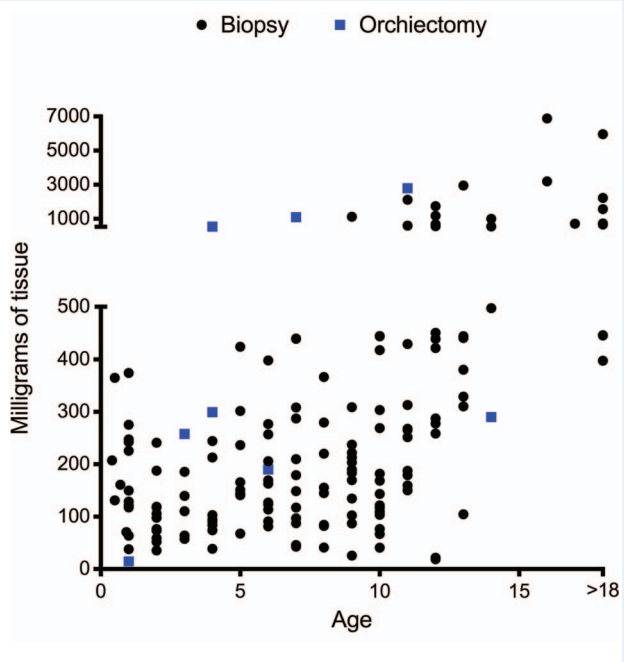

Figure 2 Amount of testicular tissue obtained for cryopreservation. Patient tissues were weighed before freezing. Testicular tissue was obtained either though a testicular biopsy (black circles; $n = 154$) or through orchiectomy (blue squares; $n = 8$).

ranged from 5 months to 34 years. All patients and families were counseled that freezing sperm is the standard of care method for fertility preservation. Twenty-three patients over the age of 13 were determined to be prepubertal based on Tanner staging, were unable to bank sperm, or decided to freeze testicular tissue in addition to sperm. For some of those patients, we processed tissue for sperm and for immature testicular tissue banking, as previously described (Picton *et al*., 2015). The average amount of tissue collected from the 162 cases for which data were available was 411.3 mg (SD = 837.3 mg, range 14.4 mg–6880.2 mg) (Fig. 2). Tissue biopsy weights were not recorded for patients at Ben Gurion University ($n = 27$). The average time from tissue removal to processing in the lab was 62 minutes (SD = 48 minutes, range 5 minutes–3 hours 45 minutes, $n = 51$) for tissue removed at the University of Pittsburgh, 38 minutes (SD = 14 minutes, range 15 minutes–1 hour, $n = 10$) for tissues removed at Mayo Clinic, 18 hours 7 minutes (SD = 9 hours 5 minutes, range 6 hours 47 minutes–91 hours 25 minutes, $n = 101$) for tissues removed at coordinated centers in the United States and shipped to Pittsburgh for processing and freezing. Exact time was not recorded for tissues removed at Ben Gurion University (Be'er Sheva, Israel), but the sample was transported to processing lab immediately after removal ($n = 27$). Overall, tissue from 152 patients were frozen at University of Pittsburgh, tissue from 10 patients were frozen at Mayo Clinic, and tissue from 27 patients were frozen at Ben Gurion University. Indications for testicular tissue freezing were malignancies (118 patients), blood disorders (45 patients), and other conditions (26 patients) described in Table 2. No unanticipated adverse events were reported. Rate of infection was 2.5% and rate of post-operative bleeding was 1.3%, which are in normal range for testicular biopsy and are anticipated risks (Dieckmann *et al*., 2005, Uijldert *et al*., 2017).

Previous chemotherapy exposure

Thirty nine percent (74 out of 189) of patients had started their medical treatment before freezing tissue. Sixteen percent (30 out of 189) of patients had received non-alkylating chemotherapy, whereas 23% (44 out of 189) of patients had received alkylating chemotherapy. Data on the dose of previous alkylating chemotherapy exposure was available for 40 out of 44 patients (average CED = 2821 mg/m2, SD = 1734, range 500–7000 mg/m2, $n = 40$; Tables 2 and 3).

Presence of undifferentiated spermatogonia in testes of cancer patients.

In 137 out of 189 cases, research tissues were available to confirm the presence of germ cells by PAS hematoxylin staining (137 samples; Fig. 3a–c; 16 samples for quantification of spermatogonia, Tables 2 and 4) or immunofluorescence staining for DDX4 (120 samples; Fig. 3g–l and Tables 2 and 3) and/or UTF1 (137 samples; Figure 3d–f, j–l and Tables 2 and 3). Germ cells were confirmed by one or more methods in 132 out of 137 patient samples tested (Tables 2–4, Figs 3 and 4).

We used UTF1 and DDX4 immunofluorescence to quantify the number of undifferentiated spermatogonia and total germ cells in the patient samples. A previous exposure to non-alkylating or alkylating chemotherapy did not impact the number of UTF1+ or DDX4+ spermatogonia/tubule cross section compared with patients that did not have a previous exposure ($P > 0.05$, Table 3 and Fig. 4). The number of UTF1+ and DDX4+ spermatogonia per tubule cross section increased with age, including a sharp rise at age 11 ($P < 0.05$), and this was true regardless of previous chemotherapy exposure (Table 3). The same age-related trend is seen with A_{dark} and A_{pale} spermatogonia in a limited series of 16 patients (Table 4). There was also no age-corrected statistical difference in UTF1 or DDX4 positive cells per tubule cross section between different diagnoses. Patients with testicular involvement (Gonadoblastoma, testicular lesions, and partial androgen insensitivity syndrome) were excluded from this analysis. The average age for those patients was 5.3 years (SD = 4.4, $n = 4$). None of those patients had received previous chemotherapy and they had an average of 0.42 UTF1+ spermatogonia/tubule cross section (SD = 0.54, range 0–1.15) and 0.37 DDX4+ spermatogonia/tubule cross section (SD = 0.69, range 0–1.40). Among the five patients with no germ cells, one was having an orchiectomy due to testicular lesions and a history of testicular torsion; three were receiving treatments for Sickle cell anemia and Thalassemia that are known to impact fertility (Singer *et al*., 2015, Smith-Whitley, 2014); and one was diagnosed with Ewing sarcoma, which to our knowledge, is not known to impact fertility.

Discussion

Cryopreservation of testicular tissue for fertility preservation is an experimental procedure that is gaining traction with tissues frozen for over 700 patients worldwide based on our own experiences and published reports (Radford *et al*., 1999, Bahadur *et al*., 2000, Brook *et al*.,

Table II Indication for testicular tissue cryopreservation.

Malignancies	# of patients	Age, mean ± SD (range)	% (n) of patients exposed to non-alkylating chemo prior to biopsy	% (n) of patients exposed to alkylating chemo prior to biopsy	Previous CED exposure (mg/m^2), mean ± SD (range)	UTF1+ cells/ cross section (±SD)	n	DDX4+ cells/ cross section (±SD)	n	Spermatogonia /cross section (±SD)	n
Bone and muscle	38	8.5 ± 4.4 (0.4–22)	10.5 (4)	23.7 (9)	1999 ± 1215 (732–4392)	2.76 ± 3.04	30	3.18 ± 4.42	28	1.81 ± 0.89	6
Hematologic	44	9.7 ± 5.7 (2–34)	22.7 (10)	63.3 (28)	3017 ± 1839 (500–7000)	2.08 ± 2.59	28	3.40 ± 4.48	22	3.91 ± 2.04	6
Brain and nervous system	31	6.9 ± 3.7 (1–13)	22.6 (7)	19.4 (6)	3520 ± 1659 (2000–6000)	2.08 ± 1.71	22	1.71 ± 1.22	21	2.81 ± 2.61	2
Genitourinary	5	9.6 ± 9.2 (4–26)	60 (3)	0 (0)	0	2.52 ± 2.58	4	2.05 ± 2.88	4	No data	0
Blood disorders											
Sickle cell disease	15	8.2 ± 5.9 (1–21)	0 (0)	0 (0)	0	1.30 ± 1.32	12	0.99 ± 1.02	11	No data	0
Beta thalassemia	15	7.3 ± 3.7 (1–12)	0 (0)	0 (0)	0	1.89 ± 1.44	11	1.15 ± 0.75	9	No data	0
Anemia[a]	12	6.2 ± 3.8 (0.9–11)	8.3 (1)	8.3 (1)	1214	1.29 ± 1.32	9	1.89 ± 2.88	8	No data	0
Myelodysplastic Syndrome	3	7.3 ± 5.5 (1–11)	33.3 (1)	0 (0)	0	4.86 ± 5.66	2	17.63	1	No data	0
Other conditions											
Wiskott-Aldrich Syndrome	6	3.0 ± 3.4 (0.5–9)	0 (0)	0 (0)	0	0.93 ± 0.64	5	1.22 ± 1.23	4	0.92	1
Testicular torsion/ benign tumor	2	7.5 ± 9.2 (1–14)	50 (1)	0 (0)	0	3.30 ± 4.66	2	0	1	No data	0
Other[b]	18	6.6 ± 5.3 (0.5–16)	22.2 (4)	0 (0)	0	2.46 ± 2.54	12	2.48 ± 4.01	11	0.69	1

CED, cyclophosphamide equivalent dose; UTF1, undifferentiated embryonic cell transcription factor; DDX4, DEAD-box helicase 4

[a]Including Aplastic anemia, Fanconi's anemia, and Diamond Blackfan anemia.
[b]Diagnoses include Farber lipogranulomatosis, hyper IGM syndrome, chronic granulomatous disease, combined immune deficiency, partial androgen insensitivity syndrome, MHC class II deficiency, IPEX syndrome, unilateral abdominal testis, DOCK8 immunodeficiency syndrome, hemophagocytic lymphohistiocytosis, and gonadal dysgenesis

Table III UTF1 and DDX4 positive cells per tubule cross section based on previous chemotherapy exposure.

Age	No chemotherapy				Non-alkylating chemotherapy				Alkylating chemotherapy			
	UTF1+ cells/cross section (±SD)	n	DDX4+ cells/cross section (±SD)	n	UTF1+ cells/cross section (±SD)	n	DDX4+ cells/cross section (±SD)	n	UTF1+ cells/cross section (±SD)	n	DDX4+ cells/cross section (±SD)	n
<1	1.53 ± 0.87	5	1.98 ± 1.01	5	No data	0	No data	0	No data	0	No data	0
1	0.63 ± 0.61	7	0.61 ± 0.63	7	1.41 ± 0.21	2	1.38 ± 0.80	2	0.50	1	No data	0
2	1.42 ± 0.92	4	0.95 ± 0.03	2	0.95 ± 0.92	2	0.48 ± 0.67	2	2.30 ± 0.97	3	2.22 ± 1.31	3
3	1.11 ± 0.18	2	1.69 ± 0.09	2	1.34 ± 0.22	2	1.54 ± 1.47	2	No data	0	No data	0
4	2.12 ± 1.53	4	1.69 ± 1.61	3	1.33 ± 0.16	2	1.95 ± 0.11	2	0.54 ± 0.33	4	0.82 ± 0.57	4
5	0.89 ± 0.64	7	0.70 ± 0.84	5	No data	0	No data	0	No data	0	No data	0
6	2.03 ± 0.43	5	1.61 ± 0.66	5	0.49 ± 0.38	2	0.46 ± 0.52	2	No data	0	No data	0
7	1.57 ± 1.04	8	0.94 ± 1.05	7	No data	0	No data	0	0.74 ± 0.64	4	0.13 ± 0.12	3
8	1.05 ± 0.48	3	0.82 ± 0.74	3	0.30	1	0.52	1	1.90	1	2.47	1
9	1.60 ± 0.99	7	1.64 ± 0.96	6	0.89	1	0.29	1	1.33 ± 1.32	3	1.60 ± 1.80	3
10	2.55 ± 1.36	8	2.11 ± 1.96	7	0.02	1	0.02	1	1.34 ± 0.90	4	1.70 ± 0.53	3
Mean (n)	1.54 ± 1.05	60	1.34 ± 1.15	52	0.94 ± 0.56	13	0.95 ± 0.87	13	1.19 ± 0.94	20	1.34 ± 1.15	17
11	2.87 ± 1.76	9	4.30 ± 3.17	7	8.86	1	17.63	1	4.08	1	7.54	1
12	3.81 ± 2.56	7	5.26 ± 5.91	6	2.99 ± 1.75	2	5.84 ± 3.51	2	3.39 ± 4.31	3	1.21 ± 1.17	2
13	5.18	1	11.42	1	3.22 ± 4.62	3	2.16 ± 2.21	3	6.95 ± 9.40	2	0.28	1
14	7.46 ± 2.11	3	14.33 ± 0.06	2	No data	0	No data	0	1.67	1	2.38	1
>16	2.76 ± 4.39	3	2.06 ± 3.52	3	7.42 ± 5.65	2	15.04 ± 3.41	2	3.64 ± 4.08	2	3.68 ± 3.43	2
Mean (n)	3.84 ± 2.74	23	5.68 ± 5.25	19	4.92 ± 4.17	8	8.2 ± 6.9	8	4.12 ± 4.56	9	2.85 ± 2.85	7

UTF1, undifferentiated embryonic cell transcription factor; DDX4, DEAD-box helicase 4

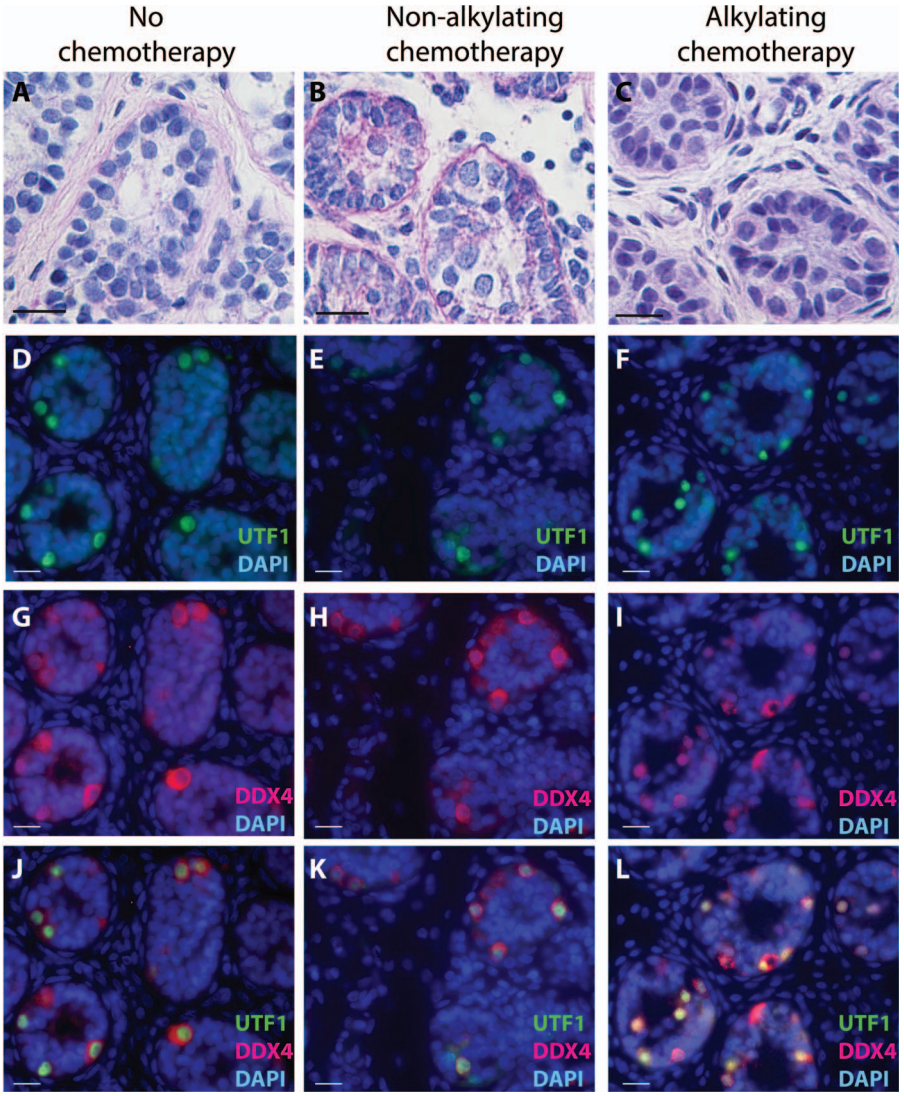

Figure 3 Confirming the presence of germ cells in testicular tissues obtained from patients. (A-C) One hundred and thirty seven patients' samples were stained with Periodic Acid-Schiff and hematoxylin to identify undifferentiated A_{dark} and A_{pale} spermatogonia. The testicular tissues were also stained for undifferentiated embryonic cell transcription factor 1 (UTF1), an established marker of undifferentiated spermatogonia **(D–F** and **J–L)** as well as the pan-germ cell marker DEAD-box helicase 4 (DDX4) **(G–I** and **J–L)**. UTF1 (green) and/or DDX4 (red) immunostaining was confirmed in 132 out of 137 patient tissues that were available for research, including patients who had received previous non-alkylating **(B, E, H, K)** or alkylating **(C, F, I, L)** chemotherapy treatment. Scale bar = 10 μm.

2001, Keros *et al*., 2007, Ginsberg, 2011, Sadri-Ardekani *et al*., 2011, Wyns *et al*., 2011, Goossens *et al*., 2013, Picton *et al*., 2015, Pietzak Iii *et al*., 2015, Onofre *et al*., 2016, Ho *et al*., 2017, Poganitsch-Korhonen *et al*., 2017, Heckmann *et al*., 2018, Stukenborg *et al*., 2018,). Here we show that it is feasible to deploy a standardized protocol for experimental testicular tissue freezing through a coordinated network of recruitment sites. We also demonstrated that it is feasible to provide centralized tissue processing and freezing services for institutions (e.g. children's hospitals) that do not have the infrastructure or expertise to provide fertility services on site. The number of patients recruited at each individual center ranged from 5 to 51, which is probably too small for any single center to make inferences of statistical significance. The coordinated centers approach enhanced our power to characterize the population of patients seeking testicular tissue cryopreservation by age, diagnosis, or previous chemotherapy exposure and determine how those parameters impacted the number of potentially therapeutic spermatogonia in patient samples.

All patient tissues shipped to the central processing center in Pittsburgh were processed within 26 hours, with the exception of one sample that was processed after 91 hours due to a shipping error. A previous study reported that immature non-human primate testicular tissues that had been cold stored for 24 hours could be xenografted and initiate spermatogenesis up to the spermatocyte stage (Jahnukainen *et al*., 2007). Human testicular tissues have been cold stored for up to 3 days at 4°C without altering tissue morphology, Sertoli cell morphology, number of spermatogonia, or number of apoptotic cells

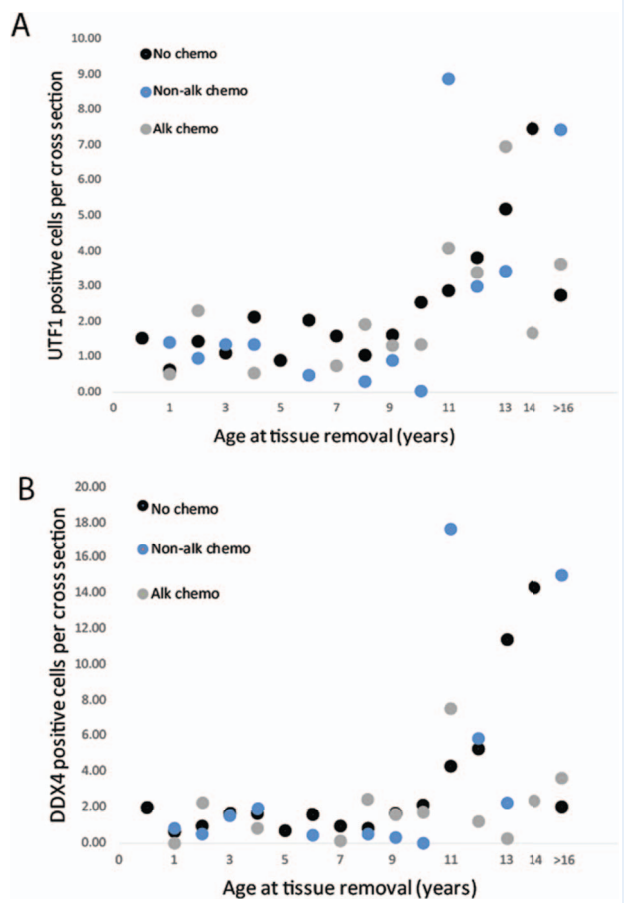

Figure 4 Number of UTF1+ and DDX4+ cells per cross section of a seminiferous tubule, based on previous chemotherapy exposure. (A) Quantification of UTF1+ spermatogonia per cross section of a seminiferous tubule. **(B)** Quantification of DDX4+ spermatogonia per cross section of a seminiferous tubule. Black circles = no previous chemotherapy treatment, blue circles = previous non-alkylating chemotherapy treatment, grey circles = previous alkylating chemotherapy treatment.

Table IV Number of spermatogonia per tubule cross section by age.

Age	A_{dark} spermatogonia/cross section (±SD)	A_{pale} spermatogonia/cross section (±SD)	Spermatogonia/cross section (±SD)	n
0–3	0.62 ± 0.09	0.44 ± 0.33	1.05 ± 0.38	4
4–7	1.62 ± 0.47	0.71 ± 0.82	2.33 ± 2.28	3
8–11	1.99 ± 0.39	0.97 ± 0.37	2.96 ± 0.73	5
12–18	2.72 ± 1.78	1.16 ± 0.99	3.89 ± 2.72	4

(Faes and Goossens, 2016). Collectively, these data suggest that some period of cold storage during shipping may be acceptable. However, more studies are needed to determine the length of cold storage prior to cryopreservation that is compatible with tissue viability and function.

Ideally, fertility preservation procedures would take place before the start of treatment. However, experience of our center and others is that many patients request fertility preservation after treatment of their medical condition has already begun (Chung *et al.*, 2004, Brannigan and Sandlow, 2008, Ho *et al.*, 2017, Heckmann *et al.*, 2018, Stukenborg *et al.*, 2018). This circumstance may be due to ineffective counseling (either not presented by the medical team or not heard by the patient/family) or inability to make a decision in the compressed timeframe between diagnosis and treatment. Among patients enrolled through our coordinated network of centers, 39% (74 out of 189) had received some form of chemotherapy before requesting fertility preservation. Since more than one-third of our patients had received some form of chemotherapy, it was important to determine whether these patients still have spermatogonia in their testicular tissue.

We showed by immunohistochemistry that 132 of 137 patients for whom research tissues were available for analysis had undifferentiated spermatogonia present in their testicular tissue. Our results show that the number UTF1+ and DDX4+ cells increase with age. There was no statistical difference for number of UTF1+ and DDX4+ cells between different diagnoses or between patients exposed to previous chemotherapy versus those that did not receive previous treatment. However, those results must be interpreted with caution because spermatogonia in chemotherapy-treated samples were not tested functionally due to the limited size of samples allocated to research. Recently, reference values for spermatogonia quantity in testes of healthy children were published (Masliukaite *et al.*, 2016), indicating a trend toward decreased spermatogonia number over the first 3 years of life; an increase until the age 6 or 7; a plateau until the age of 11 years; and a sharp incline after that. Our data are partially consistent with those observations. We observe that the number of UTF1+ and DDX4+ spermatogonia/tubule cross section increases steadily until about age 11, and then rises sharply. The apparent difference in the first decade of life could be that we used molecular markers for this analysis instead of the morphological descriptions of A_{dark} and A_{pale} spermatogonia. However, our analysis of A_{dark} and A_{pale} spermatogonia in a limited series of 16 patients tend to support our observation of an age-related increase in undifferentiated spermatogonia, which may have implications for how those tissues are used in future fertility applications.

Freezing reproductive cells/tissues for patients with previous chemotherapy exposure may raise questions about the safety of using those samples for reproduction. In 2011, Stahl *et al.* (2011) observed a modest, but statistically significant increase in congenital abnormalities among males with a history of cancer diagnosis. In contrast, Nielsen *et al.* (2018) reported that children of young cancer survivors (i.e. patients who were exposed to gonadotoxic treatments) are not at a higher risk of chromosomal abnormalities than their siblings. Meirow *et al.* (2016) reported that previous chemotherapy exposure was not associated with adverse outcomes from transplanted ovarian tissue in women.

For adult cancer patients, the current recommendation is to wait 18–24 months after completing therapy before attempting to have a family (Nangia *et al.*, 2013). This allows time for damaged mitotic and meiotic germ cells to be cleared from the testis and new spermatogenesis

to arise from stem cells, which are less susceptible to chemotherapy and radiation-induced DNA damage (Meistrich, 1986, Meistrich, 1993). There are a few reports of men who have fathered children during chemotherapy treatment (Kroner and Tachumi, 1977, Blatt *et al*., 1980, Gulati *et al*., 1986). Out of the four babies born, three were healthy but one had a heart murmur and failure to thrive.

Patient/family and physician awareness of the reproductive side effects of medical treatments as well as options for preserving fertility have improved during the past decade. Spermatogonial stem cell transplantation from frozen/thawed testicular cell suspensions have been reported for seven Hodgkin's disease patients in the UK, but fertility outcomes from that study have not been reported (Radford, 2003). Therefore, there is insufficient evidence to support removing the experimental designation from testicular tissue/cell cryopreservation at this time. However, this topic should be revisited as clinical evidence accumulates describing the safety of testicular tissue cryopreservation. This is important to maximize access to care for all patients, as some states in the United States are beginning to require fertility preservation coverage for standard procedures (http://www.allianceforfertilitypreservation.org/advocacy/state-legislation).

Our experience indicates that testicular tissue biopsy and cryopreservation is feasible in patients ranging from 5 months to 34 years of age. We recommend that all patients be counseled and referred for fertility preservation (standard or experimental) before gonadotoxic treatments begin. However, our data suggests that it is possible to retrieve and preserve testicular tissue with spermatogonia for patients who are in the early stages of their treatment. This may expand access to fertility preservation care for some patients.

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Authors' roles

H.V.P., K.A.P., K.G., S.R.S., M.S., B.P.H., L.D., S.D., E.L., A.F.P., and M.A.A. conducted the experiments. H.V.P., K.G., B.P.H., S.K.M., A.F.P., T.C., and K.E.O. analyzed and interpreted the data. H.V.P. and K.E.O. wrote and revised the manuscript. R.C., G.M.M., P.J.F., T.M.J., J.S.S., M.N.M., M.H., L.S.S., J.M., L.K., Y.G., E.R., M.S., C.F.G., P.P.R., J.I.S., and K.E.O. contributed to the conception and study design.

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Conflict of interest

The authors have declared that no conflict of interest exists.

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