Human Reproduction, Vol.25, No.1 pp. 37–41, 2010
Advanced Access publication on October 27, 2009 doi:10.1093/humrep/dep371

human reproduction

ORIGINAL ARTICLE *Andrology*

An experimental protocol for fertility preservation in prepubertal boys recently diagnosed with cancer: a report of acceptability and safety

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BACKGROUND: Gonadal damage is a consequence of therapy for pediatric malignancies. Prepubertal males have no semen or mature spermatozoa, posing a challenge for fertility preservation. Testicular tissue cryopreservation is a potential option but is still experimental. We report on a pilot protocol that offered testicular biopsy cryopreservation to families of prepubertal boys with newly diagnosed malignancy. The aims were to determine the acceptability and safety of this procedure.

METHODS: Parents of prepubertal boys with diagnoses at highest risk for treatment-related gonadal damage were offered the option of testicular cryopreservation. Half of the biopsy was frozen for the subject's potential future use and the remainder used for research. Data on negative intraoperative and/or 7 day post-operative sequelae of testicular biopsies were assessed. Two to four weeks later, parents were asked to complete a questionnaire on factors influencing their decision to have the biopsy or not.

RESULTS: Since January 2008, 24 boys have met the eligibility criteria but three required immediate treatment and were excluded. Sixteen of 21 families (76%) consented to testicular biopsy, indicating the prospective acceptability of this option to parents of boys aged 3 months to 14 years; 14 underwent the procedure without any negative intra- or post-operative sequelae. Although the time at diagnosis is stressful, families can give thoughtful consideration to this option. Factors such as religion, finance, ethics and the experimental nature of cryopreservation did not play a major role in decision-making.

CONCLUSIONS: Parents of prepubertal boys with cancer are willing to pursue testicular tissue cryopreservation at diagnosis, and testicular biopsy caused no acute adverse effects.

Key words: testicular cryopreservation / prepubertal males / fertility / pediatric malignancy

Introduction

Over the last several decades, survival rates for childhood cancer have steadily increased. With the overall cure rate for pediatric malignancies now approaching 80%, estimates indicate that one in every 640 young adults in the USA will be a survivor of childhood cancer (Hewitt *et al.*, 2003). Unfortunately, many survivors struggle with medical late effects of their treatment, including infertility (Howell and Shalet, 1998, 2001; Bath *et al.*, 2002; Larsen *et al.*, 2003; Wallace *et al.*, 2005; Sklar *et al.*, 2006). Gonadal damage is a relatively common consequence of the treatments used to cure pediatric cancer. The extent of cytotoxic germ cell damage depends on the specific agents used and the cumulative doses received. Alkylating agents are the most common class of drugs known to affect gonadal function and their impact has been studied extensively (Meistrich *et al.*, 1992; Rivkees and Crawford, 1988). Additionally, the testes have a very low threshold for radiation exposure, and even small doses are known to be gonadotoxic (Rowley *et al.*, 1974; Ash, 1980).

As treatment regimens for pediatric malignancies have improved, more and more survivors are entering their reproductive years

(Mertens *et al*., 2001). Maintenance of fertility is extremely important with regard to long-term quality of life for these survivors (Schover *et al*., 2002; Jeruss and Woodruff, 2009). Consideration must be given to whether a child's fertility is likely to be impacted by his or her treatment. Ideally, this should occur before the onset of therapy, when a window of opportunity may exist to preserve the patient's future reproductive potential (Chen *et al*., 1996; Kliesch *et al*., 1996; Opsahl *et al*., 1997; Jeruss and Woodruff, 2009). Pubertal males may produce a semen sample prior to starting gonadotoxic therapy and the sperm are cryopreserved for future use. Unfortunately, prepubertal males pose a particular challenge for fertility preservation. These boys cannot produce semen for cryopreservation. In addition, although the germ cells of the prepubertal testis include spermatogonial stem cells (SSCs), they do not yet have mature spermatozoa. For these at risk prepubertal boys, current practice does not provide any options for fertility preservation at diagnosis. A potential approach to this issue is the use of cryopreserved testicular tissue. Although significant strides have been made in animal research in this area, translational use of testicular tissue cryopreservation in humans remains experimental (Bahadur *et al*., 2000; Wallace *et al*., 2005). Ideally, prepubertal testicular tissue could be acquired and banked prior to initiating gonadotoxic cancer therapy. Years later, once the patient is ready to begin a family, this tissue could then be thawed and the germ cells reimplanted into the patient's own testes to continue full maturation there (Brinster and Zimmermann, 1994, Brinster, 2007). Alternatively, the stored cells could be matured *in vitro* until they can be used for ICSI or be reimplanted into the testes. Thawed testes tissue could also be used potentially for grafting purposes (Wyns *et al*., 2007).

In rodents, spermatogonial transplantation has resulted in restored spermatogenesis, and mice have reproduced *in vivo* (Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994). Although steady progress has been made in fertility-based animal research with SSCs, hurdles remain for transferring this science into the clinical setting for prepubertal boys newly diagnosed with cancer.

Infertility is an issue many families struggle with as they agree to initiate chemotherapy for their sons. This manuscript reports on our experience to date with a pilot protocol aimed at offering testicular cryopreservation to families of newly diagnosed prepubertal boys. We have considered several key clinical points, including the acceptability of this procedure at a stressful time, beliefs and factors that influence the decision-making of parents of prepubertal boys with cancer regarding fertility, the ability to perform a testicular biopsy without negative sequelae, and the logistics of tissue storage for both assisted reproduction and research purposes.

Materials and Methods

A multidisciplinary team was assembled to conduct testicular cryopreservation including a Pediatric Oncologist, Research Nurse, Pediatric Urologist, Reproductive Endocrinologist, Andrology Laboratory Technician and Laboratory Research Scientists. This team encompassed all of the essential personnel necessary to carry out the diverse and complex procedures of testicular tissue procurement, tissue cryopreservation and the laboratory science required for successful SSC expansion. Utilizing the expertise and infrastructure of this interdisciplinary team, a research protocol was developed for testicular tissue acquisition and distribution. After full committee review, the Institutional Review Board at The Children's Hospital of Philadelphia (CHOP) approved the pilot study.

Families of prepubertal boys newly diagnosed with Stage IV neuroblastoma, rhabdomyosarcoma, osteosarcoma or Ewing sarcoma were offered the opportunity for testicular biopsy/cryopreservation. Diagnoses of histological variants of these sarcomas, and those whose treatment would include sarcoma-like therapy with agents that place the male at high risk for infertility, were also eligible. Such patients were the target of this pilot given that their therapy would be highly gonadotoxic. Pubertal males with one of these diagnoses who attempted to bank sperm but failed were also eligible for this study. Patients with a coagulopathy, cryptorchidism or testicular involvement of their tumor were excluded.

The consent process carefully detailed 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. Once parental consent was obtained, an open testicular biopsy was performed by a urologist at CHOP during a procedure when the patient was already under general anesthesia for their clinical care, i.e. primary tumor biopsy or resection, central line placement and/or bone marrow aspirates/biopsies. In order to minimize the risk of additional exposure to anesthesia, a separate operative procedure for the testicular biopsy alone was not allowed per the approved protocol. The biopsy procedure always occurred before any cancer therapy was initiated.

In the operating room, testicular biopsy specimens were divided immediately. Half of the specimen (about 40 mm^3) was sent for storage at the Hospital of the University of Pennsylvania (Penn Fertility Care) for potential use by the patient at a later date. A freezing protocol currently in place for adult human testicular tissue cryopreservation was implemented for the current proposal, and has been published in the literature for use with prepubertal testicular tissue (Keros *et al*., 2007; Wyns *et al*., 2007). Specifically, 5% dimethyl sulfoxide is used as a cryoprotective agent in conjunction with a slow programmable freezer. This method has been demonstrated to preserve prepubertal testicular tissue, specifically cell structure and viability (Keros *et al*., 2007). The other half of the specimen (about 40 mm^3) was divided into two portions. The larger specimen (39 mm^3) was used for research purposes to advance the techniques for isolating and culturing human SSCs, as well as studies of gene expression in testicular germ cells (Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994; Kubota *et al*., 2004; Oatley *et al*., 2006; Brinster, 2007). The remaining tissue was reserved for histological analysis.

During the operative procedure and after the testicular biopsy patients were followed for any adverse outcomes, including excessive pain, bleeding or infection. Events were tracked and recorded for 1 week after the procedure, a time frame well beyond the typical 2–3 day recovery time that is associated with this type of biopsy.

Parents of prepubertal males approached as part of this pilot study (regardless of whether they decided to allow a testicular biopsy or not) were asked to complete a questionnaire concerning their beliefs about fertility and what factors influenced their decision. Those parents who actually chose to cryopreserve their son's tissue were also asked questions regarding their experience with the procedure itself. The questionnaire used in the current study was modified from our recently published method employed with pubertal males and their parents (Ginsberg *et al*., 2008). Questionnaires were administered within 2–4 weeks following the operative procedure so that the parent would have adequate recall of the events surrounding the decision. The questionnaire explored the following areas: occupation and educational level of parents, beliefs about whether cancer therapy would impact fertility, timing of initial conversations with health care providers about testicular cryopreservation, initial reaction to the idea of tissue cryopreservation, and what factors influenced the decision to cryopreserve or not.

Results

Since January 2008, 24 boys met eligibility criteria for this study. Three of these boys were not approached because their clinical condition demanded therapeutic intervention before the study team could reasonably speak to the family about the study. Of the 21 eligible boys (median age of 5 years, range 3 months to 14 years) approached for this experimental protocol, 16 families consented to the testicular biopsy (76%) (Table I). Fourteen of the 16 actually underwent testicular biopsy. For two patients the histological analysis of the frozen tumor biopsy was not consistent with malignancy and therefore no testicular biopsy was performed. The 76% rate of acceptance demonstrates the willingness of these families to participate in testicular cryopreservation.

Once a subject was identified as a possible testicular cryopreservation candidate, the interdisciplinary team worked together to make contact with the treating oncologist, to explain the study procedures and obtain consent, to confirm scheduling of the procedure and to coordinate retrieval of the tissue specimens from the operating room. The infrastructure in place for this process worked efficiently, and testicular biopsies were successfully obtained on all 14 prepubertal boys with no intra- or post-operative adverse events. Specifically, no patient had to return to the operating room for bleeding, was treated for orchitis, or sustained loss of a testis secondary to infection or bleeding. Furthermore, post biopsy there were no reports of excessive pain.

All 21 parents agreed to answer the study specific questionnaire including the two whose sons did not need to go through with the biopsy and the five who refused the testicular biopsy. Our observations indicate that when first informed of their child's cancer diagnosis, the majority of parents did have an understanding that treatment may affect fertility (Table II). Sixty-eight percent of parents who agreed to the testicular biopsy felt that the possibility of freezing tissue for future use was 'a great idea for my son', whereas all of those who refused the biopsy indicated 'not sure if this is right' (Table II). A larger percentage of those who chose not to have the biopsy endorsed the idea that parents are too overwhelmed at diagnosis to hear about testicular tissue cryopreservation (80 versus 31%). Those who did choose biopsy indicated that, although it can be overwhelming, the option needs to be discussed and, whereas there never may be the ideal time, as early as possible is best.

For all 21 parents who answered the questionnaire, religious beliefs, financial aspects and ethical issues did not appear to be major factors in the decision-making process (Table III). The experimental nature of the cryopreservation process also did not play a significant role in the decision-making process. However, the fact that frozen testicular tissue has not yet been used for successful human pregnancies was considered by a larger percentage of parents who opted against the biopsy than those who agreed. Of the 16 who agreed to the biopsy, all endorsed the concept that 'fertility is important to preserve, even though no guarantees were given regarding the ultimate

Table I Age and diagnosis of prepubertal boys whose parents were approached about testicular biopsy and cryopreservation

Patient	Age at diagnosis	Diagnosis	Parental consent to biopsy
1	3 months	Pleuropulmonary sarcoma	Yes
2	3 years	Neuroblastoma	No
3	3 years	Neuroblastoma	Yes
4	14 years*	Ewing sarcoma	No
5	9 months	Pleuropulmonary sarcoma	Yes
6	8 years	Ewing sarcoma	Yes
7	2 years	Rhabdomyosarcoma	Yes
8	1 year	Rhabdomyosarcoma	No
9	9 years	Synovial sarcoma	Yes
10	5 years	Rhabdomyosarcoma	No
11	10 years	Ewing sarcoma	Yes
12	5 years	Langerhans cell histiocytosis	Yes**
13	10 years	Ewing sarcoma	Yes
14	4 years	Malignant rhabdoid tumor	Yes
15	4 years	Rhabdomyosarcoma	No
16	11 years	Neurofibroma	Yes**
17	8 years	Rhabdomyosarcoma	Yes
18	8 years	Osteosarcoma	Yes
19	2 years	Neuroblastoma	Yes
20	6 years	Neuroblastoma	Yes
21	2 years	Neuroblastoma	Yes

*Attempted but unsuccessful sperm banking.
**Consented, but then did not need biopsy.

Table II Initial reactions of parents to testicular cryopreservation protocol

	Agreed to biopsy (n = 16) (%)	Refused biopsy (n = 5) (%)
Potential for infertility		
At diagnosis, thought treatment would or might affect fertility	75	80
Timing of discussion		
Think parents are too overwhelmed at diagnosis to hear about testicular tissue cryopreservation?	31	80
Initial reaction to idea of testicular cryopreservation		
'Great idea, this is right thing for my child'	68	0
'Not sure if this is something that is right for my child'	19	100
'No way, I know that I do not want to do this'	13	0

Table III Factors influencing the decision of parents for prepubertal son to undergo testicular biopsy, with tissue cryopreservation

Factor	Agreed to biopsy (n = 16)* (%)	Refused biopsy (n = 5)* (%)
Religious beliefs	19	20
Ethical issues	25	0
Financial considerations	25	0
Too overwhelmed by diagnosis	44	80
Limited time to decide	69	60
Risk of testicular biopsy	88	60
Experimental nature of freezing procedure	44	20
Frozen testicular tissue never used in humans to achieve pregnancy	38	60
Health of frozen tissue when thawed	50	40
Hopeful that science will advance	100	20
Worth opportunity, even though no guarantees	100	20

*Percentage of parents who affirmed the factor was considered.

outcome'. Moreover, of the 16 families that accepted the testicular cryopreservation protocol, all indicated post biopsy that 'they made the right choice, even if the tissue cannot be used in the future to restore their son's fertility'. Based upon the high rate of acceptability, strong positive nature of the initial observations from the questionnaires, the demonstrated safety of the testicular biopsy procedure, and confidence in the unique infrastructure in place to support the process, we are encouraged to continue this project.

Discussion

Gonadotoxic consequences of therapy can be a daunting prospect for newly diagnosed males with cancer. Sperm banking has become the gold standard for fertility preservation in pubertal males. However, because of the physiologic limitations of the immature testis, prepubertal males do not currently have an option for fertility preservation at diagnosis. Testicular biopsy and tissue cryopreservation holds promise for this cohort of patients. Additional scientific advances are still needed to translate successes in animal research to human clinical practice.

Because this study requires a testicular biopsy at the time of cancer diagnosis, parental desire and acceptability of testicular tissue cryopreservation as well as the safety of this procedure were of primary importance. Researchers have interviewed 318 parents regarding their acceptance of such an idea (van den Berg *et al*., 2007): they asked parents to think hypothetically about the following scenario, 'If there was an experimental procedure available at diagnosis, would you allow your sons to undergo a testicular biopsy in an attempt to collect SSCs?', and asked them to imagine themselves back at the time of diagnosis when answering this question. At diagnosis, SSC collection by means of testicular biopsy was theoretically approved by 61% of these parents (van den Berg *et al*., 2007). These data indicate that the transfer of methods used in current animal experiments on SSC collection and transplantation into clinical care is highly desired by parents of prepubertal boys with cancer. The high acceptance rate of our current prospective pilot protocol (76% chose to go through with the biopsy) affirms and surpasses the hypothetical acceptance shown in this earlier research. Families are interested in this option and are willing to undergo the procedure in real clinical practice, even when there are no clinical guarantees.

Although our current sample size is small, our first year of experience with this pilot protocol is highly encouraging. Development of the proper infrastructure and an interdisciplinary team is at the cornerstone of the pilot's success. This experimental protocol requires collaboration between clinicians and research scientists. It was important to identify experts who had the knowledge and the physical resources to support the proposed research. Prior to any patient enrollments, great care was taken to cultivate our relationships with these experts and to procure the appropriate equipment and media for both the laboratory aims of the study as well as storage of the specimens for future clinical use. With each patient enrolled on the study, the team learned how to improve our processes for identification and recruitment of eligible subjects who were at high risk for infertility, how to communicate both within our team and with other health care providers involved with the patient's care, and how to coordinate the acquisition and distribution of the tissue for clinical use and research. Flexibility was crucial, but we found that with the appropriate infrastructure and a committed interdisciplinary team, this type of approach can be successful.

Several barriers were encountered that merit consideration. First, oncology providers do not automatically consider fertility preservation options as part of the standard workup of a newly diagnosed prepubertal male. To ensure that this opportunity is offered to all eligible prepubertal boys who meet the proposal's selection criteria, our intake team must be diligent in screening patient lists and new patient referrals to the Division. Once identified, the team must contact the patient's caregiver as early as possible, making them aware of the study and finding the appropriate time to approach the family for consent.

An additional potential barrier to the 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. The questionnaire data from our pilot demonstrate that, although the time at diagnosis is stressful, families want to be presented with options and are able to make thoughtful decisions about fertility preservation.

From a safety perspective, there have been no acute adverse effects of the testicular biopsy, and this procedure is well tolerated. Although the long-term impact of testicular biopsy on newly diagnosed males with cancer is not currently known, there are long-term data on cryptorchid boys who have had testicular biopsies performed during orchiopexies (Patel *et al*., 2005). In this cohort, a total of 112 patients who had previously undergone orchiopexy and a testicular biopsy at a mean age of 8.6 years were asked to return for long-term follow-up. The mean age at follow-up was 18.6 years. All patients underwent an

exam and bilateral scrotal ultrasound. On ultrasound, no patient had evidence of testicular atrophy or testicular damage related to testis biopsy. Moreover, a semen sample was collected and 57 of the 112 patients underwent measurement of antisperm antibodies. None of the 57 semen samples showed evidence of antisperm antibodies. There was no evidence of additive testicular damage in prepubertal boys who had testicular biopsies (Patel *et al.*, 2005). The technique for testicular biopsy is the same as that used in the current research.

Over the last several decades, reproductive scientists have continued to make remarkable strides in developing fertility preservation options and expanding the cadre of assisted reproduction technologies that are available for achieving successful live births. If the pace of reproductive advances to date is any indicator of future successes, there is reason to be hopeful that the laboratory techniques developed for utilizing cryopreserved testicular tissue to restore fertility can be translated for use in human subjects.

Acknowledgements

The authors thank Dr Christos Coutifaris, Chief, Division of Reproductive Endocrinology and Infertility, for his collaboration.

Funding

Ethel Foerderer Award, CHOP (J.P.G.), NICHD grant HD 061217 (J.P.G.), NICHD grant HD 052728 (R.L.B.), and the Robert J. Kleberg, Jr. and Helen C. Kleberg Foundation (R.L.B.).

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Submitted on June 23, 2009; resubmitted on September 22, 2009; accepted on September 24, 2009