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FERTILITY PRESERVATION

Evaluation of ovarian and testicular tissue cryopreservation in children undergoing gonadotoxic therapies

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Abstract
Objective Ovarian and testicular tissue cryopreservation are the only fertility preservation options for sexually immature individuals. Because of their experimental nature, it is important to determine safety and possible bundling with other medicallyindicated procedures.
Study design Prospective observational.
Results Cryopreservation indications included cancer in 75 % of females and 50 % of males, while non-cancer indications included various hematological conditions. Similar numbers of females (12/28) and males (3/9) underwent prior chemotherapy. Females underwent laparoscopic (27/28) or robotic (1/28) approaches while incisional biopsy was used in males. Bundling of ovarian and testicular harvesting with other medicallyindicated procedures was performed in 42 % and 22 %, respectively. The operative time inclusive of bundled procedures was similar (1.6±0.1 vs. 0.9±0.3 h) but the discharge time was significantly longer for females than males (10.4±0.6 vs. 4.6±0.6 h, $p<0.05$) due to frequent bundling of medically-indicated procedures in females. All procedures were successfully completed without complications or significant blood loss.
Conclusions Pediatric gonadal tissue cryopreservation can be combined with other medically-indicated procedures to minimize the potential inconvenience, additional anesthetic risks, and costs.

Keywords Hematology/oncology · Children · Cryopreservation · Fertility preservation

Capsule Ovarian and testicular cryopreservation is feasible alone or in combination with other procedures in children.

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Introduction

Increased incidence in childhood cancer, with an estimated 10,700 cases in 2010 for ages 0–14 years in the U.S., as well as improvement of overall 5-year survival to around 80 %, have resulted in a rise in the number of long-term cancer survivors [2]. Treatment regimens for malignancies and blood dyscrasias commonly include gonadotoxic chemotherapy and radiotherapy, with significant impact on future fertility potential [36]. Furthermore, loss of fertility has been shown to significantly compromise quality-of-life, especially in younger cancer survivors [49]. Chemotherapy regimens containing alkylating agents such as cyclophosphamide and busulfan, as well as radiotherapy, commonly used in the pediatric population, have considerable gonadotoxic effects, and can result in ovarian failure in >80 % of children, especially in the preconditioning setting for hematopoietic stem cell transplantation (HSCT) [9,24,36,48]. Preconditioning chemotherapy with or without total body irradiation prior to HSCT is also associated with a high rate of gonadal failure in males [39]. Female, long-term survivors of childhood cancer may undergo normal pubertal development but commonly show signs of diminished ovarian reserve, which may adversely affect the likelihood of future pregnancy [19,23,41]. The utility of HSCT is increasing because of expanding indications in non-cancer hematological and immunological conditions [8]. As a result, the number of child survivors with compromised gonadal function has been rising.

Recent recommendations by the American Society for Clinical Oncology have supported the use of fertility preservation technologies in patients undergoing potentially gonadotoxic therapies [21]. A number of fertility preservation strategies have been developed utilizing embryo, oocyte, sperm and gonadal tissue cryopreservation [36,37]. However, because of sexual immaturity, gamete cryopreservation is impractical in the prepubertal pediatric age group, and can only be used in postpubertal children [12,31,32,39]. Since ovarian or testicular cryopreservation is not dependent on the production of mature oocytes or sperm, these procedures are the only practical, yet experimental, options for fertility preservation in children [30]. While there are no reports of success with testicular tissue freezing, several recent studies have shown restoration of ovarian function, and/or live births resulting from transplantation of cryopreserved ovarian tissue [3,10,25,26,28,29].

Given the experimental nature of the gonadal tissue cryopreservation procedures, their safety should be evaluated prior to the acceptance into the clinical practice of pediatric hematology-oncology, and these procedures should be bundled with other medically indicated procedures when possible. Thus we performed the current study to evaluate the safety and technical feasibility of gonadal cryopreservation procedures and their bundling with other procedures in a pediatric patient population.

Materials and methods

Subjects

This study was approved by the Institutional Review Board. Study subjects were 28 females and 9 males who were referred by their hematologist-oncologists, or in one case, pediatric endocrinologist, for fertility preservation. All subjects provided written informed consent or, in the case of minors, parental consent was obtained. In addition, written assent was obtained from all minors aged 9–13. In all cases, medical and/or surgical oncologist or hematologists' approval was required before the patients were admitted to the study. Only after thorough informed procedure consent, multiple discussions and consultations with the oncologist and after a surgical risk assessment that the subjects were allowed to undergo tissue-harvesting procedures. Parents were clearly explained the experimental nature of the procedure and especially in the case of boys lack of any documented success. Parents were well informed that though minimally invasive, the procedure might not result in future restoration of fertility. Subject demographic, treatment characteristics, operative data including surgical complications, estimated blood loss, anesthesia time, and discharge time were recorded. We used the National Institutes of Health criteria for pediatric human subjects research and hence included those under the age of 21. Assessment of gonadal maturity in females and males was performed in both genders as part of a routine workup prior to surgical consultation (Table 1). Whenever possible the procedures were bundled with a Porta-A-Cath (Smiths Medical, Dublin, OH) insertion for chemotherapy administration.

Tissue harvesting and cryopreservation

The ovarian tissue harvesting technique has been previously described by our laboratory [26,27,31]. All procedures were performed laparoscopically with one being assisted with the Da Vinci Robot (Intuitive Surgical Inc., Sunnyvale, CA).

For testicular tissue harvesting preoperative assessment was made by history and physical examination to exclude any co-existing testicular conditions potentially affecting future fertility. The left testis was usually chosen for biopsy since fewer (future) testicular disorders affecting fertility occur on the right side. Biopsy was sometimes done at the same time as port placement for subsequent chemotherapy. After exposing the testis a portion representing up to approximately 15 % of the volume was excised in the antero-

Table 1 Characteristics and indications for fertility preservation in females (n=28)

Characteristics	
Age at diagnosis (years)	13.9±1.5; (range: 2.3, 20.9)
Age at tissue harvesting (years)	14.6±1.2
Length of cryopreservation (years)	5.2±0.8
Prior chemotherapy	50 % (12/24)
Indications for fertility preservation	
Cancer diagnosis	75 % (21/28)
Acute myelogenous leukemia	3
Acute lymphoblastic leukemia	4
Bladder sarcoma	1
Breast infiltrating ductal adenocarcinoma	1
Ewing sarcoma	1
Hodgkin's lymphoma	3
Myelodysplastic syndrome	1
Myeloproliferative disease	1
Non-Hodgkin's lymphoma	1
Ovarian papillary serous adenocarcinoma	1
Primitive neuroectodermal tumor	1
Teratoma	1
Undifferentiated sarcoma	1
Yolk sac tumor	1
Non-cancer diagnosis	25 % (7/28)
Aplastic anemia	1
Diamond-Blackfan anemia	1
Severe combined immunodeficiency syndrome	1
Sickle cell anemia	1
Systemic lupus erythematosus	1
Thalassemia major	1
Triploidy (46XX/69XXY)	1

superior portion of the testis and immediately divided into portions for routine histological examination, scientific study and cryopreservation for future fertility procedures. The capsule was closed and the testis was returned to the scrotum.

The details of the cryopreservation techniques have been described previously [28]. In all malignancy cases small samples of gonadal tissue were also sent for routine pathological assessment to rule out any gonadal involvement by the primary cancer.

Statistics

Means±SEM are reported. A t-test was used for comparison of means while the chi-squared test was considered for analysis of proportions. A p value <0.05 was used as statistically significant.

Results

Female subject characteristics and indications for cryopreservation

A summary of baseline characteristics for female subjects is shown in Table 1. Twelve patients were previously reported [31]. Seventeen subjects had regular menstruation (mean age 17.8±0.8 years), two showed irregular menstruation (mean age 17.9±1.2 years), and nine subjects were prepubertal (mean age 5.5±1.3 years). The mean age at diagnosis was 13.9±1.5 years and ranged from 2.3 to 20.9 years. The mean age at cryopreservation surgery was 14.6±1.2 years and ranged from 2.3 to 21.0 years. Fifty percent (n=12) of patients underwent some chemotherapy prior to fertility preservation consultation. The mean length of tissue cryopreservation was 5.2±0.8 years and thus far no subject has requested tissue transplantation. Only three girls had preoperative analysis of AMH levels yielding a mean value of 1.6 ±1.0 ng/ml at an average age of 20.0±1.4 years. These levels are sufficient to consider ovarian cryopreservation but low for the given age reflecting the history of recent chemotherapy in two of these girls. Of note AMH levels can temporarily be reduced by even chemotherapy agents that do not damage ovarian primordial follicle reserve as AMH is produced from developing follicles and the latter are more sensitive to chemotherapy. Hence the lower AMH levels may not reflect reduced reserve. In fact these patient had received non-alkylating agents and the primordial follicle densities were comparable to age-matched controls [33].

Fertility preservation indications included diagnosed malignancy in 75 % of females (n=21) and non-cancer indications in 25 % (n=7) of subjects (Table 1). The most common cancer diagnoses were leukemia in (n=7), including acute myelogenous leukemia (n=3) and acute lymphoblastic leukemia (n=4). Lymphoma was also commonly seen (n=4), including Hodgkin lymphoma (n=3) and non-Hodgkin lymphoma (n=1). Other cases included bladder sarcoma, breast infiltrating ductal adenocarcinoma, Ewing's sarcoma, myelodysplastic syndrome, myeloproliferative disease, ovarian papillary serous adenocarcinoma, primitive neuroectodermal tumor, ovarian immature teratoma, undifferentiated sarcoma of the pelvis and a yolk sac tumor of the ovary. In case of ovarian cancer, gynecologic oncologist performed full evaluation pre and intra-op to rule out contralateral ovarian involvement if there was one. The non-cancer diseases included hereditary anemia (n=4), which included aplastic anemia (n=1), Diamond-Blackfan anemia (n=1), sickle cell anemia (n=1), and thalassemia major (n=1). Other diseases included severe combined immunodeficiency syndrome, systemic lupus erythematosus, and a diploidy/triploidy (46XX/69XXY). All subjects underwent ovarian cryopreservation because of

impending chemotherapy except one subject with diploidy/triploidy who underwent bilateral prophylactic oophorectomy for potential risk of gonadoblastomas.

Male subject characteristics and indications for cryopreservation

Baseline characteristics of nine male subjects are summarized in Table 2. The mean age at diagnosis was 5.0±2.4 years with a range of 0.1 to 12.7 years. Furthermore, the mean age at cryopreservation surgery was 7.0±1.5 years with a range of 1.0 to 12.8 years. Three subjects had undergone previous chemotherapy. The mean length of tissue cryopreservation was 1.4±0.1 years. Tanner stage was 2±1 at the time of the procedures. Only one subject was reportedly capable of erection and none was capable of ejaculation.

Indications for cryopreservation are summarized in Table 2. Neoplasia was diagnosed in 45 % of subjects (n=4) (Acute myelogenous leukemia, Ewing sarcoma, Hodgkin's lymphoma and optic glioma) and blood dyscrasias were the main disorders in the other 55 % (n=5) (hyper IgM syndrome [n=1], sickle cell anemia [n=2], and thalassemia major [n=2]).

Cryopreservation procedure and outcomes

Surgical approaches, findings, and outcomes are summarized in Table 3. All surgeries were performed under general anesthesia. For females, a standard laparoscopic procedure was used in 27 subjects while a robotically assisted laparoscopy was used in one subject. Partial oopherectomy was used in 21.4 % (n=6), unilateral oophorectomy in 71.4 % of females (n=20), and bilateral oophorectomy in 7.1 % of females (n=2). One of the latter subjects underwent prophylactic bilateral oophorectomy at time of tissue harvesting. All males underwent open biopsy procedures (n=9) and gonadectomies were not performed in males.

Table 2 Characteristics and indications for fertility preservation in males (n=9)

Characteristics	
Age at diagnosis (years)	5.0±2.4; (range: 1.0, 12.8)
Age at tissue harvesting (years)	7.0±1.5
Length of cryopreservation (years)	1.4±0.1
Prior chemotherapy	33.3 % (3/9)
Indications for fertility preservation	
Cancer diagnosis	45 % (4/9)
Acute myelogenous leukemia	1
Ewing sarcoma	1
Hodgkin's lymphoma	1
Optic glioma	1
Non-cancer diagnosis	55 % (5/9)
Hyper IgM syndrome	1
Sickle cell anemia	2
Thalassemia major	2

Table 3 Operative characteristics of gonadal tissue harvesting

	Females (n=28)	Males (n=9)	P-value
Estimated blood loss (ml)	≤10	≤5	
Operation time (hours)	2.3±0.4	0.9±0.3	NS
Anesthesia time (hours)	4.9±1.2	1.6±0.3	<0.05
Discharge time (hours)	23.2±8.9	4.6±0.6	<0.05
	Bundled (n=15)	Unbundled (n=22)	P-value
Operation time (hours)	2.5±0.5	1.1±0.2	NS
Anesthesia time (hours)	4.8±1.2	1.7±0.3	NS

All surgical procedures were performed with no complications and minimal blood loss. The mean operative time ("skin-to-skin inclusive of bundled procedures") was longer for females but this did not reach statistical significance between females (2.3±0.4 h) and males (0.9±0.3 h). Blood loss was minimal or none (≤10 ml) in both genders. Anesthesia time (intubation to extubation) was significantly greater for female subjects compared to male subjects (4.9±1.2 vs. 1.7±0.3 h, p<0.05). Discharge time was significantly reduced for males with a mean of 23.2±8.9 h compared to a mean in females of 10.4±0.6 h (p<0.05). Two male subjects who had been diagnosed with acute myelogenous leukemia and Hodgkin lymphoma underwent additional hospital stay for 31 days and 6 days respectively, for medically indicated treatments. Similarly, one female subject diagnosed with acute myelogenous leukemia underwent additional hospitalization for 4 days for medically indicated treatments.

All bundled procedures included insertion or removal of Porta-A-Cath/Broviac devices used for intravenous chemotherapy delivery. When we analyzed the bundled procedures, we found that 13 females (46.4 % of total) and two males (22.2 % of total) underwent additional operative procedures resulting in mean operative time of 2.5±0.5 h for bundled cases versus 1.1±0.2 h for unbundled cases. In addition, mean anesthesia time was 4.8±1.2 h for bundled cases and 1.7±0.3 h for unbundled cases. The mean operating time difference of bundled vs. unbundled procedures did not reach statistical significance, presumably because the small sample size.

Comment

This study evaluated feasibility of the ovarian and testicular cryopreservation in pediatric patients undergoing gonadotoxic therapy for cancer or underlying blood dyscrasias. We also demonstrated that gonadal tissue harvesting procedures could be potentially bundled with other medically indicated

procedures and still be safely performed as an outpatient procedure in children of both genders. Because fertility preservation is a key component of the care of young people with cancer [21], our study may provide further assurance in considering these experimental strategies in children. Bundling of medically indicated procedures with gonadal tissue harvesting can potentially reduce risks, cost, and inconvenience.

In our study, nearly all subjects were scheduled to undergo a variety of gonadotoxic treatment regimens, including chemotherapy, pelvic radiation, or HSCT, with the exception of a few female cases presenting for fertility preservation prior to oophorectomy. Many chemotherapy agents used for pediatric cancers are known to be gonadotoxic, including cyclophosphamide [36,44], busulfan [9], and other alkylating agents [23,24,44]. Recent human ovarian xenografting experiments combined with clinical data, also indicated that doxorubicin has significant gonadotoxicity [4,42]. A meta-analysis demonstrated high risk of ovarian failure (70–100 %) for females undergoing radiotherapy and chemotherapy for HSCT [22]. Craniospinal irradiation has also been shown to promote premature menarche and result in significant fertility deficits [5–7] while abdominal irradiation resulting in direct ovarian exposure has been shown to result in premature ovarian failure [48]. Some studies have recommended that only patients who receive high risk for gonatotoxic treatments be referred for cryopreservation [21]. However, currently it is difficult to predict and categorize the likelihood of immediate or future gonadal failure with precision due to many variables involved such as age, type and dose of chemotherapy and how long the individual may wait before attempting pregnancy. Because of this, fertility preservation experts tend to err on the side of offering these options rather than not. It is therefore highly important that we establish the safety profile of fertility preservation procedures.

Studies of ovarian harvesting in prepubertal children have generally shown these procedures to be performed with safe outcomes. A study of 14 prepubertal boys undergoing testicular biopsy demonstrated positive post-operative outcomes as well as insight into parental factors influencing a decision to undergo harvesting [17]. Furthermore, a study of 52 prepubertal patients (mean age: 6.43±3.32 years) and 10 peripubertal patients (mean age: 14±1.23 years) reported no significant complications [50]. While our study was different in reporting the bundling with other medical procedures, our overall safety findings of male patients were in accordance with other studies. Similarly, the overall safety of surgical cryopreservation procedures in females in our study supports findings found by others. A study of 31 female patients, with 20 under the age of 21 with various cancer and non-cancer diagnoses, reported no complications after ovarian cryopreservation [34]. Similarly, analysis of ovarian tissue cryopreservation in 19 patients showed no adverse outcomes with younger females [11]. Evaluation of 23 female patients with a mean age of 13.5 years showed the procedure could be performed with a median postoperative stay of 1 day and no delay of medical treatment [16]. Analysis of 17 patients with benign ovarian tumors who underwent ovarian tissue cryopreservation showed that surgical tumor resection could be performed with tissue preservation [15]. Studies regarding the psychological aspects of gonadal tissue harvesting in prepubertal children have also shown that the majority of patients and their families are amenable to such medical options [20,35,46].

A concern for tissue transplantation, especially in childhood cancer survivors, is the reintroduction of cancerous tissue to patients [1]. Several biomarkers and histological methods can be employed to detect cancer metastasis in harvested tissue [24]. Nevertheless, the routine use of immunohistochemical analysis may not be adequate in identifying cancerous cells in harvested tissue [14]. For the procedure used in this study, routine specimens were sent to pathology to screen for neoplasia, which was shown to be negative in all cases. For subjects whose ovarian tissues are found to harbor malignant cells, methods of in vitro growth of primordial follicles are under development [45]. If these approaches succeed in humans, primordial follicles can be isolated from cryopreserved ovarian tissue [30], and grown to maturity in vitro to be utilized for in vitro fertilization and embryo transfer. However since these procedures have not yet entered in clinical use, all subjects undergoing tissue harvesting are advised that in vitro gamete maturation is only a theoretical possibility.

Subjects in this study were referred for gonadal tissue cryopreservation as the only viable fertility preservation method available to them. Fewer than 15 live births have been attributed to transplantation of cryopreserved ovarian tissue worldwide [44]. Testicular cryopreservation also remains an experimental method and thus far no successful attempts of transplantation have been reported in humans. However, a number of studies in animals evaluating testicular cryopreservation and transplantation have shown these techniques to be viable. Ex vivo transplantation of testicular tissue in murine hosts has shown functional spermatogenesis indicating that testicular tissue retains the ability to regenerate structural support necessary for germ cell development [13]. Cryopreservation of testicular tissue followed by subsequent transplantation resulted in restoration of testicular function in a variety of animal hosts, including mice, rabbit and chicken [18,40,43]. A recent murine study has succeeded in maturation of spermatogonia in testicular tissue in vitro resulting in the generation of viable offspring, in addition to the survival of testicular tissue for 2 months in vitro [38]. These data support the possibility of maturing spermatogonia in human pediatric testicular tissue in vitro, which may be an important consideration in prepubertal males. Furthermore, strategies have been defined in animal

studies where injection of single cell suspension of testicular tissue into seminiferous tubules or direct transplantation resulting in restoration of testicular function [47]. The latter approach may also allow identification and elimination of any malignant cell from the testis prior to transplantation. Nevertheless the clinical utility of cryopreserved testicular tissue still remains theoretical at the present time and all study participants are advised as such.

The use of additional procedures for experimental protocols, especially during treatment for other potentially life-threatening illnesses, raises the concern of added morbidity. However, our study showed that both ovarian and testicular tissue harvesting could be accomplished safely and in the ambulatory setting. In addition, for the first time, we showed that these procedures can be bundled with other medically-indicated procedures further minimizing risk of additional anesthesia and procedures, as well as potentially the cost and inconvenience. Additional long-term, prospective studies with adequate follow-up and larger sample size would further support the safety, feasibility and indications for tissue cryopreservation in pediatric patients.

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