ABSTRACT: Young boys undergoing gonadotoxic treatments are at high risk of spermatogonial stem cell (SSC) loss and fertility problems later in life. Stem cell loss can also occur in specific genetic conditions, eg, Klinefelter syndrome (KS). Before puberty, these boys do not yet produce sperm. Hence, they cannot benefit from sperm banking. An emerging alternative is the freezing of testicular tissue aiming to preserve the SSCs for eventual autologous transplantation or in vitro maturation at adult age. Many fertility preservation programmes include cryopreservation of immature testicular tissue, although the restoration procedures are still under development. Until the end of 2018, the Universitair Ziekenhuis Brussel has frozen testicular tissues of 112 patients between 8 months and 18 years of age. Testicular tissue was removed in view of gonadotoxic cancer treatment (35%), gonadotoxic conditioning therapy for bone marrow transplantation (35%) or in boys diagnosed with KS (30%). So far, none of these boys had their testicular tissue transplanted back. This article summarizes our experience with cryopreservation of immature testicular tissue over the past 16 years (2002-2018) and describes the key issues for setting up a cryopreservation programme for immature testicular tissue as a means to safeguard the future fertility of boys at high risk of SSC loss.

KEYWORDS: Cryopreservation, fertility preservation, human, immature, testicular tissue

Introduction

Although sperm banking is being offered for already half a century to preserve fertility in adult men,1 fertility preservation in young boys was nonexistent until more than 15 years ago. With the growing population of long-term survivors of childhood cancer or other life-threatening diseases,2–4 the side effects of gonadotoxic treatments or genetic conditions became more apparent. One of these major side effects is lifelong subfertility or infertility. Treatments for patients with cancer involve chemotherapy and/or radiotherapy which target rapidly dividing cells, like the constantly dividing spermatogonial stem cells (SSCs).5 Spermatogonia have been reported to be more sensitive to chemotherapy6 and radiotherapy7,8 compared with mature germ cells and Leydig cells. The severity of the gonadal damage varies substantially depending on the maturity of the testicular tissue as well as the type of drug or combination of drugs, the cumulative dose, the duration and the site of radiation therapy.9,10 The most damaging chemotherapeutic agents are the alkylating ones,11 where radiation doses >4 Gy may result in permanent gonadal damage.12,13 In addition, patients with haematological disorders – including sickle cell disease (SCD) and thalassemia – require high-dose chemotherapy and/or total body irradiation as a conditioning therapy for bone marrow transplantation which are associated with a high risk of infertility.14,15 Fractionated total body irradiation has been associated with a <20% chance of gonadal recovery.16 Moreover, patients diagnosed with SCD are often treated with hydroxyurea, an antineoplastic agent reported to decrease sperm quality in adults17,18 and to deplete the spermatogonial pool in prepubertal boys.19 Klinefelter syndrome (KS), first described by Klinefelter et al20 in 1942, is the most common chromosomal aberration found in infertile men, affecting between 1 in 500 and 1 in 1000 newborn males. Klinefelter syndrome is characterized by 1 or more extra X chromosomes. A total of 80% of the patients with KS carry the non-mosaic 47,XXY karyotype, whereas the remaining 20% have higher grade aneuploidies or mosaicisms.21–23 The testicular tissue of patients with KS shows a progressive degeneration characterized by severe germ cell loss leading to impaired spermatogenesis, extensive fibrosis, hyalinization of the seminiferous tubules, and Leydig cell hyperplasia. This testicular failure is associated with increased follicle-stimulating hormone (FSH) and decreased inhibin B (INHB) and anti-Müllerian hormone (AMH) levels over time.24,25 Because young boys do not produce mature spermatozoa yet, sperm banking is not an option. The possibilities for children affected by cancer or other life-threatening diseases to become a father later in life have long been limited to sperm donation and adoption. Because the awareness of being infertile has a dramatic impact on the quality of life, other fertility preservation strategies were welcome. In 1994, the hope to give these men a genetically own child was raised by a report on SSC cryopreservation and transplantation in mice.26,27 Sterile mice, which were transplanted with SSCs from a fertile mouse, were able to produce offspring carrying...
the donor genotype. In the next 25 years, methods for cryopreservation of SSCs and testicular tissue have been optimized and translated to the human. Protocols for transplantation of SSCs and testicular tissue need further adaptation to become a clinical application. In 2002, the Universitair Ziekenhuis (UZ) Brussel was the first hospital worldwide initiating a clinical fertility preservation programme for young boys at high risk of losing their SSCs. This testicular tissue bank collected samples from 112 patients between 2002 and 2018. At present, several centres across the world offer fertility preservation to young boys. More centres are interested in setting up a testicular tissue bank, but without the help of experienced teams, this remains a real challenge. With this article, we aim to share the knowledge that we have built up during more than 15 years and to provide useful information to all centres wanting to set up a clinical programme for fertility preservation in young boys at high risk of SSC loss.

**Materials and Methods**

**Patients eligible for testicular tissue banking**

At the UZ Brussel, patients under 18 years of age and with a ≥80% risk of infertility are eligible for inclusion in the fertility preservation programme. For adolescents who are able to produce a semen sample by masturbation, sperm banking followed by assisted reproductive techniques (ART) at adulthood is offered. If masturbation is not possible, alternative methods such as assisted ejaculation techniques (penile vibrostimulation and electrostimulation) or testicular sperm extraction (TESE) could be proposed. Testicular tissue sampling and banking is the only fertility preservation option for prepubertal and peripubertal boys who do not produce mature spermatozoa yet. Also, adolescents who are unable to provide a semen sample or who are azoospermic could benefit from testicular tissue cryopreservation. Autotransplantation of the frozen-thawed SSCs or testicular tissue as well as in vitro spermatogenesis could be future options to restore fertility at adulthood. As the cure of the cancer or haematological disorder is the main concern, testicular tissue cryopreservation is preferably performed shortly after diagnosis to avoid any delay in treatment.

Due to the massive germ cell depletion occurring in KS boys, they were thought to be eligible for fertility preservation and, in 2009, the UZ Brussel also started to include patients with KS. As spermatogenesis in mosaic cases is less affected, only patients with KS with a non-mosaic karyotype (assessed on peripheral lymphocytes) were enrolled. To have a homogeneous group of patients, patients with KS receiving testosterone therapy were excluded as well as patients with KS with a history of cryptorchidism, because cryptorchidism is an additional reason for a lower spermatogonia number. At first, the aim was to cryopreserve spermatozoa. Therefore, boys with KS (n = 30) were followed until the first signs of puberty. Every 4 months, pubertal development was assessed according to Marshall and Tanner staging. Testicular volume was measured with the Prader orchidometer and the serum levels of FSH, INHB, and testosterone were measured. In case the testicular volume was >6 mL, spermaturia was investigated in morning urine samples. Testicular tissue banking was only proposed when one or a combination of the following criteria were observed:

- No further testicular growth;
- An initial serum INHB concentration below the lower reference value;
- Decreased serum INHB concentration;
- Increased FSH concentration (at least above 10 mU/L);
- Azoospermia evidenced in a semen sample obtained after masturbation, penile vibrostimulation, electrostimulation, or TESE.

As these peripubertal boys with KS presented with none or very limited numbers of spermatogonia, later on, boys with KS were referred at diagnosis. Testicular tissue of 4 prepubertal patients with KS (aged 4-7 years) has been banked.

**Patient management and follow-up**

Patients eligible for enrolment in the fertility preservation programme are referred by paediatric oncologists and haematologists to the oncofertility centre of the UZ Brussel. The oncofertility centre consists of a multidisciplinary and closely cooperating team of specialists and nurses with expertise in oncology and fertility. During consultation, the patient and his parents are informed about the available options to safeguard the patient’s future fertility. It is mandatory to inform the patient (using adapted terminology) and his parents that these fertility preservation and restoration methods are still experimental as they have not yet proven to be successful in humans. Furthermore, banking of testicular tissue does not guarantee that this material could be used for future fertility restoration. Autotransplantation is not yet safe enough for patients diagnosed with malignancies as it carries a great risk of reintroducing residual malignant cells back into the patient via the graft. Strategies to effectively eliminate these contaminating malignant cells from the cryopreserved cell suspension or testicular tissue are not available to date. In the future, these patients could benefit from in vitro spermatogenesis in combination with ART to avoid the risk of reintroducing the malignancy back into the patient. The patient and his parents should also be informed about the possible complications related to the testicular tissue sampling procedure itself (sensitive scrotum, local bleeding, and wound infection).

An informed consent signed by the parents and the patient himself (if more than 12 years of age) is necessary (Supplementary File). By signing this consent form, they comply with the removal and banking of testicular tissue. They also have the opportunity to agree or disagree with the use of a small part (<10%) of the biopsy for scientific research and/or to release the banked tissue for research after the legal maximal storage period of 10 years (in Belgium) or after the patient’s
death. After signing the informed consent, changes to the original consent form can still be requested by the parents and by the patient himself when he has reached legal age (18 years in Belgium).

Once a year, patients enrolled in the fertility preservation programme return to our centre to monitor their development. Patient follow-up includes the following:

- Weight, height, body mass index and blood pressure;
- Testicular volume measurement with a Prader orchidometer and/or testicular ultrasound;
- Evaluation of the gonadal function and development by analysing the serum levels of luteinizing hormone, testosterone, oestradiol, FSH, and INHB;
- Estimation of the degree of skeletal maturation through bone age and bone density assessment by medical imaging.

**Testicular tissue sampling**

Testicular tissue samples are to be retrieved under general anaesthesia, preferably in combination with other interventions requiring anaesthesia (like central line placement for chemotherapeutic drug administration or bone marrow aspiration). To minimize the risk for postoperative fibrosis and to preserve endocrine testicular function maximally, a single large volume biopsy is preferred over multiple biopsy sampling. Depending on the volume of the testes (for small testes proportionally more testicular tissue is removed) and according to the parents’ wish, one entire testis, one half of a testis or smaller fragment(s) (10%-25% of the testis) can be biopsied. The testicular tissue is taken from the lower pole of the largest testis.

**Testicular tissue cryopreservation**

After testicular tissue sampling, the major part (>90%) of the testicular tissue biopsy is cut into small fragments of approximately 6 mm³, cryopreserved, and stored for potential later fertility restoration purposes. Testicular tissue is cryopreserved by slow freezing in cryopreservation medium consisting of Dulbecco’s modified Eagle’s medium (DMEM/F12; 31330-038; Invitrogen, Merelbeke, Belgium) supplemented with 1.4M dimethylsulfoxide (DMSO; D2650; Sigma, Bornem, Belgium), 0.15M sucrose (10274; BDH Laboratory Supplies, Poole, UK), and 10% human serum albumin (HSA; 10064; VitroLife, Gothenburg, Sweden). Two testicular tissue pieces are placed per vial. The vials are first equilibrated for 15 minutes on ice water and then placed in an isopropyl alcohol freezing container (55710-200; Mr Frosty Freezing Container, Mr Frosty, VWR, Leuven, Belgium) which is put in a −80°C freezer. Samples are cooled at a rate of −1°C/min. When −80°C is reached (after at least 4 hour), the cryovials are stored in nitrogen gas (Figure 1) to prevent cross-contamination between the cryopreserved samples.

**Spermatogonia scanning in testicular tissue samples**

To evaluate the usefulness of testicular tissue banking, 1 fragment (10 mm³) of the biopsied testicular tissue is fixed in alcohol formalin acetic acid fixative (AFA; VWRSAFA0022A630007; VWR, Oud-Heverlee, Belgium) for at least 1 hour. After embedding in paraffin, 3 consecutive 5-µm-thick sections are made at 3 different depths of the fragment (with at least 50 µm between 2 depths) to have an overall view of the testicular tissue fragment. For each depth, 1 slide is stained with haematoxylin and eosin to evaluate the tubular integrity and progression of spermatogenesis. Consecutive slides are stained for immunohistochemistry for melanoma-associated antigen 4 (MAGE-A4) (mouse monoclonal antibody, kindly provided by Giulio Spagnoli, University of Basel, Switzerland) as a positive marker and VIMENTIN (mouse monoclonal antibody clone V9; M0725; Dako, Heverlee, Belgium) as a negative marker for germ cells. After deparaffinization and rehydration, slides are washed in phosphate-buffered saline (PBS; 70011-036; Life-technologies, Gent, Belgium) for 5 minutes. Endogenous peroxidases are blocked by incubation in 0.3% hydrogen peroxide (H₂O₂) for 30 minutes. Antigen retrieval is performed in citrate buffer, using a water bath at 95°C for 75 minutes. The slides are cooled at room temperature for 30 minutes. Non-specific binding is blocked by incubating...
slides with 4% normal goat serum (NGS; B304; Tebu-Bio, Boechout, Belgium) for 30 minutes. Slides are subsequently incubated overnight at 4°C with the primary antibody (1/200). Colour reaction is achieved using a staining kit (Dako Real Envision Detection System; K5007; Dako, Heverlee, Belgium) containing dextran coupled with peroxidase molecules and goat secondary antibodies against mouse immunoglobulins. Chromogen reaction is performed using 3,3’-diaminobenzidine tetrahydrochloride supplied with the kit (Dako Real Envision Detection System). Nuclei are counterstained with haematoxylin, and slides are dehydrated and mounted. Histological examinations are performed on an Olympus IX81 inverted light and fluorescence microscope (Olympus, Aartselaar, Belgium) and digital images are made using a digital camera (CC-12 Soft Imaging System; Olympus). In case no spermatogonia are observed in the examined sections, 1 additional fragment is thawed, fixed, and examined. The result is communicated to the patient and his parents by the fertility specialist.

Results

Regulatory framework

Our fertility preservation programme has been approved by the Institutional Review Board of the UZ Brussel. The testicular tissue collection and banking was performed after informed consent was obtained. The UZ Brussel is insured for the testicular tissue biopsy procedure and the costs for the procedure and storage are covered by the Belgian national health insurance. In Belgium, the legal maximal storage period for gametes in human reproductive tissue is 10 years. After the storage period, the UZ Brussel carries out the last instructions mentioned in the informed consent (release the banked tissue for research or destroy the banked tissue). Because storage for several decades may be needed, an extension of the legal storage period can be requested. The testicular tissue samples are prepared and cryopreserved under the guidelines of EU tissue directive.

Patients enrolled in the fertility preservation programme of the UZ Brussel

At the end of 2018, the testicular tissue bank of the UZ Brussel contained samples from 112 patients (58% acceptance rate). Of these 112 patients, 78 (70%) boys required high-risk gonadotoxic treatments. Patients with cancer as well as patients needing conditioning treatment for bone marrow transplantation accounted each for 35% (Figure 2A). Although the aim is to cryopreserve testicular tissue before initiation of gonadotoxic treatment, 17 patients already received some treatment before testicular tissue banking was considered. In total, 6 patients had been treated with low-risk therapy for a previous cancer. Only when the cancer relapsed or due to poor response to the original therapy, they needed more severe treatment and, therefore, were referred to the fertility department. Another 22 patients with blood-borne diseases had been treated with hydroxyurea before testicular tissue banking, which could also have an effect on germ cell survival. In addition, we have banked testicular tissue from 34 patients with KS (30%) (Figure 2A). Almost all patients with KS were peripubertal or postpubertal when enrolled in the fertility preservation programme, as our first aim was to bank mature spermatozoa. Unfortunately, none of the patients with KS showed ongoing spermatogenesis, and thus testicular tissue was obtained through TESE (unilateral or bilateral, single or multiple, open or micro-TESE).

Apart from banking testicular tissue for clinical use, most of the parents (69%) agreed to donate <10% of the biopsy for scientific research. In total, 17% of the parents did not consent for further experimental research after the legal maximal storage period and 19% of the parents did not consent for further experimental research in case their son would die. The decision of the parents concerning further experimental research after the legal maximal storage period and after patients’ death was not specified in 13% and 28% of the informed consents, respectively (Table 1). So far, 10 patients have died due to neuroblastoma, long-term granulomatous disease, testicular cancer, lymphoma, SCD, leukaemia (n = 3), rhabdomyosarcoma, another unspecified cancer. In answer to the parents’ wish, the stored testicular tissue of 5 patients was made available for scientific research purposes, whereas the banked testicular tissue of
3 patients was discarded. For the other 2 patients, the informed consent did not mention the parents’ choice concerning scientific research after the patients’ death. Their testicular tissues are kept in storage until the parents have been contacted.

Testicular tissue samples

Most of the parents chose to have one half of a testis (47%) or a smaller biopsy (34%) removed. In total, 19% of the patients, all requiring gonadotoxic treatment, had one whole testis removed for fertility preservation according to the parents’ wish (Figure 3A) and some of these patients got a testicular prosthesis implanted after remission. These results are in line with a Dutch questionnaire from which it was concluded that parents prefer smaller biopsy over hemicastration.76 In 45%, the left testis was biopsied, and in 42%, the right one. Bilateral biopsies (13%) were only performed in patients with KS (Figure 3B). No minor or major adverse complications occurred during or after the surgery and none of the patients needed a second intervention.

All patients included in our fertility preservation programme tested negative for cytomegalovirus, human immunodeficiency virus or hepatitis B/C. However, if patients would have tested positive, their samples would have been kept in a separate storage tank to avoid pathogen transmission to other cryopreserved testicular tissue samples.

In general, the testicular tissue of patients affected with cancer and haematological disorders contained spermatogonia (Figure 4A and B). Spermatogonia numbers were not significantly different between patients previously exposed to chemotherapy or hydroxyurea and patients who did not receive chemotherapy or hydroxyurea before testicular tissue preservation.27 However, in 2 patients with SCD and who had been treated with hydroxyurea prior to testicular tissue banking, no spermatogonia were observed (Figure 4E). Previous studies investigating the spermatogonial quantity in patients with SCD treated with hydroxyurea demonstrated a reduced or totally depleted spermatogonial pool.19,77 Testicular tissue of patients with KS hardly contained spermatogonia, even at the very young age of 4 years. Very few spermatogonia (0.03-0.06 spermatogonia/semiferous tubule) were detected in testicular tissue biopsies from prepubertal patients with KS73 (Figure 4C).

Discussion

This article describes the clinical fertility preservation programme of the UZ Brussel for young boys at high risk of SSC loss and provides useful information to centres interested in setting up an immature testicular tissue bank.

According to our own experience, but also in other centres offering fertility preservation, there is a great interest to bank testicular tissue for later use.49,76,78-80 However, because this fertility preservation method is experimental and involves surgery on young patients, one must be aware to offer testicular tissue banking only to those patients who are at high risk of becoming infertile and who could benefit from fertility restoration at adulthood.

It is of great importance that patients with cancer are referred to a fertility specialist before initiation of any gonadotoxic treatment, because even low-risk chemotherapy and/or radiotherapy can induce SSC loss. This would not be a problem in first instance, because their fertility might recover spontaneously after treatment. It could, however, become problematic if the patient relapses and needs a second and more severe treatment. Studies revealed that spermatogonia numbers in prepubertal boys exposed to alkylating agents were significantly reduced.11,19

Table 1. Consents concerning scientific research conducted on the removed testicular tissue.

| Consent Type | SCIENTIFIC RESEARCH ON SMALL PART (<10%) OF TESTICULAR TISSUE BIOPSY (%) | SCIENTIFIC RESEARCH ON BANKED TESTICULAR TISSUE AFTER LEGAL MAXIMAL STORAGE PERIOD (%) | SCIENTIFIC RESEARCH ON BANKED TESTICULAR TISSUE AFTER PATIENT’S DEATH (%) |
|-------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| Consented   | 69                                              | 70                                              | 53                                              |
| Did not consent | 20                                              | 17                                              | 19                                              |
| Not mentioned in informed consent | 11                                              | 13                                              | 28                                              |
However, another study showed that previous exposure to alkylating or non-alkylating chemotherapy had no impact on spermatogonial quantity. More studies should be conducted to elucidate whether testicular tissue banking for fertility preservation is beneficial to patients already exposed to alkylating agents before banking. However, besides the cancer treatment, the cancer itself could also affect spermatogonial quantity, but this still needs to be investigated.

The number of spermatogonia is also negatively affected by treatment with hydroxyurea. At this moment, it is not sure whether this effect is reversible and whether fertility can recover spontaneously. A retrospective multicentre study revealed that at least one or a combination of sperm parameters (volume of ejaculate, sperm concentration, total sperm count, forward motility, sperm viability, and sperm morphology) were deteriorated in adults treated with hydroxyurea. Recently, it was demonstrated that the spermatogonial quantity was reduced in testes of prepubertal boys after hydroxyurea treatment. However, as all patients with SCD received hydroxyurea, it cannot be excluded that the disease itself causes spermatogonial loss. Prospective long-term studies are needed to get better insights into the factors causing the reduced spermatogonial quantity. For now, patients with SCD and their parents should be informed about their reduced potential for successful fertility restoration. In our cryopreservation programme, the presence of spermatogonia in the testicular tissue samples is demonstrated using the marker MAGE-A4, but other markers are also suitable to detect human spermatogonia.

Autotransplantation of cryopreserved testicular tissue samples is the most promising restoration strategy. Recently, Fayomi et al demonstrated that autotransplantation of frozen-thawed prepubertal testicular tissue (pieces of 9-20 mm³, which is larger than in our cryopreservation protocol: 6 mm³) in pubertal rhesus macaques resulted in graft-derived sperm and offspring. However, for patients diagnosed with malignancies, this approach holds a great risk for malignant relapse due to the potential presence of residual malignant cells in the cryopreserved sample. Assessing possible malignant contamination in the cryopreserved samples is of immense importance, although it is not performed along with testicular tissue banking in the UZ Brussel. We opt to assess malignant cell contamination at the time autotransplantation is requested by a patient with a child wish. If malignant cells are detected, SSC transplantation could be considered.

Even if the risk of infertility or the chances of future fertility restoration are 20% or lower, more than 25% of the parents still opt to preserve their sons’ fertility through testicular tissue banking. We do not recommend testicular tissue banking for patients receiving low-risk treatments, as banking requires surgery on young patients and may reduce the chances for spontaneous recovery of spermatogenesis.

In addition to boys with cancer or haematological disorders, patients with KS were also thought to be eligible for fertility preservation by means of testicular tissue cryopreservation. Apart from a seldom spermatogonium, most of the KS samples were sclerotic and lost every potential for fertility restoration at...
adulthood. A recent study highlighted that spermatogenesis in testicular tissue of patients with KS are lost already at very young age (<4 years) and that the outcomes of a TESE procedure performed at early adolescent age are not better compared with TESE performed in adults. Furthermore, it is difficult to predict which patients with KS will have a positive TESE outcome, as clinical or hormonal biomarkers are lacking. Therefore, we do not longer recommend testicular tissue banking for fertility preservation in young patients with KS.

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Author Contributions
A.B. was involved in conception and design of the study, acquisition of data, analysis and interpretation of data, drafting of the manuscript and critical approval of the final manuscript. H.T. was involved in critical revision of the manuscript and approval of the final manuscript. E.G. was involved in conception and design of the study, analysis and interpretation of data, critical revision of the manuscript and approval of the final manuscript.

Ethical Approval
Patient data collection has been approved by the Institutional Review Board of the UZ Brussel (B.U.N. 143201630620, B.U.N. 143201732183, and B.U.N. 143201731260).

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Supplemental Material
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