Andrology

Spermatogonial stem cell preservation and transplantation: from research to clinic

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STUDY QUESTION: What issues remain to be solved before fertility preservation and transplantation can be offered to prepubertal boys?

SUMMARY ANSWER: The main issues that need further investigation are malignant cell decontamination, improvement of in vivo fertility restoration and in vitro maturation.

WHAT IS Known ALREADY: Prepubertal boys who need gonadotoxic treatment might render sterile for the rest of their life. As these boys do not yet produce sperm cells, they cannot benefit from sperm banking. Spermatogonial stem cell (SSC) banking followed by autologous transplantation has been proposed as a fertility preservation strategy. But before this technique can be applied in the clinic, some important issues have to be resolved.

STUDY DESIGN, SIZE DURATION: Original articles as well as review articles published in English were included in a search of the literature.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Relevant studies were selected by an extensive Medline search. Search terms were fertility preservation, cryopreservation, prepubertal, SSC, testis tissue, transplantation, grafting and in vitro spermatogenesis. The final number of studies selected for this review was 102.

MAIN RESULTS AND THE ROLE OF CHANCE: Cryopreservation protocols for testicular tissue have been developed and are already being used in the clinic. Since the efficiency and safety of SSC transplantation have been reported in mice, transplantation methods are now being adapted to the human testes. Very recently, a few publications reported on in vitro spermatogenesis in mice, but this technique is still far from being applied in a clinical setting.

LIMITATIONS, REASONS FOR CAUTION: Using tissue from cancer patients holds a potential risk for contamination of the collected testicular tissue. Therefore, it is of immense importance to separate malignant cells from the cell suspension before transplantation. Because biopsies obtained from young boys are small and contain only few SSCs, propagation of these cells in vitro will be necessary.

WIDER IMPLICATIONS OF THE FINDINGS: The ultimate use of the banked tissue will depend on the patient’s disease. If the patient was suffering from a non-malignant disease, tissue grafting might be offered. In cancer patients, decontaminated cell suspensions will be injected in the testis. For patients with Klinefelter syndrome, the only option would be in vitro spermatogenesis. However, at present, restoring fertility in cancer and Klinefelter patients is not yet possible.

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Key words: clinic / fertility / spermatogonial stem cell / transplantation / prepubertal boys
Introduction

Throughout Europe, more and more institutions are now offering fertility preservation to prepubertal boys facing the loss of spermatogonial stem cells (SSCs). At UZ Brussel, we started banking testicular tissue in 2002 and since then, tissue from 47 boys has been stored (Table I). One of these patients was a 4-year-old boy, who was suffering from drepanocytosis. His parents came to our fertility department in 2002 to discuss the fertility preservation options for their son. The boy had been treated with hydroxyurea for 6 months and amoxicillin daily per os. When the boy was under anaesthesia for placement of the portacath, a testicular biopsy was taken. A small piece of the tissue was prepared for histological examination, while the major part was stored for later use. The boy recovered well and in 2011, the parents returned to discuss the possibility of transplantation.

Although fertility preservation options have been discussed extensively in the literature, some important information is still lacking. Clinicians wanting to start testicular tissue banking in their centre often turn to us if they have questions regarding patient inclusion, cryopreservation protocols or transplantation strategies. Therefore, our aim in the present review was to compile these questions and find answers or discuss potential solutions.

In the near future, patients will be referred back for transplanting their banked SSCs, therefore there is an urgent need to resolve unknown issues, so that we are able to counsel and treat our patients and their parents appropriately.

Methods

We conducted an extensive Medline search using following search terms: fertility preservation, cryopreservation, prepubertal, SSC, testis tissue, transplantation, grafting and in vitro spermatogenesis. Original articles as well as review articles published in English were included. The final number of studies selected for this review was 102.

<table>
<thead>
<tr>
<th>Malignant diseases</th>
<th>$n$</th>
<th>Non-malignant diseases</th>
<th>$n$</th>
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<tr>
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<td>Drepanocytosis</td>
<td>14</td>
</tr>
<tr>
<td>Testicular cancer</td>
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<td>Klinefelter syndrome</td>
<td>10</td>
</tr>
<tr>
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<td>Thalassemia</td>
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<tr>
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<td>Granulomatus disease</td>
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<tr>
<td>B-cell lymphoma</td>
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<td>Ideopathic medullar aplasia</td>
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<td>Rhabdomyosarcoma</td>
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<tr>
<td>Anaplastic ependymoma</td>
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<td>Ewing sarcoma</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Nasopharynx carcinoma</td>
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<tr>
<td>Total</td>
<td>17</td>
<td>Total</td>
<td>30</td>
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$n$: samples collected and stored since 2002.

Results

Who should be offered preservation of SSC?

The first aim should always be to store sperm cells. In case the ejaculate does not contain mature sperm cells, testicular sperm extraction (TESE) might be an option. However, when the testes are still immature, a testicular biopsy will be taken. Several groups of patients might benefit from SSC banking.

Patients facing gonadotoxic treatments

Treatment regimens for cancer patients involve chemotherapy and radiotherapy which target rapidly dividing cells. Although there is no active production of spermatozoa in the prepubertal testis, it is not a quiescent period of testicular development. The SSCs are constantly dividing to populate the growing seminiferous tubules and are thus targets for gonadotoxic treatments (Jahnukainen et al., 2011a).

Acute lymphoblastic leukaemia (ALL), being the most common cancer type in children, involves treatment with low-risk regimens (Jahnukainen et al., 2011a). Long-term follow-up of childhood ALL survivors indicates that treatment not involving high-dose cyclophosphamide does not totally deplete SSCs and that spermatogenesis is re-initiated from the surviving stem cell population (Nummio et al., 2009; Jahnukainen et al., 2011b). However, one-fifth of the children with ALL experience relapse requiring intensive therapy including alkylating agents, testicular irradiation and/or haematopoietic stem cell transplantation, bringing them in the high risk population for permanent sterility. When these patients want to bank testicular tissue before a subsequent—and more severe—therapy, a substantial reduction in spermatogonia may have occurred.

Spontaneous recovery of spermatogenesis is possible but this depends on the survival of SSCs and their ability to differentiate. Gonadal damage is related to the type of drugs, the total dose, the fractionation schedule and the field of treatment. Irradiation doses of 0.1–1.2 Gy can cause impairment of spermatogenesis, while doses of more than 4 Gy might cause permanent damage (Wallace et al., 2005). Treatment regimens may thus be categorized into low- and high-risk treatments. High-risk treatments involve whole-body irradiation, pelvic or testicular radiotherapy, chemotherapy conditioning for bone marrow transplantation and treatment with alkylating drugs (Wallace et al., 2005). However, not all patients receiving alkylating agents are at high risk. The cumulative dose is the major risk factor for permanent sterility (Table II). Patients receiving these treatments should be counselled for fertility preservation options.

Next to cancer patients, patients suffering from blood diseases are often treated with chemo- and radiotherapy as a conditioning therapy for bone marrow transplantation. These patients are at high risk for lifelong sterility and should thus be offered testicular tissue banking.

Other drugs, such as hydroxyurea, which are administered to patients with drepanocytosis, could also influence testicular function. Long-term treatment with hydroxyurea can affect fertility (Berthaut et al., 2008). Oligo-and azoospermia was reported in four patients who received hydroxyurea starting from childhood to adulthood (Lukusa and Vermylen, 2008). The impact of these treatments on the ability to restore spermatogenesis in fertility preservation methods is currently unknown.
At the moment, follow-up data on childhood cancer survivors are scarce. In a follow-up study in 51 long-term survivors of childhood ALL, testicular size and semen quality were not altered in patients treated with <10 g/m² cyclophosphamide. However, decreased serum-free testosterone levels were observed which are indicative of impaired Leydig cell function. On the other hand, no or very few spermatozoa were observed in patients who received higher doses of cyclophosphamide or testicular irradiation (Jahnukainen et al., 2011b). Sertoli cell impairment was observed 18 years after receiving combination therapy in a 31-year-old azoospermic man (Bar-Shira et al., 2001). Unfortunately, the remainder of testicular tissue might be a better option. Testicular cell suspensions contain several cell types differing in size, shape and water content, requiring different freezing conditions and media compositions for optimal preservation of viability and functionality. The type of cryoprotectant, its concentration and the cooling rate could all influence cell survival. For SSC transplantation (SSCT) it is particularly important that the SSCs survive the freezing procedure and maintain their functionality. Therefore, protocols have been established focusing on the preservation of spermatogonia (Avarbock et al., 1996; Izadyar et al., 2002; Frederickx et al., 2004; Sà et al., 2012). Frozen-thawed bovine spermatogonia were able to colonize the mouse testis, although with lower efficiency compared with the fresh population (Izadyar et al., 2002).

However, the cryopreservation of testicular tissue might be a better option. Testicular tissue freezing preserves the cell–cell contacts, but requires more permeable cryoprotectants. The best preservation of morphology and hormonal activity was achieved when dimethylsulphoxide (DMSO) was used to freeze adult testicular tissue (Keros et al., 2005). The feasibility of the selected protocol was later confirmed using prepubertal testicular tissue (Keros et al., 2007). Most of the centres perform tissue freezing, leaving the options open to perform testicular tissue grafting, SSCT or in vitro maturation (IVM). It is not clear whether enzymatic digestion of frozen-thawed tissue would result in better viability compared with digestion before freezing. It is possible that testicular tissue is better adapted to the hypoxic

### Table II

<table>
<thead>
<tr>
<th>Alkylating agent</th>
<th>Gonadotoxic cumulative dose</th>
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<tr>
<td>Cyclophosphamide</td>
<td>19 g/m²</td>
</tr>
<tr>
<td>Busulphan</td>
<td>600 mg/kg</td>
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<td>Melphalan</td>
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<td>Procarbazine</td>
<td>4 g/m²</td>
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<tr>
<td>Ilofsamide</td>
<td>42 g/m²</td>
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<tr>
<td>Chlorambucil</td>
<td>1.4 g/m²</td>
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</table>

**Patients with Klinefelter syndrome**

Apart from gonadotoxic treatments, sterility can also occur in patients with Klinefelter syndrome (KS). KS is caused by the presence of one or more extra X chromosomes (80% have 47,XXY, while the remaining 20% represent higher grade chromosome aneuploidies or mosaicism). KS represents the most common genetic cause of azoospermia and occurs in 1 in 600 newborn males. KS patients have a relatively normal germ cell number in the testis at birth, but this gradually declines during childhood (Wikström et al., 2004). In adult life the syndrome is associated with extensive fibrosis and hyalinization of the seminiferous tubules, and Leydig cell hyperplasia. Patients experience hypergonadotrophic hypogonadism and inhibin B levels are undetectable (Akslaaede et al., 2006). Most of the patients are diagnosed because of their fertility problems. In 50% of the patients spermatogonia can be collected using TESE which can be used in assisted reproduction techniques (ARTs) (Staessen et al., 1996; Tournaye et al., 1996; Friedler et al., 2001; Verveave et al., 2004). Unfortunately, the remaining other half of the patients cannot father their own children. Cryopreservation of testicular tissue before SSC loss could be an option for these patients. We started banking testicular tissue from KS boys (age range: 13–16 years) in 2009. So far, testicular tissue from ten patients has been cryopreserved and spermatogonia were observed in about half of them, an observation that is in agreement with a Finnish study (Wikström et al., 2004; Van Saen et al., 2012).

**Clinical testicular biopsy**

**How to biopsy testicular tissue?**

The first aim should always be to retrieve spermatozoa. Spermatozoa can be collected by masturbation, penile vibrostimulation (Brackett et al., 1999) or electroejaculation (Seager and Halstead, 1993). However, if sperm collection is not possible, testicular tissue recovery might be performed. For patients receiving gonadotoxic treatment, the biopsy can be taken under general anaesthesia at the time of placing the central line. For Klinefelter patients, the tests should be biopsied as soon as possible after diagnosis. We opt to take a large biopsy from the lower pole of the largest testis (Gies et al., 2012). The technique of a single large volume biopsy instead of the multiple biopsy sampling method is preferred to avoid the risk of post-operative fibrosis and to preserve maximal endocrine testicular function. We propose to remove at least half of one testis, for two reasons: (i) testes of children are small and contain only very few SSCs. To obtain enough SSCs to recolonize an adult testis, small biopsies might be inadequate and (ii) to have enough testicular tissue for several fertility restoration methods/trials.

While the majority of the biopsy should be kept for later use, a small testis fragment (6 mm³) should be fixed for histological analyses. Performing analyses for tubular integrity and the presence of SSCs is recommended.

There is a slight risk of bleeding or infection and the area may become discoloured and this should clear up within a few days of the procedure. In general, the risks for acute and late-onset complications of testicular biopsy in children are considered low and hence elective biopsies are frequently obtained in children, for example children with leukaemia (Chan et al., 1988) or even in children with genetic disorders (Vogels et al., 2008).
conditions after freezing, since only the ‘strongest’ cells will survive the freezing procedure.

Which protocol is optimal?
Most of the centres offering testicular tissue banking use a controlled slow freezing protocol with DMSO as cryoprotectant (Keros et al., 2007; Wyns et al., 2011). As this method is time consuming and requires expensive equipment, a user-friendly and time-saving uncontrolled slow-freezing protocol was proposed. After having proved the effectiveness in a mouse model (Baert et al., submitted for publication), the uncontrolled slow-freezing method using 1.5 M DMSO and 0.1 M sucrose was validated using adult human testis tissue (Baert et al., submitted). This protocol did not only preserve the seminiferous epithelium and the interstitial compartment at a (ultra)structural level, but the spermatogonia also maintained the potential to divide. More research is required to confirm these findings using human prepubertal tissue.

An alternative to conventional freezing is vitrification. Vitrification involves the use of increasing concentrations of cryoprotectants and ultrarapid cooling to avoid ice crystal formation. Recently, the efficiency of vitrification was explored using immature human testicular tissue. The tubular integrity and the proliferation potential of spermatogonia could be maintained (Curaba et al., 2011).

Although difficult to assess, the effect on tissue functionality has to be investigated by xenotransplantation or IVM. The effect of uncontrolled slow freezing of non-human primate testicular tissue has been evaluated by xenografting. Different conditions were compared and freezing with 1.4 M DMSO yielded better graft survival than 0.7 M DMSO and ethylene glycol (Jahnukainen et al., 2007). However, xenotransplantation of primate or human tissue to the mouse testis does not restore full spermatogenesis. In human xenografts, at least early germ cell differentiation can be observed up to the spermatocyte level (Van Saen et al., 2012). Eventually, xenotransplantation to higher animal species, such as primates, can be considered. Recently, IVM of mouse testicular fragments has been reported, but the efficiency was very low (Sato et al., 2011). At present, this culture method is not yet reported for human tissue, but it may be an option to assess functionality after cryopreservation in the future.

As long as there is no efficient way to evaluate the functionality of human testicular tissue after freezing, it is difficult to conclude which of the tested protocols is optimal. It has been shown that cell viability does not necessarily correspond to the functional capacity of the SSCs (Frederick et al., 2004).

Storage or transport of tissue before cryopreservation
In some cases immediate cryopreservation is not possible or wanted. This may occur when, after procurement, the tissue has to be transported to a nearby hospital or laboratory. Therefore, short-term preservation of testis tissue may be required before cryopreservation. The effect of storage medium, tissue sample size and hypothermia have been investigated on porcine tissue (Yang and Honaramooz, 2010; Yang et al., 2010). The results showed that testicular cells can be preserved for 6 days at 4°C and maintain a viability of more than 80%. The tissue sample size did not affect viability. In contrast, the medium had a significant effect on viability, with Leibovitz medium + 20% fetal bovine serum yielding the highest scores. Cooling primate testicular tissue for 24 h at 4°C before grafting could improve graft outcome. Cooled grafts were significantly heavier, showed higher graft survival and more seminiferous tubules containing spermatogenesis compared with samples grafted immediately (Jahnukainen et al., 2007). To optimize the conditions for transporting human prepubertal testis tissue, these experiments should be repeated with human material.

Transplantation: different patients, different strategies
Two transplantation strategies are currently being studied: the injection of SSCs and the grafting of testicular tissue: the latter has the advantage that SSCs can remain within their natural niche, thereby preserving interactions between the germ cells and their supporting cells. Therefore, grafting may be regarded as a first-choice strategy. Only in cases where the risk for malignancy recurrence is real, transplantation of SSCs by injection might be proposed. An alternative approach would be to mature SSCs in vitro in combination with ART. But as mentioned earlier, this method has not been studied in men thus far (Fig. 1).

Grafting
Where to graft? Tissue can be grafted to an ectopic or homotopic location. Initially, in mouse models, grafting was performed to ectopic sites, such as in the peritoneal space, the ear or under the skin on the back (Boyle et al., 1975; Schlatt et al., 2002). Full spermatogenesis was reported in xenografts from several species (Honaramooz et al., 2002; Schlatt et al., 2002, 2003, Honaramooz et al., 2004; Oatley et al., 2004; Snedaker et al., 2004; Rathi et al., 2006, Zeng et al., 2006; Abrishami et al., 2010), but never in marmoset (Schlatt et al., 2002; Wistuba et al., 2004). The higher temperature at these ectopic sites compared with the scrotum was suggested to be the cause of either sclerosis of the graft or meiotic arrest. Consequently, tissue was grafted to the scrotum (Leutjens et al., 2008). A recent study in which testicular fragments were autologously grafted to several locations in the irradiated primate body showed that spermatogenesis could only be re-established when the graft was placed in the scrotum (Jahnukainen et al., 2012). However, although many fragments were transplanted, a very low graft survival was reported. Transplantation of the tissue under the tunica albuginea of the testis (intratesticular grafting) might improve the efficiency because, in mice, this technique proved highly efficient (Van Saen et al., 2009). Compared with ectopic xenografting (Goossens et al., 2008a), xenotransplantation of prepubertal human testis tissue into the mouse testis also resulted in better graft survival and initiation of germ cell differentiation (Van Saen et al., 2011).

Are additional interventions necessary? In the mouse, a considerable loss of SSCs after intratesticular grafting was reported (Van Saen et al., 2011). This loss resulted from degeneration of tubules in the centre of the graft, possibly due to hypoxia during the first days after grafting, with the first 4 days after transplantation being critical (Van Saen et al., in press). To reduce this SSC loss, testicular tissue fragments should be kept as small as possible and/or early blood supply to the grafted tissue should be stimulated. Vascular endothelial growth factor (VEGF) is an important regulator of angiogenesis during development as well as in adulthood (Holmes and Zachary, 2005). VEGF-treated grafts showed increased graft weight and more tubules contained...
Elongating spermatids. These VEGF-treated grafts tended to have better vasculature compared with control grafts (Schmidt et al., 2006). Next to its role in angiogenesis, VEGF also regulates SSC homeostasis (Caires et al., 2012). Prior in vitro treatment with VEGF prevented germ cell death and stimulated differentiation in bovine grafts (Caires et al., 2009). Other factors which stimulate angiogenesis and germ cell survival could also improve graft outcome. Treatment with sphingosine-1-phosphate, for example, resulted in a higher blood vessel density in ovarian transplants (Soleimani et al., 2011). However, it is not clear which treatment strategy should be applied: in vitro stimulation of the tissue fragments with VEGF prior to grafting, injections with VEGF at the graft site at the time of grafting or a combination of both. Further research is needed to elucidate the role of these factors and treatment strategies in reducing SSC loss after grafting.

When to graft? At present, it remains unknown whether testicular grafts are better supported by an immature prepubertal environment or by a mature one. The influence of the recipients’ maturation state (pre-pubertal, pubertal or post-pubertal) on the efficiency of re-establishing spermatogenesis still has to be explored.

Initiation and conservation of spermatogenesis depends on the constant and controlled interaction of hormones. High FSH levels stimulate Sertoli cell proliferation and maturation, while LH induces Leydig cell maturation and subsequent androgen production. The endocrine environment at the time of grafting is important for proper re-establishment of spermatogenesis. For ectopic grafting in animal models, high FSH and LH levels were obtained by castration (Schlatt et al., 2002). However, for obvious reasons, castration is not considered in the human. Because high levels of FSH and LH are observed just before puberty, this may be a good time for autologous grafting. However, at that time point, there may still be uncertainty about the health prognosis and the final impact of gonadotoxic treatment on fertility. Indeed, patients’ fertility can recover spontaneously, sometimes even after more than 10 years (Lampe et al., 1997). Therefore, transplantation in adulthood would be preferable. Adult patients will return to the fertility clinic when
natural conception does not occur. In contrast to other adult men, these patients have undergone testicular biopsy or (hemi)-orchietomy and had gonadotoxic treatment. Hence, they have already higher levels of FSH and LH, making both ectopic and homotopic grafting also feasible at adult age.

Increased FSH and LH levels might change the micro-environment in the remaining testicle allowing proliferation and maturation of Sertoli cells and Leydig cells. As a result, spontaneous fertility restoration might be advanced and grafting might not be necessary at all. A good follow-up of the patients who have banked testis tissue is thus extremely useful and indispensable for good counselling.

*How long will grafts last?* Evidence shows that spermatogenesis in ectopic grafts may not be everlasting. Due to the lack of an excretory system in the grafts, spermatoozoa and fluid may accumulate causing damage to the epithelium. At present, it is unclear whether macrophages will remove these excess sperm cells as they do after vasectomy. If not, spermatoozoa will undergo ageing and lose their fertilization capacity over time. Therefore, the ideal grafting period should be determined allowing the collection of spermatoozoa before these processes begin. In mouse ectopic grafts, the increase in FSH levels correlated with the damage of the seminiferous epithelium. Changes in the serum FSH levels could thus be used to determine the ideal time for sperm retrieval from the grafted tissue (Schlatt et al., 2003). Ectopic grafting always has to be combined with ART.

Also for intratesticular grafts, it is still an open question whether connections can be established between grafted and endogenous tubules, ensuring a functional excretory system. As part of the endogenous tubules are removed—and thus pulled apart—before placing the graft, this is theoretically possible. However, after grafting in rodent models, the presence of donor-derived spermatoozoa in the epididymis has not been reported and, thus, the chance for natural reproduction may be very small.

Fertilizing capacity after grafting. Little is known about the functionality of the sperm generated in the grafts. Only a few groups have addressed this question using mouse and rabbit donor tissue. ICSI using sperm retrieved from ectopic and intratesticular mouse allografts demonstrated that the spermatoozoa were able to support full-term pregnancy (Schlatt et al., 2003; Ohta and Wakayama, 2005). It was also possible to obtain offspring using rabbit sperm that had developed in intratesticular xenografts (Shinohara et al., 2002) and porcine sperm extracted from ectopic xenografts in mice (Nakai et al., 2010). Normal blastocyst development in vitro was obtained after performing ICSI with sperm from ectopic porcine and monkey xenografts (Honaramooz et al., 2004, 2008). As it is not possible to perform such experiments using sperm obtained from human xenografts, research on large mammals (e.g. non-human primates) will be of great value.

**SSC injection**

In contrast to grafting strategies, natural conception might be possible after the injection of SSCs into seminiferous tubules. This technique was first described in the mouse almost 20 years ago (Brinster and Zimmerman, 1994) and has since been used widely in both fundamental and translational research. Five years later, SSC injection was first proposed as a fertility preservation strategy (Bahadur and Ralph, 1999).

**How to transplant SSCs?** In the mouse, a testicular cell suspension containing SSCs is introduced through the efferent duct in the rete testis. This allows the filling of several seminiferous tubules with one single puncture (Ogawa et al., 1997). Because of differences in anatomy and consistency (texture) of the tissue, larger testes, for example bovine, primate, human, are not easy to inject via the efferent ducts. The rete testis, which can be visualized by ultrasound, proved to be a better injection site (Schlatt et al., 1999). Compared with a single injection, multiple injections of acellular solutions did not improve the filling efficiency of human cadaver testes (Ning et al., 2012). We experienced that testses from aging donors (aged 40–65 years) were more difficult to inject than the ones from aged donors (aged 65–83 years), because the older testes have less tension. Since testes of boys who were treated with testicular irradiation or with high doses of alkylating agents do not contain differentiated germ cells (Relander et al., 2000), their testes may have a consistency similar to those from the aged donors. Because the inner testicular pressure in testes in vivo might be higher, the infusion technique might have to be adapted to be applicable in a clinical setting. As these experiments are not possible in the human, experiments on primates or other large mammals are of high value. Very recently, rhesus monkey SSCs have been used for autologous and allogeneic transplantation. SSC infusion was performed using ultrasound-guided rete testis injections. Cells were injected under slow constant pressure and chased with saline. After having reached maturity, mature sperm cells could be found in the ejaculate in 60% of the prepubertal recipients and sperm cells were able to fertilize oocytes in ICSI. This demonstration of functional donor spermatogenesis following SSCT in non-human primates is an important milestone in the translation of SSC transplantation to a clinical setting (Hermann et al., 2012).

**Are additional interventions necessary?** Only SSCs will relocate to the basement membrane, while the somatic and more advanced germ cells will be eliminated primarily through uptake and phagocytosis by Sertoli cells (Parreira et al., 1998). The quantity of cells injected (and thus the fraction of SSCs in the injected suspension) is the determining factor for the success of transplantation.

Several enrichment strategies have been proposed. The first attempts made use of sedimentation velocity in combination with differential plating (Morena et al., 1996; Dirami et al., 1999), but the efficiency was improved when sorting techniques [using magnetic-activated cell sorting (MACS) or a fluorescence-activated cell sorter (FACS)] were employed. In mice, highly enriched SSC populations were obtained using the following marker sets: β1-integrin+/α6-integrin+/c-kit+/α7-integrin− (Shinohara et al., 2000) or MHC-I+/Thy-1+/c-kit− (Kubota et al., 2003). During the last few years many other markers for spermatogonia have been discovered (for review: Phillips et al., 2010). Inhibitor of DNA binding 4 (ID4) might be the marker that is most specific for SSCs (Oatly et al., 2011). Human SSCs share several markers with mouse SSCs: α6,-integrin, GFRα1 and THY1. However, other markers are not shared: α7-integrin, TSPY1, CD133 and SSEA4 (Dym et al., 2009). The search for (more) specific SSC markers is ongoing. But for clinical
applications, a stringent selection to obtain only SSCs might not be needed. In the injured testis, undifferentiated spermatogonia might revert to SSCs and restore spermatogenesis (Nakagawa et al., 2007).

However, even if spermatogonia could be highly enriched, for clinical application it might still be unsatisfactory. Indeed, biopsies obtained from young boys are small and contain only few SSCs. Therefore, the propagation of these cells in vitro will be necessary. In 2009, such a culture system was described starting from adult testicular tissue (Sadri-Ardekani et al., 2009). Two years later, the same group validated their results on prepubertal testis tissue (Sadri-Ardekani et al., 2011). They obtained a more than 18 000-fold increase in SSC number after 2 months of culture. As it has been estimated that a 1300-fold increase would be adequate to repopulate an adult human testis, a 1-month period of culture should be sufficient. Before this culture system can be applied in the clinic, more research has to be carried out on the (epi)genetic stability of the cells in culture, the competence of these cells to repopulate the basal membrane and the fertilizing capacity of the spermatozoa generated from SSCs propagated in vitro. Also, animal-derived media components need to be replaced by clinical grade components.

When to transplant SSCs? In mice, it has been reported that colonization efficiency is higher when SSC transplantation is performed in immature recipients compared with adult recipients who have been sterilized by chemotherapy (Ogawa et al., 1999). However, because in these experiments, immature recipients did not receive a sterilizing treatment the validity of the results can be questioned. The study of Hermann et al. (2012) showed that spermatozoa could be obtained both after autologous transplantation of SSCs in either prepubertal or adult recipients. This is an important finding for any future clinical application, because it implies that SSC transplantation may be postponed for several years until it becomes clear whether the patient is infertile.

In contrast to spermatogenesis established in testicular tissue grafts, spermatogenesis might be retained lifelong after SSC injection. Indeed, serial transplantation experiments in mice showed that SSCs were still able to self-renew and to colonize recipient mouse seminiferous tubules after at least nine successive transplantations (Ryu et al., 2006). As SSCs in human have different characteristics than those in rodents, similar experiments in animals more closely related to humans should be conducted.

Fertilizing capacity after SSC transplantation. A number of studies evaluating the efficiency of fertilization after SSCT have been performed. Compared with fertile controls, the fertilization capacity of spermatozoa obtained after SSCT was reduced both after in vivo conception and IVF, but not after ICSI (Goossens et al., 2003). The lower fertilization rate after IVF could be explained by a reduced motility of the spermatozoa as was shown by computer-assisted motility analysis (Goossens et al., 2008b). Live born offspring obtained after SSCT were found to be healthy. They showed normal weights and lengths and were fertile. Numerical chromosomal aberrations could not be detected in spermatozoa from transplanted males, or in their offspring (Goossens et al., 2010).

Since epigenetic modifications are essential for several developmental events, it is important that epigenetic characteristics are established correctly during spermatogenesis and in the offspring. A study examining the methylation pattern in mouse spermatozoa did not reveal any differences in three studied genes (Goossens et al., 2009). The general methylation status and specific histone modifications were studied in post-transplantation germ cells in a stage-dependent manner and were no different compared with control spermatogenesis. However, a deficient expression of two histone modifications was found after SSCT (Goossens et al., 2011). Although H4K5ac and H4K8ac expression in early germ cells differed from controls, the acetylation of H4 was correct at the time when histones have to be exchanged for protamines (in spermatids). The relationship between epigenetic changes and spermatogenesis is still largely unknown. It is still unclear why some modifications occur only in certain stages and what role they play, for example H4 acetylation in spermatogonia and spermatocytes. It is therefore interesting to carry out fundamental research into the exact functions of these modifications and their impact on germ cell development or fertility.

It would also be worthwhile to perform efficiency and safety studies after autologous transplantation in larger mammals.

Other unanswered questions

What if there is a risk that the biopsy contains malignant cells? Many paediatric malignancies are capable of metastasizing through the blood, with a potential risk for contamination of the collected testicular tissue. The transplantation of as few as 20 leukaemic cells could cause malignant recurrence in rats (Jahnukainen et al., 2001). In the human, the threshold number of malignant cells able to cause malignant relapse when transplanted to the testis is unknown. Therefore, it is of immense importance to detect even the slightest contamination of the testicular tissue. In case of contamination, the separation of SSCs from malignant cells before transplantation is necessary. For this purpose, the use of MACS and/or FACS for depleting cancer cells from mouse and human testicular cell suspensions has been studied but was reported to be insufficient (Fujita et al., 2005 and 2006; Geens et al., 2007). Also, cell selection by selective matrix adhesion was not sufficiently efficient (Geens et al., 2011). The poor specificity of spermatogonial surface markers and the aggregation of germ and leukaemic cells was the limiting factor in positive selection of germ cells. Applying singlet discrimination can circumvent miss-sorting resulting from cell clumping (Hermann et al., 2011). Also, immunophenotypic variation among lymphoblastic leukaemia cells can hamper the efficient removal of leukaemic cells. This immunophenotypic variation could be caused either by the enzymatic treatment or by the intratesticular environment. Therefore, a combination of both positive and negative selection will be necessary to remove all cancer cells and prevent leukaemia transmission (Hou et al., 2007a).

Since decontamination results are not conclusive, other decontamination strategies should be tested. It is possible that the culture system for propagating SSCs does not support the growth of malignant cells, but this still needs to be proved.

Xenografting could offer an alternative. Germ cells produced in ectopic xenografts could then be used in ART. However, up to now only spermatogonial survival was achieved in ectopic human prepubertal xenografts (Goossens et al., 2008b). Xenografting also raises ethical and safety concerns. An important issue for public health with regard to xenotransplantation is the risk of unintended introduction of xenogeneic infections into the human population. Endogenous retroviruses
exist as proviral DNA in the germ lines of all mammals. These retroviruses remain as an inherited part of the genetic structure and do not cause disease in the host species. However, they are able to infect cells from other species (Chapman, 2009).

Xenotransplantation could, however, serve to detect contamination in cryopreserved tissue (Kim et al., 2001; Hou et al., 2007b). While classical histology failed to detect malignant cells in ovarian tissue from leukaemic patients, grafting and quantitative real-time PCR showed the presence of contaminated cells (Dolmans et al., 2010). Quantitative real-time PCR could be a very sensitive technique to detect residual minimal diseases using patient-specific primers encoding the rearranged B- or T-cell receptor of the malignant clone (Geens et al., 2007). Still the question remains of how safe transplantation will be. The absence of contaminating cells in the screened piece of tissue cannot guarantee their absence in another part of the tissue. Therefore, for patients at risk for malignant contamination, autologous testicular tissue grafting will not be possible, so that the only option to restore fertility will be by transplanting cell suspensions.

**What in case of atrophied testes at the time of transplantation?** Apart from malignant diseases, sterility because of germ cell loss can also occur in patients with KS (47,XXY). Fifty per cent of these boys still have germ cells in their testes around puberty. Nevertheless, these germ cells will disappear after puberty (Wikström et al., 2004). Whether SSC transplantation or grafting may be an option in boys with KS remains unknown because the majority of them will show degenerated testes at adult age (Akslaede et al., 2006). Alternatively, SSCs may be cultured in vitro to produce sperm for ART, for example ICSI. In the initial attempts to achieve in vitro spermatogenesis, spermatogenesis was co-cultured with Sertoli cells. Haploid cells were detected by flow cytometry and spermatid-specific markers were expressed in rat and bull cultures (Lee et al., 2001; Iwanami et al., 2006). However, their functionality could not be proved as no viable offspring were produced after performing microinjection into activated oocytes (Iwanami et al., 2006). The spatial arrangement of testicular cells in their natural environment cannot be achieved by the conventional culture methods. A three-dimensional soft-agar-culture system was designed to mimic the in vivo features of the testis. When mouse SSCs were cultured in a three-dimensional soft agar culture system in the presence of somatic testicular cells, differentiation up to the level of post-meiotic cells was achieved (Stukenborg et al., 2008). Spermatozoa were observed when cell suspensions were cultured in the presence of gonadotrophins (Stukenborg et al., 2009). However, the functionality and safety of these in vitro produced spermatozoa still needs to be proved. Recently, Sato et al. (2011) reported the production of spermatozoa in organ culture in which fresh and frozen-thawed testis tissue fragments of mouse pups were cultured on agar half-soaked in medium. Round spermatids and spermatozoa were collected from cultured tissue fragments and used for round spermatid injection and ICSI, respectively. Live progeny were born.

So far, in vitro generation of human sperm cells from SSCs has not been reported. Late spermatids could be produced from late pachytene spermatocytes and secondary spermatocytes in co-cultures obtained from azoospermic patients. The in vitro matured spermatids were microinjected into oocytes, but showed low fertilization rates and chromosomal abnormalities were found in all generated embryos (Sousa et al., 2002). The initiation of meiosis in vitro remains the critical event to achieve full spermatogenesis. More recently, haploid human cells were generated using isolated CD49f+ cells from an azoospermic patient (Riboldi et al., 2012). Future research is definitely needed to establish full human spermatogenesis in vitro.

In vitro spermatogenesis could also be an alternative to circumvent the transmission of malignant cells to patients at risk for malignant contamination in their testis, for example leukaemic boys. However, in contrast to SSC transplantation, this strategy entails the need for ART.

**Conclusion**

Taking into account the progress that already has been made and the barriers that still have to be overcome, the first patients who will probably undergo SSC transplantation are those who suffered from non-malignant diseases (for example sickle-cell anaemia treated by bone marrow transplantation) as for these patients there will be no risk of re-introducing malignant cells into the testis. Grafting testicular tissue is a relatively easy technique but the question remaining is how much, and where, the tissue should be grafted. As long as there is no solution for the early hypoxia seen in testicular tissue grafts, necrosis will occur during the first few days after grafting (Van Saen et al., in press). Because the testicular fragments are small and might contain only few SSCs, the loss of even a few of these cells may have a substantial impact on fertility restoration. Also, as shown in a recent study on primates, multiple tissue fragments should be grafted to have a realistic chance of retrieving spermatozoa (Jahnukainen et al., 2012). Therefore, we would recommend transplanting multiple tissue fragments to different sites on the body: under the skin, in the scrotum and in the testis. It would also be useful to keep some of the stored tissue for a—possible—second grafting or for other strategies (e.g. in vitro spermatogenesis, xenografting).

At this point in time, restoring fertility in patients who have been treated for cancer is a bridge too far. The frozen-thawed biopsy has to be enzymatically digested to enable the removal of malignant cells using cell sorting strategies or differential cell culture. However, at present, this is not possible. Once the tissue is digested, grafting is no longer feasible, and the only remaining option is to inject the cell suspension in the testis. As in the mouse, it has been estimated that only 12% of the SSCs can colonize the recipient testis (Nagano, 2003) and, because biopsies taken from boys are small, SSCs should be propagated in vitro prior to injection to increase the chance for fertility restoration. However, to date, the safety aspects of the in vitro culture systems, as regards (epi)genetic stability, remain to be examined.

For patients with KS, yet another strategy is required, because 47,XXY testes may become atrophied by adult age. Grafting and cell injection are thus inappropriate approaches. The only option would be in vitro differentiation followed by ICSI. As research on in vitro spermatogenesis in mice is only starting, this method will probably not be applicable for humans in the years to come.

This review started with the presentation of a boy who had been suffering from a non-malignant disease and who later returned to the fertility department to ask about fertility restoration strategies. His indication would have favoured the grafting of testicular tissue fragments. However, as the boy was still pubertal at the time of
consultation, the impact of gonadotoxic treatment remained unclear. Therefore, we advised the boy and his parents to wait a few more years and return for transplantation only if normal fertility appeared to be compromised.

**Authors’ roles**

E.G. designed the review concept, performed the research and drafted the review, D.V.S. and H.T. performed the literature research and revised the review.

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**Conflict of interest**

None declared.

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