Autologous spermatogonial stem cell transplantation in man: current obstacles for a future clinical application

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Fertility preservation is becoming an important issue in the management of the quality of life of prepubertal boys undergoing cancer treatment. At present, the only theoretical option for preservation of fertility in these boys is the preservation of the spermatogonial stem cells for autologous intratesticular stem cell transplantation. In animal models, this technique has shown promising results. However, before translation to the clinic, some major concerns should be evaluated. Improving the efficiency of the technique is one of the first goals for further research, besides evaluation of the safety of the clinical application. Also, the cryopreservation of the spermatogonial stem cells needs extra attention, since this first step will be crucial in the success of any clinical application. Another concern is the risk of malignant contamination of the testicular tissue in childhood cancer patients. Extensive research in this field and especially on the feasibility of decontaminating the testicular tissue will be inevitable. Another important, though overlooked, issue is the prevention of damage to the testicular niche cells. Finally, xenografting and in vitro proliferation/maturation of the spermatogonia should be studied as alternatives for the transplantation technique.

Keywords: fertility preservation; autologous intratesticular stem cell transplantation; stem cell cryopreservation; childhood cancer survivors; animal models

Spermatogonial Stem Cell Transplantation: a Method for Fertility Restoration in Cancer Survivors?

Childhood cancer survivors

The gradual progress in the understanding and the treatment of cancer can ensure the majority of children to survive their malignancy. Even though not all cancer treatments impair fertility, a significant number of childhood cancer survivors are at risk of sterility due to the loss of spermatogenic cells after treatment, especially prepubertal boys treated with high-dose chemotherapy, total body irradiation and/or irradiation involving the genital region. The nature and amount of damage to the gonads is dependent on the patient’s gender, the age at the time of treatment, the field of treatment, the total irradiation dose and fractionation schedule and the total dose and nature of chemotherapy agents received (Waring and Wallace, 2000; Wallace and Thomson, 2003; Fossá and Magelssen, 2004).

Thomson et al. (2002) reported a case–control study, indicating that 30% (10 patients out of 33) of male childhood cancer survivors were azoospermic at adolescent or adult age. Of these azoospermic survivors, five had been treated for Hodgkin’s disease with alkylating agents known to be gonadotoxic (chlorambucil, procarbazine and vinblastine). Two patients had received total body irradiation [for acute lymphoblastic leukaemia (ALL) and for B-cell non-Hodgkin lymphoma]; two had been treated for Ewing’s sarcoma with ifosfamide and one had received direct testicular irradiation for ALL with testicular relapse. Moreover, in the non-azoospermic group, sperm concentration was significantly lower than that of controls.

Even though the primary objective is to cure a child affected by malignancy, sterility is a major problem in the context of ‘quality of life’. The inability to father genetically own children might have high impact on the psychological well-being of the patient in later adulthood (Schover, 2005; Van den Berg et al., 2007). At present, there are no options to preserve fertility that can be offered to prepubertal boys. The only option for fertility preservation could be spermatogonial stem cell preservation. Cryopreservation of spermatogonial stem cells before the start of any cancer therapy followed by autologous intra-testicular transplantation of these stem cells after cure is a hypothetical option that is currently...
thoroughly studied by a few research groups, mainly in rodent models. Figure 1 shows the theoretical set-up of this preservation strategy.

This strategy might eventually be useful for all males with cancer. Whereas in prepubertal boys the most commonly occurring malignancies are acute lymphoblastic leukemia (ALL) and brain tumours, males aged between 15 and 45 are most frequently affected by testicular and lymphomatous neoplasms (Colpi et al., 2004; Magelssen et al., 2006).

The most modern oncological protocols for malignant lymphomas, such as ABVD (doxorubicin, bleomycin, vinblastine, dacarbazine), do not contain alkylating agents and are therefore less gonadotoxic. Post-treatment recovery of spermatogenesis can be expected in >90% of patients. However, the repair can take up to 20 years (Fosså and Magelssen, 2004). For testicular tumours, the main chemotherapeutic treatment regimens are cisplatin-based. This agent is relative spermiotoxic and therefore leads to temporary or permanent azoospermia in almost all patients. Moreover, it has a synergic interaction with the spermotoxicity of radiotherapy (Colpi et al., 2004; Fosså and Magelssen, 2004). In general, male cancer patients diagnosed between the age of 15 and 45 years, have a 15-year cumulative probability of post-treatment paternity of only ±32% (Fosså et al., 2005).

Even though the primary fertility-preserving measure for adolescent and adult men is semen cryopreservation, the percentage of cancer patients actually having their semen frozen before the start of the cancer treatment is quite low (Tournaye et al., 2004). Moreover, patients may already be azoospermic at the moment of cancer diagnosis due to factors related to the malignancy. Especially, in patients with testicular cancer, azoospermia may be encountered (Berthelsen and Skakkebaek, 1983; Fosså and Magelssen, 2004). Therefore, adult men could also benefit from other techniques for fertility preservation, including the spermatogonial stem cell transplantation.

Also patients suffering from non-malignant diseases could benefit from the spermatogonial stem cell transplantation strategy. In severe cases of sickle cell disease or beta-thalassemia major for example, treatment may include total body irradiation or severe chemotherapy for the eradication of bone marrow cells, followed by hematopoietic stem cell transplantation (Brachet et al., 2007; Resnick et al., 2007). Therefore, these patients are also at risk of infertility after the treatment.

Most of the results of the studies on spermatogonial stem cell transplantation look very promising, and, therefore, a future clinical application is hoped for by many clinicians, patients and parents. However, many questions and obstacles still should be solved in order to be able to help the first patient.

**Spermatogonial stem cell transplantation**

The spermatogonial stem cells are the male germline stem cells. They can self renew to maintain the stem cell population and produce large numbers of differentiating cells of the spermatogenic line, eventually leading to mature spermatozoa that will transmit the genome to the next generation. Spermatogonial stem cell transplantation was first introduced in the mouse in 1994 by Brinster et al. (Brinster and Avarbock, 1994; Brinster and Zimmerman, 1994) as an assay for stem cell function. Spermatogonia from a fertile donor mouse were injected into the seminiferous tubules of a sterile recipient. The donor spermatogonia were able to colonize the seminiferous tubules of the recipients and, in some cases, induce active spermatogenesis. Soon after these first studies, more experiments were performed by different groups, using both fresh and frozen--thawed samples in different
species, including primates (Avarbock et al., 1996; Schlatt et al., 2002). These encouraging results suggest that this method might also be successful in the human and that, if this technique could be clinically applied, it may be able to restore fertility in some patients (Brinster, 2007; Goossens and Tournaye, 2007).

**Spermatogonial Stem Cell Transplantation in Human?**

**Efficiency and safety**

The efficiency of the spermatogonial stem cell transplantation is highly associated with the number of stem cells injected (Dobrinski et al., 1999a). However, spermatogonial stem cells represent only a small proportion of the total testicular cells [estimated around 0.03% in mouse; in human this percentage is assumed to be higher (Meistrich and van Beek, 1993; Tegelenbosch and de Rooij, 1993)]. Moreover, if applied in a clinical setting, one small prepubertal testis at the most could be removed for cryopreservation and future autologous testicular stem cell transplantation. Therefore, enrichment of stem cells in the suspensions for transplantation may be necessary in a clinical set-up.

In mice, the initial method for transplantation was intratubular microinjection at multiple places in the seminiferous tubules (Brinster and Zimmerman, 1994). Later, other methods for injection, which have proven as efficient, were applied: injection into the efferent duct or into the rete testis (Ogawa et al., 1997; Russell et al., 1998). In primates and human, the most promising infusion technique for germ cells is ultrasound guided multiple injection into the rete testis since injection of the seminiferous tubules or the efferent duct proved inefficient in larger testes (Schlatt et al., 1999). Brook et al. (2001) modified the method of injection into the rete testis by introducing the fluid by drip feed under gravity. With this method and by using multiple injection sites, +55% of the tubules of human cadaver testes could be filled.

Although the spermatogonial stem cell transplantation has been shown to produce live offspring in mice, some safety concerns should be taken into consideration. Some safety concerns were also observed. The live born pups showed developmental retardation of a quarter of a day, no major external abnormalities were observed. The live born pups were able to produce normal litter sizes and offspring with normal development, at least until the third generation. One possible explanation for the reduced litter sizes after in vivo conception might be associated to a lower sperm concentration and/or motility after transplantation. A detailed analysis of the motility kinematics and concentrations of spermatozoa obtained after transplantation showed decreased motility patterns (Goossens et al., 2008a). The developmental retardation in the first generation offspring, however, may suggest imprinting disorders. Extended research in this field is therefore mandatory before any possible clinical application.

**Cryopreservation**

In a clinical set-up, a period of at least 5 years will probably elapse between removal of the testicular tissue and retransplantation to the patient. Therefore, cryopreservation of prepubertal testicular tissue or testicular cell suspensions is inevitable. An optimal freezing protocol would be of great benefit for fertility preservation and would certainly have a positive effect on further development of the spermatogonial stem cells. Most studies on cryopreservation of testicular cells and tissue aimed at preserving spermatozoa for future use in intracytoplasmic sperm injection (ICSI) (Hovatta et al., 1996; Oates et al., 1997; Crabbe et al., 1999). However, in case of clinical use of the spermatogonial stem cells, cryopreservation protocols should be adapted in particular to these cells. The first successful transplantation after cryopreservation of murine testicular cell suspensions was reported by Avarbock et al. (1996). Three years later, testicular cell suspensions of rabbit, dog and hamster were efficiently frozen/thawed using a simple but effective, non-controlled rate freezing protocol with the cryoprotectant dimethylsulphoxide (DMSO) (10%) (Dobrinski et al., 1999b; Ogawa et al., 1999). Izadyar et al. (2002) studied the cryopreservation of bovine type A spermatogonia, the class of spermatogonia that contain the spermatogonial stem cells. The best protocol in their study was similar to that reported by Dobrinski et al. (1999a,b) and Ogawa et al. (1999), but they included sucrose (0.07 M) as cryoprotectant. With this protocol, almost 70% of the type A spermatogonia survived freeze/thawing and maintained proliferative capacity. Recently, primate testicular cell suspensions were frozen by Hermann et al. (2007). They also applied the non-controlled rate freezing protocol with DMSO (10%) and reported cell viability of 58% after thawing. An overview of the studies on the cryopreservation of testicular cell suspensions with the applied freezing protocols and their respective cell viability outcome after thawing can be found in Table I.

In some cases, it might be important to preserve not only the spermatogonutria but also the niche cells together with all the cell–cell contacts within the tissue (Keros et al., 2005; Ogawa et al., 2005). Since the development and structure of the testis in prepubertal boys is not similar to that in adult men, protocols should be adapted for this tissue. Recently, two groups reported freezing protocols for pre-pubertal human testicular tissue that could well preserve the structural integrity. Kvist et al. (2006) used a slow freezing protocol, adapted from a cryopreservation protocol for ovarian cortex (Newton et al., 1996), to freeze testicular tissue from prepubertal boys with cryptorchidism. They used 1.5 M ethylene glycol (EG) and 0.1 M sucrose as cryoprotectants, whereas Keros et al. (2007) applied slow programmed freezing with DMSO (5%) as cryoprotectant.

Primary research on the survival and proliferation of frozen/thawed testis tissue pieces as allo- or xenografts has already been performed in some animal models. These studies will be discussed below.

**Malignant contamination**

One of the major risks associated with autologous transplantation in cancer patients is the possibility of re-introducing malignant cells to the patient. The majority of paediatric malignancies are capable of metastasizing through the blood, making the risk of contamination of the collected testicular tissue considerably high (Jahnukainen et al., 2006). Isolation of the spermatogonial stem cells from malignant cells before transplantation will therefore be inevitable in a clinical set-up. Recently, two groups studied the use of magnetic- and/or fluorescence-activated cell sorting...
Table I. Overview of the studies on cryopreservation of testicular cell suspensions in various animal models.

<table>
<thead>
<tr>
<th>(Im)mature</th>
<th>Species</th>
<th>Cryoprotectant</th>
<th>(Non-)controlled</th>
<th>Freezing rate</th>
<th>Viability after thawing (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 NA Mouse</td>
<td>20% DMSO</td>
<td>Non-controlled</td>
<td>Insulated container − 70 °C, LN2</td>
<td>30</td>
<td>Avarbock et al. (1996)</td>
<td></td>
</tr>
<tr>
<td>2 Immature Rabbit</td>
<td>10% DMSO</td>
<td>Non-controlled</td>
<td>Insulated container − 70 °C, LN2</td>
<td>63–82</td>
<td>Dobrinski et al. (1999b)</td>
<td></td>
</tr>
<tr>
<td>3 Mature Hamster</td>
<td>No cryoprotectant</td>
<td>Non-controlled</td>
<td>Insulated container − 80 °C, LN2</td>
<td>43</td>
<td>Ogawa et al. (1999)</td>
<td></td>
</tr>
<tr>
<td>4 Immature Bovine</td>
<td>1.4 M glycerol</td>
<td>Controlled</td>
<td>Start: 5 °C, −1 °C/min to −80 °C, −50 °C/min to −120 °C, LN2</td>
<td>35.5 ± 3.3</td>
<td>Izadyar et al. (2002)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.4 M DMSO (10% FCS)</td>
<td>Controlled</td>
<td>Start: 5 °C, −5 °C/min to −80 °C, −50 °C/min to −120 °C, LN2</td>
<td>28.6 ± 3.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.4 M DMSO (10% FCS)</td>
<td>Controlled</td>
<td>Insulated container − 80 °C, LN2</td>
<td>49.3 ± 4.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.4 M DMSO (10% FCS)</td>
<td>Controlled</td>
<td>Insulated container − 80 °C, LN2</td>
<td>54.9 ± 3.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.4 M DMSO (10% FCS)</td>
<td>Controlled</td>
<td>Insulated container − 80 °C, LN2</td>
<td>38.9 ± 4.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.4 M DMSO (20% FCS)</td>
<td>Controlled</td>
<td>Insulated container − 80 °C, LN2</td>
<td>48.5 ± 5.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.4 M DMSO, 0.07 M sucrose</td>
<td>Non-controlled</td>
<td>Insulated container − 80 °C, LN2</td>
<td>68.3 ± 2.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.4 M DMSO, 0.14 M sucrose</td>
<td>Non-controlled</td>
<td>Insulated container − 80 °C, LN2</td>
<td>66.5 ± 3.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.4 M DMSO, 0.21 M sucrose</td>
<td>Non-controlled</td>
<td>Insulated container − 80 °C, LN2</td>
<td>65.8 ± 4.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 Mature Mouse</td>
<td>DMSO (% NA)</td>
<td>Non-controlled</td>
<td>Insulated container − 80 °C, LN2</td>
<td>67.4 ± 5.9</td>
<td>Kanatsu-Shinohara et al. (2003a,b)</td>
<td></td>
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<tr>
<td>6 Immature Mouse</td>
<td>1.5 M DMSO</td>
<td>Non-controlled</td>
<td>Insulated container − 80 °C, LN2</td>
<td>36.0</td>
<td>Frederickx et al. (2004)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Controlled</td>
<td>Start: room temp, − 5 °C/min to −7 °C, hold 15 min + seeding, −0.3 °C/min to −80 °C, LN2</td>
<td>47.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Start: room temp, − 5 °C/min to −7 °C, hold 15 min + seeding, −0.3 °C/min to −40 °C, LN2</td>
<td>57.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.5 M EG</td>
<td>Controlled</td>
<td>Start: room temp, − 5 °C/min to −7 °C, hold 15 min + seeding, −0.3 °C/min to −40 °C, LN2</td>
<td>67.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 Mature Rhesus monkey</td>
<td>10% DMSO</td>
<td>Non-controlled</td>
<td>Insulated container − 80 °C, LN2</td>
<td>58 ± 4.4</td>
<td>Hermann et al. (2007)</td>
<td></td>
</tr>
</tbody>
</table>

EG, ethylene glycol; DMSO, dimethylsulfoxide; LN2, liquid nitrogen; NA, information not available.
(MACS and/or FACS) for depleting cancer cells from murine and human testicular cell suspensions.

Fujita et al. (2005) reported restoration of fertility in sterile mice by transplanting spermatogonial stem cells, isolated from leukaemic mice, without inducing leukaemia in the recipient mice. They used positive selection of germ cells by flow cytometry for H-2Kb/H-2Kd (MHC class I) negative and CD45 (surface marker for leukaemic cells) negative cells. In a human in vitro model, they confirmed that MHC class I and CD45 could also be used as markers for the isolation of germ cells from leukaemia and lymphoma cells in a human set-up (Fujita et al., 2006). Our study, however, did not corroborate these promising results. In the mouse, after a combination of MACS and FACS sorting for H-2Kb negative, α6-integrin positive cells, we demonstrated that still ~0.4% of malignant cells remained in the sorted fractions and that these cells were able to develop colonies in vitro. Moreover, after transplantation of the sorted fractions to 20 recipient mice, one of the recipients developed malignancy. Also, in our human set-up, FACS sorting for HLA-A,B,C negative cells, proved not sufficiently efficient for a total depletion of malignant cells from testicular cell suspensions, as demonstrated by FACS analysis, in vitro culture and PCR analysis (Geens et al., 2007).

Moreover, the translation of these techniques from the animal model to the human is difficult, since to date, contrary to rodents, specific markers for human spermatogonial stem cells are not yet described.

One of the main concerns for malignant cell depletion is probably the level of decontamination required. Jahnukainen et al. (2001) demonstrated in a rat model that transplantation of as few as 20 leukaemic cells could cause malignant recurrence in the recipient animal, and also in our study (Geens et al., 2007), we found the occurrence of tumours in >40% of mice after transplantation with suspensions contaminated with 5–39 malignant cells. In the human, the threshold number of malignant cells able to cause malignant relapse when transplanted to the testis is not known. Therefore, it is utterly important that even the slightest contamination of the testicular tissue can be detected. For this issue, the experience in the detection of minimal residual disease (MRD) in haematological diseases might be of great interest, the experience in the detection of minimal residual contamination of the testicular tissue can be detected. For this reason, the experience in the detection of minimal residual disease (MRD) in haematological diseases might be of great interest. For the detection of MRD in the clinic, molecular techniques have been developed with sensitivity up to 10^6. This means that one malignant cell can be detected among 10^6 normal cells (Willemse et al., 2002). The most sensitive techniques are PCR-based, relying on the break-point regions of leukaemia-specific chromosomal aberrations or on the immunoglobulin or T-cell receptor gene rearrangements (Jolkowska et al., 2007).

Patients with non-malignant haematological diseases, obviously, are not at risk of malignant contamination of their testis tissue. Since this important obstacle of the technique does not apply to them, they might be the first patients to actually benefit from the spermatogonial stem cell transplantation.

**In vitro culture of spermatogonia**

Although still theoretical, solutions for some of the problems mentioned above are being suggested. Possibly the most promising of them is the in vitro culture and expansion of spermatogonial stem cells. Culturing these stem cells in vitro could increase the efficiency of the transplantation technique dramatically, since a high number of pure stem cells can be injected. Moreover, using a spermatogonial stem cell-specific culture protocol, it might be an efficient method of depleting malignant cells from germ cells. On the other hand, long-term spermatogonial stem cell culture could be useful for studying spermatogenesis mechanism and might have important implications for developing new technology in transgenesis (Nagano et al., 2001; Hamra et al., 2002). Long-term culture may even be an alternative for cryopreservation.

Nagano et al. (1998) were the first group to report long-term survival of mouse spermatogonial stem cells in vitro. Moreover, they suggested that by blocking spermatogonial differentiation, the in vitro maintenance of the stem cells could be improved (Nagano et al., 2003). Kanatsu-Shinohara et al. (2003a) reported not only long-term survival but also proliferation of spermatogonial stem cells. In the presence of glial cell line-derived neurotrophic factor (GDNF), epidermal growth factor, basic fibroblast growth factor and leukaemia inhibitory factor, gonocytes isolated from neonatal mouse testis proliferated up to 10^14-fold over a 5-month period. The cells maintained their functional capacity, as proven by the restoration of fertility after transplantation of the cultured cells to infertile recipient mice.

Kubota et al. (2004) developed a culture system consisting of a serum-free medium and mouse feeder cells. With this system, they demonstrated the positive effect of GDNF and stem cell factor (SCF) on stem cell maintenance. Further research on serum-free conditions by Kanatsu-Shinohara et al. revealed that mouse spermatogonial stem cells could proliferate in the complete absence of serum or somatic feeder cells. However, when both serum and feeder cells were omitted, the cells could not expand (Kanatsu-Shinohara et al., 2005a).

Recently, the genetic and epigenetic characteristics of the spermatogonial stem cells during long-term culture were under research. During >2 years of continuous in vitro culture, the spermatogonial stem cells maintained their euploid karyotype and androgenetic imprint. Moreover, they were able to induce normal spermatogenesis and yield fertile offspring after intratesticular transplantation. However, the telomeres gradually shortened during culture, suggesting that the cells are not immortal (Kanatsu-Shinohara et al., 2005b). Even though the in vitro culture of mouse spermatogonial stem cells is achieved by several groups, this technique is not described in any other species yet. More research in this field is therefore needed.

**In vitro maturation/differentiation of spermatogonia**

Apart from a spermatogonial stem cell-specific culture protocol, in vitro differentiation of spermatogonial stem cells and the use of in vitro derived male haploid gametes for ICSI could be an option to restore fertility while omitting malignant relapse. Many groups have focused their studies on male germ cell differentiation in vitro, mostly using tissue or organ culture (for review: see Staub, 2001). In a recent study, however, Feng et al. (2002) reported the in vitro derivation of spermatocytes and spermatids from a telomerase-immortalized murine spermatogonial stem cell line. The cell line retained the geno- and phenotypical characteristics of murine type A spermatogonia. Treatment with murine SCF stimulated the cells to undergo differentiation to primary spermatocytes and even to complete meiosis and form haploid...
genetically modified by different maturation processes (Bahadur, 2004). Without the advantage of an immortalized cell line, germ cell survival and differentiation seems to require co-culture with somatic cells, especially Sertoli cells. More research will be needed to identify the specific factors required for a more efficient and complete in vitro spermatogenesis. Moreover, special attention will have to be paid to the genetic and epigenetic status of the in vitro matured cells, since germ cells have a high risk of being genetically modified by different maturation processes (Bahadur, 2004).

Xenografting

The use of a xenogenic intermediate host could be an alternative option to avoid the transfer of malignant cells back to the patient. Xenografting of human ovarian cortex under the skin or kidney capsule of recipient mice has already proven to support the development of primordial follicles up to metaphase II (Weissman et al., 1999; Aubard et al., 2003; Gook et al., 2004, 2005).

Even though testicular tissue has some disadvantages regarding grafting (thick tunica, complex vascular architecture and high sensitivity to ischaemia (Nugent et al., 1997)), promising results have been reported recently. Complete spermatogenesis has been observed after grafting testicular tissue from immature rodents and rabbits. Fertile offspring could be obtained through assisted reproduction techniques, using sperm obtained from these grafts (Honaramooz et al., 2002; Schlatt et al., 2003). Grafting testicular tissue of mature donors, however, did not yield good results (Schlatt et al., 2002; Geens et al., 2006), suggesting that immature tissue might have a better ability to adapt to short periods of ischaemia or be more effective in inducing angiogenesis in the host.

Not only the developmental status of the tissue, but also the structural organization of the seminiferous epithelium, might be an important factor determining the success of xenografting. Grafting of immature macaque testis tissue (with a structural organization similar to rodents) into a host mouse resulted in the production of fertilization-competent sperm (Honaramooz et al., 2004), whereas after grafting marmoset testis tissue a mitotic arrest was observed at the spermatogonial level (Schlatt et al., 2002; Wistuba et al., 2004).

Our group found that after grafting adult human testicular tissue to immunodeficient recipient mice, spermatogonia could be maintained over a period of >195 days (Geens et al., 2006). Wyns et al. (2007) demonstrated the survival of 14.5% of the initial spermatogonial population after grafting testicular tissue pieces from cryptorchid testes from young boys into the scrotum of mice. However, the tissue pieces were only transplanted for a period of 3 weeks before analysis and obviously no spermatogenesis was observed in the grafts. Our group recently xenografted testicular tissue of two prepubertal patients, suffering from severe sickle-cell anaemia and in need to undergo chemotherapy and bone marrow transplantation, onto the back of six Swiss nude mice. The xenografts were evaluated 4 and 9 months after grafting. Spermatogonia could be detected by immunohistochemistry with melanoma-associated antigen MAGE-A4 antibodies and Sertoli cells could be visualized by vimentin staining. At the time of evaluation, all the grafts were well-preserved. In the majority of the seminiferous tubules, only Sertoli cells could be observed, but some surviving spermatogonia could still be detected 4 and 9 months after grafting (Goossens et al., 2008c).

The effect of freezing and thawing of testicular tissue on the survival, proliferation and maturation capacity of the grafted tissue pieces is also an important factor requiring thorough study. Recently, some reports on this subject have been published. Our group compared two freezing protocols, already tested for freezing testicular cell suspensions; a controlled rate protocol with EG (1.5 M) and sucrose (0.07 M) as cryoprotectants and a non-controlled protocol with DMSO (1.5 M) and sucrose (0.07 M). Although murine tissue pieces grafted after freezing and thawing with both protocols displayed a similar, high number of seminiferous tubules containing spermatozoa, the protocol with DMSO was found better, because it could better preserve the structure within the tissue (Goossens et al., 2008b). Jahnukainen et al. (2007) also compared the cryoprotectants DMSO and EG. After grafting of frozen/thawed testicular tissue from immature rhesus monkey to nude mice, they concluded as well that DMSO in sufficient concentrations (1.4 M) was the best cryoprotectant to be used for freezing testis tissue, in order to retain the capacity to initiate spermatogenesis. The same cryoprotectant (DMSO, 0.7 M), supplemented with sucrose (0.1 M), was chosen by Wyns et al. (2007) for freezing immature human testicular tissue pieces before grafting. Freezing and thawing of the tissue before grafting did not induce sclerosis in the grafts. Spermatogonia could survive for 3 weeks, but a significant loss of these precious cells, compared with fresh grafted tissue pieces, was observed. An overview of the applied freezing protocols can be consulted in Table II.

Before considering a possible translation of the technique to the clinic, some safety issues should be evaluated. One of the most important concerns is probably the risk of zoonosis, especially since the mature germ cells would be used for fertilization (Patience et al., 1998).

Apart from being a system for the proliferation and differentiation of spermatogonial stem cells, xenografting could also be an assay for testing the risk of malignant contamination of the testicular tissue (Hou et al., 2007). If over time no malignant cells are observed in the xenografts, tissue of the same testis may be autotransplanted or autografted to a cured patient.

Damage to the somatic environment

The sensitivity of the germ cells for cancer treatment has been well discussed. Meanwhile, little is known about the damage that could occur to the somatic elements of the testis. Moderate doses of radiation have shown to induce mild Leydig cell impairment, resulting in elevated levels of luteinizing hormone and low to normal serum testosterone. Some studies, however, suggest that this impairment may be clinically important (Howell and Shalet, 2001).

Zhang et al. (2006) transplanted spermatogonial stem cells from untreated prepubertal rats into irradiated adult rat testes. These transplanted stem cells were able to colonize the basement membrane but could not differentiate to more mature spermatogenic cells, unless testosterone levels in the transplanted rats were suppressed. Their study suggests that the transplantation of functional spermatogonia to a testicular environment that has been exposed to cytotoxic therapy may be limited by somatic niche cell damage. This damage, however, might possibly be prevented or reversed by hormonal treatment or somatic cell transplantation.
### Table II. Overview of the studies on allo/xenografting of testicular tissue after freezing and thawing.

<table>
<thead>
<tr>
<th>(Im)mature</th>
<th>Species</th>
<th>Cryoprotectant</th>
<th>(Non-)controlled</th>
<th>Freezing rate</th>
<th>Outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Immature Mouse</td>
<td>1.5 M EG, 0.7 M sucrose</td>
<td>Controlled</td>
<td>Start: 5°C, −5°C/min to −7°C, hold 15 min + seeding, −0.3°C/min to −80°C, LN2</td>
<td>32% of tubules containing spermatozoa, 42% of tubules damaged after grafting (3–5 months after grafting)</td>
<td>Goossens et al. (2008b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5 M DMSO, 0.7 M sucrose</td>
<td>Controlled</td>
<td></td>
<td>32% of tubules containing spermatozoa, 32% of tubules damaged after grafting (3–5 months after grafting)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Immature Rhesus monkey</td>
<td>No cryoprotectant</td>
<td>Non-controlled</td>
<td>Start: on ice, −0.5°C/min to −20°C, seeding at −13°C, LN2</td>
<td>7% survival (3 months after grafting)</td>
<td>Jahnukainen et al. (2007)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.4 M EG</td>
<td>Non-controlled</td>
<td></td>
<td>13% survival (5 months after grafting)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.7 M DMSO</td>
<td>Non-controlled</td>
<td></td>
<td>30% survival (3 months after grafting)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.4 M DMSO</td>
<td>Non-controlled</td>
<td></td>
<td>73% survival (5 months after grafting)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Immature Human (cryptorchid)</td>
<td>0.7 M DMSO, 0.1 M sucrose</td>
<td>Controlled</td>
<td>Start: 0°C, hold 9 min, −0.5°C/min to −8°C, hold 5 min + seeding, hold 15 min, −0.5°C/min to −40°C, hold 10 min, −7°C/min to −80°C, LN2</td>
<td>82.19 ± 16.46% of sections after cryo, compared with 93.38 ± 6.00% in fresh tissue showed good morphology (3 weeks after grafting)</td>
<td>Wyns et al. (2007)</td>
</tr>
</tbody>
</table>

### Ethical considerations

If it would be possible to overcome all the obstacles described above and if this new technology proved safe, some ethical considerations will remain. One of the first difficulties that will occur is the need to obtain proper informed consent from the parents of young boys for preserving their fertility is within the spermatogonial stem cells. Many research groups have therefore started research on these particular cells. The autologous spermatogonial stem cells have, within high promise towards a clinical application. However, important questions may need to be answered before any clinical use of these particular cells. The autologous spermatogonial stem cells to a prepubertal testis (Tournaye et al., 2003a,b), and, therefore, the decision on the timing of the transplantation of the spermatogonial stem cells, could be crucial for the physical and mental well-being of the patient. It is clear that the surgery should be optimally timed with regard to the necessary cancer treatments. However, the rights of the child should not be forgotten. In this decision, the parents will play a very important role; however, the rights of the child should not be forgotten. The child’s well-being is of utmost importance. The patient is in full remission (Bahadur et al., 2000). In mice it has been shown that transplantation success of frozen–thawed stem cells suspensions was more efficient in pre-pubertal than in adult recipients (Kamata-Shinohara et al., 2003a,b), and therefore, the cell transplantation is supported by grants from the Research Foundation—Flanders (FWO-Vlaanderen), the research council of the Brussels Free University (UZ Brussels). University (UZ Brussels), Mieke Geens is a Research Assistant at the Research Foundation—Flanders (FWO-Vlaanderen). Outside the University Hospital of the Brussels Free University, Avarbock MR, Brinster CJ, Brinster RL. Reconstitution of spermatogenesis from frozen spermatogonial stem cells. Nat Med 2003; 9(11):139–142. Funding

Acknowledgements

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Geens et al.


Spermatogonial stem cell transplantation


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