

In search of the most efficient fertility preservation strategy for prepubertal boys

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Abstract

Fertility preservation strategies are currently being developed for boys facing spermatogonial stem cell (SSC) loss. However, it is not clear yet which transplantation strategy would be the best choice. Therefore, the aim of the work presented in this thesis was both to compare these strategies and to study how to improve their efficiency.

The efficiency to restore spermatogenesis after transplantation of SSCs or testicular tissue was evaluated. In addition, we investigated the potential of transplanted adult bone marrow stem cells (BMSCs) to repopulate the testis. We aimed to improve the efficiency of human intratesticular xenografting by exogenous administration of FSH. Since spermatogonial loss was observed in human intratesticular xenografts, we finally evaluated whether early cell death was the cause of this loss.

Compared to SSC transplantation, more donor-derived spermatogenesis was observed after intratesticular tissue grafting. Human SSCs were able to survive for at least 12 months inside the mouse testis and meiotic activity was observed. However, the attempt to improve germ cell survival and induce full differentiation by the exogenous administration of FSH failed. Spermatogonia-specific apoptosis could not explain the SSC loss.

Differentiation towards the germ line was not observed after intra-testicular injection of BMSCs, neither did we observe any protective effect for SSC loss.

Intra-testicular tissue grafting seems to be the most efficient fertility preservation strategy. However, this strategy can not be applied in patients at risk of malignant contamination. For these patients SSC transplantation should be performed after decontamination of the cell suspension.

Keywords: Fertility, grafting, spermatogonial stem cells, testis, transplantation.

Introduction

In Belgium, one in three men and one in four women will develop cancer before the age of 70. In 2006, 0.6% of all cancers occurred in children, with leukemia being most common (www.kankerregister.org). Survival rate has increased to more than 80% thanks to advancements in treatment regimens (Murk and Seli, 2012). However, future fertility may be compromised, not only because of the malignancy itself, but more frequently, because of the treatment regimen involving chemotherapy and/or radiotherapy (Ortega and Tournaye, 2012).

The recovery of sperm production after gonadotoxic regimens depends on the survival of the spermatogonial stem cells (SSCs) and their ability for further differentiation. At puberty, spermatogenesis starts from these SSCs. They differentiate into B-spermatogonia, spermatocytes, spermatids and eventually spermatozoa. As long as the SSCs are not affected by the gonadotoxic treatment, spermatogenesis can be re-established. Recovery to normozoospermic levels can occur within 1 to 3 years if low doses were used, but at higher doses azoospermia can be more prolonged or even become permanent (Meistrich, 2009).

Table I. — Indications for high risk therapy.

| Diagnosis | Gonadotoxic therapy |
|---|---|
| Leukemic testicular relapse | Testicular irradiation |
| Hematopoietic stem cell transplantation Conditioned with total body irradiation Conditioned with chemotherapy | Testicular irradiation High cumulative dose of alkylating agents (cyclophosphamide, busulphan, melphalan, thiotepa) |
| Lymphomas | Procarbazine-containing regimens |
| Hodgkin disease | MEPP (mechlorethamine, oncovin/vincristine, procarbazine and prednisone) regimen |
| Metastatic or high-risk sarcomas | High cumulative dose of alkylating agents (cyclophosphamide, ifosfamide) Possible testicular irradiation |

Acute leukemia is the most common indication for allogeneic hematopoietic cell transplantation (Baker et al., 2010). Treatment of hematological malignancies and some nonmalignant disorders of the hematopoietic system may also involve bone marrow transplantation (de Rooij et al., 2002). The conditioning treatment preceding any hematopoietic transplantation involves total body irradiation and/or high-dose chemotherapy, which may destroy ongoing spermatogenesis and may even eradicate the stem cell population in the testis. Table I gives an overview of current indications for applying high-gonadotoxicity risk therapy during childhood.

While adult patients can preserve semen before starting therapy, prepubertal boys do not have this option because complete spermatogenesis is still lacking. Therefore, for these young patients, fertility preservation methods are being developed and studied in animal models. These methods involve the cryostorage of SSCs and subsequent transplantation of these stem cells. There are two options to retransplant the cryopreserved SSCs: either the introduction of a suspension into the seminiferous tubules or grafting of a piece of testicular tissue. For patients who did not have their SSCs cryopreserved before therapy, an alternative option could be the differentiation of adult stem cells, such as bone marrow stem cells (BMSCs), after their transplantation to the testis. In the meantime, few centres have already started a cryopreservation program for boys facing gonadotoxic therapy. Within a few years, these boys may return and request transplantation.

Currently, it is not clear which of the methods will be the best strategy for restoring fertility in these patients. Therefore, the general aim of this

thesis was to explore the current options for fertility preservation in prepubertal boys.

1. Exploring different fertility preservation strategies

In a first part of this study the best transplantation strategy for patients who have their SSCs cryopreserved before therapy was studied. Secondly, the feasibility of BMSCs to differentiate towards the germ line after injection in the testis was evaluated as a fertility restoration strategy for patients who did not cryopreserve SSCs.

1.1. SSCT and intratesticular tissue grafting in a mouse model

Introduction

SSCT was first described in 1994 and involves the injection of a testicular cell suspension into the testis of an infertile recipient. SSCs were able to colonize the basement membrane and re-establish spermatogenesis in different species using fresh as well as frozen-thawed cell suspensions (Brinster and Avarbock, 1994; Brinster and Zimmerman, 1994; Avarbock et al., 1996; Ogawa et al., 1999; Honaramooz et al., 2002a; Honaramooz et al., 2003; Izadyar et al., 2003; Trefil et al., 2006; Kim et al., 2008; Hermann et al., 2012). Live births were obtained after natural mating in rodents (Goossens et al., 2006) and goats (Honaramooz et al., 2003). The colonization efficiency of the technique is very low (Dobrinski et al., 1999). This may be due to the fact that SSCs need to find a ‘new’ niche since the

contact with their natural niche has been lost. Moreover, enzymatic digestion can remove surface molecules, such as β_1 -integrin, which are important for relocation of the SSCs to the basement membrane (Kanatsu-Shinohara et al., 2008).

An alternative for SSCT is testicular tissue grafting (TTG). This has the advantage that SSCs are transplanted within their natural niche/environment. In 2002, Honaramooz et al. reported testicular tissue grafting as a new option for male germ line preservation (Honaramooz et al., 2002b). In contrast to the absence of spermatogenesis after SSCT between phylogenetically more distant species, the initiation and maintenance of full spermatogenesis and steroidogenesis was observed in grafted testicular tissue. These positive results were attributed to the maintenance of the SSCs in their natural environment, which conserves the balance between germ cells and supporting cells. Moreover, initiation of spermatogenesis could be supported by the native supporting Sertoli cells, which is not the case in SSCT. Ever since this first report, ectopic testicular tissue xenografting has been performed using testicular tissue from several mammalian species (Honaramooz et al., 2002b; Schlatt et al., 2002; Schlatt et al., 2003, Honaramooz et al., 2004; Oatley et al., 2004; Snedaker et al., 2004; Rathil et al., 2006, Zeng et al., 2006; Abrishami et al., 2010). However, a meiotic block was reported in marmoset xenografts (Schlatt et al., 2002; Wistuba et al., 2004).

In most studies concerning tissue transplantation, TTG was performed to an ectopic location. However, in a clinical setting orthotopic transplantation would be more acceptable for the patient. Intratesticular grafting has been reported by Shinohara et al. (2002) and pups were born after intracytoplasmic sperm injection (ICSI) using sperm isolated from these grafts.

At present, it is not clear which of the described techniques would be the best option as fertility preservation method. Therefore, in the first part of this study a comparison was made between SSCT and intratesticular tissue grafting in a mouse model.

Materials and methods

Testicular tissue from 5 to 7-days-old green fluorescent protein (GFP)⁺ fertile donor mice was digested by a two-step enzymatic protocol to obtain a cell suspension which was injected through the rete testis in the testis of a sterilized GFP recipient mouse. Six to eight weeks before transplantation, recipient mice were sterilized by an intraperitoneal injection of busulfan, a chemotherapeuticum. When rete testis injection was unsuccessful because of a technical

failure, the testicular parenchyma was blindly injected through the tunica albuginea. In the contralateral testis a testicular tissue piece (1.5 mm³) was inserted through a fine incision in the tunica albuginea. Four months after transplantation, recipient animals were killed by cervical dislocation, their testes collected and weighted. The testes were screened under a light microscope with UV light for the presence of green fluorescent colonies and their length was determined. GFP-immunostaining was used to confirm the presence of donor-derived spermatogenesis.

Results and conclusion

The mean weight of the grafted testes (62.1 ± 12.7 mg) was significantly higher than that after rete (31.0 ± 10.7 mg ; $P < 0.0001$) and blind injection (22.8 ± 1.9 mg ; $P = 0.0077$). In 10 out of 14 rete-injected testes (71%) and in 3 of the 6 blindly injected testes (50%), green fluorescence was found after evaluation under UV light, while green fluorescence was observed in all of the grafted testes. Significantly more tubules were filled with donor spermatogenesis after grafting (122.1 ± 45.6 mm) than after rete injection (62.2 ± 80.4 mm; $P = 0.0033$) or after blind injection (1.21 ± 1.4 mm; $P = 0.0003$). The immunohistochemical staining for GFP showed tubules with donor spermatogenesis next to the tubules showing endogenous GFP spermatogenesis in testes injected through the rete. All testes in which a testicular piece was grafted were positive for GFP (Figure 1).

There was no difference in the colony length in testes which received a fresh (93.8 ± 21.8 mm) or frozen-thawed (84.8 ± 36.3 mm) testicular tissue piece (Van Saen et al., 2009a).

These results show that intratesticular tissue grafting is the most efficient fertility preservation strategy at this moment.

1.2. BMSCCT as a fertility preservation strategy

Introduction

Both abovementioned methods imply the cryopreservation of SSCs before starting therapy. Currently, not all prepubertal patients being at risk for stem cell loss bank testicular tissue. Sometimes, there will be no time to cryopreserve SSCs or testicular tissue because patients are in urgent need to start the gonadotoxic treatment, patients do not have access to a banking facility, or their parents do not accept banking due to its experimental character. When these patients face infertility at adult age, they can not rely on fertility preservation methods which involve autologous transplantation of SSCs.

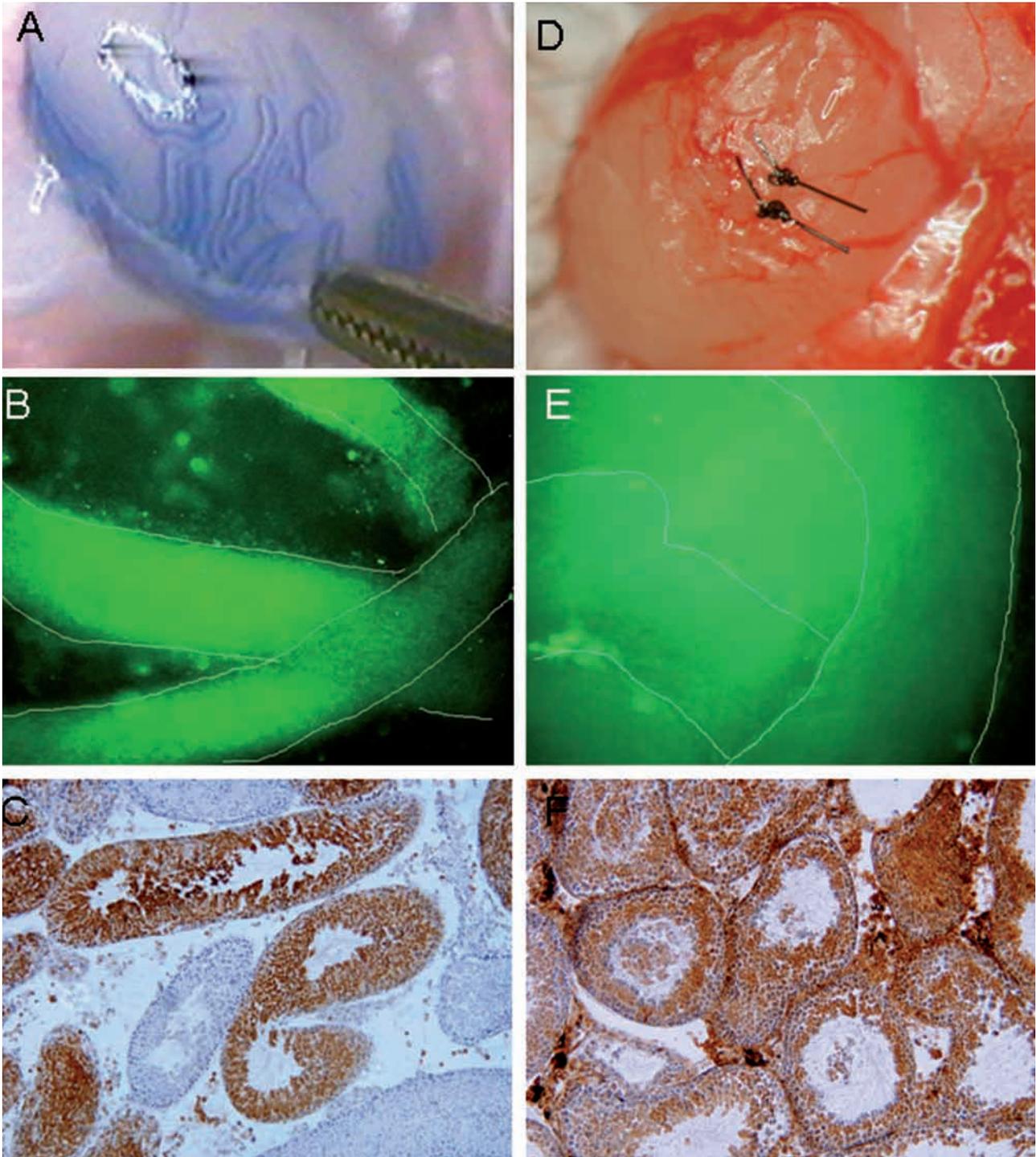


Fig. 1. — Comparison between spermatogonial stem cell transplantation (A-C) and intratesticular grafting in a mouse model (D-F). Testis after injection of a testicular cell suspension (A). Green fluorescent tubules containing donor-derived spermatogenesis were observed four months after transplantation. The walls of the tubules are aligned (B). The presence of donor-derived spermatogenesis was confirmed by GFP staining. GFP⁺ tubules are visible next to GFP tubules containing endogenous spermatogenesis (C). Testis after insertion of a testicular tissue piece (D) and the detection of green fluorescence by UV-light (E) and immunohistochemistry (F).

For these patients alternative options should be developed.

This raised the question whether other stem cells can colonize the niche and initiate spermatogenesis. Bone marrow stem cells (BMSCs) are easy to obtain and their plasticity has been widely reported (Grove et al., 2004). Upon stimulation with retinoic acid (RA), mouse and human BMSCs can differen-

tiate to male germ cells *in vitro* (Nayernia et al., 2006; Hua et al., 2009). *In vivo* differentiation of BMSCs to testicular cell types (Sertoli cells, germ cells and Leydig cells) was achieved without prior *in vitro* treatment (Lue et al., 2007).

In this part of the study, we wanted to evaluate the differentiation capacity of BMSCs towards germ cells after injection in a sterilized testis.

Materials and methods

BMSCs and SSCs were isolated from adult and prepubertal GFP⁺ mice respectively. In a first experiment, 6 to 8 weeks after busulfan administration, mice (n = 12) were transplanted by injecting SSCs and BMSCs in the efferent duct. Four months after transplantation the testes were collected and evaluated for the presence of GFP by UV light and immunohistochemistry, as described earlier. The protective effect of BMSCT against testicular gonadotoxicity was also evaluated. Recently, it was observed that bone marrow transplantation could rescue long-term fertility in sterilized female mice. Although bone marrow-derived immature oocytes were observed in the ovaries after transplantation, all of the offspring were generated by the recipient's germ line (Lee et al., 2007). Testes (n = 12) were injected with BMSCs one week after the busulfan injection, because Lee et al. (2007) described a maximal benefit in their mating experiment when BMSC transplantation was performed 1 week after cytotoxic treatment. In a third study group, a mating experiment was set up in which fertile control mice (n = 3), sterile mice (n = 5), and BMSCs transplanted mice (n = 7) were mated with wild type females one week after vehicle (control mice) and busulfan injection (sterile mice). Their ability to sire pups in wild type female mice was evaluated.

Results and conclusion

In the group transplanted 6-8 weeks after busulfan administration, green fluorescence was found in 10 out of 12 SSCT testes (83%). The mean colony length was 166.8 ± 146.9 mm. There was no green fluorescence observed in the BMSCs injected testes. No GFP⁺ spermatogenesis was found after BMSCT. Occasionally GFP⁺ cells were observed in the lumen of some tubules or in the interstitium.

In the group transplanted one week after busulfan administration, no GFP⁺ cells were found. In the mating experiment, the fertile control group showed a normal reproductive profile with normal litter sizes. The sterile group showed a normal profile during the first month after transplantation, due to transient endogenous spermatogenesis not yet destroyed by chemotherapy. All females became pregnant during this first month, but only two mice achieved a second pregnancy. The transplanted group only showed a normal profile during the first month. Thereafter, both the sterile and the transplanted mice stopped reproducing. However, the busulfan-sterilized group regained fertility 4 months after chemotherapy, whereas the BMSCT group did not show any signs of restoring fertility. Nine months after transplantation, histologic examina-

tion showed that spermatogenesis was restored in the busulfan-treated group, while testes in the BMSCT group showed atrophy. Limited spermatogenesis was present as shown by hematoxylin-eosin (HE) staining and no GFP⁺ cells were observed after immunostaining (Van Saen et al., 2009b; Figure 2).

Injection of BMSCs in the testis did not lead to differentiation towards the male germ cell line.

2. Improving intratesticular xenografting

In the second part of this study, the efficiency of human intratesticular xenografting was evaluated. We tried to improve germ cell survival and differentiation by the exogenous administration of follicle stimulating hormone (FSH). The role of apoptosis in spermatogonial loss observed in xenografts was also explored.

2.1. Human intratesticular xenografting

Introduction

Spermatogonial survival (Goossens et al., 2008) and recently differentiation up to primary spermatocytes (Sato et al., 2010) were reported in ectopic xenografts using prepubertal and neonatal human tissue, respectively. In an attempt to improve the results after grafting, long-term survival of spermatogonia and differentiation up to pachytene spermatocytes was observed when immature testicular tissue was transplanted in the peritoneal bursa inside the scrotum, after castration of the acceptor mouse. Spermatid-like structures could be observed but their presence could not be confirmed by immunohistochemistry using meiotic and post-meiotic markers (Wyns et al., 2008).

Having in mind the positive results of intratesticular tissue grafting using mouse testis, we wanted to explore the efficiency of human intratesticular xenografting. We aimed at evaluating the effects of cryopreservation and donor age on spermatogonial survival and differentiation capacity after intratesticular prepubertal grafting.

Material and methods

Human testicular tissue was obtained from four patients, aged 3, 5, 12 and 13 years. All patients underwent testicular biopsy before receiving a conditioning chemotherapy for bone marrow transplantation. One-half of a testis was removed from the patient, and 90% of this testicular biopsy was cryopreserved for later use. Testicular tissue was frozen using an uncontrolled slow freezing protocol with cryopreservation medium containing DMSO and sucrose as cryoprotectants (Wyns et al., 2007). Vials were

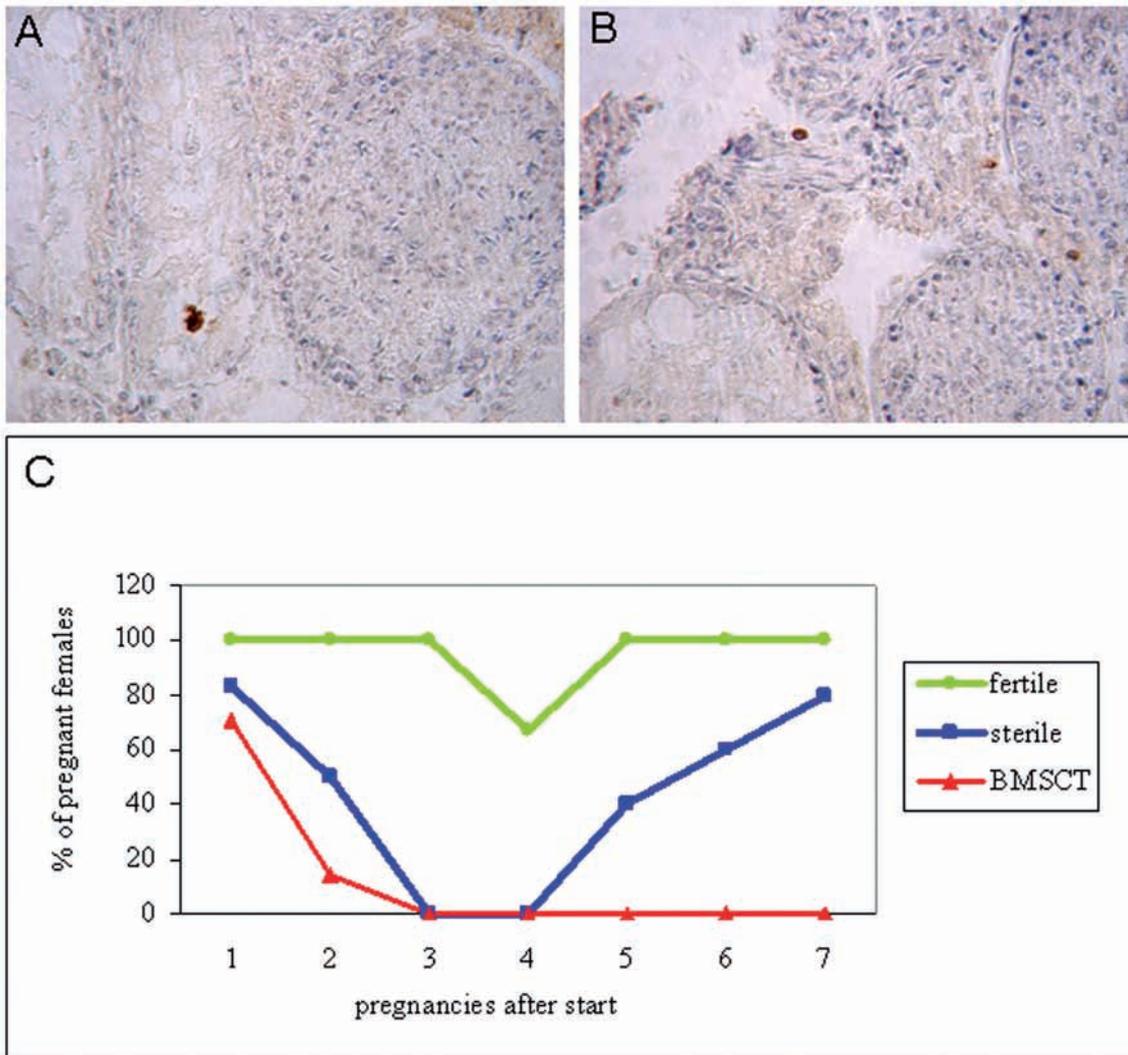


Fig. 2. — Differentiation capacity of bone marrow stem cells after injection in the testis. GFP⁺ cell in the testicular lumen after injection of a bone marrow stem cell suspension (A). GFP⁺ cell in interstitium after BMSCT (B). Percentage of mice which had a successful pregnancy during a 7-month period when mated with fertile control, sterilized control or bone marrow injected mouse (C). The fertile group was used to determine the number of pregnancies.

stored in liquid nitrogen and thawed in a water bath at 37°C immediately before transplantation. Testicular tissue pieces (fresh and/or frozen-thawed) were transplanted to the testis of 4 to 6-week-old Swiss Nu/Nu mice (n = 13). Mice were killed either 4 or 9 months after transplantation by cervical dislocation. The human testicular graft was localized by hematoxylin-eosin staining. The integrity of the graft and germ cell survival was evaluated by immunohistochemistry using a Sertoli cell-specific marker, vimentin, and a germ cell specific marker, MAGE-A4 (Figure 3A,B). The presence of differentiation and meiotic activity in the grafts was evaluated by respectively hematoxylin-eosin and staining for boll, a meiotic marker. The maturity of the Sertoli cells in the grafts was evaluated by the expression of Anti-Mullerian hormone (AMH). Immature Sertoli cells secrete AMH, while its ex-

pression is lost in mature Sertoli cells (Rajpert-De Meyts et al., 1999). Therefore, loss of expression of this marker in a testicular tissue graft is an indication for Sertoli cell maturation (Rathi et al., 2008).

Results and conclusion

A variable integrity was observed in the different recovered xenografts, ranging from totally sclerotic tissue to 89.1% intact tubules. Germ cell survival was also variable between the different grafts, but grafts from older patients showed a higher germ cell survival compared to the grafts from younger patients. Before grafting, in the 3- and 5-year-old boys, spermatogonia were the most advanced germ cells present. After grafting, Sertoli cells showed an immature phenotype and spermatogonia were the most advanced germ cells. No expression of Boll was observed.

In the 12- and 13-year-old boys, spermatogenesis was already initiated at the time of biopsy and proceeded up to the spermatocyte level. In the fresh grafts differentiation up to primary spermatocytes and secondary spermatocytes was observed after four and nine months respectively. The presence of meiotic activity was confirmed by Boll expression. In the frozen-thawed grafts, no spermatocytes could be observed except in the graft from the 12-year-old boy.

AMH expression was examined in the donor tissue before grafting and after grafting. Before grafting, AMH was expressed less intensely in the older donors compared to the young donors, indicating maturity of the Sertoli cells in the older donors. After grafting, AMH expression was observed in all grafts. However, it was observed that in tubules with ongoing differentiation, AMH expression was very weak (Van Saen et al., 2011).

Human spermatogonia are able to survive in the mouse testis for at least 9 months. Differentiation up to the spermatocyte level occurred mostly in tubules containing mature Sertoli cells, but full spermatogenesis was not present.

2.2. *The effect of FSH on intratesticular human xenografts*

Introduction

Induction and maintenance of spermatogenesis is dependent on the continuous and controlled interaction of several hormones in the hypothalamic-pituitary-testis axis. In response to FSH, Sertoli cells secrete several factors necessary for the progression of spermatogonia into spermatozoa (Sofikitis et al., 2008). It has been reported that FSH regulates spermatogonial survival by the prevention of germ cell apoptosis (Ruwanpura et al., 2008). During the first two months after grafting, an initial decrease in spermatogonia has been reported in ectopic bovine grafts (Rathi et al., 2005). In our previous study a spermatogonial loss was also observed after intratesticular tissue grafting.

When testicular tissue is transplanted ectopically, recipient animals are castrated in order to achieve high FSH and luteinizing hormone (LH) levels mimicking the situation just before puberty. These high postcastration levels of FSH and LH are likely responsible for the advancement of testicular maturation as observed in rhesus monkey xenografts (Honaramooz et al., 2004). Exogenous administration of gonadotrophins was effective in the induction of maturation in infant monkey xenografts and promotes postmeiotic differentiation in equine xenografts (Rathi et al., 2006 and 2008). However,

when intratesticular transplantation has to be performed, castration is not an option. FSH and LH levels should thus be increased in a different way.

In our next study we evaluated whether exogenous administration of human recombinant FSH could have a positive influence on the outcome of intratesticular human xenografts. We hypothesized that (1) FSH could prevent germ cell loss in the xenografts and (2) that it could stimulate postmeiotic differentiation in prepubertal xenografts.

Materials and methods

Human testicular tissue (fresh or frozen-thawed) from six patients, aged 2.5-12.5 years old was transplanted to the testis of 4-to-6-week-old Swiss nu/nu mice (n = 31). Some of the transplanted mice from the fresh as well as from the frozen-thawed group were injected subcutaneously with 16 IU rhFSH. The first injection was given one day before transplantation. After transplantation, injections were given three times a week. Mice were killed by cervical dislocation 9-12 months after transplantation. Tubule integrity, germ cell survival and the presence of differentiation was evaluated in the human grafts by immunohistochemistry and histology.

Results and conclusion

In the untreated group 90% of the fresh human grafts and 75% of the frozen-thawed grafts were recovered. No statistical difference was found in tubule integrity and germ cell survival between the different treatment groups. Although, unexpectedly a tendency towards a lower germ cell survival was observed in the FSH-treated grafts (Figure 3C). Human donor tissue and all human grafts were analyzed to record the most advanced germ cell stage. In the four youngest patients (2.5-5.5 years old) spermatogonia were the most advanced germ cells present at the time of grafting. However, in the two older patients (12.0- and 12.5-years-old) meiotic cells were already present. Differentiation up to pachytene spermatocytes was observed in grafts from older patients as well as in grafts from younger boys in both untreated as FSH-treated grafts. A statistical difference in the distribution among groups of Sertoli cell only (SCO) tubules, tubules with spermatogonia and meiotic cells as most advanced cell types was observed. More tubules containing only Sertoli cells were observed in frozen-thawed grafts, and more tubules with meiotic cells were present in fresh grafts. There was no clear influence of FSH treatment on meiotic differentiation. For an overview of the results see table II (Van Saen et al., 2013a).

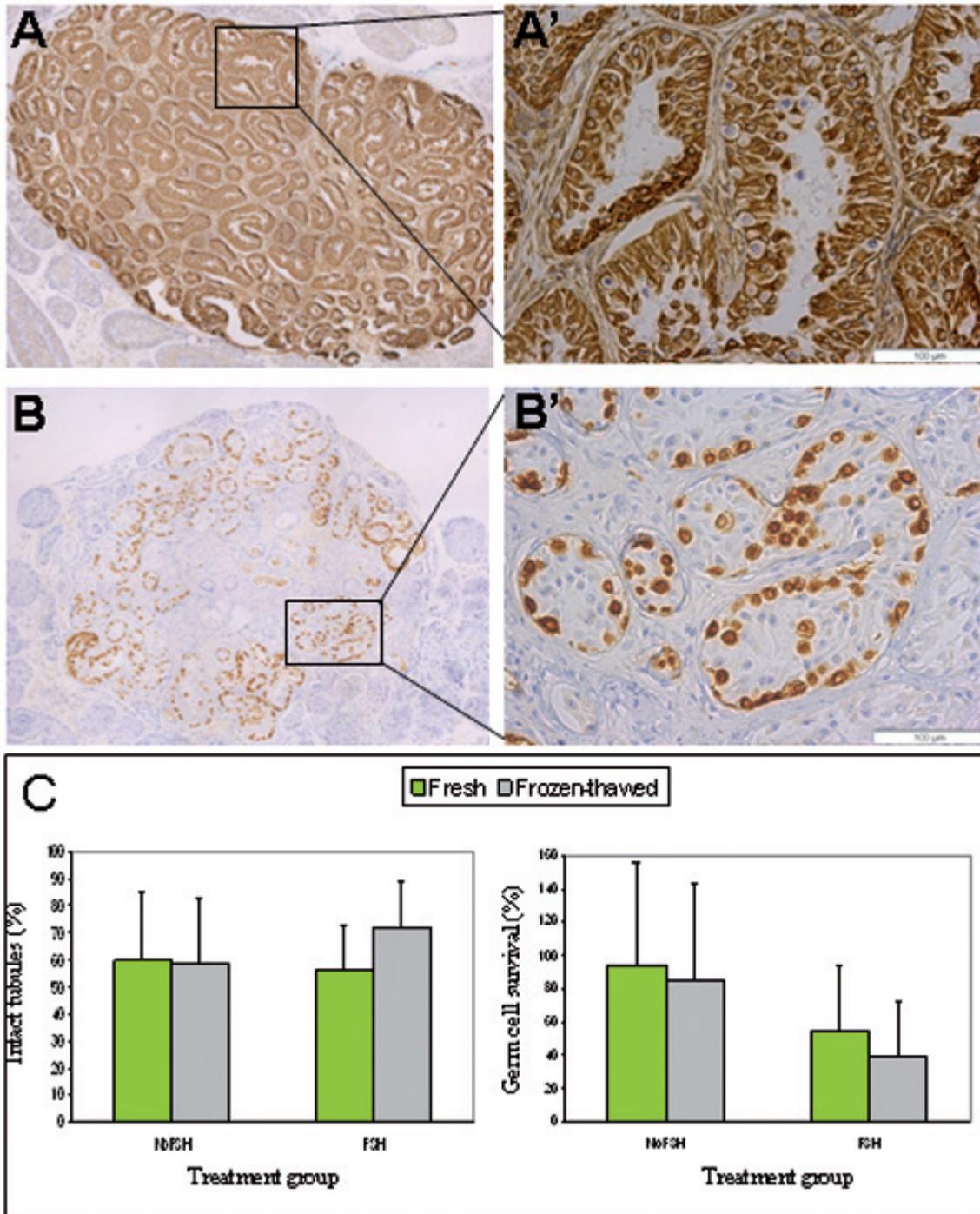


Fig. 3. — Human testicular xenografting.

Tubule integrity of the human testicular tissue graft was determined by vimentin staining using a human specific antibody (A). Germ cell survival was determined by MAGE-A4 staining (B). The right pictures represent a detail of the left pictures. Germ cell survival in untreated and FSH-treated grafts (fresh and frozen-thawed) (C).

2.3. The role of apoptosis in germ cell loss

Introduction

In the last part of this study we evaluated the role of apoptosis in germ cell loss occurring in testicular tissue grafts. Human spermatogonia were able to survive in the mouse testis for at least 9 months and limited differentiation occurred up to the spermatocyte level.

However, in the human intratesticular xenografts, spermatogonial loss was observed. Spermatogonial loss was also reported in ectopic bovine xenografts. However, no significant difference was observed in the number of apoptotic germ cells per tubule between xenografts and controls 2 months after transplantation in the bovine (Rathi et al., 2005).

Table II. — Influence of FSH on xenografts: overview of results.

| | Untreated grafts | | FSH-treated grafts | |
|-----------------------------------|------------------|---------------|--------------------|---------------|
| | Fresh | Frozen-thawed | Fresh | Frozen-thawed |
| Mouse grafts | | | | |
| Recovery | 100.0 | 100.0 | 100.0 | 100.0 |
| Tubules with full spermatogenesis | 64.1 ± 0.7 | 53.3 ± 13.7 | 61.4 ± 12.5 | 56.3 ± 7.3 |
| Human grafts | | | | |
| Recovery | 90.0 | 75.0 | 87.5 | 85.7 |
| Intact tubules (%) | 59.9 ± 25.3 | 56.0 ± 16.9 | 59.0 ± 23.9 | 72.3 ± 16.6 |
| Germ cell survival (%) | 93.7 ± 62.3 | 53.4 ± 40.5 | 83.9 ± 59.5 | 39.2 ± 32.7 |
| Differentiation | | | | |
| Sertoli-cell-only | 56.1 ± 26.6 | 70.4 ± 16.9 | 61.1 ± 30.2 | 76.9 ± 35.9 |
| Spermatogonia | 40.9 ± 24.4 | 28.5 ± 15.9 | 37.2 ± 28.6 | 21.1 ± 32.7 |
| Meiotic cells | 3.0 ± 3.4 | 1.1 ± 2.0 | 1.8 ± 3.7 | 2.0 ± 3.3 |

In analogy to intratesticular tissue grafting, embryonic mesencephalic dopamine neurons have been transplanted to the striatum to treat Parkinson's disease. However, low survival of the grafted neurons seriously affected the efficiency of this strategy (Brundin and Bjorklund, 1987). Although it was thought that cell death in these grafts was of necrotic nature, it was found that important apoptotic cell death occurred within the first 7 days after transplantation (Sortwell et al., 2000). We wanted to evaluate whether the observed spermatogonial loss was caused by apoptosis during the first days after intratesticular tissue grafting. Because of the scarcity of human prepubertal tissue available for research, this study was performed in a mouse model.

Materials and methods

GFP⁺ donor tissue was grafted to the testes of GFP-sterilized mice. At different time points after transplantation the presence of apoptosis was evaluated in intratesticular tissue grafts. Spermatogonia-specific apoptotic cell death was evaluated by flow cytometry using the annexin V assay. The development and general occurrence of cell death was evaluated by immunostaining for GFP and deoxynucleotidyl transferase mediated dUTP nick end labelling (TUNEL) assay.

Results and conclusion

Higher numbers of apoptotic spermatogonia were observed 4 and 10 days after transplantation compared to 1 and 7 days after transplantation. None of the early time points, however, showed statistical difference with the prepubertal control. No difference was observed between the late time points (1

and 2 months after transplantation mutually) and between the late time points and the fertile controls. Less apoptotic spermatogonia were observed 1 month after transplantation compared to 4 and 10 days after transplantation. The number of apoptotic spermatogonia observed 2 months after transplantation did not reach significance with the other time points. Histology showed that tubules in the centre of the graft were degenerating one day after transplantation. In the centre of most of the grafts, remnants of degenerated tubules, which have totally lost their GFP expression, were visible 4 days after transplantation. By 7 and 10 days after transplantation, sclerosis was observed in the middle of the graft and tubules started to increase in size replacing empty space at the centre of the graft. Statistically increased numbers of TUNEL⁺ tubules were observed 1 day after transplantation compared to the other early time points (Figure 4).

Spermatogonia-specific apoptosis does not explain the important spermatogonial stem cell loss observed after intratesticular tissue grafting, but probably results from degeneration of tubules in the centre of the graft due to hypoxia during the first days after intratesticular grafting (Van Saen et al., 2013b).

General discussion

We concluded that both SSCT and intratesticular tissue grafting may be useful techniques for future clinical application. However, after grafting, more extensive donor-derived spermatogenesis was observed. Hence, intratesticular tissue grafting may be suggested as being the best option.

The better results of TTG can be attributed to the fact that SSCs are transplanted within their natural

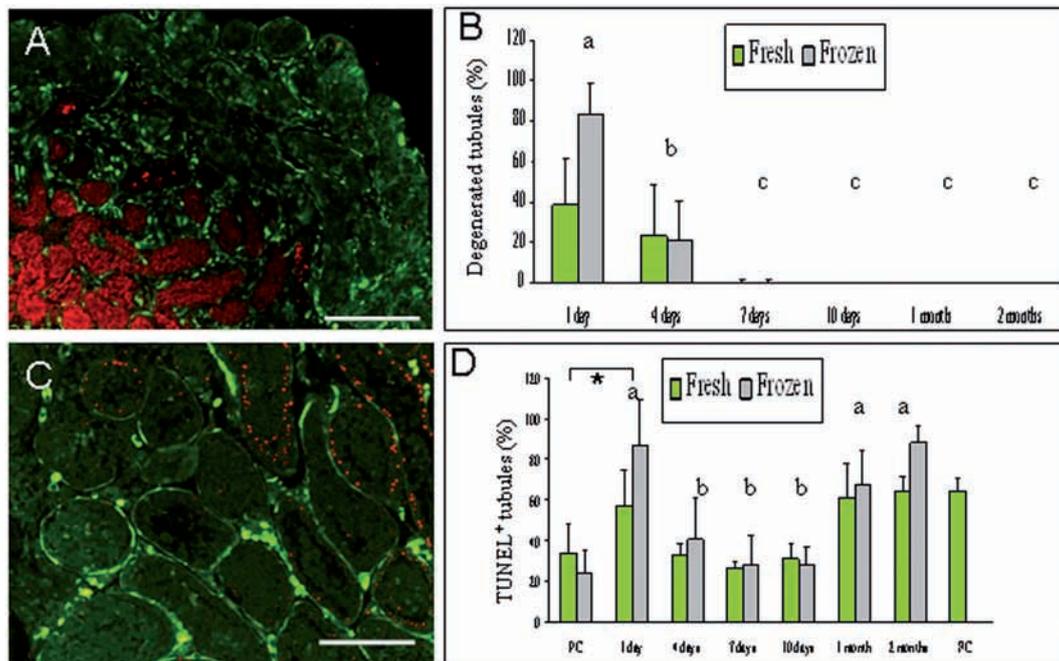


Fig. 4. — Cell death in intratesticular mouse grafts. Global cell death was evaluated by double staining for TUNEL and GFP. Intratesticular graft four days after transplantation. Degenerated tubules stain totally red (A). Percentage of total degenerated tubules (B). Intratesticular graft one month after transplantation (C). The percentage of TUNEL⁺ tubules was determined (D).

environment, preserving both cell contacts and intercellular interactions. After SSCT, SSCs need to find their way back to a new niche, which could explain the low colonization rates reported after SSCT.

Technically, intratesticular tissue grafting is a relatively simple procedure, while SSCT requires a complex and adapted infusion method in humans. Due to anatomical differences, the reintroduction of a suspension seems more difficult in the human than in the mouse. Different injection methods were evaluated in cadaver testes from large animals. Ultrasound guidance of the injection needle was helpful in localizing the rete testis. Intratesticular rete testis injection using a gravity feed was the best and least invasive technique yielding maximal infusion efficiency (Schlatt et al., 1999; Ning et al., 2012).

Until now, postmeiotic germ cells were not observed in human xenografts (Geens et al., 2006; Schlatt et al., 2006; Goossens et al., 2008; Sato et al., 2010; Van Saen et al., 2011, 2013a). Ectopic xenografting was neither successful in generating postmeiotic cells using marmoset testicular tissue (Wistuba et al., 2004). However, orthotopic grafting resulted in complete spermatogenesis in marmoset grafts (Wistuba et al., 2006; Luetjens et al., 2008). This suggests that orthotopic transplantation may be effective in a human application too. Besides, in the perspective of a clinical application, orthotopic transplantation, compared to ectopic transplantation, would be more acceptable for the patient. Orthotopic autotransplantation of cryopreserved

ovarian cortical tissue has already been performed successfully and several live births have been reported (Donnez and Dolmans, 2011).

When TTG will be performed in a clinical setting, attention needs to be paid on re-establishing the blood supply after transplantation. Degeneration of tubules in the centre of the graft was seen during the first four days after transplantation. This has important implications concerning the size of the transplanted tissue fragments. To enable maximum survival of the tissue fragment inside the testis, testicular fragments should not be too large. The administration of angiogenesis-inducing factors may improve the efficiency of intratesticular grafting. The beneficial action of VEGF has been reported in ectopic bovine testicular grafts (Schmidt et al., 2006; Caires et al., 2009). However, the number of blood vessels detected by immunohistochemical staining for von Willebrand factor was not increased in VEGF-treated xenografts compared to untreated xenografts (Schmidt et al., 2006). Sphingosine-1-phosphate (S1P) was also found to be a modulator of angiogenesis (Siess, 2002). The local administration of S1P to subcutaneous ovarian xenografts could induce the angiogenic process resulting in reduced necrosis and tissue hypoxia. More blood vessels were observed in the S1P-treated ovarian transplants (Soleimani et al., 2011).

A major drawback of TTG is the risk of reintroducing malignant cells into the testis. Therefore, it is important to remove all cancer cells from the tes-

ticular tissue before autologous transplantation can be performed. However, isolating single cells from tissue without damaging the structural integrity of the tissue is not possible. For SSCT, it is theoretically possible to remove malignant cells from the cell suspension before injection in the testis. Decontamination of single cell suspensions has been investigated by several groups, including ours. Elimination of malignant cells from a testicular cell suspension is based on positive selection of germ cells and/or negative selection of cancers cells (Fujita et al., 2005 and 2006; Geens et al., 2007; Hou et al., 2007). Another option to eliminate malignant cells would be the *in vitro* expansion of SSCs using culture conditions that specifically enrich SSCs but eliminate the malignant cells.

Although FACS may be effective in depleting malignant cells from single cell suspensions, a major concern is the substantial SSC loss during this procedure. Increasing the number of SSCs before transplantation will be essential. Successful strategies for *in vitro* expansion of SSCs have been reported in rodents (Kanatsu-Shinohara et al., 2003) and recently also in adult men as well as in prepubertal boys (Sadri-Ardekani et al., 2009 and 2011).

When cryopreserved SSCs are not available, an alternative option could theoretically be the generation of SSCs from adult stem cells, e.g. BMSCs. However, only a few donor GFP⁺ BMSCs were observed after injection in the lumen of seminiferous tubules in our study. We injected the mononuclear fraction of the bone marrow, containing both hematopoietic stem cells and mesenchymal stem cells (Ryabov et al., 2006). Lassalle et al. (2008) transplanted total bone marrow cells as well as a hematopoietic stem cell enriched fraction. Their results corroborate ours, since they too did not observe any colonization of the seminiferous tubules. As in our study, some donor cells were observed, but these cells had the characteristics of hematopoietic stem cells. It is however suggested now that only mesenchymal stem cells have the potential to differentiate into testicular cells. Recently, it was shown that mesenchymal stem cells can differentiate into somatic cells after injection in the testis, while no differentiation was observed when hematopoietic stem cells were injected (Yazawa et al., 2006). A mesenchymal-specific culture medium was also used for the *in vitro* derivation of male germ cells from bone marrow cells (Nayernia et al., 2006).

Given the current contradictory evidence, it is obvious that more research will be necessary before BMSCT can be viewed as a potential source for fertility restoration. The preservation of SSCs, followed by SSCT or TTG seems to be much more promising. Therefore, the storage of SSCs before

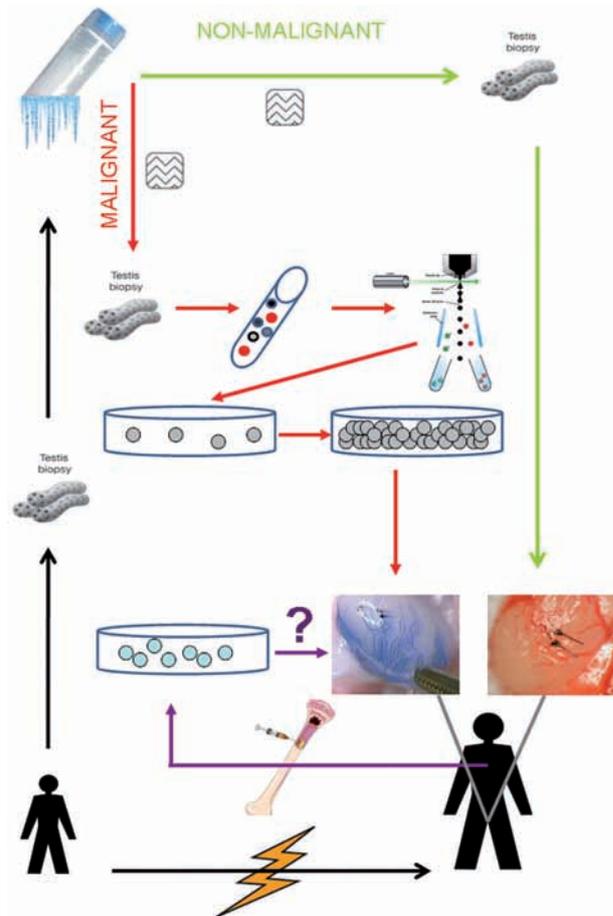


Fig. 5. — Overview of fertility preservation options for prepubertal boys.

The choice of the fertility preservation strategy will be patient-specific. Freezing a testicular biopsy is nowadays the standard procedure in our centre for patients who want to cryopreserve their SSCs before undergoing gonadotoxic treatment. If the patient was suffering from a nonmalignant disease, intratesticular tissue grafting can be performed (green line). If however, the patient has a risk of malignant cell contamination in the testis, the frozen-thawed biopsy will be enzymatically digested. Thereafter, malignant cells can be removed by flow cytometry. The SSC population will be enriched *in vitro* and SSCT will be performed (red line). If there were no SSCs stored, adult stem cells could theoretically be isolated and injected in the testis. However, this procedure has not been proven successful so far.

starting a gonadotoxic treatment should be offered to all boys at high risk for sterility and in which spermatogenesis is not yet completed. A patient-specific evaluation will eventually determine the choice of the fertility preserving method. We propose the next scheme to determine the best option for each individual patient (Figure 5).

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What is already known

Adult stem cells are becoming more and more important for regenerative medicine purposes. The adult stem cells of the testis, i.e. the spermatogonial stem cells, are the specific precursors for the germ-cell line. From puberty onwards, they will undergo mitosis and meiosis and eventually produce mature spermatozoa. Before puberty they are relatively quiescent because their main mitotic activity is related to cell renewal. Nevertheless, spermatogonial stem cells can disappear either because of gonadotoxic treatments, i.e. chemotherapy, or because of genetic conditions associated with germ cell loss, e.g. 47,XXY Klinefelter's syndrome. In order to cope with germ cell loss conditions, spermatogonial stem cell banking has been introduced in recent years. Eventually, these spermatogonial stem cells need to be transferred back into the testis for reinitiation of spermatogenesis whenever stem cell loss occurred. In animal models two approaches for transferring these stem cells have been described: either transplantation of stem cell suspensions into the seminiferous tubules or grafting of testicular tissue including these stem cells. The work presented in this thesis aimed at comparing the efficiency of these two strategies. Additionally, it was examined whether other types of adult stem cell, i.e.

bone marrow stem cells, have the potential to transdifferentiate into another type, in casu spermatogonial stem cells. This trans-differentiation strategy could be useful for those patients who lost their testicular stem cells but did have any preventive strategy to cope with stem cell loss.

What is new from this research

In a human-to-mouse xenograft model it appeared that intra-testicular grafting was more efficient than spermatogonial stem cell infusion for initiating spermatogenesis. Although human spermatogonial stem cells were able to survive in xenografts, full differentiation up to the stage of spermatozoon was lacking. The addition of recombinant FSH to xenografted mice did not improve differentiation, neither did it improve the spermatogonial stem cell survival in the grafts.

While in mice bone marrow-derived oocytes were obtained in earlier studies, in the current study no spermatogonial stem cells could be obtained from bone marrow stem cells after mouse-to-mouse transplantations. Neither was there any protective effect on stem cell loss as was described earlier for the ovary.

Which questions will this new findings arise

After transplanting testicular stem cells either by infusion or by grafting, spermatogonial stem cell survival is limited and may affect restoration of spermatogenesis. Since in this study spermatogonia-specific apoptosis was not found to be the main cause of stem cell loss, future research should focus on the nature of this loss. While after infusion no meiotic activity was observed in the xeno-transplanted stem cells, in the xenografts limited meiotic activity was observed. However, so far neither in this work nor in the work of others, full spermatogenesis could be established. Further research should tell us why spermatogenic process is hampered.