Male fertility preservation before gonadotoxic therapies

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Abstract

Background: Recent advances in cancer therapy have resulted in an increased number of long-term cancer survivors. Unfortunately, aggressive chemotherapy, radiotherapy and preparative regimens for bone marrow transplantation can severely affect male germ cells, including spermatogonial stem cells (SSCs), and lead to permanent loss of fertility. Different options for fertility preservation are dependent on the pubertal state of the patient.
Methods: Relevant studies were identified by an extensive Medline search of English and French language articles.
Results: Sperm cryopreservation prior to gonadotoxic treatment is a well established method after puberty. In case of ejaculation failure by masturbation, assisted ejaculation methods or testicular tissue sampling should be considered. Although no effective gonadoprotective drug is yet available for *in vivo* spermatogonial stem cell (SSC) protection in humans, current evidence supports the feasibility of immature testicular tissue (ITT) cryopreservation. The different cryopreservation protocols and available fertility restoration options from frozen tissue, i.e. cell suspension transplantation, tissue grafting and *in vitro* maturation, are presented. Results obtained in humans are discussed in the light of lessons learned from animal studies.
Conclusion: Advances in reproductive technology have made fertility preservation a real possibility in young patients whose gonadal function is threatened by gonadotoxic therapies. The putative indications for such techniques, as well as their limitations according to disease, are outlined.

Key words: Fertility, chemotherapy, radiotherapy, cryopreservation, immature testicular tissue.

Introduction

Due to remarkable advances in the treatment of cancer, we have seen great improvements in long-term survival rates of pediatric and reproductive-age male patients (Steliarova-Foucher *et al*., 2004). Unfortunately, fertility in adult life may be severely impaired by these treatments (Howell and Shalet, 1998; Brougham *et al*., 2003; Wallace *et al*., 2005). For this reason, development of gonadal cryobiology techniques is essential for fertility preservation.

Since the incidence of cancer is increasing at a rate of 2% per year in adolescents and 1.1% in children (Stiller *et al*., 2006), and gonadotoxic treatments are also used to successfully treat benign diseases such as drepanocytosis, thalassemia major, aplastic anemia, nephrotic syndrome and systemic autoimmune diseases, the population affected by fertility-threatening therapies is on the rise.

As a result of aggressive but effective chemo- and radiotherapeutic intervention, between 70% and 80% of children with oncological diseases survive their malignancies (Ries *et al*., 2004; Brenner *et al*., 2007).

Although these treatments are highly effective, a major concern is their adverse impact on fertility. Currently available drugs to prevent testicular damage from cytotoxic therapy have not proved helpful in humans so far. However, improved therapeutic regimens using less gonadotoxic protocols (Kulkarni *et al*., 1997; Radford *et al*., 1994; Tal *et al*., 2000) could allow more patients to preserve their germ stem cell pool and enable spontaneous recovery of spermatogenesis. Unfortunately, use of these protocols is not always possible without compromising the chances of recovery from cancer.

Loss of fertility in adult life is a major and psychologically traumatic consequence (Schover *et*

al., 1999) and fertility preservation options should therefore be proposed to these patients.

After puberty, cryopreservation of sperm is a well established method of fertility preservation. For prepubertal boys, however, very few options exist to protect their fertility, besides choosing therapies that are less toxic to their gonads. Advances in assisted reproduction technologies (ART) and increasing interest in *in vivo* and *in vitro* gamete maturation have focused on preserving immature gametes and thus germ stem cells before sterilizing treatments, in the hope of developing new techniques allowing use of stored immature gametes in the future.

Understanding the physiology of the testicular stem cell, and the self-renewal and differentiation events leading to the development of mature and functional sperm cells, may help to elucidate the impact of chemotherapeutic drugs and radiation on germ cells, as well as the potential options to decrease testicular damage and improve fertility restoration approaches.

Thus, after first summarizing the physiology of the testis, and the acute and long-term effects of cancer therapies on male fertility, this review will examine the current state of the art with respect to male fertility preservation and restoration strategies.

Physiology of spermatogenesis

Spermatogenesis is a cyclic and continuous process, with distinct phases: mitosis, meiosis and spermiogenesis ongoing throughout the entire male life span. It takes place within the seminiferous tubule, composed of a basal membrane surrounded by a layer of peritubular cells. The tubule contains seminiferous epithelium populated by different types of germ cells, according to pubertal state, and Sertoli cells, playing a nursing role for the germ cells (Griswold, 1998) through secretion of various factors, such as Glial cell line-derived neurotrophic factor (GDNF), stem cell factor (SCF), Ets related molecule (ERM), leukemia inhibitory factor (LIF) and bone morphogenetic protein 4 (BMP4). The number of functional Sertoli cells determines final sperm production, because the number of germ cells supported by the Sertoli cells is finite (Orth *et al*., 1988).

Normal spermatogenesis relies on the presence of an intact diploid spermatogonial stem cell (SSC) capable of self-renewal through mitotic amplifying divisions, and differentiation involving two meiotic divisions followed by maturation into haploid spermatids, the latter being transformed into spermatozoa.

SSC homeostasis is regulated by intrinsic gene expression and extrinsic signals, including soluble factors and adhesion molecules from the surrounding microenvironment, known as the stem cell niche (Ogawa *et al*., 2005). Diffusion of paracrine factors secreted by interstitial Leydig cells or peritubular myoid cells is also involved.

Study of SSCs has been hampered because of their scarcity (0.03% of the total number of germ cells in the adult mouse (Tegelenbosch and De Rooij, 1993)) and lack of specific markers for their isolation, since SSCs are not morphologically different from other spermatogonia, but are functionally distinct. However, culture systems that maintain a SSC population for extended periods of time and allow experimental modifications of added growth factors, combined with transplantation assays demonstrating their biological capacity to self-renew and differentiate, have enhanced our understanding of the cellular and molecular characteristics of SSCs (Kubota *et al*., 2004a, 2004b; Oatley and Brinster, 2006).

Spermatogenesis begins at puberty, although spermatogenic events leading to germ cell degeneration before the haploid stage is reached already occur in the prepubertal testis (Nistal and Paniagua, 1984).

Before puberty, the seminiferous tubule consists of Sertoli cells and different types of spermatogonia, including stem cells and differentiating type A and type B spermatogonia (from the age of 4) (Nistal and Paniagua, 1984). Two types of undifferentiated spermatogonia, in extensive contact with the basement membrane, can be distinguished by morphological criteria (Clermont, 1966): the type A *dark* spermatogonium functioning as a reserve stem cell which, under normal conditions, is mitotically quiescent, and the type A *pale* spermatogonium known as the active stem cell, which through regular mitotic divisions generates differentiating germ cells and also maintains the stem cell population (Clermont, 1972). Type B spermatogonia, characterized by chromatin clumps in the nucleus and a centrally placed nucleolus, have the least contact with the basement membrane and are committed to spermatogenic differentiation. Their number slowly increases until the age of 8-9 years, after which a period of marked spermatogonial proliferation occurs. When they move into the core of the germinal epithelium and are separated from neighboring cells by expansions of Sertoli cells, they are known as preleptotene spermatocytes. Primary spermatocytes appear after the last spermatogonial division and undergo the first meiotic division at the beginning of puberty.

Spermarche, defined as the onset of sperm production, occurs at a median age of 13.4 years (range: 11.7-15.2), when median testicular volume is 11.5 ml (range: 4.7-19.6). Most boys achieve spermarche prior to the age of peak height velocity and before being able to produce an ejaculate (Nielsen *et al*., 1986). In the absence of ejaculation,

spermaturia, defined as the presence of sperm in the urine, may be a useful tool to detect initiation of spermatogenesis (Schaefer *et al*., 1990).

Impact of gonadotoxic therapy on germ cells

Since rapidly dividing cells are the target of chemo- and radiotherapy, these treatments act not only on cancer cells, but also on germ cells during spermatogenesis. Little is known about the effects of gonadotoxic treatments on the immature testis, as fertility cannot be assessed before puberty, but cytotoxic damage to the testis has been extensively studied after puberty.

Among the germ cells, differentiating spermatogonia proliferate the most actively and are thus extremely susceptible to cytotoxic agents, although the less active stem cell pool may also be depleted (Bucci and Meistrich, 1987).

As a result, the seminiferous epithelium becomes damaged and the population of stem cells that normally differentiate to produce sperm after puberty either becomes depleted or unable to differentiate, leading to prolonged or even permanent azoospermia due to destruction of the germ cells (for review, see Schrader *et al*., 2001; Howell and Shalet, 2001). The severity and duration of cytotoxic agent-induced long-term impairment of spermatogenesis correlate with the number of type A spermatogonia that are destroyed (Meistrich, 1986), but remain unpredictable because of variable individual sensitivities (Naysmith *et al*., 1998).

After a cytotoxic insult, recovery of sperm production depends on the survival and ability of mitotically quiescent stem spermatogonia (type A *dark*) to transform into actively dividing stem and differentiating spermatogonia (type A *pale*) (van Alphen *et al*., 1988).

Gonadal impairment and fertility prognosis following chemotherapy

Chemotherapeutic agents manifest their cytotoxic effect by interrupting essential cell processes such as DNA synthesis and folate metabolism in rapidly dividing cells.

Although the prepubertal testis does not complete spermatogenesis, there is evidence that cytotoxic treatment given to prepubertal boys may affect fertility (Rivkees and Crawford, 1988; Mackie *et al*., 1996; Kenney *et al*., 2001). The presence of a steady turnover of early germ cells that undergo spontaneous degeneration before the haploid stage is reached (Muller and Skakkebaeck, 1983; Kelnar *et al*., 2002) may explain why the prepubertal state does not offer any protection against the deleterious effects of chemotherapy.

The somatic compartment of the testis may be more resistant to chemotherapeutic treatment, since these cells have a low or absent mitotic rate. Nevertheless, increased concentrations of LH and symptomatic reductions in testosterone concentrations (Howell *et al*., 1999), both signs of Leydig cell impairment, have been described, but the mechanism of this impairment after chemotherapy is not known. Evidence of Sertoli cell functional impairment following chemotherapy, responsible for germ cell differentiation inhibition where germ cells have survived, have also been reported (Bar-Shira Maymon *et al*., 2004).

The extent of damage is dependent on the agent administered, the dose delivered, the combination of cytotoxic drugs and the potential synergic interaction of radiotherapy, which complicates identification of the specific toxicity of each individual agent (for review, see Trottmann *et al*., 2007). Long-term fertility prognoses following treatment with different chemotherapeutic agents used in childhood and best estimates of fertility after chemotherapy for common childhood cancers were recently updated (Wyns *et al*., 2010).

Gonadal impairment and fertility prognosis following radiation

Besides killing germ cells, including dividing spermatogonia and SSCs (de Rooij and Russel, 2000), radiotherapy causes a block in spermatogonial differentiation, which may be attributed to damage to the somatic compartment (Zhang *et al*., 2007).

Radiation induces germinal depletion in a dose-dependent manner (Rowley *et al*., 1974) and the more immature cells are the most radiosensitive. Doses as low as 0.1-1.2 Gy damage dividing spermatogonia and result in oligozoospermia. Radiation doses over 4 Gy cause a more permanent detrimental effect and may result in complete sterility.

Fractionated radiotherapy increases seminiferous tubule damage, with doses greater than 1.2 Gy resulting in permanent azoospermia (Ash, 1980). The observed activation of reserve stem cells after a gonadotoxic insult demonstrates that a single insult is less damaging to the seminiferous epithelium than multiple insults of lower intensity (Ash, 1980).

Testicular irradiation with doses above 20 Gy is associated with Leydig cell dysfunction in prepubertal boys, while Leydig cell function is usually preserved up to 30 Gy in adults (Shalet *et al*., 1989).

Apart from dose and fractionation, other factors such as source, field of treatment, type of radiation,

age and individual susceptibility influence the gonadotoxicity of irradiation.

Complete recovery of testicular function after radiotherapy, evidenced by a return to pretreatment numbers of spermatozoa, is dependent on the dose administered. Indeed, it usually occurs within 9-18 months following a dose of ≤ 1 Gy, 30 months for 2-3 Gy and 5 years or more for doses of ≥ 4 Gy, if sterility is not permanent (for review, see Howell and Shalet, 2005).

Fertility preservation options

Three different approaches may be considered:

1. Minimizing testicular damage from cancer treatment or protecting SSCs *in vivo*.
2. Cryopreserving sperm prior to gonadotoxic treatment.
3. Cryopreserving testicular tissue prior to gonadotoxic treatment in the form of either a cell suspension, tissue fragments or a whole organ.

In vivo SSC protection

Little is known about the mechanisms by which cancer treatment damages spermatogenesis, especially in the prepubertal testis, since tubular damage cannot be evidenced before puberty. In order to reduce the deleterious effects of gonadotoxic therapies, different strategies have been tested, such as testicular shielding and use of cytoprotective drugs.

Limiting radiation exposure by shielding or removing the testes from the radiation field should be implemented whenever possible (Wallace *et al*., 2005; Ishiguro *et al*., 2007).

Gonadal protection through hormonal suppression is based on the principle that disruption of gametogenesis renders the gonads less sensitive to the effects of cytotoxic drugs or irradiation. Promising results were obtained in rodents (for review, see Shetty and Meistrich, 2005), but not in non-human primates (Boekelheide *et al*., 2005) or humans (Johnson *et al*., 1985; Waxman *et al*., 1987; Redman and Bajorunas, 1987; Fossa *et al*., 1988; Kreuser *et al*., 1990; Brennemann *et al*., 1994), except in one clinical trial (Masala *et al*., 1997) where only moderate stem cell death was induced by chemotherapy. By contrast, stimulating spermatogonial proliferation by FSH might be an option, as shown in monkeys (van Alphen *et al*., 1989; Kamischke *et al*., 2003).

Anti-apoptotic agents such as sphingosine-1-phosphate (Suomalainen *et al*., 2003; Otala *et al*., 2004) and AS101 (Carmely *et al*., 2009), as well as various other cytoprotective substances (Lirdi *et al*., 2008; Okada *et al*., 2009), have also been used with partial success in rodents.

In summary, no effective gonadoprotective drugs are so far available for use in humans. Studies aimed at identifying factors regulating spermatogonial proliferation are therefore required to find novel targets for *in vivo* SSC protection.

Sperm cryopreservation

General considerations

Cryopreservation of sperm is the only established option for fertility preservation in postpubertal males. It relies on the presence of spermatozoa and the ability to ejaculate. This procedure has been performed for decades (Royère *et al*., 1996) and it is well known that spermatozoa survive long-term cryobanking. Indeed, their use through assisted reproduction techniques has led to the birth of healthy offspring more than 20 years after initial storage (Feldschuh *et al*., 2005).

Typically, it is recommended that 3 samples be provided by masturbation, with 48-72 hours between samples destined for freezing, regardless of semen quality, as long as viable spermatozoa are available. Indeed, since intracytoplasmic sperm injection (ICSI) allows pregnancy even when a single viable spermatozoon is available after thawing of frozen semen (Hovatta *et al*., 1996), poor semen quality is no longer a major concern for fertility preservation in cancer patients.

The care plan must nevertheless be individualized according to patient status at diagnosis and time available to collect an optimal number of samples. Since sperm DNA integrity may be compromised after cytotoxic treatment (Meistrich, 1993), it is strongly recommended that sperm be cryopreserved before initiation of chemotherapy or radiotherapy.

In case of failure to provide samples by masturbation, assisted ejaculation techniques, such as penile vibratory stimulation or electroejaculation, can be considered (Schmiegelow *et al*., 1998). Electroejaculation requires general anesthesia because of the pain induced by the procedure, so should not be embarked upon without serious consideration.

Specific considerations for adolescent patients

If they are able or willing to ejaculate after masturbation, sperm banking can be offered to all male adolescents newly diagnosed with cancer from 12 years of age (Bahadur *et al*., 2002). Reports on male adolescent sperm cryopreservation showed that the potential for fertility preservation in subjects as young as 13 years of age was successful in about 50% of cases (for review, see Bashore, 2006).

For physically mature patients, emotional immaturity may constitute a barrier to producing a sample on demand, and appropriate counseling and education should be available for these patients. Private consultation with adolescents, allowing for questions they may be uncomfortable asking in the presence of their parents, has an important impact on the success of sample collection (Bahadur *et al*., 2002).

Very little information is available on sperm quality in healthy adolescents. Specimens produced are often of poor quality in peripubertal patients, as many of them have only recently commenced spermarche. The time between the first clinical signs of puberty and first ejaculation ranges between 8 and 12 months and early ejaculations are marked by very small volumes, cryptozoospermia with a majority of immotile spermatozoa, if not azoospermia, and abnormal liquefaction (Janczewski and Bablok, 1985).

Cryopreservation of mature tissue

Cryopreservation of a testicular biopsy can be proposed to patients who are not able to provide a semen sample by masturbation or using an assisted ejaculation procedure. In case of an azoospermic sample, surgical retrieval of spermatozoa by TESE (testicular sperm extraction) remains an option, since it was shown that the procedure allows sperm retrieval in 50% of cases (Schrader *et al*., 2003). Cryopreservation methods for human spermatozoa extracted from testicular biopsies have been implemented for many years now (Hovatta *et al*., 1996).

Cryopreservation of immature tissue

Since prepubertal boys cannot benefit from sperm banking, a potential alternative strategy for preserving their fertility involves immature testicular tissue banking (for review, see Wyns *et al*., 2010). It is important to stress, however, that this strategy is still experimental.

As prepubertal testicular tissue contains SSCs from which haploid spermatozoa are ultimately derived, these cells can either be cryopreserved as a cell suspension (Brook *et al*., 2001) or in the form of tissue (Kvist *et al*., 2006; Keros *et al*., 2007; Wyns *et al*., 2007), in the hope that future technologies will allow their safe utilization.

Cell suspensions

Cell suspensions have been developed with a view to facilitating cryopreservation, as cell heterogeneity in tissue pieces renders tissue freezing more challenging. Preparation of cell suspensions requires mechanical and/or enzymatic digestion of tissue. The risk of tissue digestion is that cell survival may be compromised, as shown by the reduced viability of suspensions after dispersion (Brook *et al*., 2001). In addition, suppression of cell-to-cell interactions may also be deleterious for cell proliferation and differentiation (Griswold, 1998).

Post-thaw viability ranging from 29% to 82% has been reported after cryopreservation of testicular cell suspensions in various animal models (Geens *et al*., 2008).

Tissue pieces

Cryopreservation of testicular tissue pieces may be considered as an alternative method suitable for maintaining cell-to-cell contacts between Sertoli and germinal stem cells, and therefore preserving the stem cell niche necessary for their survival and subsequent maturation (Ogawa *et al*., 2005). Another advantage of this method may be preservation of the Sertoli cells, since there is evidence of their reversion to a dedifferentiated state as a consequence of chemotherapy (Bar-Shira Maymon *et al*., 2004).

Since tissue pieces also contain the interstitial compartment, including Leydig cells, their preservation can be useful to alleviate the hormonal imbalance caused by cytotoxic therapy (Howell and Schalet, 2001). Better survival rates of Leydig cells were obtained when DMSO was used (80% compared to 50% with PROH) (Keros *et al*., 2005). Structural integrity and functional capacity were demonstrated after cryopreservation and culture of fetal and prepubertal testicular tissue (Kvist *et al*., 2006; Keros, 1999; Keros *et al*., 2007), as well as after transplantation of cryopreserved fetal testicular tissue (Grischenko *et al*., 1999).

Unlike cryopreservation of isolated cells, freezing of tissue presents new problems because of the complexity of tissue architecture. Protocols must strike a balance between optimal conditions for each cellular type, depending on water content, size and shape of cells, and the water permeability coefficient of their cytoplasmic membrane. In addition, problems can arise when extracellular ice forms, as it can cleave tissues into fragments. Furthermore, rapid solute penetration of highly compacted tissue is vital to ensure high final concentrations of cryoprotectant at temperatures which will minimize cytotoxicity. These requirements necessitate optimization of freeze-thawing protocols for each cell type, since post-thaw survival and seminiferous tubule structure are profoundly affected by both the type of cryoprotectant and the freezing rates (Milazzo *et al*., 2008). DMSO, rather than EG, PROH or glycerol, was

shown to better preserve structures within tissue (Keros *et al*., 2005; Goossens *et al*., 2008a) and to be best able to retain tissue capacity to initiate spermatogenesis (Jahnukainen *et al*. 2007). According to Keros *et al*. (2007), use of slow-programmed freezing is important to maintain undamaged morphology of spermatogonia during tissue cryopreservation.

Two teams have reported freezing protocols for prepubertal human testicular tissue that have yielded good structural integrity (Kvist *et al*., 2006; Keros *et al*., 2007). The second study investigated the influence of cryopreservation protocols on normal immature human tissue (Keros *et al*., 2007). Besides good tissue and cell integrity after freezing, very good spermatogonial recovery was achieved with their best protocol (94% ± 1% intact spermatogonia after freeze-thawing and culture). This protocol, albeit slightly modified by the addition of sucrose, was therefore used by our group for further evaluation of the functional capacity of cryopreserved human immature testicular tissue after xenografting (Wyns *et al*., 2007, 2008). An overview of studies on cryopreservation of immature testicular tissue is presented in Table I.

Fertility restoration options

Fertility restoration after sperm cryopreservation

After thawing of cryopreserved semen samples, intrauterine insemination (IUI) may be considered, but it depends on the survival of a sufficient number of motile sperm to achieve good success rates. Due to the possible deterioration of semen quality after sample thawing, the often unsatisfactory initial quality of semen in cancer patients, and the limited sperm reserve prior to therapy, IVF or IVF/ICSI are generally required to restore fertility in these patients. Success rates of ART with cryopreserved sperm in male cancer survivors are comparable to other indications, and no significant increase in miscarriage or birth defect rates has so far been reported after ART with cryobanked semen from men with cancer (Sanger *et al*., 1992; Agarwal *et al*., 2004).

Fertility restoration after mature tissue cryopreservation

Use of spermatozoa from frozen testicular tissue requires assisted reproduction by ICSI. Healthy pregnancies issuing from spermatozoa extracted from cryopreserved testicular biopsies have been reported for some time now and the technique is widely used in clinical practice (Hovatta *et al*., 1996).

Fertility restoration after immature tissue cryopreservation

Storage of testicular tissue could well be an option for prepubertal boys. Indeed, besides diploid precursor germ cells, some haploid germ cells may also be found in their testes, since spermatogenesis is known to occur to some extent in the testes of boys at very early stages of pubertal development (Muller J. and Skakkebaeck, 1983; Schaefer *et al*., 1990; Hovatta, 2001). In theory, and based on the reported fertilization potential of early spermatids after microinjection into the egg (Tesarik *et al*., 1995), these haploid spermatids obtained from the testes of prepubertal boys may have reproductive potential, although this has not yet been proved.

When haploid gametes are not present in their testes, frozen diploid precursor cells provide some hope of fertility restoration in these boys. To this end, three approaches can be considered:

1. Transplantation of purified cell suspensions back to their own testes.
2. Autografting of testicular pieces or whole testes.
3. *In vitro* maturation (IVM) up to the stage at which they are competent for normal fertilization through ICSI.

None of these approaches have been demonstrated to be efficient and safe in humans as yet. These potential options have mainly been studied in animals and lessons learned from these studies will be reviewed in detail.

Testicular germ cell transplantation

In this approach, spermatogenesis is reinitiated after transplantation of isolated testicular stem cells in germ cell-depleted testes. SSCs are recognized by Sertoli cells and are relocated from the lumen onto the basement membrane of seminiferous tubules. Because stem cells have unlimited potential to self-renew and produce differentiating daughter cells, SSC transplantation offers the possibility of long-term restoration of natural fertility. It could therefore be a potential alternative to restore fertility after cancer treatment.

The technique was first described in 1994 by Brinster and Zimmermann, who developed a SSC assay in mice that identified SSCs by their ability to generate a colony of spermatogenesis after transplantation. Testicular germ cells isolated from prepubertal mouse testes were injected into the seminiferous tubules of adult mice with Sertoli cell-only syndrome induced by busulfan treatment (Brinster and Zimmermann, 1994). Normal donor spermatogenesis, recognized by developing germ cells carrying the

Table 1. — Overview of studies on cryopreservation of prepubertal human testicular tissue.

Reference	Cryoprotectant	(Non-) controlled	Freezing rate	Type of evaluation	Outcome (germ cells)	Outcome (endocrine compartment)
Kvist *et al*., 2006	EG1.5 M Sucrose 0.1 M	Slow-controlled	Start: 1°C, -2°C/min to -9°C, hold 5 min + seeding, -0.3°C/min to -40°C, -10°C/min to -140°C, LN2	Culture 2 weeks	Well preserved STs Presence of intact SG (c-kit+)	Well preserved interstitial cells Testosterone and inhibin levels similar to fresh tissue
Keros *et al*., 2007	DMSO 0.7 M	Slow-controlled	**Program 1:** Start: 4°C, hold 30 min, -1°C/min to 0°C, hold 5 min, -0.5°C/min to -8°C, seeding, hold 10 min, -0.5°C/min to -40°C, hold 10 min, -7°C/min to -70°C, LN2	Culture 24 h	70 ± 7% ISTs in frozen-cultured tissue (vs 71 ± 7% in fresh tissue and 77 ± 4% in fresh-cultured tissue) 94 ± 1% intact SG in frozen-cultured tissue (vs 93 ± 2% in fresh tissue and 83 ± 1% in fresh-cultured tissue)	Undamaged stromal structure: 80 ± 29% of frozen-cultured samples (vs 99.49 ± 0.88% of fresh samples and 97 ± 2% of fresh cultured samples)
		Rapid- controlled	**Program 2:** Start: 4°C, hold 30 min, -1°C/min to -8°C, seeding, hold 10 min, -10°C/min to -80°C, LN2		20 ± 14% ISTs in frozen-cultured tissue 50 ± 43% intact SG in frozen-cultured tissue	Undamaged stromal structure: 29 ± 28% of frozen-cultured samples
Wyns *et al*., 2007	DMSO 0.7 M Sucrose 0.1 M	Slow- controlled	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	Immediate post-thaw evaluation	0.71 ± 0.89 SG/ST in frozen-thawed tissue (vs 0.45 ± 0.35 SG/ST in fresh tissue)	Not assessed
				Xenografting 3 weeks	82.19 ± 16.46% ISTs in frozen-grafted tissue (vs 93.38 ± 6% in fresh tissue)	14.5% SG recovery after freezing and grafting
Wyns *et al*., 2008	DMSO 0.7 M Sucrose 0.1 M	Slow- controlled	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	Xenografting 6 months	55 ± 42% ISTs in frozen-grafted tissue 3.7 ± 5.5% SG recovery 21% proliferating SG Differentiation up to pachytene stage of prophase	Signs of steroidogenic activity by 3β-HSD IHC and TEM

(I)ST: (intact) seminiferous tubule; SG: spermatogonia; LN2: liquid nitrogen; HSD: hydroxysteroid dehydrogenase; IHC: immunohistochemistry; TEM: transmission electron microscopy.

lacZ gene encoding β-galactosidase (evidenced histochemically as an intracellular blue reaction), was initiated and sustained (Fig. 1).

Although this approach has yielded healthy progeny displaying the donor haplotype in animals (Brinster and Avarbock 1994), it has not yet proved successful in humans (see Progress towards human clinical application).

Lessons learned from transplantation of fresh testicular stem cells in animals

Outcome of the technique

Autologous SSC transplantation has been reported in mice (Brinster and Zimmermann, 1994), rats (Ogawa *et al*., 1999), pigs (Honaramooz *et al*., 2002a; Mikkola *et al*., 2006), goats (Honaramooz *et al*., 2003), cattle (Izadyar *et al*., 2003a), monkeys (Schlatt *et al*., 2002a) and dogs (Kim *et al*., 2008). Restoration of fertility from donor stem cells has only been achieved in mice (Brinster and Avarbock, 1994; Ogawa *et al*., 2000; Nagano *et al*., 2001a; Brinster *et al*., 2003; Goosens *et al*., 2003), rats (Hamra *et al*., 2002; Ryu *et al*., 2003; Zhang *et al*., 2003), goats (Honaramooz *et al*., 2003) and chickens (Trefil *et al*., 2006).

Heterologous transplantation does not appear to be as successful as autologous transplantation, probably because of the phylogenetic distance between species. Indeed, it seems that the farther the phylogenetic distance, the less likely the transplantation is to result in completion of spermatogenesis.

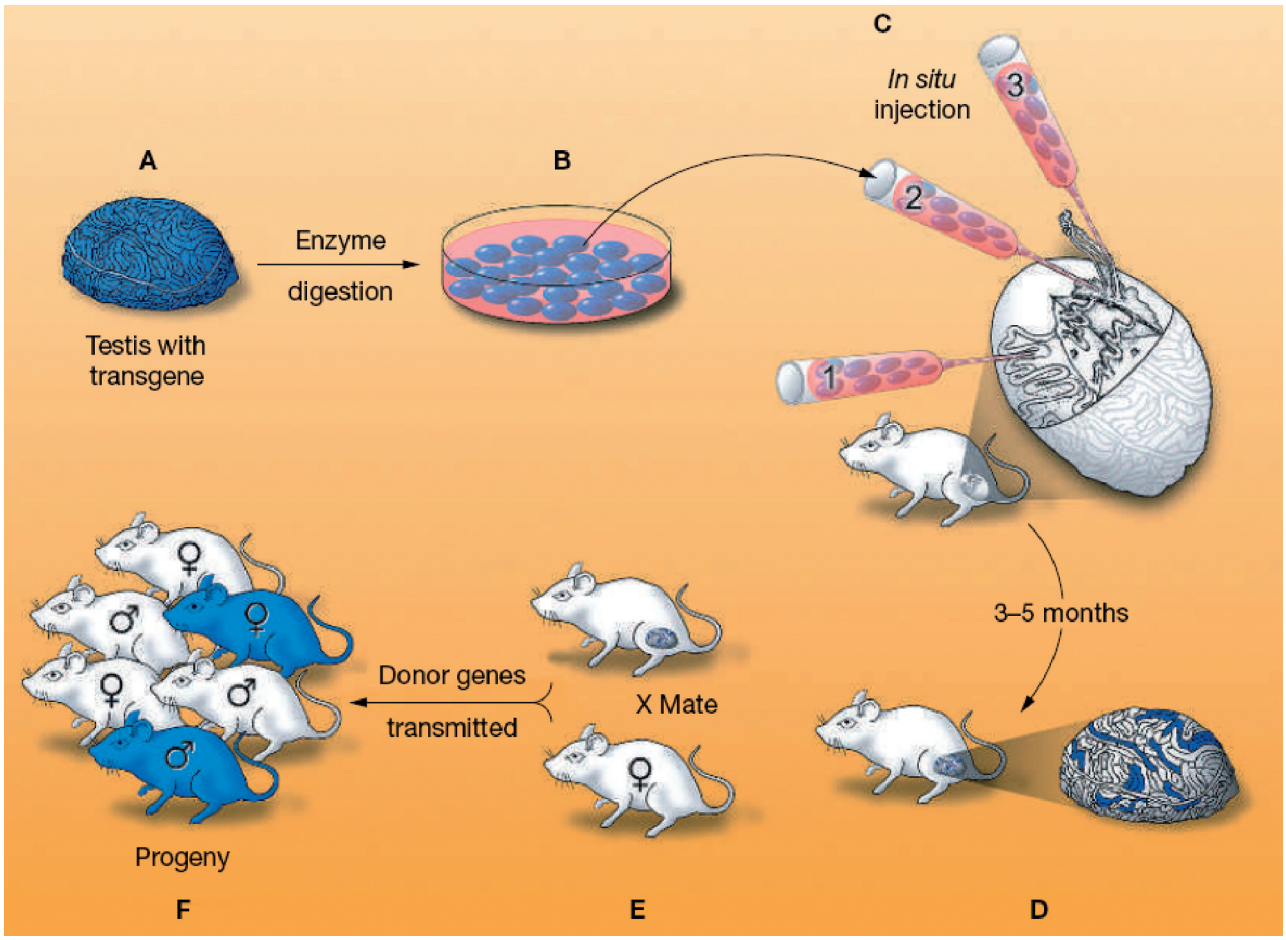

Fig. 1. — Procedure for testicular cell transplantation as developed in the mouse.
(**A**) A single-cell suspension is prepared from the testes of a fertile male expressing a reporter transgene, *Escherichia coli lacZ*.
(**B**) The testicular cells can be cultured in appropriate conditions.
(**C**) Cells are microinjected into the seminiferous tubules of an infertile recipient male. There are three methods for microinjection: the micropipette can be inserted (1) directly into the seminiferous tubules, (2) into the rete testis, or (3) into an efferent duct.
(**D**) Spermatogonial stem cells colonize the basement membrane of the tubules and generate donor cell-derived spermatogenesis, which can be stained blue using a substrate for the reporter gene product (β-galactosidase). Each blue stretch of cells in the seminiferous tubules of the recipient testis represents a spermatogenic colony derived from a single donor stem cell.
(**E**) Mating the recipient male with a wild-type female results in donor cell-derived spermatozoa fertilizing wild-type oocytes.
(**F**) Progeny with the donor haplotype are produced.
Source: Kubota and Brinster (2006) Nat Clin Pract Endocrinol Metab. 2 (2),99-108.

Indeed, SSCs from rabbits, dogs, pigs, bulls, stallions, non-human primates and humans were able to colonize the seminiferous tubules of mice and generate colonies of stem cells and cells that appeared to be early differentiating daughter spermatogonia, but could not differentiate beyond the stage of spermatogonial expansion (Dobrinski *et al*., 1999a; Dobrinski *et al*., 2000; Oatley *et al*., 2002; Nagano *et al*., 2001b; Hermann *et al*., 2007; Nagano *et al*., 2002). This suggests that the initial steps of germ cell recognition by Sertoli cells, migration to the basement membrane and initiation of cell proliferation are conserved among evolutionarily divergent species.

Efficiency of the technique

The extent of spermatogenesis was shown to depend on the number of transplanted stem cells, with an almost linear correlation (Dobrinski *et al*., 1999b, Fig. 2), and on the quantity and quality of stem cell niches in the recipient testis (Ogawa *et al*., 2000).
In rodents, the observed colonization rate was no higher than 1 out of 20 SSCs (Dobrinski *et al*., 1999b), thus showing low colonization efficiency. The colonization rate of slowly cycling primate type A *dark* spermatogonia was expected to be much lower (Jahnukainen *et al*., 2006a). Indeed, rhesus SSC engraftment efficiency was estimated to be just

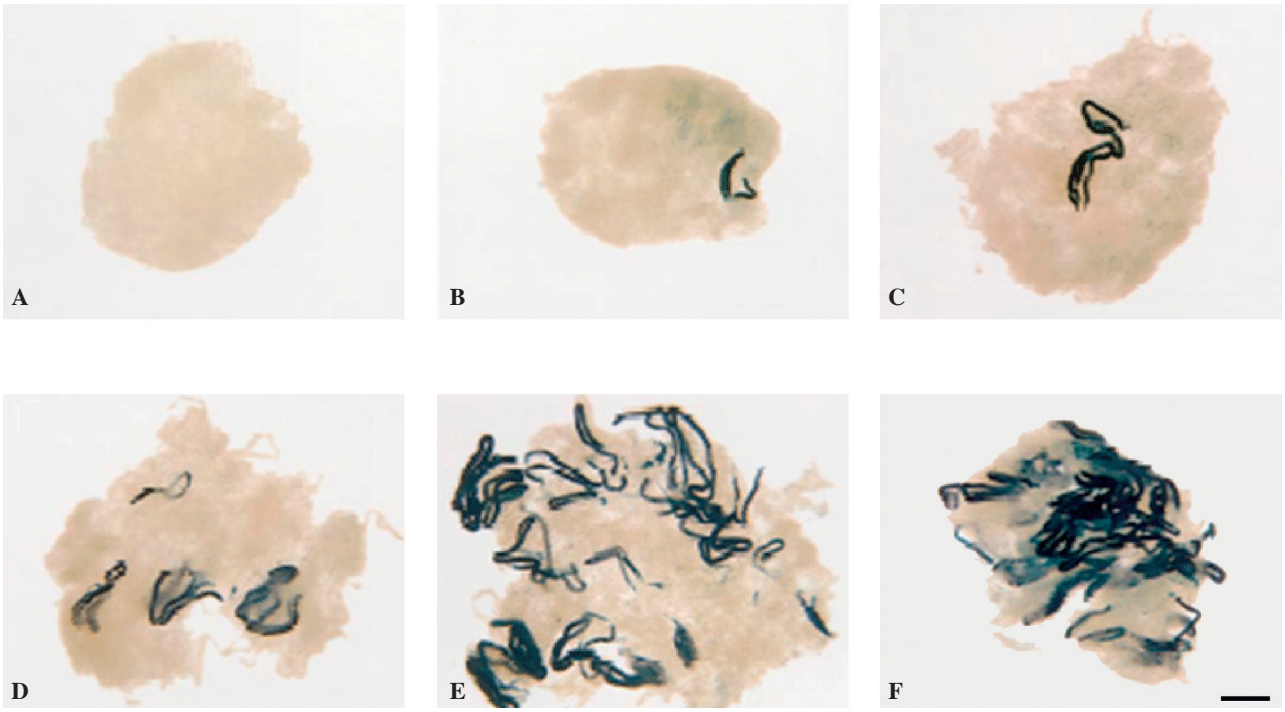

Fig. 2. — Representative pattern of colonization of recipient testes by donor-derived spermatogenic cells three months after transplantation of three different cell concentrations. **A, B:** Transplantation of 10^6 cells/ml. **C, D:** Transplantation of 10^7 cells/ml. **E, F:** Transplantation of 10^8 cells/ml. A–F: Whole mount preparations of entire recipient testes stained with X-gal. Bar= 2 mm.
Source: Dobrinski *et al*. (1999), Mol Reprod Dev 53, 142-148.

0.0015%-0.003%, hence extremely low (Hermann *et al*., 2007). Therefore, methods to increase colonization efficiency need to be developed with a view to effective clinical application.

Recipient age also appears to have an impact on colonization efficiency (Shinohara *et al*., 2001). It was suggested that the unique growth environment of the immature testis could be the result of better niche accessibility and niche proliferation due to Sertoli cell multiplication during testicular maturation and growth, and/or factors facilitating colony formation, such as differences in hormones or growth factors. Moreover, the colony expansion may be influenced by the increase in seminiferous tubule length occurring with testicular enlargement.

Techniques for SSC enrichment

Because of the small number of SSCs in a testis (2/10,000 germ cells) (Muller and Skakkebaeck, 1983), the small size of testicular biopsies recovered for fertility preservation, and the low efficiency of recolonization after transplantation, development of methods to increase the number of SSCs prior to transplantation is essential. Ideally, isolation of pure stem cells would be the most effective way of increasing the number of SSCs in a suspension and therefore transplantation efficiency (Shinohara *et al*., 1999). Adequate purification will probably be best achieved by cell sorting techniques, such as magnetic-activated cell sorting (MACS) or fluorescence-activated cell sorting (FACS) based on cell characteristics and membrane antigens. Indeed, these techniques have already been shown to improve transplantation efficiency in mice. (Shinohara *et al*., 1999, 2000, Ohta *et al*., 2000; Kubota *et al*., 2003, 2004a; Hofmann *et al*., 2005). As conserved expression of some markers of undifferentiated spermatogonia exists between mice and non-human primates (Hermann *et al*., 2007; 2009), there is hope that cell enrichment techniques may be extended to humans.

Techniques for SSC expansion

Expansion of pure stem cells in culture appears to be possible, although cell proliferation was found to be limited (Hasthorpe, 2003). Better results were achieved with expansion techniques using culture on feeder layers with a combination of growth factors, or applying serial transplantation procedures (Ogawa *et al*., 2003; Kanatsu-Shinohara *et al*., 2003a).

Until recently, strategies for *in vitro* expansion of SSCs had only proved successful in rodents (Kanatsu-Shinohara *et al*., 2003a; Kubota *et al*., 2004b; Ryu *et al*., 2005). Kanatsu-Shinohara *et al*. (2003a) were able to culture neonatal mouse testicular cells after supplementation of culture media with various growth factors and hormones. After

2 years, the cultured cells showed 10^{85}-fold logarithmic proliferation, retaining characteristic morphology and yielding fertile offspring after stem cell transplantation (Kanatsu-Shinohara *et al*., 2005). Long-term culture and propagation of human SSCs has now also been reported (Sadri-Ardekani *et al*., 2009).

Lessons learned from transplantation of frozen testicular stem cells in animals

Since high survival rates do not guarantee preservation of the functionality of frozen-thawed cells, it is important to evaluate their capacity to self-renew and differentiate through transplantation of cell suspensions. Experiments on human germ cell transplantation were not able to achieve this goal since, after 6 months' xenotransplantation to immunodeficient mice, only proliferative activity was observed (Nagano *et al*., 2002). Hence, studies in animals will help us elucidate some important considerations to be taken into account in the context of clinical application.

The potential of frozen murine testicular cells to resume spermatogenesis after transplantation was demonstrated for the first time by Avarbock *et al*. in 1996. The birth of live offspring after transplantation of frozen-thawed testicular cell suspensions provided final proof of successful cryopreservation (Kanatsu-Shinohara *et al*., 2003b). However, it appears that the functional capacity of stem cells may be compromised by cryopreservation (Frederickx *et al*., 2004). By contrast, Kanatsu-Shinohara *et al*. (2003b) did not observe decreased spermatogenic efficiency after cryopreservation.

In addition, rhesus SSCs appear to retain normal colonization capacity after freezing, since no significant difference was found in the number of produced colonies after frozen-thawed SSC transplantation compared to fresh SSC transplantation in mice (Hermann *et al*., 2007), suggesting that possible functional impairment involves germ cell differentiation rather than their ability to recolonize stem cell niches.

Progress towards human clinical application

In humans, preclinical *in vitro* studies using cadaver or surgically removed testes have demonstrated the feasibility of transplanting germ cell suspensions into testes. Fifty to 70% of seminiferous tubules were filled by means of intratubular injection (Brook *et al*., 2001) or injection into the rete testis, with needle placement controlled by ultrasonography (Schlatt *et al*., 1999). The different injection techniques described in the literature are shown in Fig. 3.

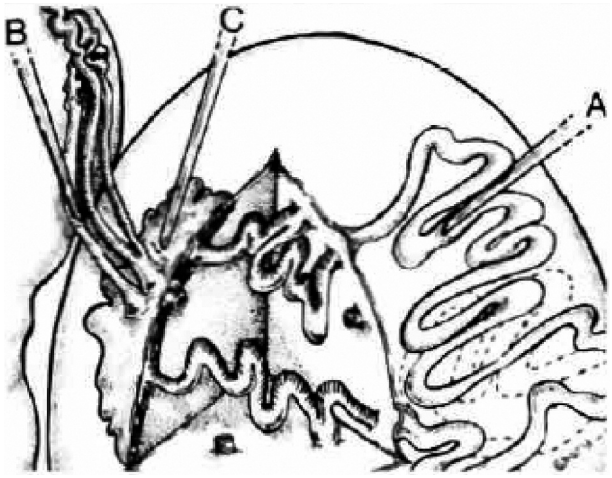

Fig. 3. — Microinjection pipette insertion sites to introduce cell suspensions into the seminiferous tubules.
A seminiferous tubule may be injected directly by inserting the micropipette (-40 ~μm) into a straight stretch of the tubule (A), into an efferent duct between the testis and the head of the epididymis (B), or into the rete testis (C).
Source: Ogawa *et al*. (1997) Int. J. Dev. Biol (41): 111-122.

A clinical trial was initiated at the Christie Hospital in Manchester (UK) in 1999 to evaluate germ cell transplantation in cancer patients. Testicular biopsies were obtained from adult males with solid tumors and cryopreserved as single-cell suspensions prior to cancer treatment. Five years after the initial report, seven out of twelve patients had undergone frozen-thawed germ cell transfer. As far as we know, no information is available on the fertility of these patients and follow-up is ongoing (Radford 2003). Drawing conclusions from this trial will nevertheless be problematic, as endogenous spermatogenesis and spermatogenesis issuing from transplanted cells will not be distinguishable.

Testicular tissue grafting

Testicular tissue grafting involves transplantation of SSCs with their intact niches and thus within their original microenvironment. Since testicular tissue grafting has not yet been reported in humans, available data will be reviewed on the basis of observations made in animals.

To date, haploid germ cells isolated from mouse testis homografts and rabbit testis xenografts have been used with ICSI to produce offspring (Shinohara *et al*., 2002; Schlatt *et al*., 2003; Ohta and Wakayama, 2005). Xenogeneic rhesus sperm generated in host mice have also been shown to be fertilization competent, allowing *in vitro* embryo development at a rate similar to that reported for *in situ* rhesus testicular sperm (Honaramooz *et al*., 2004). In view of these encouraging results in

animals, there is every hope that it will be possible, in the near future, to autograft cryopreserved testicular tissue of patients rendered sterile after fertility-threatening therapies and restore their fertility.

Lessons learned from transplantation of fresh testicular tissue in animals

Grafting of testicular tissue from several mammalian species into immunodeficient mouse hosts has resulted in varying degrees of donor-derived spermatogenesis. Complete spermatogenesis following testicular grafting has been reported in mice, rabbits, hamsters, pigs, goats, cats, bovines, horses and sheep (Shinohara *et al*., 2002; Schlatt *et al*., 2002b, 2003; Honaramooz *et al*.,2002b; Snedaker *et al*.,2004; Zeng *et al*.,2006; Schmidt *et al*., 2006a; Oatley *et al*., 2004, 2005; Ohta *et al*., 2005; Rathi *et al*., 2005, 2006; Arregui *et al*., 2008), as well as macaques (Honaramooz *et al*., 2004; Rathi *et al*., 2008). By contrast, germ cell differentiation blockage was observed in marmosets (Schlatt *et al*., 2002b; Wistuba *et al*., 2004, 2006; Jahnukainen *et al*., 2007).

The mechanisms underlying these species-specific discrepancies in spermatogenic differentiation remain unknown. Differences between host and donor gonadotropic hormones (Bousfield *et al*., 1996), leading to inefficient interaction between murine gonadotropins and grafted donor testicular tissue (Rathi *et al*., 2008) as well as species-specific structural variations in seminiferous tubule organization (Luetjens *et al*., 2005), resulting in modified paracrine interactions (Honaramooz *et al*., 2004; Wistuba *et al*., 2004), were suggested to be responsible for these differences. Moreover, the stage of germ cell development and intensity of spermatogenesis at the time of grafting also appear to be involved, since complete spermatogenesis was not reported in xenografted tissue when donor testicular tissue contained postmeiotic germ cells at the time of grafting in any species, including humans (Geens *et al*., 2006; Rathi *et al*., 2006; Schlatt *et al*., 2002b, 2006; C. Wyns, 2008, PhD thesis published by the Catholic University of Louvain, Belgium).

The reasons for the poor outcome of adult testicular tissue xenografting are so far unknown. However, studies in rodents have suggested that adult tissue could be more sensitive to ischemia than immature tissue, and that hypoxia related to the grafting procedure may be involved (Schlatt *et al*., 2002b). This hypothesis was supported by studies in bovines, showing higher expression of some angiogenic factors in grafts from younger donors (Schmidt *et al*., 2007). Furthermore, pretreatment of testicular tissue with vascular endothelial growth factor (VEGF), a potent angiogenic factor, was found to increase the number of tubules containing elongating spermatids (Schmidt *et al*., 2006b).

Variations in Sertoli cell maturation at the time of grafting, their developmental susceptibility to the detrimental influence of endocrine disruption due to the xenografting environment (Oatley *et al*., 2005; Rathi *et al*., 2008), or donor age-dependent differential gene and subsequent protein expression in donor tissue prior to grafting may also be implicated (Schmidt *et al*., 2007).

Besides causing spermatogenic differentiation impairment, xenografting has been shown to be inefficient in some species. Indeed, only 5-10% of seminiferous tubules in xenografts produced elongated or elongating spermatids in bulls (Oatley *et al*. 2004, 2005; Schmidt *et al*., 2006a), kittens (Snedaker *et al*., 2004) and horses (Rathi *et al*., 2006). Furthermore, in non-human primate testicular tissue grafts, only 2.8-4% of tubules contained mature sperm (Honaramooz *et al*., 2004; Rathi *et al*., 2008). The reasons for this low spermatogenic efficiency need to be understood in order to improve the success of this approach.

Initial germ cell loss, as reported in bovine and monkey xenografts (Rathi *et al*., 2005, 2008), could explain these poor results. Decreased expression of GDNF, involved in germ cell self-renewal, has been described in grafts (Schmidt *et al*., 2007), suggesting that the grafting procedure itself could negatively influence the number of germ cells. However, tissue culture performed prior to xenografting to increase the number of SSCs did not result in a higher percentage of seminiferous tubules with elongating spermatids at the time of graft removal (Schmidt *et al*., 2006b), indicating that other factors may be responsible for the low spermatogenic efficiency.

Lessons learned from transplantation of frozen testicular tissue in animals

An overview of studies on cryopreserved testicular tissue grafting in various animal models was recently reported by Geens *et al*. (2008). In rodents, cryopreservation of ITT led to the birth of healthy offspring (Shinohara *et al*., 2002). There is therefore every hope that this approach can be extended to humans.

A number of studies in animals designed to evaluate the effect of freezing on the functional capacity of germ cells have shown no impact on a qualitative basis (Shinohara *et al*., 2002; Honaramooz *et al*., 2002b; Schlatt *et al*., 2002b; Ohta and Wakayama, 2005; Jahnukainen *et al*., 2007; Goossens *et al*., 2008a; Van Saen *et al*., 2009). Loss of SSCs after cryopreservation was nevertheless suggested, since Ohta and Wakayama (2005)

reported lower colonization efficiency after grafting frozen-thawed testicular pieces.

Lessons learned from xenotransplantation of fresh human testicular tissue

Very few studies have been published on xenotransplantation of human testicular tissue (Geens *et al*., 2006; Schlatt *et al*., 2006; Yu *et al*., 2006). Adult testicular tissue grafting has yielded poor results, showing mainly sclerotic seminiferous tubules (and some isolated spermatogonia in 21.6-23.1% of grafts) (Geens *et al*., 2006; Schlatt *et al*., 2006).

Grafting of human ITT, either from fetuses (Yu *et al*., 2006) or prepubertal boys (Goossens *et al*., 2008b), did not result in complete spermatogenesis, although graft and germ cell survival were shown to be more favorable than in mature tissue grafts. Goossens *et al*. (2008b) observed mainly Sertoli cell-only tubules and just a few surviving spermatogonia 4 and 9 months after grafting, constituting considerable spermatogonial loss.

Lessons learned from xenotransplantation of frozen human testicular tissue

No studies have reported xenografting of cryopreserved adult testicular tissue and only two have been published on cryopreserved ITT xenotransplantation in humans (Wyns *et al*., 2007; 2008). Grafts were performed orthotopically in immunodeficient mice. After grafting frozen-thawed cryptorchid tissue for 3 weeks, we demonstrated survival of 14.5% of the initial spermatogonial population, with 32 % of these cells showing proliferative activity, not significantly different from the 17.8% in fresh tissue. The number of Sertoli cells was unchanged and 5.1% were proliferative compared to 0% in fresh tissue. Raised FSH levels in the castrated mice, the removal of some inhibitory mechanisms that normally operate in quiescent immature testes and/or other paracrine factors were suggested to play a role in the Sertoli cell multiplication. In order to study the capacity of frozen SSCs to self-renew and differentiate, long-term grafts of normal immature tissue were performed. We found 3.7% of the initial spermatogonial population remaining after freeze-thawing and 6 months' xenografting, with 21% of these cells showing proliferative activity.

Since considerable loss of spermatogonial cells occurred, it was essential to evaluate to what extent cryopreservation itself was implicated. Freezing did not appear to have a major impact on these cells. Indeed, no difference in spermatogonial cell numbers was observed between fresh and frozen-thawed testicular pieces (Wyns *et al*., 2007) and high survival rates (94 ± 1%) were obtained after freezing and culture (Keros *et al*., 2007). Regarding the effect of cryopreservation on the differentiation capacity of human SSCs, we found that the remaining spermatogonia retained the ability to reinitiate spermatogenesis, but normal differentiation beyond the prophase of the first meiosis could not be proved with appropriate germ cell markers (Wyns *et al*., 2008). We observed spermatid-like structures on hematoxylin-eosin-stained histological sections (Fig. 4), albeit slightly smaller than control spermatids ($p = 0.045$), but these structures did not show characteristic markers of postmeiotic cells or acrosome development by immunohistochemistry (IHC).

Preservation of the steroidogenic capacity of Leydig cells was evidenced by both IHC and transmission electron microscopy (Wyns *et al*., 2008).

IVM of germ cells

IVM of germ stem cells, leading to *in vitro*-derived male haploid gametes available for ICSI, circumvents the risk of reintroducing malignant cells, making this procedure potentially highly beneficial in cancer patients.

Efforts have focused on establishing optimal *in vitro* culture systems to allow male germ cells to complete meiosis and spermatid elongation in experimental conditions. So far, it has not been possible to develop a culture system that supports complete *in vitro* spermatogenesis from spermatogonia, despite several promising studies in animals (Lee *et al*., 2001; Feng *et al*., 2002; Izadyar *et al*., 2003b). A number of studies have investigated culture systems suitable for *in vitro* spermatogenesis in humans (Cremades *et al*., 2001; Sousa *et al*., 2002; Tanaka *et al*., 2003; Lee *et al*., 2007). Most studies describe culture systems using Vero cells or Vero cell-conditioned media (Cremades *et al*., 2001; Sousa *et al*., 2002; Tanaka *et al*., 2003). Normally differentiated elongated spermatids and even mature spermatozoa able to fertilize human oocytes and achieve normal embryonic development have been generated from human round spermatids (Cremades *et al*., 2001). The addition of Vero cell-conditioned medium to a mixture of different types of spermatogonial cells co-cultured with Sertoli cells, supplemented with FSH and testosterone, induced differentiation of human primary spermatocytes from non-obstructive azoospermic men into round spermatids at a rate of 3-7%, and from round spermatids into normal late spermatids at a rate of 5-32% (Sousa *et al*., 2002). Co-culture of isolated primary spermatocytes with Vero cells generated chromosomally normal round spermatids (Tanaka *et al*., 2003).

Xenogeneic Sertoli cells were also used for IVM of human male germ cells in co-culture, leading to

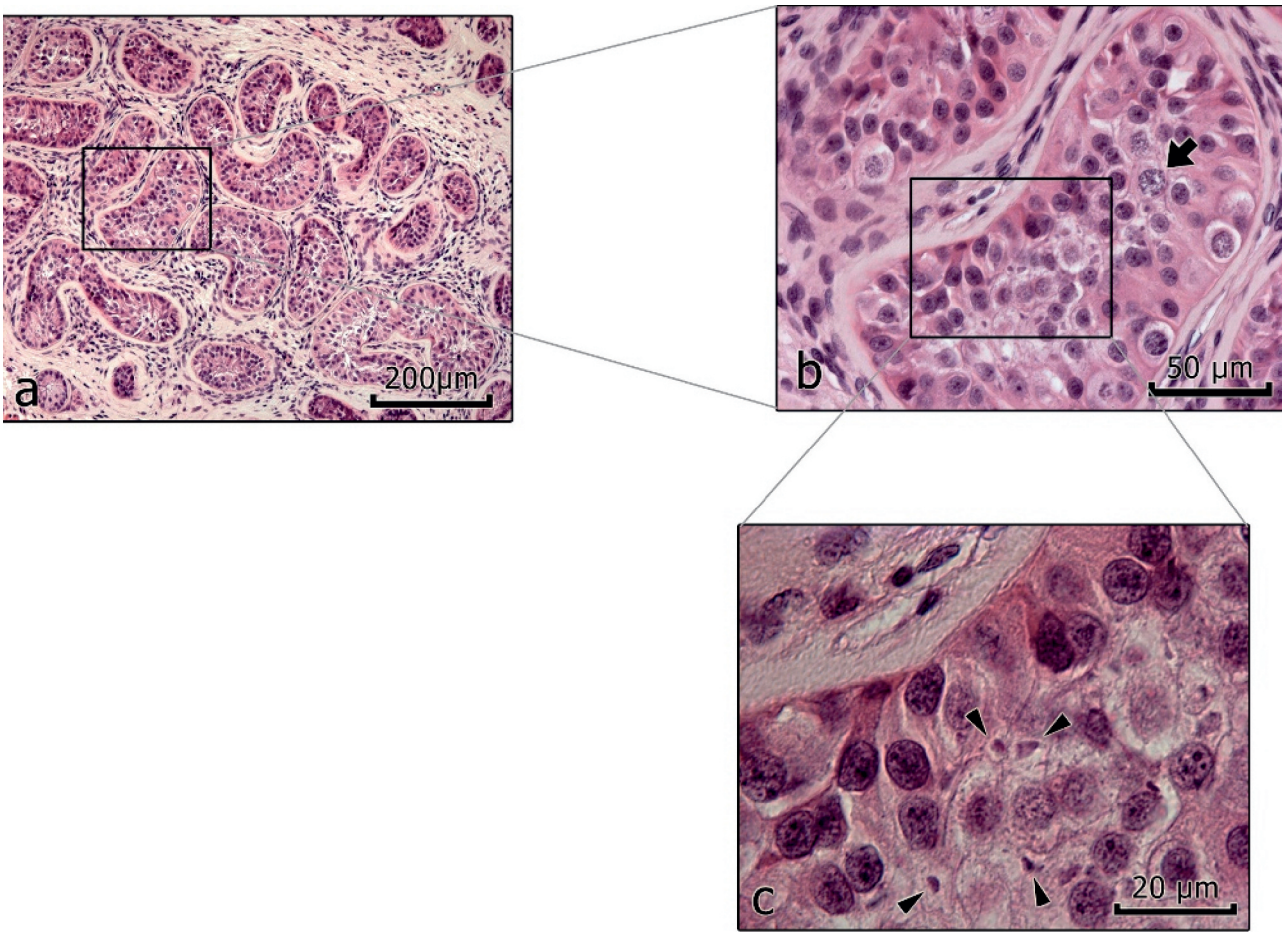

Fig. 4. — Histological appearance (hematoxylin/eosin sections) of donor testicular tissue from a 12-year-old boy after 6 months' orthotopic xenografting at ×200 magnification (a), showing pachytene spermatocytes (arrow) and spermatid-like cells (inset) at ×400 magnification (b) and spermatid-like cells at ×1000 magnification (c).

the development of human round spermatids, but not later stages of germ cell maturation (Kawamura *et al*., 2003).

Encapsulation of testicular cells dissociated from seminiferous tubules in calcium alginate, to promote and sustain interactions between germ and Sertoli cells without limiting permeability to media components, was applied with limited success to human testicular tissue from azoospermic males with maturation arrest (Lee *et al*., 2006). Although this method failed to induce spermiogenesis and did not result in pregnancy, the differentiated germ cells displayed a normal chromosomal status and were able to activate human oocytes after injection into the cytoplasm.

In vitro culture of whole human testicular tissue, allowing conservation of cellular interactions within and between seminiferous tubules and the interstitial compartment, was shown to elicit differentiation of elongated spermatids from primary spermatocytes when supplemented with rFSH and testosterone (Tesarik *et al*., 1998), but gradual apoptotic loss of meiotic and postmeiotic germ cells independent of the presence of gonadotropins was reported (Roulet *et al*., 2006).

To promote cell-to-cell communication, 3D cell culture was developed, allowing re-establishment of Sertoli and germ cell contacts within a collagen gel matrix. The system led to differentiation of spermatocytes from patients with maturational arrest into presumptive spermatids (Lee *et al*., 2007).

Induction of human meiosis and spermiogenesis in an *in vitro* culture system represents an attractive strategy for fertility restoration, which has yielded a number of healthy live births (Tesarik *et al*., 1999), but these were the result of maturation of the later stages of spermatogenesis rather than the stem cells.

Since neither the biomolecular factors nor specific microenvironment necessary for the development of each stage of spermatogenesis have yet been completely elucidated, it is unlikely that IVM of diploid stem cells into haploid spermatozoa will be technically feasible in the near future (Lee *et al*., 2006). However, as germ cell survival and differentiation appear to require co-culture with somatic cells, cryopreservation of tissue containing Sertoli cells

could be particularly useful with a view to potential fertility restoration through IVM.

Safety issues

Cancer cell contamination

The most important, life-threatening concern of this approach is the risk of reintroducing malignant cells after transplantation. Indeed, the majority of pediatric malignancies metastasize through the blood, thus carrying a high risk of malignant contamination of the testes. The risk is greater with hematological cancers, as the testes can act as sanctuary sites for leukemic cells. Indeed, it has already been shown that as few as 20 leukemic cells injected into a testis can induce a relapse of the disease (Jahnukainen *et al*., 2001). Since reintroduction of malignant cells into a patient previously cured of disease must be absolutely excluded, germ cell isolation and cell sorting methods allowing complete purification of SSCs need to be validated before safe transplantation can be contemplated. Cell sorting methods have shown promising results in animal studies, as sorting of murine germ cells allowed transplantation without reinducing leukemia (Fujita *et al*., 2005). The technique was subsequently applied to human testicular cell suspensions, but does not appear to have been entirely successful (Fujita *et al*., 2006; Geens *et al*., 2007). Table 2 summarizes existing studies on the elimination of cancer cells from testicular cell suspensions

Since no marker has yet been identified that is exclusively expressed on SSCs, allowing positive selection of these cells through cell sorting techniques, further research into surface markers in animal germ cells and their equivalents in human germ cells is needed to ensure complete elimination of cancer cells from testicular cell suspensions.

Cancer cell contamination is also a major concern in tissue autografting, since it has been reported that leukemic cells can survive cryopreservation/xenotransplantation (Hou *et al*., 2007). Therefore, testicular tissue autografting after cure can only be considered for patients in whom there is no risk of testicular metastases or who have undergone gonadotoxic therapies for non-malignant disease.

Infectious transmission

Due to the risk of infectious transmission from animals to humans (Patience *et al*., 1998), testicular xenografting should not be considered for reproductive purposes at present. This approach is nevertheless useful for the evaluation of the functional capacity of germ cells and should therefore form part of the assessment of germ cell cryopreservation protocols (Frederickx *et al*., 2004), for the understanding of testicular physiology and pathophysiology (Jahnukainen, 2006b) and for testing malignant contamination of tissue before autografting (Hou *et al*., 2007).

The risk of animal viral transmission or contamination with animal antigens or cellular membrane-binding molecules (Patience *et al*., 1998) is also present in IVM with co-culture systems using Vero cells or xenogeneic Sertoli cells, so these systems should not be used for clinical purposes.

Birth defect risks

Goossens *et al*. recently reported smaller litter size, significantly lower fetal weight and reduced length in first generation offspring after germ cell transplantation, suggesting imprinting disorders (Goossens *et al*., 2006). Further investigation is required to elucidate the underlying reasons before autotransplantation can be safely introduced into clinical practice. Apart from this study, very little information is available on potential birth defect risks after fertility restoration techniques, and observations mainly focus on IVM of diploid gametes.

Chromosomal abnormalities were found in embryos obtained after ooplasmic injection of *in vitro*-derived haploid germ cells issuing from diploid germ cells in one study. These abnormalities could be attributable to the completion of meiosis or part of the spermiogenic process under *in vitro* conditions, although the source of the immature tissue used (men with non-obstructive azoospermia) may also have played a role (Sousa *et al*., 2002).

Special attention should also be paid to the genetic and epigenetic status of *in vitro*-matured cells (Bahadur *et al*., 2000; Bahadur, 2004). Indeed, acceleration of the cytoplasmic and nuclear maturation events that occur *in vitro* in cultured male germ cells may override natural endogenous control mechanisms involved in DNA condensation and cause a disturbance in epigenetic reprogramming, resulting in aberrant gene expression, abnormal phenotypic characteristics and defects in the male gamete's capacity to fertilize the oocyte and induce normal embryonic development.

In addition, abnormalities in the expression of oocyte-activating factor or deficiencies in the functioning of the reproducing element of the centrosome of *in vitro*-derived haploid male gametes may cause fertilization failure or aberrant embryonic development after oocytoplasmic injection (for review, see Georgiou *et al*., 2007).

Although the birth of healthy offspring has been reported after IVM of immature germ cells like primary spermatocytes (Tesarik *et al*., 1999), insufficient data are currently available to allow safe clinical application.

Table 2. — Studies on isolation of germ cells with detection of cancer cell contamination.

Reference	Species	Cell sorting technique	Markers	Evaluation after cell sorting	Outcome (% of residual contamination/number of contaminated samples or mice)
Fujita *et al*., 2005	Mouse	FACS	H-2Kb/H2Db$^-$ (MCH cl I) CD45$^-$	Cell transplantation Histology: testis, bone marrow, peritoneal exudate of recipient mice	No contamination of recipient mice
Fujita *et al*., 2006	Human	FACS	MCH cl I$^-$ CD45$^-$	RT-PCR for germ cell markers (DAZL, HIWI, VASA, NANOG, STELLAR, OCT4)	1.45% K562 cells (CML) 0% K562 cells after IFγ (for induction of MCH cl I)
Geens *et al*., 2007	Mouse	MACS + FACS	H2Kb$^-$ (MCH cl I) CD49f$^+$ (α6 integrin)	FACS	0.39% H2Kb$^+$ cells
				In vitro culture	3.1% (1/32) contaminated cultures
				Cell transplantation	1/20 contaminated mice
	Human	FACS	H2Kb$^-$ (MCH cl I)	FACS *In vitro* culture PCR for B cell receptor	0.58% SB$^+$ cells 1/11 contaminated samples

MCH cl I: major histocompatibility complex class I (marker of somatic cells); α6 integrin: marker of SSCs; CD45: surface marker of leukemic cells; IFγ: interferonγ; CML: chronic myelogenous leukemia.

Ethical concerns

Learning that a child has cancer is devastating for all concerned, and treatment needs to begin quickly, leaving very little time for the impact of possible future sterility to sink in. However, the inability to father one's own children might have a huge impact on the psychological well-being of patients in adulthood (Schover, 2005; van den Berg *et al*., 2007), so it is crucial to inform them of the potential consequences of their therapy on future fertility. Ethical concerns have been expressed about ITT cryopreservation, highlighting the importance of the risk/benefit balance (Bahadur and Ralph, 1999). Because of the small size of testes from prepubertal children, immature gonadal tissue sampling may be considered too invasive a procedure. However, in the two existing studies on testicular tissue harvesting in young cancer patients (Keros *et al*., 2007; Wyns *et al*., 2007), no major surgical complications occurred during testicular biopsy. Mean biopsy volume was about 5% of testicular volume which, according to morphological studies (Muller and Skakkebaeck, 1983), should provide enough germ cells for fertility preservation. Regarding general anesthesia, since this biopsy is generally performed under the same anesthesia as that used for placement of the central line for chemotherapy, there is no additional risk involved.

When considering the benefits of tissue harvesting, the safety and effectiveness of fertility preservation and restoration procedures are essential issues. Children and their parents should be informed of the experimental nature of this approach and the fact that there is no guarantee of success (Tournaye *et al*., 2004; Bahadur, 2004; Jahnukainen *et al*., 2006a). Parental consent and the child's ascent, meaning he was given the opportunity to discuss the procedure, should be sought. As obtaining fully informed consent from children is difficult, substituted consent from parents should for now be limited to the safekeeping of tissue (Bahadur and Ralph, 1999; Bahadur *et al*., 2000).

With continued advances in potential fertility restoration strategies, ethical guidelines will need to be established with respect to harvesting, preservation and use of prepubertal testicular tissue.

Conclusion

Since post-therapy recovery of spermatogenesis remains unpredictable, it is important to inform patients facing infertility as a side effect of their treatment of all the options available to preserve their fertility (Wallace *et al*., 2005). Gamete banking should be offered to all patients of reproductive age, given the already well established and highly effective use of cryopreserved sperm and rapidly advancing experimental techniques allowing fertility restoration after immature tissue banking in animals, as well as the successes reported after ovarian tissue cryopreservation and transplantation after recovery from cancer.

Hormonal or cytoprotective drug manipulation aimed at enhancing spontaneous recovery of

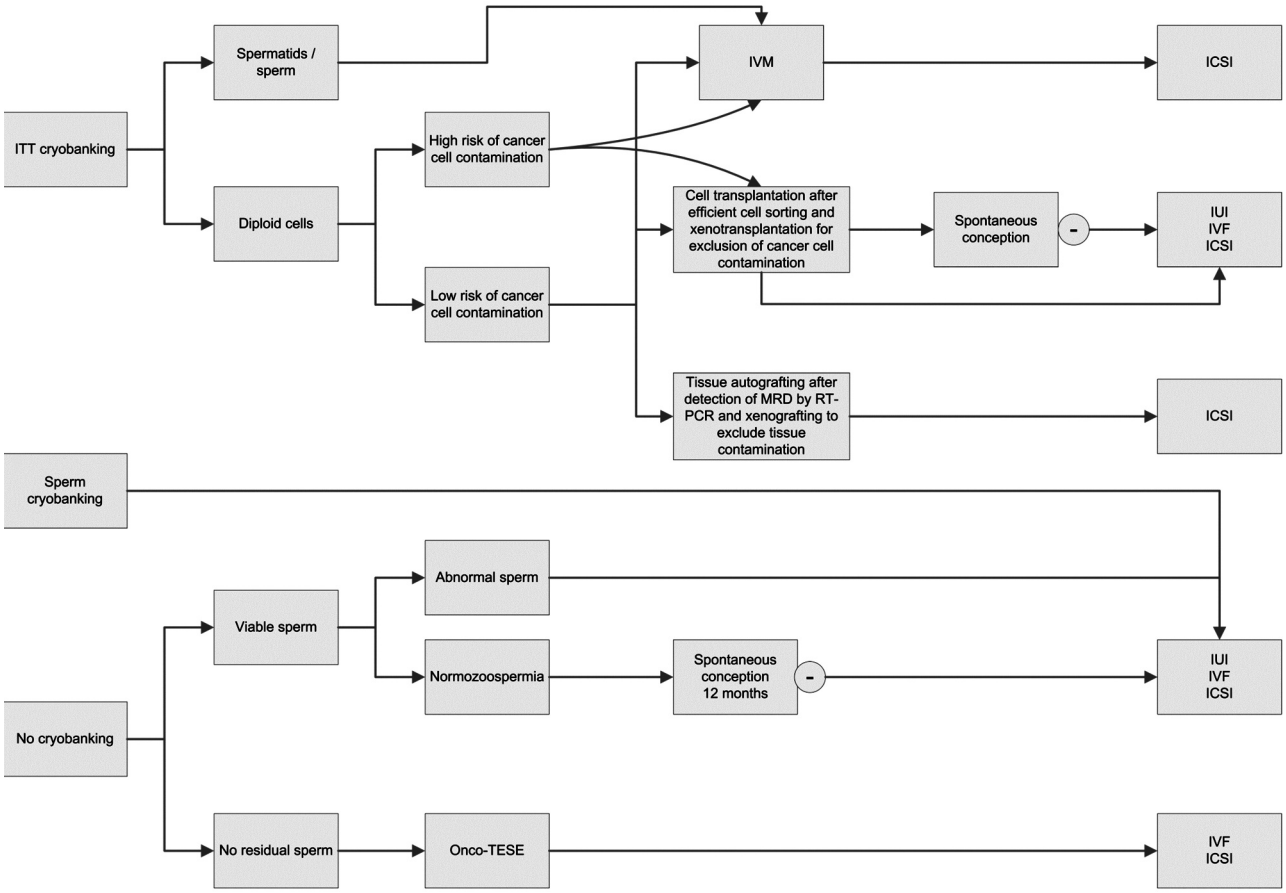

Fig. 5. — Fertility restoration strategy after gonadotoxic therapies
ITT = immature testicular tissue.

spermatogenesis remains a possibility for the future. SSC preservation offers the prospect of several realistic applications, although none is feasible in humans at this point in time. Future advances in fertility preservation technology rely on improved understanding of the cryobiology of gonadal tissue and cells.

Before considering fertility restoration options, patient selection is essential, since risks vary according to disease. No single (or simple) algorithm can so far summarize all the possible strategies for fertility preservation and restoration in case of gonadotoxic therapy in male patients, but the most appropriate course of action may be selected according to the scheme shown in Figure 5. Over the next few years, research should focus on how to extend successful experiments in animals to young boys and on the identification of the ideal microenvironment for SSC development. As germ cell survival and differentiation appear to require co-culture with somatic cells, cryopreservation of tissue containing Sertoli cells could be particularly useful with a view to potential fertility restoration through IVM.

Resolving numerous important technical issues discussed in this review should lead to safe and efficient methodologies for fertility restoration after storage of ITT, and the development of ethically accepted pilot protocols, which will then need to be submitted for further ethical approval before definitive and universal clinical implementation. Until then, samples should at least be banked after providing careful counseling and obtaining informed consent, making sure the patient understands there is no guarantee of success (Hovatta, 2003). Preservation of testicular tissue from today's prepubertal patients will allow them to consider various fertility restoration options that will emerge in the next 20-30 years, giving them hope of fathering children with their own genetic heritage.

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