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Options for fertility preservation in prepubertal boys

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BACKGROUND: Fertility in adult life may be severely impaired by gonadotoxic therapies. For young boys who do not yet produce spermatozoa, cryopreservation of immature testicular tissue (ITT) is an option to preserve their fertility, albeit still experimental. This paper covers current options for ITT cryopreservation and fertility restoration.

METHODS: Relevant studies were identified by an extensive Medline search of English and French language articles. Search terms were: gonadotoxicity, cytoprotection, cryopreservation, ITT, spermatogonia, testicular transplantation, testicular grafting and *in vitro* maturation (IVM).

RESULTS: Although no effective gonadoprotective drug is yet available for *in vivo* spermatogonial stem cell protection in humans, current evidence supports the feasibility of ITT cryopreservation before gonadotoxic treatment with a view to fertility preservation. Controlled slow freezing with dimethyl sulfoxide allows survival and proliferation of human spermatogonia after xenotransplantation, but only partial differentiation. Animal data look promising, since healthy offsprings have been obtained after transplantation of frozen testicular cell suspensions or tissue pieces. However, none of the fertility restoration options from frozen tissue, i.e. cell suspension transplantation, tissue grafting and IVM have proved efficient and safe in humans as yet.

CONCLUSION: While additional evidence is required to define optimal conditions for ITT cryopreservation with a view to transplantation or IVM, 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 childhood cancer, we have seen great improvements in life expectancy, with up to 80% of children surviving their disease, resulting in a growing population of adult long-term survivors of childhood malignancies (Ries *et al.*, 2004; Steliavora-Foucher *et al.*, 2004; Magnani *et al.*, 2006; Stiller *et al.*, 2006; Brenner *et al.*, 2007).

It is therefore estimated that, by the year 2010, 1 in 250 young adults aged 20–29 years will be a cancer survivor (Bleyer, 1990; Blatt, 1999).

Although oncological treatments are highly effective, a major concern is their adverse impact on fertility (Howell and Shalet, 1998; Brougham *et al.*, 2003; Wallace *et al.*, 2005). 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 (Viviani *et al*., 1985; Radford *et al*., 1994; Kulkarni *et al*., 1997; Tal *et al*., 2000; Van Beek *et al*., 2007) could enable spontaneous recovery of spermatogenesis, but their use is not always possible without compromising patient survival.

Since rapidly dividing cells are the target of chemo- and radiotherapy, these treatments act not only on cancer cells, but also on germ cells. Differentiating spermatogonia proliferate rapidly and are thus extremely susceptible to cytotoxic agents, although the less active stem cell pool may also be depleted (Bucci and Meistrich, 1987).

Consequently, although the prepubertal testis does not complete spermatogenesis, there is evidence that cytotoxic treatment given to prepubertal boys affects fertility (Rivkees and Crawford, 1988; Mackie *et al*., 1996; Kenney *et al*., 2001). In addition, 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 possibly explain why the prepubertal state does not offer any protection against gonadotoxic treatments.

Recovery of sperm production after a cytotoxic insult 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).

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 levels (Howell *et al*., 1999), both signs of Leydig cell impairment, have been described. Evidence of Sertoli cell functional impairment following chemotherapy, responsible for germ cell differentiation inhibition where germ cells have survived, has also been reported (Bar-Shira Maymom *et al*., 2004).

Gonadotoxicity after chemo- and/or radiotherapy

Although little is known about the effects of gonadotoxic treatments on the immature testis, as fertility cannot be assessed before puberty, cytotoxic damage has been extensively studied after puberty.

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 the identification of the specific toxicity of each individual agent. Literature on semen quality in adult cancer patients before and after therapy was recently reviewed (Trottmann *et al*., 2007).

Since this review focuses on fertility in prepubertal cancer patients, special attention was paid to studies investigating the effect of cancer therapies in children.

Table I shows long-term fertility prognoses following treatment with different chemotherapic agents used in childhood. Supplementary Table SI (see online) provides the best estimate of fertility prognosis after chemotherapy for common childhood cancers.

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 >4 Gy may result in complete sterility (for review, see Howell and Shalet, 2005). Fractionated radiotherapy increases seminiferous tubule damage, with doses >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 >20 Gy is associated with Leydig cell dysfunction in prepubertal boys (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.

Boys with acute leukemia requiring marrow-ablative chemoradiotherapy and hematopoietic stem cell transplantation (HSCT) are at extremely high risk, with 85% of adult patients found to be azoospermic after total body irradiation and cyclophosphamide administration (Anserini *et al*., 2002).

Loss of fertility: who can benefit from fertility preservation?

Loss of fertility in adult life is a major psychologically traumatic consequence. Indeed, in a quality-of-life analysis of former oncological patients, about 80% viewed themselves as potential parents, and the vast majority of younger cancer survivors saw their cancer experience as pivotal in preparing them to be better parents (Schover *et al*., 1999). Therefore, 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).

There is also considerable evidence that gonadotoxic treatments, such as HSCT, can cure a variety of non-malignant disorders in children, so fertility preservation should not be reserved solely for boys with cancer (Slavin *et al*., 2001; Vassiliou *et al*., 2001; Jacobson *et al*., 2004; Passweg and Rabusin, 2008; Rabusin *et al*., 2008; Kazadi *et al*., 2009). It should also be considered for benign conditions where seminiferous tubule degeneration is expected over time, such as Klinefelter syndrome (Arce and Padrón, 1980; Aksglaede *et al*., 2006). The indications for immature testicular cryopreservation in case of malignant and non-malignant disease are summarized in Table II.

Methods

We conducted an extensive Medline search. Search terms were: gonadotoxicity, cytoprotection, cryopreservation, immature testicular tissue (ITT), spermatogonia, testicular transplantation, testicular grafting and IVM.

A total of 471 articles from 1973 to 2009 were initially selected from 4045 papers. Figure 1 shows a flow diagram detailing the results of the literature search and selection of studies. Since the topic is innovative, original articles of any design and review articles published in English and French were suitable for inclusion. Selection criteria were based on the main outcome of interest, i.e. fertility restoration options from ITT, and aimed to synthesize the state of current knowledge for potential clinical applicability in humans. For concerns connected to the main subject, e.g. *in vivo* spermatogonial stem cell (SSC) protection, the goal was to introduce the reader to the literature, rather than provide an exhaustive review. The final number of studies referenced in this review was 193.

Table I Long-term fertility prognosis following treatment with different agents

Good	Moderate	Poor
Azathioprine	Thiotepa	Cyclophosphamide (>7.5 g/m^2) (Meistrich *et al.*, 1992)
Fludarabine	Gemcitabine	Ifosfamide (>60 g/m^2) (Williams *et al.*, 2008)
Methotrexate	Cisplatin	Mustine, carmustine
6-mercaptopurine	Oxaliplatin	Busulfan
	Carboplatin	Chlorambucil (>1.4 g/m^2)
Vincristine	Doxorubicin	Melphalan (140 mg/m^2)
Vinblastine	Dacarbazine	Chlormethine
Bleomycin	Cytosine-arabinoside (cytarabine)	Procarbazine (>4 g/m^2) (Bokemeyer *et al.*, 1994)
Actinomycin-D	Daunorubicin	Cisplatin (>600 mg/m^2) (Petersen *et al.*, 1994; Pont and Albrecht, 1997)
Etoposide	Mitoxantrone	Mechlorethamine

Adapted from Meirow and Schenker, 1995; Howell and Shalet, 2001.

Table II Indications for immature testicular cryopreservation in case of malignant and non-malignant disease

Malignant	Non-Malignant
• Leukemia • Hodgkin's disease • Non-Hodgkin's lymphoma • Myelodysplastic syndromes • Solid tumors • Soft tissue sarcoma	(1) HSCT in case of: • hematological disorders: thalassemia major, sickle cell disease, aplastic anemia, Fanconi anemia • primary immunodeficiencies • severe autoimmune diseases unresponsive to immunosuppressive therapy: juvenile idiopathic arthritis, juvenile systemic lupus erythematosus, systemic sclerosis, immune cytopenias • osteopetrosis • enzyme deficiency disease: Hurler's syndrome (2) Risk of testicular degeneration • Klinefelter syndrome

HSCT, hematopoietic stem cell transplantation.

Results

Fertility preservation options before gonadotoxic therapies

Two different approaches may be considered: (i) minimizing the testicular damage from cancer treatment or protecting SSCs *in vivo*; (ii) cryopreserving testicular tissue prior to gonadotoxic treatment in the form of either cell suspension, tissue fragments or whole organ.

In vivo SSC protection

In order to reduce the deleterious effects of gonadotoxic therapies, different strategies have been tested, such as testicular shielding and the 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; Redman and Bajorunas, 1987; Waxman *et al.*, 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 follicle-stimulating hormone (FSH) might be an option as shown in monkeys by Van Alphen *et al.* (1989) and to a lesser extent, in a randomized trial comparing GnRH antagonists and recombinant human FSH (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) and various other cytoprotective substances (Lirdi *et al.*, 2008; Okada *et al.*, 2009) have also been used with partial success in rodents.

In conclusion, 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.

Immature gamete cryopreservation

Since prepubertal boys cannot benefit from sperm banking, a potential alternative strategy for preserving their fertility involves storage of

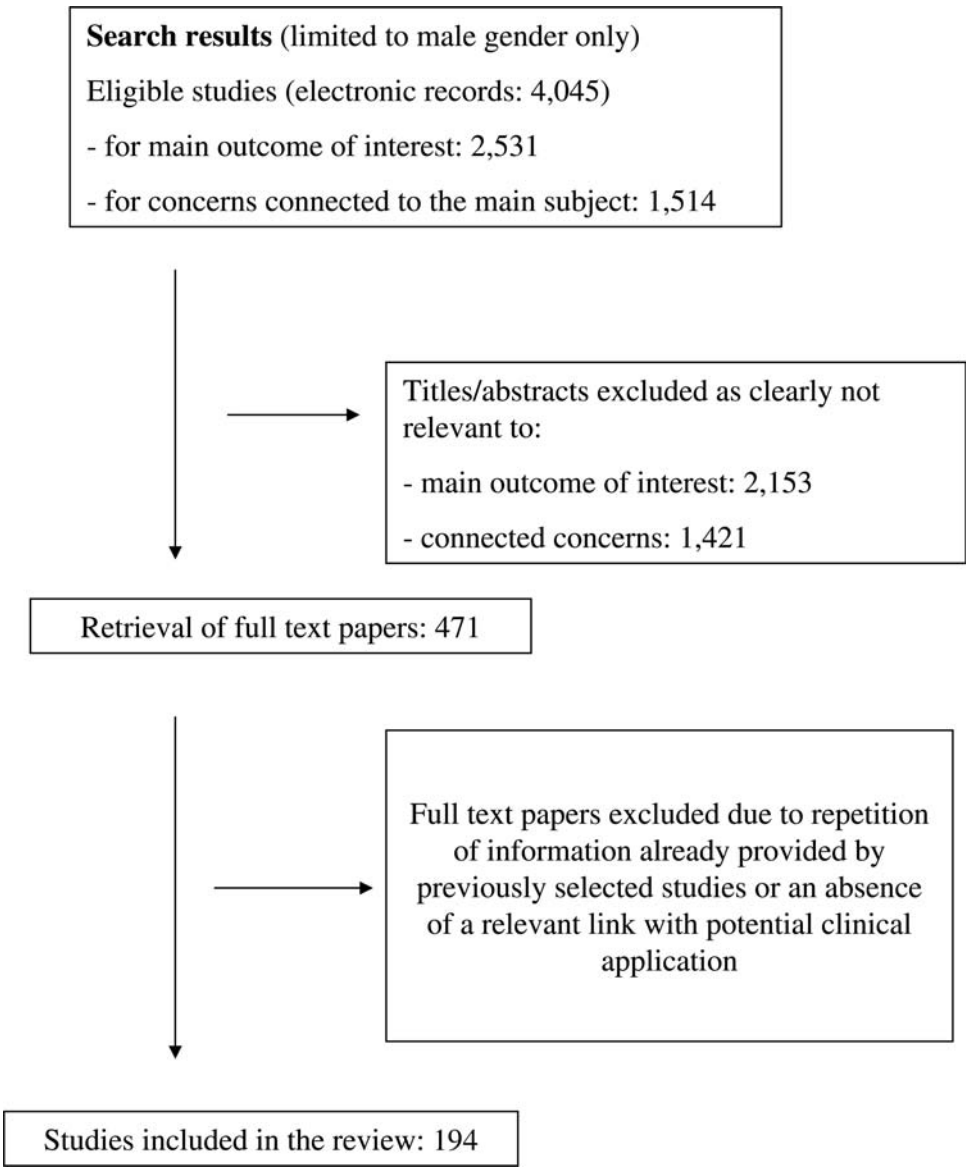

Figure 1 Flow diagram of search and inclusion criteria for studies in the review.

testicular tissue, in the hope that future technologies will allow its safe utilization (for review, see Tournaye *et al*., 2004). 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). It is also noteworthy that in 20% of Tanner stage II boys, spermiation has already started (Schaefer *et al*., 1990), allowing cryopreservation of haploid gametes.

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, compromising cell survival (Brook *et al*., 2001) and cell-to-cell interactions necessary for cell proliferation and differentiation (Griswold, 1998).

In various animal models, post-thaw viability of 29–82% was reported (Geens *et al*., 2008). For human testicular cell suspensions, post-thaw viability of up to 60% was achieved, regardless of cryoprotective agent (Brook *et al*., 2001; Hovatta *et al*., 2001). Whether it is better to produce cell suspensions before or after cryopreservation is still a matter of debate, however.

Tissue pieces. Cryopreservation of testicular tissue pieces may be considered as an alternative method capable of 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). Other advantages 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), and Leydig cells, whose preservation may be useful to alleviate the hormonal imbalance caused by cytotoxic therapy (Howell and Shalet, 2001).

Better survival rates of Leydig cells were obtained when dimethyl sulfoxide (DMSO) was used (80% compared with 50% with propanediol (PROH)) (Keros *et al*., 2005). Structural integrity and functional capacity were demonstrated after cryopreservation and culture of fetal and prepubertal testicular tissue (Keros, 1999, 2007; Kvist *et al*., 2006), as well as after transplantation of frozen-thawed fetal testicular tissue (Grischenko *et al*., 1999).

Because of the complexity of the tissue architecture, cryopreservation protocols must strike a balance between optimal conditions for each cellular type, depending on the 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 that minimize cytotoxicity. Post-thaw survival and seminiferous tubule structure are profoundly affected by both the type of cryoprotectant and freezing rates (Milazzo *et al*., 2008), so optimization of freeze-thawing protocols is mandatory. DMSO, rather than ethylene glycol (EG), PROH or glycerol, was shown to better preserve structures within tissue (Keros *et al*., 2005; Goossens *et al*., 2008a) and best maintain tissue capacity to initiate spermatogenesis (Jahnukainen *et al*. 2007). Furthermore, slow-programmed freezing better protects spermatogonial morphology (Keros *et al*., 2007).

Two teams have reported freezing protocols for prepubertal human testicular tissue, both yielding good structural integrity (Kvist *et al*., 2006; Keros *et al*., 2007). Using different cooling and freezing rates, Keros *et al*. (2007) observed a difference mainly in terms of survival of spermatogonia, with 94% of intact spermatogonia found after freeze-thawing and culture with their best protocol. We therefore applied this protocol, albeit slightly modified by the addition of sucrose, for further evaluation of the functional capacity of cryopreserved human ITT after xenografting. Quantitative determination of the number of spermatogonia and Sertoli cells after freeze-thawing showed an absence of cell loss. After orthotopic xenografting of cryopreserved human ITT, preservation of spermatogonia able to proliferate was demonstrated (Wyns *et al*., 2007; 2008). An overview of all studies on cryopreservation of ITT is presented in Supplementary Table SII (see online).

Whole testis. Due to the small number of SSCs contained in a testicular biopsy and the small size of a child's testis, it is possible that cryopreservation of a whole testis may be more appropriate, with a view to later organ autografting. Cryopreservation methods for whole testes need to be developed, however, as has been done for whole ovaries (Courbière *et al*., 2006; Jadoul *et al*., 2007; Martinez-Madrid *et al*., 2007).

Fertility restoration after immature tissue cryopreservation

In the light of results obtained from animal studies, frozen diploid precursor cells may provide some hope of fertility restoration in prepubertal boys in the absence of haploid gametes. Three approaches may be considered: transplantation of purified cell suspensions back to their own testes, autografting of testicular pieces or whole testes, or IVM up to a stage at which they are competent for normal fertilization through intracytoplasmic sperm injection (ICSI).

None of these approaches have proved 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 to germ cell-depleted testes. SSCs are recognized by Sertoli cells and relocate 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.

The technique was first described in 1994 by Brinster and Zimmermann, who developed an SSC assay in mice that identified SSCs by their ability to generate a spermatogenic colony 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 lacZ gene encoding β-galactosidase, was initiated and sustained.

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*., 1999a), 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. Rat gonocytes produced mature spermatozoa after xenogeneic transplantation to the testes of mice (Clouthier *et al*., 1996), but qualitative and quantitative abnormalities of sperm were observed (Russell and Brinster, 1996). Abnormal spermatozoa were also found when hamster germ cells were transplanted to mice, probably reflecting the limited ability of mouse Sertoli cells to fully support hamster germ cells (Ogawa *et al*., 1999b).

SSCs from all other mammalian species examined (i.e. 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 what looked like early differentiating daughter spermatogonia, but could not differentiate beyond the stage of spermatogonial expansion (Dobrinski *et al*., 1999a; Dobrinski *et al*., 2000; Nagano *et al*., 2001b; Nagano *et al*., 2002; Oatley *et al*., 2002; Hermann *et al*.,

2007). One study nevertheless demonstrated some early meiotic spermatocytes after transplantation of porcine male germ cells to mice (Choi *et al*., 2007). This suggests that the initial steps of germ cell recognition by Sertoli cells, migration to the basement membrane, initiation of cell proliferation and possibly some early steps of differentiation are conserved among evolutionarily divergent species.

Efficiency of the technique. The extent of spermatogenesis has been shown to depend on the number of transplanted stem cells, with an almost linear correlation (Dobrinski *et al*., 1999b), and on the quantity and quality of stem cell niches in the recipient testis (Ogawa *et al*., 2000; Ohta *et al*., 2000).

In rodents, the observed colonization rate was no higher than 1 of 20 SSCs (Dobrinski *et al*., 1999b), thus showing low colonization efficiency. The colonization rate of slowly cycling type A dark spermatogonia in primates was expected to be much lower (Jahnukainen *et al*., 2006a), estimated to be as low as 0.0015–0.003% in rhesus monkeys (Hermann *et al*., 2007). Recipient age appears to have an impact on colonization efficiency, since more and larger spermatogenic colonies were generated in preadolescent recipient mouse testes than in adult testes (Shinohara *et al*., 2001). Better niche accessibility and niche proliferation due to Sertoli cell multiplication, elements facilitating colony formation and an increase in seminiferous length during testicular enlargement may be involved. This should be taken into account to ensure optimal transplantation time in clinical practice.

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, increasing the number of SSCs prior to transplantation is essential. Ideally, isolation of pure stem cells would be the most effective method to increase the number of SSCs in a suspension and therefore transplantation efficiency. Indeed, Shinohara *et al*., 1999 observed that a 10-fold enrichment of SSCs produced up to a 10-fold increase in the number of colonies formed in recipients.

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. These techniques have already been shown to improve the transplantation efficiency in mice (Shinohara *et al*., 1999, 2000; Hofmann *et al*., 2005). So far, the highest level of SSC enrichment has been achieved based on Thy1 expression (Kubota *et al*., 2003, 2004a). As conserved expression of some markers of undifferentiated spermatogonia (PLZF, GFR-α1 and Thy-1) 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 has been found to be limited (Hasthorpe, 2003). Better results were achieved using culture on feeder layers with a combination of growth factors, or applying serial transplantation procedures (Kanatsu-Shinohara *et al*., 2003a; Ogawa *et al*., 2003).

So far, strategies for *in vitro* expansion of SSCs have only proved successful in rodents (Kanatsu-Shinohara *et al*., 2003a; Kubota *et al*., 2004b; Ryu *et al*., 2005). Using various growth factors and hormones, such as β-estradiol, progesterone, epidermal growth factor, fibroblast growth factor (bFGF), leukemia inhibitory factor (LIF) and glial-cell derived neurotrophic factor (GDNF), a 2×10^{14}-fold expansion in total neonatal mouse testicular cell number was achieved over ~160 days (Kanatsu-Shinohara *et al*., 2003a). After 2 years, the cultured cells showed 10^{85}-fold logarithmic proliferation, retaining the characteristic morphology and yielding fertile offspring after stem cell transplantation (Kanatsu-Shinohara *et al*., 2005).

A number of studies have shown that SSC self-renewal is critically dependent on GDNF (Kubota *et al*., 2004b, Kanatsu-Shinohara *et al*., 2008) and that bFGF co-operates with GDNF for rodent SSC growth (Hofmann *et al*., 2005; Kanatsu-Shinohara *et al*., 2008; Wu *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 for clinical application.

The potential of frozen murine testicular cells to resume spermatogenesis after transplantation was demonstrated for the first time by Avarbock *et al*., 1996. Live birth of offspring achieved after transplantation of frozen testicular cell suspensions provided final proof of successful cryopreservation (Kanatsu-Shinohara *et al*., 2003b). While it appears that the functional capacity of mouse SSCs may be compromised by cryopreservation (Frederickx *et al*., 2004), this was not observed by Kanatsu-Shinohara *et al*. (2003b). Moreover, rhesus SSCs retained normal colonization capacity after freezing and transplantation in mice (8.42 colonies per 10^6 frozen-thawed viable cells, not significantly different from 4.64 colonies per 10^6 fresh viable cells) (Hermann *et al*., 2007), suggesting that possible functional impairment due to cryopreservation 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).

A clinical trial was initiated in Manchester (UK) in 1999 to evaluate the germ cell transplantation in cancer patients but, as far as we know, no information is available on the fertility of these patients (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 generate offspring (Shinohara *et al*., 2002; Schlatt *et al*., 2003; Ohta *et al*., 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 (Honaramooz *et al*., 2002b; Schlatt *et al*., 2002b, 2003; Shinohara *et al*., 2002; Snedaker *et al*.,2004; Oatley *et al*., 2004, 2005; Ohta *et al*., 2005; Rathi *et al*., 2005, 2006; Schmidt *et al*., 2006a; Zeng *et al*., 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 differences in spermatogenic differentiation remain unknown, but some hypotheses can be proposed.

First, differences between host and donor gonadotropic hormones (Bousfield *et al*., 1996) may lead to inefficient interaction between murine gonadotrophins and grafted donor testicular tissue. Supplementation with exogenous gonadotrophins could therefore be useful. Indeed, xenografts of ITT from rhesus monkeys to mice treated with exogenous gonadotrophins, showed some degree of sperm differentiation, compared with blockage at the spermatogonial level observed in untreated mice (Rathi *et al*., 2008). However, observations from two different studies do not support this hypothesis, since autologous grafts of marmoset tissue (Wistuba *et al*., 2006) and xenografts of marmoset and horse ITT to gonadotrophin-supplemented recipient mice (Wistuba *et al*., 2004; Rathi *et al*., 2006) showed blockage in germ cell differentiation. These conflicting results suggest that species-specific differences in gonadotrophins are not the only explanation for differentiation impairment.

Second, as suggested by studies on testicular tissue xenografts from macaques and marmosets, species-specific structural differences in seminiferous tubule organization (Luetjens *et al*., 2005), resulting in modified paracrine interactions, might explain differences in germ cell differentiation within grafts (Honaramooz *et al*., 2004; Wistuba *et al*., 2004).

Third, the initiation and extent of differentiation may be influenced by the stage of germ cell development and intensity of spermatogenesis at the time of grafting. Indeed, complete spermatogenesis was not reported in xenografted tissue when donor testicular tissue contained post-meiotic germ cells at the time of grafting in any species, including humans, and most grafts regressed or contained degenerated tubules (Schlatt *et al*., 2002b, 2006; Geens *et al*., 2006; Rathi *et al*., 2006). We also observed degenerative changes when adult human testicular tissue was grafted orthotopically to immunodeficient mice (C. Wyns, 2008, PhD thesis published by the Catholic University of Louvain, Belgium; Fig. 2).

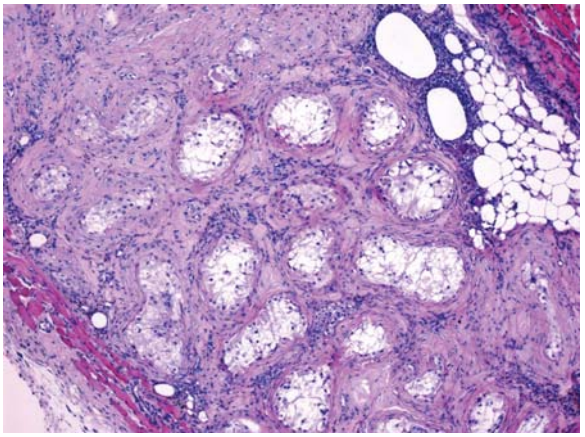

Figure 2 Histological appearance (hematoxylin–eosin sections) of donor testicular tissue from a 44-year-old man after 3 weeks' orthotopic xenografting at x200 magnification. Most tubules show degenerative changes, i.e. sclerosis, while the remaining contain mainly Sertoli cells.

The reasons for the poor outcome of adult testicular tissue xenografts 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, a potent angiogenic factor, was found to increase the number of tubules containing elongating spermatids (Schmidt *et al*., 2006b).

Nevertheless, the angiogenesis hypothesis is probably insufficient to explain this poor outcome, since donor age-dependent variations in germ cell differentiation have also been observed in immature donors (Oatley *et al*., 2005; Rathi *et al*., 2008).Variations in Sertoli cell maturation at the time of grafting, or their developmental susceptibility to the detrimental influence of endocrine disruption due to the xenografting environment, may account for the inability of these cells to support germ cell differentiation, and may thus be involved in the age-dependent variations found (Oatley *et al*., 2005; Rathi *et al*., 2008). 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 that freezing does not appear to affect the functional capacity of frozen germ cells on a qualitative basis (Honaramooz *et al*., 2002b; Schlatt *et al*., 2002b; Shinohara *et al*., 2002; 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 (Geens *et al*., 2006; Schlatt *et al*., 2006) and some isolated spermatogonia in 21.6–23.1% of grafts (Geens *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 in humans 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 with 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 is essential to evaluate to what extent cryopreservation itself is implicated. Freezing does not appear to have a major impact. Indeed, we did not observe a decrease in spermatogonial cell numbers between fresh and frozen-thawed testicular pieces ($P = 0.267$) (Wyns *et al*., 2007). Furthermore, Keros *et al*. (2007) obtained a very high survival rate ($94 \pm 1\%$) of spermatogonia after freezing and culture. 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 found spermatid-like structures on hematoxylin–eosin-stained histological sections (Fig. 3), albeit slightly smaller than control spermatids ($P = 0.045$), but these structures did not show characteristic markers of post-meiotic cells or acrosome development by immunohistochemistry (IHC).

Signs of preservation of the steroidogenic capacity of Leydig cells, both by IHC, revealing the steroidogenic enzyme 3-β hydroxysteroid-dehydrogenase, and by transmission electron microscopy (TEM), were also observed (Wyns *et al*., 2008).

IVM of germ cells

In vitro maturation (IVM) of germ stem cells, leading to *in vitro*-derived male haploid gametes available for ICSI, circumvents the risk of reintroducing the 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. Early research on meiotic differentiation *in vitro* was reviewed by Staub (2001).

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). The study by Feng *et al*. (2002) is noteworthy, since these authors achieved *in vitro*-derived spermatocytes and spermatids from murine SSCs, with geno- and phenotypical characteristics of type A spermagonia, using telomerase-immortalized cell lines in the absence of supportive cells.

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

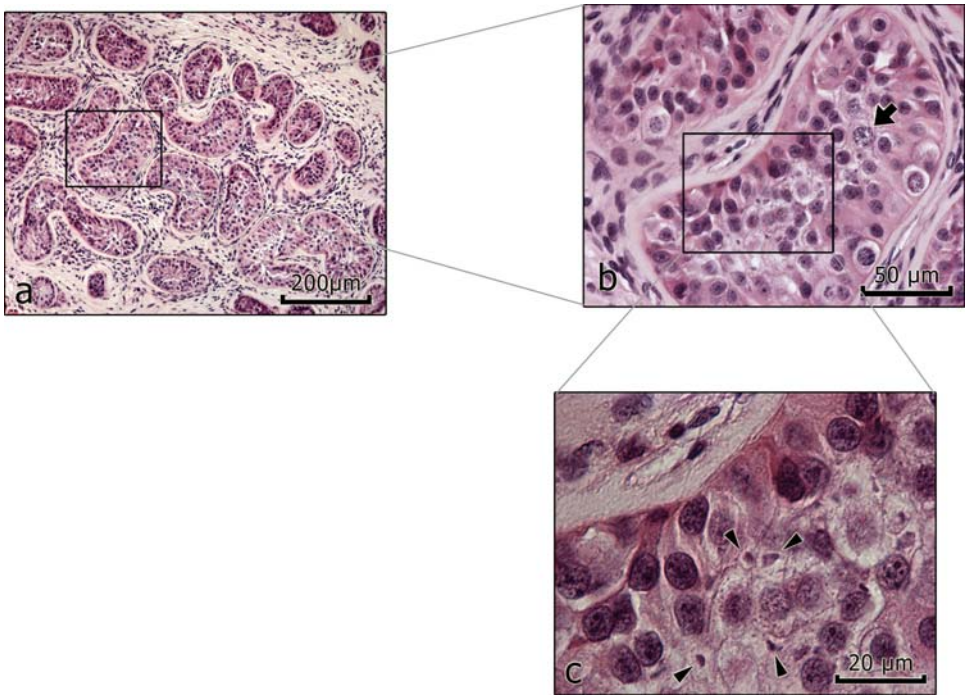

Figure 3 Histological appearance (hematoxylin–eosin sections) of donor testicular tissue from a 12-year-old boy after 6 months' orthotopic xenografting at x200 magnification (**a**), showing pachytene spermatocytes (arrow) and spermatid-like cells (inset) at x400 magnification (**b**) and spermatid-like cells at x1000 magnification (**c**).

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 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 of azoospermic male 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 normal chromosomal status and were able to activate human oocytes after injection into the cytoplasm. The advantage of such a culture system is its absence of toxicity, high permeability, biodegradability and three-dimensional (3D) support (Zimmermann *et al*., 2000). It could therefore be considered a promising technique for human application.

In vitro culture of whole human testicular tissue, allowing conservation of cellular interactions within and between seminiferous tubules and the interstitial compartment, could elicit differentiation of elongated spermatids from primary spermatocytes when supplemented with rFSH and testosterone (Tesarik *et al*., 1998). Using whole human testicular fragment culture, germ cell differentiation appeared to follow the same timing as *in vivo* spermatogenesis, but gradual apoptotic loss of meiotic and post-meiotic germ cells, independent of the presence of gonadotrophins, was observed (Roulet *et al*., 2006).

To promote cell–cell communication, 3D cell culture was developed. Dissociated testicular cells were embedded, allowing aggregation and re-establishment of Sertoli and germ cell contacts within a collagen gel matrix, leading to differentiation of spermatocytes from patients with maturational arrest into presumptive spermatids (Lee *et al*., 2007). The exact mechanism by which the collagen matrix supports *in vitro* spermatogenesis is unknown, but it is likely that it retains growth or humoral factors secreted by Sertoli cells in close proximity to germ cells.

While Sertoli cells appear to play a key role in the regulation of growth/proliferation of spermatogonial cells and early stages of spermatogenesis in *in vitro* culture, this does not seem to be the case for later stages. Indeed, almost 22% of cultured human round spermatids displayed flagellar growth without the presence of other germ cells or Sertoli cells, but their fertilizing and developmental capability (atypical flagellar growth with no cytoplasm observed surrounding the flagellum) remained markedly reduced (Aslam and Fishel, 1998).

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

Table III 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 *In vitro* culture Cell transplantation	0.39% H2Kb$^+$ cells 3.1% (1/32) contaminated cultures 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: interferon-γ; CML: chronic myelogenous leukemia.

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. Future research should focus on the identification of specific factors and signaling pathways that are present only in the testis, supplying an ideal microenvironment for full spermatogenic maturation.

Safety issues

Cancer cell contamination. The most important, life-threatening concern of spermatogonial transplantation is the risk of reintroducing malignant cells. 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 disease relapse (Jahnukainen *et al.*, 2001).

Therefore, germ cell isolation and cell-sorting techniques enabling complete purification of SSCs need to be validated before safe transplantation can be contemplated. While cell-sorting methods have shown promising results in animal studies (Fujita *et al.*, 2005), the same cannot be said of humans (Fujita *et al.*, 2006; Geens *et al.*, 2007). Table III summarizes the existing studies on the elimination of cancer cells from testicular cell suspensions.

One of the reasons for suboptimal cell sorting may be that the surface antigens are shared by other stem cells, namely hematopoietic stem cells (Spangrude *et al.*, 1988) involved in hematological cancers. Immunophenotyping malignant cells from each patient, followed by inclusion of patient-specific cancer antigens for cell sorting, should therefore improve the success of the technique. For this purpose, we strongly advise storing patient blood and/or tumor samples before therapy and applying techniques for minimal residual disease (MRD) assessment, allowing detection of malignant cells among normal cells, with a sensitivity as high as 10^{-5}–10^{-6} (Jolkowska *et al.*, 2007).

Dym *et al.* (2009) recently reviewed all currently known phenotypic markers for human and rodent spermatogonia, but no specific marker has yet been identified that is exclusive to SSCs, allowing positive selection of these cells through cell-sorting techniques. Further research on surface markers should focus on the complete elimination of cancer cells from cell suspensions before sorted preparations can be safely transplantated.

Cancer cell contamination is also a major concern in tissue autografting. Since it has been reported that leukemic cells can survive cryopreservation/xenotransplantation and increase the incidence of generalized leukemia in the nude mouse host (Hou *et al.*, 2007), and suggested that damage to testicular tissue or disturbance of vessel barriers in cryopreserved tissue may enhance hematogenous invasion of surviving malignant cells in tissue grafts, 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

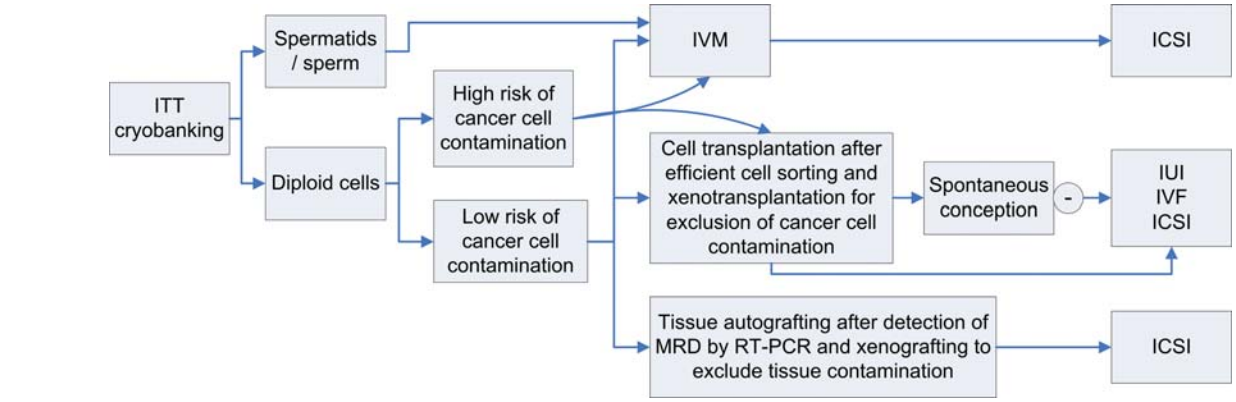

Figure 4 Fertility restoration strategy after gonadotoxic therapies in prepubertal boys. ITT, immature testicular tissue.

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 *et al*., 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*. (2003) recently reported smaller litter size, significantly lower fetal weight and reduced length in first generation mouse offspring after germ cell transplantation, suggesting imprinting disorders (Goossens *et al*., 2006). Further investigation is therefore 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. 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 in the study (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 failure in fertilization 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.

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 genetic 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, which must therefore be done for good reason (Tournaye *et al*., 2004; Jahnukainen *et al*., 2006a). However, in the two available 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. Furthermore, in a follow-up study of cryptorchid boys who had undergone testicular biopsy during orchidopexy, no adverse long-term effects were reported (Patel *et al*., 2005). 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 fertility restoration (Bahadur, 2004; Tournaye *et al*., 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

Providing young people undergoing gonadotoxic treatment with adequate fertility preservation strategies is a challenging area of reproductive medicine, but every patient should be given the chance to consider fertility-sparing options because the detrimental effect of such therapy on gonadal function remains unpredictable. Hormonal or cytoprotective drug manipulation aimed at enhancing spontaneous recovery of 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 the fertility restoration options, patient selection is essential, since risks vary according to disease. No single (or simple) algorithm can, thus far, summarize all the possible strategies for fertility preservation and restoration in the case of gonadotoxic therapy in prepubertal boys, but the most appropriate course of action may be selected according to the scheme shown in Fig. 4. 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.

Although testicular grafting looks promising in the light of animal experiments and appears to be technically easier and more efficient than SSC transplantation (Van Saen *et al*., 2009), this approach should only be considered for patients in whom there is no risk of malignant contamination of the tissue. The most promising approach will probably involve orthotopic autografting of immature testicular tissue or whole testes, stored prior to gonadotoxic therapy for benign pathologies or, more cautiously, non-hematological and non-metastasizing cancers. Resolving numerous important technical issues discussed in this review should lead to safe and efficient methodologies for fertility restoration after storage of preserved gametes, 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 that 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.

Authors' Roles

C.W. wrote the paper and discussed the material in depth after selecting and interpreting the relevant studies for this review. M.C. the performed histological analysis of ITT during all our experiments, provided figures and was involved in tissue cryopreservation. B.V. was responsible for ITT cryopreservation and banking. A.V.L. supervised the laboratory components during immature testicular tissue cryopreservation. J.D. reviewed the paper and contributed to the discussion.

Supplementary Data

Supplementary data are available at http://humupd.oxfordjournals.org/.

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References

Aksglaede L, Wikström AM, Rajpert-De Meyts E, Dunkel L, Skakkebaek NE, Jull A. Natural history of seminiferous tubule degeneration in Klinefelter syndrome. *Hum Reprod Update* 2006; **12**:39–48.

Anserini P, Chiodi S, Spinelli S, Costa M, Conte N, Copello F, Bacigalupo A. Semen analysis following allogeneic bone marrow transplantation. Additional data for evidence-based counselling. *Bone Marrow Transplant* 2002;**30**:447–541.

Arce B, Padrón S. Spermatogenesis in Klinefelter's syndrome. *Reproduction* 1980;**4**:177–184.

Arregui L, Rathi R, Zeng W, Honaramooz A, Gomendio M, Roldan ER, Dobrinski I. Xenografting of adult mammalian testis tissue. *Anim Reprod Sci* 2008;**106**:65–76.

Ash P. The influence of radiation on fertility in man. *Br J Radiol* 1980; **53**:271–278.

Aslam I, Fishel S. Short-term culture and cryopreservation of spermatogenic cells used for human in-vitro conception. *Hum Reprod* 1998;**13**:634–638.

Avarbock MR, Brinster CJ, Brinster RL. Reconstitution of spermatogenesis from frozen spermatogonial stem cells. *Nature Med* 1996;**2**:693–696.

Bahadur G. Ethics of testicular stem cell medicine. *Hum Reprod* 2004; **19**:2702–2710.

Bahadur G, Ralph D. Gonadal tissue cryopreservation in boys with paediatric cancers. *Hum Reprod* 1999;**14**:11–17.

Bahadur G, Chatterjje R, Ralph D. Testicular tissue cryopreservation in boys. Ethical and legal issues. *Hum Reprod* 2000;**15**:1416–1420.

Bar-Shira Maymom B, Yogev L, Marks A, Hauser R, Botchan A, Yavetz H. Sertoli cell inactivation by cytotoxic damage to the human testis after cancer therapy. *Fertil Steril* 2004;**81**:1391–1394.

Blatt J. Pregnancy outcome in long-term survivors of childhood cancer. *Med Ped Oncol* 1999;**33**:29–33.

Bleyer WA. The impact of childhood cancer on the United States and the world. *Cancer* 1990;**40**:355–367.

Boekelheide K, Schoenfeld HA, Hall SJ, Weng CC, Shetty G, Leith J, Harper J, Sigman M, Hess DL, Meistrich ML. Gonadotropin-releasing hormone antagonist (cetrorelix) therapy fails to protect nonhuman primates (Macaca arctoides) from radiation-induced spermatogenic failure. *J Androl* 2005;**26**:222–234.

Bokemeyer C, Schmoll HJ, van Rhee J, Kuczyk M, Schuppert F, Poliwoda H. Long-term gonadal toxicity after therapy for Hodgkin's and non-Hodgkin's lymphoma. *Ann Hematol* 1994;**68**:105–110.

Bousfield GR, Butney VY, Gotschall RR, Baker VL, Moore WT. Structural features of mammalian gonadotropins. *Mol Cell Endocrinol* 1996; **125**:3–19.

Brennemann W, Brensing KA, Leipner B, Boldt I, Klingmuller D. Attempted protection of spermatogenesis from irradiation in patients with seminoma by D-tryptophan-6 luteinizing hormone releasing hormone. *Clin Investig* 1994;**72**:838–842.

Brenner H, Steliarova-Foucher E, Arndt V. Up-to-date monitoring of childhood cancer long-term survival in Europe: methodology and application to all forms of cancer combined. *Ann Oncol* 2007; **18**:1561–1568.

Brinster RL, Avarbock MR. Germline transmission of donor haplotype following spermatogonial transplantation. *Proc Natl Acad Sci USA* 1994; **22**:11303–11307.

Brinster RL, Zimmermann JW. Spermatogenesis following male germ-cell transplantation. *Proc Natl Acad Sci USA* 1994;**91**:11298–11302.

Brinster CJ, Ryu BY, Avarbock MR, Karagenc L, Brinster RL, Orwig KE. Restoration of fertility by germ cell transplantation requires effective recipient preparation. *Biol Reprod* 2003;**69**:412–420.

Brook P, Radford J, Shalet S, Joyce A, Gosden R. Isolation of germ cells from human testicular tissue for low temperature storage and autotransplantation. *Fertil Steril* 2001;**75**:269–274.

Brougham MF, Kelnar CJ, Sharpe RM *et al.* Male fertility following childhood cancer: current concepts and future therapies. *Asian J Androl* 2003;**5**:325–337.

Bucci LR, Meistrich ML. Effects of busulfan on murine spermatogenesis: cytotoxicity, sterility, sperm abnormalities, and dominant lethal mutations. *Mutat Res* 1987;**176**:259–268.

Carmely A, Meirow D, Peretz A, Albeck M, Bartoov B, Sredni B. Protective effect of the immunomodulator AS101 against cyclophosphamide-induced testicular damage in mice. *Hum Reprod* 2009;**1**:1–8.

Choi YJ, Park JK, Lee MS, Ahn JD, Hwang KC, Song H, Kim JH. Long-term follow-up of porcine male germ cells transplanted into mouse testes. *Zygote* 2007;**15**:325–335.

Clouthier DE, Avarbock MR, Maika SD, Hammer RE, Brinster RL. Rat spermatogenesis in mouse testis. *Nature* 1996;**381**:418–421.

Courbière B, Odagescu V, Baudot A, Massardier J, Mazoyer C, Salle B, Lornage J. Cryopreservation of the ovary by vitrification as an alternative to slow-cooling protocols. *Fertil Steril* 2006;**41**:1243–1251.

Cremades N, Sousa M, Bernabeu R, Barros A. Developmental potential of elongating and elongated spermatids obtained after in-vitro maturation of isolated round spermatids. *Hum Reprod* 2001;**16**:1938–1944.

Dobrinski I, Avarbock MR, Brinster RL. Transplantation of germ cells from rabbits and dogs into mouse testes. *Biol Reprod* 1999a; **61**:1331–1339.

Dobrinski I, Ogawa T, Avarbock MR, Brinster RL. Computer assisted image analysis to assess colonization of recipient seminiferous tubules by spermatogonial stem cells from transgenic donor mice. *Mol Reprod Dev* 1999b;**53**:142–148.

Dobrinski I, Avarbock MR, Brinster RL. Germ cell transplantation from large domestic animals into mouse testes. *Mol Reprod Dev* 2000; **57**:270–279.

Dym M, Kokkinaki M, He Z. Spermatogonial stem cells: mouse and human comparisons. *Birth Defects Res* 2009;**87**:27–34.

Feng LX, Chen Y, Dettin L, Pera RA, Herr JC, Goldberg E, Dym M. Generation and in vitro differentiation of a spermatogonial cell line. *Science* 2002;**297**:392–395.

Fossa SD, Klepp O, Norman N. Lack of gonadal protection by medroxyprogesterone acetate-induced transient medical castration during chemotherapy for testicular cancer. *Br J Urol* 1988;**62**:449–453.

Frederickx V, Michiels A, Goossens E, De Block G, Van Steirteghem AC, Tournaye H. Recovery, survival and functional evaluation by transplantation of frozen-thawed mouse germ cells. *Hum Reprod* 2004; **19**:948–953.

Fujita K, Ohta H, Tsujimura A, Takao T, Miyagawa Y, Takada S, Matsumiya K, Wakayama T, Okuyama A. Transplantation of spermatogonial stem cells isolated from leukemic mice restores fertility without inducing leukemia. *J Clin Invest* 2005;**115**:1855–1861.

Fujita K, Ohta H, Tsujimura A, Miyagawa Y, Kiuchi H, Matsuolka Y, Takao T, Takada S, Nonomura N, Okuyama A. Isolation of germ cells from leukemia and lymphoma cells in a human in vitro model: potential clinical application for restoring human fertility after anticancer therapy. *Cancer Res* 2006;**66**:11166–11171.

Geens M, De Block G, Goossens E, Frederickx V, Van Steirteghem A, Tournaye H. Spermatogonial survival after grafting human testicular tissue to immunodeficient mice. *Hum Reprod* 2006;**21**:390–396.

Geens M, Van de Velde H, De Block G, Goossens E, Van Steirteghem A, Tournaye H. The efficiency of magnetic-activated cell sorting and fluorescence-activated cell sorting in the decontamination of testicular cell suspensions in cancer patients. *Hum Reprod* 2007;**22**:733–742.

Geens M, Goossens E, De Block G, Ning L, Van Saen D, Tournaye H. Autologous spermatogonial stem cell transplantation in man: current obstacles for a future clinical application. *Hum Reprod Update* 2008; **14**:121–129.

Georgiou I, Pardalidis N, Gianaakis D, Saito M, Watanabe T, Tsounapi P, Loutradis D, Kanakas N, Karagiannis A, Baltogiannis D *et al.* In vitro spermatogenesis as a method to bypass pre-meiotic or post-meiotic barriers blocking the spermatogenetic process: genetic and epigenetic implications in assisted reproductive technology. *Andrologia* 2007; **39**:159–176.

Goossens E, Frederickx V, de Block G, Van Steirteghem A, Tournaye H. Reproductive capacity of sperm obtained after germ cell transplantation in a mouse model. *Hum Reprod* 2003;**18**:1874–1880.

Goossens E, Frederickx V, de Block G, van Steirteghem A, Tournaye H. Evaluation of in vivo conception after testicular stem cell transplantation in a mouse model shows altered post-implantation development. *Hum Reprod* 2006;**21**:2057–2060.

Goossens E, Frederickx V, Geens M, De Block G, Tournaye H. Cryosurvival and spermatogenesis after allografting prepubertal mouse tissue: comparison of two cryopreservation protocols. *Fertil Steril* 2008a;**89**:725–727.

Goossens E, Geens M, De Block G, Tournaye H. Spermatogonial survival in long-term human prepubertal xenografts. *Fertil Steril* 2008b; **90**:2019–2022.

Grischenko VI, Keros VA, Bondarenko VA. Effect of human fetotesticular tissue transplantation on functional state of reproductive system at some male infertility forms. *Probl Cryobiol* 1999;**4**:73–76.

Griswold MD. The central role of Sertoli cells in spermatogenesis. *Cell Dev Biol* 1998;**9**:411–416.

Hamra FK, Gatlin J, Chapman KM, Grellhesl DM, Garcia JV, Hammer RE, Garbers DL. Production of transgenic rats by lentiviral transduction of

male germ-line stem cells. *Proc Natl Acad Sci USA* 2002; **99**:14931–14936.

Hasthorpe S. Clonogenic culture of normal spermatogonia: in vitro regulation of postnatal germ cell proliferation. *Biol Reprod* 2003; **68**:1354–1360.

Hermann BP, Sukhwani M, Lin CC, Sheng Y, Tomko J, Rodriguez M, Shuttleworth JJ, McFarland D, Hobbs RM, Pandolfi PP *et al.* Characterization, cryopreservation, and ablation of spermatogonial stem cells in adult rhesus macaques. *Stem Cell* 2007;**25**:2330–2338.

Hermann BP, Sukhwani M, Simorangkir DR, Chu T, Plant T, Orwig KE. Molecular dissection of the male germ cell lineage identifies putative spermatogonial stem cells in rhesus macaques. *Hum Reprod* 2009; **1**:1–13.

Hofmann MC, Braydich-Stolle L, Dym M. Isolation of male germ-line stem cells; influence of GDNF. *Dev Biol* 2005;**279**:114–124.

Honaramooz A, Megee SO, Dobrinski I. Germ cell transplantation in pigs. *Biol Reprod* 2002a;**66**:21–28.

Honaramooz A, Snedaker A, Boiani M, Scholer HR, Dobrinski I, Schlatt S. Sperm from neonatal mammalian testes grafted in mice. *Nature* 2002b; **418**:788–781.

Honaramooz A, Behboodi E, Meege SO, Overton SA, Galantino-Homer H, Echelard Y, Dobrinski I. Fertility and germline transmission of donor haplotype following germ cell transplantation in immunocompetent goats. *Biol Reprod* 2003;**69**:1260–1264.

Honaramooz A, Li M-W, Penedo CT, Meyers S, Dobrinski I. Accelerated maturation of primate testis by xenografting into mice. *Biol Reprod* 2004; **70**:1500–1503.

Hou M, Andersson M, Eksborg S, Söder O, Jahnukainen K. Xenotransplantation of testicular tissue into nude mice can be used for detecting leukemic cell contamination. *Hum Reprod* 2007; **22**:1899–1906.

Hovatta O. Cryopreservation of testicular tissue in young cancer patients. *Hum Reprod Update* 2001;**7**:378–383.

Hovatta O. Cryobiology of ovarian and testicular tissue. *Best Pract Res Clin Obstet Gynaecol* 2003;**17**:331–342.

Howell S, Shalet S. Gonadal damage from chemotherapy and radiotherapy. *Endocrinol Metab Clin North Am* 1998;**27**:927–943.

Howell SJ, Shalet SM. Testicular function following chemotherapy. *Hum Reprod Update* 2001;**7**:363–369.

Howell SJ, Shalet SM. Spermatogenesis after cancer treatment: damage and recovery. *J Natl Cancer Inst Monogr* 2005;**37**:712–717.

Howell SJ, Radford JA, Ryder WDJ, Shalet SM. Testicular function after cytotoxic chemotherapy: evidence of Leydig cell insufficiency. *J Clin Oncol* 1999;**17**:1493–1498.

Ishiguro H, Yasuda Y, Tomita Y, Shinagawa T, Shimizu T, Morimoto T, Hattori K, Matsumoto M, Inoue H, Yabe H *et al.* Gonadal shielding to irradiation is effective in protecting testicular growth and function in long-term survivors of bone marrow transplantation during childhood or adolescence. *Bone Marrow Transplant* 2007;**39**:483–490.

Izadyar F, Den Ouden K, Stout TA, Stout J, Coret J, Lankveld DP, Spoormakers TJ, Colenbrander B, Oldenbroek JK, Van der Ploeg KD *et al.* Autologous and homologous transplantation of bovine spermatogonial stem cells. *Reproduction* 2003a;**126**:765–774.

Izadyar F, Den Ouden K, Creemers LB, Posthuma G, Parvinen M, de Rooij DG. Proliferation and differentiation of bovine type A spermatogonia during long-term culture. *Biol Reprod* 2003b;**68**:272–281.

Jacobson DA, Duerst R, Tse W, Kletzel M. Reduced intensity haemopoietic stem-cell transplantation for treatment of non-malignant diseases in children. *Lancet* 2004;**364**:156–162.

Jadoul P, Donnez J, Dolmans MM, Squifflet J, Lengele B, Martinez-Madrid B. Laparoscopic ovariectomy for whole human ovary cryopreservation: technical aspects. *Fertil Steril* 2007;**87**:971–975.

Jahnukainen K, Hou M, Pettersen C, Setchell B, Söder O. Intratesticular transplantation of testicular cells from leukemic rats causes transmission of leukemia. *Cancer Res* 2001;**61**:706–710.

Jahnukainen K, Ehmcke J, Söder O, Schlatt S. Clinical potential and putative risks of fertility preservation in children utilizing gonadal tissue or germline stem cells. *Peditatr Res* 2006a;**59**:40R–47R.

Jahnukainen K, Ehmcke J, Schlatt S. Testicular xenografts: a novel approach to study cytotoxic damage in juvenile primate testis. *Cancer Res* 2006b; **66**:3813–3818.

Jahnukainen K, Ehmcke J, Hergenrother SD, Schlatt S. Effect of cold storage and cryopreservation of immature non-human primate testicular tissue on spermatogonial stem cell potential in xenografts. *Hum Reprod* 2007;**22**:1060–1067.

Johnson DH, Linde R, Hainsworth JD, Vale W, Rivier J, Stein R, Flexner J, Van Welch R, Greco FA. Effect of a luteinizing hormone releasing hormone agonist given during combination chemotherapy on posttherapy fertility in male patients with lymphoma: preliminary observations. *Blood* 1985;**65**:832–836.

Jolkowska J, Drewich K, Dawidowska M. Methods of minimal residual disease (MRD) detection in childhood haematological malignancies. *J Appl Genet* 2007;**48**:77–83.

Kamischke A, Kuhlmann M, Weinbauer GF, Luetjens M, Yeung CH, Kronholz HL, Nieschlag E. Gonadal protection from radiation by GnRH antagonist or recombinant human FSH: a controlled trial in a male nonhuman primate (Macaca fascicularis). *J Endocrinol* 2003; **179**:183–194.

Kanatsu-Shinohara M, Ogonuki N, Inoue K, Miki H, Ogura A, Toyokuni S, Shinohara T. Long-term proliferation in culture and germline transmission of mouse male germline stem cells. *Biol Reprod* 2003a;**69**:612–616.

Kanatsu-Shinohara M, Ogonuki N, Inoue K, Ogura A, Toyokuni S, Shinohara T. Restoration of fertility in infertile mice by transplantation of cryopreserved male germline stem cells. *Hum Reprod* 2003b; **18**:2660–2667.

Kanatsu-Shinohara M, Ogonuki N, Iwano T, Lee J, Kazuki Y, Inoue K, Miki H, Takehashi M, Toyokuni S, Shinkai Y *et al.* Genetic and epigenetic properties of mouse male germline stem cells during long-term culture. *Development* 2005;**132**:4155–4163.

Kanatsu-Shinohara M, Muneto T, Lee J, Takenada M, Chuma S, Nakatsuji N, Horiuchi T, Shinohara T. Long-term culture of male germline stem cells from hamster testes. *Biol Reprod* 2008;**78**: 611–617.

Kawamura H, Kaponis A, Tasos A, Giannakis D, Miyagawa I, Sofikitis N. Rat Sertoli cells can support human meiosis. *Hum Reprod* 2003; **18**(Suppl. 1): 78 Presented in the 19th Annual Meeting of ESHRE, Madrid, Spain, 2003.

Kazadi Lukusa A, Vermylen C, Vanabelle B, Curaba M, Brichard B, Chantrain C, Dupont S, Ferrant A, Wyns C. Bone marrow transplantation or hydroxyurea for sickle cell anemia: long term effects on semen variables and hormone profiles. *Ped Hematol Oncol* 2009;**26**:186–194.

Kelnar CJ, McKinnell C, Walker M, Morris KD, Wallace WH, Saunders PT, Fraser HM, Sharpe RM. Testicular changes during infantile 'quiescence' in the marmoset and their gonadotrophin dependence: a model for investigating susceptibility of the prepubertal human testis to cancer therapy? *Hum Reprod* 2002;**17**:1367–1378.

Kenney L, Laufer MR, Grant FD, Frier H, Diller L. High risk of infertility and long term gonadal damage in males treated with high dose cyclophosphamide for sarcoma during childhood. *Cancer* 2001; **91**:613–621.

Keros V. Cryopreservation of human fetus testicular tissue and low temperature bank creation for further application in human transplantology. *Probl Cryobiol* 1999;**3**:54–58.

Keros V, Rosenlund B, Hultenby K, Aghajanova L, Levkov L, Hovatta O. Optimizing cryopreservation of human testicular tissue: comparison of protocols with glycerol, propanediol and dimethylsulphoxide as cryoprotectants. *Hum Reprod* 2005;**20**:1676–1687.

Keros V, Hultenby K, Borgström B, Fridström M, Jahnukainen K, Hovatta O. Methods of cryopreservation of testicular tissue with viable spermatogonia in pre-pubertal boys undergoing gonadotoxic cancer treatment. *Hum Reprod* 2007;**22**:1384–1395.

Kim Y, Turner D, Nelson J, Dobrinsji I, McEntee M, Travis AJ. Production of donor-derived sperm after spermatogonial stem cell transplantation in the dog. *Reproduction* 2008;**136**:823–831.

Kreuser ED, Hetzel WS, Hautmann R, Pfeiffer EF. Reproductive toxicity with and without LHRHA administration during adjuvant chemotherapy in patients with germ cell tumors. *Horm Metab Res* 1990;**22**:494–498.

Kubota H, Avarbock MR, Brinster RL. Spermatogonial stem cells share some, but not all, phenotypic and functional characteristics with other stem cells. *Proc Natl Acad Sci USA* 2003;**100**:6487–6492.

Kubota H, Avarbock MR, Brinster RL. Culture conditions and single growth factors affect fate determinations of mouse spermatogonial stem cells. *Biol Reprod* 2004a;**71**:722–731.

Kubota H, Avarbock MR, Brinster RL. Growth factor essential for self-renewal and expansion of mouse spermatogonial stem cells. *Proc Natl Acad Sci USA* 2004b;**101**:16489–16494.

Kulkarni SS, Sastry PS, Saikia TK, Parikh PM, Gopal R, Advani SH. Gonadal function following ABVD therapy for Hodgkin's disease. *Am J Clin Oncol* 1997;**20**:354–357.

Kvist K, Thorup J, Byslov AG, Hoyer PE, Mollgard K, Yding Andersen C. Cryopreservation of intact testicular tissue from boys with cryptorchidism. *Hum Reprod* 2006;**21**:484–491.

Lee DR, Kaproth MT, Parks JE. In vitro production of haploid germ cells from fresh and frozen-thawed testicular cells of neonatal bulls. *Biol Reprod* 2001;**65**:873–878.

Lee DR, Kim K-S, Yang YH, Oh HS, Lee SH, Chung TG, Cho JH, Kim HJ, Yoon TK, Cha KY. Isolation of male germ stem cell-like cells from testicular tissue of non-obstructive azoospermic patients and differentiation into haploid male germ cells in vitro. *Hum Reprod* 2006; **21**:471–476.

Lee JH, Gye MC, Choi KW, Hong JY, Lee YB, Park DW, Lee SJ, Min CK. In vitro differentiation of germ cells from nonobstructive azoospermic patients using three-dimensional culture in a collagen gel matrix. *Fertil Steril* 2007;**87**:824–833.

Lirdi LC, Stumpp T, Sasso-Cerri E, Miraglia SM. Amifostine protective effect on cisplatin-treated rat testis. *Anat Rec* 2008;**291**:797–808.

Luetjens CM, Weinbauer GF, Wistuba J. Primate spermatogenesis: new insights into comparative testicular organization, spermatogenic efficiency and endocrine control. *Biol Rev Camb Philos Soc* 2005;**80**:475–488.

Mackie EJ, Radford M, Shalet SM. Gonadal function following chemotherapy for childhood Hodgkin's disease. *Med Pediatr Oncol* 1996;**27**:74–78.

Magnani C, Pastore G, Coebergh JW, Viscomi S, Spix C, Steliarova-Foucher E. Trends in survival after childhood cancer in Europe, 1978–1997: report from the Automated Childhood Cancer Information System project (AGCIS). *Eur J Cancer* 2006;**42**:1981–2005.

Martinez-Madrid B, Camboni A, Dolmans MM, Nottola SA, Van Langendonckt A, Donnez J. Apoptosis and ultrastructural assessment after cryopreservation of whole human ovaries with their vascular pedicle. *Fertil Steril* 2007;**87**:1153–1165.

Masala A, Faedda R, Alagna S, Satta A, Chiarelli G, Rovasio PP, Ivaldi R, Taras MS, Lai E, Bartoli E. Use of testosterone to prevent cyclophosphamide-induced azoospermia. *Ann Intern Med* 1997; **126**:292–295.

Meirow D, Schenker JG. Cancer and male fertility. *Hum Reprod* 1995; **10**:2017–2022.

Meistrich ML, Wilson G, Brown BW, da Cunha MF, Lipshultz LI. Impact of cyclophosphamide on long-term reduction in sperm count in men treated with combination chemotherapy for Ewing and soft tissue sarcomas. *Cancer* 1992;**70**:2703–2712.

Mikkola M, Sironen A, Kopp C, Taponen J, Sukura A, Vilkki J, Katila T, Andersson M. Transplantation of normal boar testicular cells resulted in complete focal spermatogenesis in a boar affected by the immotile short-tail sperm defect. *Reprod Dom Anim* 2006;**41**:124–128.

Milazzo JP, Vaudreuil L, Cauliez B, Gruel E, Massé L, Mousset-Siméon N, Macé B, Rives N. Comparison of conditions for cryopreservation of testicular tissue from immature mice. *Hum Reprod* 2008;**23**:17–28.

Muller J, Skakkebaeck N. Quantification of germ cells and seminiferous tubules by stereological examination of testicles from 50 boys who suffered sudden death. *Int J Androl* 1983;**6**:143–156.

Nagano M, Brinster CJ, Orwig KE, Ryu BY, Avarbock MR, Brinster RL. Transgenic mice produced by retroviral transduction of male germ-lie stem cells. *Proc Natl Acad Sci USA* 2001a;**98**:13090–13095.

Nagano M, McCarrey JR, Brinster RL. Primate spermatogonial stem cells colonize mouse testes. *Biol Reprod* 2001b;**64**:1409–1416.

Nagano M, Patrizio P, Brinster RL. Long-term survival of human spermatogonial stem cells in mouse testes. *Fertil Steril* 2002; **78**:1225–1233.

Oatley JM, de Avila DM, McLean DJ, Griswold MD, Reeves JJ. Transplantation of bovine germinal cells into mouse testes. *J Anim Sci* 2002;**80**:1925–1931.

Oatley JM, de Avila DM, Reeves JJ, McLean DJ. Spermatogenesis and germ cell transgene expression in xenografted bovine testicular tissue. *Biol Reprod* 2004;**71**:494–501.

Oatley JM, Reeves JJ, McLean DJ. Establishment of spermatogenesis in neonatal bovine testicular tissue following ectopic xenografting varies with donor age. *Biol Reprod* 2005;**72**:358–364.

Ogawa T, Dobrinski I, Brinster RL. Recipient preparation is critical for spermatogonial transplantation in the rat. *Tissue Cell* 1999a;**31**:461–472.

Ogawa T, Dobrinski I, Avarbock MR, Brinster RL. Xenogeneic spermatogenesis following transplantation of hamster germ cells to mouse testes. *Biol Reprod* 1999b;**60**:515–521.

Ogawa T, Dobrinski I, Avarbock MR, Brinster RL. Transplantation of male germ line stem cells restores fertility in infertile mice. *Nat Med* 2000; **6**:29–34.

Ogawa T, Ohmura M, Yamura Y, Sawada H, Kubota Y. Expansion of murine spermatogonial stem cells through serial transplantation. *Biol Reprod* 2003;**68**:316–322.

Ogawa T, Ohmura M, Ohbo K. The niche for spermatogonial stem cells in the mammalian testis. *Int J Hematol* 2005;**82**:381–388.

Ohta H, Wakayama T. Generation of normal progeny by intracytoplasmic sperm injection following grafting of testicular tissue from cloned mice that died postnatally. *Biol Reprod* 2005;**73**:390–395.

Ohta H, Yomogida K, Dohmae K, Nishimune Y. Regulation of proliferation and differentiation in spermatogonial stem cells: the role of c-kit and its ligand SCF. *Development* 2000;**127**:2125–2131.

Okada FK, Stumpp T, Miraglia SM. Carnitine reduces testicular damage in rats treated with etoposide in the prepubertal phase. *Cell Tissue Res* 2009;**337**:269–280.

Otala M, Suomalainen L, Pentikäinen MO, Kovanen P, Tenhunen M, Erkkilä K, Toppari J, Dunkel L. Protection from radiation-induced male germ cell loss by sphingosine-1-phosphate. *Biol Reprod* 2004; **70**:759–767.

Passweg JR, Rabusin M. Hematopoetic stem cell transplantation for immune thrombocytopenia and other refractory autoimmune cytopenias. *Autoimmunity* 2008;**41**:600–665.

Patel RP, Kolon TF, Huff DS, Carr MC, Zderic SA, Canning DA, Snyder HM III. Testicular microlithiasis and antisperm antibodies following testicular biopsy in boys with cryptorchidism. *J Urol* 2005; **174**:2008–2010.

Patience C, Takeuchi Y, Weiss RA. Zoonosis in xenotransplantation. *Curr Opin Immunol* 1998;**10**:539–542.

Petersen PM, Hansen SW, Giwercman A, Rorth M, Skakkebaek NE. Dose-dependent impairment of testicular function in patients treated with cisplatin-based chemotherapy for germ cell cancer. *Ann Oncol* 1994;**5**:355–358.

Pont J, Albrecht W. Fertility after chemotherapy for testicular germ cell tumor. *Fertil Steril* 1997;**68**:1–5.

Rabusin M, Andolina M, Maximoca N, Paediatric Autoimmune Diseases Working Parties. Haematopoietic SCT in autoimmune diseases in children: rationale and new perspectives. *Bone Maroow Transplant* 2008;**41**:S96–S99.

Radford J. Restoration of fertility after treatment for cancer. *Horm Res* 2003;**59**:21–23.

Radford JA, Clark S, Crowther D, Shalet SM. Male fertility after VAPEC-B chemotherapy for Hodgkin's disease and non-Hogkin's lymphoma. *Br J Cancer* 1994;**69**:379–381.

Rathi R, Honaramooz A, Zeng W, Schlatt S, Dobrinski I. Germ cell fate and seminiferous tubule development in bovine testis xenografts. *Reproduction* 2005;**130**:923–929.

Rathi R, Honaramooz A, Zeng W, Turner R, Dobrinski I. Germ cell development in equine testis tissue xenografted into mice. *Reproduction* 2006;**131**:1091–1098.

Rathi R, Zeng W, Megee S, Conley A, Meyers S, Dobrinski I. Maturation of testicular tissue from infant monkeys after xenografting into mice. *Endocrinology* 2008;**19**: (Epub ahead of print).

Redman JR, Bajorunas DR. Suppression of germ cell proliferation to prevent gonadal toxicity associated with cancer treatment. *Workshop on Psychosexual and Reproductive Issues Affecting Patients with Cancer*. New York: American Cancer Society, 1987, 90–94.

Ries LA, Eisner MP, Kosary CL *et al.* (eds). *Childhood Cancer by the ICCC. SEER Cancer Statistics Review*. Bethesda, MD: National Institute, 2004, 1975–2001.

Rivkees SA, Crawford JD. The relationship of gonadal activity and chemotherapy-induced gonadal damage. *JAMA* 1988;**259**:2123–2125.

Roulet V, Denis H, Staub C, Tortorec AL, Delaleu B, Satie AP, Patard JJ, Jégou B, Dejucq-Rainsford N. Human testis in organotypic culture: application for basic or clinical research. *Hum Reprod* 2006; **21**:1564–1575.

Rowley MJ, Leach DR, Warner GA, Heller CG. Effect of graded doses of ionizing radiation of the human testis. *Radiat Res* 1974;**59**:665–678.

Russell LD, Brinster RL. Ultrastructural observations of spermatogenesis following transplantation of rat testis cells into mouse seminiferous tubules. *J Androl* 1996;**17**:615–627.

Ryu BY, Orwig KE, Avarbock MR, Brinster RL. Stem cell niche development in the postnatal rat testis. *Dev Biol* 2003;**263**:253–563.

Ryu BY, Kubota H, Avarbock MR, Brinster RL. Conservation of spermatogonial stem cell renewal signaling between mouse and rat. *Proc Natl Acad Sci USA* 2005;**102**:14302–14307.

Schaefer F, Marr J, Seidel C, Tilgen W, Schärer K. Assessment of gonadal maturation by evaluation of spermaturia. *Arch Dis Child* 1990; **65**:1205–1207.

Schlatt S, Rosiepen G, Weinbauer C, Rolf C, Brook P, Nieschlag E. Germ cell transfer into rat, bovine, monkey, and human testes. *Hum Reprod* 1999;**14**:144–150.

Schlatt S, Foppiani L, Rolf C, Weinbauer GF, Nieschlag E. Germ cell transplantation into X-irradiated monkey testes. *Hum Reprod* 2002a; **17**:55–62.

Schlatt S, Kim S, Gosden R. Spermatogenesis and steroidogenesis in mouse, hamster and monkey testicular tissue after cryopreservation and heterotopic grafting to castrated hosts. *Reproduction* 2002b; **124**:339–346.

Schlatt S, Honaramooz A, Boiani M, Schöler HR, Dobrinski I. Progeny from sperm obtained after ectopic grafting of neonatal mouse testes. *Biol Reprod* 2003;**68**:2331–2335.

Schlatt S, Honaramooz A, Ehmcke J, Goebell PJ, Rübben H, Dhir R, Dobrinski I, Patrizio P. Limited survival of adult human testicular tissue as ectopic xenograft. *Hum Reprod* 2006;**21**:384–389.

Schmidt JA, de Avila JM, McLean DJ. Grafting periode and donor age affect the potential for spermatogenesis in bovine ectopic testis xenografts. *Biol Reprod* 2006a;**75**:160–166.

Schmidt JA, de Avila JM, McLean DJ. Effect of vascular endothelial growth factor and testis tissue culture on spermatogenesis in bovine ectopic testis tissue xenografts. *Biol Reprod* 2006b;**75**:167–175.

Schmidt JA, de Avila JM, McLean DJ. Analysis of gene expression in bovine testis tissue prior to ectopic testis tissue xenografting and during the grafting period. *Biol Reprod* 2007;**76**:1071–1080.

Schover LR. Motivation for parenthood after cancer: a review. *J Natl Cancer Inst Monogr* 2005;**34**:2–5.

Schover LR, Rybicki LA, Martin BA, Bringelsen KA. Having children after cancer. A pilot survey of survivors' attitudes and experiences. *Cancer* 1999;**86**:697–709.

Shalet SM, Tsatsoulis A, Whitehead E, Read G. Vulnerability of the human Leydig cell to radiation damage is dependent upon age. *J Endocrinol* 1989;**120**:161–165.

Shetty G, Meistrich ML. Hormonal approaches to preservation and restoration of male fertility after cancer treatment. *Natl Cancer Inst Monogr* 2005;**34**:36–39.

Shinohara T, Avarbock MR, Brinster RL. β1- and α6-integrin are surface markers on mouse spermatogonial stem cells. *Proc Natl Acad Sci USA* 1999;**96**:5504–5509.

Shinohara T, Orwig KE, Avarbock MR, Brinster RL. Spermatogonial stem cell enrichment by multiparameter selection of mouse testis cells. *Proc Natl Acad Sci USA*. 2000;**97**:8346–8351.

Shinohara T, Orwig KE, Avarbock MR, Brinster RL. Remodeling of the postnatal mouse testis is accompanied by dramatic changes in stem cell number and niche accessibility. *Proc Natl Acad Sci USA* 2001; **98**:6186–6191.

Shinohara T, Inoue K, Ogonuki N, Kanatsu-Shinohara M, Miki H, Nakata K, Kurome M, Nagashima H, Toyokuni S, Kogishi K *et al.* Birth of offspring following transplantation of cryopreserved immature testicular pieces and in-vitro microinsemination. *Hum Reprod* 2002; **17**:3039–3045.

Slavin S, Nagler A, Aker M, Shapira MY, Cividalli G, Or R. Non-myeloablative stem cell transplantation and donor lymphocyte infusion for the treatment of cancer and life-threatening non-malignant disorders. *Rev Clin Exp Hematol* 2001;**5**:135–146.

Snedaker AK, Honaramooz A, Dobrinski I. A game of cat and mouse: xenografting of testis tissue from domestic kittens results in complete cat spermatogenesis in a mouse host. *J Androl* 2004;**25**: 926–930.

Sousa M, Cremades N, Alves C, Silva J, Barros A. Developmental potential of human spermatogenic cells co-cultured with Sertoli cells. *Hum Reprod* 2002;**17**:161–172.

Spangrude GJ, Heimfeld S, Weissman IL. Purification and characterization of mouse hematopoietic stem cells. *Science* 1988;**241**:58–62.

Staub C. A century of research on mammalian male germ cell meiotic differentiation in vitro. *J Androl* 2001;**22**:911–926.

Steliavora-Foucher E, Stiller C, Kaatsch P, Berrino F, Coebergh JW, Lacour B, Parkin M. Geographical patterns and time trends of cancer

incidence and survival among children and adolescents in Europe since the 1970s. (the ACCIS project): an epidemiological study. *Lancet* 2004;**364**:2097–2105.

Stiller CA, Desandes E, Danon SE, Izarzugaza I, Ratiu A, Vassileva-Valerianova Z, Steliavora-Foucher E. Cancer incidence survival in European adolescents. 1978–1997. Report from the Automated Childhood Cancer Information System project. *Eur J Cancer* 2006;**42**:2006–2018.

Suomalainen L, Hakala JK, Pentikäinen V, Otala M, Erkkilä K, Pentikäinen MO, Dunkel L. Shingosine-1-phosphate in inhibition of male germ cell apoptosis in the human testis. *J Clin Endocrinol Metab* 2003;**88**:5572–5579.

Tal R, Botchan A, Hauser R, Yogev L, Paz G, Yavetz H. Follow-up of sperm concentration and motility in patients with lymphoma. *Hum Reprod* 2000;**15**:1985–1988.

Tanaka A, Nagayoshi M, Awata S, Matawari Y, Tanaka I, Kusunoki H. Completion of meiosis in human primary spermatocytes through in vitro cocultures with Vero cell. *Fertil Steril* 2003;**79**:795–801.

Tesarik J, Guido M, Mendoza C, Greco E. Human spermatogenesis in vitro: respective effects of follicle stimulating hormone and testosterone on meiosis, spermiogenesis, and Sertoli cell apoptosis. *J Clin Endocrinol Metab* 1998;**83**:4467–4473.

Tesarik J, Bah Ceci M, Ozcan C, Greco E, Mendoza C. Restoration of fertility by in vitro spermatogenesis. *Lancet* 1999;**353**:555–556.

Tournaye H, Goossens E, Verheyen G, Frederickx V, De Block G, Devroey P, Van Steirteghem A. Preserving the reproductive potential of men and boys with cancer: current concepts and future prospects. *Hum Reprod Update* 2004;**10**:525–532.

Trefil P, Micakova A, Mucksova J, Hejnar J, Poplstein M, Bakst MR, Kalina J, Brillard JP. Restoration of spermatogenesis and male fertility by transplantation of dispersed testicular cells in the chicken. *Biol Reprod* 2006;**75**:575–581.

Trottmann M, Becker AJ, Stadler T, Straub J, Soljanik I, Schlenker B, Stief CG. Semen quality in men with malignant diseases before and after therapy and the role of cryopreservation. *Eur Urol* 2007; **52**:355–367.

van Alphen MM, van de Kant HJ, de Rooij DG. Repopulation of the seminiferous epithelium of the rhesus monkey after X irradiation. *Radiat Res* 1988;**113**:487–500.

van Alphen MM, van de Kant HJ, de Rooij DG. Protection from radiation-induced damage of spermatogenesis in the rhesus monkey (Macaca mulatta) by follicle-stimulating hormone. *Cancer Res* 1989; **49**:533–536.

Van Beek RD, Smit M, van den Heuvel-Eibrink MM, de Jong FH, Hakvoort-Cammel FG, van den Bos C, van den Berg H, Weber RFA, Pieters R, de Muinck Keizer-Schrama S. Inhibin B is superior to FSH as a serum marker for spermatogenesis in men treated for Hodgkin's lymphoma with chemotherapy during childhood. *Hum Reprod* 2007; **22**:3215–3222.

Van den Berg H, Repping S, van der Veen F. Parental desire and acceptability of spermatogonial stem cell cryopreservation in boys with cancer. *Hum Reprod* 2007;**22**:594–597.

Van Saen D, Goossens E, de Block G, Tournaye H. Regeneration of spermatogenesis by grafting testicular tissue or injecting testicular cells into the testes of sterile mice: a comparative study. *Fertil Steril* 2009; **91**:2264–2272.

Vassiliou G, Amrolia P, Roberts IA. Allogeneic transplantation for haemoglobinopathies. *Best Pract Res Clin Haematol* 2001;**14**:807–822.

Viviani S, Santoro A, Ragni G, Bonfante V, Bestetti O, Bonadonna G. Gonadal toxicity after combination chemotherapy for Hodgkin's disease. Comarative results of MOPP vs ABVD. *Eur J Cancer Clin Oncol* 1985;**21**:601–605.

Wallace WH, Anderson RA, Irvine DS. Fertility preservation for young patients with cancer: who is at risk and what can be offered? *Lancet Oncol* 2005;**6**:209–218.

Waxman JH, Ahmed R, Smith D, Wrigley PF, Gregroy W, Shalet S, Crowther D, Rees LH, Besser GM, Malpas JS *et al.* Failure to preserve fertility in patients with Hodgkin's disease. *Cancer Chemother Pharmacol* 1987;**19**:159–162.

Williams D, Crofton PM, Levitt G. Does ifosfamide affect gonadal function? *Pediatr Blood Cancer* 2008;**50**:347–351.

Wistuba J, Mundry M, Luetjens CM, Schlatt S. Cografting of hamster (Phodopus sungorus) and marmoset (callithrix jacchus) testicular tissues into nude mice does not overcome blockade of early spermatogenic differentiation in primate grafts. *Biol Reprod* 2004; **71**:2087–2091.

Wistuba J, Luetjens CM, Wesselmann R, Nieschlag E, Simoni M, Schlatt S. Meiosis in autologous ectopic transplants of immature testicular tissue grafted to Callithrix jacchus. *Biol Reprod* 2006;**74**:706–713.

Wu Z, Falciatori I, Molyneux LA, Richardson TE, Chapman KM, Hamra FK. Spermatogonial culture medium: an effective and efficient nutrient mixture for culturing rat spermatogonial stem cells. *Biol Reprod* 2009;**81**:77–81.

Wyns C. *Male Fertility Preservation after Gonadotoxic Treatment. Ph.D. Thesis*. Belgium: Catholic University of Louvain, 2008.

Wyns C, Curaba M, Martinez-Madrid B, Van Langendonckt A, Wese F-X, Donnez J. Spermatogonial survival after cryopreservation and short-term orthotopic immature human cryptorchid testicular tissue grafting to immunodeficient mice. *Hum Reprod* 2007;**22**:1603–1611.

Wyns C, Van Langendonckt A, Wese F-X, Donnez J, Curaba M. Long-term spermatogonial survival in cryopreserved and xenografted immature human testicular tissue. *Hum Reprod* 2008;**23**:2402–2414.

Yu J, Cai ZM, Wan HJ, Zhang FT, Ye J, Fang JZ, Gui YT, Ye JX. Development of neonatal mouse and fetal human testicular tissue as ectopic grafts in immunodeficient mice. *Asian J Androl* 2006;**8**: 393–403.

Zeng W, Avelar GF, Rathi R, Franca LR, Dobrinski I. The length of the spermatogonic cycle is conserved in porcine and ovine testis xenografts. *J Androl* 2006;**27**:527–533.

Zhang Z, Renfree MB, Short RV. Succesful intra- and interspecific male germ cell transplantation in the rat. *Biol Reprod* 2003;**68**:961–967.

Zimmermann U, Mimietz S, Zimmermann H, Hillgartner M, Schneider H, Ludwig J, Hasse C, Hasse A, Rothmund M, Fuhr G. Hydrogel-based non-autologous cell and tissue therapy. *Biotechniques* 2000; **29**:564–581.

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