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Cryopreservation of testicular tissue or testicular cell suspensions: a pivotal step in fertility preservation

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BACKGROUND: Germ cell depletion caused by chemical or physical toxicity, disease or genetic predisposition can occur at any age. Although semen cryopreservation is the first reflex for preserving male fertility, this cannot help out prepubertal boys. Yet, these boys do have spermatogonial stem cells (SSCs) that able to produce sperm at the start of puberty, which allows them to safeguard their fertility through testicular tissue (TT) cryopreservation. SSC transplantation (SSCT), TT grafting and recent advances in *in vitro* spermatogenesis have opened new possibilities to restore fertility in humans. However, these techniques are still at a research stage and their efficiency depends on the amount of SSCs available for fertility restoration. Therefore, maintaining the number of SSCs is a critical step in human fertility preservation. Standardizing a successful cryopreservation method for TT and testicular cell suspensions (TCSs) is most important before any clinical application of fertility restoration could be successful.

OBJECTIVE AND RATIONALE: This review gives an overview of existing cryopreservation protocols used in different animal models and humans. Cell recovery, cell viability, tissue integrity and functional assays are taken into account. Additionally, biosafety and current perspectives in male fertility preservation are discussed.

SEARCH METHODS: An extensive PubMED and MEDline database search was conducted. Relevant studies linked to the topic were identified by the search terms: cryopreservation, male fertility preservation, (immature)testicular tissue, testicular cell suspension, spermatogonial stem cell, gonadotoxicity, radiotherapy and chemotherapy.

OUTCOMES: The feasibility of fertility restoration techniques using frozen-thawed TT and TCS has been proven in animal models. Efficient protocols for cryopreserving human TT exist and are currently applied in the clinic. For TCSs, the highest post-thaw viability reported after vitrification is 55.6 ± 23.8%. Yet, functional proof of fertility restoration in the human is lacking. In addition, few to no data

are available on the safety aspects inherent to offspring generation with gametes derived from frozen-thawed TT or TCSs. Moreover, clarification is needed on whether it is better to cryopreserve TT or TCS.

WIDER IMPLICATIONS: Fertility restoration techniques are very promising and expected to be implemented in the clinic in the near future. However, inter-center variability needs to be overcome and the gametes produced for reproduction purposes need to be subjected to safety studies. With the perspective of a future clinical application, there is a dire need to optimize and standardize cryopreservation and safety testing before using frozen-thawed TT of TCSs for fertility restoration.

Key words: cryopreservation / testicular tissue / testicular cell suspensions / slow freezing / vitrification / fertility restoration / prepubertal boys

Introduction

Long-term preservation and storage of biological samples outside the body is achieved through cryopreservation. At low temperatures, the biological metabolism of living cells dramatically diminishes thereby stopping enzymatic and chemical reactions. Hence, long-term preservation of cells and tissues is possible. This strategy is of key importance for scientific research and many modern clinical therapies, including reproductive medicine. This emerging field encompasses cryopreservation of gametes (sperm and oocytes), embryos and reproductive cells [testicular cell suspensions (TCSs)] and tissues [ovarian tissue and testicular tissue (TT)] (Fuller and Paynter, 2004).

In clinical practice, ensuring the patient's health is the primary need. However, to face infertility in adult life undermines a patient's psychological well-being (Schover *et al.*, 1999). Fertility preservation is of great importance to guarantee the quality of life of these patients (Oosterhuis *et al.*, 2008). Reproductive stem cell loss begets from a variety of conditions including chemo- and radiotherapy (Thomson *et al.*, 2002), genetic and congenital conditions such as Klinefelter' syndrome (KS; 47,XXY) (Aksglaede *et al.*, 2006) and cryptorchidism (Schroeder *et al.*, 2013).

In pre- and peripubertal girls, cryopreservation of ovarian cortical tissue, followed by transplantation at adult age, has demonstrated the ability to restore fertility and to generate live births (De Vos *et al.*, 2014; Demeestere *et al.*, 2015).

For adult men and boys with ongoing spermatogenesis, sperm banking must always be offered as a first line treatment (Palermo *et al.*, 1992). In men, this is a validated and non-invasive procedure to preserve fertility (Tournaye *et al.*, 2014). Assisted reproduction techniques with semen cryopreserved before the onset of gonadotoxic treatment has shown good fertility outcome (Agarwal *et al.*, 2004; Anderson *et al.*, 2015). However, for prepubertal boys not producing sperm, this option does not exist. Nonetheless, these boys do have spermatogonial stem cells (SSCs) able to produce sperm from puberty onwards, giving them the alternative to cryopreserve TT or TCSs to safeguard their chances of having offspring (Goossens *et al.*, 2013).

Cryopreservation of a testicular biopsy provides several options after thawing: (i) autologous SSC transplantation (SSCT); (ii) grafting TT to the testis or to a heterotopic area; or (iii) *in vitro* spermatogenesis (IVS) (Sato *et al.*, 2011; Zhou *et al.*, 2016). Alternatively, TCSs can be cryopreserved, although this excludes the possibility of a graft. To keep all options available, TT sampling has been recommended. These fertility restoration techniques have been successfully applied in several animal models (Dobrinski *et al.*, 1999a; Shinohara *et al.*, 2002; Hermann *et al.*, 2012; Sato *et al.*, 2013) and are considered to be very promising for future clinical applications (Fig. 1).

Who should be offered SSC preservation?

Infertility can have a dramatic psychosocial impact during adulthood. For a large group of male patients without the alternative of sperm cryopreservation, SSC banking represents an option to prevent this distress. Several groups of patients might benefit from SSC banking.

Patients facing cancer treatment

Of children diagnosed with cancer, 80% are expected to survive their disease (Hudson, 2010). Since 30% of male childhood cancer survivors are azoospermic at adult age (Thomson *et al.*, 2002), TT banking is recommended.

A variety of cancer conditions indicate for TT banking. Children and adolescents with testicular cancer, leukemia or Ewing sarcoma are at the highest risk of developing permanent sterility after cancer treatment (Tournaye *et al.*, 2014).

The effect of chemotherapy on spermatogenesis varies substantially depending on the combination of drugs used and potential negative effects on future fertility should be followed up continuously during adolescence. Nitrogen mustard derivatives, alkylating drugs and cisplatin seem to have the most detrimental effect on germ cell proliferation (Loren *et al.*, 2013; Tournaye *et al.*, 2014).

Irradiation injures the germ-cell pool in a dose-dependent manner (Rowley *et al.*, 1974; Wallace *et al.*, 2005). Doses as low as 0.1 Gy to more than 4 Gy can result in oligozoospermia or even complete sterility (Howell and Shalet, 2005; Wyns *et al.*, 2010). Testicular irradiation with doses over 20 Gy might cause Leydig cell dysfunction in prepubertal boys (Shalet *et al.*, 1989). Moreover, dose fractionation and most importantly the cumulated dose are major risk factors for permanent sterility (Ash, 1980; Tournaye *et al.*, 2014).

Although spermatogenesis is not yet active in prepubertal boys, the testicular environment is not quiescent. There is a constant turnover of germ cells and SSCs continuously divide to populate the growing seminiferous tubules. Since chemotherapy kills dividing cells, the SSCs of children are also at risk (Chemes, 2001; Jahnukainen *et al.*, 2011; Goossens *et al.*, 2013).

Patients with life-threatening non-malignant diseases

Gonadotoxic treatments are also used as a conditioning treatment and/or to cure a variety of life-threatening non-malignant conditions (Slavin *et al.*, 2002; Passweg and Rabusin, 2008; Lukusa *et al.*, 2009). Common treatments for drepanocytosis, thalassemia, idiopathic medulla aplasia and granulomatous disease are full body radiotherapy to deplete the blood stem cell line before hematopoietic stem cell transplantation and hydroxyurea regimens (Bernaudin *et al.*, 2007;

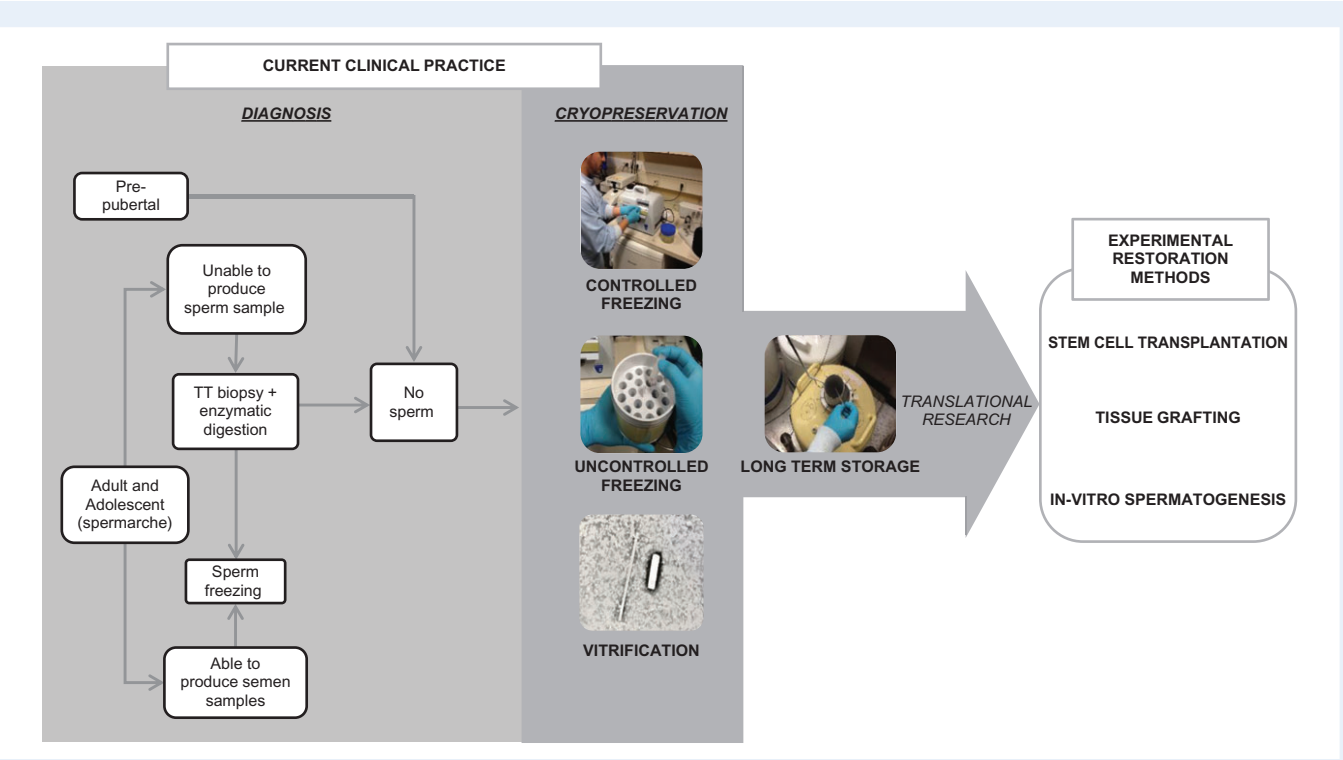

Figure 1 Clinical set-up for spermatogonial stem cell (SSC) preservation in prepubertal, adolescent and adult patients at high risk of infertility. Before the start of the gonadotoxic treatment or expected SSC loss, according to the age and pubertal stage, tissue or semen samples are retrieved. SSCs are cryopreserved during the time of therapy and recovery. Different cryopreservation techniques (e.g. controlled and uncontrolled freezing and vitrification) are being optimized to effectively preserve these samples. Later when the patient is in full remission and fertility restoration techniques are available, tissue pieces or cell suspensions can be thawed for autologous transplantation or *in vitro* spermatogenesis. In the best-case scenario, SSCs could (recolonize the seminiferous tubules and) reinitiate spermatogenesis, leading to mature spermatozoa.

Picton *et al.*, 2015). Any condition requiring bone marrow transplantation involves a high infertility risk (>80%) for prepubertal boys (Sadri-Ardekani and Atala, 2014a).

Additionally, patients dealing with severe autoimmune diseases (e.g. juvenile systemic lupus and systemic sclerosis) (Anserini *et al.*, 2002; Oktay and Oktem, 2009; Sadri-Ardekani and Atala, 2014a) that necessitate administration of high dose chemotherapy may opt for TT cryopreservation.

Patients enduring genetic and congenital conditions

Of men experiencing azoospermia, 15% suffer from KS (Van Assche *et al.*, 1996; Foresta *et al.*, 1999). KS is the most common sex chromosome abnormality in humans (1/600 live births). Yet, only 10% of KS patients are diagnosed before puberty (Bojesen *et al.*, 2003). In a retrospective study for TT banking, analysis of seven non-mosaic 47,XXY adolescents, aged between 13 and 16 years, demonstrated massive fibrosis and hyalinization of the testis. Spermatogonia were detected in five out of seven patients. Only in the youngest patient, the spermatogonia were located in non-degenerating seminiferous tubules (Van Saen *et al.*, 2012a). Indeed, early development of the testis appears to be normal in boys with KS, yet, SSC depletion occurs in mid-puberty, leading to infertility (Gies *et al.*, 2012a). In adult KS patients, spermatogenic foci may remain, although spermatozoa can be retrieved by TESE in less than 50% (Tournaye *et al.*, 1996; Sciurano *et al.*, 2009). This implies that freezing of semen samples or TT sampling should be offered to boys with KS preferably before puberty, at the onset of puberty, or as soon as it is diagnosed (Wikström *et al.*, 2007; Gies *et al.*, 2012a,b; Van Saen *et al.*, 2012a,b). However, to this date, no clinical parameters are available to efficiently diagnose and detect patients who might benefit from these techniques (Gies *et al.*, 2012b). Retrieval of SSCs in prepubertal boys with KS should therefore still be viewed as experimental and patients and their parents must be counseled accordingly (Van Saen *et al.*, 2012a,b).

Cryobiology and cryopreservation-induced cell death

SSC cryopreservation is a cost-effective and efficient method to preserve genetic material for decades (Lee *et al.*, 2014a). Success in tissue and/or cell cryopreservation is built upon the understanding of biophysical fundamentals underlying any cryobiological protocol (Fuller and Paynter, 2004).

As summarized in Fig. 2, along cooling, cells and tissues lose osmotic equilibrium within their medium. Extracellular medium starts freezing with temperatures around −5°C, yet, the cytoplasm remains unfrozen. Between −5 and −10°C, cells supercool and the growth of extracellular ice leads to an increase of solute (electrolyte) concentration in the extracellular medium. The cells equilibrate with the

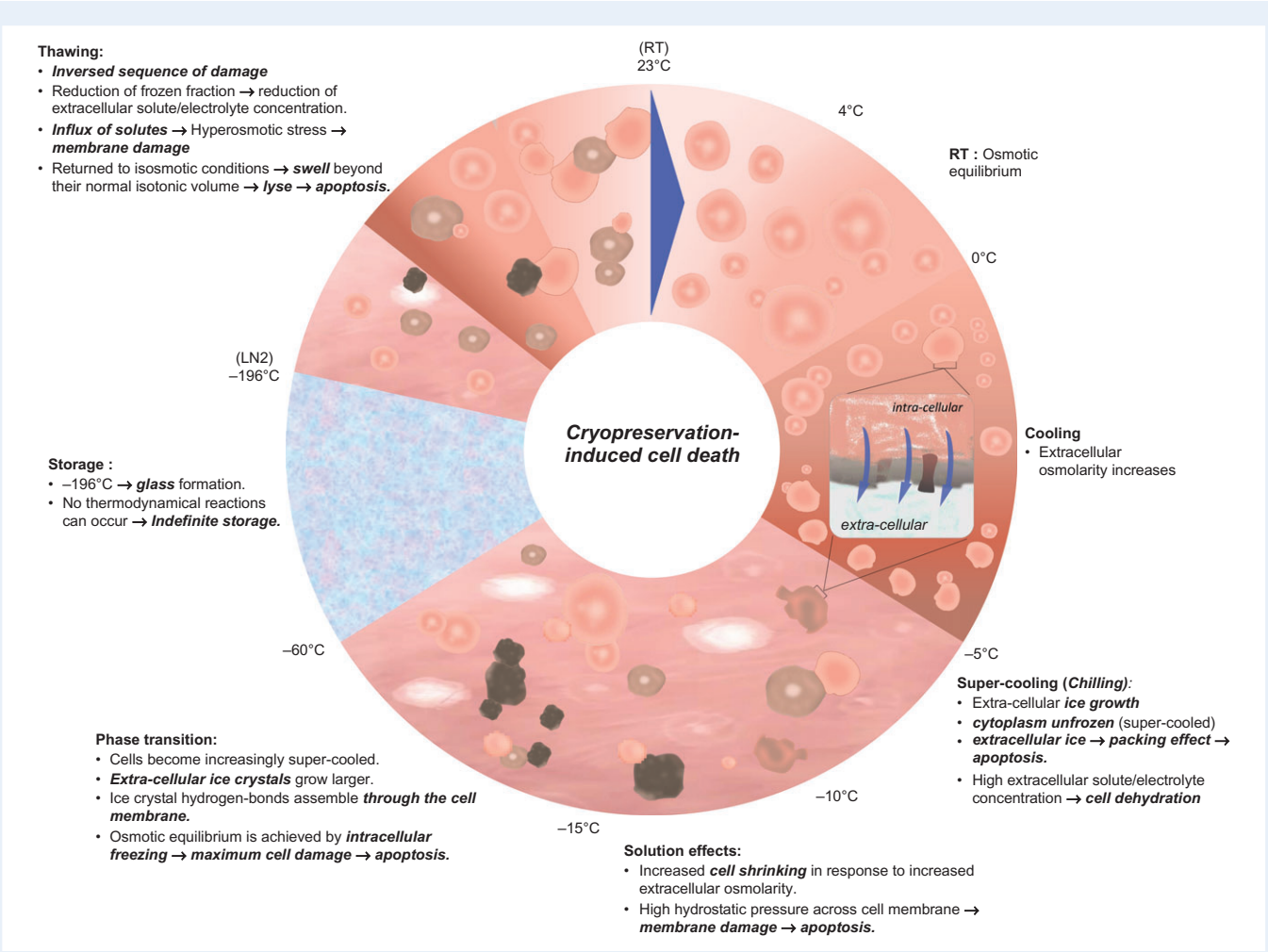

Figure 2 Schematic of physical events underlying the freezing, storing and thawing.

medium by losing water causing severe cell dehydration and shrinkage. Between −10 and −15°C, the extracellular ice expands, increasing cell-ice and cell–cell contacts. These lead to a packing effect and may result in cell damage. The major hurdle for cells to surpass is the water to ice phase transition. Indeed, between −15 and −60°C, cells become increasingly supercooled. Extracellular ice crystals grow larger, and exceptionally, ice crystal hydrogen-bonds assemble through the cell membrane, leading to osmotic equilibrium via intracellular freezing. Intracellular ice freezing is considered the major degree of cryopreservation-induced cell damage. Hence, the ability of cells and tissues to endure the lethality of this intermediate zone (between −15 and −60°C), that they must traverse twice during cooling and warming, is crucial for their survival (Mazur, 1970, 1977).

There is evidence that the intrinsic response of cells to cryopreservation is different depending upon whether the cells are part of a tissue or whether they are isolated in a cell suspension. Indeed, scaling up of cryopreservation from a microscopic cellular level to a macroscopic tissue level will introduce heat and mass transfer phenomena (Karlsson and Toner, 1996). Heat transfer limitations relate to thermal conductivity of a tissue sample. Generally it is more difficult to achieve rapid cooling and warming rates in tissues compared with cell suspensions, implying non-uniform rates of cooling throughout a tissue sample. Hence, the temperature will change more slowly in the interior of the sample compared with the surface. Mass transfer and the subsequent redistribution of water are determined by membrane-limited water transport in the case of individual cells, whereas cell–cell interactions and the diffusive processes must be taken into consideration for multicellular tissues (Levin *et al.*, 1977; Karlsson and Toner, 1996).

To improve cell survival during freezing and thawing, cryoinjury caused by intracellular ice crystal formation has to be avoided. Addition of cryoprotective agents (CPAs) and controlling freezing and thawing rates are intended to diminish the width of the possible damage window during cryopreservation by lowering ice formation temperatures to −40°C. Two classes of CPAs exist, permeating CPAs (e.g. dimethyl sulfoxide [DMSO], glycerol, formamide and propanediol) and non-permeating CPAs including sugars (e.g. sucrose, trehalose, dextran, lactose and d-mannitol) and high molecular weight compounds (e.g. polyethylene glycol and hydroxyethyl starch). Their cryoprotective action consists of decreasing the concentration of solutes and increasing membrane stability during the dehydration and rehydration phases (Lee *et al.*, 2014b).

Vitrification may be a promising alternative to freezing. It leads to utrarapid liquid solidification and thus, avoids ice formation. In this technique, very high concentrations of CPAs are combined with ultra-rapid cooling rates (>10^6 °C/min) with the intention of preventing crystal formation in the solution and allowing the cells to shrink slowly enough to avoid membrane damage. This is followed by direct plunging of the samples into liquid nitrogen to avoid phase transition damage (Yavin and Arav, 2007).

The major steps in a cryopreservation process can be summarized as follows: (i) addition of CPAs to cells/tissues before cooling; (ii) cooling of the cells/tissues toward a low temperature (e.g. −196°C, the liquid nitrogen temperature) at which the cells/tissues are stored; (iii) warming the cells/tissues; and (iv) removal of the CPAs from the cells/tissues after thawing.

Animal studies have underlined the pivotal role of viable and functional SSCs on the efficiency of any of the prospective fertility restoration procedures (TTG, SSCT and IVS). Comparisons of functional assays using fresh to cryopreserved samples indicate loss of SSCs during the cryopreservation procedure. Thus, the effectiveness of the cryopreservation procedures is critical and should be improved (Izadyar *et al.*, 2002a,b; Shinohara *et al.*, 2002; Frederickx *et al.*, 2004; Baert *et al.*, 2012; Hermann *et al.*, 2012; Jahnukainen *et al.*, 2012; Sá *et al.*, 2012; Pacchiarotti *et al.*, 2013; Sato *et al.*, 2013).

This review describes the basics of male reproductive tissue and cell cryopreservation. It gives an overview of existing cryopreservation protocols used in different animal models. Important parameters such as cell recovery, cell viability, tissue integrity and functional assays are taken into account. Additionally, biosafety and current perspectives in male fertility preservation are discussed. As an increasing number of centers start to cryopreserve TT samples, the paramount goal of this work is to inform the reader about the need to develop and standardize a proper procedure to cryopreserve TT and TCSs in order to optimize the success rates of future clinical procedures (Ruutiainen *et al.*, 2013; Picton *et al.*, 2015).

Methods

A broad PubMED and MEDline database search was conducted. Search terms were: cryopreservation, male fertility preservation, (immature)testicular tissue, testicular cell suspension, spermatogonial stem cell, gonadotoxicity, radiotherapy and chemotherapy. An initial search query lead to a total of 4154 papers associated to the topic. Selection criteria based on relevance to the topic, outcome of interest (i.e. percentage of viable cells after testicular cell suspension cryopreservation, tissue morphology, and functional test by SSCT) and potential clinical application, resulted in a total of 352 scientific and review articles. Finally, 54 studies linked to the main subject of interest, published in English or French between 1996 and 2015, were referenced in this review (Fig. 3).

Results

Testicular tissue cryopreservation

In the 1990s, cryopreservation of TT emerged as a safe and effective means to facilitate the treatment of couples with azoospermia. From one procedure, it enables the storage of enough spermatozoa for multiple In-vitro fertilization (IVF)/Intra-cytoplasmic sperm injection (ICSI) cycles while reducing patient costs and risks of repeated

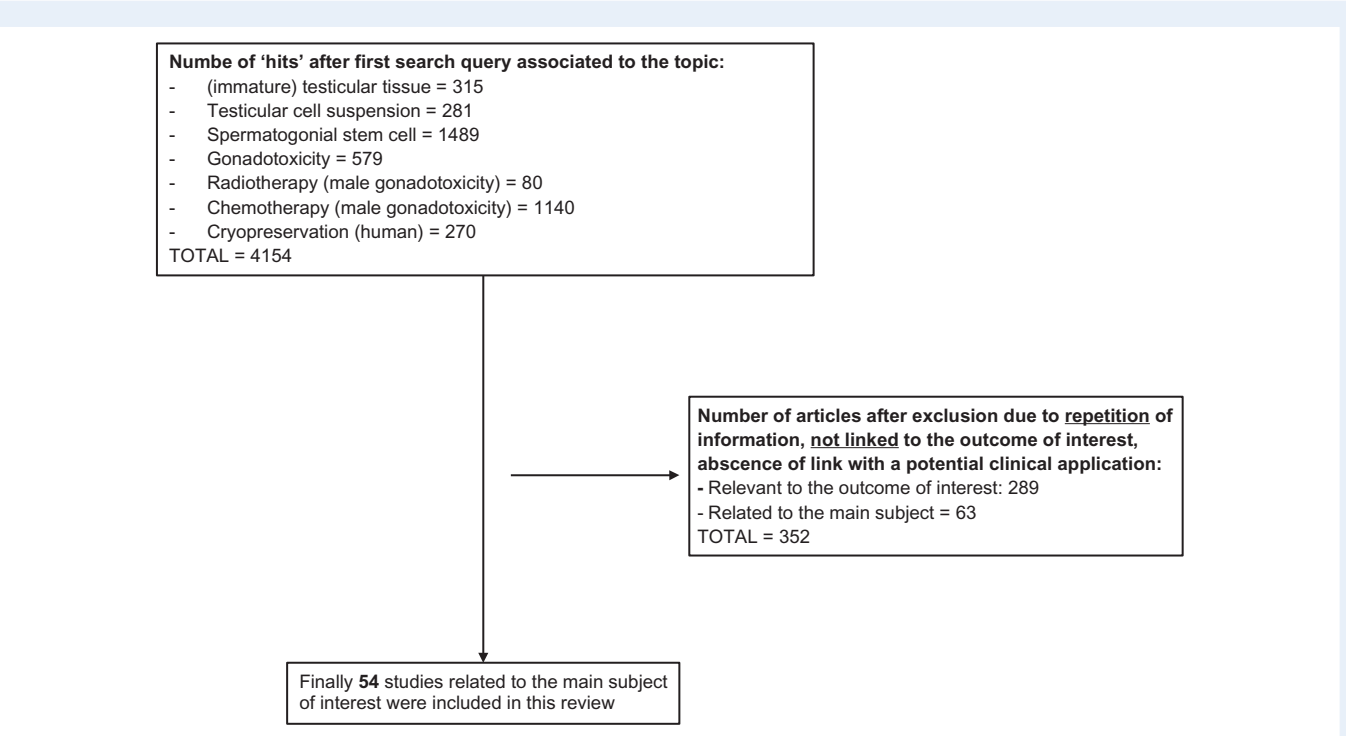

Figure 3 Analysis diagram for inclusion and exclusion criteria of studies in this review.

surgeries (Hovatta *et al.*, 1996; Trombetta *et al.*, 2000; Garg *et al.*, 2008). The main purpose of early TT cryopreservation protocols was thus to preserve the most mature stages of spermiogenesis. The freezing media contained glycerol as this is used worldwide as the preservative of choice for spermatozoa, although it is not optimal for TT (Crabbe *et al.*, 1999). However, the pioneering work of Brinster and colleagues brought TT cryopreservation in a new light. By elegantly showing that spermatogenesis could be restored by transplanting cryopreserved SSCs in the seminiferous tubules of sterilized mice (Avarbock *et al.*, 1996), it was quickly recognized that TT cryopreservation could also be helpful in preserving the fertility of prepubertal boys who are undergoing gonadotoxic chemotherapy or radiotherapy (Nugent *et al.*, 1997). Therefore, adapted TT cryopreservation protocols were needed with a focus on preserving SSCs and their supportive cells rather than mature germ cells. To find such, the rationale was to strive for minimal cryoinjury and maximal cell recovery. This posed a challenge, owing to the various cell types in TT, each differing in dimension, complexity, water permeability, and hence requiring different optimal cryopreservation protocols. The major achievements made during the last two decades regarding tissue preparation, cooling, cryoprotection, warming after storage and storage vessels to cryopreserve TT are reviewed here.

Tissue preparation

The macroscopic physical dimension of the tissue is a major point to be defined in a cryopreservation protocol. It is key to achieving equal distribution with rapid in and out diffusion of CPAs and uniform rates of temperature change to limit cryoinjury (Karlsson and Toner, 1996). This is especially true for vitrification, as the sample size is a critical variable in the probability of successful solidification of the aqueous milieu of the cells/tissue into a non-crystalline glassy phase. It is also important in the prevention of devitrification which occurs during warming and is characterized by the formation of ice crystals (Yavin and Arav, 2007). Yet, few data exist on the effect of sample size on TT cryopreservation. Nevertheless, some important observations have been made. For instance, it seemed that results of the studies differed depending on the donor species. It was shown that whole testes with pricked tunica albuginea were better preserved than testes with intact tunica, testes without tunica and testis halves, after rapid freezing of immature mouse TT (Gouk *et al.*, 2011). In agreement, another rodent study proved that the original tissue size can influence the outcome of TT cryopreservation as immature rat tissue showed fewer morphological alterations when 7.5 mg fragments were slow frozen compared to 15 mg fragments, albeit using a similar cryopreservation protocol (Travers *et al.*, 2011). Nevertheless, the cell viability of immature porcine testicular tissues undergoing the same cryopreservation treatment was not affected by the original size of the tissue fragment (5, 15, 20 or 30 mg) (Abrishami *et al.*, 2010). Also, for the cryopreservation of tissue from prepubertal boys, varying fragments sizes (2–9 mm^3) were successfully frozen (Keros *et al.*, 2007; Wyns *et al.*, 2007, 2008; Curaba *et al.*, 2011a; Poels *et al.*, 2013, 2014; Picton *et al.*, 2015). As evidenced by studies using whole ovaries, it would be interesting to see if whole human testes can be cryopreserved (Pfeifer *et al.*, 2014).

Cooling, cryoprotection and warming after storage

The two most commonly used cryopreservation procedures are slow (controlled or uncontrolled) freezing and vitrification. Rapid freezing has been reported as well, although without great success (Gouk *et al.*, 2011). Slow freezing and vitrification differ mainly in concentrations of CPAs and cooling rates used. Slow freezing with controlled-freezing rates allows modifying the soaking temperature to master cell dehydration before reaching temperatures at which intracellular ice nucleation occurs, thereby minimizing the probability of harmful intracellular ice crystal formation. A recent study found that soaking at −9°C better preserved the ability of frozen-thawed mouse SSCs to generate haploid germ cells after *in vitro* maturation than −7°C and −8°C (Arkoun *et al.*, 2015). Initiating ice-nucleation manually after soaking is often performed during controlled slow freezing as an additional protective step, although this may not be necessary and may even be harmful (Milazzo *et al.*, 2008; Baert *et al.*, 2013). Further down the line, slow freezing with either controlled or uncontrolled cooling rates takes advantage of the regulatory properties of extracellular ice formation to gradually dehydrate cells when the temperature slowly decreases. This further reduces the risk of intracellular ice. In addition to aspects related to the freezing rate, slow freezing avoids cytotoxicity because this procedure generally requires low CPA concentrations. Freezing solutions yielding good results in animal and human models contain penetrating CPAs like DMSO (0.7–3 M), ethylene glycol (EG, 1.5 M) or glycerol (1 M) with or without the addition of sucrose (0.05–0.1 M) or serum (5–80%) (Table I). As discussed above, the formation of extracellular ice, may not pose a problem for cryopreservation of cell suspensions, but is likely to be the main problem in tissues. Therefore, during vitrification, ice crystal formation is bypassed by using ultrafast cooling rates and higher concentrations of CPAs, e.g. 6.2 M EG combined with 24 M polyvinylpyrrolidone and 0.3 M trehalose. Often a combination of multiple CPAs is used to minimize their individual cytotoxicity (2.1–2.6 M DMSO besides 2.7–2.8 M EG with or without 0.5 M sucrose and 20–25% serum) (Table I). This should, in theory, avoid injuries associated with mechanical disfiguration. Incomplete vitrification, though, it may induce the damaging mechanism associated with fast cooling rates. Because cells are only partially dehydrated at fast cooling rates, cell injury is attributed to intracellular ice formation or membrane rupture due to strong osmotic fluxes (Mazur, 1970; Muldrew and McGann, 1994).

An overview of the most compelling mammalian TT cryopreservation protocols is given in Table I and include mouse, rat, pig, bovine, monkey and human studies. Some of these present interesting and promising results on post-thaw cell and tissue integrity, and show the ability to purify viable SSCs for further use (Milazzo *et al.*, 2008; Gouk *et al.*, 2011; Wu *et al.*, 2011, 2014; Unni *et al.*, 2012; Gholami *et al.*, 2013; Pacchiarotti *et al.*, 2013; Yango *et al.*, 2014; Zhang *et al.*, 2015). However, these evaluations are merely indicative for the quality of the cryopreservation protocol. Testicular cell activity assessment by *in vitro* culture or transplantation assays should be included in TT cryopreservation studies, as cell and tissue integrity do not necessarily correctly predict the functionality of the testicular cells, hence it may lead to false positive or negative discrimination (Jahnukainen *et al.*, 2007; Zeng *et al.*, 2009; Baert *et al.*, 2012). Using animal models, several protocols have proven to preserve immature TT. In rodents, maintenance of tissue integrity and activity

Table I Overview of experimental cryopreservation protocols for mammalian testicular tissue.

Protocol	Species	(Im) mature	Cryoprotectant	Freezing rate	Main outcome	Reference
Controlled	Mouse	Mature	1.5 M DMSO + 0.1 M sucorse + 1% HSA	Start: 0°C, −2°C/min to −7°C, seeding, −0.3°C/min to −40°C, −10°C/min to −140°C, LN2	Spz in isografts	Schlatt *et al.* (2002)
		Immature	1.5 M DMSO + 0.1 M sucorse + 1% HSA	Start: 0°C, −2°C/min to −7°C, seeding, −0.3°C/min to −40°C, −10°C/min to −140°C, LN2	Spz in isografts	Schlatt *et al.* (2002)
			DMSO + FCS (concentrations not mentioned)	Start: 4°C, −2°C/min to −7°C, hold 10 min, −0.3°C/min to −30°C, LN2	Offspring using spz from allografts	Shinohara *et al.* (2002)
			1.5 M DMSO + 0.05 M sucrose + 10% FCS	Start: 5°C, −2°C/min to −9°C, hold 7 min, −0.3°C/min to −40°C, 25°C/min to −150°C, LN2	Maintenance of tissue integrity and activity in organotypic tissue culture, spz in allografts	Milazzo *et al.* (2008, 2010), Travers *et al.* (2011)
			0.7 M DMSO + 5% HSA	Start: 4°C, −1°C/min to −0°C, hold 5 min, −0.5°C/min to −8°C, seeding + hold 10 min, −0.5°C/min to −40°C, hold 10 min, −7°C/min to −70°C, LN2	Spz in allografts	Yildiz *et al.* (2013)
	Rat	Mature	1 M DMSO + 20% FBS	Start: RT, −2°C/min to −9°C, hold 5 min + seeding, −0.3°C/min to −40°C, 10°C/min to −140°C, LN2	Preservation of testicular cells	Unni *et al.* (2012)
		Immature	1.5 M DMSO + 0.05 M sucrose + 10% FCS	Start: 5°C, −2°C/min to −9°C, hold 7 min, −0.3°C/min to −40°C, 25°C/min to −150°C, LN2	Maintenance of tissue integrity and activity in organotypic tissue culture	Travers *et al.* (2011)
			1 M DMSO + 20% FBS	Start: RT, −2°C/min to −9°C, hold 5 min + seeding, −0.3°C/min to −40°C, 10°C/min to −140°C, LN2	Preservation of testicular cells	Unni *et al.* (2012)
	Porcine	Immature	1 M glycerol + 5% FBS	Start: 4°C, −1°C/min to −0°C, hold 5 min, −0.5°C/min to −8°C, hold 15 min + seeding, −0.5°C/min to −40°C, hold 10 min, −7°C/min to −80°C, LN2	Spz in xenografts	Abrishami *et al.* (2010)
	Human	Mature	0.7 M DMSO + 5% patient serum	Start: 4°C, −1°C/min to −0°C, hold 5 min, −0.5°C/min to −8°C, hold 15 min + seeding, −0.5°C/min to −40°C, hold 10 min, −7°C/min to −80°C, LN2	Maintenance of tissue integrity and activity in organotypic tissue culture	Keros *et al.* (2005)
			0.5 M EG + 20% FBS	Start: RT, −2°C/min to −9°C, hold 5 min + seeding, −0.3°C/min to −40°C, 10°C/min to −140°C, LN2	Preservation of testicular cells	Unni *et al.* (2012)
			1.4 M DMSO + 10% HSA + 1% Dextran	Start: 5°C, −1°C/min to −80°C,−50°C/min to −120°C, LN2	Preservation of testicular cells	Pacchiarotti *et al.* (2013)
		Immature	1.5 M EG + 0.1 M surcorse + 10% HSA	Start: 1°C, −2°C/min to −9°C, hold 5 min + seeding, −0.3°C/min to −40°C, −10°C/min to −140°C, LN2	Maintenance of tissue integrity and activity in organotypic tissue culture	Kvist *et al.* (2006)
			0.7 M DMSO + 5% HSA	Start: 4°C, −1°C/min to −0°C, hold 5 min, −0.5°C/min to −8°C, seeding + hold 10 min, −0.5°C/min to −40°C, hold 10 min, −7°C/min to −70°C, LN2	Maintenance of tissue integrity and activity in organotypic tissue culture	Keros *et al.* (2007); Pietzak *et al.* (2015)
			0.7 M DMSO + 0.1 M surcorse + 10% HSA	Start: 0°C, hold 9 min, −0.5°C/min to −8°C, hold 5 min + seeding, hold 15 min, −0.5°C/min to −40°C, hold 10 min, −7°C/min to −80°C, LN2	Maintenance of tissue integrity and activity in xenografts	Wyns *et al.* 2007, (2008); Curaba *et al.* (2011a); Poels *et al.* (2013, 2014)
Uncontrolled	Mouse	Immature	1.5 M DMSO + 0.07 M sucrose	Insulated container in −80°C, LN2	Spz in allografts	Goossens *et al.* (2008), Baert *et al.* (2012)
			Cell Banker 1	Vial in −80°C, LN2	Offspring using spz from organotypic culture	Yokonishi *et al.* (2014)
	Sheep	Immature	3 M DMSO + 20% FBS	Insulated container in −80°C, LN2	Spz after xenografting	Pukazhenthi *et al.* (2015)
	Deer	Immature	1.5 M DMSO + 80% FBS	Insulated container in −80°C, LN2	Spc in xenografts	Pothana *et al.* (2015)
	Bovine	Mature	1.4 M DMSO + 0–20% NCS	Insulated container in −80°C, LN2	Preservation of SSCs	Wu *et al.* (2011, 2014)
		Immature	2.8 M DMSO + 20% FBS	Insulated container in −80°C, LN2		Devi *et al.* (2014)

					Maintenance of tissue integrity and activity in organotypic tissue culture	
			1.5 M DMSO + 0.2 M trehalose + 10% FBS	2 h in −20°C, −80°C, LN2	Preservation of testicular cells	Zhang *et al.* (2015)
	Monkey	Immature	1.4 M DMSO + 10% FCS	In −20°C, seeding at −13°C, to −20°C, LN2	Spz in autografts	Jahnukainen *et al.* 2007, (2012)
	Human	Mature	1.5 M DMSO + 0.15 M sucrose + 10% HSA	Insulated container in −80°C, LN2	Maintenance of tissue integrity, preservation of cell dynamics in propagation culture	Baert *et al.* (2013), (2015)
			1.28 M DMSO + 25% FBS	Insulated container in −80°C, LN2	Preservation of SSCs	Yango *et al.* (2014)
Vitrification	Mouse	Immature	2.8 M DMSO + 2.8 M EG + 25% HSA	Straw, LN2	Maintenance of tissue integrity and activity in organotypic tissue culture	Curaba *et al.* (2011b)
			6.77 M EG + 0.6 M sucrose	Straw-in-straw, LN2	Preservation of SSCs	Gouk *et al.* (2011)
			2.1 M DMSO + 2.7 M EG + 0.5 M sucrose + 20% FBS	SSV	Spz in allografts, Spz in organotypic culture	Baert *et al.* (2012), Dumont *et al.* (2015)
			2.1 M DMSO + 2.7 M EG + 20% FBS + 0.5 M sucrose	Vial, LN2	Preservation of SSCs	Gholami *et al.* (2013)
			Stem Cell Keep	Vial, LN2	Offspring using spz from organotypic culture	Yokonishi *et al.* (2014)
	Porcine	Immature	2.1 M DMSO + 2.7 M EG + 0.5 M sucrose + 20% FCS	SSV	Spz in xenografts	Abrishami *et al.* (2010)
			35% EG + 5% PVP + 0.3 M trehalose	SSV	Offspring using spz from xenografts	Kaneko *et al.* (2013)
	Monkey	Immature	2.1 M DMSO + 2.7 M EG + 0.5 M sucrose + 25% HSA	Straw, LN2	Maintenance of tissue integrity and activity in xenografts	Poels *et al.* (2012)
	Human	Immature	2.8 M DMSO + 2.8 M EG + 25% HSA	Straw, LN2	Maintenance of tissue integrity and activity in organotypic tissue culture	Curaba *et al.* (2011a)
			2.1 M DMSO + 2.7 M EG + 0.5 M sucrose + 25% HSA	Straw, LN2	Maintenance of tissue integrity and activity in xenografts	Poels *et al.* (2013)

DMSO, dimethyl sulfoxide; EG, ethylene glycol; FBS, fetal bovine serum; HSA, human serum albumin; LN2, liquid nitrogen; NCS, newborn calf serum; spz, spermatozoa; SSC, spermatogonial stem cell; SSV, solid-surface vitrification.

in organotypic cultures was reported using slow freezing and vitrification (Travers et al., 2011; Curaba et al., 2011b). Complete germ cell differentiation was observed by *in vitro* maturation of vitrified-thawed TT fragments (Dumont et al., 2015), as well as after grafting slow-frozen mouse and rat samples and vitrified-thawed mouse samples (Schlatt et al., 2002; Milazzo et al., 2010; Baert et al., 2012; Yildiz et al., 2013). Importantly, true validation of slow freezing as a means to preserve TT functionality came with the publication from Shinohara et al. (2002) in which they reported birth of mouse offspring from sperm retrieved from samples grafted after thawing. Moreover, healthy offspring were produced with sperm grown from slow-frozen or vitrified-thawed immature mouse TT in an organotypic culture setup (Yokonishi et al., 2014). These achievements from the rodent model have been confirmed in higher mammals. Slow frozen bovine, sheep and deer TT tissue gave promising results in an organotypic culture or xenografting approach after thawing (Devi et al., 2014; Pothana et al., 2015; Pukazhenthi et al., 2015). Grafted immature porcine and non-human primate immature TT pieces that were cryopreserved by slow freezing or vitrification were able to sustain spermatogenesis (Jahnukainen et al., 2007, 2012; Abrishami et al., 2010; Poels et al., 2012). Furthermore, normal reproductive development of pigs produced from sperm that was retrieved from vitrified-thawed xenotransplanted immature TT was reported (Kaneko et al., 2013, 2014). For human tissue, studies focusing on slow freezing have resulted in well-established protocols using programmable freezers that are able to keep the tissue integrity and activity in organotypic cultures or long-term xenografts (Kvist et al., 2006; Keros et al., 2007; Wyns et al., 2007, 2008; Curaba et al., 2011a; Poels et al., 2013, 2014). To maximize the quality and integrity of human TT during cryopreservation, there is a continuous search to improve or replace current protocols. In recent years, faster and cheaper alternatives are on the rise. Similar results to programmed slow freezing were observed with vitrified-thawed immature human TT (Curaba et al., 2011a; Poels et al., 2013). In addition, our group has published an uncontrolled slow freezing protocol able to protect the integrity of adult human TT and maintain cell dynamics in long-term propagation culture, with results similar to fresh controls (Baert et al., 2013, 2015). However, confirmation with immature TT is still warranted.

Upon removal from storage, CPAs that have reached the internal compartments of cells must diffuse back through numerous membranes in the tissue regardless of whether ice crystal formation occurred during freezing or was circumvented by vitrification. Earlier studies pointed out that consistent cooling and warming rates, for instance slow cooling followed by slow thawing, or fast cooling followed by fast thawing, can improve cell/tissue survival after cryopreservation. However, as mentioned before, cell damage in tissues can occur during warming due to the heat and mass transfer limitation (CPA cytotoxicity or recrystallization/devitrification) (Mazur, 1970; Karlsson and Toner, 1996). Therefore, optimal procedures for thawing and CPA removal are also critical for tissue survival after cryopreservation. However, there has been very limited research addressing these issues. Tissues are generally removed from storage by rapid warming and gradual removal of loaded CPAs. The ambient temperature for warming can have great impact as thawing of adult bovine tissue at 37°C and 97–100°C resulted in better cell viability and spermatogonial survival compared to that at 4°C (Wu et al., 2011).

Storage vessels

Different TT freezing protocols have been developed over the years using either straws or vials to store cryopreserved TT. The choice of the vessel was mostly made based on previous literature and the logistic situation. To our knowledge, only the group of Rives performed a comparative study in which they found that the morphology of immature rat TT was better protected using vials compared to straws (Travers et al., 2011).

In contrast to freezing, studies dealing with vitrification carefully consider which vessel to be used during cryopreservation: an open or closed device. Open devices are devices allowing direct contact of the sample with the cooling solution, typically liquid nirogen (LN_2). Using open devices, the cooling rates achieved are in general approximately 20,000–30,000°C/min which favors good vitrification of the sample (Scholz, 2012). Indeed, open vitrification systems, i.e. open straw, have been successfully employed to preserve the integrity and activity in organotypic culture and long-term xenografting of immature mouse, monkey and human TT after cryopreservation (Curaba et al., 2011b; Poels et al., 2012, 2013). The problem is that direct contact with the cooling solution introduces a risk of pathogen transmission to the sample during cooling and a high risk of cross contamination in the container. In closed systems on the other hand, the sample is not in contact with the cooling solution during freezing or storage, thereby, tackling the problem of contamination. One drawback of a closed system is that the cooling rate is much lower and therefore requires higher concentrations of CPAs to prevent ice crystal formation. This makes the protocols potentially more dangerous for cells due to the cytotoxicity of CPAs (Scholz, 2012). However, two closed vitrification systems have been described for TT cryopreservation with exciting results. Firstly, vitrification of immature mouse TT was successfully performed by simply plunging the vial containing the CPA-submerged samples in LN_2 (Gholami et al., 2013; Yokonishi et al., 2014). Secondly, vitrification was achieved by solid-surface vitrification. This procedure includes exposing TT samples to a vitrification solution before placing them on a sterile aluminum boat floating on LN_2, and transferring the samples into precooled vials followed by plunging them into LN_2 (Abrishami et al., 2010). Using this approach, the functionality of immature mouse and porcine TT could be maintained during cryopreservation (Baert et al., 2012; Kaneko et al., 2013, 2014; Dumont et al., 2015). Our group studied the feasibility of vitrifying mature human tissue with a protocol proven to preserve the functionality of immature porcine TT upon xenografting (Abrishami et al., 2010). However, we observed signs of cryoinjury to the human TT and therefore solid-surface vitrification needs further optimization for human applications (Baert et al., 2013).

Testicular cell suspension cryopreservation

Since the introduction of the SSCT technique in 1994 (Brinster and Zimmermann, 1994), cryobanking of TCSs has been proposed as a plausible solution for fertility preservation in prepubertal boys (Avarbock et al., 1996; Frederickx et al., 2004). Indeed, TCS cryopreservation might facilitate SSC survival bypassing the hurdles of heat and mass exchange encountered with tissue cryopreservation (Wyns et al., 2010). However, the handling and cryopreservation of TCSs, requires exposition to high concentrations of digestive and cryoprotective solutions which may interfere with cell recovery,

viability and functionality. Yet, the ability of TCSs to endure long-term cryopreservation (>14 years) was evaluated by Wu *et al.* (2012). Maintenance of viability and the ability to colonize and reestablish spermatogenesis was shown in recipient seminiferous tubules after SSCT. Importantly, in non-human primates, successful regeneration of spermatogenesis has been reported in the rhesus. Sperm obtained after SSCT with cryopreserved TCSs permitted the generation of embryos by *in vitro* fertilization (Hermann *et al.*, 2012).

Tissue preparation

In contrast to cryopreservation of tissues, isolation of single cell suspensions requires enzymatic digestion which might influence germ cell viability and change the biophysical properties of the cells, increasing cell sensitivity to the cryopreservation process (Karlsson and Toner, 1996). Isolation of mouse testicular cell populations rely on a two-step enzymatic digestion based on the activity of collagenase and trypsin (Bellvé *et al.*, 1977). Evidence of cell stress is the reduced viability and functionality of cell suspensions after digestion and re-suspension (Griswold, 1998; Brook *et al.*, 2001; Joyce *et al.*, 2001). Mechanical tissue disaggregation is an efficient and time-effective alternative to enzymatic digestion. High cell numbers, viability and spermatogonial enrichment were reported after mechanical disaggregation of TT (Schneider *et al.*, 2015). However, the cell viability in the samples was highly variable among suspensions, suggesting the need for further optimization of this method.

Cooling, cryoprotection and warming after storage

Regardless the disaggregation method, TCSs contain (immature) Sertoli cells, Leydig cells, myoid cells and only a small percentage of SSCs. Similar to other tissue-specific stem cells, SSCs are rare and represent only 0.03% of all germ cells in the rodent testes (Phillips *et al.*, 2010). This heterogeneous cell population varies in function, size, water content, and membrane permeability. Together with the scarcity of SSCs, this renders the development of an efficient cryopreservation procedure a real challenge. Literature reports a post-thaw viability ranging from 29 to 68%, depending on the cryopreservation method used and the type of cell concentration and viability assessment. An overview of these studies is shown in Table II.

In a first attempt to cryopreserve mouse SSCs, simple cryopreservation procedures for cultured somatic cells were extrapolated to TCSs (Avarbock *et al.*, 1996). This same cryopreservation method was used for rabbit and dog TCSs (Dobrinski *et al.*, 1999a). After freezing and thawing, cells were transplanted into recipient mice. These donor cells were able to colonize mouse testes but did not differentiate beyond the stage of spermatogonia as a consequence of the phylogenetic distance between donor and recipient animals. Yet, this provided strong evidence that SSCs of many mammalian species could be preserved for long periods using a similar cryopreservation protocol (Avarbock *et al.*, 1996; Ogawa *et al.*, 1999; Dobrinski *et al.*, 1999a, 2000). This was unexpected as cryopreservation methods for mature spermatozoa differ between species. Subsequently, a first attempt to establish a freezing protocol for crude human TCSs was reported by Brook *et al.* (2001). Human TCSs were isolated and cryopreserved through a controlled-rate freezing method with glycerol as CPA. Yet, an overall low cell viability was observed. Furthermore, no clinical-grade requirements nor functional assays were included in this study.

Additionally, information on the optimal cooling rate specific for SSCs remains scant. SSCs are presumed to have a comparable size and nucleus/cytoplasm ratio to lymphocytes, for which an optimal cooling rate of 10°C/min has already been defined (Thorpe *et al.*, 1976; Frederickx *et al.*, 2004). However, development of a cryopreservation method specific for purified bovine type A spermatogonia resulted in enhanced survival rates when using DMSO and an uncontrolled cooling rate of 1°C/min, which is the optimal rate for hematopoietic stem cells (Grilli *et al.*, 1980; Donaldson *et al.*, 1996; Izadyar *et al.*, 2002b). This study highlighted the advantage of an uncontrolled-rate freezing to the non-linear cooling rate (Izadyar *et al.*, 2002b). Indeed, up to the point of ice formation (Fig. 2), high cooling rates increase the risk of 'fast cooling damage' (Izadyar *et al.*, 2002b). After that point, uncontrolled freezing increases the freezing rate approaching −80°C/min at temperatures between −40°C and −60°C (Liu *et al.*, 2000) minimizing the risk of damage. In addition, frozen-thawed type A spermatogonia retained their ability to colonize the testis of a recipient mouse demonstrating that long-term preservation of type A spermatogonia (including SSCs) is possible without apparent harmful effects to their function. To circumvent the risk of 'fast cooling damage', Frederickx *et al.* (2004) reported controlled-freezing of TCSs at a rate below 1°C/min. On the one hand, slow controlled-freezing leads to better cell dehydration and reduced intracellular ice formation (Mazur, 1990), but on the other, this causes an increase in intracellular and extracellular solute concentrations, stressing the cell membrane by extreme shrinkage (Mazur and Rigopoulos, 1983). After thawing and transplantation, cell survival was acceptable and reinstallation of spermatogenesis was achieved but the efficiency could be improved.

Given the low viability achieved with controlled and uncontrolled methods, intracellular ice crystal formation could still be the main cause for cell damage. Indeed, achieving optimal freezing rates for SSCs is a real hurdle. The rate has to be slow enough to prevent production of intracellular ice and yet rapid enough to minimize the time that cells are exposed to solution effects (Mazur, 1984). Thus, other options need to be considered. An elegant study by Sá *et al.* (2012) showed 55.6 ± 23.8 % viability for human adult diploid testicular cell suspensions after performing vitrification in open pulled straw (OPSs). To this date, this is the best recovery rate reported in the human model. Its advantage was due to the benefits of evading ice formation through vitrification. However, OPS vitrification requires direct contact between LN_2 and the medium containing SSCs. This contact carries a potential hazard for transmission of infective agents, rendering this method difficult to implement in the clinical practice (Martino *et al.*, 1996; Vajta *et al.*, 1998).

DMSO, a widely used CPA, has been found to promote cell survival during cryopreservation. DMSO cryoprotective properties are observed at a concentration of 1.5 M (Table II) (Frederickx *et al.*, 2004). DMSO is a slow permeating molecule that penetrates rather freely across cell membranes and prevents intracellular ice crystal formation (Akkök *et al.*, 2011). Such concentrations have proven to allow supercooled cells to freeze well above −40°C thereby shortening the time window of possible damage during cryopreservation (Mazur, 1984; Karlsson *et al.*, 1993). Yet, due to its toxicity, the high concentration and long exposure of DMSO could have several consequences when used in a clinical application (Brayton, 1986; Zambelli *et al.*, 1998). Pacchiarotti *et al.* (2013) prompted an investigation to

Table II Overview of experimental cryopreservation protocols for mammalian testicular cell suspensions.

Protocol	Species	(Im) mature	Cryoprotectant	Freezing rate	Viability after thawing (%) ± STDV	Main outcome	Reference
Controlled	Mouse	Immature	1.5 M DMSO	Start: room temp, −5°C/min to −7°C, hold 15 min + seeding, −0.3°C/min to −80°C, LN2	48 ± 6	Donor derived spermatogenesis	Frederickx *et al.* (2004)
				Start: room temp, −5°C/min to −7°C, hold 15 min + seeding, −0.3°C/min to −40°C, LN2	56 ± 2		
			1.5 M EG	Start: room temp, −5°C/min to −7°C, hold 15 min + seeding, −0.3°C/min to −40°C, LN2	60 ± 8		
	Bovine	Immature	1.4 M glycerol (10% FCS)	Start: 5°C, −1°C/min to −80°C, −50°C/min to −120°C, LN_2	34 ± 5	Recipient testicular colonization	Izadyar *et al.* (2002a,b)
				Start: 5°C, −1°C/min to −80°C, −50°C/min to −120°C, LN_2	29 ± 4		
			1.4 M DMSO (10% FCS)	Start: 5°C, −1°C/min to −80°C, −50°C/min to −120°C, LN_2	55 ± 4		
				Start: 5°C, −5°C/min to −80°C, −50°C/min to −120°C, LN_2	39 ± 4		
	Human	Adult[a]	10% HAS, 10% DMSO, 1% Dextran	Start: room temperature, 4°C for 10 minutes, −1°C/min to −80°C, −50°C/min to −120°C, LN_2	33 ± 5	No functional assay performed	Pacchiarotti *et al.* (2013)
Uncontrolled	Mouse	Mature	3 M DMSO	Insulated container −70°C, LN_2	3 ± 4	Donor derived spermatogenesis resulting in offspring	Wu *et al.* (2012)
			DMSO	Insulated container −80°C, LN_2	67 ± 6	Donor derived spermatogenesis resulting in offspring	Kanatsu-Shinohara *et al.* (2003)
		Immature	1.5 M DMSO	Insulated container −80°C, LN_2	36 ± 2	Donor derived spermatogenesis	Frederickx *et al.* (2004)
			1.5 M DMSO (10% FCS) 200 mM dissacharide (*trehalose*)	Insulated container −80°C, LN_2	N/A	Donor derived spermatogenesis	Lee *et al.* (2014b)
			3 M DMSO	Insulated container −70°C, LN_2	12 ± 4	Donor derived spermatogenesis resulting in offspring	Wu *et al.* (2012)
	Rat	Mature	3 M DMSO	Insulated container −70°C, LN_2	2 ± 2	N/A	Wu *et al.* (2012)
	Hamster	Mature	1.5 M DMSO	Insulated container −70°C, LN_2	43 ± 8	xenogeneic spermatogenesis	Ogawa *et al.* (1999)
	Rabbit	Mature	3 M DMSO	Insulated container −70°C, LN_2	13	N/A	Wu *et al.* (2012)
	Rabbit	Mature Immature	1.5 M DMSO	Insulated container −70°C, LN_2	63 ± 22	Recipient testicular colonization	Dobrinski *et al.* (1999a,b)
	Dog	Mature Immature	1.5 M DMSO	Insulated container −70°C, LN_2	63 ± 22	Recipient testicular colonization	Dobrinski *et al.* (1999a,b)
	Boar	Immature	1.5 M DMSO (10% FCS) 200 mM dissacharide (*trehalose*)	Insulated container −80°C, LN_2	62 ± 3	Recipient testicular colonization	Lee *et al.* (2014a)
	Bovine	Immature	No cryoprotectant (10% FCS)	Insulated container −80°C, LN_2	36 ± 3	Recipient testicular colonization	Izadyar *et al.* (2002a,b)
			1.4 M DMSO (10% FCS)	Insulated container −80°C, LN_2	49 ± 5		
					60 ± 5		
			1.4 M DMSO (20% FCS)	Insulated container −80°C, LN_2	49 ± 5		
			1.4 M DMSO, 0.07 M sucrose		68 ± 3		

		1.4 M DMSO, 0.14 M sucrose			67 ± 3		
		1.4 M DMSO, 0.21 M sucrose			66 ± 4		
	Baboon	Mature	3 M DMSO	Insulated container −70°C, LN_2	13	N/A	Wu *et al.* (2012)
	Rhesus monkey	Mature	10% DMSO	Insulated container −80°C, LN_2	58 ± 4	Recipient testicular colonization	Hermann *et al.* (2007)
Vitrification	Human	Adult	(1)1.1 M DMSO, 1.34 M EG, 10% HSA (2)0.67 M S, 2.3 M DMSO, 3.0 M EG, 10%HSA	open pulled straw (OPS) vitrification	56 ± 24	No functional assay performed	Sá *et al.* (2012)
N/A	Human	Adult Fetal	1.28 M DMSO 25% FCS	−1°C/min, LN_2	75 ± 4 67 ± 4	No functional assay performed	Yango *et al.* (2014)

DMSO: dimethyl sulfoxide, EG: ethylene glycol, FCS: fetal bovine serum, HSA: human serum albumin, N/A: information not available, LN_2: liquid nitrogen.
[a]Sexual reassignment

develop an effective clinical-grade procedure for the cryopreservation of human testicular cells and/or tissue under current Good Tissue Practice and Manufacturing practices regulations. DMSO, dextran and human serum albumin (HSA) were the components of the cryopreservation medium. This study reported DMSO to be suitable for safe and effective handling of human testicular cells and tissues, as it has been validated in human cord blood (Rubinstein *et al.*, 1995), bone marrow (Odavic *et al.*, 1980) and peripheral blood stem cell freezing (Körbling and Freireich, 2011) and other clinical applications (Pacchiarotti *et al.*, 2013). Yet, this study used TT and cells from sexual reassignment patients, hence, further studies are needed to determine whether these findings obtained from hormone-treated patients can be generalized to other patients. Studies aiming to reduce, replace or combine DMSO with less toxic CPAs have not found a better substitute for DMSO in terms of recovery, survival and functionality of frozen-thawed cells (Table II) (Izadyar *et al.*, 2002b; Frederickx *et al.*, 2004; Pacchiarotti *et al.*, 2013; Lee *et al.*, 2013a, 2014b).

Addition of polymeric non-penetrant CPAs is believed to protect the cell by forming a viscous shell stabilizing cell membranes during dehydration and rehydration (Anchordoguy *et al.*, 1987; Sum *et al.*, 2003; Lee *et al.*, 2014b). Supplementing cryoprotective media with different sugar molecules (glucose, fructose, mannose, galactose, trehalose) increased post-thaw viability in mouse SSCs (Izadyar *et al.*, 2002b; Lee *et al.*, 2013b, 2014b). Uncontrolled freezing with a basic medium supplemented with fetal calf serum, DMSO and 50 mM trehalose (a high molecular weight sugar) resulted in a significantly increased post-thaw cell viability and preserved the functional capacities of mouse SSCs during one week of culture compared to cells frozen using the same freezing method without trehalose (Lee *et al.*, 2013b). The use of a higher concentration of trehalose (200 mM) in a serum-free cryopreservation medium confirmed its effectiveness and revealed a concentration-dependent increase of the proliferation capacity of the frozen-thawed cells. Additionally, employing a similar cryopreservation medium has proven to be effective in the cryopreservation of porcine SSCs, thus demonstrating effectiveness in a higher animal model (Lee *et al.*, 2014a). Yet, no studies have observed the influence of different CPA sugar molecules on human TCS.

Storage at −196°C is favored for TCSs. In theory, at this temperature, cryopreserved cells and tissues can endure storage for centuries (Mazur, 1984; Swain and Smith, 2010). Yet, to this date, the sole proof for effectively storing functional SSCs for a long-term was given by the birth of fertile offspring from mouse SSCs which had been stored for approximately 14 years (Wu *et al.*, 2012).

In the same way as freezing, thawing can cause cellular and extracellular damage although in an inversed sequence (crystal formation, ice formation and rehydration). Cryopreserved TCSs are often thawed by immersion in a 37°C water bath, which was found to be better than thawing in ice water (Frederickx *et al.*, 2004). This corroborated the hypothesis that rapid warming avoids growth of ice crystals, which may have occurred during rapid cooling (Mazur, 1970). CPA removal procedures are also critical factors for cell survival after cryopreservation. However, thawing is often poorly controlled. Clinical grade cryopreservation and banking require standardization, automation and safety assessment of all the technological steps including thawing. In view of the implementation of a clinical

procedure, controlled thawing is still an unexplored alternative (Gurina et al., 2015).

Storage vessels

To the best of our knowledge there are no studies that compare different vessels (e.g straws, vials) and it is therefore difficult to make recommendations. Yet, Saragusty et al. (2009) described how volume and cell concentration affect cell viability after cryopreservation. With ice growth, the intra-vessel pressure increases, causing the cells to pack in the unfrozen part of the medium and provokes cell destruction by a 'pack effect'. The larger 'surface-area-to-volume ratio' in vials compared to straws might reduce this 'pack effect' allowing easier extracellular ice expansion (Saragusty et al., 2009).

It remains a matter of debate, whether it is better to cryopreserve TT or TCSs (Wyns et al., 2010). Pacchiarotti et al. (2013) and Yango et al. (2014) stated that TT cryopreservation should be recommended. However, SSEA-4+ cells (a spermatogonial population) exhibited differential sensitivity to cryopreservation based on whether they were cryopreserved as TTs or as TCSs. Unfortunately, no functional proof was provided.

How to assay the functionality of SSCs?

It has been shown that cell viability does not necessarily correspond to the functional capacity of the SSCs (Frederickx et al., 2004; Jahnukainen et al., 2007; Zeng et al., 2009). In mammals, re-establishment of spermatogenesis is the only available system by which the stem cell function can be assessed. True validation of slow freezing and vitrification to preserve SSC functionality has been proven through TT tissue grafting and SSCT. In mouse (Shinohara et al., 2002; Frederickx et al., 2004; Wu et al., 2012) and higher mammals (Jahnukainen et al., 2007, 2012; Abrishami et al., 2010; Poels et al., 2012; Devi et al., 2014; Pothana et al., 2015; Pukazhenthi et al., 2015) spermatogenesis and offspring have been achieved after these procedures (Tables I and II). Functional frozen-thawed SSCs in suspension will generate colonies of donor-derived spermatogenesis after SSCT (Avarbock et al., 1996; Ogawa et al., 1999; Dobrinski et al., 1999b; Izadyar et al., 2002a,b; Frederickx et al., 2004; Hermann et al., 2007). The number of functional SSCs relates to the colony formation after transplantation (Nakagawa et al., 2007). Full proof for SSC functionality and fertility restoration is the production of donor-derived offspring (Kanatsu-Shinohara et al., 2003; Wu et al., 2012). SSCT with frozen-thawed cultured human SSCs to immunodeficient mice have demonstrated the ability of these cells to colonize the recipients tubules (Sadri-Ardekani et al., 2009).

Despite the promising results obtained in different models, the variability of methods used to assess cell viability and, more importantly, functionality hinders the translation to human SSCs. Additionally, the efficiency of the cryopreservation protocols can only be evaluated by transplantation assays, but this is not yet possible for human TT and TCSs. Thus, as long as there is no efficient way to evaluate the functionality for human SSCs after freezing, it is difficult to conclude which of the tested protocols is optimal.

Clinical, biological and genetic biosafety in cryopreservation of SSCs

Although fertility can be restored in mice having undergone SSCT or TTG, the question remains whether this process could be applied in a clinic in a safe and acceptable manner. So far, only a few reports have addressed safety issues related to the clinical implementation of male fertility restoration strategies using SSCs.

Cancer cell contamination

Many hematopoietic malignancies are capable of metastasizing through the blood. Testicular biopsies from cancer patients may contain malignant cells and thus, the risk for cancer relapse exists when transplanting these samples. For these patients, the preservation and transplantation of cancer-cell-free testicular cell suspensions is a requirement. Assessing the risks of transplanting carcinogenous cells into a cured patient is a critical step in the translation of the proposed fertility restoration techniques into a clinical application. In rats, transplanting only 20 leukemic cells to the testis, caused malignant relapse (Jahnukainen et al., 2001). In the human, the threshold number of malignant cells able to cause malignant relapse when transplanted to the testis is unknown. Therefore, it is of immense importance to detect contamination in the TT samples (Hou et al., 2007; Goossens et al., 2013). In case of contamination, the separation of SSCs from malignant cells before transplantation is necessary. Cell sorting techniques such as magnetic-activated and/or fluorescence-activated cell sorting, selective matrix adhesion, and selective cell culturing aiming to deplete cancer cells have been studied. Yet, in mice and human models the reported results are insufficient. The lack of specific SSC surface markers and the aggregation of germ and leukemic cells are limiting factors in positive selection of germ cells (Fujita et al., 2006; Geens et al., 2007, 2011; Dovey et al., 2013; Goossens et al., 2013; Valli et al., 2014). A pilot study by Sadri-Ardekani and Atala (2014b) reported a testicular cell culture system not only efficient for propagation of SSCs but also for eliminating contaminating acute lymphoblastic leukemai (ALL) cells. Culturing testicular cells in combination with ALL cells permitted the elimination of ALL cells after 26 days of culture. Anyhow, for patients at risk of malignant contamination, autologous TTG will not be possible, so the only option to restore fertility will be by transplanting cell suspensions or IVS.

Storage of TTs and TCSs in LN_2 holds the risk that samples become infected with agents, especially viral agents, inadvertently released into storage tanks from other (infected) samples (Fuller and Paynter, 2004). Despite the fact that the LN_2 liquid phase provides a more stable temperature for storage, storing in the vapor phase appears to be safer (Tedder et al., 1995; Fountain et al., 1997).

Genetics

Unfortunately, compared with the increasing body of evidence illustrating the effectiveness of SSCT and TTG in reproductive terms, only a few studies have addressed safety concerns regarding the influence on genetic and epigenetic modifications in germ cells. After SSCT and TTG with fresh samples, neither numerical chromosomal alterations, nor epigenetic modifications were detected in spermatozoa (Goossens et al., 2009, 2010, 2011). Extended cell manipulation and exposition to digestive and cryoprotective solutions, plus the hurdle of cryopreservation itself, may induce modifications in the germ

line cells, e.g. changes in DNA methylation, or chromosomal abnormalities. Indeed, the disruption of SSCs from their niche might influence the establishment of correct epigenetic patterns and thus the development of spermatozoa arising from the possibly altered SSC (Goossens *et al.*, 2011). Oocytes fertilized by spermatozoa arising from epigenetically altered SSCs develop abnormally. Only a few reports have addressed the influence of cryopreservation on genetic and epigenetic changes in SSCs. DMSO was found to induce modifications in the cell's epigenetic profile (Iwatani *et al.*, 2006; Kawai *et al.*, 2010). Wu *et al.* (2012) evaluated the ability of TCSs to endure long-term cryopreservation (>14 years). Cultured mouse and rat SSCs re-established complete spermatogenesis and produced fertile mouse progeny without apparent genetic or epigenetic errors. Also, genetic stability and spermatogenic function of adult mouse SSCs were reported after transplanting cultured and cryopreserved TCSs into germ cell-ablated recipients (Yuan *et al.*, 2009). For TTs, cryopreservation has reported no deleterious effect on meiotic recombination and synapsis (Li *et al.*, 2009). In non-human primates, sperm obtained after SSCT using cryopreserved TTCs was reported in the rhesus. The functional ability of donor-derived sperm to fertilize rhesus oocytes by *in vitro* fertilization and to stimulate early embryo development suggested that the sperm were functionally normal. Unfortunately, no surrogacy, thus, no proof of normal embryo development in the offspring was reported (Hermann *et al.*, 2012). Further studies need to be conducted to confirm and examine the potential genetic or epigenetic changes in SSCs and offspring using frozen-thawed prepubertal TT or TCS samples.

Conclusion and future recommendations

Testicular toxicity is an inevitable long-term consequence of several therapeutic oncological regimens, leading to gonadal failure or sterility in many patients. Cryopreservation of TT is an option for fertility preservation when other techniques such as cryopreservation of ejaculated sperm are not available or applicable. Given the limited number of SSCs in the testes, an optimal cryopreservation protocol is a prerequisite for the successful clinical application of any fertility restoration strategy (Frederickx *et al.*, 2004; Phillips *et al.*, 2010).

Until now, the Univeristaire Ziekenhuis (UZ) Brussel has stored TT from more than 90 prepubertal boys. Recently, Picton *et al.* (2015) divulged the outcome of a survey distributed to 24 hospitals in Europe and Israel prior to December 2012, where they stated that more than 260 young patients had already undergone TT retrieval for fertility preservation. Of the responding hospitals, 50% actively offer TT cryobanking for fertility preservation in boys and adolescents and the remainder were seriously considering install a tissue-based fertility preservation program. However, a recent study of 23 French regional sperm and tissue banks recorded considerable inter-center variations in practices involving young patients seeking to preserve their fertility before cancer therapy, demonstrating that new fertility preservation strategies such as immature TT cryopreservation are still underused (Daudin *et al.*, 2015). In consequence, germ cell banking is not universally practiced in pediatric oncology centers and practices differ between centers (Anderson *et al.*, 2015). Furthermore, cryopreservation of human prepubertal testicular tissue or cells has not yet resulted in sperm production, neither in vivo nor *in vitro*, and therefore, albeit their clinical application, none of the proposed cryopreservation protocols have been fully validated.

Effective protocols for human TT are currently applied in the clinic (Keros *et al.*, 2005; Kvist *et al.*, 2006; Keros *et al.*, 2007; Wyns *et al.*, 2007; Baert *et al.*, 2013). To date, controlled slow freezing protocols using 0.7 to 1.5 M DMSO, 0.1 M sucrose and 5 to 10% HSA for adult and prepubertal human TT have reported encouraging results, including the preservation of testicular cells, the maintenance of TT integrity and activity in organotypic tissue culture (Table I).

For cancer patients, tissue could contain malignant cells and thus, the risk of cancer relapse exists when using these samples. Thus, cancer cell-free TCSs would be the best option to preserve and restore fertility for these patients (Sá *et al.*, 2012). Currently, OPS vitrification, including high concentrations of DMSO (1.1 to 2.8 M), non-penetrant CPAs and 10% HSA has resulted in the highest cell viability. However, this technique is at risk of introducing infectious agents, thus, lacks the option of a clinical application. In contrast, a controlled slow freezing protocol, produced under effective clinical-grade guidelines for the cryopreservation of human testicular cells, although achieving a lower cell viability, was conferred by Pacchiarotti *et al.* (2013) as an alternative in view of a clinical application.

Recommendations for the future

Optimization of cryopreservation protocols requires the refinements to the freezing and thawing rates, the osmotic conditions, the choice and concentration of CPAs, and the equilibration times in the CPAss. Improvements in all these factors might result in better survival and functionality of human tissue and cell samples permitting successful fertility restoration. An optimal cryopreservation protocol for human TT and TCS should benefit from the experience gathered in different animal models. These results should be judiciously used for the optimization and standardization of a clinical-grade cryopreservation protocol. For instance, alternatives to reduce, replace or combine toxic CPAs with less toxic factors such as antioxidant reagents and antiapoptotic factors are still unexplored alternatives which should be prompted (Aliakbari *et al.*, 2016). Additionally, the use of convenient and inexpensive techniques, such as uncontrolled freezing or vitrification, should be studied as an alternative to controlled freezing (Baert *et al.*, 2012). More importantly, information regarding the efficiency of these protocols and the influence on genetic and epigenetic modifications in germ cells after the cryopreservation procedure must be carefully studied.

For cancer patients, several burdens regarding biosafety lead to the question whether it is better to cryopreserve cell suspensions or TT. Later, the subsequent functional restoration of cryopreserved samples is a challenging task. Indeed, male fertility strategies such as SSCT, TTG and IVS are all promising experimental approaches to restore fertility (Orwig and Schlatt, 2005; Hermann *et al.*, 2012; Faes *et al.*, 2013; Nickkholgh *et al.*, 2014). Finally, since SSC cryopreservation has a major impact on the outcome of fertility restoration techniques, national guidelines and recommendations for good tissue banking would be helpful (Picton *et al.*, 2015).

Authors' roles

J.O. and Y.B. were involved in the conception and design of this review, drafting of the article and final approval. K.F. was involved in revision of the article and final approval. E.G. was involved in the conception and design of this review, revision of the article, and final approval.

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Conflict of interest

The authors declare that no competing interests exist.

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