<div style="text-align:center; background:blue; color:white;">**PERSPECTIVE**</div>

FREEZING PROTOCOLS FOR THE CRYOPRESERVATION OF IMMATURE TESTICULAR TISSUE – A SYSTEMATIC REVIEW

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Abstract

Increasing numbers of childhood cancer survivors reach adulthood making therapy induced infertility a growing concern. Sperm cryopreservation is not possible prior to puberty. Testicular tissue cryopreservation has been proposed as an alternative fertility preservation method for prepubertal males but no standardised cryopreservation procedure for immature tissue has been agreed to date. Here we review the current literature of cryopreservation protocols to determine which method best preserves the morphology and function of immature testicular tissue; and to examine which tissue intervention, grafting or tissue culture, is mostly likely to restore fertility. Embase, Medline, and Web of Science were systematically searched using relevant MeSH headings and search terms for testis, cryopreservation, and fertility preservation. This systematic search returned 4748 unique entries which were screened for relevance. Eleven studies were found to be eligible and were included in the systematic review. We found that cryopreservation protocols differ in freezing rate and cryoprotectant media, the optimum combination of which for ITT has yet to be determined. Further investigations must be carried out to decipher which method best preserves tissue integrity and function and which application method is most likely to induce spermatogenesis.

Keywords: cryopreservation; fertility preservation; oncofertility; testis.

INTRODUCTION

As a result of breakthroughs in science and medicine, the past 50 years has seen a dramatic increase in five year survival following paediatric cancer from as little as 39% 50 years ago (1), to more than 80% today (2). This notable reduction in mortality means a higher number of patients are reaching adulthood and the long-term health effects of treatment are becoming more evident. Today, estimates suggest approximately one in every 530 adults is a long term survivor of childhood cancer (2).

Infertility is one potential repercussion of gonadotoxic therapies used to treat malignancy that can affect survivors into adulthood. As more evidence uncovers the psychosocial effects of infertility (3), a shift is occurring in the field of paediatric oncology to bring fertility into the conversation and fully inform patients and their parents of treatment consequences.

Adults undergoing gonadotoxic therapies should be offered fertility preservation (FP) prior to the commencement of their treatment. Embryo, oocyte, and sperm cryopreservation are well established techniques for serving this

function (4). Unfortunately, these techniques are not practical and often impossible for children who have not yet reached reproductive maturity. Ovarian tissue cryopreservation (OTC) has become available in recent years for younger females or those unable to delay treatment. This FP technique, though still experimental, has resulted in over 130 live births to date (5, 6). Unlike females, who are born with all the gametes they will ever have, males continuously produce new gametes in the form of spermatozoa from the onset of puberty for the remainder of their life (7). This poses additional challenges in preserving fertility of males who have not yet reached puberty and begun the process of spermatogenesis. The most promising resolution is cryopreservation of immature testicular tissue (ITT) prior to gonadotoxic therapy (8, 9, 10) and restoration of spermatogenesis by transplantation of spermatogonial stem cells (SSCs) (11, 12), tissue grafts (13, 14, 15, 16), or in vitro spermatogenesis (17, 18, 19, 20, 21, 22, 23, 24), following treatment.

Testicular function and gonadotoxic therapies

Spermatozoa production takes place in the seminiferous tubules (STs) of the testis and takes approximately 72 days from beginning to the formation of mature spermatozoa (25). The germinal epithelium of the STs consists of SSCs and Sertoli cells, which are present from the basal layer through to the tubule lumen. SSCs are undifferentiated spermatogenic cells with pluripotent potential that most commonly differentiate into spermatogonia (SG), the precursor cells to spermatozoa (26). The distinct location where the SSCs reside in the seminiferous epithelium is also known as the SSC niche (27). Sertoli cells secrete growth factors and nourishment to support SSCs in their transition to mature spermatozoa (28).

SSCs divide continuously and give rise to new SSCs but their rapid proliferation can cause them to be unwanted targets of chemo- and radiotherapy (25). If SSCs are completely depleted, establishment or restoration of spermatogenesis is not possible. The loss may be attributed to the cytotoxic effects of alkylating agents (29, 30), radiation exposure (31), or to a lesser extent non-alkylating agents (30). There also appears to be a correlation between an increased risk of infertility and patient age, pathology, and individual sensitivities to treatment side effects (29, 31, 32, 33).

The somatic cells of the testis are less sensitive to therapy induced damage (32, 34) and as a result, they can continue to produce testosterone and induce the secondary characteristics of male puberty.

Current practices in prepubertal male fertility preservation

Cryopreservation of spermatozoa is the gold standard FP method for males. Unfortunately, this is not possible in prepubertal males and in many cases is not possible in puberty where patients are unable or unwilling to produce a sperm sample (35). Testicular tissue cryopreservation (TTC) involves the biopsy of tissue prior to- or in the early stages of treatment (9). Tissue is then cryopreserved and stored with the intention of using it to initiate or restore fertility in the future.

At present, TTC is primarily carried out on boys receiving gonadotoxic therapies for malignant conditions with small numbers of centres offering it for non-malignant conditions, including Klinefelter syndrome and haemoglobinopathies (36). Evidence is increasing that supports the safety of the surgical procedure, though there are reports of postoperative complications at a rate of 2-3% (9, 10, 37). The biopsied tissue is then divided into cubes and portioned. One portion is taken for morphological analysis and to check for malignant contamination and the rest is cryopreserved for future use (37, 38). Occasionally and with consent, some samples are retained for research (10).

To date, there have been no live births as a result of cryopreserved ITT (9, 39). However, as no FP alternatives exist for underage males, the procedure is now carried out in specialized centres globally, in anticipation of future medical advances allowing for it to restore fertility in future (9, 10, 39).

Future fertility restoration

There are several potential options for fertility restoration following the thawing of cryopreserved testicular tissue. The three most promising methods currently in development are: testicular tissue grafting, SSC transplantation and in vitro spermatogenesis/maturation (IVM).

Transplantation or grafting of tissue is theoretically advantageous as this preparation preserves not only SSCs but also their supporting cells and the cell-cell interactions of

the testes (40, 41). Spermatogenesis by grafting has now been achieved in several animal species (42, 43, 44, 45, 46), although it has yet to be reported in humans (47). Grafting tissues to ectopic sites is often accompanied with high germ cell loss, sclerosis and poor graft survival, often attributed to the higher temperatures experienced at ectopic locations than in the scrotum where endogenous spermatogenesis occurs (47, 48, 49). However, high rates of SSC loss are also associated with orthotopic grafting (50, 51), possibly due to hypoxic ischemia leading to tissue degeneration and cell apoptosis in the days immediately succeeding the transplant (52).

Live births have been reported in non-human primates as a result of both xeno- (53) and autologous ectopic grafting (45), both achieved through intracytoplasmic sperm injection (ICSI). There are no reports of donor derived spermatozoa in recipient epididymal structures and it is not likely that testicular grafts are capable of forming a functional excretory system with established epididymal structures. On that basis, ICSI will always be required to produce offspring where fertility is restored by testicular grafting, as it is unlikely that natural reproduction would be possible.

Reimplantation of tissue or cells from patients with malignancies comes with a risk of reintroducing malignancy after reimplantation. Several studies have highlighted this risk in SSC or tissue transplantations (44, 54, 55, 56). Methods to sort malignant cells from testicular cell cultures (57) and cell suspensions (56) have been promising. Unfortunately, no robust decontamination methods are currently available, highlighting the need for further research into the safety and effectiveness of these approaches. Until a clinically accepted protocol confirming the safety of testicular matter transplants is established, the only option for patients at risk of contamination is in vitro spermatogenesis, also known as IVM. Endogenous spermatogenesis is a complex process, involving cell-cell interactions between spermatogenic cells and somatic Sertoli cells, as well as release of gonadotrophins and growth factors (25, 28). Reproducing the development in vitro is a difficult and expensive process and the patient would require IVF or ICSI at a fertility clinic (58). Although complete spermatogenesis has not been achieved from human ITT to date, two publications report getting to the spermatid stage (20, 59).

Another potential option is SSC transplantation. Briefly, this involves microinjection of SSCs into the tubule lumen whereupon the cells migrate to the basement membrane (BM) and colonize the SSC niche of the recipient testis, initiating endogenous spermatogenesis (60). It has been successfully demonstrated in animals and is recognised as the only option that could result in a natural conception (60, 61, 62). However, SSC culture and propagation involves cryopreservation of isolated cells rather than tissue and is not applicable to this review.

To date, each of the three fertility restoration methods have yielded promising results using animal models (42, 43, 60, 63, 64). The next challenge is to reproduce results in humans. As for which method is most likely to dominate in future, the evidence suggests the optimal protocol will vary between individual cases and that patient age, condition and choice should be considered (65). As such, it is likely these three approaches will develop in parallel going forward.

Aims and objectives

This is an early attempt to conclude which cryopreservation protocol best preserves ITT for the potential restoration of fertility in the future. A systematic review has been carried out and relevant articles were appraised and compared in order to draw an informed conclusion to the question: which cryopreservation protocol best preserves ITT for the potential restoration of fertility in the future?

Eleven studies investigating ITT cryopreservation are included in this qualitative synthesis (13, 14, 15, 16, 17, 18, 19, 20, 22, 23, 24), the protocols of which are discussed. The protocols differ from one another in freezing rate and the cryoprotectant method applied. After tissues had been thawed, some were cultured and others were xenografted onto the testis of mice. The effects, consequences and outcomes of each of these will be discussed.

MATERIALS AND METHODS

Literature search

A systematic literature search was conducted on Embase, Medline and Web of Science databases using the PICO framework (66). Relevant MeSH headings and keywords for testis, cryopreservation, and fertility preservation

Table 1. Summary of papers included in the systematic review.

First author (Ref.)	Year	Participant number, n	Mean age (years ± SD)	Age range (years)	Cryopreservation protocol as per (ref.)
Kvist (18)	2006	8	2.4	1-5.5	Kvist et al. (18)
Keros (19)	2007	5	7.2 ± 5.2	2-12	Keros et al. (19)
Wyns (14)	2007	11	Not disclosed	2-12	Wyns et al. (14)
Wyns (15)	2008	5	11.8 ± 2.9	7-14	Wyns et al. (14)
Curaba (25)	2011	2	9.0 ± 4.2	6-12	Curaba et al (25)
Poels (16)	2013	10	8.1 ± 4.0	2-12	Wyns et al. (14)
Poels (17)	2014	6	8.3 ± 5.6	2-15	Wyns et al. (14)
de Michele (20)	2017	3	8.3 ± 5.5	2-12	Wyns et al. (14)
de Michele (21)	2018	5	7.0 ± 4.8	2-12	Wyns et al. (14)
Medrano (23)	2018	4	11.3 ± 3.1	7-14	Baert et al. (9)
Portela (24)	2019	9	10.1 ± 2.3	6-14	Keros et al. (19)

were included. Searches were limited to English. The last date included in the search was 28/06/2020.

Selection criteria

The main inclusion criteria for publications included in the review was: i) presentation of original data on cryopreservation of testicular tissue; ii) participants of prepubertal or peripubertal sexual maturity. The main reasons articles were excluded were for falling into the following categories: i) animal/non-human study; ii) sperm cryopreservation; iii) mature adult tissue; iv) participants had Klinefelter syndrome; v) patients were undergoing gender reassignment; vi) surgical technique/safety report; vii) case report; viii) review with no original data.

RESULTS

Study characteristics

The initial search returned 7,325 articles, 2,577 of which were removed for being duplicate entrees. The remaining 4,748 titles were screened and a further 4,357 records were excluded for not meeting in inclusion criteria. 391 publications were identified, and the abstracts reviewed. Sixty two articles were selected for a full-text review. Of these a further 51 were excluded as studies focusing on cryopreservation of testicular cell suspensions were not considered relevant or studies did not specify the cryopreservation protocol used.

Eleven studies were selected for qualitative synthesis. A summary can be found in Table 1.

The study selection is outlined in Figure 1 using a PRISMA flow chart (67). A summary of the publications included can be found in Table 2.

DISCUSSION

Testicular tissue cryopreservation

Whether mature spermatozoa are cultivated from immature testis by IVM or testicular tissue graft, one step they share is cryopreservation at the time of biopsy. According to a recent survey, the number of tissues currently in storage is marginally smaller than the total number of tissues collected since the first programme was established in 2002 (39). Despite decades of research and a growing number of centres offering TTC, no standardized protocol for the cryopreservation of ITT exists. Cryopreservation of mature testicular specimens has been around since the 1990s as a step to treat couples with azoospermia (68). Unfortunately, these protocols are not suitable for ITT as protocols for mature samples are focused on preserving spermatozoa rather than preserving SSCs and their supporting cells, as is required to induce spermatogenesis in immature samples (18, 69).

Comparison of methods

Before appraising the results of studies, a comparison of study methodologies was carried out. The primary inclusion criterion for this review was that studies be conducted on

Figure 1. PRISMA flow diagram illustrating the article selection for the systematic review following the scheme of Moher et al., 2009 (67).
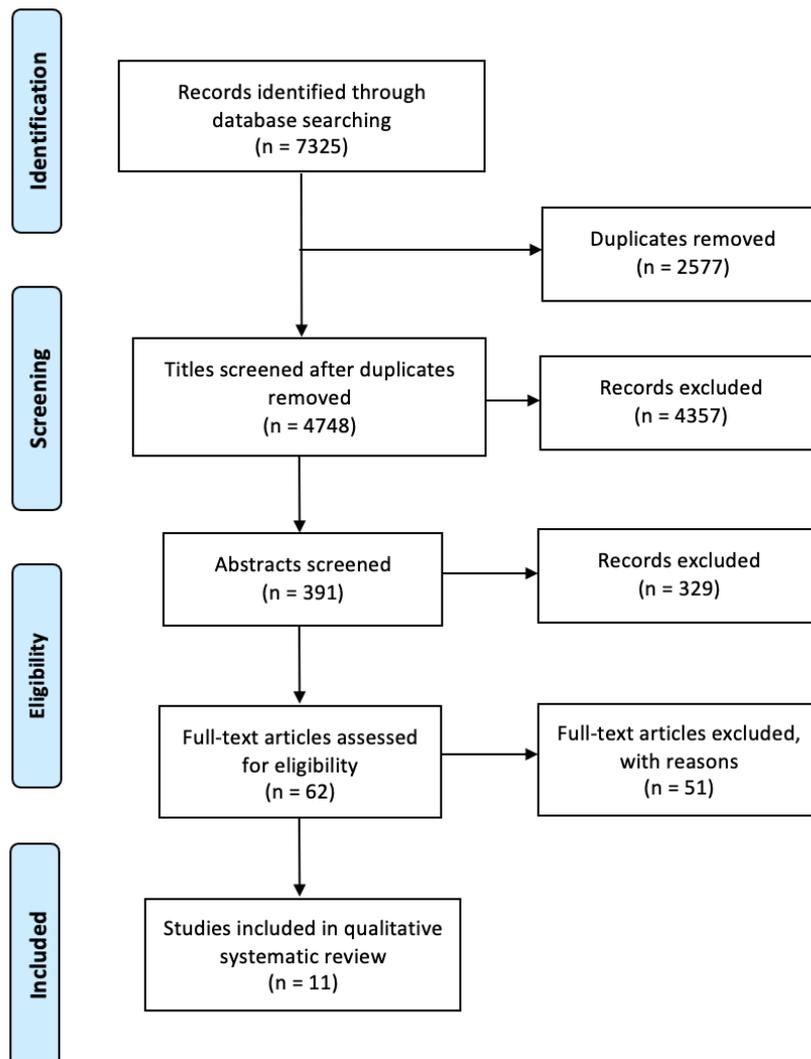
immature testicular tissue. Natural variation in genital development led to the establishment of a five point scale, known as the Tanner Stages, in order to chronicle an individual's progress through puberty, where 1 indicates no physical signs of puberty and 5 is full development (70). Alternatively, the Johnsen score quantifies spermatogenesis where 10 indicates maximum spermatogenesis and 1 indicates complete absence of germ cells (71). While all patients across studies had immature tissue at the time of biopsy and freezing, the level of immaturity varied. Some studies refer to patient testicular maturity at the time of biopsy by referencing Tanner stages (14, 24), some by Johnsen scores (22, 23) or both (19, 20). Others make no reference at all (13, 15, 16, 17, 18). Age ranges from 12 months to 15 years across studies. Tanner stages varied from 1 to 3 and Johnsen

scores from 1 to 8, suggesting that although all tissues could be classified as immature, they may not be directly comparable. Patients of more advanced age, Tanner stage and/or higher Johnsen score are more likely to have a greater quantity of SSCs and SG present in tubules (72), theoretically increasing the likelihood that some cells will survive the cryopreservation and thawing process.

There is also variability in the size of tissue fragments that were frozen. Though many studies cut fragments to ~1 mm^3 (15,16, 17, 22, 23), some fragments were as large as 8 or 9 mm^3 at the time of freezing. It remains to be proven in humans that the size of tissue fragments affects how quickly and effectively the cryoprotectant media diffuses in and out of tissues (73), but there is evidence of this in animals (74).

Table 2. Outline of cryopreservation protocols for immature testicular tissue.

Author (ref)	Year	Type	Cryomedia	Equili-bration	Start	Step 1	Step 2	Step 3	Step 4	Step 5	Step 6	Step 7	Step 8	Step 9
Kvist et al. (18)	2006	CSF	Leibovitz L-15 medium; 1.5 M EG; 0.1 M sucrose; 10 mg/mL HSA	2°C; 10 mins	1°C	−2°C/min to −9°C	Pause 5 mins	Seeding	−0.3°C/min to −40°C	−10°C to −140°C	LN$_2$ (−196°C)			
Keros et al. (19)	2007	CSF	5% DMSO; 5% HSA; HBSS;	4°C; 30 mins	(Cryo I) 4°C	−1°C/min to 0°C	Pause 5 mins	−0.5°C/min to −8°C	Seeding	Pause 10 mins	−0.5°C/min to −40°C	Pause 10 mins	−7°C/min to −70°C	LN$_2$ (−196°C)
					(Cryo II) 4°C	−1°C/min to −8°C	Seeding	Pause 10 mins	−10°C/min to −80°C	LN$_2$ (−196°C)				
Wyns et al. (14)	2007	CSF	0.7 M DMSO; 0.1 M sucrose; 10 mg/mL HSA	4°C; 30 mins	4°C	0°C for 9 mins	−0.5°C/min to −8°C	Pause 15 mins	Seeding	Pause 15 mins	−0.5°C/min to −40°C	Pause 10 mins	−7°C/min to −80°C	LN$_2$ (−196°C)
Curaba et al.(25)	2011	V	MEM/Gluta-max I; 2.8 M DMSO; 2.8M EG; 25 mg/mlLHSA	0°C; 25 mins	0°C	LN$_2$ (−196°C)								
Poels et al. (16)	2013	V	Leibovitz L-15; 15% DMSO; 15% EG; 0.5 M sucrose; 25 mg/mL HSA	4°C; 10 mins	4°C	LN$_2$ (−196°C)								
Baert et al. (9)	2013	USF	0.7M DMSO; 0.15 M sucrose; 10% HSA	0°C; 15 mins	0°C	−1°C/min to −80°C	LN$_2$ (−196°C)							

Abbreviations: CSF, controlled slow freezing; USF, uncontrolled slow freezing; V, vitrification; EG, ethylene glycol; HSA, human serum albumin; DMSO, dimethyl sulfoxide; HBSS, Hanks' Balanced Salt Solution; LN$_2$, Liquid nitrogen.

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Methodological heterogeneity also existed post-thaw during tissue evaluation. Anti-MAGE-A4 antibodies were used in all studies as a marker to quantify SG and anti-Ki67 was used in all bar one study to look for proliferating cells. Portela et al. (23) used an anti-PCNA antibody as a proliferation marker. Although Ki-67 has been shown to be more specific than PCNA in the study of some cancer cells (75), for the purpose of these experiments, it is unlikely there would be any significant variation in results.

Perhaps the most consequential variation between studies is the method of tissue culture. While most studies culture tissues in vitro (17, 18, 19, 20, 22, 23), four studies xeno-grafted tissues into mice (13, 14, 15, 16). Furthermore, within each of those groups there are additional variants in length of culture/graft and composition of culture media. When it comes to carrying out analysis on tissues all studies used similar methodologies with little to no variation with respect to section thickness, staining and tissue grading procedures. The most commonly shared outcome measure across studies was ST integrity and all studies followed a grading system described by Keros et al. (69). Good/intact morphology was classified as good adhesion of cells to the BM, good cell cohesion and no sclerosis. However, it must be noted that the methodological similarities are few and perfect comparisons between different freezing protocols are not possible. Therefore, in order to come to a conclusion in relation to the study question, methodological variability and heterogeneity has been taken into account.

Cryoprotectant method

CPAs are essential for protecting cells and tissues against cryoinjury caused by ice crystal formation during the freezing process. Rather than freezing as a solvent, water freezes as pure substance, separating from its solutes and freezing in crystals. Injury is a result of this exclusion process and is classified as either mechanical or chemical. Mechanical damage is caused by the mechanical force of ice crystals distorting the shape of cells and chemical damage, or "solution effects" injury is the result of chemical and osmotic effects caused by an increasing concentration of solutes in the residual, unfrozen water (76).

CPAs dissolve in water and lower its melting point whilst also being relatively non-toxic to cells. When CPAs are added to a freezing protocol, as water freezes into ice crystals the concentration of CPA in the unfrozen water increases, restricting the formation of further ice crystals as the temperature lowers. The result is liquid pockets between crystals where cells survive. At -100°C and lower, the residual liquid solidifies into a glass-like structure, preserving the cell architecture at that time point for the duration of storage (77).

Glycerol, a permeating CPA, is the CPA of choice for preservation of mature spermatozoa. It protects stability and structure of the membrane and causes few alterations to sperm quality (78). When glycerol was applied to ITT, it resulted in significant damage to the tubule basal compartment (69). The most promising CPAs applied to ITT are also common in OTC and are often applied in the same concentrations (79). This is indicative of CPAs performing differently on tissue preparations to single cell preparations. This is further evidenced by DMSO, a widely used CPA for OTC and TTC, causing deleterious effects in sperm cryopreservation (80).

In 2006, Kvist et al. published a comparison of two ITT cryopreservation protocols (17). Eight patients aged 12-66 months underwent surgery for cryptorchidism and a portion of the biopsied tissue was retained for research. Boys with cryptorchidism are at risk of infertility later in life due to SSC loss as a result of the testes being exposed to the normal body temperature (81). Immediately after biopsy, samples were transported in testis culture media at room temperature for 30 min. Tissue fragments were equilibrated in one of two cryomedia before being slow frozen in a programmable freezer (Table 2). Both cryomedia contained ethylene glycol [EG] (1.5 mol/L), sucrose (0.1 mol/L) and human serum albumin [HSA] (10 mg/mL). EG is a permeating CPA meaning its small molecular size allows it to cross the cell membrane to reduce cell dehydration and ice growth from inside the cell, while sucrose is non-permeating, acting outside the cell (77). The first cryomedia was supplemented with Leibovitz L-15 medium and the second with phosphate buffered saline (PBS). Tissues were later thawed and samples cryopreserved in each of the two cryomedia were either fixed or cultured for 2 weeks in culture media, the same as control samples which had not been cryopreserved. The morphology of both fresh and cryopreserved uncultured and cultured samples were examined

as was testosterone and inhibin B production from both fresh and cryopreserved cultured samples. A c-kit was used for the immunohistochemical detection of SG.

The study demonstrated that structural and functional characteristics of cryopreserved ITT could be maintained and found no observable difference comparing the morphology of fresh and cryopreserved samples. Somatic cell function measured by hormone production also returned similar results between groups. Leibovitz medium, known to support the growth of embryonic cells in explant cultures, was expected to better preserve tissues than the PBS supplemented cryomedia (82). However, there was also no significant difference in outcomes between the two cryomedia. Importantly, although the CPAs applied in this study showed promising results, neither cryomedia solution is applied elsewhere in the literature. The cryomedia and freezing schedule used in this study was modelled on the authors' previous success for preservation of ovarian tissue (83). However, reports that dimethyl sulfoxide (DMSO), an alternative permeating CPA to EG, is more suitable for animal ITT resulted in its wider application and greater prominence in the literature (84, 85). Lastly, cryopreserved samples in this study were only stored at -196°C for one hour meaning the tissues were subject to little of the degenerative effects associated with long term storage. Further to this, no attempt to quantify SG was made, the cell most important for future restoration of fertility.

All other studies in the review employ DMSO as the primary component in the cryomedia, either in isolation (18, 23) or alongside sucrose (13, 14, 15, 16, 19, 20, 22). This preparation has been shown to effectively preserve the structure of the tubule basal compartment and is efficient at maintaining SG, Sertoli cells and the stromal compartment when examined by transmission electron microscopy (TEM) (18, 69). Keros et al. applied a DMSO based medium in their 2007 publication comparing two freezing protocols (18). The first and more successful, Cryo I, was initially developed two years previously for the preservation of adult tissue with a focus on preserving SG (69). This was compared to another protocol (Cryo II) developed for fetal testicular tissue in 1999 (86). The cryomedia in both cases consisted of 5% DMSO (0.7 mol/L) and 5% HSA which was equilibrated with tissues for 30 min at 4°C. Cryo I and Cryo II differed exclusively in freezing rate (Table 2). As Cryo I was verified to better preserve tissues, the protocol is applied clinically today in multiple FP centres (9, 39). It is also the program of choice in one other publication included in this review.

Portela et al. (23) applied the Cryo I protocol and attempted to induce in vitro spermatogenesis. Tissues from nine males aged six to 14 were cultured for five weeks and the functionality of the frozen-thawed cultures were compared to fresh-cultured controls. The experiment failed to induce IVM of spermatozoa but tissue structure, endocrine function and SG proliferation were maintained equally in frozen-thawed and fresh tissue cultures. This is evidence that the applied protocol and DMSO based cryomedia minimises cryopreservation induced damage and has a negligible effect on the culturing capacity of the tissues.

In 2007, Wyns et al. modified the program with the addition of 0.1 M sucrose (13). This modified protocol was subsequentially applied in several experiments investigating xenografting (10, 14, 15, 16) and tissue culture (19, 20, 21), as well as being applied clinically in FP programs (10). When applied as a CPA, sucrose exerts protective effects by electrostatically interacting with membrane phospholipids, providing stabilization (87). The decision to include sucrose into the protocol is unsurprising as it was previously shown to have positive cryopreservation outcomes on a variety of cell types, including SSCs (88). This protocol also included modifications to the rate of freezing (Table 2).

Interestingly, both protocols were compared by Baert et al. and although this study used mature adult tissues, the result provides an insight into the effects of sucrose in the cryomedia. Although both protocols performed similarly on cell integrity score, samples cryopreserved with additional sucrose had a significantly greater percentage of tubules survive the process compared to fresh controls. This was not found in the absence of sucrose. Cell proliferative activity, measured by the PCNA index, indicate the proliferative activity of samples preserved using sucrose was equal to the controls. Samples without sucrose performed significantly worse. Lastly, sucrose appeared to have some protective effects on interstitial cell apoptosis, though it was not significant. The authors theorise the absence of sucrose in the Keros et al. Cryo I program could result in

insufficient cell dehydration. They also note, however, that tissues frozen with sucrose still express cryoinjury and ultrastructural changes compared to fresh controls. They suggest a higher concentration of DMSO could improve the extent of cryoinjury. Higher concentrations of DMSO had also been suggested to enhance the differentiating capacity of SSCs in non-human primates (89). However, increasing DMSO from 0.7 M to 1.5 M in this study proved ineffective (8). Nevertheless, the experiments carried out in this paper would need to be reproduced using ITT before the true effects of sucrose on ITT is known. Furthermore, more comparisons of CPA combinations should be explored in order to find the optimum cryomedia for human ITT cryopreservation.

Freezing rate

In cryobiology, samples are slow-frozen or vitrified. Slow-freezing requires lower concentrations of CPA and takes hours. Over the past two decades, several groups have published slow-controlled (8, 13, 15, 17, 18, 41, 69, 90) and slow uncontrolled (8) freezing protocols for ITT. Controlled slow freezing (CSF) is the favoured approach for ITT but uncontrolled slow freezing (USF) is considered an inexpensive, convenient and time saving alternative (49, 51, 89). Slow freezing protocols often include manual ice nucleation or "seeding" (13–20, 23). Ice nucleation can be described as initiation of ice crystal formation. It is thermodynamically favourable for new water molecules to bond to established, growing ice crystals than it is to form new ones (91). Seeding involves clamping the sides of a vial with forceps that have been cooled in liquid nitrogen to initiate controlled crystal formation (92).

In contrast to slow freezing, vitrification is ultra-rapid freezing in high concentrations of CPA. Vitrification's success in the fertility field is growing, in particular with human oocytes where the technique has increased survival after thawing to over 84% (93). However, vitrification's use with ITT has been limited to a small number of studies (8, 15, 24). In OTC, vitrification has resulted in two live births to date (94, 95) but slow-freezing remains the preferred approach resulting in over 130 live births (5). The greater success of slow-freezing preparations in OTC is the likely cause of researchers investing greater interest in slow freezing for TTC.

Freezing tissues is more challenging than freezing cell suspensions due to the complex diversity of cell types and the heat exchange through tissues during the freeze and thawing processes. CPA diffusion rate and changing osmolarity are also considerations. In the aforementioned study, Keros et al. compared two cryopreservation protocols using the same cryomedia, differing exclusively in freezing rate (18). Both protocols maintained the temperature at −8°C for 10 min after seeding but the latter protocol took less time to -8°C, cooling 1°C/min compared to 0.5°C/min (Table 2). After seeding, the former protocol again took a longer time to cool and the results were significantly different. For Cryo II, the faster of the two protocols, only 20±14% of STs remained intact after freezing, a significant reduction compared to fresh and Cryo I samples, at 71±7% and 70±7% respectively. There was also a significantly greater number of tubules with central and tubular necrosis in the Cryo II group. Fresh, fresh cultured, and Cryo I groups all had similar percentages of SG survival and SG detachment from surrounding cells. The Cryo II group scored significantly worse with only 50±43% of SG with normal undamaged morphology. This is compared to 93±2% and 94±1% in the fresh and Cryo I samples, respectively. Because tissues cryopreserved using the Cryo I protocol scored better overall, its use has been continued in clinically practice and other publications (9, 23). As the Cryo I and Cryo II programs differed exclusively in freezing rate, the paper is a good demonstration of how important freezing rates are in cryobiology.

Slow freezing permits time for changing CPA concentration to equilibrate with the changing osmolarity of the extracellular matrix. It minimizes extracellular ice formation and moderates increasing CPA concentration from inducing chemical damage as the freezing progresses. Equilibration times at the beginning and throughout the cooling period are therefore included in many freezing protocols. Of the studies in this review, initial equilibration times range from 10 to 30 min and additional holding times range from 5 to 15 min (13, 17, 18). Kvist et al. soaked tissues for 5 min at -9°C prior to seeding. Wyns et al. also included a 5-min soak prior to seeding and a 15-minute soak afterward, both at -8°C. Keros et al. (Cryo I) included the post-seeding soak at -8°C and an earlier pause was carried out at 0°C for 5 min. However,

subjecting the tissues to CPAs for these prolonged pauses prior to the completion of the cryopreservation exposes tissues to potential cytotoxicity. The USF protocol employed by Medrano et al. does not apply these holding times to tissues before −80°C when it is held for 24 h. This protocol was designed by Baert et al. who compared it to the CSF programs by Keros et al. and Wyns et al. (8). Although the comparisons were carried out on adult tissues, they found the USF program to be superior to both CSF protocols, which they suggest is down to a shorter equilibration time and alternative freezing rate. To study the effects of freezing rate on ITT preservation going forward, further experiments need to be carried out focusing on freezing rates and equilibration times.

Vitrification of human testicular tissue has been explored but not implemented clinically. Of the two publications that trialed vitrification, neither found it had an improvement on slow-freezing, but neither was it found to perform worse (15, 24). Curaba et al. suggested the technique could be a slow freezing alternative when it was shown to preserve tubule integrity, SG survival and proliferative capacity in vitro (24). However, it was not possible to quantify results or report a functional evaluation due to small participant numbers (n=2) and lack of tissues. Poels et al. pursued and in vivo model to test their vitrification protocol (15). After xenografting frozen-thawed samples into mice for six months they found similar ST integrity, SG recovery and proliferative activity of intratubular cells and SG in vitrified and slow-frozen grafts. It is evident from these publications that vitrification should be investigated further. Although no evidence so far suggests it can improve on CSF or USF it is theoretically advantageous in that it requires no special equipment and costs less. As vitrification also lowers the threat of ice crystal formation, theoretically, it could better preserve the SSC niche.

In both published vitrification protocols, the cryomedia included equal amounts DMSO and EG (15, 24). Vitrification's greatest caveat is that the method requires higher CPA concentrations, thus exposing tissues to a greater likelihood of experiencing toxic side-effects (92). Based on preliminary findings on vitrified ITT (15, 24) and one study by Baert et al. comparing the technique to slow-freezing on adult tissues (8), the method appears equal in its ability to preserve tissues. Unfortunately, none

of these studies have been extensive enough to draw any stable conclusion and the method requires further exploration. The same is true for determining the perfect slow-freezing rate for ITT. What is evident is that freezing rate and the correct blend of CPAs are both critical factors for effective cryopreservation and the best combination has yet to be established.

Integrity and function of tissues and cells

The effectiveness of cryopreservation protocols is measured by survival and functional capacity of the cells and tissues after thawing. Several methods are used to assess morphology and structural integrity of tissues after a biopsy including light microscopy, TEM, and immunohistochemistry (18, 51). For a complete comparison, analyses should be carried out on fresh, fresh-cultured or fresh-grafted tissue, and frozen-thawed tissue where applicable.

Kvist et al. carried out analyses of frozen-thawed tissues after culturing them for two weeks. The results were then compared to fresh tissue and fresh tissue cultures. They observed no difference in morphology between samples. They also observed a similar tubular diameter between fresh tissue and frozen-thawed cultures and confirmed the presence of SG in all groups. Hormone production was measured by testosterone and inhibin B secretion in culture. Both were reduced in frozen-thawed samples but neither reached a statistical significance. This was the first study to demonstrate frozen-thawed human ITT samples could maintain structure and function equal to fresh tissue (17). However, a quantification of preserved cells and tubules were not included in the results. Consequently, it is difficult to compare this protocol to others with precision.

Keros et al. similarly showed tissues could be preserved following CSF and unlike the previous study the morphological analysis included STs and SG quantification. As previously mentioned, one of the protocols, Cryo II, failed to protect tissues against cryoinjury but the alternative protocol, Cryo I, returned satisfactory results. Today, it is clinically applied across the globe including a large multicentre program between the United States and Israel (9, 39). No structural difference was observed between fresh, fresh cultured, and frozen cultured tissues. SG ultrastructure was comparable across groups and all had good attachment of SG and Sertoli cells to the BM. These results were echoed by Portela et al. who

applied the same protocol before culturing tissues for 5 weeks, attempting to initiate in vitro spermatogenesis. The experiment failed to produce mature spermatozoa, however, tissue structure, endocrine function and SG proliferation were maintained after cryopreservation to match fresh tissue cultures. This study also included an analysis of hormone production and found testosterone levels remained equal to fresh-cultured tissues for the duration of the culture period (23).

In 2007, Wyns et al. (13) published their alternative freezing procedure which was subsequently applied in several experiments investigating frozen-thawed xenografting (10, 14, 15, 16) and culture experiments (19, 20, 21), as well as being clinically applied in FP programs (10). Cryomedia consisted of DMSO (0.7 mol/L), HSA (10 mg/mL) and was additionally supplemented with 0.1 M sucrose. After retrieving from storage, the tissue was thawed and xenografted onto the scrotum of mice where it matured for 3 weeks. Overall morphology of frozen-thawed tissue did not have major signs of cryoinjury compared to fresh-grafted tissue. Well preserved structural integrity was found in 82.2±16.5% of tubules from frozen-grafted tissues compared to 93.4±6.0% in fresh-grafted controls and there were no signs of fibrosis or sclerosis in either group. Interestingly, although they found the number of SG decreased from 0.55±0.52 per tubule in fresh-grafted controls to 0.08±0.13 in test samples, they found the percentage of SG that continued to proliferate higher at 32% in frozen-grafts than in controls (17.8%), but this did not reach significance. Overall, cell proliferative activity jumped from 0.07±0.06 per ST in fresh tissue to 2.27±2.11 in frozen-grafted samples (13). However, the purpose of this study was not to assess germ cell differentiation but rather to identify a xenografting model for future research. The study was soon succeeded by two subsequent examinations of long-term xenografts of a 6 month duration (14, 15). Similarly, histological and immunohistological analyses returned similar results for fresh grafted and frozen grafted tissue with respect to tubule integrity, SG recovery, SG proliferation and germ cell differentiation (14, 15).

The same protocol has also been applied in several studies interested in organotypic cultures for the initiation of in vitro spermatogenesis (19, 20, 21). The first such study by de Michele et al.

successfully provided a cultured microenvironment that preserved tissue viability during culture for 139 days (19). Two culture media were compared, one containing testosterone (M1) and another containing human chorionic gonadotrophin (M2), which acts on Leydig cells to stimulate endogenous testosterone production. Follicle stimulating hormone and retinoic acid were included in both media to help recreate an environment analogous to the natural physiological niche (96). Both media performed similarly against all parameters of interest. Decent ST structure, somatic cell function, and spermatogonial proliferation continued for the duration of the culture period. Testosterone production indicated Leydig cell function and Sertoli cell maturation was demonstrated by a reduction in anti-Müllerian hormone expression as the culture progressed (19).

Whether tissues were cultured in vitro or xenografted in their respective publications, neither method has achieved complete spermatogenesis to date. Be that as it may, the results of these preliminary studies are evidence that freezing and thawing ITT tissues can preserve the tissue ultrastructure and function.

Tissue application

As previously mentioned, there are three promising routes to fertility restoration after freezing and thawing ITT. SSC transplantation involves isolating cells prior to freezing and is not covered in this review. In vitro culture and tissue grafting have both been explored after cryopreservation using the protocols previously mentioned. Both approaches are capable of preserving tissue integrity and ultrastructure but both have failed to produced mature spermatozoa (13, 14, 15, 16, 18, 22, 23). Nevertheless, research in both areas continues in the hope of inducing spermatogenesis. It is difficult to determine at this point if grafting or IVM will be the method of choice to restoring fertility after cryopreservation of ITT in the future. Both are capable of preserving tissue integrity and functional capacity but both result in progressive germ cell loss over time. In order to better compare which approach has more promise, further research needs to be conducted where the freezing and thawing conditions are the same and the functional outcomes of the tissues can be compared. However, it is likely that the exact method of tissue application will depend on patient circumstance.

In summary, cryopreservation of ITT is essential for the future restoration of fertility in pediatric cancer patients. Published protocols differ from each other in freezing rate and CPA application but it is unclear yet which protocol best preserves the structure and function of ITT after it has been thawed. Optimised application of the tissue has also yet to be determined.

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