Article

What is the best protocol to cryopreserve immature mouse testicular cell suspensions?

Jaime Onofre [a],*, **Katrien Faes** [a], **Prashant Kadam** [a], **Elena Vicini** [b], **Ans M. M. van Pelt** [c], **Ellen Goossens** [a]

[a] *Biology of the Testis, Research Laboratory for Reproduction, Genetics and Regenerative Medicine, Vrije Universiteit Brussel (VUB), Laarbeeklaan 103, Brussels, 1090, Belgium*
[b] *Department of Histology and Medical Embryology, University of Rome 'La Sapienza', Via A. Scarpa, 14 00161 Rome, Rome, Italy*
[c] *Center for Reproductive Medicine, Women's and Children's Hospital, Academic Medical Center, University of Amsterdam, Meibergdreef 9, 1105 AZ Amsterdam, Amsterdam, The Netherlands*

Jaime Onofre is a Doctoral candidate for Medical Sciences at the Vrije Universiteit Brussel. He completed his training and Masters degree in Human Reproduction and assisted reproductive technologies at the Biomedical sciences faculty of the University Paris Diderot. He is currently preparing his doctoral thesis in the optimization, safety and clinical translation of fertility preservation strategies to avoid and cure male infertility.

KEY MESSAGE
Testicular tissue cryopreservation is offered to prepubertal boys whose fertility is at stake. If the tissues present a risk of contamination, e.g systemic cancers, testicular cell suspensions must be generated and cryopreserved. As human prepubertal tissue is scarce, prepubertal mouse testes were used to find an optimal cryopreservation protocol.

A B S T R A C T

Research question: From a clinical perspective, which parameters grant optimal cryopreservation of mouse testicular cell suspensions?
Design: We studied the effect of different cryopreservation rates, the addition of sugars, different vessels and the addition of an apoptotic inhibitor on the efficiency of testicular cell suspension cryopreservation. After thawing and warming, testicular cell suspensions were transplanted to recipient mice for further functional assay. After selecting the optimal cryopreservation procedure, a second experiment compared the transplantation efficiency between the selected freezing protocol and fresh testicular cell suspensions.
Results: Multiple- and single-step freezing did not differ significantly in terms of recovered viable cells (RVC) ($33 \pm 28\%$ and $38 \pm 25\%$). The addition of sucrose did not result in a higher RVC ($33 \pm 20\%$). Cells frozen in vials recovered better than those frozen in straws ($52 \pm 20\%$ versus $33 \pm 20\%$; $P = 0.0049$). The inclusion of an apoptosis inhibitor (z-VAD[Oe]-FMK) significantly increased the RVC after thawing ($61 \pm 18\%$ versus $50 \pm 17\%$; $P = 0.0480$). When comparing the optimal cryopreservation procedure with fresh testicular cell suspensions, a lower RVC ($63 \pm 11\%$ versus $92 \pm 4\%$; $P < 0.0001$) and number of donor-derived spermatogonial stem cell colonies per testis (34.04 ± 2.34 versus 16.78 ± 7.76; $P = 0.0051$) were observed.
Conclusion: Upon freeze–thawing or vitrification–warming, and assessment of donor-derived spermatogenesis after transplantation, Dulbecco's modified Eagle's medium supplemented with 1.5M dimethyl-sulphoxide, 10% fetal calf serum and 60 µM of Z-VAD-(OMe)-FMK in vials at a freezing rate of –1°C/min was optimal.

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* Corresponding author.
 E-mail address: Jaime.Onofre.Meza@vub.be (J Onofre).

Introduction

Gonadotoxic therapy, such as chemotherapy and radiation, might result in subfertility, leading to the need for fertility-preservation techniques (Hudson, 2010; Jahnukainen et al., 2015). In women wishing to undergo fertility preservation, ovarian tissue preservation combined with transplantation of ovarian cortex fragments has been successful, and has led to live births (Demeestere et al., 2015). For men and adolescent boys, sperm banking is the gold standard for fertility preservation. Prepubertal boys who cannot yet produce sperm do not have this option (Hudson, 2010; Tournaye et al., 2014), although they do have spermatogonial stem cells (SSCs). This opens up the possibility of cryopreserving testicular tissue containing SSCs to safeguard their chances of having their fertility restored in the future.

Effective protocols to cryopreserve human testicular tissue exist (Baert et al., 2013; Keros et al., 2005), and several fertility centres worldwide already offer testicular tissue cryopreservation to patients before fertility-compromising therapy. Testicular tissue cryopreservation and grafting is the most efficient strategy to preserve and restore fertility in animal models (Van Saen et al., 2009). In cases of systemic cancers, however, autologous grafting represents a high risk of reintroducing cancer cells in the patient (Jahnukainen et al., 2001). As such, standard testicular tissue cryopreservation could be carried out in view of subsequent generation of testicular cell suspensions (TCSs) from which cancerous cells could be depleted, while SSCs would be propagated *in vitro* for spermatogonial stem-cell transplantation (SSCT) (Giudice et al., 2017; Goossens et al., 2013). In future clinical application, cryopreservation of in-vitro propagated TCS is required during safety testing before SSCT (Figure 1).

Currently, the highest post-thawing–warming viability of human TCSs was obtained after vitrification (55 ± 24%). The recovery rate of viable SSCs is unknown as no functional proof was provided (Sá et al., 2012), highlighting the need for an effective procedure for SSC preservation.

In the present study, we conducted a thorough screening of different cryopreservation methods with the aim of designing an efficient, reproducible and clinically friendly cryopreservation protocol for TCSs containing SSCs. Our primary goal was to increase the number of viable SSCs after thawing–warming as it is vital to the success of SSCT. As human prepubertal tissue is scarce, prepubertal mouse TCSs were used.

Materials and methods

Experimental design

The present study was conducted in two parts. The experimental design (Part 1 and Part 2) is presented in Figure 2A and Figure 2C.

Animals and testicular cell isolation

Approval for this study was given by the Animal Care and Use Committee of the Vrije Universiteit Brussel on 14 October 2013 (approval number 13-216-1), and on 27 February 2017 (approval number 14-216-3). Recipient and donor mice were obtained by crossing male inbred C57BL with female inbred SV129 green fluorescence protein (GFP). Male GFP negative (GFP⁻) F1-hybrids were used as recipients, whereas neonatal (aged 6–10 days) GFP-positive (GFP⁺) F1-hybrid pups were used as donors. Each replicate consisted of one single donor testis taken from one mouse and 12 replicates were considered per condition. Donor testicular cells were isolated as previously described (Ogawa et al., 1997) with minor modifications. Briefly, after weighing, testicular tissue was incubated in Dulbecco's modified Eagle's medium (DMEM)/F12 containing 1 mg/ml collagenase IV (C5138; Sigma-Aldrich, Diegem, Belgium) and 30 mg/ml desoxyribonuclease (DNAse; DN25; Sigma-Aldrich) for 20 min in a 37°C shaking water bath. After incubation, the testicular tubules were washed twice with 10 ml of DMEM/F12 by centrifugation for 5 min at 600 g. Subsequently, 10 ml DMEM/F12 solution supplemented with 0.25% trypsin (T4665; Sigma-Aldrich) and 1 mM ethylenediaminetetracetic acid (E6511; Sigma-Aldrich) was added to the cell pellet and incubated in a 37°C shaking water bath for 5 min. The trypsin reaction was halted by adding 4% fetal calf serum (FCS) (10500-056; Life Technologies) and 30 mg/ml DNAse. The solution was filtered through a 40 μm nylon mesh cell strainer (35234; BD Falcon, Leuven, Belgium), centrifuged for 5 min at 600 g and the supernatant removed.
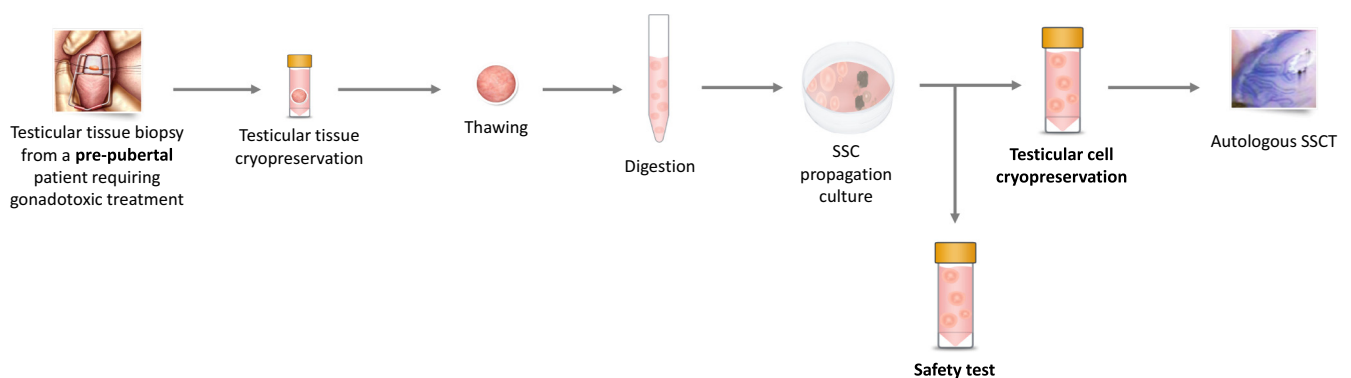

Figure 1 – Strategy to preserve fertility in prepubertal cancer patients. For restoring fertility, the biopsy will be enzymatically digested. Spermatogonial stem cell (SSC) numbers will be increased during propagation *in vitro* for later autotransplantation. To safeguard the success of fertility restoration, cultured testicular cells must be successfully cryopreserved during the lag-time of safety screening. SSCT, spermatogonial stem-cell transplantation.
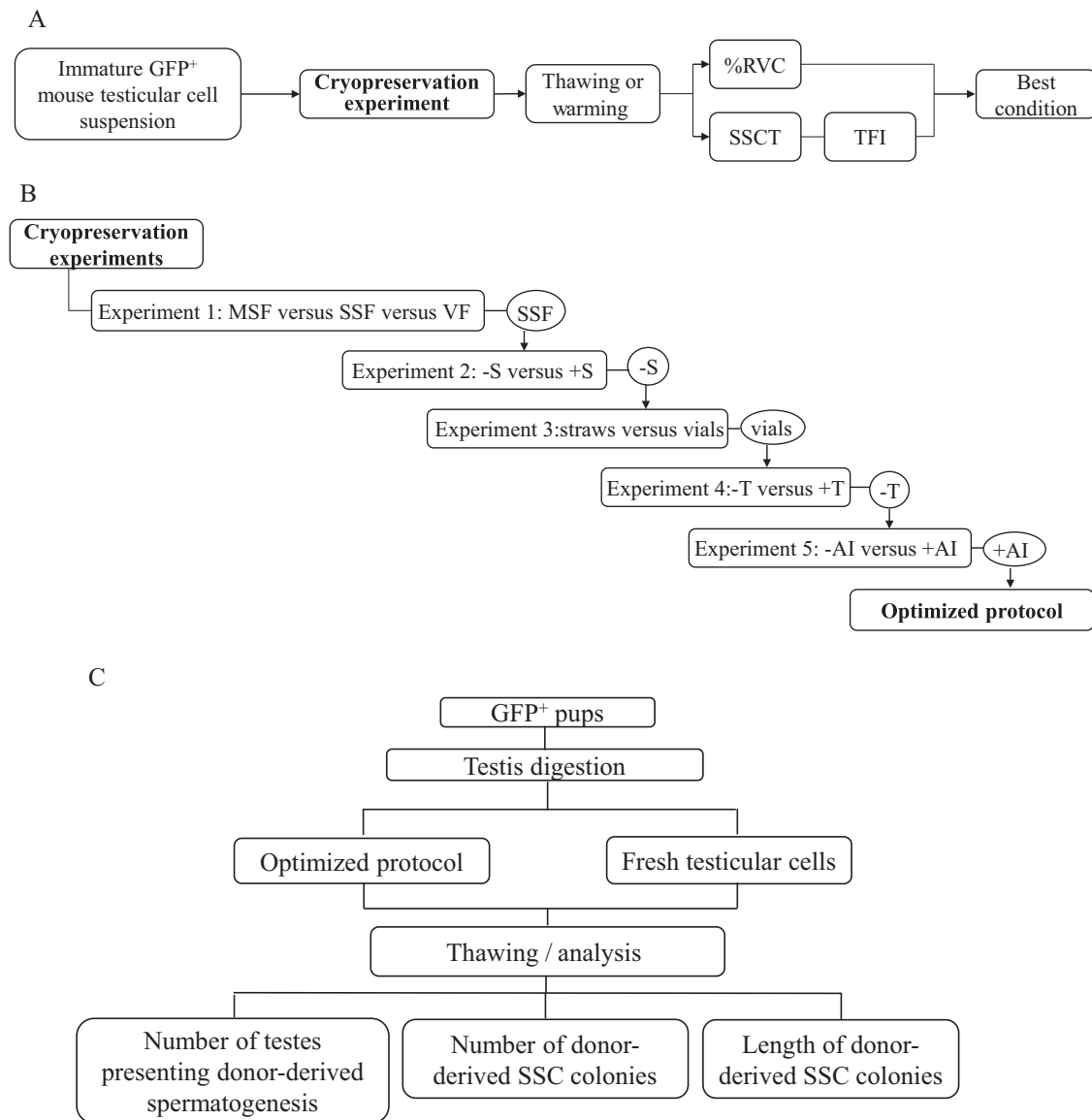
Figure 2 – Experimental design. For each experimental replicate, cell isolation, freezing–thawing or vitrification–warming and transplantation of cells was carried out. After freezing–thawing or vitrification–warming, the percentage recovery of viable cells (%RVC) was reported and the testicular cell suspensions were transplanted (SSCT) to recipient mice. After 12 weeks, transplanted testes were collected and the tubular fertility index (TFI) was analysed (A). The optimization of the cryopreservation protocol was performed step-wise, it included five independent experiments. Experiment 1 compared a multiple (MSF) and single (SSF) controlled-slow freezing protocols and vitrification (VF). A second experiment evaluated the influence of a non-permeating cryoprotective agent, sucrose (S). A third experiment compared two types of cryopreservation vessels (straws versus vials). The fourth experiment considered the addition of a high molecular weight cryoprotective agent, trehalose (T) and the fifth experiment investigated the addition of an apoptotic inhibitor (AI), z-VAD(Oe)-FMK (B). In a second set of experiments the optimized cryopreservation protocol was compared with fresh testicular cell suspensions (C). Description of the relevant experimental conditions for each cryopreservation protocol used in the present study (D). FCS, fetal calf serum; GFP, green fluorescence protein. SSC, spermatogonial stem cell.

Cryopreservation and thawing–warming

After isolation, testicular cells were suspended in 10 µl injection medium (DMEM/F12, 10% penicillin streptomycin [15140-122; Life Technologies]) per mg of testicular tissue resulting in TCS with a concentration of $1–2 \times 10^6$ cells/ml.

On the basis of the cryopreservation protocol developed by Frederickx et al. (2004), further modifications were tested (**Figure 2B**).

The first experimental group focused on the influence of different freezing rates. Testicular cell suspensions were cryopreserved in 200 µl cryopreservation medium as described by Frederickx et al. (2004) (DMEM/F12 supplemented with 1.5M dimethyl sulfoxide [DMSO] and 10% FCS) with a multiple-rate controlled slow freezing programme (MSF) or a controlled slow freezing rate programme (SSF) using a biofreezer (DB1 Freezer; Biotronics Ltd, Rotselaar, Belgium) to cool cells stored in 0.5 ml CBS cryostraws (14650; Fertipro NV,

D

Protocol	Permeable CPA	Non-permeable CPA	Vessel	Cryopreservation rate
MSF	1.5M DMSO	10% FCS	Straws	5°C/min until –7°C, equilibration 15 min, –0.3°C/min until –40°C
SSF	1.5M DMSO	10% FCS	Straws	–1°C/min until –40°C
VF	0.75M DMSO + 0.67M EG / 1.05M DMSO + 1.35M EG	250 mM sucrose + 10% FCS	Straws	plunged into LN$_2$ (vitrification)
+S	1.5M DMSO	70 mM sucrose + 10% FCS	Straws	–1°C/min until –40°C
vials	1.5M DMSO	10% FCS	Vials	–1°C/min until –80°C
+T	1.5M DMSO	200 mM trehalose + 10% FCS	Vials	–1°C/min until –80°C
+AI	1.5M DMSO	60 µM Z VAD(OMe)-FMK + 10% FCS	Vials	–1°C/min until –80°C

Fig. 2 – (continued)

Beernem, Belgium). Vitrification was achieved by exposing 200 µl of the TCS to 200 µl of a DMEM/F12 based vitrification solution, including 0.75M DMSO and 0.67 M ethylene glycol for 10 min, followed by a 5-min wash by centrifugation at 600 g and resuspension in 100 µl of injection medium. Cells were then incubated in 200 µl of a second vitrification solution with a final concentration of 1.05 M DMSO, 1.35 M ethylene glycol, 250 mM sucrose and 10% FCS for 5 min. A total of 200 µl/straw were loaded into cryostraws and subsequently plunged into LN$_2$ (Lee et al., 2014; Rodrigues et al., 2008).

In the second group of experiments, the influence of a non-permeant cryoprotective agent (CPA) was evaluated by adding 70 mM of sucrose to the original cryopreservation medium. Cells were frozen by means of a biofreezer with a controlled slow freezing rate of –1°C/min until –40°C (Pan et al., 2017). In the third group, the influence of the type of cryopreservation vessel was investigated (cryovials versus cryostraws). The TCSs were cryopreserved as described by Frederickx et al. (2004) using 1.8 ml vials (NUNC377267; VWR, Leuven, Belgium). An isopropyl alcohol container (479–3200; Mr Frosty Freezing Container; VWR) was used for cooling and cells cooled in a –80°C freezer overnight. These containers provide a slow freezing rate of about –1°C/min. In the fourth group, the influence of the high molecular weight sugar trehalose (T9531, Sigma-Aldrich, Diegem, Belgium) was studied by adding 200 mM to the original cryopreservation medium (Buchanan et al., 2004; Lee et al., 2013a). Vials were used to freeze the TCS using an isopropyl alcohol container (–1°C/min), which was put in a –80°C freezer overnight. In the final set of experiments, 60 µM of Z-VAD(OMe)-FMK (ab120487; Abcam, Cambridge, UK) was included in the original cryoprotective medium to study the effect of an apoptosis inhibitor (Ha et al., 2016). An isopropyl alcohol container (–1°C/min) was used to freeze the TCSs overnight in a –80°C freezer.

All experimental groups were carried out step-wise and based on the results of the previous experiment. For each condition, every cryostraw or cryovial was loaded with 200 µl of the cell suspension containing 1–2x10^6 cells/ml. The cryomedium was added drop-wise

to the cell suspension in a 1:1 (v/v) ratio leading to the above final concentrations. For all procedures, pre-cooled (in ice water) cryopreservation medium was used. After cooling, cryostraws or cryovials were stored in LN$_2$ for a period ranging from 2 weeks to 3 months. Thawing–warming was carried out in a 37°C water bath for 30 s according to Frederickx et al. (2004). Protocol details are shown in **Figure 2D**.

Cell counting and viability assessment

Concentration and viability were assessed with a Tali® image-based cytometer (T10796; Life Technologies, Ghent, Belgium) at three time points: immediately after cell isolation, after the addition of CPA, and subsequent to the freeze–thawing or vitrification–warming procedure. A total of 10 µl of the cell suspension were diluted by a factor of 10 with injection medium. One microliter of the Tali® Dead cell red-viability kit (A10786; Life Technologies) was added. After a 5-min incubation in the dark, 25 µl of this solution was loaded onto a Tali® slide (T10794; Life Technologies).

After thawing–warming, the percentage recovered viable cells (RVC) was calculated as follows:

$$\%RVC = \frac{\text{No. of viable cells after thawing or warming}}{\text{No. of viable cells after isolation}} \times 100$$

Transplantation experiments

Six to eight weeks before the transplantation procedure, endogenous spermatogenesis in recipient mice was suppressed by treatment with busulfan (40 mg/kg). Before SSCT, mice were anesthetized with 150 µl of a mixture of 0.1 mg/ml Medetor (Pfizer Animal Health NV, Louvain-La-Neuve, Belgium) and 0.75 mg/ml ketamine (Santé Animale, Libourne, France), dissolved in saline solution. After disinfection of

the incision area with cedium chlorhexidini alcoholicus 0.5% (BE351513; Laboratoires Gifrer Barbezat, France), the abdomen was opened and the testes were exteriorized. After thawing, cells were washed, assessed for concentration and viability, then resuspended in injection medium and 0.5 ml FCS (FCS; 10500-056; Life Technologies)] to obtain a concentration of 10–20 x 10^6 cells/ml. The goal was to inject 2 x 10^5 cells (10 μl) of the TCS. Transplantations were carried out under a stereomicroscope as previously described (Frederickx et al., 2004). Briefly, the efferent duct was immobilized and the microinjection pipette introduced through the efferent duct until the tip reached the rete testis. Trypan blue dye was added to the tip of the pipette to visualize the entry of the injected solution in the seminiferous tubules. Immediately after transplantation, mice were injected with a 100 μl of an antibiotic solution [1900 μl physiological saline serum containing 100 μl of Baytril 2.5 % (Bayer, Diegem, Belgium)].

Immunohistochemistry and histological analysis

Twelve weeks after transplantation, recipient males were killed by cervical dislocation. The testes were collected, decapsulated, fixed in acetic-formol-alcohol (PFAFA0060AF59001; Labonord, Rekkem, Belgium) and embedded in paraffin. From each testis, 5-μm thick serial sections were cut with a microtome (SM2010R; Leica, Brussels, Belgium). Slides were deparafinized in xylene and rehydrated in a descending series of isopropanol (100%, 100%, 90% and 70%) followed by a 5-min wash in phosphate buffered saline (PBS) (70011051; Life Technologies). Endogenous peroxidases were blocked in 0.3% hydrogen peroxide (H3410-500 ml; Sigma-Aldrich) for 30 min, after which the sections were incubated with 3% normal goat serum (039B304; tebu-bio, Boechout, Belgium) for 30 min. Then, the slides were incubated overnight with the primary mouse anti-GFP antibody (1/200; sc-9996; tebu-bio) at 4°C. The next morning, the sections were washed three times with PBS for 5 min followed by incubation with a rabbit anti-mouse secondary antibody (K5007; Dako, Heverlee, Belgium) for 1 h at room temperature. After three washes with PBS, 3,3'-diaminobenzidine (1/50; K5007; Dako) was added to visualize the immunoreactivity. All slides were counterstained with haematoxylin. The sections were dehydrated in a mounting series of alcohol (70%, 90%, 100% and 100%) and in xylene. Finally, slides were mounted using acrytol mounting medium (100406; Surgipath, Labonord) and analysed under an Olympus IX 81 inverted bright field microscope (IX81S1F-3, Aartselaar, Belgium). Adult GFP$^+$ mouse testicular tissue sections, with and without the addition of primary mouse anti-GFP antibody were used as positive and negative controls, respectively. Thirty serial cross-sections per testis (with a 100 μm shift between each slide) were blindly analysed to assess the tubular fertility index (TFI), which is the percentage of tubules containing donor-derived spermatogenesis (Paniagua and Nistal, 1984) (Figure 3B–3E).

Whole-mount testes assay

After isolation and decapsulation, testicular tubules were fixed in 4% paraformaldehyde (PFA) (47608; Sigma-Aldrich, Machelen, Belgium) for 2 h at 4°C and washed in PBS. Tubules were incubated in 1 M glycine (CAS56-40-6; Calbiochem, Leuven, Belgium) for 30 min at room temperature. After incubation, tubules were mounted and spread onto a slide with SlowFade® Gold antifade reagent (S36939, Life Technologies) according to Gassei et al. (2015), and analysed in the GFP channel of a fluorescence microscope (Gassei et al., 2015). Donor-derived colonies were visualized by GFP auto-fluorescence using a fluorescence microscope (Nikon Eclipse Ti-S, Nikon, city, Belgium) and a confocal microscope (Leica Microsystems, Inc., Buffalo Grove, IL, USA) to verify its characteristics. The number and length of colonies was measured by computer-assisted morphometry software (Image J, NIH). Donor-derived colony counts were obtained from manual counts from magnified digital images based on the observation that colonies have distinctive, tapered, lightly stained areas at their edges where cells are still spreading horizontally along the seminiferous tubules and vertical differentiation is incomplete (Figure 4D–4F) (Dobrinski et al., 1999). The number of colonies per testis was defined as the total number of genuine, lightly staining colony edges present in each sample. Green fluorescent segments smaller than 0.1 mm in length were excluded as these do not comply with the definition of a colony (Dobrinski et al., 1999; Nagano et al., 1999).

Statistics

GraphPad Prism, version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA), was used for data presentation and statistical analyses. All data are presented as means ± SD. Data were tested for normality using the Shapiro–Wilk test, and the statistical differences between the different cryopreservation protocols evaluated with the non-parametric Kruskal–Wallis test (Dunn's post-hoc) for %RVC, t-test for donor-derived colony activity and chi-squared for the number of testis presenting donor-derived spermatogenesis. $P < 0.05$ was considered significant.

Results

Part 1

Recovery of viable testicular cells after cryopreservation and thawing–warming

Cryopreservation and thawing–warming of cells generally results in cell loss caused by cryopreservation-induced cell damage. To improve the recovery of TCSs, we investigated various cryopreservation conditions and compared the percentage of recovered RVC before and after cryopreservation and thawing–warming. Absolute numbers of RVC before and after freezing–thawing or vitrification–warming are shown in Table 1. The percentage RVC obtained after each cryopreservation protocol is presented in Figure 3A.

Influence of freezing rates. After freezing–thawing, no significant differences were observed when comparing multiple (MSF 1.2 x 10^5 ± 1.1 x 10^5 RVC; 33 ± 28%) with single-step freezing programmes (SSF 1.7 x 10^5 ± 1.3 x 10^5 RVC; 38 ± 25%). In contrast, vitrification resulted in a significantly lower RVC (3.3 x 10^4 ± 3.8 x 10^4; 10 ± 14%; $P < 0.0001$) compared with the other protocols. For further optimization, SSF was chosen as this protocol was more time– and cost-efficient compared with MSF.

Influence of adding a non-permeable cryoprotective agent. Addition of 70 mM sucrose (+S) to the cryopreservation medium resulted in an immediate decrease in the number of viable cells (3.3 x 10^5 ± 1.8 x 10^5; $P = 0.0047$) compared with freshly isolated cells. After freezing–thawing, RVC was 33 ± 20% (1.2 x 10^5 ± 8.0 x 10^4). As the addition of sucrose did not improve the cryopreservation outcome, further cryopreservation procedures were carried out without sucrose.
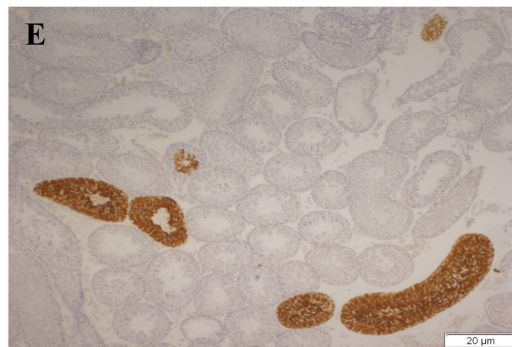
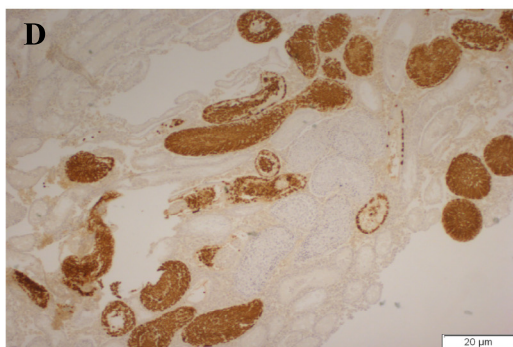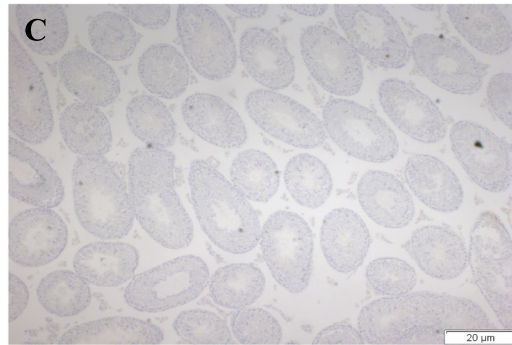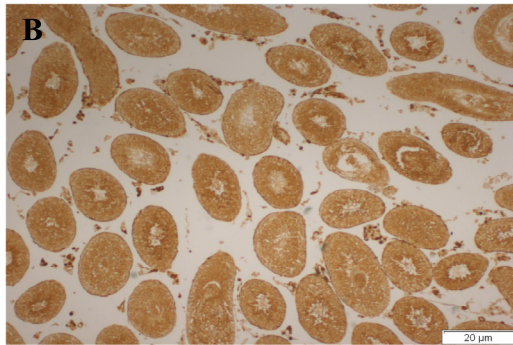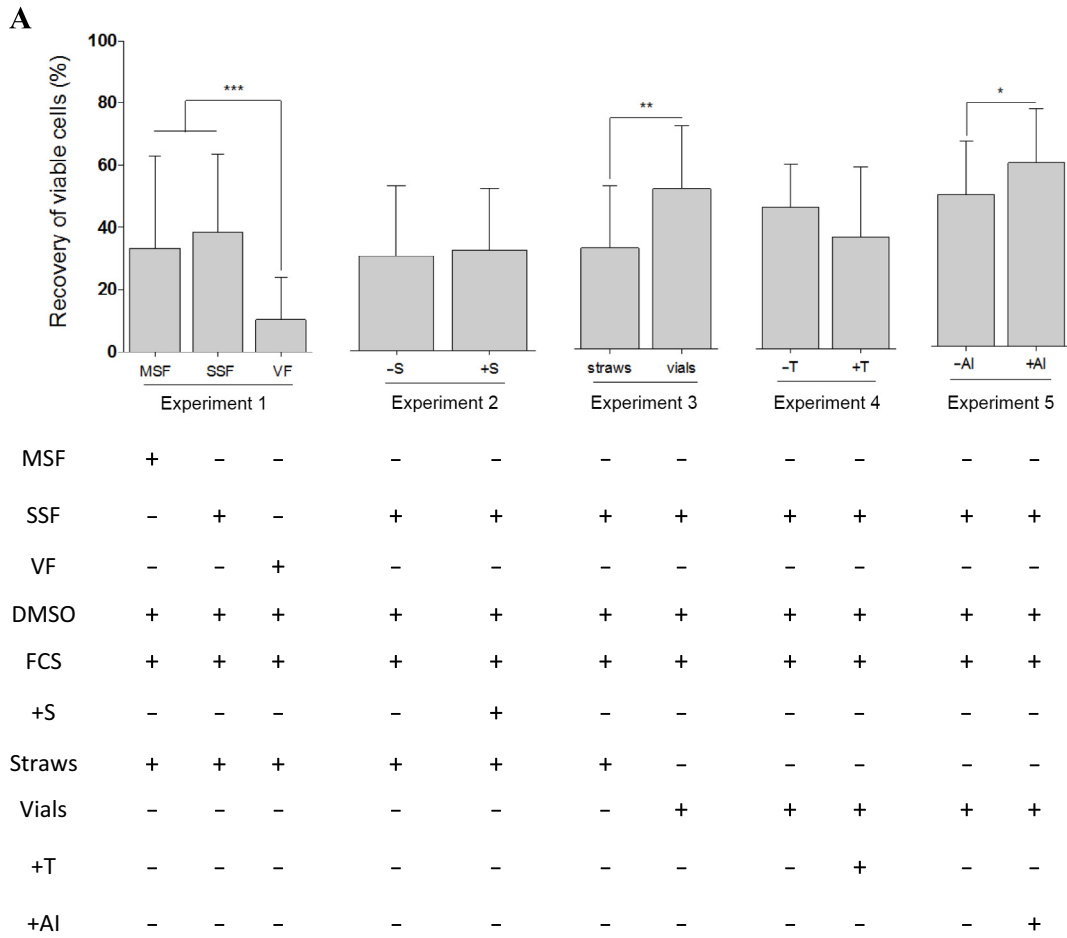|	MSF	SSF	VF	−S	+S	straws	vials	−T	+T	−AI	+AI
MSF	+	−	−	−	−	−	−	−	−	−	−
SSF	−	+	−	+	+	+	+	+	+	+	+
VF	−	−	+	−	−	−	−	−	−	−	−
DMSO	+	+	+	+	+	+	+	+	+	+	+
FCS	+	+	+	+	+	+	+	+	+	+	+
+S	−	−	−	−	+	−	−	−	−	−	−
Straws	+	+	+	+	+	+	−	−	−	−	−
Vials	−	−	−	−	−	−	+	+	+	+	+
+T	−	−	−	−	−	−	−	−	+	−	−
+AI	−	−	−	−	−	−	−	−	−	−	+

Figure 3 – Stepwise optimization of the cryopreservation protocol included the comparisons of different freezing rates (Experiment 1), sugars (Experiments 2 and 4), cryopreservation vessels (Experiment 3) and the addition of an apoptosis inhibitor (Experiment 5). (A) Spermatogonial stem-cell transplantation (SSCT) outcome was observed via anti-green fluorescence protein (GFP) immunohistochemistry. Adult mouse GPF$^+$ testicular tissue with (B) and without (C) addition of primary antibody were used as positive and negative controls respectively. SSCT using freshly isolated testicular cells before cryopreservation (D). SSCT using cells cryopreserved with the optimal cryopreservation protocol (plus apoptosis inhibitor [AI]) (E). Results in (A) are expressed as means ± SD, for each protocol n = 12 testicular cell suspensions were treated. *P = 0.0480, **P = 0.0049, ***P < 0.0001 by Kruskal–Wallis multiple comparisons test (Dunn's *post-hoc*). S, sucrose; T, trehalose; DMSO, dimethyl sulfoxide; FCS, fetal calf serum; MSF, multiple-rate controlled slow freezing programme; SSF, controlled slow freezing rate programme, VF, vitrification.

Influence of the cryopreservation vessel. Cells frozen in vials recovered significantly better than those frozen in straws, $1.0 \times 10^5 \pm 5.0 \times 10^4$ (33 ± 20%) cells were recovered when freezing in straws whereas $2.0 \times 10^6 \pm 1.2 \times 10^5$ (65 ± 16%) cells were recovered when frozen in vials with an RVC of 52 ± 20% (P = 0.0049). Consequently, the subsequent trials were performed using vials.

Influence of adding a high molecular-weight non-permeable cryoprotective agent. The addition of trehalose did not influence cell recovery. A 37 ± 23% RVC was achieved. Consequently, the following procedures were carried out using medium without trehalose.

Influence of adding a broad-spectrum anti-apoptotic factor. The addition of Z-VAD-(OMe)-FMK to the cryopreservation medium enhanced cell viability and RVC. After freezing and thawing, a significant (P = 0.0480) improvement in the number of viable cells ($2.6 \times 10^5 \pm 8.5 \times 10^4$ versus $2.3 \times 10^5 \pm 9.2 \times 10^4$) was achieved, yielding a RVC of 61 ± 18% versus 50 ± 17%, with and without Z-VAD-(OMe)-FMK, respectively.

Spermatogonial stem cell transplantation assay

To evaluate the presence and direct effects of cryopreservation on the SSC population, TCSs were transplanted into germ-cell depleted recipient mice before and after the freeze–thaw and vitrification–warming cycle for each experimental condition. The TFI was then determined by the percentage of GFP$^+$ tubules (**Figure 3B–3E**). In line with an increase in cell viability, we found the highest TFI 12 weeks after transplantation for cryopreservation experiments, including vials and the apoptotic inhibitor (9% and 7%, respectively). The percentage of TFI did not increase in the other conditions. The average number of transplanted cells and TFI are presented in **Table 2**.

Part 2

In part two, the optimal cryopreservation protocol from part one (1.5 M DMSO, 10% fetal calf serum and 60 μM of Z-VAD-[OMe]-FMK in vials at a freezing rate of –1°C/min) was compared with fresh TCSs. The number of SSCs and their colonizing activity was assessed by the number and length of SSC colonies after transplantation (**Figure 4**). After digestion of testicular tissues, fresh TCSs presented a RCV of 92 ± 4%, whereas cryopreservation of TCSs using 1.5M DMSO + 10% FCS + Z-VAD-(OMe)-FMK in vials resulted in 63 ± 11% RVC after thawing, showing no difference with the RVC achieved in Part 1.

The number of testes with donor derived spermatogenesis after SSCT was the same in both fresh and cryopreserved TCSs. A significant difference (P = 0.0051) was found in the numbers of SSC colonies per testes (34.04 ± 2.34 versus 16.78 ± 7.76 for fresh and cryopreserved TCSs, respectively), indicating a loss of 51% of initial SSCs as a result of the freezing procedure. No differences were found in the length of the surviving colonies (3.94 ± 2.47 versus 2.95 ± 0.37 mm for TCSs before and after freeze–thawing, respectively) (**Table 3**).

Table 1 – Absolute number of viable cells in prepubertal mouse testicular cell suspensions after cell isolation, addition of cryoprotective agent and cryopreservation–thawing or warming under different cryopreservation protocols.[a]

Experimental group	Protocol	n	Number of viable cells before cryopreservation	After CPA addition	After freeze–thawing or vitrification–warming
1	MSF	12	$3.3 \times 10^5 \pm 1.6 \times 10^5$	$3.2 \times 10^5 \pm 1.3 \times 10^5$	$1.2 \times 10^5 \pm 1.1 \times 10^5$[c]
	SSF	12	$4.2 \times 10^5 \pm 2.6 \times 10^5$	$4.7 \times 10^5 \pm 2.9 \times 10^5$	$1.7 \times 10^5 \pm 1.3 \times 10^5$[c]
	VF	12	$4.2 \times 10^5 \pm 2.5 \times 10^5$	$3.8 \times 10^5 \pm 1.7 \times 10^5$	$3.3 \times 10^4 \pm 3.8 \times 10^4$[c]
2	–S	12	$3.8 \times 10^5 \pm 9.7 \times 10^4$	$2.9 \times 10^5 \pm 1.2 \times 10^5$	$1.1 \times 10^5 \pm 8.4 \times 10^4$[c]
	+S	12	$3.9 \times 10^5 \pm 9.0 \times 10^5$	$3.3 \times 10^5 \pm 1.8 \times 10^5$[b]	$1.2 \times 10^5 \pm 8.0 \times 10^4$[c]
3	straws	12	$4.0 \times 10^5 \pm 2.0 \times 10^5$	$3.3 \times 10^5 \pm 1.5 \times 10^5$	$1.0 \times 10^5 \pm 5.0 \times 10^4$[c]
	vials	12	$4.0 \times 10^5 \pm 2.0 \times 10^5$	$2.7 \times 10^5 \pm 2.3 \times 10^5$	$2.0 \times 10^5 \pm 1.2 \times 10^5$[c]
4	–T	12	$3.6 \times 10^5 \pm 8.8 \times 10^4$	$2.7 \times 10^5 \pm 1.2 \times 10^5$	$1.6 \times 10^5 \pm 5.2 \times 10^4$[c]
	+T	12	$3.8 \times 10^5 \pm 1.5 \times 10^5$	$2.6 \times 10^5 \pm 2.4 \times 10^5$	$1.3 \times 10^5 \pm 7.8 \times 10^4$[c]
5	–AI	12	$4.6 \times 10^5 \pm 9.0 \times 10^4$	$3.4 \times 10^5 \pm 1.5 \times 10^5$	$2.3 \times 10^5 \pm 9.2 \times 10^4$[c]
	+AI	12	$4.5 \times 10^5 \pm 1.0 \times 10^4$	$3.1 \times 10^5 \pm 2.4 \times 10^5$	$2.6 \times 10^5 \pm 8.5 \times 10^4$[c]

AI, apoptotic inhibitor; CPA, cryoprotective agent; MSF, multiple-rate controlled slow freezing program; S, sucrose; SSF, slow freezing rate programme, T, trehalose; VF, vitrification.

[a] For protocol details see **Figure 2D**.

[b] P = 0.0047 by Kruskal–Wallis test (compared with the number of cells after isolation).

[c] P < 0.0001 by Kruskal–Wallis test (compared with the number of cells after cell isolation).

Experiment	Protocol	Average number of transplanted cells ± SD	Number of testes with donor-derived spermatogenesis/total number of transplanted testes	Average testicular weight at analysis (mg)	TFI (%)[c]
1	Fresh	$1.2 \times 10^5 \pm 8.1 \times 10^4$	4/6[d]	30.70 ± 8.65	40[p]
	MSF	$1.4 \times 10^5 \pm 7.1 \times 10^4$	2/5[e]	28.30 ± 5.23	3[q]
	SSF	$1.0 \times 10^5 \pm 1.2 \times 10^5$	2/5[e]	28.05 ± 5.73	3[q]
	VF	$1.2 \times 10^5 \pm 9.1 \times 10^4$	0/4[f]	18.56 ± 5.19	0[q]
2	Fresh	$1.4 \times 10^5 \pm 1.6 \times 10^5$	3/4[g]	31.55 ± 0.78	30[r]
	–S	$1.5 \times 10^5 \pm 1.6 \times 10^5$	2/5[h]	29.72 ± 4.64	2[s]
	+S	$1.1 \times 10^5 \pm 1.0 \times 10^5$	2/5[h]	25.75 ± 3.89	1[s]
3	Fresh	$2.1 \times 10^5 \pm 1.9 \times 10^5$	7/9[i]	31.89 ± 4.20	35[t]
	straws	$2.2 \times 10^5 \pm 1.2 \times 10^5$	4/9[j]	29.53 ± 4.38	2[u]
	vials	$1.2 \times 10^5 \pm 4.2 \times 10^4$	6/8[j,k]	28.25 ± 6.38	9[u]
4	Fresh	$1.0 \times 10^5 \pm 9.1 \times 10^4$	2/4[l]	31.50 ± 3.54	37[v]
	–T	$1.7 \times 10^5 \pm 1.0 \times 10^5$	2/5[l,m]	31.15 ± 0.21	5[w]
	+T	$1.8 \times 10^5 \pm 7.1 \times 10^4$	1/5[n]	26.20 ± 6.79	0[w]
5	Fresh	$1.2 \times 10^5 \pm 8.4 \times 10^4$	4/5[o]	32.33 ± 2.79	33[x]
	–AI	$1.4 \times 10^5 \pm 9.2 \times 10^4$	3/5[o]	30.20 ± 7.22	6[y]
	+AI	$1.4 \times 10^5 \pm 9.6 \times 10^4$	3/4[o]	31.75 ± 1.53	7[y]

Table 2 – Testis with donor-derived spermatogenesis.[a,b]

AI, apoptosis inhibitor; MSF, multiple-rate controlled slow freezing program; S, sucrose; SSF, slow freezing rate programme; T, trehalose; VF, vitrification.

[a] For protocol details see **Figure 2D**.

[b] Significance is indicated by the presence of a different superscript within each experimental group.

[c] Tubular fertility index (TFI) expresses the percentage of tubules containing complete donor-derived spermatogenesis.

After spermatogonial stem-cell transplantation, the number of testis with donor derived spermatogenesis was significantly higher in testis transplanted with fresh testicular cell suspensions for groups 1–4 respectively: [d]vs[e] $P = 0.0116$; [d]vs[f] $P < 0.0001$; [g]vs[h] $P = 0.0011$; [i]vs[j] $P = 0.0038$; [l]vs[n] $P = 0.0003$ by chi-squared test. Likewise, single (SSF) and multiple (MSF) step controlled freezing as vials and medium without trehalose (–T) presented a significantly higher number of testes with donor-derived spermatogenesis than their counterparts: [e]vs[f] $P < 0.0001$; [j]vs[i,k] $P = 0.0062$; [l,m]vs[n] $P = 0.0098$ by chi-squared test. TFI: [p]vs[q] $P < 0.0001$; [r]vs[s] $P = 0.0046$; [t]vs[u] $P < 0.0001$; [v]vs[w] $P = 0.0088$; [x]vs[y] $P = 0.0339$ by Kruskal–Wallis multiple comparisons test (Dunn's *post-hoc*).
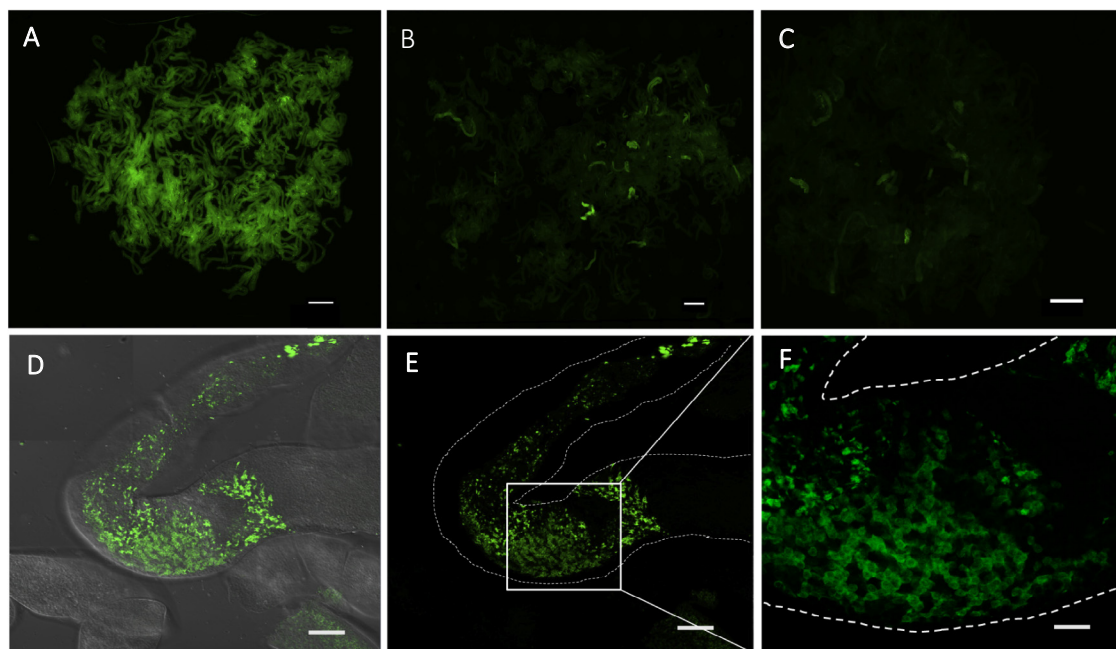

Figure 4 – Spermatogonial stem cell (SSC) colony formation upon transplantation before and after freeze–thawing. Image of a whole mount adult green fluorescence protein (GFP+) mouse testis (A) obtained 12 weeks after SSCT with fresh (B) and frozen–thawed cells with the optimized protocol (C). Donor-derived colonies were defined as distinctive, tapered, green fluorescent areas greater than 0.1 mm length (D, E). The dynamics of SSC colony formation, cells spreading horizontally (propagation) and vertically (differentiation) along the seminiferous tubules were observed (F). The scale bar represents 2 mm (A, B, C); 60 μm (D, E); 25 μm (F).

Table 3 – Number and length of donor-derived spermatogonial stem-cell colonies in testes transplanted with cryopreserved cells with the optimal protocol and fresh testicular cell suspensions. ,		
	Freshly digested testicular cell suspensions	Freeze–thawed (optimized protocol)
Percentage recovery of viable cells (%RVC)	92 ± 4[c]	63 ± 11
Number of testis with donor-derived spermatogenesis (%)	3/3 (100%)	4/4 (100%)
Number of colonies/testis	8.33 ± 3.06[d]	3.25 ± 0.96
Number of colonies/10^5 cells injected	4.08 ± 0.28	2.01 ± 0.93
Number of colonies/testis corrected for transplantation efficiency (12%)[f]	34.04 ± 2.34[e]	16.78 ± 7.76
Length of colonies (mm)	3.94 ± 2.47	2.95 ± 0.37

RVC, recovered viable cells.

[a] Values are mean ± SD.

[b] t-test was used to assess significant differences.

[c] $P < 0.0001$.

[d] $P = 0.0236$.

[e] $P = 0.0051$.

[f] Number of colonies corrected for the colonization efficiency of 12% (Nagano et al., 1999).

Discussion

Cryopreservation of isolated TCSs is a prerequisite to SSCT in patients with a systemic cancer. Unfortunately, testicular tissue cryopreservation and subsequent grafting presents the risk of reintroducing malignant cells and thus cancer relapse (Jahnukainen et al., 2001). As an alternative for these patients, TCSs generated from stored testicular tissue can be digested and cultured to allow for SSC isolation and propagation. During the mandatory safety testing that will be needed before SSCT can be considered, cultured cells will have to be cryopreserved again (**Figure 1**). It is still unclear, however, what cryopreservation technique better protects SSC: tissue cryopreservation or single cell cryopreservation. Throughout the cryopreservation process, cells respond differently depending on whether they are part of a tissue or whether they are in suspension and cell suspensions have been developed with with the aim to facilitate cryopreservation. Furthermore, cell heterogeneity in tissues renders freezing more challenging as a result of the structural limitation of heat and mass transfer. This heterogeneity, however, also maintains important cell–cell contacts that can safeguard the cells through cryopreservation and thawing events (Karlsson et al., 1993; Wyns, 2013). In contrast, cryopreservation of single cell suspensions requires direct exposure to enzymatic and CPA solutions, which can change the biophysical properties of the cells, resulting in higher cell sensitivity to cryopreservation events (Brook et al., 2001).

The end goal is to preserve SSCs, for which there is a gap in knowledge and optimal freeze–thawing procedures have yet to be defined. As such, identifying the key characteristics that influence cell viability during the freeze–thaw cycle will aid in the preservation of these valuable cells. Some important criteria have already been identified; for example, some evidence suggests that SSC are more resistant to cryopreservation stress compared with differentiated germ cells (Lee et al., 2013b). Furthermore, the cooling rate during cryopreservation can be inferred from their cell size and nucleus–cytoplasm ratio, which in these cells is very similar to hematopoietic stem cells for which an optimal cooling rate of 1°C/min was already defined (Berz et al., 2007; Buchanan et al., 2004; Mazur, 1970; Rodrigues et al., 2008).

In the present study, no difference in RVC was observed between single and multiple step controlled slow freezing (33 ± 28% and 38 ± 25%, respectively). These results were lower than those reported by Izadyar et al. (2002) and Frederickx et al. (2004), who achieved 50% and 70% cell viability after freezing–thawing, respectively. The nearly 30% difference could be explained by slight differences in the protocol. Frederickx et al. (2004) achieved multiple step freezing using a Planer Kryo 10 biofreezer (Planer products, Gaithsburg, USA), whereas, for this study, a DB1 biofreezer (Biotronics Ltd, Rotselaar, Belgium) was used. The efficiency of the DB1 biofreezer may be lower owing to its single chamber system, allowing the samples to cool by intermittent soaking of the chamber in LN2 until the desired temperature is reached. The Planer Kryo 10 offers a multi-chamber system, each with a controlled temperature, which is ideal for multiple step cryopreservation protocols.

As Frederickx et al. (2004) showed that cooling to –40°C increased cell recovery, but not viability, we adjusted the cooling rate to –1°C/min, as per Izadyar et al. (2002), reaching a temperature of –40°C. This reduces the time during which cells remain vulnerable to ice formation, potentially reducing damage sustained by ice crystal formation (Mazur, 1984). Furthermore, side-effects such as CPA toxicity and cell shrinkage may become less severe as a result of altered freezing rates (Gilkey and Staehelin, 1986).

The vitrification procedure used in this study was based on the study by Baert et al. (2013), which was designed for mouse testicular tissue biopsies. In contrast to controlled freezing, poor cell viability and survival were achieved. This may be a result of the longer equilibration time or incomplete vitrification (crystallization) (Baert et al., 2012). As SSCs are quite resistant to cryopreservation stress, we hypothesized that the reduced viability after vitrification could be the result of significant somatic cell death (Kanatsu-Shinohara et al., 2003; Lee et al., 2013b). The transplantation assay, however, showed that functional SSCs were not recovered after vitrification.

It has been reported that the addition of the non-permeating CPA sucrose significantly enhanced survival and viability of bovine spermatogonia after thawing, yielding 70% viability (Izadyar et al., 2002). It was suggested that non-penetrating CPAs, such as sucrose, trehalose and dextrane) may offer enhanced membrane stability, helping the cell to support variances in osmolarity while equilibrating with the cryoprotective medium. Moreover, non-penetrating CPAs may diminish the physical damage encountered during ice-nucleation (Acker and McGann, 2003; Jain and Roy, 2009; Karlsson et al., 1993; Lee et al., 2014). Nevertheless, addition of sucrose did not improve post-thaw

cell recovery and viability. Similarly, Lee et al. (2013b) recently showed that adding trehalose to the cryomedium has a concentration-dependent effect on mouse SSC recovery and proliferation capacity after freeze–thawing (Jain and Roy, 2009; Lee et al., 2014). Trehalose is a complex disaccharide known to facilitate cellular integrity as a stress-response factor. Notably, it allows for efficient preservation of mammalian cells (Eroglu et al., 2000; Jain and Roy, 2009). In the present study, TCSs cryopreserved in the presence of trehalose displayed decreased cell viability and very low SSC activity after SSCT. Taken together, our results strongly suggest that SSC are sensitive to osmotic variations in the cryomedium. Indeed, it has been reported that improved cell survival stemming from the addition of sugar molecules is achieved by preculturing cells in cryopreservation medium with a given molecule (Agca et al., 2005; Mazur and Cole, 1989; Seijo, 2000). Including a 10–15 min incubation time before initiating cryopreservation would allow the cells to recover from the osmotic stress caused by the addition of sugars (Izadyar et al., 2002). As we did not include an equilibration time, our protocol may have predisposed the testicular cells to osmotic shock, resulting in decreased cell recovery. Any further optimization should, therefore, consider the influence of equilibration times with non-permeating CPAs on SSC cryopreservation.

Additionally, we investigated the difference in viability between cryovials and cryostraws, which necessitated a change in cryopreservation device as our biofreezer does not function with vials. Alternatively, an isopropyl-container was used, resulting in a significantly improved cell survival compared with cryostraws cooled in a biofreezer (52% ± 20 versus 33% ± 20 RVC), which may be due to a reduction in mechanical damage to the cells (Saragusty et al., 2009). Indeed, during freezing, extracellular ice nucleation and expansion increases the intra-vessel pressure, which combined with extracellular ice nucleation, may apply pressure to the cells to the unfrozen part of the medium causing cell crushing by a 'pack effect'. As a result, the larger the surface area with which cells are in contact in the medium, the greater the damage (Saragusty et al., 2009). Storing 200 µl in a 1.8 ml vial permits a higher surface-area-to-volume ratio compared with storing 200 µl in a 0.5 ml cryostraw (Gilkey and Staehelin, 1986; Saragusty et al., 2009). The pressure created by ice crystals might thus be released in the empty vessel space, diminishing the 'pack effect' and resulting in an increase in cell viability and recovery. It would be of interest to use vials in combination with a programmed controlled freezing rate to further increase viability.

Finally, the mitochondrial caspase 9 apoptotic pathway is thought to be responsible for cryopreservation-induced delayed-onset cell death (Bissoyi et al., 2014). The apoptotic inhibitor Z-VAD-(OMe)-FMK is the most suitable candidate owing to its broad spectrum inhibitory proprieties against caspases and its irreversible effect on cells (Bissoyi et al., 2014; Ha et al., 2016; Peter and Linde-Forsberg, 2003). A few studies on mouse spermatogonia frozen with a cryomedium, including an anti-apoptotic factor, have been published (Ha et al., 2016; Lee et al., 2013a). In our study, Z-VAD-(OMe)-FMK led to significantly higher recovery of viable cells (61 ± 18%). Nevertheless, the number of viable cells recovered after thawing can still be improved. Additional attention could be given to the thawing process, which is as important as freezing. In this study, samples were thawed in a 37°C water bath, which is considered a standard procedure. Controlled thawing is an unexplored, yet promising alternative that deserves to be studied (Gurina et al., 2015). Also, further study of vitrification is warranted as it is a fast and easy way to cryopreserve cell samples.

Furthermore, it has been shown that cell viability does not correlate with the functional capacity of the SSCs (Frederickx et al., 2004). The SSCT assay, as we used in this study, is the gold standard to verify and quantify the presence, proliferation and differentiation capacity of donor SSCs after freeze–thawing procedures. Consideration, however, must be given to primary testicular cell propagation culture in future studies, as it is a fundamental step for the future clinical application of the SSCT technique (Sadri-Ardekani et al., 2009).

Although immature mouse SSCs can be cryopreserved and retain spermatogenic function upon freeze–thawing, the present results may not be easily extrapolated to humans owing to the different structure and biology of the human testis (Kanatsu-Shinohara et al., 2003). Therefore, additional research will be necessary to develop a suitable clinical grade procedure with xenofree cryopreservation media (Hermann et al., 2012; Karlsson and Toner, 1996; Tournaye et al., 2004).

In conclusion, using a controlled slow-freezing method including 1.5M DMSO, 10% FCS and 60 µM of Z-VAD-(OMe)-FMK in DMEM as cryoprotection medium in vials leads to the highest recovery of viable cells (61%) currently reported. Importantly, the superiority of the proposed cryopreservation protocol was confirmed by SSCT. When comparing the number of donor-derived stem cell colonies after transplantation of cryopreserved and fresh TCSs, a recovery of 49% of the original SSCs could be shown (Table 3). These cryopreservation conditions are relevant for the eventual translation of this cryopreservation protocol to human TCSs, in the hope of supporting a future clinical application.

REFERENCES

Acker, J.P., McGann, L.E., 2003. Protective effect of intracellular ice during freezing? Cryobiology 46, 197–202. doi:10.1016/S0011-2240(03)00025-7.

Agca, Y., Mullen, S., Liu, J., Johnson-Ward, J., Gould, K., Chan, A., Critser, J., 2005. Osmotic tolerance and membrane permeability characteristics of rhesus monkey (Macaca mulatta) spermatozoa. Cryobiology 51, 1–14. doi:10.1016/j.cryobiol.2005.04.004.

Baert, Y., Goossens, E., van Saen, D., Ning, L., in't Veld, P., Tournaye, H., 2012. Orthotopic grafting of cryopreserved prepubertal testicular tissue: in search of a simple yet effective cryopreservation protocol. Fertil. Steril. 97, 1152–7–2. doi:10.1016/j.fertnstert.2012.02.010.

Baert, Y., Van Saen, D., Haentjens, P., In't Veld, P., Tournaye, H., Goossens, E., 2013. What is the best cryopreservation protocol for human testicular tissue banking? Hum. Reprod. 28, 1816–1826. doi:10.1093/humrep/det100.

Berz, D., McCormack, E.M., Winer, E.S., Colvin, G.A., Quesenberry, P.J., 2007. Cryopreservation of hematopoietic stem cells. Am. J. Hematol. 82, 463–472. doi:10.1002/ajh.20707.

Bissoyi, A., Nayak, B., Pramanik, K., Sarangi, S.K., 2014. Targeting cryopreservation-induced cell death: a review. Biopreserv. Biobanking 12, 23–34. doi:10.1089/bio.2013.0032.

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

Buchanan, S.S., Gross, S.A., Acker, J.P., Toner, M., Carpenter, J.F., Pyatt, D.W., 2004. Cryopreservation of stem cells using trehalose: evaluation of the method using a human hematopoietic cell line. Stem Cells Dev. 13, 295–305. doi:10.1089/154732804323099226.

Demeestere, I., Simon, P., Dedeken, L., Moffa, F., Tsepelidis, S., Brachet, C., Delbaere, A., Devreker, F., Ferster, A., 2015. Live birth after autograft of ovarian tissue cryopreserved during childhood. Hum. Reprod. 0, 2107–2109. doi:10.1093/humrep/dev128.

Dobrinski, I., Ogawa, T., Avarbock, M.R., Brinster, R.L., 1999. Computer assisted image analysis to assess colonization of recipient seminiferous tubules by spermatogonial stem cells from transgenic donor mice. Mol. Reprod. Dev. 53, 142–148. https://doi:10.1002/(SICI)1098–2795(199906)53:2<142::AID-MRD3>3.0.CO;2-O.

Eroglu, A., Russo, M.J., Bieganski, R., Fowler, A., Cheley, S., Bayley, H., Toner, M., 2000. Intracellular trehalose improves the survival of cryopreserved mammalian cells. Nat. Biotechnol. 18, 163–167. doi:10.1038/72608.

Frederickx, V., Michiels, A., Goossens, E., De Block, G., Van Steirteghem, A.C., Tournaye, H., 2004. Recovery, survival and functional evaluation by transplantation of frozen-thawed mouse germ cells. Hum. Reprod. 19, 948–953. doi:10.1093/humrep/deh154.

Gassei, K., Valli, H., Orwig, K.E., 2015. Whole-mount immunohistochemistry to study spermatogonial stem cells and spermatogenic lineage development in mice, monkeys, and humans. Methods Mol. Biol. 1210, doi:10.1007/978-1-4939-1435-7_15.

Gilkey, J.C., Staehelin, L.A., 1986. Advances in ultrarapid freezing for the preservation of cellular ultrastructure. J. Electron Microsc. Tech. 3, 177–210. doi:10.1002/jemt.1060030206.

Giudice, M.G., de Michele, F., Poels, J., Vermeulen, M., Wyns, C., 2017. Update on fertility restoration from prepubertal spermatogonial stem cells: how far are we from clinical practice? Stem Cell Res. 1–7. doi:10.1016/j.scr.2017.01.009.

Goossens, E., Van Saen, D., Tournaye, H., 2013. Spermatogonial stem cell preservation and transplantation: from research to clinic. Hum. Reprod. 28, 897–907. doi:10.1093/humrep/det039.

Gurina, T.M., Pakhomov, A.V., Polyakova, A.L., Legach, E.I., Bozhok, G.A., 2015. The development of the cell cryopreservation protocol with controlled rate thawing. Cell Tissue Bank. doi:10.1007/s10561-015-9533-6.

Ha, S.J., Kim, B.G., Lee, Y.A., Kim, Y.H., Kim, B.J., Jung, S.E., Pang, M.G., Ryu, B.Y., 2016. Effect of antioxidants and apoptosis inhibitors on cryopreservation of murine germ cells enriched for spermatogonial stem cells. PLoS ONE 11, doi:10.1371/journal.pone.0161372. e0161372.

Hermann, B.P., Sukhwani, M., Winkler, F., Pascarella, J.N., Peters, K.A., Sheng, Y., Valli, H., Rodriguez, M., Ezzelarab, M., Dargo, G., Peterson, K., Masterson, K., Ramsey, C., Ward, T., Lienesch, M., Volk, A., Cooper, D.K., Thomson, A.W., Kiss, J.E., Penedo, M.C., Schatten, G.P., Mitalipov, S., Orwig, K.E., 2012. Spermatogonial stem cell transplantation into rhesus testes regenerates spermatogenesis producing functional sperm. Cell Stem Cell 11, 715–726. doi:10.1016/j.stem.2012.07.017.

Hudson, M.M., 2010. Reproductive outcomes for survivors of childhood cancer. Obstet. Gynecol. 116, 1171–1183. doi:10.1097/AOG.0b013e3181f87c4b.

Izadyar, F., Matthijs-Rijsenbilt, J.J., den Ouden, K., Creemers, L.B., Woelders, H., de Rooij, D.G., 2002. Development of a cryopreservation protocol for type a spermatogonia. J. Androl. 23, 537–545. doi:10.1002/j.1939-4640.2002.tb02276.x.

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

Jahnukainen, K., Mitchell, R.T., Stukenborg, J.B., 2015. Testicular function and fertility preservation after treatment for haematological cancer. Curr. Opin. Endocrinol. Diabetes Obes. 22, 217–223. doi:10.1097/MED.0000000000000156.

Jain, N.K., Roy, I., 2009. Effect of trehalose on protein structure. Protein Sci. doi:10.1002/pro.3.

Kanatsu-Shinohara, M., Ogonuki, N., Inoue, K., Ogura, A., Toyokuni, S., Shinohara, T., 2003. Restoration of fertility in infertile mice by transplantation of cryopreserved male germline stem cells. Hum. Reprod. 18, 2660–2667. doi:10.1093/humrep/deg483.

Karlsson, J.O., Toner, M., 1996. Long-term storage of tissues by cryopreservation: critical issues. Biomaterials 17, 243–256. doi:10.1016/0142-9612(96)85562-1.

Karlsson, J.O., Cravalho, E.G., Borel Rinkes, I.H., Tompkins, R.G., Yarmush, M.L., Toner, M., 1993. Nucleation and growth of ice crystals inside cultured hepatocytes during freezing in the presence of dimethyl sulfoxide. Biophys. J. 65, 2524–2536. doi:10.1016/S0006-3495(93)81319-5.

Keros, V., Rosenlund, B., Hultenb, K., Aghajanova, L., Levkov, L., Hovatta, O., 2005. Optimizing cryopreservation of human testicular tissue: comparison of protocols with glycerol, propanediol and dimethylsulphoxide as cryoprotectants. Hum. Reprod. 20, 1676–1687. doi:10.1093/humrep/deh797.

Lee, Y.-A., Kim, Y.-H., Kim, B., Jung, M.-S., Auh, J., Seo, J., Park, Y.-S., Lee, S., Ryu, B., 2013b. Cryopreservation of mouse spermatogonial stem cells in dimethylsulfoxide and polyethylene glycol. Biol. Reprod. 89, 109. doi:10.1095/biolreprod.113.111195.

Lee, Y.A., Kim, Y.H., Kim, B.J., Kim, B.G., Kim, K.J., Auh, J.H., Schmidt, J.A., Ryu, B.Y., 2013a. Cryopreservation in trehalose preserves functional capacity of murine spermatogonial stem cells. PLoS ONE 8, doi:10.1371/journal.pone.0054889. e54889.

Lee, Y.A., Kim, Y.H., Ha, S.J., Kim, B.J., Kim, K.J., Jung, M.S., Kim, B.G., Ryu, B.Y., 2014. Effect of sugar molecules on the cryopreservation of mouse spermatogonial stem cells. Fertil. Steril. 101, doi:10.1016/j.fertnstert.2013.12.033.

Mazur, P., 1970. Cryobiology: the freezing of biological systems. Science 168, 939–949.

Mazur, P., 1984. Freezing of living cells: mechanisms and implications. Am. J. Physiol. 247, C125–C142.

Mazur, P., Cole, K.W., 1989. Roles of unfrozen fraction, salt concentration, and changes in cell volume in the survival of frozen human erythrocytes. Cryobiology 26, 1–29. doi:10.1016/0011-2240(89)90030-8.

Nagano, M., Avarbock, M.R., Brinster, R.L., 1999. Pattern and kinetics of mouse donor spermatogonial stem cell colonization in recipient testes. Biol. Reprod. 60, 1429–1436. doi:10.1095/biolreprod60 .6.1429.

Ogawa, T., Arechaga, J.M., Avarbock, M.R., Brinster, R.L., 1997. Transplantation of testis germinal cells into mouse seminiferous tubules. Int. J. Dev. Biol. 41, 111–122.

Pan, C., Yu, S., Zhang, P., Wang, B., Zhu, Z., Liu, Y., Zeng, W., 2017. Effect of sucrose on cryopreservation of pig spermatogonial stem cells. J. Integr. Agric. 16, 1120–1129. doi:10.1016/S2095-3119(16)61489-2.

Paniagua, R., Nistal, M., 1984. Morphological and histometric study of human spermatogonia from birth to the onset of puberty. J. Anat. 139, 535–552.

Peter, A.T., Linde-Forsberg, C., 2003. Efficacy of the anticaspase agent zVAD-fmk on post-thaw viability of canine spermatozoa. Theriogenology 59, 1525–1532. doi:10.1016/S0093-691X(02)01206-2.

Rodrigues, J.P., Paraguassú-Braga, F.H., Carvalho, L., Abdelhay, E., Bouzas, L.F., Porto, L.C., 2008. Evaluation of trehalose and sucrose as cryoprotectants for hematopoietic stem cells of umbilical cord blood. Cryobiology 56, 144–151. doi:10.1016/j.cryobiol.2008.01.003.

Sá, R., Cremades, N., Malheiro, I., Sousa, M., 2012. Cryopreservation of human testicular diploid germ cell suspensions. Andrologia 44, 366–372. doi:10.1111/j.1439-0272.2012.01290.x.

Sadri-Ardekani, H., Mizrak, S.C., Van Daalen, S.K.M., Korver, C.M., Roepers-Gajadien, H.L., Koruji, M., Hovingh, S., De Reijke, T.M., de la Rosette, J.J., Van Der Veen, F., de Rooij, D.G., Repping, S., van Pelt, A.M.M., 2009. Propagation of human spermatogonial stem cells in vitro. JAMA 302, 2127–2134. doi:302/19/2127 [pii]\r10.1001/jama .2009.1689.

Saragusty, J., Gacitua, H., Rozenboim, I., Arav, A., 2009. Do physical forces contribute to cryodamage? Biotechnol. Bioeng. 104, 719–728. doi:10.1002/bit.22435.

Seijo, G., 2000. Effects of preculture with sucrose and aba on cell suspensions water status and its relation with vitrification resistance. Rev. Bras. Fisiol. Veg. 12, 166–180. doi:10.1590/S0103 -31312000000200007.

Tournaye, H., Goossens, E., Verheyen, G., Frederickx, V., De Block, G., Devroey, P., Van Steirteghem, A., 2004. Preserving the reproductive potential of men and boys with cancer: current concepts and future prospects. Hum. Reprod. Update 10, 525–532. doi:10.1093/humupd/ dmh038.

Tournaye, H., Dohle, G.R., Barratt, C.L.R., 2014. Fertility preservation in men with cancer. Lancet 384, 1295–1301. doi:10.1016/S0140 -6736(14)60495-5.

Van Saen, D., Goossens, E., De Block, G., Tournaye, H., 2009. Regeneration of spermatogenesis by grafting testicular tissue or injecting testicular cells into the testes of sterile mice: a comparative study. Fertil. Steril. 91, 2264–2272. doi:10.1016/ j.fertnstert.2008.02.100.

Wyns, C., 2013. Fertility preservation: current prospects and future challenges. Gynecol. Endocrinol. 29, 403–407. doi:10.3109/09513590 .2012.754872.