What is the best cryopreservation protocol for human testicular tissue banking?

Y. Baert¹,*, D. Van Saen¹, P. Haentjens², P. In’t Veld³, H. Tournaye¹,4, and E. Goossens¹

¹Biology of the Testis, Research Laboratory for Reproduction, Genetics and Regenerative Medicine, Vrije Universiteit Brussel, Laarbeeklaan 103, Brussels 1090, Belgium ²Centre for Outcomes Research, Universitair Ziekenhuis Brussel, Laarbeeklaan 101, Brussels 1090, Belgium ³Department of Pathology, Vrije Universiteit Brussel, Laarbeeklaan 103, Brussels 1090, Belgium ⁴Centre for Reproductive Medicine, Universitair Ziekenhuis Brussel, Laarbeeklaan 101, Brussels 1090, Belgium

*Correspondence address. Tel: +32-2-477-46-44; Fax: +32-2-477-46-32; E-mail: yoni.baert@vub.ac.be

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STUDY QUESTION: Is there a better alternative to the conventional cryopreservation protocols for human testicular tissue banking?

SUMMARY ANSWER: Uncontrolled slow freezing (USF) using 1.5 M dimethylsulphoxide (DMSO) and 0.15 M sucrose as cryoprotectants appears to be a user-friendly and efficient method for the cryopreservation of human testicular tissue.

WHAT IS KNOWN ALREADY: Currently, time-consuming controlled slow freezing (CSF) protocols that need expensive equipment are commonly used for human testicular tissue banking. USF and vitrification are cryopreservation techniques that were successfully applied in several animal models but need further exploration with human tissue.

STUDY DESIGN, SIZE, DURATION: Fragments (n = 160) of testicular tissue from 14 patients undergoing vasectomy reversal were assigned to a fresh control group or one of the following cryopreservation procedures: CSF using DMSO at a concentration of 0.7 or 1.5 M in the presence (+S) or absence of sucrose (−S), USF using either 0.7 or 1.5 M DMSO combined with sucrose, solid-surface vitrification (SSV) or direct cover vitrification (DCV).

MATERIALS, SETTING, METHODS: Light microscopic evaluations were performed to study apoptosis, germ cell proliferation ability, spermatogonial survival, coherence of the seminiferous epithelium and integrity of the interstitial compartment after cryopreservation. Ultrastructural alterations were studied by scoring cryodamage to four relevant testicular cell types.

MAIN RESULTS AND THE ROLE OF CHANCE: The USF 1.5 M DMSO + S protocol proved not solely to prevent cell death and to preserve seminiferous epithelial coherence, interstitial compartment integrity, SG and their potential to divide but also protected the testicular cell ultrastructure. A significant reduction in the number of SG per tubule from 21.4 ± 5.6 in control tissue to 4.9 ± 2.1, 8.2 ± 5.4, 11.6 ± 5.1, 8.8 ± 3.9, 12.6 ± 4.4 and 11.7 ± 5.7 was observed after cryopreservation combined with at least one other form of cryoinjury when using CSF 0.7 M DMSO − S, CSF 0.7 M DMSO + S, CSF 1.5 M DMSO + S, USF 0.7 M DMSO + S, USF 0.7 M DMSO + S, SSV and direct cover vitrification (DCV), respectively (P < 0.001).

LIMITATIONS, REASONS FOR CAUTION: Supplementary research is required to investigate the effect on tissue functionality and to confirm this study’s findings using prepubertal tissue.

WIDER IMPLICATIONS OF THE FINDINGS: An optimal cryopreservation protocol enhances the chances for successful fertility restoration. USF, being an easy and cost-effective alternative to CSF, would be preferable for laboratories in developing countries or whenever tissue is to be procured from a diseased child at a site distant from the banking facility.

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Key words: cryopreservation / electron microscopy / germ cells / immunohistochemistry / male infertility
**Introduction**

The number of men surviving cancer at a young age has increased dramatically in the past 40 years thanks to early detection and improved cancer treatments. Nowadays, the overall cure rates for paediatric malignancies approach 80% and are still improving (Ginsberg, 2011). Unfortunately, as most of these patients receive polychemotherapy and/or irradiation, a major concern is impairment or loss of their fertility due to spermatogonia (SG) depletion (Howell and Shalot, 2005). Cancer treatment during childhood is assumed to impair fertility in about one-third of male childhood cancer survivors (Rendtorff et al., 2010). Not surprisingly, fertility preservation is an important issue in regard to the long-term quality of life. Cryopreservation of small fragments of testicular tissue is an experimental strategy for fertility preservation in cases where cryopreservation of sperm is not applicable (see Geens et al., 2008; Schlatt et al., 2009 for review).

Post-pubertal and adult cancer patients could also benefit from the upcoming fertility preservation strategy, since some of them may already be azoospermic at the moment of sperm banking as a result of a spermatogenic arrest caused by pretreatment or factors related to their malignancy (Sabanegh and Ragheb, 2009; Dohle, 2010). Other potential indications include systemic or haematological non-cancerous diseases which require gonadotoxic treatments (Geens et al., 2008), gonadectomy (Jahnukainen et al., 2006), Klinefelter’s syndrome (Paduch et al., 2009), Yq deletions (Krausz and Forfi, 2006) and cryptorchidism (Kivist et al., 2006).

At present, controlled slow freezing (CSF) using dimethylsulphoxide (DMSO) as a cryoprotectant is the method used to cryopreserve immature testicular tissue in animal models (Schlatt et al., 2002; Shinohara et al., 2002; Luetjens et al., 2008; Milazzo et al., 2010). In rodents, micro-insemination using sperm retrieved from grafted cryopreserved pre-pubertal testicular tissue has even led to the birth of healthy offspring (Shinohara et al., 2002). Because these findings are supportive for a clinical application, several teams started freezing human testicular tissue using a programmable freezer. Kivist et al. (2006) reported a CSF method, initially developed for cryopreserving human ovarian tissue, to be feasible for the cryopreservation of human testicular tissue too. More recently, Keros et al. (2007) reported a programmed freezing protocol with induced ice nucleation using 0.7 M DMSO for prepubertal testicular tissue, which was originally developed for adult testicular tissue (Keros et al., 2005), yielding good structural integrity. Wyns et al. (2008) adapted this protocol by adding 0.1 M sucrose and observed survival of prepubertal human SG able to proliferate and initiate differentiation in frozen–thawed xenografts. It is, therefore, the first-choice technique to cryopreserve immature testicular tissue in their reproductive program (Wyns et al., 2011). Nevertheless, since CSF requires expensive computerized equipment and the process is time-consuming, a more convenient alternative would be preferable.

Several publications reported uncontrolled slow freezing (USF) to be a successful cryopreservation strategy for testicular tissue of different animal species (Honaramooz et al., 2002; Ohta and Wakayama, 2005; Jahnukainen et al., 2007; Baert et al., 2012). However, USF has not yet been considered for human testicular tissue, despite the fact that this method is inexpensive, convenient and time-saving, and thus, possibly a good substitute for CSF.

Recent studies also propose vitrification, which is an ultrarapid ice-free cryopreservation technique, as a potential alternative method for prepubertal testicular tissue cryopreservation. The group of Wyns reported preservation of immature mouse (Curaba et al., 2011), immature non-human primate (Poels et al., 2012) and immature human testicular tissue (Poels et al., 2013) by vitrification using open straws. In the present study, we developed a new open vitrification technique, termed DCV, based on a promising study on ovarian tissue cryopreservation (Zhou et al., 2010). Despite many advantages, concerns have been raised regarding the biological safety of open vitrification systems, as liquid nitrogen may mediate the transfer of pathogenic agents (Bielanski and Vajta, 2009). The closed solid-surface vitrification (SSV) system would be a safer option for clinical application. SSV was reported to yield good preservation of prepubertal mouse (Baert et al., 2012) and piglet testicular tissue (Abrihami et al., 2010). So far, no study determined whether human testicular tissue can be successfully preserved by DCV or SSV.

The present study was designed to explore USF, SSV and DCV for cryopreservation of human testicular tissue and to compare these new methods with conventional CSF. To evaluate the efficiency of freezing and vitrification, we systematically examined human testicular tissue by light microscopy and transmission electron microscopy.

**Materials and Methods**

**Donor testicular tissue**

A testicular tissue biopsy measuring ~50 mm³ was obtained from 14 adult patients undergoing a vasectomy reversal after written informed consent. All men had normal spermatogenesis, as proven by histology. The biopsied specimens were immediately transported to the laboratory on ice, washed to remove any residual blood and cut into 6–8 mm³-sized fragments for the freezing procedures or 3–4 mm³ in case vitrification was performed. The fragments were allocated to different groups until each group contained 20 fragments. Fresh tissue pieces were immediately fixed and subsequently used as non-cryopreserved controls for light microscopic or electron microscopic evaluations. The remaining pieces were prepared for cryopreservation (Fig. 1). This study was approved by the internal review board of the UZ Brussel (99/131D).

**Controlled slow freezing**

Testicular tissue was cryopreserved using three CSF protocols, two USF protocols and two vitrification methods. CSF was performed by placing two tissue pieces in each cryovial filled with sterile Hanks’ balanced saline solution (HBSS; 14175-129; Life Technologies, Merelbeke, Belgium), containing 0.7 M DMSO (D2650; Sigma-Aldrich, Bornem, Belgium) without sucrose (−S) and 5 mg/ml human serum albumin (HSA; 10046; Vitrolife, Göteborg, Sweden) or with 0.1 M sucrose (+S; 10274–5c; VWR, Leuven, Belgium) and 10 mg/ml HSA as described by Keros et al. (2007) and Wyns et al. (2008), respectively. As a third CSF procedure, we modified the latter protocol by using 1.5 M instead of 0.7 M DMSO to test whether an increased final DMSO concentration would enhance its protective effect on tissue. Equilibration was performed at +4°C for 30 min. Using a programmable freezer (Planer Kryo 10 series, Planer Products, Middlesex, UK), the vials were cooled at 1°C/min with holding at 0°C for 5 min, followed by cooling at 0.5°C/min until −8°C. At this temperature, the program was put on hold for 10 min to allow manual seeding. The program continued at a rate of 0.5°C/min until −40°C, held for 10 min, and continued to −70°C at 7°C/min, with subsequent plunging to liquid nitrogen. The samples were thawed according to Keros et al.
(2007) or Wyns et al. (2008), in a water bath at 37°C until the ice melted (2 min) and then washed twice for 5 min in sterile HBSS on ice or, when applicable, three times in a reversed sucrose gradient solution (0.1, 0.05 and 0 M sucrose). Afterwards, the fragments were immediately fixed for light or electron microscopy.

### Uncontrolled slow freezing

The USF 1.5 M DMSO + S protocol described earlier by our team for mouse tissue contained a slightly higher sucrose concentration (Baert et al., 2012). In each cryovial, two tissue pieces were equilibrated in cryoprotective medium for 15 min on ice water. The cryomedium for USF consisted of Dulbecco’s minimal essential medium/F12 (DMEM/F12; 31330-095; Life Technologies) supplemented with 1.5 M DMSO, 0.15 M sucrose and 10% HSA. An USF 0.7 M DMSO + S protocol was added in order to study possible DMSO toxicity at higher concentration. The cryovials were placed in an isopropyl alcohol container (479–3200; Mr Frosty Freezing Container; VWR) and put in a −80°C freezer. Samples were cooled with an uncontrolled rate of ~1°C/min. When −80°C was reached, cryovials were transferred to liquid nitrogen for storage. The samples were thawed at 37°C in a water bath for 2 min as described in Baert et al. (2012), washed twice in DMEM/F12 supplemented with 10% HSA to dilute the cryoprotectant and fixed for further assessment.

### Vitrification

Testicular tissue samples were vitrified and warmed as previously described for prepubertal mouse testicular tissue (Baert et al., 2012). The testicular fragments were exposed to a DMEM/F12-based vitrification solution containing 1.05 M DMSO and 1.35 M ethylene glycol (EG; E-9129; Sigma-Aldrich) for 10 and 2.1 M DMSO and 2.7 M EG for 5 min. At the second step, the vitrification solution was supplemented with 20% HSA. The following step differed between the two vitrification methods. For SSV, the tissue pieces were transferred to an aluminium floater partially immersed in liquid nitrogen to allow vitrification. Afterwards, two vitrified samples were transferred to a liquid nitrogen-cooled cryovial before the storage in a liquid nitrogen tank. For DCV, the fragments were immediately put in a cryovial and directly exposed to liquid nitrogen. Samples were warmed according to Baert et al. (2012) by adding a pre-warmed solution (37°C, DMEM/F12 + 0.5 M sucrose + 20% HSA) to the cryovials and by keeping the samples at 37°C for 2 min. Samples were washed in DMEM/F12 with 20% HSA for 2 min at 37°C and finally immersed in a fixative for light or electron microscopy.

### Light microscopy

Samples for light microscopy were fixed in a hydrosafe fixative (R10 S7-16-60; Labonord, Rekkem, Belgium). This is an alcohol-based formaldehyde mixture. After fixation, the samples were embedded in paraffin and cut into 5 μm-thick serial sections for immunohistochemistry. Three sections separated by 40 μm were evaluated for each marker. For the purpose of quantifying cells, three random fields were imaged per section. Inhibin α (M3609; Dako, Heverlee, Belgium) was used as a marker for active Sertoli cells (SCs). This marker also allowed assessing the effect of cryopreservation on the seminiferous epithelial integrity by determining the percentage of intact tubules. Normal seminiferous epithelium consists of SCs which extend from the basal membrane to the luminal layer of the tubule and show a good adhesion between each other, with germ cells and with the basement membrane (Mnuk and Cheng, 2004). A disrupted epithelium is characterized by ruptured cell–cell and cell–matrix connections (Keros et al., 2005). Immunohistochemical stainings for steroidogenic acute regulatory protein (StAR; sc-25806; Tebu-bio, Boechout, Belgium), a Leydig cell (LC)-specific marker, were used to study the integrity of the interstitial tissue after cryopreservation by counting the number of intact LC clusters per tubule (Gotch et al., 1984). Such intact clusters (ICs) are seen as a small or larger group of LCs in close contact with the surrounding connective tissue and in the vicinity or around blood vessels (Davidoff et al., 2009). Staining procedures for inhibin α and StAR were performed as previously described (Van Saen et al., 2011).

Terminal deoxynucleotidyl transferase-mediated nick end-labeling (TUNEL), with in situ end-labeling of fragmented DNA, was used to evaluate apoptosis. The percentage of TUNEL-stained tubular and interstitial nuclei to all nuclei in the tubules and interstitium was recorded. A TUNEL assay was performed with the DeadEnd Colorimetric TUNEL System (Promega, Leiden, The Netherlands). The sections were deparaffinized and rehydrated, followed by fixation in 4% paraformaldehyde for 15 min. They were incubated with 20 μg/ml of protease K for 10 min at room temperature, and then the sections were rinsed with phosphate buffered saline and kept in the equilibration buffer for 10 min at room temperature. Thereafter, they were incubated with 100 μl of a working terminal deoxynucleotidyltransferase (TdT) reaction mix [containing recombinant TdT (rTdT) enzyme] in a humid atmosphere for 1 h at 37°C. The reaction was terminated by washing the slides for 15 min. The sections were then immersed in 0.3% hydrogen peroxide for 5 min.
at room temperature to inactivate endogenous peroxidase and subsequently incubated with 100 μl of streptavidin for 30 min at room temperature. Finally, the sections were stained with diaminobenzidine (DAB). Positive controls consisted of incubating sections with Dnase I. A negative control was performed by incubating the section with a TdT reaction mix without the rTdT enzyme.

Cell proliferation ability was assessed by detection of proliferating cell nuclear antigens (PCNAa) in the basal germ cells of the cryopreserved fragments. PCNAa is a central component of the DNA replication machinery and is also involved in many cell processes, such as DNA recombination and repair, chromosome assembly and cell cycle progression via its protein–protein interactions. Therefore, it has been promoted as a good predictive marker for cryoinjury (Milazzo et al., 2010). The PCNA index was estimated as the percentage of PCNA-positive germ cells located at the basement membrane. Germ cells in the basal region of the seminiferous tubule were recognizable by their large, rounded nuclei, while SCs have an ovoid or triangular-shaped nucleus (Mruk and Cheng, 2004). The average number of ubiquitin carboxy-terminal hydrolase L1 (UCHL1)-positive SG per tubule was determined to study and compare the actual spermatogonial survival after cryopreservation. UCHL1 has been employed in various animal models and the human to identify SG (von Kopylow et al., 2010). The cross-sections were deparaffinized and rehydrated in descending series of alcohol. Peroxide activity was quenched using 0.3% H2O2 (for PCNA stainings) or 3% H2O2 (for UCHL1 stainings) in methanol for 30 min. Sections were placed in citrate buffer (0.01 M, pH 6.0) for 75 min in a 95°C water bath (for PCNA stainings) or for 4 min in a microwave at 500 W (for UCHL1 stainings) and cooled for 30 min at room temperature. Non-specific binding was blocked in 10% normal goat serum for 30 min at room temperature. Sections stained for PCNA underwent an additional permeabilization step with 0.1% Triton X-100 for 30 min at room temperature. The primary antibody of PCNA (1/400, NA03; VWR) or UCHL1 (1/2500, 7863-0504; Bioconnect, Huissen, The Netherlands) was added to the sections and incubated at 4°C overnight or for 2 h, respectively. A horse-radish peroxidase-labelled secondary anti-mouse antibody (K5007; Dako Real Envision Detection System, Dako) was added and incubated at room temperature for 1 h. DAB was used as a chromogen.

All sections were counterstained with haematoxylin, rehydrated and mounted. Examinations were performed on an inverted light microscope (Olympus IX81). Digital images were captured with a digital camera (CCD 8 Soft Imaging System, Olympus) at a final magnification of ×200 for Inhibin α and StAR stained slides or ×400 for PCNA, UCHL1 and TUNEL-stained slides.

**Transmission electron microscopy**

Samples for electron microscopy were fixed in 2.5% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.4) at 4°C. Fresh and cryopreserved samples of ±1 mm² were post-fixed in 1% osmium-tetroxide and 2% uranyl acetate. Next, the samples were dehydrated through an ascending series of ethanol, immersed in propylene oxide for solvent substitution and embedded in a Polysciences Inc., Eppelheim, Germany). Ultrathin sections (50–100 nm) were cut with an ultracut ultra-microtome (Reichert-Jungh, Depew, NY, USA), mounted on a copper grid and contrasted with uranyl acetate and lead citrate. Ultrastructural examinations were performed using a Tecnai 10 Philips electron microscope. Digital images were taken at magnifications between ×1850 and ×12500 using a mega view G2 CCD camera (SIS-company, Munster, Germany). Images were saved in TIFF format with a resolution of 1376 × 1032 and viewed with the Olympus Soft Imaging Viewer (version 5.1, Olympus Soft Imaging Solutions) software. A semi-quantitative analysis of the cell integrity was performed by studying the ultrastructural morphology of 20 SG, SCs, LCs and myoid cells (MCs) in each fresh and cryopreserved sample. As defined by Trump et al. (1974), subcellular alterations characteristic of acute cryopreservation injury range from reversible changes (dilated endoplasmatic reticulum; dense mitochondria with enlarged cristae) to those which are irreversible (high-amplitude swollen mitochondria containing flocculent densities; distorted membrane systems). When there were no signs of cryoinjury (intact cell) or when reversible morphological alterations were observed (influenced cell), testicular cells were categorized as undamaged. If irreversible cryoinjury was noted, testicular cells were considered as damaged. The proportions of undamaged (intact and influenced) and damaged testicular cells were calculated in each sample.

**Statistical analyses**

Data are presented as mean values ± standard deviation, and statistical analyses were conducted using one-way analysis of variance with post hoc Bonferroni adjustments (IBM SPSS Statistics Version 19, IBM Corporation, Somers, NY, USA). A P-value of <0.05 was considered statistically significant.

**Results**

**Apoptosis, germ cell proliferation ability and SG survival**

Sections stained by TUNEL were analysed to study apoptosis after cryopreservation. The frequency of apoptotic cells in the tubular compartment was not different from the control for all tested protocols. The mean value of apoptotic cells in the interstitium slightly increased after cryopreservation, though it did not reach statistical significance (Fig. 2).

Cell proliferation ability was assessed by estimating the PCNA index (PCNA-positive basal germ cells to all basal germ cells) in the different experimental groups (Fig. 3A, B and E). In addition, the average number of UCHL1-positive SG per tubule was determined to study spermatogonial survival after cryopreservation (Fig. 3C–E). When compared with the fresh control (PCNA index of 36.8 ± 9.7% and 21.4 ± 5.6% SG per tubule), PCNA indices and the number of SG decreased significantly to 18.9 ± 9.0% (P < 0.001) and 4.9 ± 2.1 (P < 0.001) after CSF 0.7 M DMSO + S and to 23.9 ± 5.0% (P < 0.02) and 11.6 ± 5.1% (P < 0.001) after CSF 1.5 M DMSO + S, respectively. Higher PCNA indices were obtained with CSF 0.7 M DMSO + S (25.4 ± 6.7%), USF 0.7 M DMSO + S (28.9 ± 10.0%), SSV (41.4 ± 14.0%) and DCV (36.2 ± 17.3%). Compared with the control, our data nevertheless reveal a significant loss (P < 0.001) of SG per tubule after CSF 0.7 M DMSO + S (8.2 ± 5.4%), USF 0.7 M DMSO + S (8.8 ± 3.9%), SSV (12.6 ± 4.4) and DCV (11.7 ± 5.7). In the fragments cryopreserved with USF 1.5 M DMSO + S, the PCNA index (32.4 ± 13.1%) and the number of SG per tubule (16.9 ± 8.7) were not affected compared with the fresh control (Fig. 3E).

**Seminiferous epithelium coherence and interstitial compartment integrity**

The average percentage of intact and distorted tubules was determined on inhibin α immunostained sections. On average in the fresh tissue fragments, 89.7 ± 5.8% of the tubules was considered to have a structurally normal epithelium. The tissue samples subjected to CSF 0.7 M DMSO + S (74.5 ± 8.0%, P < 0.05), USF 0.7 M DMSO + S (72.4 ± 11.7, P < 0.005), SSV (71.0 ± 21.6%, P <
0.002) and DCV (59.6 ± 20.9%, P < 0.001) displayed severe deformation of the seminiferous epithelium compared with the control, characterized by a rupture of cell–cell and cell–matrix connections. CSF and USF could preserve the epithelial morphology when using 1.5 M DMSO and sucrose (Fig. 4A–C). Surprisingly, also CSF using 0.7 M DMSO without the addition of sucrose could preserve the tubules.

Immunohistochemistry for StAR allowed visualization of the LC clusters to study the impact of the cryoprotocols on the integrity of the interstitium. In the control tissue fragments, 1.9 ± 0.4 LC clusters per tubule showed IC morphology and normal connectivity to surrounding interstitial components. This number was not statistically different after cryopreservation (Fig. 4D–F).

**Figure 2** Protection from apoptosis. Apoptotic cells in a fresh (A) and SSV-treated fragment (B) detected by TUNEL. Negative control incubated without terminal transferase (C). Positive control treated with DNase I (D). No significant differences in apoptosis were observed between fresh and cryopreserved fragments (E). A red arrow in A and B indicates a negative cell. A black arrow in A and B indicates a positive cell. Results in (E) are expressed as means ± SD and were compared with the values obtained from a fresh control (Fr). In all groups, n = 10 fragments. CSF, controlled slow freezing; DCV, direct cover vitrification; DMSO, dimethylsulphoxide; S, sucrose; SSV, solid-surface vitrification; USF, uncontrolled slow freezing; Vi, vitrification.

Protection of testicular cell ultrastructure

The impact of the different cryopreservation procedures on testicular cell morphology was studied semi-quantitatively at high resolution using the transmission electron microscopy.

The majority of testicular cells in fresh fixed tissues was undamaged; up to 66.7 ± 29.3% of LCs, 90.0 ± 17.3% of MCs, 100.0 ± 0.0% of SG and 93.3 ± 11.5% of SCs. Surviving testicular cells in CSF 0.7 M DMSO − S, CSF 1.5 M DMSO + S, both USF and both vitrification groups that have an undamaged ultrastructure were observed in frequencies not different from the control. CSF 0.7 M DMSO + S-cryopreserved fragments contained a percentage of cells showing
an undamaged morphology not different from the control for all but one cell type, i.e. SG. Using the latter protocol, the SG that survived cryopreservation exhibited distinct ultrastructural signs of cryoinjury, thereby reducing the percentage of undamaged SG significantly to 33.3 ± 2.9% (Fig. 5A–E).

**Discussion**

In the present study, we explored USF, SSV and DCV as new cryopreservation methods for human testicular tissue and compared them with the conventional CSF. Our data demonstrate that only USF 1.5 M DMSO + S proved to efficiently preserve human testicular tissue.

Cryopreservation of human testicular tissue by CSF, being the method of choice in current fertility preservation programmes, is based on a lengthy slow-rate cryopreservation procedure requiring an expensive controlled rate freezer and liquid nitrogen supply. Moreover, different cycles have to be performed in series for this type of freezing. On the contrary, USF only takes up about half the time of the CSF procedure and requires a rather inexpensive small −80°C freezer, which might be portable, and isopropyl alcohol containers to place cryovials in it. The vitrification technique needs just ±
Figure 4. Preservation of the seminiferous epithelial coherence and interstitial compartment integrity. Tubules of a specimen cryopreserved with USF 0.7 M DMSO + S showing an intact (IT) or distorted (DT) epithelium that were stained for inhibin α at low (A) and high (B) magnification. Tissue treated with CSF 0.7 M DMSO + S, USF 0.7 M DMSO + S and both vitrification protocols showed a poor outcome, as indicated by significantly decreased percentages of intact tubules (C). Cryoinjury was not prominent when the capacity to preserve the interstitial compartment was evaluated by StAR immunostaining (D). High magnification image of an IC and distorted cluster of LCs in an USF 0.7 M DMSO + S cryopreserved fragment (E). Every tested cryoprotocol resulted in a comparable number of LC clusters per tubule showing a normal structure (F). Insets in (A) and (D) represent negative controls. Results in (C) and (F) are shown as means ± SD and were compared with values obtained from fresh controls (Fr). In all groups, n = 20 fragments. *P < 0.05; **P < 0.005; ***P < 0.002; ****P < 0.001. CSF, controlled slow freezing; DCV, direct cover vitrification; DMSO, dimethyl-sulphoxide; LC, Leydig cell; S, sucrose; SSV, solid-surface vitrification; USF, uncontrolled slow freezing; Vi, vitrification.
30 min of preparation and allows an immediate transfer to liquid nitrogen, thereby, completely eliminating the need for cryopreservation equipment. USF and vitrification are, therefore, potentially useful techniques not only when the procurement site is distant from the banking site, but also for laboratories in developing countries (Vajta and Nagy, 2006).

So far, data comparing CSF, USF and vitrification for testicular tissue are limited. In mice, Milazzo et al. (2008, 2010) tested distinct CSF and USF protocols to determine the optimal parameters for immature mouse testicular tissue cryopreservation. They showed the superiority of CSF 1.5 M DMSO + S in preserving seminiferous cord morphology, cell proliferation ability and functional integrity among tested conditions. In our previous work, we have performed comparative investigations of USF 1.5 M DMSO + S and SSV for prepubertal mouse testicular tissue. We showed that SSV resulted in success rates comparable with the USF protocol in maintaining testicular cell ultrastructure, tubular morphology and tissue function (Baert et al., 2012). In piglets, Zeng et al. (2009) compared cell viability and in vivo developmental potential after CSF 1.3 M DMSO − S, USF 1.3 M DMSO − S and a closed vitrification system. No significant differences were observed between these three methods. In agreement, results from comparative investigations by Abrishami et al. (2010) demonstrated SSV to be as efficient as CSF 1.3 M DMSO + S in cryopreserving piglet testicular tissue. The group of Wyns showed their vitrification system using open cryostraws to be as efficient as CSF 0.7 M DMSO + S in preserving the integrity of prepubertal mouse (Curaba et al., 2011) and prepubertal human testicular tissue (Poels et al., 2013).

In this study, we used two well-established CSF protocols that were developed by the group of Keros et al. (2005) and Wyns et al. (2008) for freezing human testicular tissue. Like Keros et al. (2005), we were able to preserve the tubular epithelial structure and interstitial cells in...
human testicular tissue with CSF using 0.7 M DMSO – S. However, we were unable to preserve the SG. TUNEL staining did not reveal more apoptotic cells in the tubules, but analyses targeting the SG showed a reduced cell proliferation ability and spermatogonial loss when using CSF 0.7 M DMSO – S. This discrepancy in results can be due to the use of MAGE-A4 as a SG marker by Keros et al. Given that primary spermatocytes also express this protein makes it plausible that Keros et al. have included these cells in their analysis (Aubry et al., 2001). The SG that did survive and all studied somatic cell types displayed a comparable ultrastructure to control cells. Multiple factors may have contributed to the less favourable results of CSF 0.7 M DMSO – S. First, it was reported that the induced ice-nucleation step included in this CSF protocol may not be necessary and may even be harmful (Milazzo et al., 2008). Second, the absence of sucrose in the cryopreservation medium of this CSF method could have resulted in insufficient cell dehydration. Macromolecules, such as sugars, can act as a non-permeable cryoprotectant, increasing water withdrawal from cells and, thus, counteracting intracellular ice crystal formation. It was reported that the addition of sucrose as a cryoprotectant improved the cryopreservation outcome of embryos (Van den Abbeel et al., 1994), ovarian tissue (Amorim et al., 2010) and spermatogonial stem cells (Izadyar et al., 2002). However, in this study, CSF 0.7 M DMSO + S-frozen tissue still exhibited cryoinjury: a reduction in tubules with a coherent seminiferous epithelium, poor spermatogonial survival and the surviving SG showing clear ultrastructural changes compared with the control. A third factor concerns the low concentration of DMSO (0.7 M). This may cause insufficient dehydration and eventually the formation of harmful intracellular ice crystals. Elevating the concentration to 1.5 M DMSO may improve protection against tissue cryoinjury (Milazzo et al., 2008; Milazzo et al., 2010). Surprisingly, we found no improvement in the outcome when cryopreserving human testicular tissue by CSF 1.5 M DMSO + S.

The USF 1.5 M DMSO + S protocol was developed earlier by our team for testicular tissue preservation in a mouse model (Goossens et al., 2008; Baert et al., 2012). Apparently, this USF protocol can also maintain human testicular tissue during cryopreservation, since the present results showed no major differences between USF 1.5 M DMSO + S-frozen tissue and fresh control tissue. Reasons why USF 1.5 M DMSO + S gave better results than any CSF protocol could be the freezing rate itself or the shorter equilibration time. Prolonged exposure to DMSO before storage can be harmful (Fleming and Hubel, 2006). The testicular cells in both fresh and USF tissue pieces, which were categorized using the TEM as damaged, displayed structural changes which could result from hypoxia and handling of the material before fixation.

Vitrification aims at removing a high proportion of cellular water and solidification of the solution without crystal formation. To achieve this, it is necessary to use both high cooling rates and high concentrations of cryoprotectants. The SSV technique was applied because it is a closed system and thereby it minimizes the potential hazard of pathogenic contamination of samples. Furthermore, it provides enough space for tissue (Abrishami et al., 2010). In order to give a vitrifiable concentration of total cryoprotectants while decreasing their individual specific toxicity, DMSO and EG were combined. SSV could not protect human testicular tissue; more seminiferous tubules were found displaying a ruptured epithelium after cryopreservation and SSV had a negative impact on the spermatogonial number. We hypothesize that this may be due to stress on the cells as a result of mechanical forces generated by the extracellular ice. Incomplete vitrification, and thus, crystallization, may have occurred because in SSV high cooling rates are difficult to attain. High cooling rates are easier to obtain with DCV in which liquid nitrogen is directly applied to the testicular tissue. Nevertheless, by using the latter procedure, the outcome of vitrification did not improve. Vitrification is a promising technology in testicular tissue cryopreservation, but still in an initial stage. Further optimization of this technique should be considered. Elevating concentrations of cryoprotectants and/or reducing tissue size may help to achieve complete vitrification (Yavin and Arav, 2007).

Because normal prepubertal testicular tissue is difficult to obtain, we cryopreserved adult human testicular tissue. Protocols similar to those described here have been used successfully to preserve prepubertal testicular tissue from various animals (Honaramooz et al., 2002; Jahnukainen et al., 2007; Goossens et al., 2008; Abrishami et al., 2010; Baert et al., 2012). Yet the newly introduced protocols for human testicular tissue cryopreservation should be tested for cryopreserving prepubertal human testicular tissue. A study in rats already highlighted the different sensitivity of immature tissue to the selection of the cryoprotectants and their concentrations (Unni et al., 2011). Furthermore, functional integrity evaluation of cryopreserved tissue is fundamental to validate a freezing–thawing/cooling–warming procedure, since previous studies concluded that a high survival rate of morphologically normal SG does not necessarily imply that those SG are actually functional (Frederickx et al., 2004; Jahnukainen et al., 2007; Zeng et al., 2009). The opposite may also be true; our previous study showed that cryoinjured tissue retained its function and could recover after transplantation (Baert et al., 2012). It remains, thus, important to assess cell or tissue functionality by xenografting or by in vitro assays.

In conclusion, this study showed that USF 1.5 M DMSO + S is an efficient alternative to CSF for human testicular tissue. USF 1.5 M DMSO + S yielded the best results in preventing apoptosis and protecting the tubular epithelium coherence, interstitial compartment integrity, cell proliferation ability, spermatogonial number and testicular cell ultrastructure. However, further studies should be conducted to evaluate cell or tissue functionality and to confirm the present findings using prepubertal testicular tissue.

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**Authors’ roles**

Y.B.: conception and design of the study, acquisition of samples for analysis, interpretation of data, drafting of the article and final approval for submission. D.V.S.: acquisition of samples for analysis and revision of the article. P.H.: interpretation of data and revision of the article. P.I.V.: transmission electron microscopy-sample acquisition, discussion of the results and revision of the article. H.T.: conception and design of
the study and final approval for submission. E.G.: conception and design of the study, interpretation of data, discussion of the results, revision of the article and final approval for submission.

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Conflict of interest

None declared.

References


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None declared.

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