Comparison of conditions for cryopreservation of testicular tissue from immature mice

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BACKGROUND: Cryopreservation of immature testicular tissue could be considered as a major step in fertility preservation for young boys with cancer. In the present study, eight different freezing protocols were evaluated in immature mice testis. METHODS: Testis from six-day-old mice were frozen using either 1,2-propanediol (PROH) or dimethylsulphoxide (DMSO: D) at 1.5 M. Different cooling rate curves were tested: (i) controlled slow protocol with seeding (CS) or (ii) without seeding (CS−), (iii) controlled rapid protocol and (iv) non-controlled protocol. Cryodamage of seminiferous cords was semi-quantitatively determined, establishing a scoring of alterations. Cell viability and apoptosis induction were assessed on testicular cell suspensions immediately after digestion (D0) and after a 20-h culture period (D1). Cells recovered after digestion of 100 mg tissue and the rate of living and non-apoptotic cells were quantified at D0 and D1. A long-term culture (9 days) of testis pieces was carried out for the protocol offering the best survival. Testosterone production, intratubular cell proliferation and tubule growth were assessed. RESULTS: DMSO produced optimal results in the different cooling rate curves tested when compared with PROH. Optimal results were obtained for the DCS− procedure (P < 0.05). Testosterone production, tubule growth and cell proliferation of post-thaw pieces were similar to fresh samples. CONCLUSIONS: Testis freezing with 1.5 M DMSO in a CS− procedure was found to maintain not only immature testicular tissue architecture, but also viability of testicular cells, endocrine and partial exocrine functions of the testis. Semi-quantitative evaluation of seminiferous cord cryodamage can be effectively used to rapidly screen optimal freezing conditions and as a possible quality control in a human application.

Keywords: cryopreservation; male germ cell; immature testicular tissue

Introduction

For several years, paediatric oncologists have included fertility preservation in the management of childhood cancer treatment. Therefore, ovarian tissue banking may be proposed in young and adolescent girls as well as ejaculated spermatozoa cryostorage in sexually mature boys (for review, Tournaye et al., 2004). However, even if banking of immature testicular tissue has been frequently described as a major step in fertility preservation of young boys with cancer, different strategies have been suggested including completion of spermatogenesis by in vitro maturation or after germ cell transplantation or testicular fragment grafting (for review, Tournaye et al., 2004; Jahnukainen et al., 2006). However, to date, no culture system may offer a complete in vitro spermatogenesis starting from spermatogonia, despite several promising studies in experimental animals (Boitani et al., 1993; Lee et al., 2001; Feng et al., 2002; Izadyar et al., 2003). In contrast, testicular stem cell transplantation has provided the most promising results in animal models, with restoration of fertility in mice (Brinster and Avarbock, 1994), rats (Hamra et al., 2002; Zhang et al., 2003) and non-rodent species as well (for review, Jahnukainen et al., 2006). After cryopreservation,
transplantation of stem cells also allowed to overcome male infertility in mice (Avarbrock et al., 1996; Kanatsu-Shinohara et al., 2003). To date, complete spermatogenesis was also achieved with fresh immature testis fragments of mouse hamster, rabbit, bull, pig, goat, cat and monkey, after grafting into immuno-deficient mice (Honaramooz et al., 2002, 2004; Schlatt et al., 2002, 2003; Shinohara et al., 2002; Snedaker et al., 2004; Oatley et al., 2005). Similarly, xenografting of frozen–thawed mouse and rabbit prepubertal testicular pieces showed restored spermatogenesis (Schlatt et al., 2002; Shinohara et al., 2002). However, xenografting of fresh (Yu et al., 2006) and post-thaw (Wyns et al., 2007) human immature testicular tissue has failed to allow a complete meiotic process.

Immature testicular tissue may be cryopreserved in three different ways: (i) testicular cell suspension, (ii) testicular pieces (for review, Tournaye et al., 2004) and (iii) entire gonad. Cell suspension preparation requires mechanical and/or enzymatic digestion of the tissue with the risk to compromise cell survival during the procedure of freezing and thawing. Furthermore, the suppression of cell-to-cell interactions can also be deleterious for cell proliferation and differentiation. Thus, storage of testicular tissue pieces could be considered as an alternative method suitable for maintaining cell-to-cell contacts between Sertoli and germinal stem cells and therefore to preserve the stem cell niche necessary for their survival (Ogawa et al., 2005). Storage of whole testis has not, to our knowledge, been reported but may be an option for whole organ vascular transplantation, as described for ovary (Courbiere et al., 2006). Cryopreservation of whole testis or testicular pieces offers the opportunity to use the frozen–thawed tissue in the different applications described above.

In contrast, to date, very few studies have been dedicated to the comparison of freezing–thawing procedures for adult (Jezeck et al., 2001; Keros et al., 2005) or prepubertal (Kvist et al., 2006; Jahmukainen et al., 2007; Keros et al., 2007) testicular tissue. However, it is absolutely necessary to establish an optimal procedure for freezing and thawing immature testicular tissue before application in humans. Since August 2004, the French Law for Assisted Reproductive Techniques has allowed cryostorage of human female and male gonads in situations where treatment may alter fertility prematurely. However, even if ovary storage is considered as routinely performed, some reserves are expressed concerning immature testicular tissue. More data are required in animal models to validate the procedure in humans. The same recommendations were made by the French Federation of CECOS (Centre d’Etude et de Conservation des Œufs et du Sperme humains) organising in France sperm cryopreservation before gonadotoxic treatment (unpublished data). Considering these different points, we decided to evaluate several protocols for cryopreservation of immature testicular tissue in a mouse model. The choice of this model was not only based on ease of availability, but also because immature mice testis is currently reported in studies performed on germ cells or testicular tissue cryostorage and is recognised to be a suitable model for germ cell transplantation and xenografting. Therefore, freezing protocols developed in our study could be compared with published data. In the present study, viability, apoptosis and morphology of immature mice testicular tissue were assessed in eight storage protocols. A screening method based on semi-quantitative evaluation of post-thaw seminiferous cord morphology was developed in order to select optimal freezing–thawing conditions. The protocol offering the best parameters of evaluation was tested further in a long-term in vitro culture of frozen–thawed seminiferous cords.

Materials and Methods

**Mice and testis collection**

Timed pregnant female mice CD-1 strain, at 15 days of gestation, were purchased from Charles River Breeding Laboratories (L’Arbresle, France). For each experiment, 3 or 4 litters of 6 or 7 days old mice were sacrificed by decapitation. Testes were excised and rinsed in Hank’s balanced solution at 4°C (HBSS, Sigma-Aldrich, Saint-Quentin Fallavier, France). The tunica albuginea was immediately removed and testes were then maintained in the same transport medium as used for ovarian tissue (Brahma I, Cryobiosystem, Paris, France) at 4°C until all samples were collected (between 30 and 60 min, average 40 min). Brahma I was used because this medium is validated for human tissue transport before cryopreservation and may also be used for human testes. For one experiment, 20 testes were used: 10 for fresh control and 10 for one freezing assay (2 for histology and 8 for viability and apoptosis tests). Testes distribution between groups was randomly performed and the number of freezing conditions tested in one experiment depended on the number of testes available. For each condition tested in one experiment, testes were weighed (between 2 and 3 mg) and then frozen in one cryotube. An outline of the experiments is shown in Fig. 1.

**Figure 1:** Outline of the experimental procedure using immature mice

For fresh controls, testes were fixed just after decapsulation for histology analysis. Enzymatic digestion was performed just after weighing for viability tests. TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling; D0, day of thawing and digestion; D1, 20 h after digestion and cell culture.
Cryopreservation

Two cryoprotective media and four different freezing protocols were compared. The number of fresh controls and freezing assays for each protocol tested are shown in Table I. The freezing media consisted of Leibovitz® L-15 medium (EUROBIO, Courtabeuf, France) supplemented with 1.5 mol/l dimethylsulphoxide (DMSO, Sigma-Aldrich) or 1.5 mol/l 1,2-propanediol (PROH, Sigma-Aldrich), plus 0.05 mol/l sucrose (Sigma-Aldrich) and 10% (v/v) fetal calf serum (FCS, EUROBIO).

After removal of tunica albuginea, testes were placed in 1.8 ml cryovials (CRY25, Dutscher, Brunath, France) containing 1.5 ml cryoprotective medium and were equilibrated at 4°C for DMSO (D) and at room temperature for PROH (P). For the non-controlled freezing protocol (NC), testes were cooled directly in a constant -80°C freezer during 24 h. For the controlled freezing protocols, testes were frozen in a programmable Minicool 40PC freezer (Air Liquide, Paris, France) and the following programmes were used: (i) controlled slow-freezing with seeding (CS+): start at 2°C/min to −9°C, 2 min of soaking, then manual seeding for ice crystal nucleation, 0.3°C/min to −40°C, 25°C/min to −150°C; (ii) controlled slow-freezing without seeding (CS−): as for the CS+ protocol but without seeding; (iii) controlled rapid-freezing (CR): start at 20°C, 5°C/min to −8°C, 10°C/min to −25°C, 25°C/min to −150°C. The CS programme is used in our laboratory for ovary cryopreservation (data not published) since three years (Gosden et al., 1994; Poirot et al., 2002). The CR programme is employed in our laboratory (CECOS) for sperm cryopreservation since 1982 and was proposed previously by Bahadur et al. (2000). After each freezing procedure, samples were plunged and stored into liquid nitrogen at −196°C.

Samples were thawed rapidly in a 37°C water bath. The cryomedium was removed in a four-stage procedure. Testes were left for 5 min at each step, first in 1.0 mol/l DMSO (or PROH), 0.05 mol/l sucrose, 10% FCS, second in 0.5 mol/l DMSO (or PROH), 0.05 mol/l sucrose, 10% FCS and finally to Leibovitz medium without cryoprotectants.

Cell isolation by enzymatic digestion

A two steps enzymatic procedure was performed to isolate cells, just after the freezing–thawing procedure, for viability and apoptosis tests. For fresh controls, digestion occurred just after decapsulation and weighing. Testes were first placed in 2.5 ml of HBBS without calcium and magnesium containing 0.5 mg/ml collagenase IV (Sigma-Aldrich) and 0.08 mg/ml Dnase I (DN-EP, Sigma-Aldrich) and incubated for 5 min at 33°C in a shaking water bath operated at 80 cycles per min. Then, trypsin (0.3 mg/ml) (Sigma-Aldrich), EDTA (1 mM) (Sigma-Aldrich) and DNase I (0.06 mg/ml) were added to the media and the dispersed seminiferous cords were incubated for 10 min, using the conditions described above. The trypsin reaction was stopped by adding 10% FCS and precipitates (if present) were removed with a pipette.

<table>
<thead>
<tr>
<th>Freezing protocols</th>
<th>DMSO</th>
<th>PROH</th>
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<tbody>
<tr>
<td>Controlled slow-freezing without seeding</td>
<td>n = 6</td>
<td>n = 6</td>
</tr>
<tr>
<td>Controlled slow-freezing with seeding</td>
<td>n = 5</td>
<td>n = 5</td>
</tr>
<tr>
<td>Controlled rapid-freezing</td>
<td>n = 4</td>
<td>n = 4</td>
</tr>
<tr>
<td>Non-controlled freezing rate</td>
<td>n = 6</td>
<td>n = 4</td>
</tr>
</tbody>
</table>

DMSO, dimethylsulphoxide; PROH, propanediol.

Detection of dead cells

Cell viability was determined by a trypan blue (Sigma-Aldrich) exclusion test and by propidium iodide (PI) labelling of dead cells evaluated with the annexin test (see below). Microscopic evaluation was carried out no later than 10 min after the end of incubation and ~500 cells for trypan blue and 300 cells for PI were counted.

Detection of plasma membrane phosphatidylycerine externalization

The apoptosis-associated translocation of phosphatidylycerine from the inner to the outer leaflet of the plasma membrane was assessed with the use of fluorescein isothiocyanate (FITC)-labelled Annexin V, a calcium-dependent phospholipid-binding protein with a high affinity for phosphatidylycerine. PI was used for the identification of dead cells. This examination was performed using the Annexin-V-FLUOS Staining Kit (Roche, Meylan, France) and the procedure recommended by the manufacturer for staining of cell suspensions. The slides were screened using a ×100 objective in an epifluorescence microscope (DMRDX, Leica, Reuil-Malmaison, France). An FITC/Rhodamine double band-pass filter set was used to count dead (red with a green membrane) and early apoptotic (only green membrane) cells. The visualization was combined with the use of white-light to count non-apoptotic living cells (no fluorescent labelling).

Detection of DNA fragmentation

Cells were washed in phosphate-buffered saline (0.01 M phosphate buffer, 0.0027 M KCl, 0.13 M NaCl), fixed 15 min at 4°C in 4% paraformaldehyde (Sigma-Aldrich), spread onto dry slides (Polysine®, Menzel-Gläser®, Braunschweig, Germany) and stored at −20°C. Fixed smears of cells were processed for terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labelling (TUNEL) using a Cell Death Detection Kit (Roche Molecular Biology, Penzberg, Germany). TUNEL labelling was carried out according to the manufacturer’s instructions.

TUNEL-positive nuclei were green due to the addition of FITC-labelled dUTP to the exposed ends of multiple DNA fragments resulting from the apoptosis-induced internucleosomal DNA breakage. A counterstaining with PI (Roche Molecular Biology) was used to visualize in red TUNEL-negative nuclei. For each slide, 500 cells were counted.
**Light microscopy**

Tests were fixed in Bouin’s solution for 18 h at room temperature and then dehydrated in ethanol and embedded in paraffin. Ten sections (4 μm) from each testis were cut at intervals of 20 μm and stained with hemalun and eosin. Histological examinations were performed using a conventional light microscope (Diaplan, Leica Microsystems). Serial digital images were recorded at magnification of ×1000. Histological characteristics of the frozen–thawed testes were compared with those of control testes, which were fixed immediately after decapsulation. For each cryopreservation procedure tested, four independent samples were scored using two slides. Slides were coded for blinded analysis by one person and counts were performed by two other observers.

The integrity and the structural changes of fresh controls and frozen–thawed sections were evaluated semi-quantitatively. Nuclei of intratubular cells (spermatagonia and Sertoli cells) were scored as follow: (i) distinction between Sertoli cells and spermatagonia nuclei was scored as 0 if easy, 1 if difficult and 2 if impossible, (ii) observation of nucleoli was scored as 0 if easy (visible in >40% of cells) and scored as 1 if indistinguishable (in the case of pyknotic nuclei present in a large number and very condensed), (iii) nuclei condensation was scored as 0 if absent or present in only 1 nucleus, as 1 if <40% of nuclei were condensed and as 2 if >40% were pyknotic. Therefore, a total absence of nuclei alteration was scored as 0 and the worst score for nuclei morphology was 5. The epithelium (constituted by intratubular cells) was scored as follow: (i) detachment of cells from the basement membrane was scored as 0 if absent, as 2 if partial and as 3 if total or observed on >75% of the circumference, (ii) gap formation and shrinkage were scored as 0 if absent, as 2 if slight and as 3 if more obvious. Therefore, as for nuclei, epithelium morphology was scored from 0 to 5. The global score for each seminiferous cord section was the sum of nuclei and epithelium morphology and consequently was between 0 and 10. For each testis, the global score was the mean of scores for 30 seminiferous cords sections. For the different tested procedures (and for the control), each datapoint corresponds to the mean of global scores obtained from four testes cryopreserved with the same protocol, but corresponding to four distinct experiments (not the same day, with mice from different litters and with media newly prepared for each experiment).

A seminiferous cord structure was judged to be good when epithelium and nuclei scores where both ≤1. For fresh controls and freezing protocol, four histological evaluations were performed.

**Testosterone assessment**

Testosterone was measured separately in the culture media from Days 3, 5, 7 and 9 by direct radioimmunoassay using the testosterone kit (ImmunoTech Beckman-Coulter, Roissy, France). The samples were assayed in duplicate according to the recommendations of the manufacturer. The assay had a lower limit of sensitivity of 0.04 ng/ml with in average 7.2 and 10.7% of intra and inter-variations, respectively.

**Morphological evaluation after culture**

Structural changes of fresh and frozen–thawed cultured testes were evaluated using a digital image analysis system (LAS, Leica, Germany) connected to an inverted microscope (DM4000, Leica). Slides were coded for blinded analysis and counts were performed by two observers. The diameter of at least 10 cross-sectioned tubules was assessed on Days: D0, D3, D5, D7 and D9 for frozen–thawed and fresh cultured testes. The total number of intratubular cells (spermatagonia, Sertoli cells and spermatocytes I) was also counted at the same time on 10 cross-sectioned tubules. A tubule was defined as cross-sectioned when the ratio between the longest diameter and the diameter perpendicular to the longest one was evaluated between 1 and 1.5. Only cells with visible nuclei were counted.

**PCNA immunostaining**

Four micrometer sections of paraffin-embedded tissue were deparaffinised in xylene and rehydrated in ethanol bath series. The sections were processed for proliferating cell nuclear antigen (PCNA) antibody, as a marker of cellular proliferation ability, according to the manufacturer recommendations (Zymed Laboratories, San Francisco, CA, USA). After substrate reaction, stained cells were briefly counterstained with haematoxylin (Merck, France) and mounted with an aqueous mounting medium (Glycergel®, Dako, France). Stained cells were analysed under a light microscope (DMRD®, Leica, Germany) at ×400 magnification. Ten sections of tubules were evaluated for each day of culture (fresh and cryopreserved tissue). PCNA immunostaining was also carried out on fresh testis removed from male mice sacrificed on Days 7, 10, 12, 14 and 16 after delivery, theoretically corresponding, respectively, to D0, D3, D5, D7 and D9 of cultured testes.

**Statistical analysis**

Calculated values used for statistical analysis of results are shown in Table II. Two main criteria were chosen to evaluate the eight protocols tested: (i) proportion of non-apoptotic living cells after freezing–thawing procedure and digestion, at D0 or D1, that depend on cell recovery after digestion, viability evaluated by trypan blue test and proportion of early apoptotic cells (Annexin V), (ii) morphological alterations of seminiferous cords semi-quantitatively evaluated. Means of recovery and viability and apoptosis induction tests for the different freezing protocols were compared with results obtained with the fresh control of the day of the experiment or between D0 and D1, by a non-parametric test of Wilcoxon. Mean results of cell viability assessed with trypan blue and PI were compared for the different conditions by a paired sample t-test. Histological scores were compared using a Mann–Whitney U-test. The presence of a relationship between the global semi-quantitative evaluation of
morphological alterations for the different cryopreservation protocols tested and the proportions of non-apoptotic living cells was evaluated with Spearman’s correlation coefficient. Testosterone measurement and histological evaluation after culture were compared by a non-parametric test of Wilcoxon. Data are presented as mean ± SD and a P-value < 0.05 was considered to be significant.

Results

Cell recovery after digestion and after a 20-h culture period
The percentages of cell counted after thawing and digestion when compared with the fresh control are shown in Fig. 2. Recovery after digestion (Rd) significantly varied between the different freezing protocols (P < 0.001). Rd was not significantly different in DCS− condition compared with control. However, Rd significantly decreased under the other conditions (Fig. 2). For samples with low values of Rd, precipitates formed during the second step of digestion were generally more important probably due to an increase of cellular lysis.

Cell recoveries after a 20-h culture period did not differ significantly between the eight freezing procedures tested.

Cell viability
For the different protocols tested and for fresh controls, cell viability did not significantly vary between the two viability tests (trypan blue or PI, 0.10 < P < 0.50). Therefore, trypan blue test values were used for analysis of results. After digestion of non-cryopreserved testes (controls) >95% (95.45 ± 1.03) of cells were viable. At D0, the frequency of dead cells significantly increased for PROH whatever the protocol tested (Fig. 3A). For DMSO, the viability was significantly decreased in, only, DCR (P < 0.05).

Apoptosis
The frequencies of early apoptotic cells (Annexin V test) are represented in Fig. 3B. Just after thawing (D0), the rate of early apoptotic cells was not different from the control for DCS− and DNC conditions, and significantly increased for DCR (P < 0.01), PCR (P < 0.05) and PNC (P < 0.05) protocols. For the other protocols, the mean value of early apoptotic cells was moderately increased even if it did not reach a significant level. After a 20 h culture period (D1), the frequency of annexin-positive cells did not increase compared with D0 values in the different freezing conditions. However, signs of early apoptosis were significantly detected in the controls, suggesting an effect of culture condition on early apoptosis induction.

On D0, the proportion of cells with fragmented DNA (assessed by TUNEL method), in the different freezing protocols, was similar to controls, even when percentages of necrotic

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**Table II.** Calculated values used for statistical analysis of data.

<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>Definition</th>
<th>Calculations</th>
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<tbody>
<tr>
<td>N cell D0 (10⁶/100 mg of tissue)</td>
<td>Number of cells recovered after digestion of 100 mg of testicular tissue.</td>
<td>(Cd × Vd × 100)/Wt</td>
</tr>
<tr>
<td>Rd (%)</td>
<td>Cell recovery after digestion = proportion of cells recovered for each freezing protocol tested, when compared with fresh controls of the experiment.</td>
<td>N cell D0 for the tested protocol/N cell D0 for the control × 100</td>
</tr>
<tr>
<td>Rec (%)</td>
<td>Cell recovery after culture = proportion of cells recovered after culture.</td>
<td>N cell cult D0/N cell cult D1 × 100</td>
</tr>
<tr>
<td>N cell D1 (10⁶/100 mg of tissue)</td>
<td>Number of cells recovered after digestion and culture of 100 mg of testicular tissue.</td>
<td>N cell × % Liv TB</td>
</tr>
<tr>
<td>N Liv cell (10⁶/100 mg of tissue)</td>
<td>Number of living cells just after digestion of 100 mg of testicular tissue (N Liv cell D0) or after a 20H culture period (N Liv cell D1).</td>
<td>N Liv cell × Liv TB</td>
</tr>
<tr>
<td>% Ap− Liv cell (%)</td>
<td>Proportion of non-apoptotic living cells just after digestion (% Ap− Liv cell D0) or culture (% Ap− Liv cell D1) for the freezing protocol tested, when compared with the fresh control of the experiment.</td>
<td>N Liv Ap−/N Liv for the tested protocol</td>
</tr>
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**Figure 2:** Cell recovery after enzymatic digestion when compared with the fresh controls in the experiment (100%) Each value corresponds to the mean ± SD of percentages obtained for each procedure (n = 4 for DCR, PCR and PNC; n = 5 for DCS− and PCS +; n = 6 for DCS+, DNC− and PCS−, n = 15 for fresh control). Results were compared with values obtained with each fresh control. NS, *P < 0.05; **P < 0.01; ***P < 0.001 versus fresh control. D, dimethylsulphoxide; P, propanediol; CR, controlled rapid; NC, not controlled; S+, with seeding; S−, without seeding

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cells were high (Fig. 3C). However, on D1, the rate of late apoptotic cells significantly increased in protocols with PROH ($P < 0.01$ for PCS$-$, $P < 0.05$ for PCS$+$, $P < 0.05$ for PCR, $P < 0.05$ for PNC) and also for DCR condition ($P < 0.05$).

**Proportion of non-apoptotic living cells**

The frequency of non-apoptotic living cells significantly decreased for all freezing protocols evaluated (Fig. 4). However, testes frozen in medium containing DMSO had a significantly higher percentage of non-apoptotic living cells when compared with medium including PROH ($P < 0.01$ for NC and $P < 0.05$ for CS$-$, CS$+$ and CR cooling rate curves). For the two cryoprotectants, CS$-$ offered the optimal results and CR the worst.

Values from global semi-quantitative evaluation of morphological alterations for the different cryopreservation protocols tested were negatively correlated with proportions of non-apoptotic living cells ($r = -0.93$, $P < 0.01$) (Fig. 4). For the two cryoprotectants, CS$-$ again offered best results but the worst were obtained with NC cooling.

**Morphology of the fresh and cryopreserved testis**

Weakly altered tissues (Fig. 5B) only presented alteration of the seminiferous epithelium with small gaps due to the shrinkage of the cytoplasm of some cells. Nuclei were well-preserved and the distinction between spermatogonia and Sertoli cells was generally easy. Moderately damaged cords (Fig. 5C and D) displayed larger gaps and cell-to-cell connection disruption with, at times, a partial detachment from myoid cells of the basal membrane. Rounding of Sertoli cells nuclei was observed and distinction between intratubular cells was more difficult. Finally, severe cryo-injuries (Fig. 5G–I) were characterized by major detachment from the basal compartment and by the presence of numerous pyknotic nuclei (round, small and dark).

Approximately 88.6 $\pm$ 8.2% of seminiferous cords from the fresh tissue was evaluated as being structurally normal (Fig. 5A). In the remaining 11.4% of cords, alterations were slight and the morphology was, also, considered to be good (both epithelium and nuclei scores $\leq$ 1). Many of these changes probably resulted from hypoxia and handling during the preparation of the material before fixation.

Fresh and DCS$-$ cryopreserved testis generally exhibited a similar morphology. For DCS$-$, 47.4 $\pm$ 21.9% of cords were undamaged and 95.0 $\pm$ 4.2% were judged to be well-preserved. Main damages concerned a moderate alteration of the epithelium with formation of small gaps (38.3 $\pm$ 16.6%) or presence of a small proportion of cells with condensed nuclei (22.5 $\pm$ 16.3%).

For DNC, PCS$-$ and DCS$+$, respectively, 73.4 $\pm$ 8.9, 50.0 $\pm$ 8.8 and 46.5 $\pm$ 35.0% of cords had no or little morphological impairment. Alteration of the epithelium was the main structural anomaly observed, with formation of large gaps, in 26.7 $\pm$ 8.9, 41.7 $\pm$ 12.7 and 37.6 $\pm$ 22.3% of cords for DNC, PCS$-$ and DCS$+$, respectively. Nuclei alterations were generally moderate for DNC and PCS$-$, however, for DCS$+$, 18.9 $\pm$ 12.6% of cords had a high proportion of condensed nuclei. These nuclei were generally rounded and distinction between Sertoli and spermatogonia nuclei was impossible for 19.6 $\pm$ 28.5% of cords.

For DCR, histological appearance was judged to be good for 25.0 $\pm$ 12.7% of cords. Most of sections presented an altered epithelium and a detachment of intratubular cells from the basement was observed in 10.0 $\pm$ 8.4% of cords. In 90.0% of cords most of nuclei were rounded and distinction between Sertoli and spermatogonia was difficult (44.2 $\pm$ 11.5%) or impossible (45.8 $\pm$ 17.4%). However, proportion of condensed nuclei was generally nil (41.7 $\pm$ 13.2%) or low (51.7 $\pm$ 9.8%).

Major alterations in histological appearance were found for PCS$+$, PCR and PNC protocols in 35.6 $\pm$ 27.0, 49.8 $\pm$ 32.6 and 81.9 $\pm$ 18.9% of the tubules, respectively, partial or total detachment of intratubular cells from the basement membrane was observed. Epithelium was generally altered and nuclei were often pyknotic (rounded and condensed). Only 9.3 $\pm$ 9.6 and 0.8 $\pm$ 1.6% of cords were well-preserved for PCS$+$ and PCR freezing procedures, respectively. The lower...
quality in terms of tissue morphology was observed for the PNC freezing protocol, with 100% of poorly preserved cords.

Testosterone production of the fresh and cryopreserved testes after culture

The production of testosterone by the fresh and cryopreserved cultured testes is shown in Fig. 6. The frozen–thawed immature testis secreted on average similar quantity of testosterone than was produced by the fresh samples. Although on Days 7 and 9, the testosterone production tended to be higher for the frozen–thawed samples, the difference did not reach a statistically significant value. Furthermore, the production of testosterone increased from D0 to D9.

Morphology of the fresh and frozen–thawed testes after culture

The mean diameter of the seminiferous tubules increased between Day 0 and Day 9 in fresh and cryopreserved cultured tubules (Fig. 7). The total number of intratubular cells was higher on Day 9 compared with Day 3 (Fig. 7) in fresh and cryopreserved cultured tubules. However, the difference did not reach a statistically significant value. On Day 9, as spermatocyte was the more advanced stage observed for germ cells, we concluded that in long-term organ cultures only Sertoli cells, spermatogonia and sometimes spermatocytes were present inside tubules. Spermatocytes I at lepotoene and zygotene stages were observed in fresh (14.9% of intratubular cells) and post-thaw (5.0% of intratubular cells) tissues. Some spermatocytes I at early pachytenie were only detected in fresh samples (see below).

PCNA expression in vivo and on fresh and frozen–thawed testes after culture

PCNA was intensely expressed on Day 7 post-partum (pp) (Fig. 8). PCNA labelling interested Sertoli cells and spermatogonia. Then, the number of cells labelled with PCNA antibody decreased until Day 10 post-delivery. An increase of PCNA expression was observed from Day 12 to Day 16. Spermatocytes I at lepotoene, zygotene and pachytenie stages were labelled (Fig. 9).

After in vitro culture of fresh and cryopreserved testes, PCNA expression followed the same profile as observed in vivo. The level of expression was reduced, more specifically for fresh cultured testis. However, the difference did not reach a statistically significant value.

Discussion

In the present study, two cryoprotective agents and four cooling rates were tested in immature mice testis. In previous works regarding testicular tissue cryopreservation, the number of protocols tested was never high, generally due to the complex and time consuming methodology employed. For immature testicular tissue, a single protocol has generally been proposed (Schlatt et al., 2002; Shinohara et al., 2002) or when comparisons were performed, no more than four different freezing–thawing procedures have been tested (Kvist et al., 2006; Jahnukainen et al., 2007; Keros et al., 2007). Our study demonstrates that six-day-old immature mouse testes are well-preserved when frozen with CS− programme, in the Leibovitz® L15 medium containing 1.5 M DMSO, 0.05 M sucrose and 10% FCS. DMSO provides superior cryoprotection as compared with PROH, probably due
to its low molecular weight and high tissue penetration. Based on our results, both cooling rate and type of cryoprotective agent employed were critical. This observation is in agreement with previous data reporting that DMSO produces better results than PROH, glycerol (Keros et al., 2005) or ethylene glycol (Jahnukainen et al., 2006). Nevertheless, the choice of the cryoprotectant is not currently clearly established, as mentioned in a recent report concerning testicular fragments of prepubertal patients well-preserved using ethylene glycol (Kvist et al., 2006). The CS cooling has been generally proposed for testicular tissue freezing (Schlatt et al., 2002; Shinohara et al., 2002; Keros et al., 2005, 2007; Kvist et al., 2006), as confirmed in our study. Nevertheless, in contrast to most other published freezing protocols (Schlatt et al., 2002; Keros et al., 2005, 2007; Kvist et al., 2006), seeding did not appear to be necessary and could even be potentially harmful. Temperature, soaking time before seeding or manual seeding duration are possibly critical and should be further tested. Our freezing procedure was proposed for cryopreservation of testis pieces. In our experiments, immature mice testis was not cut before freezing. However, epididymis as well tunica albuginea were removed, the size of prepared

Figure 5: Seminiferous cord morphology after cryopreservation with the following conditions (A) control; (B) DCS −; (C) PCS −; (D) DNC; (E) DCS +; (F) DCR; (G) PCS +; (H) PNC and (I) PCR. Scale bar: 10 μm. (A) Fresh control: sertoli cells (small ovoid nuclei arranged perpendicular to the basement membrane; St) and type A spermatogonia (large spherical nuclei; Sg) make up the seminiferous epithelium. Sertoli cell cytoplasm fills the future lumen of the seminiferous tubules. (B−D) Slightly altered cord: nuclei are well-preserved (rare pyknotic nuclei) and distinction between Sertoli cells and spermatogonia is easy, the epithelium is slightly altered with some gaps. (E and F) Altered cord: all nuclei are rounded and distinction between Sertoli cells and spermatogonia is not possible, a moderate increase of the proportion of pyknotic nuclei is observed and the epithelium is strongly altered with large gaps at the emplacement of the future lumen. (G−I) Very highly altered cord: distinction between Sertoli cells and spermatogonia is impossible with major increase in the proportion of pyknotic nuclei. (H and I) The epithelium is retracted with a total detachment of intratubular cells from the basement membrane

Figure 6: Testosterone concentration (mean ± SD) in tissue culture medium throughout a 9 day period with fresh and post-thaw testis tissue (DCS − protocol)
testis for cryopreservation was small with a weight no more than 3 mg, close to the size reported in a human application by Keros et al. (2005, 2007) and Kvist et al. (2006). Freezing of testis pieces allows to maintain cell-to-cell interaction, as well as exocrine (spermatogenesis) and endocrine (Leydig cells) functions of the testis.

In our study, we also developed a rapid screening method allowing evaluation of numerous freezing–thawing parameters in order to select optimal conditions for cryopreservation of prepubertal testicular tissue. This method is based on histological semi-quantitative evaluation of morphological alterations of seminiferous cords. Our approach was validated by comparing the morphology and the viability of testicular cells for different freezing–thawing procedures. Thus, an increase of morphological alterations between the different protocols tested was correlated with a decrease in the amount of non-apoptotic living cells recovered after enzymatic digestion of frozen–thawed testicular tissue \((r = -0.93, P < 0.01, \text{Fig. 4})\).

Light or electron microscopy have been used to evaluate cryopreservation protocols of ovarian tissue (Hovatta et al., 1996; Gook et al., 1999; Hreinsson et al., 2003; Bedaiwy et al., 2006) as well as adult (Jezeck et al., 2001; Keros et al., 2005) and prepubertal (Kvist et al., 2006; Keros et al., 2007) testicular tissue. Jezeck et al. (2001) showed by semi-quantitative evaluation of morphological modifications that poor cryopreservation conditions had a significant impact on the structure of adult rat seminiferous epithelium, more particularly on the basal compartment with a ‘folding’ or disruption of the lamina propria associated to a notable damage of Sertoli cells and spermatogonia (nuclear damage, increased vacuolization, swelling and shrinkage of the cytoplasm). Semi-quantitative histological evaluation was also performed on frozen–thawed adult human testicular tissue (Keros et al., 2005). The most typical damage reported was rupture of the cell-to-cell connections inside the seminiferous tubules, mainly in the basal compartment. In our study, we attempted to use the same approach and to evaluate morphological alterations caused by freezing and thawing procedures using a semi-quantitative scoring method. Different histological features were evaluated and compared with fresh controls, taking into account recovery and viability values. We attempted to establish a correlation between the different morphological changes observed and individual cell survival. Furthermore, a recent report of Keros et al. (2007) using a global architectural evaluation of human seminiferous tubules supported our results concerning the possible impact of cryopreservation protocol on the structure of immature testicular tissue.

In general, cell recovery after digestion was lower, as tissues were altered, according to morphological characteristics of seminiferous cords. Therefore, a severe trauma due to inadequate conditions of cryopreservation may have led to a massive and rapid necrosis process, with a quick lysis of most cells. Among recovered cells, the percentage of living cells on the day of thawing was generally high for good protocols, such as DCS− (>90%). In contrast, proportion of dead and necrotic cells was particularly elevated for PCR (59%) and PNC (32%) procedures (Fig. 3A). As apoptosis is a long and progressive process, often taking 12–24 h (Collins et al., 1997), most of these dead cells may be necrotic. Considering that freezing–thawing procedures could induce apoptosis, we attempted to detect early marker of apoptosis on the day of thawing with Annexin V marker and late stage of apoptosis process after a 20-h culture period with TUNEL assay. As expected, DNA fragmentation, detected by TUNEL assay, only significantly increased after a 20-h culture period and mostly for poorer cryopreservation procedures (PCS−, DCR, PCR, PNC and PCS+) (Fig. 3C). Percentages of living Annexin V-positive cells were significantly increased just after thawing and digestion, but only for the DCR, PCR and PNC protocols (Fig. 3B). Programmed cell death following freezing and thawing may compromise long-term cell survival and proliferation. The inclusion of an apoptotic inhibitor in cryomedia may improve cryopreservation outcome (Baust et al., 2000, 2001).

Our histological evaluation method allows the rapid testing of several conditions in combination. Thus, we can envisage testing and changing one or more of the following parameters: (i) sample transport conditions, (ii) size of the tissue fragment, (iii) cryomedium composition, (iv) equilibration procedure, (v) freezing programme and (vi) thawing procedure. Furthermore, post-thaw histological evaluation may also be a rapid and effective tool to assess the quality of frozen fragments, in terms of likely subsequent use, within the context of a human clinical application.

However, for conditions that preserve testicular morphology, this approach is not sufficient to conclude the
The impact of the procedure on the functionality of the cryopreserved testicular tissue (Kvist et al., 2006; Jahnukainen et al., 2007). Xenografting into immunodeficient hosts (Shinohara et al., 2002; Jahnukainen et al., 2007; Goossens et al., 2007) or in vitro culture (Keros et al., 2005; Kvist et al., 2006) of testicular tissue is then necessary to detect differences in spermatogonial stem cell potential as well as to assess the endocrine function integrity of the tissue. In our study, the endocrine function of Leydig cells was maintained after a long-term culture of frozen–thawed testes. The release of LH into the medium stimulated testosterone synthesis. This result confirmed that our protocol maintains the hormonal activity of Leydig cells, in agreement with previous studies using a different procedure for immature testis cryopreservation (Keros et al., 2005, 2007; Kvist et al., 2006). Furthermore, the increase of the seminiferous cord diameter, also described by Kvist et al. (2006), as well as the presence of more numerous intratubular cells (Sertoli cells, spermatogonia and spermatocytes I) after 9 days of culture supported the ability of our protocol to preserve not only somatic cells but also germ cells. The expression of PCNA marker in culture was comparable between fresh and frozen–thawed tissue and also respected the in vivo kinetic expression. The increase of tubule size and of the number of intratubular cells are in agreement with a cell proliferation process. Sertoli cells and spermatogonia maintained their ability to proliferate. However, it is not excluded that a minor part of PCNA-positive cells observed could be in repair process and not in S-phase. PCNA was intensely expressed on D0 of culture (Day 7 pp) corresponding to primitive type A spermatogonia and Sertoli cell proliferation stage (Bellve et al., 1977). Then, PCNA expression decreased until D3 (Day 10 pp), in agreement with the decrease of the proliferative ability of Sertoli cells described in vivo. An increase of PCNA expression was observed from D5 to D9 (Day 12–16 pp): in vivo, type A and type B spermatogonia are present by Day 10 and meiotic prophase is initiated, with the germ cells reaching the early and late pachytene stages by Days 14 and 18 (Bellve et al., 1977). In our fresh and post-thaw culture system, spermatogonia were able to differentiate and enter into meiosis. An initiation of spermatogenesis was
observed with the presence of spermatocytes I at leptotene and zygotene stages by Days 9. However, the percentage of cells at leptotene stages was lower in post-thaw tissue. Furthermore, spermatocytes I did not reach the pachytene stage in the frozen–thawed samples. Germ cells may undergo some alterations not detected by our evaluations or additional time may be necessary to reach pachytene stage for frozen–thawed tissue. Our culture system was not maintained after 9 days of culture because beyond this culture time, necrosis increased seriously in seminiferous cords, with more difficulties to analyse the different cells present in the tubules and their distribution.

In conclusion, evaluation of morphological alterations of seminiferous cords and individual cell survival in cryopreserved immature mice testes, showed the superiority of (i) CS$_2$ when compared with other freezing procedures and (ii) DMSO when compared with PROH. For the DCS$_2$ condition, in vitro culture of frozen–thawed seminiferous tubules confirmed the preservation of Leydig cells endocrine function, the proliferative ability of germ cells and Sertoli cells as well as the potential of germ cells to initiate in vitro meiosis. The optimal protocol should be tested in other species and finally in human samples.

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References


Figure 9: PCNA immunostaining (brown nuclei) on fresh and post-thaw (DCS$_2$) testicular tissue on Days 0 (A: post-thaw tissue), 5 (B: post-thaw tissue, D: fresh tissue) of culture. On Days 0 (A) and 5 (B), only spermatogonia (Sg) and Sertoli cells (St) were observed and expressed PCNA. On Day 9, spermatocytes I at leptotene (Le) and zygotene (Zy) stages were detected on fresh and post-thaw samples. Early pachytene (EP) cells were only detected on fresh samples. Scale bar =10 μm.


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