Ovarian tissue freezing: slow freezing versus vitrification

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Rationale and perspectives of cryopreserving and transplanting ovarian tissue

- Fertility can be maintained after curing disease where treatment may harm ovarian function

- And will lead to menstrual cycles and an endogenous hormone production in contrast to other fertility preserving methods
Chemotherapy and gonadotoxicity

Risk of inducing detrimental effects on the gonad

- The specific chemotherapeutic drug used
- Dose of chemotherapy
- Duration of chemotherapy
- Age of woman
### Candidate diseases for ovarian cryopreservation

<table>
<thead>
<tr>
<th>Cancer patients</th>
<th>Adjunctive oophorectomy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast cancer</td>
<td>Endometriosis</td>
</tr>
<tr>
<td>Cervical cancer</td>
<td></td>
</tr>
<tr>
<td>Hodgkin’s lymphoma</td>
<td></td>
</tr>
<tr>
<td>Non-Hodgkin’s lymphoma</td>
<td>Autoimmune diseases</td>
</tr>
<tr>
<td>Osteosarcoma</td>
<td>Collagen vacular diseases (SLE)</td>
</tr>
<tr>
<td>Ewing’s sarcoma</td>
<td>Acute Glomerulonephritis</td>
</tr>
<tr>
<td>Wilm’s tumor</td>
<td>Behcet’s disease</td>
</tr>
<tr>
<td><strong>Bone marrow transplant patients</strong></td>
<td><strong>Ovarian diseases</strong></td>
</tr>
<tr>
<td>Leukemia (?)</td>
<td>BRCA-1 and -2 mutations</td>
</tr>
<tr>
<td>Aplastic anemia</td>
<td>Turner’s Syndrome</td>
</tr>
<tr>
<td>Sickle cell anemia</td>
<td></td>
</tr>
</tbody>
</table>
Only the ovarian cortex is cryopreserved
Preparation of human ovarian tissue for cryopreservation
CRYOPRESERVATION PROTOCOL

(KLT. Schmidt et al., Hum Reprod. 2003)

- Cryoprotectant: 1.5 mol/l Ethyleneglycol
  0.1 mol/l Sucrose
  10 mg/ml HSA

- Temperature profile:
  1. Equilibration - rotation (1-2 °C in 30 min)
  2. -2 °C/min indtill – 9 °C.
  3. Manuel seeding
  4. – 0.3 °C/min until – 40 °C
  5. –10 °C/min until – 140 °C
  6. Liquid nitrogen (– 196 °C)
Percentage of morphological healthy follicles before and after cryopreservation
6 ovaries in each group

**Cryopreservation media:**
- I: Leibowitz medium, 10 % FCS & 1.5 M DMSO
- II: Leibowitz medium, 10 % FCS & 1.5 M Ethyleneglycol
- III: PBS, 0.1 M sucrose & 1.5 M DMSO
- IV: PBS, 0.1 M sucrose & 1.5 M Ethyleneglycol

<table>
<thead>
<tr>
<th></th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>BEFORE</td>
<td>97 ± 0.5</td>
<td>97 ± 0.7</td>
<td>97 ± 0.8</td>
<td>97 ± 1.3</td>
</tr>
<tr>
<td>AFTER</td>
<td>32 ± 11</td>
<td>31 ± 11</td>
<td>47 ± 11</td>
<td>63 ± 8</td>
</tr>
</tbody>
</table>
Freeze-thawed mouse ovary implanted under the kidney capsule of an ovariectomized mouse for 2 weeks.
Primordial follicles in the ovarian cortex from a 12 year old girl
Human ovarian tissue transplanted under the skin of immunodeficient ovarieectomised mice for 4 weeks.
Transplanting ovarian cortical tissue to ovariectomised immunodeficient nude mice for four weeks.

Tissue from 42 women (49 transplantations) showed surviving follicles and resulted in no apparent disease development in all cases.

Frozen thawed human ovarian cortex implanted under the skin of ovariectomized SCID mice for 4 weeks.
Transport of ovarian tissue – 6 year old girl 20 hours on ice prior to cryopreservation

Human ovarian tissue transplanted under the skin of ovariectomised SCID mice for 4 weeks
Equilibration in cryoprotectant for 30 min at 0 °C on a shaking table

Lessons learned: temperature at equilibration
Number of patients with cryopreserved ovarian tissue at University Hospital of Copenhagen

(Sep 2009: 385 patients)
Three step thawing procedure

I
0.75 M Ethyleneglycol
0.2 M sucrose in PBS
10 min

II
0.1 M sucrose in PBS
10 min

III
PBS
10 min
Orthotopic transplantation of ovarian tissue
Transplantation of frozen/thawed ovarian tissue: successful pregnancies

- Belgium 2004 – ♀
- Israel 2005 – ♀
- Denmark 2007 – ♀
- Belgium 2007 – ♀
- Denmark 2008 – ♂
- Denmark 2008 – ♀

All healthy babies
Transplantation of frozen/thawed ovarian cortex with cryopreservation immediately after recovery

<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>Age (years)</th>
<th>Proportion of tissue transplanted (%)</th>
<th>Lifespan (months)</th>
<th>No. remaining cryopreserved cortex</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Non-Hodgkin’s Lymphoma</td>
<td>32</td>
<td>A: 20</td>
<td>A: 45</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>B: 35</td>
<td>B: 21</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Hodgkin’s Lymphoma</td>
<td>28</td>
<td>A: 40</td>
<td>A: 54</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>B: 30</td>
<td>B: 6</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Hodgkin’s Lymphoma</td>
<td>25</td>
<td>A: 60</td>
<td>A: 26</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>D: 40</td>
<td>D: 37</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Paroxymal Nocturnal haemoglobin</td>
<td>19</td>
<td>33</td>
<td>17</td>
<td>24</td>
</tr>
<tr>
<td>5</td>
<td>Aplastic anaemia</td>
<td>25</td>
<td>40</td>
<td>12</td>
<td>10</td>
</tr>
</tbody>
</table>
Mean and individual FSH concentrations following ovarian autotransplantation in 12 women.
Overall results of assisted reproduction after transplantation of frozen/thawed ovarian tissue
(September 2009)

<table>
<thead>
<tr>
<th>Category</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. cycles</td>
<td>47</td>
</tr>
<tr>
<td>Follicles asp.</td>
<td>49</td>
</tr>
<tr>
<td>No. oocytes</td>
<td>35</td>
</tr>
<tr>
<td>No. Fertilized</td>
<td>21</td>
</tr>
<tr>
<td>No. transferred</td>
<td>14</td>
</tr>
<tr>
<td>Pos. hCG</td>
<td>6</td>
</tr>
<tr>
<td>Children born</td>
<td>3</td>
</tr>
</tbody>
</table>
Vitrification of human ovarian cortical tissue

**General considerations:**

- The type and mixture of the cryoprotectants
- The size of pieces of tissue to be cryopreserved
- The speed of cooling (direct emission in liquid N$_2$)
- The need of clinical verification – time period
Diffusion distances of cryoprotectants in ovarian tissue
Very different from conditions in oocytes and embryos

Slow freezing versus vitrification
Slow freezing and vitrification of human ovarian cortical tissue

General considerations:

- The texture of human cortical tissue is very different to most animal tissue
- which may affect the penetration rate of the cryoprotectants and optimal conditions
- and models developed by the use of animal tissue may not be used clinically in connection with human tissue and *vice versa*
Vitrification versus controlled-rate freezing in cryopreservation of human ovarian tissue

Experimental set-up

Keros V et al., *Hum Reprod*, 2009;24:1670
Vitrification versus controlled-rate freezing in cryopreservation of human ovarian tissue

<table>
<thead>
<tr>
<th>Step</th>
<th>DMSO (mol/l)</th>
<th>PrOH (mol/l)</th>
<th>Ethyleneglycol (mol/l)</th>
<th>PVP</th>
<th>Temp (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.35</td>
<td>0.35</td>
<td>0.35</td>
<td>-</td>
<td>Room</td>
</tr>
<tr>
<td>2</td>
<td>0.7</td>
<td>0.75</td>
<td>0.75</td>
<td>-</td>
<td>Room</td>
</tr>
<tr>
<td>3</td>
<td>1.4</td>
<td>1.5</td>
<td>1.5</td>
<td>10 %</td>
<td>+ 4</td>
</tr>
</tbody>
</table>

Duration of each step was either 5 or 10 min

Keros V et al., *Hum Reprod*, 2009;24:1670
Controlled-rate freezing in cryopreservation of human ovarian tissue

- **PrOH-protocol**
  1,5 mol/l propanediol, 0,1 mol/l sucrose, 25 mg/ml HSA in PBS
  
  *Equilibration at room temperature*

- **Ethylene glycol-protocol**
  1,5 mol/l ethylene glycol, 0,1 mol/l sucrose, 10 mg/ml HSA in PBS

  *Equilibration at +4 °C*

Keros V et al., *Hum Reprod*, 2009;24:1670
Vitrification versus controlled-rate freezing in cryopreservation of human ovarian tissue

Results:

- Based on tissue from 20 women and using morphological characteristics evaluated by light and electron microscopy
- The study concluded that vitrification was comparable to slow freezing in terms of preserving follicles in human ovarian tissue
- However, it appears that the ovarian stroma retained a better morphological integrity after vitrification

Clinical implication – Vitrification is not yet applied in a clinical setting

Keros V et al., Hum Reprod, 2009;24:1670
Human ovarian tissue vitrification versus conventional freezing: morphological, endocrinological, and molecular biological evaluation

<table>
<thead>
<tr>
<th>Step</th>
<th>DMSO (M)</th>
<th>PrOH (M)</th>
<th>Ethylene-glycol (M)</th>
<th>Acetamide (M)</th>
<th>Temp (°C)</th>
<th>Time (min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12.5 % of final concentration</td>
<td>Room</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>25 %</td>
<td>Room</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>50 %</td>
<td>+ 4</td>
<td>15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>2.62</td>
<td>1.31</td>
<td>0.0075</td>
<td>2.60</td>
<td>+ 4</td>
<td>15</td>
</tr>
</tbody>
</table>

The cortical tissue is plunged directly into liquid nitrogen

Controlled-rate freezing in cryopreservation of human ovarian tissue

- **DMSO-protocol**
  1.5 mol/l, 0.1 mol/l sucrose, 10 % SSS in Leibovitz medium

  *Equilibration ice cold*

Human ovarian tissue vitrification versus conventional freezing: morphological, endocrinological, and molecular biological evaluation

- During a 16 day long culture period there were no difference in oestradiol and progesterone secretion

- PCR detection of GAPDH mRNA showed a significant reduced expression in the vitrified cortical tissue

- Morphology:

Isachenko V et al., Reproduction, 2009;138:319
A number of other studies also suggest that there is not too big a difference between vitrification and slow-freezing.

- The primordial follicle is pretty resistant to freezing (as is the mature oocyte and testicular tissue).
- The oocyte and the granulosa cells are metabolically relatively inactive at the early resting stage.
- Easy penetrable cryoprotectants is required at conditions which minimize toxicity (low temperature).
The size of the frozen/thawed cortical pieces used for transplantation

- Most vitrification studies use pieces of cortical tissue considerably smaller than those employed clinically

- Keros V et al. 2009 used pieces of: 1 x 1-2 x 5-8 mm

- Isachenko V et al. 2009 used pieces of 0,3 – 1 x 1-1,5 x 0,7 – 1 mm

- Small pieces of cortical tissue facilitate quick penetration of cryoprotectant and build on experience from oocytes and embryos, but
Vitrification of ovarian tissue: factors that need clarification before implementation in a clinical setting

- The importance of the size of the cortical fragments for subsequent functioning of the tissue
- How to obtain sufficient cooling rates in a clinical setting
Survival of primordial follicles after grafting fresh or frozen-thawed cortical tissue from sheep ovaries to SCID mice

**Slow-freezing protocol:** 1.5 M DMSO, 10% FSC, Leibowitz medium

<table>
<thead>
<tr>
<th>Graft type</th>
<th>Primordial follicles</th>
<th>No. ± SEM</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Control</td>
<td>192 ± 47</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>2) Fresh</td>
<td>68 ± 11</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>3) Frozen-thawed</td>
<td>54 ± 12</td>
<td>28</td>
<td></td>
</tr>
</tbody>
</table>

The vast majority of follicles are lost following transplantation

Conclusions

- Ovarian cryopreservation including transport of tissue prior to cryopreservation is now a clinical option.

- In combination with ART results from Denmark suggests that ovarian cryopreservation do present a clinical relevant way of preserving fertility.

- Efficacy of vitrification of ovarian tissue in a clinical setting still requires development.

- Research to enhance transplantation efficiency is warranted.
Collaborators

**University Hospital of Copenhagen:**
- Anders Nyboe Andersen
- Anne Loft
- The Fertility Clinic
- Lene Lundwald
- Christian Ottesen
- Department of Gynaecology

**Laboratory of Reproductive Biology:**
- Anne Grete Byskov
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- Kirsten Tryde Schmidt
- Tiny Roed
- Inga Husum
- Marjo Westerdahl
- Stine Gry Kristensen

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**University Hospital of Odense:**
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- Per Emil Rasmussen
- The Fertility Clinic
- Department of Gynaecology

The Danish Medical Research Council

The Danish Cancer Foundation