Embryo / blastocyst cryopreservation: which embryo, which blastocyst and which method

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Cryopreservation of embryos
To preserve fertility
To increase cumulative pregnancy rates
To prevent multiple pregnancy rates

Cryopreservation in ART
To preserve fertility
To increase cumulative pregnancy rates
To prevent multiple pregnancy rates
Cumulative birth rates –
Addition in live births from freezing-thawing transfer (689 couples)

Lundin and Bergh 2007

Total cumulative rate live birth
Fresh + frozen

Vitrification / slow freeze
Development
Morphology
 Survival
 Pregnancy
Slow cooling / freezing

Vitrification

Slow cooling
- Cooling rates: 0.3 °C / min
- Controlled ice crystal formation (nucleation / "seeding")
- Thawing rate

Vitrification
- Cooling rates: 2,000 - 20,000 °C / min
- No ice crystal formation
- Toxic CPA concentrations?
- Thawing (warming) rate

Slow cooling
- At a certain temperature the kinetic energy of the molecules will become lower than the binding energy
  └→
- Molecules will start to organise into clusters that may grow into structures (crystals)
- They will try to organise into the energetically most favourable positions
Vitrification ("Glass transition")

- If the cooling occurs fast enough, the molecules never reach their energetically preferred position.
- They will form a glassy state: a non-equilibrium, amorphous, disordered state of extremely high viscosity.
- The transition to glass is a function of cooling rate and solute concentration.

20 °C Vitrification

Slow cooling

Temp (°C)

-120
-40
-7

ICE

GLASS

Conc. of solute (CPA)

LIQUID

Vitrification occurs
Factors that affect cell survival:

- Species
- Development stage
- Type of cryoprotectant
- Method of cryopreservation

Seeding (nucleation)

Initiation of ice formation in a controlled manner
- slow ice propagation through the solution

Avoids the damage of supercooling
- uncontrolled ice formation

Ice crystallisation

Removes water from the solution
- higher salt conc. in solution
- water passes out of cells
- higher osmotic pressure within the cell
- cryoprotectant moves into cells
• Also during slow cooling the cells will be exposed to very high concentrations of solutes, similar to during vitrification.
• However, during slow freezing this occurs at low temperatures, when the cells are less active.

Embryo selection in FER cycles – what should we freeze??

Embryo “quality” criteria – fresh vs. cryopreservation survival

• PN morphology
• no MNB
• 4 cells (~8) –
• even sized cells
• < 20 (~30?)% fragmentation
• first cleavage before 25-27-hours
• 1 nucleus / cell
Number of cells (prefreeze) Sahlgrenska University Hospital (n=458)

<table>
<thead>
<tr>
<th>Cell survival</th>
<th>100%</th>
<th>60-80%</th>
<th>&lt; 50%</th>
<th>mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 cells (n=320)*</td>
<td>55%</td>
<td>18%</td>
<td>27%</td>
<td>69.1</td>
</tr>
<tr>
<td>5 cells (n=94)*</td>
<td>37%</td>
<td>24%</td>
<td>39%</td>
<td>60.0</td>
</tr>
<tr>
<td>6 cells (n=44) #</td>
<td>34%</td>
<td>32%</td>
<td>32%</td>
<td>63.6</td>
</tr>
</tbody>
</table>

* p= 0.002, # p= 0.009

Implantation vs. number of prefreeze cells

5572 embryos
- 2 cells frozen day 2 7.2%
- 4 cells frozen day 2 16.9%
- 4 cells frozen day 3 5.5%
- Non-intact 4 cells day 2 <11%
- Fresh 4 cells day 2 16.6%

~ 30% implantations lost due to cryopreservation (30-35% SU) Edgar et al 2000

2003-2006 (n= 1393 SET) Sahlgrenska University Hospital

<table>
<thead>
<tr>
<th>Survival, %</th>
<th>Implantation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 *</td>
<td>232/967 (24)</td>
</tr>
<tr>
<td>70-90 *</td>
<td>56/325 (17)</td>
</tr>
<tr>
<td>60</td>
<td>9/63 (11)</td>
</tr>
<tr>
<td>40-50</td>
<td>7/65 (14)</td>
</tr>
</tbody>
</table>

* p= 0.011
Predictive factors for outcome of frozen embryo transfers

- 822 double embryo transfers
- 420 single embryo transfers
- Delivery rate 18.7% vs. 14.3%

- Predictive factors:
  - Woman’s age
  - Embryo quality (≥ 4 cells, intact after thawing)

Frozen-thawed SET - Predictive factors for live birth

- 622 single embryo transfer cycles
- 16% live birth

- Independent predictive factors:
  - Fertilisation method (IVF)
  - Embryo survival

Embryo morphology and survival rates

(640 4-cell embryos frozen separately on day 2)

<table>
<thead>
<tr>
<th>Cell survival</th>
<th>100%</th>
<th>75%</th>
<th>&lt; 50%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade 4:1+4:2A (n=435)</td>
<td>46% *</td>
<td>15%</td>
<td>39%</td>
</tr>
<tr>
<td>Grade 4:2B (n=160)</td>
<td>36% *</td>
<td>15%</td>
<td>49%</td>
</tr>
<tr>
<td>Grade 4:2c (n=45)</td>
<td>53%</td>
<td>10%</td>
<td>37%</td>
</tr>
</tbody>
</table>

A= <20% fragm
B= irregular cell size
C= slightly granular

*p= 0.034 for
100% 4:1+4:2A vs. 4:8
Take home message
- cleavage stage embryos

- Prefreeze embryo characteristics influence survival rates after cryopreservation

- Survival rates after cryopreservation affects implantation rates

- I.e. Prefreeze embryo characteristics does affect implantation rates

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Early cleavage and survival rates
(297 embryos frozen separately on day 2)

<table>
<thead>
<tr>
<th>Cell survival</th>
<th>100%</th>
<th>&gt; 50%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early cleavage</td>
<td>52%</td>
<td>14%</td>
</tr>
<tr>
<td>Late cleavage</td>
<td>59%</td>
<td>11%</td>
</tr>
</tbody>
</table>

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Influence of media (culture and cryo)

<table>
<thead>
<tr>
<th>Culture/Freezing</th>
<th>CM1+FM1 (HEPES)</th>
<th>CM2+FM2 (PBS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N=</td>
<td>1321</td>
<td>305</td>
</tr>
<tr>
<td>100%</td>
<td>44.9%</td>
<td>32.8%</td>
</tr>
<tr>
<td>75-90</td>
<td>12.3%</td>
<td>15.4%</td>
</tr>
<tr>
<td>% GQE</td>
<td>51%</td>
<td>56%</td>
</tr>
</tbody>
</table>
Embryo selection in FER cycles – what should we freeze??

Artificial shrinkage ("collapse")

Prefreeze blastocyst scoring variables vs. survival and implantation rates:
- Development rate (morula, early blastocyst, expanded blastocyst day 5 - 6)
- Morphology
### Blastocyst quality and success rates

<table>
<thead>
<tr>
<th>Blastocyst quality</th>
<th>Bad</th>
<th>Good</th>
<th>Morula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitrification warming cycles</td>
<td>113</td>
<td>184</td>
<td>59</td>
</tr>
<tr>
<td>Survival 24 hours</td>
<td>54%</td>
<td>80%</td>
<td>63%</td>
</tr>
<tr>
<td>Impl. / transferred embryo</td>
<td>6%</td>
<td>22%</td>
<td>18%</td>
</tr>
</tbody>
</table>

(P. vanderzwalmen, Prague 2007, Cremedes et al, 2008)

### Extended culture before or after cryopreservation? Calculation……..

- 100 GQE embryos
- 45 blastocysts
- 36 survive (80%)
- 20 implant (55%)

- 100 GQE embryos
- 60 cryo quality
- 40 survive >75% (65%)
- 10 implant (25%)

### Vitrification vs. Slow freezing
Cleavage stage embryos

Vitrification vs. slow freezing - Survival rate

Loutradi et al., Fertil Steril, 2007

Cleavage stage embryos

Vitrification vs. slow freezing

- RCT
  - 466 donated human day 3 embryos
  - Cryoloop vitrification (PROH+ethylene glycol) or slow freezing

- Survival rate:
  - Vitrification vs. slow freezing 95% vs. 89% (p=0.02)

Balaban et al., Hum Reprod, 2008

Cleavage stage embryos

Pregnancy/transfer rate

<table>
<thead>
<tr>
<th></th>
<th>Vitrified %</th>
<th>Slow-freezing %</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rama Raju, 2005 8-cells embryos, ET day 3</td>
<td>35 (14/40)</td>
<td>17.4 (4/23)</td>
<td>NS</td>
</tr>
<tr>
<td>Kuwayama, 2005 4-cells embryos, ET day 5</td>
<td>27 (136/504)</td>
<td>32 (172/536)</td>
<td>NS</td>
</tr>
</tbody>
</table>
Summary
Cryopreservation of early cleavage stage embryos

- RCTs indicate higher survival rate with vitrification as compared with slow freezing
- ...but similar pregnancy rates
- Controlled studies show similar (or better?) perinatal outcome after slow freezing as compared with fresh IVF
- No adverse effect on children born after vitrification has been reported, but experience is limited

Blastocysts Survival rate
Vitrification vs. slow freezing

Loutradi et al., Fertil Steril, 2007
### Blastocysts

**Pregnancy/transfer rate**

<table>
<thead>
<tr>
<th></th>
<th>Vitrified</th>
<th>Slow-freezing</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Huang, 2005</td>
<td>53.8%</td>
<td>No controls</td>
<td></td>
</tr>
<tr>
<td>Blastocysts</td>
<td>7/13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kuwayama, 2005</td>
<td>53%</td>
<td>51%</td>
<td>NS</td>
</tr>
<tr>
<td>blastocysts</td>
<td>(2516/4745)</td>
<td>(50/98)</td>
<td></td>
</tr>
</tbody>
</table>

### Blastocysts

**Vitrification & Neonatal outcome**

<table>
<thead>
<tr>
<th></th>
<th>Fresh blastocysts</th>
<th>Vitrified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Live born</td>
<td>208</td>
<td>147</td>
</tr>
<tr>
<td>Major and minor defects:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (%)</td>
<td>4 (2.0)</td>
<td>2 (1.8)</td>
</tr>
</tbody>
</table>

Takahashi et al., Fertil Steril 2005

### Summary

**Cryopreservation of blastocysts**

- RCTs indicate higher survival rate with vitrification as compared with slow freezing
- ……but similar pregnancy rates
- No adverse effect on children born after vitrification has been reported, but experience is very limited
Vitrification

<table>
<thead>
<tr>
<th>Pro’s</th>
<th>Con’s</th>
</tr>
</thead>
<tbody>
<tr>
<td>• No intracellular ice</td>
<td>• Not so fast if many embryos</td>
</tr>
<tr>
<td>• Very fast, if few embryos</td>
<td>• Potentially more toxic</td>
</tr>
<tr>
<td>• High survival rates</td>
<td>• Possible contamination</td>
</tr>
<tr>
<td>• Low cost??</td>
<td>• Other risks? (child outcome?)</td>
</tr>
</tbody>
</table>

Conclusions

• Vitrification may be an alternative to slow freezing – but still experimental
• Associated with higher survival rates than slow freezing – but not increased LBR / PR
• Prospective trials needed to confirm this and to evaluate pregnancy rates and outcomes
• Small number of births and few controlled studies

The search for excellence…..
In the lab (cryo)

• Remember that cryopreservation is (can be) an important contribution to live birth rate!
• Morphology (before and after cryopreservation) – cryopreserve good quality embryos / blastocysts – transfer preferably intact
• Vitrification vs. Slow-freeze?
Thank you!

Damages of low temperature

- Low temperature per se – e.g. phase transitions in membranes, denaturation of proteins
- Direct effects of freezing – intracellular ice formation, membrane damages
- Indirect effects of freezing – changes in ionic interactions (high salt concentrations), cellular ultrastructure changes (dehydration)
Resultat slow cooling day 2 - SU

- **Survival rate**
  - Intact: ~50%
  - 75–100%; ~70%
- **Implantation rate** 23%
- **Live Birth rate** 18%

Cryoprotectants

- Dimethyl sulfoxide (DMSO)
- Glycerol
- Ethylene glycol (EG)
- 1,2-propanediol (PrOH)

Embryo criteria (day 2) - FER

<table>
<thead>
<tr>
<th>Fresh transfer</th>
<th>Cryopreservation</th>
</tr>
</thead>
<tbody>
<tr>
<td>• no MNB</td>
<td>• no MNB</td>
</tr>
<tr>
<td>• 4 cells (−8)</td>
<td>• 4 cells (−8)</td>
</tr>
<tr>
<td>• even sized cells</td>
<td>even sized cells</td>
</tr>
<tr>
<td>• &lt; 20% fragmentation</td>
<td>&lt; 20% fragmentation</td>
</tr>
<tr>
<td>• first cleavage before</td>
<td>first cleavage before</td>
</tr>
<tr>
<td>25-27 hours</td>
<td>25-27 hours ±</td>
</tr>
<tr>
<td>• 1 nucleus / cell</td>
<td>• 1 nucleus / cell</td>
</tr>
</tbody>
</table>