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

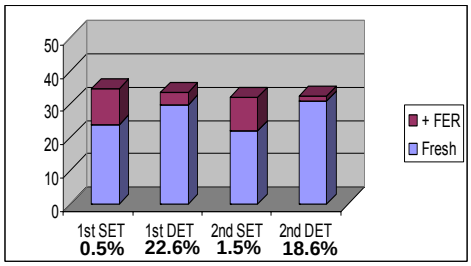
Cryopreservation in ART

To preserve fertility

To increase cumulative pregnancy rates

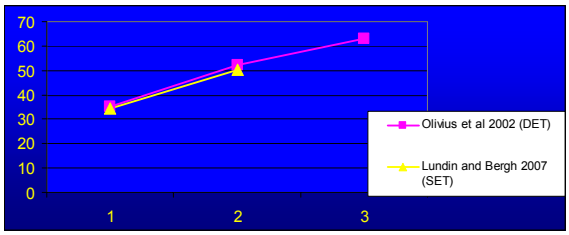
To prevent multiple pregnancy rates

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

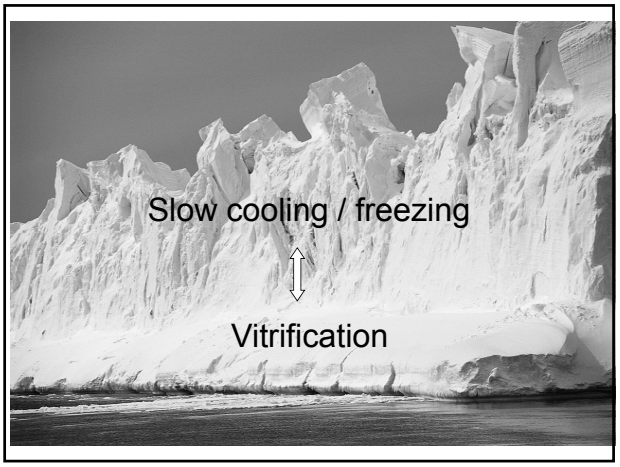
Development

Morphology

⇩

Survival

Pregnancy



Slow cooling	Vitrification
• Cooling rates: 0.3 °C / min • Controlled ice crystal formation (nucleation / ”seeding”) • Thawing rate	• Cooling rates: 2.000 - 20.000 °C / min • No ice crystal formation • Toxic CPA concentrations? • Thawing (warming) rate

Slow cooling

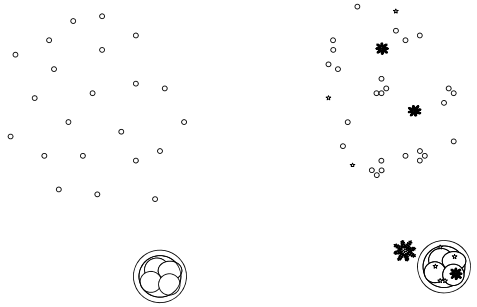
- At a certain temperature the kinetic energy of the molecules will become lower than the binding energy

⇩

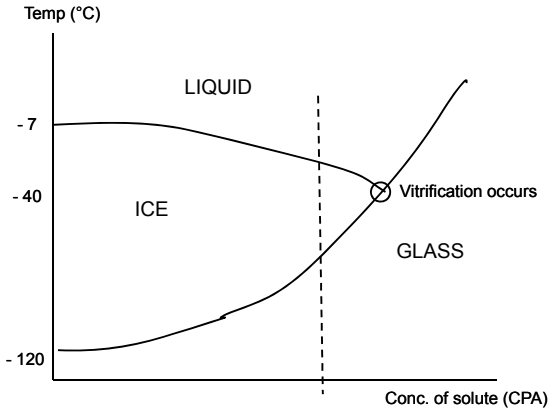
- Molecules will start to organise into clusters that may grow into structures (crystals)
- They will try to organise into the energetically most favourable positions

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 °CVitrification Slow cooling



Factors that affect cell survival:

- ❖Species
- ❖Development stage
- ❖Type of cryprotectant
- ❖Method of cryopreservation

Seeding (nucleation)

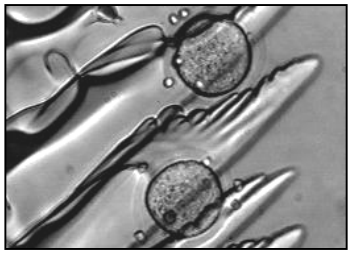
Initiation of ice formation in a controlled manner
⟹ slow ice propagation through the solution

Avoids the damage of supercooling
⟹ uncontrolled ice formation

Ice crystallisation

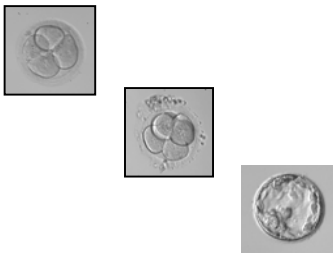
Removes water from the solution

- 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)

Cell survival	100%	60-80%	< 50%	mean
4 cells (n=320)* #	55%	18%	27%	69.1
5 cells (n=94)*	37%	24%	39%	60.0
6 cells (n=44) #	34%	32%	32%	63.6

* p= 0.002, # p= 0.009

Implantation vs. number of prefreeze cells

5572 embryon

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

Survival, %	Implantation (%)
100 *	232/967 (24)
70-90 *	56/325 (17)
60	9/63 (11)
40-50	7/65 (14)

* 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)

Salumets et al 2006

Frozen-thawed SET -Predictive factors for live birth

- 622 single embryo transfer cycles
- 16% live birth
- Independent predictive factors:
  - Fertilisation method (IVF)
  - Embryo survival

Olivius et al 2008

Embryo morphology and survival rates

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

Cell survival	100%	75%	< 50%
Grade 4:1+4:2A (n=435)	46% *	15%	39%
Grade 4:2B (n= 160)	36% *	15%	49%
Grade 4:2c (n= 45)	53%	10%	37%

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

*p= 0.034 for 100% 4:1+4:2A vs. 4:B

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

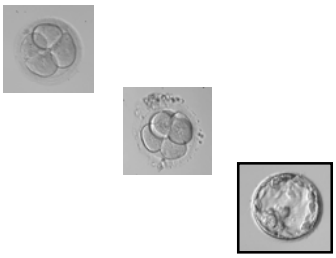
Early cleavage and survival rates
(297 embryos frozen separately on day 2)

Cell survival	100%	> 50%
Early cleavage	52%	14%
Late cleavage	59%	11%

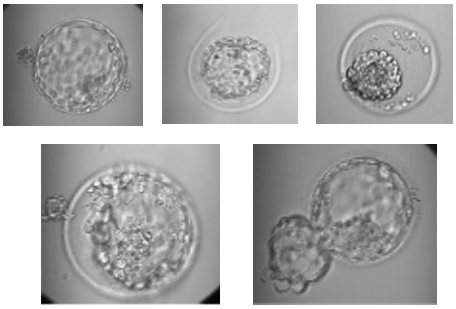
Influence of media (culture and cryo)

Culture/ Freezing	CM1+FM1 (HEPES)	CM2+FM2 (PBS)
N=	1321	305
100%	44.9%	32.8%
75-90	12.3%	15.4%
% GQE	51%	56%

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

Blastocyst quality	Bad	Good	Morula
Vitrification warming cycles	113	184	59
Survival 24 hours	54%	80%	63%
Impl. / transferred embryo	6%	22%	18%

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

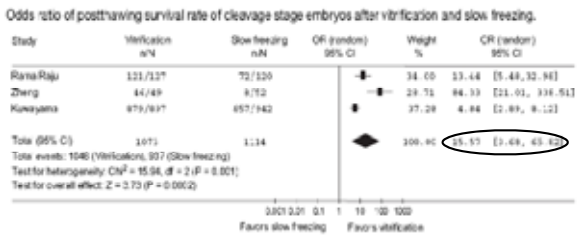
Extended culture before or after cryopreservation ? Calculation…….

- 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

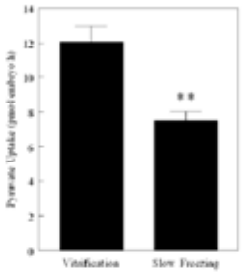


Loutradi et al, Fertil Steril, 2007

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



Pyruvate uptake by cryosurvived embryos

Cleavage stage embryos
Pregnancy/transfer rate

	Vitrified %	Slow-freezing %	P-value
Rama Raju, 2005 8-cells embryos, ET day 3	35 (14/40)	17.4 (4/23)	NS
Kuwayama, 2005 4-cells embryos, ET day 5	27 (136/504)	32 (172/536)	NS

Early cleavage stage embryos Vitrification & slow freezing Neonatal outcome

426 articles

Slow freezing
25 articles
>25,000 infants

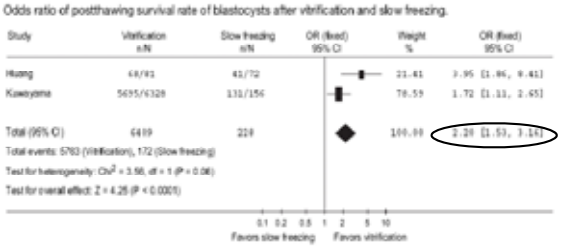
Vitrification
2 articles
11 + 69 deliveries

Wennerholm, ESHRE 2008

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

	Vitrified %	Slow-freezing %	P-value
Huang, 2005 Blastocysts	53.8 (7/13)	No controls	
Kuwayama, 2005 blastocysts	53 (2516/4745)	51 (50/98)	NS

Blastocysts
Vitrification & Neonatal outcome

	Fresh blastocysts	Vitrified
Live born	208	147
Major and minor defects:		
Total (%)	4 (2.0)	2 (1.8)

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

Pro´s	Con´s
• No intracellular ice • Very fast, if few embryos • High survival rates • Low cost??	• Not so fast if many embryos • Potentially more toxic • Possible contamination from N2 • Other risks ? (child outcome?)

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

Fresh transfer	Cryopreservation
• no MNB	• no MNB
• 4 cells (– 8) +	• 4 cells (– 8) +
• even sized cells +	• even sized cells +
• < 20% fragmentation	• < 20% fragmentation
• first cleavage before 25-27 hours +	• first cleavage before 25-27 hours ±
• 1 nucleus / cell +	• 1 nucleus / cell ?