Embryo/blastocyst cryopreservation: laboratory aspects

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Etienne Van den Abbeel PhD

Introduction

Three laboratory areas that warrant consideration and discussion

Optimizing embryo development in culture
Selecting the most viable/normal embryo for transfer
Optimizing cryopreservation procedures

Introduction

Cryopreservation of human embryos: Why?

- Increase efficiency of ART
- Tool to reduce multiple pregnancies
- Transfer in natural cycle
- Fertility preservation

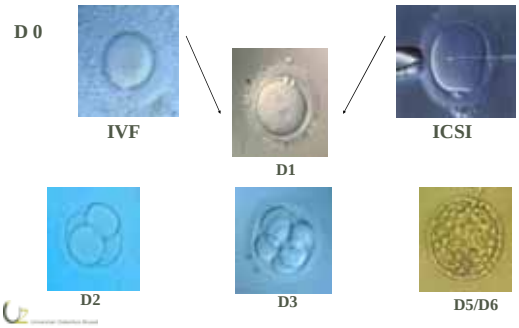
→ Efficient cryopreservation programmes?

Learning objectives

Cryopreservation programmes

- **Strategies to assess/select embryos/blastocysts before freezing and after thawing**
  - → Freezing policy
  - → Assessment of survival/transfer
- **Cryopreservation procedure**
  - → Vitrification >>> slow controlled-rate freezing ??
- **Storage**

Freezing policy



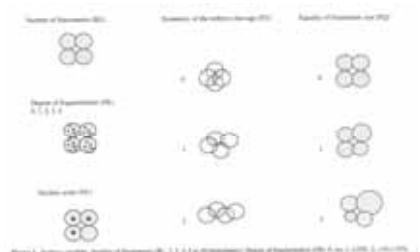
Freezing policy

Freezing strategies

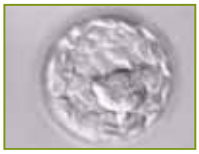
- Two strategies
  - S1: freezing before morphology becomes a substantial factor: one-cell two pronucleate stage freezing
  - S2: optimizing fresh transfer allowing the morphologically best embryos to be transferred: two- to 16-cell stage freezing or blastocyst stage freezing

Which embryos to freeze?

Freezing policy

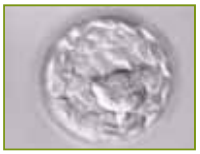


Freezing policy




Freezing policy



Freezing policy

Conclusion

Risk to throw out the child with the bathwater before freezing!!

Uniform reporting of the cryo data (Jones et al, 1995) (Hum Reprod 10, 2136-2138)

- Cycle cryopreservation rate (cycles with cryo/cycles with fresh transfer)
- Fresh embryo transfer rate (embryos transferred fresh/2PN embryos)
- Embryo cryopreservation rate (embryos frozen/2PN embryos)

Learning objectives

Cryopreservation programmes

Strategies to assess/select embryos/blastocysts before freezing and after thawing

- Freezing policy
- Assessment of survival/transfer

Cryopreservation procedure

- Vitrification >>> slow controlled-rate freezing ??

Storage

Assessment of survival/transfer

- Damaged embryos have a lower implantation potential than fully intact ones
  - → Speirs et al (1996) (Hum Reprod 11 (suppl 1) 107-192)
  - → Van den Abbeel et al (1997) (Hum Reprod 12, 2006-2010)
  - → Burns et al (1999) (Fertil Steril 72, 527-532)
  - → Edgar et al (2000) (Hum Reprod 15, 175-179)
  - → Guérif et al (2002) (Hum Reprod 17, 1321-1326)
  - → Pal et al (2004) (Fertil Steril
  - → Gabrielsen et al (2005) (RBM online 12, 70-76)
  - → Tang et al (2006) (Hum Reprod 21, 1179-118)
  - → Edgar (2007) (RBM Online 14, 718-723)

To what extent intermediate-stage embryos may loose cells after thawing without subsequent viability loss?

Assessment of survival/transfer

Table 3. Outcome from single cryopreserved embryo transfer (SCET) in women under 36 years in relation to number of surviving blastomeres (day-2 embryos cryopreserved at the 4-cell stage).

Pre-freeze blastomeres	*Post-thaw blastomeres*	*SCET*	*FH*	*Implantation rate %*
4	4	615	160	26.0
4	3	131	36	27.5
4	2	85	8	9.4

FH = fetal heart beat.

Assessment of survival/transfer

Cell loss in human day 3 embryos and implantation in single frozen embryo transfers

(Van Landuyt and Van den Abbeel , 2004-2007: 547 single FRET cycles, cryo day 3, ET day 4)

Table I. Clinical pregnancy rates (%) in relation to cell loss after freezing

	Cell stage before freezing				
N° cells damaged	6	7	8	>8	Total
0	12/64 (18.8)	20/82 (24.4)	30/175 (17.1)	15/61 (24.6)	77/382 (20.2)
1	1/5 (20.0)	3/19 (15.8)	9/43 (20.9)	7/19 (36.8)	20/86 (23.3)
2	0/2 (0.0)	4/10 (40.0)	2/17 (11.8)	0/5 (0.0)	6/34 (17.6)
>2	1/3 (33.3)	0/3 (0.0)	2/10* (20.0)	0/8 (0.0)	3/24 (12.5)

*2 pregnancies after transfer of embryos with 50% cell loss (4/8)

Assessment of survival/transfer

Resumption of mitosis in frozen/thawed embryos is capable of selecting the viable embryos for transfer

Van Der Elst et al (1997) Hum Reprod 12, 1513-1521)
Ziebe et al (1998) (Hum Reprod 13, 178-181)
Van den Abbeel et al (2000) (Hum Reprod 15, 373-378)
Tiitinen et al (2001) (Hum Reprod 16, 1140-1144)
Guérif et al (2002) (Hum Reprod 17, 1321-1326)
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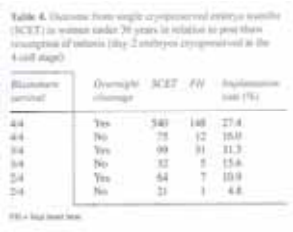
Assessment of survival/transfer



Assessment of survival/transfer

Cell loss in human day 3 embryos, resumption of mitosis and implantation in single frozen embryo transfers
Van Landuyt and Van den Abbeel, 2004-2007: 547 single FRET cycles, cryo day 3, ET day 4)

	Fully intact embryos	Damaged embryos
% Compact (24h)	72.4	72.1
% Pregnant	29.9	28.8
% Not compact (24h)	27.6	27.9
% Pregnant	11.1	11.6

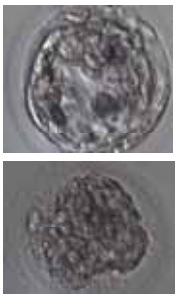
Assessment of survival/transfer

Cryopreservation of blastocysts

Morphological survival blastocyst stage

- Fully intact (ET)
- Moderately damaged (ET)
- Severely damaged (some ET)
- Degenerated (no ET)

Assessment of survival/transfer

UZBrussel results
Survival and transfer rate

Table I. Survival and transfer rates according to the day of vitrification and blastocyst quality

	N warmed	N survived (%)	N transferred (%)
Day 5 VIT	329	262 (79.6)	242 (73.6)
Day 6 VIT	97	74 (76.3)	66 (68.0)
Day 5 Early	159	136 **(85.5)**[a]	128 **(80.5)**[b]
Day 5 Advanced	170	126 **(74.1)**[a]	114 **(67.1)**[b]
Day 5 ICM A	99	73 (73.7)	68 (68.7)
Day 5 ICM B	71	53 (74.6)	46 (64.8)

a) $p < 0.05$ b) $p < 0.01$

Assessment of survival/transfer

Cryopreservation of blastocysts
Developmental potential in-vitro of thawed blastocysts

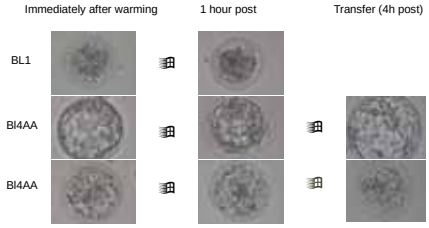
→ Early blastocysts
- Capability of expansion
  Van den Abbeel et al 2005) (Hum Reprod 20, 2939-2945)
  Guerif et al (2003) (Theriogenology 60, 1457-1466)

→ Advanced blastocysts
- Capability of re-expansion
  Van den Abbeel et al 2005) (Hum Reprod 20, 2939-2945)
  Guerif et al (2003) (Theriogenology 60, 1457-1466)
  Shu et al 2008 (Fertil Steril,)

→ Expansion/reexpansion: 4 hours or overnight?

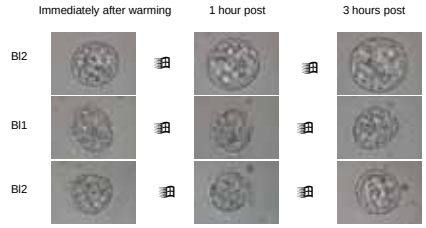
Blastocyst at UZBrussel

- Assessment of expansion/re-expansion after warming



Blastocyst vitrification at UZBrussel

- Assessment of expansion/re-expansion after warming



Assessment of survival/transfer

Assessment of survival/transfer

Developmental potential — Morphological survival

Dilemma: thawing policy?

→ How many embryos/blastocysts to thaw?
- Developmental stage
- Expected survival rates
- Transfer policy

Risk to throw out the child with the bathwater after thawing!!

Learning Objectives

Cryopreservation programmes:

Strategies to assess/select embryos/blastocysts before freezing and after thawing

- Freezing policy
- Assessment of survival/transfer

Cryopreservation procedure

- Vitrification >>> slow controlled-rate freezing ??

Storage

Introduction

Cryopreservation of reproductive cells

↓

Stopping biological time

↓

-196°C

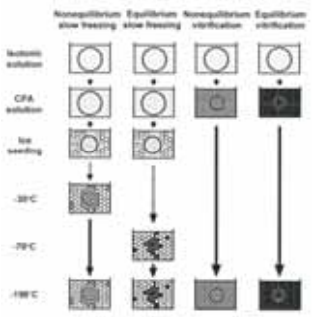
↓

Lethal intra-cellular ice formation

↓

Fate of cellular water?

Slow freezing versus vitrification



Challenges of freezing/stated advantages

Challenges:

→ When cells cooled slowly, their survival depends on cooling rate and/or warming rate.

→ Extra (and intra) cellular ice christals

→ Cells may be killed by slow cooling to ~0°C.

→ Solution effects.

→ Expensive equipment required.

Advantages:

→ Robust

→ Simple

Efficient freezing programmes?

- Frozen embryos have a lower implantation potential than fresh ones
- Not all embryos survive the procedure with all cells intact
- Damaged embryos have a lower implantation potential than fully intact ones

Further optimization?

- Possible?
  → Edgar et al RBM Onine (2009): Effect of sucrose concentration
- Indicated?
  → Vitrification is a better cryopreservation strategy than freezing?

Stated advantages of vitrification

- No extra and intra cellular ice crystal formation
- Dehydrate cell before cooling (no solution effects injury)
- Cool rapidly to “outrun” chilling injury
- Flexibility

Claims made for vitrification

- Very quick
- Low cost
- Very simple
- It can work better than freezing
- Safe

Do we have the evidence to support these claims?

Claims made for vitrification

Is vitrification a very quick procedure?

- Equilibration step and Vitrification step
- Warming step and several dilution steps
- One to one approach

Claims made for vitrification

Is vitrification a low cost procedure?

No biological freezers required

Flexibility: manpower

Vitrification media and devices:

→ Commercial companies

- Expensive devices!
- Expensive media formulations!

Claims made for vitrification

Is vitrification a simple procedure ?

- Probability of vitrification:

$$\frac{\text{Cooling and warming rates} \times [\text{CPA}]}{\text{Volume}}$$

- Succesfull vitrification depends on “sufficient” penetration of permeating CPA’s and “sufficient” dehydration by non-permeating CPA’s during the equilibration and vitrification step
  - Permeability characteristics of oocytes to water and CPA
    - Temperature and time dependency
    - Variability amongst oocytes

Claims made for vitrification

Can vitrification work better than freezing?

Meta analysis

Review papers

Vitrification of embryos: freezing versus vitrification

Loutradis et al (Fertil Steril 90, 186-193, 2008)

Systematic review and meta analysis on vitrification versus slow freezing of human embryos

Kolibianakis et al (Current opinion in OB/GYN 21, 270-274, 2009)

Cryopreservation of human embryos by vitrification or slow freezing: which one is better?

→ Vitrification as compared with slow freezing, appears to be better in terms of post-thawing survival rates both for cleavage-stage embryos and for blastocysts

→ Postthawing blastocyst development of embryos cryopreserved in the cleavage stage is significantly higher with vitrification as compared with slow freezing

→ No significant difference in clinical pregnancy rates per transfer could be detected between the two cryo methods

Claims made for vitrification

Is vitrification a safe procedure?

→ Cross contamination when using open devices?

→ Long term LN2 storage (vapour storage) of apparently vitrified, minimal-volume (<1µl) samples

- Spontaneous devitrification possible

→ Cryoprotectants are NOT neutral

- Biological (long term) effects of vitrification?

→ Children follow-up? >2000 deliveries

- Perinatal outcome (~ 900 children)
  - Mukaida et al, 2009; Rama Raju et al, 2009; Wennerholm et al, 2009

Learning objectives

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Storage

Storage of gametes/embryos

Quality and storage

Risk assessment

Unavoidable risks (Earthquakes, fire …)

Compliance with standards (Eu directives)

Avoidable risks

- Injury to personnel
- Loss of stored material
- Damage to stored material
- Misidentification of stored material

Storage of gametes/embryos

Quality and storage

→ Physical security of vessels and specimens
- Secure (assessed/cameras)
- Locked
- Ventilation
- Risk registers (fire etc…)

→ Liquid nitrogen supply and staff safety
- Continued supply (fail-safe systems)
- Oxygen measurements
- Burn wounds

Storage of gametes/embryos

Quality and storage

→ The relative safety of the containment system (vials or straws)
- Explosion of containment systems
- Cooling/warming rates and containment systems
- Cross contamination
  - Closed systems
  - Cryopreservation procedure
  - Sterile liquid nitrogen
  - Patient screening before cryopreservation

Storage of gametes/embryos

Quality and storage

→ The type of nitrogen storage
- Vapour phase storage or liquid phase storage
  - Temperature control/variations/autofilling
  - Sedimentation in liquid storage
  - Cleaning of storage containers

→ The suitability of equipment to do the job
- Finite lifespan of storage containers

→ Witnessing and security of labelling

→ Early warning and monitoring systems

General conclusions

- The aim of a cryopreservation programme should be to have fully intact embryos after thawing. However, also damaged embryos can give rise to live births
- Vitrification appears to be a better cryopreservation procedure than freezing
- Resumption of mitosis or further development in-vitro of frozen-thawed surviving embryos is capable of selecting the viable embryos for transfer. However, also not further cleaving embryos (intact ones and non-intact ones) can give rise to live births
- Freezing and thawing policy: risk to throw out the child with the bathwater before freezing and after thawing
- Centres should begin a cryo risk management process and identify areas of highest risk. An early warning system should be mandatory. This has to be affordable, manageable, easy to use and implemented alongside other risk reduction strategies