ESHRE Campus workshop

CRYOBIOLOGY AND CRYOPRESERVATION OF HUMAN GAMETES AND EMBRYOS

Prague, Czech Republic

13 & 14 April 2007

Contents

Organisation

Faculty

- K. Lundin (Deputy Coordinator of the SIG embryology)
- C. Magli (Deputy Coordinator of the SIG embryology)
- D. Royère (Past-Coordinator of the SIG Embryology)
- E. Van den Abbeel (Coordinator of the SIG embryology)

Local organiser

- Milan Mrazek (Czech Republic)

Faculty

- S. Al-Hasani (Germany)
- K. Lundin (Sweden)
- C. Magli (Italy)
- L. Rienzi (Italy)
- D. Royere (France)
- R. Sucha (Czech Republic)
- H. Tournaye (Belgium)
- E. Van den Abbeel (Belgium)
- J. Van der Elst (Belgium)
- P. Van der Zwalmen (Belgium)
- H. Woelders (The Netherlands)

Course description

In ART programs world wide, there are several topics of concern that impact greatly on patients and are of great concern for clinicians and scientists alike: reducing multiple pregnancies and male and female gamete cryopreservation.

Each topic independently brings its own need for ongoing discussion and research;

(1) the scientific community as well as the public is concerned about high-order multiple births and clearly the only remedy to almost avoid multiple birth is to transfer only one embryo which should result in more embryos being cryopreserved. However, national and international registers indicate that the results obtained with cryopreserved embryos are substantially lower than with fresh ones; (2) storing the male gamete is currently an accepted clinical procedure; (3) it is also clear that storing the female gamete can have great impact in reproductive medicine. Despite some successful clinical trials, there are several problems associated with the cryopreservation of the female gamete.

To optimize the cryopreservation of human embryos and gametes clinical embryologists worldwide have empirically modified basic procedures. Often these modifications were introduced without any justification and moreover this has not led to a substantial improvement of the results.

Therefore understanding basic principles of cryobiology and cryopreservation will help scientists and clinicians to optimize protocols for providing multiple options to the patients.

The SIG embryology is offering a two-day course aimed at clinical embryologists, clinical staff and other members with an interest in the fundamental cryobiology and understanding of human embryo and gamete cryopreservation. The course will attempt to describe the scientific properties of cryobiology, how they have been developed and how they are applied. The course will also describe the current status of human embryo and gamete cryopreservation and the factors influencing the cryopreservation outcome.

Learning objectives

At the conclusion of this course, the participant should be able to:

- apply the fundamental principles of cryobiology to improve current cryopreservation procedures
- to understand the factors that influence the cryopreservation outcome
- summarize current cryopreservation practices in ART

Program – 13 April 2007

08.30 – 09.00: Welcome and registration

Session I: Fundamental cryobiology

09.00 – 09.45: ***H Woelders (The Netherlands)***
Fundamental cryobiology for clinical embryologists

09.45 – 10.30: ***E Van den Abbeel (Belgium)***
Basic principles of freezing and vitrification

10.30 – 11.00: Coffee break

Session II: Human embryo cryopreservation

11.00 – 11.30: ***D Royere (France)***
Human embryo cryopreservation: a review of clinical issues related to the success rate

11.30 – 12.00: ***K Lundin (Sweden)***
Embryo characteristics influencing outcome of human embryo cryopreservation

12.00 – 12.30: ***C Magli (Italy)***
Cryopreservation of biopsied embryos after preimplantation genetic diagnosis or screening

12.30 – 13.30: Lunch break

Session III: Vitrification

13.30 – 14.15: ***P Van der Zwalmen (Belgium)***
Laboratory aspects of the vitrification of human embryos

14.15 – 14.45: ***S Al-Hasani (Germany)***
Three years of routine vitrification of human zygotes: is it still fair to advocate slow-rate freezing?

14.45 – 15.15: Coffee break

15.15 – 15.45: *R Sucha (Czech Republic)*
Vitrification of eggs and blastocysts: the Czech experience.

15.45 – 17.00: ***Commercial companies presentations***
Presentations of vitrification systems, carriers and results obtained

Program – 14 April 2007

Session IV: Cryopreservation of human gametes

08.30 – 09.15: ***J Van der Elst (Belgium)***
Aspects of the storage of the female gamete

09.15 – 10.00: ***L Rienzi (Italy)***
Biological and physiological aspects of the cryopreservation of human oocytes

10.00 – 10.30: Coffee break

10.30 – 11.00: ***H Tournaye (Belgium)***
Clinical aspects of the cryopreservation of the human male gamete

11.00 – 11.30: ***J Van der Elst (Belgium)***
Storing in reproductive medicine and the EU directives

Session V: General discussion

11.30 - 12.30: Vitrification: will it replace conventional freezing techniques in ART?

Fundamental cryobiology for clinical embryologists

Henri Woelders

Wageningen UR, Animal Sciences Group
Lelystad

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Fundamental Cryobiology

In this presentation:

Explain some aspects of fundamental cryobiology

and how this fundamental knowledge can help us to understand and improve methods for freezing embryos and gametes

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Fundamental Cryobiology

A great variety of terminology can be encountered:

- Cryogenic storage
- Freezing
- Slow freezing
- (ultra) rapid freezing
- Vitrification
- Drying
- Freeze-drying
- Glass transition

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Fundamental Cryobiology

They all relate to the same physico-chemical relationships

But are not necessarily the same thing.

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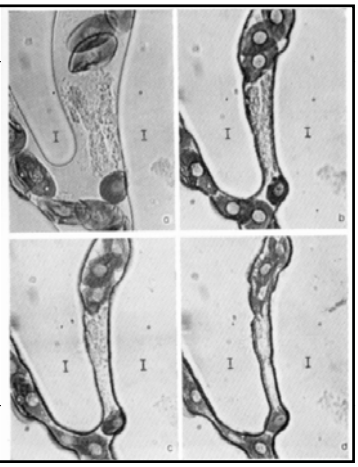
Slow Freezing

Erythrocytes at

-1,5 -5;

- 7 -10 ºC.

Rapatz & Luyet, 1960



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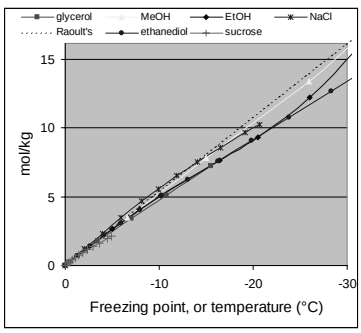

Woelders and Chaveiro 2004

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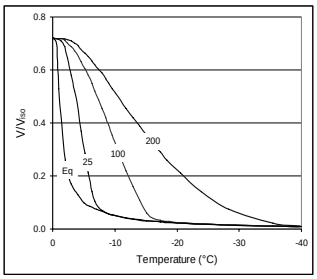
Slow Freezing



Mazur, 1963; Mazur and Koshimoto, 2002; see also Liu et al., 1997

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Slow Freezing

Water freezes (extracellularly) as pure ice
An unfrozen fraction remains that contains all solutes

- The volume of unfrozen fraction ↓
- Water content ↓
- Solute (salt) concentration ↑
- Osmotic pressure ↑
- Viscosity ↑

While IIF is prevented!

At some point of temperature and concentration ➔Glass transition

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Glass transition

Glass transition means that a liquid becomes solid in an amorphous state. The lateral mobility of molecules becomes practically zero.

A glass is stable because (by definition) molecules have lost the ability of translation movement. No significant biological or chemical changes will take place.

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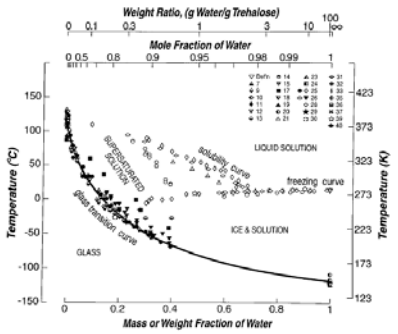
Phase diagram

Phase diagram of trehalose-water

Chen et al. 2000



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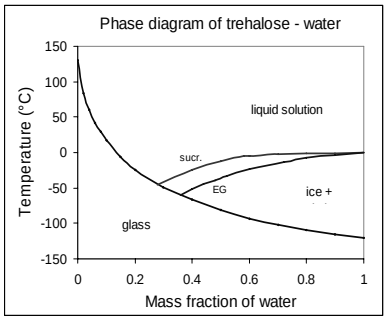
Phase diagram

Phase diagram of trehalose-water



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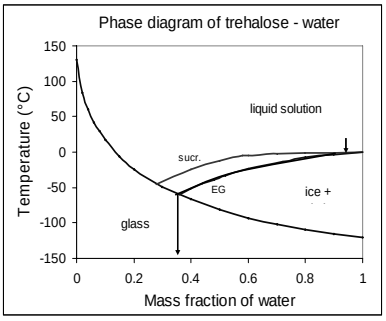
Slow-Freezing



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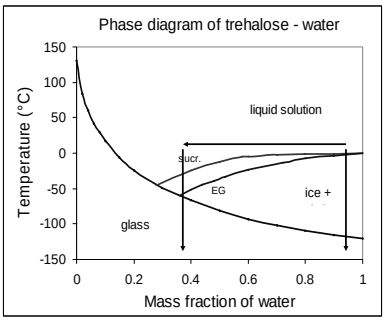
Vitrification



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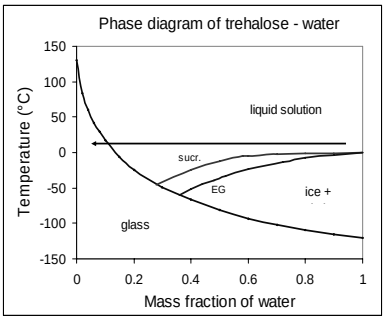
Drying (and/or increase solutes)



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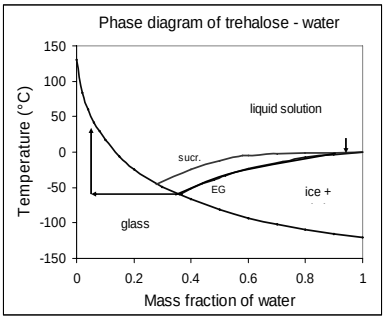
Freeze Drying



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Fundamental Aspects

The common denominator is the phase diagram shown earlier and the fact that any storage requires glass transition

A glass is stable because (by definition) molecules have lost the ability of translation movement.

Still, a glass at very low temperatures is much more stable than a glass at ambient temperatures.

For cold storage you must go to glass transition while preventing IIF

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Cryoprotective Agents (CPAs)

To reach glass transition, water content must be low

Or….Solute concentrations must be very high.

Negative effects of very high salt:
- Salt loading
- Destabilisation of proteins
- Cell shrink excessively

By replacing part of the solutes by permeable solutes this can be alleviated

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CPAs

Examples of such compounds:

■ propane triol	glycerol
■ propane diol	propylene glycol (PG)
■ ethane diol	ethylene glycol
■ butane diol	
■ ethanol	
■ methanol	
■ dimethyl sulfoxide	DMSO

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CPAs

Impermeable CPAs can be used in addition to permeable CPAs
For instance macromolecules that increase the viscosity

- PVP
- PVA
- Ficoll
- Hexa-ethyl Starch (HES)

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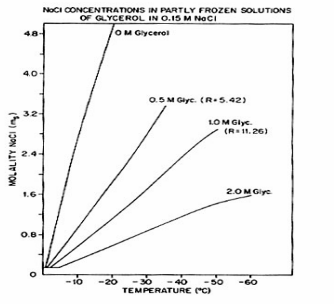
CPAs

Mazur &
Rigopoulos, 1983



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CPAs from nature

How to make the intracellular solute concentration high?

1. We use membrane permeable solutes (CPAs)
2. Nature has another trick:
   When frost sets in or hibernation starts, cold hardy plants and animals produce high intracellular concentrations of sugars, like trehalose

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CPAs from nature

We can mimic this trick by loading cells with trehalose:

e.g. by

- Electroporation
- Injection (e.g. into oocytes, Eroglu et al, 2003)
- Using membrane pores (Staph aureus hemolysin. Genetically modified so they become switchable from open to close by addition or removal or addition of zinc^{2+} ions; This has successfully been applied to freeze fibroblasts

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Osmotic events

- Cells shrink in hyper-osmotic; swell in hypo-osmotic solution
- Cells shrink and swell when CPA is added
- Cells shrink during ice formation (slow freezing method)
- Cells swell and shrink when CPA is removed

All these osmotic events can be predicted, and optimised, when we know:

V_w	Cell water volume
A	Membrane surface area
L_p	Membrane permeability for water
P_s	Membrane permeability for permeant solute (CPA)

and the respective activation energies (E_a) of L_p and P_s

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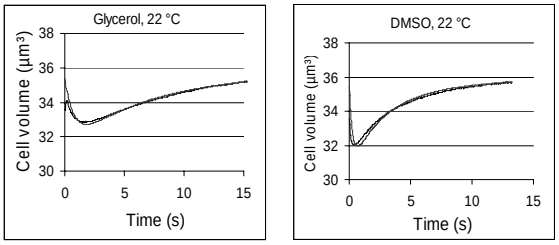
Measuring membrane permeability

Spermatozoa: Stopped flow fluorometry

Volume changes of sperm after adding cryoprotectant



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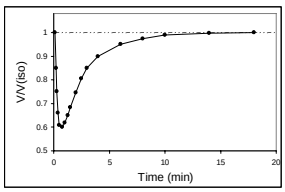
Measuring membrane permeability

Embryo's

- Transfer the embryos in medium with e.g. 10% glycerol.
- Video recording of Shrink-swell cycles
- Image analysis → measure/calculate the volume of the embryo



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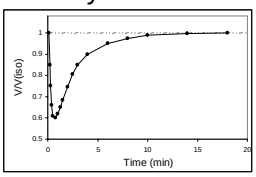
Measuring membrane permeability

- Use equations that describe membrane flux

$$\frac{dV_w}{dt} = L_{p(T)}.A.R.T.\Delta M$$

$$\frac{dV_{CPA}}{dt} = P_{s(T)}.A.\Delta M_i$$



- Fitting of the measured shrink-swell curve with these equations will render values for L_p en P_s
- By doing this at three different temperatures, one can use an 'Arrhenius plot' to calculate the temperature dependence.

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Apply Fundamental cryobiology:

Addition and removal of CPA
In vitrification ànd slow freezing

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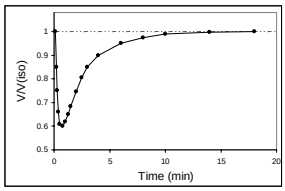
Volume excursion after adding CPA

- Adding CPA leads to shrink-swell cycle
- Magnitude of volume excursion depends on
  - Ratio of Lp and Ps
  - Concentation of CPA
- May harm cells when exceeding osmotic tolerance limits



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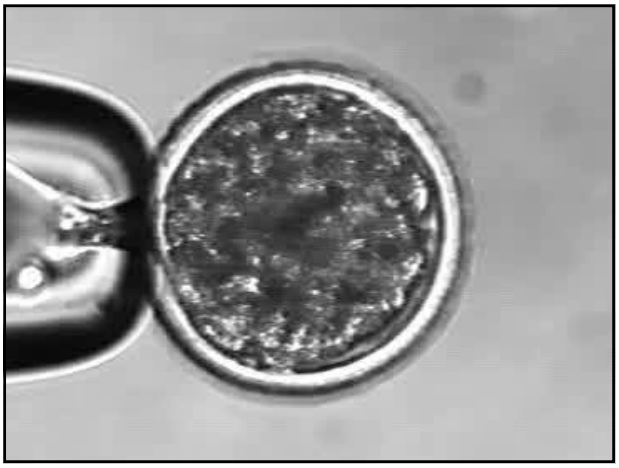
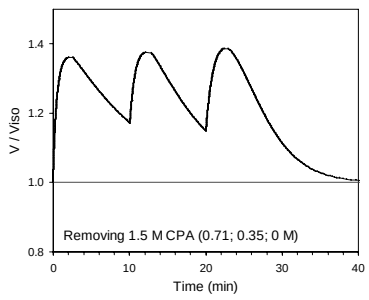


Volume excursions Removal of CPA



Human oocytes:

Lp = 0.55; Ps = 8.00

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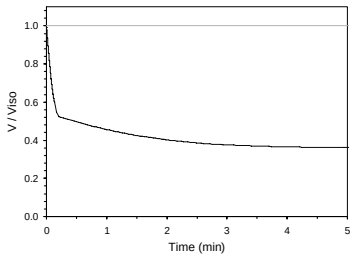
Volume excursions Addition of vitrification solution



Human oocytes:

Lp = 0.55; Ps = 8.00

Oocytes are equilibrated with:

7.5% EG + 7.5% DMSO

Then, placed in:

15% EG + 15% DMSO + 0.5 M Sucrose

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Volume excursions

- Such simulations can show what happens in terms of cell volume but also in terms of intra and extracellular concentrations of solutes.
- For instance it can be shown that the intracellular concentration of EG and DMSO of human oocytes in Vitrification Solution is not reduced by using the non-permeant CPA sucrose.
- What happens to the cells depends on type of cell, type of CPA, concentration of CPA and non-permeant solutes, etc.
- Therefore, these simulations help make decisions on type of CPA and protocol.

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Apply Fundamental cryobiology:

The cooling rate in slow freezing methods

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Slow Freezing: The Cooling Rate

The purpose of slow freezing is:

Increase solute concentration to glass transition while preventing IIF

IIF is prevented by cooling slow enough to allow the cells to dehydrate enough to prevent intracellular supercooling.

The question arises: How slow is “slow-freezing”

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Cooling Rate

At a too high cooling rate

- IIF
- Pore erosion caused by a too rapid water efflux
- Too abrupt (ultra)structural morphological change (very rapid shrinking)

At a too low cooling rate

- Cells shink to a too low volume
- Cytoplasma becomes too high salt
- Unfavourable conditions last longer

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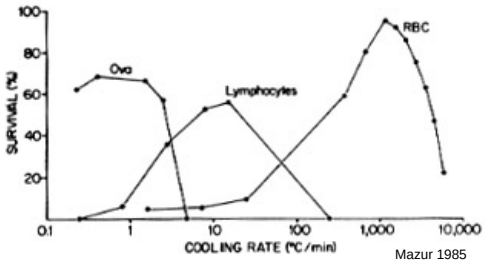
Cooling Rate

Optimal rate somewhere between ‘too slow’ and ‘too fast’



Mazur 1985

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Cooling rate; theoretical modelling

Mechanisms of cryo damage are related to osmotic changes during freezing and thawing, and to the resulting flux of water across the cell membrane

These events can be modelled mathematically

To do this, one must know the values of V_w, A, and L_p en P_s

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Theoretical Model

Mazur 1963	Assumed linear cooling (constant cooling rate)
Liu et al. 2000	Also assumed linear cooling, but included movement of CPA

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Theoretical Model

Theory predicts that a linear freezing programme is not optimal.

Woelders and Chaveiro (2004) developed a model without assuming a linear freezing programme

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New Theoretical Model (Woelders and Chaveiro 2004)

Boundary conditions:

- Cooling rate must always be as high as possible
- But not too high to cause IIF
- Membrane flux of water and CPA must not be too strong
- Transmembrane osmotic pressure difference must remain within limits

These boundary conditions can be expressed mathematically

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New Theoretical Model

$$\frac{dV_w}{dt} = L_{p(T)} ART \frac{p}{1.86}$$

$$\frac{dV_{CPA}}{dt} = P_{s(T)} A \left(\frac{\bar{V}_{CPA}\, c_{CPA(ini)}}{c_{s(ini)} + c_{CPA(ini)}} \times \frac{-\theta}{1.86} - \frac{V_{CPA(T)}}{V_{W(T)}} \right)$$

$$CR_{(T)} = L_p \cdot ART \cdot p \cdot (\theta + p)^2 / (N^4 \cdot 1.86^2)$$

$$L_{p(T)} = e^{\left[\ln\left(L_{p(295)}\right) + \frac{E_a}{R \cdot 295} - \frac{E_a}{RT} \right]} \qquad P_{s(T)} = e^{\left[\ln\left(P_{s(295)}\right) + \frac{E_a}{R \cdot 295} - \frac{E_a}{RT} \right]}$$

c = molar conc.
m = molal conc.
p = permissible supercooling
E_a = activation energy
L_p = hydraulic conductivity
P_s = permeability CPA
θ = Celsius temperature

Woelders et al, 2004

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New Theoretical Model

Due to the chosen boundary conditions, the model predicts the 'optimal' freezing programme, in which:

- The cooling rate is always as high as possible (to prevent so-called "slow cooling damage")
- While conditions that could lead to "fast cooling damage" are precluded.

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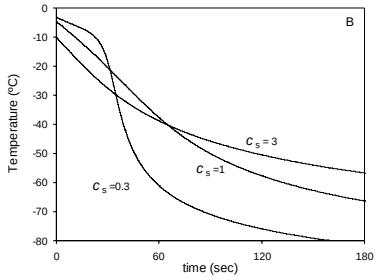
New Theoretical Model

Bovine spermatozoa (Woelders and Chaveiro 2004)



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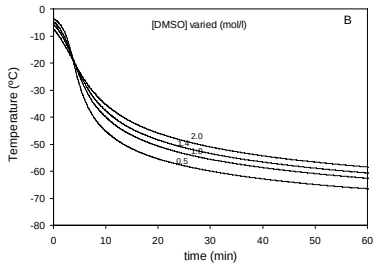
New Theoretical Model

Human blood stem cells (Woelders & Lagenberg unpublished)



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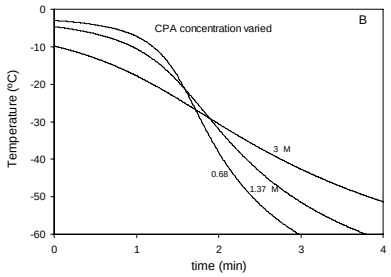
New Theoretical Model

Bovine morulae (preliminary estimates of L_p, P_s en E_a)



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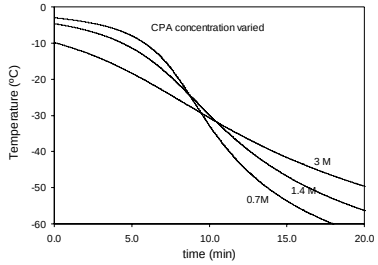
New Theoretical Model

Human oocytes (Woelders, unpublished)



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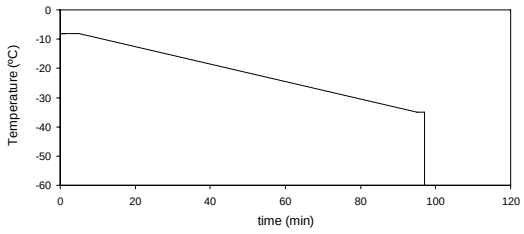
Present slow freeze method

Embryos, oocytes



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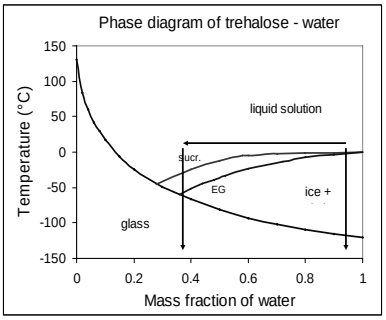
Vitrification



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Vitrification

No importance of Cooling Rate?

- Very high cooling and thawing rate enable vitrification with lower CPA concentrations
- Ultrarapid cooling to outrun:
  - Spindle depolymerization (but see Stachecki et al 2004; Rienzi et al 2005)
  - Lipid lateral phase separation
  - Other hypothermia induced changes

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Vitrification

Very high cooling and thawing rate by techniques like

- Minimal volume (cryoloop o.p.s., cryotop etc.
- Solid surface freezing (ops, cryotop)
- N_2 at freezing point versus at boiling point (Vitmaster)

Applications, e.g.

- Semen vitrified without any CPA (Isachenko et al 2003)
- Oocytes vitrified with relatively low CPA concentration (e.g. Lucena et al. 2006; Antinori et al. 2007)

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Vitrification human oocytes

Year	1st author	Method	Clinical/Experim.
2004	Fuchinoue	GV, Taxol, Vitrification	Exp.
2005	Kuwayama	Vitrification Cryotop	Babies
2005	Kyono	Vitrification	Baby
2006	Lucena	Vitrification Cryotop	Pregnancies
2006	Isachenko	Aseptical Vitrification	Exp.
2006	Chen GA	Vitrification	Baby
2006	Chen ZJ	Vitrification Cryoloop	Babies
2006	Selman	Vitrification	Pregnancies
2007	Kuwayama	Vitrification Cryotop	Review
2007	Antinori	Vitrification Cryotop	Babies

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Conclusions

Understanding of fundamental aspects of freezing and thawing enables us:

- To explore new ideas
- To optimise existing (slow-cooling) methods
- To optimise combinations of CPA concentration-cooling curve such that optimal results may be obtained with the lowest possible CPA concentration

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Basic principles of cryopreservation and vitrification

Etienne Van den Abbeel
Prague 2007

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Vrije Universiteit Brussel
Centrum voor Reproductieve Geneeskunde

Outline of the presentation

- **Introduction**
- **Basic principles of freezing**
- **Conclusions**
- **Basic principles of vitrification**
- **Conclusions**
- **Prospects for the future**

Introduction
Importance of human oocyte cryopreservation

- Oocyte donation programmes
- Malignancy
- Social
- Ethical

→ **Efficient oocyte cryopreservation procedures**

Introduction
Importance of human embryo cryopreservation

- Increase the efficiency of ART

↓

Efficient embryo cryopreservation procedures

↑

- Tool to reduce multiple pregnancies

Human oocyte and embryo cryopreservation

Data from international and national surveys

↓

Not all oocytes and embryos survive the cryopreservation with (all) cells intact

↓

Implantation potential of cryopreserved oocytes and embryos is lower than for fresh embryo's

↓

Improve cryopreservation procedures

Introduction

Cryopreservation of reproductive cells

↓

Stopping biological time

↓

-196°C

↓

Lethal intra-cellular ice formation

↓

Fate of cellular water

↙ ↘

Equilibrium (quasi-equilibrium) cooling Non-equilibrium cooling

Introduction

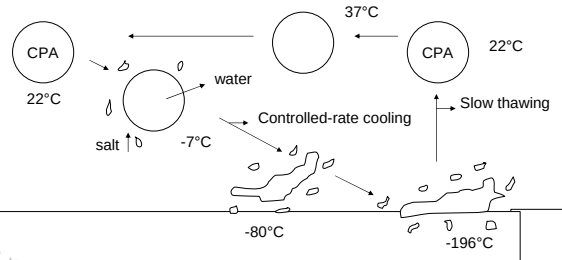
Basic definitions

Cryobiology: is the branch of biology that studies life at below-normal temperatures

Cryopreservation: is the process of preserving or storing living systems in a viable condition at low temperatures for future use by freezing or by vitrification (= ice-free cryopreservation)

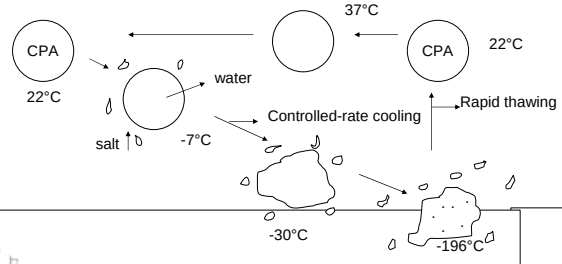
Basic principles of freezing

Equilibrium cooling (controlled-rate cooling)



Basic principles of freezing

Quasi-equilibrium cooling (interrupted controlled-rate cooling)



Basic principles of freezing

Principle variables of freezing

→ The effect of cooling rates

→ Likelihood of intracellular ice formation

→ Cell volume excursions during cooling

→ Osmotic responses to CPA solutions

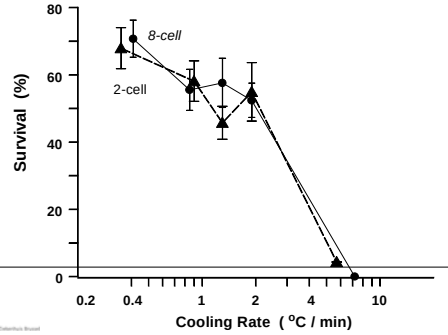
→ Chilling injury

Basic principles of freezing

Principle variables of freezing

→ The effect of cooling rates

Survival of Cryopreserved Mouse Embryos



data of Whittingham et al. 1972 Science 178:411

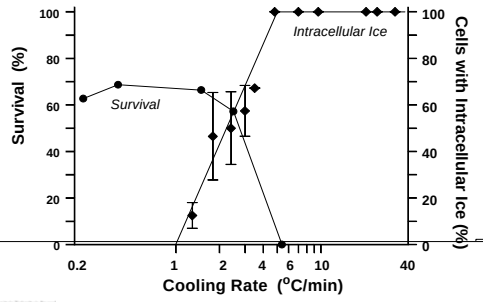
Basic principles of freezing

Principle variables of freezing

→ Likelihood of intracellular ice formation

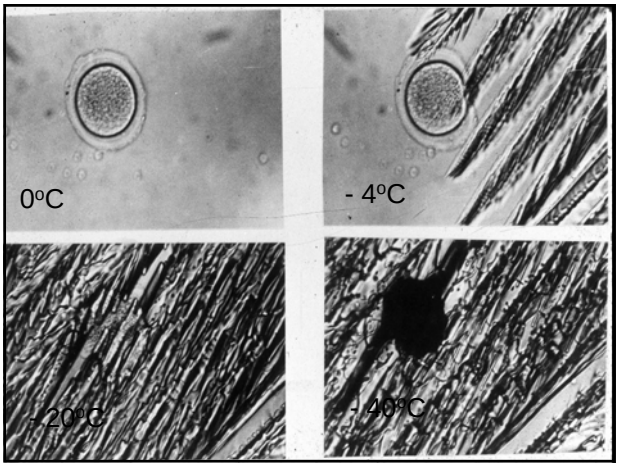
13 titel 2-4-2007

Intracellular Ice Formation in Mouse Oocytes



data of Leibo et al. 1978 Cryobiology 15:257

14 titel 2-4-2007



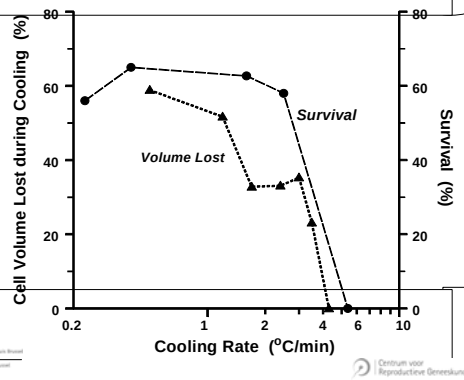
Basic principles of freezing

Principle variables of freezing

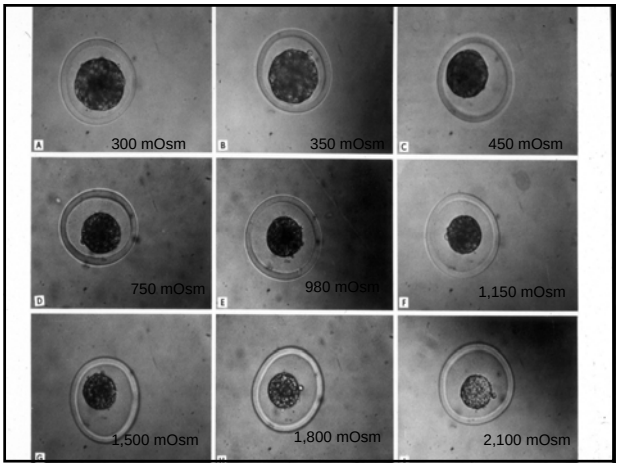
→ Cell volume excursions during cooling

16 titel 2-4-2007

Cell Volume and Survival of Mouse Oocytes



17 titel 2-4-2007



Basic principles of freezing

Principle variables of freezing

→ Osmotic responses in CPA solutions

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Cryoprotective Additives

- Methanol 32
- Ethylene Glycol 62
- Propylene Glycol 76
- Dimethyl Sulfoxide 78
- Glycerol 92

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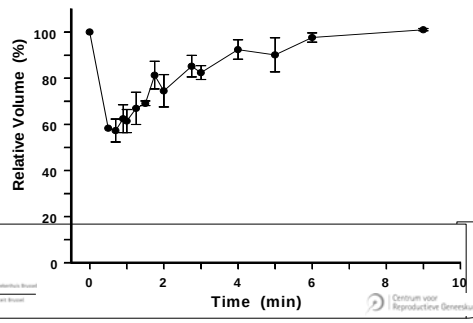
Mouse Ooocytes in 1.5 M Ethylene Glycol



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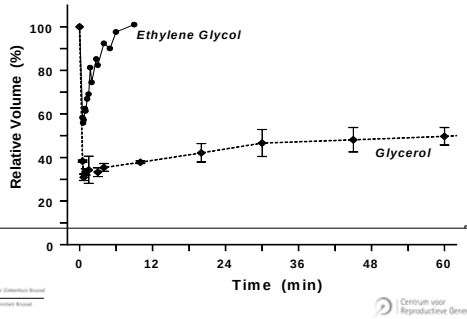
Mouse Ooocytes in 1.5 M CPA Solutions



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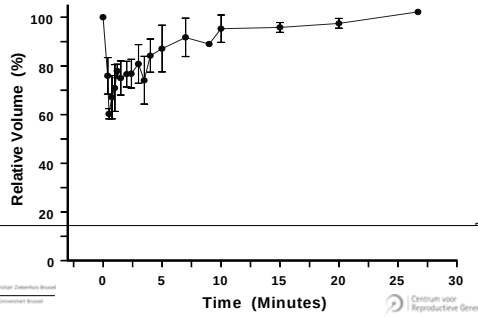
Human Oocytes in 1.5 M Ethylene Glycol



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Basic principles of freezing

Principle variables of freezing

→ Chilling injury

Damage between 30°C and -7°C without freezing

(cytoskeletal elements, membranes, lipids)

24 titel 2-4-2007

Conclusions on freezing

- → When cells cooled slowly, their survival depends on cooling rate and/or warming rate.
- → Various chemicals may act as cryoprotectants (CPAs).
- → Cells may be killed by cooling to ~0°C.
- → Cells may survive freezing but be killed by osmotic shock.
- → Expensive equipment required.
- → Long procedures.

Rationale of vitrification

- → Slow cooling allows time for cell dehydration at subzero temperatures
- → Vitrification:
  - Dehydrate cell before cooling
- → Vitrification:
  - Cool rapidly to “outrace” chilling injury

Basic principles of vitrification

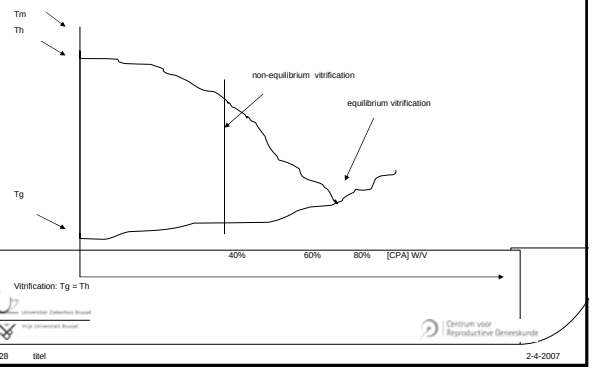
Definition of vitrification:

Solidification of a solution without ice chrystal formation

(Nucleation temperature (Th) = glass formation temperature (Tg))

B Luyet (1948; 1949)

Basic principles of vitrification



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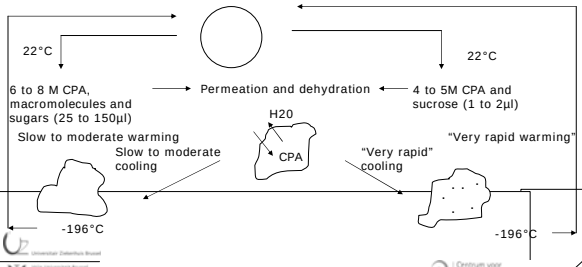
Basic principles of vitrification

Non-equilibrium cooling (cooling by direct plunging into LN2)

Equilibrium vitrification **Non-equilibrium vitrification**



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Basic principles of vitrification

Probability of vitrification

↓

Cooling and warming rates x viscosity (CPA)/Volume solution

(Yavin and Arav, 2007)

30 titel 2-4-2007

Basic principles of vitrification

Succesfull vitrification of human oocytes, embryos and blastocysts depends on a correct interplay between a “sufficient” high cooling rate, “sufficient” extracellular CPA, “sufficient” permeation or concentration of a penetrating cryoprotectant, “sufficient” dehydration by a non-penetrating cryoprotectant, and a “sufficient” high warming rate

Basic principles of vitrification

Principle variables of vitrification

→ The effect of cooling and warming rates

→ Permeability of cells to water and CPA

→ CPA toxicity

→ Osmotic responses in CPA solutions

Basic principles of vitrification

Principle variables of vitrification

→ The effect of cooling and warming rates

- Concentration of CPA low or near zero: cooling and warming rates >1.000.000°C/min
  - Technically a challenge

Basic principles of vitrification

Principle variables of vitrification

→ The effect of cooling and warming rates

- Concentration of CPA very high: low cooling and warming rates (20 to 2000°C/min)
  - CPAs toxic
  - Volumes of vitrification solutions 25 to 150 µl
  - Safe storage
  - Closed systems

(Fahy et al, 1984; Rall et al, 1985)

Basic principles of vitrification

Principle variables of vitrification

→ The effect of cooling and warming rates

- Concentration of CPA moderate: cooling and warming rates from 15.000 to 30.000°C/min
  - Small volumes used (1 to 2 µl): safety?
  - Direct contact between cells (milieu) and LN2: safety?

(Vajta et al, 1996, 1997)

- Special devices used for vitrification (EM grids, cryo top, hemi straw, cryoloop, OPS, SSV, cryo leaf….)

Basic principles of vitrification

Principle variables of vitrification

→ **Permeability of cells to water and CPA**

- **Glyc<EG<DMSO<PG**
- Variability amongst oocytes and embryos
- Oocytes<zygotes<embryos<blastocysts

Basic principles of vitrification

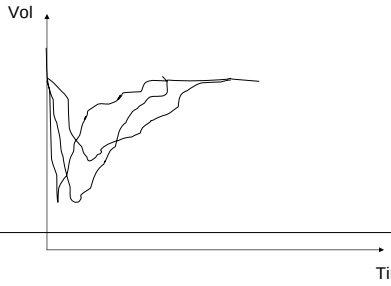
Principle variables of vitrification

→ **Permeability of cells to water and CPA**

- Glyc<EG<DMSO<PG
- **Variability amongst oocytes and embryos**
- Oocytes<zygotes<embryos<blastocysts

Basic principles of vitrification

Osmotic response in 1.5M CPA



Basic principles of vitrification

Principle variables of vitrification

→ **Permeability of cells to water and CPA**

- Glyc<EG<DMSO<PG
- Variability amongst oocytes and embryos
- **Oocytes<zygotes<embryos<blastocysts**

Basic principles of vitrification

Principle variables of vitrification

→ CPA toxicity
- Type and concentration of CPA
  - PG, EG, DMSO, Glyc ….
- Temperature of exposure

Basic principles of vitrification

Principle variables of vitrification

→ Osmotic responses to CPA solutions
- Osmotic tolerance limits of cells to be vitrified
  - Stepwise addition or one-step addition of CPAs?

Benefits of vitrification as compared to freezing

→ Very simple procedure?
→ Reduces the time of the cryopreservation procedure?
→ No ice crystallization
→ Eliminates the costs of expensive programmable freezing equipment?
→ Flexibility

Variables of vitrification that can profoundly influence its effectiveness

- → Technical proficiency of the embryologist
- → The device that is used for vitrification
- → Direct contact of the LN2 and the vitrification solution can be a source of cross contamination (EU?)
- → Concentration and type of CPA and the temperature of exposure
- → Risk of crystallization during storage or warming

Applications of vitrification

Vitrification may offer more options than slow cooling for:

Oocytes

Embryos

Blastocysts

Conclusions

Recent published data on the “vitrification” of human oocytes, embryos and blastocysts indicate that vitrification “apparently” works and produces even somewhat “better” results than conventional freezing

Conclusions

→ **Vitrification to improve survival and viability of human oocytes, embryos and blastocysts**

- How?
- Safety?
- Efficiency?

Prospects for the future

→ Equilibrium vitrification in low toxicity solutions

- Injected CPAs (Trehalose or other CPAs)

→ Alteration of oocyte, embryo or blastocysts

- Stabilization of cytoskeletal elements
- Alteration of membrane permeability (aquaporins)
- Artificial shrinking of advanced blastocysts

Human embryo cryopreservation : A review of clinical issues related to success rate

D Royère, Médecine et Biologie de la Reproduction, CHU Bretonneau, UMR6175 Inra / Cnrs / Haras / Université de Tours

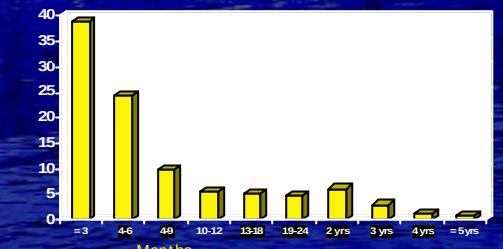
FET : clinical issues related to success rate

- **Factors involved in FET issue**
  - **FET cycle itself**
  - **OPU cycle source of frozen embryo(s)**
  - **Patients characteristics**
- Factors involved in pregnancies issued from FET
  - Comparing fresh and frozen embryo transfers
  - Analysing FET issues as a function of development stage
  - Related to OPU cycle

Factors involved in FET issue

- Report on French Data Base Fivnat / FET 2001-2002
- 14 247 registered FET
  - FET cycle analysis
- 9286 FET related to OPU
  - Patient age
  - Infertility factor
  - Ovulation treatment
  - ART procedure

Time lag between OPU and FET



Mean interval : 9,5 ± 12,8 months; 63 % 6 months, 79 % year following OPU

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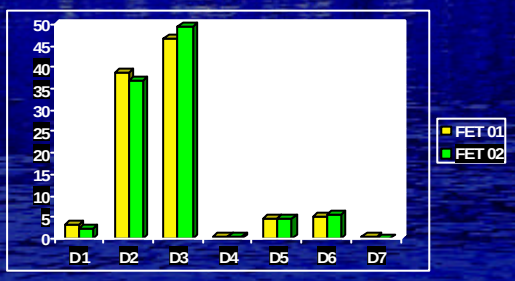
Day of freezing / FET



Total Day2 - Day3 : 85.9 %
Total Day5 - Day7 : 10,6 %

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FET cycle

	2001	2002	Total
Natural cycle	10,3	10,7	10,5
Stimulated cycle	**59,5**	**55,9**	**57,9**
Hormonal Replacement cycle + GnRHa	3,6	3,8	3,7
Hormonal replacement cycle – GnRHa	26,4	28,2	26,2
Other / Unprecised	0,0	1,3	0,6

P<0,001

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FET cycle : ovulation treatment

	2001	2002	Total	
CC	5,1	3,0	4,2	
CC-HMG	2,4	1,1	1,9	P<0,001
HMG	7,2	4,2	5,9	
FSH only	**81,4**	**90,8**	**85,2**	
FSH + CC ou HMG	0,8	0,8	0,8	

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FET cycle : Ovulation triggering (hCG) depending on the cycle type

	2001	2002	Total	
Natural cycle	50,0	43,2	47,1	
Stimulated cycle	**96,9**	**93,1**	**95,2**	
Hormonal replacement cycle + GnRHa	8,2	4,0	6,2	P<0,001
Hormonal replacement cycle – GnRHa	0,6	1,1	0,8	

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FET : Results (1)

	2001	2002	Total	
Transfers (% / thawing)	88,4	86,7	87,6	NS
Clin Pregnancy Rate CPR (/ thawing)	14,1	15,4	14,7	P<0,05
CPR (/ transfer)	16,0	17,8	16,8	P<0,01
Thawed embryos : mean (SD)	2,6 (1,4)	2,6 (1,6)	2,6 (1,5)	NS
Transferred embryos : mean (SD)	2,02 (0,75)	1,97 (0,75)	2,00 (0,75)	P<0,01

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FET : Results (2)

	2001	2002	Total	
Thawed embryos : mean (SD)	2,6 (1,4)	2,6 (1,6)	2,6 (1,5)	NS
Tranferred embryos / transfer : mean (SD)	2,02 (0,75)	1,97 (0,75)	2,00 (0,75)	P<0,01
Transferred embryos / thawing : mean (SD)	1,79 (0,95)	1,71 (0,97)	1,75 (0,96)	P<0,01
% thawed embryo used : mean (SD)	74,0 (34,9)	71,8 (35,9)	73,0 (35,2)	P<0,01

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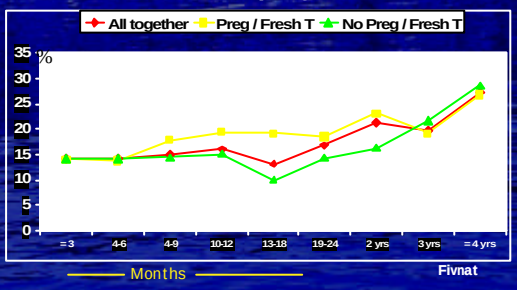
CPR as a function of time lag between OPU and FET and prognostic of OPU



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P<0,001

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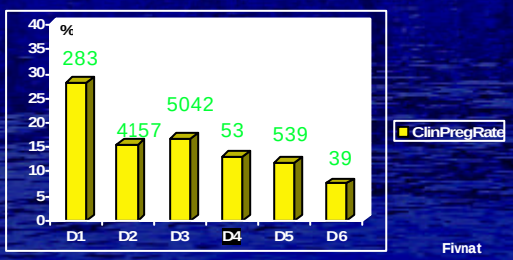
Clinical Pregnancy rate depending of the day of freezing



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p<0,001

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Clinical pregnancy rate as a function of FET cycle treatment

	N	% Cycles	CP Rate	
Natural cycle	1112	10,5	16,4	
Stimulated cycle	**6523**	**57,9**	**15,5**	
Hormonal replacement cycle + GnRHa	431	3,7	15,8	
Hormonal replacement cycle - GnRHa	3226	26,2	**12,3**	P<0,001
Other / NA	69	0,6	20,3	

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Clinical pregnancy Rate as a function of ovarian stimulation

	N	% Cycles	% CPR
CC	249	4,2	15,5
CC-HMG	113	1,9	14,2
HMG	379	5,9	12,7
FSH only	**5261**	**85,2**	**16,0**

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NS

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Influence of embryo recovery on implantation

	Implantation	Failure	
Thawed embryos : mean (SD)	2,7 (1,4)	2,6 (1,5)	P<0,01
Transferred Embryons	2,12 (0,70)	1,99 (0,77)	P<0,01
% thawed embryos used mean (SD)	84,2 (23,0)	70,4 (37,3)	P<0,01

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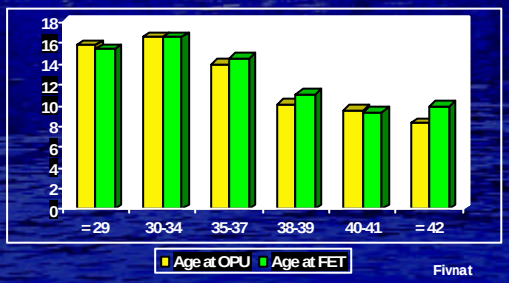
Clinical pregnancy rate as a function of the number of embryo transferred

	2001	2002	% Transfer	CPR
1	23,3	26,6	24,8	11,4
2	**53,9**	**51,6**	**52,9**	**18,4**
3	20,3	20,1	20,2	19,2
= 4	2,4	1,7	2,1	18,6

P<0,001 P<0,001

Fivnat

Clinical pregnancy rate as a function of age at OPU and age at FET



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The influence of age at OPU is more relevant than age at FET

Clinical pregnancy rate as a function of age at OPU and age at FET

Age at FET	Age at OPU					
	= 29	30-34	35-37	38-39	40-41	= 42
= 29	15,4					
30-34	18,8	16,4				
35-37		19,4	13,9			
38-39		16,7	16,7	9,9		
40-41				13,3	8,7	
= 42					20,7	8,3

Age at FET : NS (p=0,22) **Age at OPU : p<0,02**

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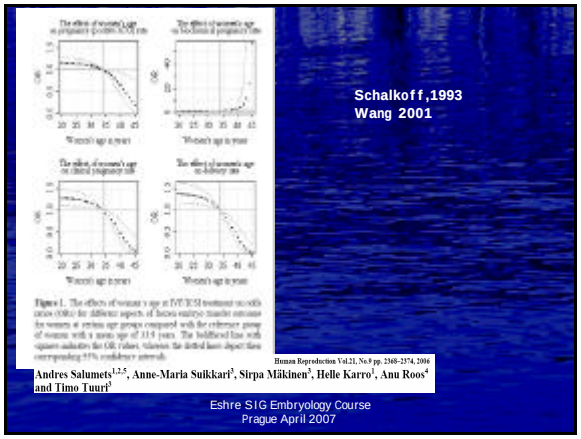

Schalkoff,1993
Wang 2001

Human Reproduction Vol.21, No.9 pp. 2368–2374, 2006

Andres Salumets[1,2,5], Anne-Maria Suikkari[3], Sirpa Mäkinen[3], Helle Karro[1], Anu Roos[4] and Timo Tuuri[3]

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TABLE 2. Risk of subsequent pregnancy loss, relative risks, and 95% confidence intervals by oocyte source, embryo type, and woman's age among 148,494 pregnancies conceived through assisted reproductive technology, United States, 1999–2002

Analytical group	No. of pregnancy losses	Total no.	Total risk of pregnancy loss*	95% confidence interval	Probability (%) of subsequent pregnancy loss at specific gestational weeks†								Relative risk‡	95% confidence interval
					6	8	10	12	16	20	24	32	40		
Total	41,878	148,494	28.6	28.4, 28.8	14.4	9.3	5.1	3.4	2.2	1.2	0.54	0.45	0.16	Not applicable	
Patient eggs															
Fresh embryos by maternal age (years)															
<33	8,967	40,938	22.3	21.8, 22.7	10.2	6.9	4.2	3.1	2.2	1.2	0.51	0.41	0.12	Referent	
33–34	4,455	18,770	24.1	23.4, 24.7	11.6	7.8	4.5	3.3	2.3	1.2	0.46	0.35	0.22	1.06	1.05, 1.12
35–37	7,027	26,309	27.1	26.5, 27.7	13.8	9.3	5.2	3.5	2.3	1.3	0.55	0.45	0.16	1.22	1.19, 1.25
38–40	6,631	18,341	36.6	35.9, 37.3	21.5	14.1	7.1	4.4	2.5	1.3	0.64	0.53	0.12	1.64	1.61, 1.68
41–42	2,767	5,468	51.2	49.8, 52.6	34.0	23.1	11.7	6.7	3.6	1.8	0.80	0.73	0.46	2.30	2.24, 2.36
>42	995	1,581	63.3	60.9, 65.7	44.7	30.8	13.5	5.3	2.0	1.1	0.23	0.23	—§	2.85	2.73, 2.97
Thawed embryos by maternal age (years)															
<33	2,080	6,388	32.8	31.7, 34.0	13.2	7.4	3.7	2.4	1.5	0.72	0.18	0.13	—	1.47	1.42, 1.54
33–34	1,017	3,072	33.3	31.7, 35.0	13.9	7.2	3.8	2.5	1.4	0.78	0.23	0.23	—	1.50	1.42, 1.58
35–37	1,413	3,934	36.4	34.8, 37.9	17.1	10.1	5.4	3.5	2.1	1.1	0.64	0.64	—	1.63	1.56, 1.71
38–40	979	2,356	42.3	40.1, 44.5	23.3	14.4	7.5	4.3	2.7	1.6	1.2	1.2	0.81	1.90	1.80, 2.01
41–42	284	668	43.4	39.7, 47.3	22.8	11.4	4.2	1.9	1.1	0.27	—	—	—	1.95	1.79, 2.13
>42	177	350	50.9	45.7, 56.3	31.5	21.1	12.1	6.2	4.1	1.8	0.61	—	—	2.29	2.06, 2.54
Donor eggs															
Fresh embryos	3,775	16,304	23.6	22.9, 24.3	12.0	7.3	4.2	2.9	1.9	1.1	0.60	0.59	0.22	1.06	1.02, 1.10
Thawed embryos	1,311	4,025	32.9	31.5, 34.4	14.6	8.8	4.1	2.6	1.6	0.79	0.38	0.30	—	1.48	1.41, 1.55

Pregnancy Loss among Pregnancies Conceived through Assisted Reproductive Technology, United States, 1999-2002

Sherry L. Farr[1], Laura A. Schieve[1], and Denise J. Jamieson[1]

American Journal of Epidemiology Advance Access published March 19, 2007

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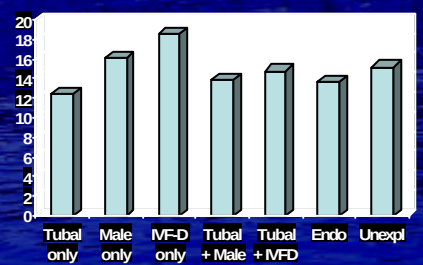
Clinical pregnancy rate as a function of infertility factors



P=0,04

Fivnat

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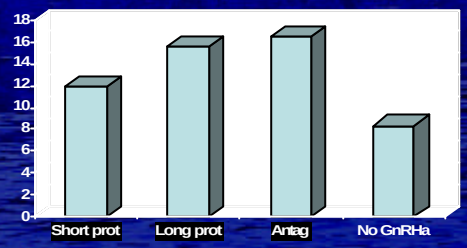
Clinical pregnancy rate as a function of ovarian stimulation for OPU



P < 0,01

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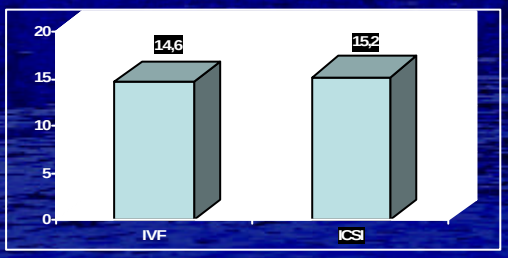
Clinical pregnancy rate as a function of ART procedure



NS

Fivnat

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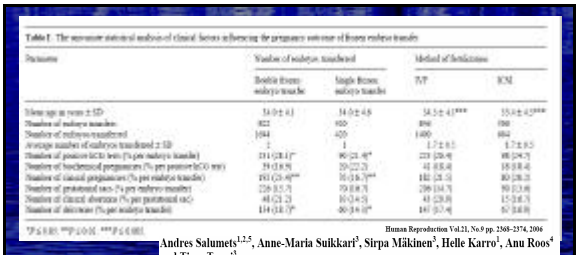

Human Reproduction Vol.21, No.9 pp. 2368–2374, 2006

Andres Salumets[1,2,5], Anne-Maria Suikkari[3], Sirpa Mäkinen[3], Helle Karro[1], Anu Roos[4] and Timo Tuuri[3]

Van Steirteghem, 1994
Kowalik, 1998
Mandelbaum, 1998
Simon, 1998

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Table 2. Parameters examined in relation to the implantation rate. Individual parameters are either expressed in all embryos transferred (expressed) or not expressed in any of the embryos transferred (non-expressed). The relative risk describes the ratio of the expressed to the non-expressed parameter. The *P*-value tests the difference in implantation rates for the expressed and non-expressed parameters. NS = not statistically significant; CI = confidence interval.

Parameters	*Implantation rate (95% CI)/n*				*Relative risk*	*P-value*
	Expressed	n	*Non-expressed*	n	*(95% CI)*	
Four or more cells at freezing	0.14 (0.10, 0.18)	383	0.06 (0.03, 0.11)	187	2.3 (1.2, 4.6)	0.017
Resumption of mitosis	0.14 (0.11, 0.18)	381	0.06 (0.02, 0.20)	85	2.4 (0.7, 8.3)	NS
Survival of all blastomeres	0.12 (0.09, 0.16)	498	0.05 (0.02, 0.15)	61	2.5 (0.8, 7.6)	NS
Resumption of mitosis including more than 2 cells	0.17 (0.12, 0.24)	188	0.07 (0.04, 0.12)	259	2.4 (1.3, 4.6)	0.005
Six or more cells at transfer	0.20 (0.13, 0.31)	105	0.06 (0.04, 0.10)	406	3.2 (1.7, 6.1)	<0.0001
Child in previous fresh cycle	0.16 (0.11, 0.23)	216	0.08 (0.06, 0.11)	603	1.9 (1.2, 3.0)	0.007
IVF/ICSI	0.12 (0.08, 0.16)	519	0.09 (0.07, 0.13)	300	1.2 (0.8, 2.0)	NS
Assisted hatching	0.15 (0.11, 0.19)	351	0.07 (0.05, 0.10)	468	2.1 (1.3, 3.3)	0.002
Age less than 36 years	0.12 (0.09, 0.15)	559	0.07 (0.04, 0.12)	260	1.6 (0.9, 2.8)	NS

A Gabrielsen[1], J Fedder[2], L Agerholm[2]

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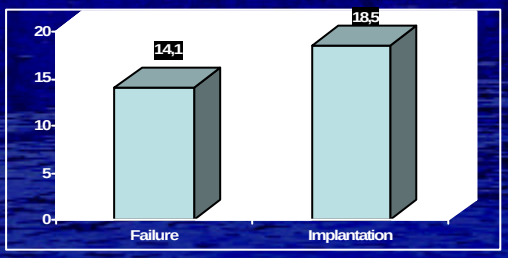
Clinical pregnancy rate as a function of the fresh embryo transfer issue



P<0,001

Fivnat

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A Gabrielsen[1], J Fedder[2], L Agerholm[2]

Wang, 2001
Osmanagaoglu, 2004

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FET : clinical issues related to success rate

- Factors involved in FET issue
  - FET cycle itself
  - OPU cycle source of frozen embryo(s)
  - Patients characteristics
- **Factors involved in pregnancies issued from FET**
  - **Comparing fresh and frozen embryo transfers**
  - **Analysing FET issues as a function of development stage**
  - **Analysing FET issues as a function of OPU cycle**

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Material & Methods

- Files FET pregnancies/ period 1996-2002: 4138
- Included (discarding biochemical ones & non available issues) : 3632
- Parameters assessed: % Spontaneous Abortion, Ectopic Pregnancy, Medical termination, Multiple Pregnancy, Prematurity, Hypotrophy, Perinatal death, Malformations, Sex ratio
- The number of fœtus was taken in account

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Pregnancy issues following Frozen Embryo Transfer (FroET) as compared to Fresh Embryo Transfer (FreET)

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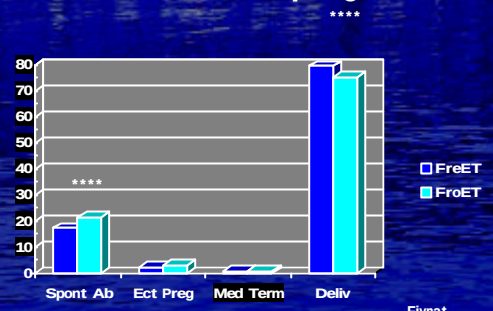
Issues of clinical pregnancies

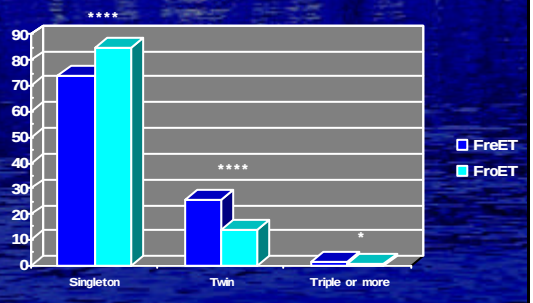


SA : 21.6 vs. 17.3 %, p < O.0001

Issues of clinical pregnancies

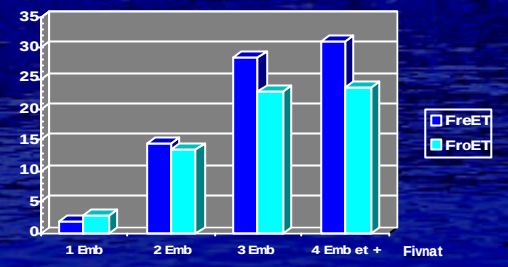
- Increase in pregnancy wastage Aytoz, 1999
- Clinical abortion rate 20-25%,
  - Kowalik, 1998
  - Aytoz, 1999
  - Van den Abbeel, 2000
  - Salumets, 2003

Multiple Pregnancy Rate (after reduction)



MPR 14.8 vs. 26.7 %, p < 0.0001

Multiple Pregnancy Rate as a function of the number of embryos transferred



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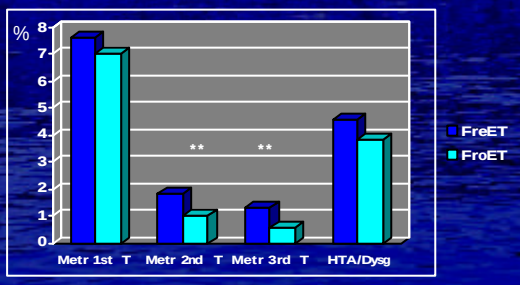
Morbidity during pregnancy



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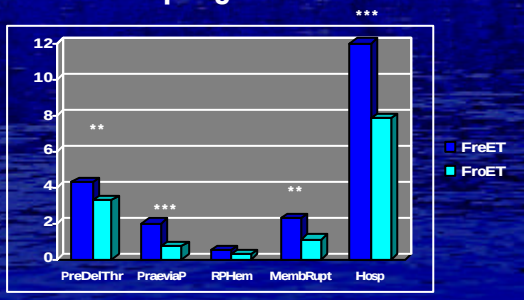
Pathologies during singleton pregnancies



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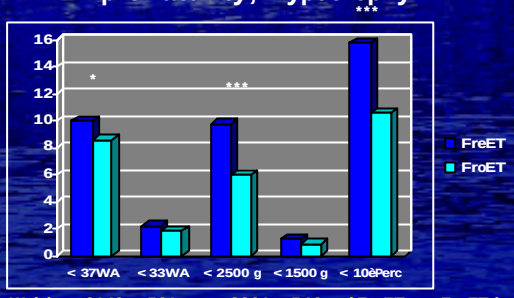
Characteristics of singleton neonates : prematurity, hypotrophy



Weight : 3148 ± 561 g vs. 3291 ± 546 g (FreET vs. FroET)

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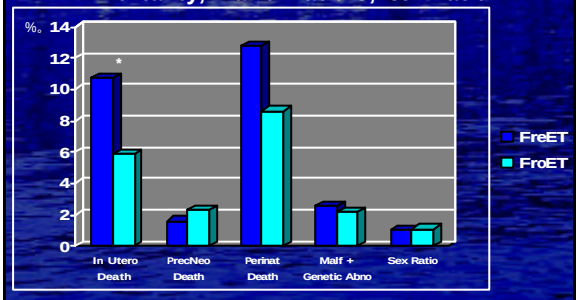
Characteristics of singleton neonates : mortality, malformations, sex ratio



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Issues of pregnancies after FET depending on the development stage (embryo = FET vs. Blastocyst = FBT)

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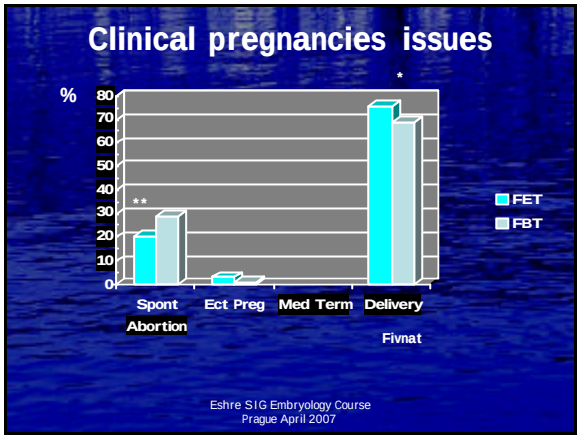
Clinical pregnancies issues
%
80
70
60
50
40
30
20
10
0
*
**
FET
FBT
Spont Abortion
Ect Preg
Med Term
Delivery
Fivnat
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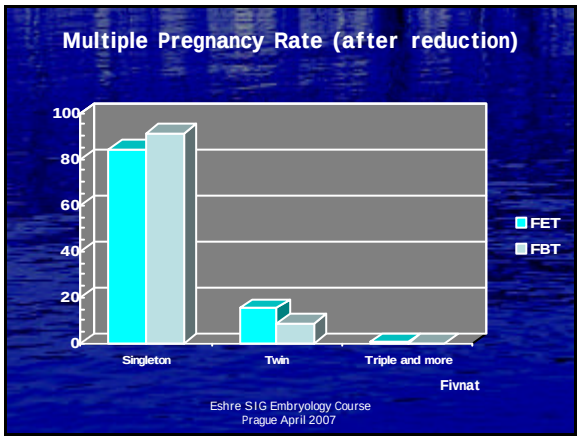
Multiple Pregnancy Rate (after reduction)
100
80
60
40
20
0
FET
FBT
Singleton
Twin
Triple and more
Fivnat
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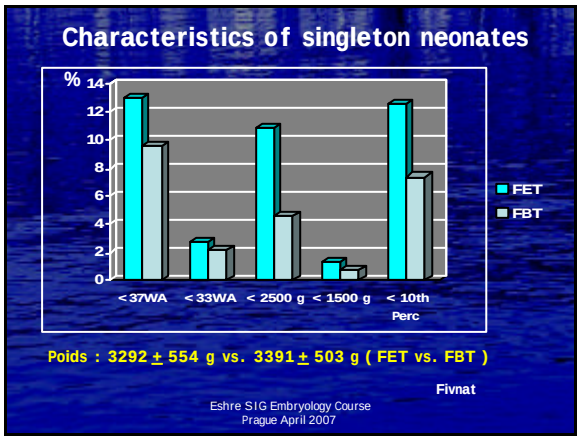
Characteristics of singleton neonates
%
14
12
10
8
6
4
2
0
FET
FBT
< 37WA
< 33WA
< 2500 g
< 1500 g
< 10th Perc
Poids : 3292 ± 554 g vs. 3391 ± 503 g (FET vs. FBT)
Fivnat
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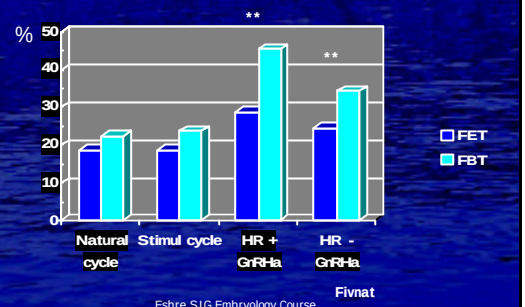
Rate of spontaneous abortions as a function of stimulation and development stage



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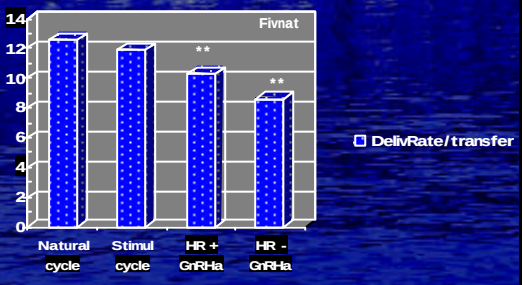
Delivery rate as a function of cycle treatment



Such difference was not explained by the ovulatory status of the patients

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Spontaneous abortion rate as a function of embryo survival after thawing

- At least one good survival embryo
  (100% intact blastomeres)
  OR = 0,701 [0,504 – 0,974]
  Van den Abbeel, 1997
  SurvBlast EPL 17.3% vs. 42.9% Partial
- Blastocyst vs. embryo
  OR = 1,352 [0,945 – 1,933]

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Pregnancy issues after FET related to OPU

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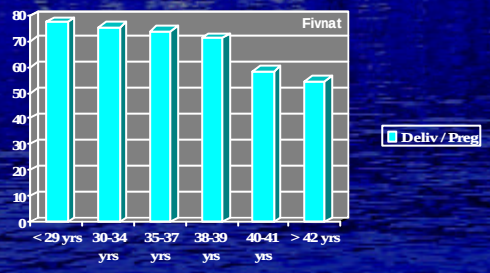
Influence of age at OPU



Age at OPU and Spontaneous Abortion Rate :
OR = 1,15 [1,03 – 1,29]

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Which OPU parameters were related to issue of pregnancies following FET ?

- No influence of
  - ART procedure (IVF or ICSI)
  - The number of frozen embryos
  - Pregnancy following OPU
  - Sperm concentration

on Spontaneous Abortion rate as well as Multiple Pregnancy Rate

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Conclusions (1)

- General evolution of pregnancies following FET remain similar to pregnancies issued from OPU
- Multiple Pregnancy Rate however is lower after FET
- Patient age at OPU is the major prognostic factor for FET pregnancy issue.

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Conclusions (2)

- The less favourable prognostic of pregnancies issued from FET cycles using Hormonal replacement could not be related to patient ovulatory status.
- The lower frequency of various complications in singleton pregnancies, as compared to fresh transfer's (Precocious Delivery Threatening, Praevia Placenta, membrane Rupture, Prematurity, Hypotrophy, In Utero Death) was not explained by patient age but might refer rather to their fertility status when embryos may be frozen.

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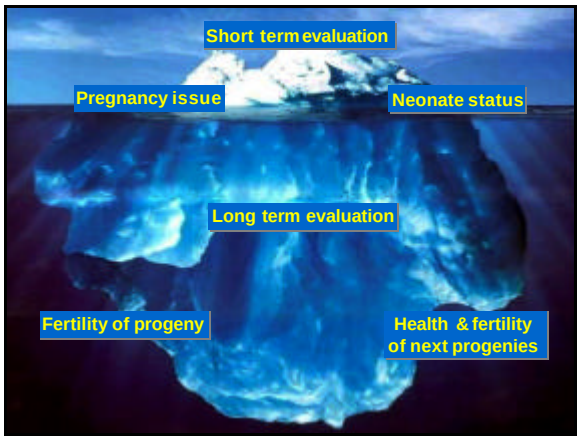


Thanks
for your
attention !
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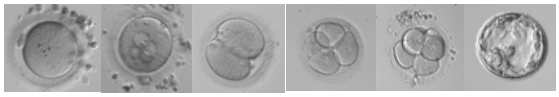
Embryo characteristics influencing outcome of human embryo cryopreservation

Kersti Lundin
Reproductive Medicine
Sahlgrenska University Hospital
Göteborg, Sweden

Overview

- Embryo quality
- Embryo scoring variables vs. embryo freezing variables
- Number of cells
- Morphology
- Early cleavage
- ICSI / IVF
- Media



What is "embryo quality"?

How do we define "embryo quality"?

What are we measuring?

Development variables
Implantation potential
Live birth

⇕

Nuclear status / cytoplasmic status / metabolic status / environment / chromosomal status

Variables for embryo selection

- Oocyte assessments
- Zygote scoring
- Cleavage rates
- Morphology (fragmentation, cell size)
- Number of nuclei
- Metabolic / genetic status?

Embryo selection criteria

- 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

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

Implantation vs. number of 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

Edgar et al 2000

Survival and cleavage rate vs. implantation rates (n=654 cycles)

- 100% blastomere survival ⟹ 22% IR
- 50-75% blastomere survival ⟹ 7% IR
- Cleaved embryos ⟹ 20% IR
- Uncleaved ⟹ 3% IR
- Intact + Cleaved embryos ⟹ 27% IR

Guerif et al 2002

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)

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

SU: Frozen-thawed transfer - Predictive factors for live birth

- 622 <u>single</u> 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)

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= 0.15
100% v.s <100% and
75/100 vs. < 75

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

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

ICSI vs. IVF –
Sahlgrenska Hospital 2003-06

Survival rates

- ICSI: 46% > 60% survival
- IVF: 49% > 60% survival

Implantation – single embryo transfers

- ICSI: 18.9% (709 SET)
- IVF: 24.5% (795 SET)

Frozen-thawed transfer - Predictive factors for live birth

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

Influence of culture medium

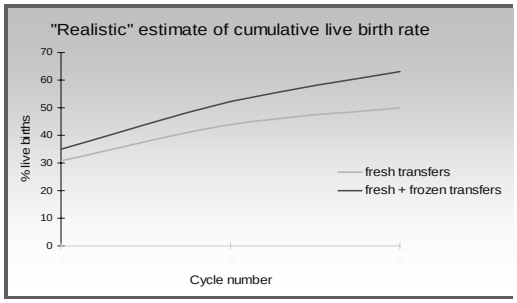
Freezing	CM1+FM1	CM1+FM1	CM2+FM2	CM2+FM1
Thawing	CM1+TM1	CM2+TM2	CM2+TM2	CM2+TM1
N=	1321	483	305	68
100%	44.9%	47.0%	32.8%	39.7%
75-90	12.3%	9.9%	15.4%	14.7%
% GQE	51%		56%	

FM1 / TM1 = Hepes-based freezing/thawing medium
FM2 / TM2 = PBS-based freezing/thawing medium

Embryo selection criteria

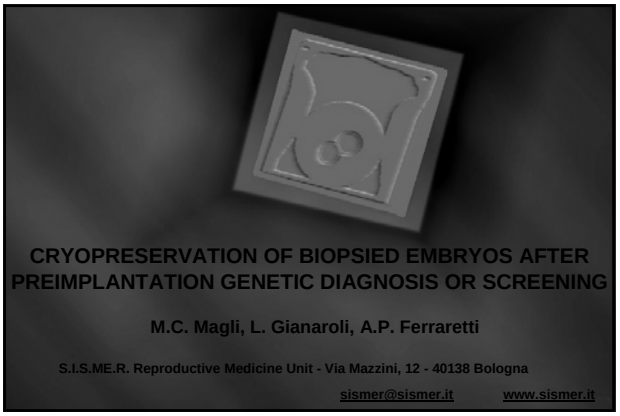
Fresh transfer	Cryopreservation
• PN morphology	• PN morphology ?
• no MNB	• no MNB
• 4 cells (– 8)	• 4 cells (– 8) +
• even sized cells	• even sized cells
• < 20 (-30?)% fragmentation	• < 20 (-30?)% fragmentation
• first cleavage before 25-27-hours	• first cleavage before 25-27-hours
• 1 nucleus / cell	• 1 nucleus / cell ?

Cumulative birth rates - Addition in live births from freezing-thawing transfers



Conclusion

- In a high-quality program, transfer of good quality cryopreserved embryos yield a pregnancy rate which is a **considerable** addition to that of fresh transfers
- Although the overall lower pregnancy and implantation rates compared to fresh transfers indicate that embryo quality is affected, success rate after transfer of a single intact embryo may be comparable to transfer of a fresh embryo



CRYOPRESERVATION OF BIOPSIED EMBRYOS AFTER PREIMPLANTATION GENETIC DIAGNOSIS OR SCREENING

M.C. Magli, L. Gianaroli, A.P. Ferraretti

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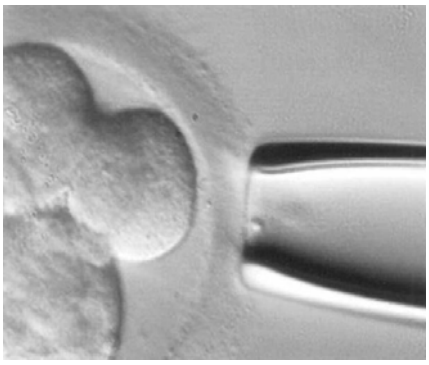
sismer@sismer.it www.sismer.it

PGD



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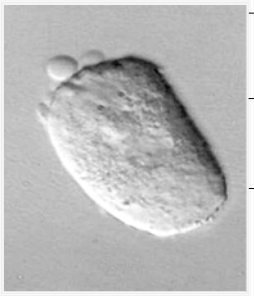
PGD



Aneuploidy

Euploid	33%
Aneuploid	67%

Structural abnormalities

Euploid/balanced	19%
Aneuploid/unbalanced	81%

Single gene disorders

	Recessive	Dominant
Wild type	25%	50%
Healthy carrier	50%	-
Affected	25%	50%

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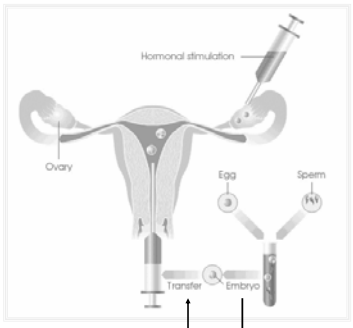
PGD



- Embryo biopsy
- Genetic analysis

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BIOPSIED EMBRYO CRYOPRESERVATION

The availability of an efficient cryopreservation program is especially important in the case of biopsied embryos

The embryos diagnosed as transferrable by PGD are of particular value as the possibility of storing them avoids to have to couple the need of repeating all the steps of a PGD cycle.

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BIOPSIED EMBRYO CRYOPRESERVATION

Chromosomally abnormal embryos
> 7 cells and <5% fragmentation

Slow-freezing w/ PROH in 0.1M sucrose

Frozen on the afternoon of day 3

Thawed two days later

Magli MC, Gianaroli L, Fortini D, Ferraretti AP, Munné S (1999) Impact of blastomere biopsy and cryopreservation techniques on human embryo viability. Hum Reprod 14, 770-773

S.I.S.ME.R. VISION 2000

BIOPSIED EMBRYO CRYOPRESERVATION

Embryo freezing

Embryos were frozen using 1,2-propanediol (PROH) and 0.1M sucrose as cryoprotectants in HEPES-buffered HTF medium (Lassalle *et al*., 1985). HSA was used to supplement both freezing and thawing (25 mg/ml) solutions.

1- 1.5 mol/l PROH for 10 min at room temperature
2- 1.5 mol/l PROH with 0.1 mol/l sucrose and loaded individually into plastic straws.

Cooling:
- start at 20°C, at a rate of –2°C/min to –7°C
- manual seeding was induced manually
- 0.3°C/min to –30°C
- 50°C/min to –150°C
- plunging and storage in liquid nitrogen

Magli MC, Gianaroli L, Fortini D, Ferraretti AP, Munné S (1999) Impact of blastomere biopsy and cryopreservation techniques on human embryo viability. Hum Reprod 14, 770-773

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BIOPSIED EMBRYO CRYOPRESERVATION

Embryo thawing

- Exposure of straws to air for 30 s
- immersion in a water bath at 30°C for 45 s.

PROH was removed by serial transfer into:
- 1.0 M PROH + 0.2 M sucrose at room temperature for 5 min
- 0.5 M PROH + 0.2 M sucrose at room temperature for 5 min
- 0.2 M sucrose for 10 min.

Rehydration was completed by transfer to HEPES-buffered HTF medium for 10 min.

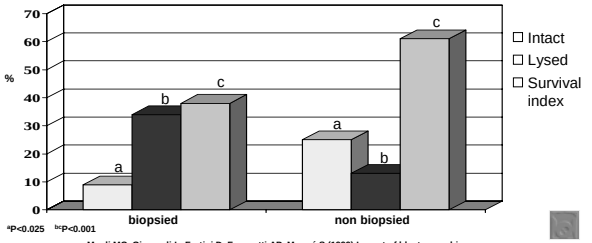
Embryos were transferred to culture medium at 37°C before being assessed for blastomere survival.

Magli MC, Gianaroli L, Fortini D, Ferraretti AP, Munné S (1999) Impact of blastomere biopsy and cryopreservation techniques on human embryo viability. Hum Reprod 14, 770-773

S.I.S.ME.R. VISION 2000

BIOPSIED EMBRYO CRYOPRESERVATION

The cryopreservation of biopsied embryos has given disappointing results when performed at the cleavage stage.



[a]P<0.025 [b]P<0.001

Magli MC, Gianaroli L, Fortini D, Ferraretti AP, Munné S (1999) Impact of blastomere biopsy and cryopreservation techniques on human embryo viability. Hum Reprod 14, 770-773

S.I.S.ME.R. VISION 2000

BIOPSIED EMBRYO CRYOPRESERVATION

Embryos derived from abnormal fertilization
> 6 cells and <20% fragmentation

Slow-freezing w/ DMSO

Frozen on the morning of day 3

(No indication about thawing)

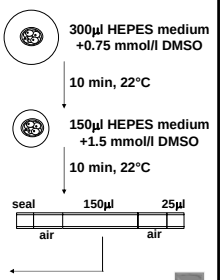
Joris H, Van den Habbeel E, De Vos A, Van Steirteghem A (1999) Reduced survival after human embryo biopsy and subsequent cryopreservation. Hum Reprod 14, 2833-2837.

S.I.S.ME.R. VISION 2000

BIOPSIED EMBRYO CRYOPRESERVATION

Joris et al., 1999

The cryoprotectant solution was made-up in HEPES-buffered Earle's medium supplemented with 0.5% w/v HSA (further referred to as HEPES medium). Embryos were first incubated in 300 µl HEPES medium with 0.75 mmol/l DMSO for 10 min at 22°C. The embryos were then transferred to a 150 µl droplet of HEPES medium with 1.5 M DMSO and incubated for 10 min at 22°C. The embryos were loaded into plastic ministraws [0.25 ml, Pailette Souple, Industrie de la Médecine Vétérinaire (IMV), L'aigle, Air Liquide, Machelen, Belgium]. The loading of the straws was done as follows: 25 µl of HEPES medium was aspirated into the straw and then some air was aspirated. Next, 150 µl HEPES medium with 1.5 mmol/l DMSO containing the embryos was aspirated, followed by the aspiration of another air bubble. Subsequently, HEPES medium was aspirated until the cotton plug of the straw became wet. The open end of the straw was closed with powder (IMV, Air Liquide). Up to three embryos were loaded into a straw. The loaded straws were transferred to a programmable freezer (Minicool 40 PC, Air Liquide) and placed horizontally in the freezing chamber. The controlled freezing procedure started after all the straws had been loaded. Cooling from 22°C to −7°C was done at a rate of 2°C/min. This temperature was kept for 5 min. At this point the seeding was performed by touching the straws with a liquid-nitrogen-(LN_2)-cold forceps at the level of an air bubble. After another 5 min at −7°C, the temperature was lowered to −80°C at a rate of 0.3°C/min and to −100°C at 10°C/min. The straws were then plunged into LN_2. Straws were stored vertically in LN_2 filled containers (GT40, Air Liquide).



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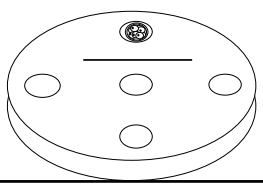
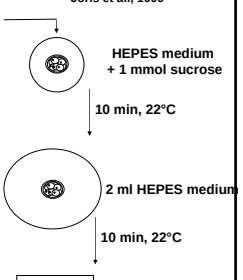
BIOPSIED EMBRYO CRYOPRESERVATION

Joris et al., 1999

For thawing the straws were taken from the LN_2 and transferred to the programmable freezer that had first been cooled to −100°C. After a 5 min holding period at −100°C the straws were warmed to 4°C at 4°C/min. After a 10 min holding period the straws were taken from the chamber and the content of the straws was expelled in HEPES medium containing 1 mmol/l sucrose (Sigma). After an incubation of 10 min at 22°C the embryos were transferred to 2 ml of HEPES medium without sucrose and further incubated for 10 min at 22°C. After two additional rinsing steps the embryos were put into culture.

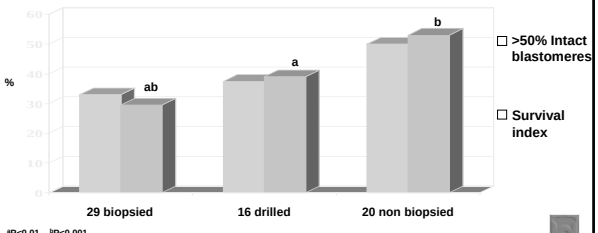




S.I.S.ME.R. VISION 2000

BIOPSIED EMBRYO CRYOPRESERVATION

The cryopreservation of biopsied embryos has given disappointing results when performed at the cleavage stage.



[a]P<0.01 [b]P<0.001

Joris H, Van den Habbeel E, De Vos A, Van Steirteghem A (1999) Reduced survival after human embryo biopsy and subsequent cryopreservation. Hum Reprod 14, 2833-2837.

S.I.S.ME.R. VISION 2000

BIOPSIED EMBRYO CRYOPRESERVATION

Standard method - freezing

Initially, embryos were frozen using 1,2-propanediol (PROH) and sucrose as cryoprotectants in HEPES-buffered HTF medium (Lassalle *et al*., 1985). HSA was used to supplement both freezing (10 mg/ml) and thawing (4 mg/ml) solutions.

Embryos were equilibrated in 1.5 mol/l PROH for 10 min at room temperature before being transferred to 1.5 mol/l PROH with 0.1 mol/l sucrose and loaded individually into plastic straws.

Cooling was conducted in programmable freezer at a rate of –2°C/min to –7°C, at which point seeding was induced manually. Cooling was then continued at rates of –0.3°C/min to –30°C and –50°C/min to –150°C before plunging and storage in liquid nitrogen.

Jericho E., Wilton L, Gook DA, Edgar DH (2003) A modified cryopreservation method increases the survival of human biopsied cleavage stage embryos. Hum Reprod 18, 568-571.

S.I.S.ME.R. VISION 2000

BIOPSIED EMBRYO CRYOPRESERVATION

Standard method - thawing

Embryos were thawed rapidly by removing straws from storage, exposure to air for 30 s and immersion in a water bath at 30°C for 45 s.

PROH was removed by serial transfer into 0.75 mol/l PROH in the presence of 0.2 mol/l sucrose at room temperature for 5 min followed by 0.2 mol/l sucrose for a further 5 min. Rehydration was completed by transfer to sucrose-free HEPES-buffered HTF medium for 10 min.

Embryos were transferred to culture medium at 37°C before being assessed for blastomere survival.

Jericho E., Wilton L, Gook DA, Edgar DH (2003) A modified cryopreservation method increases the survival of human biopsied cleavage stage embryos. Hum Reprod 18, 568-571.

S.I.S.ME.R. VISION 2000

BIOPSIED EMBRYO CRYOPRESERVATION

Modified method

Adjustments were made to both the freezing and thawing solutions but not to the freezing or thawing rates.

Freezing

- **The concentration of PROH in the freezing solutions remained at 1.5 mol/l, but the sucrose concentration was doubled to 0.2 mol/l.**

- **The HSA protein supplement was replaced by 20% (vol:vol) heat inactivated maternal serum.**

Jericho E., Wilton L, Gook DA, Edgar DH (2003) A modified cryopreservation method increases the survival of human biopsied cleavage stage embryos. Hum Reprod 18, 568-571.

S.I.S.ME.R. VISION 2000

BIOPSIED EMBRYO CRYOPRESERVATION

Modified method

Thawing

- In order to maintain the osmotic buffering effect of increased sucrose during rehydration the concentration was increased to 0.3 mol/l during the first thawing steps.

- **Thawed embryos were rehydrated by sequential transfer to 0.75 mol/l PROH + 0.3 mol/l sucrose (5 min), 0.3 mol/l sucrose (5 min), and 0.2 mol/l sucrose (10 min). The concentration of HSA during thawing was 20 mg/ml.**

- **Rehydration was completed by transfer to sucrose-free HEPES buffered HTF medium (10 min) and embryos were transferred to culture medium at 37°C prior to assessment.**

Jericho E., Wilton L, Gook DA, Edgar DH (2003) A modified cryopreservation method increases the survival of human biopsied cleavage stage embryos. Hum Reprod 18, 568-571.

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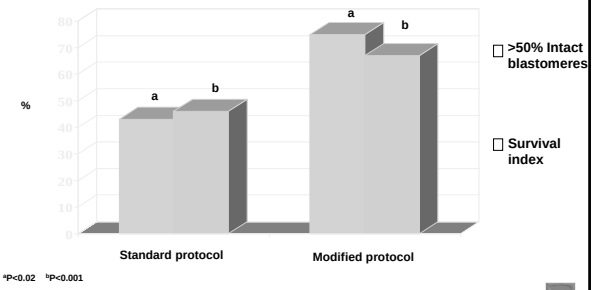
BIOPSIED EMBRYO CRYOPRESERVATION



[a]P<0.02 [b]P<0.001

Jericho E., Wilton L, Gook DA, Edgar DH (2003) A modified cryopreservation method increases the survival of human biopsied cleavage stage embryos. Hum Reprod 18, 568-571.

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BIOPSIED EMBRYO CRYOPRESERVATION

Implantation of biopsied embryos cryopreserved using the modified method.

No. Thawed cycles	**41**
No. Transferred cycles (%)	**36 (88)**
No. Total pregnancies	**8**
No. Clinical pregnancies	**6**
(%) per transferred cycle	**(16.7)**
(%) per thawed cycle	**(14.6)**
Implantation rate (%)	**(12)**

Jericho E., Wilton L, Gook DA, Edgar DH (2003) A modified cryopreservation method increases the survival of human biopsied cleavage stage embryos. Hum Reprod 18, 568-571.

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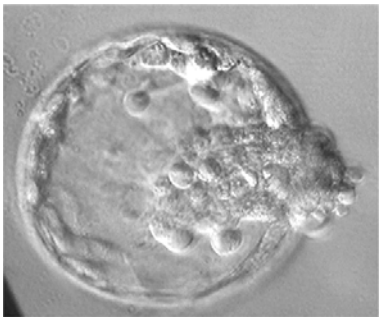
BIOPSIED EMBRYO CRYOPRESERVATION



The freezing protocol was applied to 18 blastocysts diagnosed as abnormal by PGD.

After thawing, 11 survived (61%) and 8 hatched *in vitro*.

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BIOPSIED EMBRYO CRYOPRESERVATION

AIM OF THE STUDY

- To grow biopsied embryos which were diagnosed as normal or healthy carriers after PGD (for aneuploidy, translocations or genetic disorders) to the blastocyst stage and freeze them.

- To analyze the outcome of the thawing cycles.

Magli MC, Gianaroli L, Grieco N, Cefalù E, Ruvolo G, Ferraretti AP (2006) Cryopreservation of biopsied embryos at the blastocyst stage. Hum Reprod 21, 2656-2660.

S.I.S.ME.R. VISION 2000

BIOPSIED EMBRYO CRYOPRESERVATION

MATERIALS AND METHODS

49 patients → **89 frozen blastocysts**

Thawing cycles

34 patients **47 thawed blastocysts**

The cryopreservation solutions were based on the use of HEPES-buffered T6 medium supplemented with 10% HSA.

Magli MC, Gianaroli L, Grieco N, Cefalù E, Ruvolo G, Ferraretti AP (2006) Cryopreservation of biopsied embryos at the blastocyst stage. Hum Reprod 21, 2656-2660.

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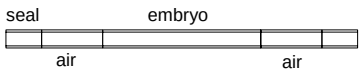
PROH - FREEZING: SOLUTIONS

1) **T6 HEPES + 25mg/ml HSA** ⟹ **1-2 min**

2) **1.5M PROH** ⟹ **10 min**

3) **1.5M PROH + 0.1M sucrose**
Straw loading ⟹ **1 min**



Magli MC, Gianaroli L, Grieco N, Cefalù E, Ruvolo G, Ferraretti AP (2006) Cryopreservation of biopsied embryos at the blastocyst stage. Hum Reprod 21, 2656-2660.

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PROH - FREEZING

Starting temperature	**+ 18°C**
Step 1	**- 2.0°C/min to -7.0°C**
Step 2	**seeding at -7.0°C**
Step 3	**- 0.3°C/min to -30°C**
Step 4	**- 50°C/min to -154°C**

LN_2

Magli MC, Gianaroli L, Grieco N, Cefalù E, Ruvolo G, Ferraretti AP (2006) Cryopreservation of biopsied embryos at the blastocyst stage. Hum Reprod 21, 2656-2660.

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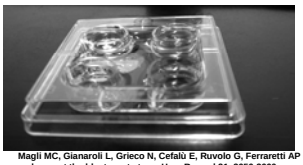
PROH - THAWING

Performed in the late afternoon of the day preceding the transfer

1- 1.0M PROH + 0.2M sucrose ⟹ 5 min

2- 0.5M PROH + 0.2M sucrose ⟹ 5 min

3- 0.2M sucrose ⟹ 5 min



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RESULTS

		Conventional cycles
No. Thawed blastocysts	47	92
No. Survived blastocysts (%)	25 (53)	87 (58)
No. Transferred cycles (%)	18 (53)	63 (68)
No. Clinical pregnancies	7	18
(%) per transferred cycle	(39)	(29)
(%) per thawed cycle	(21)	(20)
Implantation rate (%)	(32)	(20.7)
No. Abortions	2	5
No. Infants born	6	13

Magli MC, Gianaroli L, Grieco N, Cefalù E, Ruvolo G, Ferraretti AP (2006) Cryopreservation of biopsied embryos at the blastocyst stage. Hum Reprod 21, 2656-2660.

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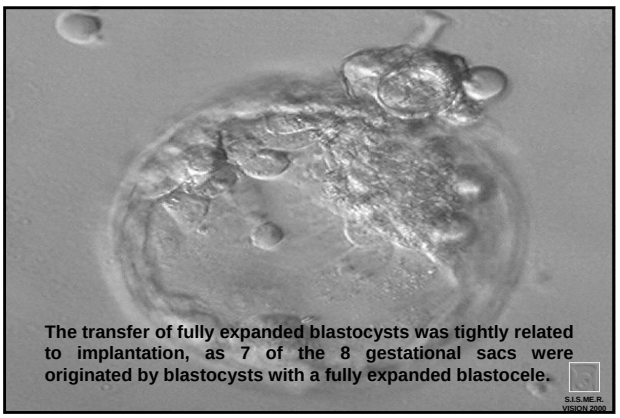

The transfer of fully expanded blastocysts was tightly related to implantation, as 7 of the 8 gestational sacs were originated by blastocysts with a fully expanded blastocele.

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Cumulative data in PGD and conventional IVF/ICSI cycles

	PGD cycles	IVF/ICSI cycles	
Patients (*n*)	34	88	
Age in years (M ± SD)	35.9 ± 4.4*	33.8 ± 3.4*	P<0.005
Oocytes [*n* (M ± SD)]	416 (12.2 ± 2.9)	899 (10.2 ± 3.3)	
Inseminated oocytes (*n*)	416	695	
Fertilized oocytes [*n* (%)]	337 (81)	542 (78)	
Embryos [*n* (%)]	310 (92)	515 (95)	
Biopsied embryos (*n*)	272	–	
PGD normal [*n* (%)]	140 (51)	–	
Transferred embryos [*n* (%)]	54 (17)**	141 (27)****	P<0.001
Cryopreserved embryos [*n* (%)]	47 (15)***	150 (29)*****	P<0.001
Transferred cycles (*n*)	29[a]	75[b]	
Clinical pregnancies [*n* (%)]	13 (45)	23 (31)	
Implantation rate (%)	25.9	19.9	

Magli MC, Gianaroli L, Grieco N, Cefalù E, Ruvolo G, Ferraretti AP (2006) Cryopreservation of biopsied embryos at the blastocyst stage. Hum Reprod 21, 2656-2660.

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Cumulative data in PGD and conventional IVF/ICSI cycles

	PGD cycles	IVF/ICSI cycles
Transferred cycles—cumulative (*n*)	47	138
Transferred embryos—cumulative [*n* (M ± SD)]	79 (1.8 ± 0.5)	228 (1.6 ± 0.5)
Clinical pregnancies—cumulative (*n*)	20	41
Percentage per transferred cycle	43	30
Percentage per patient	59	47
Abortions—cumulative (*n*)	5	13
Implantation rate (%)—cumulative	29.1	20.2
Take-home baby rate per patient (%)	44	32

Magli MC, Gianaroli L, Grieco N, Cefalù E, Ruvolo G, Ferraretti AP (2006) Cryopreservation of biopsied embryos at the blastocyst stage. Hum Reprod 21, 2656-2660.

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CONCLUSIONS

The culture of spare biopsied embryos to the blastocyst stage has two advantages:

1) it permits the selection of the most viable embryos for cryopreservation by discarding those having developmental arrest. In this way the number of frozen embryos is kept to a minimum.

2) According to the results in this study, the blastocyst stage is especially convenient for a successful cryopreservation program contributing a figure of 11% to the cumulative clinical pregnancy rate.

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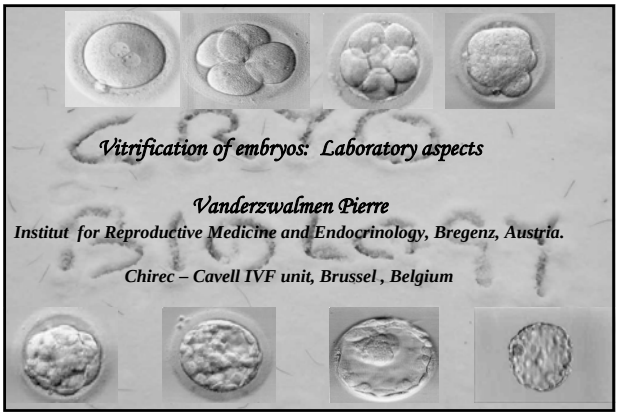


Vitrification of embryos: Laboratory aspects

Vanderzwalmen Pierre
Institut for Reproductive Medicine and Endocrinology, Bregenz, Austria.

Chirec – Cavell IVF unit, Brussel , Belgium

Outline of the presentation

Frequent questions from the laboratory part:

- Vitrification as an alternative to slow freezing procedure: Why?
- Which protocol for vitrification? Is it possible to apply a standardized vitrification protocol ?
- How to approach the critical aspects of vitrification ?
  - Which CP?
  - Exposure time to CPs? In one step or several steps?
  - Which embryo carriers?
  - Non aseptic or aseptic embryo carrier devices?
- One decade experience with vitrification of human embryo : Is it still a place for programmable automatic freezing system?
- Conclusions

« Crystallization is incompatible with living systems and should be avoided whenever possible » Luyet (1937)

Vitrification: an alternative to slow freezing protocol. Why ?

Controlled ice crystal formation during freezing is a KEY factor in determining the viability of oocytes and embryos following freezing and thawing.

ICE CRYSTALS should never be allowed to appear and grow inside the cells

How to reduce the negative effects of ice crystal formation that occured during a slow freezing protocol?

Alternative that eliminate ice cystal formation

VITRIFICATION

Ice free cryopreservation of mouse embryos at –196 C by vitrification.
„in vitro" development in DMSO – EG-sucrose-acetamide
Rall-Fahy Nature 1985

Vitrification: definitions

Vitrification is a process by which a liquid is solidified into a non-crystalline (glassy) phase by lowering rapidly the temperature below the „glass transition temperature (Tg) and greatly increasing the viscosity

Glass

A solid with the molecular structure of a liquid, strictly an extremely viscous liquid with many mechanical properties of a solid.

Which protocol for vitrification?
Is it possible to apply a standardized vitrification protocol ?

From the definition of vitrification:

Vitrification is obtained by combining:

the use of concentrated solutions

with rapid cooling,

to avoids the formation of ice.

Factors that influence the probability of successful vitrification

$$\frac{\text{Cooling – warming rates} \times \text{viscosity}}{\text{Volume of the sample}} \quad \text{Type of embryos - cells}$$

1 Use of high concentration of permeable cryoprotectant

Vitrification **CP: 5M-7M**

Depress the freezing/melting point.
Increase the viscosity.

Slow freezing **CP: 1.5M**

Permeable, Low MW: Ethylene glycol - DMSO - Erythritol
Propanediol - Glycerol

Non Permeable, Low MW: Sucrose Trehalose

High MW: Ficoll, PEG

Two solutions
E.G.
E.G.-DMSO

Macromolecules (Ficoll, polyethylene glycol) ?beneficial effect?

- Encapsulate the embryos in a vitrification layer solution

1 Exposure to cryoprotectants

Step 1: To protect the intracellular part

SC ***(not vitrified solution)***

	EG	DMSO	Sucrose	Ficoll 400	Osmolarity
VS1	10% (1,8M)	10% (1,4M)	/	/	**3500**

Step 2: To protect the intra and extracellular parts

(vitrified sol)

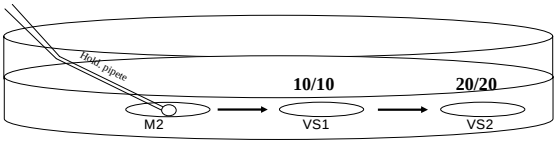
	EG	DMSO	Sucrose	Ficoll 400	Osmolarity
VS2	20% (3,6M)	20% (2,8M)	0,75M	25µM	**6770**

How embryos react in VS1 and VS2 ?

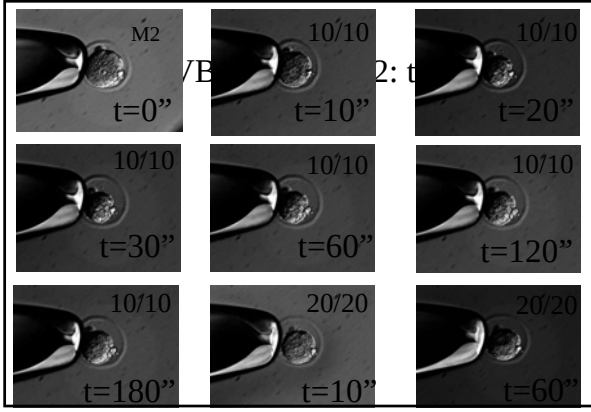
Volume analysis in relation with the exposure to cryoprotectant solutions

Ectors F. & Vanderzwalmen P. GIGA - ULg

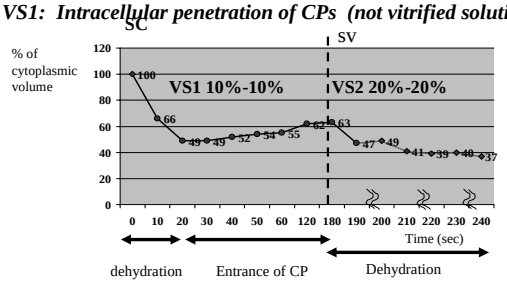
Evaluation of cytoplasmic volume in VS1 (2PN; FVB) 3 droplets of M2, VS1 & VS2 under oil



Embryo is maintained with a holding pipette
One picture / 10”

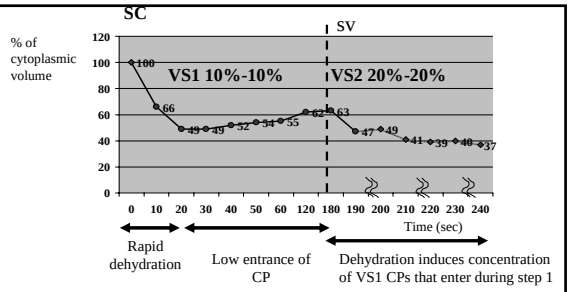


VS1: Intracellular penetration of CPs (not vitrified solution)



VS2 permits intra & extracellular vitrification by:

1. ***Dehydration of the cell to protect the embryo***
2. ***Vitrification of the extracellular compartment***



The final $[CPs]_i$ will be determined by the time of exposure in VS1 and the rate of dehydration in VS2

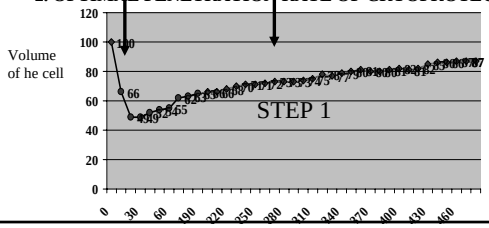
The success of cryopreservation protocols depends on:

- **1. OPTIMAL DEHYDRATION** process when cells are exposed to hypertonic conditions. *(! Don't touch Osmotically inactive H2O)*

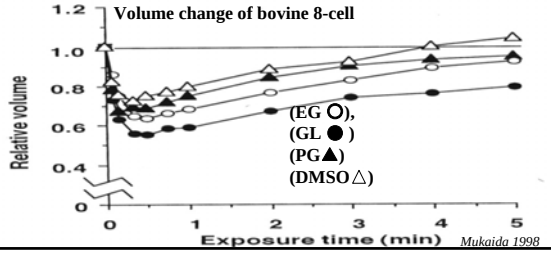
  Fonction of the concentration of VS1

  Hydraulic conductivity: Cell 's permeability to H2O

- **2. OPTIMAL PENETRATION RATE OF CRYOPROTECTANT.**



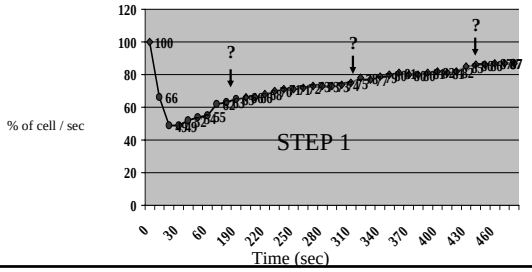
- *Type and concentration of cryoprotective additives (CPs)*
  - permeability property of the CPs (ethylene glycol > glycerol)
  - concentration (higher CPs, faster it will permeate the cell)
  - temperature of the CPs
  - type of CPs

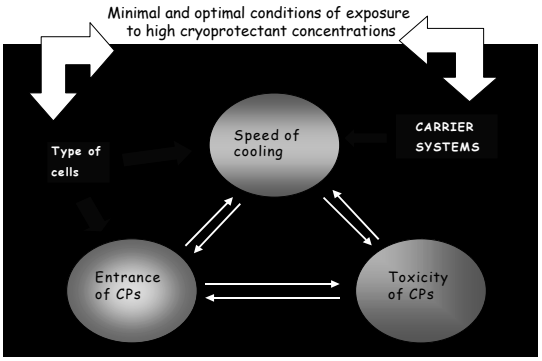


The success of cryopreservation protocols depends on:

↖ **3. OPTIMAL EXPOSURE TIME TO THE CPs**

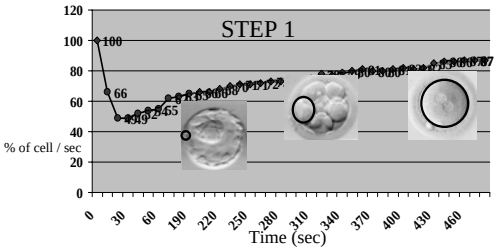
How long the cells have to be exposed to VS1 solution in order to obtain a successful vitrification (avoid toxic effect) ?





↖ **3. OPTIMAL EXPOSURE TIME TO THE CPs**

Determined by a number of biophysical factors

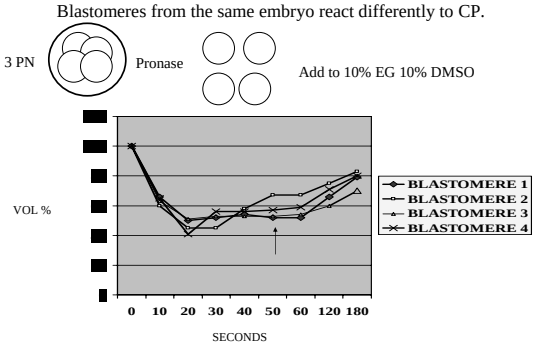


Largest cell in the body (130μm) with high water content and one single membrane

Largest cells have a low surface area to volume ratio, hence they are less efficient at taking up CP and at loosing water

Determined by a number of biophysical factors

Blastomeres from the same embryo react differently to CP.



2 Use of high speed of cooling

Vitrification

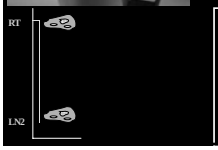
1 Volume of the CP drop

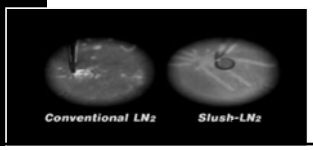
Size of the sample
(0.5ml → µl → pl)

critical cooling rate:
The rate of cooling which produces vitrification

e of embryo carrier

of LN2





CARRIER SYSTEMS (I)

2,000°C/ min

French mini-straws. (Vanderzwalmen 1986, Vanderzwalmen 1997,Chen *et al.*, 2000; Yokota *et al.*, 2000, 2001, Vanderzwalmen *et al.*, 2002)

Small straws with thin walls. 10,000°C C/min

(1) Open-pulled straws *(OPS)* (Vatja ,Kuleshova *et al.*, 1999; Chen *et al.*, 2000)

(2) Flexipet-denuding pipette *(FDP)* (Liebermann *et al.*, 2002)

CARRIER SYSTEMS (Ii)

Increase the speed of temperature conduction: small volume of CP, 20.000°C/min

contact with LN2

Direct contact between a small volume of vitrification solution and LN2.

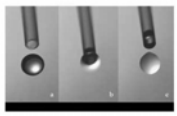
(1) Electron microscope copper grid (EM grids)(Martino 1996, Hong *et al.*, 1999; Park *et al.*, 2000; Chung *et al.*, 2000; Wu *et al.*, 2001; Son *et al.*, 2003; Yoon *et al.*, 2003)

(2) Cryoloop *(CL)* (Lane *et al.*, 1999; Mukaida *et al.*, 2001, 2003; Liebermann & Tucker, 2002; Reed et al., 2002; Liebermann & Tucker, 2003)

(3) Hemi-straw system *(HS)* (Vanderzwalmen *et al.*, 2000; Kuwayama & Kato, 2000; Liebermann & Tucker, 2002; Sugioka *et al.*, 2003; Vanderzwalmen *et al.*, 2003)

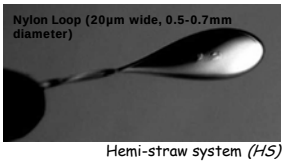
(4) Cryotop (Kuwayama et al., 2005)

Open Pulled Straw (OPS)
Vajta 1998



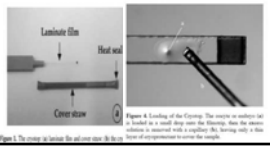
Cryoloop *(CL)*

(Lane *et al.*, 1999; Mukaida *et al.*, 2001, 2003; Liebermann & Tucker, 2002; Reed et al., 2002; Liebermann & Tucker, 2003)



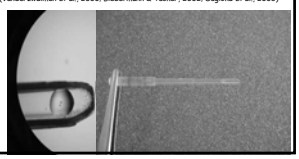
Cryotop

(Kuwayama & Kato, 2000)



Hemi-straw system *(HS)*

(Vanderzwalmen *et al.*, 2000; Liebermann & Tucker, 2002; Sugioka *et al.*, 2003)



B U T

The disadvantage of the ultra-rapid vitrification procedures is that the vitrification solution comes in direct contact with liquid nitrogen during cooling .

Source of contamination: bacterial, fungi, viruses

(Tedder 1995, Fountain 1997, Bielanski 2000, Fountain 1997, Bielanski 2003.)

Contamination in Liquid Nitrogen Storage Vessels

- **during distribution**
- **during storage:**
  - ***From the LN2***

ice formed in the atmosphere above liquid nitrogen

ice formed on the walls of Dewars.

  - ***From the samples***

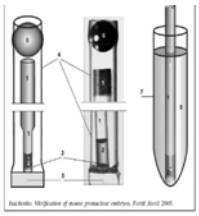
Aseptic vitrification

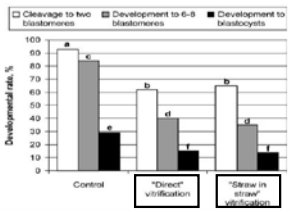
Isolation of the sample

Vitrification of mouse pronuclear embryos after polar body biopsy without direct contact with liquid nitrogen *FS 2005*

Aseptic technology of vitrification of human pronuclear oocytes using open-pulled straws *Hum Reprod 2005*

("straw in straw" vitrification) *Isachenko V*





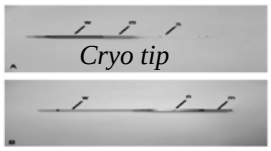
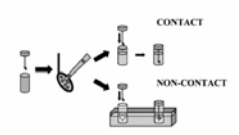


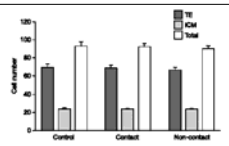
Table 4. Survival, pregnancy and delivery rates after single embryo transfer of human blastocysts vitrified with either the Cryotop or the CryoTip method.

	Cryotop	CryoTip
Survived/vitrified rate (%)	221/227 (97)	82/88 (93)
Pregnancy/transfer rate (%)	131/221 (59)	42/82 (51)
Delivery/transfer rate (%)	113/221 (51)	39/82 (48)

No significant differences between corresponding values were found.

Comparison of open and closed methods for vitrication of human embryos and the elimination of potential contamination Kuwayama 2005

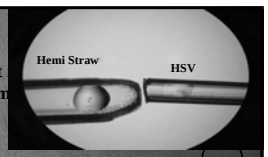


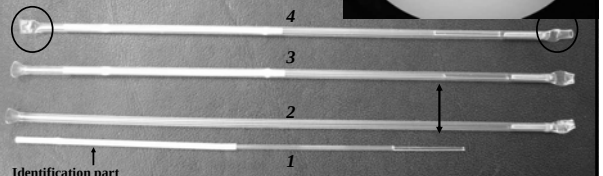


Vitrication of mouse pronuclear oocytes with no direct liquid nitrogen contact Larman 2006

(HSV)

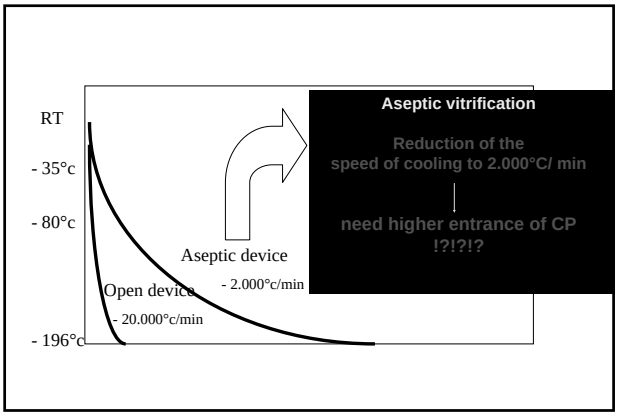
The High Security Vitrification kit allow complete isolation of em

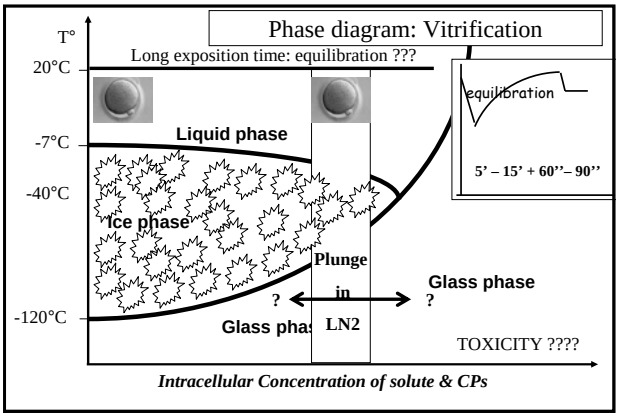


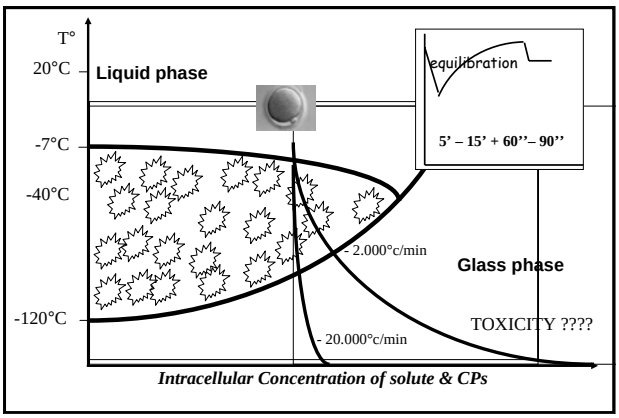


1 Mini Hemi Straw
2 250µl high security straw
3 Mini Hemi-straw inside de high security straw
4 Complete sealed system (VHS)

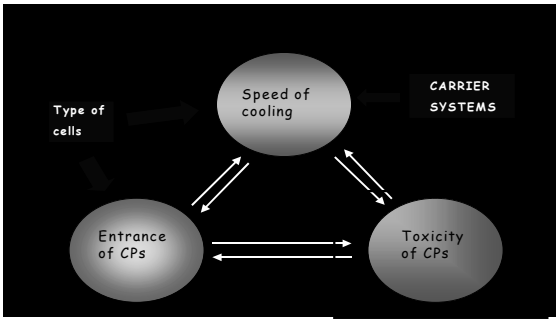
Vanderzwalmen 2006


RT
- 35°c
- 80°c
- 196°c
Aseptic device
- 2.000°c/min
Open device
- 20.000°c/min
Aseptic vitrification
Reduction of the speed of cooling to 2.000°C/ min
need higher entrance of CP
!?!?!?


Phase diagram: Vitrification
T°
20°C
Long exposition time: equilibration ???
equilibration
5' – 15' + 60''– 90''
Liquid phase
-7°C
-40°C
Ice phase
Plunge in LN2
Glass phase
?
?
-120°C
Glass phas
TOXICITY ????
Intracellular Concentration of solute & CPs


T°
20°C
Liquid phase
equilibration
5' – 15' + 60''– 90''
-7°C
-40°C
- 2.000°c/min
Glass phase
-120°C
TOXICITY ????
- 20.000°c/min
Intracellular Concentration of solute & CPs

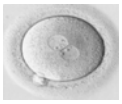
Key objective: Balance between <u>maximizing the cooling</u> rate and

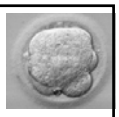


Storage, warming and rehydration, differ only slightly between the two procedures

the main difference exists in :

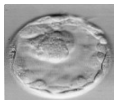
- **Exposure to cryoprotectants**
- **Fast Cooling to subzero T°**
- **Fast Warming : warming is one of the most important steps**
  - **Function of the time of exposure to the CPs and type of cells**



One decade experience with vitrification of human embryo






Ice free cryopreservation of mouse embryos at –196 C by vitrification.
„in vitro“ development in DMSO – EG-sucrose-acetamide
Rall-Fahy Nature 1985

A simple and efficient procedure for preservation of mouse embryos by vitrification.
„in vivo“ development and births of mice after using Glycerol-Propanediol as CP
Scheffen-Vanderzwalmen Cryo-letter 1986

Pregnancies following transfer of cattle embryos preserved by vitrification.
„in vivo“ development and births of calves after using Glycerol-Propanediol as CP
Massip-Vanderzwalmen Cryo-letter 1986

A simple method for mouse embryo cryopreservation in a low toxicity vitrification solution, without appreciable loss of viability.
„in vivo development“ in a solution of Propanediol-Ethylene Glycol
Kasai –Koni JRF 1990

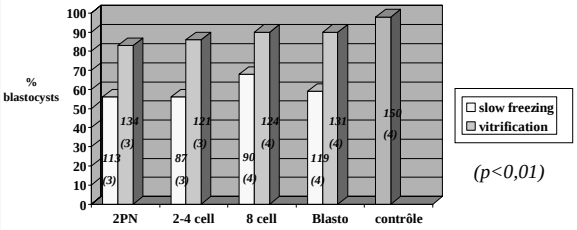
Are programmable freezers still needed in the embryo laboratory?

Dr Gábor Vajta

RBM Online - Vol 12 No 6. 2006 779–796

Slow freezing vs vitrification

C 57BL/6 mice: zygotes to blastocysts



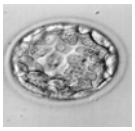
(p<0,01)

Vitrification provides better results than slow freezing for all developmental stages

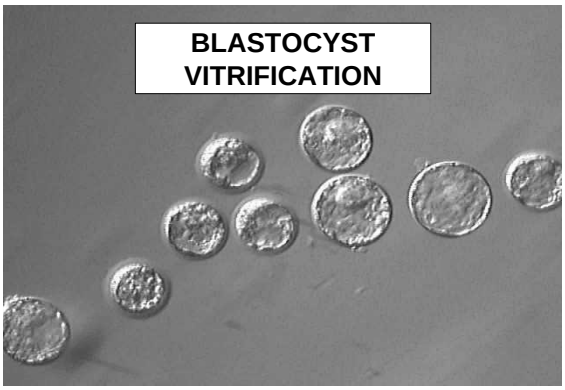
Ectors Vanderzwalmen: Giga Transgenic unit 2005 Walker et al 2004 SMR- A PART Paris 2006

Slow freezing vs vitrification : human

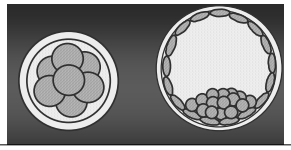
retrospective studies



	slow freezing	***Vitrification***
Nb transfers	254	254
Survival *(after 3 h)*	96%	98%
Implantation	30%	33%
Ong. preg/ vitrif	43%	49%
	Liebermann FS 2006	
Nb transfers	51	35
Survival *(after 3 h)*	86%	100%
Implantation	7%	43% p< 0.05
Ong. preg / transf	18%	27% p< 0.05
	Stehlik RBMO 2005	
Nb transfers	80	84
Survival	60%	95%
Implantation	4%	15% p< 0.05
Ong. preg / transf	17%	35% p< 0.05
	Rama Raju RBMO 2005	



Advantages of blastocyst cryopreservation



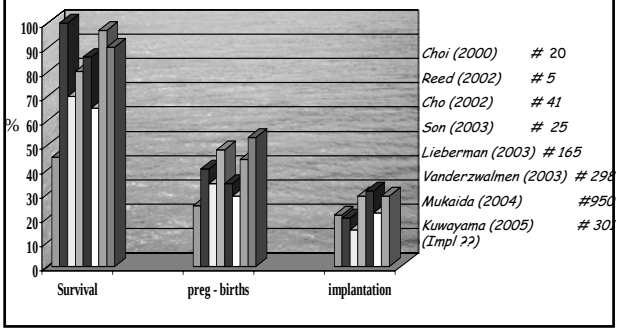
(1) Small volume and high number of blastocyst cells (Cryobiologically)

(2) Selection of the best embryos (biochemically and Cytogenetically)

Pregnancies and births after vitrification on day 5 and day 6.

- **Blastocysts after <u>IVM</u>, IVF and culture**
  Kyono 2005
- **Blastocysts after <u>assisted hatching</u> and/or <u>PGD</u>**
  Zech 2005 Escriba FS 2006
- **Blastocysts after <u>previous slow freezing</u> on day 3**
  Hiraoka 2006
- **<u>Revitrification</u> of blastocysts**
  Son 2005
- **Blastocysts after IVF and culture to day 5**
  (Vanderzwalmen 1997, Choi 2000, Mukaida 2002, Reed 2002, Cho 2002, Son 2003, Liebermann 2003, Kuwayama 2005, Stehlik 2005)

Vitrification of blastocysts: overview of the litterature (end 2004)



Important differences between reports: which factors affect the results ?

- Method of vitrification:
  - Solutions of CPs and nature of CPs.
  - Embryo carriers .
- Selection of embryo for vitrification. (policy of selection: quality)
- Developmental stage:
  - Blastocysts: Degree of blast. cavity expansion.
  - Cell characteristic (S/V, membrane permeability, diffusivity)
- « in vitro » culture conditions: reduction of cryo-tolerance.
- Day of transfer : 3 h or 24 h after thawing.
- Assisted hatching ZP :still a matter of debate!
- Policy of transfers and vitrification

Important differences between reports: which factors affect the results?

Solutions of CPs

Vitrification of blastocysts		
Sol. EG – DMSO	**7.5 / 15**	**10 / 20**
# Vitrification	**38**	**45**
# blastocysts	**102**	**92**
Survival at 24 h	**46 (45%)**	**72 (78%) p< 0.001**
# Transfers	**28 (74%)**	**36 (80%)**
# Ongoing pregnancies		
vitrification cycles	**6 (16%)**	**19 (42%) p< 0.01**
Implantation rates	**13%**	**27%**

Important differences between reports: which factors affect the results ?

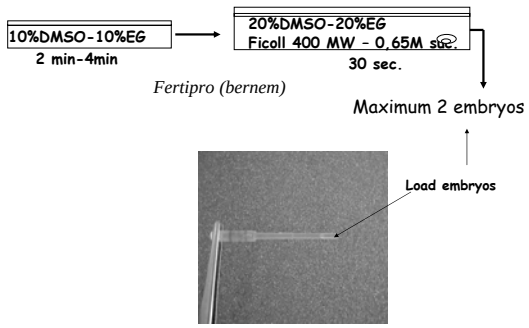
Embryo carriers

0,25 ml straw

Hemi Straw

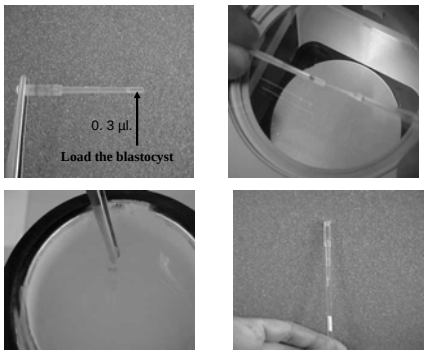
High Security Vitrification Straw

Hemi-straw system (HS)

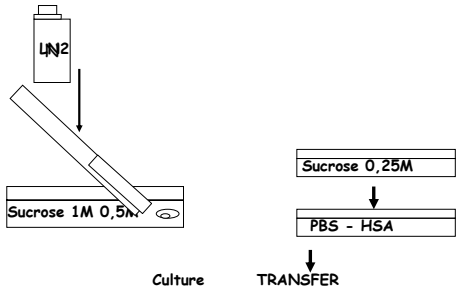


Hemi-Straw

« Vitriplug »

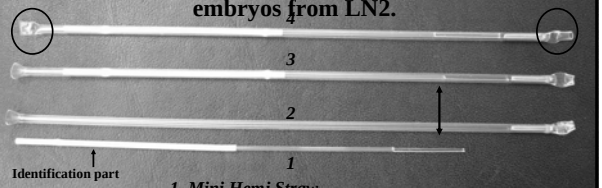


Hemi-straw system (HS)



(HSV)

The High Security Vitrification kit is simple to handle and allow complete isolation of embryos from LN2.



1 Mini Hemi Straw
2 250µl high security straw
3 Mini Hemi-straw inside de high security straw
4 Complete sealed system (VHS)

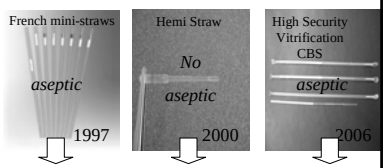
Vanderzwalmen 2006

Important differences between reports: which factors affect the results?

Embryo carriers

One decade experience with vitrification of human blastocysts: overall results

Evolution of the vitrification procedures according to the blastocyst carriers



Cooling/ warming	1.000°C/ 800°C	20.000°C/ 18.000°C	2.000°C/ 18.000°C
N° vitrification-warming cycles	61	459	34
% embryo development after 24h	51%	67%	76%
Birth - Ongoing Pregnancy/vit cycles (%)	16.3%	27%	32%
Implantation rates (transf blast)	14.7%	17%	20%

Important differences between reports: which factors affect the results?

Blastocysts quality

432 Vitrification-warming cycles of blastocysts : effect of the quality

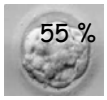
Hemi Straw DMSO /EG 10 / 20 (Fertipro - Beernem)

Embryo quality	*Bad quality*	*Good quality*	*Mix quality*
N° vitrification-warming cycles	**113**	**184**	**135**
N° vitrified-warmed embryos	**361**	**442**	**481**
Survival after 24 hours	**53.7%[a]**	**79.9%**	**66.5%[b]**
Mean embryos transferred	**2.2**	**2.0**	**1.9**
Ongoing pregnancy – birth per: vitrification-warming cycle	**(12) 10.6%**	**(65) 35.3%**	**(36) 26.6%**
Implantation (baby) per: Transferred embryos	**6.2%**	**22.4%**	**17.3%**
Vitrified embryos	**10.9%**	**9.8%**	**1.4%**

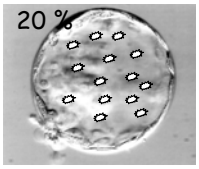
SIMAF Chirec

Important differences between reports: which factors affect the results?

Degree of expansion of the blastocoelic cavity



10% / 10%	2 – 3 min.
20% / 20%	30 sec.
Carrier system: 0.25ml straw	

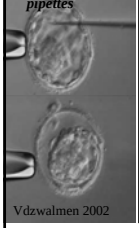


Solutions to reduce the negative effect of the cavity:

- **1 Reduce the volume of the blastocoelic cavity**

- **2 Increase the exposure time to the CPs solution**

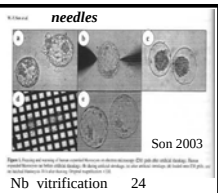
Needles or pipettes



Vdzwalmen 2002

Nb vitrification	123
Survival:	78%
Ong. Preg.	32%
Implantation	23%

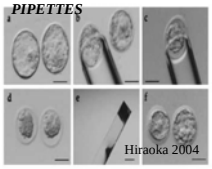
needles



Son 2003

Nb vitrification	24
Survival:	90%
Ong. Preg.	48%
Implantation	29%

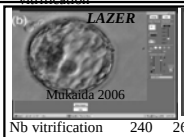
PIPETTES



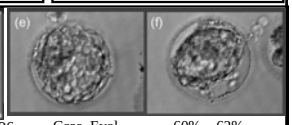
Hiraoka 2004

Nb vitrification	28
Survival:	98%
Ong. Preg.	50%
Implantation	33%

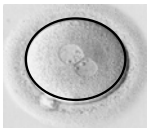
LAZER



Mukaida 2006



Nb vitrification	240	26	Gros. Evol.	60%	62%
Survie:	97%	98%	Implantation	46%	49%



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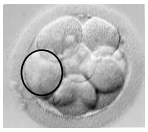

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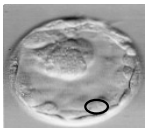
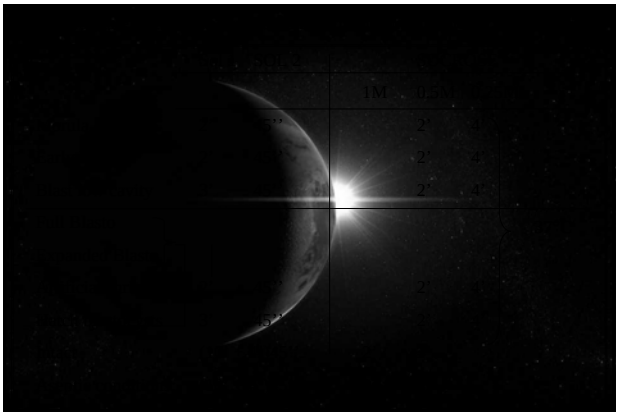


2 Increase the exposure time to the CPs solution

blastocyst cells: Small volume comparing to cavity and 8 cell embryo / Faster protected

BUT: Toxicity with such strategy for blastocysts ?????



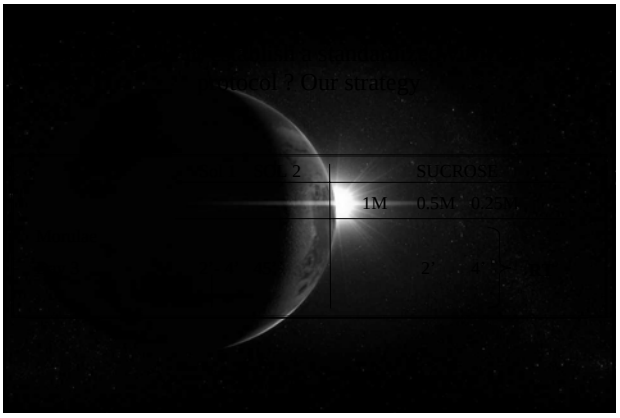
Vitrification of morulas (hemi-Straw)

Sol. EG – DMSO	**7.5 / 15 and 10 / 20**
# Vitrification cycles	59
# morulas	140
Blastocysts after 24 h	88 (63%)
# Transfers	51
# Ongoing pregnancies	16
transfer	31%
vitrification cycles	27%
Implantation rates	18%

Vitrification of embryos with 6 to 8 blastomeres

(hemi-Straw)

Sol. EG – DMSO	**7.5 / 20**	**10 / 20**
# Vitrification cycles	14	36
# day 3 embryos	30	84
Survival at 24 h	18 (60%)	54 (64%)
# Transfers	10 (71%)	30 (83%)
# Ongoing pregnancies		
vitrification cycles	6 (43%)	14 (39%)
Implantation rates	33%	26% NS



Clinical results of vitrification of day 2 – 3 human embryos

	Cryostraw Mukaida 1998	Cryoloop (1) Desai 2007	Cryoloop (2) Raju 2005	Cryotop Nagata clinic
Temp.	Room(25–27°C)	Warm stage(37°C)	Warm stage(37°C)	Room(25–27°C)
equilibration Step	EG.F.S. 20 :20%EG (2min)	7.5%EG+7.5%DMSO (2min)	10%EG (5min)	7.5%EG+7.5%DMSO (5–10min*)
Vitrification Step	EG.F.S. 40 :40%EG (1min)	15%EG+15%DMSO+F.+S. (35sec)	40%EG+S. (30sec)	15%EG+15%DMSO+S. (1min)
Cooling system	Vapor phase LN_2 (3min), then plunged into LN_2	Plunged into LN_2 directly (Ultra-rapid cooling)	Plunged into LN_2 directly (Ultra-rapid cooling)	Plunged into LN_2 directly (Ultra-rapid cooling)
Warming Step	One step 0.5M S. (5min)	Two steps 0.25M S. (2min) 0.125M S. (3min)	Four Steps 1M S. (2.5min) 0.5M S. (2.5min) 0.25M S. (2.5min) 0.125M S. (2.5min)	Two Steps 1M S. (1min) 0.5M S. (3min)

EG.: Ethylene Glycol, F.: Ficoll, S.:Sucrose

*: The duration of equilibration is adjusted according to the time needed for re-expansion of the vitrified embryos.

Clinical results of vitrification of day 2 – 3 human embryos

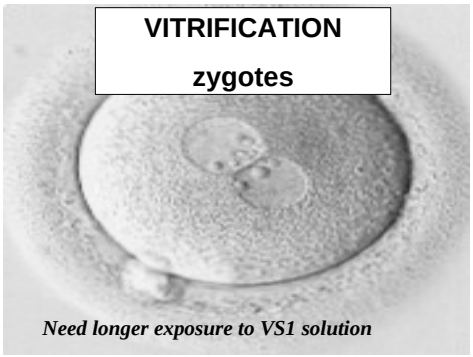
	Cryostraw	Cryoloop(1)	Cryoloop(2)	Cryotop
Age		34.1±4.5	31.3±4.5	35.0±4.5
No. of cycles	127	77	40	604 346 patients.
Survival rate		201/236 85%	121/127 95%	1701/1774 95.9%
Cleavage rate*	486/661 76%	184/236 78%		1289/1774 72.7%
Pregnancy rat	34/127 26.8%	34/77 44.2%	14/40 35.0%	164/604 27.2%
Implantation rate		40/201 19.9%	18/121 14.9%	192/1442 13.3%
Delivery rate*	22/127 17%		13/40 32.5%	118/604 19.5%

*: Including survival and further cleavage rate

Cryoloop(1): Reported by Desai in 2007, Cryoloop(2): Reported by Raju in 2005.

VITRIFICATION

zygotes



Need longer exposure to VS1 solution

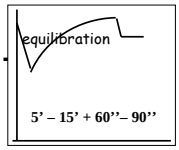


Table 1. Survival and development rates of human pronuclear (PN) embryos cryopreserved by either slow cooling or vitrification using the Cryotop method.

	Slow cooling	*Vitrification*
Survived/cryopreserved rate (%)	1730/1944 (89)[a]	5881/5881 (100)[b]
Cleaved/surviving rate (%)	1557/1730 (90)[a]	5469/5881 (93)[b]
Blastocyst/cleaved rate (%)	796/1557 (51)[a]	3058/5469 (56)[b]
Blastocyst/cryopreserved rate (%)	796/1944 (41)[a]	3058/5881 (52)[b]

[a,b]Values within rows with different superscripts are significantly different ($P < 0.01$).

« Comparison of open and closed methods for vitrication of human embryos and the elimination of potential contamination » Kuwayama RBMonline 2005

Three years of routine vitrification of human zygotes: is it still fair to advocate slow-rate freezing? *Dr Safaa Al-Hasani* RBMOnline - *Vol 14. No 3. 2007 288-293*

Cryotop	# Vitrification cycles	92
DMSO/EG	Survival	89%
	Clinical preganancy rate	28.2%
5 to 15 `min	Implantation	15.6%

10 Patients (hyperstimulation)

Comparison of zygotes development in fresh or after vitrification-warming

MII 224

2 PN 161

VITRIFICATION Day 1

DMSO-EG 10-20

6' – 9'

HS and HVS

76 zygotes

WARMING – CULTURE TO DAY 5

zygotes 68

Survival	**64 (94%)**
# blasto	**29 (45%)**
# Grade AA	**16 (24%)**
# Transfers	**10**
Average Bl	**2.2**
Clinical Preg	**5**
Ongoing Preg	**4 (40%)**
Implantation	**18%**

CULTURE TO day 5

VITRIFICATION Day 5

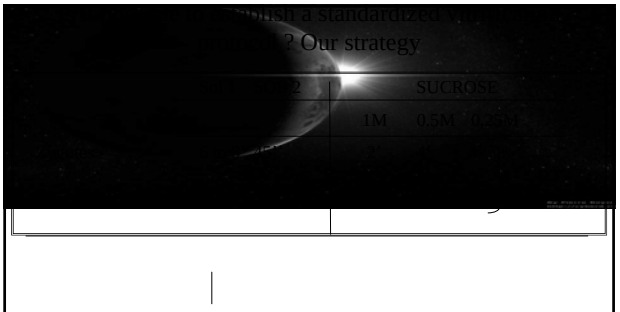
85 zygotes

blastocyts 34 (40%)

grade AA 25 (29%)

Vitrification

CHIREC – ZECH ART units



Encouraging results but:

Vitrification has suffered from different drawbacks:

- The use of high concentration of CP.
- Possible disease transmission mediated by liquid nitrogen.
- Commercial interest in marketing slow freezing (equipment and material) was not motivated to propagate a method that can be performed in a simple foam box and with some primitive handmade tools.

Trouble shootings

- No control of the temperature of the CPs .
- Not enough LN2 in the Dewar flask when plunging the embryo carrier. Be sure that the whole device is immersed in LN2.
- Evaporation of the micro-drop: loading the VS2 medium just before the carrier is plunge in LN2.
- Accidental warming:
  - Search embryos in the tank when storage in vapor.
  - Dewar flask and sucrose solution should be placed nearby
  - Handle the carrier in a wrong way
- Embryo stucked on the surface of the carrier (HVS, cryotop): volume of CPs is too small

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- *Zinst M*
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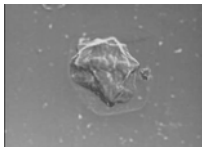
- *Dr Puissant F*
- *Delval A*
- *Vanderzwalmen S*
- *Dr Ectors F*

Vitrification of human oocytes and embryos



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- Luyet 1937 wrote that crystallization is incompitable with living systems and should be avoided whenever possible

Introduction

- Cryopreservation of human oocytes, zygotes, cleavage stage embryos and blastocysts has progressed to become a useful adjunct to human IVF-ET programs

Vitrification, an ultra rapid cooling technique, offers an interesting perspective in the attempts to develop the optimal cryopreservation procedure for human oocytes and embryos

Up to now scientific results have proved that vitrification is at least equal or significantly better than results obtained by traditional slow-cooling method

Conventional cryopreservation versus ultra-rapid vitrification

- Basic principles of cryopreservation
- Traditional method vs. rapid freezing
- Vitrification, cooling rates and
- Difficulties or disadvantages of vitrification
- Safety of the procedure and straws & vials (LN2 contamination)
- Cryoprotectant of the vitrification
- State of the art

Main principles of the vitrification in ART:

- Guarantee of fertilization (oocyte)
- High survival rate after warming
- Increasing the success rate through a significant high cumulative pregnancy rate

Steps of Cryopreservation

- Equilibration in the cryoprotectant
- Freezing process
- Storage in LN_2
- Thawing (warming) process
- Removal of the cryoprotectant
- Culture in the physiological milieu

Factors influencing the success of cryopreservation

1. Possible temperature shocks (+ 15°C or -5°C)
2. Possible changes in the plasma membrane
3. Selection of the right cryoprotectant
4. Dehydration: intensity and time
5. Critical cell volume
6. Solute concentration
7. Cooling rate
8. Thawing rate

Temperature shock

This happened if the cells cooled too fast (also without ice crystalization)

Temperature shock

This happened if the cells cooled too fast (also without ice crystalization)

This shock starts at the plasma membrane due to:

- Shrinkage of the different parts of the membrane
- Mechanical effect
- Reduction of the volume

Characteristics of cryoprotectants

- High soluble in water
- Relative low molecular weight (> 400)
- Fast cell permeability
- Conjunction with water to built stable H_2 bridges
- With high concentration should be non-toxic
- Reducing the freezing point of the extracellular fluid
- Low influx of the intracellular water to avoid the sudden shrinkage of the cell.

Cryoprotectants

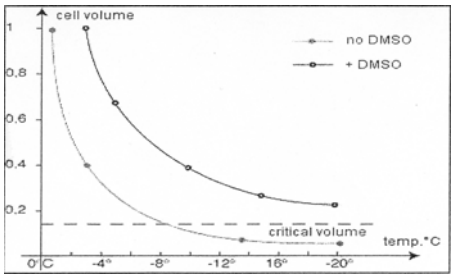
1-Permeable (MG 400)

- Methanol CH_3OH (32)
- Ethanol C_2H_5OH (46)
- Ethylenglycol $C_2H_4(OH)_2$ (62)
- 1-2 Isopropanol $C_3H_6(OH)_2$ (76)
- Glycerol $C_3H_5(OH)_2$ (92)
- DMSO (CH_3) SO (78)

2-Non permeable (MG > 10000)

- Polyethylenglycol (PEG; 8000)
- Polyvinyl pyrrolidone (PVP; 40000)
- Ficoll (70000 or 400000)
- Sucrose

Cryoprotective & critical volume



Cryprotectant concentration and solute concentration during freezing (exp. DMSO)

Isotonic saline solution (9g NaCl/Liter)

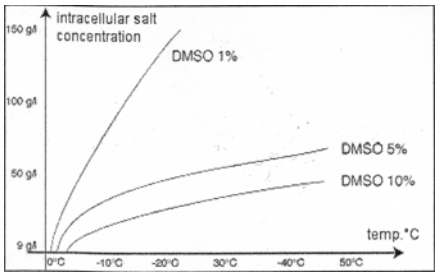
replaced by	replaced by	replaced by
1% DMSO	*5% DMSO*	*10%DMSO*

It will reach a concentration of 50g/Liter

by

-5°C	*-20°C*	*-50°C*

DMSO concentration and solute concentration during freezing



Action of the cryoprotectant

Penetration of cryoprotectant in the cell and partially replacing the

↓

intracellular water

↓

dehydration of the cell

Cooling rate

- Avoiding temperature shock
- Avoiding damage effect of the cell during dehydration
- Avoiding damage of the colloidal milieu of the cell

Cooling rate

Optimal cooling rate,

if the cell gives the maximum

amount of the intracellular water to avoid the intracellular ice crystal formation

Cooling rate

Optimal cooling rate is dependent on the critical volume of the cell which can be defined as:

- The permeability of the cell membrane to the water
- Large membrane surface
- The relation between cell surface to the cell volume according to these phenomenon each cell has its cool rate

Thawing rate

The thawing rate is closely related to the cooling rate in general: the fast thawing is preferable

- Thawing rate has no influence to the slow freezing

The most important principle of the cryopreservation of the oocytes and embryos is:

The formation of ice crystals which should be avoided during the process of freezing of the cells and tissues

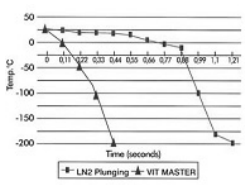
- Intracellular crystal formation creates lethal factores through unwanted physical and chemical events they may injure the cell during cryopreservation process

Two techniques were developed

- Controlled, slow freezing (slow-rate freezing) *(Whittingham et al., 1972)*
- Ultra-rapid freezing Vitrification procedure *(Rall & Fahy, 1985)*

Freezing in the liquid nitrogen LN_2 (Vitrification)

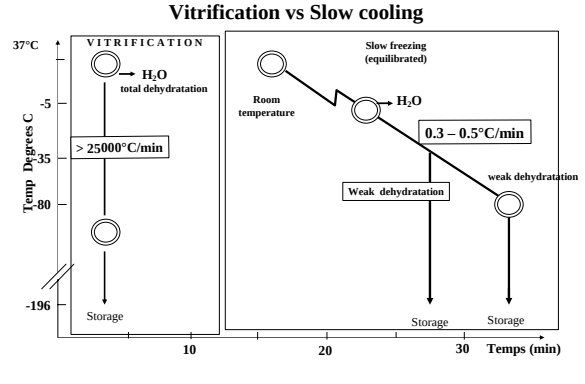
Physical definition:
solidification of a solution to be similar to the state of the glass



- The physical definition of vitrification is the solidification of a solution (water is rapidly cooled and formed into a glassy, vitrified state from the liquid phase) at low temperature, not by ice crystallization but by extreme elevation in viscosity during cooling.

Fahy 1984

-In contrast to slow-rate freezing protocols, during vitrification the entire solution remains unchanged and the water does not precipitate, so no ice crystals are formed.



Slow freezing vs. ultrarapid

	Traditonal	Vitrification
CPA-Concentration	1.5 M	3.0- 5.0 M
Volume	0.3-1.0 ml	< 1 µl
Contact with N_2 & the cell	no	yes
Cooling rate	~ 0.5°C /min	15.000- 50.000 °C/min
Freezing	slow	ultrarapid
Thawing/ Warming	slow	rapid
Time consuming	≥ 180 min.	2 sec.
Dehydration	not controlled	controlled

Slow freezing vs. ultrarapid

	Traditonal	Vitrification
Reduced osmotic injury	no	yes
Zona pellucida fracture	possible	no
Ice crystal formation	yes	no
Seeding	yes	no need
Procedure	Complicated	simple
Device	yes	no need
Costs	high	less

Terminology

Instead of Freezing → Vitrification

Instead of Thawing → Warming

Historical review

- It was described at the end of the 18th Century

(Tammann, 1898)

- Vitrification of mouse embryos at -196°C

(Rall & Fahy, 1985; Ali and Shelton, 1993)

- Blastocyst development from bovine oocytes

(Martino et al. 1996)

- Blastocyst development, Pregnancies, deliveries from human vitrified oocytes, zygotes, cleaved eggs and blastocyst

Why we prefer the vitrification procedure now ?

- There is no mechanical injury (extracellular crystal formation)
- Less osmotic stress for the cell
- No intracellular crystal formation
- Less labor in thelaboratory daily work
- Simple protocol
- It is useful for cells like oocytes and blastocyst which have less success with slow freezing
- No need for expensive device

Cooling rate and vitrification (importance)

- High cooling rate needs high concentration of cryoprotectants
- There is a practical limit to achieve high cooling rate which correlates with biological limit of the cryoprotectant of cells during vitrification
- For this reason it is important to find a balance between a maximum cooling rate and a minimum concentration of cryoprotectant.

Example for cooling rates

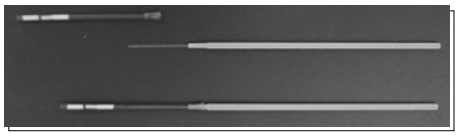
A) ~ 2500°C/ min by using 0.25 ml straws. Thick straw and large volume of medium do not allow a high cooling rate and thawing rate

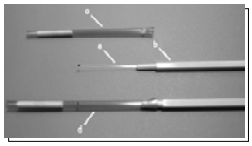
B) ~20000-25000°C/min by using a carrier which allows a very small volume which can get a direct contact with LN_2

Cell carrier systems

- open- pulled straws (OPS)	*Kuleshova et al. 1999; Chem et al. 2000*
- French ministraws	*Chem et al. 2000; Yokota et al. 2000, 2001; Vanderzwalmen et al. 2002*
- Hemi-straw system (HSS) or Cryotop also referred as minimum volume cooling method (MVC)	*(Kuwayama & Kato 2000, Vanderzwalmen et al. 2000, 2003; Liebermann & Tucker 2002; Stehlik et al. 2003*
- Flexipet-denuding pipette (FDP)	*Liebermann et al. 2002*
- Electron microscope copper grid	*Hong et al. 1999, Park et al. 2000; Son et al. 2003; Yoon et al. 2003*
- Cryo loop (CL)	*Lane et al. 1999; Mukaida et al. 2001; Reed et al. 2002*

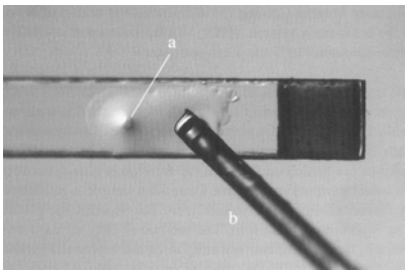
Ultra-rapid Vitrification container: Cryotop





a: Transparent fine Polypropylene sheet (0.8mm x 2cm)
b: Plastic handle
c: Cover cap
d: Cover Top part during storage in LN_2

Cryotop



Critical side of Vitrification

- Very high cooling rate
- Protection against potentially toxic chemicals
  (Vitrification needs high concentration of permeable cryoprotectant to induce a intracellular vitrification)
  (which is sometimes biologically and technically difficult and problematic)
- There is a direct contact between the vitrification medium and LN_2 which is eventually a source of contamination.

Solution for Vitrification

1) To reach a high cooling rate, special container or carrier should be used
2) To minimize the toxicity of the cryoprotectant, at least two different types of cryoprotectant should be used in a stepwise incubation and two different concentration *(lower-strength and full-strength)*
3) LN_2 as a source of contamination ?

Contamination during LN_2 storage

- Virus and bacteria can survive the LN_2 at -196°C like Hepatitis- and HIV-virus ?
- Extra tank for vitrified material
- Infected material should not be frozen

What are the different solutions for vitrification ?

- Permeable cryoprotectant for the cell membranes *(Glycerol, Ethylenglycol, DMSO)*
- Non- permeable cryoprotectant *(Sugar, Proteines, Polymere)*

What are the characteristics of the vitrification solutions

Essential substances: permeable cryoprotectant

- The solution should have the power for dehydration
- The possibility to reduce the freezing point
- These cryoprotectants should be non-toxic

Non-permeable substances (Sucrose, Trehalose)

- Dehydration of the cell through osmosis
- Reduction of the swelling shock during rewarming
- Support the process of dehydration through reduction of the incubation time
- The sucrose leads to increase of the viscosity of the solution

- Additives with large molecular weights, such as disaccharides like sucrose, do not penetrate the cell membrane, but they can significantly reduce the amount of cryoprotectant required as well as the toxicity of EG by decreasing the concentration required to achieve a successful cryopreservation of human oocytes and embryos

The non-permeating sucrose acts as a osmotic buffer to reduce the osmotic shock that might occures as a result from the dilution of the cryoprotectant after cryostorage

Equilibration in the protective substance

A) Time
- The incubation should be short as possible
- Two-step equilibration is important to reduce the toxicity

B) Temperature
- The fast entrance and the degree of toxicity of the cryoprotectant can be influenced by temperature
- Equiliberation at 37°C avoid the re-expansion of the cell especially the first step of warming
- It is advisable to equilibrate between 22 and 25°C

Is the technique of vitrification standarized to be adopted in IVF centers?

All the developmental stages are now vitrified sucessfully
These are some technical difficulties…….

a- Type and concentration of the cryoprotectant
b- Variability in the volume of the media or the carrier
c- Temperature of the solution during equilibration
d- Type of vitrification container
e- Skillness of the embryologist

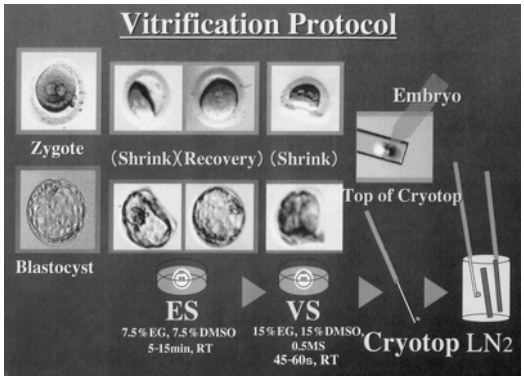
Successful vitrification

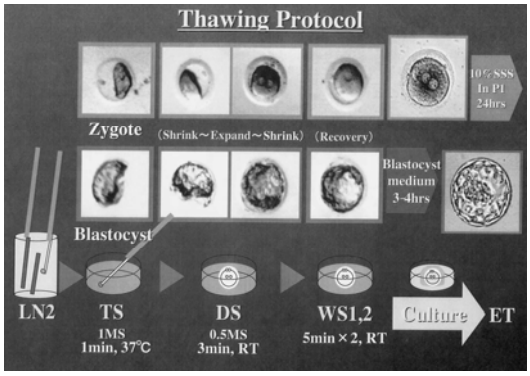
- High cooling rate (>25.000 °C)
- Fast cooling period (< 2 sec.)
- Low volume (< 1µl)

This will lead to avoid crystal formation

Vitrification of a water inside cells can be achieved in three ways

- Increasing the speed of temparature conduction
- Increasing the concentration of cryoprotectant
- Reduce the volume of cryoprotectant (<1µl).
- Very rapid cooling rates from 15,000 to 30,000 C/min can be achieved (e.g. ΔT from 25 C to -196 C = 221 C/0.5 sec = 26520 C/min)


Vitrification Protocol
Zygote
(Shrink)(Recovery)(Shrink)
Embryo
Top of Cryotop
Blastocyst
ES
7.5%EG, 7.5%DMSO
5-15min, RT
VS
15%EG, 15%DMSO,
0.5MS
45-60s, RT
Cryotop LN2


Thawing Protocol
10%SSS
In PI
24hrs
Zygote
(Shrink~Expand~Shrink)
(Recovery)
Blastocyst medium
3-4hrs
Blastocyst
LN2
TS
1MS
1min, 37℃
DS
0.5MS
3min, RT
WS1,2
5min×2, RT
Culture
ET

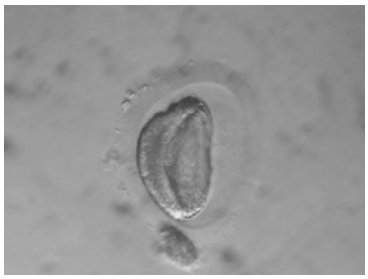


Table 2. In vitro and in vivo survival rates of 2PN stage embryos after cryopreservation by vitrification or conventional slow freezing methods.

Cryo-Method	No.T. Cycles	No.(%) Zygote Thawed	Surviving	> 2 cells	No.(%) Transfers	Pregnacies
Vitrification	9	30	30 (100)	28 (93)	9 (27/28)	3/7 (43)
Slow Freezing	40	177	157 (89)	141 (90/80)	40 (134/141)	10/40 (25)

Towako Kato Ladies' Clinic

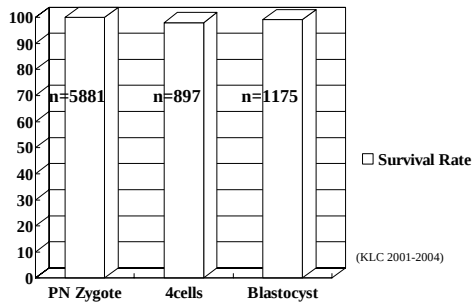


Fig. 1 Post-thaw survival rates of human PN zygotes, 4-cells stage embryos and blastocysts after vitrification

Our preliminary results

No. of Cycles	No. of 2PN vitrified	No. of 2PN warmed(cycles)	No. & % 2PN survived	No. & % Preg.
95	**484**	**150 (48)**	**135(90)**	**20 (41.7)***

•Four deliveries, 3 biochemical
•Two twins, one with 3 implantations

Results

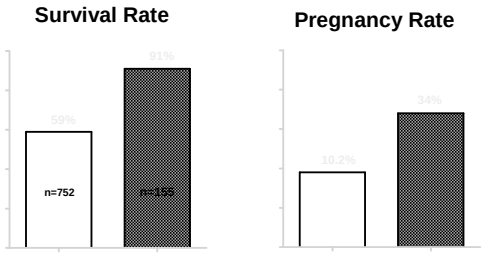
		Slow-cooling (1/00 – 11/05)	Vitrification (3/04 – 01/07)
Patients	(n)	**752**	**211**
2 PN oocytes	(n)	**3.616**	**1.035**
thawed	(n)	**1.438**	**363**
survived	(n)	**848** (59 %)	**335** (92 %)
increase (suvival rate)		**+ 32 (Percentage points)**	
Cycles	(n)	**583**	**129**
Implantation rate	(%)	**111 (7.3%)**	**69 (20.5%)**
Pregnancy Rate	(%)	**10.2%**	**36.4%**
increase (Implantation rate)		**+ 63 (Percentage points)**	

Results

		Vitrification (3/04 – 04/06)
Patients with Cryo-ET	(n)	**33**
Cycles	(n)	**37**
2 PN oocytes vitrified	(n)	**163**
2 PN oocytes warmed	(n)	**133**
2 PN oocytes survived	(n)	**121**
Survival rate	(%)	**91**
Embryo-Transfers	(n)	**37**
Embryos / Transfer	(n)	**2,8**
positive hCG	(n)	**20**

		Vitrification (3/04 – 04/06)
Clinical pregnancies	(n)	**17**
Implantation rate	(%)	**20,5**
Pregnancy rate / ET	(%)	**54**
Ongoing pregnancies	(n)	**7**
biochemical pregnancies	(n)	**3**
Abortions	(n)	**2**
Births	(n)	**8**
Twins	(n)	**3**
Triplets	(n)	**1**

Lübeck Results (till 07/2006)



Possible explanations for high success rate:

- **High survival rate**
- **No intracellular crystal formation**
- **No sublethal effects**
- **Programmed cycle**

Open Questions

1-Toxicity of the Cryoprotactants
2-Type of the Carrier
3-LN2 Contamnination

Future Aspects

- In case of OHSS and PCO
- All 2PN cryopreservation

- Quality is evolution
- Vitrification is a revolution

Prof. van der Elst

19.1.2007

Summary

- The vitrification procedure is easy to be done
- Much less costs
- It is the procedure of the first choice in the future
- The survival rate is very high with all different stages of development
- It may be need to be standardized
- It needs skillness of the embryologist

Vitrification of eggs and blastocysts: the Czech experience

Suchá R.

Institute of reproductive medicine and endocrinology
Pilsen, CZ

Prague April 13th, 2007

Oocyte cryopreservation

- **Animal** – improved breeding programs , preserved endangered species, research application (genetic enginering, embryo clonning)
- **Human**
  - To avoid long term embryo cryopreservation
  - In case of failure to obtain sperm
  - To avoid synchronization in oocyte-donation cycles and to repeat infectious-disease screen
  - Ethical opposition to embryo CP (Italy)
  - Strategy for fertility preservation
    - Delay childbearing
    - Diagnosis of cancer and sterilizing therapies (radio, chemo)
    - .....

Successful CR methods must avoid

- Ice-crystal formation
- Solution effect
- Osmotic shock

⇒Additional chemicals to avoid cell damage = cryoprotectants (CP)

- ⇒Permeating - **PROH, EG, Glycerol, DMSO** (form hydrogen bonds with water to prevent ice crystallization and solution effect, diluting the remaining electrolytes)
- ⇒Non-permeating – **sucrose,** trehalose (remain extracellular - dehydrating intracellular space, prevent ice-crystal formation, prevent osmotic shock and swelling during thawing)

MII oocyte

- Relatively limited success with oocyte storage compared to embryo
- Short life span
- Unique structural features
  - Zona pellucida
  - Cortical granules
  - Microtubular meiotic spindel
  - Condensed chromosomes
- Only about 300 children have been born through the fertilization of frozen oocyte

History

- 1977 – 1st success with mouse oocyte freezing
- 1986 – 1st human pregnancy after human oocyte CP (slow freezing technique) (Chen)
- Very few live birth reported during pre-ICSI era (ICSI 1992 – Palermo)
- 1995 – human oocyte vitrification (Hunter) (65% surv. Rate, no further development)
- 1997 – 1st life birth from thawed oocyte fertilized by ICSI (slow freezing) (Porcu)
- 1999 – 1st birth from vitrified human oocytes (Kuleshova)
- 2003 – 1st large series of human oocyte vitrification published (Yoon)

What makes the mammalian oocyte so difficult to cryopreserved compared to the fertilized oocyte and embryo?

- Oocyte physiology
  - Differences in plasma membrane permeability to water and CP
  - Idiosyncratic physiology of the oocyte itself

Intracellular calcium

- Second messenger in somatic cells
- Involved in events followed sperm penetration of the oocyte
  - First Ca_i transient – initiation of cortical granule fusion with oolemma → zona hardening

Zona hardening

- Preventing polyspermy
- Reflects that the oocyte has started to undergo activation

Cryopreservation of oocytes → induces zona hardening

- exposure oocyte to CP → increase in Ca_i
- need of ICSI for fertilization
- no optimal development following oocyte cryopreservation

CP – PROH, EG and DMSO – induces immediate ↑ of Ca_i

- EG
  - Smallest and shortest increase
- PROH
  - Most protracted elevation

Extracellular derived Ca_i ⇒ using medium devoid of calcium

DMSO

- removal of calcium from medium is not effective
- intracellular derived Ca_i (mitochondria or ER)

→ DMSO is not suitable CP for oocytes

→ Calcium in CP media needs to be evaluated

Changes in energy metabolism and enzyme leakage

- SF induces a greater decrease in oxydative metabolism than VF
- SF – decrease in the uptake of the central nutrient pyruvate
- VF – imparts less stress on the cell and less impact on the energy metabolism
- Measuring the appearance of the LDH in the medium – SF – releasing of LDH into medium X VF has almost no impact on this parameter $\Rightarrow$ damage to cell physiology with SF

Proteomic analysis of frozen/thawed oocytes X unfrozen controls

- Some proteins are affected after SF
  - Chronic exposure to PROH
    - $\rightarrow$ aberrations in the proteome
    - $\rightarrow$ loss of 70% of the cortical granules $\rightarrow$ results in the activation of the oocyte
- Vitrified oocytes appear to be similar to controls

Meiotic spindle and cytoskeleton

- Functional meiotic spindle is essential for completion of meiosis and correct complement of genetic material in the oocyte
- Cooling, CP and cryopreservation $\rightarrow$ induce microtubule depolymerization

Slow freezing

- **Spindle disappears** during the thawing but reforms in the majority of oocytes within 3 hours → ICSI after **spindle recovery time** → oocyte aging
- Deteriorated mitochondria and loss of intermediate filaments following thawing – **damage of the cytoskeleton** → risk of abnormal cell division and chromosome desegregation (aneuploidy)

Vitrification

- The meiotic **spindle remains intact** → ICSI can be done soon after thawing
- **Minimal damage of the cytoskeleton**, only the intermediate filaments affected, which are subsequently reorganized by the time of the first cleavage division

Cobo et al. 2001

- The incidence of chromosomal abnormalities in human embryos that were obtain from cryopreserved oocytes was no different than that of control embryos (FISH analysis)

Slow freezing X Vitrification????

Lane and Gardner 2001:

- **Embryo development** following oocyte cryopreservation
- Superiority of VF over SF in every parameter measured
  - Survival rate
  - Fertilization rate
  - Blastocyst/oocyte
  - Blastocyst cell number

Cil et al. 2006:

- A meta-analytic comparison of oocyte vitrification success rate with slow freezing and the SART IVF data with unfrozen oocytes.
- Reports and literature - Jan 1986 - May 2006
- 34 reports of SF and 10 reports of VF
- Age/year matched SART success rate

	VF 1998-2005	SF 1996-2005	SART 1998-2003
FR	74,2% (637/859)	64,9% (2478/3818)	
Clin.preg. /thawed oocytes	4,5% (61/1354)	2,3% (153/3720)	
Clin.preg. /ET	45,5% (61/134)	20,6% (153/742)	50,8% (12836/25252)
Live birth/ET	41,2% (49/119)	15,6% (110/706)	43,9% (11076/25252)
IR	17,1% (81473)	10,1% (185/1828)	

MII : Overall Results

Slow freezing

attempts	Oocyte	Survival	Cleavage	Pregn	Implant/thawed oocytes *not per embryo tansferred*
625	4.727	2.715	1.301	53	57
		57%	48%	8%	1.2%

***Chen (1986** 1988), Van Uem (1997), Porcu (1997 1998 1999)), Borini (1998), ucker (1996 98), Polak (1998),Young (1998), Porcu (2000 Winslow (2001), Cha (1999), Wurfel (1999), Chen (2002), Chia (2002), Quintans (2002), Marina (2003), Boldt (2003), Fabbri (2004)*

Vitrification

attempts	Oocytes	Survival	Cleavage	Pregn	Implant/thawed oocytes *not per embryo transferred*
292	1.925	1.628	1.202	76	66
		85%	74%	26%	3.4%

***Cha (1998)**, Hong (1999), Kuleshova (1999), Chung (2000), Park (2000), Kuwayama (2000),Cher (2000), Yoon (2000), Wu (2001), Yoon (2003), Katayama (2003), Liebermann (2003), Kuwayama (2005), Kim (2005), Ruvalcaba (2005), Chian (2005), Lucena (2005), Okimura (2005)*

How to improve viability after vitrification?

- **Use various types of cryo-containers and vitrification methods** (Kuwayama – Cryotop)
- **Use less toxic CP** (Chian – DMSO → PROH)
- **Apply different types or concentrations of cryoprotectants – add CP in several steps** (Vandenzwalmer)
- **Use higher cooling rate – Slush Nitrogen** (Yoon)

- Compounds for high osmolarity of CPA
  - polymers (Ficoll, Dextran, PVP, hyaluronic acid, PVA)
  - protein (BSA)
  - sugar (sucrose, trehalose)
- Introduce cytoskeletal stabilizers - taxol and cytochalasin B

Kuwayama M. :
Highly efficient vitrification for cryopreservation of human oocytes and embryos: The Cryotop method

Theriogenology 67 (2007), 73-80.

Two-step equilibration in a vitrification solution
EG / DMSO + sucrose + Cryotop technique

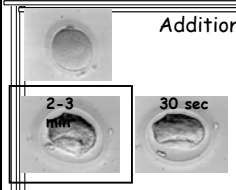
MII
- number of vitrified oocytes - 111
- survival rate - 95%
- fertilization rate (ICSI) - 91%
- development BC stage - 50%
- PR/ET (2.2 embryos/ET) - 41%

Chian :
High survival rates and pregnancies of human oocytes following vitrification: preliminary report (Fertil steril 2005)

- Replacement of DMSO by PROH
- Exposure solution PROH – EG 7.5 /7.5% 5 (one step) 37°C
- Vitrification solution (PROH – EG 15/15%)
- Cryoleaf
- Warming: Suc 1M 37°C 1 min, Suc 0.5 M 3 min
- Clinical pregnancy rate 44%

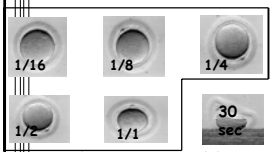
Vanderzwalmen P.:
Influence of the addition of cryoprotectant to the oocytes in one or several steps on the embryo development.

Addition of CP in one step or in several steps



Oocytes	metaphase II
·N°	81
·Intact after vitrification	64 (79%)
·2PN after ICSI	48 (75%)
·Cleavage on D3	32 (50%)
Grade A –B	7 (22%)
Grade C + ND	25 (78%)

10%DMSO-10%EG



Oocytes	metaphase II
·N°	47
·Intact after vitrification	38 (81%)
·2PN after ICSI	27 (71%)
·Clevage à J3	
Grade A –B	12 (44%)
Grade C + ND	15 (55%)

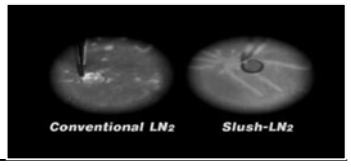
By doing steps addition of CP the maximum oocyte volume change was reduced.

Yoon:

Survival rate of human oocytes and pregnancy outcome after vitrification using slush nitrogen in assisted reproductive technologies (Fertil Steril 2007)

- Applying negative pressure with a vacuum → $LN_2 \Rightarrow SN_2$ – mixture of liquid and solid N_2
  - Lower internal temperature – 210°C without vaporization
  - SR 85,1%, FR 77,4%, PR/ET 43,3%, IR 14,2%

Liquid nitrogen (LN_2) *vs.* Slush nitrogen(SN_2)



Temperature	**-196°C**	**-210°C**
Vaporization	**Yes**	**No**
Cooling Rate	**-20,000°C / min**	**-135,000°C / min**
CPA concentration	**High**	**Low**
Cryo-damage	**low**	**very low**

Oocyte cryopreservation

- Children follow up
- Normal development and growth of the children born from frozen oocytes

Conclusion I

- Human oocytes – delicate architecture but freezable
- Healthy children have been born (chromosomally normal embryos originate from frozen oocytes)
- Constantly improving technique and results
- ⇒ Oocyte freezing should be considered as routine assisted reproductive technology when used appropriately

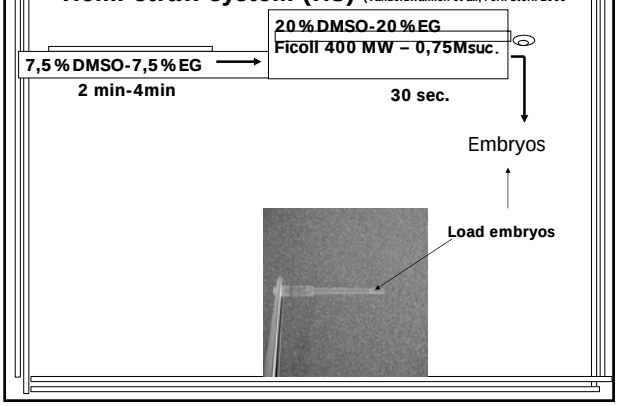
Conclusion II

- Oocyte **vitrification** results are more and more promising
- For consistent results and worldwide use:
  - Commercially available tools (inexpensive?)
  - Ready to use solutions
  - Easy to learn and apply efficient aseptic method

Czech vitrification experience

- Oocyte vitrification
- Blastocyst vitrification

Hemi-straw system (HS) (Vanderzwalmen *et al.*, Feril Steril 2000



Institute of reproductive medicine and endocrinology in 2006 (Pilsen, CZ)

• Blastocyst VF cycles	41
• No of BC thawed	133
• No of BC transfered	92
• Ø No BC/ET	2,2
• Survival rate	69%
• Clinical PR/ET	46% (19/41)
• IR	27% (25/92)

Aspects of storage of the female gamete

Josiane Van der Elst

Universitair Ziekenhuis Brussel
Vrije Universiteit Brussel
Centrum voor Reproductieve Geneeskunde

ESHRE, Prague, April 2007

Outline lecture

- Oocytes and ovarian tissue
- Cryobiological aspects
- Clinical application
- Ethical aspects
- Legal aspects

2

Oocyte and ovarian tissue

- Silent primordial follicles in stroma
- Initiation of growth
- Follicle and oocyte development
  - → primary, secondary, preantral, antral, Graafian follicles
  - → oocyte growth,maturation
- Ovulation of mature oocyte

3

Outline lecture

- Oocytes and ovarian tissue
- Cryobiological aspects
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- Ethical aspects
- Legal aspects

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Cryobiological aspects

- Oocyte = one-cell system
- Vulnerability
  - → Large cell (100 µm diameter)
  - → Low surface-to-volume ratio
  - → Zona pellucida
  - → Spindle
  - → Chromosomes attached

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Defining cryobiological problems : 80's

- low overall survival rate after freezing
- zona pellucida hardening reducing fertilisation rate
- disruption of meiotic spindle
- reports on genetic abnormalities
  - → polyploidy (non extrusion of 2nd polar body)
  - → aneuploidy

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Handling cryobiological problems: 90's

- Adaptation of freezing - thawing protocols
  - → PROH > DMSO
  - → PROH-high sucrose > PROH-low sucrose
  - → Recuperation time after thawing (2-3h) (Polscope)
  - → Choline substitution for Na
  - → Emerging vitrification > slow freezing
- Adaptation of fertilisation method
  - → ICSI to overcome zona hardening, after 1992

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New horizons: 2000 's

- Vitrification boom
- New cocktails of cryoprotectants (EG, PROH, sucrose)
- New freezing devices
  - → EM grids
  - → Cryoleaf
  - → Open pulled straw
  - → Cryotop
  - → Cryotip
  - → Vitriplug

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Outline lecture

- Oocytes and ovarian tissue
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Clinical application – Phase I – the 80's

- Original enthusiasm - beginners luck ?
  - → 1986, 87
  - → Quickly two Births after oocyte cryopreservation
  - → Using protocols for embryo cryopreservation
  - → not reproducible, discovery of problems

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Clinical application – Phase II – the 90's

- The hard times
  - → Embryo cryopreservation had become the method of choice worldwide
  - → Pronuclear oocyte freezing good results and acceptable as pre - embryo
  - → Few groups continuing on real mature oocyte freezing
  - → Driven by moral, legal and religious concerns
  - → Germany, Italy, South America

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Clinical application – Phase II – the 90's

- The hard times
- Time of PROH protocol improvements leading to better clinical outcome
  - Survival 55 - 60%
  - Fertilisation 60 - 65%
  - Pregnancy rate per transfer around 20%
  - Implantation rate per embryo < 10%
  - Implantation rate per thawed oocyte < 2 %

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Clinical application – Phase II – the 90's

- The hard times
- Time of first clinical vitrification reports emerging (Italy, Japan, Korea)
  - Higher survival rates 70 – 90 %
  - Pregnancy rate per transfer > 20%
  - Implantation rate per embryo > 10%
  - Implantation rate per thawed oocyte < 5 %

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Clinical application – Phase III – the 00's

- The new times
- Renewed interest in oocyte cryopreservation
  - → Large oocyte donor programmes
  - → Legal constraints
  - → Cancer survivors
  - → Technical bravoure?
  - → Wildlife ?

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Clinical application – Phase III – the 00's

- Larger clinical series being published
  - → vitrification and slow freezing
  - → catching up with embryo freezing
    - Increasing pregnancy rate (above 30 %)
    - Increasing implantation rate per embryo (> 20%) and per thawed oocyte (> 10%)
- Prospective randomised study between slow freezing and vitrification for different age groups lacking today

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Clinical application – children

- > 150 live births (Winslow)
- 1 congenital anomaly

  → ventricular septal defect)
- No intellectual or developmental deficit

  → N= 16 children

  → 3 y follow up

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Clinical application – indications 2007

- Female oncology patients
- Oocyte donor programmes –(quarantine)
- Objections against embryo freezing
- Unforeseen availability of oocytes (no sperm, rescue of insemination)

- Single women
- Delaying child bearing
- Escape of reproductive ageing
- Oocytes for research (cloning)

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Clinical application: round up

- Oocyte freezing is no first line treatment in daily ART practice today
- Place to be defined in patient - tailored reproductive medicine
- Still to be considered experimental treatment
- Oversight of institutional review board needed
- Children follow up needed

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Outline lecture

- Oocytes and ovarian tissue
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- Clinical application
- Ethical aspects
- Legal aspects

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Ethical aspects

- Oocyte banking: commercial aspects
- Legal enforcement
- Reproductive tourism
- Destination at reaching reproductive age limit
- Disposition in case of separation, divorce or abandonment
- Destination post-mortem
- Promise of young age: forever young!

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Outline lecture

- Oocytes and ovarian tissue
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- Clinical application
- Ethical aspects
- Legal aspects

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Legal aspects

- National laws
  - → Time limits for reproduction
  - → Oocyte donation allowed?
  - → Anonymity?
  - → Post-mortem?
- Claims
- European Cell and Tissue Directives

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Outline lecture

- Oocytes and ovarian tissue
- Cryobiological aspects
- Clinical application
- Ethical aspects
- Legal aspects

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Cryobiological aspects

- Follicle = unit of survival
- Different cell types
  - → Oocyte
  - → Granulosa cells
  - → Surrounding stroma cells
- Vulnerability
  - → Dissociation of cell types during freezing-thawing
  - → Developing follicles

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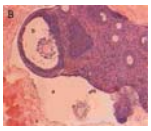
Cryobiological problems

- Ischaemia
  - → data from xenograft studies
  - → massive loss of follicles after transplantation

- Limited antral follicle development
- Oocyte quality (immaturity)
- Short term functioning of grafts



25

Outline lecture

- Oocytes and ovarian tissue
- Cryobiological aspects
- Clinical application
- Ethical aspects
- Legal aspects

26

Methodology

Laparoscopic ovary removal (one ovary)

- → Strip cortex (1-2mm) containing reserve of oocytes
- → Cut into 5x5 mm pieces
- → Computer controlled slow freezing
- → After thawing majorily primordial follicles survive
- → Taking into account follicular kinetics (Gougeon) the earliest expected follicular growth from primordial follicles is around 4- 5 months post transplantation

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Clinical application

- Autologous grafting
  - → Orthotopic sites
  - → Heterotopic sites
- Follow-up by echography, hormone levels

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Clinical application

Orthotopic autologous transplantation

- Oktay, 2000
  - 2x hMG stimulation
  - 2x ovulation
- Radford, 2001
  - one dominant follicle after 7 months
- Schmidt, 2005
  - 3 patients
  - IVF
  - ET in two patients, no pregnancies

29

Clinical application

Heterotopic autologous transplantation

- Oktay, 2004
  - Abdominal subcutis
  - 8 ICSI attempts, 20 oocytes, 1 four cell embryo, no pregnancy
- Wolner – Hanssen, 2005
  - Fore -arm
  - Two follicles, growth ended
- Demeestere, 2006
- Rosendahl, 2006

30

Clinical application

Orthotopic autologous transplantation

- Donnez, 2004
  - Hodgkin
  - Transplant to peritoneum below hilus remaining ovary
  - Spontaneous pregnancy
- Meirow, 2005
  - Non -Hodgkin
  - Transplant under cortex remaining ovary
  - Pregnancy after IVF

Baby Tamara

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Clinical indications 2007

- Female oncology patients
  - → Type of cancer, severity and prognosis
  - → Pre - or post - pubertal
  - → Age limit
  - → Existing children
  - → Psychological support
- Non malignant disease
  - → Turner syndrome
- Escape of reproductive ageing?

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Outline lecture

- Oocytes and ovarian tissue
- Cryobiological aspects
- Clinical application
- Ethical aspects
- Legal aspects

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Ethical aspects

- Safeguarding fertility: reality versus hope
- No dangerous delay of cancer treatment
- Risk of reintroduction of malignancy
- Psychological support
- Destination of tissue post-mortem (delicate)
- Use for oocyte donation allowed (unconditional altruism)
- Minor children(co-decision of parents or guards)

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Outline lecture

- Oocytes and ovarian tissue
- Cryobiological aspects
- Clinical application
- Ethical aspects
- Legal aspects

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Legal aspects

- National law
- European Cell and Tissue Directives
- Minor children

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ESHRE SIG EMBRYOLOGY COURSE:
"Cryobiology and cryopreservation of human gametes and embryos"
Prague April 13th – 14th, 2007

Biological and physiological aspects of the cryopreservation of human oocytes

Laura Rienzi

Center for Reproductive Medicine
European Hospital – Roma

Gamete cryopreservation

Significant differences exist in the ability to store male and female gametes (extreme flexibility for males, severe restrictions for females).

Considerable efforts have been expended in oocyte cryopreservation.

Slow freezing (in spite of its limitation) has become standarized with a considerable industrial and commercial background.

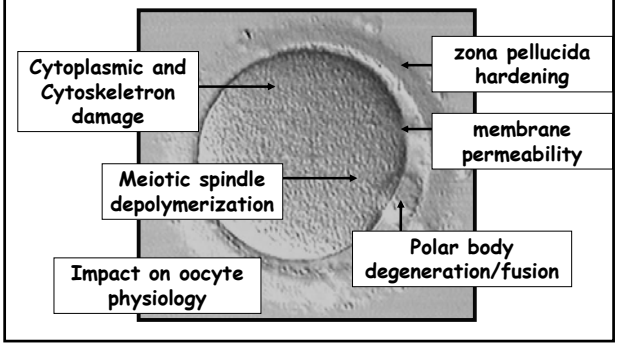
Oocyte cryopreservation

Successful pregnancies have been reported by different centers after frozen oocyte-derived embryo transfer.

The viability of the oocytes after thawing and of the competence of the deriving embryos have been highly variable, and in general have been reported to be relatively poor.

There is a pressing need to develop efficient way to cryopreserve oocytes.

Possible injuries to the cell during cryopreservation



PRINCIPLES OF SLOW FREEZING

1. Low levels of cryoprotectants.
2. Slow controlled rates of cooling.
3. Slow dehydration of cells to minimize ice crystal formation and damage.

Author	Study	Patients	Clinical pregncncies	Abortions	Children born	Live births	Freezing protocol
Chen 1986	IVF	7	2	0	2	2	SF DMSO
Chen, 1988	IVF	2	2	0	1	1	SF DMSO
Van Uem, 1987	IVF	2	2				O
Siebzehnruebl, 1989	IVF	10	2				0.1M
Porcu, 1997	ICSI	1	1				0.1M
Polak de Fried, 1998	ICSI	1	1				0.1M
Wurfel, 1999	ICSI	1	1	0	2	1	SF PrOH 0.1M
Porcu, 1999	ICSI	96	16	3	16	13	SF PrOH
Porcu, 1999	ICSI	1	1	0	2	2	SF PrOH
Porcu, 2000	ICSI	23	3	0	3	3	SF PrOH
Winslow, 2001	ICSI	33	12	2	16	10	SF PrOH
Kyono, 2001	ICSI	1	1	0	1	2	SF PrOH
Chen, 2002	ICSI	1	1	0	2	2	SF PrOH 0.3M
Quintans, 2002	ICSI	12	6	4	2	2	SF choline
Fosas, 2003	ICSI	7	4	0	2	2	SF PrOH 0.3M
Boldt, 2003							SF choline 0.3/0.5M
Borini, 2004							SF PrOH 0.1M
Huttelova, 2004							SF PrOH
Miller, 2004							SF PrOH
Notrica, 2004							SF PrOH 0.1 M
Chen, 2004	ICSI	1	1	0	1	2	SF PrOH 0.1/0.3M
Chen, 2005	ICSI	21	7	0	8	7	SF PrOH 0.3M
Levi Setti, 2005	ICSI	1	1	0	1	2	SF PrOH 0.3M
Tjer, 2005	ICSI	1	1	0	1	2	SF PrOH 0.3M
Borini, 2006	ICSI	146	18	3	15	15	SF PrOH 0.1M
Levi Setti, 2006	ICSI	120	18	6	13	13	SF PrOH 0.3M

…only 5 live births before ICSI

107 live births after 10 years…

SLOW FREEZING

Variable	Slow Freezing literature 1996-2004	Fresh oocytes Cornell University
Age, mean	33.7	33.6
Fertilization rate	64.9 (2,478/3,818)	76.7 (2,788/3,637)
Clinical pregnancies per thawed oocyte	0.023 (153/6720)	-
Clinical Pregnancies per injected oocytes	0.040 (153/3818)	0.075 (272/3637)
Clinical Pregnancies per transfer	20.6 (153/742)	68.5 (272/397)
Implantation rate	10.1 (185/1828)	39.8 (436/1095)

Oktay *et al.*, 2006

Italian experience
Multicentre study

Cycles	1292
Frozen oocytes	9584
Thawed oocytes	5210
Survived oocytes	2821
Injected oocytes	2504
Fertilized oocytes	1677
Obtained embryos	1481
Pregnancies	86

~ 60.6 thawed oocytes to obtain 1 pregnancy

Scaravelli, Istituto Superiore di Sanità, Rome - Italy

Italian experience
Multicentre study

Sucrose Concentration	Survival Rate	Fertilization Rate	Cleavage Rate	Implantation Rate
0,1 mol/l	39.6	58.8	88.0	9.6*
0,3 mol/l	66.5*	71.4	88.5	4.6
Total	54.1	67.0	88.3	5.8

Sucrose Concentration	Transfers	Pregnancy	Pregnancy/ Transfer	Pregnancy/ Cycles
0,1 mol/l	212/479	40	18.9	8.4
0,3 mol/l	441/813	46	10.4	5.7
Total	653/1292	86	13.2	6.7

Scaravelli, Istituto Superiore di Sanità, Rome - Italy

PRINCIPLES OF VITRIFICATION

1. High levels of cryoprotectants.
2. Extremely fast rates of cooling.
3. No ice crystal formation or damage; straight to a glass.

VITRIFICATION

Author	Study	Patients	Clinical pregncncies	Abortions	Ongoing pregnancies	Gestational sacs	Freezing protocol
Kuleshova, 1999	ICSI	4	1	0	1	1	VF
Cha, 1999	ICSI	1	1	0	1	1	VF
Yoon, 2003	ICSI	34	6	0	7	6	VF
Katayama, 2003	ICSI	2	2	0	2	2	VF
Chian, 2005	ICSI	25	11				EAF
Kim, 2005	ICSI	13	7				ds
Ruvalcaba, 2005	ICSI	NA	8				
Okimura, 2005	ICSI	NA	12				
Lucena, 2006	ICSI	73	13				OP
Kuwayama, 2005	ICSI	29	12				OP
Kyono, 2005	ICSI	1	1	0	1	1	VF CRYOTOP
Selman, 2006	ICSI	7	2	0	2	2	VF OPS

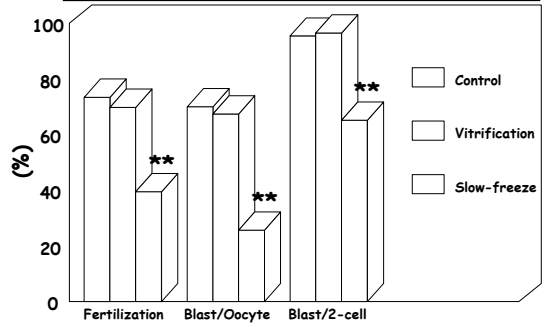
36 live births
30 ongoing pregnancies
in 3 years…

VITRIFICATION

Variable	Fresh oocytes Cornell University	Vitrification literature 2003-2005
Age, mean	33.6	32.3
Fertilization rate	76.7 (2,788/3,637)	74.2 (637/859)
Clinical pregnancies per thawed oocyte	-	4.5×10^{-2} (61/1354)
Clinical Pregnancies per injected oocytes	7.5×10^{-2} (272/3637)	7.2×10^{-2} (61/859)
Clinical Pregnancies per transfer	68.5 (272/397)	45.5 (61/134)
Implantation rate	39.8 (436/1095)	17.2 (81/473)

Oktay *et al.*, 2006

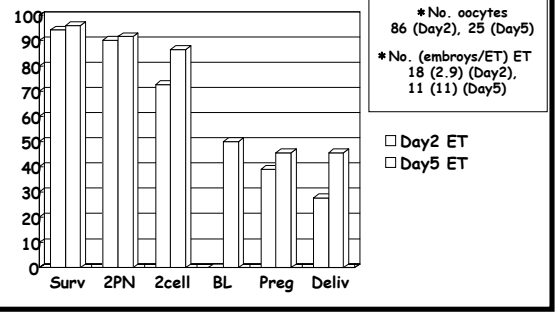
CRYOPRESERVATION OF MOUSE OOCYTES



Lane and Gardner, 2001

VITRIFICATION

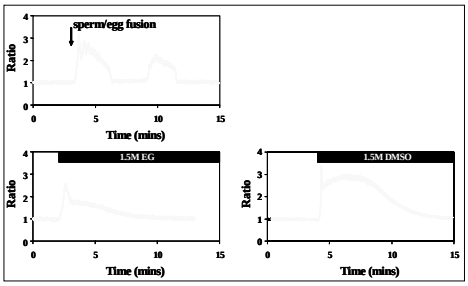
Masa Kuwayama, ESHRE, 2006



Factors that may influence the efficiency of oocyte cryopreservation

1) CRYOPROTECTANTS and intracellular Ca^{2+}
2) TEMPERATURE and meiotic spindle
3) CRYOPRESERVATION PROTOCOL and oocyte metabolism
4) CRYOPRESERVATION PROTOCOL and oocyte protein profile

Effect of cryoprotectants on intracellular Ca2+



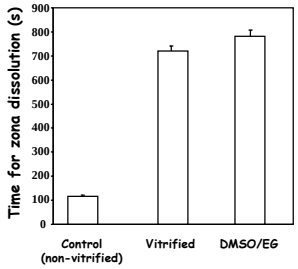
Larman et al., 2006

VITRIFICATION: cryoprotectants

Increase in intracellular Ca2+ triggers activation:

- ✔ Block to polyspermy
- ✔ Completion of meiosis and start of mitotic divisions
- ✔ Down-regulation of cell cycle proteins
- ✔ Apoptosis

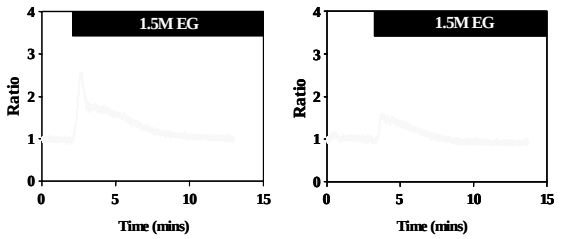
Increase in Ca2+ and zona hardening



Larman et al., 2006

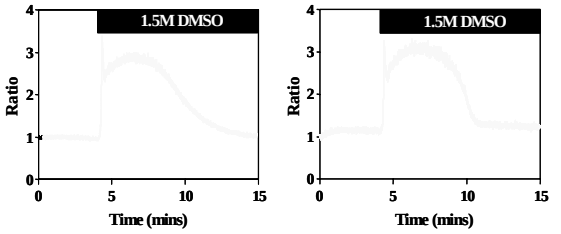
n = greater than 60 oocytes per treatment with 3 replicates

Effect of removing extracellular Ca2+ on EG challenge



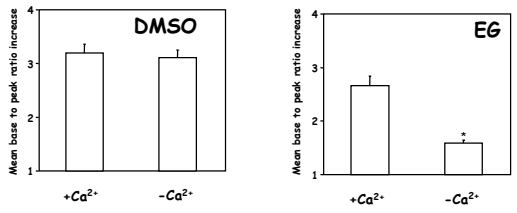
Larman et al., 2006

Effect of removing extracellular Ca2+ on DMSO challenge



Larman et al., 2006

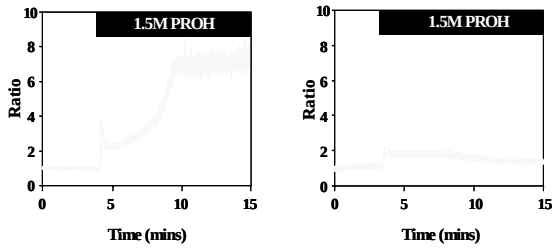
Effect of removing extracellular Ca2+ on cryoprotectant-induced Ca2+ release



n = 12 for each treatment with 4 replicates *; p= <0.01

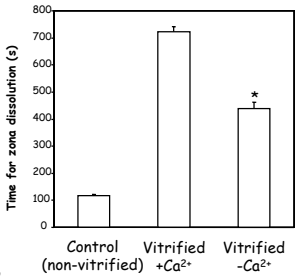
Larman et al., 2006

Propanediol increases intracellular Ca2+



Larman et al., 2006

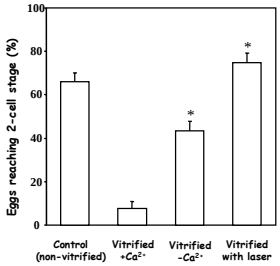
Removing extracellular Ca2+ reduces zona hardening



Larman et al., 2006

n = greater than 60 oocytes per treatment with 3 replicates *; p = <0.01

Removing extracellular Ca2+ increases fertilization rates



Larman et al., 2006

n = greater than 200 oocytes per treatment with 3 replicates *; p = < 0.01

Factors that may influence the efficiency of oocyte cryopreservation

2) TEMPERATURE and meiotic spindle

Meiotic Spindle analysis *SLOW FREEZING*

AIM OF THE STUDY

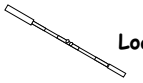
Non invasive visualization (Polscope system) of the meiotic spindle in living human oocytes to follow the behavior at different steps of the freezing and thawing procedures.

SOURCE OF OOCYTES

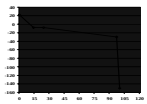
Only supernumerary fresh MII human oocytes with a detected meiotic spindle obtained after ovarian stimulation from consenting patients undergoing ICSI cycles were included.

Rienzi *et al.*, Human Reproduction, 2004

FREEZING PROCEDURE				
SOLUTIONS	Culture Medium	PBS	1.5 M PrOH	1.5 M PrOH + 0.1 sucrose
TEMPERATURE	37°C	RT	RT	RT



Loading and cooling



THAWING PROCEDURE					
SOLUTIONS	1.0 M PrOH + 0.1 M sucrose	0.5 M PrOH + 0.1 M sucrose	0.1 M sucrose	PBS	Culture Medium
TEMPERATURE	RT	RT	RT	RT	37°C

Meiotic Spindle analysis
SLOW FREEZING

Freezing procedure

During the freezing procedure, the meiotic spindle remained detectable in all of these oocytes (n°=56) up to the end of incubation in freezing solution.

The meiotic spindle signal intensity increased progressively during oocyte dehydration.

Meiotic Spindle analysis
SLOW FREEZING

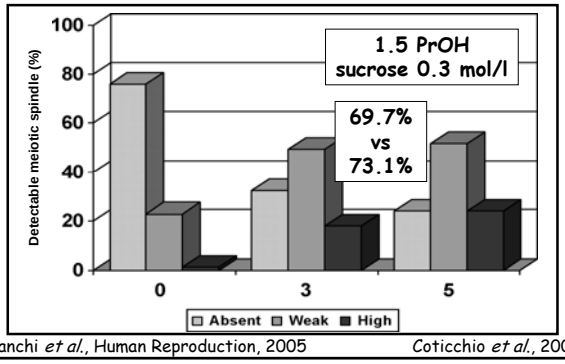
Thawing procedure

Meiotic Spindle	1.0 M PrOH+0.1 M sucrose	0.5 M PrOH+0.1 M sucrose	0.1 M sucrose	PBS	Medium 37°C
Detected	10 31.3%	8 25%	0 0%	0 0%	32 100%
Not detected	22 68.7%	24 75%	32 100%	32 100%	0 0%

At the end of the thawing procedure there was not a single survived oocyte in which the meiotic spindle could be detected.

The meiotic spindle reappeared again after incubation at 37°C in culture medium.

Meiotic Spindle analysis
SLOW FREEZING



Bianchi *et al.*, Human Reproduction, 2005 Coticchio *et al.*, 2006

Meiotic Spindle analysis
SLOW FREEZING

These studies have shown that slow freezing brings disruption of the oocyte meiotic spindle.

All meiotic spindles of cryopreserved metaphase II oocytes are products of de novo assembly in the post thaw period (both protocols: sucrose 0.1 and 0.3 mol/l).

Three hours of in vitro culture are necessary to obtain a good signal intensity.

Nevertheless, increased sucrose concentration seems to better preserve the meiotic spindle configuration.

Meiotic Spindle analysis
VITRIFICATION

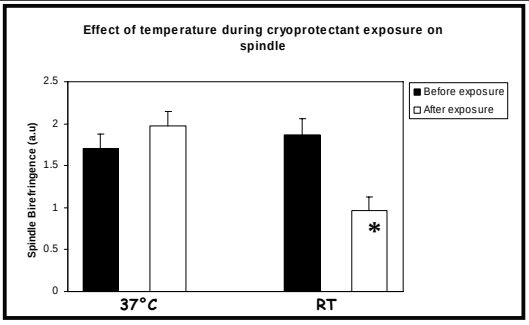
MEIOTIC SPINDLE AND VITRIFICATION TEMPERATURE:

Non invasive visualization of the meiotic spindle in living mouse oocytes following cryoprotectant exposure at room temperature (RT) and 37°C.

Non invasive visualization of the meiotic spindle in living mouse and human oocytes following vitrification procedure performed at 37°C.

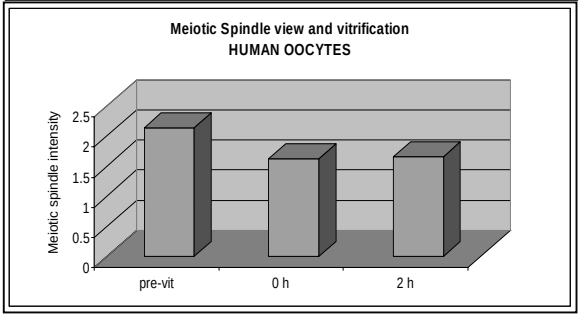
Colorado Centre for Reproductive Medicine & European Hospital Rome

Meiotic Spindle analysis
VITRIFICATION



Larman, personal comunication

Meiotic Spindle analysis *VITRIFICATION*



Rienzi et al., personal observation

Factors that may influence the efficiency of oocyte cryopreservation

3) CRYOPRESERVATION PROTOCOL and oocyte metabolism

OOCYTE METABOLISM post-cryopreservation

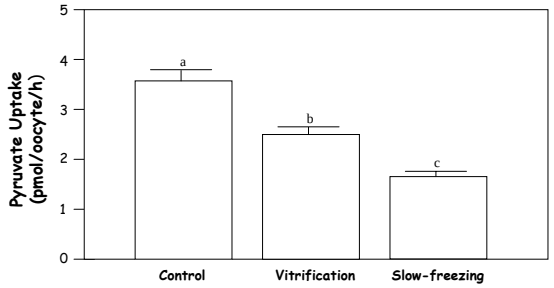
METABOLISM MONITORING THROUGH PYRUVATE UPTAKE (mouse oocytes):

Mouse oocytes and developing embryos following slow freezing were metabolically impaired compared with those that were vitrified

…although vitrification was also associated with a decrease in nutrient utilization by the oocyte compared to controls the decrease was significantly smaller than that induced by slow freezing.

Lane and Gardner., 2001; Lane et al., 2002

OOCYTE METABOLISM post-cryopreservation



Lane and Gardner., 2001;

Factors that may influence the efficiency of oocyte cryopreservation

4) CRYOPRESERVATION PROTOCOL and oocyte protein profiles

OOCYTE PROTEIN PROFILE post-cryopreservation

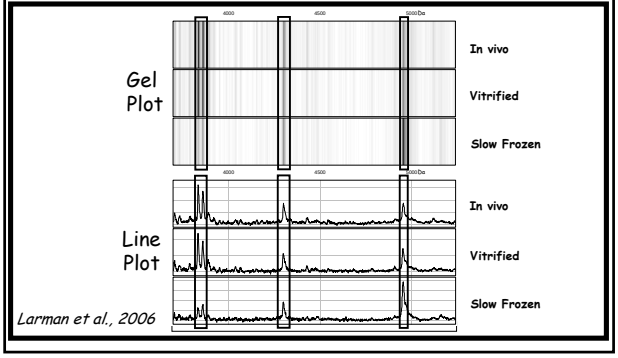
PROTEOMIC ANALYSIS OF OOCYTE PROTEIN PROFILES (mouse oocytes) by SELDI-TOF MS:

Mouse oocytes following slow freezing revealed major alterations compared with those that were vitrified.

Vitrified oocyctes appeared to be similar to the non-cryopreserved control oocytes...

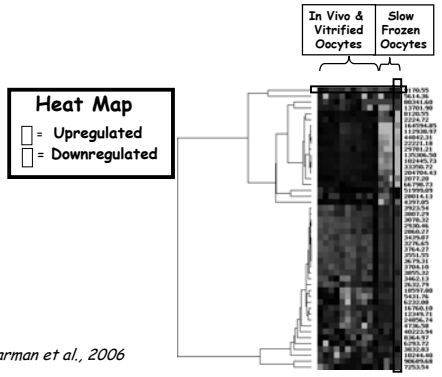
Larman et al., 2006

OOCYTE PROTEIN PROFILE post-cryopreservation



Larman et al., 2006

Hierarchical Clustering of Anionic Protein Profile



Larman et al., 2006

OOCYTE CRYOPRESERVATION
CONCLUSIONS

- High survival rate can be obtained with the actual oocyte cryopreservation protocols (~90% with vitrification procedure and ~60% with slow freezing procedure). Deriving embryo viability has still to be determined.

- Vitrification is fast and is a relatively easy technique (~150 sec *vs* ~100 min for slow freezing).

- Vitrification seems to expose the oocytes to lower risk for the meiotic spindle, to less stress on the cell metabolism and no relevant changes in protein profiles are reported.

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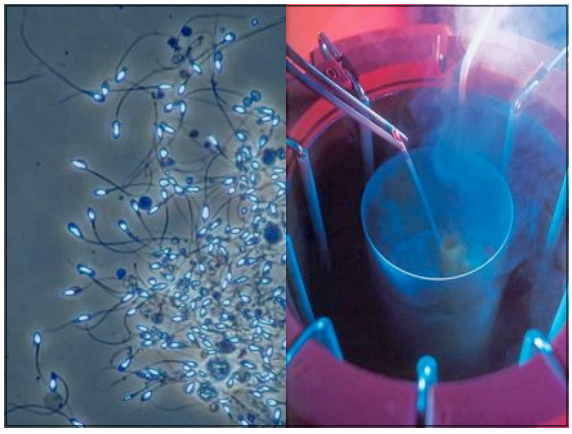
Clinical aspects of the cryopreservation of the human male gamete

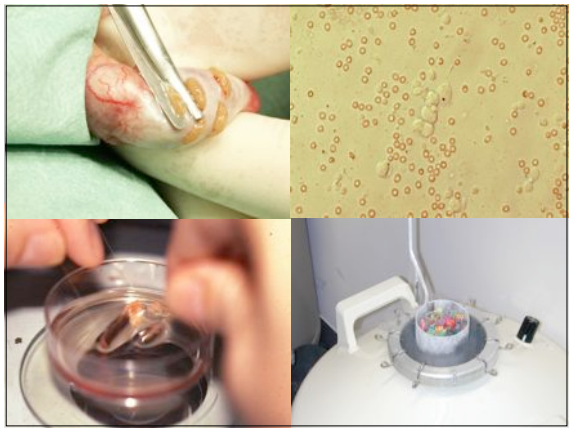
Herman Tournaye, M.D. Ph.D.
Centre for Reproductive Medicine Brussels

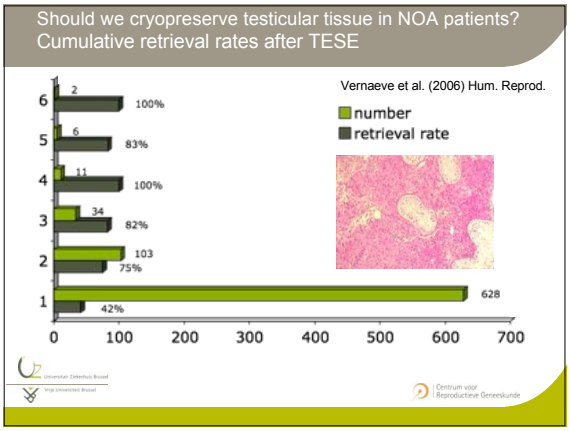


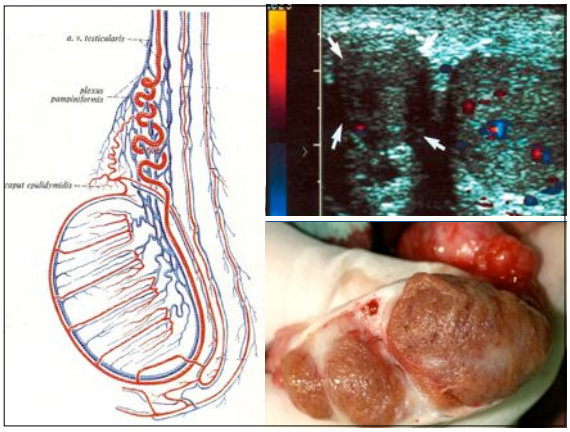
Cryopreservation of ejaculated spermatozoa

Well-established and accepted strategy

- to quarantine donor semen
- to preserve fertility potential before starting gonadotoxic treatments
- to ensure availability of sperm for ART (cryptozoospermia, business men, ….)




Should we cryopreserve testicular tissue in NOA patients?
Cumulative retrieval rates after TESE
Vernaeve et al. (2006) Hum. Reprod.
number
retrieval rate
6
5
4
3
2
1
2
100%
6
83%
11
100%
34
82%
103
75%
628
42%
0
100
200
300
400
500
600
700



Human Reproduction Vol.19, No.12 pp. 2822–2830, 2004
Advance Access publication October 18, 2004

Should diagnostic testicular sperm retrieval followed by cryopreservation for later ICSI be the procedure of choice for all patients with non-obstructive azoospermia?

G.Verheyen[1], V.Vernaeve, L.Van Landuyt, H.Tournaye, P.Devroey and A.Van Steirteghem

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[1]To whom correspondence should be addressed. E-mail: greta.verheyen@az.vub.ac.be

BACKGROUND: This was a retrospective study to determine if diagnostic testicular biopsy followed by cryopreservation should be the procedure of choice for all patients with testicular failure. **METHODS:** The first part of the study analysed 97 ICSI cycles scheduled with frozen–thawed testicular sperm for 69 non-obstructive azoospermia (NOA) patients. The second part focused on a subgroup of 32 patients who underwent 42 ICSI cycles with frozen and 44 cycles with fresh testicular sperm. Sperm characteristics, fertilization, embryo quality, pregnancy and implantation rates were evaluated. **RESULTS:** Part I: The average time needed to find sperm was 113 min per cycle and 17 min per individual sperm. Fertilization rate, embryo transfer rate, ongoing pregnancy and implantation rates were 58.4%, 83%, 20.8% and 11.3%, respectively. Part II: The search time per sperm was higher ($P = 0.016$) in frozen (18 min) than in fresh suspensions (13 min). A higher embryo transfer rate was observed in fresh cycles than in frozen cycles (93.2% vs 76.2%, $P = 0.028$). Fertilization, ongoing pregnancy and implantation rates were comparable for the two groups. **CONCLUSIONS:** Even in a programme with low-restrictive criteria for patient allocation and for sperm cryopreservation, diagnostic testicular biopsy followed by cryopreservation can be the procedure of choice for patients with testicular failure.

Should we cryopreserve testicular tissue in NOA patients?

Table V. Comparison of sperm characteristics in the ICSI cycles with fresh (44 cycles) and frozen (42 cycles) testicular sperm of 32 non-obstructive azoospermia (NOA) patients

	Fresh TESE	Frozen TESE	Mann–Whitney
Cycles	44	42	
Search time/cycle (min)	81	110	$P = 0.053$
Search time/sperm (min)	13	18	$P = 0.016$
% oocytes injected with motile sperm	82.3	83.7	NS
Cycles injected with only motile sperm (%)	33/44 (75)	31/42 (74)	NS[a]
Cycles injected with only immotile sperm (%)	3/44 (7)	4/42 (10)	NS[a]
COC/cycle	10.5 ± 6.2	9.3 ± 5.2	NS
Metaphase II/cycle	9.1 ± 5.8	7.6 ± 4.2	NS
% 2PN	58.0 ± 24.2	59.3 ± 25.5	NS
% 1PN	7.0 ± 11.0	7.8 ± 19.2	NS
% ≥ 3PN	3.6 ± 8.3	1.9 ± 4.9	NS

[a]Chi-square test.

Verheyen et al. 2004 Hum Reprod

Should we cryopreserve testicular tissue in NOA patients?

Table VI. Results of embryo transfer, pregnancy and implantation rates after ICSI with fresh (44 cycles) and frozen (42 cycles) testicular sperm of 32 non-obstructive azoospermia (NOA) patients

	Fresh TESE	Frozen TESE	Chi-square
Cycles	44	42	
Transfers (%)	41 (93.2)	32 (76.2)	$P = 0.028$
Embryos/ET	2.6	2.5	NS
Pos hCG/cycle (%)	9/44 (20.4)	8/42 (19.0)	NS
Pos hCG/ET (%)	9/41 (21.9)	8/32 (25.0)	NS
Clinical PR/cycle (%)	7/44 (15.9)	6/42 (14.3)	NS
Clinical PR/ET (%)	7/41 (17.1)	6/32 (18.7)	NS
Implantation rate (%)	8/105 (7.6)	6/81 (7.4)	NS

ET, embryo transfer; Pos, positive; PR, pregnancy rate.

Verheyen et al. 2004 Hum Reprod

Journal of Andrology, Vol. 26, No. 6, November/December 2005

Severe Hypospermatogenesis in Cases of Nonobstructive Azoospermia: Should We Use Fresh or Frozen Testicular Spermatozoa?

RON HAUSER,* LEAH YOGEV,* AMI AMIT,† HAIM YAVETZ,* AMNON BOTCHAN,* FUAD AZEM,* JOSEPH B. LESSING,* AND DALIT BEN-YOSEF†

*From the *Institute for the Study of Fertility and the †IVF Unit, Lis Maternity Hospital, Tel Aviv Sourasky Medical Center, Sackler Faculty of Medicine, Tel Aviv University, Israel.*

ABSTRACT: The aim of this comparative clinical study was to examine whether the fertilizing potential of frozen-thawed testicular sperm in the most severe cases of hypospermatogenesis is reduced compared with fresh testicular sperm. The results could determine the necessity of using fresh testicular sperm cells, which mandates involving the spouse by performing simultaneous in vitro fertilization intracytoplasmic sperm injection (IVF-ICSI) treatment in this subgroup of nonobstructive azoospermia (NOA) patients. We studied 13 couples in which the husband was diagnosed as having NOA and few motile testicular sperm cells or only immotile testicular sperm cells were isolated by testicular sperm extraction (TESE). Each couple underwent both an ICSI cycle, in which fresh testicular sperm that were retrieved shortly beforehand were injected, and a consecutive cycle, which used frozen-thawed sperm that were retrieved in the original TESE procedure but were cryopreserved and stored until use. We found that motility was lost during the freezing and thawing process in some cases, which resulted in significantly more cycles with only immotile sperm cells for injection in the frozen-thawed sperm group (38.5%) than in the fresh sperm group (7.7%; $P < .05$). Availability of only immotile sperm cells significantly reduced fertilization rates in both fresh and frozen-thawed groups, but the respective overall fertilization rate (44.9% vs 41.1%) and quality of embryos and pregnancy rate (18.2% vs 15.4%) were not significantly different between groups. Implantation rates were more favorable in the fresh sperm group (10.5% vs 5.9%), but not significantly so. We conclude that, although cryopreservation does impair motility, which results in significantly more cycles with only immotile sperm cells for ICSI in the most severe forms of hypospermatogenesis, fertilization and pregnancy rates are not significantly compromised.

Key words: Testicular sperm extraction, fertilization, implantation, IVF-ICSI.

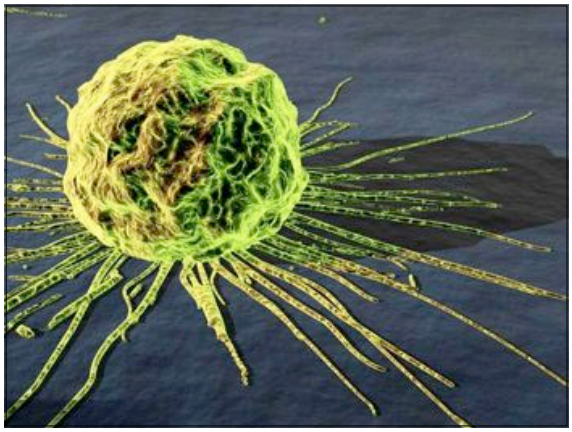
J Androl 2005;26:772–778

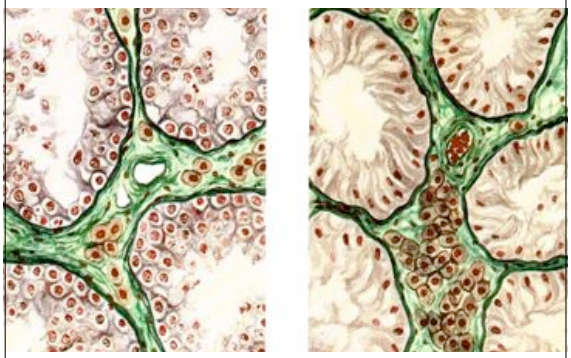
Should we cryopreserve testicular tissue in NOA patients?

Yes, we should

- similar outcome after ICSI
- but…works for 4 out of 5 patients
- counsel your patient towards back-up TESE

Verheyen et al. 2004 Hum Reprod





Azoospermia at diagnosing cancer

Variable prevalence: 3 - 100 %

Chapman et al. 1981:	14 / 14 (100%)
Lass et al. 1998:	40 / 231 (17%)
Kelleher et al. 2001:	31 / 930 (3.3%)

ADULT UROLOGY

"ONCO-TESE": TESTICULAR SPERM EXTRACTION IN AZOOSPERMIC CANCER PATIENTS BEFORE CHEMOTHERAPY—NEW GUIDELINES?

M. SCHRADER, M. MÜLLER, N. SOFIKITIS, B. STRAUB, H. KRAUSE, M. SCHOSTAK, AND K. MILLER

TABLE I. *Patients with testicular germ cell tumors and azoospermia before chemotherapy*

Clinical Stage	Patients with Azoospermia (n)	Patients with Successful Sperm Retrieval (n)	Patients with Maturation Arrest (JS 3–5) (n)	Patients with SCOS (JS 1–2) (n)
I	2	2/2	0/2	0/2
IIA–IIB	8	3/8	3/8	2/8
>IIC	4	1/4	0/4	3/4

Key: JS = Johnsen score; SCOS = Sertoli cell-only syndrome. Histologic examination results according to clinical tumor stage; classification into clinical tumor stages followed World Health Organization guidelines.

ADULT UROLOGY

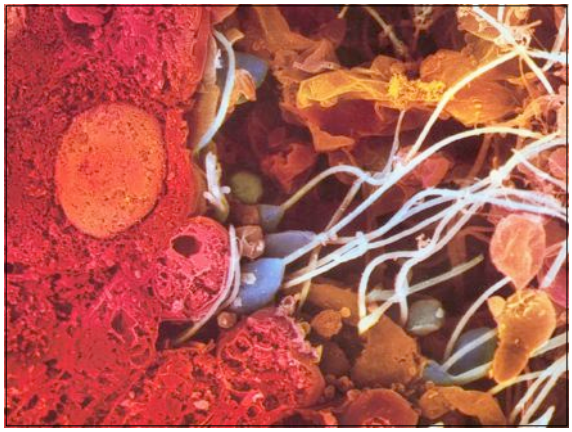
"ONCO-TESE": TESTICULAR SPERM EXTRACTION IN AZOOSPERMIC CANCER PATIENTS BEFORE CHEMOTHERAPY—NEW GUIDELINES?

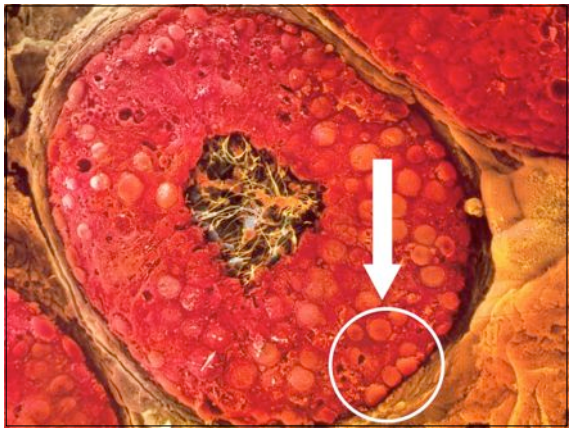
M. SCHRADER, M. MÜLLER, N. SOFIKITIS, B. STRAUB, H. KRAUSE, M. SCHOSTAK, AND K. MILLER

TABLE III. *Patients with Hodgkin's/non-Hodgkin's disease and azoospermia before chemotherapy*

Disease	Patients with Azoospermia (n)	Patients with Successful Sperm Retrieval (n)	Patients with Maturation Arrest (JS 3–5)	Patients with SCOS (JS 1–2)
Hodgkin's disease	7	3/7	2/7	2/7
Non-Hodgkin's disease	10	5/10	3/10	2/10

Key: JS = Johnsen score; SCOS = Sertoli cell-only syndrome. Histologic examination results according to the disease type.





Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 11298–11302, November 1994
Developmental Biology

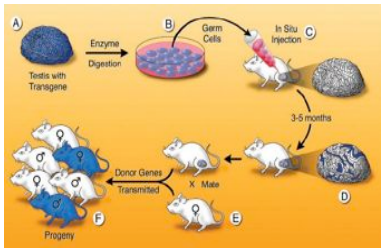
Spermatogenesis following male germ-cell transplantation

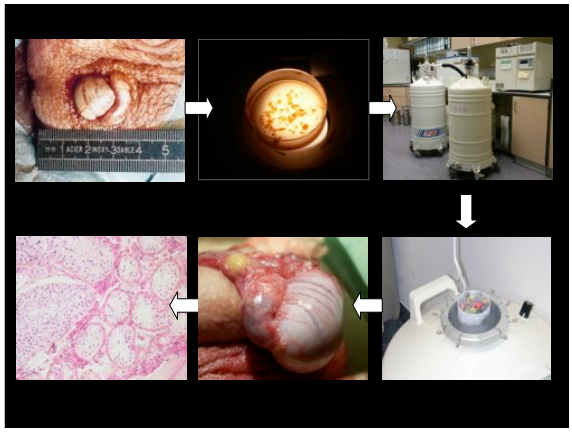
(spermatogonia/stem cells/testes/transgenic mice)

RALPH L. BRINSTER* AND JAMES W. ZIMMERMANN†

Laboratory of Reproductive Physiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA 19104

Contributed by Ralph L. Brinster, August 11, 1994





Cryopreservation of testicular tissue in adults

BMJ VOLUME 319 9 OCTOBER 1999 www.bmj.com

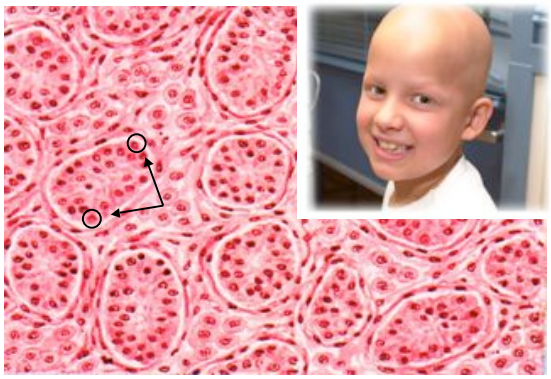
Fertility after treatment for cancer

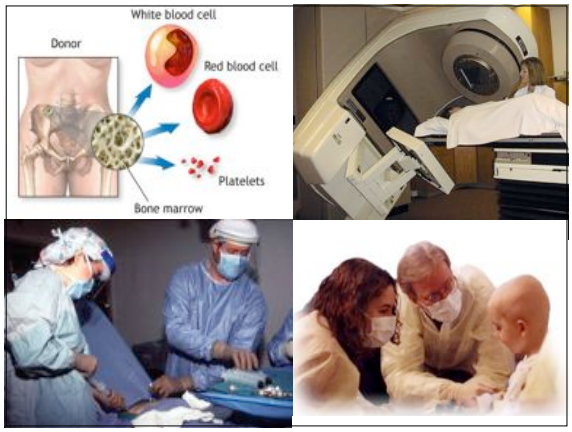
Questions remain over ways of preserving ovarian and testicular tissue

11 men have had testicular tissue harvested and cryopreserved as a single cell suspension (J A Radford et al, British Cancer Research meeting, Edinburgh, July 1999, and PF Brook et al, unpublished), and five who have now successfully completed treatment for cancer have had this material injected back into the donor testis. Results of follow up semen analysis are awaited with interest.

JA Radford *senior lecturer in medical oncology*
SM Shalet *professor of endocrinology*
Christie Hospital, Manchester M20 4BX

BA Lieberman *consultant gynaecologist*
St Mary's Hospital, Manchester M13 0JH





Where do we stand anno 2007 ?

- Technically feasable ?
  - transplantation protocol
  - storage protocol
- Reproductive efficiency ?
- Reproductive safety ?

Where do we stand anno 2007 ?

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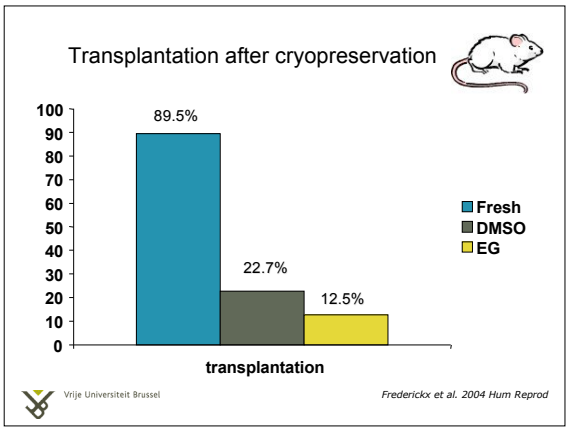
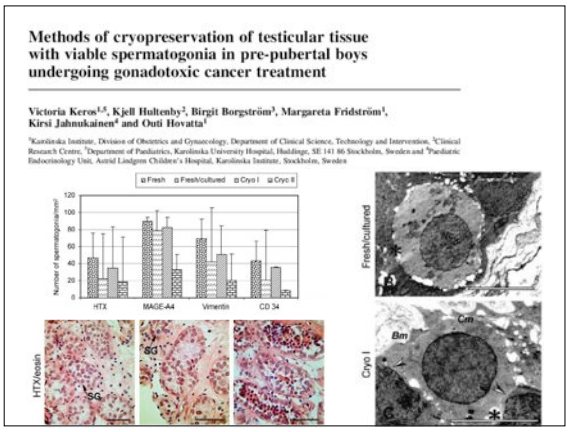
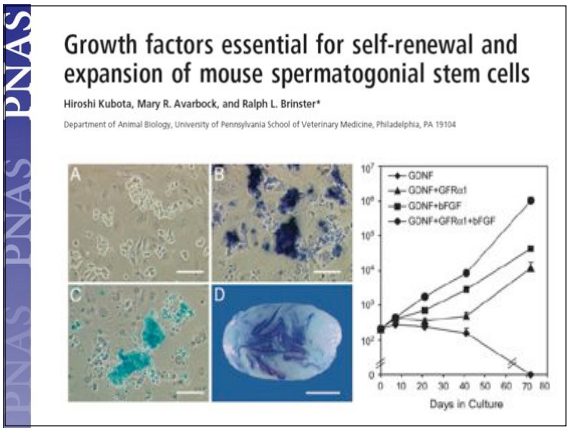

Transplantation after cryopreservation
100
90
80
70
60
50
40
30
20
10
0
89.5%
22.7%
12.5%
Fresh
DMSO
EG
transplantation
Vrije Universiteit Brussel
Frederickx et al. 2004 Hum Reprod


Methods of cryopreservation of testicular tissue with viable spermatogonia in pre-pubertal boys undergoing gonadotoxic cancer treatment
Victoria Keros[1,5], Kjell Hultenby[2], Birgit Borgström[3], Margareta Fridström[1], Kirsi Jahnukainen[4] and Outi Hovatta[1]
Fresh
Fresh/cultured
Cryo I
Cryo II
HTX
MAGE-A4
Vimentin
CD 34
HTX/eosin
Fresh/cultured
Cryo I
Cm
Bm


PNAS
Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells
Hiroshi Kubota, Mary R. Avarbock, and Ralph L. Brinster*
Department of Animal Biology, University of Pennsylvania School of Veterinary Medicine, Philadelphia, PA 19104
A
B
C
D
GDNF
GDNF+GFRα1
GDNF+bFGF
GDNF+GFRα1+bFGF
Days in Culture

Where do we stand anno 2007 ?

- Technically feasable ?
  - transplantation protocol
  - storage protocol
- Reproductive efficiency ?
- Reproductive safety ?

[CANCER RESEARCH 61, 706–710, January 15, 2001]

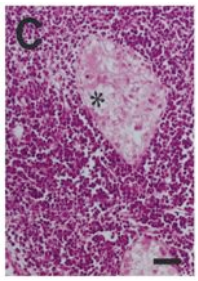
Intratesticular Transplantation of Testicular Cells from Leukemic Rats Causes Transmission of Leukemia[1]

Kirsi Jahnukainen,[2] Mi Hou, Cecilia Petersen, Brian Setchell, and Olle Söder

Pediatric Endocrinology Unit, Karolinska Institutet, Karolinska Hospital, 171 76 Stockholm, Sweden [K. J., M. H., C. P., B. S., O. S.], and Department of Pediatrics, University of Turku, 20520 Turku, Finland [K. J.]

ABSTRACT

A rat T-cell leukemia model was used to study the safety of germ cell transplantation as a mean of preventing infertility in males undergoing gonadotoxic cancer treatment. Donor germ cells were harvested from the testes of terminally ill leukemic rats and were either used directly or cryopreserved and thawed before transplantation by rete testis microinjection. All rats transplanted with testicular cells from leukemic donors developed signs of terminal rat T-cell leukemia, whereas control animals remained healthy. Cryopreservation of the donor germ cells caused a 3- to 6-day delay in the terminal phase of leukemia. When a known number of leukemic cells were mixed with germ cells and microinjected into the testis, the rate of appearance of terminal leukemia was directly related to the number of transferred leukemic lymphoblasts. As few as 20 leukemic cells were able to cause a cancer relapse resulting in terminal leukemia 21 days after transplantation in three of five transplanted animals. Our results demonstrate that germ cell transplantation with the presently used techniques is not safe enough for clinical use. Improved methods for purging testicular specimens of cancer cells or totally new approaches with transient xenogenetic host models to detect contamination of malignant cells must be developed before this techaique can be offered to patients without fear of disease relapse.

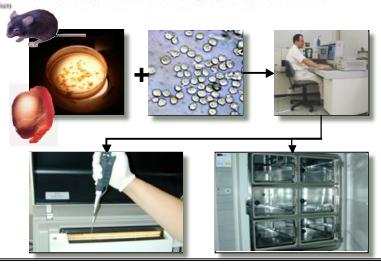


Human Reproduction Vol.22, No.3 pp. 733–742, 2007
Advance Access publication November 2, 2006

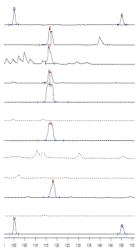
The efficiency of magnetic-activated cell sorting and fluorescence-activated cell sorting in the decontamination of testicular cell suspensions in cancer patients

M.Geens[1,3], H.Van de Velde[2], G.De Block[1], E.Goossens[1], A.Van Steirteghem[2] and H.Tournaye[2]

[1]Research Centre for Reproduction and Genetics, [2]Centre for Reproductive Medicine, University Hospital and Medical School, Vrije Universiteit Brussel, Brussels, Belgium



Malignant decontamination by FACS (HLA1-)



0,05% SB	HLA-type 1 positivity (%)		Tumorgrowth in culture (%)		PCR
patient	pre sort	post sort	pre sort	post sort	B-cell
1b	6.42	0.32	100	50	positive
2b	9.85	1.54	100	25	positive
3b	5.64	0.02	75	50	positive
4b	4.56	0.11	75	25	positive
5b	8.41	**0.00**	100	**0**	**negative**
6b	5.31	0.40	100	50	positive
average	**6.698**	**0.398**	**91.7**	**33.3**	

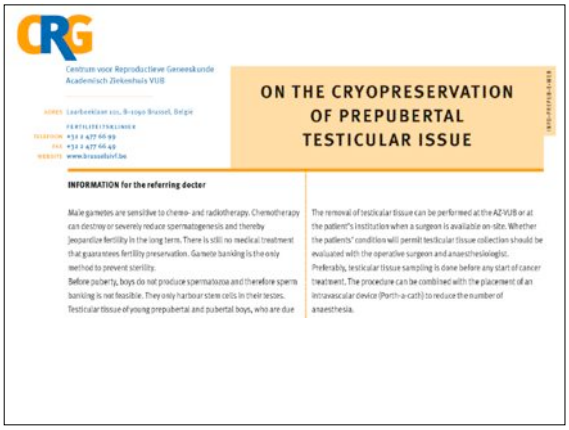
Clinical application Brussels

inquiries: n=13

accepted	n=5	sickle cell anemia (2) thalassemia major leukemia idiopathic aplastic anemia
refused	n=1	leukemia (pretreated)

parents declined after counselling

n=4	leukemia
n=2	sickle cell anemia
n=1	idiopathic aplastic anemia



Centrum voor Reproductieve Geneeskunde
Academisch Ziekenhuis VUB

ON THE CRYOPRESERVATION OF PREPUBERTAL TESTICULAR ISSUE

INFORMATION for the referring doctor

Male gametes are sensitive to chemo- and radiotherapy. Chemotherapy can destroy or severely reduce spermatogenesis and thereby jeopardize fertility in the long term. There is still no medical treatment that guarantees fertility preservation. Gamete banking is the only method to prevent sterility.
Before puberty, boys do not produce spermatozoa and therefore sperm banking is not feasible. They only harbour stem cells in their testes. Testicular tissue of young prepubertal and pubertal boys, who are due

The removal of testicular tissue can be performed at the AZ-VUB or at the patient's institution when a surgeon is available on-site. Whether the patients' condition will permit testicular tissue collection should be evaluated with the operative surgeon and anaesthesiologist.
Preferably, testicular tissue sampling is done before any start of cancer treatment. The procedure can be combined with the placement of an intravascular device (Porth-a-cath) to reduce the number of anaesthesia.

Testicular tissue banking in adolescents

- only if banking of ejaculate failed
- wet preparation before banking:

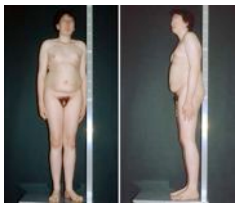
  freezing protocol for
  spermatozoa vs. testicular stem cells

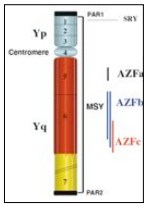
Testicular tissue banking in adolescents

Cell and Tissue Banking (2006) 7:105–112

Sperm cryopreservation in male infertility due to genetic disorders

Csilla Krausz* and Gianni Forti
Andrology Unit, Department of Clinical Physiopathology





When should we cryopreserve testicular tissue ?

- we should cryopreserve testicular tissue in ICSI candidates with non-obstructive azoospermia
- we may consider cryopreserving testicular tissue in boys and men undergoing sterilising treatments

- we should maybe cryopreserve testicular tissue in pubertal azoospermic Klinefelter's boys and young adults with Yq deletions or Klinefelter's syndrome

Centrum voor Reproductieve Geneeskunde

14

Storing in reproductive medicine and the EU Directives

Josiane Van der Elst

Universitair Ziekenhuis Brussel
Vrije Universiteit Brussel
Centrum voor Reproductieve Geneeskunde

ESHRE CampusPrague, April 2007

Outline lecture

- Political background
- Directive 2004/23/EC
- Directive 2006/17/EC (TD1)
- Directive 2006/86/EC (TD2)
- EACC

European Union

- The European Union was founded in 1957
- political treaties between the member states define strategy
- European directives are made in relation to these treaties
- Directives are made and decided upon by European Institutions

European Treaties

- Treaty of Rome (1957) (EEC)
  - → economic cooperation
  - → no formal basis for measures in field of public health
- Treaty of Maastricht (1992) (EU)
  - → European citizenship
  - → + defense, justice, public health

    Article 129: information, education, surveillance

4

European Treaties

- Treaty of Amsterdam (1999)
  - → + public health protection
  - → Article 152 (former article 129) provides legal tools to ensure a high level of human health protection
- EU Directive 2004/23/EC on tissues and cells fits in a larger EU framework of safeguarding public health

5

European Institutions

- The EU decision - making process involves three main institutions
  - → Council of Ministers
  - → European Parliament
  - → European Commission

6

How is EU law making done

- Three major steps
- Legislative initiative - Law proposal
  - → proposal prepared by the European Commission
- Legislative decision - making process
  - → the formal proposal is examined by the EP and the Council of Ministers
  - → Co-decision procedure
- Legislative execution
  - → EC orders transposition into national law
  - → EC follows up implementation

7

The making of 2004/23/EC

- Initiative
  - → Proposal for EU Tissues and Cells Directive 2002
- Co-decision EP and Council
  - → Law adopted in 2004 after Publication in Official Journal of EU on 7 April 2004
- Execution
  - → Comes into force on 7 April 2006
  - → Transposition into national laws by 7 April 2006
  - → Art. 28 : Technical directives to be developed through committees

8

Directive 2004/23/EC

2004/23/EC Mother Directive
Into force on 7 April 2006

2006/17/EC Technical directive 1 (TD1)
donation, procurement, testing
Into force on 1 November 2006

2007/86 / EC Technical directive 2 (TD2)
coding, processing, preservation, storage and distribution
Into force on1 September 2007

9

Outline lecture

- Political background
- Directive 2004/23/EC
- Directive 2006/17/EC (TD1)
- Directive 2006/86/EC (TD2)
- EACC

10

Directive 2004 / 23 / EC

A legal document on setting standards of quality and safety for the donation, procurement, testing, coding, processing, preservation, storage and distribution of human tissues and cells intended for human application including haematopoietic peripheral blood, umbilical- cord (blood) and bone-marrow stem cells, reproductive cells (eggs, sperm), foetal tissues and cells and adult and embryonic stem cells in order to prevent transmission of infectious diseases

11

Directive 2004/23/EC

- Tissue establishments (ART centres) have to fullfill all safety and quality criteria to comply with the directive and technical directives
- National competent authorities have to
  - → Set up licensing system for tissue establishments
  - → Organize inspections
  - → Report back to European Commission

12

Outline lecture

- Political background
- Directive 2004/23/EC
- Directive 2006/17/EC (TD1)
- Directive 2006/86/EC (TD2)
- EACC

13

Requirements of TD1

full donor documentation on donation, procurement, testing

→ voluntary

→ unpaid

→ informed consent

→ unique donor identification

→ medical history

→ laboratory testing results

14

Donor documentation

- all records entered into registry
- clear and readable
- protected
- accessible for authority
- to be kept for minimum 30 years

15

Laboratory testing

- Partner donation, direct use
  - → No testing necessary
- Partner donation (not direct use)
  - → Anti - HIV 1,2
  - → Hepatitis B
    - HBsAg
    - Anti-HBc
  - → Anti - HCV -Ab
    - Possible exception for intra uterine insemination

16

Laboratory testing

- Donation other than by partners (sperm, oocyte donors)
  - → Serological screening HIV, HBV, HCV
  - → + Syphilis
  - → + Chlamydia (sperm donors)
  - → Quarantine (sperm)
  - → genetic screening for autosomal recessive genes prevalent in the donor's ethnic background

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Cryopeservation

- Serology obliged when freezing and storing reproductive cells or embryos
- Separate storage obliged
  - → when positive tests
  - → When results unavailable at moment of storage
    - CBS system
    - Separate storage tanks for separate serological profiles

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Outline lecture

- Political background
- Directive 2004/23/EC
- Directive 2006/17/EC (TD1)
- Directive 2006/86/EC (TD2)
- EACC

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Requirements of TD 2

- Quality Management System
- Air quality
- Traceability
- Coding
- Notification of adverse reactions and events

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QMS

→ Quality Management System
- organization,
- personnel,
- facilities,
  - Air quality
- equipment
- documentation and records

→ Examples ISO 9001, 15189; HFEA Standards

→ EUTD > QMS

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Air quality requirements

Working zone: air quality A

Background : air quality D

Less stringent environment in case of:

- final process of product sterilisation foreseen
- air quality requirements detrimental effect on tissues or cells
- route of application to the body low risk of transmitting infection
- technical incompatibility

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Traceability

- donor ID
- donation ID
  - storage for at least 30 years
- Data on materials, reagents used that can influence safety and quality of tissues and cells such as media lot and batch numbers
  - storage for at least 10 years

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Coding

→ unique internal code always needed

→ unique European coding system

- only needed for non partner donation
- European coding system not yet decided on
- decision foreseen for 2007
- implementation for 2008

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Notification of adverse reactions - events

- definition of adverse reaction – adverse event
  - → relating only to transmission of disease?
  - → or also to quality and safety ?
    - e.g. OHSS; fertilisation failure?

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Outline lecture

- Political background
- Directive 2004/23/EC
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Is there help to listen and talk to Europe ?

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EACC: an ESHRE offer you can't refuse

- European Assisted Conception Consortium
- Joint venture between ESHRE and HFEA
  - → ESHRE = European Society for Human Reproduction and Embryology
  - → HFEA = Human Fertilisation and Embryology Authority (UK
- Not-for-profit initiative
- Established at ESHRE 2005 Copenhagen

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EACC Objectives

- Bring together IVF professionals and national authorities from European member states
  - To share learning and best practice
  - Provide advice to member states
- Communication to European Commission
  - → present joint position of regulators and practitioners
  - → give expert advice to EC

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EACC Members

Per member state / three members

→ Two practitioners
- one clinician
- one embryologist

→ One regulator

Please check ESHRE Website – link to EACC - membership:
check for members of your country; they are your representatives

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EACC Executive Committee

Five members

→ Three practitioners

- Anna Veiga
- Ioannis Messinis
- Josiane Van der Elst

→ Two regulators

- Angela McNAb (chair)
- Bernard Loty

New board to be voted for ESHRE 2007

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EACC activities untill now

Full Consortium meetings (for members)

ESHRE Public session

Meetings with European Commission

ESHRE Website

direct link EACC

EACC Newsletter

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