Universitair Ziekenhuis Gent

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Cryoinjuries on gametes and embryos

ESHRE Course, Lisbon Portugal, 2010

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AIMS

Everything you need to know (as a clinical embryologist) on gamete and embryo cryobiology and what you probably forgot …or what you did not know…

Consequences of embryo cryopreservation/injuries for the implantation potential of oocytes and embryos/safety

Oocytes and embryos: efficient cryopreservation programmes?

Dilemma

Which strategy is better for our patients: freezing or vitrification?

Specific aims

Specific learning objectives:

Avoiding injuries: understanding en knowing what you are doing

- Basic principles of freezing
- Basic principles of vitrification
- Efficiency and safety of freezing and vitrification

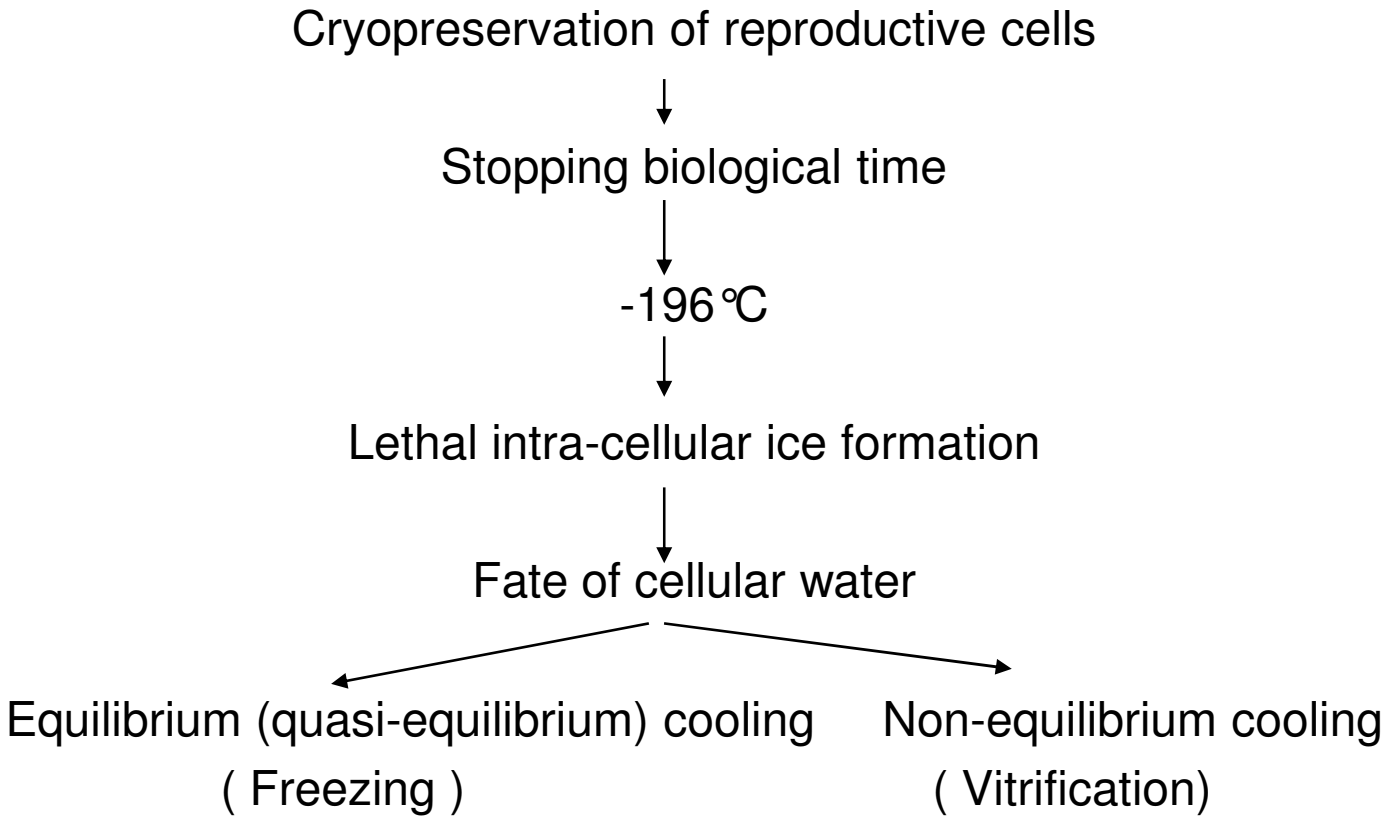
Situation in reproductive medicine

- Not all oocytes and embryos/blastocysts survive the cryopreservation procedure, in embryos and blastocysts often intact blastomeres coexist with damaged ones (Cellular injury)
  - **Embryos/blastocysts with cell loss implant less well than intact ones**
- Oocytes require ICSI to be fertilized
- Effects of cryopreservation on oocyte and embryo metabolism and physiology (Subcellular injury)
  - **Biological effects of cryopreservation**

It should be the aim to have intact embryos after cryopreservation

Cryopreservation should be biologically neutral to oocytes and embryos/blastocysts

Cryopreservation



Cryopreservation

Freezing

- **ice**

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

Vitrification

- **glass**

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

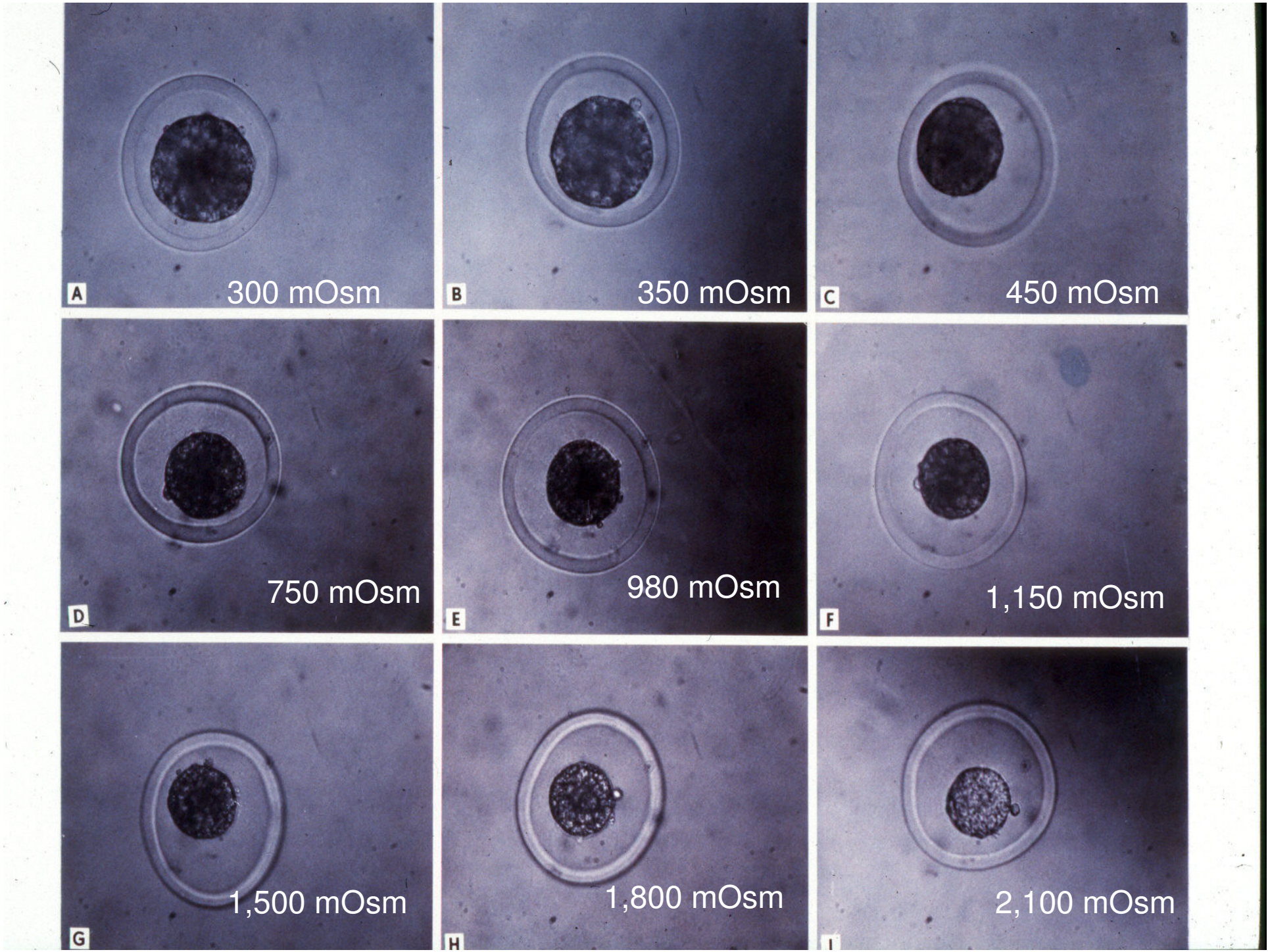
Osmotic events

- 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:

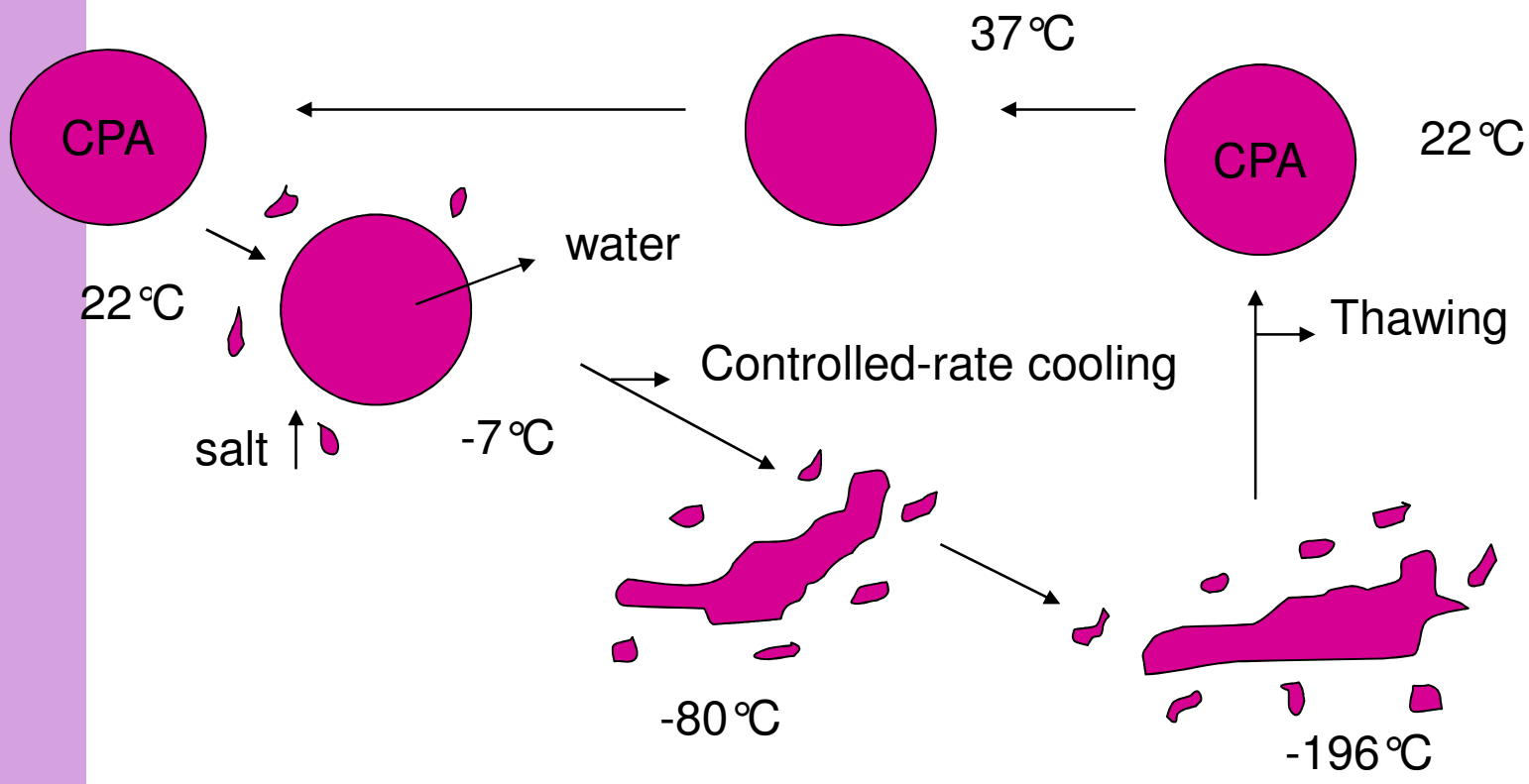
V_w	Cell water volume
A	Membrane surface area
L_p	Membrane permeability for water
P_s	Membrane permeability for permeant solute (CPA)

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


A
300 mOsm
B
350 mOsm
C
450 mOsm
D
750 mOsm
E
980 mOsm
F
1,150 mOsm
G
1,500 mOsm
H
1,800 mOsm
I
2,100 mOsm

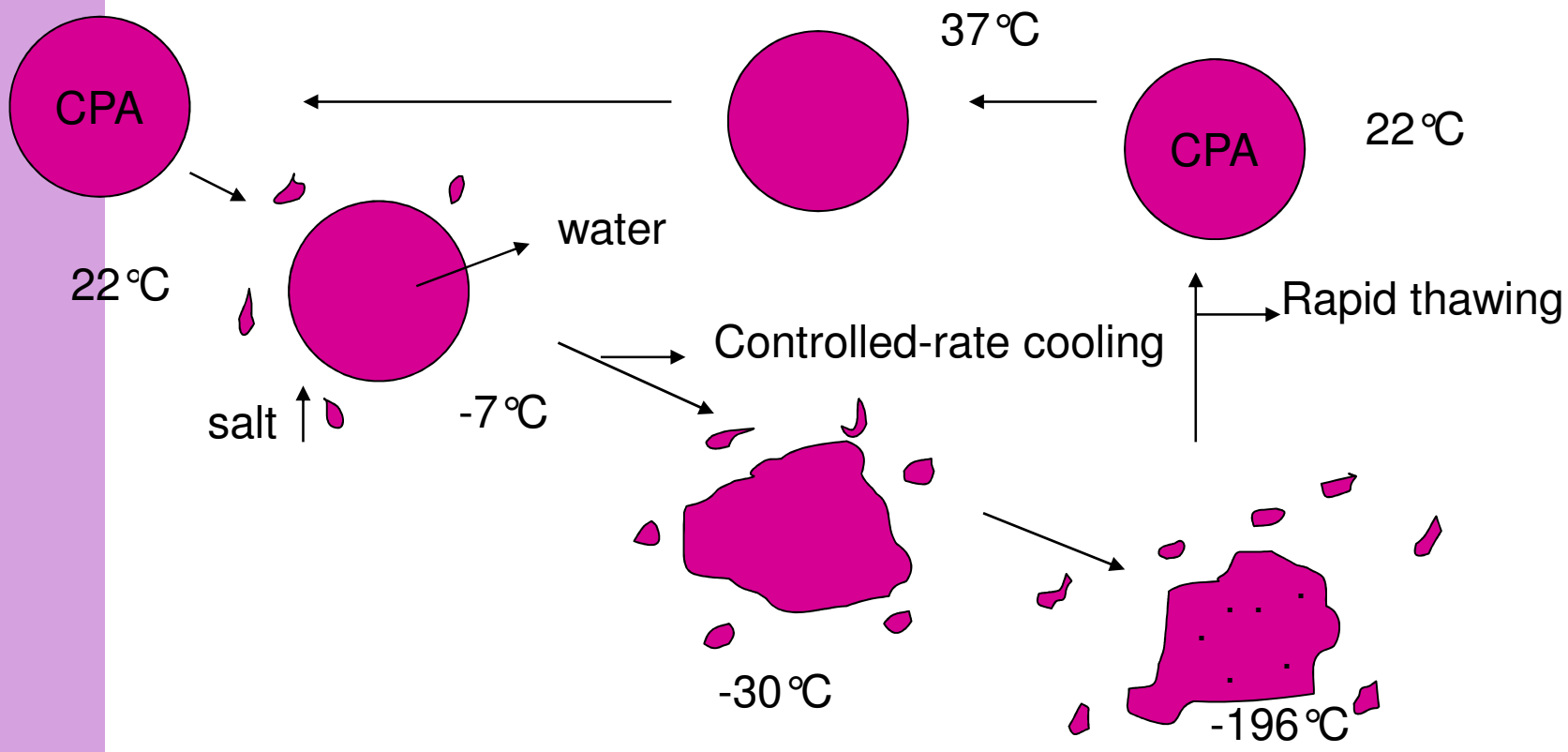
Basic principles of freezing

Equilibrium cooling (controlled-rate cooling)



Basic principles of freezing

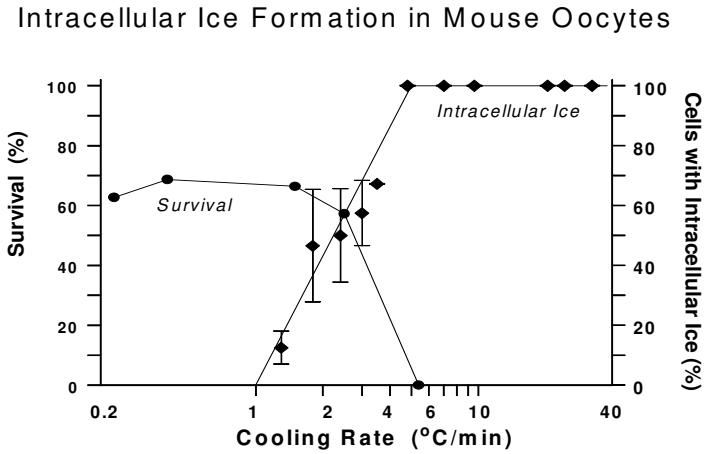
Quasi-equilibrium cooling (interrupted controlled-rate cooling)



Basic principles of freezing

Summary: variables of freezing

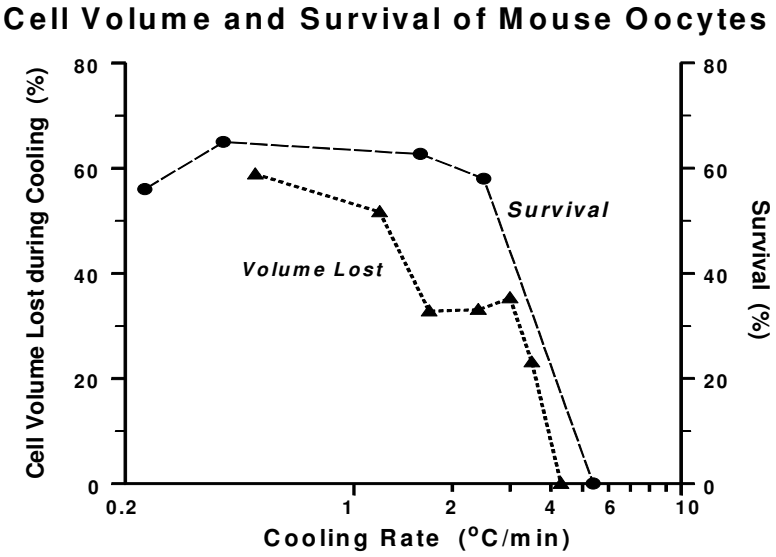
- The effect of cooling rates (1) (likelihood of intracellular ice formation) (dependent on S/V ration and Hydraulic Conductivity Lp)

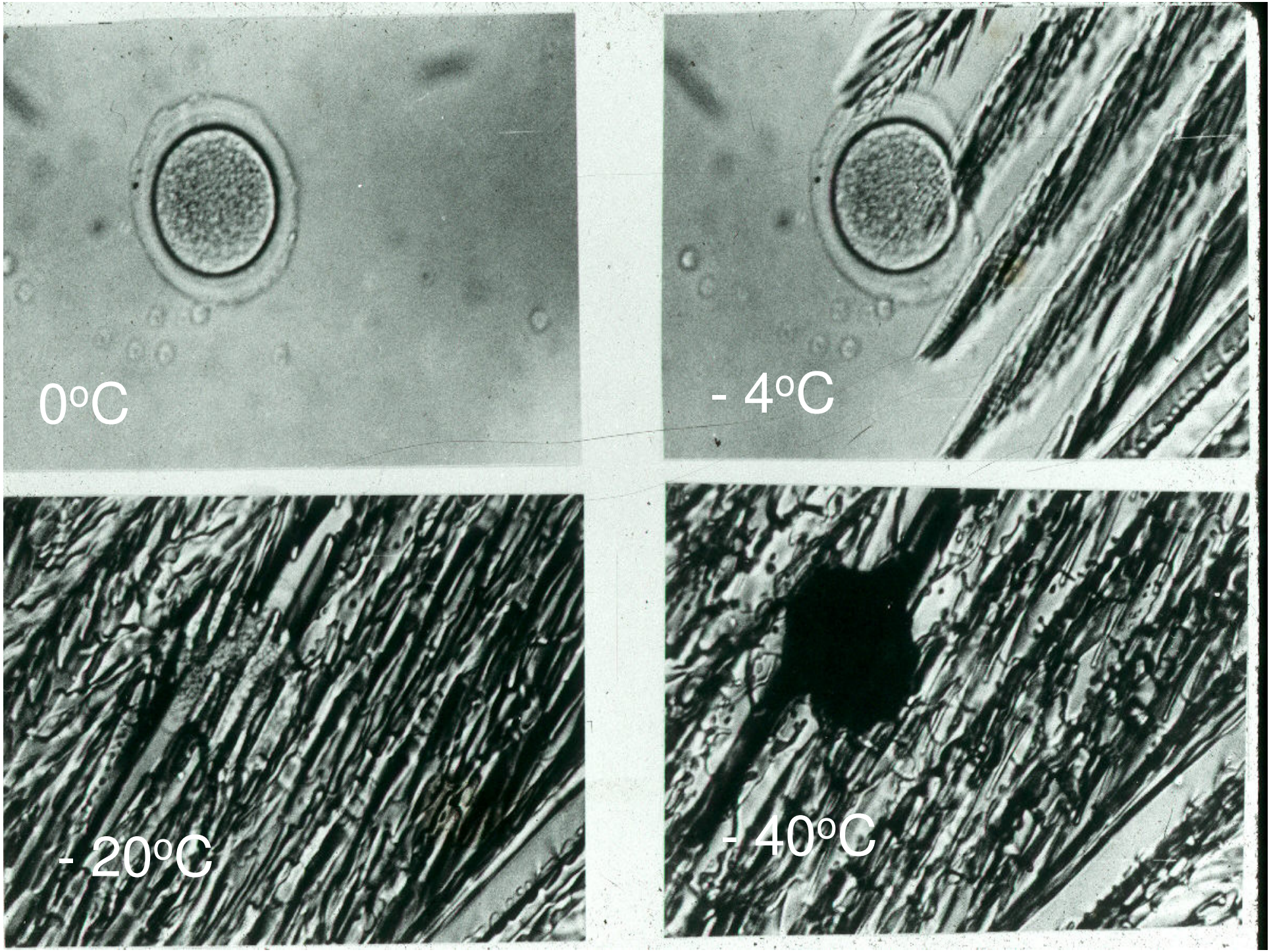


data of Leibo et al. 1978 Cryobiology 15:257

Basic principles of freezing

Cell volume excursions during cooling (2)




0°C
- 4°C
- 20°C
- 40°C

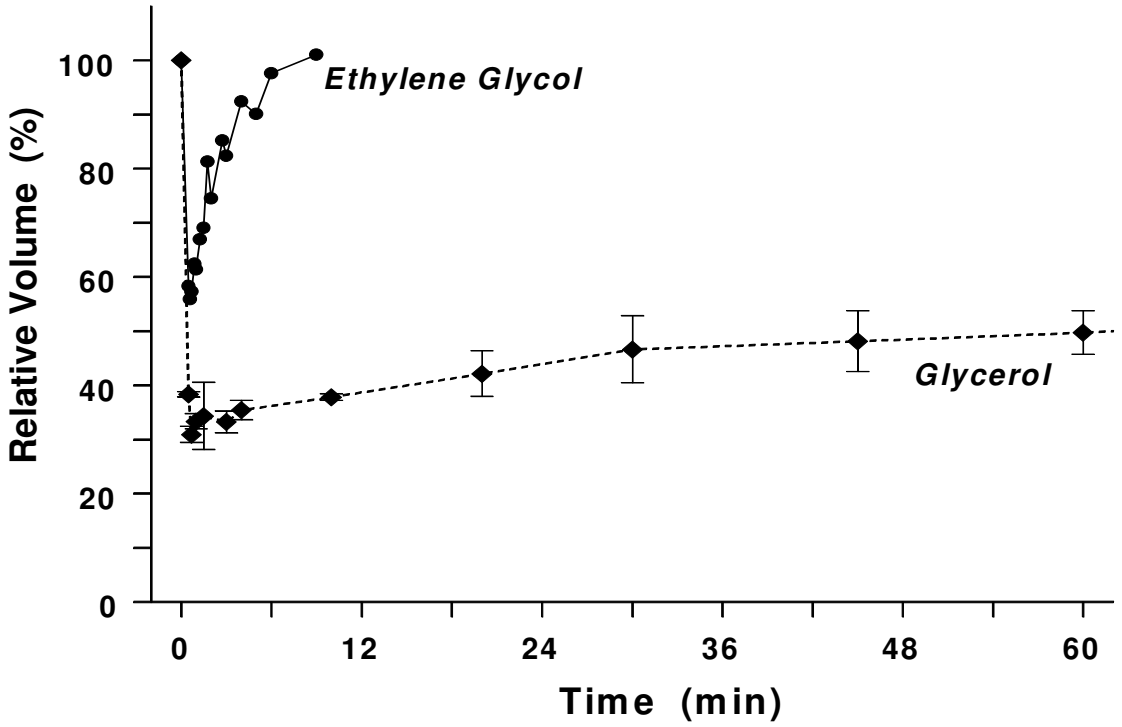
Cryoprotective Additives

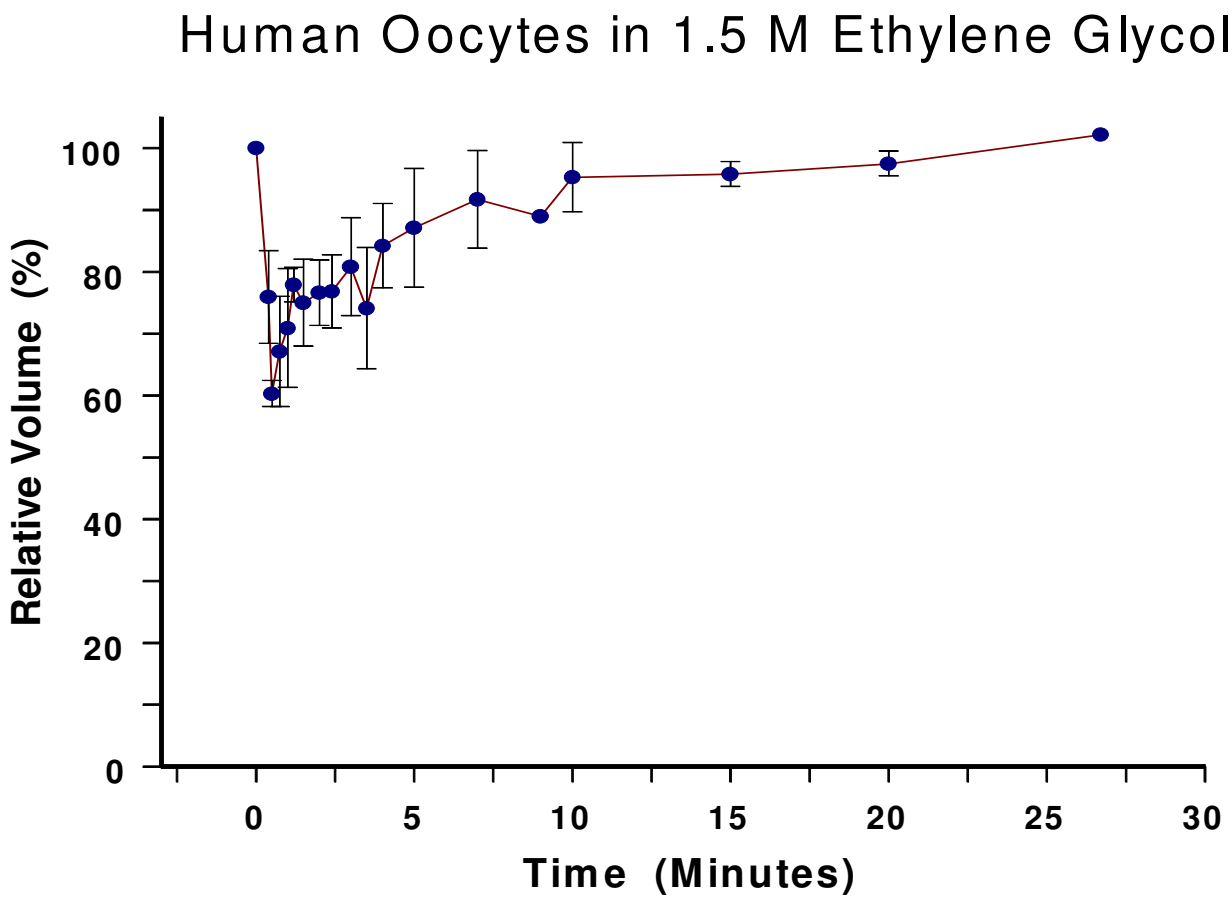
Other variables of freezing (1)

- Osmotic responses in CPA solutions

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

Mouse Ooocytes in 1.5 M CPA Solutions





Basic principles of freezing

Other variables of freezing (2)

- Chilling injury

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

(cytoskeletal elements, membranes, lipids)

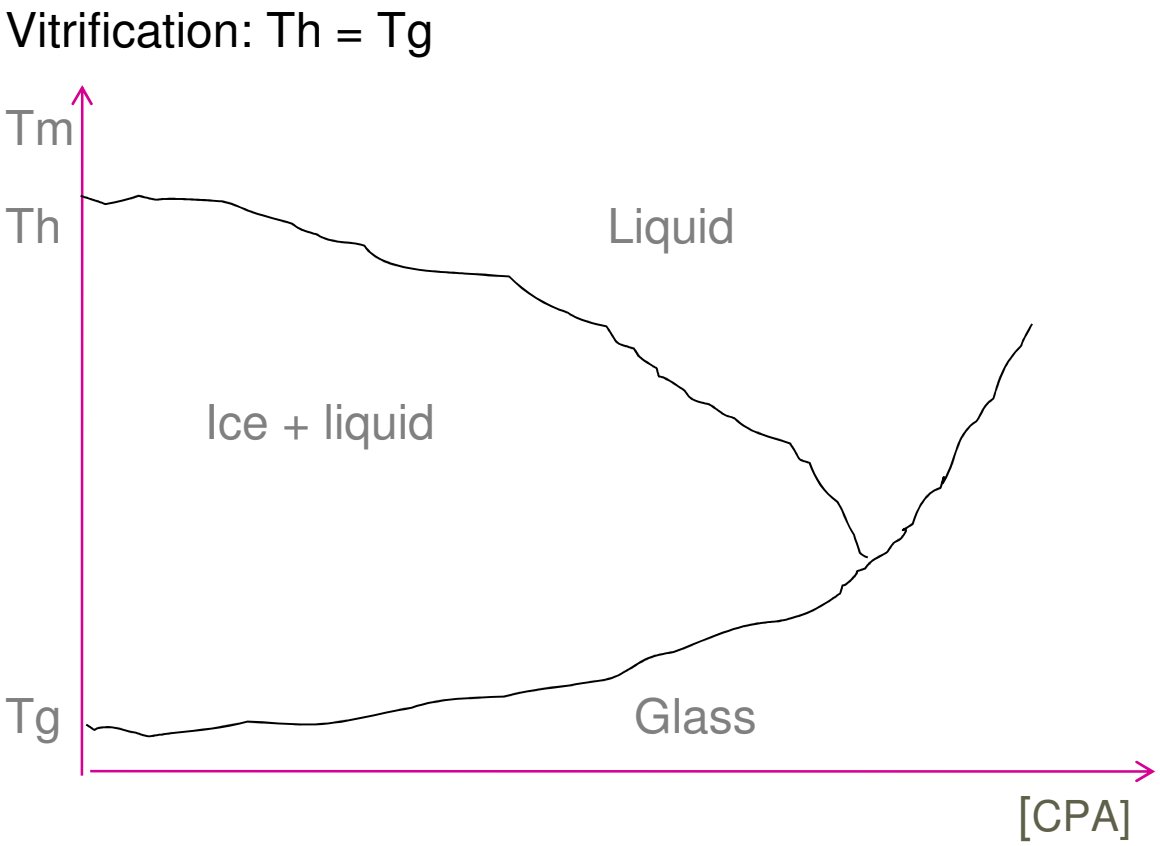
Conclusions on freezing

- 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 stress.
- Expensive equipment required.
- Long procedures.

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

Basic principles of vitrification



Basic principles of vitrification

Probabilty of vitrification

- The effect of cooling and warming rates

$$\frac{\text{Cooling/warming rates x [CPA]}}{\text{Sample Volume}}$$

Equilibrium "true" vitrification: high [CPA], cooling rate independent, vol >100µl

Non-equilibrium vitrification (minimal volume vitrification): low [CPA], high cooling rates, vol < 1µl

Basic principles of vitrification

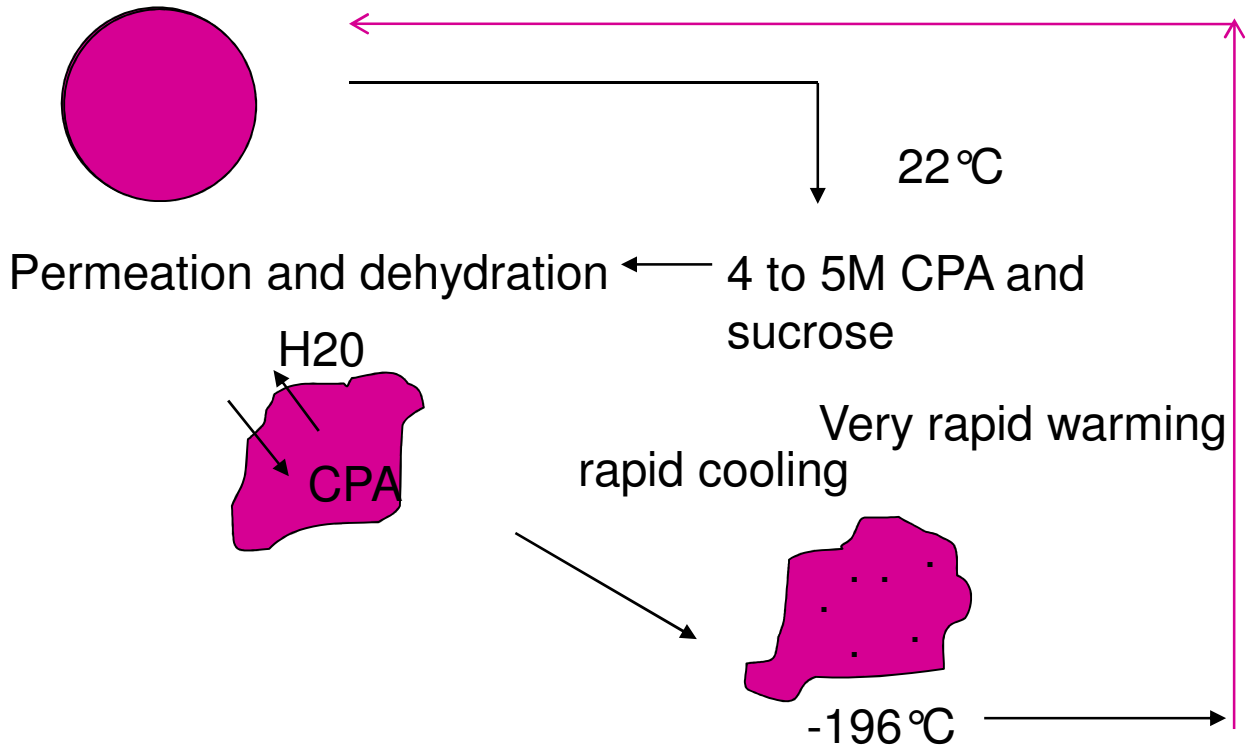
1997 Vajta et al

- Minimal Volume Vitrification (= Vitrification method # true vitrification)

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

Basic principles of vitrification

Non-equilibrium vitrification (minimal volume vitrification)



Basic principles of vitrification

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

Variables of vitrification

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

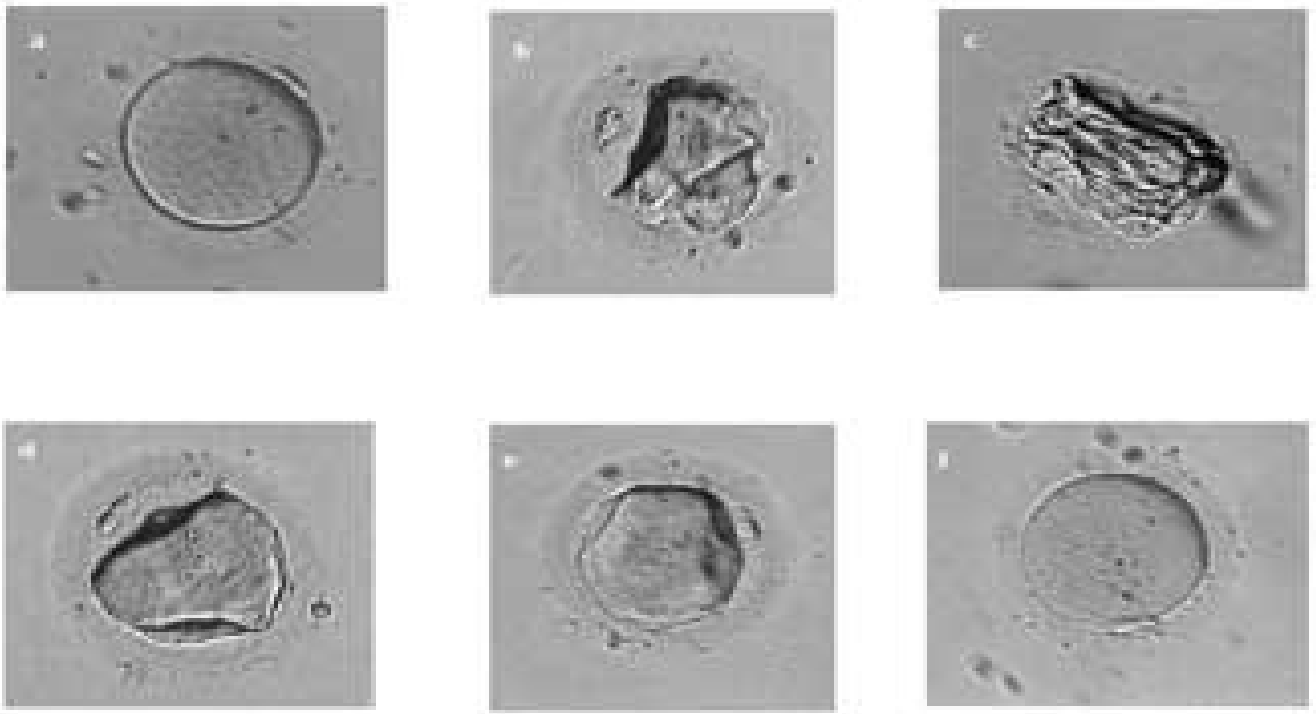
Basic principles of vitrification

Variables of vitrification

- Osmotic responses to CPA solutions

  Osmotic tolerance limits of cells to be vitrified

Osmotic responses to CPA solutions



Conclusions on vitrification

Benifits of vitrification

- Very simple procedure?
- Reduces the time of the cryopreservation procedure?
- Flexibility
- No ice crystallization?
- Eliminates the cost of expensive programmable freezing equipment?
- Efficient

Conclusions on vitrification

Variables of vitrification that can profoundly influence its effeciency:

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

Efficiency: cellular loss after cryopreservation

Embryos	Blastocysts
No survival	
< 50% survival	Severely damaged
> 50% survival	Moderately damaged
100% survival	

Efficiency: cellular loss after cryopreservation

Conclusion

Damaged embryos have a lower implantation potential than fully intact ones

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

It should be the aim to have fully intact embryos after thawing/warming

Efficiency: cellular loss after cryopreservation

Conclusion

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

Van Der Elst et al (1997) Hum Reprod 12, 1513-1521)
Ziebe et al (1998) (Hum Reprod 13, 178-181)
Van den Abbeel et al (2000) (Hum Reprod 15, 373-378)
Tiitinen et al (2001) (Hum Reprod 16, 1140-1144)
Guérif et al (2002) (Hum Reprod 17, 1321-1326)
Edgar (2007) (RBM Online 14, 718-723)

Efficiency: meta-analysis vitrification versus freezing

Embryos and blastocysts

Loutradi et al 2008

Kolibianakis et al 2009

AbdelHafez et al 2010

Efficiency: meta-analysis vitrification versus freezing

Conclusions on embryos

- Vitrification as compared with slow freezing, appears to be better in terms of post-thawing survival rates both for cleavage-stage embryos and for blastocysts
- Postthawing blastocyst development of embryos cryopreserved in the cleavage stage is significantly higher with vitrification as compared with slow freezing
- No significant difference in clinical pregnancy rates per transfer could be detected between the two cryo methods
- Conventional freezing of human blastocysts has been carried out with no satisfactory results. Results obtained from vitrification of blastocysts document the fact that vitrification is the perfect alternative to conventional freezing

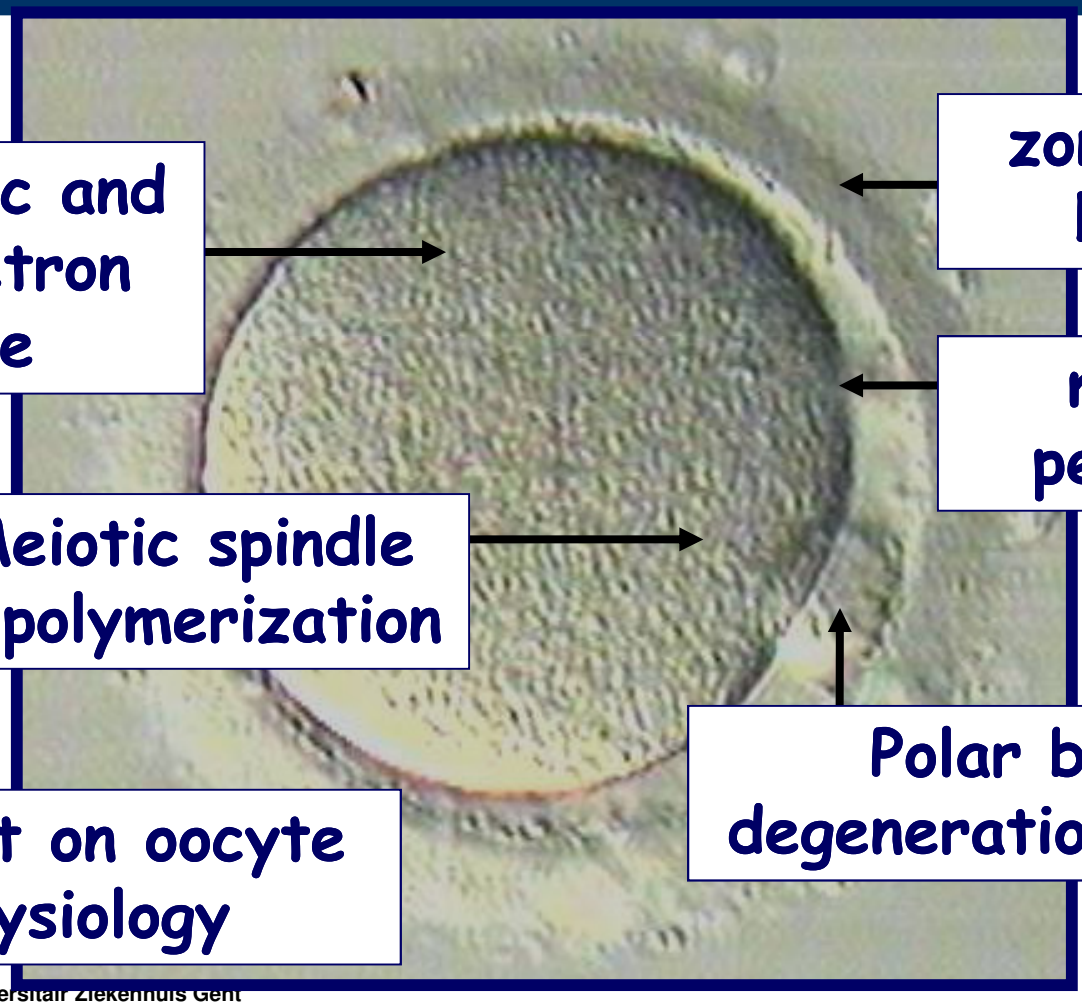
Efficiency: cellular loss after cryopreservation

MII Oocytes

No survival

100% survival

Possible injuries to the cell during cryopreservation



Cytoplasmic and Cytoskeletron damage

zona pellucida hardening

membrane permeability

Meiotic spindle depolymerization

Polar body degeneration/fusion

Impact on oocyte physiology

Efficiency: meta-analysis vitrification versus freezing

MII Oocytes

Oktay et al 2006;2008

Gook and Edgar, 2007

Noyes et al, 2009

Efficiency: meta-analysis vitrification versus freezing

Conclusions on MII Oocytes

- The results obtained suggest that vitrification/warming is currently the most efficient means of oocyte cryopreservation in relation to subsequent success in establishing pregnancy
- ...” Perhaps one hesitation regarding oocyte cryopreservation partially lies in the commercial interests of companies promoting premature elective fertility preservation. In addition, competing interests exist for those who manufacture oocyte storage containersand/ or oocyte cryopreservation culture media. Clearly, clinicians and embryologists need to be cognizant of these latter conflicts as more publications appear in the literature”...(Noyes et al 2009)

Safety of cryopreservation: Effects on oocyte/embryo physiology?

Temperature

Cryoprotectants

Cryopreservation procedure

Safety of cryopreservation: Effects on oocyte/embryo physiology?

Temperature and meiotic spindle

Cryoprotectants and intracellular Ca

Cryoprotectants and meiotic/mitotic spindles

Cryoprotectants and oocyte genotoxicity

Cryoprotectants and oocyte mitochondrial distribution

Cryoprotectants and microfilaments

Cryoprotectants and gene expression

Cryopreservation of mature oocytes

CRYOPRESERVATION and oocyte metabolism

- As compared to fresh controls both vitrification and freezing have an impact on oocyte metabolism
- Vitrification has less impact that freezing

(Lane and Gardner (2001)

Cryopreservation of mature oocytes

CRYOPRESERVATION and oocyte protein profile

- As compared to in vivo controls, the protein profile of oocytes was affected after freezing but not after vitrification

(Larman et al, 2006)

Cryopreservation of embryos

CRYOPRESERVATION and embryo metabolism

- Stokes et al, 2007
  - Embryo cryopreservation safe

- Balaban et al, 2008
  - Vitrification better than freezing (=more neutral)

Safety: children born

Wennerholm et al, 2009

- Data concerning infant outcome after slow freezing of embryos was reassuring. Properly controlled follow-up studies of neonatal outcome are needed after slow freezing of blastocysts and after vitrification of early cleavage-stage embryos, blastocysts and oocytes. In addition child long-term follow-up studies for all cryopreservation techniques are essential

Take home messages

- It should be the aim of a cryopreservation programme to have fully intact oocytes and embryos after thawing/warming
- The technique of vitrification offers several advantages for the IVF laboratory, however there are still some technical challenges associated with the technique
- As compared to freezing, the immediate morphological survival is higher after vitrification in different developmental stages
- The clinical outcome after vitrification appears to be better than after freezing
  - Vitrification provided a significant clinical breakthrough for the preservation of oocytes and blastocysts
- It is my opinion that vitrification is the future for cryopreservation and more significant developments should be expected in the near future to make the technique more robust and safe