# SRBT WEBINAR

# A Vitrified Future in Cryobiology: A Tribute to the Pioneers

**Presenters:**
**Mitchel Schiewe, PhD**
**Greg Fahy, PhD**
**Zsolt Peter Nagy, MD PhD**

**Moderator:**
**Sue Gitlin, PhD**
**Chairman SRBT Education Committee**

July 22, 2014

# Mitchel Schiewe, PhD

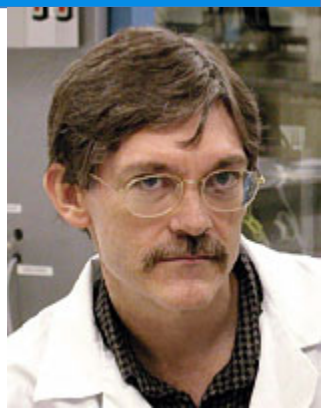
Scientific Lab Director at Southern California Institute for Reproductive Sciences in Newport Beach and Technical Director to the California Cryobank.

Doctorate attained in 1989 involving ART applications in domestic animal models for non-domestic species which included the  Vitrification of sheep blastocysts.

Published 38 peer-reviewed papers and over 100 scientific abstracts

2-time Chairman of RBPG, involved in the formation of SRBT

# Greg Fahy, PhD



Chief Scientific Officer, 21st Century Medicine, Inc

Originator of the first practical method of Cryopreservation by vitrification and the inventor of computer based systems to apply this technology to whole organs.
Bachelor of Science degree in Biology from the University of California at Irvine and a Ph.D. from the Medical College of Georgia in Augusta.

His scholarly work is highly published and he holds more than 15 patents.

# Zsolt Peter Nagy, MD, PhD

Scientific and Laboratory Director at Reproductive Biology Associates (RBA), in Atlanta.

MD and Ob&Gyn specialty degrees at Semmelweis Medical University in Budapest. PhD Free University of Brussels (VUB): “ICSI: technical and biological aspects to increase efficiency”.

Involved in studies on novel egg/embryo viability assessment methods.  Investigated the basic and clinical aspects of cryopreservation, especially oocyte vitrification that has contributed to the development of largest donor oocyte cryo-bank in North-America.

Current Chairman of Alpha as well as serving SART, and the SRBT board. He is author /co-author of over 200 publications.

# A Vitrified Future in Cryobiology: A Tribute to the Pioneers

## ASRM / SRBT

## Cryobiology Education

# Learning Objectives

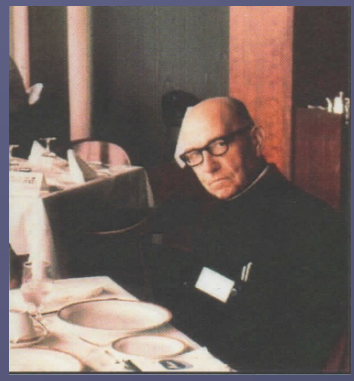
- At the conclusion of this presentation, participants should be able to:

1. Have a historical reference to cryobiological principles and developments, the investigators who devised them and when they were applied to ART;
2. Understand the basic principles behind effective cryopreservation, from freezing to vitrification, of embryos and oocytes;
3. Become familiar with the history behind various cryoprotectants and freezing protocols;
4. Gain an appreciation of how far the technology has evolved in 50+ years, to being the highly efficient process it is today, where vitrified embryo transfer cycles are more effective than fresh ET, and where Egg Banking is becoming a viable alternative.

# Stanley celebrare la vita in Italia !

## He loved educating students

# Early Cryobiology - Cells



- Luyet (1937)
  - "The vitrification of organic colloids and of protoplasm"
- Luyet and Hodapp (1938)
  - "Revival of frog's spermatozoa vitrified in liquid air"
- Luyet & Gehenio (1940)
  - "Life and death at low temperatures"

Vitrification (VTF) = solidification without crystallization

(*Luyet applied Kinetic VTF w/o cryoprotectants*)

- Solution supercools, becomes viscous and forms a transparent glass.
- proposed that vitrification might be useful for cryopreservation.

# Luyet's Approach to VTF

- Father Luyet was a Biophysicist and the 1st President of the Society for Cryobiology (1964).
- What he knew:

1) Vitrification of living systems might be attainable by "outrunning" ice growth using high cooling rates ("the main problem is to secure a high cooling velocity.")

2) Vitrification is easier at low water content and high viscosity

3) Devitrification and/or recrystallization is the main cause of death after rapid cooling.

BUT: He used agents like sucrose and ethylene glycol as ***osmotic dehydrating agents only***, thus missing their essential role as the key to successful vitrification!

# Tissue Vitrification According to Luyet

VOL. 7, NO. 126 BIODYNAMICA MAY, 1950

## RESUMPTION OF HEART-BEAT IN CHICK EMBRYO FROZEN IN LIQUID NITROGEN

FREDERICO GONZALES AND BASILE LUYET
DEPARTMENT OF BIOLOGY, ST. LOUIS UNIVERSITY, ST. LOUIS, MISSOURI

36-48-hour chick embryos were exposed to 30% EG for ~ 6 min, then dried in air for 2-4 min, immersed in LN2, and then in 40°C Tyrode solution; 38/65 embryos developed at least some degree of heartbeat

VOL. 7, NO. 143 BIODYNAMICA DEC., 1953

## GROWTH OF NERVE TISSUE AFTER FREEZING IN LIQUID NITROGEN

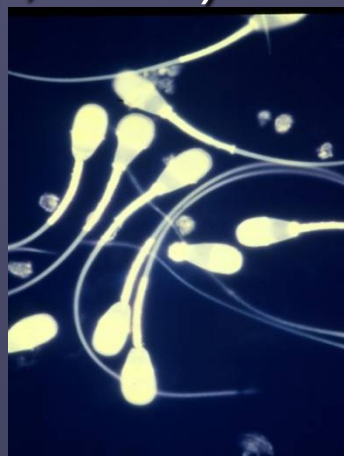
B. LUYET AND F. GONZALES
INSTITUTE OF BIOPHYSICS, ST. LOUIS UNIVERSITY*
ST. LOUIS, MISSOURI

5-day old chick brain was cut into 1 $mm^3$ pieces and exposed to 60% EG for one min, then transferred to LN2 and thawed in room temperature Ringer's; the explants produced normal neural outgrowths but with slightly lower abundance than controls

EG = ethylene glycol; LN2 = liquid nitrogen

# Early Cryobiology - Sperm

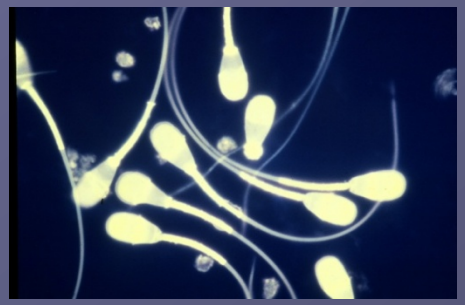
- Early pioneers included:
  - Hammond (1930): cooling Rabbit sperm to 0ºC, then fertilized oocytes and produced offspring
    - He had the foresight to suggest that the transportation of cooled sperm by airplane could allow for the worldwide distribution of genetic material.
  - Shettles (1940): cryosurvival rates varied among men, <10%
  - Hoagland and Pincus (1942): applied a vitrification procedure developed in the frog (Luyet and Hodapp, 1938) to human sperm
  - Parkes (1945): noted that human sperm survived at higher rates when cooled in a large volume versus small volume; thus indirectly determining that cooling rate is correlated to survival.



# Progress in Cryobiology – Sperm

- In the late 1940s, a student of Professor Alan Parkes, Chris Polge, accidentally discovered that glycerol, not sugars, interacted favorably with albumin to protect membranes in avian sperm cooling down to -79°C

(Polge, Smith & Parkes, 1949)



- Bunge & Sherman (1953) proved the fertilizing capacity of frozen human sperm
  - Reported first 4 human pregancies using glycerol

Progress in the cryobaking of cattle and human followed in the 1950s and 1960s/1970s, respectively

# Early Cryobiology - Oocyte

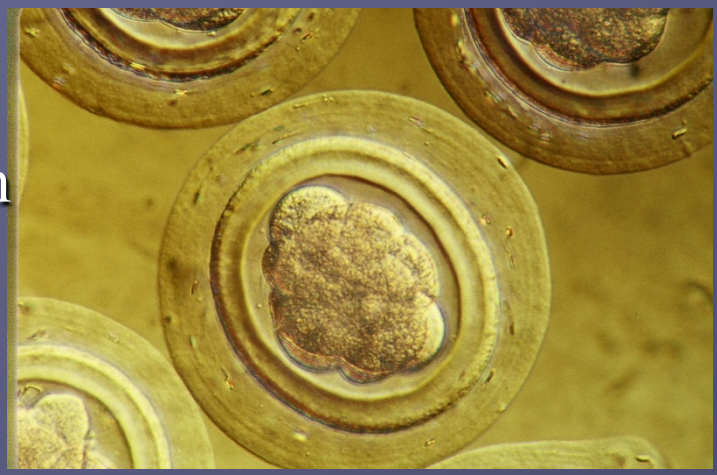
MC Chang – Student of Prof. J. Hammond/worked w/G.Pincus

- Pioneer in IVF and oocyte cooling/freezing
  - Rabbit oocytes (Chang, 1947,1954)
- Recognized the importance of cooling rate to maintaining gamete viability, documented artificial activation by rapid cooling and produced litters from embryos stored at 0°C.
  - Mouse ovaries / oocytes (Chang, 1958)
- First offspring produced from sub-zero cooling to -10°C for 10 min./IVF. Extended periods of cooling ('Hold') or lower temp's proved detrimental to ova.
- Mentored: R. Yanagimuchi

  * One of the greatest Reproductive Biologist of our time

# Early Cryobiology - Embryo

Audrey Smith, a Physician whom worked with Christopher Polge in Dr. Alan Parkes lab in the 1940's, co-discovering the beneficial effects of Glycerol.

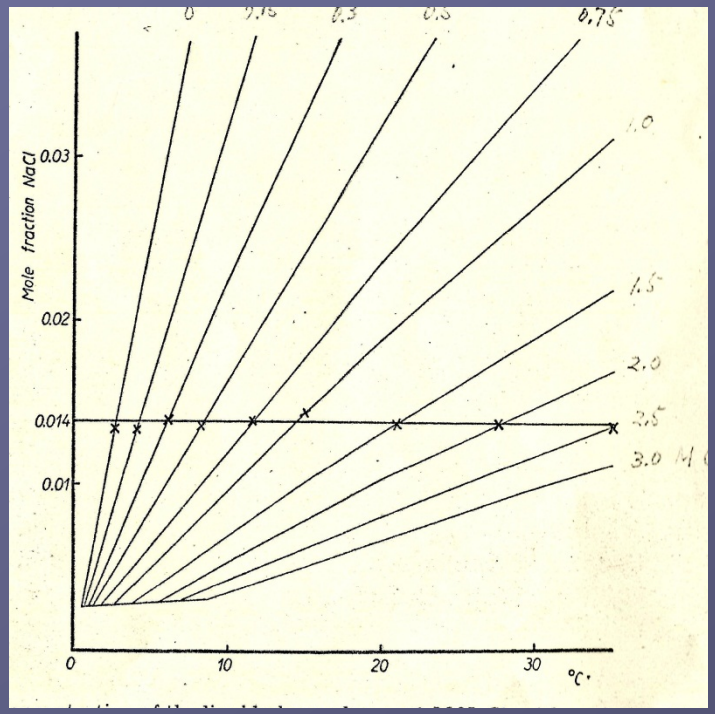


- Rabbit embryos (A Smith, 1952)
  - Poor animal model choice due to the low permeability of cleavage-stage embryos to glycerol
  - Partial success cooling to -79°C, followed by some development post-thaw, but no offspring (Ferdows et al., 1958; Smith, 1961).

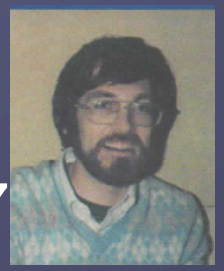
# Why Does Freezing Work? A Thermodynamic Understanding

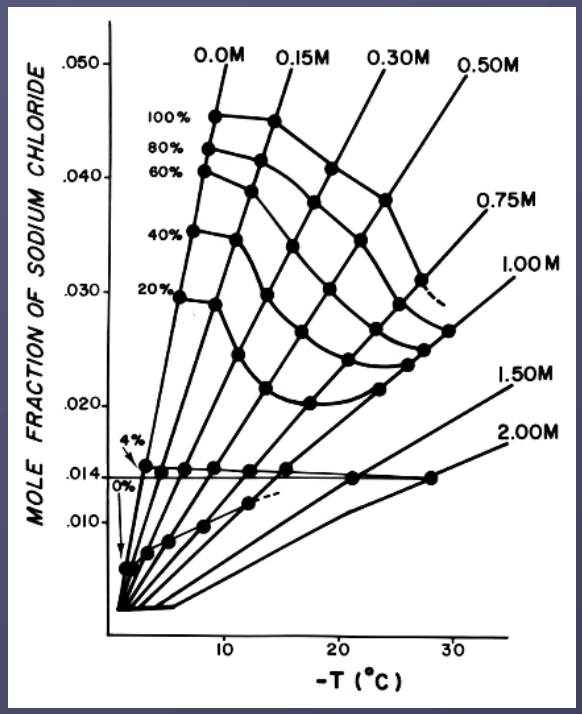
Increased understanding of cryobiology was provided by the theory of colligative cryoprotection devised by J.E. Lovelock.

- Lovelock JE,1953, BBA, 11: 28-36.




Fahy & Karow, Cryo '77



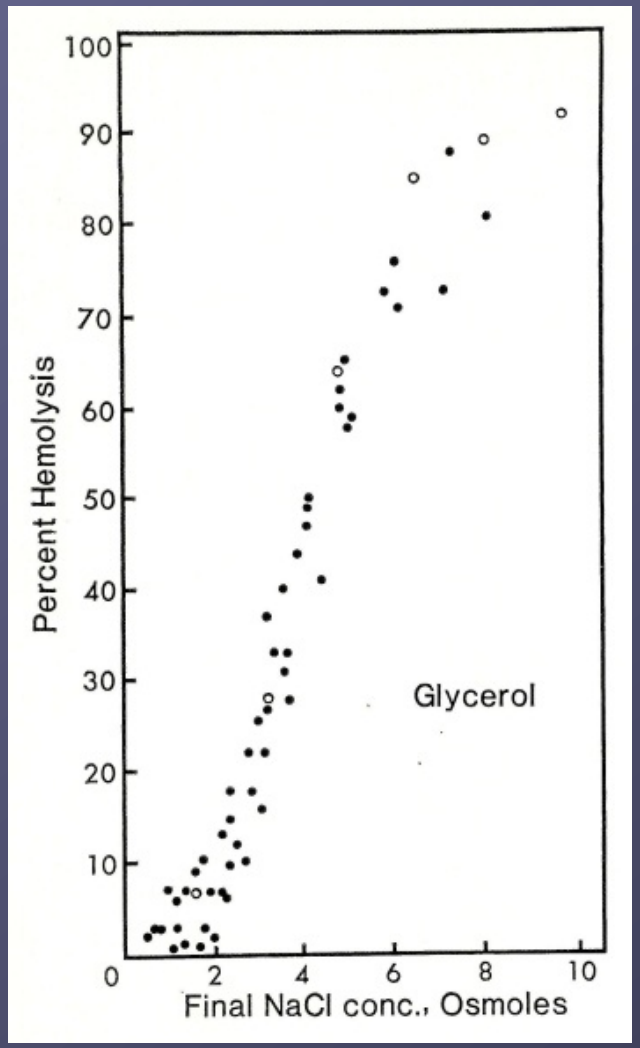


# Why Does Freezing Work?
# A Confirmation of the Theory

Meryman HT, 1960-80's {Head, Transplant Lab, ARC}

- "Osmotic stress as a mechanism of freezing injury" (Cryobiology, 1971)

"Freezing Injury from 'Solution Effects' and its Prevention by Natural or artificial Cryoprotection" Cryobiology 14: 287-302, 1977

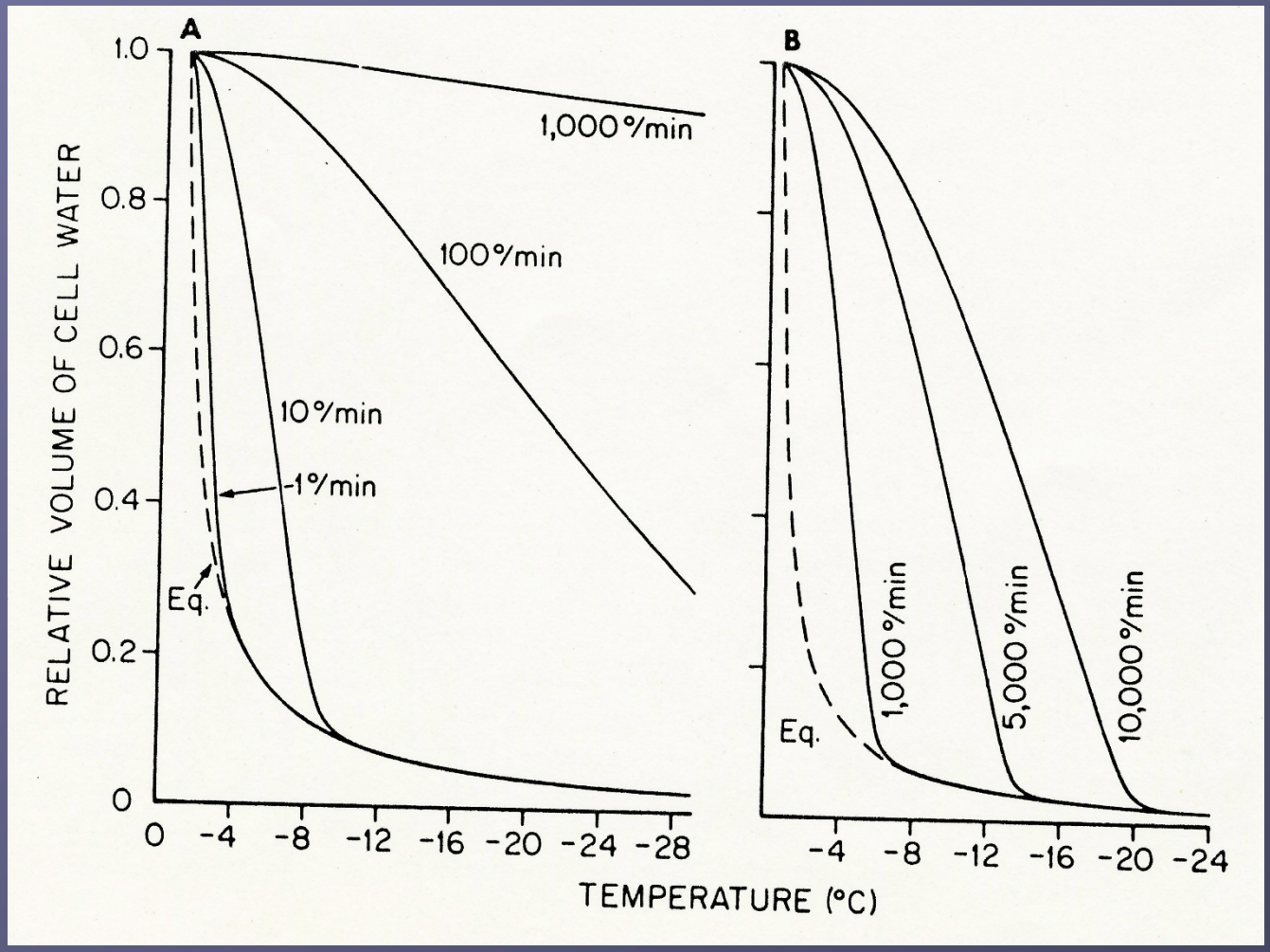


# Why Does Freezing Work? A Kinetic Understanding

- Peter Mazur – Harvard U - Magna Cum Laude graduate
  - determined that the kinetics of cellular water loss during subzero cooling is a function of T, cooling rate, cell membrane permeability and the cell surface-to-volume ratio. (Mazur, 1963).
  - The role of cell membranes in the freezing of yeast and other small cells (Mazur, 1965)
  - Interactions of cooling velocity, T, and warming velocity on the survival of frozen and thawed yeast (Mazur and Schmitt, 1968)
  - Cryobiology: the freezing of biological systems (1970)
- Permitted rapid progress in freeze-preserving more complicated multicellular, mammalian embryos

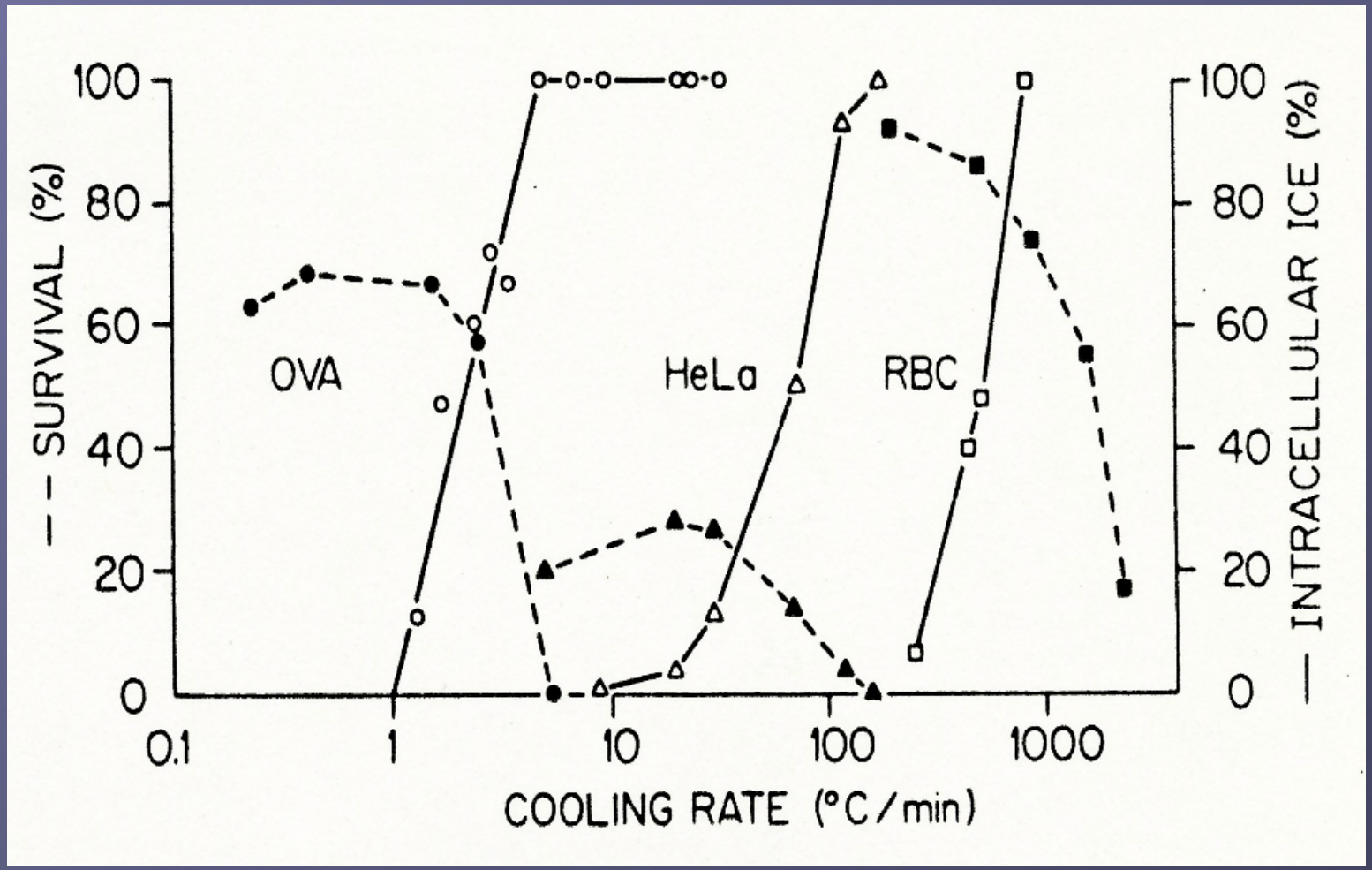
# Mazur's "Two Factor" Theory

As cooling rate increases, intracellular supercooling Increases.



# Mazur's "Two Factor" Theory

As intracellular supercooling increases, the odds of Intracellular ice formation increase.



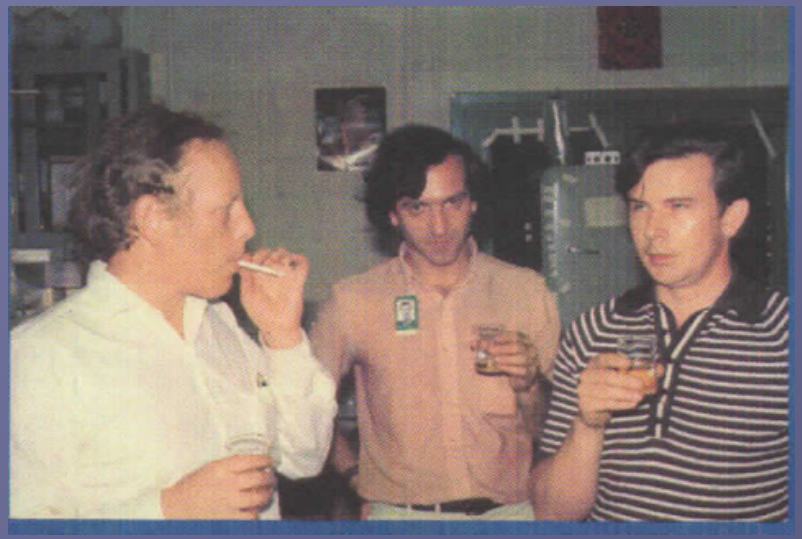
# Explaining the Existence of Optimum Cooling Rates

- Factor One: Solution effect /osmotic stress
  - ↓ cooling rate reduces risk of IIF but increases risk of damage from excessive cell shrinkage or extended exposure to ↑ [solute], thus cooling too slowly is potentially harmful.

- Factor Two: IIF from excessive supercooling
  - ↑ cooling rate → inadequate dehydration → intracellular crystalization during cooling and recrystalization during warming could occur, both of which may cause injury.

(Mazur, 1963, 1970; Leibo et al., 1974)

# History Made – Uniting Minds / Continents

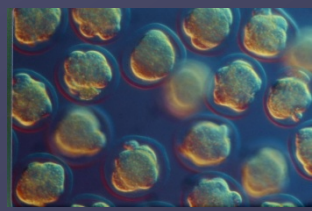


Oak Ridge Laboratory, TN
A government Nuclear testing lab; they supported Dr. Mazur's research in the hope that he would determine a way to preserve healthy bone marrow.


David Whittingham initiated mouse embryo freezing studies in 1969-1971. After publishing a 1971 Abstract w/o live births, he was recruited to The Oak Ridge Laboratory

Maximum post-thaw viability was achieved using a 'slow' cooling (0.22°/min) / 'slow' warming (<100°C/min) procedure with DMSO as the cryoprotectant.
(Whittingham, Leibo and Mazur; 1972)

(Wilmut, 1972)

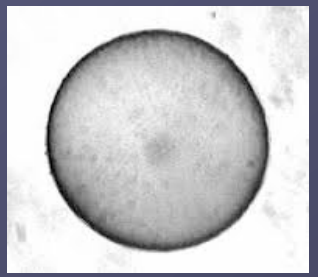


# Historic Embryo Cryo-Successes - I

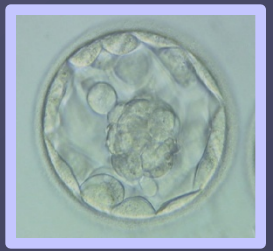
- Mouse (Whittingham, Leibo and Mazur, 1972; Wilmut,1972)
- Cow (Wilmut and Rowson, 1973)

  - FET birth of Frostie II  1953 AI -"FROSTIE I">

- Rabbit (Whittingham and Adams, 1974)
- Rat (Whittingham, 1975)
- Goat (Bilton and Moore, 1976)
- Horse (Yamamoto et al., 1982)
- Human 8-cell (Trounson and Mohr, 1983)



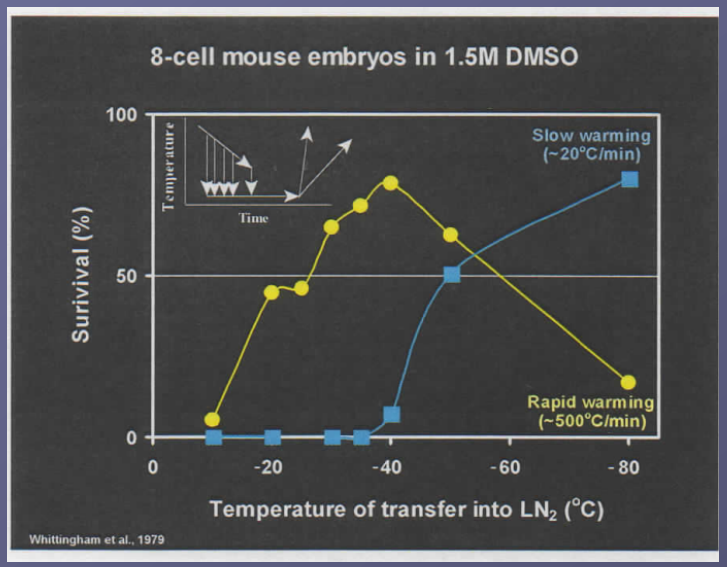
  - Blastocyst: (Cohen et al., 1985;
    Fehilly et al., 1985)



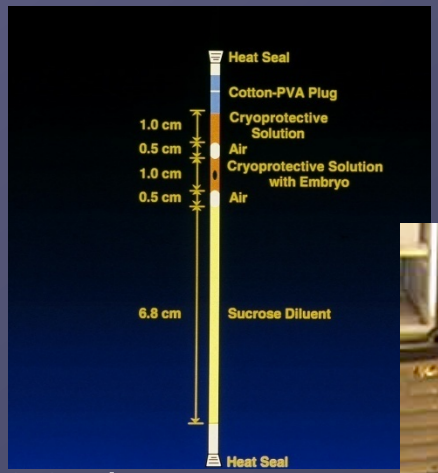
# Historic Embryo Cryo-Successes - II

- Sheep (Willadsen et al., 1976, 1977, 1978)
- Mouse (Whittingham et al., 1979)

‘Rapid’ freeze / ‘Rapid’ thaw



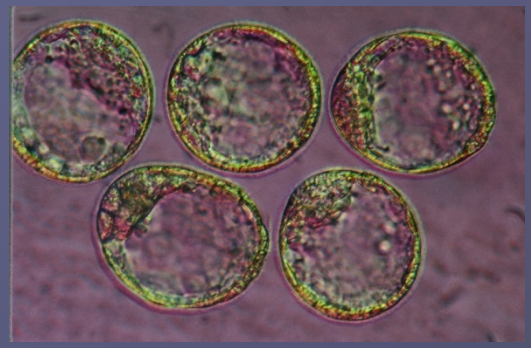

Modern freeze program

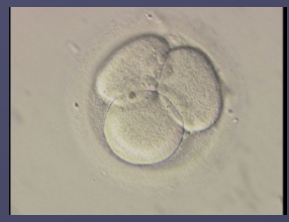



- Cow
  - 1-step straw: (Leibo et al., 1983, 1984)

# Alternative Cryoprotectants- Glycols

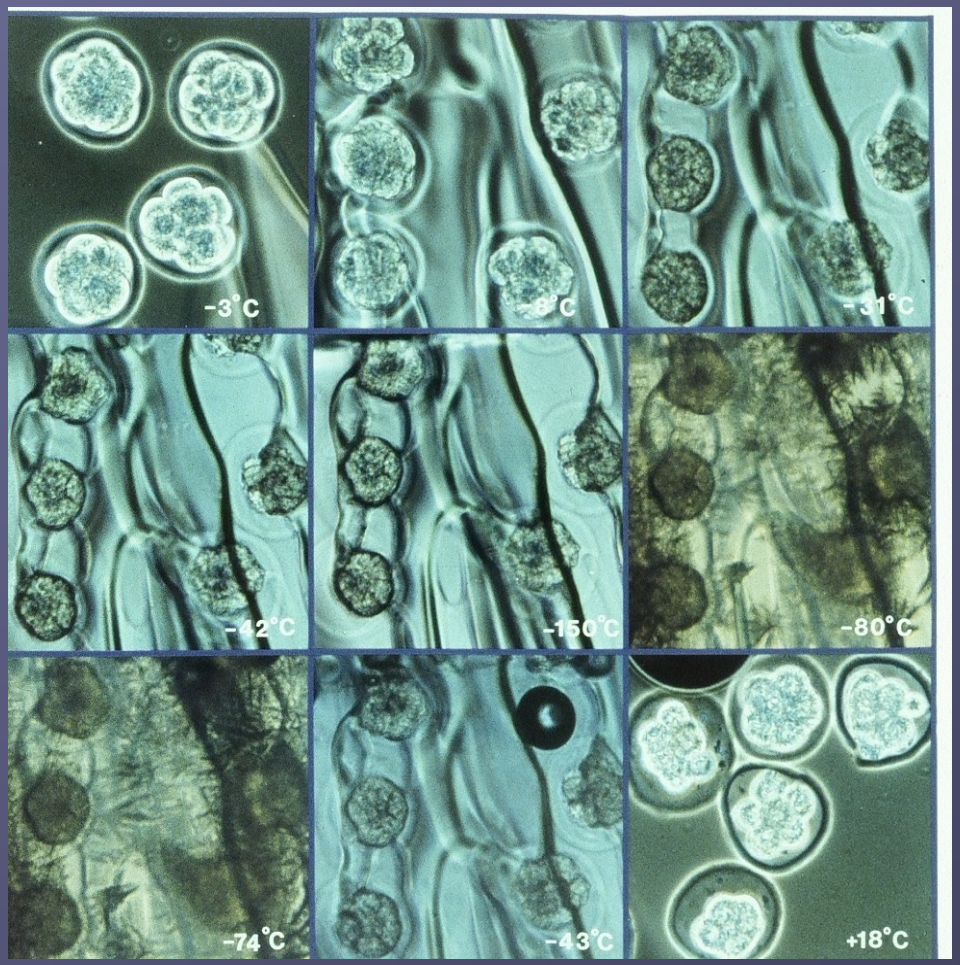
- Mouse – Cleavage stage to Blastocysts
  - Glycols (Kasai et al., 1981)
  - PPG (Renard et al., 1984)
- Cow - Blastocysts
  - (PPG: Renard et al., 1985)
- Rabbit
  - (PPG: Renard et al., 1984)
- Sheep - Blastocysts
  - (PPG: Wildt et al., 1986)
- Human – 2PN , 2-cell to 8-cell
  - (PPG: (Lassalle et al, 1985; Testart et al., 1986)





# Impact of Cryomicroscopy

- Cryomicroscopic observations enhanced our applied knowledge of the physiochemical processes behind the cryophysics.
  - Mouse model (Rall et al., 1980, 1984; Rall and Polge, 1984)
  - Bovine model (Lehn-Jensen and Rall, 1984)




Courtesy of Bill Rall

# Cryo-Principle to Cellular Survival

- Cryoprotective agents substantially ↑ viscosity of intracellular regions, causing water diffusion to cease and the liquid cytoplasm to form a metastable glass (vitrify) upon rapid cooling.

Rall et al., 1983

- If the [cryoprotectant] is sufficiently high, the crystallization of water molecules in the extracellular medium is inhibited completely, and the solution becomes vitrified.

Fahy, 1984

# Vitrification – A Novel Technique

- A concept to tissue preservation established (Fahy et al., 1984).
  - A goal of Dr. Fahy's was whole organ perfusion and cryopreservation

Greg Fahy and Bill Rall collaborated at the American Red Cross Blood Bank Labs, Bethesda,MD between 1984-86.

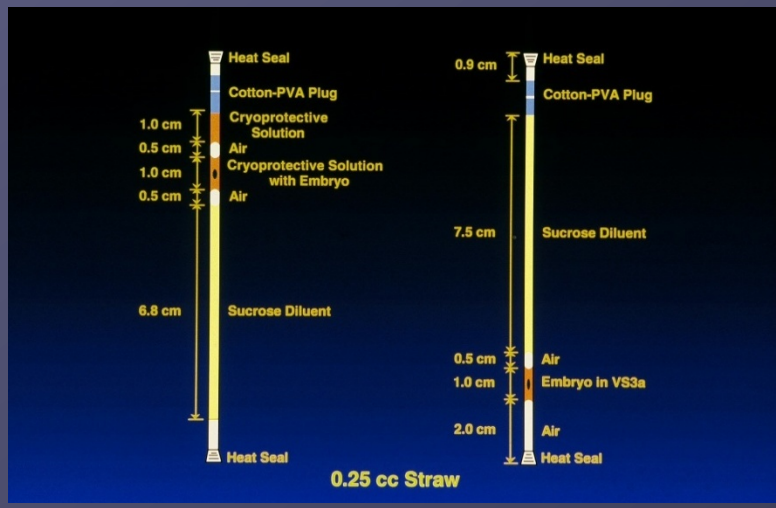
- Dr. Rall's clandestine experiments with mouse embryos were ongoing behind cold room doors (Rall and Fahy., 1985), and "Embryo Vitrification" success achieved!

Refer to PG07-ASRM, 2010

# Vitrification – A Practical Approach

- VS1 : a high molarity solution of 2.53M DMSO, 2.36 M Acetamide, 1.2M PPG and 5.4%PEG required a refrigerated working T to reduce solution toxicity effects (Rall and Fahy, 1985).
- VS3a: 6.5M glycerol solution with 6% BSA proved effective under room T conditions and live offspring were produced:
  - Mouse, 1986

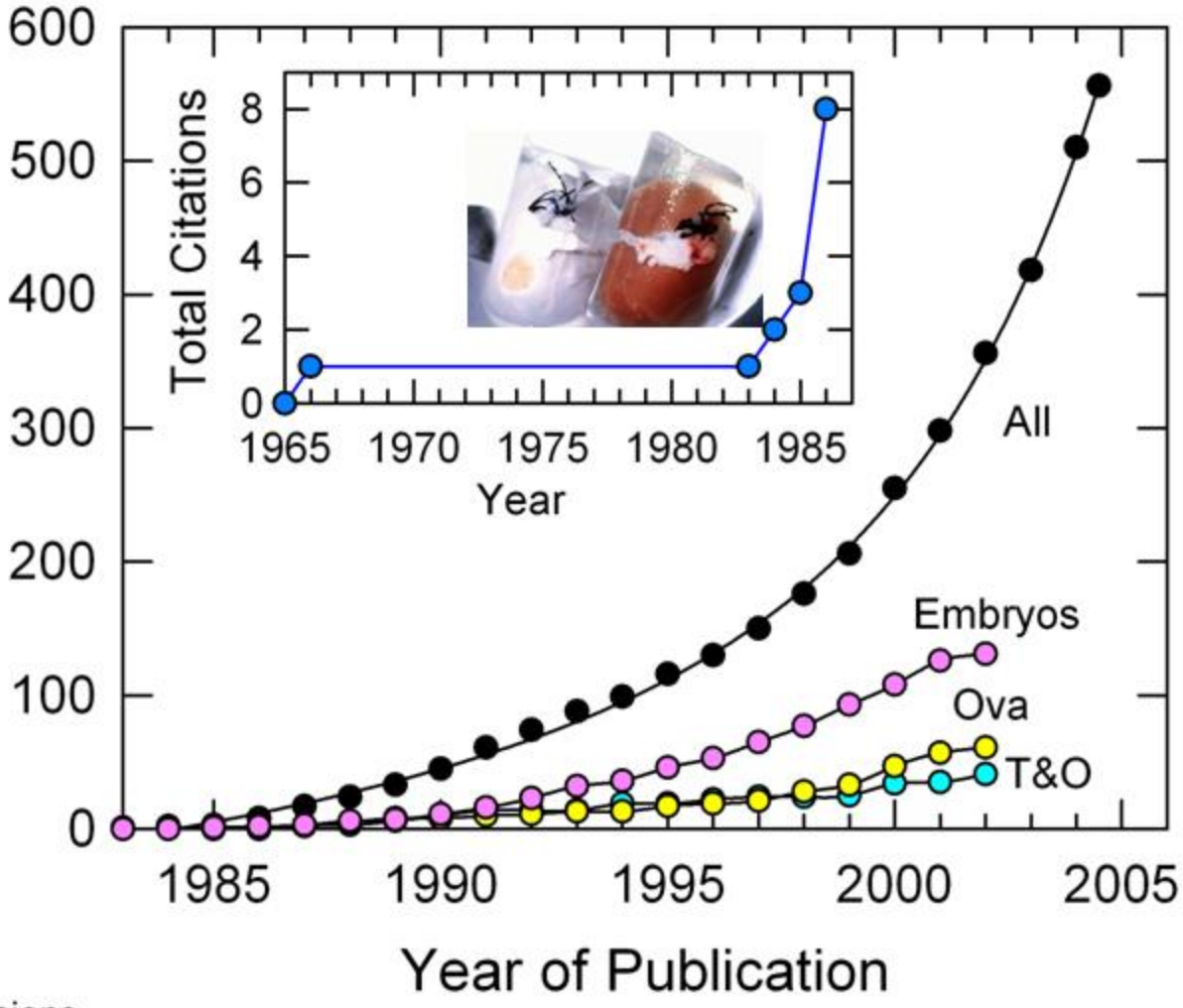
  (Rall, Woods, Whittingham)
  - Cow, 1988/89 (Rall and Leibo)
  - Sheep, 1988/89 (Schiewe)




(Schiewe, 1989)

# The Revival and Modern Application of Vitrification

Cumulative *PubMed* Entries for Vitrification as a Method of Cryo-preservation*

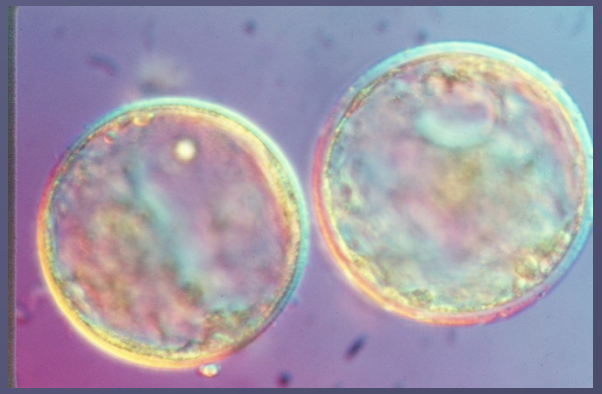



*Prior to PubMed Citation Revisions

Modified from Fahy et al., 2006 and Fahy and Rall, 2007;
Image from Fahy et al., 1984, with modifications

# Vitrification-Early Success

- Different vitrification methods/solutions were applied successfully in the :
  - Mouse (Scheffen, Van der Zwalmen and Massip, 1986)
  - Rabbit (Smorag et al., 1989)
  - Cattle (Massip et al., 1986, 1987)
  - Goat (Yuswiati and Holtz, 1990)
  - Sheep (Szell et al., 1990)

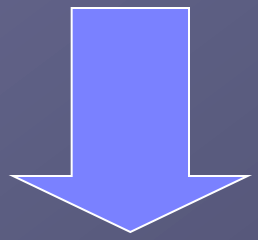


  - Ultimately, high molar VS containing a combination of ethylene glycol, DMSO and other macromolecules have proven to be effective and less toxic (Ishimori et al., 1992a, 1992b; Kasai et al., 1992).

# Oocyte Freezing History

1986: Slow freeze, DMSO (Chen, Australia)
1987: Slow freeze, DMSO (Van Uerm, West Germany)
1989: Slow freeze, PROH and DMSO (Siebzegnrubi, West Germany)

1993-95 SF / PROH and Sucrose
ICSI expts. (Gook et al.)

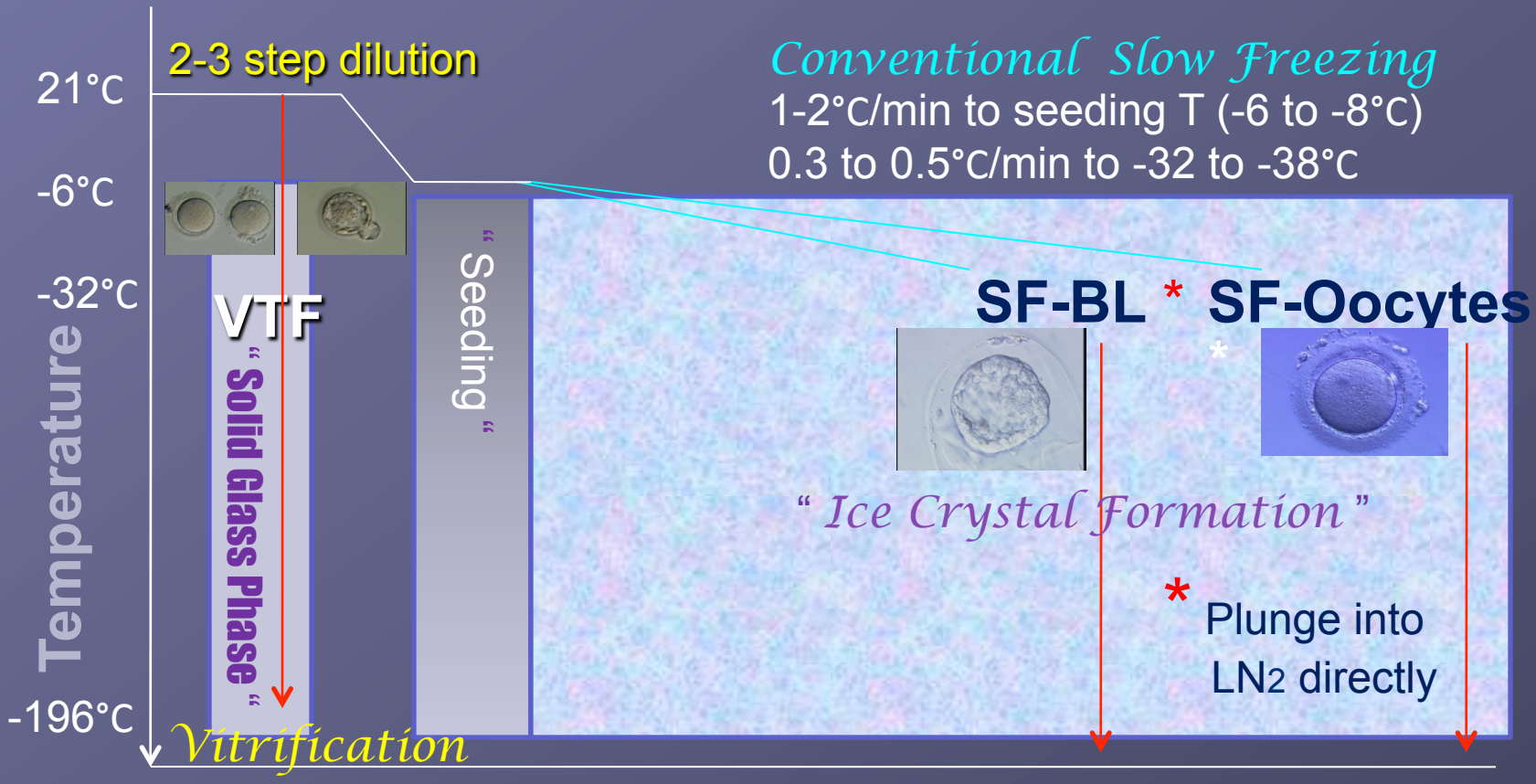


Eight years

1997: Slow freeze, PROH and Sucrose - ICSI (Porcu, Italy)
1998: Slow freeze, PROH and Sucrose - Immature/Donor oocytes (Tucker, USA)
1999: Vitrification, EG and Sucrose - open pulled straws (Kuleshova, Australia)
2000: Vitrification: EG and Sucrose - electron microscope grid (Toon, Cha, Korea)
2003: Vitrification, EG, DMSO and Sucrose - Cryotop™ (Katayama, USA)
2003: Slow freeze, Choline-based medium (Quintans, Argentina)

Schiewe et al, ASRM 2010

# Schematic Comparison of Slow-Freezing to Vitrification Techniques



# Smith's First Warning

"Luyet's practical demonstrations of vitrification [and] devitrification ... depend on the doubtful assumption that transparency is a criterion of the vitreous state. . . . There is no definite proof that protoplasm has . . . ever been vitrified. To prove the existence of the vitreous state, the absence of crystals must be demonstrated . . . ."

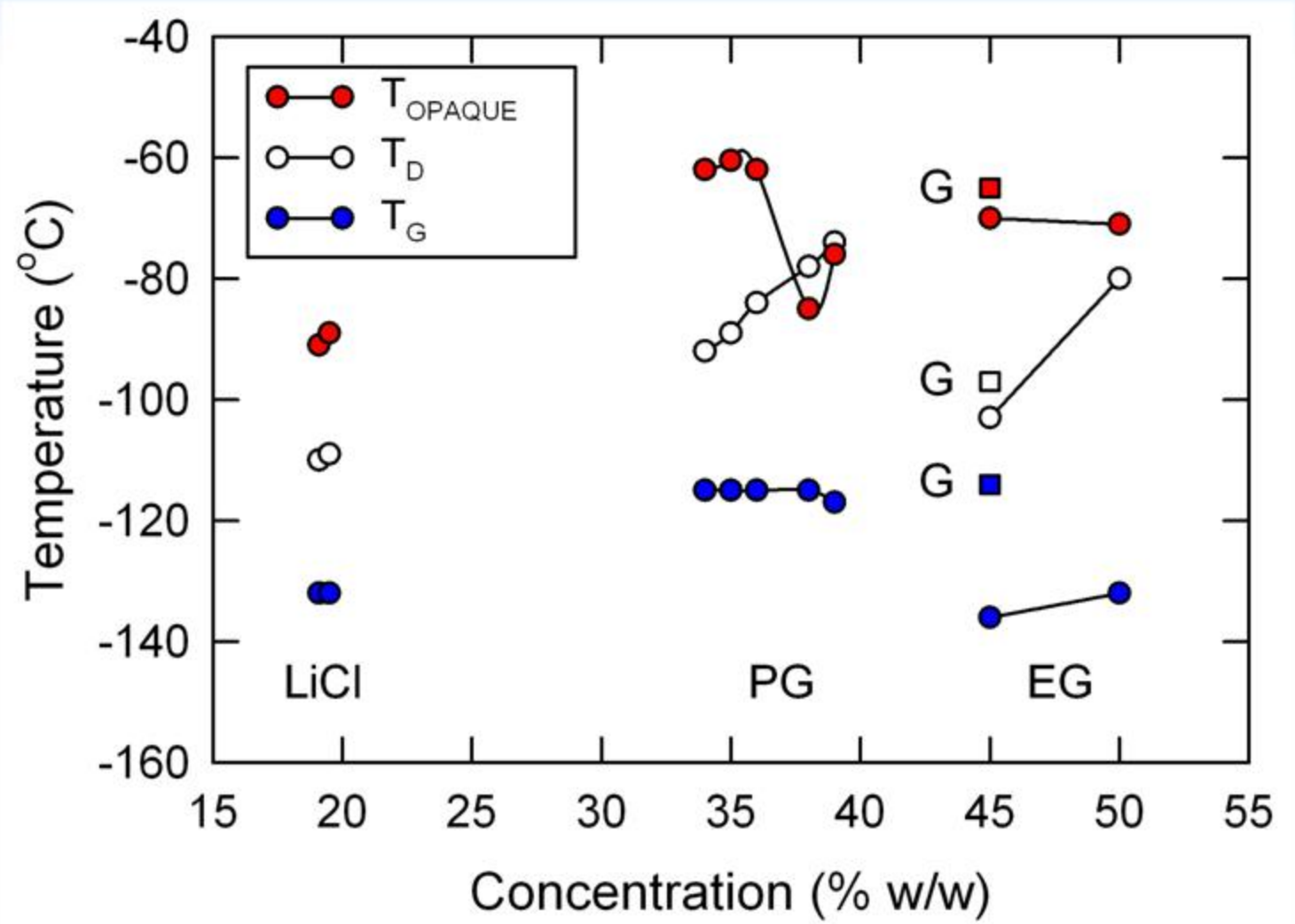
-- Audrey U. Smith, 1954

# Smith's Second Warning

"Luyet . . . postulated that the revival of a cell after freezing and thawing could be used as a criterion of whether vitrification had occurred. . . . [But] It would appear that the survival of cells after ultrarapid cooling and rewarming can be explained without evoking intracellular vitrification."

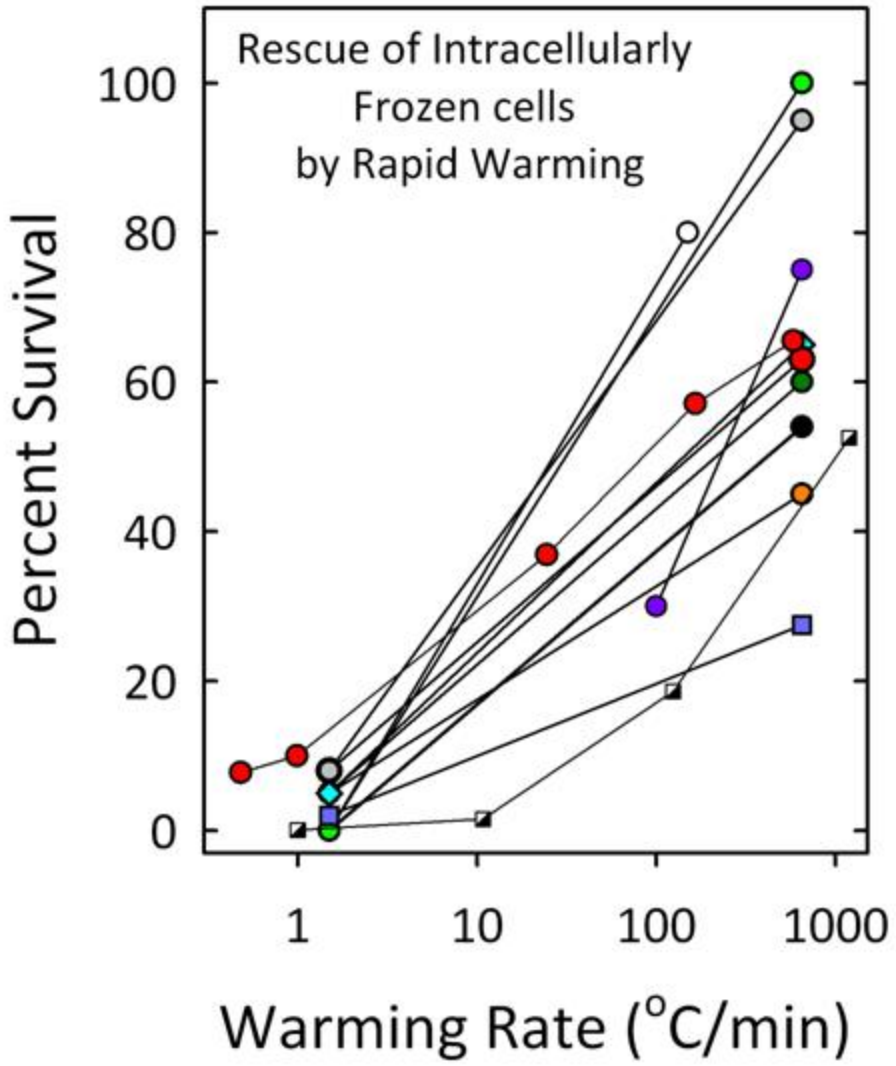
-- Audrey U. Smith, 1954

# Transparency vs Devitrification




Drawn from the data of Forsyth and MacFarlane, 1986

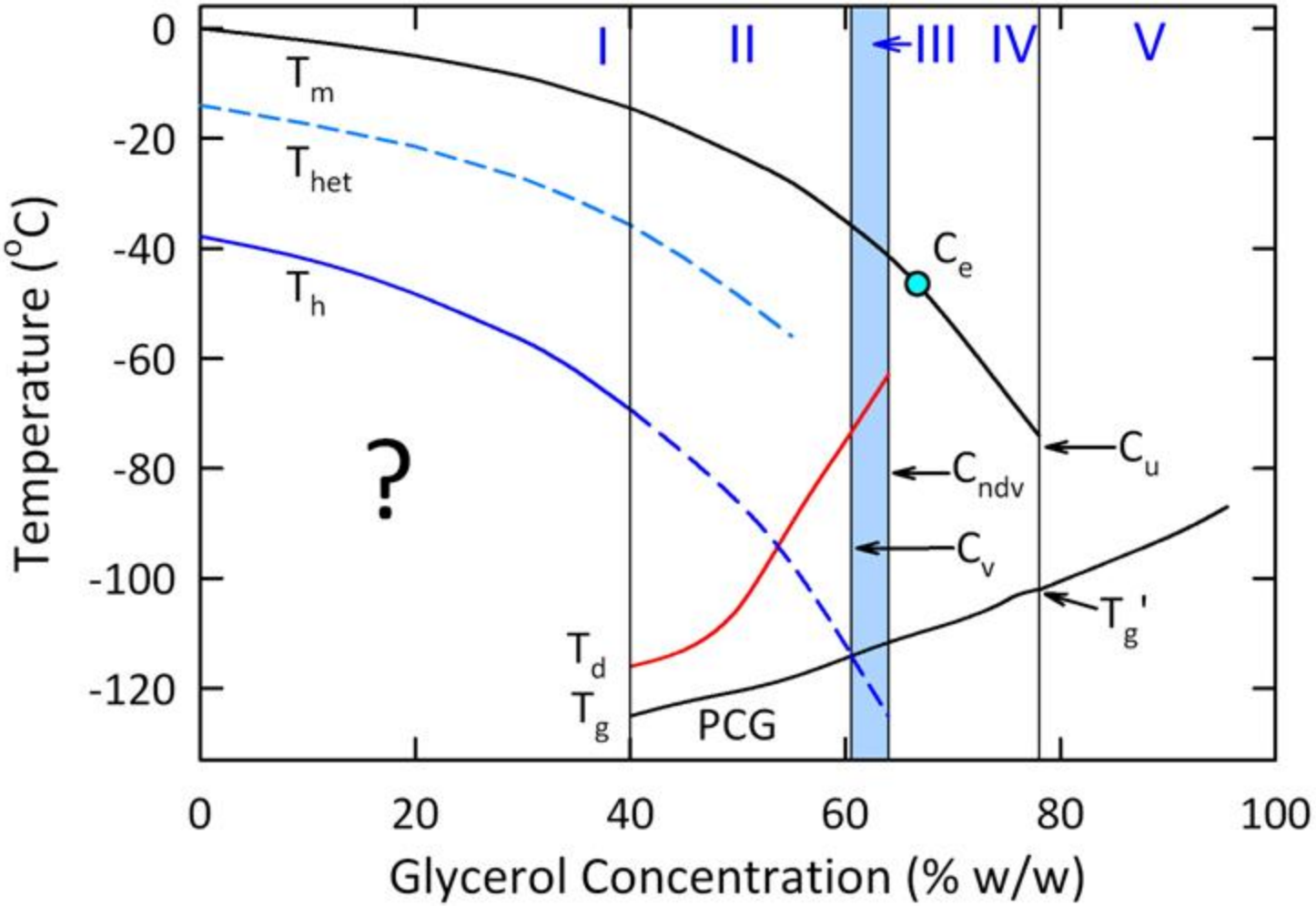
# IIF Can be Survived if Warming is Rapid




- Lily pollen
- Neurospora spores
- Mouse and rabbit embryos plunged from -40°C
- CHO tissue culture cells
- Human red cells in 2M glycerol
- V79 tissue culture cells
- Human erythrocytes
- Mulberry cells
- Ascites cells
- Bacteria
- Cultured carrot cells
- Mouse and human stem cells

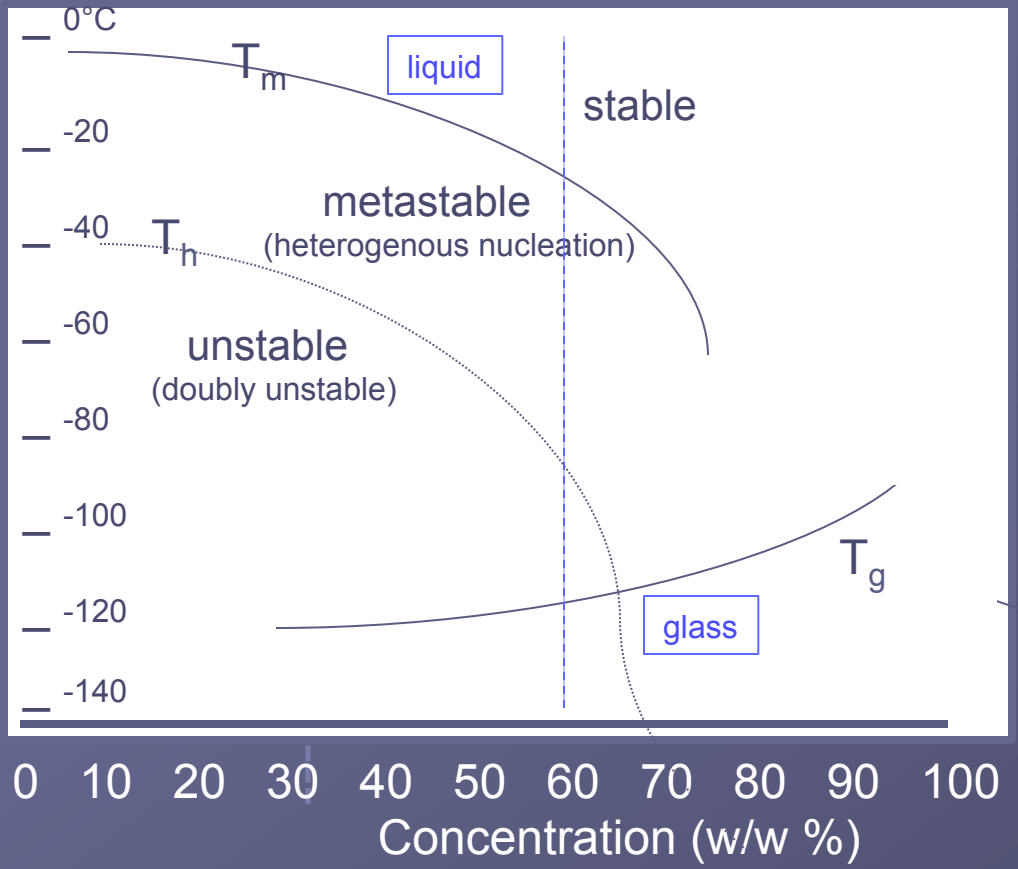
For details and original references,
See Fahy, 1987

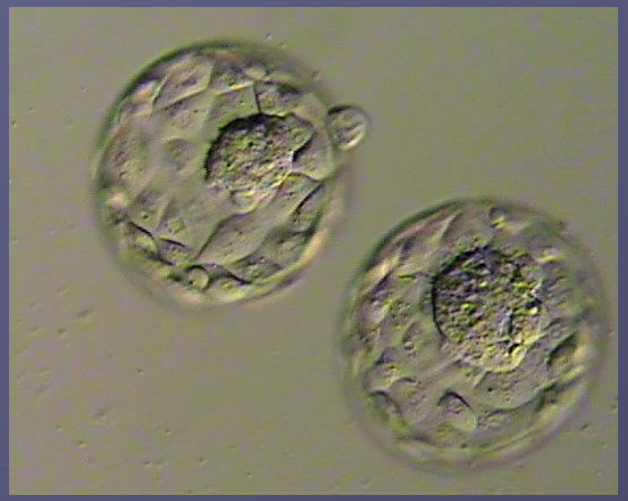
# The Risk of Nucleation and Devitrification Depends Strongly on Concentration




Modified from Fahy et al., 1984, and from Mullen and Fahy, 2010

# Thermodynamics of Vitrification

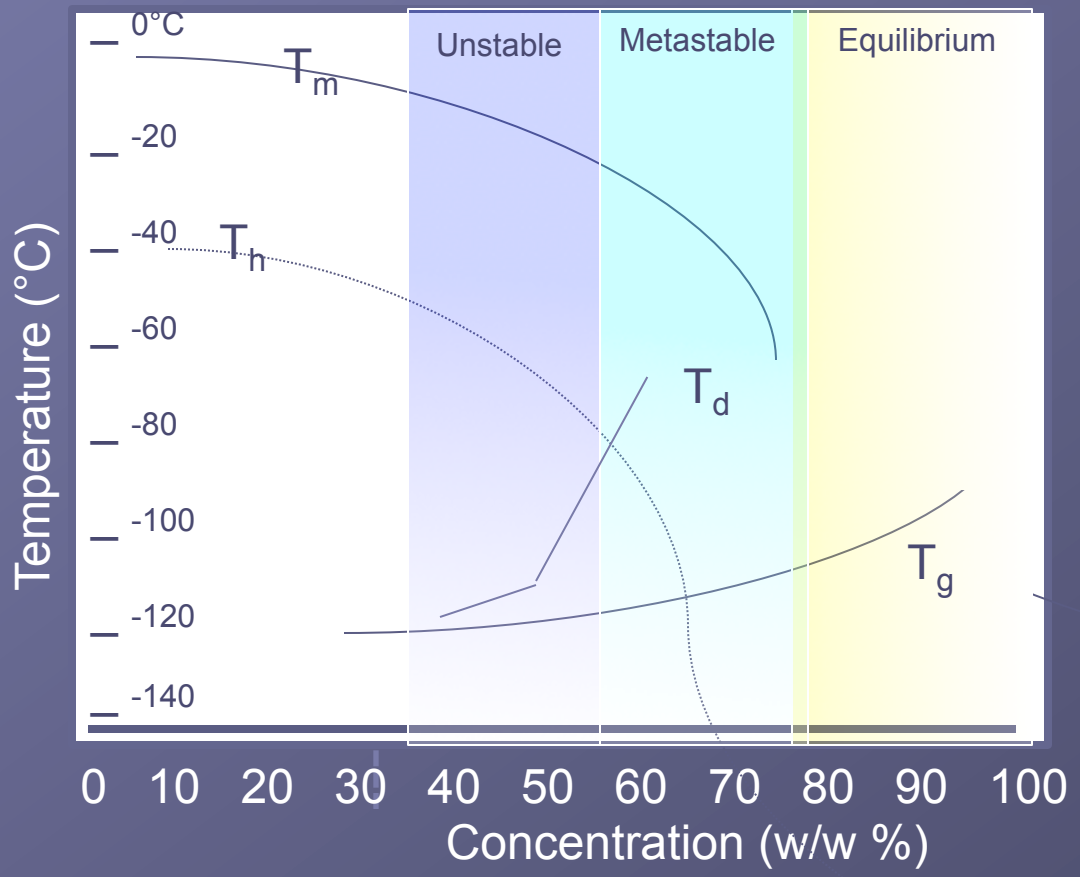


Thermodynamic lines for melting points ($T_m$), heterogeneous nucleation ($T_h$) and glass transition ($T_g$) are approximated trends.

(Adapted from Dr. Brian Wowk's Talk; 50th Society for Cryobiology, 2013)

# Types of Vitrification




It is important to understand the VTF system you are using:

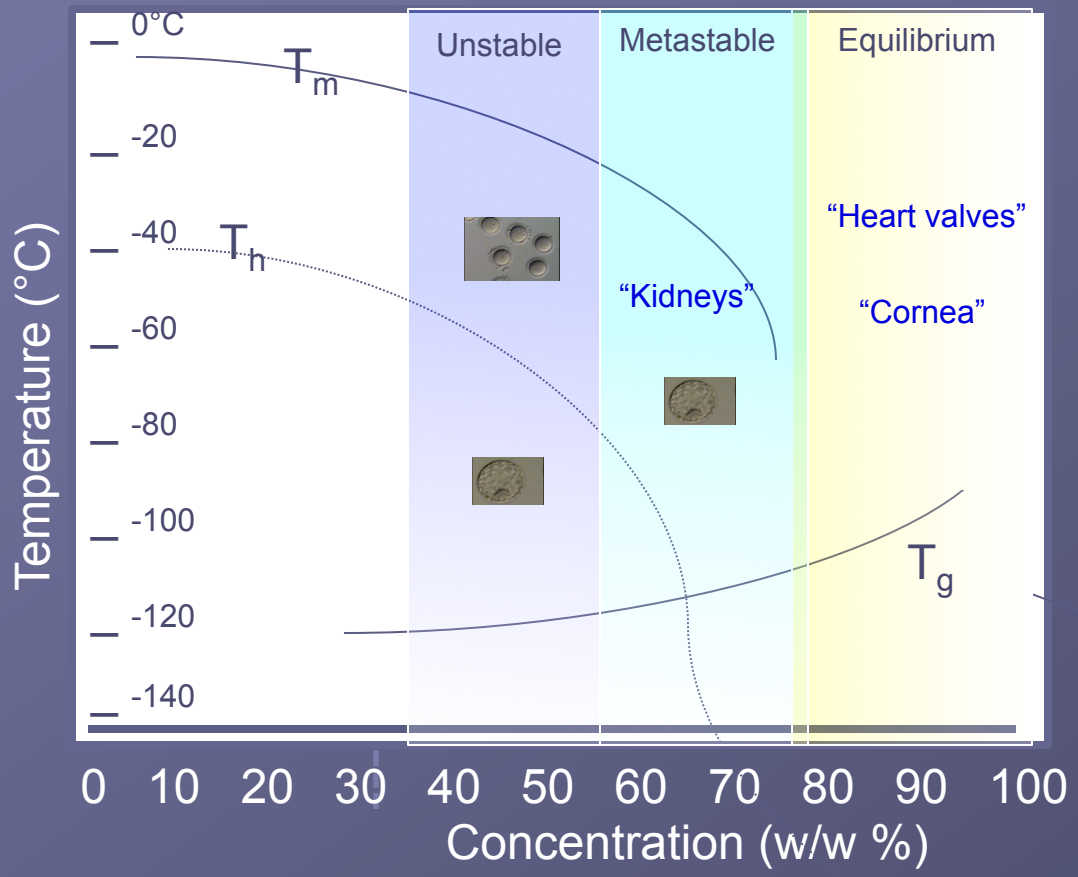
-Open systems with ultra-rapid cooling and warming can effectively use a lower concentration CPA's, but technical variation in warming can Increase susceptability to ice growth

-Closed, aseptic systems with lower cooling rates and rapid warming in excess of 2800°C/min (Mazur and Seki, 2010) can achieve similar success, but are more metastable at higher [CPA]

Thermodynamic lines for melting points ($T_m$), heterogeneous nucleation ($T_h$) and glass transition ($T_g$) are approximated trends.

(Adapted from Dr. Brian Wowk's Talk; 50th Society for Cryobiology, 2013)

# Types of Vitrification




The variety of micro-devices used In ART with micro-volumes have achieved high rates of success with both unstable and metastable forms of EQ-VTF. Other types of tissues have also been successfully vitrified in higher volume systems, when metastable and full EQ-VTF is applied

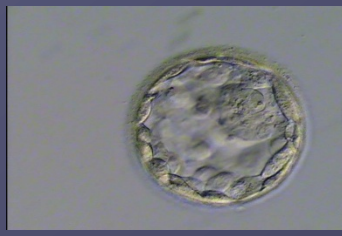
Whereas , small cells with little intracellular water, like sperm, have proven to survive Kinetic VTF in the absence of CPA (+0.5M Sucrose)

Thermodynamic lines for melting points ($T_m$), heterogeneous nucleation ($T_h$) and glass transition ($T_g$) are approximated trends.

(Adapted from Dr. Brian Wowk's Talk; 50th Society for Cryobiology, 2013)

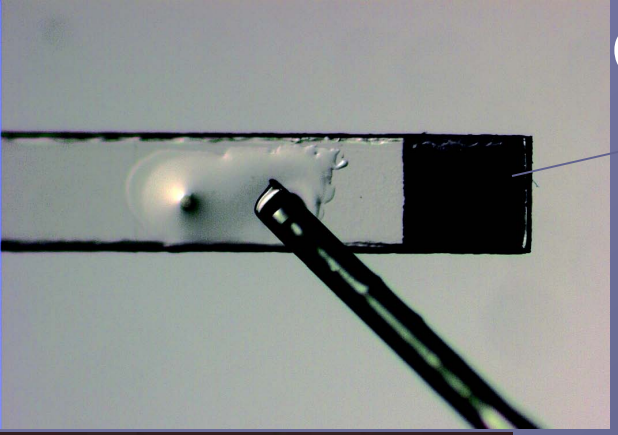
# Vitrification – Container Type?

- 0.25 mL French straw, 1-step *in situ* dilution (Rall et al., 1987; Schiewe et al, 1991)
- Open-pulled straw (OPS: Vajta et al., 1997)
- Cryo-loop (Lane et al.,1999a, 1999b)
- EM grid (Park et al.,1999)
- Nylon Mesh (Matsumoto et al., 2001)
- Cryotop (Kuwayama et al. 2000, 2005b, 2007)
- CryoTip™ (Kuwayama et al. 2005a)

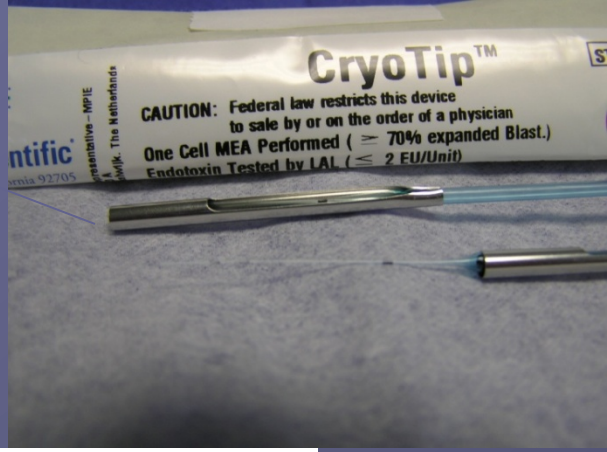


# VTF Devices

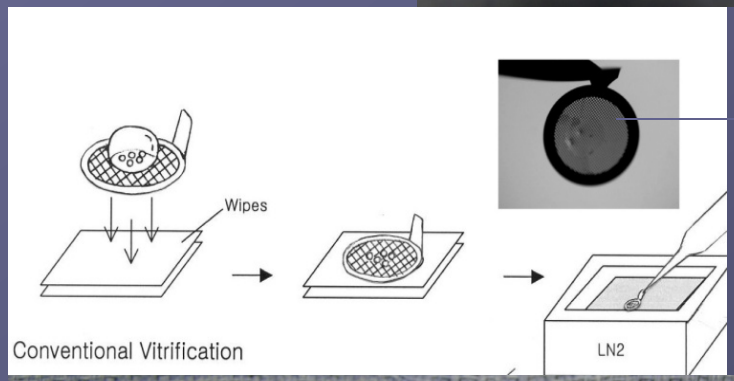
CryoTop



CryoTip

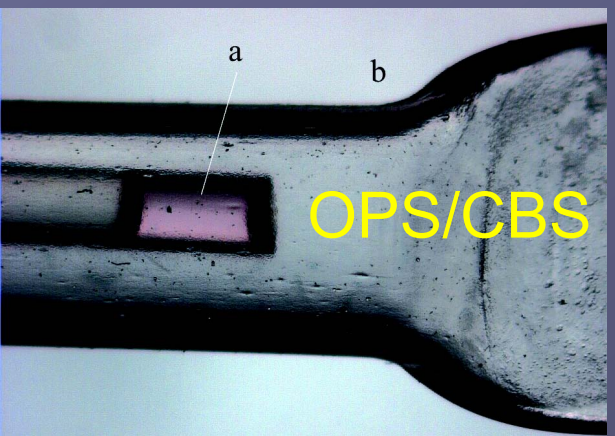



Cryo Loop

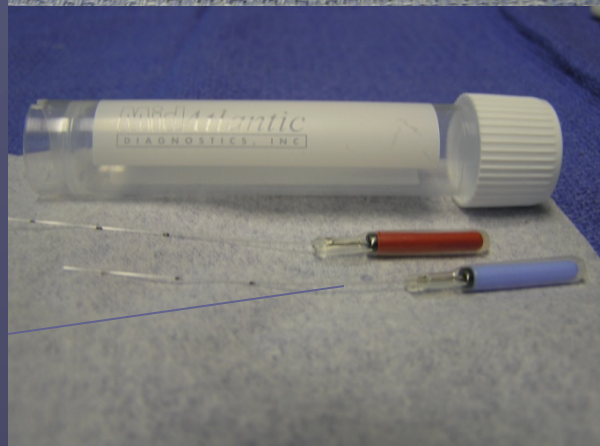

EM Grid

microSecure




OPS/CBS

CryoLeaf



Cryopette

# BL VTF using Cryo-loops: Artificial shrinkage of the blastocoele improved survival and pregnancy outcomes

Mukaida et al., 2006

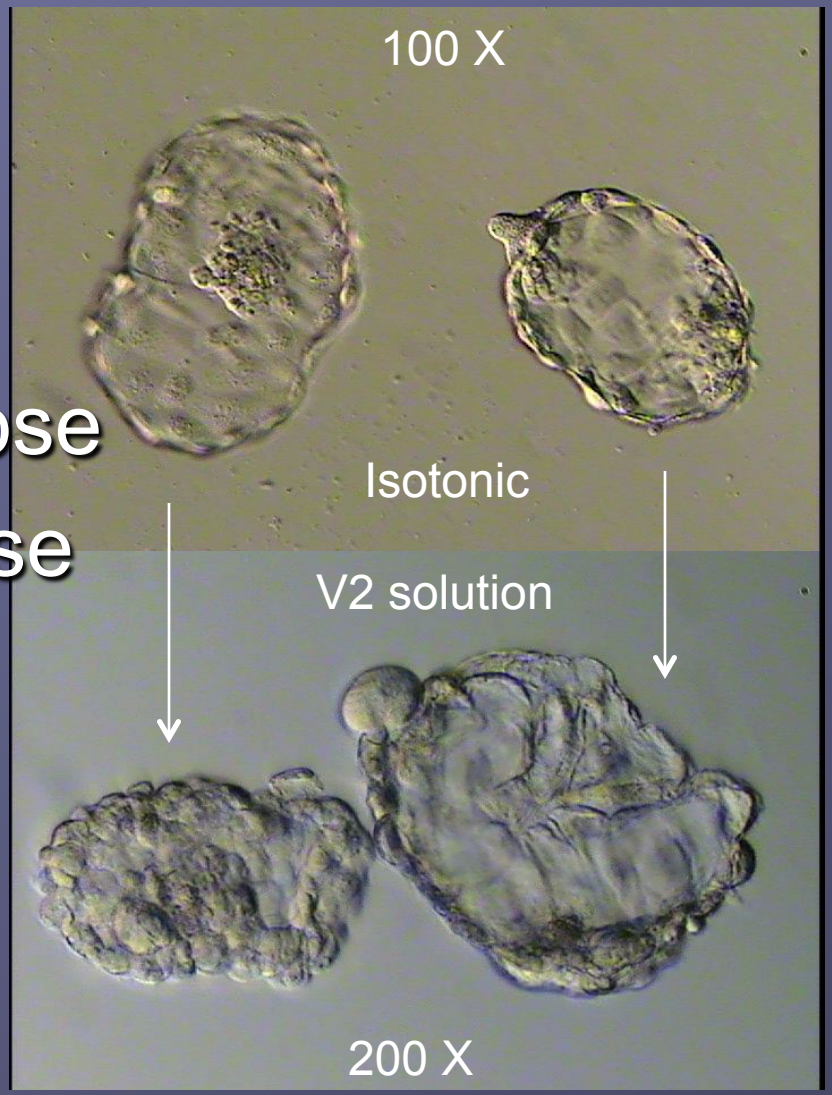
| | # VTF | Survival Rate | Implantation Rate |
|---|---|---|---|
| Intact BL | 569 BL | 86% | 21% |
| Pre-collapsed | 502 BL | 97%* | 47%* |

ESHRE 2010: 10 years experience: >98% survival
High live birth rates, normal child health/well being

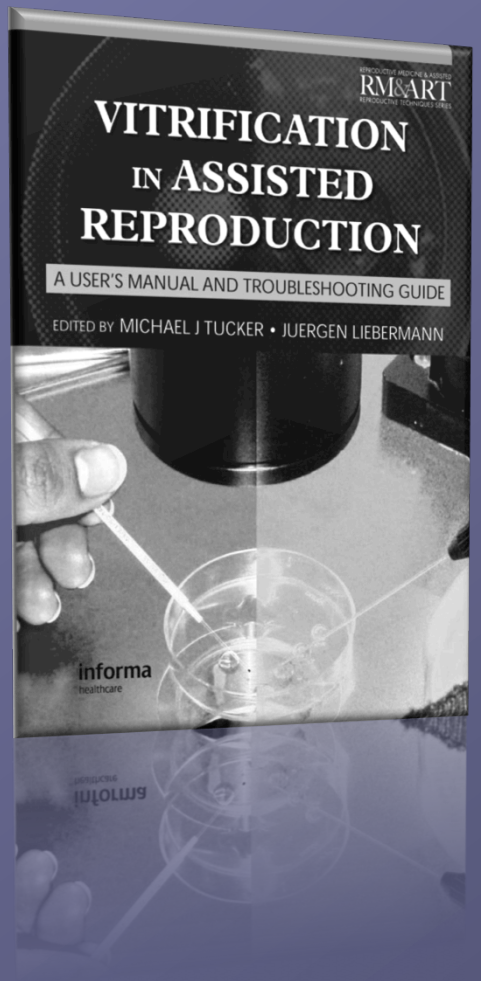
# Artifical BL Collapse or not?

- LZD collapse
- No collapse

Hatched BL Model



# Retrospective Data from Blastocyst Cryopreservation Program at Fertility Centers of Illinois (Chicago), Where Vitrification (VIT) Applied from January 2004 to August 2009




| Technique | VIT |
|---|---|
| Patient age (years) | 34.5 ± 5.0 |
| No. of thaw cycles | 1611 |
| Transfers | 1597 |
| Blastocysts thawed | 3205 |
| Blastocysts survived (%) | 3091 (96.4) |
| Blastocysts transferred | 3062 |
| Mean no. blastocysts transferred | 1.9 |
| Implantations (%) | 909 (29.7) |
| Positive pregnancy/thaw (%) | 788 (48.9) |
| Positive pregnancy/FET (%) | 788 (49.3) |
| Clinical pregnancy/thaw (%) | 678 (42.1) |
| Clinical pregnancy/FET (%) | 678 (42.5) |
| Ongoing pregnancy/FET (%) | 567 (35.5) |
| Live births | 502 (264 girls/238 boys) |

FET = frozen embryo transfer

*Liebermann and Tucker (2006) Fertility & Sterility 86; 20-26; Liebermann J (2009) RBMOnline 19, Suppl. 2,*

(Slide courtesy of J. Liebermann, FCI)

# Aseptic Device that Separates Blastocysts from $LN_2$ (HSV)

| Day of Development | Day 5 | Day 6 |
|---|---|---|
| Patient age (years) | 34.1 ± 5.1 | 34.3 ± 4.4 |
| No. of cycles | 191 | 203 |
| No. of transfers | 191 | 201 |
| No. of blastocysts thawed | 385 | 399 |
| No. of blastocysts survived (%) | 375 (97.4) | 388 (97.2) |
| No. of blastocysts transferred | 372 | 385 |
| Mean no. of blastocysts transferred | 1.9 | 1.9 |
| No. of implantations (%) | 145 (39.0)[a] | 97 (25.2)[a] |
| No. of positive pregnancies/FET (%) | 119 (62.3)[b] | 86 (42.8)[b] |
| No. of clinical pregnancies/FET (%) | 102 (53.4)[c] | 74 (36.8)[c] |
| Ongoing pregnancies/FET (%) | 99 (51.8)[d] | 71 (35.3)[d] |

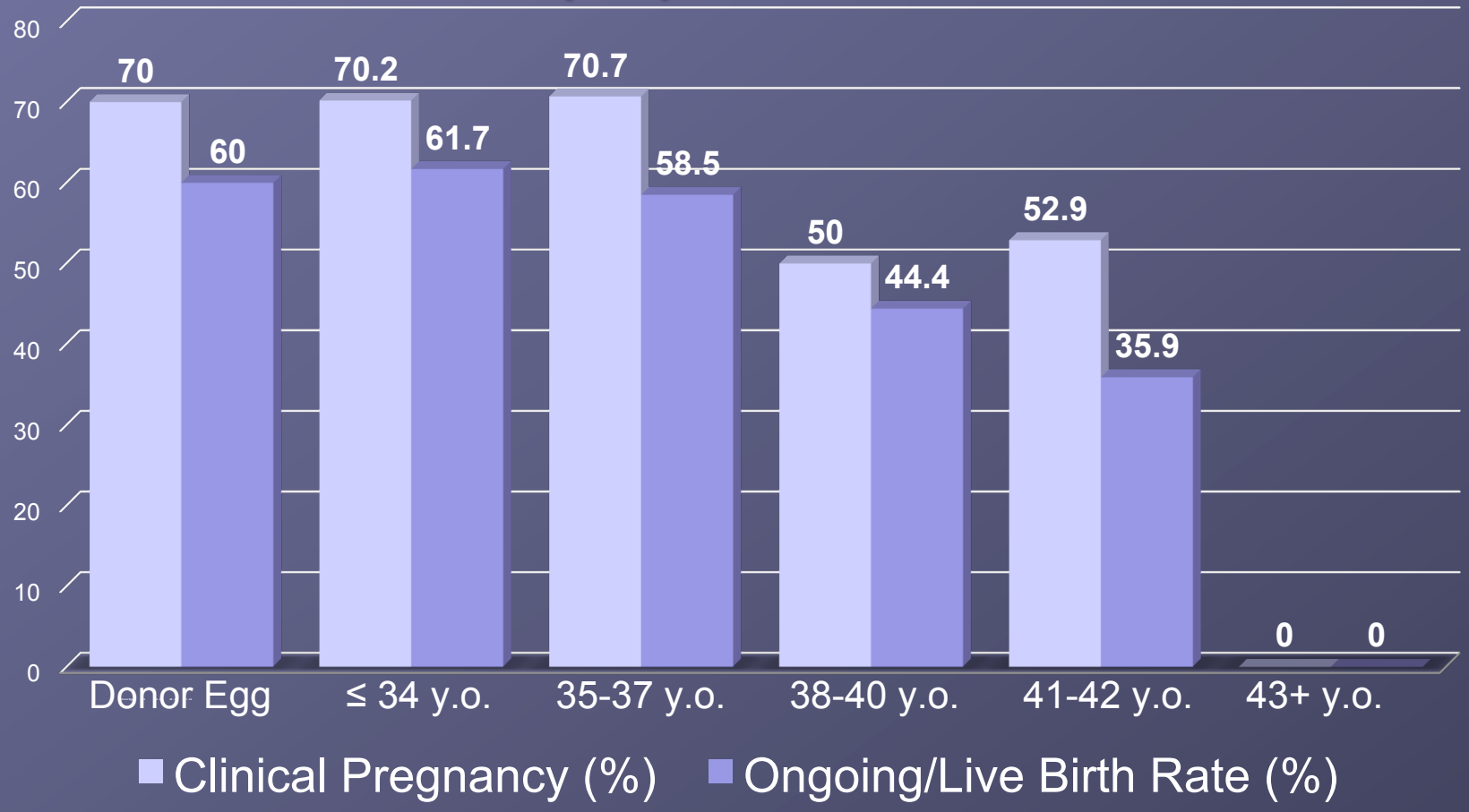
[a,b,c,d]P<0.01

(Slide courtesy of J. Liebermann, FCI)

# Non-PGS VFET Pregnancy Outcomes
# Dr. Robert E. Anderson, MD/SCCRM

## MicroSecure VTF / I.C.E. non-DMSO VS ; 2011-2013

*** No BL collapse pre-VTF: 96% BL survival**

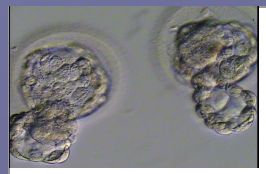



Schiewe et al, ASRM 2014

SCIRS Southern California Institute for Reproductive Sciences

# PGS-VFET Pregnancy Outcomes
# Dr. Robert E. Anderson, MD/SCCRM

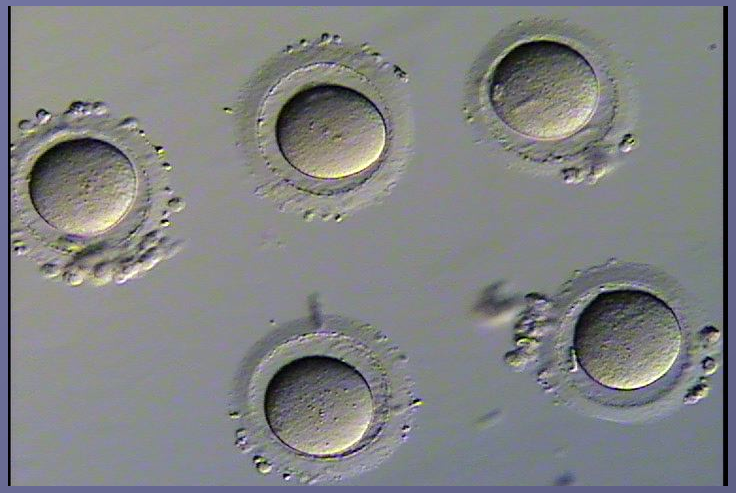
- VFET of euploid Blastocysts, with 99% survival of biopsied embryos



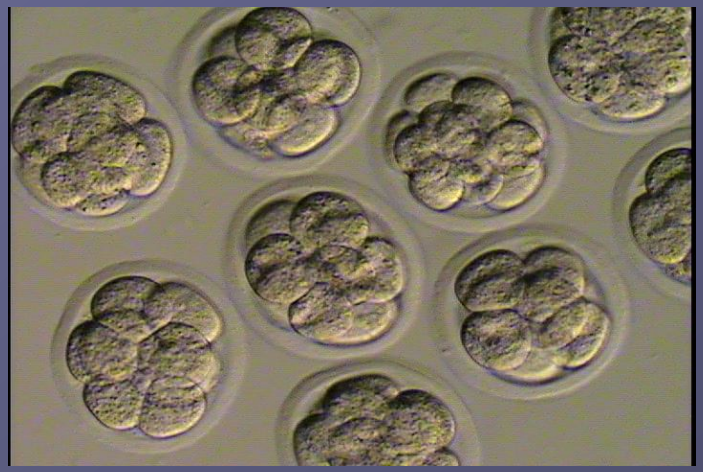



Schiewe et al, ESHRE 2014

# VTF Success/Post-warming



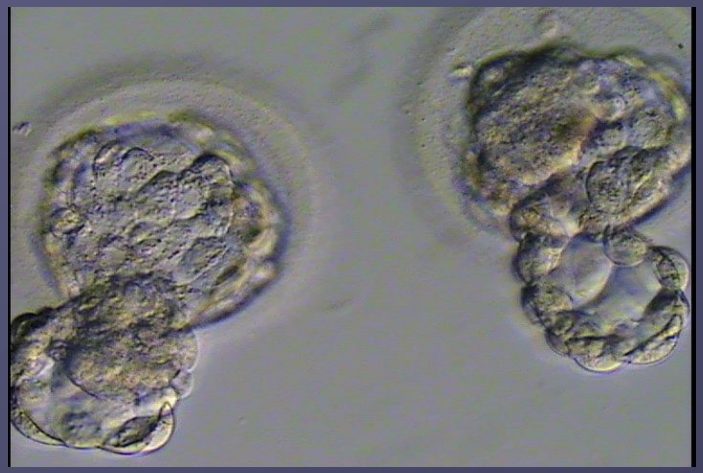
Oocytes



Cleavage stages

Blastocyst

PGS-BLs



PGS = preimplantation genetic screening

# OOCYTE VITRIFICATION: WHERE ARE WE AT TODAY?

## CLINICAL RESULTS

Dr. ZP Nagy

# Why to cryopreserve eggs?

- Government restrictions / legislation
- Fertility preservation
  - Medical
  - Social
- Emergency cycle management
  - Failure to obtain sperm
- Elective cycle management
  - Ethical/Religious reasons
  - Ovarian hyperstimulation syndrome (OHSS)
- Donor egg banking

# Numbers of clinics and cycles performed with slow freezing, vitrification, and fresh cycles

Levi Setti et al, Human oocyte cryopreservation with slow freezing versus vitrification. Results from the National Italian Registry data, 2007-2011. Fertil Steril. 2014

| | Slow freezing | | | Vitrification | | | Total cycles | | | Fresh cycles | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Year | Center | Cycles | Range | Center | Cycles | Range | Centers | Cycles | Range | Center | Cycles | Range |
| 2007 | 85 | 2,426 | 1–270 | 30 | 568 | 1–252 | 93 | 2,994 | 1–270 | 181 | 40,005 | 2–1,415 |
| 2008 | 88 | 2,625 | 1–304 | 41 | 659 | 1–225 | 104 | 3,284 | 1–304 | 185 | 44,037 | 5–1,599 |
| 2009 | 88 | 1,916 | 1–165 | 60 | 1,186 | 1–162 | 114 | 3,102 | 1–325 | 180 | 47,911 | 7–1,702 |
| 2010 | 75 | 1,097 | 1–81 | 69 | 1,344 | 1–143 | 109 | 2,441 | 1–191 | 174 | 52,661 | 7–1,847 |
| 2011[a] | 64 | 863 | 1–80 | 88 | 1,644 | 1–145 | 120 | 2,507 | 1–177 | 179 | 56,086 | 2–1,897 |
| **Total** | **109** | **8,927** | **1–805** | **102** | **5,401** | **1–718** | **146** | **14,328** | **1–1,255** | **204** | **240,700** | **2–8,460** |

# Thawed/warmed or fresh oocytes per live born baby following slow freezing, vitrification, and fresh cycles

Levi Setti et al, Human oocyte cryopreservation with slow freezing versus vitrification. Results from the National Italian Registry data, 2007-2011. Fertil Steril. 2014

| Year | **SF** | n | **VT** | n | **Fresh** | n | OR (95% CI), SF (SF = 1) vs. VT | *P* value, SF vs. VT | *P*value |
|---|---|---|---|---|---|---|---|---|---|
| 2007 | 12,573/ 199 | **63.2** | 2,317/ 50 | **46.3** | 234,004/ 6,476 | **36.1** | 1.37 (1.00–1.83) | .047 | <.001 |
| 2008 | 13,592/ 237 | **57.4** | 2,949/ 63 | **46.8** | 256,293/ 7,479 | **34.3** | 1.23 (0.93–1.63) | .148 | <.001 |
| 2009 | 10,821/ 155 | **69.8** | 5,707/ 134 | **42.6** | 285,042/ 8,037 | **35.5** | 1.65 (1.31–2.09) | <.001 | <.001 |
| 2010 | 6,068/ 101 | **60.1** | 6,906/ 141 | **49.0** | 312,481/ 9,281 | **33.7** | 1.23 (0.95–1.59) | .113 | <.001 |
| 2011 | 4,860/ 86 | **56.5** | 8,625/ 172 | **50.1** | 333,618/ 8,733 | **38.2** | 1.13 (0.87–1.47) | .361 | <.001 |
| **Overall** | 47,914/778 | **61.6** | 26,504/ 560 | **47.3** | 1,421,438/ 40,006 | **35.5** | 1.31 (1.17–1.46) | <.001 | <.001 |

# Fertility Preservation
## *MEDICAL*

Five years' experience using oocyte vitrification to preserve fertility for medical and nonmedical indications. Garcia-Velasco JA1, Domingo J, Cobo A, Martínez M, Carmona L, Pellicer A. FS 2013

6 babies

| | Oncological |
|---|---|
| Nº patients FP | 361 |
| Nº Patients using v. oocytes | 11 |
| Mean age at vitrification | 31.9 ± 5.1 |
| Mean age at warming | 36.1 ± 6.1 |
| Nº oocytes warmed | 69 (6.2 ± 0.1) |
| Survival rate | 88.6 |
| Nº embryos transferred | 2 ± 0.1 |
| Nº patients with surplus embryos | 5 (45.5) |
| CPR/patient | 6 (54.5) |
| OPR/patient | 5 (45.5) |
| Live birth | 4 |
| Ongoing pregnancies | 1 |

*Updated 5-years experience of applying oocyte vitrification for Fertility Preservation at IVI.*

Slide courtesy of Dr. Cobo

# Fertility Preservation
## *SOCIAL*

Five years' experience using oocyte vitrification to preserve fertility for medical and nonmedical indications. Garcia-Velasco JA1, Domingo J, Cobo A, Martínez M, Carmona L, Pellicer A. FS 2013

6 babies

| | Non oncological |
|---|---|
| Nº patients FP | 907 |
| Nº Patients using v. oocytes | 35 |
| Mean age at vitrification | 35.9 ± 4.2 |
| Mean age at warming | 38.1 ± 2.8 |
| Nº oocytes warmed | 250 (7.0 ± 3.5) |
| Survival rate | 92.3 |
| Nº embryos transferred | 2 ± 0.7 |
| Nº patients with surplus embryos | 22 (62.9) |
| CPR/patient | 15 (42.8) |
| OPR/patient | 11 (31.4) |
| Live birth | 8 |
| Ongoing pregnancies | 3 |

*Updated 5-years experience of applying oocyte vitrification for Fertility Preservation at IVI.*

Slide courtesy of Dr. Cobo

# Oocyte vitrification for emergency cycle management and other causes

| | No semen sample the day of OPU | Gynecological causes |
|---|---|---|
| Nº of patients | 18 | 74 |
| Mean age | 34.9 ± 3.6 | 37.9 ± 3.9 |
| Nº of embryo transfers | 18 (100) | 68 (91.9) |
| Nº of vit. Oocytes | 188 (9.56 ± 1.5) | 899 (10.3 ± 4.1) |
| Survival rate | 172 (91.8) | 758 (84.3) |
| Mean number of ET | 1.9 ± 0.5 | 1.9 ± 0.4 |
| **Implantation rate** | 41.7 | 37.1 |
| Pregnancy rate | 11 (61.1) | 41 (60.3) |
| **Clinical pregnancy rate** | 11 (61.1) | 40 (58.0) |
| Ongoing pregnancy rate | 11 (61.1) | 35 (51.4) |

Cobo et al, 2011

# Outcomes for patients who choose to electively cryopreserve part of their oocytes (fresh / cryo)

| RBA, 2013 | FRESH CYCLE | WARMING CYCLE |
|---|---|---|
| # PATIENTS / CYCLES | 37/42 | 34/34 |
| AVG. AGE ± S.D. | 32.6 ± 3.70 | 33.6 ± 3.51 |
| AVG. # OOCYTES RETRIEVED | 31.6 | - |
| AVG. # MII OOCYTES ±S.D. | **22.8** ± 10.9 | - |
| AVG. # MII VITRIFIED ± S.D. | **13.1** ± 8.9 | - |
| AVG. # MII WARMED ± S.D. | | 9.6 ± 7.48 |
| SURVIVAL RATE ±S.D. (n) | - | 82.9% |
| AVG. No. OOCYTES ICSI ±S.D. | 9.2 ± 5.31 | 8.0 ± 6.72 |
| **FERTILIZATION RATE ±S.D. (n)** | **71.5%** | **77.9%** |
| **BLASTOCYST RATE ±S.D. (n)** | **43.0%** | **49.8%** |
| AVG. # EMBRYOS TRANSFERRED | 1.4 | 1.7 |
| IMPLANTATION RATE (n) | 16% | 25% |
| CLINICAL PREGNANCY RATE (n) | **33%** | **41%** |

# Oocyte vitrification in the management of OHSS

## Patients at risk of OHSS

| | |
|---|---|
| **Nº patients** | **44** |
| Age (mean ± SD) | 32.8 ± 3.3 |
| Nº of vitrified oocytes (mean ± SD) | 593 (16.9 ± 5.1) |
| Nº of warmed oocytes (mean ± SD) | 450 (12.9 ± 5.0) |
| **Survival N(%)** | **378 (84.0)** |
| Nº of embryo transfers (%) | 33 (94.3) |
| **Implantation rate** | **24/70 (34.3)** |
| Mean number of embryos transferred ± SD | 2.2 ± 0.6 |
| Pregnancy rate/transfer | 22/33 (66.6) |
| **Clinical pregnancy rate** | **18/33 (54.5)** |
| Miscarriage rate | 4/18 (22.2) |
| Ongoing pregnancy rate/transfer | 14/33 (42.4) |

Slide courtesy of Dr. Cobo

# Egg-banking in ovum donation. RCT

| | Egg- bank | Fresh | P value |
|---|---|---|---|
| Number of subjects | 295 | 289 | |
| MII oocytes retrieved | 3286 (11.1 ±3.2) | 3185 (11.0 ±2.8) | 0.634 |
| Survival rate | 3039 (92.5) | - | - |
| Oocytes inseminated | 3039 (10.3±2.9) | 3185 (11.2 ±3.4) | 0.091 |
| Fertilization rate (2PN) | 2256 (74.2) | 2334 (73.3) | 0.393 |
| Top quality day-3 | 1098 (36.1) | 1201 (37.7) | 0.198 |
| Clinical Pregnancy Rate | 50.2% | 49.8% | NS |

*Cobo et al Hum Reprod. 2010*

# MEB experience on donor egg banking

- Donation cycles 1,035
- M2 vitrified 23,060 (22.3/don)
- Recipient cycles 3,424
- M2 Warmed 21,462 (6.3/R.)
- Survival 88%
- Fertilization 78%
- Pregnancy (clinical) 52%

# Practical aspects of vitrification

- Contamination?
- “Double Vitrification”
- How many eggs “needed”? (Fert. Pres.)
- Live birth / safety

# Viral screening of spent culture media and liquid nitrogen samples of oocytes and embryos from hepatitis B, hepatitis C, and human immunodeficiency virus chronically infected women undergoing in vitro fertilization cycles

Ana Cobo, Ph.D., José Bellver, M.D., María José de los Santos, Ph.D., and José Remohí, M.D.
IVI-Valencia, Institut Universitari IVI, Valencia, Spain

Fertility and Sterility® VOL. 97 NO. 1 / JANUARY 2012

SCM OPU

SCM embryo culture

LN remains from OV or EV

LN from storage tank

Egg Bank

**TABLE 1**

Viral screening for chronically infected patients according to different types of samples and ART procedures.

| Patient | Type of sample | No. of assays | Type of virus | ART procedure | PCR analysis |
|---|---|---|---|---|---|
| 1 | SCM | 1 | HIV | OPU | Negative |
| 2 | SCM | 1 | HCV | OPU | Negative |
| 3 | SCM | 3 | HCV | OPU | Negative |
| | | | | | |
| | SCM | 1 | HBV | OPU | Negative |
| | LN | 1 | HBV | Embryo vitrification | Negative |
| 24 | FF | 1 | HIV | OPU | Negative |
| | SCM | 1 | HIV | IVF/ET | Negative |
| | LN | 1 | HIV | Embryo vitrification | Negative |

*Cobo. Viral screening of culture media and LN. Fertil Steril 2012.*

# Vitrified Embryos From Vitrified Eggs ("Double Vitrification")

| 100 patients (Cryo Egg Bank) | Cryo Embryo |
|---|---|
| Number of warmed embryos | 190 |
| Survived | 189 (99%) |
| No of Es for ET (x) | 176 (1.8)* |
| Pregnancies (Clinical) | 53 (53%) |
| Implantation / FCA | 68 (39%) |
| Miscarriages | 12 |
| Live births (limited data) | 33 |
| Girls | 15 |
| Boys | 18 |

* Four of these embryos were biopsied in the first cycle, then vitrified

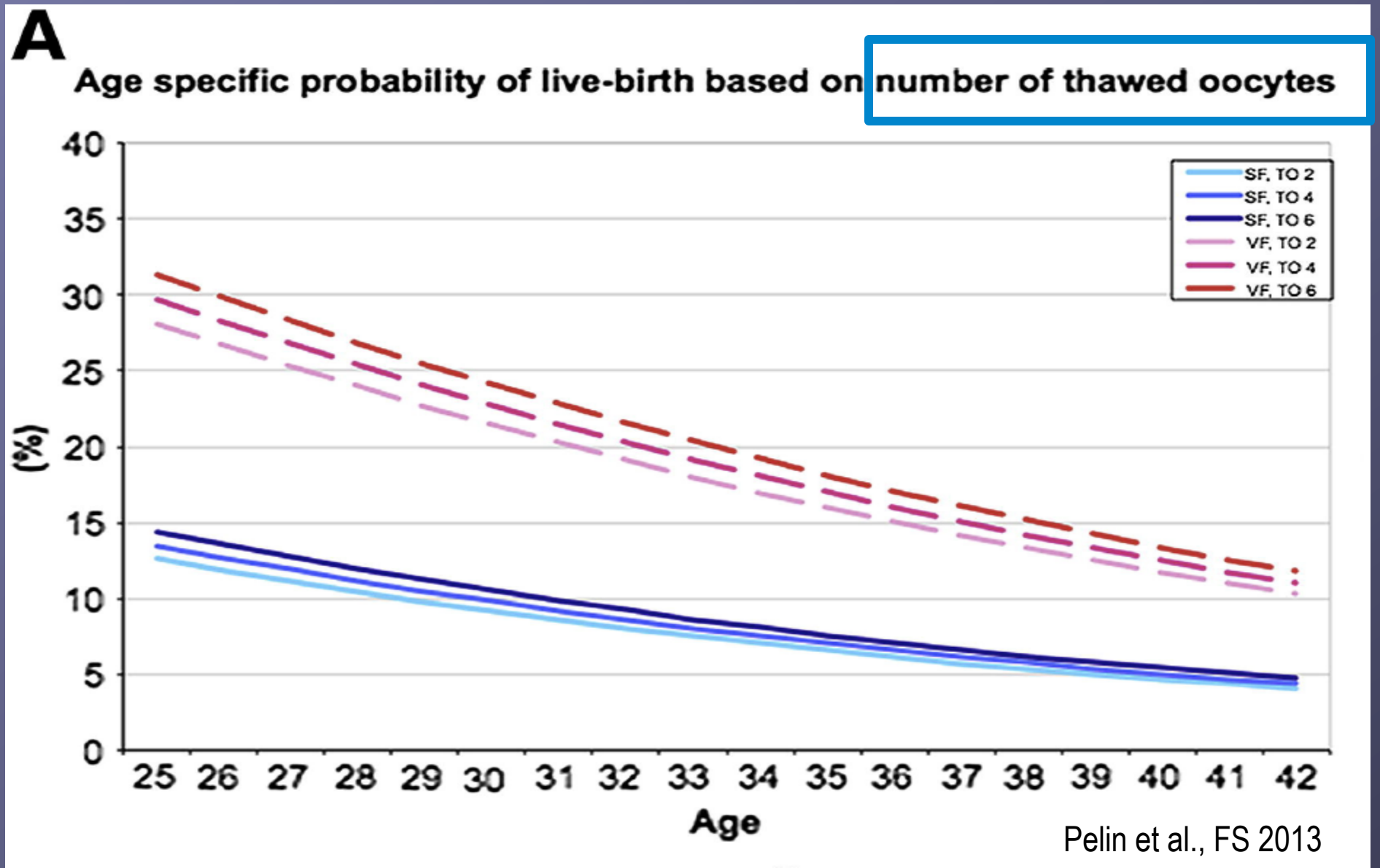
# How many eggs? RCT: IVF patients 30–39 years

Prospective controlled study to evaluate laboratory and clinical outcomes of oocyte vitrification obtained in in vitro fertilization patients aged 30 to 39 years. Chang CC, Elliott TA, Wright G, Shapiro DB, Toledo AA, Nagy ZP. FS 2013

| | Young **30–36 y (n=11)** | Advanced **37–39 y (n=11)** | *P* |
|---|---|---|---|
| Patient age (mean±SD) | 32.9 ±1.9 | 37.9 ±0.8 | <.01 |
| **Survival rate (%)** | **82.5** | **76.4** | NS |
| Fertilization rate (%) | 70.1 | 62.9 | NS |
| Day 3 good Embryo (%) | 55.6 | 40.4 | <.05 |
| Embryos transferred | 24 (2.18) | 29 (2.64) | NS |
| **Clinical pregnancies (%)** | **7/11 (63.6)** | **3/11 (27.3)** | NS |
| Implantations (%) | 10/24 (41.7) | 6/29 (20.7) | NS |
| Take home babies (%) | 6/11 (54.5) | 2/11 (18.2) | NS |
| No. of live births | 8 | 3 | – |
| **Oocyte to Live birth (%)** | **8/97 (8.2)** | **3/89 (3.3)** | NS |

Chang et al FS, 2013

# Age-specific probability of live birth with oocyte cryopreservation: an individual patient data meta-analysis




Pelin et al., FS 2013

# Live Birth Data from Egg Cryo from RBA

## Including deliveries until the end of 2011

| | Fresh Donor | Cryo Donor |
|---|---|---|
| No. of patients / Deliveries | 58 | 257 |
| Recipient Age | 39.9 ±5.6 | 41.3 ± 4.5 |
| Live births (infants born) | 91 | 338 |
| Term delivery 37 weeks | 28 | 188 |
| Congenital anomaly* | 3 | 5 |
| All deliveries | 2659.4 ±690.9 | 2938.3 ± 770.0 |
| Singleton/twin/triplet deliveries | 26/31/1 | 178 / 77 / 2 |
| Term deliveries | 3361.2 ±677.2 | 3518.8 ± 585.2 |

Down sy. 2xHemangioma

Congenital anomalies: heart murmur, 1 baby died at 2 months with multiple complications, cleft lip/palate, club foot, spina bifida (TAB)

# CONCLUSION: Vitrification has changed our way of work

## Deferred embryo transfer

- Elevated P4 levels
- Risk of OHSS
- Endometrial impairment
- LR in AMA
- ArrayCGH

ASSISTED REPRODUCTION

J Assist Reprod Genet (2010) 27:357–363

**Can fresh embryo transfers be replaced by cryopreserved-thawed embryo transfers in assisted reproductive cycles? A randomized controlled trial**

Abbas Aflatoonian · Homa Oskouian · Shahnaz Ahmadi · Leila Oskouian

**Evidence of impaired endometrial receptivity after ovarian stimulation for in vitro fertilization: a prospective randomized trial comparing fresh and frozen–thawed embryo transfer in normal responders**

*Bruce S. Shapiro, M.D., Ph.D.,[a,b] Said T. Daneshmand, M.D.,[a,b] Forest C. Garner, M.Sc.,[a,b] Martha Aguirre, Ph.D.,[a] Cynthia Hudson, M.S.,[a] and Shyni Thomas, B.Sc.[a]*

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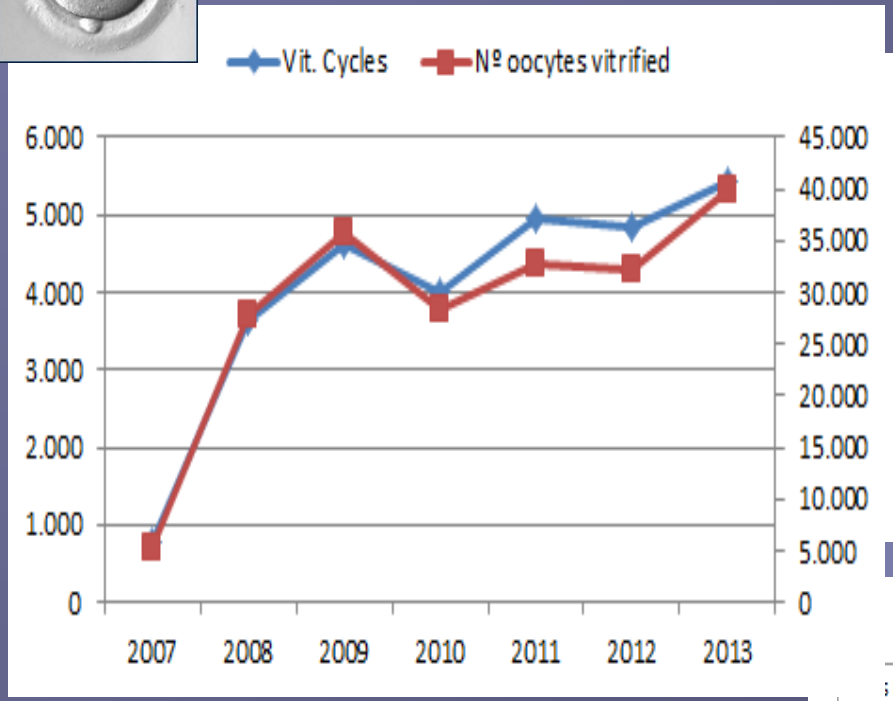
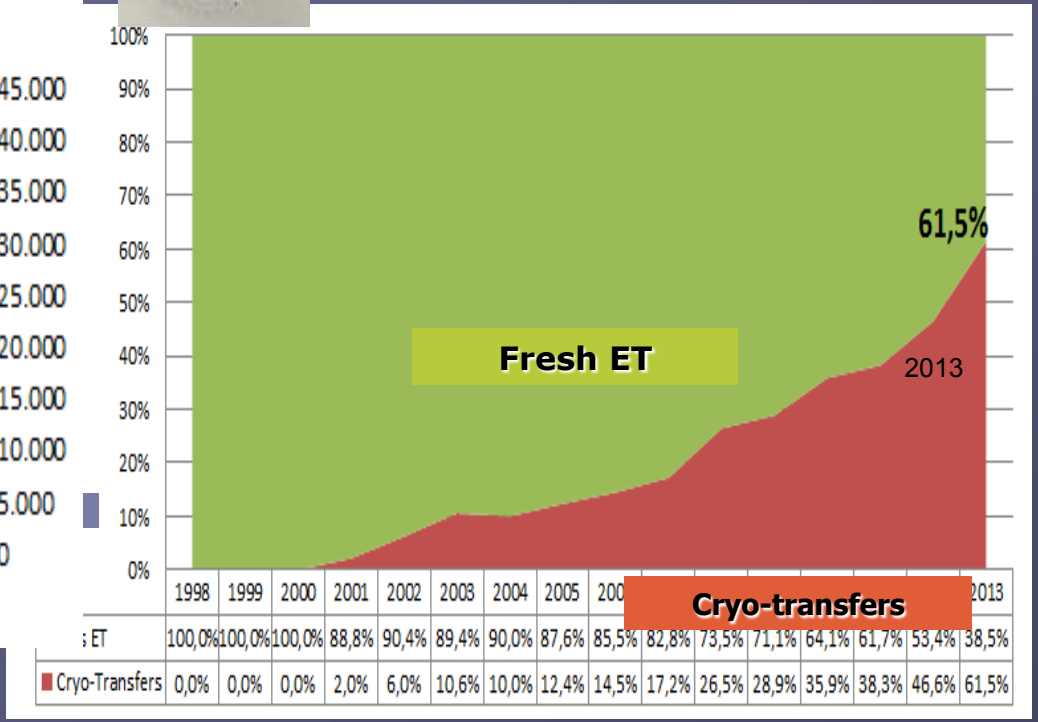
Slide courtesy of Dr. Cobo

# The Future (is now): Efficient cryopreservation changes IVF practice




Distribution of Cryotransfers




Graphs courtesy of Dr. Cobo

- Improvements?
  - Solutions, devices, procedures?

# Thank you


To the memories of Dr. Stanley Leibo and the other Legends in Cryobiology