Vitrification Techniques & Outcomes: More than 10 Years of Clinical Application

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IrvineScientific

Outline




Cryopreservation in general

Cryoprotectant

Basics of vitrification & devices

Published results on vitrification

About 20% (700,000/3.5Mill) of all offspring born worldwide from IVF cycles are from oocyte and embryo cryopreservation.

Cryopreservation: What are the aims?

Arrest the metabolism which could then be reversed

Maintain structural & genetic integrity

Achieve acceptable survival rate after thawing

Method must be reliable & repeatable

Cryopreservation in General: What is involved?

I. Initial exposure to cryoprotectant (intracellular water has to be removed – gradual dehydration)

II. Cooling (slow / rapid) to subzero temperature (-196°C)

III. Storage

IV. Thawing / Warming (gradual rehydration)

V. Dilution and removal of the cryoprotectant agent and replacement of the cellular and intracellular fluid at precise rate

VI. Recovery & return to a physiological environment

ICE CRYSTALS

(even a small amount will destroy the cellular structure)

should not appear and grow inside the cells or tissues of the specimen being cryopreserved

Evolution of Cryopreservation relative to ART

1972	1973/74	1983
Slow freezing of mouse embryos	Slow freezing of domestic animal embryos	Slow freezing of human embryos

Evolution of Cryopreservation relative to ART

Slow freezing of human embryos

Slow freezing of human oocytes

1985 **1989** **1993**

Vitrification of mouse embryos

Vitrification of mouse oocytes

Vitrification of bovine blastocysts

Evolution of Cryopreservation relative to ART

Ultrarapid vitrification with EM grids

Kuleshova *et al.* 1999 [First Live birth from vitrified oocytes]

1996 **1997** **1999**

OPS ultrarapid vitrification

Cryoloop ultrarapid vitrification

EM = electron microscope
OPS = open pulled straw

Ongoing controversy over appropriate stage at which to freeze the embryo

Oocytes	Zygote	Cleavage-Stage	Blastocyst-Stage
DAY 0	DAY 1	DAY 3	DAY 5

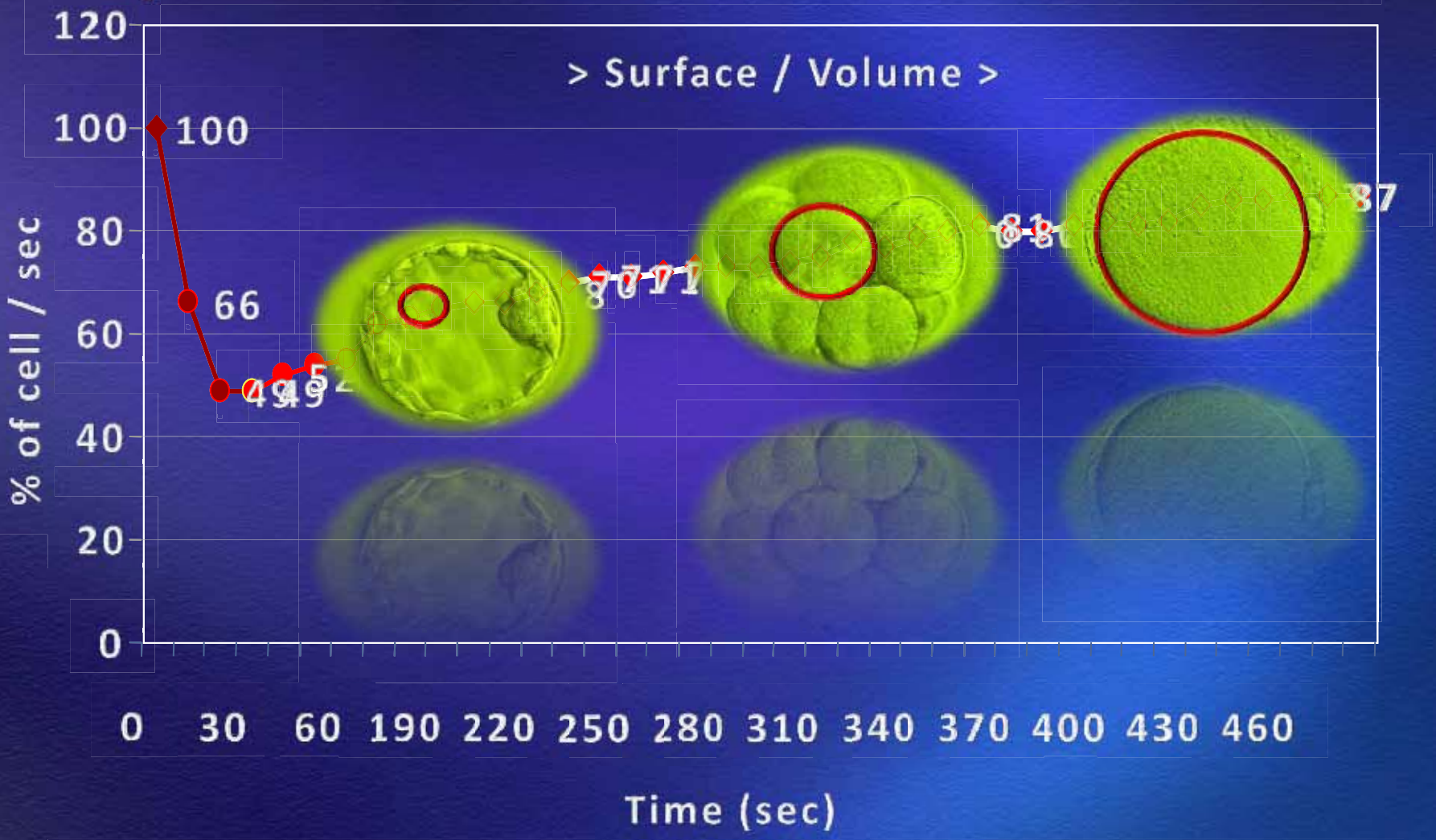
SURVIVAL AFTER CRYOPRESERVATION

LOW → HIGH

LOW HIGH

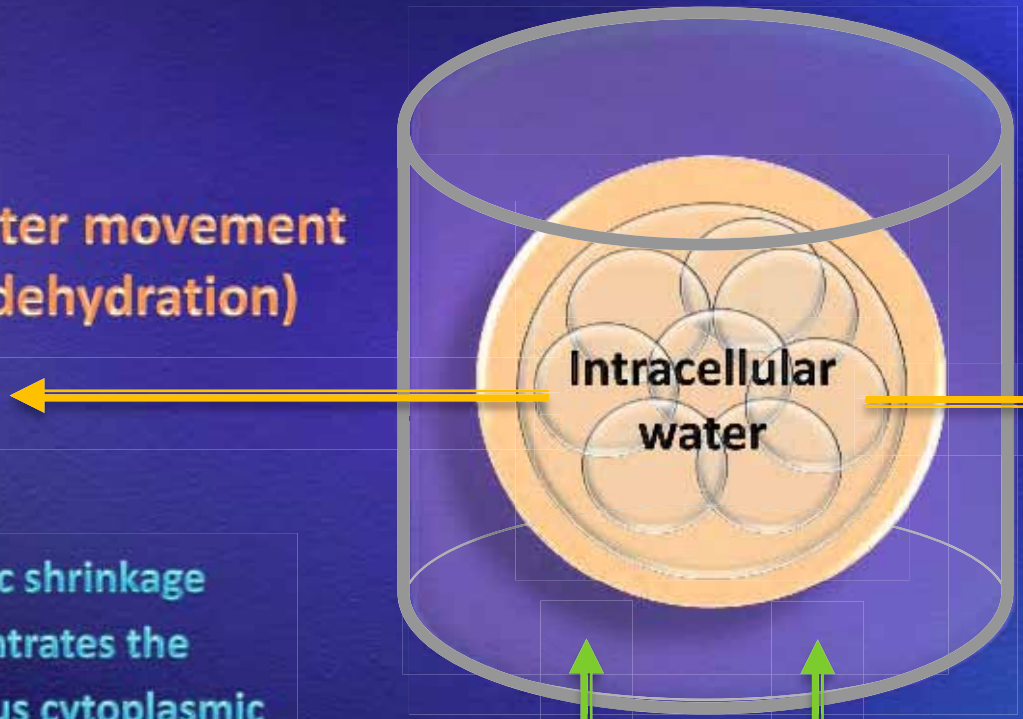
!The value of cryopreservation at the later stage combines the advantage of longer culture to select viable embryos!

Optimal Dehydration: Surface / Volume ratio



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

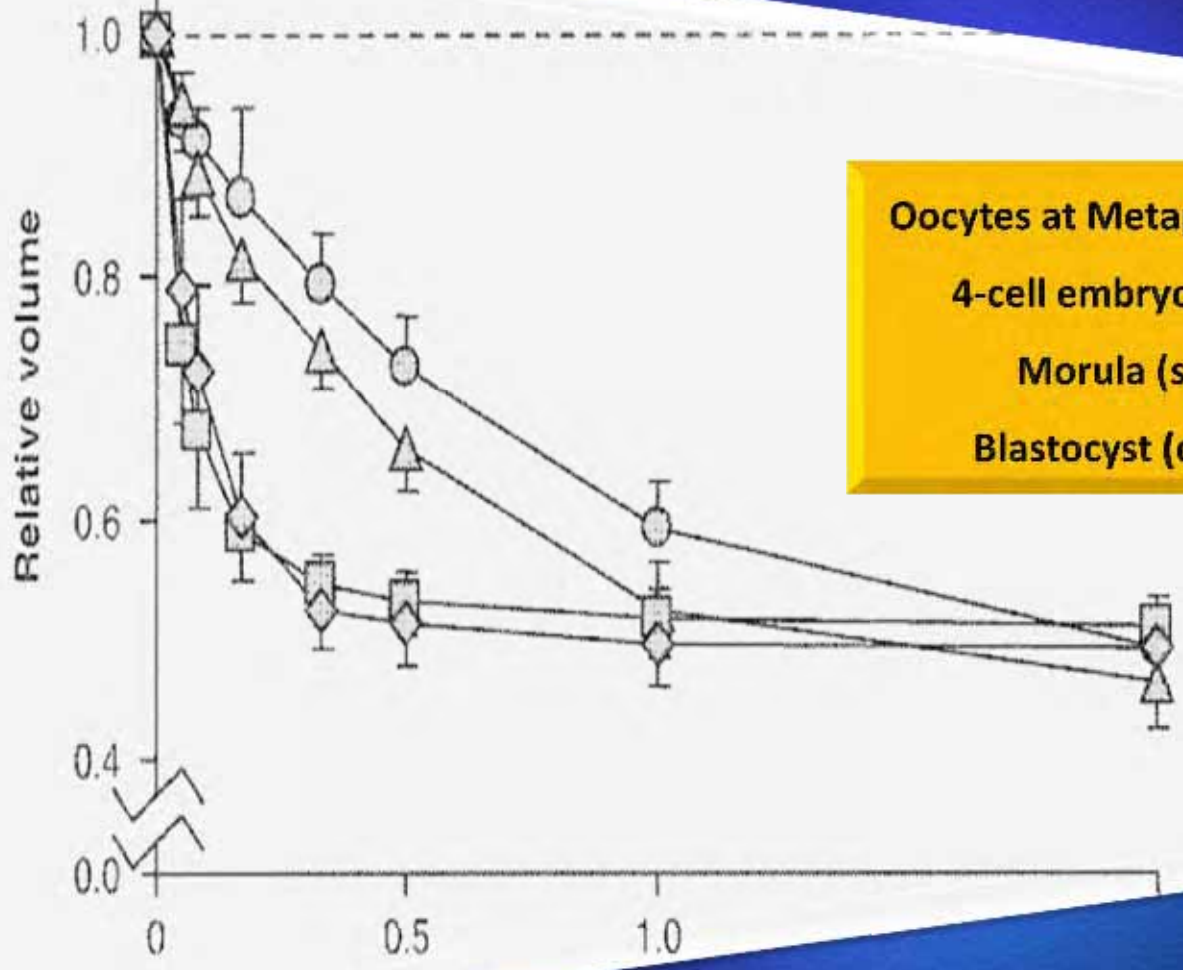
Volumetric / Osmotic Contractions



Osmotic shrinkage concentrates the endogenous cytoplasmic macromolecules and greatly increases the likelihood of intracellular vitrification.

Partial permeation of cryoprotectant inside the cell

Changes in volume at different stages in PB1 medium containing sucrose



Oocytes at Metaphase II (circle)

4-cell embryo (triangles)

Morula (squares)

Blastocyst (diamonds)

Edashige *et al.*, 2006

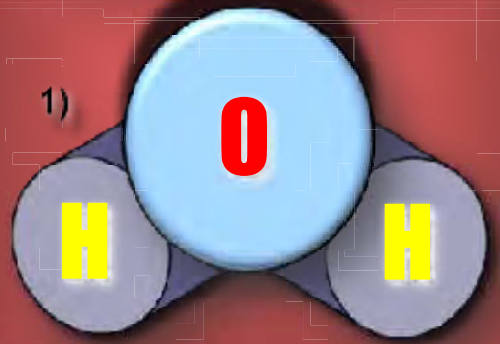
Two basic Cryopreservation - Techniques

Traditional slow freezing
(Whittingham *et al.*, *Science*; 1972; Wilmut, *Life Sci*, 1972)

Vitrification
(Rall & Fahy, *Nature*; 1985)

VITRIFICATION

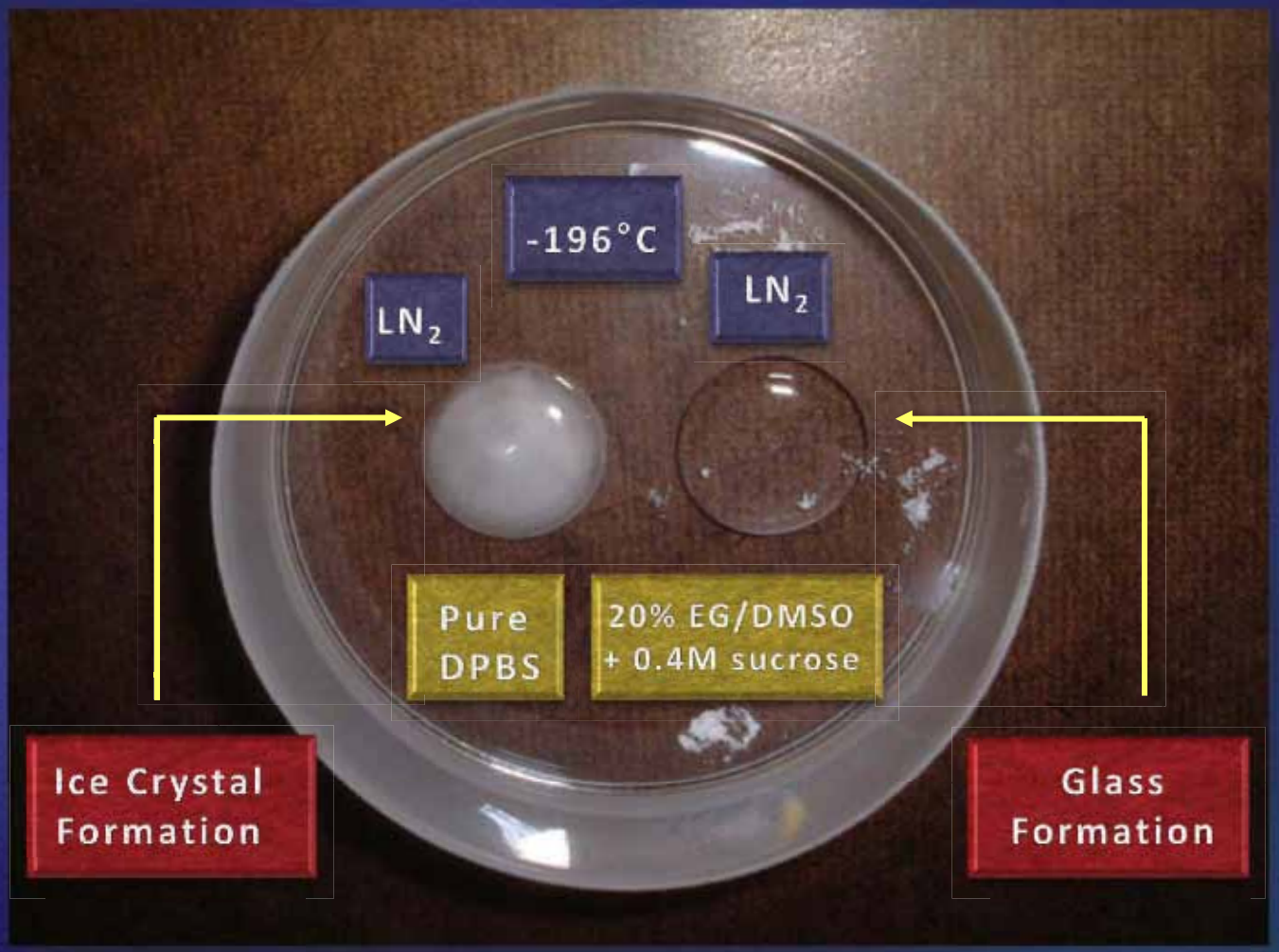
1)



2) Rapid cooled *(ultrahigh speed)* and formed into a

3) Glassy, vitrified state *(amorphous phase)* by extreme elevation in viscosity (up to 10^{14} Pa s) during cooling

4) Result: arrest of molecular diffusion and chemical processes including degradation & aging ("State of suspended animation")

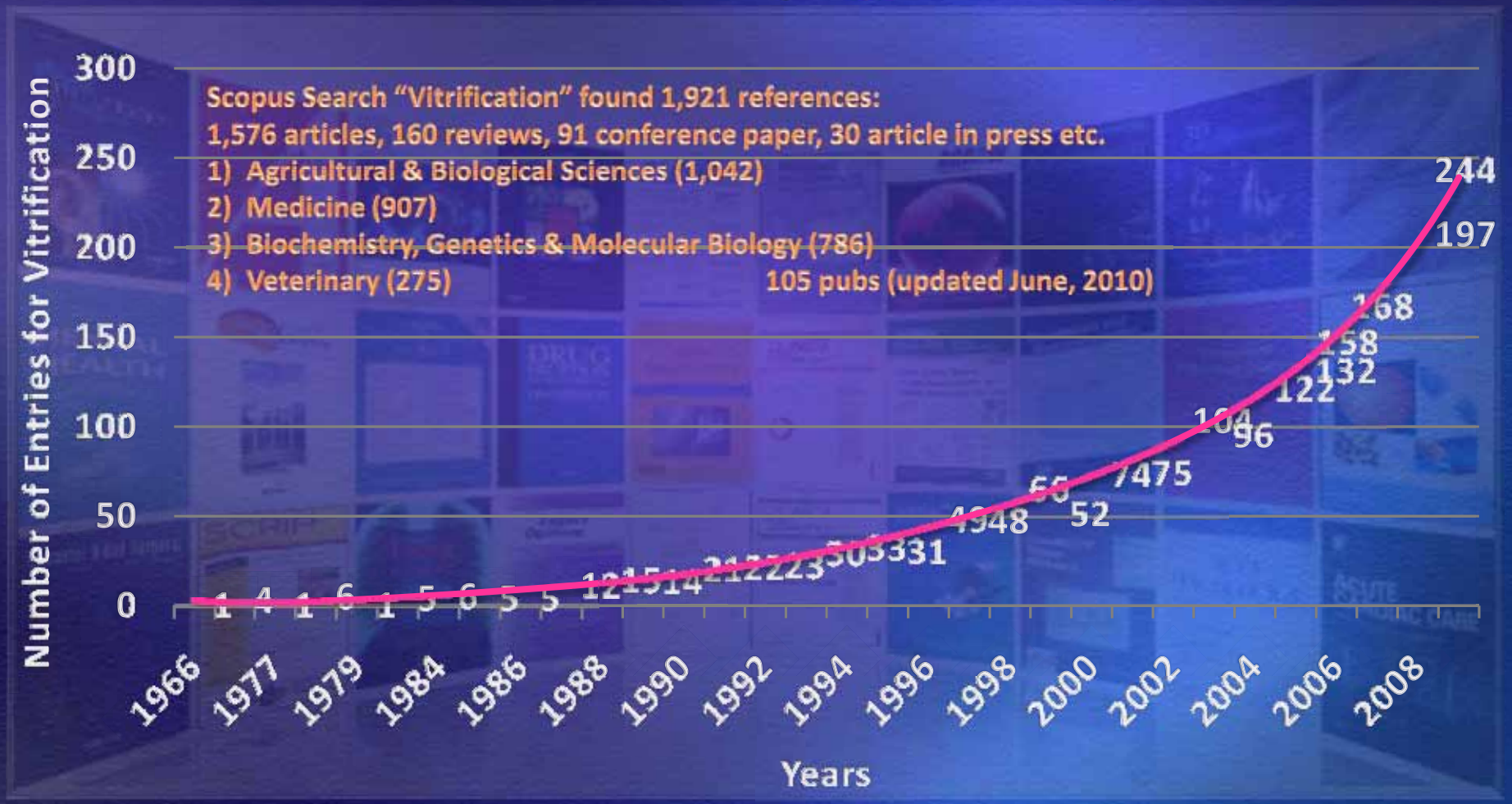


Physical Definition: solidification of a solution without ice crystal formation (solution is rapidly cooled and converted into a glass-like amorphous solid that is free of any crystalline structures) at low temperature by extreme elevation in viscosity during cooling

Slow cooling vs. Vitrification

	Traditional	Vitrification
CPA-concentration	1.5M	3.0 – 5.0M
Volume	0.3 – 1.0 ml	< 1µl
Cooling rate	0.3°C/min	>2,500°C/min
Duration (required time)	> 90min	10 to 15min
Seeding & controlled rate freezing	YES	NO
Could be completed by one person within minutes	NO	YES
Minimize osmotic injuries/stress	NO	YES
Ice crystallization	YES	NO
Procedure	COMPLEX	SIMPLE & FLEXIBLE
Equipment	YES (FREEZER)	NO
Running cost	EXPENSIVE	INEXPENSIVE

Data from from the Scopus Library, the world's largest abstract and citation database of research literature and quality web sources, offers access to 245 million references and 27 million abstracts from over 15,000 peer-reviewed journals.



The explosion of interest in the use of vitrification is beginning to affect clinical embryo storage

Type of Cryoprotectant

Intracellular: Low molecular weight; can permeate into the cells, lower freezing point; replace water in cells, is a small neutral solute, it can prevent or reduce ice-crystal formation during freezing-thawing procedure

Extracellular: Hypertonic solution is required for releasing intracellular water, i.e. sucrose is added to control & minimize excessive release (dehydration) or influx of water (rehydration) to prevent excessive osmotic shrinkage or swelling during the CPA addition or removal

Type of Cryoprotectant

Sole/Mixture

Concentration

Time of Exposure

Temperature during Exposure

TOXICITY

1)

- Reduce the extent of crystallization (ability to block the ability of water to freeze)

2)

- Replace intracellular water (some)
- Helps maintain cell volume

3)

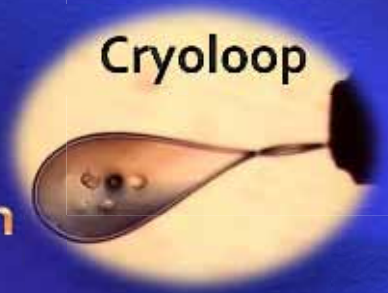
- Ability to decrease cryoinjuries

"New" Technique

Programmable Cell Freezer

Carriers for Vitrification

Traditional embryo cryopreservation can be seen as a highly positive contribution to overall patient treatment, but there are documented limitations to the current methods



Carrier Systems

1) **Hemi-straw system** (Kuwayama & Kato, 2000; Vanderzwalmen *et al.*, 2000; Liebermann & Tucker, 2002; Sugioka *et al.*, 2003; Vanderzwalmen *et al.*, 2003)
2) **Electron microscope copper grid** (Hong *et al.*, 1999; Park *et al.*, 2000; Chung *et al.*, 2000; Wu *et al.*, 2001; Son *et al.*, 2003; Yoon *et al.*, 2003)
3) **Cryoloop** (Lane *et al.*, 1999; Mukaida *et al.*, 2001, 2003; Liebermann & Tucker, 2002; Reed *et al.*, 2002; Liebermann *et al.*, 2003)
4) **Flexible micropipettes *(FDP, Stripper tip)*** (Liebermann *et al.*, 2002; Walker *et al.*, 2004)
5) **French ministraws** (Chen *et al.*, 2000; Yokota *et al.*, 2000, 2001; Vanderzwalmen *et al.*, 2002)
6) **Open-pulled straws** (Kuleshova *et al.*, 1999; Chen *et al.*, 2000)
7) **Cryotop** (Kuwayama *et al.*, 2005; Lucena *et al.*, 2006; Liebermann & Tucker, 2006)
8) **Solid surface vitrification on a metal block** (Bagis *et al.*, 2005)
9) **Cryotip** (Kuwayama *et al.*, 2005)
10) **High Security Vitrification Kit *(HSV)*** (Liebermann, 2009)
11) **Fibreplug; Rapid-I; Cryopette**
12) **Nylon Mesh** (Nakashima *et al.*, 2010)

"CLOSED" vs "OPEN" SYSTEMS

A. "Closed" systems (no direct contact with LN2) are:

- CryoTip,
- High Security Vitrification Straws (HSV)
- Rapid-I, Cryopette

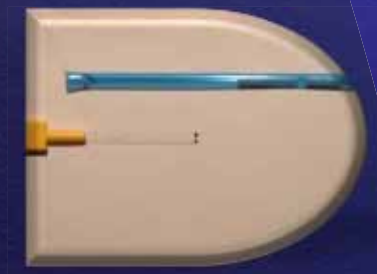
B. "Open" systems (direct contact with LN2) are:

- Microscope cooper grid
- Cryoloop,
- Cryotop or Cryolock,
- Cryoleaf
- Fibreplug

"CLOSED" vs "OPEN" SYSTEMS (cont.)

A. "Closed" systems (no direct contact with LN2) provide:

- Lower cooling rates (1,500 to 5,000°C/min) because of the reduced heat transfer by the carrier wall

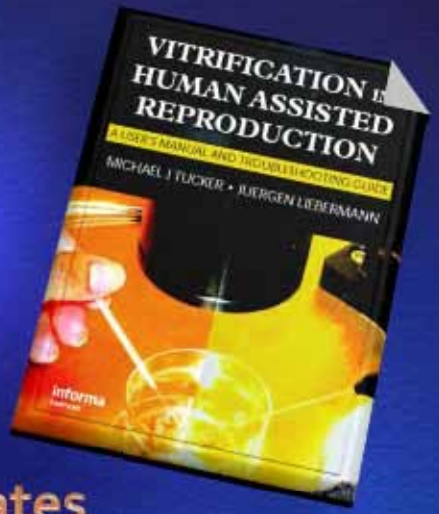


B. "Open" systems (direct contact with LN2) provide:

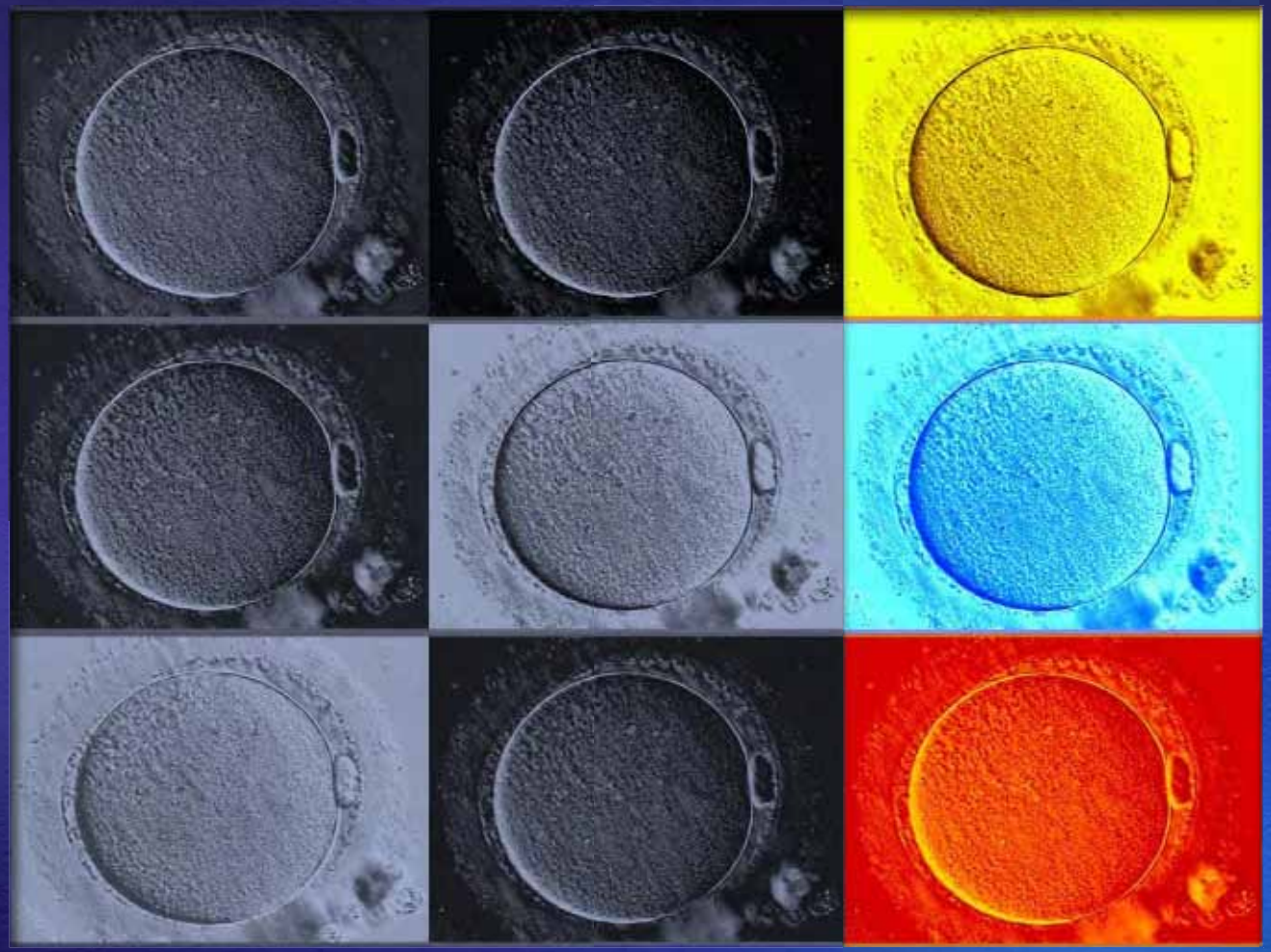
- Higher cooling rates (>20,000°C/min) because of no reduced heat transfer

Successful Vitrification

- Short cooling time (<2sec)
- High cooling/warming rates
- Allows the use of lower concentrations of cryoprotectants, thereby diminishing toxicity & osmotic injury
- Minimal sample volume (submicroliter), that helps to minimize the probability of nucleation for ice formation




Recently published results on vitrification of oocytes, zygotes, cleavage stage embryos and blastocysts



1651 – William Harvey

–

Everything comes from the egg

"ex ovo omnia"


Live Births
FERTILIZATION
SURVIVAL
~ 1000
4239/5502
(77.0%)
6757/7811
(87.0%)

More than 20 Years of Oocyte Cryopreservation

Over the last two decades great success in embryo freezing was achieved, however; the efficiency of oocyte cryopreservation has remained low mainly because of low rates of survival, fertilization, and cleavage. However, what makes oocytes so unique comparing to embryos besides differences in cell size and membrane permeability?

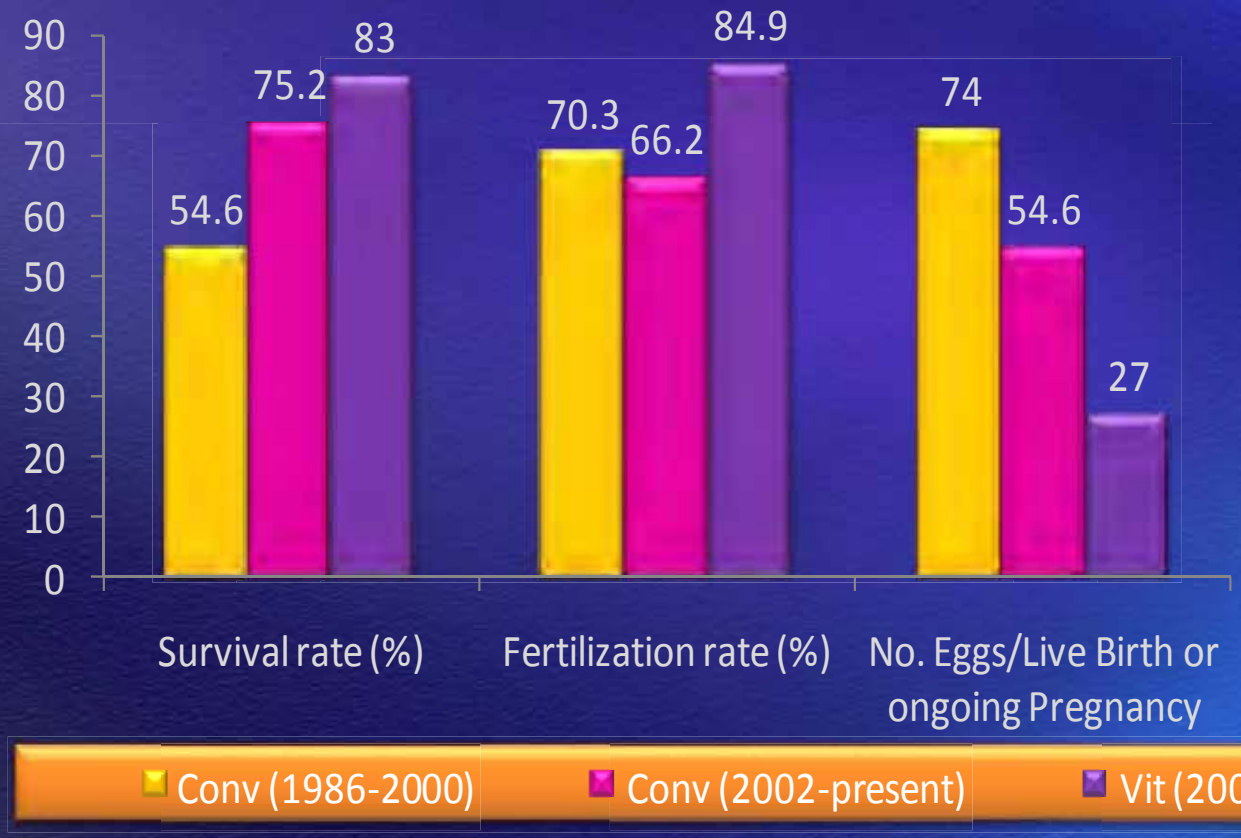
1) Maternal DNA is held suspended in the cytoplasm on the meiotic spindle & not within the protective confines of the nuclear membrane

2) Damage in the DNA and microtubles could explain the limited success of oocyte

3) Oocyte is arrested in a state primed for activation

4) Changes in its environment can cause parthenogenetic activation

Oocyte Cryopreservation: Historical & Recent Results

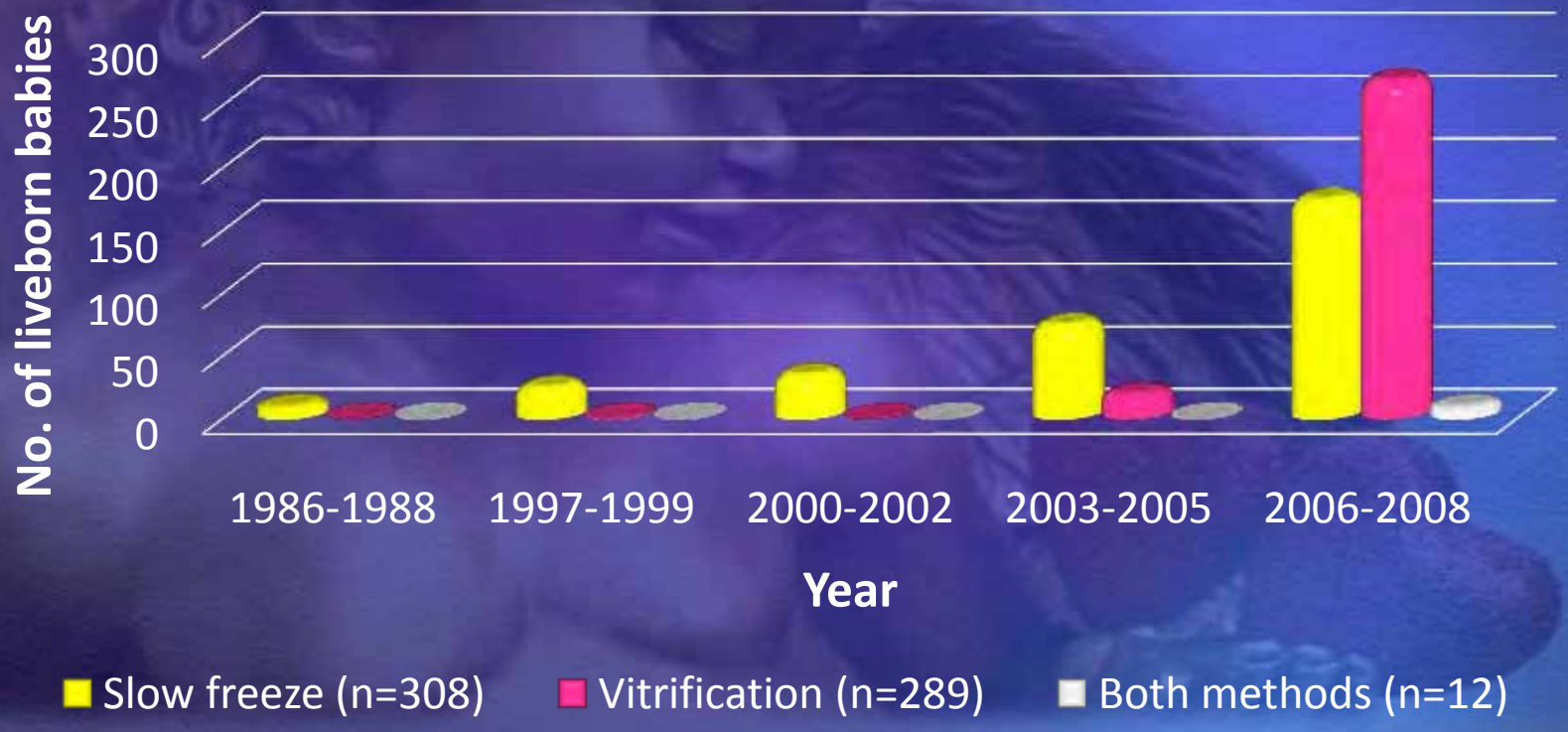


Conv (1986-2000)
- Chen (1986)
- Tucker (1998)
- Porcu (2000)

Conv (2000-present)
- Quintans (2002)
- Fosas (2003)
- Boldt (2003)
- Jain (2005)
- Chamayou (2006)

Vit (2003-present)
- Yoon (2003)
- Katayama (2003)
- Kuwayama (2005)
- Lucena (2006)
- Cobo (2008)

Number of live births from reports of slow-frozen and vitrified, then thawed-warmed, fertilized and transferred oocytes from 1986 to 2008 (adopted from Noyes *et al.*, 2009)



Cumulatively, there were 609 live born babies. Slow freezing reports span from 1986 (Chen, 1986) to 2008 while vitrification birth reports began in 1999 (Kuleshova *et al.*, 1999). Although 13 years later than the first slow-freeze birth, the number of reported babies born as a result of vitrified oocytes is now approaching that of slow-frozen oocytes.

Noyes *et al.*, 2009

Noyes, Porcu, Borini, 2009 RBMOnline 18; 769-776

Over 900 oocyte cryopreservation babies born with no apparent increase in congenital anomalies.

308 babies from slow freezing

289 babies from vitrification

327 other live births were verified

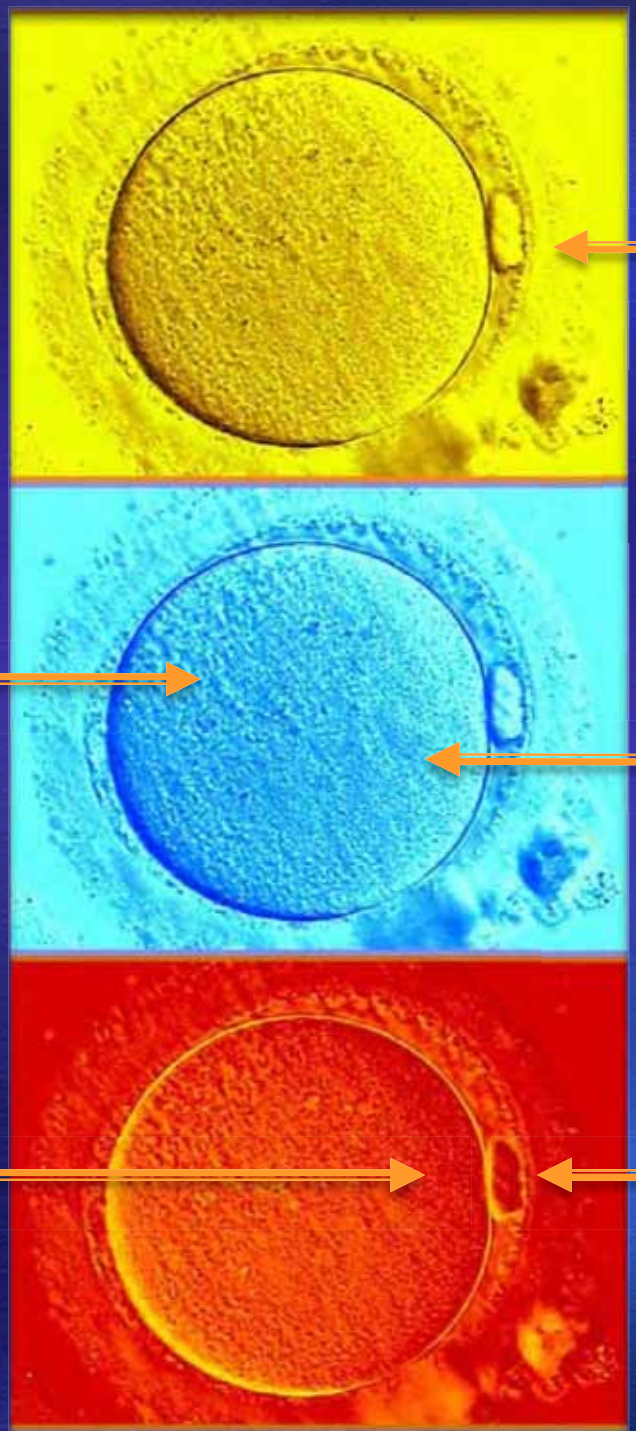
Compared with congenital anomalies occurring in naturally conceived infants, no difference was noted.

1.3% (12) were noted to have birth anomalies:

- 3x ventricular septal defects
- 1x choanal & 1x biliary atresia
- 1x Rubinstein-Taybi syndrome
- 1x Arnold-Chiari syndrome
- 1x cleft palate,
- 3x clubfoot
- 1x skin haemangioma

Oocyte Cryo:
Areas of Concern

Impact on oocyte physiology



Safety Issues

Zona pellucida hardening

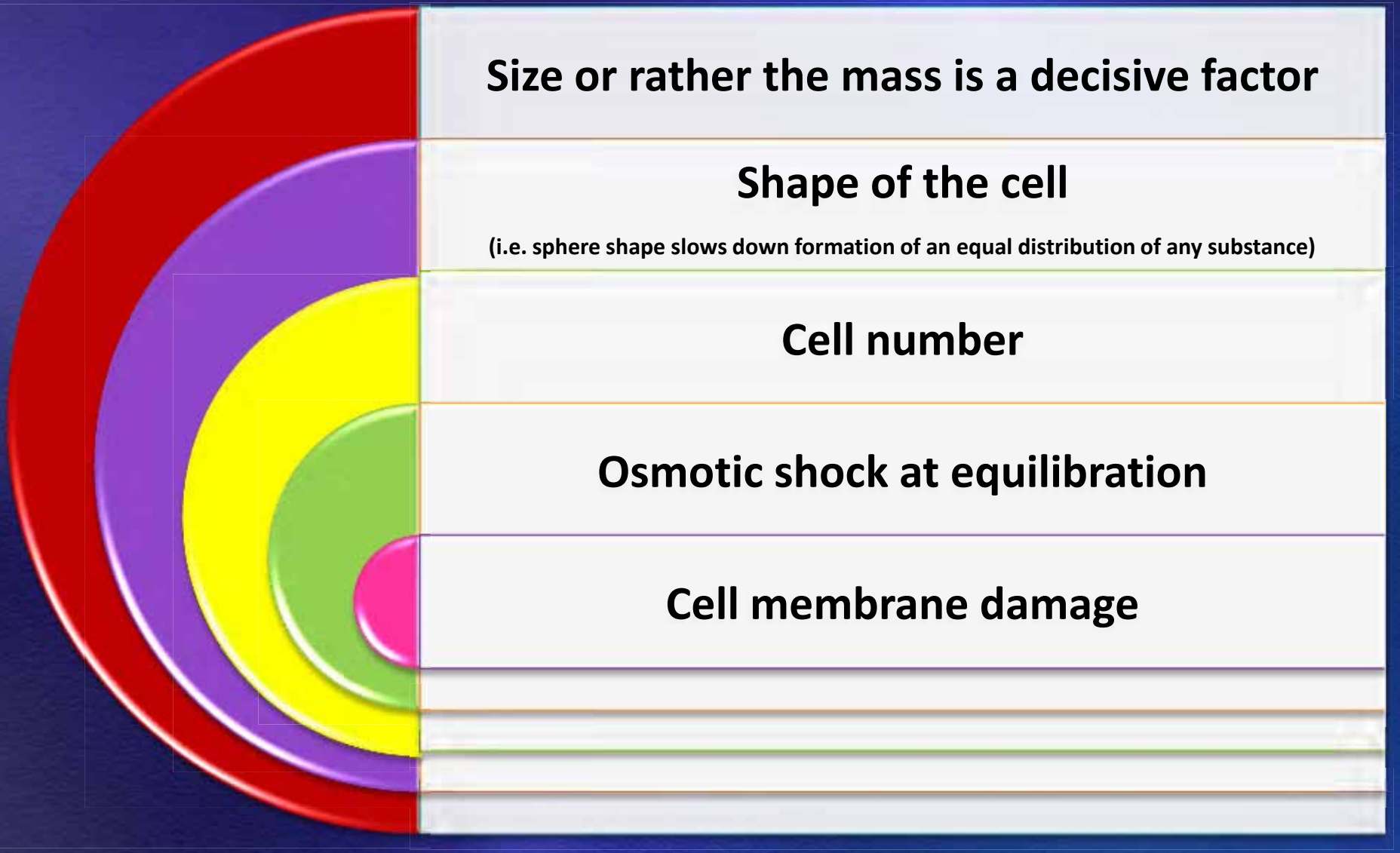
Cytoplasmic and cytoskeletal damage

Membrane permeability

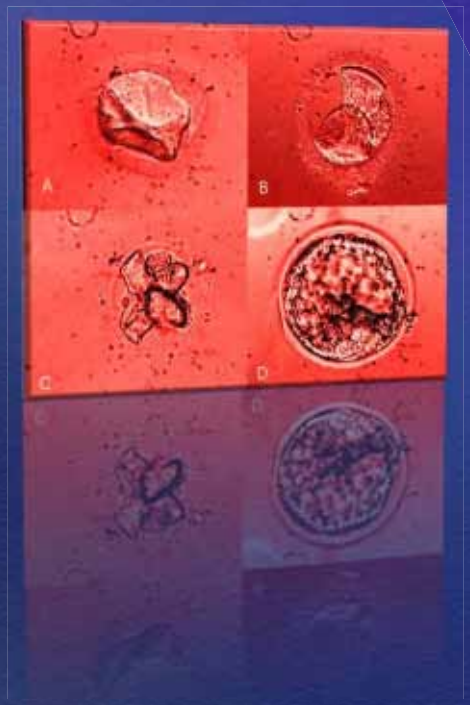
Meiotic spindle depolymerization

Polar body degeneration/fusion

Factors that contribute to the sensitivity of cryoinjuries



Pathway for the movement of water & cryoprotectants



- For successful cryopreservation, smooth movement of water and CPA through the plasma membrane is essential.
- For most cell types, simple diffusion through the lipid layer is the way of moving water through the plasma membrane - limited permeability
- In contrast, if water channels (called aquaporine) are involved, then the membrane is extremely permeable by water.

Membrane Permeability

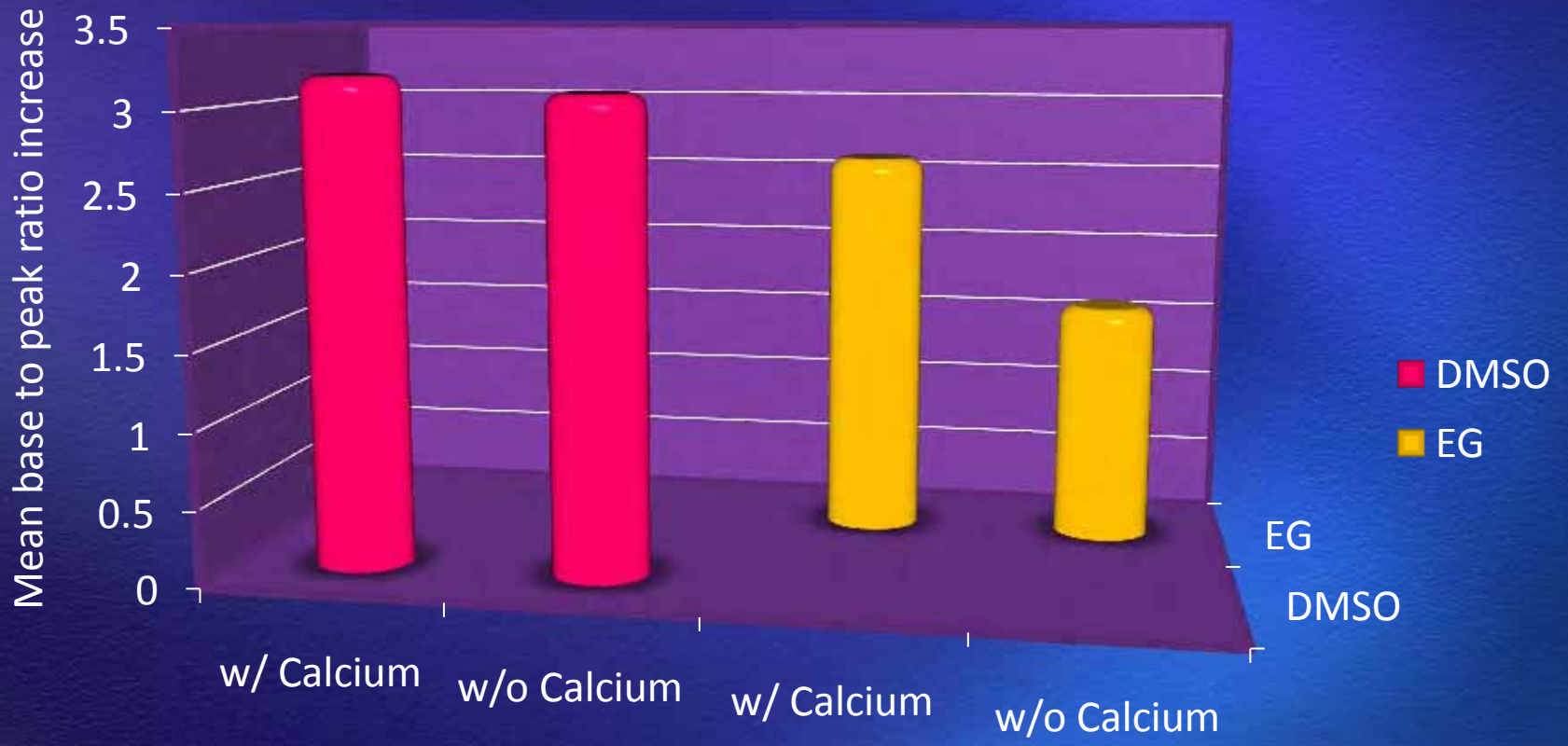
Mouse 2-4-cell stage embryos have a low permeability to water similar to oocytes

Morulae and blastocysts have high water permeability

In general:

High permeability to water with lower Arrhenius activation energy (E_a) suggests the movement by facilitated diffusion through channel processes, whereas lower permeability with higher E_a is suggestive of movement via a channel-independent process, i.e. by simple diffusion

Cryoprotectant and Calicum Release



Larman *et al.*, 2006

Removing external calcium failed to affect the intracellular increase caused by DMSO

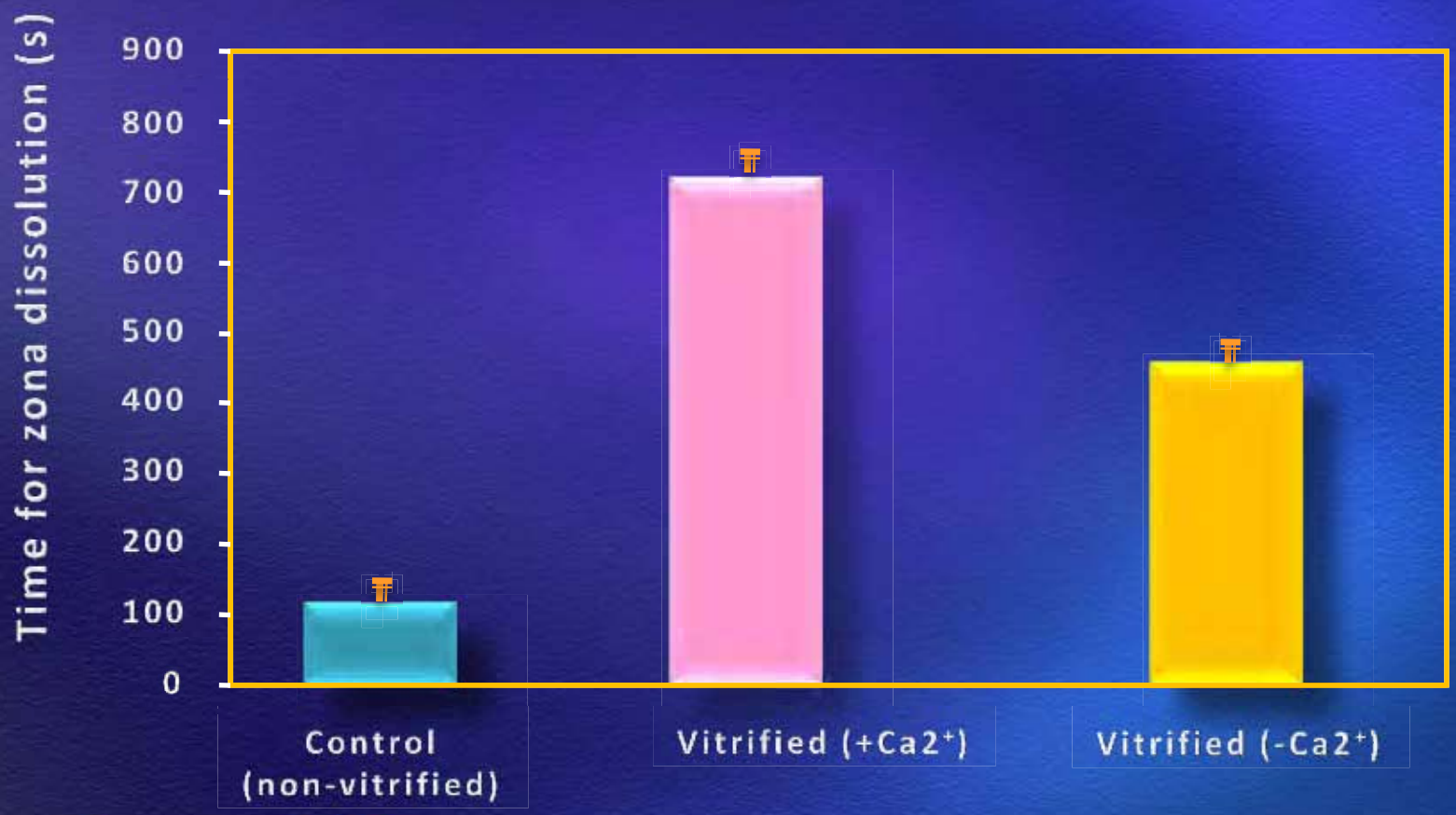
DMSO is using intracellular calcium

EG is using calcium from the media via influx across the plasma membrane

In contrast, removal of external calcium significant reduces the intracellular calcium rise caused by EG

Impact on Zona Pellucida

Zona hardening assessment using 1% w/v chymotrypsin



Larman *et al.*, 2006

n > 60 oocytes/treatment - 3 replicates

Metabolic 'Health'

Impact on oocyte physiology

Which Technique?

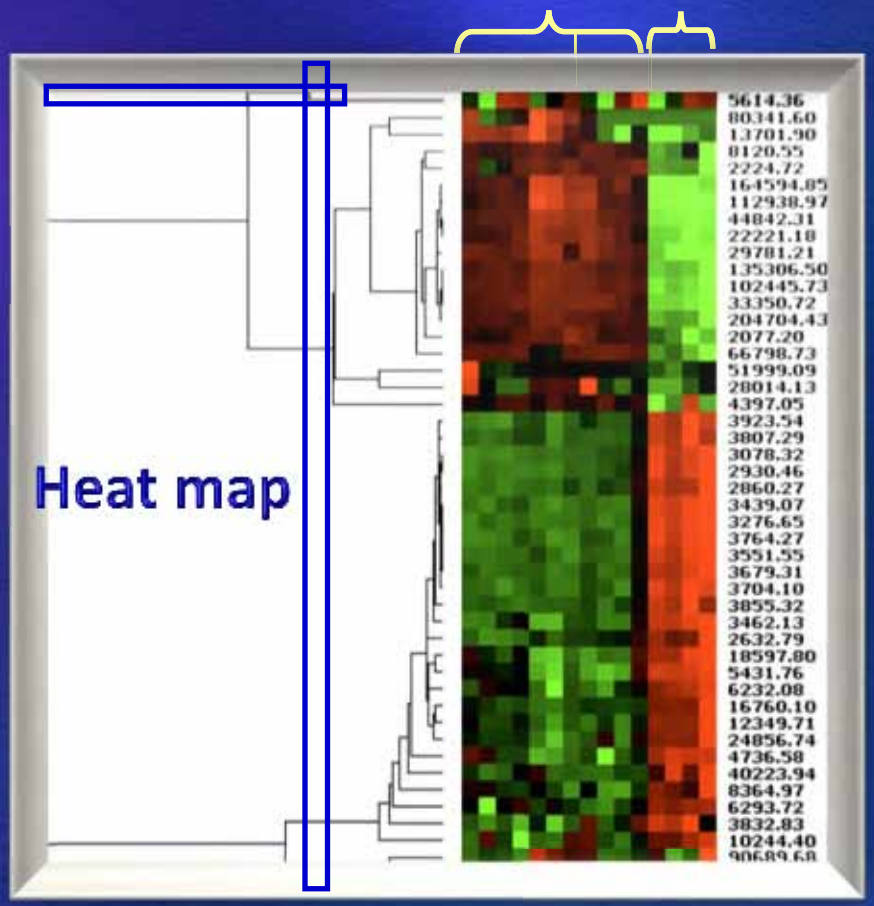
Impact on oocyte physiology

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

Hierarchical clustering of anionic protein profile

= Upregulated

= Downregulated



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.

Gardner *et al.*, 2006

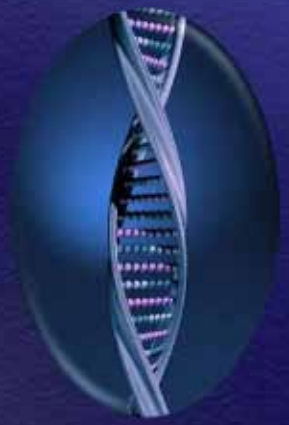
Which Technique?

Variable	Slow-freezing literature 1996-2005	Vitrification literature 2003-2005
Age, mean	33.7	32.3
Fertilization rate	64.9 (2,478/3,818)	74.2 (637/859)
Clinical pregnancies per thawed oocyte	2.3 $x10^{-2}$ (153/6720)	4.5 $x10^{-2}$ (61/1354)
Clinical pregnancies per injected oocytes	4.0 $x10^{-2}$ (153/3818)	7.2 $x10^{-2}$ (61/859)
Clinical pregnancies per transfer	20.6 (153/742)	45.5 (61/134)
Implantation rate	10.1 (185/1828)	17.2 (81/473)

Oktay *et al.*, 2006

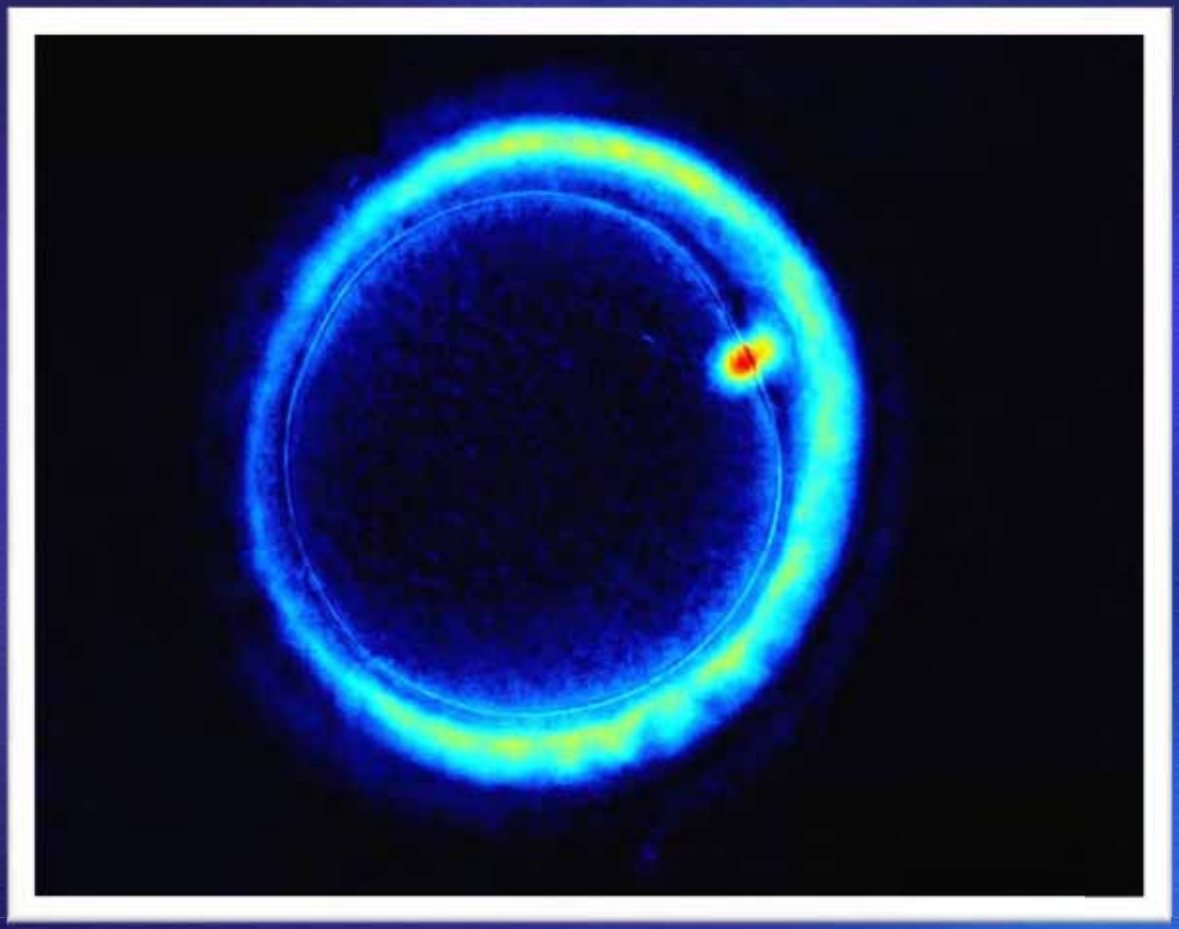
Slow freezing or vitrification: Survival & meiotic spindle

Cryopreservation method	No. of thawed cycles	No. of oocytes thawed/warmed	Survival rate (%)
Slow freezing	1,683	1,275	75
Vitrification	948	899	95



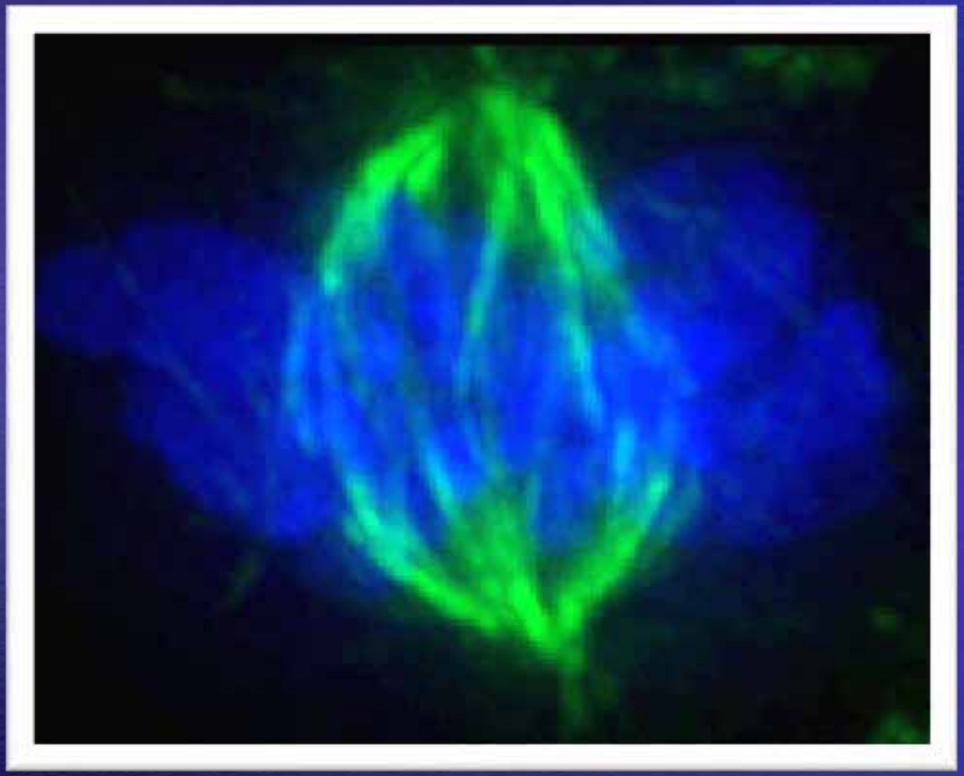
Spindle recovery is faster after vitrification than slow freezing.

Chen SU & Yang YS, 2009. Slow freezing or vitrification: their effects on survival and meiotic spindles, and the time schedule for clinical practice. Tawain J Obstet Gynecol; 48:15-22.



The microtubules of meiotic spindles are vulnerable to the thermal changes and will depolymerize. After incubation at 37°C, the microtubules repolymerize. Spindle recovery is faster after vitrification than slow freezing!

Chen & Yang, 2009



Blue - condensed chromosomes

Green - Spindle

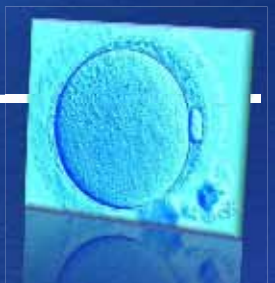
Immediately after warming of vitrified MII oocytes, beta-tubulin is depolymerized and chromatin remains condensed on the metaphase plate. Within a 2-hour period, beta-tubulin repolymerizes, forming morphologically normal metaphase spindles with properly aligned chromatin.

Gomes *et al.*, 2008

Comparison of survival and embryonic development in human oocytes cryopreserved by slow-freezing and vitrification

Cryopreservation protocol	No. of oocytes	No. of surviving (%)	Fertilization rate (%)	Cleavage rate (%)	High quality embryos on Day 3 (%) Grad 1 & 2	Blastocyst development (%)	Abnormal meiotic spindle & chromosome configuration (%)
Slow freezing + 0.3mol/l sucrose	123	75 (61.0)	46 (61.3)	25 (54.4)	6/25 (24.0)	3/25 (12.0)	25/64 (39.1)
Vitrification Kit from MediCult Cryoleaf method (7.5% EG / PROH) + 15% + 0.5mol/l sucrose	292	268 (91.8)	182 (67.9)	142 (78.0)	60/142 (42.3)	47/60 (33.1)	11/62 (17.7) Control: 3/18 (16.7)
P Value		<0.01	NS	<0.01	<0.01	<0.05	

Cao YX, Xing Q, Li L, Cong L, Zhang ZG, Wei ZL, Zhou P. Comparison of survival and embryonic development in human oocytes cryopreserved by slow-freezing and vitrification. Fertility & Sterility, 2009; 92:1306-11.



Human oocyte vitrification: High pregnancy rates when carried out in fertile women (31.7±3.0)

Vitrification method EM gold grid	# of warming cycles	# of oocytes warmed	Survival (%)	Fertilization (%)	Clin. pregnancy/ warming cycle (%)	Implantation (%)
1.5M EG/5.5M EG+1M Suc	20	395	320 (81.0)	208/285 (72.3)	16/20 (80.0)	24 (45.3)
IR per warmed/injected oocytes						6.4/9.1
Live-birth per warmed/injected oocytes						5.1/7.2

Kim T, Laufer LR, Hong SW. Vitrification of oocytes produces high pregnancy rates when carried out in fertile women. Fertility & Sterility 2010, 93;467-474.

Embryo development of fresh versus vitrified metaphase II oocytes after ICSI: a prospective randomized sibling-oocyte study (n=40)

Vitrification method	Total # of oocytes # of sibling vitrified/warmed oocytes	Survival (%)	FR (%) per warmed oocyte	# of ET (%)	cPR/cycle (%)	IR (%)
Cryotop						
7.5% EG/DMSO & 15% EG/DMSO+1M Suc	244 124	120/124 (96.8)	95/124 (76.6)	39/40 (97.5)	15/40 (37.5)	19/93 (20.4)
					54/124 (43.5%)	69/318 (21.7%)
Fresh sibling oocytes	120		100/120 (83.3)	Excellent quality embryos on day 2 (fresh vs vitrified) 52.0 vs 51.6%		

Rienzi *et al.* Embryo development of fresh versus vitrified metaphase II oocytes after ICSI: a prospective randomized sibling-oocyte study. Human Reproduction 2010,25:66-73

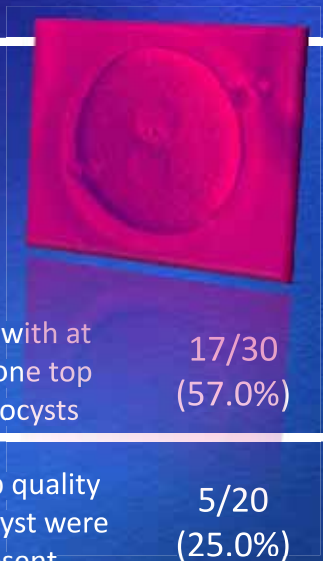
Summary of clinical results for vitrified/warmed zygotes

No. of warmed zygotes	339
No. of survived zygotes (%)	302 (89%)
No. of cleaved embryos (%)	243 (80.5%)
No. of patients who underwent ET (%)	103
No. of transferred embryos	2.3
No. of pregnancies (%ET)	38/103 (36.9%)
No. of clinical pregnancies (%ET)	29/103 (28.2%)
Implantation rate (%)	38/243 (15.6%)

Al-Hasani *et al.*, RBMOnline 2007; 14:288-293.

Vitrification of zygotes in a closed system: sibling analysis on blastocyst formation rate of vitrified vs. fresh zygotes (n=19)

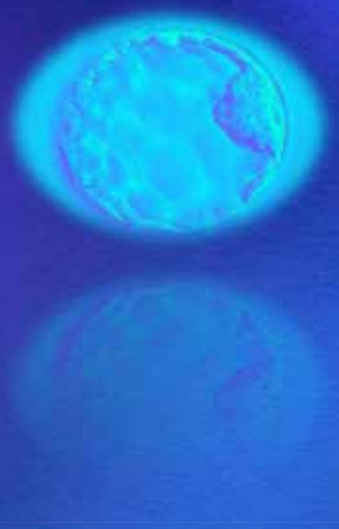
Vitrification method	Total # of zygotes # of sibling vitrified/warmed zygotes	Survival (%)	1) Developmental potential up to the blastocyst stage 2) Top quality blastocysts	# of ET (%)	cPR/ET (%)	IR (%)
"Vitrisafe" (closed system)						
5% EG/DMSO 10% EG/DMSO 20% EG/DMSO + 0.5M Suc	348 184	176/184 (96.0)	1) 27% vitrified vs. 21% control 2) 14% vitrified vs. 18% control			
53 vitrified/warming cycle	542	521/542 (96.0%)	1) 175/521 (34.0%) 2) 83/175 (47.0%)	N=30 with at least one top blastocysts	17/30 (57.0%)	18/53 (34.0%)
				No top quality blastocyst were present	5/20 (25.0%)	6/40 (15%)



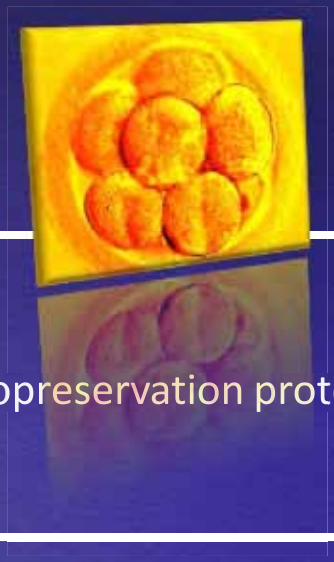
Vanderzwalmen *et al.*,: Oral presentation at the 26th Annual Meeting of the European Society of Human Reproduction & Embryology. Human Reproduction 2010, Vol. 25 Supplement 1 i103:O-261

Validation of media and devices for vitrification of human embryos: in search of an optimum (ART Lab Award at ESHRE 2008, Barcelona, Spain)

Vitrification protocol	Morphological survival after thawing (%)	
	Cleavage embryos	Blastocysts (Day 5/6)
"Open system" (Cryotop) with DMSO/EG/sucrose	76	86.4
"Closed system" (Cryotip) with DMSO/EG/sucrose	76.9	74.1
"Closed system" (CBC-Vit) with DMSO/EG/sucrose	79.3	90.9
"Closed system" (CBC-Vit) with 1,2-PG/EG/sucrose	60	26.9
P Value		<0.005



Guns Y, Vandermonde A, Vitrier S, Sterckx S, Devroey P, Van den Abbeel E, Van Der Elst J. Validation of media and devices for vitrification of human embryos: in search of an optimum. Oral presentation at ESHRE 2008; O-134 pp. i55.



Randomized controlled trial comparing vitrification versus slow freezing of human cleavage stage embryos

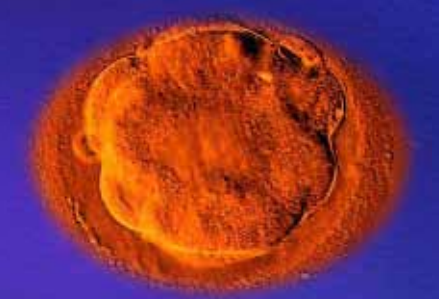
Cryopreservation protocol	Survival of Cleavage Stage Embryos (n=265)	Transfer performed of initial cycles (%)	PR per Transfer %)	PR per initiated cycle (%)
Slow freezing (1.5M 1,2-propanediol + 0.1M Sucrose CBS Straws	56/81 (69.1%)	27/55 (45.5%)	4/27 (14.8%)	4/55 (7.3%)
Vit Kit w/ DMSO (Irvine) Carrier (HSV)	89/93 (97.8%)	61/72 (84.7%)	13/61 (21.3%)	13/72 (18.0%)
Vit Kit w/o DMSO (Vitrolife) Carrier (HSV)	89/91 (97.8%)	51/62 (82.2%)	9/51 (17.6%)	9/62 (14.5%)

Fasano *et al.*,: Oral presentation at the 26th Annual Meeting of the European Society of Human Reproduction & Embryology. Human Reproduction 2010, Vol. 25 Supplement 1 i13:O-31

Neonatal outcome after vitrified day 3 embryo transfer:

A preliminary study.

No. of warmed embryos	907
No. of survived zygotes (%)	817 (90.7%)
Clinical pregnancy rate (%)	36.8%
Implantation rate (%)	18.1%
Miscarriage rate (%)	7.7%
Live birth rate (%)	24.2%
Congenital birth defect rate (%)	1.18%



Results were comparable with pregnancies using fresh embryo transfers.

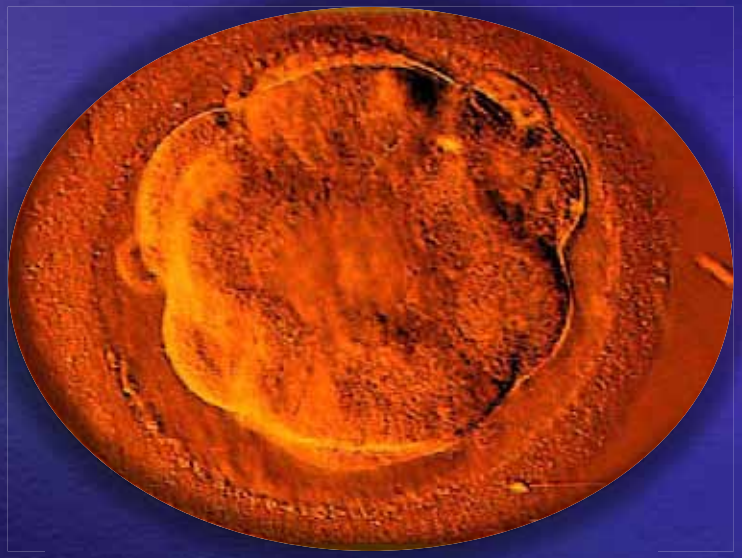
Raju *et al.*, 2009 *Fertility & Sterility* 92, 143-148.

Clinical pregnancy and live births after transfer of embryos vitrified on day 3

Age (yrs)	Less than 38	38-42
No. of ETs	200	70
Clinical pregnancy rate (%)	45%	29%
Implantation rate (%)	24%	13%
No. of deliveries	66	
Twin rate (%)	18.2%	
Preterm (%)	13.6%	
Total no. of infants born	78	

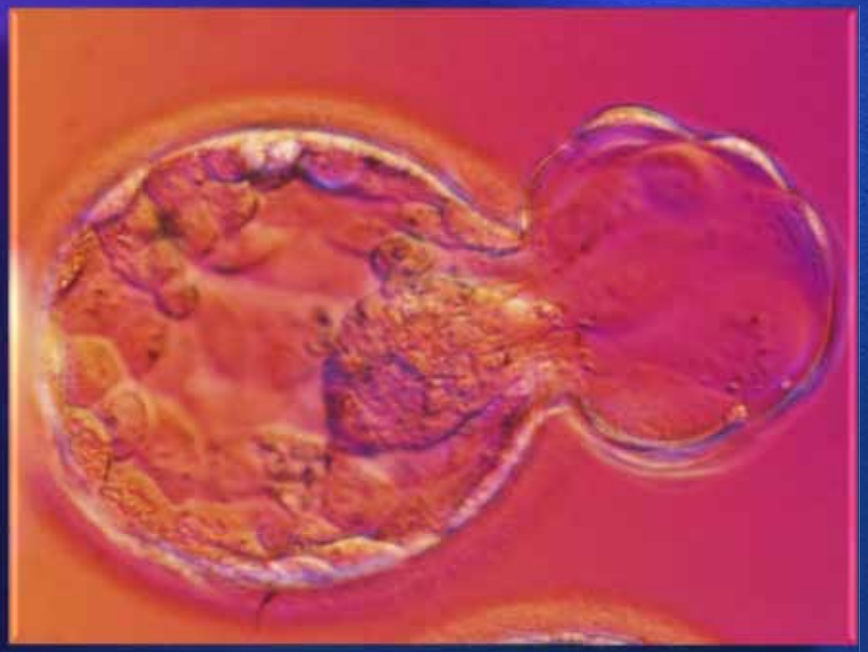
(these data attest to the efficacy and safety of the vitrification technique of human embryos at the 6- to 8-cell stage.

Desai *et al.*, 2010 RBMOnline; Webpaper 2009/4702



1) Vitrification is more benefit for the developmental ability of the thawed embryos and more suitable for the cryopreservation of day 3 cleavage stage embryos (Li *et al*., 2007).
2) Further evidence that vitrification imparts less trauma to cell and is, therefore, a more effective means of cryopreserving the human embryo than conventional slow freezing (Balaban *et al*., 2008).

Vitrification at FCI (Chicago) 01/2004 – 07/2010



I. Approximately 120 hours (day five) into development the healthy human embryo should be at the blastocyst stage comprised of some 50 to 150 cells, of which about 20 to 30% make up the inner cell mass (ICM), the remainder making up the trophectoderm (TE)

II. The higher cell number allows better compensation for cryo-injuries, which results in greater viability and faster recovery

III. The cytoplasmatic volume of the cells is lower, thus the surface-volume ratio is higher, and that in turn makes the penetration of the cryoprotectant faster

IV. On average fewer embryos per patient were frozen-stored, but each one when thawed has a greater potential for implantation

Important to know what to freeze .....!

(1) It has been observed that ICM's are preferentially damaged by cryopreservation

(2) The observed clinical outcomes directly related to ICM quality

Data from Marius Meintjes, PhD; Presbyterian/Harris Methodist ARTS Program, Dalles, TX

Therefore, effort to improve blastocyst cryopreservation should focus to a significant degree on ICM survival by proper selection of blastocysts

Compounds

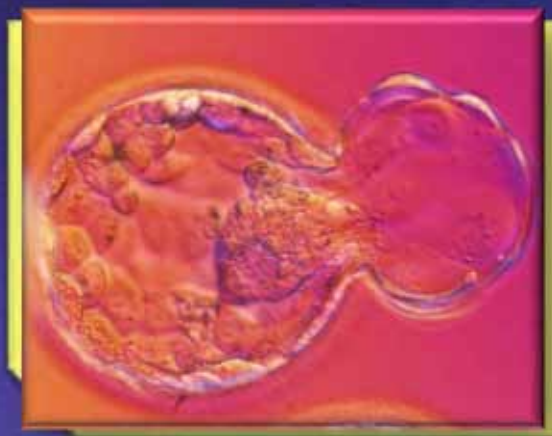
- Ethylene glycol; EG [$C_2H_4(OH)_2$; Molecular mass 62g/mol]

- Dimethyl sulfoxide; DMSO [$(CH_3)_2SO$; Molecular mass 78g/mol]

- Sucrose; [$(C_{12}H_{22}O_{11})$; Molecular mass 342g/mol]

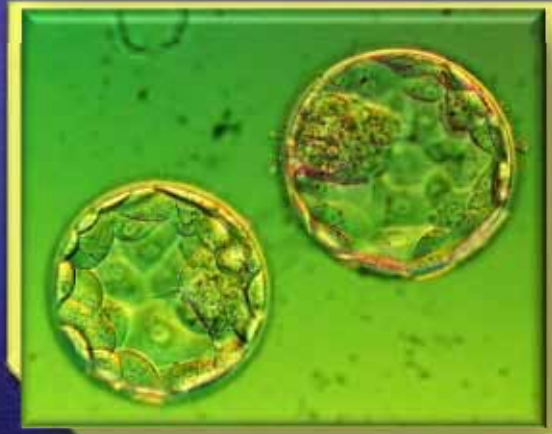
- Protein (Serum Substitute Supplement; SSS)

- .....dissolved in Hepes-HTF or M199



Note on the vitrification solution:

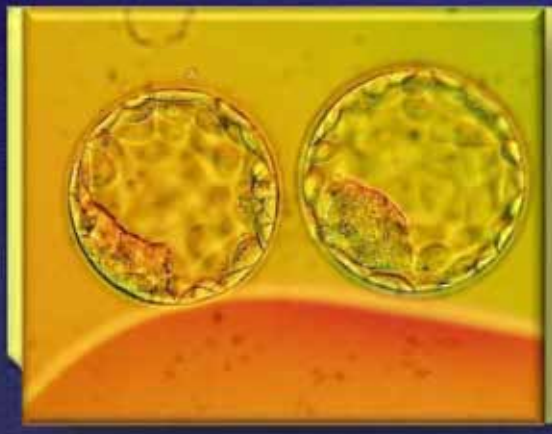
Recent publications have shown that the use of relatively high concentration of cryoprotectants such as 15% ethylene glycol (EG) used in an equimolar mixture with dimethyl sulphoxide (DMSO) had no negative effect on the perinatal outcome of blastocyst transfer using vitrification, when compared to fresh blastocyst transfer.



Takahashi K, Mukaida T, Goto T, Oka C. Perinatal outcome of blastocyst transfer with vitrification using cryoloop: a 4-year follow-up study. *Fertil Steril* 2005;84:88-92.

Liebermann J, and Tucker M. Comparison of vitrification and conventional cryopreservation of day 5 and day 6 blastocysts during clinical application. *Fertil Steril* 2006;86:20-26.

Liebermann J Vitrification of human blastocysts: An update. RBMOnline 2009



Note on the vitrification solution containing DMSO:

Kartberg et al., 2008 observed that DMSO-containing vitrification solution leads to less chemical injury upon prolonged exposure compared with the DMSO-free vitrification solution, possibly due to stabilizing the osmolarity in the cells and better embryo membrane integrity. Kartberg A-J, Hamniliki F, Arvidsson T, Stavreus-Evers A, Svalander P. Vitrification with DMSO protects embryo membrane integrity better than solutions without DMSO. RBMOnline 2008; 17, 378-384.

Dehydration & Rehydration

Dehydration in two steps prior to vitrification

- Equilibration solution (7.5% (v/v) CPA's [EG:DMSO])
- Vitrification solution (15% CPA's [EG:DMSO] (v/v) + 0.5 sucrose)

Load device for vitrification (Carrier)

Plunge into liquid nitrogen (LN2)

Thawing (dilution of CPA's & rehydration in three steps with decreasing sucrose concentration)

- Thawing solution (1.0M sucrose)
- Dilution solution (0.5M sucrose)
- Washing solution (zero sucrose)

Vitrification Solution

- Modified M-199 + 20% SSS
- <u>Equilibration solution (ES):</u>

  7.5% EG/DMSO
- <u>Vitrification solution (VS):</u>

  15% EG/DMSO + 0.5M Sucrose

Vitrification-Workplace

- Microscope
- Styrofoam filled with LN2
- Patient sheet for documentation
- Stripper tip
- A cane with an attached goblet
- Carrier
- 60 mm culture dish

Note: **All blastocysts were vitrified without artificial shrinkage before the cryopreservation procedure**

Thawing-Warming Solution

- **Thawing solution (TS):**

  1.0M Sucrose

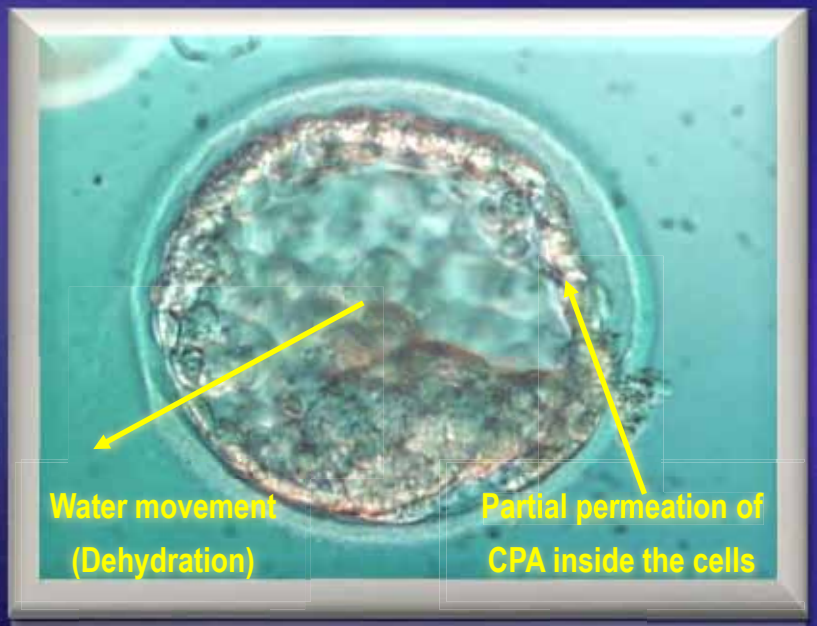
- **Diluent solution (DS):**

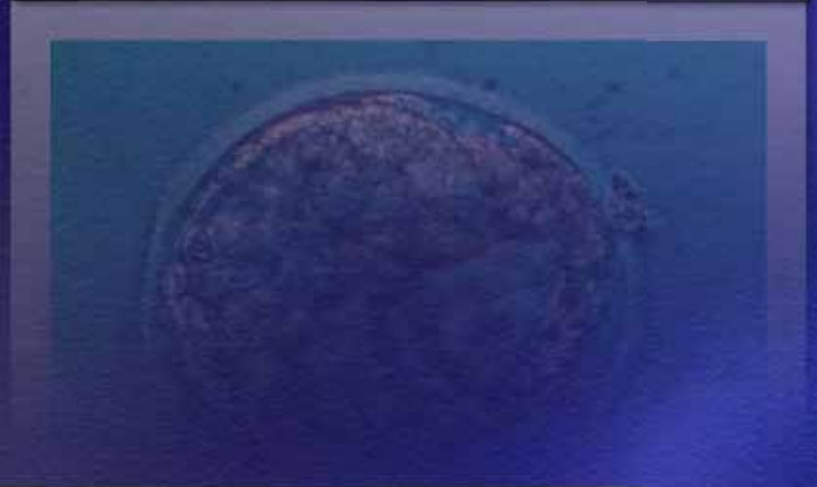
  0.5M Sucrose

- **Holding solution (HS):**

  modified M-199 + 20% SSS

Summary of what is important to observe

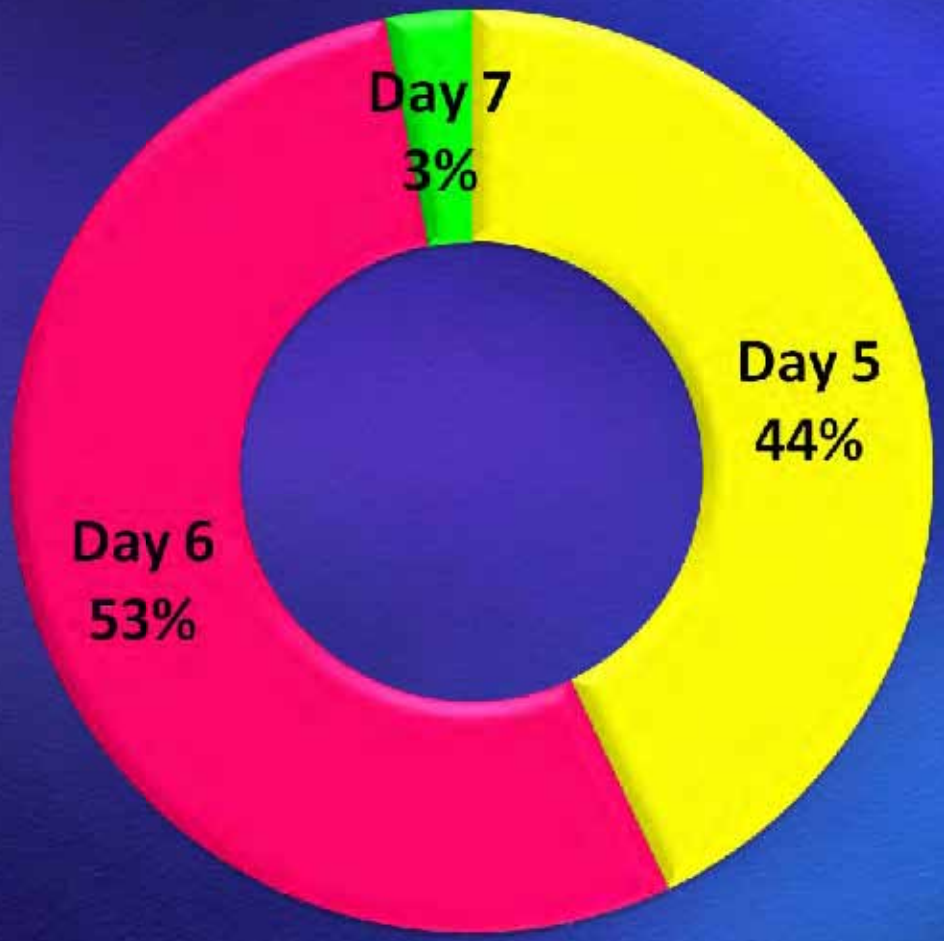




A. <u>During vitrification</u> - when the cells are starting to re-expand connect drops to get a higher concentration of cryoprotectant

B. <u>During warming</u> – when cells are starting to shrink connect drops to get a lower concentration of sucrose & to allow re-expansion

11,430 blastocysts from 3,232 pts between 01/2004 – 07/2010 were vitrified (average age 33.7±4.9)



Blastocyst Vitrification at the
Fertility Centers of Illinois (Chicago)

Retrospective data from the blastocyst cryopreservation program at the Fertility Centers of Illinois, Chicago, where vitrification (VIT) technology was applied from 01/2004 – 07/2010.

Technique	VIT
Patient's age (y)	34.7 ± 5.1
No. of warmed cycles	1995
No. of transfers	1977
No. of blastocysts warmed	3898
No. of blastocysts survived (%)	3767 (96.6)
No. of blastocysts transferred	3725
Mean no. of blastocysts transferred	1.9
No. of implantations (%)	1126 (30.2)
No. of positive pregnancies/warm (%)	975 (48.9)
No. of positive pregnancies/VET (%)	975 (49.3)
No. of clinical pregnancies/warm (%)	857 (43.0)
No. of clinical pregnancies/VET (%)	857 (43.3)
Ongoing pregnancies/VET (%)	721 (36.5)
No. of Livebirths	630 (326 girls & 304 boys)

Liebermann & Tucker (2006) Fertility & Sterility 86; 20-26; Liebermann J (2009) RBMOnline; http://www.rbmonline.com/Article/4328

A comparison of retrospective data from the blastocyst cryopreservation program at the Fertility Centers of Illinois, Chicago of vitrified day-5 and day-6 from 01/2004 – 07/2010.

Day of development	Day 5	Day 6
Patient's age (y)	34.6 ± 5.3	34.8 ± 4.9
No. of warmed cycles	971	1024
No. of transfers	968	1009
No. of blastocysts warmed	1955	1943
No. of blastocysts survived (%)	1887 (96.5)	1880 (96.7)
No. of blastocysts transferred	1863	1862
Mean no. of blastocysts transferred	1.9	1.8
No. of implantations (%)	640 (34.4)[a]	486 (26.1)[a]
No. of positive pregnancies/warm (%)	543 (55.9)[b]	432 (42.2)[b]
No. of positive pregnancies/VET (%)	543 (56.1)[c]	432 (42.8)[c]
No. of clinical pregnancies/warm (%)	473 (48.7)[d]	384 (37.5)[d]
No. of clinical pregnancies/VET (%)	473 (48.9)[e]	384 (38.1)[e]
Ongoing/delivered pregnancies/VET (%)	398 (41.1)	323 (32.0)
No. of Livebirths	367	263

[a]$P<0.05$; [b,c,d,e]$P<0.01$

Aseptic Device that separate Blastocysts from LN2 (10/07-07/2010)

Patient's age (y)	34.6 □5.2
No. of warmed cycles	666
No. of transfers	662
No. of blastocysts warmed	1275
No. of blastocysts survived (%)	1245 (97.6)
No. of blastocysts transferred	1233
Mean no. of blastocysts transferred	1.9
No. of implantations (%)	396 (32.1)
No. of positive pregnancies/VET (%)	342 (51.7)
No. of clinical pregnancies/VET (%)	303 (45.8)
Ongoing pregnancies/VET (%)	253 (38.2)
No. of Livebirths	156 (78 boys & 78 girls)

Closed-System HSV (High Security Vitrification Kit) from Cryo Bio System

Heat-insulating barrier dramatically reduces the speed of cooling (1.700 ºC/min) from aseptic device such the HSV, that separates blastocysts from LN2

Aseptic Device that separate Blastocysts from LN2 (10/07-07/2010)

Day of Development	Day 5	Day 6
Patient's age (y)	34.5 L 5.6	34.7 L 4.8
No. of warmed cycles	324	342
No. of transfers	324	338
No. of blastocysts warmed	627	648
No. of blastocysts survived (%)	614 (97.9)	631 (97.4)
No. of blastocysts transferred	609	648
Mean no. of blastocysts transferred	1.9	1.8
No. of implantations (%)	223 (36.6)[a]	173 (26.7)[a]
No. of positive pregnancies/VET (%)	186 (57.4)[b]	156 (46.2)[b]
No. of clinical pregnancies/VET (%)	164 (50.6)[c]	139 (41.1)[c]
Ongoing pregnancies/VET (%)	138 (42.6)[d]	115 (34.0)[d]
No. of Livebirths	99	57

[a,c,d]P<0.05; [b]P<0.01

FET outcome (Livebirths)

Deliveries

387 114 5

V

I 506 630 326 304

T

D5 D6 D5 D6 D5 D6

208 179 75 39 3 2

A vitrification solution with a mixture of 7.5% EG/DMSO, followed by a 15% EG/DMSO with 0.5M sucrose step is safe for clinical use by giving rise to healthy babies without abnormalities

SUMMARY ON VITRIFICATION OF BLASTOCYSTS

Vitrification of blastocysts using the cryotop/HSV is proven to be effective by achieving high implantation and pregnancy rates.

Slow growing embryos can be cryopreserved on day 6, as they yield a satisfactory survival, implantation and pregnancy rate.

Summary on Vitrification

Simplifies laboratory techniques for cryopreservation; is able to be undertaken on a more flexible basis by laboratory staff & the vitrification approach is very flexible and allows the handling of the embryos without the pressure of time

May enable more optimal timing of embryo cryopreservation, e.g., individual blastocysts may be cryopreserved at their optimal stage of development and expansion

Vitrification is a feasible alternative to traditional cryopreservation methods, because it is proven to be effective for all stages of embryo development

Embryo Cryopreservation

Photograph by Howard Cao

- Vitrification, with its increased Clinical Application, is showing a trend to greater consistency and better outcomes.
- WHEN (not if!) IVF programs overcome fear of the unknown, and take on the challenge of significant learning curve with vitrification, THEN vitrification will become clinical standard for human embryo cryopreservation.
- Different Embryonic stages will continue to be cryopreserved for differing reasons whether practical, political or personal.


In all things, be willing to
listen to people around you.
None of us is really smart
enough to go it alone.
~John clendenin

Lakefront Chicago

The IVF Lab at River North Chicago:

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