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**Advanced Current & Future Cryogenic
Technologies for ART**

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Disclosures

Founder of Innovative Cryo Enterprises LLC

We focus on all aspects of cryopreservation and offer vitrification media, advise on all things cryobiological, and consulting services.

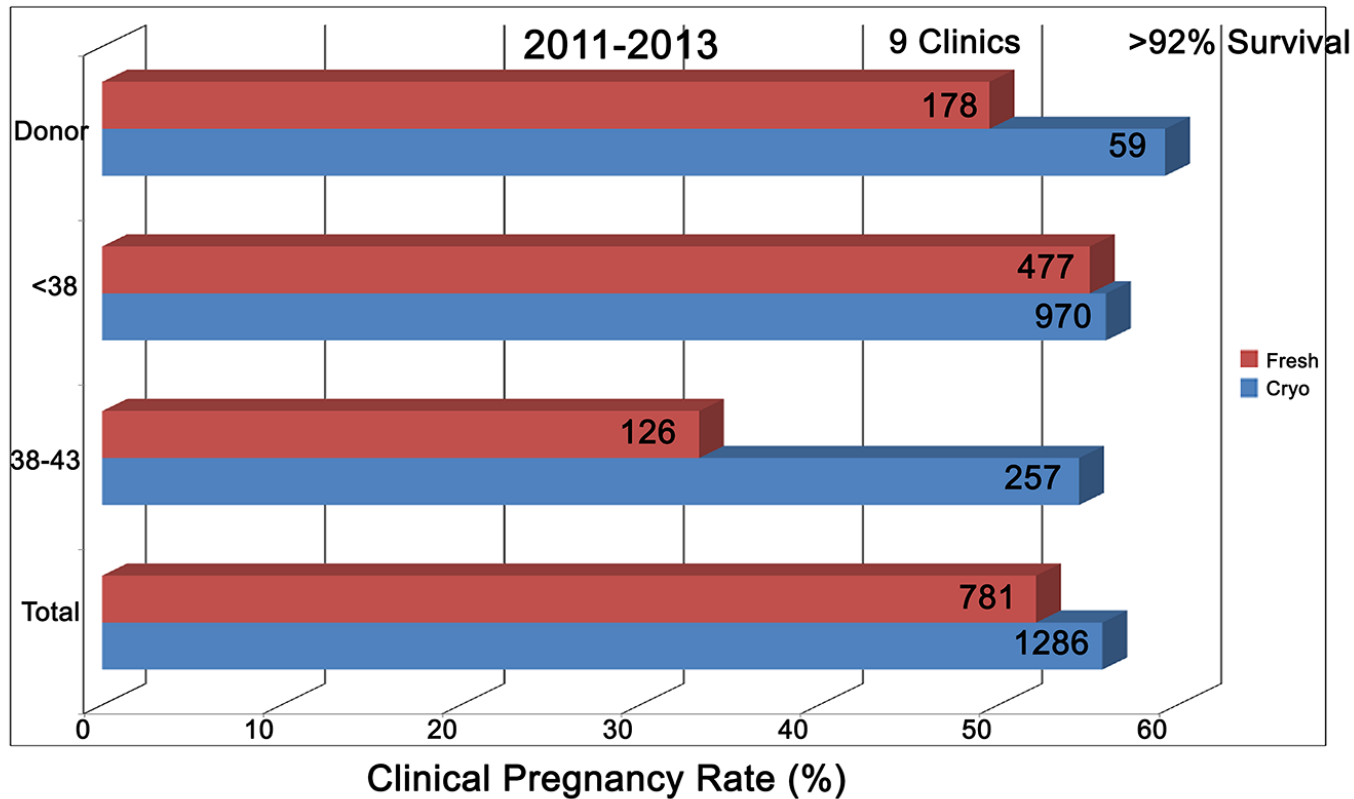
Topics

- Why vitrification
- Current vitrification practices
- How field has changed
- New advancements
- Future technology

Why Vitrification: Benefits of FET

Fresh vs. FET preg rates

Clinical Results - I.C.E. Vitrification Blastocyst



Shapiro's Conclusions

- Published evidence indicates endometrial development and receptivity are impaired by ovarian stimulation.
- Success rates and cost-efficiency are improved via a freeze-all strategy.
- National average implantation rates with FET exceed those with fresh transfer.
- Infants are generally healthier and closer to ideal birthweight following FET.

Shapiro's Conclusions cont.

- Ovarian stimulation impairs endometrial receptivity, particularly through embryo-endometrium asynchrony.
- Freeze all circumvents the compromised endometrium.
- FET is associated with many reduced maternal and perinatal risks when compared to fresh autologous transfers.
- Some or all of these risks differences appear to be due to uterine effects of COS.

Current Vitrification Practices: History

“The only method of stable and long-term (practically infinite) preservation and storage of any perishable biological materials, particularly cells, is to keep them in the glassy (vitreous) state.” Ice damage must be avoided!

Luyet and other “*pioneers of the cryobiological frontiers*” including Lovelock, Rall, Meryman, Mazur, Polge, Smith, Levitt, Farrant, and Willadsen, clearly understood some 40-70 years ago that only a glassy state would insure stable and non-lethal preservation of cells.

Luyet, The *vitrification* of organic colloids and of protoplasm, Biodynamica 1937.

Cryobiology History

Storage of gametes and embryos has used slow cooling for most of the past 40 years. (This was prior to blastocyst culture.)

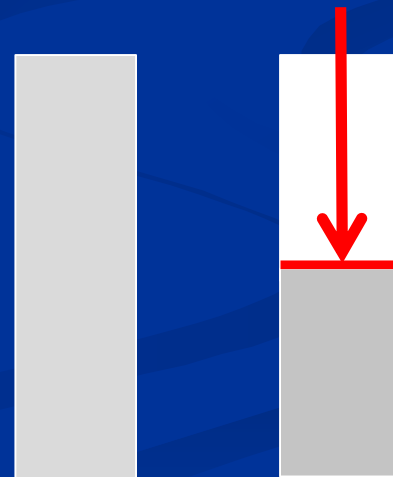
Only recently rapid cooling was used, and we refer to it as vitrification. (Because slow-cooling did not work so well for storage of blastocysts.)

In actuality, both slow- and rapid-cooling are forms of vitrification.

Vitrification: Intracellular Ice



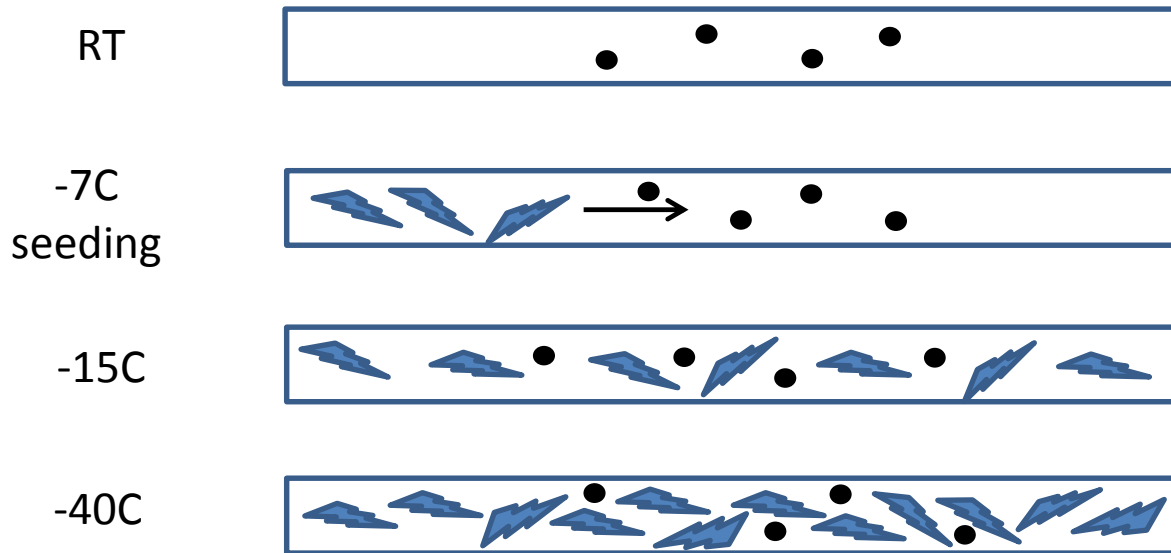
Slow-cooling consists of a vitrified portion and a portion made up of ice, whereas rapid cooling consists mainly of a vitrified portion.



Slow-Cooling: Vitrification

Both slow- and rapid-cooling are forms of vitrification!

Slow cooling 2C/min; seed; 0.3C/min to -40C; plunge in LN2



Slow-Cooling: Vitrification

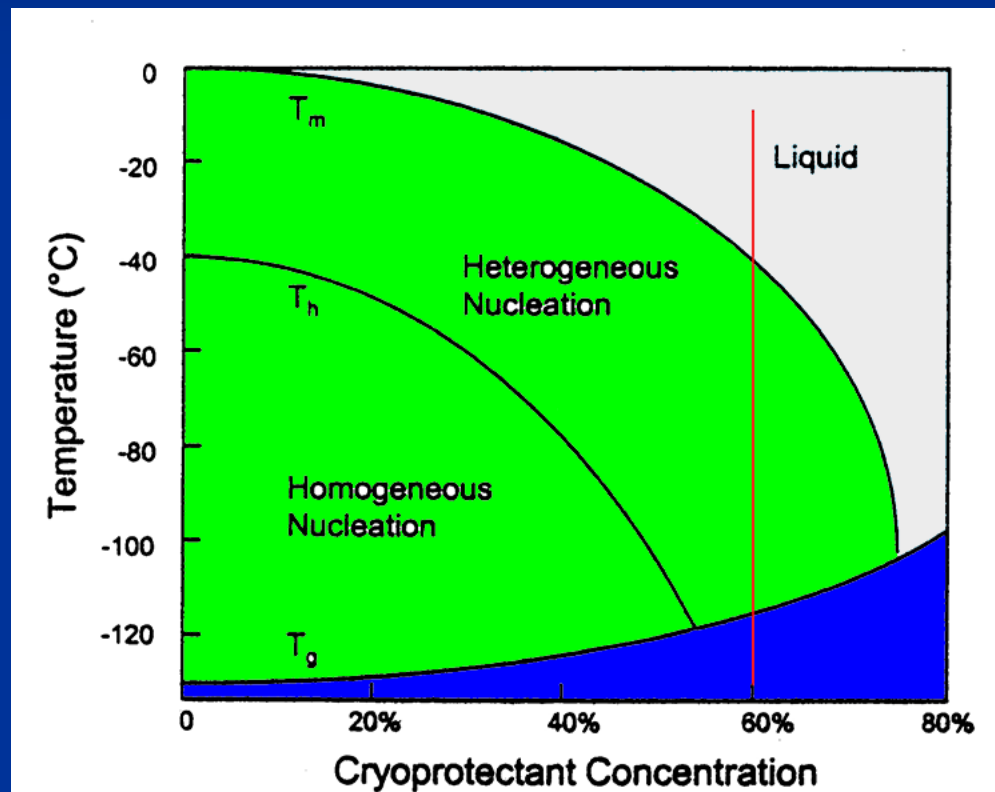
Rall & Fahy's demonstrated that mouse embryos could be vitrified and survive by cooling them in a relatively concentrated solution and cooling at either 2,500C/min or 20C/min.

This demonstrated that vitrification is ***not rate dependent***.

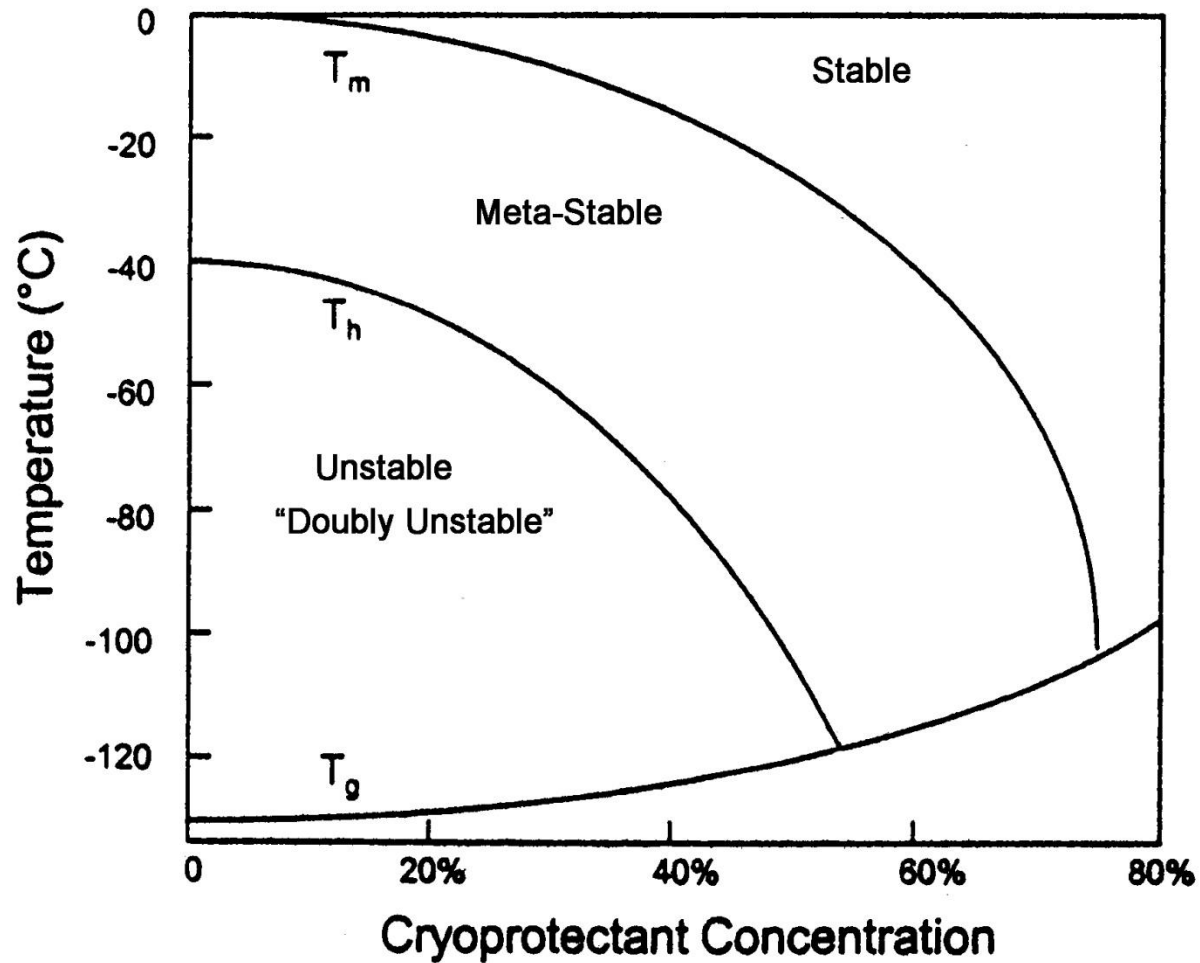
Rall & Fahy 1985 Nature

Phase Diagram

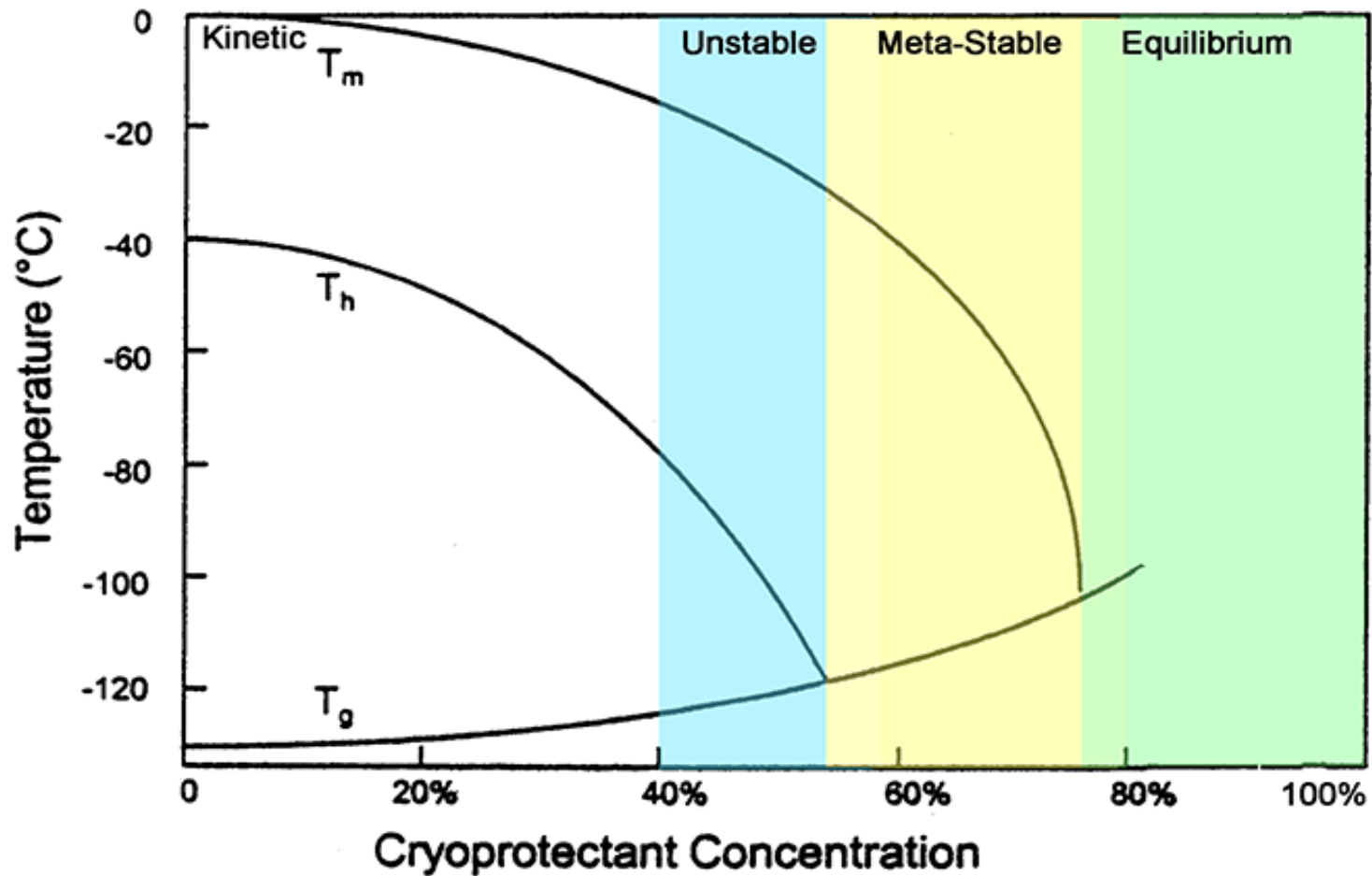
Shows liquid; liquid:solid; and solid phases of a cooled solution



Phase Diagram: Vitrification Types



Types of Vitrification



Current Vitrification Practices

There are 2 types of vitrification protocols in use today for storing human oocytes and embryos.

- DMSO/EG (EG/PrOH; most manufacturers and devices)
- DMSO-free (I.C.E. Vitrification) Note: S³ was the original version.

Equilibration to Vitrification Solutions

CPA / H₂O

Non-Equilibrium

Equilibrium

outside \neq inside

outside = inside



approx. 2-5 min

TIME

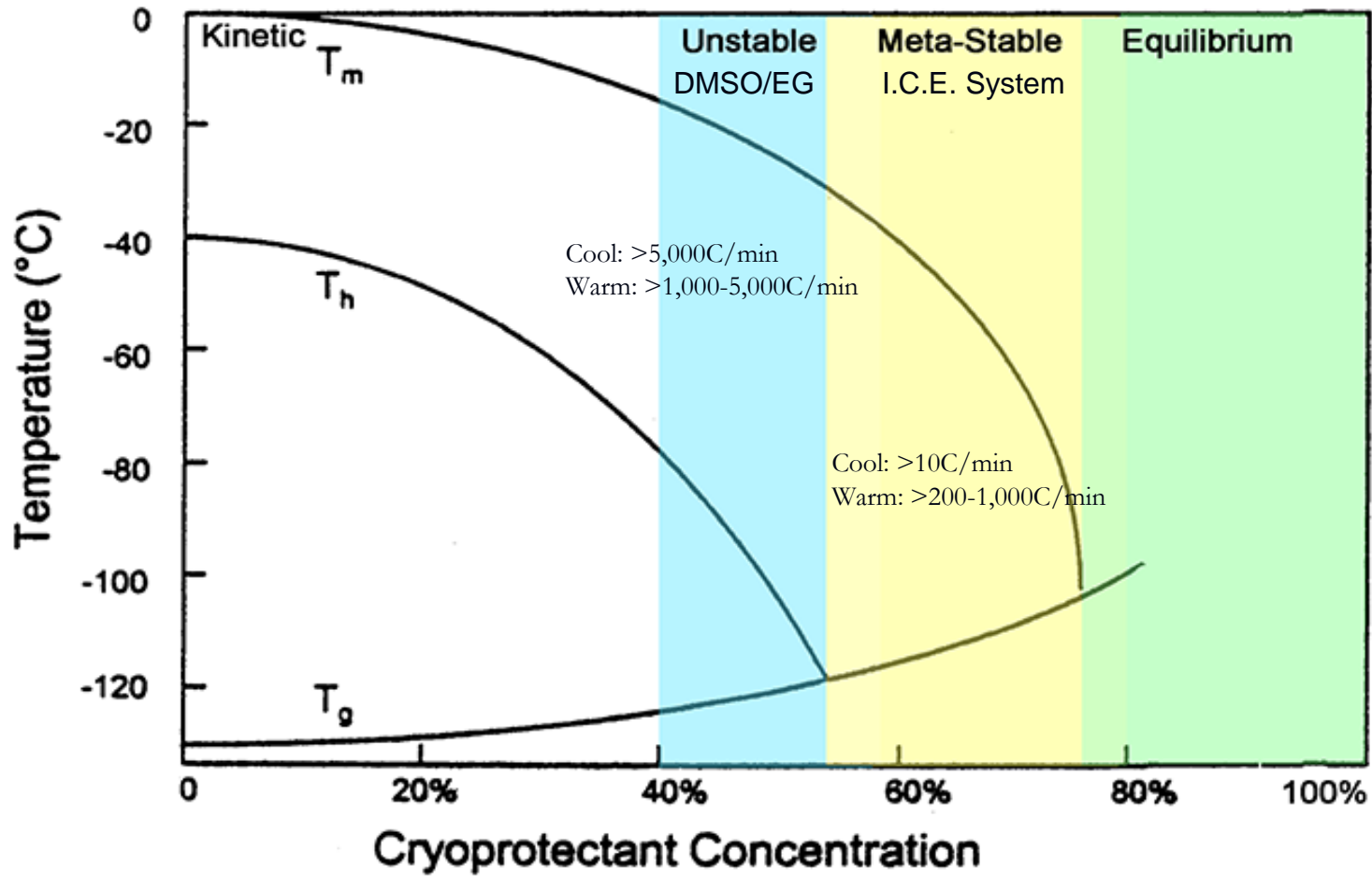
Dependant on:

- Cell Type (membrane permeability coefficient)
- Temperature
- Solution composition

Basic (Rapid-Cooling) Vitrification Procedures

	DMSO/EG	I.C.E. (DMSO-Free)
Pre Freeze	1 step (15%) 10-15 min	2 step (10-30%) 7-10 min
Freeze	1 step; 60 sec (30%)	1 step; 2-5 min (>30%)
Rehydration	3 step	5 step

Types of Vitrification



Current Vitrification Practices

DMSO/EG systems:

Widely published results. (only good results tend to be published)

ICE Vitrification:

Numerous abstracts, papers, book chapters, etc on website www.icevitrification.com Live born in 1000's.

Both types of vitrification systems:

>90% survival; pregnancy rates close to or above rates using fresh embryos in stimulated cycles.

How The Field Has Changed

- Blastocyst Culture -- Demand for better cryopreservation system
- PGD / PGS – Necessary to store cells in order to obtain results
- FET / eSET – Necessary to store cells and transfer on subsequent cycle
- Same basic technique since OPS paper. Vajta et al 1998 Mol Repro Dev

Advancements

EG/DMSO System (OPS paper) Vajta et al 1998 Mol Repro Dev

Simple Modifications:

- Devices
- Media

S³ Vitrification (ICE vitrification) Stachecki et al 2008 RBMO

Based on slow-cooling, using a large volume container

New Advancements

Cryo-Tec

Sugar

Media and buffer system

HPC (hydroxypropylcellulose)

Device

ICE Vitrification

Strict testing & high quality components

Hyaluronate

Protocol optimization

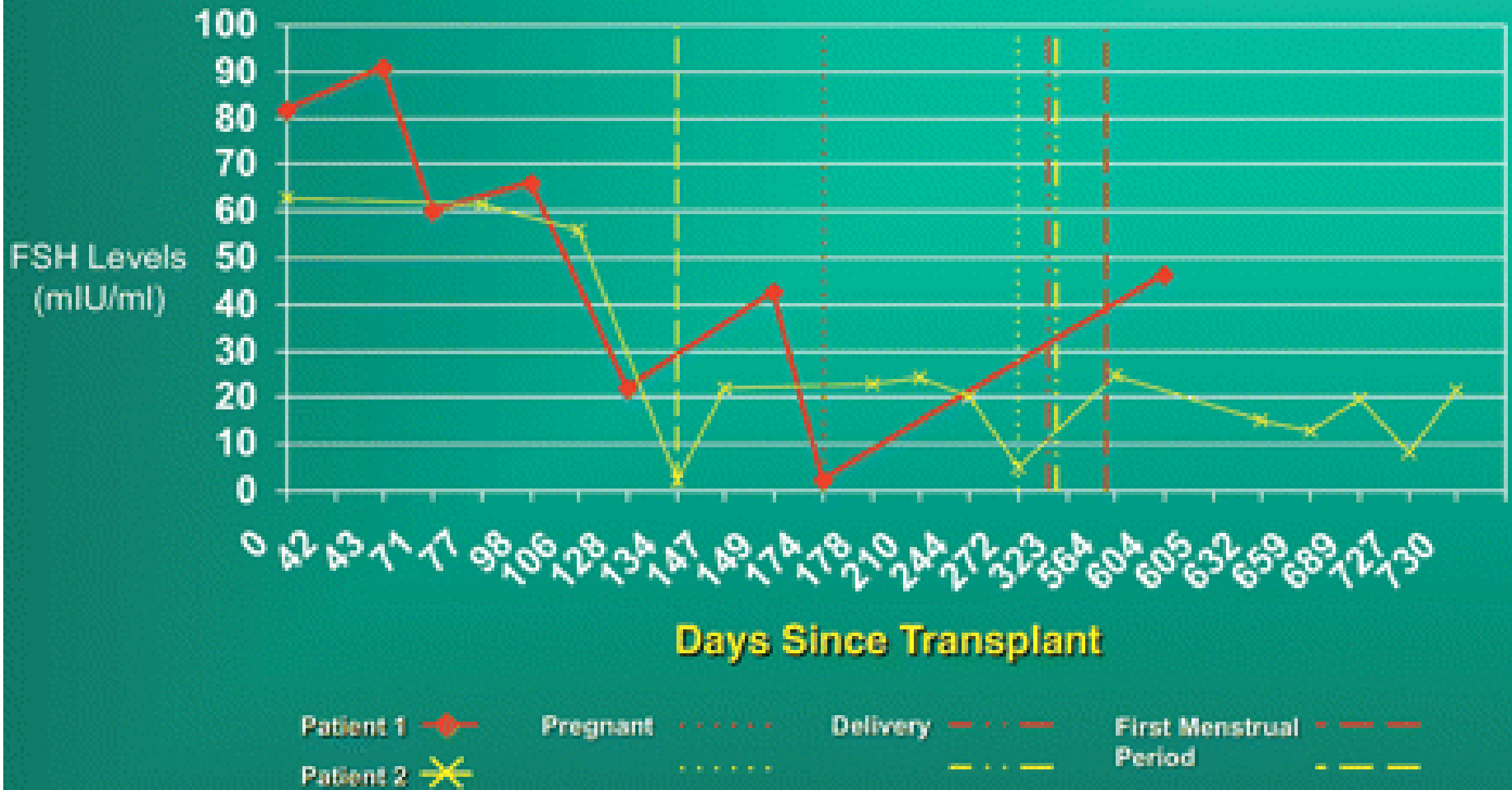
New Advancements

Ovarian Vitrification: Silber Mol Hum Reprod 2012

Fresh Transplants: 11 babies in 7 of 9 recipients.

Frozen Ovarian Tissue: 3 babies in 3 transplants over 14 years.

Transplant of Thawed Ovarian Tissue



Ovarian Vitrification: Procedure

Ovarian cortex is sliced into thin strips 1x10x10mm.

Vitrified in EG/DMSO 7.5% and then 20%

Vitrified in minimal volume on metal strips

Thawed tissue in 37C media

Grafted to ovary

Ovarian Vitrification: Procedure

Ovarian cortex analysis from 62 women with solid organ cancer; only 1 had tumor cells in ovarian cortex.

The reason for the remarkable absence of ovarian metastasis might possibly be due to the fibrous avascular nature of the ovarian cortex.

Slow-cooling vs vitrification: 41% vs >90% oocyte survival (Newton *et al.*, 1996; Gook *et al.*, 1999; Kagawa *et al.*, 2009; Silber *et al.*, 2010)

Others have had different results.

Ovarian Vitrification: Silber's Conclusions

Because we found no loss of oocyte viability (after vitrification), we hope that the frozen grafts will last as long as the fresh ones.

It is clear from our fresh grafts that a proper microsurgical technique, is necessary for cortical grafting success.

Numerous other births after implanting ovarian tissue for a total of 28 live births thus far. (Donnez *et al.*, 2004; Meirow *et al.*, 2005; Demeestere *et al.*, 2007; Andersen *et al.*, 2008; Piver *et al.*, 2009; Sanchez-Serrano *et al.*, 2010; Donnez *et al.*, 2011)

Worldwide frozen ovarian cortical tissue transplantation pregnancies.

Case #	Diagnosis	Babies	Study
1	Hodgkins	1	Donnez
2	Neurotumor	1	Donnez
3	Non-Hodgkins	1	Meirow
4	Hodgkins	1	Demeestere
5	Ewings	3	Andersen
6	Hodgkins	1	Andersen
7	POF	1	Silber
8	Hodgkins	2	Silber
9	Polyangiitis	1	Piver
10	Breast cancer	2	Pellicer
11	Sickle cell	1	Piver
12	Hodgkins	2	Revel
Totals: 12 patients		17 babies	8 centers

Ovarian Vitrification:

PrOH/EG Study Sanfillippo et al 2015 Reprod Biol Endo

Vitrification of Ovarian Tissue for Fertility Preservation

Ting et al, 2017 Ped & Adoles Oncofert Chapter

New Advancements: Sperm Vitrification

Vitrification is being investigated extensively and applied to embryos and oocytes but very seldomly to spermatozoa, with the exception of a few reports.

The question of diminished spermatozoa motility after cryopreservation is crucial, although the mechanism of sperm impairment and its mechanical and/or physical–chemical etiology remains unclear.

IIF, osmotic effects, cooling/warming damage, CPA toxicity, etc...

New Advancements: Sperm Vitrification

Technology of cryoprotectant-free vitrification of human, canine, dog and fish spermatozoa is now reality.

Interesting results were published by Argentina group regarding comparison of cryoprotectant-free and cryoprotectant-including porcine sperm vitrification in large volume.

Authors concluded that rapid warming of porcine spermatozoa vitrified without permeable cryoprotectants may preserve DNA condensation and integrity better than the other processing methods studied in this work.

Authors noted also that porcine sperm vitrification could be used to produce embryos with ICSI. Fact is that porcine ejaculate normally has volume to 500 ml and for insemination we need an extra large volume.

During our workshop here in Cologne together with our colleagues from Spain we have obtained interesting results with cryoprotectant-free vitrification of stallion sperm (not yet published results). These results were better than after standard slow freezing with cryoprotectants.

Sperm Vitrification

Sperm cannot tolerate high CPA concentrations like oocytes and embryos.

Human sperm vitrification without CPA's based on Luyet's original work. Nawroth et al 2002 Cryo Lett; Slabbert et al 2015

Others tried to improve upon this idea and Isachenko et al 2011 focused on both cooling and warming rates.

There is no evidence of additional risks for birth defects or chromosomal abnormalities after using cryopreserved sperm.

Kennedy 2015 Int Fed Fert Soc

Sperm Vitrification:

In contrast to conventional freezing, spermatozoa vitrified (in 0.5cc straws) with aseptic cryoprotectant-free technology displayed superior functional characteristics.

The motility rate, integrity rates of cytoplasmic, and acrosomal membranes were significantly higher after vitrification than after conventional freezing (76% vs 52%, 54% vs 28% and 44% vs 30%, respectively) ($p < 0.05$). Isachenko et al 2011 Clin Lab.

Vitrified sperm present similar or higher quality than the sperm frozen with slow-freezing protocols and are also fertile by means of IVF/ICSI and intrauterine insemination, although reports are still scarce. Moce et al 2015

New Advancements: Sperm Vitrification

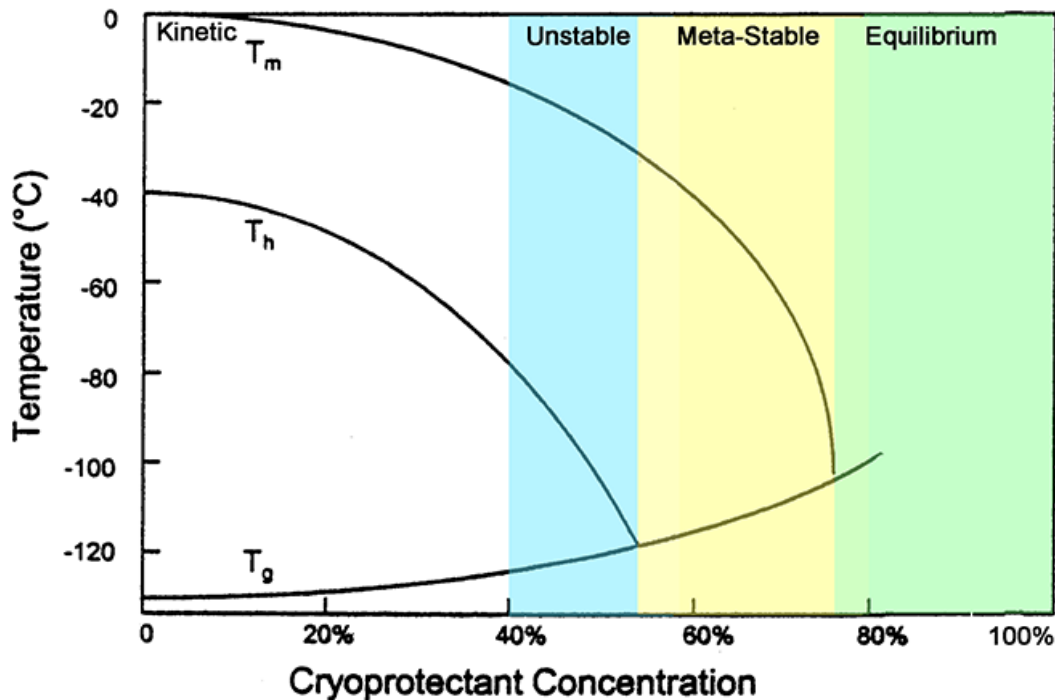
Lyophilisation and freeze-dried sperm used for ICSI have resulted in viable offspring for several animal species, however, reports of functional lyophilised human sperm are lacking.

Future Technology: Kinetic Vitrification

Krioblast: Ultra rapid cooling of around 500,000C/min.

The objective is to have a “universal” cryo protocol for cells.

Bolyukh, Katkov et al. 2012



Future Technology: Automated Vitrification

1) Genea Biomedx: Gavi

Not totally automated

Need to pipet cell into dish & plunge into LN2

Need to thaw manually

2) Essco:

Not released yet, still in development

Not totally automated either

3) Vitriscience: Automated Vitrification System

No information yet

Thank You!

