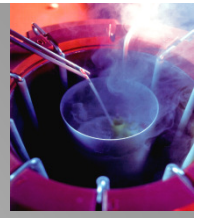
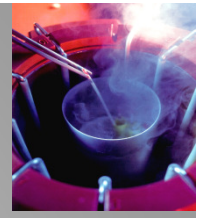


Cryobiology of vitrification

- PD Dr. med. vet., Dr. rer. biol. hum habil
- ULRICH SCHNEIDER

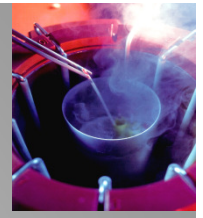


Vitrification pushed in ART as new and
as method that solves all problems with oocyte and embryo freezing

Impression that fancy and costly sets (Cryotip, Cryotop, Cryoleaf, etc.) are absolutely required to achieve good results.

Nonsense if you understand and follow the cryobiological principles

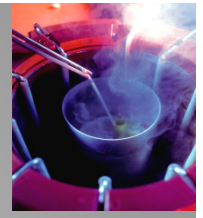
Question proprietary freezing media or patented methods



• Scientists with important contribution to vitrification

Father Basile J. Luyet – active since 1937

- attempting to arrest life and then restart it
- 3 factors control the achievement of the vitreous state
  - velocity of crystallization
  - size of the zone of crystallization temperature
  - cooling velocity sufficient to prevent the formation of crystals
- 1969 Luyet characterized his attempts of vitrification
- in retrospect as „mostly negative“



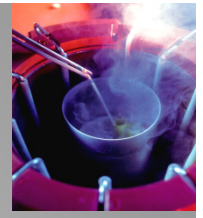
- Scientists with important contribution to vitrification

Pierre Butron – active since 1978

- Studied kinetics of glass formation on cooling
- and devitrification on warming
- key discovery:
- propylene glycol vitrifies at 30-40 WT % depending on cooling rate

Grey Fahy – active since 1972

- organ preservation by vitrification
- problem of the CPA toxicity
- toxicity neutralizer, non penetrating CPA, exponential addition and wash out of CPA



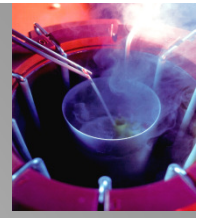
• Scientists with important contribution to vitrification

Bill Rall – active since 1980

- studying mammalian embryos under cryomicroscope
- defined homogenous nucleation temperature for embryos
- clearly demonstrated that during mod. 2step freezing embryos survive cryopreservation as a result of at least intracellular vitrification

Bill Rall and Greg Fahy – 1985

- successful vitrification of 8-cell mouse embryos



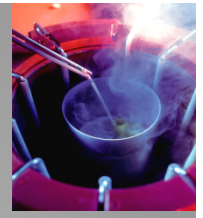
Molarity

Osmolarity

Molality

Osmolality

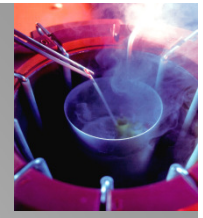
weight percent



molar solution
1 mol of solute in 1000 ml of solvent

EG MW 62.07

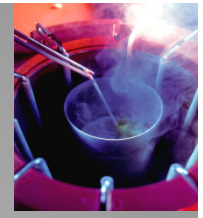
62.07 g + PBS up to the 1000 ml mark



molal solution
1 mol of solute in 1000 g of solvent

EG MW 62.07

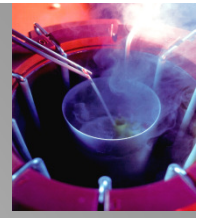
62.07 g + PBS up to 1000 g



molar concentration or molarity c defined as:

$$c = \frac{n}{V}$$

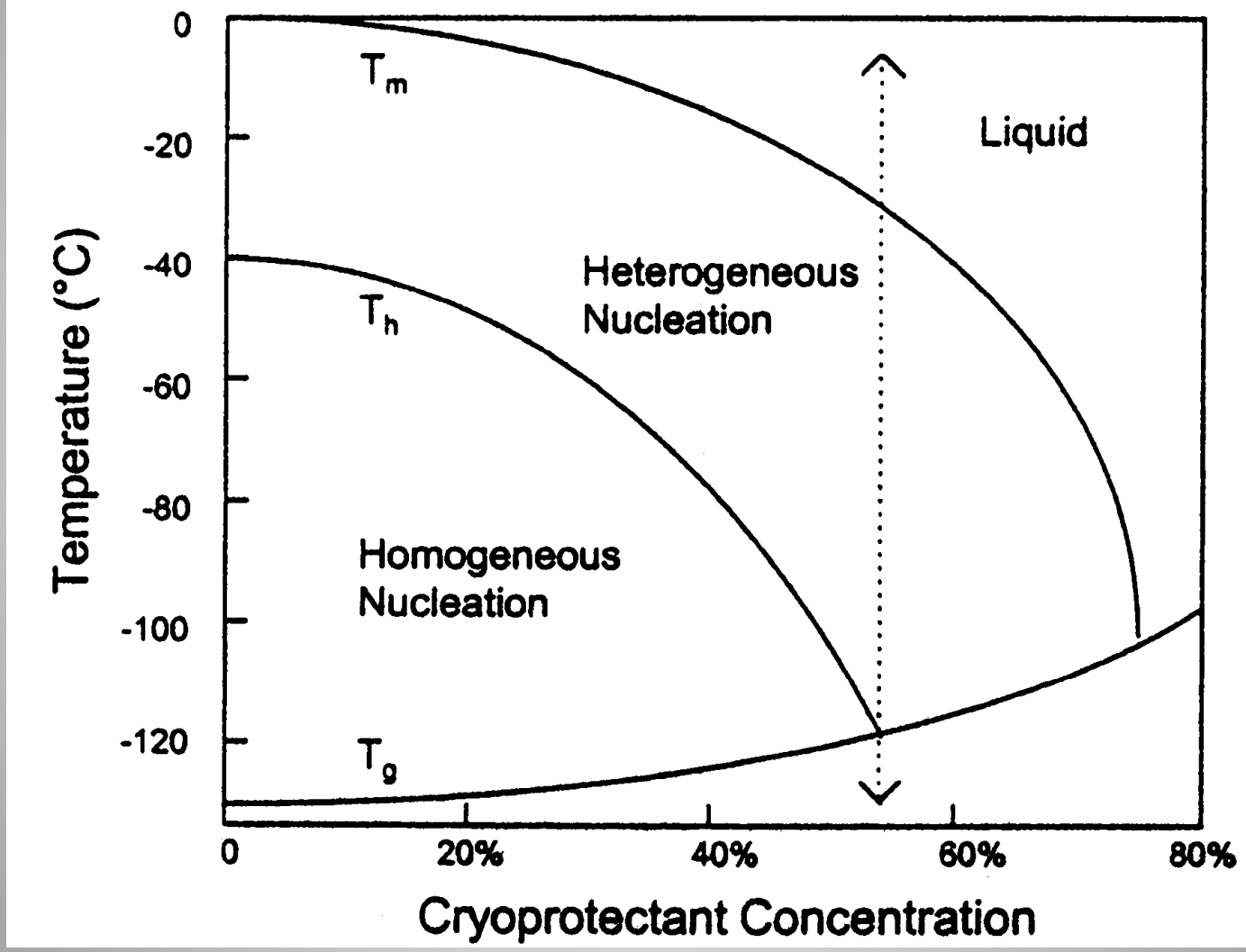
where n is the amount of solute in moles present in volume V

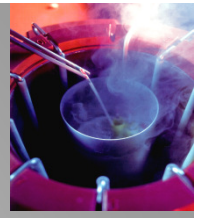


- Vitrification = „turned into glass“
  defined as

  conversion from fluid to a solid by an increase in viscosity without phase change (crystallization of water)

- This state is achieved in systems
- that are sufficiently concentrated or cooled rapidly
- where the increase in viscosity inhibits molecular rearrangement into a crystalline pattern





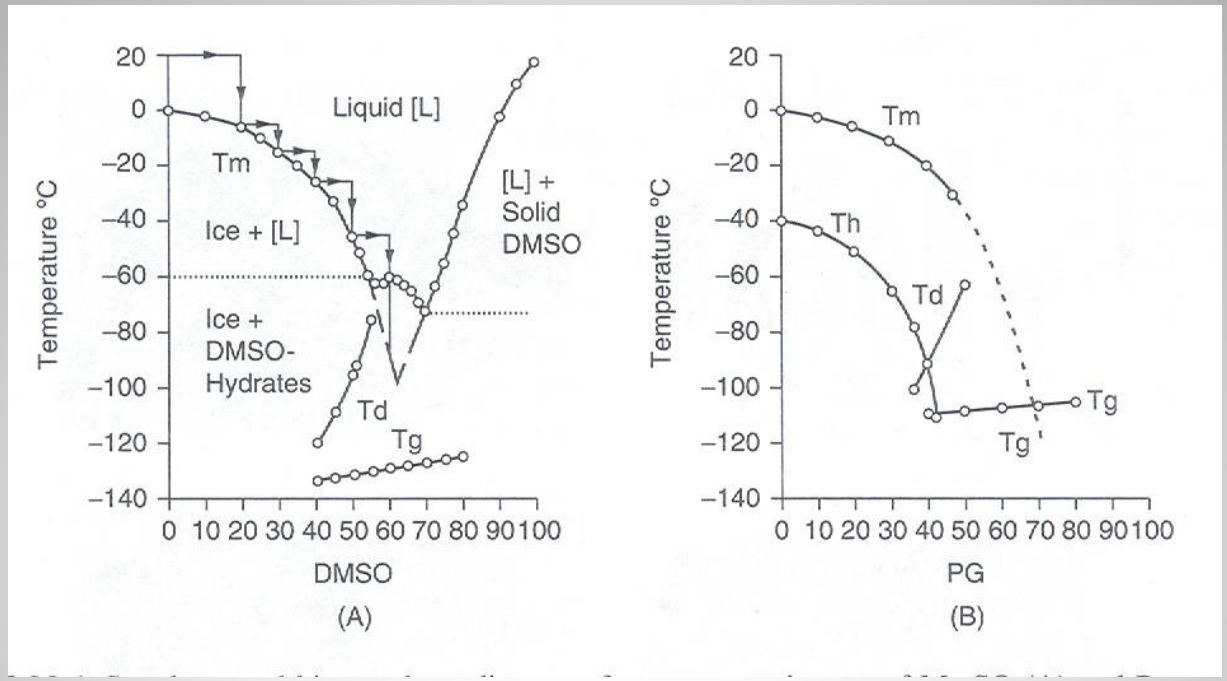
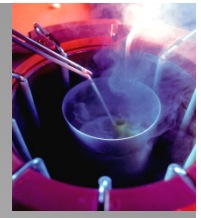
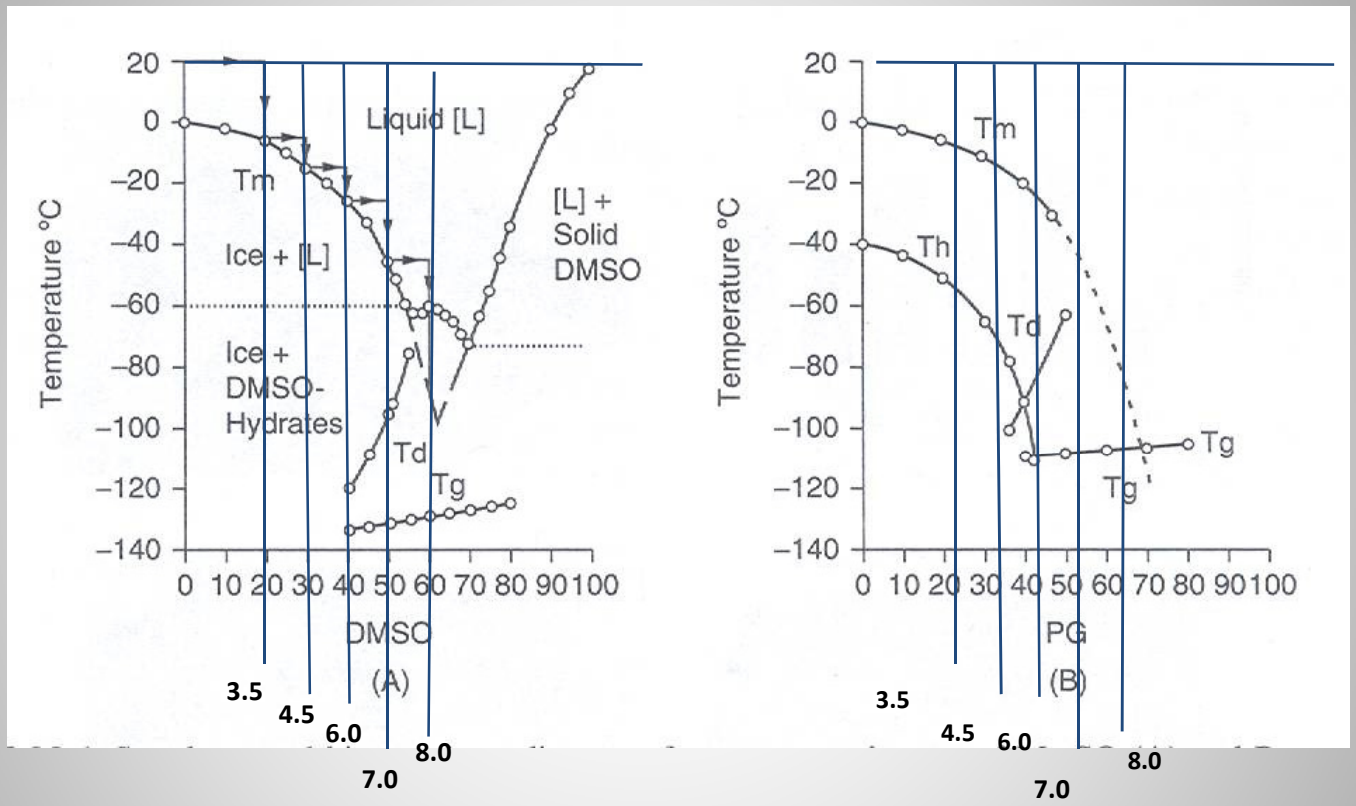


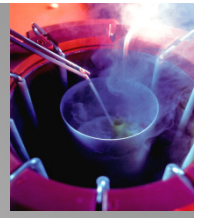
FIGURE 22.1 Supplemented binary phase diagrams for aqueous mixtures of Me_2SO (A) and Propane-1,2-diol (B) showing the principal events and phase changes associated with cooling and heating. A supplemented phase diagram combines nonequilibrium data on a conventional equilibrium phase diagram and serves to depict the important transitions inherent in cooling and warming aqueous solutions of cryoprotective solutes. Details are described elsewhere (Fahy, 1998; MacFarlane, 1987; Rasmussen and MacKenzie, 1968; Taylor, 1987) and in the text. T_m, equilibrium melting point curve (liquidus curve); T_h, homogeneous nucleation curve; T_d, devitrification curve; T_g, glass transition curve. The stepped line above the Me_2SO–H_2O liquidus T_m curve represents a scheme for incremental equilibration of a tissue with sufficient cryoprotective additive such that the system does not freeze during cooling. (See text for details.)

Life in the Frozen State, Edt. By B.J. Fuller, N. Lane, E.E. Benson, CRC Press, 2004, p. 607





Life in the Frozen State, Edt. By B.J. Fuller, N. Lane, E.E. Benson, CRC Press, 2004, p. 607



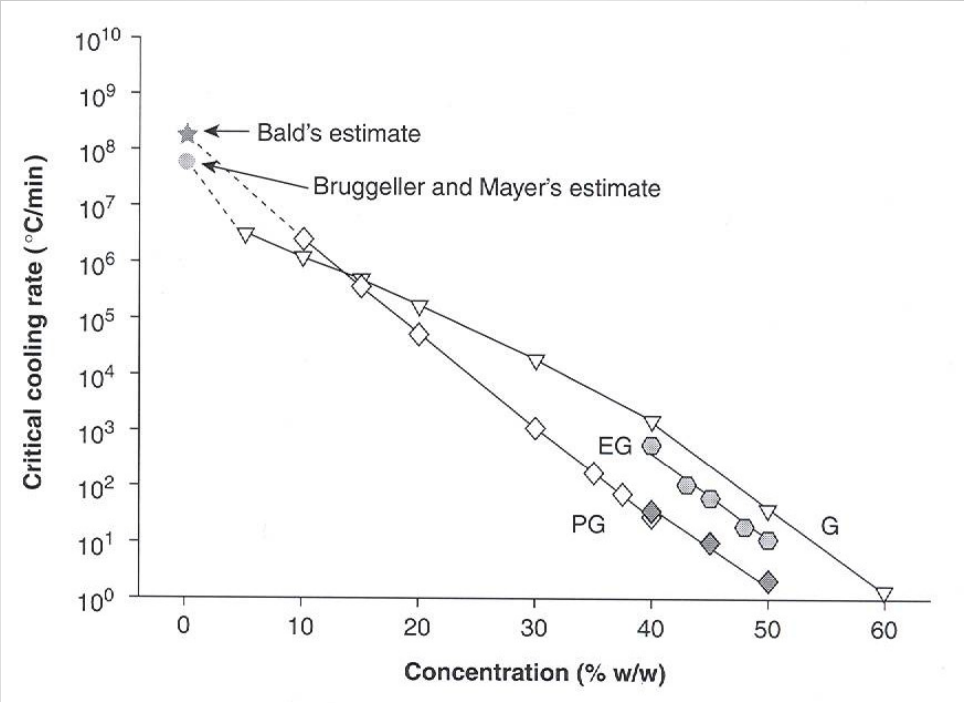


Figure 1A.5 Cooling rate required to vitrify samples ranging in concentration from 0% to 60% w/w cryoprotectant. white points, unpublished calculations of Mehmet Toner[83] (published here by permission); star, estimate of Bald;[86] filled circle, high estimate of Bruggeller and Mayer (their low estimate is one order of magnitude lower);[46] gray diamonds, corrected observed critical cooling rates for the prevention of ice in propylene glycol (PG) – water solutions (from Baudot and Odagescu);[87] G = glycerol hexagons, observed critical cooling rates for ethylene glycol (EG) – water solutions.[87]

Vitrification in Assisted Reproduction, Edt. By M.J. Tucker, J. Liebermann, 2007, p. 13

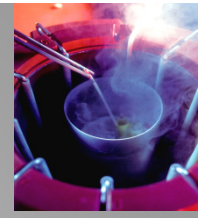


TABLE 1

Critical Cooling Rates (C/min) to Avoid Any Crystallization for ME_2SO and Other Aqueous Solutions

% (w/w) Solute	1,2-Propanediol	ME_2SO	Ethylene glycol	Glycerol
30	1200			
35	240			
40	35	500 +/- 50		
45	10	14 +/- 3	260	380
50	< 2.5	< 2.5		70

Baudot et al., Cryobiology, 40, 151-158 (2000)
Modified Table 1

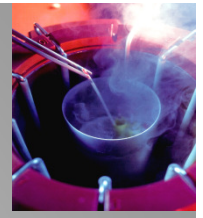


Table 1. Cooling rates for modern vitrification devices.

Device	Media (ul)	Freezing Rate
0.25cc straw	25ul	4460°C/min
Open-pulled straw	1.5ul	16,340°C/min
Cryo-Top	0.1ul	22,800°C/min
Cryo-Tip	<2ul	12,000°C/min
0.25cc straw (S^3 vit)[1]	10ul to 250ul	<100°C/min

From Kuwayama et al., (2005b).
[1]This study.

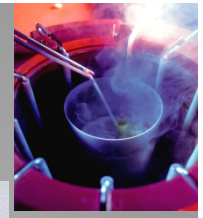


Table 1 : Vitrification Properties of Sugar-EG-Saline Solutions: How the Sugar Type and Concentration Influenced the Lowest Total Solute Concentration at Which Vitrification Would Occur

Vitrification Solution	Total solute concentration (wt%)	Sugar concentration (mol/kg)	Tg (°C)
EG-saline	59	0	-127.7
Sucrose-EG-saline	60	0.1	-126.8
Sucrose-EG-saline	61	0.5	-122.6
Sucrose-EG-saline	65	1	-118

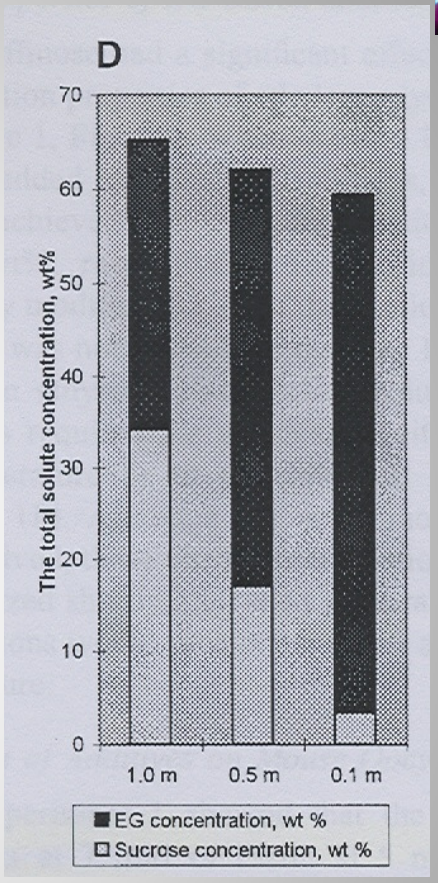
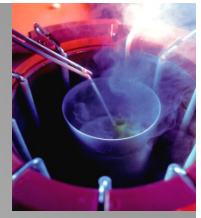


Fig.1. The lowest total solute concentration required for vitrification: (D) ethylene glycol-saline solutions.

Kuleshova et al., Cryobiology 38, 119-130 (1999) modified Table 1 and Fig. 1



We have discussed vitrification conditions of a **cell free** system

To reach vitrification conditions independant of cooling and warming rate, we need the same app. 60% solute concentration **inside the cell** also.

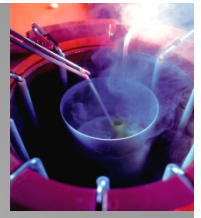


Table I. *In vitro* development of pronuclear stage embryos exposed to cryoprotectant [1,2-propanediol (PROH) or dimethylsulphoxide (DMSO) at 8.0 mol/l concentration each with 0.5 mol/l sucrose) for 45 s

Cryoprotectant	No. of embryos	Survival		Development to			
				2-cell-embryos		Blastocysts	
		n	%	*n*	%	*n*	%
DMSO	95	95	100	91	96	76	80
PROH	95	94	99	89	94	80	84
Control	113			106	94	94	83

M.A. Nowshari et al. Human Reproduction, 10 , 3237-3242, 1995

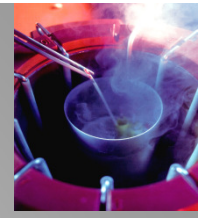


Table 2: Developmental competence of oocytes exposed to changes in external osmolality

	External osmolality (mOsmol/kg H_2O)						
	39	76	151	302	598	1262	2264
No. of oocytes	49	49	49	49	49	49	49
Percentage of 2PN embryos	63	59	71	69	69	71	69
Percentage of Good-Quality embryos	52	72	51	56	53	54	53
Mean number of cells (+/- SD)	5.9 (+/- 1.5)	6.4 (+/-1.9)	6.0 (+/-1.6)	6.8 (+/-2.0)	6.1 (+/-2.1)	7.1 (+/-1.7)	5.7 (+/-2.3)

Van den Abbeel et al., Human Reproduction, 22 (2007)

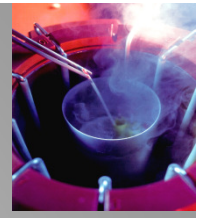
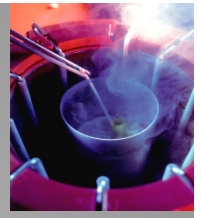


TABLE 5

Effect of Exposure of *in Vivo* Matured MII Oocytes to 0.5 or 1.0 M Sucrose at Room Temperature for 10 Min on Their Subsequent Two-Cell Formation and Parthenogenetic Activation Rates

		0.5 M			1.0 M		
	Inseminated ?	N	Two Cells %	Blastocysts %	N	Two Cells %	Blastocysts %
Sucrose at MII	Yes	10	90	40	9	89	55
Sucrose at MII	No	14	0	0	11	0	0

Kuleshova et al., Cryobiology 38, 119-130 (1999)
modified Table 5



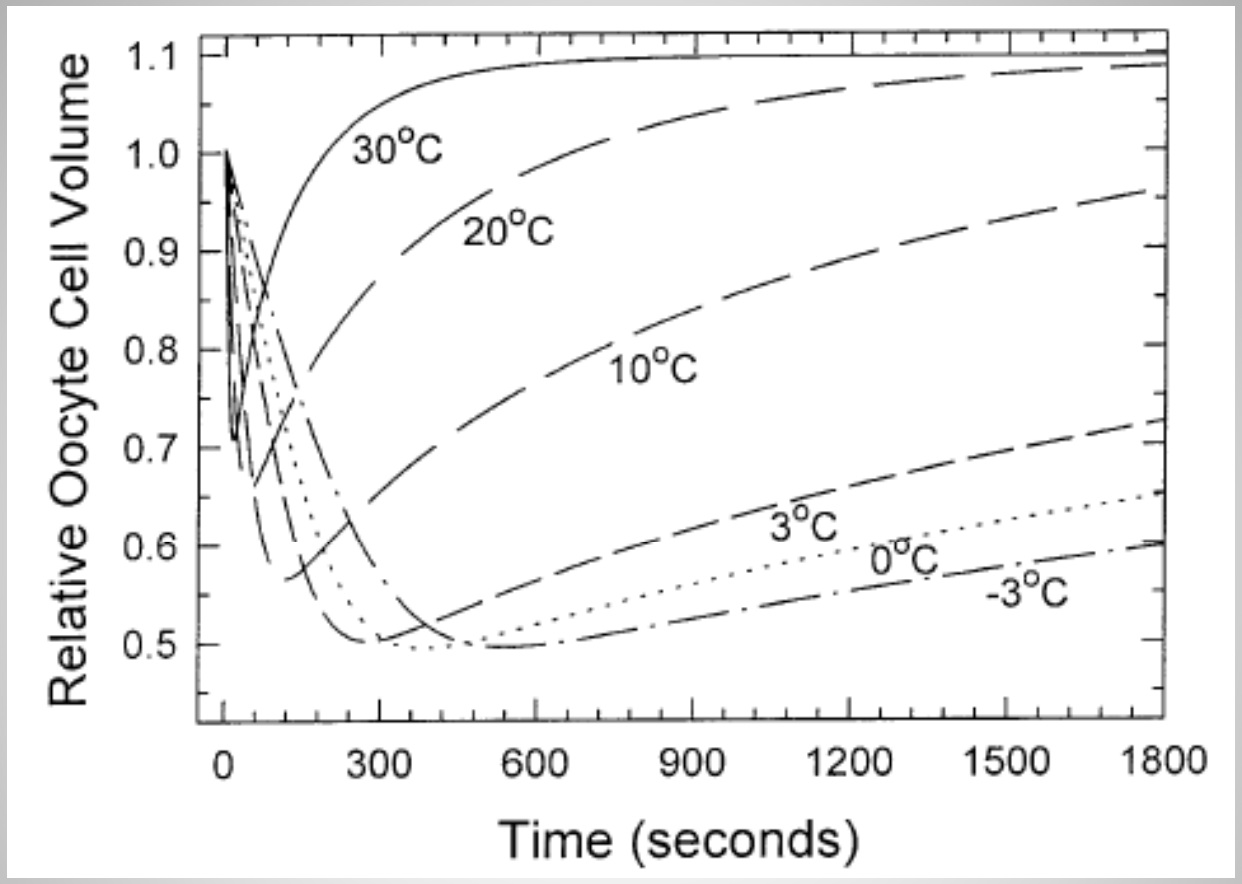
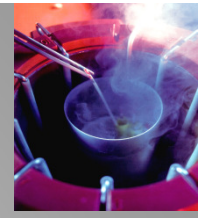
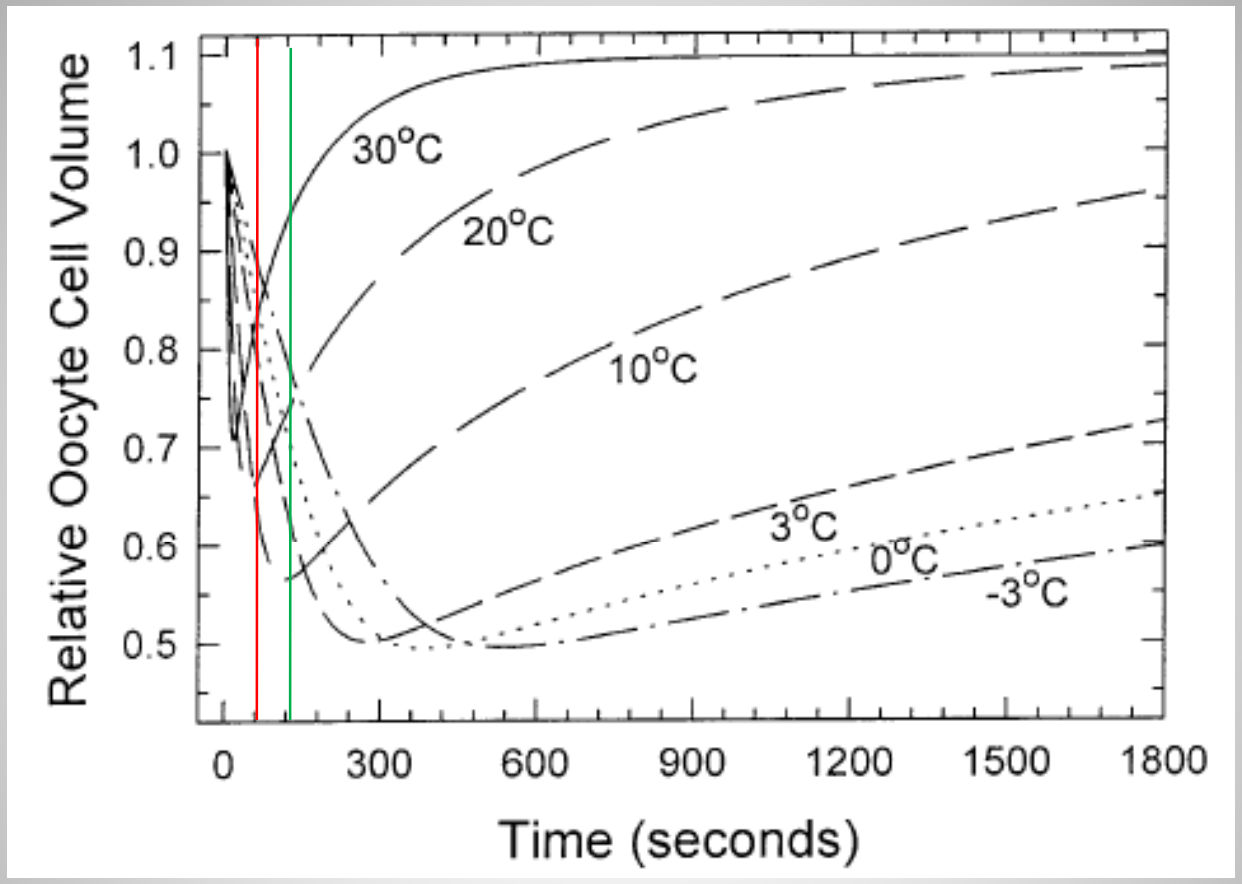


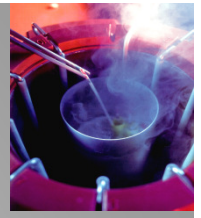
FIG. 2. Predicted responses of the kinetic cell volume responses of MII mouse oocytes in 1.5 M Me_2SO at the indicated temperatures.

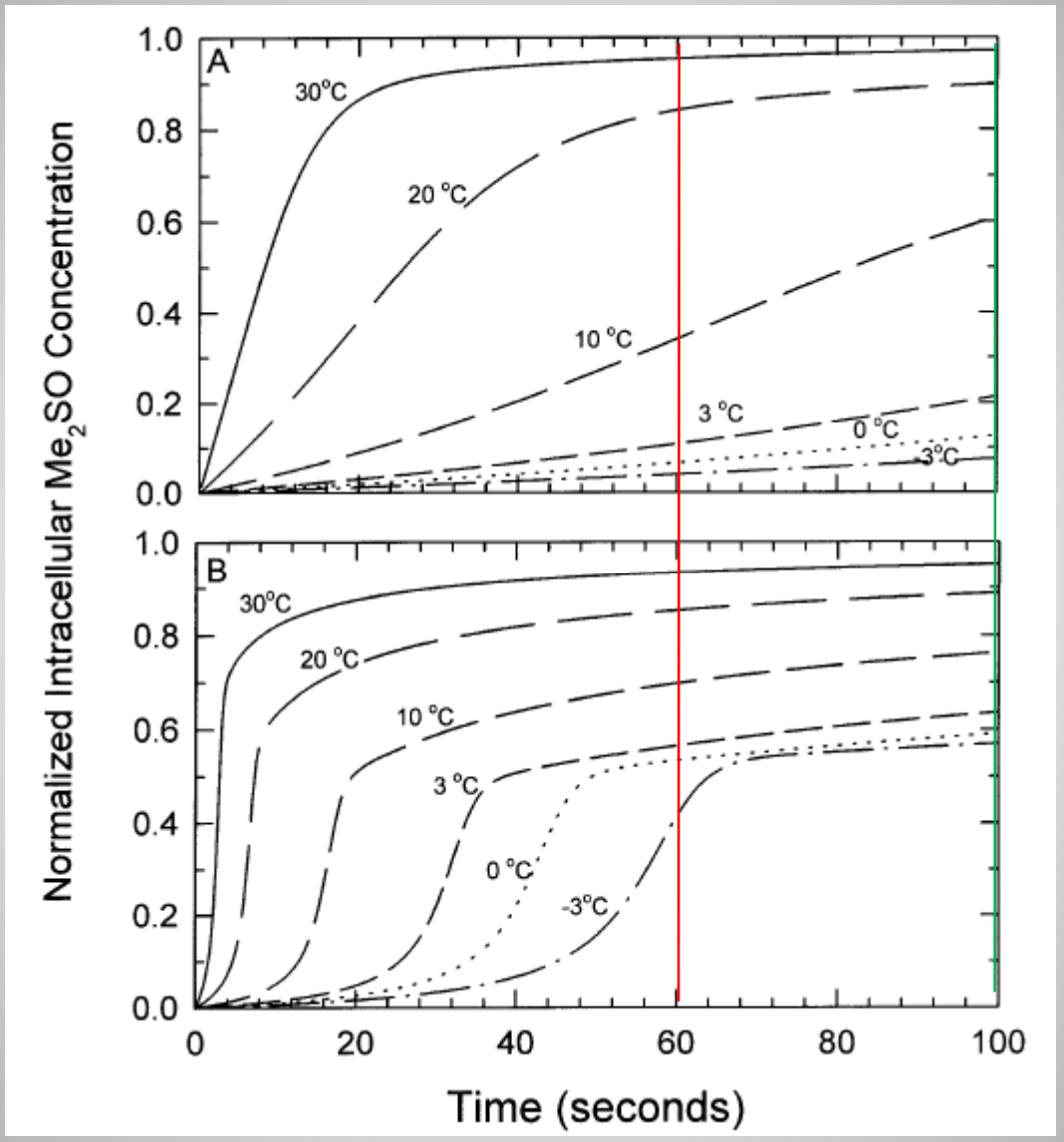
Agca, et al., Cryobiology 36, 287-300 (1998)





Agca, et al., Cryobiology 36, 287-300 (1998)





Agca, et al., Cryobiology 36, 287-300 (1998)

FIG. 6. Simulation of MII mouse oocytes after exposed to either 1.5 M (A) or 6 M (B) Me_2SO at various temperatures and the corresponding intracellular Me_2SO concentrations.

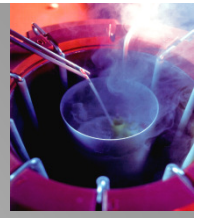
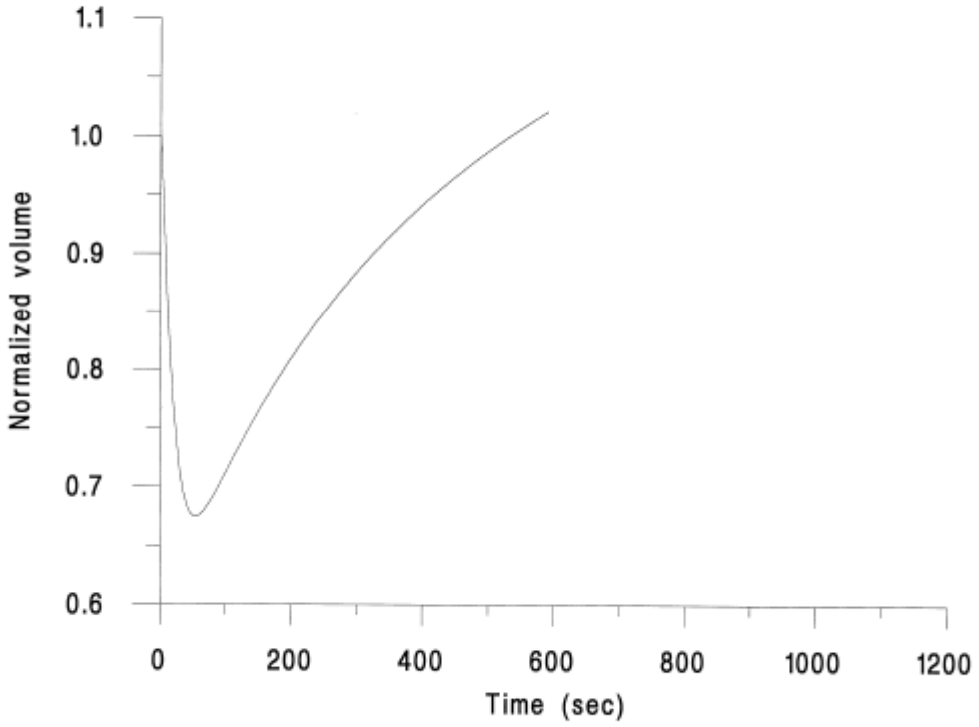
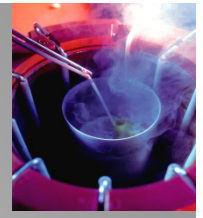


FIGURE 3

Predicted osmotic response of human oocyte exposed to 1.5 M propane-1,2-diol for 10 minutes at 24°C followed by exposure to 1.5 M propane-1,2-diol plus either 0.1 M or 0.2 M (heavy solid line) sucrose at 24°C.



Paynter. Oocyte membrane permeability and cryoprotectant. Fertil Steril 2001.

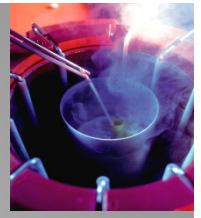


How does the volume change during the second step - exposure to 1.5 M PROH + 0.2 M sucrose?

A stays the same because no change in CPA concentration

B immediate shrinkage due to the added sucrose

C volume response in relation to gradients caused by sucrose



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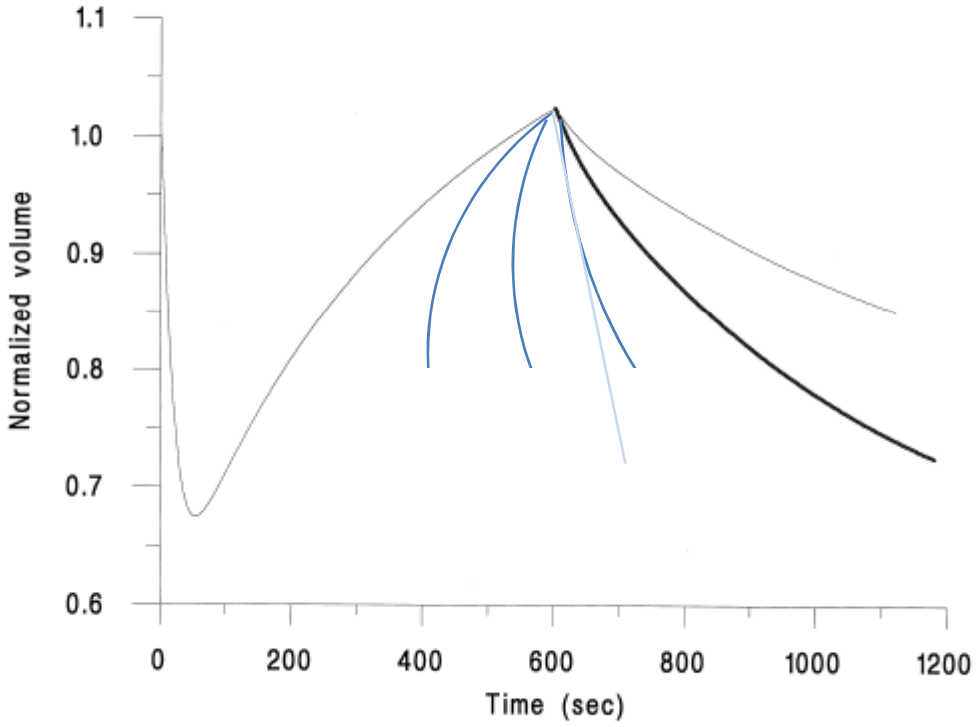
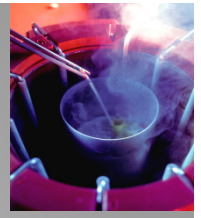
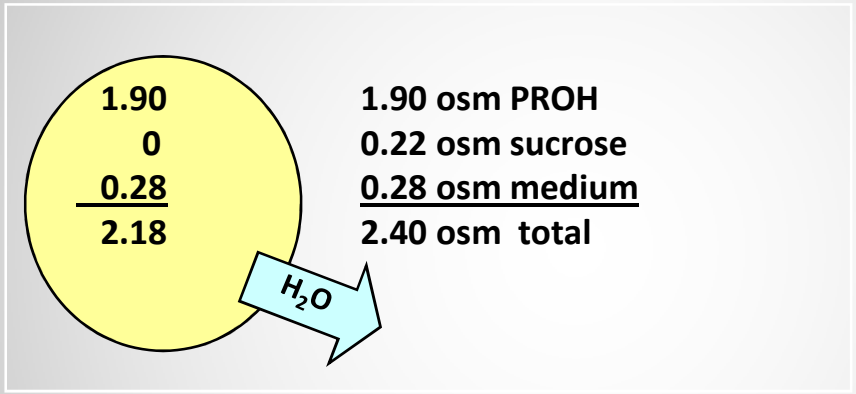
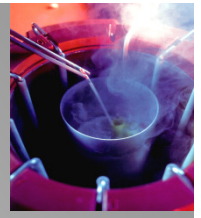
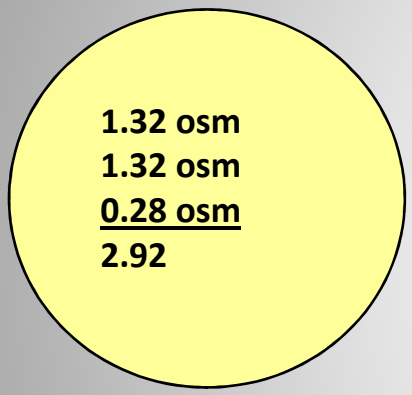


Paynter. Oocyte membrane permeability and cryoprotectant. Fertil Steril 2001.

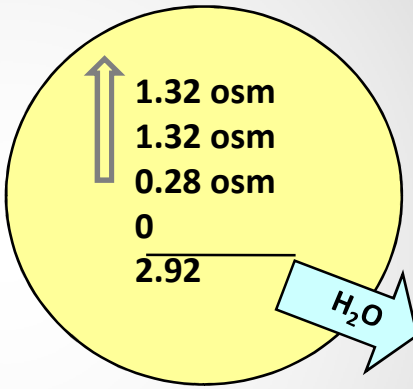








1.32 osm CPA
1.32 osm CPA
0.28 osm medium
2.92 osm total

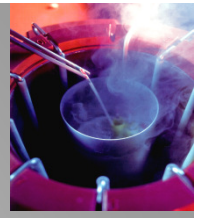


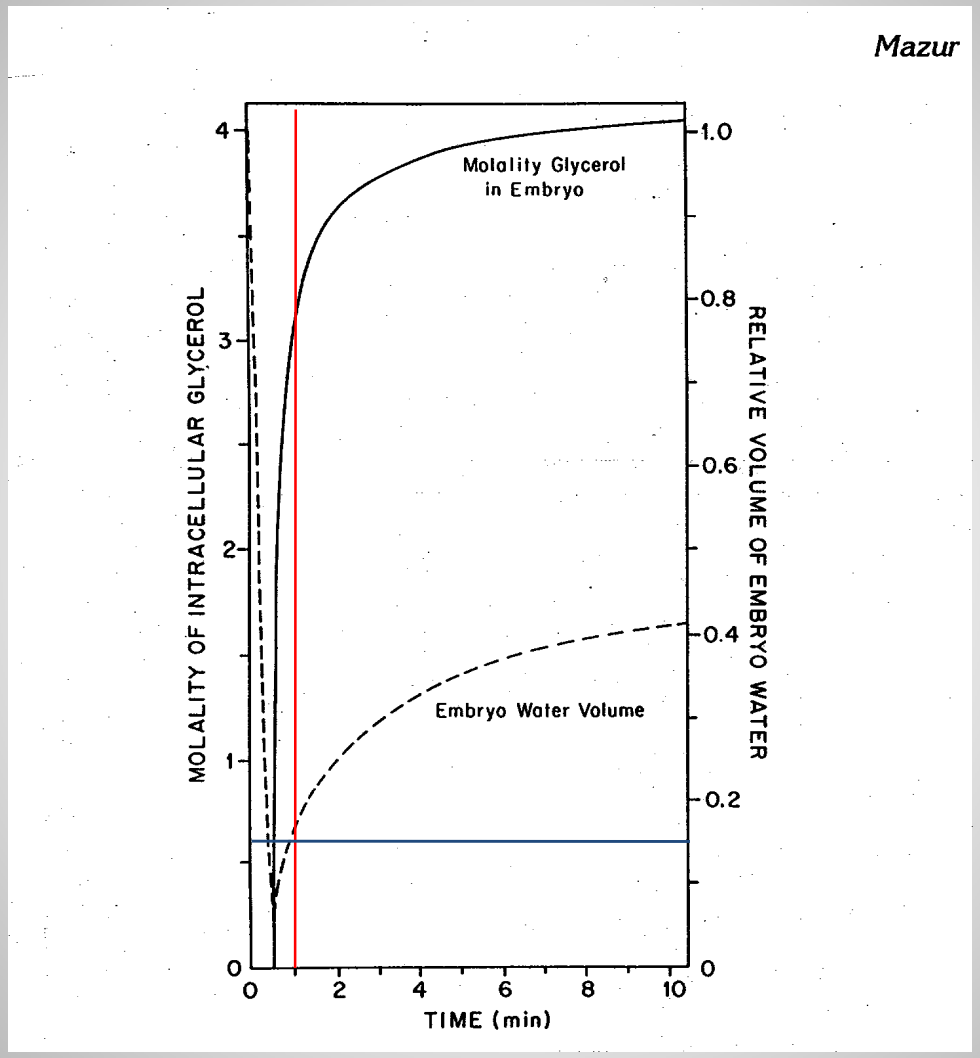
2.94 osm CPA
2.94 osm CPA
0.28 osm medium
0.58 osm sucrose
6.74 osm total

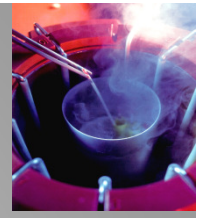
1. step

2. step

Kuwayama protocoll







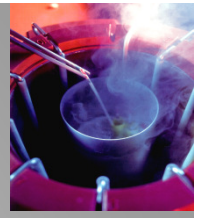
Do we need cryotips, cryotops, cryoleaf, etc.?

The underlying assumption of the use is that high survival demand the avoidance of IIF during cooling by converting water to glass, and the higher the cooling rate, the more likely that conversion.

Pragmatically it is fortunate that the devices used to achieve very high cooling rates also produce very high warming rates. Mechanistically it has resulted in confounding of whether cooling rate or warming rate are the important cooperative.

If the primary cause of injury or death in a vitrification procedure is ice recrystallisation during warming and not failure to vitrify during cooling, the potential remedies may be quite different.

Seki and Mazur, Cryobiology 59, 75-82, 2009



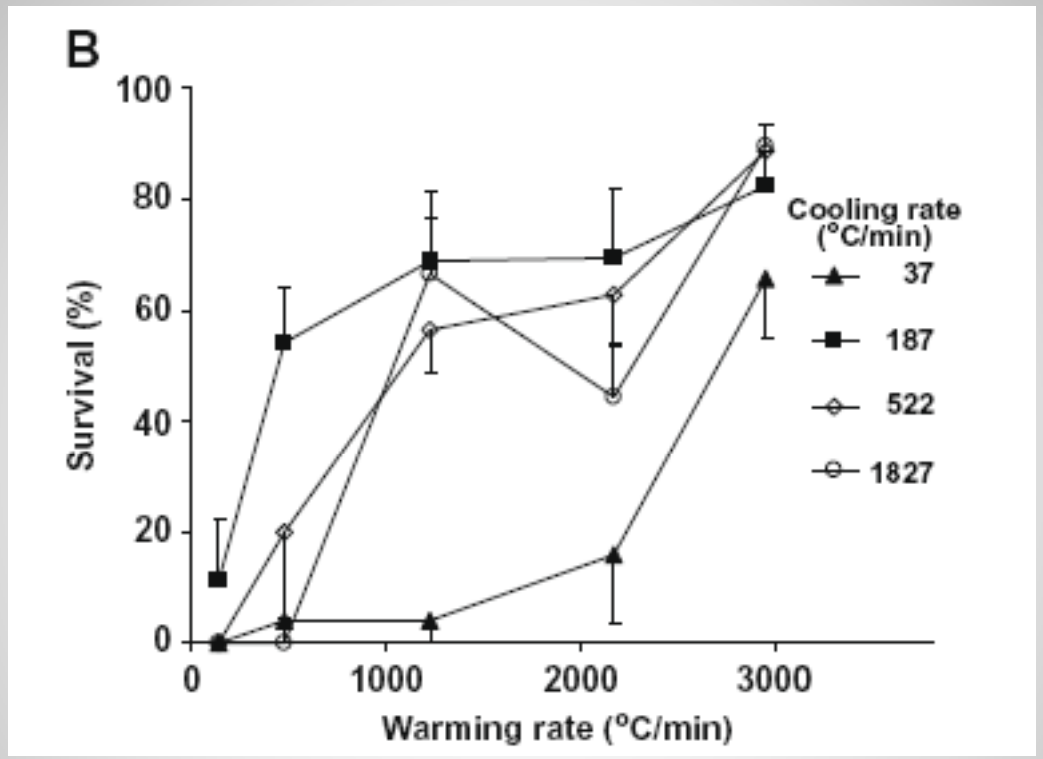
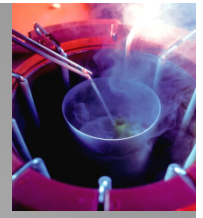


Fig. 3. (A) The survival of EAFS 10/10-vitrified oocytes warmed at 139, 476, 1220, 2170, 2950 °C/min as a function of the cooling rate. (B) The survival of EAFS 10/10-vitrified oocytes cooled at 37, 187, 522, 1827 °C/min as a function of the warming rate. The filled symbols depict conditions where the EAFS 10/10 solution froze; the open symbols depict where it vitrified.

Seki and Mazur, Cryobiology 59, 75-82, 2009



Potential developments in the field

Chemical substitution in conventional CPA to enhance glass formation e.g. methoxylation of PROH and Glycerol reduces critical cooling rate from 500°C to 50°C/min

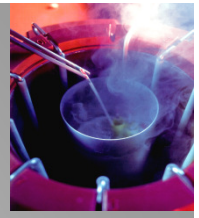
Stabilization of cytoskeleton with compounds like Taxol, Cytochalasin B

Add antinucleating agents to VS

Computer modelling of cell response during vitrification procedure

Raise intracellular sugar concentration by microinjection of Trehalose

ESHRE Campus Workshop, Athens, 25-26 September 2009



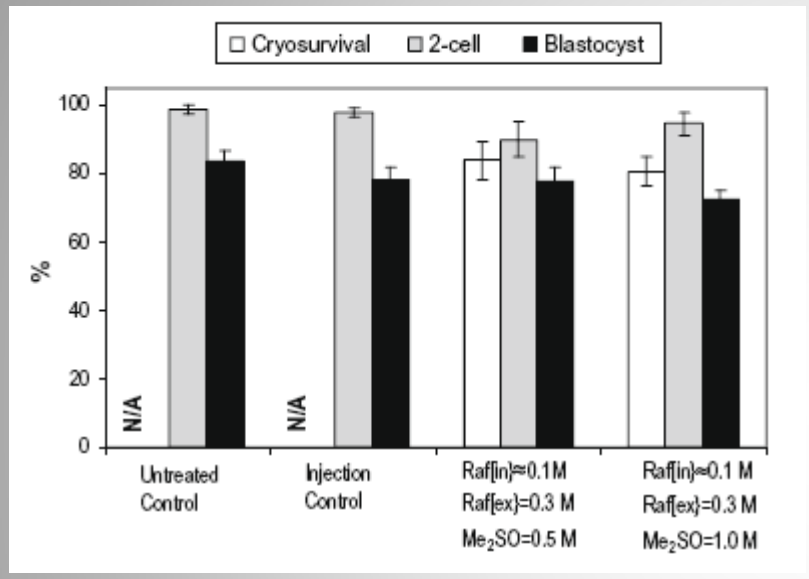


Fig. 1. Cryosurvival, fertilization, and embryonic development of mouse oocytes after cooling to −196 °C. The survival rates represent mean ± s.e.m. There was no significant difference between the groups ($p > 0.05$).

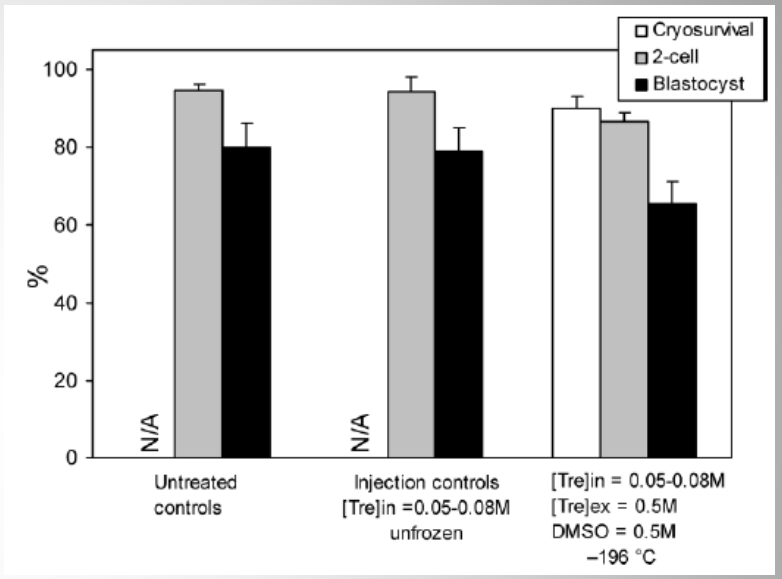


FIG. 5. Cryosurvival, fertilization, and embryonic development of mouse oocytes after cooling to −196°C. The rates represent mean ± SEM. There was no significant difference between the groups ($P > 0.05$). N/A indicates that control oocytes were not cryopreserved, and thus the cryosurvival is not applicable.

A.Eroglu, Cryobiology (2009), July 2009

Eroglu et al. Biol. Reprod., 80, 70 – 78, 2009

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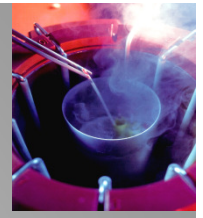

S³ Vitrifcation System: A Novel Approach To Blastocyst Freezing

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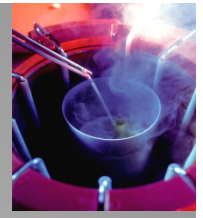
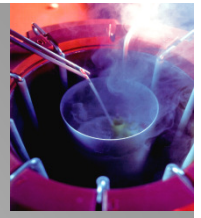


Table 1. Cooling rates for modern vitrification devices.

Device	Media (ul)	Freezing Rate
0.25cc straw	25ul	4460°C/min
Open-pulled straw	1.5ul	16,340°C/min
Cryo-Top	0.1ul	22,800°C/min
Cryo-Tip	<2ul	12,000°C/min
0.25cc straw (S^3 vit)[1]	10ul to 250ul	<100°C/min

From Kuwayama et al., (2005b).
[1]This study.



S-3 method

freezing container: „standard“ 0.25 cc straw

Cryo-Medium: PBS; cholin based medium; 20% HSA

CPA-solution
I. 10 % glycerol
II. 10 % glycerol, 20% ethylenglycol
III. 25 % glycerol, 25 % ethylenglycol

Handling:
CPA-Sol. I. 5 min. at roomtemp
CPA-Sol. II. 5 min. at roomtemp
CPA-Sol. III. up to 90 (-120) sec
until exposure to nitrogen vapor ≈ -100^{o}C
during those 90 sec load and heat seal straw

keep at -100^{o}C 2-5 min
transfer straw into liquid nitrogen

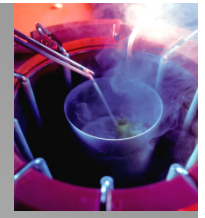
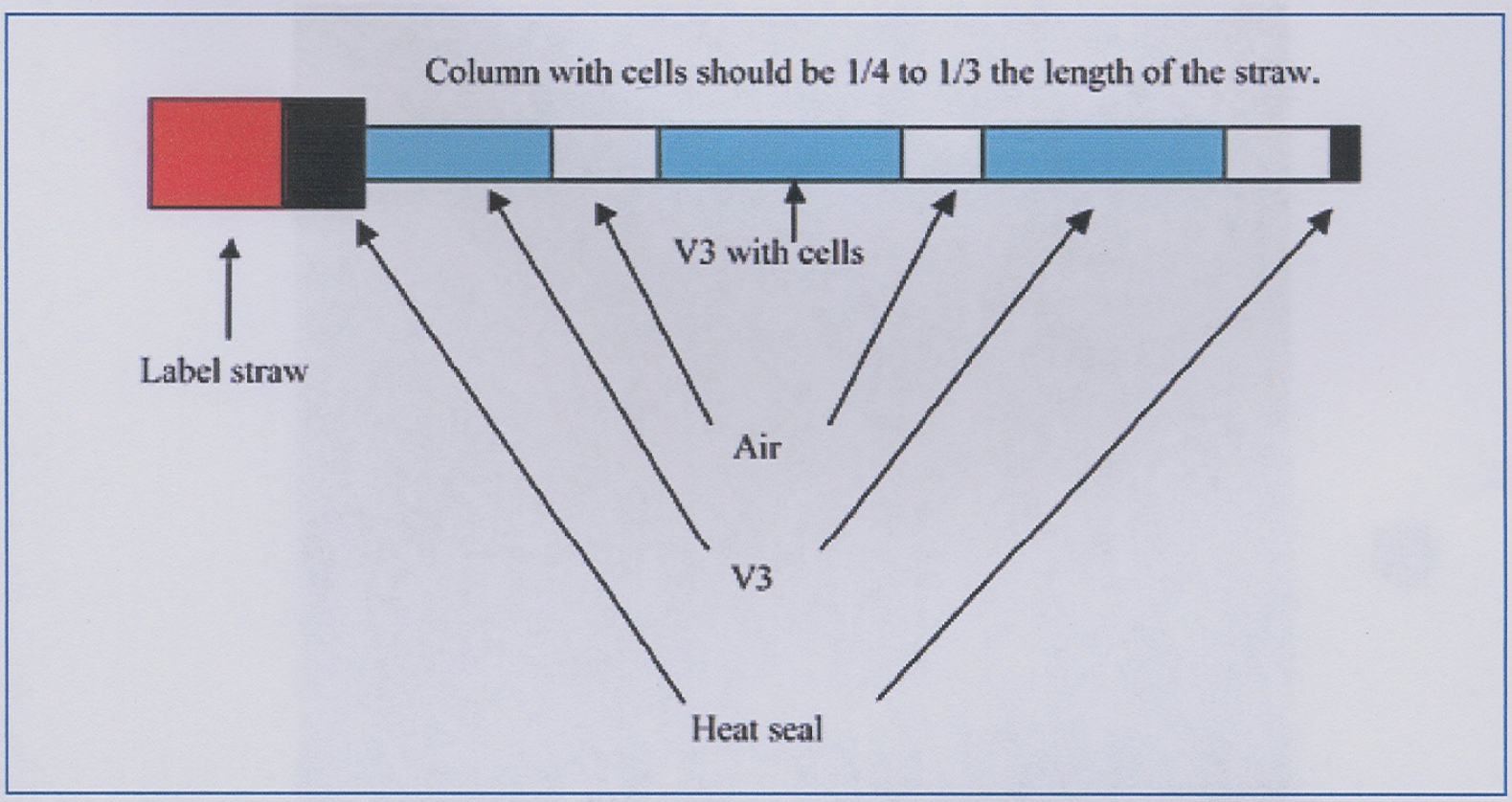
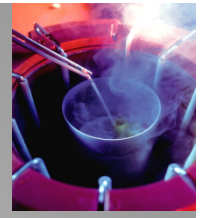


Figure 1. Straw Loading Diagram





S-3 method

Thawing straw 5 sec in air
10 sec in waterbath 20^{o} C

CPA-Dilution empty straw into cryomedium w/o CPA containing:

0.85 M sucrose, 5 min, RT
0.4 M sucrose, 5 min, RT
0.2 M sucrose, 5 min, RT
0.1 M sucrose, 5 min, RT

transfer into culture medium

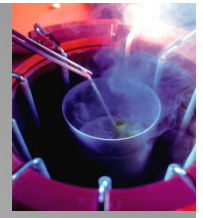
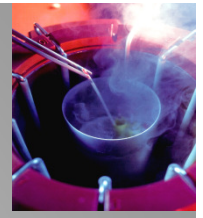


Table 3. Clinical results of S3 Vitrification on blastocysts.

Clinic	Thawed	Intact	Transfers	Replaced	FHB	Preg/Transfer
A	104	86 (83%)	45	80	47 (58.7%)	32/45 (71.1%)
B	160	141 (88.1%)	77	131	43 (32.8%)	37/77 (48.0%)
C	41	35 (85.4%)	19	35	16 (45.7%)	12/19 (63.1%)
D	566	509 (89.9%)	209	509	N/A	116/209 (55.5%)
E	13	13 (100%)	8	13	5 (38.5%)	5/8 (62.5%)
Total	884	784 (88.7%)	358	768	111 (42.8%)	202/358 (56.4%)



Conclusion

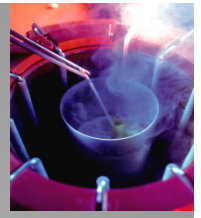
Discuss some of the cryobiological factors that influence the vitrification procedure as applied to oocytes and embryos.

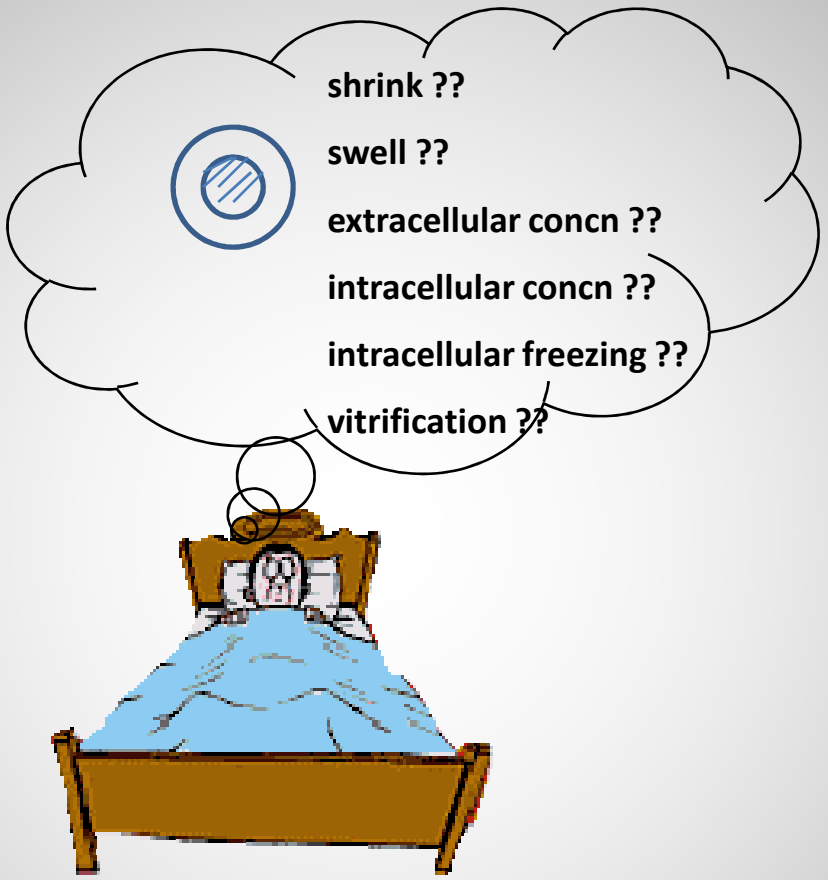
The results obtained with the cryotip are excellent, however, one has to realize that the vitrification method was tailored to the device.

There are a number of possibilities to modify the nonequilibrium vitrification method to allow easier and more secure handling also in combination with sealed 0.25ml straws.

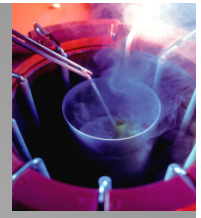
Computer modelling will optimize the addition and removal procedures for vitrification solutions.

Standard slow freezing and vitrification should be seen as alternative methods. Similar cryobiological principles apply.





...if you dream at night how a cell responds during freezing and thawing then you begin to understand cryobiology



Thank You For Your Attention !

email: ulrich.schneider@amedes-group.com