ESHRE Campus Symposium

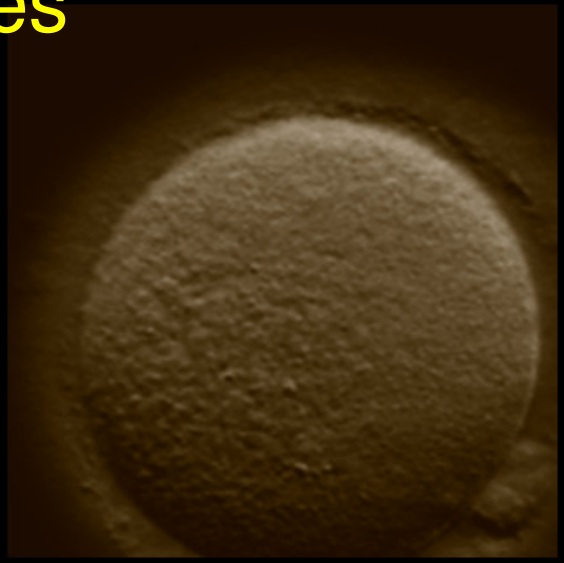
Cryobiology and cryopreservation of human gametes and embryos

Athens, Greece
25-26 September 2009

“Cryopreservation of human oocytes with slow freezing techniques ”

Giovanni Coticchio

TECNOBIOS PROCREAZIONE

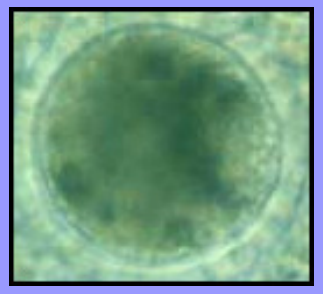


Outline

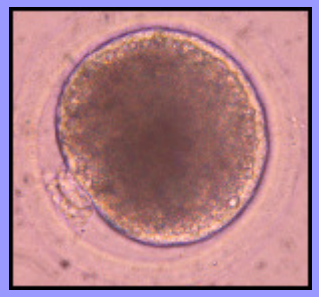
- General principles
- Safety margins: reproducibility and consistency
- Theoretical models and margins for improvements
- Safety of liquid nitrogen storage
- Overview of cryodamage
- Timeline of slow freezing: progressive improvement over time
- Clinical efficiency
  - How to measure clinical efficiency
  - Factors (age, protocol, processing timing)
  - Performance in vitro and in vivo of frozen oocytes (vs. frozen embryos and vitrified oocytes)
- Health of children

Cellular differences make oocytes of various species differently amenable to cryopreservation

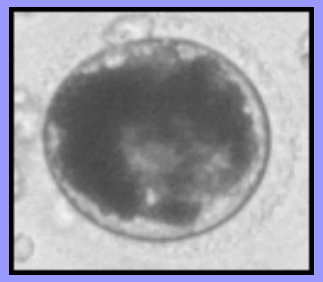
Lipid droplets, organelle aggregates



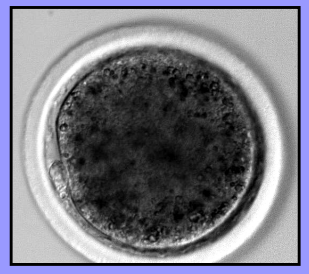
Bovine



Pig

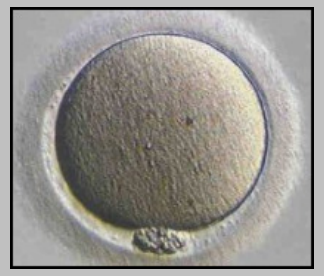


Horse



Sheep

No inclusions

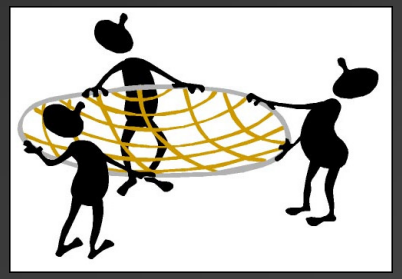


Human

Mouse

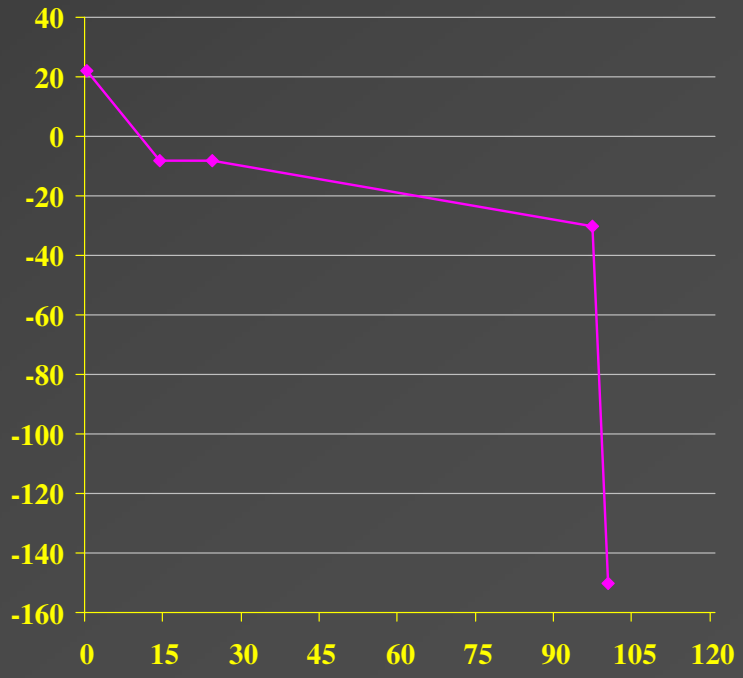
General outlook to cryopreservation by slow freezing and vitrification

	Slow freezing	Vitrification
CPA toxicity	+	+ + +
Osmotic stress	+ +	+ + +
Solution effect toxicity	+ + +	-
Intracellular ice risk	+ +	-
Accidental thawing/devitrification risk	-	+ +
Technology dependence	+ + +	+
Costs	+ + +	+ + +
Operational times	+ +	+ +
Contamination risk	-	+ +



Technology

- Robust
- Operator friendly
- Reproducible (well established in IVF labs for decades)
- Small variability
  - Intra-operator
  - Intra-laboratory
  - Inter-laboratory
- Allows monitoring of cooling process for quality assurance



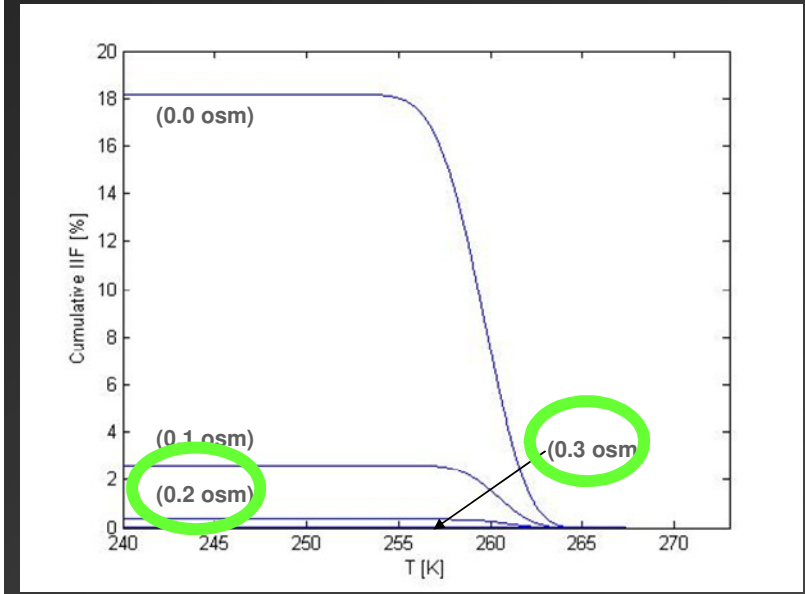
Operational times of slow freezing vs vitrification

	Slow Freezing	Vitrification
Dehydration	10-15 min	10-15 min
Loading straw/cryotop	< 2 min	< 2 min
Seeding	1 min	-
Cooling to LN2 temperature	90-100 min	-
Storage in liquid nitrogen	5 min	5 min
Warming and rehydration	30-35 min	10-15
Oocytes from different patients	May be cryopreserved at the same time	Must be vitrified separately

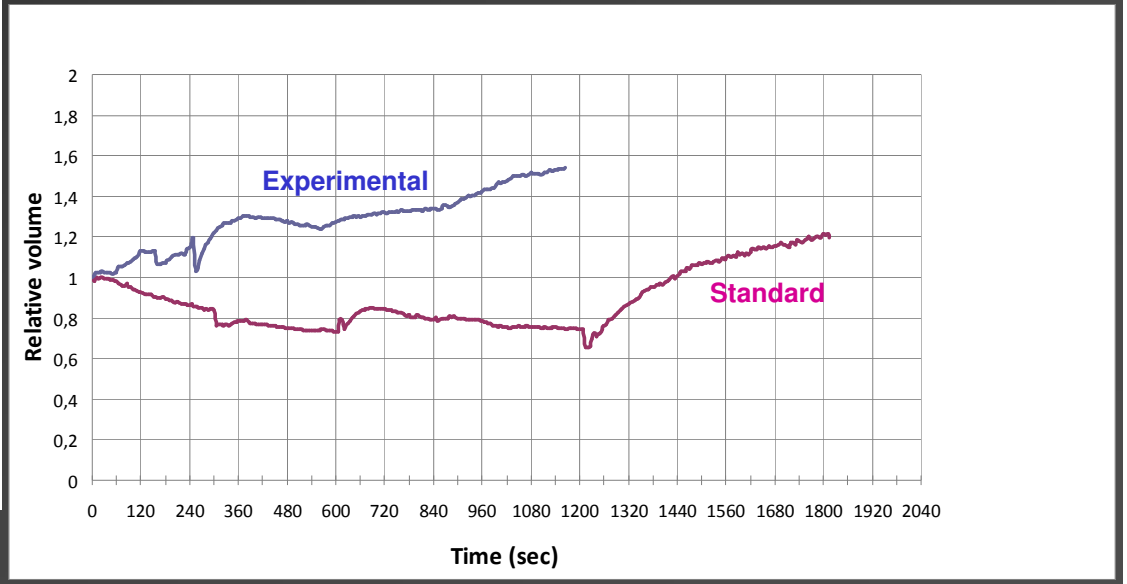
Margins of improvement

… In the development of protocols to cryopreserve human occytes ….. there is much room for improvement in several respects. …. Models based on basic principles have been developed …. they have enormous potential …. to improve recovery and reproducibility, faster processing and lower cost- all based on a fundamental understanding rather than empiricism.

Prediction of intracellular ice formation as a function of temperature with different sucrose concentration



" … the entire process of cryoprotectant addition and removal could be shortened"

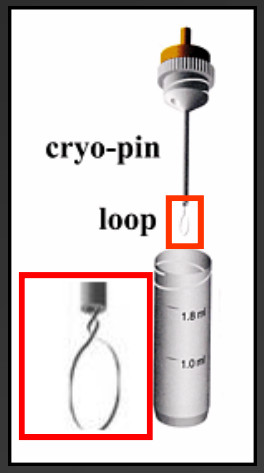


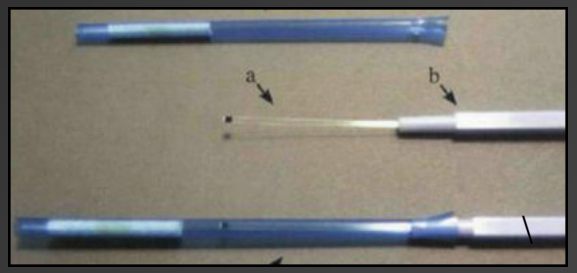
J. Mc Grath, 2009. In "Preservation of Human Oocytes" Borini & Coticchio Eds.

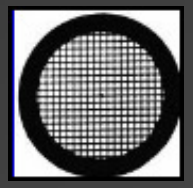
Oocyte volume changes as a function of time with different rehydration protocols

Cross contamination - Containment

CBS high security straws (used in controlled slow cooling), if sealed correctly, may be stored without danger of leakage



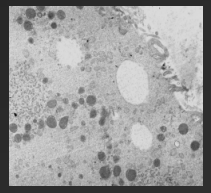
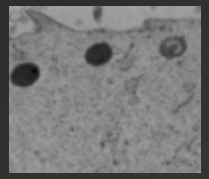
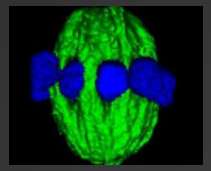




Vitrified samples, if not contained (open pulled straws, cryoloops, cryotop, cryoleaf, grids etc), are “open” to liquid nitrogen

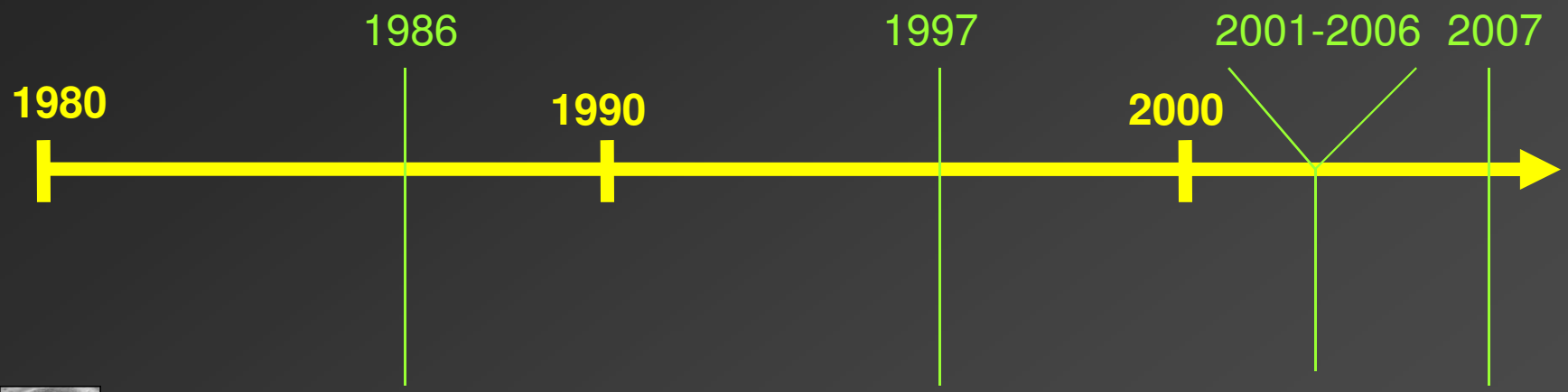
All “open systems” are potential risk

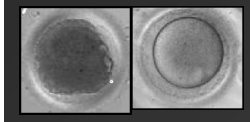
Cell damage from cryopreservation

	Protocols			
	Slow freezing		Vitrification	
	0.3M/0.3M	0.2M/0.3M	Cryoleaf	Cryotop
 Ultrastructural damage (vacuolisation)	+ +	+	+	n. a.
 Cortical granules loss	+ +	+ +	+	n. a.
 MII spindle damage	– / +	–	+ +	–

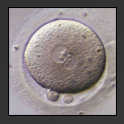
Coticchio et al., 2006; Nottola et al., 2007; Nottola et al., 2008; De Santis et 2008; Cobo et al., 2008; Bromfield et al., 2009; Coticchio et al., 2009

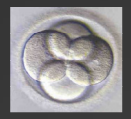
Steady outcome improvement by slow freezing over time

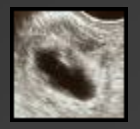




Survival	20-30%	40-50%	70-75%	70-75%
Fertilization	<40%	40-50%	70-75%	70-75%
Cleavage (4-cell)	???	???	10-15%	20-30%
Implantation per thawed oocyte	???	???	2.5-2.7%	> 5%







Clinical efficiency of oocyte cryopreservation

- "*Comparisons of techniques or approaches should ideally be made by comparing relevant outcomes in women of equivalent age within the same clinic.*"

- *"The quality of oocytes cryopreserved will impact on clinical efficiency. This serves to emphasize the importance of controlling for oocyte quality when determining the effects of cryopreservation, with parallel fresh controls being an ideal component of any study."*

- *"Because different studies may or may not include selection of embryos developed from cryopreserved oocytes, implantation rate should be calculated on the basis of thawed (or warmed, in the case of vitrification) oocytes."*

Edgar et al., 2007

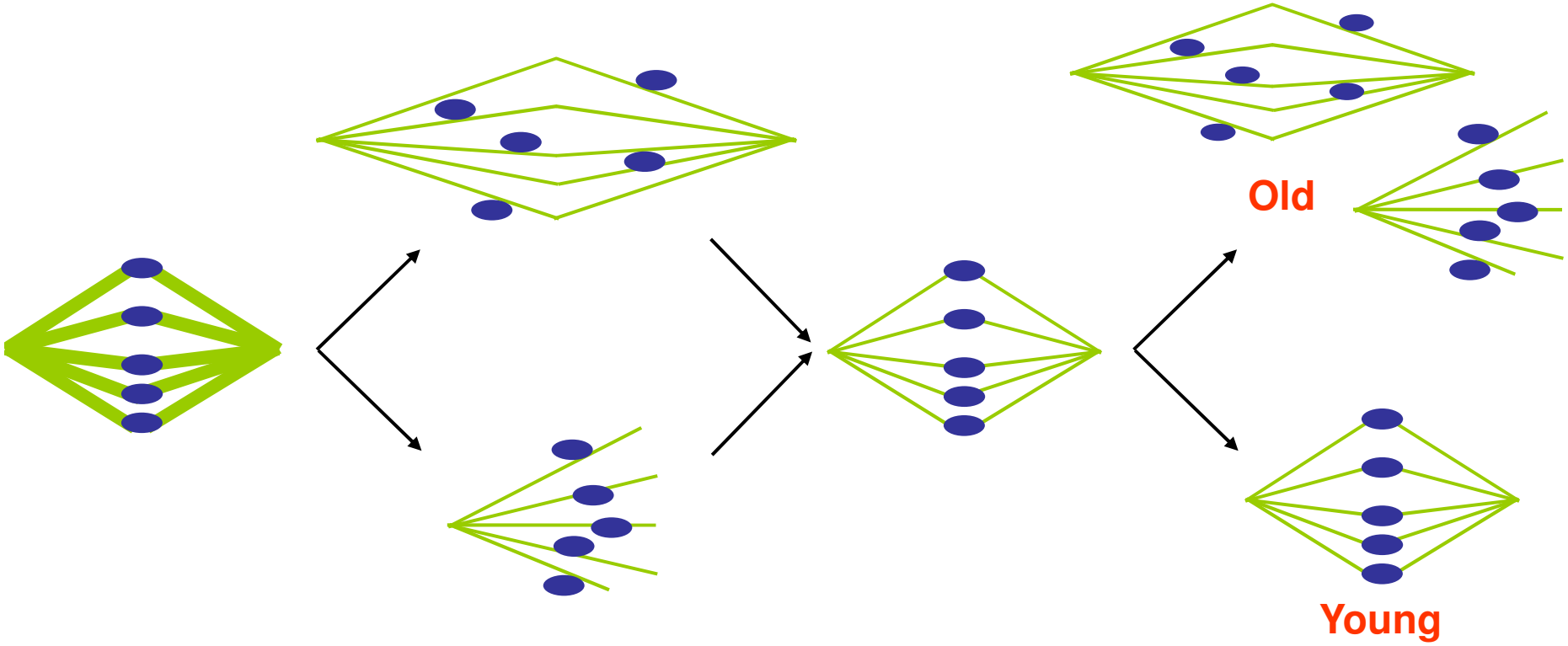
Importance of the **age factor** to appraise clinical efficiency

Collective data from three different slow freezing protocols

	Female age			
	< 35	35 – 38	39 – 40	41 – 42
Cycles	474	376	163	126
Thawed oocytes	2469	1825	849	597
Survival (%)	1712/2469 (69.3)	1269/1825 (69.5)	707/849 (68.8)	425/597 (71.2)
Fertilization (%)	1001/1326 75.5	745/1013 (73.5)	359/442 (81.2)	254/335 (75.8)
Cleavage (%)	924/ 1001 (92.3)	684/745 (91.8)	342/359 (95.3)	235/254 (92.5)
Embryo transfers	423	325	145	114
Pregnancies/cycle (%)	80/474 (16.9)	55/376 (14.6)	29/163 (17.8)	6/125 (4.8)
Pregnancies/ET (%)	80/423 (18.9)	55/325 (16.9)	29/145 (20.0)	6/114 (5.3)
Implantations (%)	103/924 (11.1)	70/682 (10.3)	40/340 (11.8)	6/235 (2.6)
Implantations / thawed oocytes (%)	103/2468 (4.2)	70/1825 (3.9)	40/849 (4.7)	6/579 (1.0)
Abortions (%)	15 (18.8)	17 (30.9)	11 (37.9)	3 (50.0)

Borini et al., unpublished

Spindle and chromosome dynamics in oocytes of patients of different age after freezing-thawing



Unfrozen

- bipolar
- integrated chromosomes
- high tubulin mass

0 h

- partial loss of bipolarity
- spindle elongation
- chromosome migration/ detachment
- loss of tubulin mass

1 h

- recovery of bipolarity
- realignment/ integration of chromosomes
- maintained low tubulin mass

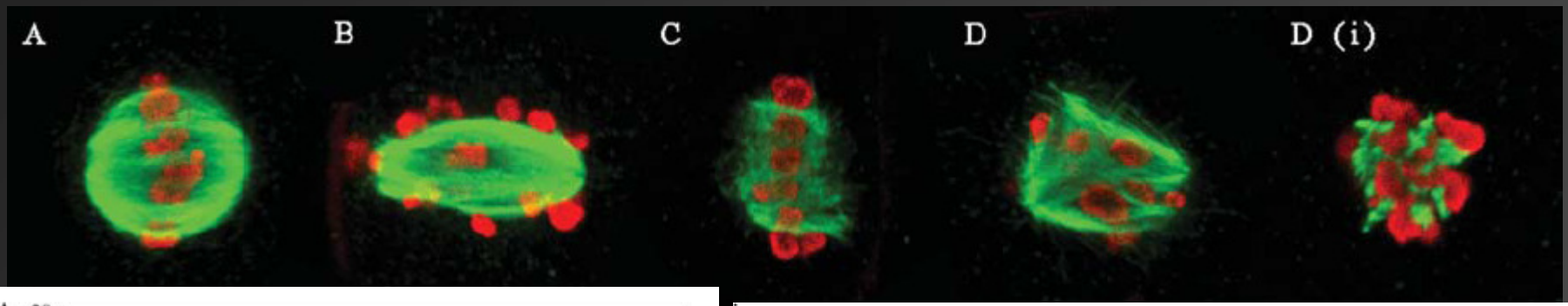
2-3 h

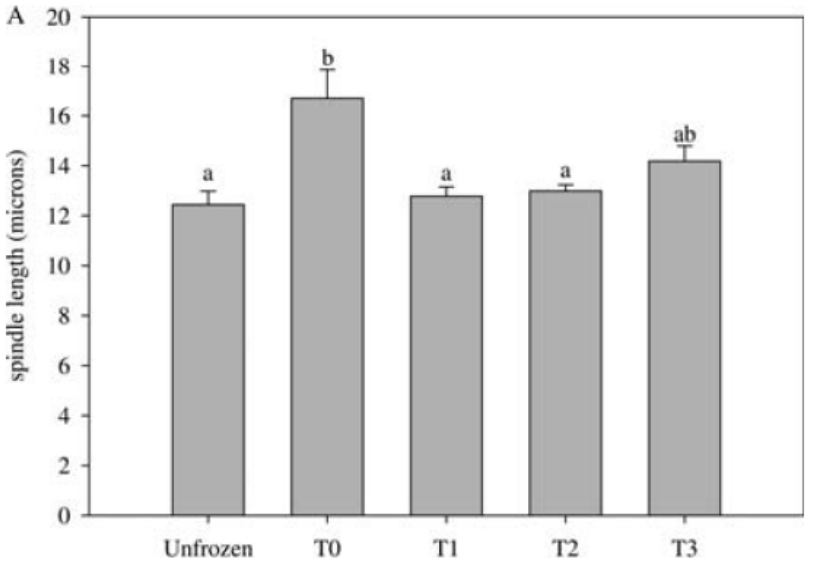
- partial loss of bipolarity
- spindle elongation
- chromosome migration/ detachment
- maintained low tubulin mass

Bromfield, Coticchio, Scjaino et al., 2009

Meiotic spindle dynamics in human oocytes following slow-cooling cryopreservation

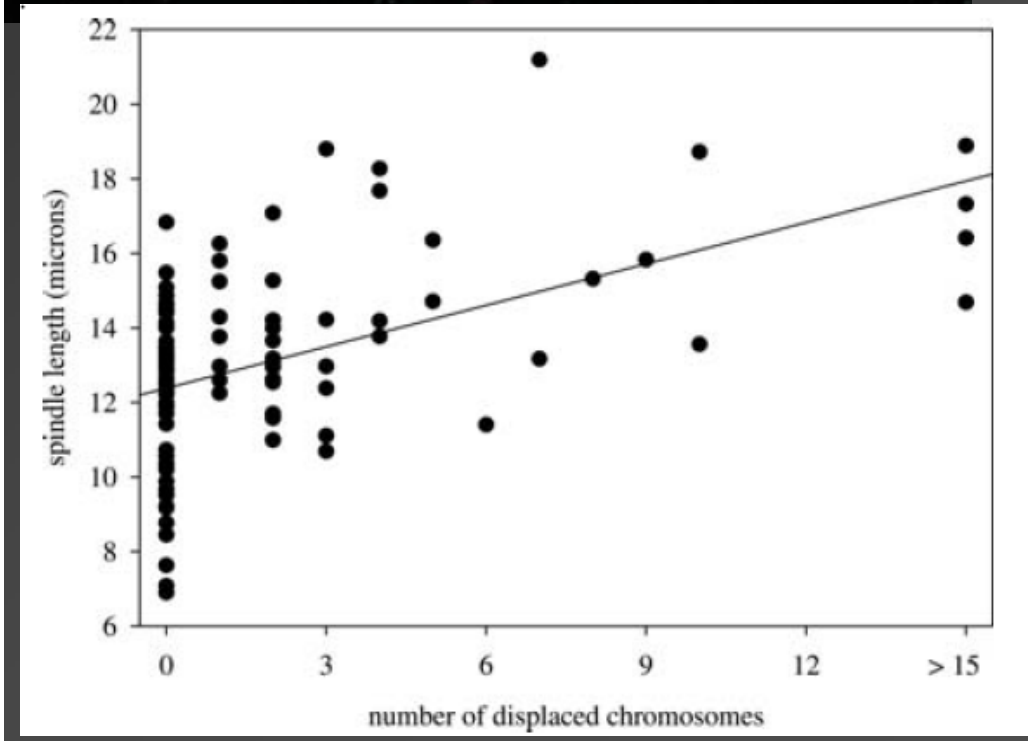
J.J. Bromfield[1], G. Coticchio[2], K. Hutt[1], R. Sciajno[2], A. Borini[2], and D.F. Albertini[1,3]



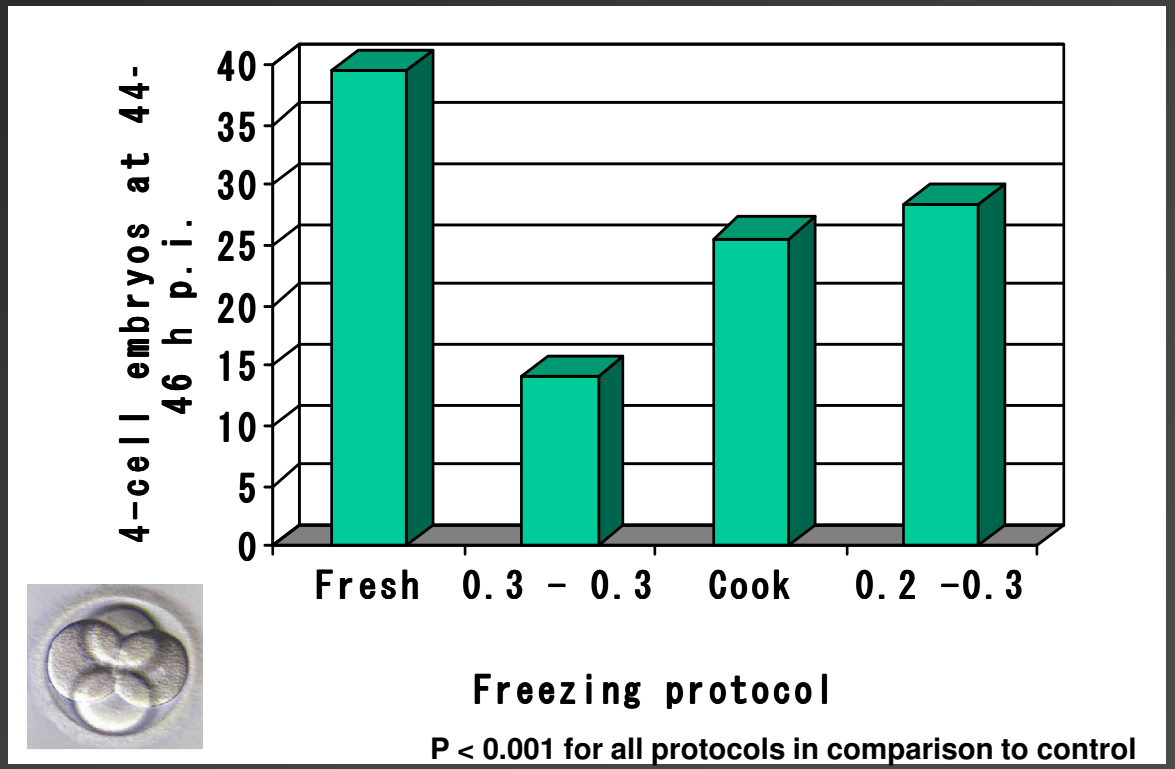


B

	Unfrozen	T0	T1	T2	T3
MT volume (μm^{-3})	814 ± 73[a]	510 ± 75[b]	582 ± 30[b]	528 ± 60[b]	463 ± 52[b]



Impact of protocol: Performance in vitro of embryos from sibling fresh and frozen oocytes



Borini et al., unpublished

Impact of protocol

	Protocol		
	0.3 – 0.3	**Cook**	**0.2 – 0.3**
Cycles	246	220	119
Thawed oocytes	1193	1229	609
Survival (%)	881/1193 (73.8)	772/1229 (62.8)	465/609 (76.4)
Fertilization (%)	559/767 (72.9)	451/611 (73.8)	268/335 (80.0)
Cleavage (%)	512/559 (91.6)	422/451 (93.6)	251/268 (93.7)
Embryo transfers	227	191	109
Pregnancies/cycle (%)	25/246 (10.2)	32/220 (14.5)	28/119 (23.5)
Pregnancies/ET (%)	25/227 (11.0)	32/191 (16.8)	28/109 (25.7)
Implantations (%)	28/512 (5.5)	40/420 (9.5)	40/249 (16.1)
Implantations / thawed oocytes (%)	28/1193 (2.3)	40/1229 (3.3)	28/609 (6.6)
Abortions (%)	4 (16.0)	5 (15.6)	8 (28.6)

Female age < 35 years

Borini et al., unpublished

Culture time before AND after freezing/thawing: a casual chance for oocyte aging?

Table 2. Efficiency of oocyte cryopreservation.

Time of pre-freeze culture

Parameter	*All (510 cycles)*	2 h *Group A (162 cycles)*	> 2 h *Group B (348 cycles)*
Clinical pregnancy rate per thawed oocyte (%)	72/2608 (2.76)[a]	46/832 (5.53)[b]	26/1776 (1.46)[c]
Gestational sacs per thawed oocyte (%)	85/2608 (3.26)[d]	54/832 (6.49)[e]	31/1776 (1.75)[f]
Clinical pregnancy rate per injected oocyte (%)	72/1379 (5.22)[g]	46/442 (10.41)[h]	26/937 (2.77)[i]

Group A: freezing within 2 h of oocyte retrieval. Group B: freezing more than 2 h after oocyte retrieval.
[a,b]$P < 0.001$; [b,c]$P < 0.001$; [d,e]$P < 0.001$; [e,f]$P < 0.001$; [g,h]$P < 0.001$; [h,i]$P < 0.001$; [a,c]$P = 0.007$; [d,f]$P = 0.004$; [g,i]$P = 0.008$.

In this study post thaw-culture varied between 2 and 5 hours. This may have a major impact on in vitro aging

Parmegiani et al., 2008

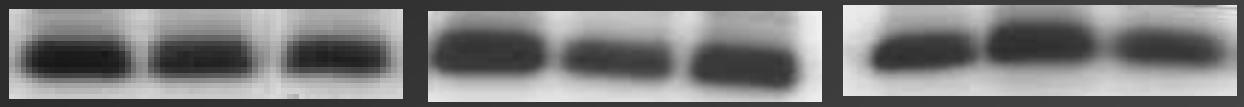
"Activity of maturation promoting factor (MPF) decreases over time in frozen-thawed human oocytes"

MPF

control | 1h post thawing | 2h post thawing

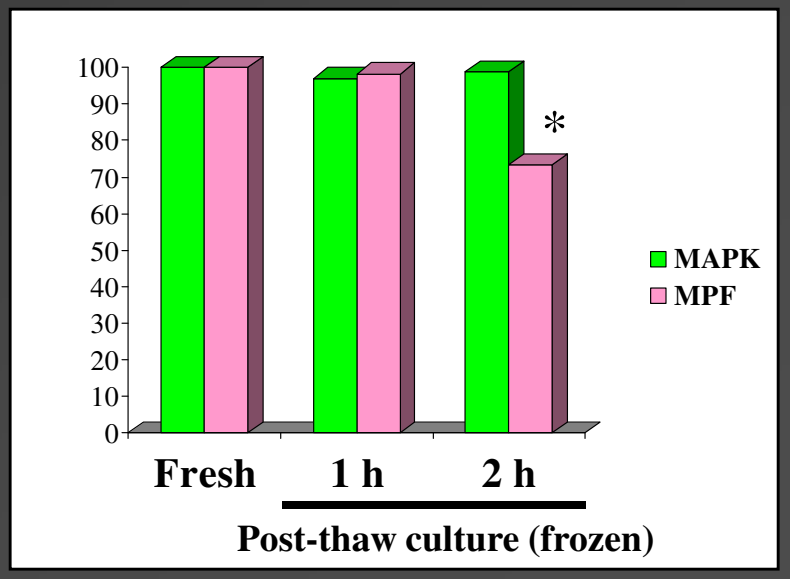
cryopreserved

MAPK



control | 1h post thawing | 2h post thawing

cryopreserved



Data from collaborative work between

Tecnobios Procreazione
Arcispedale S Maria Nuova, Reggio Emilia
Department of Physiological Biochemical Cellular Sciences, Sassari

Culture time before AND after freezing/thawing: a casual chance for oocyte aging?

0.2 M sucrose oocyte freezing protocol

	Pre-freeze + post-thaw culture time		
	< 7 h	**7 – 8 h**	**> 8 h**
Mean age	36.0	35.3	35.4
Cycles	22	54	137
Thawed oocytes	108	266	686
Survival (%)	88/108 (81.5)	210/266 (78.9)	539/686 (78.6)
Fertilization (%)	49/62 (79.0)	129/155 (83.2)	330/402 (82.1)
Cleavage (%)	47/49 (95.9)	127/129 (98.4)	310/330 (93.9)
Embryo transfers	22	54	137
Pregnancies/cycle (%)	7/22 (31.8)	15/54 (27.8)	22/137 (16.1)
Pregnancies/ET (%)	7/22 (31.8)	15/54 (27.8)	22/137 (16.1)
Implantations (%)	10/47 (21.3)	20/127 (15.7)	32/309 (10.4)
Implantations / thawed oocytes (%)	10/108 (9.3)	20/266 (7.5)	32/686 (4.7)

Borini et al., unpublished

Efficiency: slow freezing vs. vitrification

Kim, T. J., et al., 2009. Vitrification of oocytes produces high pregnancy rates when carried out in fertile women. Fertil Steril. In Press

TABLE 1

Clinical summary of thawing cycles with vitrified oocytes.

Age (y) (mean ± SD)	31.7 ± 3.0
FSH level (mIU/mL) (mean ± SD)	5.7 ± 1.1
No. of patients for warming cycles	19
No. of warming cycles[a]	20
No. of vitrified oocytes	483
No. of warmed oocytes	395
No. of survived oocytes (%)	320 (81.0)
No. of MI and GV stage oocytes after warming	35
No. of microinjected oocytes	285
No. of fertilized oocytes (%)	208 (72.3)
No. of cleaved embryos (%)	185 (89.8)
No. of ET cycles	20
No. of clinical pregnancies/ warming cycle (%)	16/20 (80.0)
No. of clinical pregnancies/ patient (%)	16/19 (84.2)
No. of implantations (%)	24 (45.3)
No. of implantation per warmed oocytes (%)	24/373[d] (6.4)

0.2 M sucrose oocyte freezing protocol

Mean Age	31.2	36.4	39.6	41.4
Thawing cycles	119	74	39	28
Thawed oocytes	609	365	213	126
Survived oocytes (%)	465 (76.4)	266 (72.9)	154 (72.3)	100 (79.4)
Micr'ed oocytes (%)	335	191	113	76
Fertilized oocytes (%)	268 (80.0)	152 (79.6)	95 (84.1)	52 (68.4)
Cleaved embryos (%)	251 (93.7)	140 (92.1)	92 (96.8)	51 (98.1)
ET cycles	109	66	37	25
Preg / thaw cycle (%)	28 (23.5)	11 (14.9)	6 (15.4)	2 (7.1)
Implantations (%)	40 (16.1)	15 (10.7)	9 (9.8)	2 (3.9)
Implantations (% per thawed oocyte)	**40 (6.6)**	**15 (4.1)**	**9 (4.2)**	**2 (1.6)**

Borini et al., unpublished

Efficiency: slow freezing vs. vitrification

	Controlled rate freezing			Controlled rate freezing			Vitrification			
Permeating Cryoprotectant	1.5M PROH			1.5M PROH (Na depleted)			5M EG	2.7M EG + 2.1M DMSO	2.7M EG + 2.0M PROH	*3.6M EG + 2.7M DMSO +0.1M Ficoll
Sucrose	0.1M	0.2M	0.3M	0.1M	0.2	0.3M	1.0M	0.5M	0.5M	1.0M
Survival (no. of thawed oocytes)	51% (4027)	71% (1451)	73% (7595)	52% (127)	60% (815)	70% (890)	75% (838)	83% # (1454)	80% (395)	96% (111)
Fertilisation	54%	80%	73%	56%	66%	72%	74%	87%	70%	-
Cleavage	85%	93%	90%	100%	84%	88%	94%	93%	53%	76%
Embryos per 100 thawed oocytes	23	53	48	29	33	44	52	76	30	51
Implantation rate	10%	17%	6%	21%	11%	13%	10%	16%	13%	61%
Implantations per 100 thawed oocytes	2.3	9.0	2.9	6.1	3.7	5.8	5.2	10.7	3.8	26

D. Gook and D. Edgar, 2009. In "Preservation of Human Oocytes" Borini & Coticchio Eds.

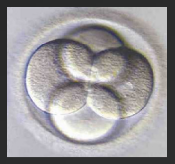
Embryo vs. oocyte slow freezing

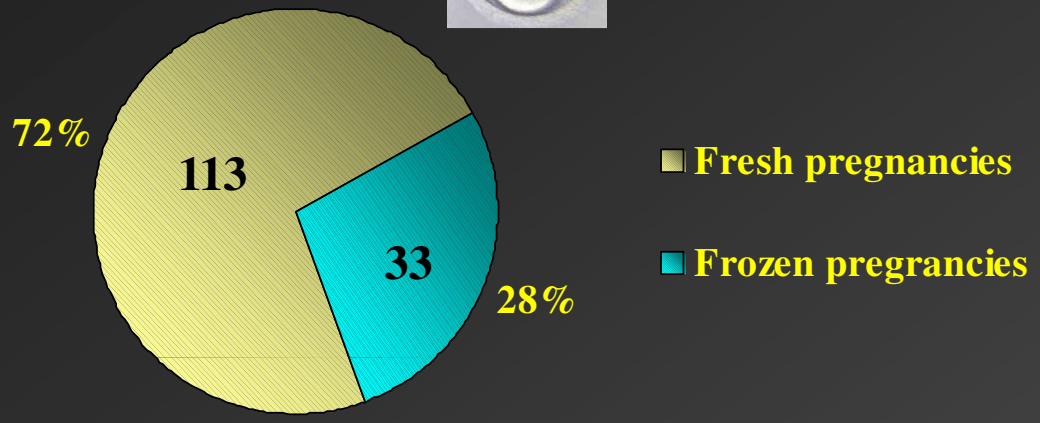
	Female age					
	< 35	35 – 38	39 – 40	41 – 42	>42	Total
Cumulative pregnancy rates from frozen embryos (1992-2004)	54	50	34	26		50
Cumulative pregnancy rates from frozen oocytes (different protocols, 2004-2008)	53	52	47	25	15	49
Cumulative pregnancy rates from frozen oocytes (0.2M sucrose protocol, 2006-2008)	63	65	40	28	17	57

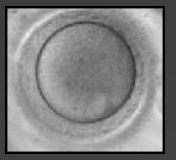
Cumulative pregnancy rates analysed by female age from cycles in which embryos or oocytes were cryopreserved

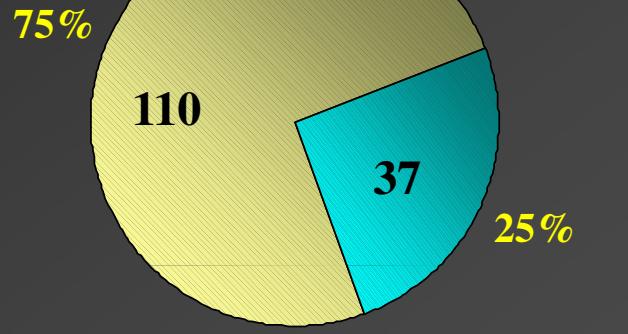
Adapted from A. Borini and M.A. Bonu. 2009, In "Preservation of Human Oocytes" Borini & Coticchio Eds.

Cumulative pregnancy rate: Frozen Embryos vs Frozen Oocytes









Standard embryo freezing protocol

Retrievals 211
Patient age <39
Fresh preg. rate / cycle
53.5%
Frozen preg. rate / cycle
37.1%
Cumulative preg. rate
73.9%

0.2 M sucrose oocyte freezing protocol

Retrievals 226
Patient age <39
Fresh preg. rate / cycle
48.6%
Frozen preg. rate / cycle
27.6%
Cumulative preg. rate

Conclusions: Controlled rate slow freezing of oocytes

- "Application of fundamental cryobiological principles is leading to a gradual but consistent improvement in outcomes, and promises further advances if the scientific focus is maintained (Fuller, 2009)"
- Highly reliable, highly reproducible, more quality assurance friendly
- No risk of contamination during storage in liquid nitrogen
- Efficiency (implantations per used oocytes) comparable to the one of frozen embryos
- The contest still open:
  - The best slow freezing and vitrification results are similar
  - No large/rigorous prospective trials have been conducted so far
- No increase in congenital abnormalities after the birth of approximately 600 babies