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*The search for excellence in IVF:
a practical approach*

ESHRE CAMPUS 2010:
Maribor Slovenia, 22-23 January 2010

**Oocyte cryopreservation: slow cooling
versus vitrification**

Laura Rienzi, Rome, Italy

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Question:

Oocyte cryopreservation:
slow cooling versus vitrification?

Vitrification!!!!!!

THANK YOU FOR THE ATTENTION

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Possible injuries



membrane permeability

Membrane permeability

Survival rates of human oocytes frozen with the same slow freezing protocol
(Lassalle et al., 1985)

60-65% 35-40% 75-80%

Aquaporin-9, a protein channel that can transport water and other solutes through the plasmalemma is expressed in rat GV-stage but not mature oocytes (Ford et al., 2000)

Permeability
Aquaporin-9 expression

+ + -
+ - +

Osmotic response to glycerol of mouse oocytes injected with Aquaporin-3 cRNA

Relative volume (%)

Time (min)

Edashige et al., 2003

Possible injuries

CG release and ZP hardening

membrane permeability

Cortical granules release

No evidence of cortical granule discharge in cryopreserved oocytes

Gook et al., 1993

Failed Fertilized

Frozen

Non-frozen

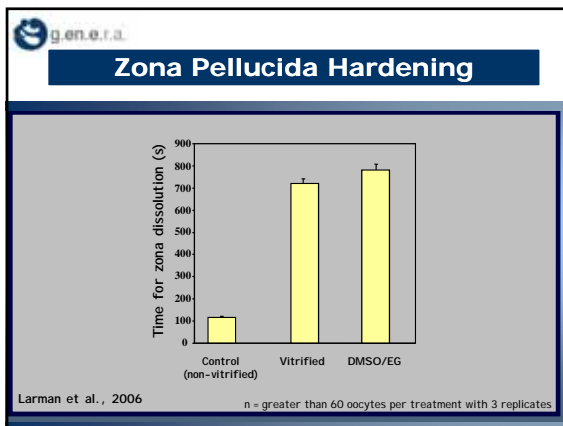
"The immunostaining examination for CG of the frozen-thawed oocytes did not reveal evidence of the premature release of CG."

Li et al., 2005

Fresh

Frozen

Ghetler et al., 2006





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Aneuploidy and PB retention

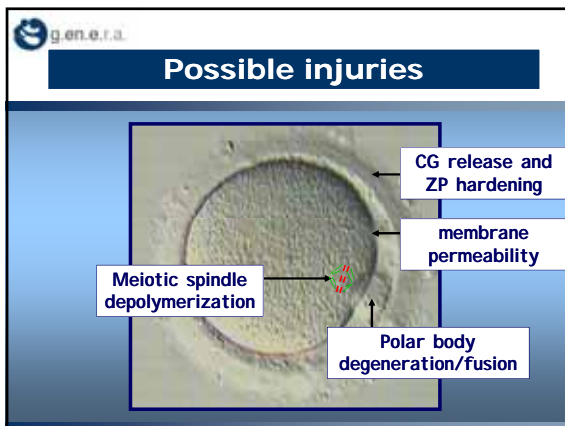
Early reports on failure of PBII extrusion and increase of aneuploidy in thawed mouse oocytes

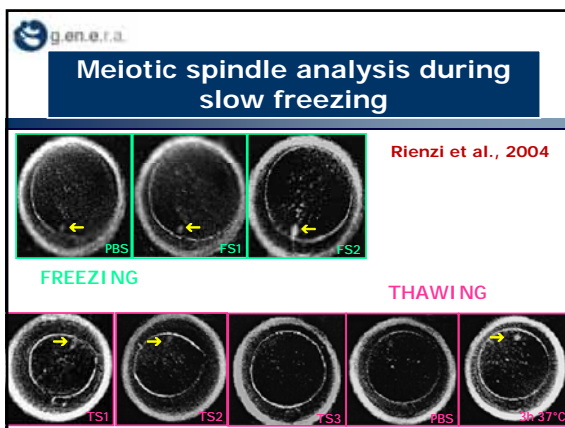
Glenister et al, 1987; Carroll et al., 1989

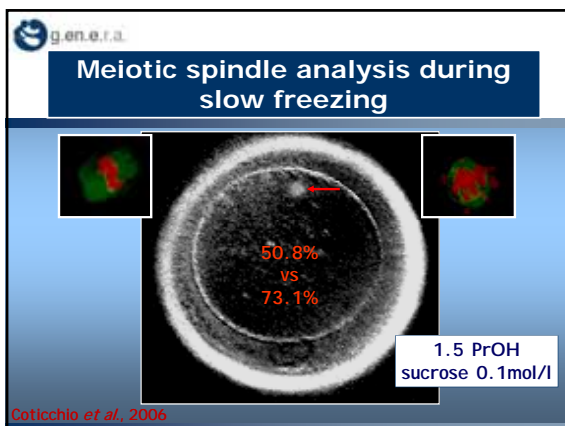
Frozen	No. of Oocytes (%)		
	Scored	% Aneuploidy	% Retention PB
+	352	6.4	2.6
-	218	8.0	4.4

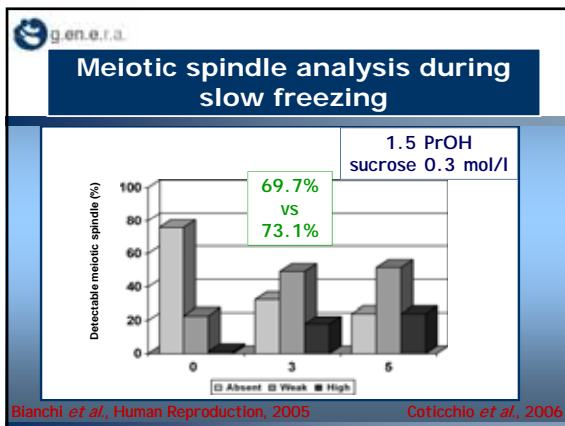
No increase in the rates of aneuploidy/digyny in parthenogenetically activated mouse oocytes after cryopreservation with DMSO/slow freezing

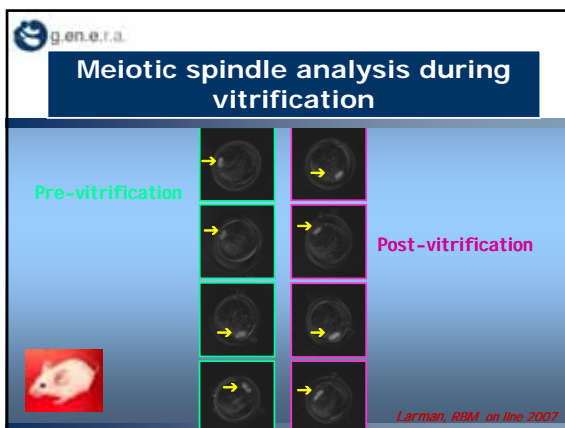
Bos-Mikich and Whittingham, 1995

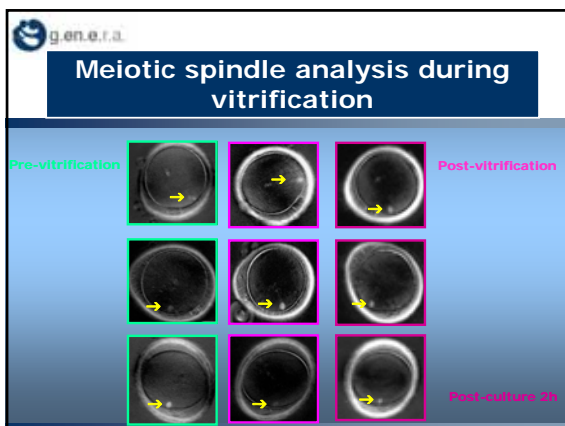


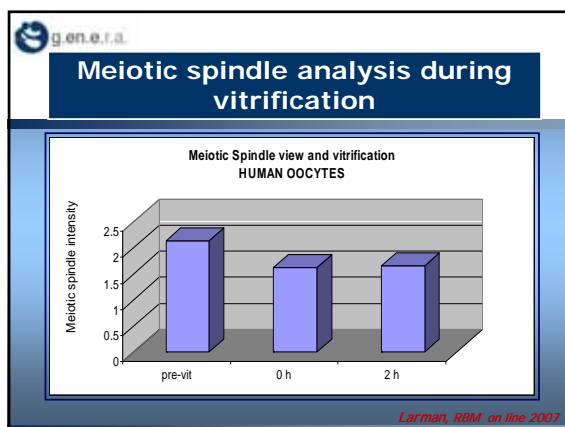












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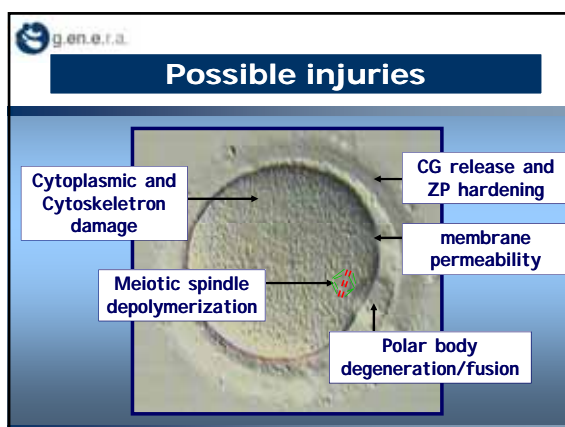
Meiotic spindle analysis during vitrification

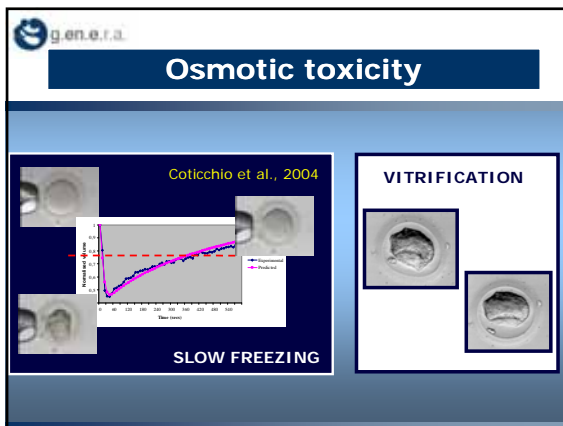
Table 2. Morphological analysis of meiotic spindle and metaphase plates

Cryopreservation protocol	Spindle shape		Metaphase plate chromosome alignment	
	Normal (butterfly shape)	Abnormal	Normal	Slightly abnormal
Control (fresh oocytes)	23 (88.3)	3 (11.5)	25 (96.2)	1 (3.8)
Slow freezing + 0.5 mol/l sucrose	17 (81.6)	4 (19.0)	19 (90.5)	2 (9.5)
Slow freezing + 0.5 mol/l sucrose	19 (82.6)	4 (17.4)	20 (87.0)	3 (13.0)
Slow freezing + choline replacement	16 (88.9)	2 (11.1)	17 (94.4)	1 (5.6)
Vitrification by Cryotop method	13 (76.3)	4 (23.5)	16 (94.1)	1 (5.9)

Values are number (percentage). There were no statistically significant differences in spindle and chromosome configurations between cryopreserved and fresh oocytes.

Cobo et al., RBM on line 2008





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Osmotic toxicity

OOCYTE OSMOTIC TOLERANCE AND OOLEMMA PERMEABILITY


Temperature of exposure influence shrinking (swelling) patterns

Oocyte shrinkage tolerance is about 30% of their initial volume

- At 22°C, EG has a lower permeability coefficient relative to DMSO and PG
- The membrane is more selective for EG and DMSO than for PG (mean reflection coefficient Sigma lower for PG)
- Permeability coefficients of individual oocytes varied substantially (inherent biological variability)

Van den Abbeel et al., 2007





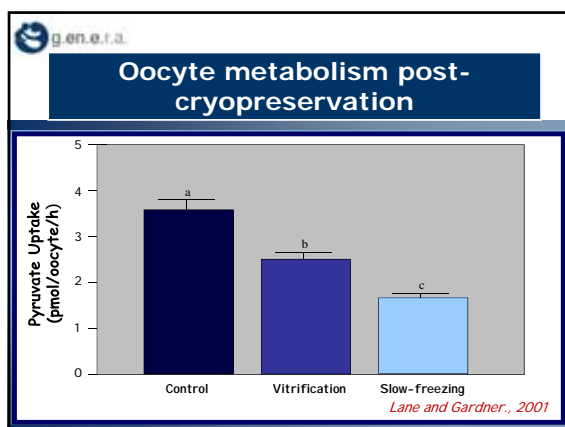
Oocyte metabolism post-cryopreservation


METABOLISM MONITORING THROUGH PYRUVATE UPTAKE (mouse oocytes):

Mouse oocytes and developing embryos following slow freezing were metabolically impaired compared with those that were vitrified

...although vitrification was also associated with a decrease in nutrient utilization by the oocyte compared to controls the decrease was significantly smaller than that induced by slow freezing.

Lane and Gardner, 2001; Lane et al., 2002





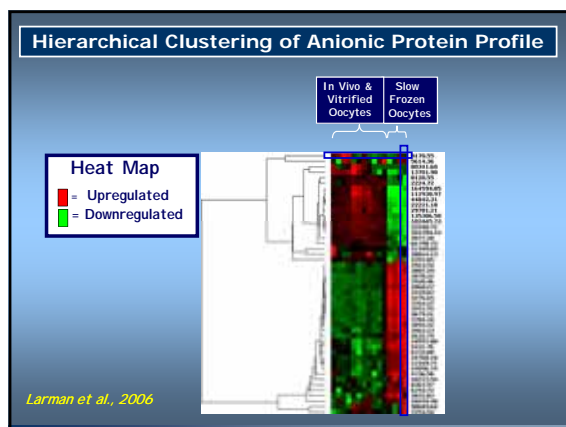
Oocyte protein profile post-cryopreservation

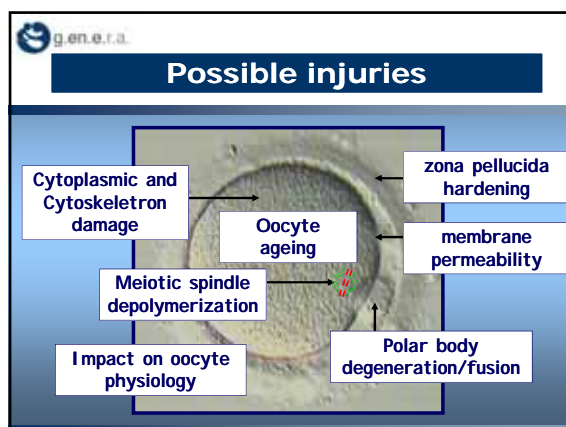
PROTEOMIC ANALYSIS OF OOCYTE PROTEIN PROFILES (mouse oocytes) by SELDI-TOF MS:

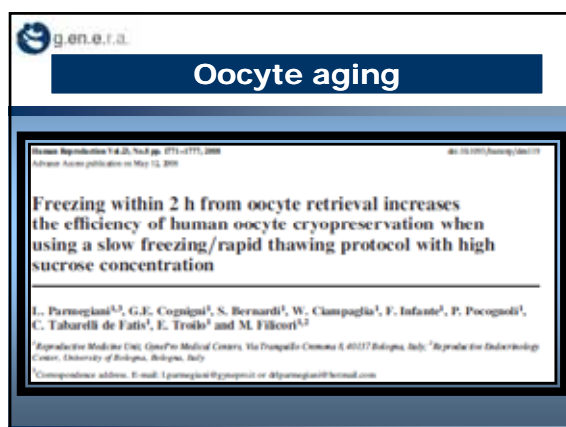
Mouse oocytes following slow freezing revealed major alterations compared with those that were vitrified.


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

Larman et al., 2006








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Oocyte safety

Oocyte cryopreservation poses certainly specific problems:

- The oolemma and not the size of MII oocyte is the key to explain the low **survival** rates obtained with slow freezing.
- Release of **cortical granules** (controversial)
- Chemical **toxicity** from cryoprotectants (type specific)
- Osmotic **toxicity**
- **Meiotic spindle** depolymerization (slow freezing)
- Oocyte **physiology** alteration (metabolism and protein profile) especially true for slow freezing

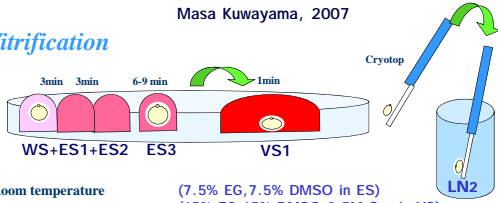

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Technical aspects

1. Cryoprotectants toxicity
(↓slow freezing,↑Vitrification)
2. Ice crystals formation and physical damage
(↑slow freezing,↓Vitrification)
3. Direct contact with nitrogen
(↓slow freezing,↑Vitrification)

Masa Kuwayama, 2007

Vitrification



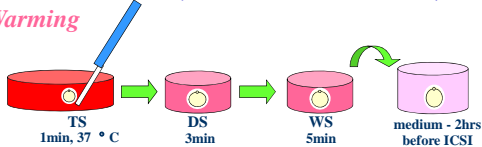
3min 3min 6-9 min 1min

WS+ES1+ES2 ES3 VS1

Cryotop LN2

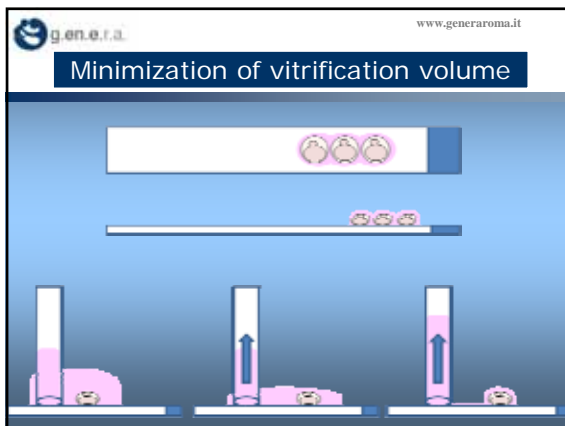
Room temperature (7.5% EG, 7.5% DMSO in ES)
(15% EG, 15% DMSO, 0.5M Suc in VS)

Warming




TS 1min, 37 °C DS 3min WS 5min medium - 2hrs before ICSI

(1M Suc in TS, 0.5M Suc in DS)









Direct contact with nitrogen

OOCYTE CONTAMINATION:


- Not sterile procedure
- Liquid nitrogen may be contaminated by the surface of straws/cryovials or other tools
- Risk of liquid nitrogen mediated disease transmission

Tedder et al., 1995; Fountain et al., 1997; Berry et al., 1998

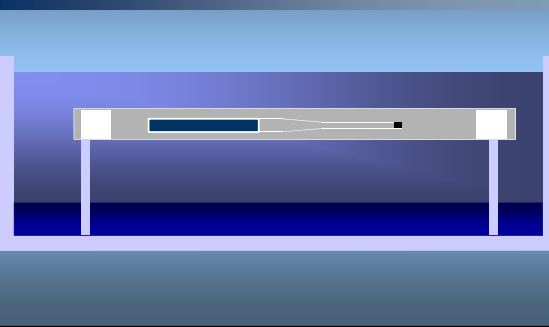
SOLUTIONS:


- Use of sealed system to avoid direct contact
- Cooling and storage in liquid nitrogen vapors

Larman et al. 2006, Cobo et al., 2007



Closed storage

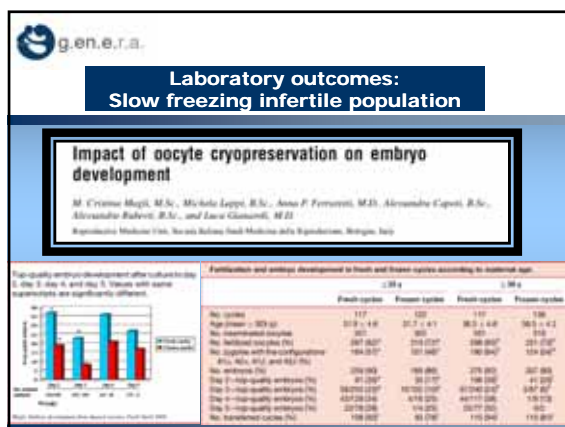




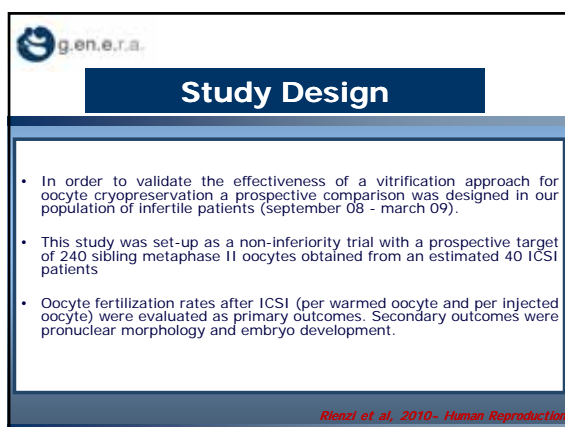
OUTCOMES


1. Laboratory OUTCOMES

2. Clinical OUTCOMES










Patient population

Table 1 Patient's baseline characteristics and fresh cycle parameters

	Patients included (N = 49)
Female age (mean years \pm SD)	35.5 \pm 4.8
Baseline FSH (mean mU/mL \pm SD)	6.44 \pm 3.1
Previous IVF attempts (mean \pm SD)	0.58 \pm 1.0
GnRH agonist long protocol (%)	31/40 (77.5)
Anovulatory protocol (%)	9/40 (22.5)
Days of stimulation (mean \pm SD)	10.8 \pm 1.95
Total gonadotrophin amount IU (mean \pm SD)	2201.65 \pm 765.7
Number of CCOCs retrieved (mean \pm SD)	13.3 \pm 4.5
Number of PHE oocytes (mean \pm SD)	10.7 \pm 3.6
Number of PHE oocytes vitrified (mean \pm SD)	6.3 \pm 2.8

CCOC, cumulus corona oocyte complex; PHE, metaphase II.


Rienzi et al., 2010 - Human Reproduction



Materials and methods

- The general idea of the study was to minimize extra stress on oocytes often related with cryopreservation procedures, namely:
 - Long exposure to Hepes buffered media, with uncertain temperature control, for oocyte denudation and selection under the inverted microscope
 - Prolonged oocyte *in vitro* culture without the protection of cumulus and corona cells
 - Oocyte ageing
- In this way, by using randomized sibling oocytes the only difference between the fresh and the vitrified group was the vitrification procedure itself followed by 2 hours of *in vitro* culture.

Rienzi et al., 2010 - Human Reproduction




Laboratory Outcomes

Table III Primary and secondary outcomes measures: fertilization, pronuclear morphology, embryo development and embryo morphology of fresh and vitrified sibling oocytes

	Fresh ICSI	Vitrified/Warmed ICSI (%)	Absolute difference (%) (95% CI)	OR (95% CI)	P
Fertilization (2PN) per sibling oocyte	100/120 (83.3) ^a	95/124 (76.6) ^a	-6.73 (-16.6 to 3.39)	0.65 (0.33 to 1.29)	0.20
Fertilization (2PN) per injected oocyte	100/120 (83.3) ^a	95/120 (79.2) ^a	-4.17 (-14.6 to 5.7)	0.76 (0.37 to 1.53)	0.50
Normal 2PN morphology	96/100 (96.0) ^a	86/95 (90.5) ^a	-5.47 (-13.4 to 1.84)	0.39 (0.08 to 1.49)	0.16
1PN oocytes	3/120 (2.5) ^a	6/120 (5.0) ^a	2.5 (-2.82 to 8.22)	2.05 (0.42 to 12.9)	0.50
3PN	1/120 (0.83) ^a	2/120 (1.66) ^a	0.83 (-3.09 to 5.1)	2.01 (0.10 to 119.9)	1
Degenerated oocytes post ICSI	1/120 (0.83) ^a	4/120 (3.34) ^a	3.51 (-1.75 to 7.47)	4.08 (0.39 to 203.5)	0.37
Day 2 embryo development	100/100 (100) ^a	91/95 (95.8) ^a	-2.11 (-7.3 to 1.9)	0.0 (0.00 to 0.23)	0.24
Excellent quality embryos	52/100 (52.0) ^a	49/95 (51.6) ^a	-0.43 (-14.2 to 13.3)	0.98 (0.53 to 1.79)	0.90
Good quality embryos	38/100 (38.0) ^a	41/95 (43.2) ^a	5.16 (-8.49 to 18.6)	1.24 (0.47 to 2.28)	0.47
Fair/poor quality embryos	10/100 (10.0) ^a	3/95 (3.16) ^a	-6.84 (-14.6 to 0.42)	0.29 (0.05 to 1.19)	0.10

^aPercentages, expressed per normal oocyte.
^bPercentages, expressed per randomized oocyte.
^cPercentages, expressed per 2PN fertilized oocyte.
^dPercentages, expressed per thawed oocyte.


Rienzi et al., 2010 - Human Reproduction



Laboratory outcomes:
Vitrification egg donation program

Comparison of concomitant outcome achieved with fresh and cryopreserved donor oocytes vitrified by the Cryotop method
Ana Cobo, Ph.D., Maribel Kerejeta, Ph.D.,* Sonia Pérez, Ph.D.,* Amparo Ruiz, M.D.,* Antonio Pellicer M.D.,* and José Remohí, M.D.**
*IVI Universidad de Valencia, Valencia, Spain, and *Kara Latham Clinic, Northborough, Minnesota, United States

Embryo quality	Vitrified	Fresh	P value
Cleavage rate day 2 embryos (%)	181/171 (84.3)	176/180 (97.8)	.002
No. of cell day 2 embryos (mean \pm SD)	3.9 \pm 1.1	3.9 \pm 1.5	.567
Good quality day 2 embryos (%)	138/181 (84.6)	138/176 (77.8)	.005
Cleavage rate day 3 embryos (%)	125/181 (77.8)	149/176 (84.8)	.008
No. of cell day 3 embryos (mean \pm SD)	6.9 \pm 2.3	6.9 \pm 2.7	.558
Good quality day 3 embryos (%)	101/125 (80.8)	120/149 (80.5)	.904
No. of embryos undergoing extended culture	78	143	
Blastocyst rate No. (%)	39/78 (48.7)	88/143 (61.5)	.069
Good quality blastocysts No. (%)	24/39 (61.5)	42/88 (47.7)	.612



OUTCOMES

1. Laboratory OUTCOMES
2. Clinical OUTCOMES



Clinical outcomes:
Slow freezing infertile population

Evidence-based clinical outcome of oocyte slow cooling


Since 1992, Dr. Borini has been Clinical Director of the Assisted Reproduction Centre "Servizio Procreatore", Bologna, Italy, and of a number of associated IVF clinics. After graduating in Medicine and Surgery at the University of Bologna, Bologna, Italy, in 1985, he completed his residency in Obstetrics and Gynaecology at the University of Bologna in 1991. He then attended the University of California Irvine, Irvine, California, as Research Fellow from October 1993 to May 1994. His research areas are embryo implantation, oocyte freezing and multiple induction of ovulation. Dr. Borini has published more than 160 research papers and acts as Chairman of CECOS (ITALY).

**Clinical outcomes:
Slow freezing infertile population**

No. of patients	749
No. of attempts with fresh or frozen-thawed embryos	762
No. of attempts transferred (mean \pm SD)	16.78 (2.2 \pm 0.3)
No. of pregnancies	285
Pregnancy rate (%) per embryo transfer	16.9 (26.7/157)
Pregnancy rate (%) per patient	38.0 (26.7/749)
Multiple pregnancy rate (%)	31.1 (45.3/267)
Gestational rate (%)	23.8 (30.6/1678)
Abortion rate (%)	3.7
Miscarriage rate (%)	11.0

No. of patients	749
No. of pregnancies	318
No. of gestational sacs	458
Cumulative pregnancy rate (%) per transfer	47.1 (41.6/874)
Cumulative pregnancy rate (%)	42.6 (31.8/749)

**Clinical outcomes:
Vitrification infertile population**

Table II Clinical outcomes of cycles performed with vitrified/warmed oocytes

	Patients included (N = 40)
Number of warmed oocytes (mean \pm SD)	3.1 \pm 0.30
Number of embryos transferred (mean \pm SD)	2.3 \pm 0.88
Number of embryo transfer performed (%)	39/40 (97.5)
Clinical pregnancy rate per cycle (%)	15/40 (37.5)
Clinical pregnancy rate per transfer (%)	15/29 (38.5)
Ongoing pregnancy rate per cycle (%)	12/40 (30.0)
Ongoing pregnancy rate per transfer (%)	12/29 (30.8)
Implantation rate (%)	19/93 (20.4)
Ongoing implantation rate (%)	16/93 (17.2)


Rienzi et al, 2010- Human Reproduction

Cumulative ongoing pregnancy rates: vitrification

Cumulative ongoing pregnancy rate achieved with oocyte vitrification and cleavage stage transfer without embryo selection in a standard infertility program

Journal:	Human Reproduction
Manuscript ID:	HUMREP-09-1133
Manuscript Type:	Original Articles
Keywords:	CRYOPRESERVATION, ONGOING PREGNANCY, OOCYTE, DCSS, FEMALE AGE
Specialty:	Infertility


Ubaldi et al., accepted Human Reproduction


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Study design

- The study was design as a prospective longitudinal cohort study.
- The baseline characteristics, embryological data, clinical and ongoing pregnancy rate were analyzed on a per cycle basis.
- The cumulative pregnancy rate obtained with fresh and vitrified oocytes from the same stimulation cycle was analyzed on a per patient basis.


Ubaldi et al., accepted Human Reproduction


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Material & methods

- All consecutives patients undergoing ICSI treatment in the Centre for Reproductive Medicine GENERA between September 2nd 2008 and May 15th 2009 were considered for this study
- Only patients with supernumerary oocytes available for cryopreservation were included. A single fresh attempt was included for each patient (special population of good responder patients).

Ubaldi et al., accepted Human Reproduction


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Laboratory results

Baseline patient's characteristics fresh and warming cycles

Baseline patient's characteristics		n patients	100%
Mean age (mean ± SD)		32.84 ± 4.89	
Mean basal FSH (mean ± SD)		6.5 ± 2.32	
Agonist protocol (%)		844 (82) (76.4%)	
Antagonist protocol (%)		19 (19) (23.4%)	
Fresh cycle laboratory outcomes			
CGC (mean ± SD)		12.9 ± 4.7	
MII (mean ± SD)		16.2 ± 5.6	
Survived MII (mean ± SD)		5.97 ± 3.40	
IFN (mean ± SD)		2.07 ± 0.94	
Top quality embryos (mean ± SD)		5.19 ± 0.90	
Embryo transferred (mean ± SD)		2.12 ± 0.79	
Embryo vitrified (mean ± SD)		6.22 ± 0.89	
Warming cycle laboratory outcomes			
Survived MII (mean ± SD)		4.13 ± 2.12	
Survived MII (mean ± SD)		3.89 ± 0.90	
Survived MII (mean ± SD)		2.47 ± 0.84	
IFN (mean ± SD)		2.18 ± 0.87	
Top quality embryos (mean ± SD)		3.47 ± 0.91	
Embryo transfer (mean ± SD)		2.80 ± 0.80	

Table 1. Baseline patient's characteristics and laboratory outcomes.

Results

Effect of patients and cycle characteristics on cumulative ongoing pregnancy rates based on Cox regression analysis

Covariate	P-value	OR	(95% CI)
Female age groups			
<34 years (reference)			
34-37 years	0.30	0.78	0.47 to 1.31
38-43 years	0.39	0.77	0.48 to 1.23
43-49 years	0.64	0.44	0.18 to 0.64
Fertility factors			
male (reference)			
idiopathic	0.75	0.90	0.48 to 1.67
androgenitis	0.19	0.39	0.09 to 1.60
ovulatory	0.54	0.51	0.07 to 3.91
total	0.81	0.93	0.52 to 1.65
combined	0.25	1.54	0.73 to 3.30
0.60	0.80	0.30	0.02 to 1.23
Number of COC	0.80	1.01	0.92 to 1.09
Number of MI oocytes	0.57	1.03	0.90 to 1.14
Incubation time prior to ICSI/vitrification	0.71	1.02	0.94 to 1.17
Sperm selection	0.41	0.79	0.45 to 1.37
Sperm quality	0.84	1.07	0.58 to 2.09

odds ratio (OR), 95% confidence interval (95% CI)

odds ratio (OR), 95% confidence interval (95% CI)

Conclusions cumulative pregnancy

- High cumulative ongoing pregnancy rates were achieved in a standard infertility program with transfers of embryos derived from fresh and subsequently vitrified eggs
- Among various infertility factors, only female age influenced significantly the outcome
- The overall efficiency justifies the application of this strategy in routine infertility work

Clinical outcomes: Vitrification egg donation program

Characteristic	Stratified	Fixed	Mixed
No. of branches	22	2	4
No. of members (approximate)	100 (7.1 ± 1.2)	4 (2.1 ± 0.4)	10 (2.1 ± 0.5)
Power = 50			
Proportionality ratio per member	10/200 (50.0)	1/1000	1/250
Proportionality ratio of the social	10/200 (50.0)	0/1000	10/250
Ratio of members (approximate)			
Multiple proportionality ratio fixed	0/10 (0.0)	1/1000	0
Proportionality ratio	0/10 (0.0)	0	0
Non-proportional proportionality ratio	0/10 (0.0)	0	0
Grouping proportionality ratio	1/100 (1.0)	1/1000	0/1000

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[illegible]

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Obstetric outcomes

Chian RC, Huang JY, Tan SL, Lucena E, Saa A, Rojas A, Castellón LA, García Amador MI, Montoya Sarmiento JE.

Obstetric and perinatal outcome in 200 infants conceived from vitrified oocytes. *Reprod Biomed online* 2008

Noyes N, Porcu E, Borini A.

Over 900 oocyte cryopreservation babies born with no apparent increase in congenital anomalies. *Reprod Biomed online* 2009

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Traditional freezing and/or vitrification?

Efficiency in donation program not compromised
(Cobo et al., 2007; Nagy et al., 2007)

Prospective randomized study with own oocytes
no difference (Rienzi et al., 2010)

The clinical pregnancy rate has doubled with the
introduction of vitrification (Tulandi et al., 2008)

Cumulative ongoing pregnancy rate with oocyte
vitrification without embryo selection in a
standard infertility program (Ubaldi et al., 2010)

Technical problems related to the safety of the technique has
still to be solved...

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CONCLUSIONS

Oocyte vitrification offers new perspectives:

- oocyte cryo-banking
- fertility preservation
- equality between genders

BUT not for general population (which of course would loose the advantages of embryo selection in the fresh cycle)

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