



CLINICA VALLE GIULIA, Rome

*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

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## Membrane permeability



60-65%



35-40%



75-80%

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

**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)



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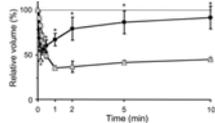

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Permeability

Aquaporin-9 expression

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



Edashige et al., 2003

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## Possible injuries




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## Cortical granules release

No evidence of cortical granule discharge in cryopreserved oocytes (Gook et al., 1993)



Frozen



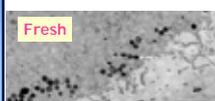
Non-frozen

"The immunostaining examination for CG of the frozen-thawed oocytes did not reveal evidence of the premature release of CG." (Li et al., 2005)

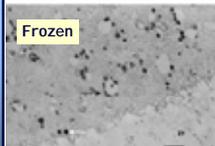
Failed Fertilized



Fresh



Frozen



Ghetler et al., 2006

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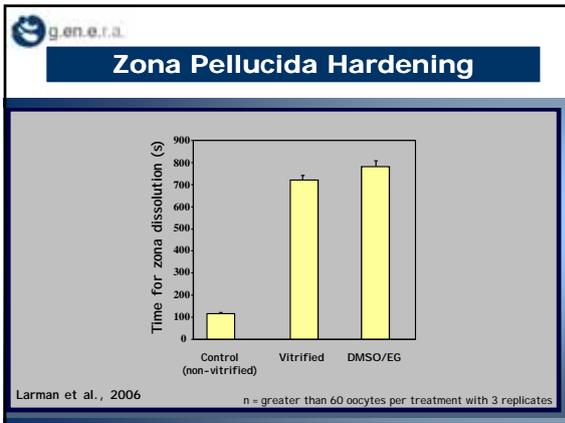
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### Aneuploidy and PB retention

Early reports on failure of PBI 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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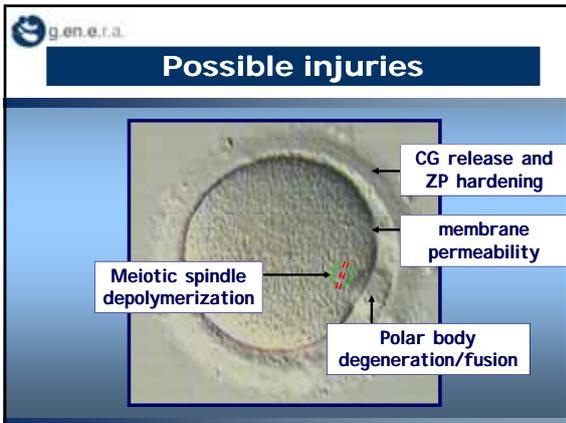
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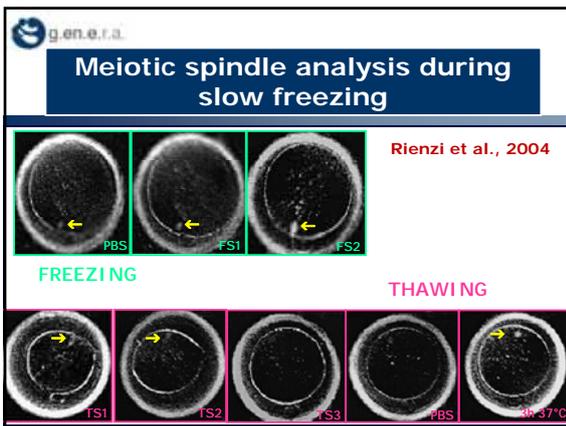
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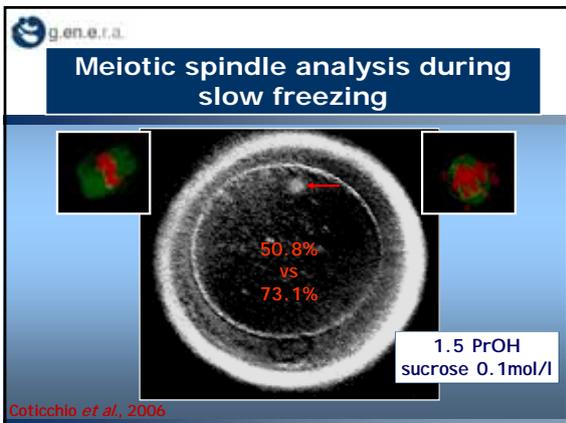
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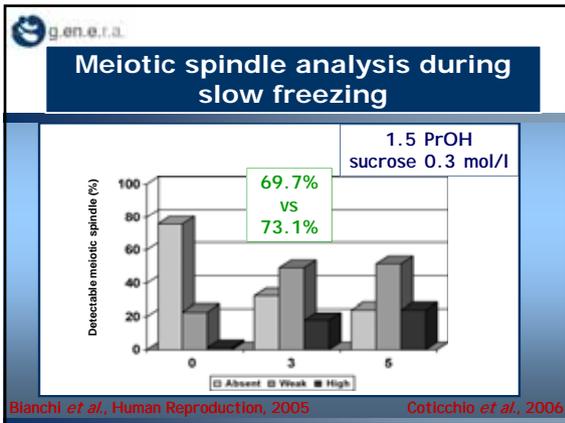
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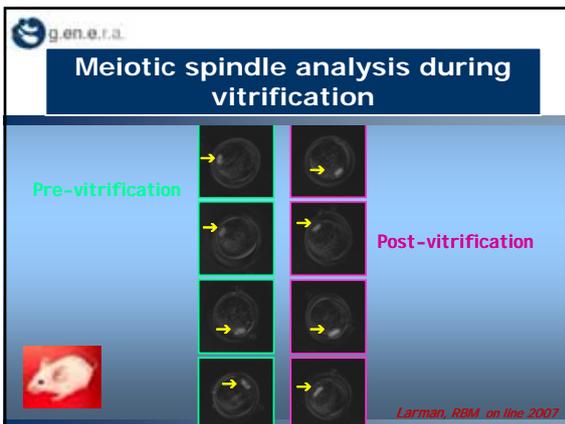
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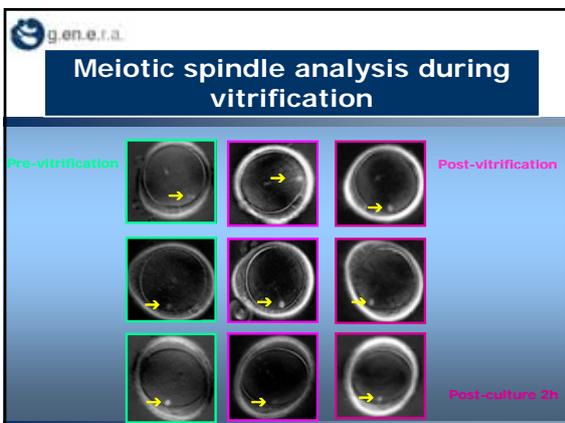
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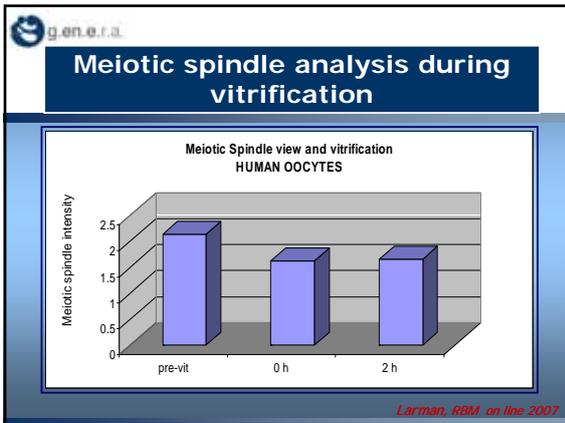
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### Meiotic spindle analysis during vitrification

Table 2. Morphological analysis of meiotic spindle and metaphase plates

Cryopreservation protocol	Spindle shape		Metaphase plate chromosome alignment	
	Normal (birefr. shape)	Abnormal	Normal	Slightly abnormal
Control (fresh oocytes)	23 (88.3)	3 (11.5)	25 (90.2)	3 (3.8)
Slow freezing + 0.3 mol/l sucrose	17 (81.6)	4 (19.0)	19 (90.5)	2 (9.5)
Slow freezing + 0.3 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 (76.5)	4 (23.5)	16 (94.1)	1 (6.3)

\*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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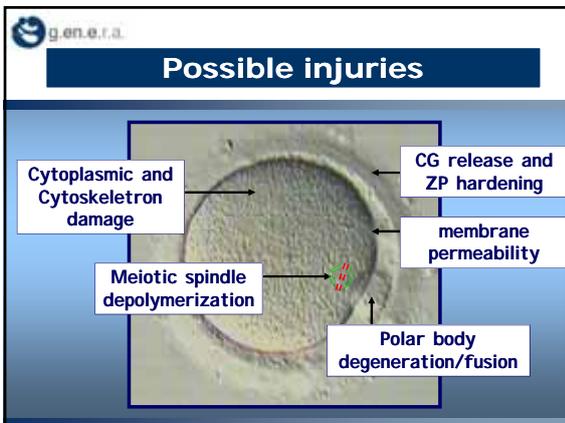
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## Osmotic toxicity

Coticchio et al., 2004

SLOW FREEZING

VITRIFICATION

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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*

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## Possible injuries

Cytoplasmic and Cytoskeleton damage

Meiotic spindle depolymerization

Impact on oocyte physiology

zona pellucida hardening

membrane permeability

Polar body degeneration/fusion

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## 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*

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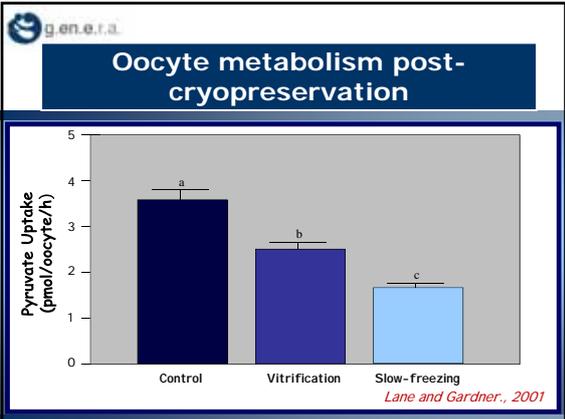
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## Oocyte protein profile post-cryopreservation

**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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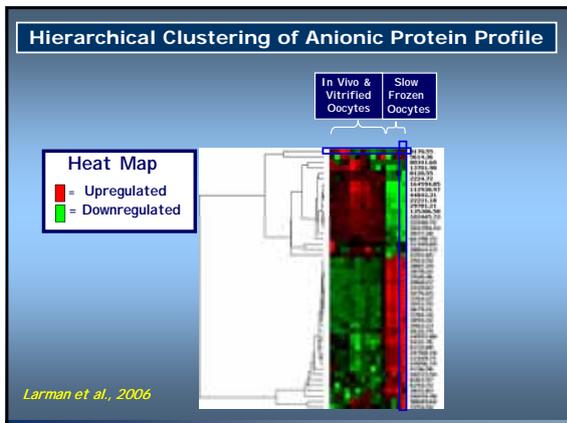
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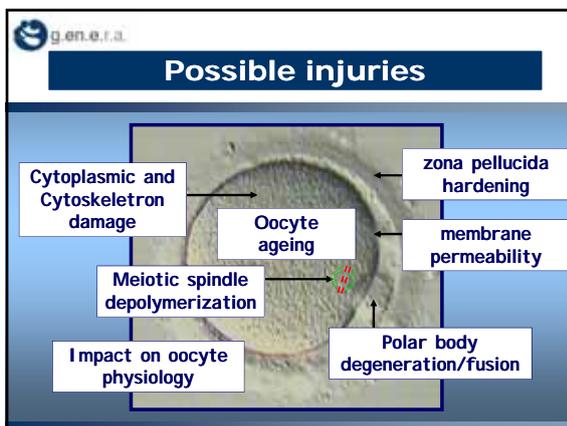
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**Oocyte aging**

*Human Reproduction* 14 (2), 2000, pp. 271-277, 2000  
 doi:10.1093/humrep/14(2)271

Freezing within 2 h from oocyte retrieval increases the efficiency of human oocyte cryopreservation when using a slow freezing/rapid thawing protocol with high sucrose concentration

L. Parmegiani<sup>1,2</sup>, G.E. Cognigni<sup>1</sup>, S. Bernardi<sup>1</sup>, W. Ciampaglia<sup>1</sup>, F. Infante<sup>1</sup>, P. Pucognoli<sup>1</sup>, C. Tabarelli de Fatis<sup>1</sup>, E. Troilo<sup>1</sup> and M. Filicori<sup>1,2</sup>

<sup>1</sup>Reproductive Medicine Unit, Gynecology Medical Center, Via Donquattrone Costanzi 8, 40137 Bologna, Italy; <sup>2</sup>Reproductive Endocrinology Center, University of Bologna, Bologna, Italy

Correspondence address: E-mail: L.parmegiani@unibo.it or M.parmegiani@unibo.it

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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)

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Masa Kuwayama, 2007

### Vitrification

3min 3min 6-9 min 1min

WS+ES1+ES2 ES3 VS1

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

Cryotop LN2

### Warming

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

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

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### Minimization of vitrification volume

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### Minimization of vitrification volume

*Cobo et al., 2009*

- Volume of 0.1 µl
- Cooling rate can be increased to -23,000°C/min
- Required CPA concentration in VS
  - 50% (v/v) to 30% (v/v)
- Osmolarity of VS
  - ~8,000 to 4,000 mosm/l

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### Safety of the procedures

Concerns

*"The most widely emphasized concerns... are toxicity and danger of contamination.*

*Unfortunately, available vitrification methods still struggle with these problems to date"*

*Son and Tan, 2009*

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 **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

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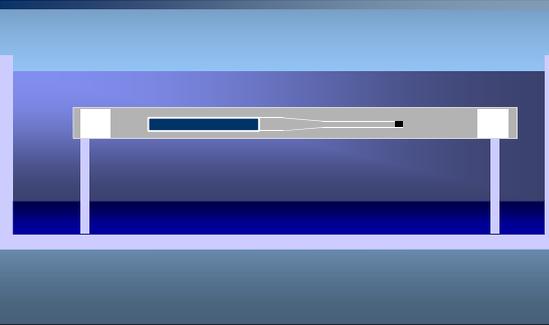
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 **Closed storage**



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 **OUTCOMES**

**1. Laboratory OUTCOMES**

2. Clinical OUTCOMES

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**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.,\* Maurizio Kuvshinov, Ph.D.,\* Sonia Pérez, Ph.D.,\* Amparo Esté, M.D.,\* Antonio Pellicer M.D.,\* and José Remohí, M.D.\**

\*IVI Universidad de Valencia, Valencia, Spain, and \*Kao Labor Clin, Nishinomiya, Minato, Tokyo, Japan

Embryo quality	Vitrified	Fresh	P value
Cleavage rate day 2 embryos (%)	101/111 (91.0)	136/140 (97.1)	.002
No. of cell day 2 embryos (mean ± SD)	2.9 ± 1.1	2.9 ± 1.5	.567
Good quality day 2 embryos (%)	138/161 (84.6)	128/130 (97.8)	.005
Cleavage rate day 3 embryos (%)	125/161 (77.6)	149/176 (84.6)	.008
No. of cell day 3 embryos (mean ± SD)	6.9 ± 2.3	6.9 ± 2.7	.558
Good quality day 3 embryos (%)	101/125 (80.8)	120/149 (80.5)	.994
No. of embryos undergoing extended culture	76	143	
Blastocyst rate No. (%)	39/76 (48.7)	88/143 (61.5)	.009
Good quality blastocysts No. (%)	24/32 (81.3)	42/60 (70)	.612

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**OUTCOMES**

1. Laboratory OUTCOMES

2. Clinical OUTCOMES

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**Clinical outcomes:  
Slow freezing infertile population**

**Evidence-based clinical outcome of oocyte slow cooling**



Since 1992, Dr Barri has been Clinical Director of the Assisted Reproduction Centre "Servizio Procreazione", 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 Barri has published more than 160 research papers and acts as Chairman of CEICOG (ITALY).

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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	(%)
Mean age (mean ±SD)		32.83 ± 4.33	
Mean basal FSH (mean ±SD)		6.1 ± 2.33	
Agonist protocol (%)		844 (82)	(79.8%)
Antagonist protocol (%)		19 (2)	(2.4%)
<b>Fresh cycle laboratory outcomes</b>			
CGC (mean ±SD)		22.9 ± 4.7	
MII (mean ±SD)		16.2 ± 3.5	
Survived MII (mean ±SD)		3.97 ± 0.80	
2PN (mean ±SD)		2.07 ± 0.84	
Top quality embryos (mean ±SD)		3.18 ± 0.90	
Embryo transferred (mean ±SD)		3.12 ± 0.79	
Embryo vitrified (mean ±SD)		6.22 ± 0.88	
<b>Warming cycle laboratory outcomes</b>			
MII (mean ±SD)		4.1 ± 2.1	
Survived MII (mean ±SD)		3.80 ± 0.80	
2PN (mean ±SD)		2.47 ± 0.84	
Top quality embryos (mean ±SD)		2.16 ± 0.81	
Embryo transfer (mean ±SD)		3.47 ± 0.91	
Embryo transfer (mean ±SD)		2.80 ± 0.80	

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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 lose the advantages of embryo selection in the fresh cycle)

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