Postępy w kriokonserwacji gamet, zarodek oraz tkanek jajnika

Dr Ricardo Faundez

Katedra Dużych Zwierząt z Kliniką

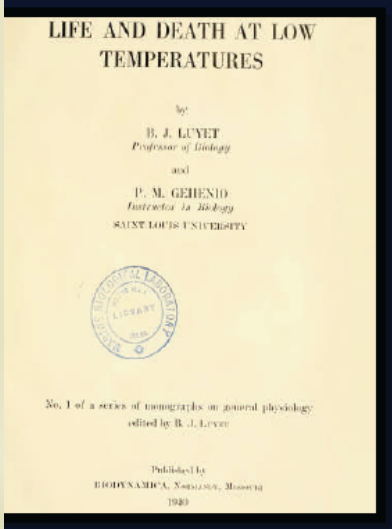
Wydział Medycyny Weterynaryjnej SGGW

*Witryfikacja

Vitrification is not a new technique

"it is more difficult to destroy a prejudice than an atom"

Albert Einstein


LIFE AND DEATH AT LOW TEMPERATURES

by

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No. 1 of a series of monographs on general physiology edited by B. J. Luyet

Published by
BIODYNAMICA, Normandy, Missouri
1940

1935 — **Slow freezing of mouse embryos**

1948 — **Slow freezing of domestic animal embryos**

1972 — **Slow freezing of human embryos**

1973/1974 — Vitrification of mouse embryos

1983 — **Slow freezing of human oocytes**

1985 — Vitrification of mouse oocytes

1989 — Vitrification of bovine blastocysts

1993 — **Ultrarapid Vitrification with EM grids**

1996 — **OPS Ultrarapid**

1997 — **Cryoloop Cryotip Ultrarapid Vitrification**

1999

Techniques

Slow Freeze	Vitrification
• Lower cryoprotectant concentration • Longer exposure time • Cryomachine • Longer to perform • Technically easier	• Higher cryoprotectant concentration • Shorter exposure time • Shorter to perform • More precise timing • More clinical expertise • Open containers

Does one method cause more cryodamage than the other?

Techniques

Cryoprotective Agents

Permeating

Affect / pass through cell membranes

Interact with and replace H_2O

Lower freezing point

↑Toxicity with ↑T° and Concentration

PROH

DMSO

Glycerol

Ethylene Glycol

↑ Increased Permeability

Non-Permeating

Do not pass through cell membranes

Create osmotic gradient / Dehydration

(High MW: >1000)

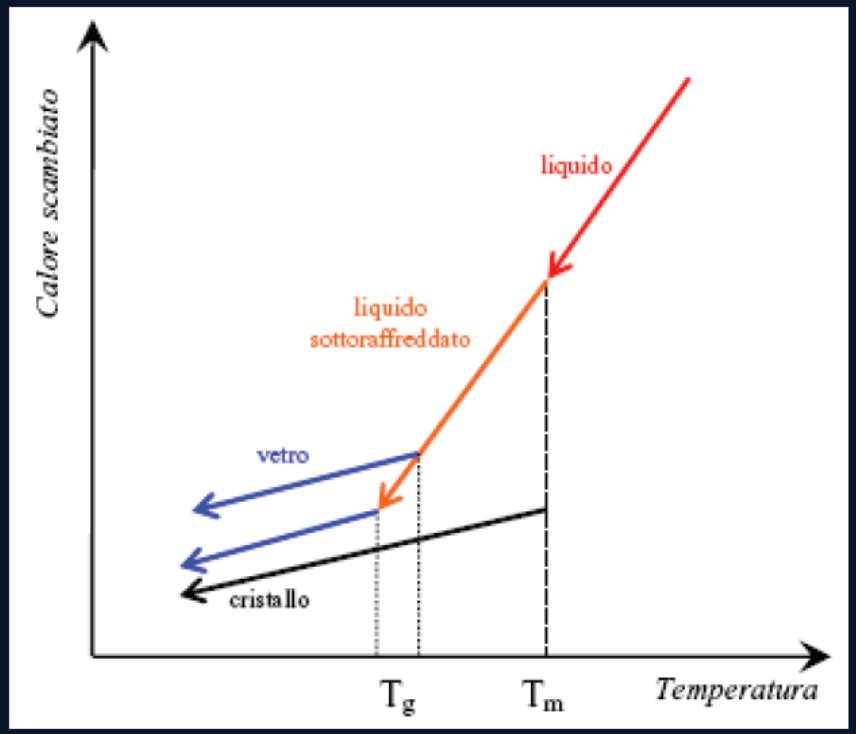
Glucose

Sucrose

Ficoll

Biophysical aspects

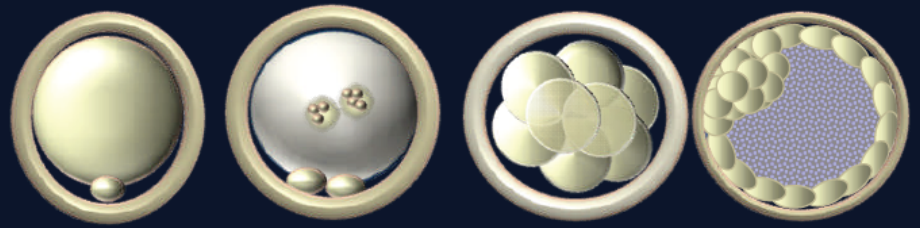
Vitrification is a pseudo second order phase transition (IUPAC Compendium of Chemical Terminology, 1997) converting a material into a glassy amorphous solid that is free from crystalline structure



Probability of vitrification =

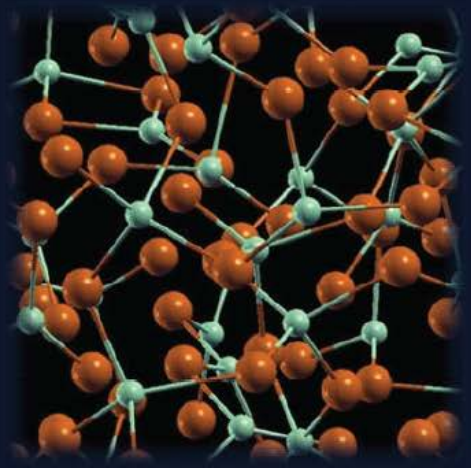
$$\frac{\textit{Cooling/warming rates} * \textit{Viscosity}}{\textit{Volume of the sample}}$$

Types of cells

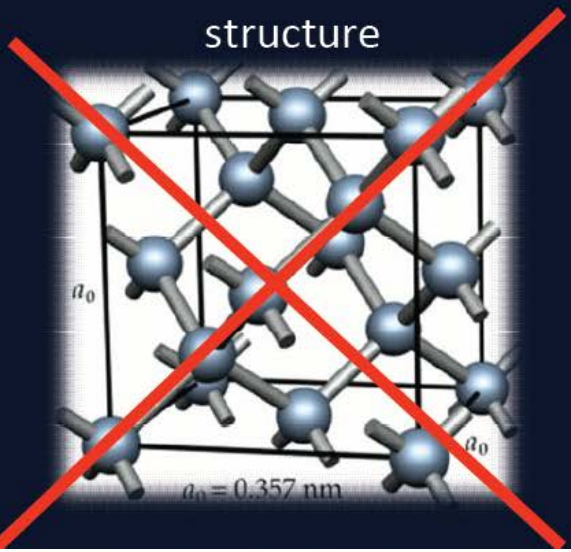


Biophysical aspects

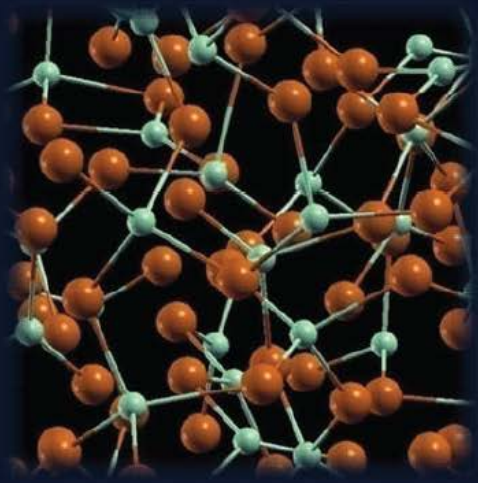
Molecular organization in a **LIQUID solution**



Molecular organization as in a ***SOLIDE*** **CRYSTAL** structure

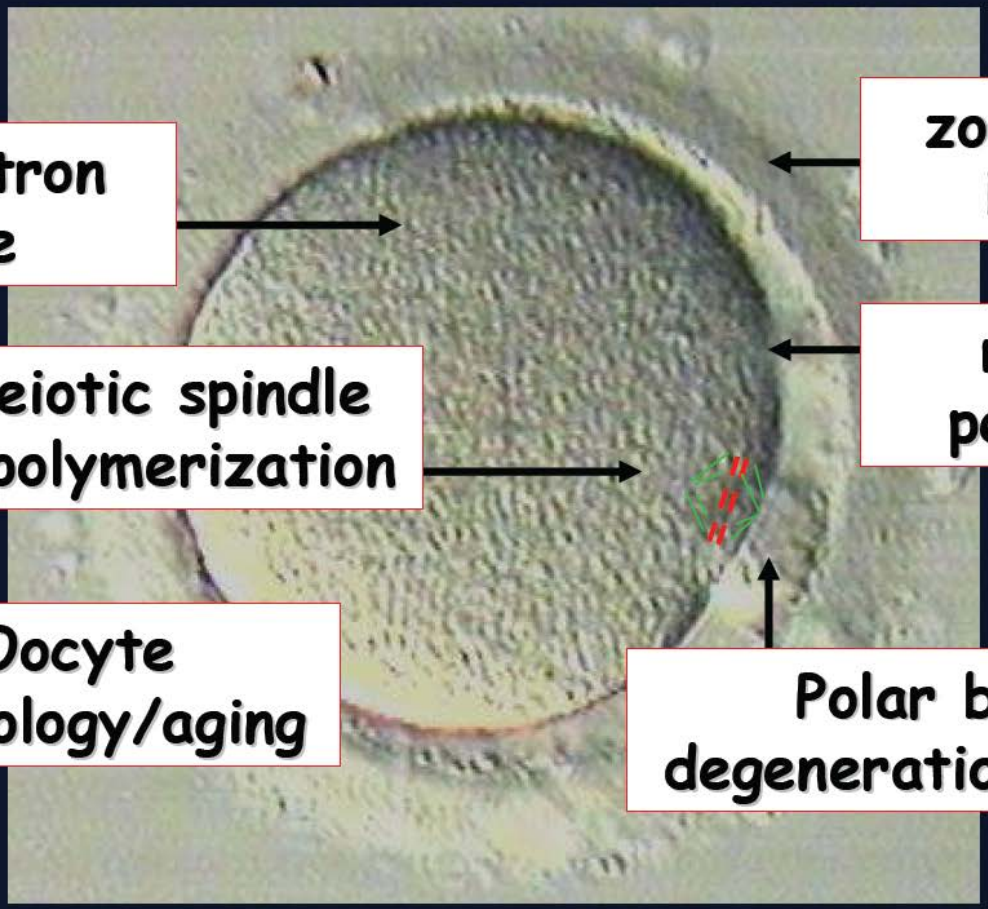


No molecular re-arrangement in a ***SOLIDE GLASSY*** *state*



Oocyte vitrification: possible injuries

"Ex ovo omnia" William Harvey 1578



Cytoskeletron damage

zona pellucida hardening

Meiotic spindle depolymerization

membrane permeability

Oocyte physiology/aging

Polar body degeneration/fusion

*Effect of cooling on oocyte structure

- Decrease of permeability of the oolema to water and cryoprotective agents

- Disassembly of the meiotic spindle apparatus

  Increase of chromosomal abnormalities

- Premature cortical granule exocytosis

  Changes in the zona pellucida resulting in reduced fertilization rate

* Slow freezing versus ultra-rapid freezing

	Traditional	Vitrification
CPA concentration	1.5 M	3.0–5.0 M
Volume	0.3–1.0 mL	<1 µL
Contact between N_2 and cell	No	Yes
Cooling rate	~0.5°C/min	~25.000–50.000°C/min
Freezing	Slow	Ultra-rapid
Thawing / warming	Slow	Rapid
Time consuming	≥180 min	1 sec
Dehydration	Not controlled	Controlled

Slow freezing versus ultra-rapid freezing

	Traditional	Vitrification
Reduced osmotic injury	No	Yes
Zona pellucida fracture	Possible	No
Ice crystal formation	Yes	No
Seeding	Yes	No need
Procedure	Complicated	Simple
Device	Yes	No need
Costs Liquid nitrogen amount Duration out Incubator	High High > 4 Hrs	Less Much less 10 min

Current comparison

Vitrified egg vs fresh (same donor) May 2006- March 2009

	Cryo oocyte	Fresh oocyte	*P*
Number of donors	81	81	NA
Number of recipients	100	91	NA
Mean age (±SD) of recipients	40.9 (±4.9)	41.2 (±4.7)	NS
Mean number of oocytes per recipients	7.1	25.28	<.001
Mean number of oocytes for ICSI	6.0	15.0	<.001
Average 2PN ICSI fertilization rate	77%	57%	<.001
Implantation Rate	52%	56%	NS
Mean number of embryos cryopreserved	1.5 (±1.5)	12.5 (±8.8)	<.001
Clinical pregnancy rate	67%	69%	NS

Nagy et al.,RBA 2009

Summary of clinical outcomes from oocyte cryopreservation using various protocols

	1.5 M PROH + 0.1 M sucrose	1.5 M PROH + 0.2 M sucrose	1.5 M PROH + 0.3 M sucrose	1.5 M PROH + 0.1 M sucrose (Na depleted)	1.5 M PROH + 0.2 M sucrose (Na depleted)	1.5 M PROH + 0.3 M sucrose (Na depleted)	Vitrification 2.7 M EG + 2.1 M DMSO + 0.5 M sucrose
Survival (%) (#)	50 (3537)	72 (926)	74 (4902)	52 (127)	62 (329)	59 (190)	91 (628)
ICSI fert (%)	54	80	73	56	58	68	91
Cleavage (%)	85	93	90	100	86	83	92
Embryos per 100 thawed oocytes	23	53	49	29	31	33	76
Implantation (%)	10	17	5	21	11	16	14
Implantations per 100 thawed oocytes	2.3	9.1	2.4	6.1	3.4	5.3	11

* Vitrification of Human Oocytes

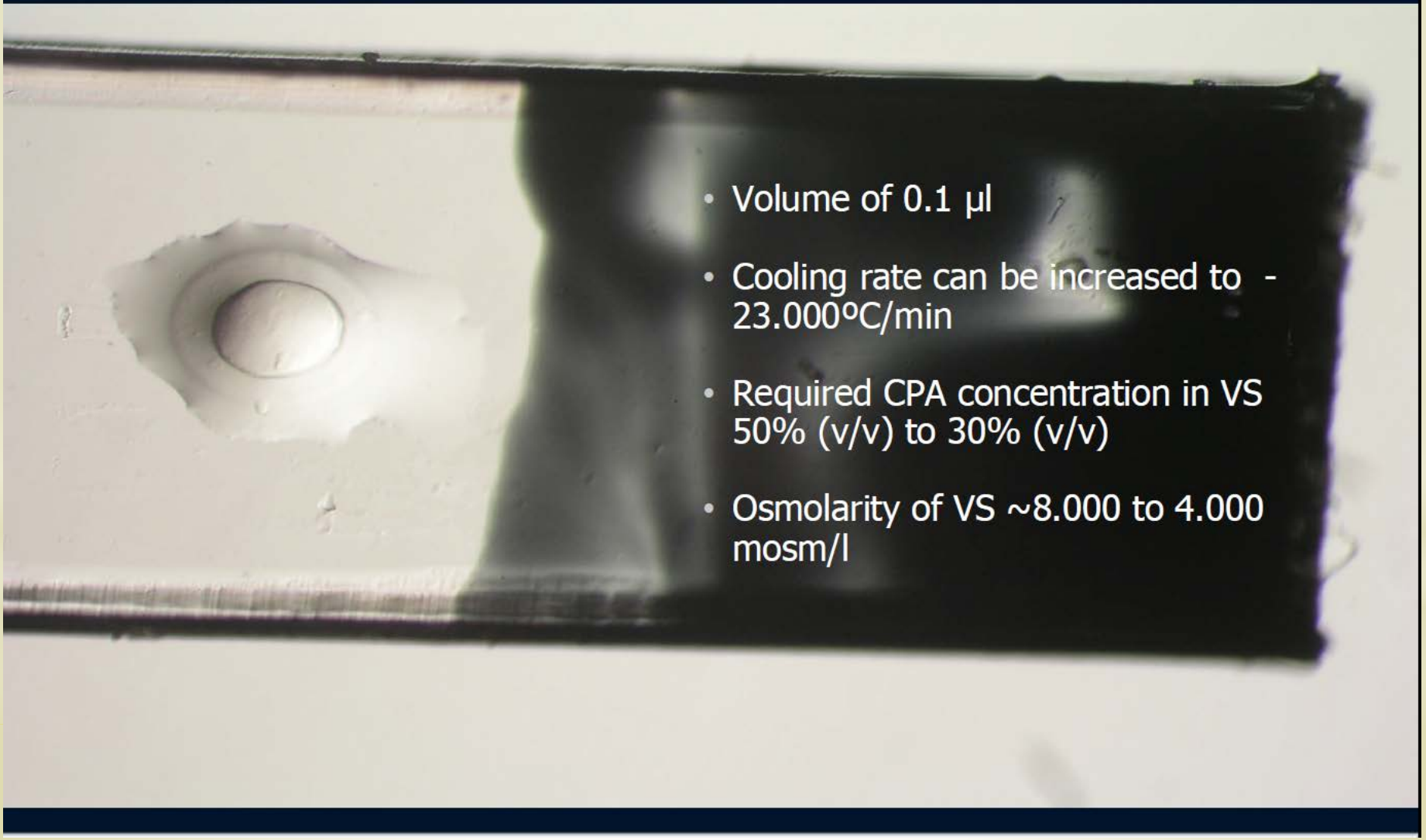
Kuwayama 2005	
Vitrified oocytes	64
Survived oocytes after warming	58(91%)
Fertilized oocytes	52(89.6%)
Embryo Transfer	29
N° of clinical pregnancy	12(41%)
Deliveries	7
Ongoing pregnancies	3

* Successful vitrification

* High cooling rate (> –50.000°C)
* Fast cooling period (<1 sec.)
* Low volume (<1 µL)
* High concentration of cryoprotectants

=> This will avoid crystal formation

Minimum volume-direct contact approach



- Volume of 0.1 µl
- Cooling rate can be increased to -23.000ºC/min
- Required CPA concentration in VS 50% (v/v) to 30% (v/v)
- Osmolarity of VS ~8.000 to 4.000 mosm/l

Laboratory outcomes: Oocyte vitrification infertile population

human reproduction

ORIGINAL ARTICLE *Embryology*

Embryo development of fresh 'versus' vitrified metaphase II oocytes after ICSI: a prospective randomized sibling-oocyte study

Laura Rienzi[1], Stefania Romano, Laura Albricci, Roberta Maggiulli, Antonio Capalbo, Elena Baroni, Silvia Colamaria, Fabio Sapienza, and Filippo Ubaldi

G.E.N.E.R.A. Centre for Reproductive Medicine, Clinica Valle Giulia, via de Notaris 2B, Rome, Italy

[1]Correspondence address. E-mail: rienzi@generaroma.it

Laboratory outcomes: Oocyte vitrification infertile population

In order to validate the effectiveness of a vitrification approach for oocyte cryopreservation a prospective comparison was designed in our population of infertile patients (september 08 - march 09).

This study was set-up as a non-inferiority trial with a prospective target of 240 sibling metaphase II oocytes obtained from an estimated 40 ICSI patients

Oocyte fertilization rates after ICSI (per warmed oocyte and per injected oocyte) were evaluated as primary outcomes. Secondary outcomes were pronuclear morphology and embryo development

Rienzi et al., Human Reproduction 2010

Laboratory outcomes: Oocyte vitrification infertile population

The general idea of the study was to minimize extra stress on oocytes often related with cryopreservation procedures, namely:

1. Long exposure to Hepes buffered media, with uncertain temperature control, for oocyte denudation and selection under the inverted microscope

2. Prolonged oocyte *in vitro* culture without the protection of cumulus and corona cells

3. 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., Human Reproduction 2010

Laboratory outcomes: Oocytes vitrification infertile population

Survival rate 96.7%

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)[b]	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)[b]	95/120 (79.2)[b]	−4.17 (−14.0 to 5.7)	0.76 (0.37 to 1.53)	0.50
Normal 2PN morphology	96/100 (96.0)[c]	86/95 (90.5)[c]	−5.47 (−13.4 to 1.84)	0.39 (0.08 to 1.49)	0.16
1PN oocytes	3/120 (2.5)[b]	6/120 (5.0)[b]	2.5 (−2.82 to 8.22)	2.05 (0.42 to 12.9)	0.50
3PN	1/120 (0.83)[b]	2/120 (1.66)[b]	0.83 (−3.09 to 5.1)	2.01 (0.10 to 119.9)	1
Degenerated oocytes post-ICSI	1/120 (0.83)[b]	4/120 (3.34)[b]	2.51 (−1.75 to 7.47)	4.08 (0.39 to 203.5)	0.37
Day 2 embryo development	100/100 (100)[c]	93/95 (97.9)[c]	−2,11 (−7.3 to 1.9)	0.0 (0.00 to 0.23)	0.24
Excellent quality embryos	52/100 (52.0)[d]	49/95 (51.6)[d]	−0.43 (−14.2 to 13.3)	0.98 (0.53 to 1.79)	0.90
Good quality embryos	38/100 (38.0)[d]	41/95 (43.2)[d]	5.16 (−8.49 to 18.6)	1.24 (0.67 to 2.28)	0.47
Fair/poor quality embryos	10/100 (10.0)[d]	3/95 (3.16)[d]	−6.84 (−14.6 to 0.42)	0.29 (0.05 to 1.19)	0.10

[a]Percentages, expressed per warmed oocyte.
[b]Percentages, expressed per inseminated oocyte.
[c]Percentages, expressed per 2PN fertilized oocyte.
[d]Percentages, expressed per deaved oocyte.

Rienzi et al., Human Reproduction 2010

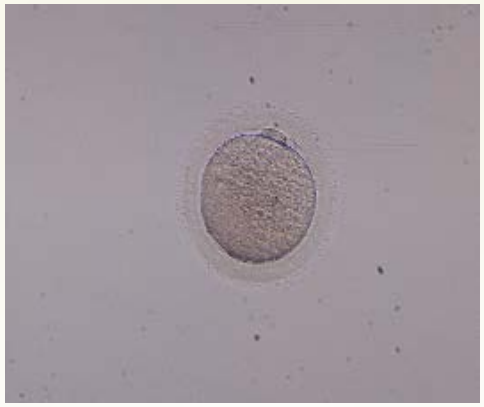
* Embryo Development of Fresh „Versus“ Vitrified Metaphase II Oocytes after ICSI: A Prospective Randomised Sibling-Oocyte Study

***Conclusion:**

Our results indicate that oocyte vitrification procedure followed by ICSI is not inferior to fresh insemination procedure, with regard to fertilization and embryo developmental rates. Moreover, ongoing clinical pregnancy is comparable with this procedure, even with a restricted number of oocytes available for inseminat-ion. We believe that these results will help the spread of vitrification for human oocytes cryopreservation.The promising clinical results obtained, in a population of infertile patients, need to be confirmed on a larger scale.

Rienzi et al., 2010, Human Reprod., 25, 66-73

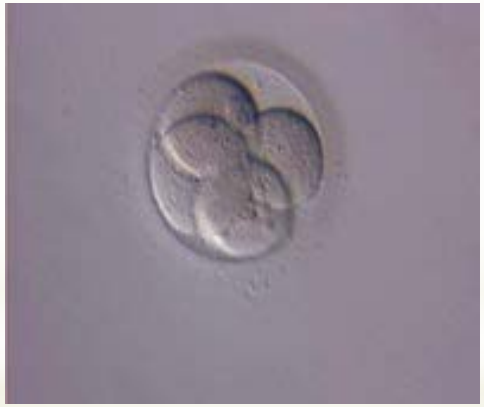
Before Vitrification



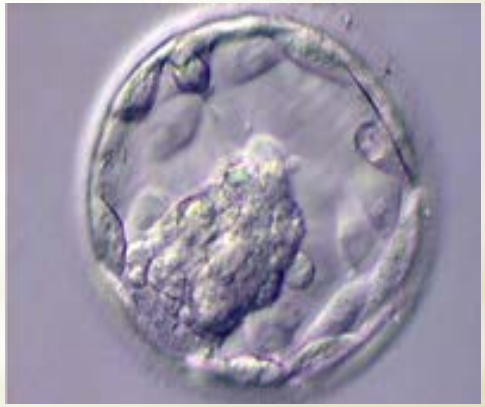
Just after Thawing

2hrs after Culture

PN stage (Day 1)



4-cell stage (Day2)



Blastocyst (Day5)

Figs. 5 Human oocytes before and after vitrification, ICSI and IVC.

Clinical outcomes: Oocyte vitrification infertile population

Human Reproduction, Vol.00, No.0 pp. 1–7, 2010
doi:10.1093/humrep/deq046

human reproduction

ORIGINAL ARTICLE *Infertility*

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

Filippo Ubaldi [1], Reno Anniballo [2], Stefania Romano [1], Elena Baroni [1], Laura Albricci [1], Silvia Colamaria [1], Antonio Capalbo [1], Fabio Sapienza [1], Gábor Vajta [3], and Laura Rienzi [1,*]

[1]G.EN.E.R.A Centre for Reproductive Medicine, Clinica Valle Giulia, Via G. De Notaris 2, 00197 Rome, Italy [2]Andrology Center 'John McLeod', via F. Petrarca, Naples, Italy [3]James Cook University, Cairns Campus, Cairns, QLD, Australia

Clinical outcomes: Oocyte vitrification infertile population

- 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., Human Reproduction 2010

Clinical outcomes: Oocyte vitrification infertile population

- 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.

Ubaldi et al., Human Reproduction 2010

Clinical outcomes: Oocyte vitrification infertile population

Table I Baseline patient's characteristics, fresh and warming cycles laboratory outcomes.

Baseline patient's characteristics	
No. patients	182
Mean age (mean ± SD)	35.81 ± 4.19
Mean basal FSH (mean ± SD)	6.5 ± 2.23
Agonist protocol (%)	143/182 (78.6%)
Antagonist protocol (%)	39/182 (21.4%)
Fresh cycle: laboratory outcomes	
COC (mean ± SD)	12.8 ± 4.7
MII (mean ± SD)	10.1 ± 3.5
Inseminated MII (mean ± SD)	2.95 ± 0.40
2 PN (mean ± SD)	2.57 ± 0.61
Top quality embryos (mean ± SD)	1.51 ± 0.96
Embryo transferred (mean ± SD)	2.52 ± 0.59
Oocyte vitrified (mean ± SD)	6.22 ± 3.08
Warmed cycles: laboratory outcomes	
Warmed MII (mean ± SD)	4.23 ± 1.23
Survived MII (mean ± SD)	3.80 ± 0.89
Inseminated MII (mean ± SD)	2.97 ± 0.16
2 PN (mean ± SD)	2.54 ± 0.65
Top quality embryos (mean ± SD)	1.47 ± 0.91
Embryo transfer (mean ± SD)	2.40 ± 0.80

COC, cumulus-oocyte complex; MII, metaphase II; PN, pronucleus.

44.6% of our patients,
39.9% of cycles

Ubaldi et al., Human Reproduction 2010

Oocyte vitrification neonatal outcomes

Neonatal data on 221 infants

Supplementary Table III. Vitrification of oocytes. Neonatal outcome.

First author, year of publication, country	Study period	Freezing protocol	Live births	Duration of gestation (weeks)	Weight (g)	Comments
Kuleshova,. 1999, Italy	1998	Vitrification	1	37	3,500	Normal female karyotype
Katayama, 2003, Japan	2002	Cryotop vitrification	1	NA	6-pound, 9-ounce	Healthy
Yoon., 2003, Korea	1997-2002	Vitrification	5 singletons 1 set of twins	NA	NA	Healthy (4 had amniocentesis, all normal)
Kuwayama, 2005, Japan	NA	Cryotop vitrification	7	NA	NA	Healthy
Kyono, 2005 Japan	NA	Vitrification	1	NA	3,000	Healthy
Antinori, 2007, Italy	2004-2006	Cryotop vitrification	3	NA	NA	Healthy
Chen, 2008, China	NA	Cryoloop Vitrification	1	38	3,090	Normal karyotype
Chian, 2008, Canada	NA	Cryoleaf or cryptop vitrification	151 singletons 49 multiples	Singletons 37+3 Multiples 35+5	Singletons 2,920±370 Multiples 2,231±550	Congenital anomalies 2.5 % (1 biliary atresia, 1 clubfoot, 1 skin hemangioma, 2 ventricular septal defects)
Total number of infants with some information of health status			221			

Infants conceived following oocyte vitrification are not associated with increased risk of adverse obstetric and perinatal outcomes

Wennerholm et al., 2009

COMPARISON OF ANEUPLOIDY RATES OF BLASTOCYST STAGE EMBRYOS DERIVED FROM FRESH AND VITRIFIED OOCYTES

	Control n=16	Vitrification n=10	P
Female Age			
Mean (+/-SD)	29.4 (+/-5.4)	28.0 (+/-1.0)	NS
# Cells per Blast			
Mean (+/-SD)	43.0 (+/-13.4)	38.0 (+/-32.8)	NS
Normal cells per blast			
Mean (+/-SD)	23.0 (+/-14.7)	20.0(+/-28.5)	NS
Total # of normal cells	368	200	NS
% Normal cell	56.5	52.6	NS
Total # of cells	688	380	NS

Oocyte cryopreservation birth 'case reports' 1986–2008

Parameter	Cryopreservation method		
	Slow-freeze	Vitrification	Both
No. of embryo transfers	1974	834	19
No. of liveborn babies	282	285	12
Baby gender (gender information available for 168 slow-freeze, 189 vitrification and 12 both methods)	99 female, 69 male	86 female, 103 male	8 female, 4 male
Birth defects	1 ventricular septal defect, 1 choanal atresia, 1 Rubenstein-Taybi syndrome	2 ventricular septal defect, 1 biliary atresia, 1 clubfoot, 1 skin haemangioma	None

Adapted Noyes N, Porcu E Borini A. Reprod BioMed Online 2009. http://www.rbmonline.com/Article/3971 [e-pub ahead of print on 8 April 2009].

Oocyte vitrification: results

Efficiency in donation program not compromised with vitrification *(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, 2008)*

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

Conclusions on Egg Banking

- Similar outcomes with fresh and frozen eggs
- Eliminating difficulty of synchronization
- Decreasing risks of disease contamination
- May prevent most of the moral/ethical questions of extra embryos
- More cost effective – 5 frozen eggs / implantation

Current results validate the use of oocyte cryo-banking for egg donation purposes

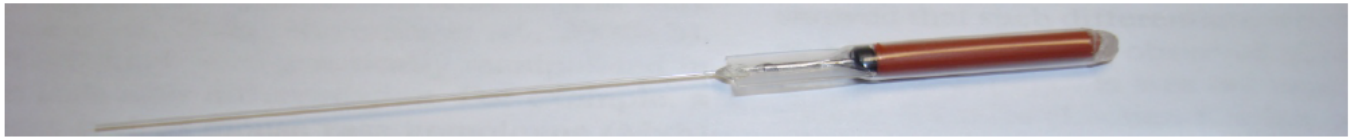
Open or Closed system?

CLOSED SYSTEMS (heat sealing):

- More "easily accepted" in daily IVF use – Prevention of "contamination.

- It works for slow-freezing for embryo/oocyte, reasonably for vitrifiation of embryos – but questionable for vitrification of oocytes.

- It may be questionable if closed system truly prevents (biological) particles passing through (material feature at -196C?)



OPEN SYSTEMS:

- It's use is questioned because of "contamination" "risk". – However, no proven evidence that contamination occurred with oocyte/embryo storage.

- It works well both for embryos and oocytes.

PN oocytes vitrification clinical outcomes

Table 1. Major outcomes of the cycles of cryopreservation and embryo transfer.

Parameter	
No. of patients	92
No. of 2PN zygotes vitrified	849
No. of 2PN zygotes warmed	339
No. of 2PN zygotes survived	302
Survival rate (%)	89
No. of cycles of embryo transfer	111
No. of completed cycles of embryo transfer	103
No. of transferred embryos	243
No. of embryo transfers	106
Mean no. of embryos per transfer	2.3
No. of positive HCG tests	41[a]
No. of biochemical pregnancies	9
No. of clinical pregnancies	29
Implantation rate (%)	15.6
Pregnancy rate (%)	36.9
Clinical pregnancy rate (%)	28.2
No. of ongoing pregnancies	15
No. of spontaneous abortions	5
No. of deliveries	9
No. of infants delivered	18
Incidence of twins	5
Incidence of triplets	2

The pregnancy rate obtained was three times higher (36.9%) than that obtained with the slow-rate freezing method (10.2%) used previously in the same centre.

Vitrification of human zygotes at the pronuclear stage is a successful and reliable method with favourable outcomes and can be recommended as a routine technique for cryopreservation of human embryos.

Al-Hasani et al., 2007

Oocyte cryopreservation for donor egg banking

Ana Cobo [a], José Remohí [a], Ching-Chien Chang [b], Zsolt Peter Nagy [b,*]

[a] *IVI-Valencia, Plaza de la Policía Local 3, 46015 Valencia, Spain;* [b] *Reproductive Biology Associates, 1150 Lake Hearn Dr., Suite 600, Atlanta, GA 30342, USA*

[*] Corresponding author. *E-mail addresses:* zsolt.peter.nagy@gmail.com, peter.nagy@rba-online.com (ZP Nagy).

Ana Cobo obtained her first degree at the University of Valle in Colombia in 1988. She obtained an MSc degree in the Biology of Reproduction at the University of Chile in 1994. After moving to Valencia, Spain in 1995, she joined Instituto Valenciano de Infertilidad (IVI) as part of the embryology staff. She obtained a Master's degree in Human Reproduction in 1998 and a PhD in 2003 at the University of Valencia, Spain. She is currently in charge of the cryobiology unit at IVI-Valencia. Her major areas of interest are oocyte and embryo cryopreservation and oocyte morphology linked to embryo development.

Table 1 2008–2009 (24 month) outcome data using vitrified donor oocytes in IVF treatment for recipients in two IVF centres.

Outcome	IVI	RBA
Donation cycles	1051	168
Recipient cycles	919	322
Age (years)	41.2 ± 4.3	41.1 ± 4.9
Total oocytes warmed (per recipient)	12,786 (12.9 ± 4.0)	2001 (6.2 ± 1.9)
Total oocytes for ICSI	11,949 (11.4 ± 3.4)	1750 (5.4 ± 1.7)
Two-pronuclei ICSI fertilization rate	8920 (74.7)	1494 (85.4)
Good-quality embryos on day 3 (per inseminated oocyte)[a]	5366/11,949 (44.9)	979/1750 (55.9)
Good-quality embryos on day 5 (per embryo subjected to extended culture)[a]	1427/3568 (40.0)	582/1185 (49.1)
Implantation rate	655/1655 (39.6)	255/577 (44.2)
Embryos cryopreserved	1915 (1.8 ± 2.0)	414 (1.3 ± 1.5)
Clinical pregnancies (per transfer)[b]	502 (55.4)	182 (56.5)
Infants born[c]	343 (180 female; 163 male)	146 (64 female; 82 male)

Values are *n*, mean ± standard deviation, *n* (mean ± standard deviation), *n* (%) or *n*/total (%). ICSI = intracytoplasmic sperm injection, IVI = Instituto Valenciano de Infertilidad; RBA = Reproductive Biology Associates.

[a] IVI and RBA embryo scoring systems are different, thus it may not be possible to directly compare these numbers.

[b] Additionally, 25 (IVI) and 21 (RBA) more clinical pregnancies from subsequent embryo cryotransfer were obtained.

[c] Additionally, 10 infants born from subsequent embryo cryotransfer at IVI (six female and four male) and 17 infants at RBA (nine female and eight male). There is no data on all infants born during this period. Four newborns at RBA had birth defects.

The study centres' experience with the storage of oocytes for donation shows a positive impact on the management of an oocyte donation programme, becoming easier and much more efficient at achieving excellent clinical results, in fact as high as obtained with fresh donor oocytes. Additionally, egg cryobanking provides the possibility of distributing oocytes among two or more recipients, without facing any difficulties of endometrial synchronization, which can also make the treatment more economical and so more affordable.

Oocyte donation as a form of IVF treatment should and will be performed only through egg cryobanking, as it provides a more efficient, safer and more affordable alternative to fresh oocyte donation.

*Korzyści witryfikacji

* znacznie wyższa skuteczność pod względem uzyskanych ciąż z zamrożonych komórek jajowych lub zarodków
* pod względem etycznym dla części pacjentek; witryfikacja umożliwia świadomy wybór i rezygnację z mrożenia nadliczbowych zarodków na rzecz zamrożenia komórek jajowych
* skuteczne leczenie niepłodności dla kobiet źle reagujących na stymulację hormonalną (tzw. low responders). Witryfikacja umożliwia bankowanie oocytów i po zebraniu i zamrożeniu kilku podejście do pełnej procedury in vitro (wzrastają szanse na powodzenie zabiegu)
* szansa na macierzyństwo dla kobiet przed leczeniem onkologicznym, bankowanie własnych komórek jajowych i ich wykorzystanie po zakończeniu leczenia onkologicznego jest szansą na urodzenie dziecka po terapii onkologicznej

*Korzyści witryfikacji

* W Polsce zazwyczaj mrozi się nadliczbową ilość zarodków powstałych w skutek zabiegu in vitro. Są to zarodki, których nie podało się podczas pierwszego transferu pacjentce.
* Nadwyżka zarodków wynika z rekomendacji medycznych dotyczących ilości jednorazowo podawanych zarodków. Przyjęto, że można podawać maksymalnie 3 zarodki (najczęściej jednak podaje się tylko 1 lub 2 zarodki). Jest to podyktowane ochroną pacjentki przed ciążą mnogą (trojaczki, czworaczki).
* Jednym z rozwiązań problemu nadliczbowych zarodków jest mrożenie komórek jajowych bez ich zapłodnienia.
* Obecna metoda mrożenia tzw. slow freezing, ma jednak sporo wad i nie jest aż tak skuteczna, jak chcieliby tego lekarze i pacjentki. Po rozmrożeniu komórek jajowych znaczna ich część obumiera. Rozwiązaniem tego problemu jest witryfikacja.
* Witryfikowane komórki jajowe można wykorzystać, jeżeli pacjentka nie zajdzie w ciążę po podaniu niemrożonych zarodków

*Witryfikacja blastocyst

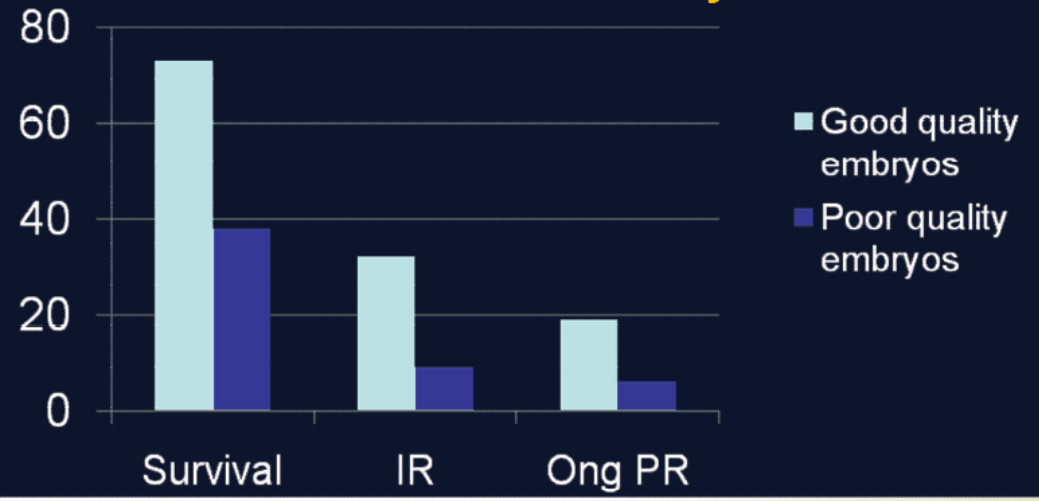
Blastocysts vitrification

Table 1: Comparison of survival, implantation and pregnancy rates according to loading device

	Loading Device	Sample Size	Survival Rate	Implantation Rate	Pregnancy Rate
Mukaida et *al*, 2001[8]	Cryoloop	N = 60	63%	--	31%
Cho, 2002 et *al* [21]	EM grid	N = 21	83%	--	34%
Reed et *al*, 2002[10]	Cryoloop	N = 54	100%	15%	--
Mukaida et *al*, 2003[9]	Cryoloop	N = 725	80%	20%	37%
Osada et *al*, 2003[11]	Cryotop	N = 580	99%	--	56%
Stehlik et *al*, 2005[12]	Cryotop	N = 41	100%	--	50%
Takahashi et *al*, 2005[19]	Cryoloop	N = 1129	86%	29%	44%
Kuwayama et *al*, 2005[18]	Cryotip	N = 5695	90%	--	53%
Liebermann et *al*, 2006[13]	Cryotop	N = 547	97%	31%	46%
Mukaida et *al*, 2008[29]	Cryoloop	N = 5412	92%	36%	49%

A number of variables determine the outcomes of blastocyst vitrification:

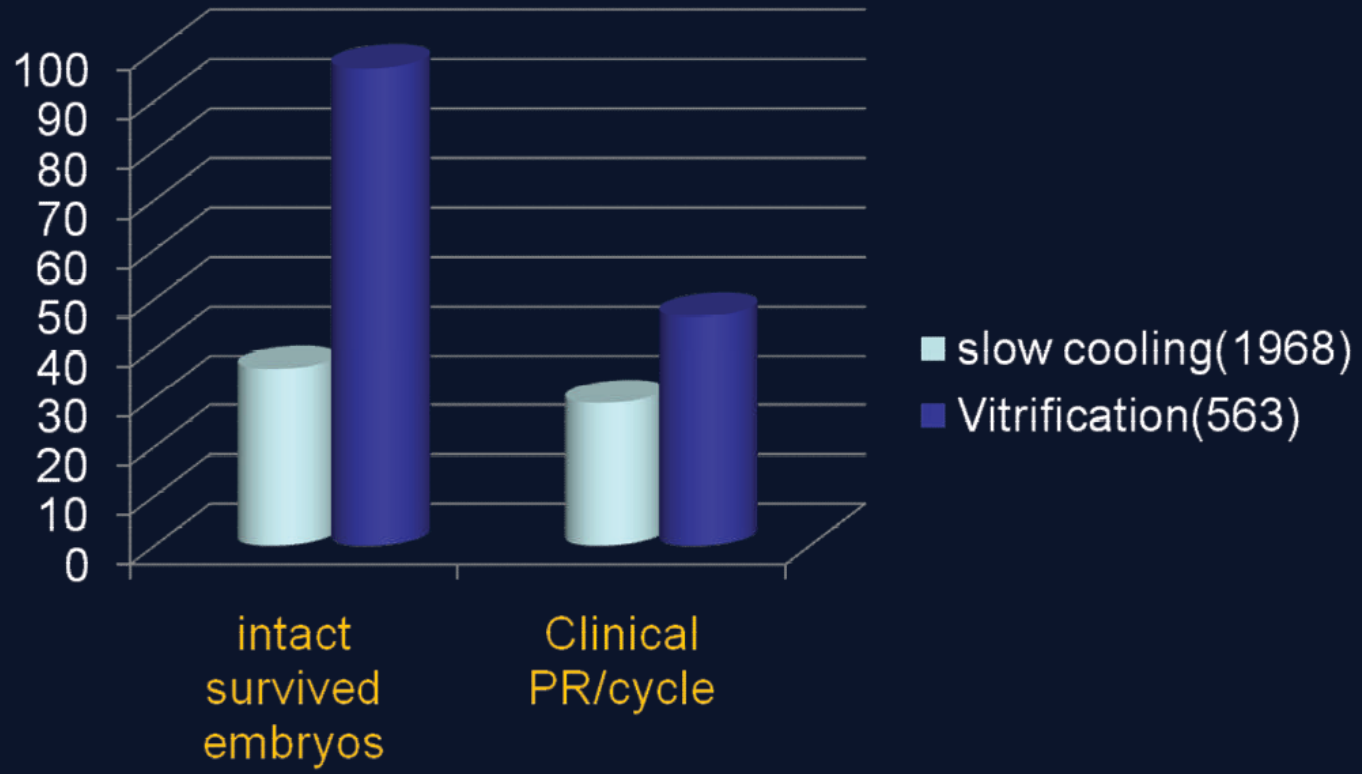
- **Pre-vitrification blastocyst selection;**
- **Post-warming blastocyst selection**



Clinical outcomes: embryos vitrification

Cryotop Vitrification method provides improved embryo survival rates with a higher proportion of intact survived embryos that should be responsible of the better clinical outcome observed.

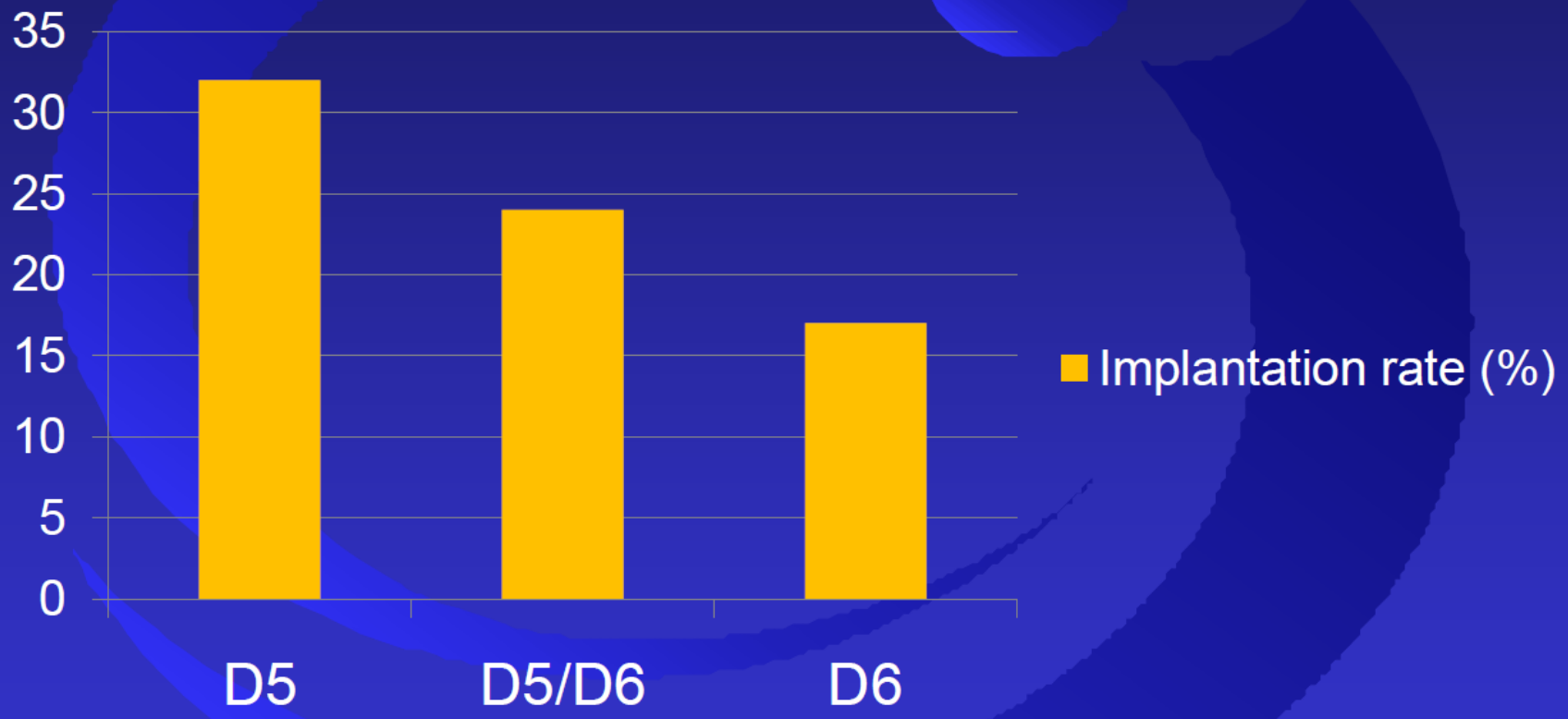
Cobo et al., ASRM 2009



Consistent and reproducible results

D5 and D6 differences (OD)

Mean age = 43, n=177



	D5 only	D5+D6	D6 only
Implantation/transfer	80/251 (32%)	8/33 (24%)	6/36 (17%)

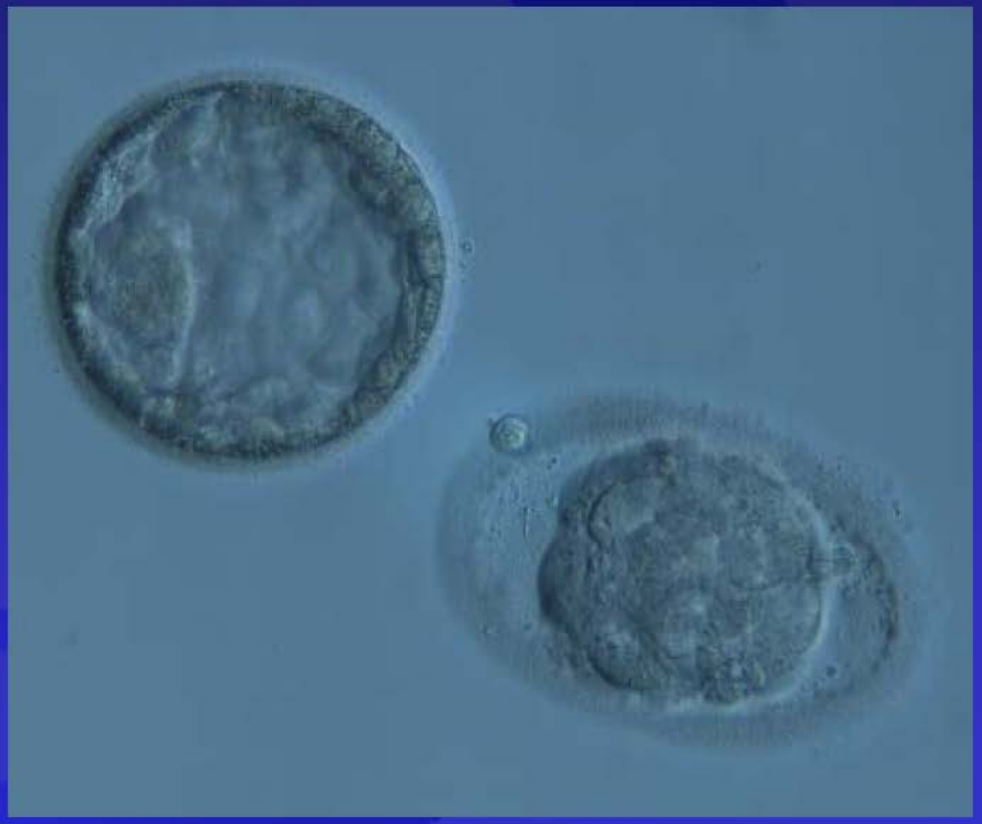
Differences not significant (D5 vs. D6: p=0.07)

Warming results: Clinical

Patient age	<35	35-37	38-40	>40	OD
Cycles	164	80	76	21	217
Pregnancies	90	31	27	7	88
Pregnancy rate	55%	39%	36%	33%	41%
Emb. Transferred (mean)	295 (1.8)	145 (1.8)	150 (2.0)	51 (2.4)	380 (1.7)
Sacs	112	35	35	10	120
Implantation rate	38%	24%	23%	20%	32%

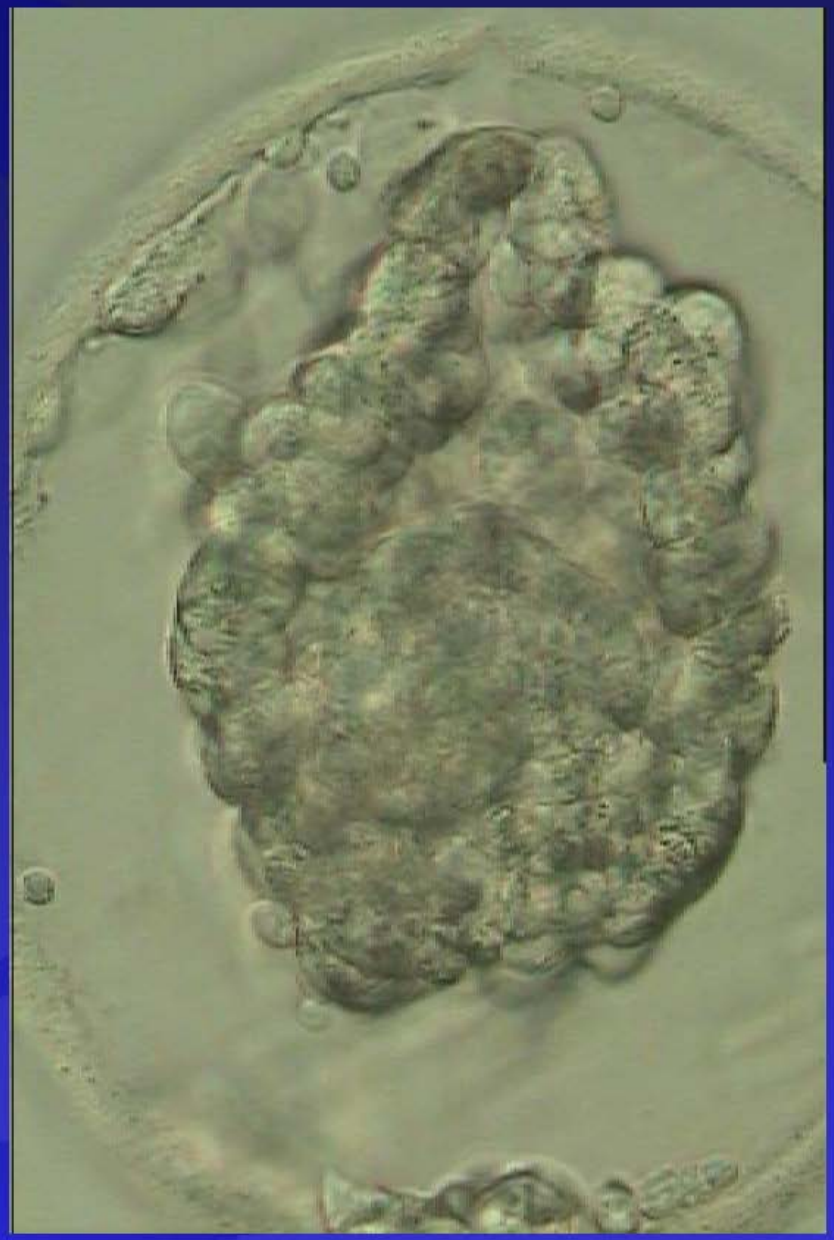
Blastocyst survival

- Blastocysts look very nice during and immediately after warming.
- Most ET's done within 1 hour of warming
- Culture and ET in 20% SSS

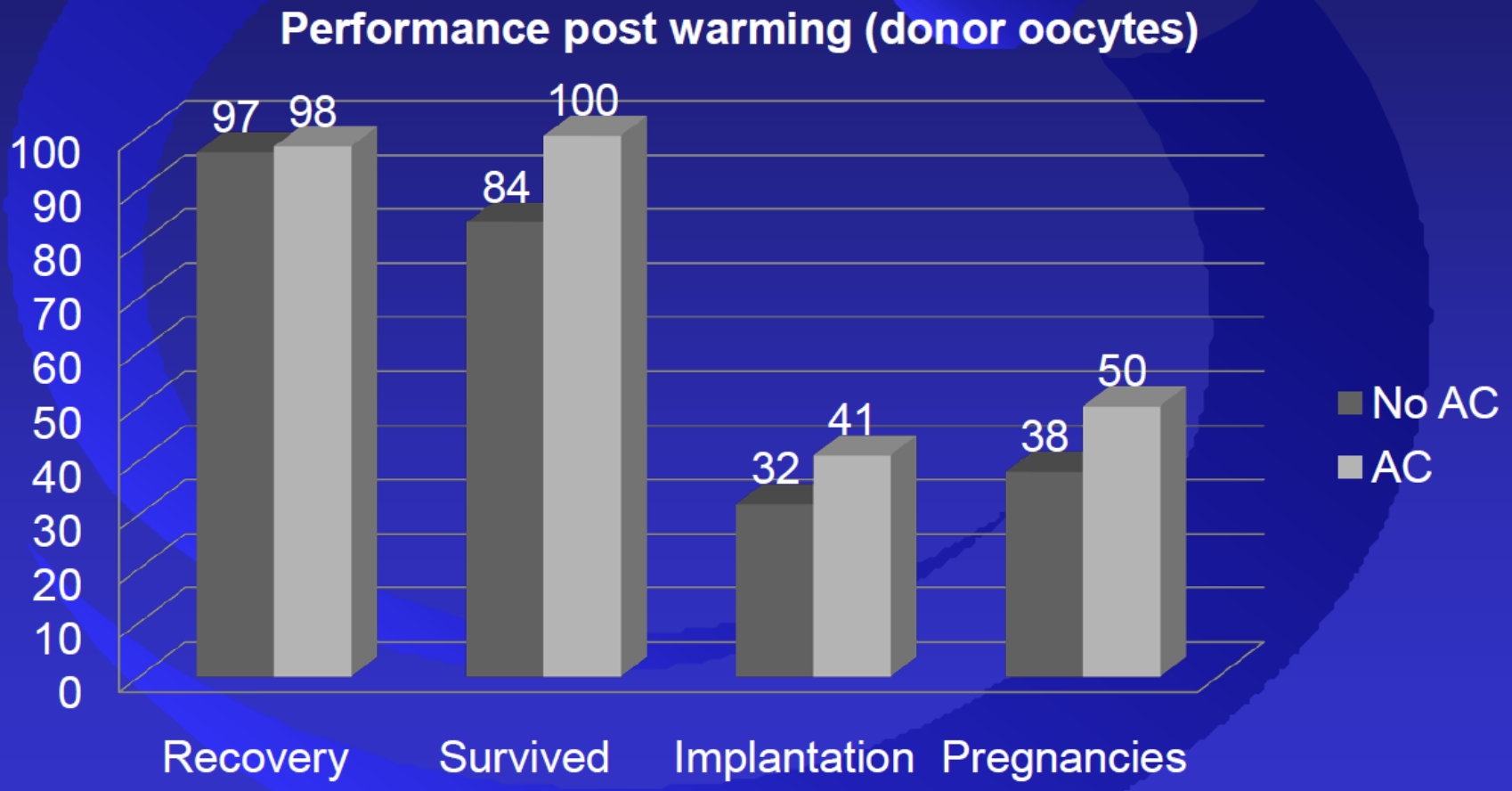


Artificial collapse of blastocysts

- Blastocysts that do not survive warming usually come out of straw fully expanded
- Collapsing will eliminate this problem
- Implemented summer 2009

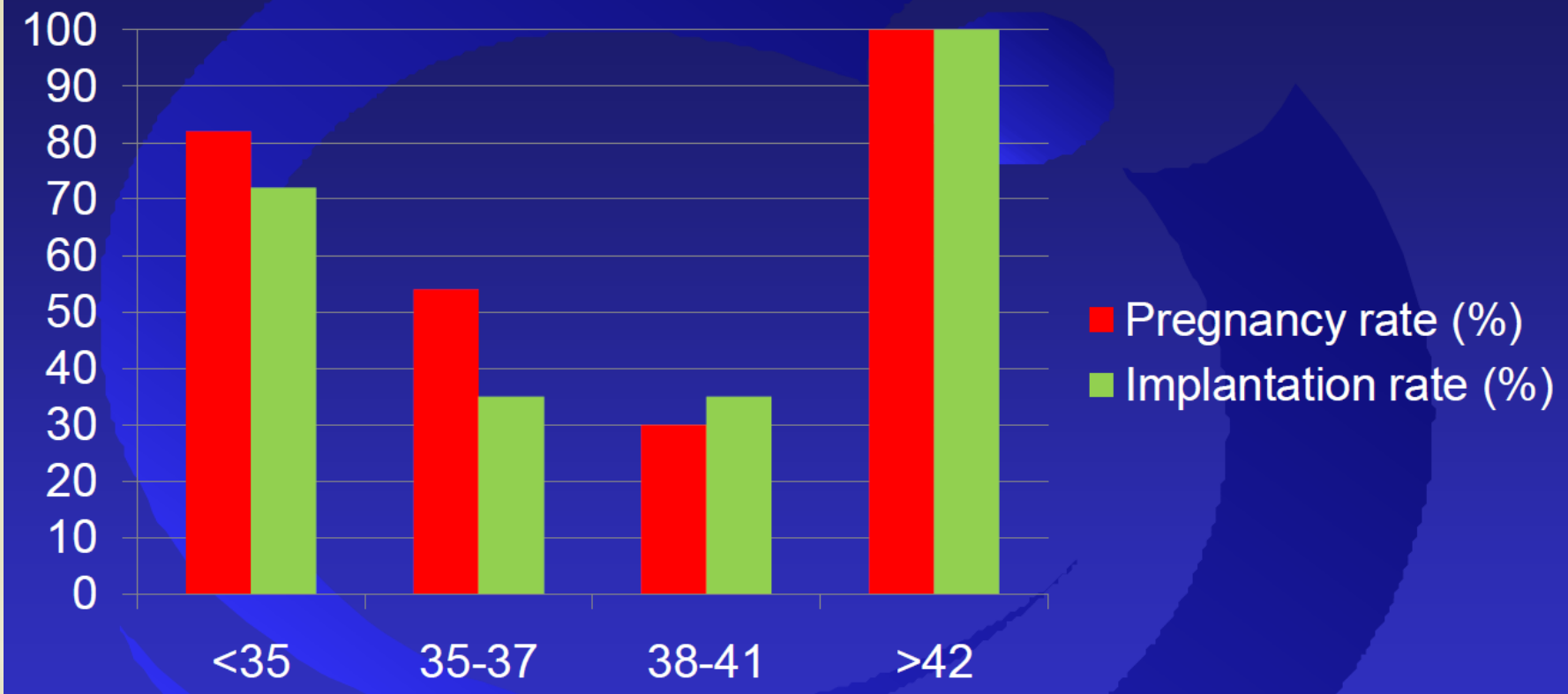


Does assisted collapse help?
Frozen cycle outcomes 2010

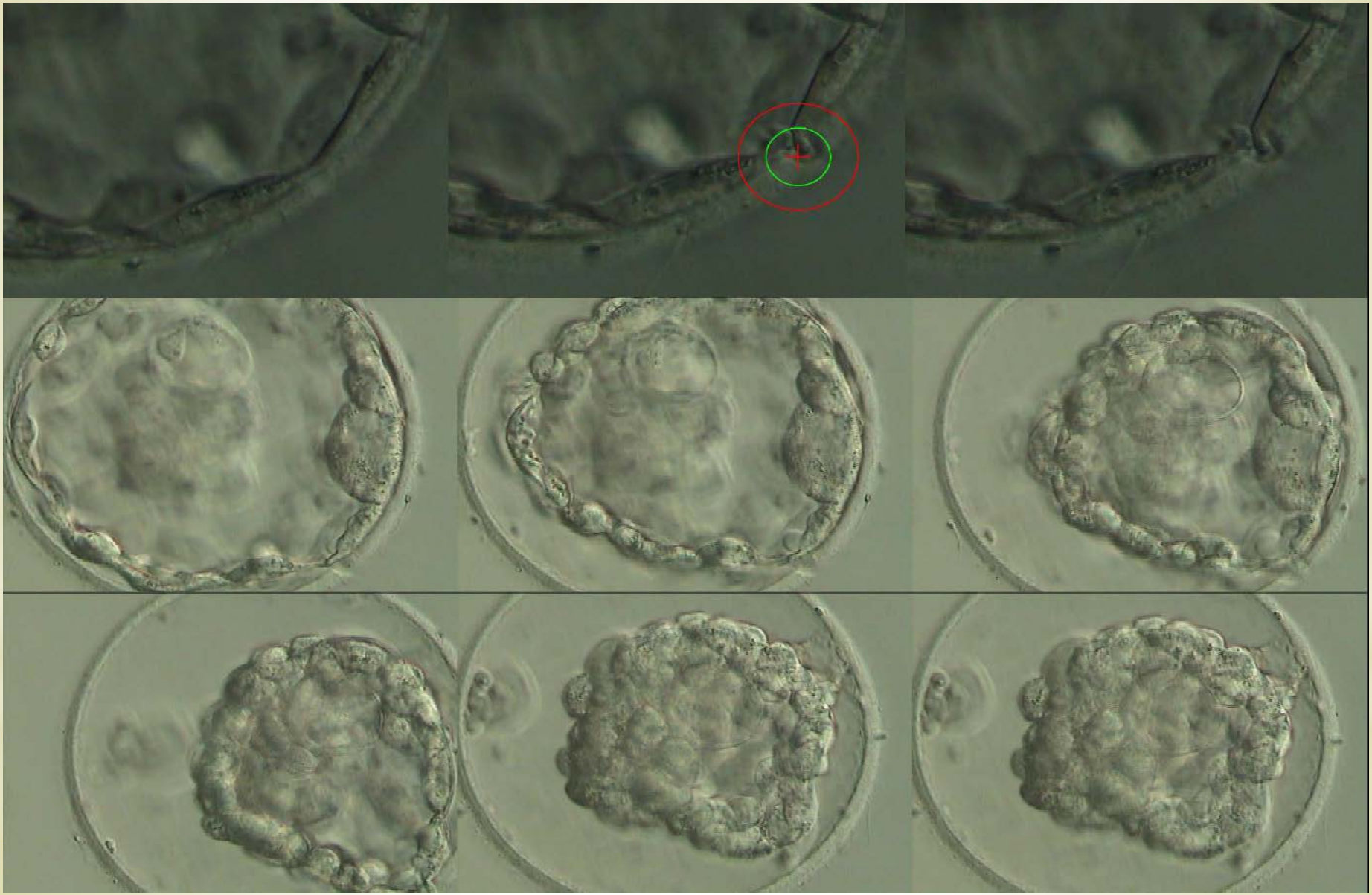


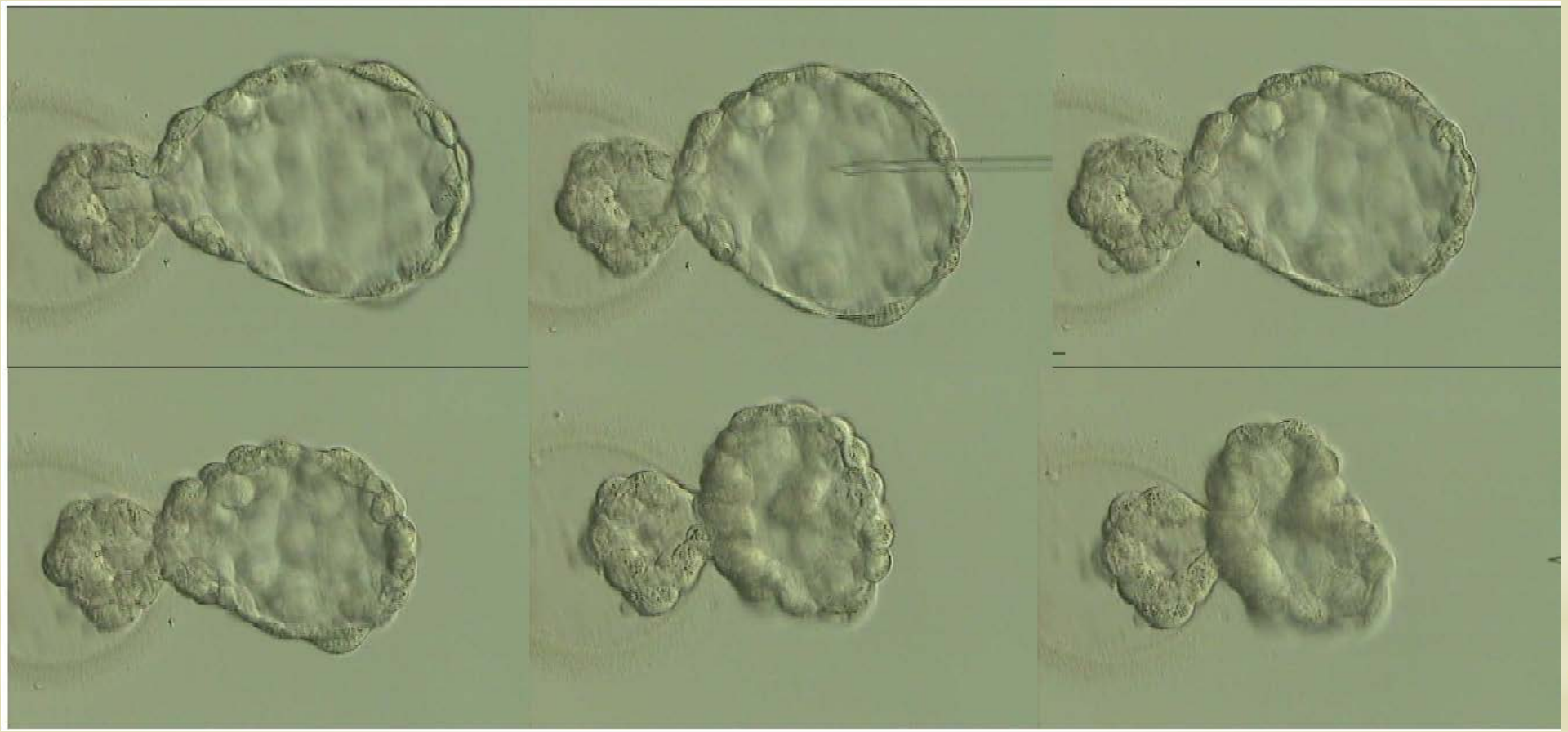
n = 58 for "no AC" group and 52 for "AC" group

Artificial Collapse



	<35	35-37	38-41	≥42
Pregnancy	14/17	7/13	3/10	1/1
Implantation	15/20	7/20	7/20	1/1
Av. transferred	1.2	1.5	2	1





Blastocyst vitrification: neonatal outcomes

252 children born after blastocysts vitrification

Supplementary Table I. Vitrification of blastocysts. Neonatal outcome.

First author, year, country	Study period	Protocol	Live births	Duration of gestation	Weight (g)	Comments
Choi, 2000, Korea	1999-2000	Vitrification	3 singletons 2 sets of twins	NA	2,700-4,080	Normal karyotypes Normal physical profiles
Yokota., 2001, Japan	NA	Vitrification	1	39	3,026	Normal karyotype No anomalies
Son., 2003, Korea	NA	Artificial shrinkage of blastocoels before vitrification	3 singletons 6 sets of twins	NA	1,950-3,550	Normal physical profiles
Takahashi., 2005, Japan	2000-2003	Cryoloop vitrification	147 children (55 singletons, 40 sets of twins, 4 sets of triplets)	38.1±2.8	2,601 ±709	Birth defects: 1.4% Boys 74; Girls 73
Hiraoka, 2006, Japan	NA	Recryopreservation by vitrification	1 set of twins	36	2,155 2,590	Healthy
Mukaida., 2006, Japan	2003-2005	Artificial shrinkage of blastocoeles before vitrification	77	NA	NA	Healthy No bias in sex ratio
Hiraoka, 2007, Japan	NA	Vitrification of hatched blastocyst	2	NA	NA	Healthy
Parriego, 2007, Spain	NA	Vitrification of biopsied blastocysts	1	38	2,830	Healthy
Total number of infants with some information on health status			252			

No statistical differences in the mean gestational age, birth weight, preterm birth rate, or congenital birth defect rates as compared with fresh blastocyst transfer

Wennerholm et al., 2009

VIT-MASTER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THAW


LN2
EUROTHERM
2132
-195


LN2
EUROTHERM
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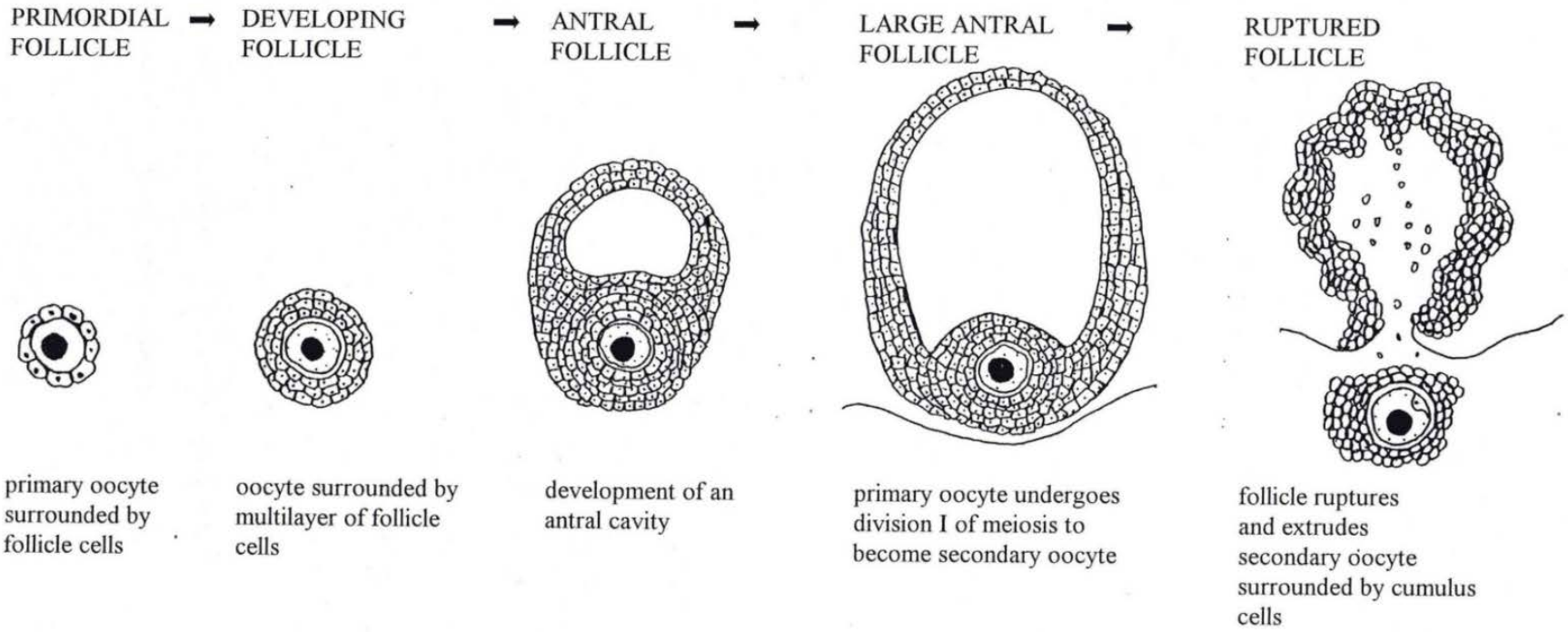
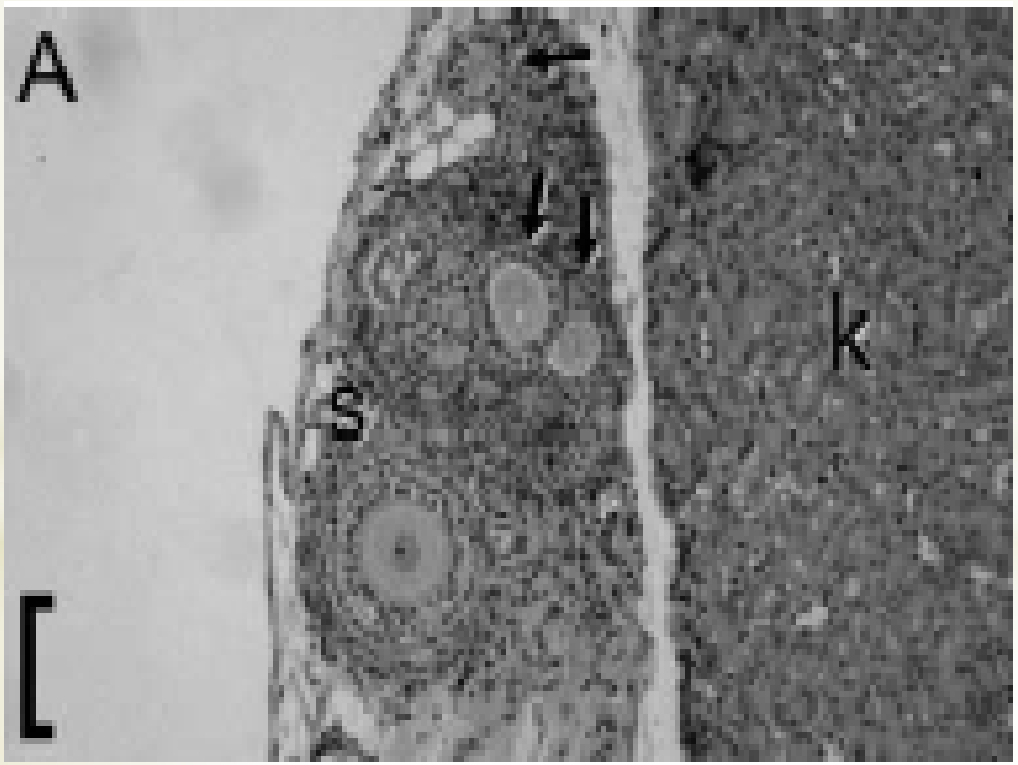
*Kriokonserwacja tkanek jajnika

* Recall that ovarian tissue contains follicles in a range of developmental stages. Most numerous are small primordial and primary follicles

Fig. 1.1 The stages of oocyte development



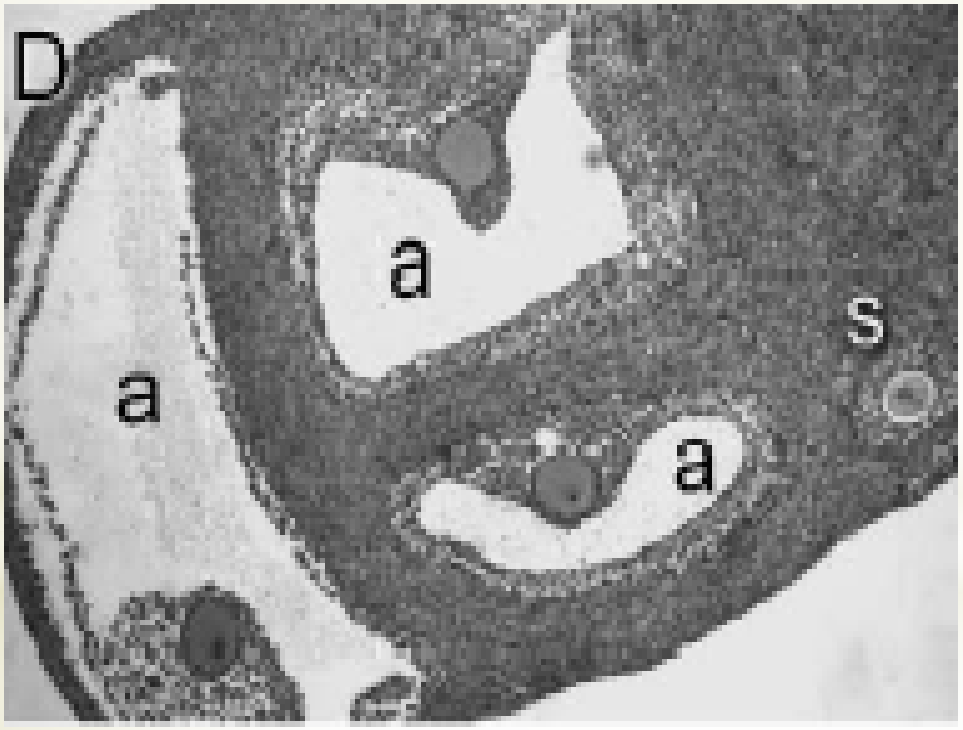
* Ovarian tissues normally cryopreserved as strips or fragments of few mm sizes



Most small follicles found in cortical tissue of ovary

Developing follicles in cortical strip; Gook D *et al.* (2004) Hum Reprod OnLine publication, October

Ovarian tissues normally cryopreserved as strips or fragments of few mm sizes

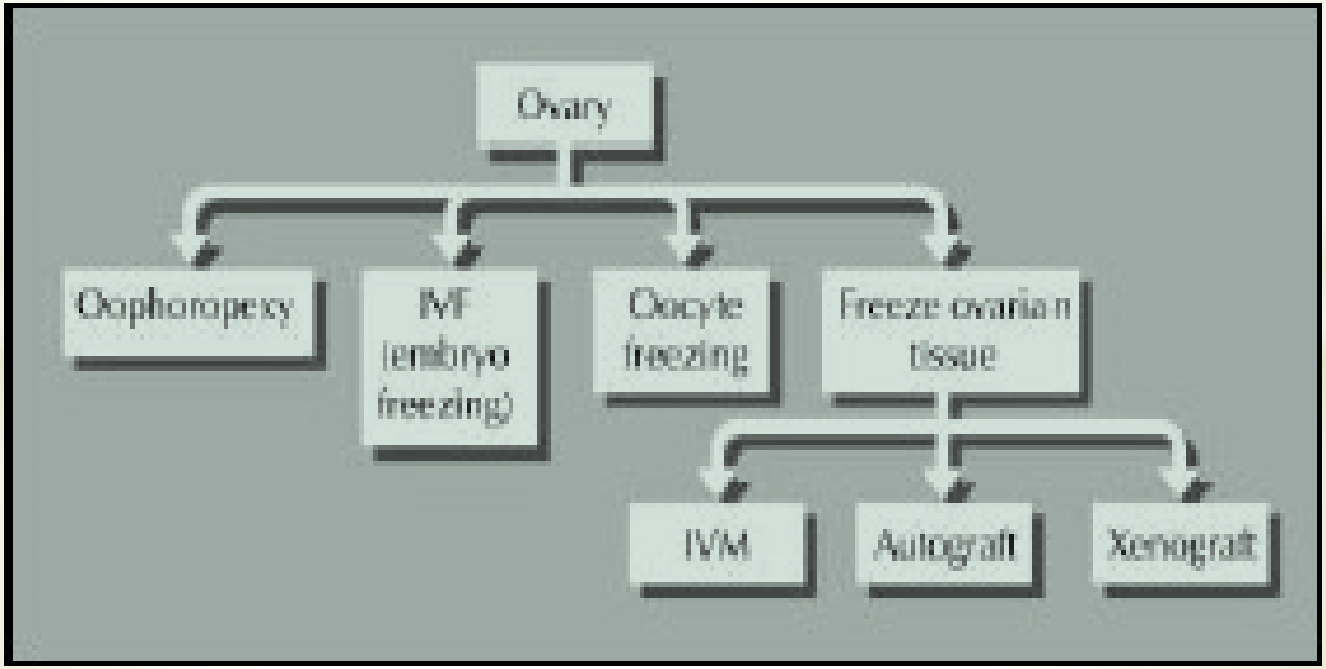


Antral follicles are rare, but can occasionally find them in the tissue

Developing follicles in cortical strip; Gook D *et al.* (2004) Hum Reprod OnLine publication, October
(These happen to be tissues developing *AFTER* cryopreservation)

* Ovarian Tissue - Recall

- In mammals, the final cohort of female reproductive cells (Oocytes) are present throughout the ovary in primordial follicles at birth.
- During sexual maturity, one dominant follicle develops and yields one mature MII oocyte per cycle



Ovarian tissue can be used as a source of immature oocytes for maturation and IVF, or as a transplant

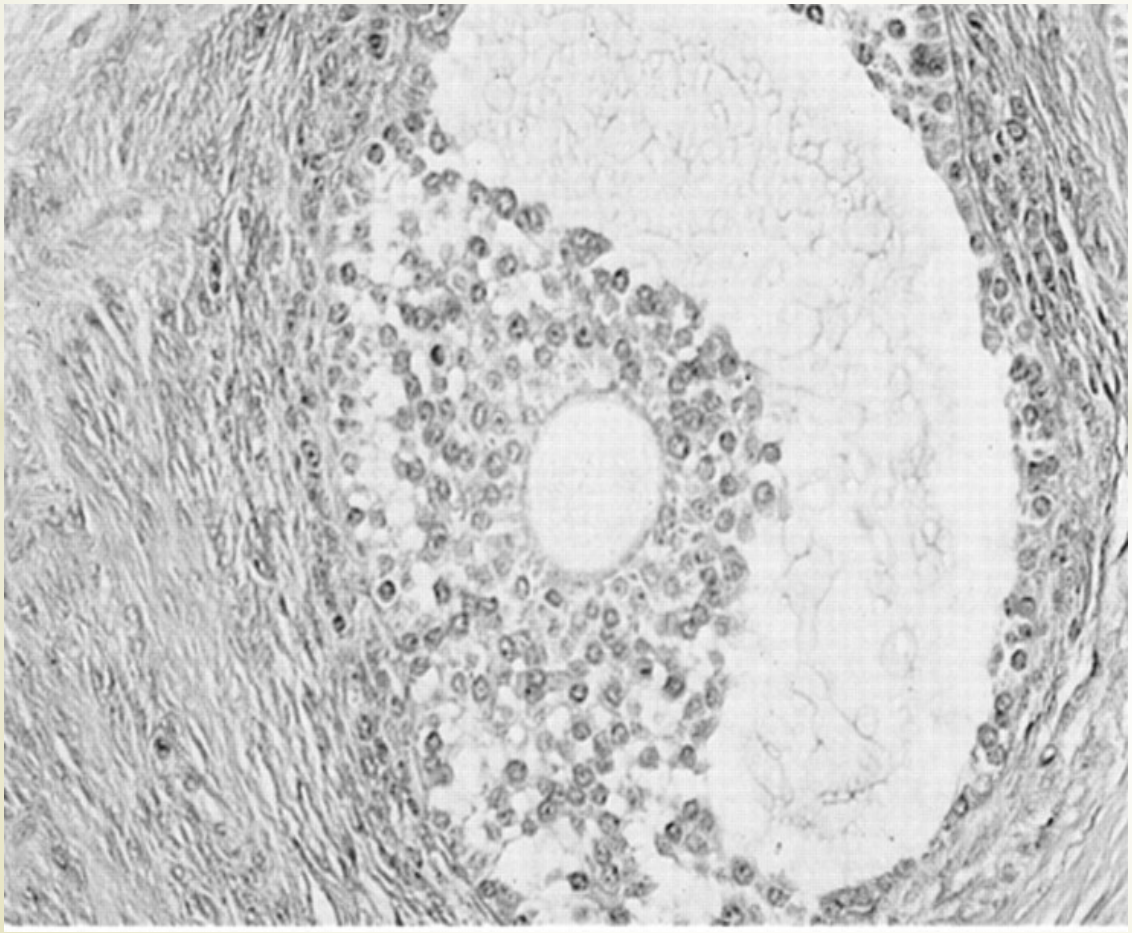
*Cryopreservation of Ovarian Tissues

- *At birth, the primordial follicle population of ovarian tissue represents the lifetime supply of oocytes
- *Primary ovarian failure may be induced in ‘young’ patients by anticancer treatments
- *Oophorectomised tissues could provide donor oocytes for clinical infertility treatment or species conservation

*Requirements of Cryopreservation

- *must preserve the ability of follicles (oocyte + supporting cells) to grow and acquire mature characteristics
- *must maintain cell-cell communications and correct sequence of hormone responsiveness

Ovarian tissue can be xenografted and assessed for follicle growth – for example primate or human ovarian tissue can be grafted into NODSCID mice under the skin (Experimental assessment only)

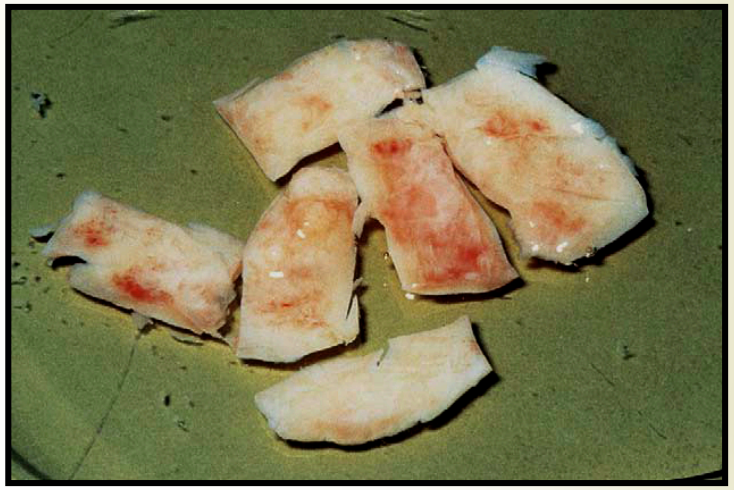


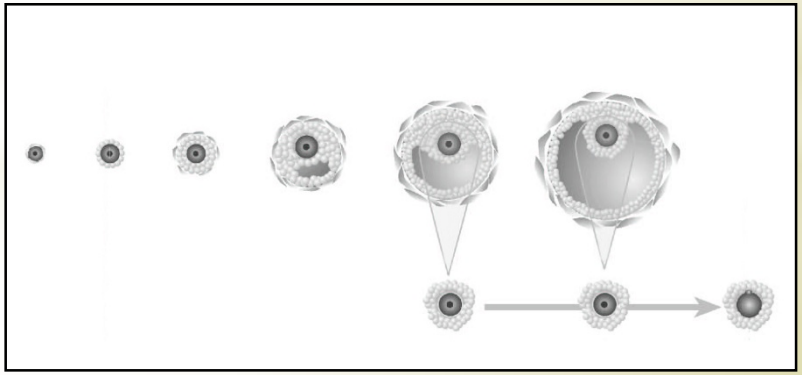
Growing follicle from ovarian tissue placed under skin

In the future Cryopreservation of „Ovarian Cortex“

* ovarian biopsy and cryopreservation

* in vitro Growth („IVG“) followed by In vitro Maturation („IVM“)





Oktay et al. 2004

*Witryfikacja tkanek jajnika

Successful vitrification of bovine and human ovarian tissue

Dr Noriko Kagawa began her career in 2000 in animal embryology and studied assisted reproductive technologies in the pig, establishing the porcine follicular growth system using severe combined immunodeficiency mice. She moved to the human IVF field in 2004 and has developed a vitrification method for mammalian ovaries. She obtained her PhD in 2005 from the Kyoto University, Japan. Her major research interests have focused on in-vitro growth of pre-antral follicles of adult mammals and vitrification of whole ovaries. She is currently the senior scientist at the world's largest human IVF unit (Advanced Medical Institute of Fertility, Kato Ladies' Clinic).

Dr Noriko Kagawa

Noriko Kagawa[1,3], Sherman Silber[2], Masashige Kuwayama[1]
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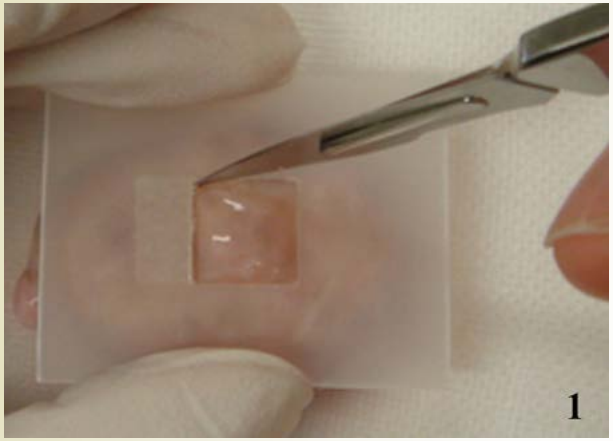
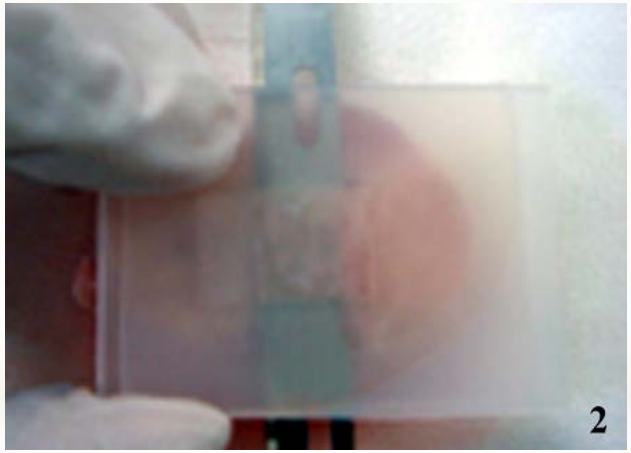
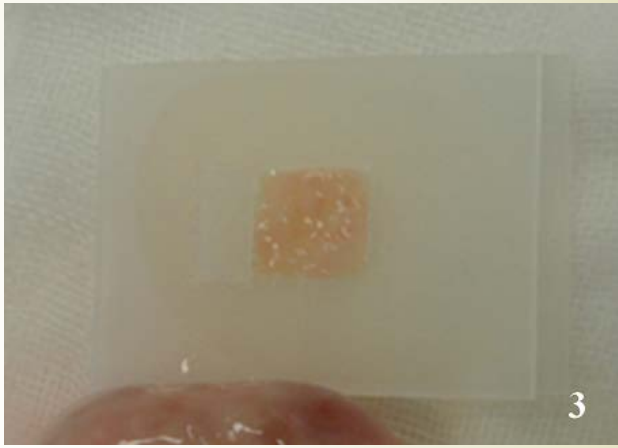
[3]Correspondence: e-mail: n-kagawa@towako-kato.com

Successful vitrification of bovine and human ovarian tissue

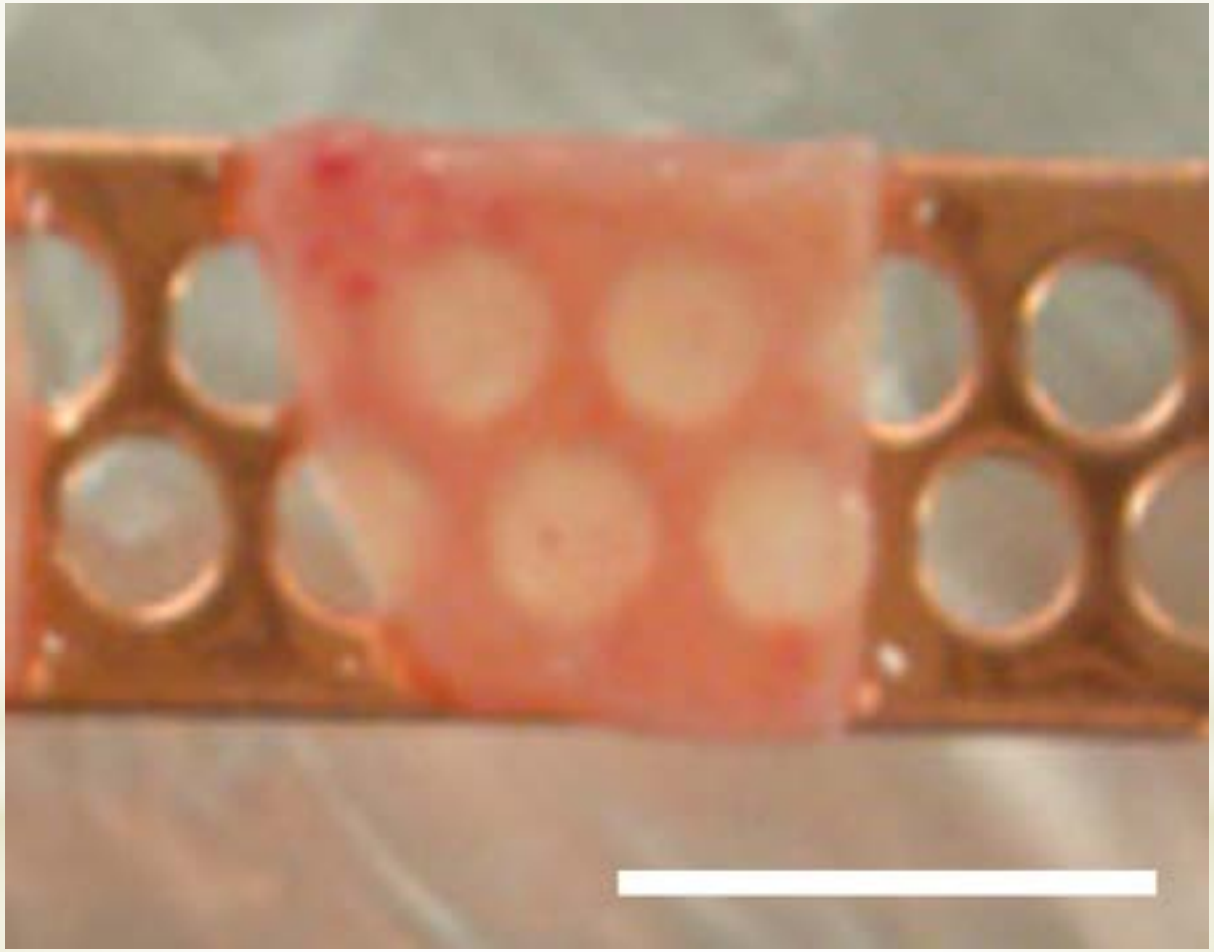






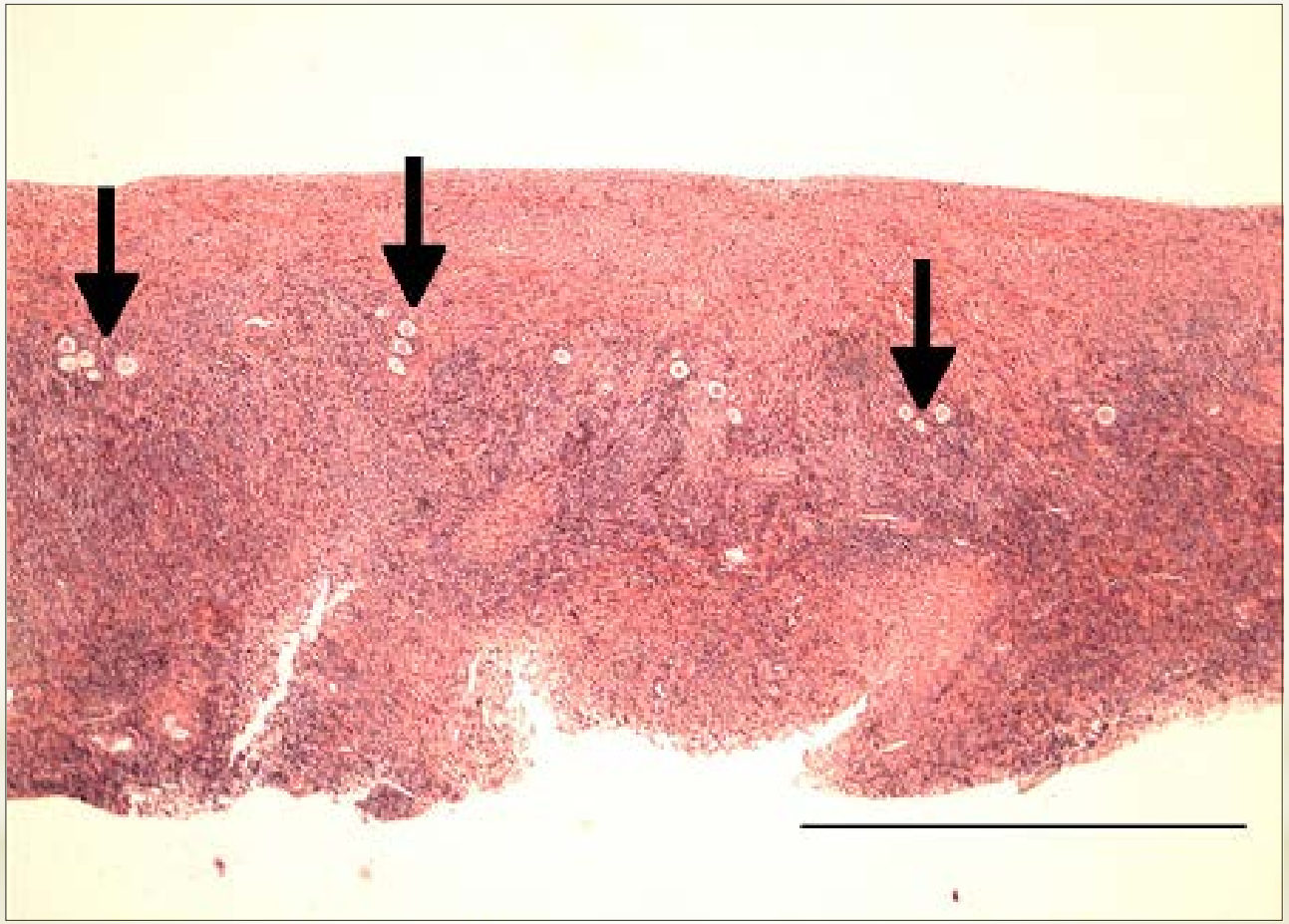
Vitrifcation procedure: the Cryotissue method.
The ovarian tissue slicer was developed, with a plate to produce 1 × 10 × 10 mm slices. (1) The tissue slicer was put on the surface of ovary. (2) Then another plate was put on the tissue slicer, the ovary was cut between the slicer and the surface of ovary by using a sharp edge. (3) The ovarian tissue was cut into 1 × 10 × 10 mm slices. Kagawa et al. RBM online, 2009

Successful vitrification of bovine and human ovarian tissue



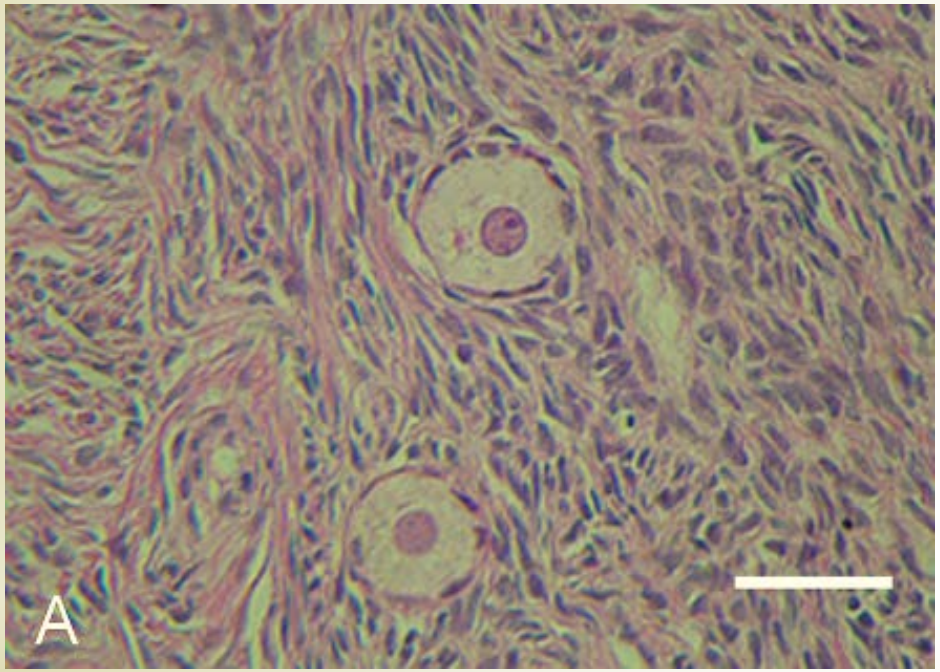
Gross morphology of vitrifed human ovarian tissue using the Cryotissue method. Vitrifed human ovarian tissue was translucent in liquid nitrogen (–196°C). Scale bar represents 10 mm.
Kagawa et al. , RBM online, 2009

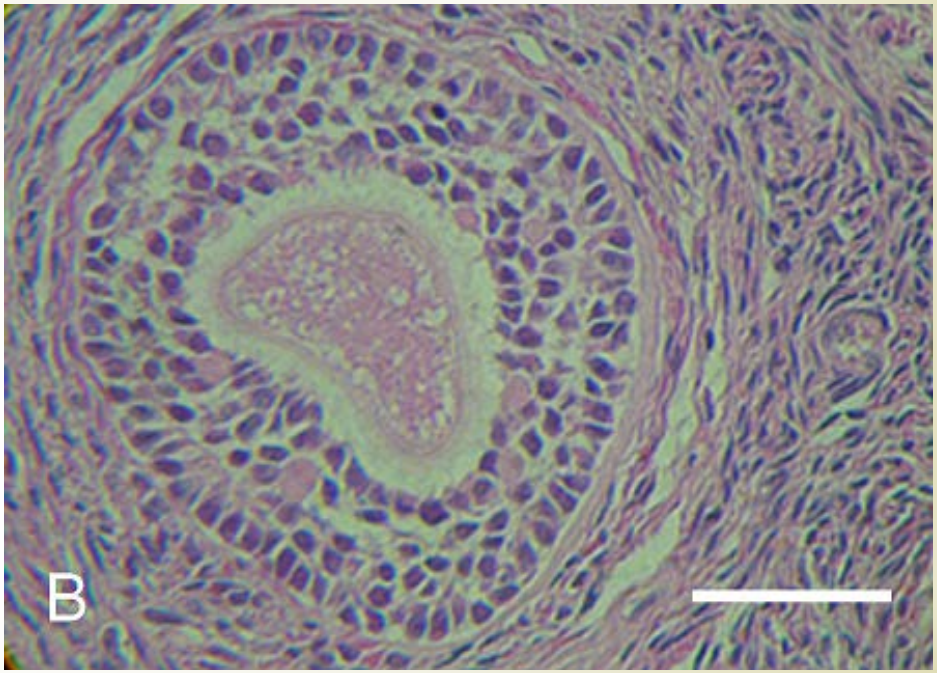
*Successful vitrification of bovine and human ovarian tissue



All oocytes (arrows) were located in the cortical area of the human ovarian tissue. Note that they were all located within 0.75 mm of the surface, allowing much thinner slices to be made than can be obtained by hand, or than have been used in previous studies. Scale bar represents 1 µm.
Kagawa et al. RBM online 2009

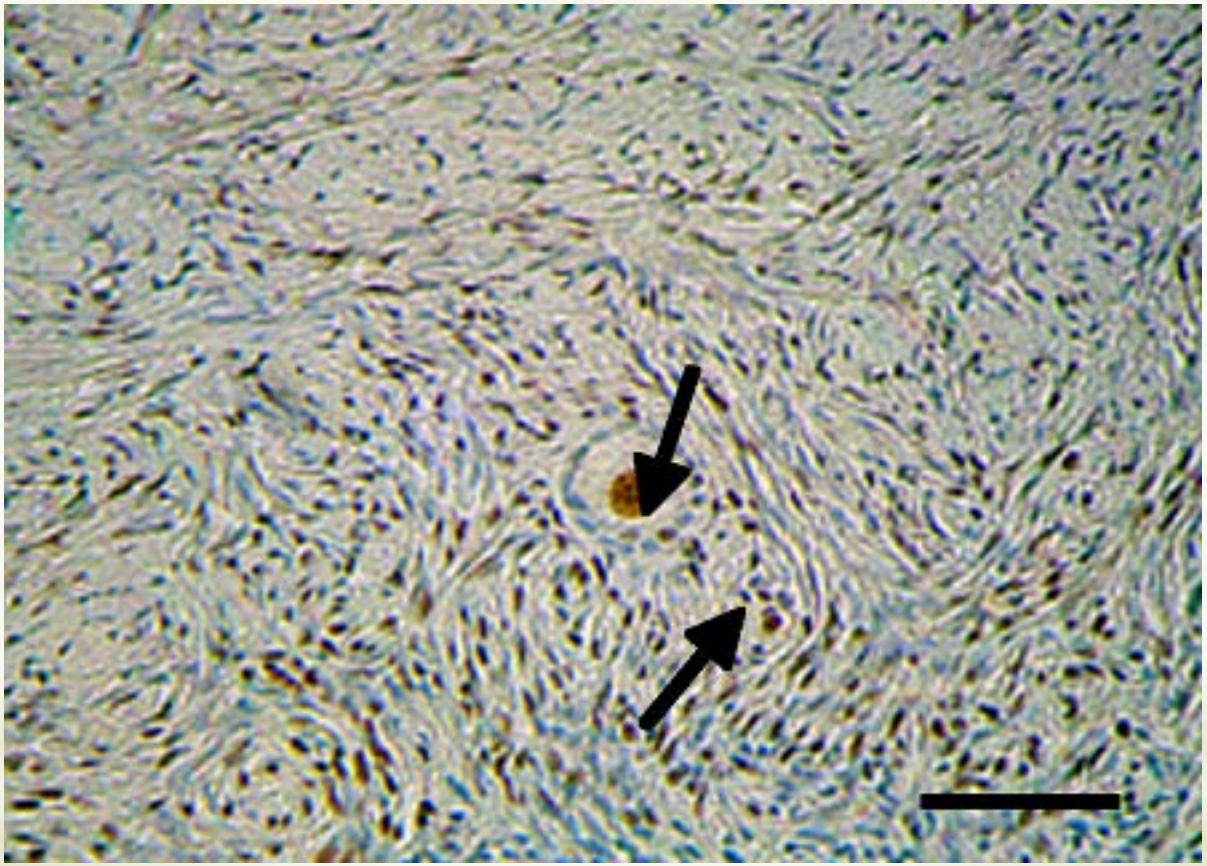
*Successful vitrification of bovine and human ovarian tissue





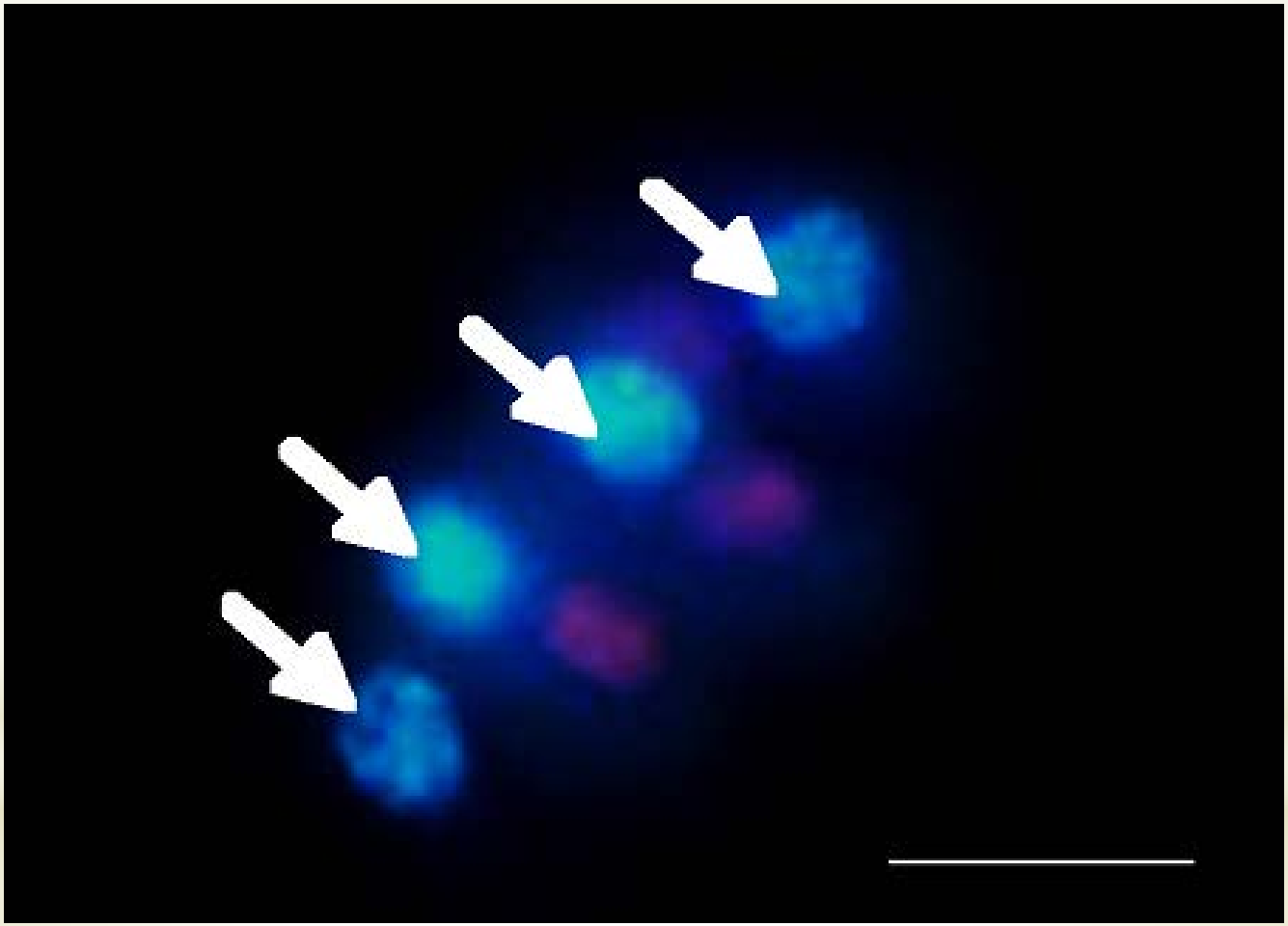
Morphologically normal oocyte in a pre-antral follicle from vitrifed-warmed human ovarian tissue. (A) Normal oocyte was surrounded by one or two layers of somatic cells in normal interstitial tissue of vitrifed ovarian tissue. Haematoxylin–eosin staining. (B) Normal oocyte was surrounded by three or four layers of somatic cells in normal interstitial tissue of vitrifed ovarian tissue. Scale bar represents 50 µm. Kagawa et al. RBM online 2009

Successful vitrification of bovine and human ovarian tissue



Histological section of vitrifed human ovarian tissue. Immunohistochemical staining for proliferating cell nuclear antigen (PCNA), a marker protein for proliferating cells. Abundant PCNA-positive proliferating cells were demonstrated in the interstitial tissue cells and a few proliferating cells were noted in granulose cells (arrows) of vitrifed human ovarian tissue. Scale bar represents 50 µm.
Kagawa et al., RBM online, 2009

Successful vitrification of bovine and human ovarian tissue



Surviving oocytes (arrows) of pre-antral follicles of vitrifed–thawed ovarian tissue in human (Hoechst/propidium iodide stain). Nuclei of living oocytes were blue. Scale bar represents 50 µm. Kagawa et al., RBM online, 2009

*Successful vitrification of bovine and human ovarian tissue

Kagawa et al., RBMOnline 2009

Survival of oocytes in vitrified-warmed human ovarian tissue

	No. of samples	Number of Oocytes (%)	
		Collected	Surviving
Vitrified	7	954 (100)	855 (89.6)

Successful vitrification of bovine and human ovarian tissue

Kagawa et al., RBMOnline 2009

Abstract:

[…] In addition, human ovarian tissue from cancer patients, and from ovary transplant donors was also vitrifed by the Cryotissue method.

After warming, high oocyte survival in human tissue (similar to bovine tissue) was obtained. These results indicate that an ultra-rapid cooling vitrifcation method has the potential for clinical use in human ovarian tissue cryopreservation. […]

CLINICAL ARTICLE

Combining ovarian tissue cryobanking with retrieval of immature oocytes followed by in vitro maturation and vitrification: an additional strategy of fertility preservation

Jack Y. J. Huang, M.D., Togas Tulandi, M.D., M.H.C.M., Hananel Holzer, M.D., Seang Lin Tan, M.D., M.B.A., and Ri-Cheng Chian, Ph.D.

Department of Obstetrics and Gynecology, McGill University Health Center, McGill University, Montreal, Quebec, Canada

Objective: To report an additional strategy of fertility preservation, which combines ovarian tissue cryobanking with retrieval of immature oocytes from excised ovarian tissue, followed by in vitro maturation (IVM) and vitrification.

Design: Retrospective analysis of case series.

Setting: University teaching hospital.

Patient(s): Women who underwent oophorectomy or ovarian wedge resection before receiving chemotherapy and/or radiotherapy.

Intervention(s): Immature oocyte retrieval, IVM, oocyte vitrification, ovarian tissue cryobanking.

Main Outcome Measure(s): Oocytes retrieved from the excised ovarian tissue, oocyte maturation rate, and number of oocytes cryopreserved by vitrification.

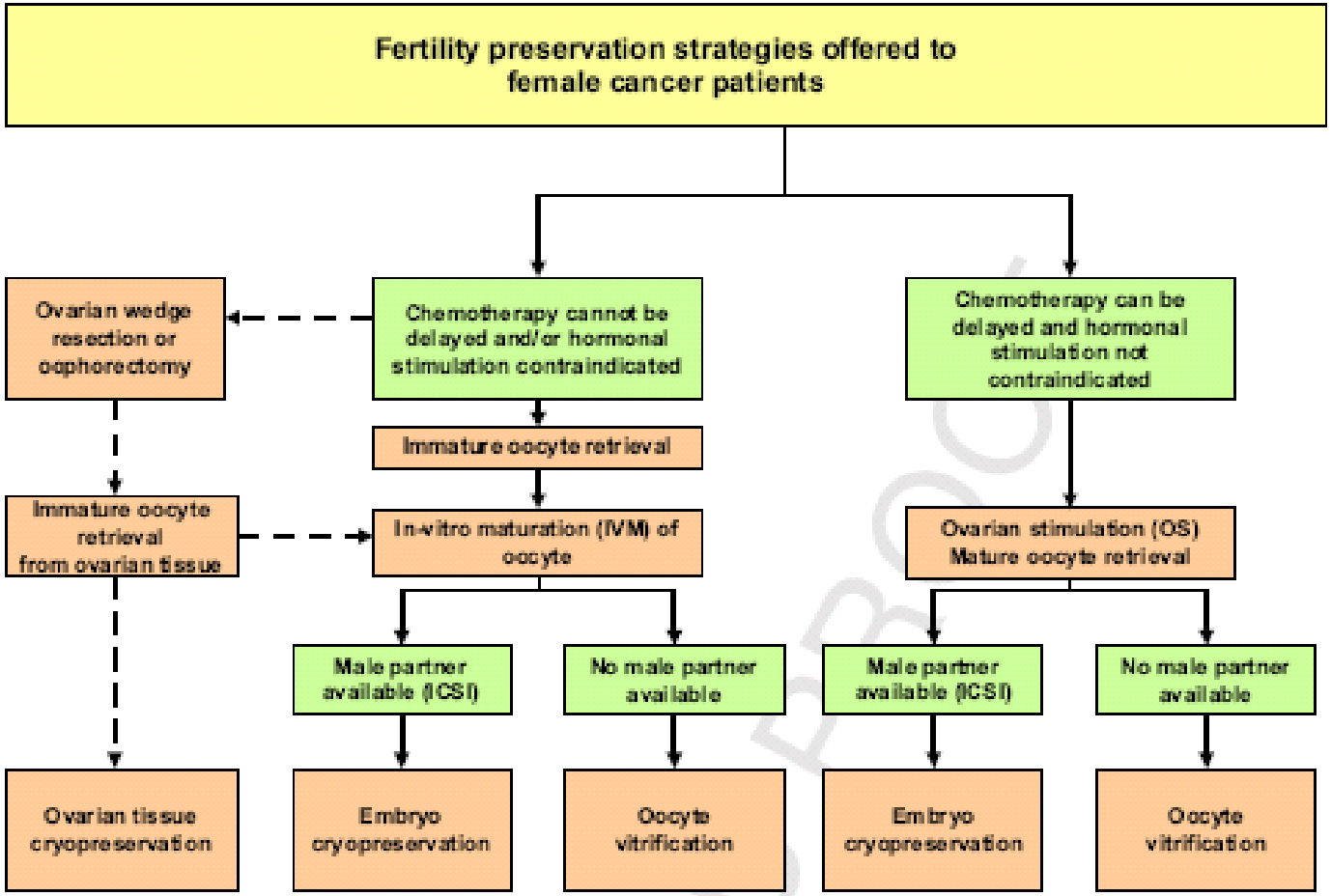
Result(s): Four consecutive patients underwent retrieval of immature oocytes from the antral follicles of the excised ovarian tissue. The mean number of immature oocytes recovered was three (1, 3, 4, and 3, respectively). The mean maturation rate following IVM was 79% (100%, 100%, 50%, and 67%, respectively). In total, eight mature oocytes were vitrified.

Conclusion(s): Oocytes can be retrieved from excised ovarian tissue, matured in vitro, and cryopreserved by vitrification. This fertility preservation technique could be combined with ovarian tissue cryobanking. (Fertil Steril® 2007; ■:■-■. ©2007 by American Society for Reproductive Medicine.)

Key Words: Fertility preservation, cryopreservation, oocyte, in-vitro maturation, vitrification, ovarian tissue cryobanking

FIGURE 1

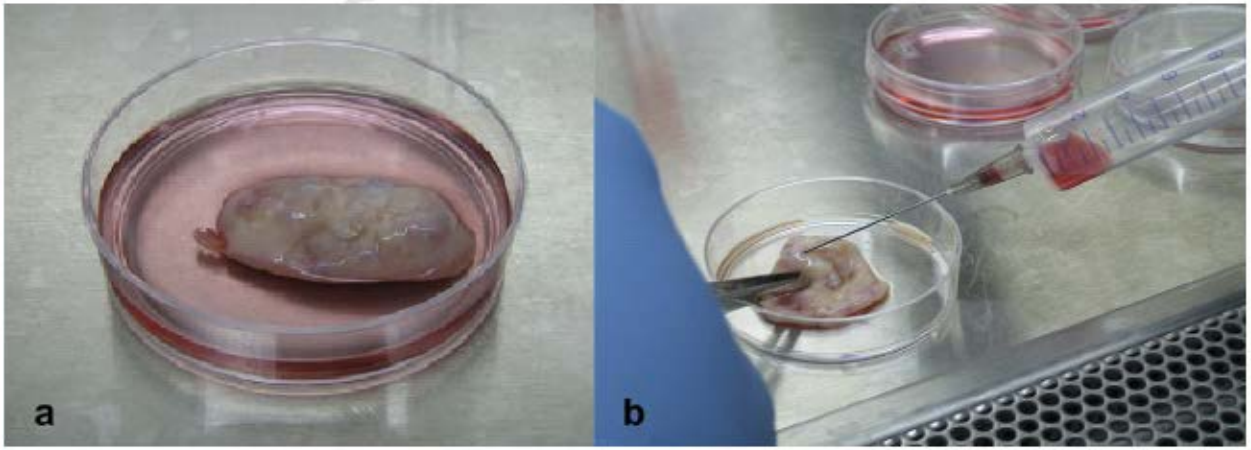
Preservation strategies offered to female cancer patients.



Chian. Vitrification of in vitro matured oocytes. Fertil Steril 2008.

FIGURE 1

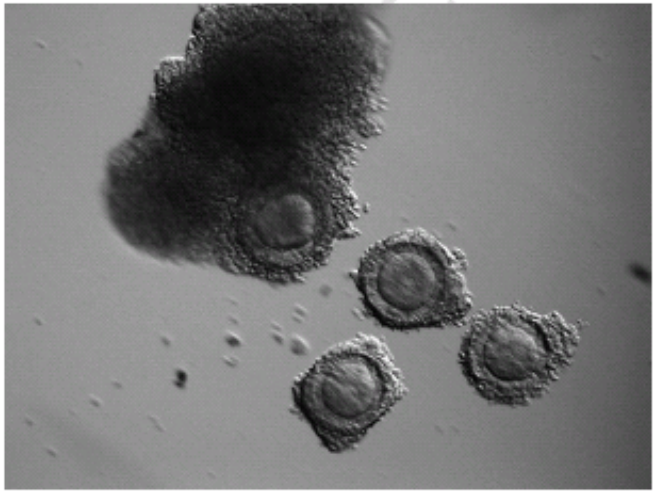
(a) The excised ovarian tissue was suspended in cold (4°C) Leibovitz L-15 medium. (b) The visible follicles were aspirated with an 18-gauge syringe needle that was attached to a 10-mL syringe.



Huang. Cryopreservation of ovarian tissue and oocytes. Fertil Steril 2007.

FIGURE 2

Four immature cumulus–oocyte complexes were retrieved from the excised ovarian tissue.



Huang. Cryopreservation of ovarian tissue and oocytes. Fertil Steril 2007.

Why do we prefer the vitrification procedure now?

* No mechanical injury (extracellular crystal formation)
* Less osmotic stress to cells
* No intracellular crystal formation
* Less labour in laboratory daily work
* Simple protocol
* Useful for oocytes and blastocysts, which have less success with slow freezing
* No need for expensive devices

*Future Aspects

- *Avoiding hyperstimulation syndrome in patients with PCOS by vitrification of all 2PN and replaced in a programmed cycle
- *Cancelling of fresh ET in case of more than 10 Follicles
- *Vitrification of all zygotes resulted from IVM programme
- *An option for cancer patients to vitrify the oocytes instead of ovarian tissue
- *In oocytes donation programme
- *Vitrification of the oocytes to postpone fertility
- *Mantains viability of specimens during long term storage

*Conclusions of vitrification

* Easy to perform
* Low cost
* Future first choice procedure
* It was shown to be superior to slow freezing procedure
* Very high survival rates of oocytes and embryos at all stages of development
* It seems that the cryotop method is the most efficient procedure
* Revitrification is possible
* Ovarian Cortex is now also possible

Dziękuje za uwagę