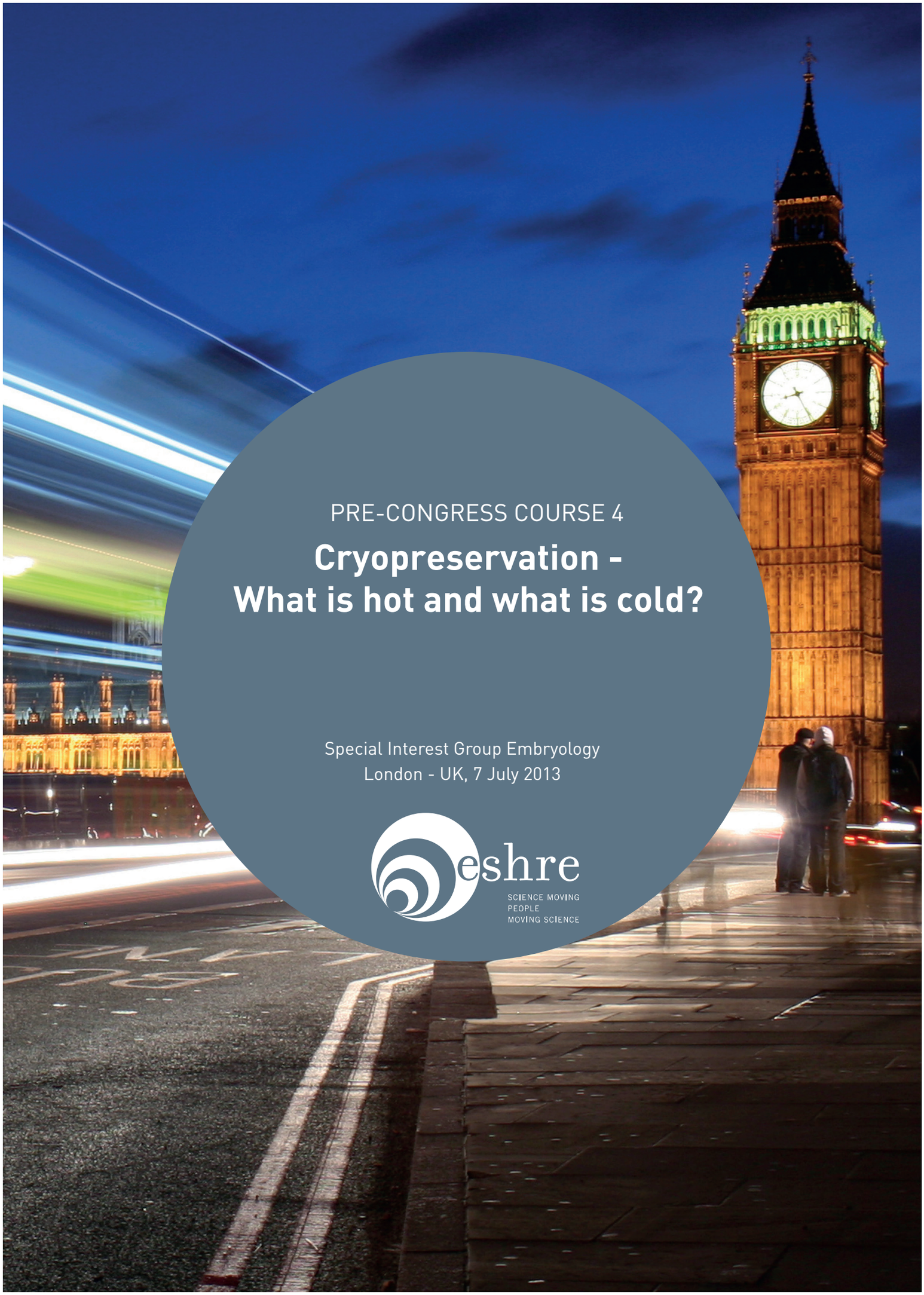


PRE-CONGRESS COURSE 4

Cryopreservation - What is hot and what is cold?

Special Interest Group Embryology
London - UK, 7 July 2013

eshre
SCIENCE MOVING
PEOPLE
MOVING SCIENCE

What is hot and what is cold?

London, United Kingdom
7 July 2013

Organised by
The ESHRE Special Interest Group Embryology

Contents

Course coordinators

Kersti Lundin (Sweden) and Cristina Magli (Italy)

Course description

This course is aimed to give an update on current and possible future practices in short and long-term cryopreservation, as well as analyse risks, utilisation rates, and outcome

Target audience

IVF lab technologists, clinical embryologists, scientists and clinicians

Scientific programme

Chairman: Kersti Lundin - Sweden
Chairman: Cristina Magli - Italy

Burning aspects of cryopreservation

09:00 - 09:30	Cryobiology with focus on gametes and embryos *Speaker to be announced*
09:30 - 09:45	Discussion
09:45 - 10:15	Cross-contamination of samples in liquid nitrogen – statistical probability or absolute certainty? *Ana Cristina Cobo Cabal - Spain*
10:15 - 10:30	Discussion
10:30 - 11:00	Coffee break

Cryopreservation , optimisation of methodology

11:00 - 11:30	Is there still a place for slow freezing in ART *David H. Edgar - Australia*
11:30 - 11:45	Discussion
11:45 - 12:15	Fresh versus frozen oocytes – are we reaching the optimum? *Maria Jose De Los Santos - Spain*
12:15 - 12:30	Discussion
12:30 - 13:30	Lunch

Chairman: Maria Jose De Los Santos - Spain
Chairman: David Edgar - Australia

Long term cryopreservation

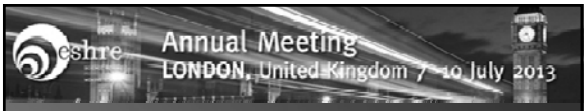
13:30 - 14:00	Cryopreservation and utilization of ovarian tissue; when, where and how *Christiani Amorim - Belgium*
14:00 - 14:15	Discussion
14:15 - 14:45	Cryopreservation of testicular tissue; when, where and how *Greta Verheyen - Belgium*
14:45 - 15:00	Discussion
15:00 - 15:30	Coffee break

A cold look at cryopreservation

15:30 - 16:00	Epigenetic events in early embryos & possible effects of culture conditions *Petra Hajkova - United Kingdom*
16:00 - 16:15	Discussion
16:15 - 16:45	Freeze-dried sperm preservation: are we looking at the future? *Luca Gianaroli - Italy*
16:45 - 17:00	Discussion
17:00 - 17:30	Business meeting Special Interest Group Embryology

Cryobiology with focus on gametes and embryos – **Amir Arav (Israel)**

Dr. Amir Arav replaces another speaker and there was not sufficient time to prepare a contribution for the syllabus.



Cross-contamination of samples in liquid nitrogen, statistical probability or absolute certainty?

Ana Cobo. Ph.D.
IVI-Valencia, Spain
ana.cobo@ivi.es
www.ivi.es

London, July 2013

IVI

CONFLICT OF INTEREST

I declare that I have no conflict of interest.

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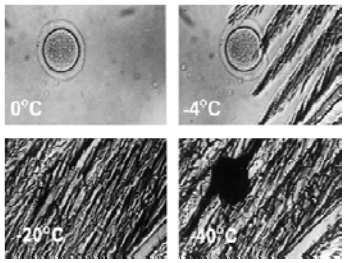
LEARNING OBJECTIVES

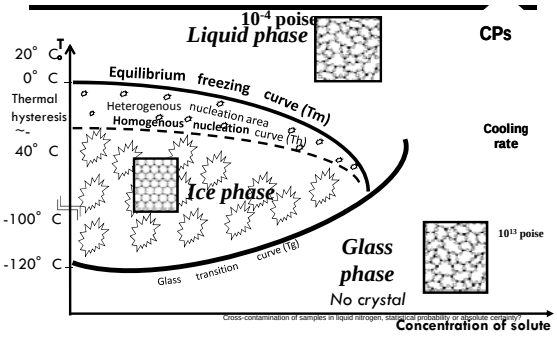
- To assess current evidence of vitrification outcome. Why is direct contact with Liquid Nitrogen required?
- To evaluate the risks of cross contamination due to direct contact with Liquid Nitrogen during vitrification and/ storing.
- To evaluate different option to reduce the hypothetical risk of cross contamination.

Ice formation



Leibo et al Theriogenology 2007

Biophysical aspects: binary phase diagram



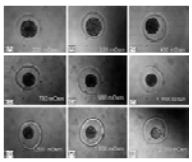
Vitrification

$$\text{Probability of vitrification} = \frac{\text{Cooling rate} \times \text{Viscosity}}{\text{Volume}}$$

Saragusty and Arav, Reproduction 2011

How to perform?

1. High viscosity ⇨ High CPA concentration



Osmotic Stress ⇕ Cell toxicity

Leibo; Theriogenology, 2008

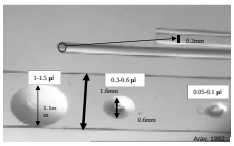
How to perform?

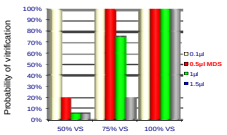
2. High cooling rate

⇩

a. Direct plunging in to the LN (open systems), use of SLN

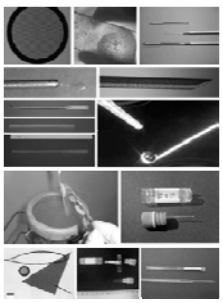
b. Use very low volume while the loading the sample MDS *(Arav, 1992)*

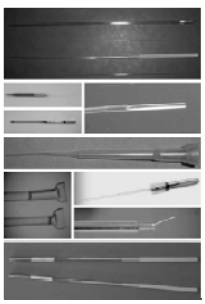




The use of minimum volume allows a reduction in the CPA concentration.

Different carrier systems





Saragusty; Reprod, 2011

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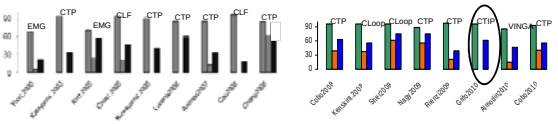
Vol. 13 Suppl. 4 2006 Reproductive BioMedicine Online; www.rbmonline.com/Article/4385
on web 15 October 2006

Contribution from 11th World Congress on Controversies in Obstetrics, Gynecology and Infertility (COGI) 'Celebration - 30 years of IVF' and Serono Symposia International Foundation

Vitrification of human mature oocytes in clinical practice

Ana Cobo[1], Gabor Vajta[2], José Remohí[3]

Open systems

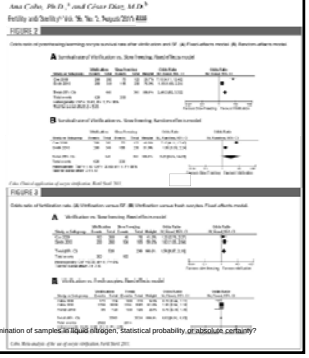


☐ Survival rate ☐ Implantationl rate ☐ Pregnancy rate

IVI

Clinical application of oocyte vitrification: a systematic review and meta-analysis of randomized controlled trials

Ana Cobo, Ph.D.,[a] and César Díaz, M.D.[b]



IVI

Hum. Reprod. Advance Access published June 30, 2010

ORIGINAL ARTICLE Embryology

Use of cryo-banked oocytes in an ovum donation programme: a prospective, randomized, controlled, clinical trial

Table IV Primary outcome, OPR, according to the type of oocytes received.

	Egg-bank	Fresh
Ongoing pregnancy rate/ITT	131 (42.7)	125 (41.7)
Ongoing pregnancy rate/cycle	131 (41.9)	125 (43.3)
Ongoing pregnancy rate/transfer	131 (49.1)	125 (48.3)

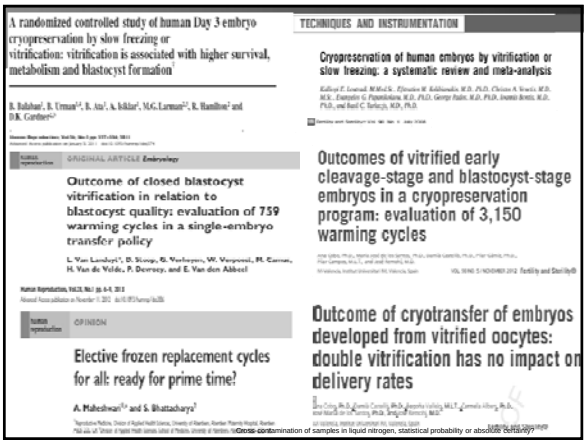
Unless otherwise indicated values are mean ± SD or n (%).
ITT, intention to treat.

OR=0.921 (0.667-1.274)

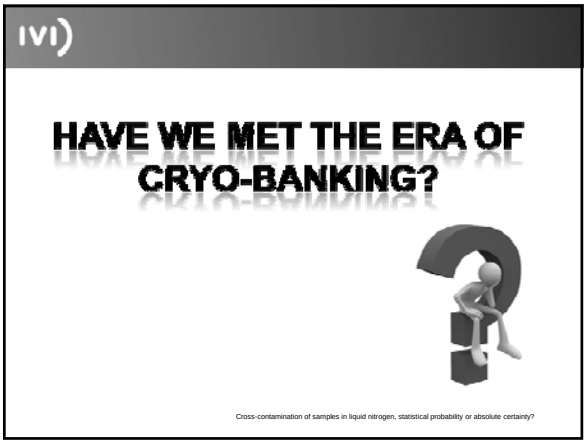
5-years experience of Egg-banking in IVI Valencia

		95% CI
Number of cycles	3,137	
Number of vitrified oocytes donated	33.096	
Number of embryo transfers/donation	2,729 (91.2)	90.1-92.3
Number of embryos replaced (Mean ± SD)	5,118 (1.87 ±1.8)	1.78-1.96
Number of embryo cryopreservation cycles/donation cycle	2,125 (67.7)	65.7-69.7
Mean number of re-vitrified embryos (Mean ± SD)	6,417 (2.1 ± 1.9)	2.0-2.2
Implantation rate	1,989 (38.8)	36.7-40.9
Clinical pregnancy /cycle	1,485 (47.3)	44.8-49.8
Clinical pregnancy /embryo transfer	1,485 (54.4)	51.9-56.9
Ongoing pregnancy /cycle	1,222 (38.9)	36.2-41.6
Ongoing pregnancy /embryo transfer	1,222 (44.7)	41.9-47.5

Cross-contamination of samples in liquid nitrogen, statistical probability or absolute certainty?



Cross-contamination of samples in liquid nitrogen, statistical probability or absolute certainty?



Cross-contamination of samples in liquid nitrogen, statistical probability or absolute certainty?

ASRM PAGES

Mature oocyte cryopreservation: a guideline

The Practice Committees of the American Society for Reproductive Medicine and the Society for Assisted Reproductive Technology

Society for Reproductive Medicine and Society for Assisted Reproductive Technology, Birmingham, Alabama

Fertility and Sterility® Vol. 99, No. 1, January 2013

There is good evidence that fertilization and pregnancy rates are similar to IVF/ICSI with fresh oocytes when vitrified/warmed oocytes are used as part of IVF/ICSI for young women. Although data are limited, no increase in chromosomal abnormalities, birth defects, and developmental deficits has been reported in the offspring born from cryopreserved oocytes when compared to pregnancies from conventional IVF/ICSI and the general population. Evidence indicates that oocyte vitrification and warming should no longer be considered experimental. This document replaces the document last published in 2008 titled, "Ovarian Tissue and Oocyte Cryopreservation." Fertil Steril 2008;89:260–4 (Fertil Steril® 2013;99:37–43. ©2013 by American Society for Reproductive Medicine.)
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Discuss: You can discuss this article with its authors and with other ASRM members at http://fertstertforum.com/goldsteinj-mature-oocyte-cryopreservation-guideline/

Cross-contamination of samples in liquid nitrogen, statistical probability or absolute certainty?

human reproduction ESHRE PAGES

Oocyte cryopreservation for age-related fertility loss†

ESHRE Task Force on Ethics and Law, including, W. Dondorp[1,8], G. de Wert[1], G. Pennings[2], F. Shenfield[3], P. Devroey[4], B. Tarlatzis[5], P. Barri[6], and K. Diedrich[7]

Human Reproduction, Vol.27, No.5 pp. 1231–1237, 2012

The recently established possibility of effectively cryopreserving functional oocytes through vitrification promises to revolutionize IVF practice. One new option is that of women using this technology to protect their reproductive potential against the threat of time. This document analyses the ethical arguments about the acceptability of this option. In an earlier statement the Task Force concluded that "oocyte freezing for fertility preservation without a medical indication should not be encouraged" (Shenfield *et al.*, 2004). In the light of new scientific developments, and after considering relevant ethical arguments, the Task Force now takes the view that oocyte cryopreservation to improve prospects of future child bearing should also be available for non-medical reasons.

Cross-contamination of samples in liquid nitrogen, statistical probability or absolute certainty?

HOWEVER.....

Potential risk of cross contamination

LN_2 mediated infections

- Papova virus,in dermatology, swabs in LN_2 (Charles et al., 1971) possible transmission between patients.
- Hepatitis B virus between blood samples(Tedder et al., 1995)
- Escherichia coli, between semen pellets (Piasecka-Serafin,1972)
- BVDV, BHV, between embryos stored in OPS straws (Bielanski et al.,2000)

Potential risk of cross contamination

LN_2 mediated infections

- Papova virus,in dermatology, swabs in LN_2 (Charles et al., 1971) possible transmission between patients.
  Sharing swabs
- Hepatitis B virus between blood samples(Tedder et al., 1995)
  Inappropriate leaky bags; 50 mL samples; millions of blood cells
- Escherichia coli, between semen pellets (Piasecka-Serafin,1972)
  Experimental: high bacterial concentration.
- BVDV, BHV, between embryos stored in OPS straws (Bielanski et al.,2000)
  Experimental; high viral concentration

Should be considered:

- Two hundred fifty million bull sperm samples cryopreserved
- Two hundred twenty thousand cryopreserved bovine embryos transferred
- Tens of millions of human sperm samples cryopreserved
- Millions of cryopreserved human embryos, and
- Embryos from thousands of cryopreserved human oocytes

None of the infections was attributable to LN2 mediated transmission.
(None of these studies used "open" methods for cryopreservation!)

Why don't we have more infections if:

- Collection of semen is not a sterile procedure
- Oocytes are contaminated with blood during collection
- Many containers are inappropriately sealed or leaky
- Usually the outer surface of straws and vials is not sterile
- Storage tools (canisters, holders) are not sterilized
- Factory derived LN_2 is not sterile
- Many pathogens survive storage in -196° C
- Cryoprotectants protect also pathogens
- Dewars aren't decontaminated regularly

Possible explanation

- The number of cells is too small

When one is considering embryos as vectors for pathogens, it is important to remember that reproductive tissues are quite different from the other tissues regulated by the FDA.

Another characteristic of reproductive tissue that differs from other donor tissue is that these reproductive tissues are usually completely free of blood, one of the most common carriers of pathogens

Pomeroy et al. Fert. Steril. 2010

- Pathogens do not attach to the zona

Zona is an efficient barrier

Fact:
No LN_2 mediated disease transmission in ART

Viral screening of spent culture media and liquid nitrogen samples of oocytes and embryos from hepatitis B, hepatitis C, and human immunodeficiency virus chronically infected women undergoing in vitro fertilization cycles

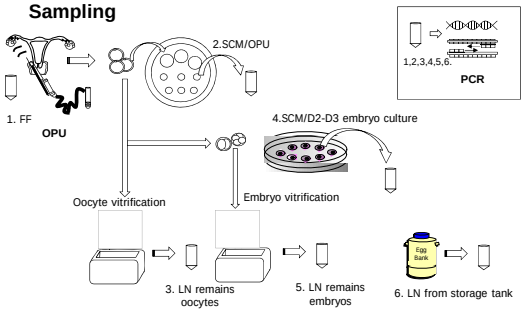
Objective: To assess the presence of viral RNA or DNA sequences in spent culture media used after ovum pick-up or embryo culture and in LN used for oocytes or embryo vitrification in seropositive patients (HIV,HBV,HCV)

N=24 patients and 63 samples

HIV	HCV	HBV	HCV + HBV
N=6	N=11	N=6	N=1

Materials and Methods:

Sampling



Viral screening of spent CM and LN samples of oocytes or embryos coming from HIV, HCV, and HBV chronically infected women undergoing IVF cycles.

Cross-contamination of samples in liquid nitrogen, statistical probability or absolute certainty?

RESULTS

Cross-contamination of samples in liquid nitrogen, statistical probability or absolute certainty?

Strategies to avoid potential cross contamination

Efficiency → **Open systems**

Safety (EUCTD) → **Closed systems for storage**

1. **During vitrification:**
   - Use of closed systems
   - LN sterilization
2. **During storage**
   - Use of vapor tanks

Cross-contamination of samples in liquid nitrogen, statistical probability or absolute certainty?

Use of closed Systems

Infertile patients autologous cycles

Effects of two vitrification protocols on the developmental potential of human mature oocytes

Alessio Paffoni *, Cristina Ouerweri, Stefania Ferrari, Liliana Restelli, Anna Elisa Nicolosi, Claudia Scarduelli, Guido Ragni

Table 3 CryoTip versus CryoTop groups: embryological data.

Characteristic	CryoTip (closed) vitrification-warming cycles (n = 51)	CryoTop (open) vitrification-warming cycles (n = 52)	P	OR (95% CI)
Warmed oocytes (total)	5.1 ± 2.0 (261)	5.1 ± 1.4 (268)	NS	
Survived oocytes	151/261 (57.9)	222/268 (82.8)	0.001	0.3 (0.2–0.4)
Inseminated oocytes (total)	2.8 ± 1.3 (151)	3.0 ± 0.8 (159)	NS	
Fertilization (2PN) rate	87/151 (57.6)	116/159 (73.0)	0.005	0.5 (0.3–0.8)
Abnormal 2PN morphology	14/87 (16.1)	2/116 (1.7)	0.001	10.9 (2.4–100.8)
Oocytes degenerated post ICSI	16/151 (10.6)	10/159 (6.3)	NS	1.8 (0.8–4.2)
Embryos on day 2 (≥2 cells)	69/87 (79.3)	112/116 (96.6)	0.001	0.1 (0.0–0.4)
Good-quality embryos on day 2	30/87 (34.5)	62/112 (55.4)	0.003	0.4 (0.3–0.9)
Cycles with no viable embryos	24/51 (47.1)	1/53 (1.9)	0.001	46.2 (7.7–926.7)
Clinical pregnancy rate/warming cycle	4/51 (7.8)	14/53 (26.4)	0.01	0.2 (0.1–0.8)
Clinical pregnancy rate/patient	4/48 (8.3)	14/49 (28.6)	0.01	0.2 (0.1–0.8)
Implantation rate	4/69 (5.8)	15/112 (13.4)	NS	0.4 (0.1–1.3)
Gestational sacs/inseminated oocyte	4/151 (2.6)	15/159 (9.4)	0.02	0.3 (0.1–0.8)
Live birth rate/warming cycle	3/51 (5.9)	11/52 (20.8)	NS	0.2 (0.0–1.0)

Values are mean ± SD or n/total (%) unless indicated otherwise.
CI = confidence interval; NS = not statistically significant; OR = odds ratio; 2PN = two pronuclei.

Cross-contamination of samples in liquid nitrogen, statistical probability or absolute certainty?

Use of closed Systems

Ovum donation

Clinical validation of a closed vitrification system in an oocyte-donation programme

Dominic Stoop *, Neelke De Munck, Eleonora Jansen, Peter Platteau, Etienne Van den Abbeel, Greta Verheyen, Paul Devroey

Table 2 Laboratory outcomes: warmed oocyte embryo transfer.

Parameter	Outcome
Warmed oocytes	123
Surviving oocytes	111 (90.2)
Fertilized oocytes	86 (77.5)
Embryos on day 3	80
Excellent quality	29 (26.2)
Good quality	24 (30.0)
Moderate quality	16 (20.0)
Poor quality	11 (13.8)
Embryos transferred	36
Recipients with embryo transfer	20 (100)
Embryos transferred	1.8 ± 0.4
Excellent quality	22 (61.1)
Good quality	11 (30.6)
Moderate quality	3 (8.3)

Values are n, n (%) or mean ± SD.

Table 3 Clinical outcomes.

Outcome	Warmed oocyte embryo transfers	Warmed oocyte frozen embryo transfers	All transfers
Warming cycles	20	3	23
Transfers	20	3	23
Clinical pregnancy rate	10/20 (50.0)	1/3 (33.3)	11/23 (47.8)
Ongoing pregnancy rate	9/20 (45.0)	1/3 (33.3)	10/23 (43.5)
Implantation rate	12/36 (33.3)	2/5 (40)	14/41 (34.1)

Values are n or n/total (%).

Cross-contamination of samples in liquid nitrogen, statistical probability or absolute certainty?

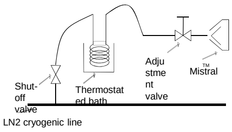
RCT evaluating a semi-close device

Clinical trials.gov identifier:NCT01745563

	SC device (N=83)	Conventional device (N=84)	OR (CI95%)	P
Nº vitrified/warmed oocytes	1019	1007		
Survival rate N (%)	920 (90.3)	928 (92.2)	0.757 (0.283-1.878)	0.240
Nº ET	74 (89.1)	73 (86.9)	-	0.325
Mean embryos transferred	1.99 ± 0.2	1.98 ± 0.3	-	0.523
Mean embryos cryopreserved	3.1 ± 1.7	3.2 ± 1.9	-	0.594
CPR/ET	44 (59.5)	41 (56.1)	0.731 (0.364-1.466)	0.721
OPR/ET	42 (56.7)	40 (54.6)	0.640 (0.322-1.269)	0.226
CPR/cycle	44 (53.0)	41 (48.8)	0.665 (0.361-1.224)	0.217
OPR/cycle	42 (50.6)	42 (50.0)	0.597 (0.321-1.110)	0.118

Cross-contamination of samples in liquid nitrogen, statistical probability or absolute certainty?

Filtration of Liquid Nitrogen



Cross-contamination of samples in liquid nitrogen, statistical probability or absolute certainty?

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ARTICLE IN PRESS

Sterilization of liquid nitrogen with ultraviolet irradiation for safe vitrification of human oocytes or embryos

Lodovico Parmegiani, B.Sc., Antonio Accorsi, B.Sc., Graciela Estela Cognigni, M.D., Silvia Bernardi, B.Sc., Enzo Troilo, B.Sc., and Marco Filicori, M.D.
Reproductive Medicine Unit, GynePro Medical Centers, Bologna, Italy; and Department of Occupational Medicine, University of Bologna

FIGURE 1

REFERENCE LABORATORY

IVI VALENCIA, S.L.

Parmegiani. UV sterilization of liquid nitrogen. Fertil Steril 2009.

IVI

2. During Storage

Storage of human oocytes in the vapor phase of nitrogen

Ana Cobo, Ph.D., Josep Ll. Romero, Ph.D., Sonia Pérez, Ph.D., María J. de los Santos, Ph.D., Marcos Meseguer, Ph.D., and José Remohí, M.D.
IVI-Universidad de Valencia, Valencia, Spain

Fertility and Sterility® Vol. ■, No. ■, ■ 2009
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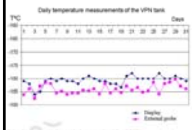
FIGURE 1

Daily temperature measurements of the vapor-phase nitrogen tank.



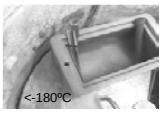
Cobo. Vapor storage for vitrified oocytes. Fertil Steril 2009.

TABLE 2

Oocyte distribution, survival, and clinical outcome.

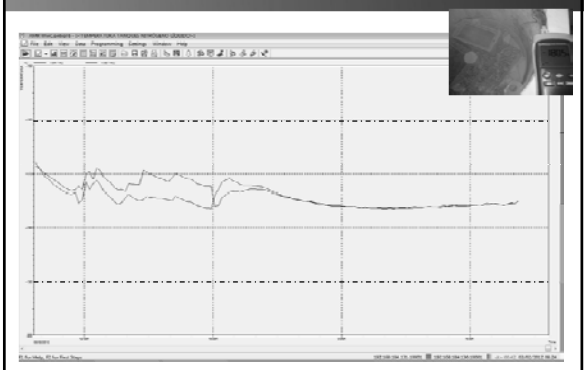
	Vapor-phase nitrogen	LN	P value
No. vitrified oocytes (mean ± SD)	464 (10.5 ± 3.1)	472 (10.0 ± 2.1)	.401
Survival no. (%)	442 (95.3)	447 (94.5)	.354
No. injected oocytes	442	447	
Normal fertilization no. (%)	325 (73.1)	320 (71.7)	.645
Abnormal fertilization no. (%)	24 (5.4)	23 (5.1)	.847
Degenerated oocytes no. (%)	25 (5.7)	27 (6.0)	.726
No. transfers	43	45	
No. embryos transferred (mean ± SD)	87 (1.9 ± 0.3)	88 (1.8 ± 0.5)	.300
Pregnancy rate/transfer	26 (60.4)	27 (60.0)	.964
Clinical pregnancy rate/transfer	25 (58.1)	24 (53.3)	.649
Clinical pregnancy rate/cycle	25 (56.8)	24 (52.2)	.657
Miscarriage rate	4 (16.0)	3 (12.5)	.727
Implantation rate — No. sacs/No. embryos transferred	35 (40.2)	30 (34.1)	.400
Multiple pregnancy rate (twin)	9 (36.0)	7 (29.1)	.490
Ongoing pregnancy rate/transfer	21 (48.8)	21 (46.6)	.902
Ongoing pregnancy rate/cycle	21 (47.7)	21 (45.6)	.902
Live births	7	5	

Cross-contamination of samples in liquid nitrogen, statistical probability or absolute certainty?



Temperature.
Handle samples under temperature glass transition temperature

Temperature monitoring of storage tanks



Conclusions

- There is no evidence of cross-contamination after cryo-transfer in ART, thus, the risk must be minimum.
- Due to the current concerns about the risk of cross contamination it is necessary to implement measures to reduce hypothetical risks, selecting the appropriate device and isolating the samples during storage


Thank you!
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SIGE precongress course: Cryopreservation - What is hot and what is cold?

ESSHRE London 2013.

Title of presentation:

Cross-contamination of samples in liquid nitrogen, statistical probability or absolute certainty?

Presented by:

Ana Cobo

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Is there still a place for slow freezing in ART?

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No commercial relationships or conflict of interest to declare

Learning objectives

- Understand the advantages and disadvantages of different approaches to cryopreservation
- Be aware of the objective evidence available when comparing slow cooling and vitrification
- Understand that slow cooling and vitrification are only approaches and that each may include multiple methods
- Appreciate that the balance of advantages and disadvantages of slow cooling may be different for different biological entities

Cryopreservation in ART

- Fertility preservation (sperm, follicles/ovarian tissue, oocytes, embryos)
- Legislative restrictions or managing oocyte donation (oocyte)
- Maximising fertility potential while minimising multiple pregnancy (embryos, oocytes)

Slow cooling or vitrification?

Slow cooling and vitrification ...

- are fundamentally similar and based on the same principles
- are not definitive descriptions since protocols for either can vary considerably

Potential advantages of slow cooling

- Significantly lower concentrations of permeating (?toxic) cryoprotectants + no DMSO
- Relative tolerance to technical variation
- Less time consuming (person hours) when many samples are being cryopreserved simultaneously
- Safety (closed systems, larger volumes)
- Safety (proven clinical outcomes)

However, vitrification ...

- Is less dependent on machinery
- Can be less time consuming (start to finish) with smaller numbers of samples (flexibility)

But, of course, ...

- The efficiency of cryopreservation is the most (although not the only) important consideration

The clinical efficiency of cryopreservation

- Survival (structural)
- Retention of developmental potential (functional)

Briefly, cryopreservation of sperm

- Vitrification may not be feasible for large volumes/ numbers of sperm (normal semen)
- Vitrification may provide advantages for small volumes/ numbers of sperm

Briefly, cryopreservation of ovarian tissue

- High survival rates of primordial follicles reported for both slow cooling and vitrification
- Suggestions that vitrification may result in improved survival of associated stromal tissue

Cryopreservation of oocytes – slow cooling

Summary of reported clinical outcomes from oocytes slow cooled in

1.5M Propanediol + 0.1M sucrose

- 4027 oocytes thawed
- 51% thawed oocytes survived
- 54% of injected oocytes fertilised
- 85% of embryos cleaved normally
- 10% of transferred embryos implanted

Gook and Edgar, Hum Rep Update 13, 591-605 (2007)

Optimal dehydration ?

Variation in Membrane Hydraulic Permeability of Human Oocytes

Membrane hydraulic permeability Lp (μm/atm/min) measured in individual oocytes at 20° C

Oocyte	1	2	3	4	5	6	7	8
Lp	0.32	0.6	1.09	0.56	0.16	0.51	0.23	0.8

Hunter et al, J Cell Physiol, 150, 175-9 (1992)

Summary of reported results using elevated sucrose methods

	0.1M suc	0.2M suc	0.3M suc
No. thawed	4027	1451	7595
Survival	51%	71%	73%
Fertilisation	54%	80%	73%
Development	85%	93%	90%
Implantation	10%	17%	6%
FH's/100 thawed oocytes	2.3	9.0	2.9

Gook and Edgar, Hum Rep Update 13, 591-605 (2007)

Slow cooling with differential sucrose concentration during dehydration (0.2M) and rehydration (0.3M)

	Survival	Fertilisation	Implantation (day 2)
Slow cooled oocytes (women ≤ 38)	75.1%	77.3%	16.7%
Fresh oocytes (women ≤ 38)	_	79.7%	17.3%

Bianchi et al, Rep BioMed Online 14, 64-71 (2007)

Slow cooling with dehydration (0.2M sucrose) at 37°C

	Survival	Fertilisation	Implantation (day 2)
Slow cooled oocytes (women < 38)	75.8%	67.6%	30.0%
Fresh oocytes (women < 38)	–	70.8%	26.0%

Gook & Edgar, J Assist Reprod Genet, 28, 1171-76 (2011)

Therefore

It is possible to achieve implantation rates with embryos derived from slow cooled oocytes similar to those achieved using fresh oocytes

Cryopreservation of oocytes – vitrification

Oocyte Vitrification
Oocyte donors (mean age 27)

	Fresh (n=219)	Vitrified (n=231)
Survival	_	97%
Fertilisation	82%	76%
Day 2 embryos	98%	94%
Mean cell No. Day 2	3.9	3.8
Blastocyst development	47%	49%

Open vitrification using EG/DMSO/Sucrose

Cobo et al, Fertil Steril, 89, 1657-64 (2008)

Oocyte Vitrification
Oocyte donors (mean age 27)

	Fresh (n=3185)	Vitrified (n=3039)
Survival	_	92.5%
Fertilisation	73.3%	74.2%
Top quality day 3 embryos	60.7%	58.4%
Implantation (day 3)	40.9%	39.9%

Open vitrification using EG/DMSO/Sucrose

Cobo et al., Hum Rep, 25, 2239-46 (2010)

Multicentre study
Unselected infertility cycles (mean age 36)

	486 warming cycles 2721 warmed oocytes
Survival	84.7%
Fertilisation	75.2%
Top quality day 2/3 embryos	48.1%
Delivery rate	26.3%
Implantation (newborn)	15.8%

Open vitrification using EG/DMSO/Sucrose

Rienzi et al., Hum Rep, 27, 1606-12 (2012)

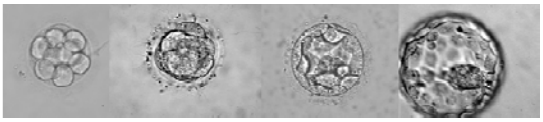
Conclusions – oocyte cryopreservation

- Embryos derived from slow cooled and vitrified oocytes can implant at similar rates to embryos derived from equivalent fresh oocytes
- Survival rates of vitrified oocytes are consistently higher than those observed with slow cooling, although widespread application may be less successful than results with a young oocyte donor population
- Unless modifications to slow cooling can achieve consistent survival rates of 80-90%, vitrification is the most efficient technique for oocyte cryopreservation
- Research into safer closed vitrification systems should continue

Cryopreservation of embryos in ART

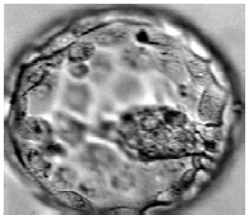
When should we transfer and cryopreserve embryos?

Periimplantation stage versus early cleavage stage



Pros	Cons
Selection of embryos with high implantation potential	?? Loss of potentially viable embryos
"Culling" of suboptimal embryos	?? Increased potential for epigenetic modification

Cryopreservation at the blastocyst stage



High success rates reported with both approaches (more reports using vitrification) but requirement for controlled comparisons due to….

- Potential differences in oocyte/embryo quality between centres
- Potential differences in selection of embryos for cryopreservation between centres

and even then..

- Introduced bias due to differential experience with the techniques may result in underperformance of one or other approach

Cryopreservation of blastocysts

	Slow cooling (9% Glycerol + 0.2M sucrose)	Vitrification (EG +DMSO + sucrose)
Survival	84%	90%
Pregnancy rate	51%	53%

Kuwayama et al, Rep BioMed Online, 11, 608-14 (2005)

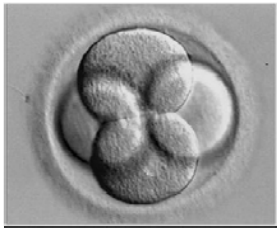
Comparison of blastocyst slow cooling and vitrification

	Slow Cooling (9% Glycerol/ 0.2M sucrose)	Vitrification (EG/DMSO/ sucrose)
Survival	518/525 (92%)	523/528 (96%)
Implantation	152/518 (29%)	160/523 (31%)

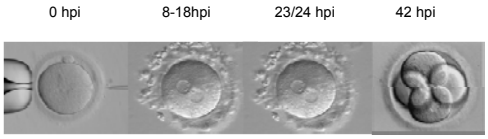
Liebermann and Tucker, Fertil Steril, 86, 20-6 (2006)

but vitrification appears to be the method of choice for cryopreserving blastocysts

Cryopreservation at cleavage stages

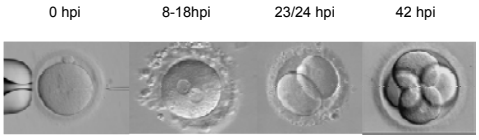


Transfer of a single embryo on day 2



Under 36 IMPLANTATION RATE = 28.6%

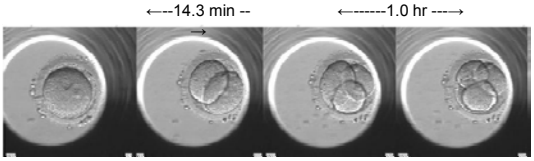
Transfer of a single embryo on day 2



Under 36 IMPLANTATION RATE = 42.5%

Lawler, Baker & Edgar, Rep Fert & Dev, 19, 482-7 (2007)

Non-invasive imaging during culture of human frozen thawed pronucleate stages predicts blastocyst formation

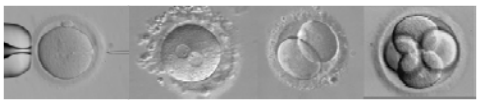


Wong et al, Nature Biotechnology, 28, 1115-21, (2010)

- Early cleavage stage embryos which survive and are transferred after cryopreservation exhibit similar implantation potential to EQUIVALENT "fresh" embryos

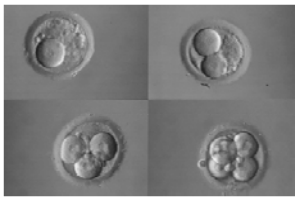
Edgar et al, Human Reproduction 15, 175-9 (2000)

0 hpi 8-18hpi 23/24 hpi 42 hpi



	Implantation rate
Fresh	42.5%
Cryopreserved	45.2%

Significance of blastomere loss: Single cryopreserved embryo transfers



	SET's	**Imp Rate**
4/4	1347	27.5%
3/4	268	26.5%
2/4	138	10.9%

Edgar et al, Rep BioMed Online, 14, 718-23 (2007)

Confident adoption of early cleavage stage SET requires highly effective cryopreservation

Slow cooling or vitrification?

Cryopreservation of cleavage stage embryos

	Slow cooling (1.5M PROH + 0.1M sucrose multistep)	Vitrification (15% EG +15%DMSO +0.5M sucrose)
Survival (≥ 50% intact)	91%	98%
Pregnancy rate	32%	27%

Kuwayama et al, Rep BioMed Online, 11, 608-14 (2005)

Outcomes of vitrification and slow cooling (Day 3 embryos)

Prospective Randomised Controlled Trial (RCT)

	Vitrification[a]	**Slow cooling[b]**	***P*-value**
Cryosurvival	222/234 (94.8%)	206/232 (88.7%)	0.02
Embryos with all blastomeres surviving	173/234 (73.9%)	106/232 (45.7%)	<0.01

[a] EG + PROH + Ficoll + Sucrose

[b] PROH + 0.1M sucrose (multistep)

Balaban et al., Hum Rep, 23, 1976-82 (2008)

Vitrification of embryos - most recent data

- Survival ≈ 95% for day 2, 3, 5 & 6 embryos
- Delivery rates similar to fresh
- Open vitrification using EG/DMSO/Sucrose

Cobo et al., Fertil Steril, 98, 1138-46 (2012)

? Optimal slow cooling

Increasing the concentration of the non permeating cryoprotectant (sucrose) during dehydration prior to slow cooling….

- ..increases the cryosurvival of unfertilised oocytes*
- ..increases the cryosurvival of biopsied cleavage stage embryos**

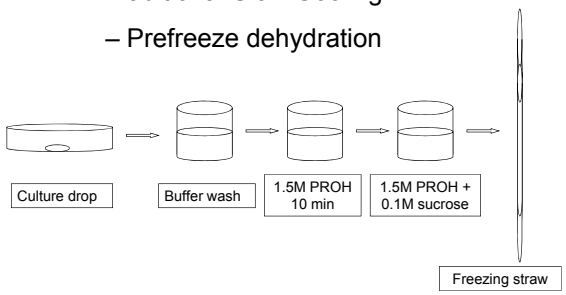
* Fabbri et al., Hum Rep, 16, 411-6 (2001)

** Jericho, Wilton, Gook & Edgar., Hum Rep, 18, 568-71 (2003)

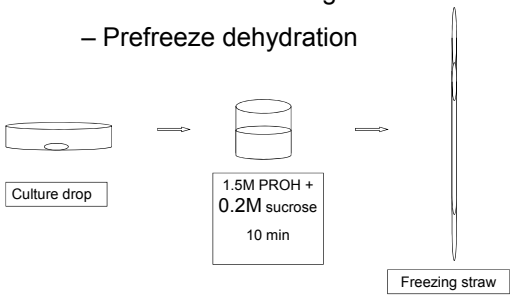
The Melbourne IVF Slow Cooling Method for Cleavage Stage Embryos

Edgar, Karani & Gook, Rep BioMed Online 19, 521-5 (2009)

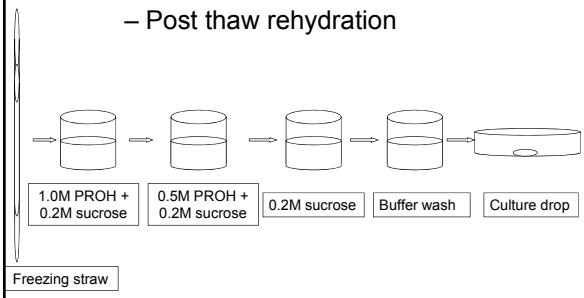
Traditional Slow Cooling – Prefreeze dehydration



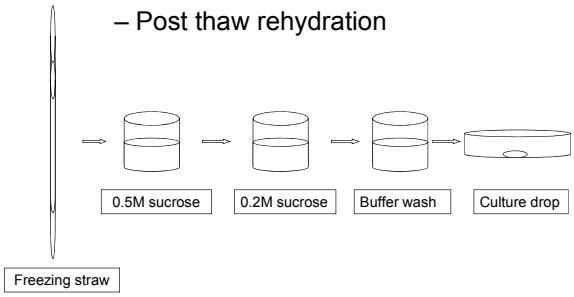
Melbourne IVF Slow Cooling Method – Prefreeze dehydration



Traditional Slow Cooling – Post thaw rehydration



Melbourne IVF Slow Cooling Method – Post thaw rehydration



Novel slow freezing methodology – key elements

Conventional	MIVF
Stepwise prefreeze addition of PROH and sucrose	One step dehydration in PROH + sucrose
Low concentration of sucrose	Elevated sucrose concentration
Stepwise removal of PROH in low sucrose	One step removal of PROH in high sucrose (“osmotic buffering”)
?? Inadequate dehydration	More rapid and complete dehydration and cryoprotection

Cryosurvival of 4-cell embryos

Blastomeres surviving	MIVF modified traditional method (0.1M sucrose / single step)	MIVF method (0.2M sucrose / single step)
≥ 50%	466 (82%)	519 (95%)
≥ 75%	423 (75%)	492 (90%)
100%	343 (61%)	441 (80%)
Total	566	548

* Edgar, Karani & Gook, Rep BioMed Online 19, 521-5 (2009)

Cryosurvival and embryo quality

Cells (42 h)	EC (24h)	Fragments	IR (<36)	Survival
4	+/-	all	31.6%	91%
< 4	+/-	all	11.0%	95%
all	+	all	32.4%	97%
all	-	all	19.5%	93%
all	+/-	< 10%	31.3%	94%
all	+/-	10-30%	21.3%	96%

Transferred embryos (< 36yrs)

	0.1 M Sucrose	0.2M Sucrose
Embryos Thawed	183	217
Embryos Transferred	139	193
FH	32	48
IR/Embryo Transferred	23.1%	24.8%
IR/ Embryo Thawed	17.5%	22.1%

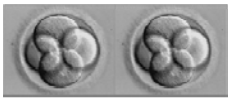
Summary of outcomes from modified method

Embryos for transfer	↑ 18%
Fully intact embryos	↑ 47%
Blastomere survival	↑ 23%

Implantations per embryo thawed ↑26%

Clinical outcomes

What is the outcome when we transfer 2 fresh 4 cell stage embryos (day 2)



Female Age	ET's	Preg Rate (FH)	*Multiple Rate*
< 36	1366	42.5%	*(40.3%)*
36-39	935	34.2%	*(31.3%)*

Women <36

100 transfers of 2 fresh 4 cell embryos	*40 births*	56 babies*
100 transfers of 1 fresh 4 cell embryo	*31 births*	31 babies
100 transfers of 1 thawed 4 cell embryo	*24 births*	25 babies
Total	*55 births*	56 babies

* but 32 of the 56 babies will be in a twin environment in utero

Melbourne IVF

- 1.8 embryos frozen for every fresh embryo transferred
- 46% of all babies born are from cryopreserved embryos
- Half of all cycles in women under 36 - at least 3 embryos cryopreserved
- 80% of all women giving birth from a fresh cycle have stored embryos

Cumulative FH pregnancy rate in first cycle

	<35	35-40
All cycles	63% (n=1857)	53% (n=1675)
At least one embryo frozen	65% (n=1402)	60% (n=1056)
At least three embryos frozen	71% (1030)	69% (n=721)

Conclusions – embryo cryopreservation

- High survival and implantation rates can be achieved with cryopreservation at early cleavage and blastocyst stages of development
- High survival and implantation rates can be achieved with optimal application of slow cooling and vitrification
- There is a paucity of RCT's comparing optimal application of slow cooling and vitrification of human embryos
- Both slow cooling and vitrification should be considered for embryo cryopreservation
- Research to improve both approaches must continue

Edgar and Gook, Human Reproduction Update, 18, 536-554 (2012)

Cryopreservation in ART

	Slow cooling	Vitrification
Sperm	+++	+++
Ovarian tissue	++	++
Oocytes	++	+++
Blastocysts	++ / +++	+++
Cleavage stage embryos	+++	+++

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Fresh versus frozen oocytes , are we reaching the optimum?

Dr. Maria J. de los Santos, PhD
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IVI Valencia

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I declare no conflict of interest

- **Lecture Outline**
  - **Introduction**
  - **Data from the past**
  - **Where we are right now**
  - **Where we would like to go**

Oocyte related Factors

Size (~ 150 µm)

Spheric shape

✓ Difficulty for uniform CPA distribution

✓ Specific membrane permeability associated to MII stage

✓ Difficult homogeneous distribution of the cryoprotectant

✓ Low cryotolerance

1 cell

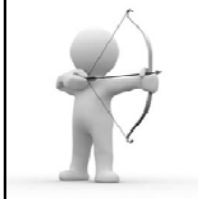
✓ "all or nothing"

Leibo Anim Repros Sci 1996
Coticchio RBMonline 2007
Gook and Edgar Mol Hum Update 2007
Baghi et al 2008. Expert Rev Med Devices 2009

Subcellular estructures

- Meiotic spindle
- Actine filaments
- Cortical granules
- Mitochondrial distribution

✓ Zona hardening
✓ Risk of oocyte activation
✓ Risk of dyginia during fertilization
✓ PB deg/fusion
✓ Risk of embryo aneuploidy

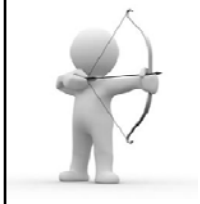


✓ **Preserve cytoplasmic structure**

- ✓ **Cellular integrity**
- ✓ **Subcellular integrity**
  - ✓ **Mitochondria**
  - ✓ **Cytoskeletal stability**
    - ✓ **Meiotic spindle**
    - ✓ **Actin filaments**

✓ **Preserve gamete physiology**

- ✓ **Metabolic activity**
- ✓ **Gene expression profile**



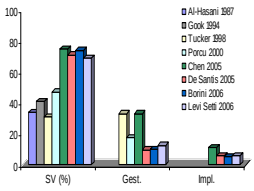
- ✓**Good clinical outcome**
  - ✓**Fertilization**
  - ✓**Preimplantational embryo development**
  - ✓**Pregnancy rates**
  - ✓**Delivery rates**
  - ✓**Healthy new borns**

Slow freezing

Chen 1986	Freezing of human oocytes

Oocyte cryopreservation using slow freezing protocols had important problems:

- ✓Highly inefficient
- ✓Low consistency
- ✓Suboptimal outcome



1st generation protocols

EQ: 1.5M PROH / 0.1 M suc

Dilution:

PROH (1.0; 0.5; 0.2 M) /0.2Msuc

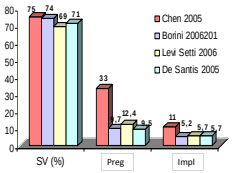
Lasalle et al, 1985

Ref.	Nº ovo	Surv. (%)
Al-Hasani et al., 1987	159	33.9
Gook et al., 1994	134	41.0
Tucker et al., 1998	311	24.0
Porcu et. al, 2000	1640	46.8
Borini et.al, 2004	737	37*

Gestación 25.4%
Implantation 16.4%

2nd generation protocols

EQ: 1.5M PROH / 0.3 M suc
Dilution:
PROH (1.0; 0.5; 0.2 M) /0.3 Msuc.
Fabbri et al, 2001



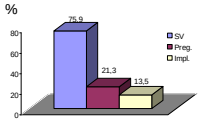
Replacement of Na^+ for Choline

EQ: 1.5M PROH HTF+ o PBS+/0.3M suc
Dilution PROH- /0.5M suc. Boldt et al 2006

Parameter	PBS	mHTF
Thaw cycles	26	28
Patients	19	19
Mean age in years (range)	32.4 (24-39)	30.0 (25-3
Transfer cycles	19	74
Eggs frozen	171	285
Eggs thawed	171	100
Survival (%)	106 (61.4)	113 (56.5)
ICSI	104	112
Fertilized (%)	68 (65.8)	76 (67.9)
Embryos transferred	47	63
Mean embryo/ET number	4.7	5.4
Mean embryo grade*	2.4	2.6
Pregnancies	5 (26.3)	9 (37.5)
Biochemical losses*	1	1
Clinical losses	0	7
Implantation sites*	5	10
Implantation rate/embryos transferred (%)	10.6	11.9
Implantation rate/eggs thawed (%)	3.0	3.5
Ongoing pregnancies	0	1
Live born deliveries	4	5
Singleton deliveries	3	4
Twin deliveries	1	1

3rd Generation protocols

EQ: 1.5M PROH / 0.3 M suc
Dilution:
PROH (1,0; 0.5; 0,2 M) /0.2 M suc.
Fabbri et al, 2001



Bianchi, et.al., 2007

Efficiency of oocyte cryopreservation: a meta-analysis

Kutluk Oktay, M.D.,[a] Aylin Pelin Cil, M.D.,[a] and Heejung Bang, Ph.D.[b]

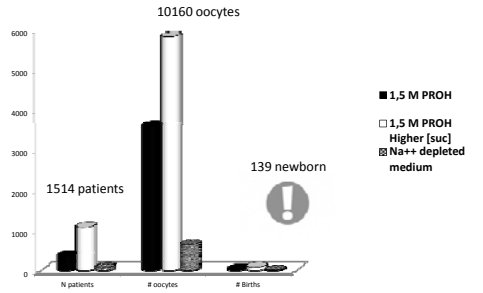
Poor results:
Survival:~**75%.** Pregnancy ~**20%**. Implanttion de **6-10%**

Human Reproduction Update, Vol.13, No.6 pp. 591–605, 2007
Advance Access publication September 10, 2007
doi: 10.1093/humupd/dmm038

Human oocyte cryopreservation

The validation has not been possible, mainly because of the scarce potential of the developed embryos, allowing a very limited utility.

From 1996-2007 using slow freezing protocols



Adapted from Gook and Adgar Hum Reproduction Update 2007

Human Reproduction, Vol.27, No.9 pp. 2571 - 2584, 2012
Advanced Access publication on July 10, 2012 doi:10.1093/humrep/des255
human reproduction ESHRE PAGES

Assisted reproductive technology in Europe, 2008: results generated from European registers by ESHRE[1]

A.P. Ferraretti[2], V. Goossens, J. de Mouzon, S. Bhattacharya, J.A. Castilla, V. Korsak, M. Kupka, K.G. Nygren, A. Nyboe Andersen, The European IVF-monitoring (EIM)[3], and Consortium, for the European Society of Human Reproduction and Embryology (ESHRE)
ESHRE Central Office, Pleemstraat 60, B-1852 Grimbergen, Belgium

Frozen oocyte replacement (FOR)

FOR was recorded by the same nine countries of 2007 report, with a total of **4080** thaws, **3359** transfers, **556** pregnancies and **358** deliveries. The majority (80%) was performed in Italy.

DELIVERY RATE OF 8.8%

Human Reproduction, Vol.27, No.9 pp. 2571 - 2584, 2012
Advanced Access publication on July 10, 2012 doi:10.1093/humrep/des255
human reproduction ESHRE PAGES

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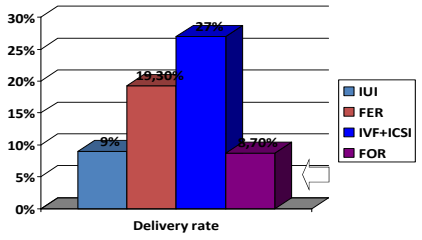


IVI

Vitrification

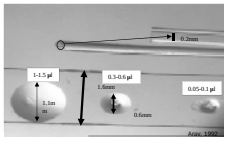
How to perform?

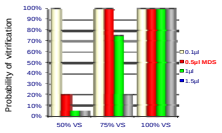
2. High cooling rate

15.000 to 30.000 ºC/min

a. Direct plunging in to the LN (open systems), use of SLN

b. Use very low volume while the loading the sample MDS
(Arav, 1992)





The use of minimum volume allows a reduction in the CPA concentration.

Human Reproduction vol 14 no 10 pp 2677–2679, 1999

CASE REPORT

Birth following vitrification of a small number of human oocytes

Lilia Kuleshova[1], Luca Gianaroli[2], Cristina Magli[2], Anna Ferraretti[2] and Alan Trounson[1,3]

17 oocytes
1-2 µl

Open Pulled Straw

Survival rate	11/17 (64.7%)
Fertilization rate	5/11 (45%)

Article

Highly efficient vitrification method for cryopreservation of human oocytes

Table 3. In-vitro survival of cumulus-enclosed or cumulus-free human oocytes after vitrification by the Cryotop method using two different cryoprotectant concentrations compared with freezing by standard equilibrium cooling. CPA = cryoprotective additive.

Cryopreservation method	CPA concentration (mol/l)	Cumulus layer	No. of oocytes cryopreserved	No. (%) with normal morphology	No. fertilized	No. (%) of blastocysts
Cryotop vitrification	6.8[d]	+	33	25 (75.8)[b]	23	8 (24.2)[b]
	6.8[d]	–	10	3 (30.0)[c]	2	0
	5.0[d]	+	64	58 (90.6)[a]	52	32 (50.0)[a]
Standard equilibrium cooling	1.5[e]	–	9	2 (22.2)[c]	0	0

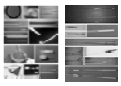
[a,b,c]Values with different superscripts within the same columns are significantly different ($P < 0.05$).
[d,e]Cryoprotectants ethylene glycol and propylene glycol respectively.

Vol. 18 Suppl. 4 2009 Reproductive BioMedicine Online; www.rbmonline.com/Article/4555
on web 15 October 2009

Contribution from 11th World Congress on Controversies in Obstetrics, Gynecology and Infertility (COGI) 'Celebration - 30 years of IVF' and Serono Symposia International Foundation

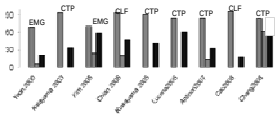
Vitrification of human mature oocytes in clinical practice

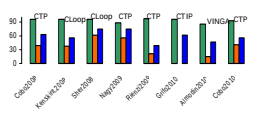
Ana Cobo[a,*], Gabor Vajta[b], José Remohí[a]



Saragusty; Reprod, 2011

Open systems





☐ Survival rate ☐ Implantationl rate ☐ Pregnancy rate

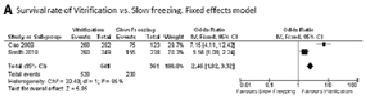
Oocyte-banking: storage problem, possible cross contamination and safety

Clinical application of oocyte vitrification: a systematic review and meta-analysis of randomized controlled trials

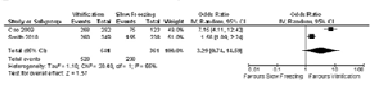
Ana Cobo, Ph.D.[a] and César Díaz, M.D.[b]

Odds ratio of postthawing/warming oocyte survival rate after vitrification and SF. (A) Fixed-effects model. (B) Random-effects model.

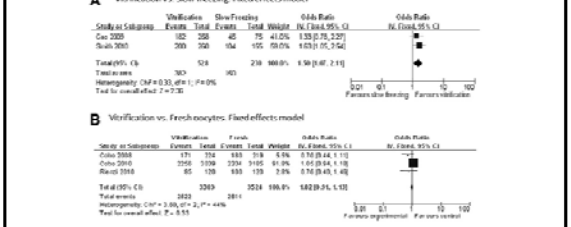
A Survival rate of Vitrification vs. Slow freezing, Fixed effects model



B Survival rate of Vitrification vs. Slow freezing, Random effects model



Odds ratio of fertilization rate. (A) Vitrification versus SF. (B) Vitrification versus fresh oocytes. Fixed effects-model.



Cobo. Meta-analysis of the use of oocyte vitrification. Fertil Steril 2011.

Table IV Primary outcome, OPR, according to the type of oocytes received.

	Egg-bank	Fresh
Ongoing pregnancy rate/ITT	131 (43.7)	125 (41.7)
Ongoing pregnancy rate/cycle	131 (44.4)	125 (43.3)
Ongoing pregnancy rate/transfer	131 (49.1)	125 (48.3)

Unless otherwise indicated values are mean ± SD or n (%).
ITT, intention to treat.

Clinical application of oocyte cryopreservation

- Infertile patients (AR)
  - Endometrial inadequacy
  - OHS risk
  - No possibility of insemination
  - Low response
  - Ethical/religious considerations
  - Legal restrictions
- Fertility preservation
  - Social freezing
  - Medical reasons
- Ovum donation

Other indications

	OHSS	No SS at ovum pick-up	Other
Nº patients	44	18	74
Age	32.8 ± 3.3	34.9 ± 3.6	37.9 ± 3.9
Nº of vit. oocytes	593 (16.9 ± 5.1)	18 (100)	68 (91.9)
Nº of warmed oocytes	450 (12.9 ± 5.0)	188 (9.56 ± 1.5)	899 (10.3 ± 4.1)
Survival	378 (84.0)	172 (91.8)	758 (84.3)
Mean ET	2.2 ± 0.6	1.9 ± 0.5	1.9 ± 0.4
Implantation rate	34.3	41.7	37.1
CPR	14 (42.4)	11 (61.1)	35 (51.4)

Accumulation of oocytes: a new strategy for managing low-responder patients

A Cobo [a,*], Nicolás Garrido [b], Juana Crespo [c], Remohí José [c], Antonio Pellicer [c]

Reproductive BioMedicine Online (2012) 24, 429–432

Table 3 Live birth rate per patient and per embryo transfer.

	LR-Accu-Vit	LR-fresh
Embryo transfers (n)	220	318
Transfer cancellations/patient (%, 95% CI)	9.1 (6.8–11.4) [b]	34.0 (29.8–38.2) [b]
Implantation rate		
n/total	110/440	138/540
% (95% CI)	25.0 (20.7–30.0)	25.6 (21.9–29.3)
Embryos transferred (mean, 95% CI)	2.0 (1.9–2.1) [c]	1.7 (1.6–1.8) [c]
Live-birth rate/embryo transfer		
n/total	73/220	108/318
% (95% CI)	33.2 (25.7–38.0)	34.0 (28.7–39.1)
Live-birth rate/patient		
n/total	73/242	108/482
% (95% CI)	30.2 (24.3–35.9)	22.4 (18.7–26.1)
Cumulative live-birth rate/patient		
n/total	88/242	114/482
% (95% CI)	36.4 (30.3–42.4) [d]	23.7 (19.9–27.4) [d]

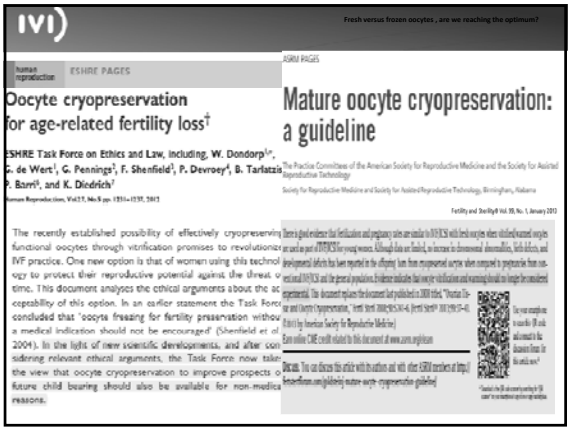
Table 3 Effect of oocyte accumulation by vitrification in the clinical outcome of low-responder PGS patients aged 41–44 years.

Outcome	≤5 MII oocytes	≥6 MII oocytes	Accumulated vitrified oocytes[a]
Mean age (SD)	42.1 (1.1)	41.7 (0.9)	42.1 (1.0)
No. of patients	87	155	105
Mean no. of MII oocytes retrieved (SD)	3.6 (1.2)	10.5 (4.5)	10.1 (3.3)
Transfers (%)	21.8	51.6[b]	59.2[c]
Mean no. of embryos transferred (SD)	1.0 (0)	1.4 (0.6)	1.4 (0.5)
PR	31.6	26.3	29.5
IR	31.6	24.3	25.6
Ongoing PR/transfer	26.3	18.8	29.5
Ongoing PR/oocyte retrieval	5.8	11.6	15.2[d]
Ongoing IR	26.3	19.8	24.4

Values are percentages unless otherwise indicated. IR = implantation rate; MII = metaphase II; PR = pregnancy rate.
[a]Patients with low ovarian response accumulating ≥6 MII oocytes in several stimulation cycles using vitrification technology.
[b]$P < 0.0001$ versus ≤5 MII oocytes.
[c]$P < 0.0001$ versus ≤5 MII oocytes.
[d]$P = 0.0391$ versus ≤5 MII oocytes.

5-years experience of Egg-banking in IVI Valencia

		95% CI
Number of cycles	3,137	
Number of vitrified oocytes donated	33,096	
Number of embryo transfers/donation	2,729 (91.2)	90.1-92.3
Number of embryos replaced (Mean ± SD)	5,118 (1.87 ±1.8)	1.78-1.96
Number of embryo cryopreservation cycles/donation cycle	2,125 (67.7)	65.7-69.7
Mean number of re-vitrified embryos (Mean ± SD)	6,417 (2,1 ± 1.9)	2.0-2.2
Implantation rate	1,989 (38.8)	36.7-40.9
Clinical pregnancy /cycle	1,485 (47.3)	44.8-49.8
Clinical pregnancy /embryo transfer	1,485 (54.4)	51.9-56.9
Ongoing pregnancy /cycle	1,222 (38.9)	36.2-41.6
Ongoing pregnancy /embryo transfer	1,222 (44.7)	41.9-47.5



ESHRE PAGES

Oocyte cryopreservation for age-related fertility loss†

ESHRE Task Force on Ethics and Law, including, W. Dondorp[1,*], G. de Wert[1], G. Pennings[2], F. Shenfield[3], P. Devroey[4], B. Tarlatzis[5], P. Barri[6], and K. Diedrich[7]

The recently established possibility of effectively cryopreserving functional oocytes through vitrification promises to revolutionize IVF practice. One new option is that of women using this technology to protect their reproductive potential against the threat of time. This document analyses the ethical arguments about the acceptability of this option. In an earlier statement the Task Force concluded that 'oocyte freezing for fertility preservation without a medical indication should not be encouraged' (Shenfield et al. 2004). In the light of new scientific developments, and after considering relevant ethical arguments, the Task Force now takes the view that oocyte cryopreservation to improve prospects of future child bearing should also be available for non-medical reasons.

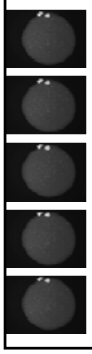
ASRM PAGES

Mature oocyte cryopreservation: a guideline

The Practice Committees of the American Society for Reproductive Medicine and the Society for Assisted Reproductive Technology

Society for Reproductive Medicine and Society for Assisted Reproductive Technology, Birmingham, Alabama

Fertility and Sterility® Vol. 95, No. 1, January 2011



Obstetric outcome in pregnancies from vitrified oocytes.

RBMOnline - Vol 16, No 5, 2008 608-610 Reproductive BioMedicine Online; www.rbmonline.com/Article/3269 on web 26 March 2008

Commentary

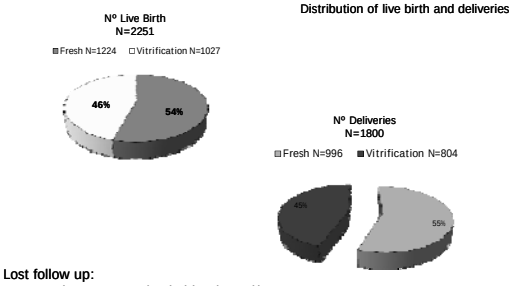
Obstetric and perinatal outcome in 200 infants conceived from vitrified oocytes

Ri-Cheng Chian[1,4], Jack YJ Huang[1], Seang Lin Tan[1], Elkin Lucena[2], Angela Saa[2], Aleiandro Roias[2], Luis Arturo Ruvalcaba

"The results indicate that the mean birth weight and the incidence of congenital anomalies are comparable to that of spontaneous conceptions in fertile women or infertile women undergoing in-vitro fertilization treatment".

"These preliminary findings may provide reassuring evidence that pregnancies and infants conceived following oocyte vitrification are not associated with increased risk of adverse obstetric and perinatal outcomes".

Distribution of live birth and deliveries



Lost follow up:
N=23 patients; 1.2% (30 babies (1.3%))

Obstetric outcome

	Fresh oocytes	Vitrified oocytes	OR(95%CI)	p value
Singleton pregnancies	77.1%(74.4-79.6)	72.9%(69.7-75.9)	0.80(0.64-1.00)	ns
Twin pregnancies	22.8% (20.2-25.4)	26.7% (23.6-29.7)		ns
>=triplets (monozygotic twining)	0%	0.4% (-0.03-0.84)	na	
Chorionic villi biopsies	1.3% (0.7-2.2)	2.4% (1.5-3.7)	1.91 (0.91-4.00)	ns
Amniocentesis	8.3% (6.7 10.2)	13.1% (10.9 15.7)	1.67 (1.23 2.28)	0.0011
Malformations (echography)	1.2% (0.7-1.5)	0.6% (0.2-1.5)	0.51 (0.18-1.48)	ns
Placental abnormalities	2.3% (1.5-3.5)	2.9% (1.9-4.4)	1.26 (0.70-2.30)	ns
Intrauterine growth retardation (IUGR)	6.7% (5.3-8.4)	5.3% (4.0-7.2)	0.79 (0.53-1.19)	ns
Diabetes	9.4% (7.7-11.4)	9.3% (7.4-11.5)	0.99 (0.72-1.37)	ns
Hypertension	12.3% (10.4-14.6)	12.8% (10.6-15.4)	1.05 (0.79-1.40)	ns
Hb<11	6.1% (4.7-7.8)	6.5% (4.9-8.5)	1.07 (0.72-1.58)	ns
2nd&3rd trim bleeding	6.3% (5.0-8.1)	8.5% (6.7-10.6)	1.37 (0.95-1.97)	ns
Membrane rupture <37th week	6.5% (5.1-8.2)	5.5% (4.1-7.3)	0.84 (0.56-1.23)	ns
Preterm del. risk <37th week	8.1% (6.5-10.0)	8.3% (6.6-10.5)	1.03 (0.73-1.46)	ns

Delivery/puerperal outcome

	Fresh oocytes	Vitrified oocytes	OR(95%CI)	p value
Gestational age	38.2 (38.0-38.4)	38.2 (38.0-38.4)		ns
Vaginal delivery	35.6% (31.5-39.7)	27.7% (23.9-31.5)		
Caesarean section	64.4% (61.5-67.6)	72.3% (69.1-75.5)	1.44 (1.16-1.76)	<0.001
Placental abnormalities	1.3% (0.7-2.2)	2.3% (1.5-3.7)	1.89 (0.90-3.94)	ns
Umbilical cord complications	0.2% (0.02-0.9)	0.5% (0.01-1.7)	2.87 (0.26-31.7)	ns
Puerperal pathology	7.0% (5.6-8.8)	6.4% (4.8-8.3)	0.90 (0.62-1.32)	ns

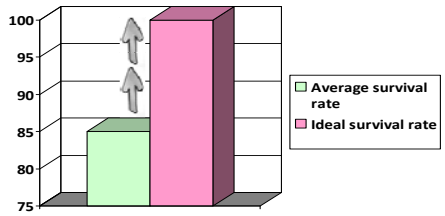
Perinatal outcome

	Fresh oocytes	Vitrified oocytes	OR(95%CI)	p value
Weight	**2871 (2834-2908)**	**2859 (2818-2901)**		**ns**
LBW <2500gr	**29.6% (27.0-32.2)**	**29.9% (27.1-32.7)**	**1.01 (0.85-1.21)**	**ns**
VLBW <1500gr	**3.7% (2.4-5.0)**	**4.0% (2.6-5.4)**	**1.07 (0.64-1.78)**	**ns**
Height	48.8 (48.7-49.0)	48.9 (46.6-49.1)		ns
Cranial perimeter	33.6 (33.5-33.8)	33.5 (33.4-33.7)		ns
Apgar 1	8.9 (8.8.-8.9)	8.8 (8.7-8.9)		ns
Apgar 5	9.6 (9.5-9.6)	9.6 (9.5-9.6)		ns
Apgar 10	9.6 (9.5-9.7)	9.6 (9.5-9.7)		ns
Malformation	**1.4% (0.9-2.2)**	**1.7% (1.0-2.1)**	**1.20 (0.61-2.32)**	**ns**
Major malformation	**0.8% (0.4-1.5)**	**0.7% (0.3-1.4)**	**0.83 (0.32-2.20)**	**ns**
Minor malformation	**0.6% (0.3-1.2)**	**1.0% (0.5-1.8)**	**1.71 (0.64-4.51)**	**ns**
Intensive care adm.	14.2% (12.3-16.3)	13.8 (11.8-16.0)	0.97 (0.76-1.23)	ns
ICU stay (days)	12.6 (10.5-14.7)	12.3 (10.0-14.5)		
Perinatal Mortality	**0.1% (0.04-0.6)**	**0.09% (0.01-0.5)**	**0.59 (0.05-6.66)**	**ns**
Healthy infant	**99.9% (98.9-100)**	**99.9% (98.9-100)**	**na**	**ns**
Female	47.5% (44.7-50.3)	53.8% (50.7-56.8)	1.29 (1.10-1.51)	0.04
Male	52.5%	46.2%		

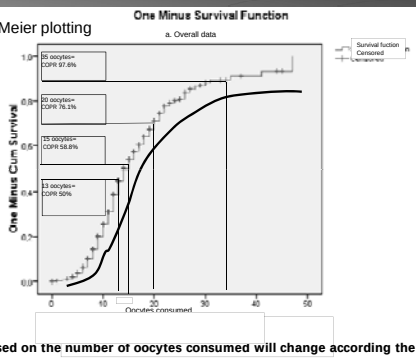
Improvements

- ✓Technical improvements
  - ✓Survival rates
  - ✓Biological viability
  - ✓Ensure standards of safety
- ✓Culture strategies after warming

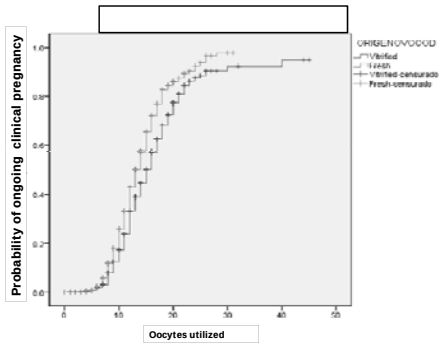
Technical improvements: Improve survival rate



Kaplan-Meier plotting



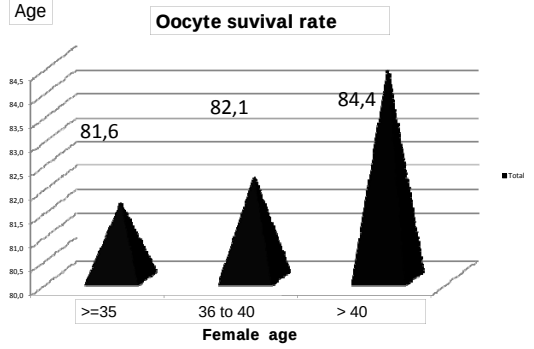
COPR based on the number of oocytes consumed will change according the survival rate.



Factors affecting survival rate

- **Age??**
- **Ovarian stimulation protocols??**
- **Oocyte quality??**

Age

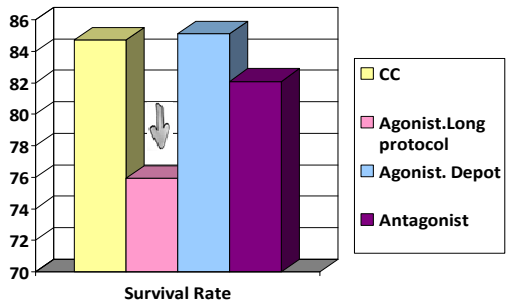


Ovarian stimulation protocols/response

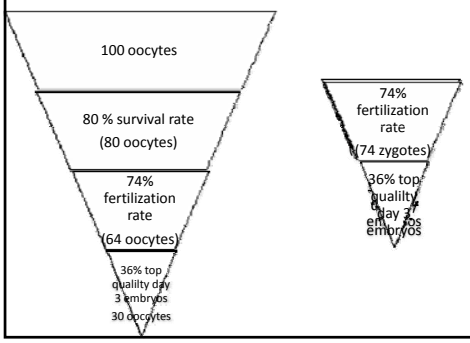
Forward logistic regression analysy to evaluate the effect of patiente and cycle characteristics on survival per oocyte

Covariate	P value	OR	95 % IC
Days of stimulation	0.554	0.9	0.67-1.24
Stimulation protocol (Agonist vs antagonist)	0.310	1.11	0.95-1.37
Serum E2day of hCG	0.552	1.00	0.99-1.00
Serum P4 day of hCG	0.350	0.46	0.08-2.36
# Retrieved oocytes	0.364	1.08	0.91-1.28
Age	0.18	1.01	0.95.1.26

Ovarian stimulation protocols



Oocyte quality



Technical improvements: biological viability or survived oocytes

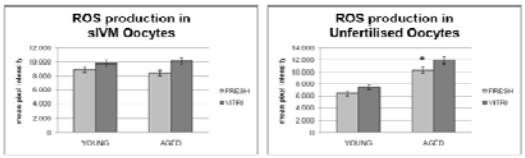
- In discarded oocytes, vitrification alters the oocyte gene expression profile Reduces proteins involved in the redox homeostasia:
  - Thioredoxin reductase I and Procollagen-lysine,
  - 2-oxoglutarate 5-dioxygenase 2

Monzo et al., Human Reprod 2012

  - Mice fresh old oocytes had increased ROS level compared with fresh young cells
  - In young oocytes vitrification caused a significant increase of ROS production, whereas vitrified old oocytes exhibited similar ROS levels to the fresh control

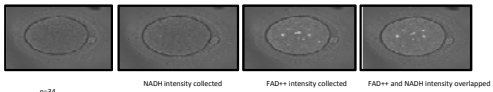
Tatone et al., 2011

IN VIVO DETECTION OF REACTIVE OXIGEN SPECIES: RESULTS

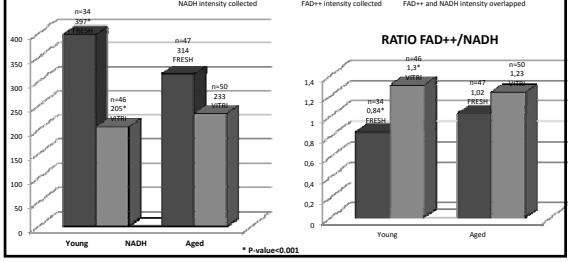


In the Unf group, aging was responsible for an increase in ROS production.

Oocyte autofluorescence measured by confocal microscopy



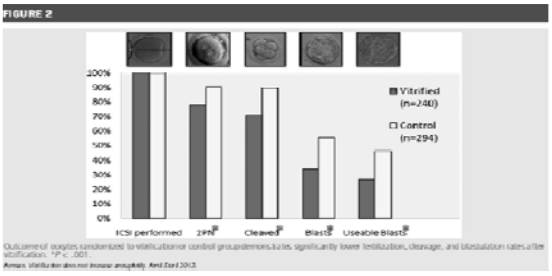
NADH intensity collected | FAD++ intensity collected | FAD++ and NADH intensity overlapped

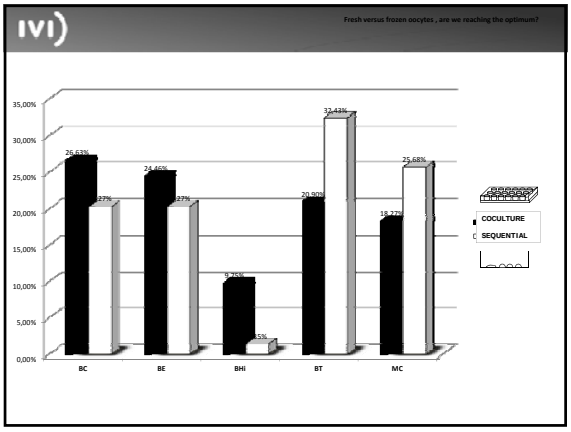


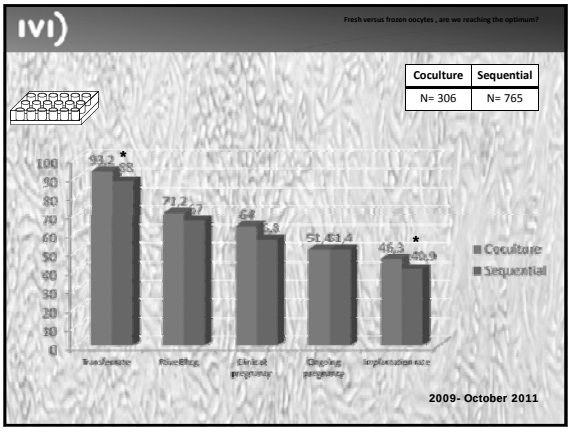
* P-value<0.001

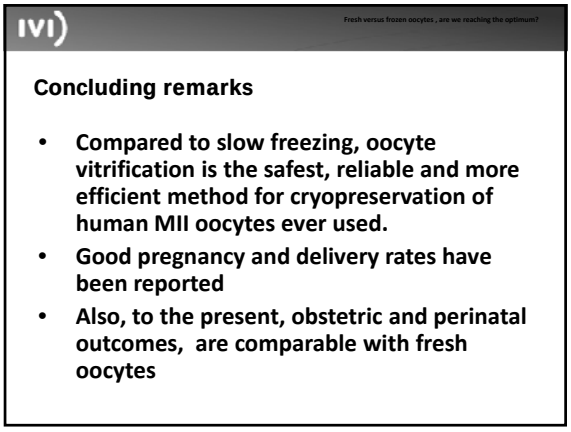
Improve culture strategies after warming

FIGURE 2



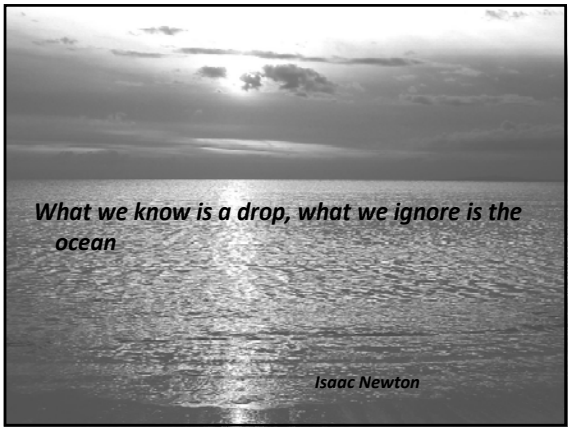

IVI
Fresh versus frozen oocytes , are we reaching the optimum?
35,00%
30,00%
25,00%
20,00%
15,00%
10,00%
5,00%
0,00%
26,63%
27%
24,46%
27%
20,90%
32,43%
25,68%
BC
BE
BHi
BT
MC
COCULTURE
SEQUENTIAL


IVI
Fresh versus frozen oocytes , are we reaching the optimum?
Coculture
Sequential
N= 306
N= 765
100
90
80
70
60
50
40
30
20
10
0
93,2
71,2
64
51
46,3
Coculture
Sequential
2009- October 2011


IVI
Fresh versus frozen oocytes , are we reaching the optimum?
Concluding remarks
• Compared to slow freezing, oocyte vitrification is the safest, reliable and more efficient method for cryopreservation of human MII oocytes ever used.
• Good pregnancy and delivery rates have been reported
• Also, to the present, obstetric and perinatal outcomes, are comparable with fresh oocytes

Concluding remarks

- **However, some improvements can be done in order to maximize this already high efficient technique:**
- **Research on:**
  - **Ovarian stimulation protocols?**
  - **Gamete/embryo physiology after vitrification**
  - **Methods of embryo culture**



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ESHRE Annual Meeting 2013
Pre-Congress Course 4
"Cryopreservation - What is hot and what is cold?"

Cryopreservation and utilization of ovarian tissue; when, where and how

Christiani A. Amorim, *VMD, PhD**
Gynecology Research Unit
IREC – UCL

Declaration

Conflict of interests: Christiani A. Amorim declares there is no conflict of interests

Lecture overview and learning objectives

- When should we cryopreserve ovarian tissue?
  - Candidates for ovarian tissue freezing: when risk of ovarian failure is high
- How should we cryopreserve ovarian tissue?
  - From biopsy collection to freezing procedure
  - What about vitrification of ovarian tissue?
- Where should we use cryopreserved ovarian tissue?
  - Techniques for ovarian tissue transplantation
- Final considerations
  - Results
  - Effect of cryopreservation and transplantation procedures on follicle survival and development
  - When transplantation of cryopreserved tissue is not advised
  - Alternatives

When should we cryopreserve ovarian tissue?

Options for preserving fertility before starting cancer treatment

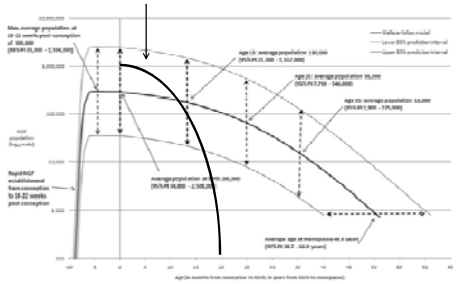
Cryopreservation	Prepuber	Immediate chemotherapy/ Risk of ovarian stimulation	Partner/ semen donor
Embryos	ϕ	ϕ	ϕ
Oocytes	ϕ	ϕ	X
Ovarian tissue	X	X	X

When chemotherapy cannot be delayed or in prepuber patients, cryopreservation of ovarian tissue is the only option

Gonadotoxicity: factors

- Chemotherapy
  - Type of agent: alkylating agents (busulfan, chlorambucil, chlormetin, ciclophosphamide, iphosphamide, melfalan, procarbazine)
  - Dosis
- Radiotherapy
  - Irradiation area (total, abdominal, pelvic)
  - Total dosis (LD_{50} < 2 Gy)
- Combination chemotherapy + radiotherapy

Chemotherapy

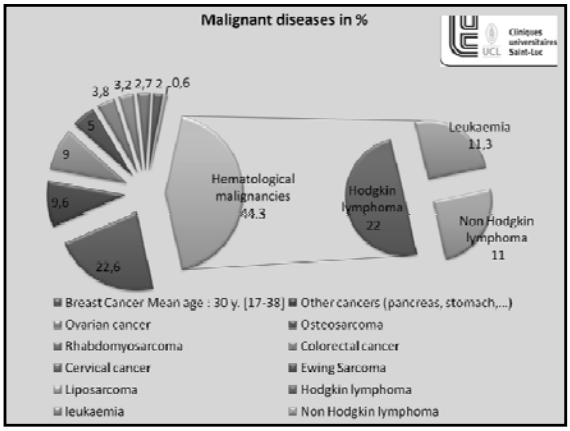


Adapted from Wallace and Kelsey. PLoS ONE, 2010

Risk of infertility after the treatment

High risk > 80%	Medium risk	Low risk < 20%
Total body irradiation	Leukaemia	Leukaemia
Pelvic irradiation	Cerebral tumor >24 Gy	Cerebral tumor <24 Gy
Bone marrow transplantation	Non- Hodgkin's lymphoma	Wilms' tumor
Hodgkin's lymphoma: alkylant	Hodgkin's lymphoma	Germinal cell tumor (no radio)
	Ewing's Sarcoma no metast.	
	Osteosarcoma	
	Hepatoblastoma	
	Neuroblastoma	

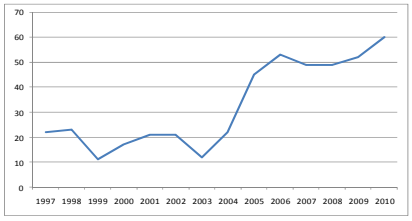
Wallace et al. Lancet 2005



Indications: benign diseases

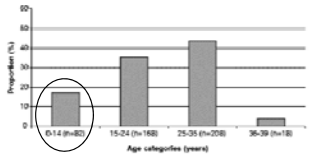
- Uni/bilateral oophorectomy:
  - Benign ovarian tumors
  - Severe endometriosis
- Risk of premature menopause:
  - Turner's syndrome
  - Family history
- Bone marrow transplantation:
  - Hematologic diseases (drepanocytosis, thalassemy, anemia aplástica)
  - Autoimmune diseases (Lupus, rhumatoid artritis)

Number of ovarian tissue cryopreservation procedures by year in our department



Dolmans et al. J Assist Reprod Genet, 2005

Percentage of patients undergoing ovarian tissue cryopreservation (n = 476) by age group



Dolmans et al. J Assist Reprod Genet, 2005

How should we cryopreserve ovarian tissue?

Conventional freezing – St Luc Hospital UCL

Ovarian biopsy:

Operation theatre (harvesting)

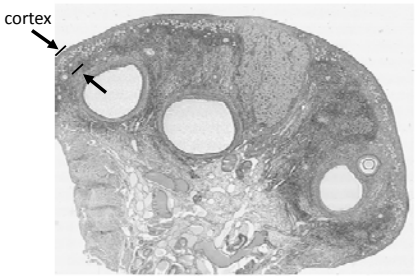
↓

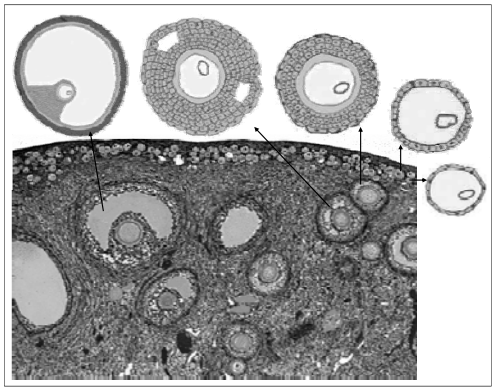
Sterile room (preparation and exposure to CPA)

↓

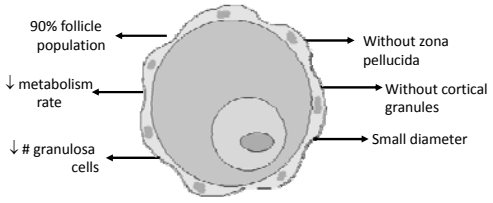
Cryobank (freezing and storage)

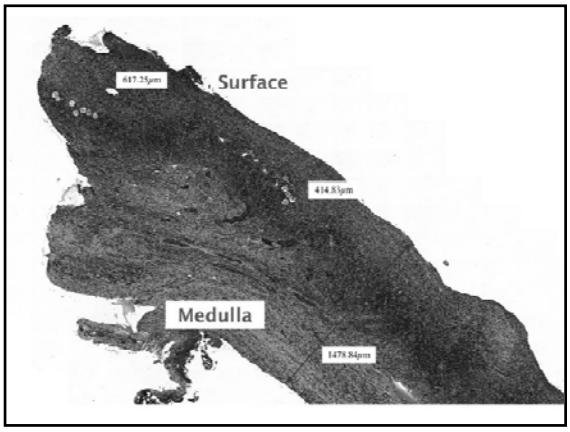
Operation theatre (biopsy harvesting)

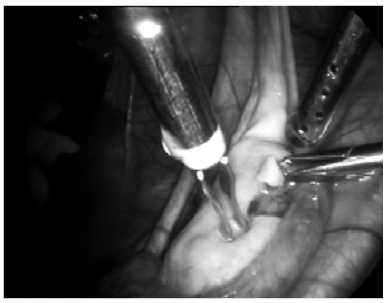




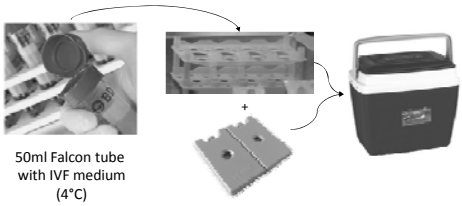
Why primordial and primary follicles?







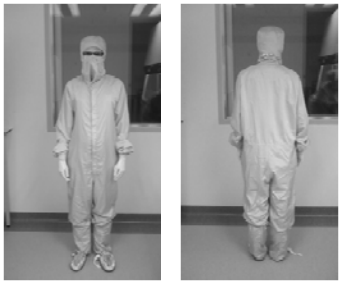
Operation theatre



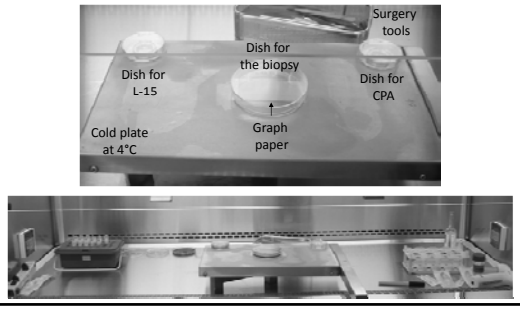
50ml Falcon tube
with IVF medium
(4°C)

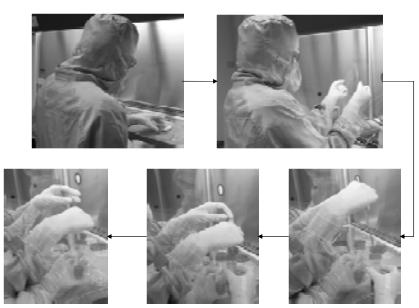
Sterile Room
(preparation and exposure to CPA)

Sterile room – preparation

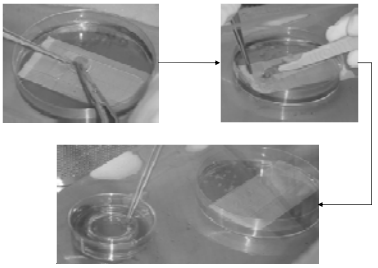


Sterile Room (exposure to CPA)

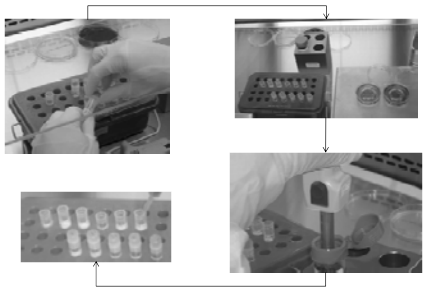


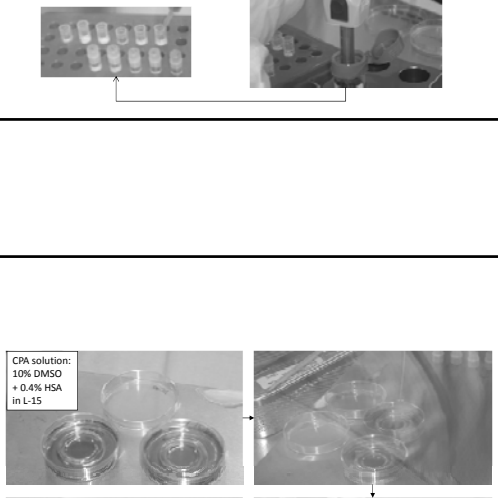


Biopsy preparation

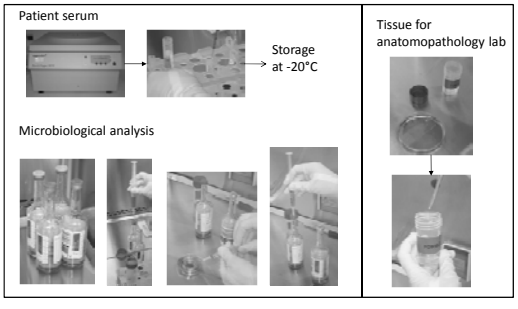


Preparation of the cryovials



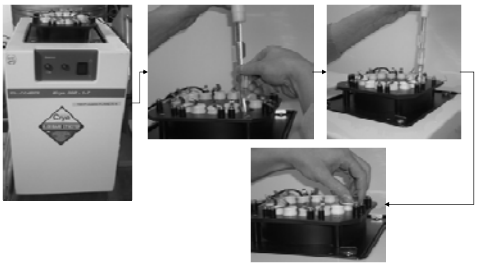


Serum, remaining biopsy and sterile room control



Cryobank: freezing procedure

Loading the Planner:



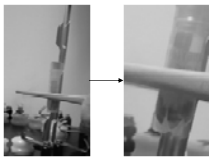
Cryobank: freezing procedure

Cooling curve:

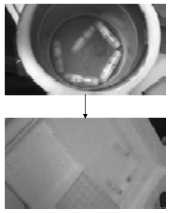
0°C
↓ -2°C/min
-8°C
(seeding + 15min)
↓ -0.3°C/min
-40°C
↓ -30°C/min
-150°C → Liquid nitrogen

Cryobank: freezing procedure

Seeding:

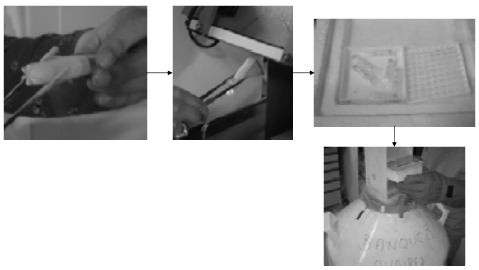


Frozen tissue:

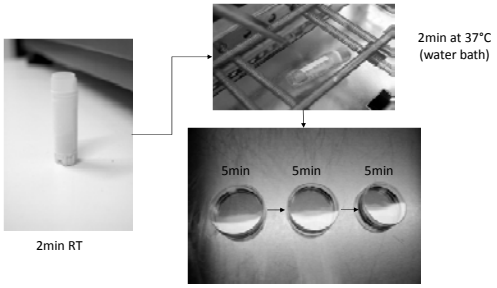


Cryobank: freezing procedure

Sealing with cryoflex and storage:



Thawing of human ovarian tissue



What about vitrification of ovarian tissue?

What about vitrification of ovarian tissue?

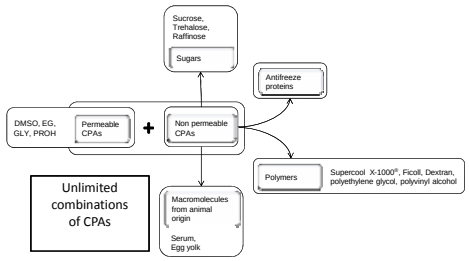
- Conventional freezing has been shown to negatively affect
  - Ovarian stroma
  - Granulosa cells
  - Theca cell formation

(*Schubert et al., 2008; Nottola et al., 2008; Keros et al., 2009*)

What about vitrification of ovarian tissue?

- Vitrification
  - Prevents intracellular ice formation (*Fahy et al., 1986)*
    - Membrane and cell organelle rupture
    - Injury caused by the solution effect
  - How can this be achieved?
    - High [CPAs]
    - High cooling rate
    - Small aqueous volume

- High [CPAs]: ↑ Toxicity
  - To decrease CPA toxicity: CPA cocktails



Vitrification – High [CPA]

- CPA cocktails
  - Stepwise addition
  - Addition at low temperatures

} ↓ CPA toxicity

Vitrification: high cooling rate

- Aim: avoid ice formation
- How it can be achieved
  - Small sample size
  - Carrier system
    - Carriers: straws, TEM copper grid, Cryotop, needle, etc
    - Carrierless: solid-surface vitrification, minimum drop size

Vitrification

Preparation of several VSs

Preparation of tiny little fragments of tissue

Stepwise addition of CPAs → Time consuming

Vitrification procedure → Great expertise

How vitrification has been working for human ovarian tissue?

Studies using human ovarian tissue

Parameter	Vitrification → warming → fixation Freezing → thawing → fixation		
	Vitrification > Freezing	Vitrification = Freezing	Vitrification < Freezing
Follicle damage (Gandolfi et al., 2006)			x
Follicle survival (Wang et al., 2008)		x	
Follicle and tissue US (Wang et al., 2008)	x		
Follicle morphology (Chang et al., 2011)		x	

Studies using human ovarian tissue

Parameter	Vitrification → warming → IVC → fixation Freezing → thawing → IVC → fixation		
	Vitrification > Freezing	Vitrification = Freezing	Vitrification < Freezing
Follicle survival and growth (Li et al., 2007; Huang et al., 2008)		x	
Follicle survival (Wang et al., 2008; Keros et al., 2009)		x	
Follicle and tissue ultrastructure (Wang et al., 2008)	x		
Stroma ultrastructure (Keros et al., 2009)	x		
Tissue histology (Isachenko et al., 2009)			x
Necrosis (Rahimi et al., 2004)		x	
Follicle morphology (Xiao et al., 2010)		x	
Follicle population and AMH expression (Oktem et al. , 2011)			x

Studies using human ovarian tissue

Parameter	Vitrification → warming → xenograft → fixation Freezing → thawing → xenograft → fixation		
	Vitrification > Freezing	Vitrification = Freezing	Vitrification < Freezing
Necrosis (Rahimi et al., 2004)		x	
Apoptotic cells in follicles and tissue (Rahimi et al., 2009)			x
Tissue revascularization (Rahimi et al., 2010)		x	
Growing follicles (Rahimi et al., 2010)			x
Growing follicles (Amorim et al., 2012)	x		

Vitrification: take home message

- Vitrification is <u>not</u> a simple and fast procedure
- So far, vitrification does not seem to be more efficient than freezing to cryopreserve human ovarian tissue

Where should we use cryopreserved ovarian tissue?

Transplantation procedure

orthotopic *vs* heterotopic site

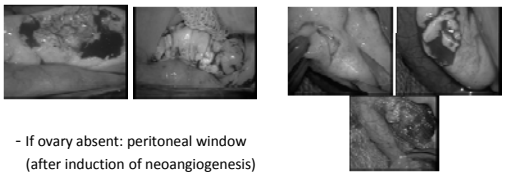
Advantages and disadvantages of heterotopic and orthotopic sites for ovarian tissue transplantation

	Heterotopic site (subcutaneous)	Orthotopic site
Advantages	• No limitation of the number of fragments transplanted • Easy transplantation procedure • Easy access for follicular monitoring and oocytes collection	• Possibility of natural conception • Restoration of fertility demontrasted • Favourable environment for follicular development
Disadvantages	• Restoration of fertility not yet demonstrated • IVF procedure required • Effect of the local environment on the oocyte quality is unknown	• Number of fragments transplanted limited by the ovarian size • Invasive transplantation procedure

Orthotopic transplantation: goal of surgery

Replace frozen-thawed tissue in an ideal environment

- If ovary present: decortication and suture or Interceed

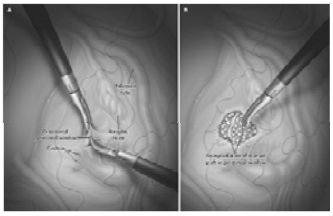


- If ovary absent: peritoneal window (after induction of neoangiogenesis)

Livebirth after orthotopic transplantation of cryopreserved ovarian tissue

Vol 364 October 16, 2004

J Donnez, M M Dolmans, D Demylle, P Jadoul, C Pirard, J Squifflet, B Martinez-Madrid, A Van Langendonckt



1st Laparoscopy
- Peritoneal window: incision in R. ovarian fossa
- Induction of neovascularization: coagulation of borders

2nd Laparoscopy
- Transplantation of fragments

Final considerations: results

Results

- Duration of the graft activity
  - > 4 years: if no chemotherapy before cryopreservation
  - ≤ 2 years: if chemotherapy before cryopreservation
- More than 20 life births
  - St Luc Hospital
    - 15 transplantations of cryopreserved ovarian tissue
    - 6 life births

→ Why not all??

Study on the quality of the eggs

ORIGINAL ARTICLE *Infertility*

IVF outcome in patients with orthotopically transplanted ovarian tissue

Marie-Madeleine Dolmans, Jacques Donnez[1], Alexandra Cacciottolo, Dominique Demylle, Christiani Amorim, Anne Van Langendonckt, and Céline Pirard

- 24 cycles: 8 empty follicles (33%)

  18 oocytes: 6 «abnormal»

  12 MII oocytes → 5 fertil
- Only 5 embryos obtained from 24 cycles

What could be affecting follicle development?

- Previous chemotherapy?
  - Some patients underwent cryopreservation before any gonadotoxic treatment
- Transplantation procedure?
- Cryopreservation procedure?

Effect of the transplantation?

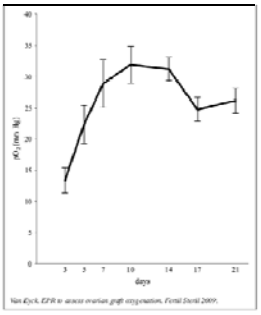
Electron paramagnetic resonance as a tool to evaluate human ovarian tissue reoxygenation after xenografting

Anne-Sophie Van Eyck, M.D.,[a] Bénédicte Francine Jordan, Pharm.D., Ph.D.,[b] Bernard Gallez, Pharm.D., Ph.D.,[b] Jean-François Heinlen, Pharm.D., Ph.D.,[a] Anne Van Langendonckt, M.Sc., Ph.D.,[a] and Jacques Donnez, M.D., Ph.D.[a]

Fertility and Sterility® Vol. 92, No. 1, July 2009

FIGURE 2

Monitoring of pO_2 values (mean ± SEM, n = 6 patients). The pO_2 levels significantly increased from day 5 to day 10. Reoxygenation kinetics were characterized by reoxygenation speed (slope = 2.4 ± 0.4 mm Hg/d; $P<.001$), specific to our graft model. The highest level obtained on day 10 was still maintained on day 14. From day 17, pO_2 values were stable until day 21 and remained higher than the value observed on day 3.



Van Eyck, EFR to assess ovarian graft oxygenation, Fertil Steril 2009.

Negative controls (day 21 post-euthanasia)

pO_2 (mmHg): 10.5 ± 1.9mmHg

// hypoxic value

Effect of transplantation?

- Initial hypoxia and ischemia (~ 3 days post-grafting) could cause:
  - ↓ follicular survival
  - ↑ follicular activation
  - Asynchrony between oocyte and follicular cells

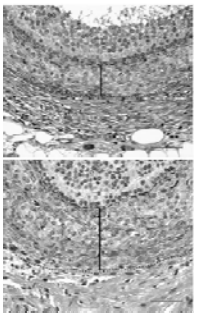
Effect of the cryopreservation?

Impact of freezing and thawing of human ovarian tissue on follicular growth after long-term xenotransplantation

K Israëlsson A. Samuelsson · Sara Duvall · Marie-Madeleine Dolmans · Alessandra Camboni · Jacques Donnez · Anne Van Langendonckt

J Assist Reprod Genet (2011) 28:1153–1165

Freezing and thawing procedures: impact on the morphology of antral follicles, specifically the thickness of the theca cell layer.



Frozen-thawed xenografted human ovarian tissue

Fresh xenografted human ovarian tissue

Conclusion

- Orthotopic reimplantation of cryopreserved ovarian tissue
  - Feasible
  - Resulted in more than 20 life births
- Avascular transplantation
  - Successful
  - May affect follicular population (hypoxia and ischemia period)
- Cryopreservation procedure
  - Successful
  - May affect follicle development → more studies

Final considerations

Transplantation of cryopreserved ovarian tissue: safety aspects

When transplantation of cryopreserved tissue <u>is not</u> advised

Risk of ovarian involvement in different cancers

High risk >11%	Medium risk 0.2-11%	Low risk < 0.2%
Leukemia	Breast cancer	Wilms' tumor
Neuroblastoma	Adenocarcinoma of uterine cervix	Non-Hodgkin's lymphoma
		Hodgkin's lymphoma
		Nongenital rhabdomyosarcoma
		Osteogenic sarcoma
		Squamous cell carcinoma of the uterine cervix
		Ewing's sarcoma

Risk of reimplantation of malignant cells!

Oktay. Hum Reprod Update, 2001

Alternatives

- *In vitro* culture of primordial follicles *(Telfer et al., 2008)*
- Artifical ovary *(Dolmans et al., 2008; Amorim et al., 2009; Vanacker et al., 2012)*

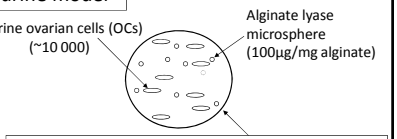
Transplantation of an alginate–matrigel matrix containing isolated ovarian cells: First step in developing a biodegradable scaffold to transplant isolated preantral follicles and ovarian cells

Biomaterials 33 (2012) 6079–6085

Julie Vanacker [a], Valérie Luyckx [a], Marie-Madeleine Dolmans [a], Anne Des Rieux [b], Jonathan Jaeger [a], Anne Van Langendonckt [a], Jacques Donnez [a,c], Christiani A. Amorim [a]

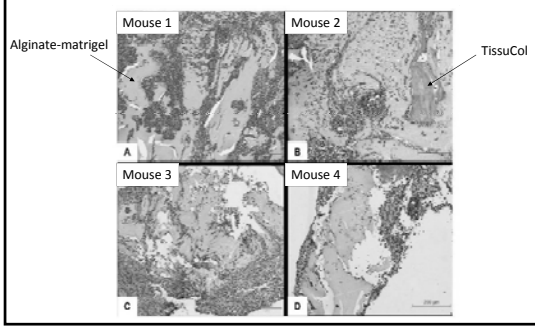
Autografts in a murine model

N=4 NMRI mice
1 week



Matrigel (1/2) + 2% irradiated alginate (Sigma) (1/2)

• Scaffold degradation
The matrix is well degraded 1 week after grafting

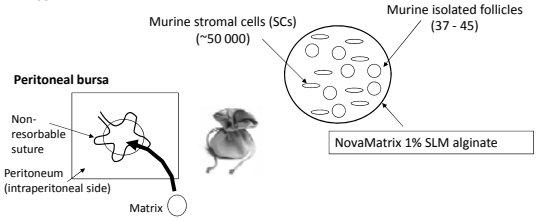


First transplantation of a biodegradable artificial ovary containing murine isolated follicles: hope for the clinical application

Vanacker J, Luyckx V, Van Langendonckt A, Donnez J, Dolmans MM, Amorim CA

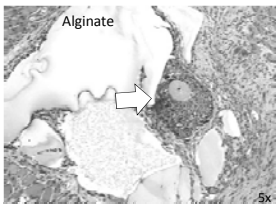
In preparation

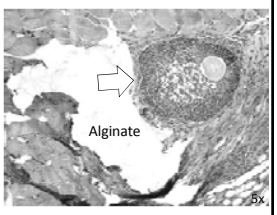
N=7 beads grafted to 6 NMRI mice
1 week



- Follicle recovery

  Grafted isolated follicles are found even at more advanced follicular stages





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Dolores Gonzalez
Olivier Van Kerk
Anne Lépage
Mira Hryniuk, *BA*

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Cryopreservation of testicular tissue: when, where and how ?

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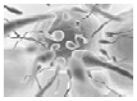
ESHRE LONDON 7 July 2013

Disclosure

I declare to have
no commercial relationships
no conflict of interest



Learning objectives

- History of (testicular) sperm freezing
- Indications for testicular sperm freezing
- When to cryopreserve testicular sperm?
- What (quality) to cryopreserve?
- How to cryopreserve testicular sperm?
- Cryodamage to testicular sperm
- ICSI with frozen-thawed testicular sperm
- Testicular freezing for prepubertal boys
- Conclusions

3

History of sperm freezing

- 1776 Spallanzani → low t° effects on human spermatozoa
- 1866 Montegazza suggested cryobanks for animal semen
- 1949 Polge used glycerol as cryoprotectant for mammalian spermatozoa
- 1950 successful use of extenders
- 1953 Sherman observed fertilization and embryo development with human sperm frozen on dry ice
- 1964 First birth after glycerol freezing of human sperm in liquid nitrogen
- 1973 First association of sperm banks (CECOS – France)
- 1976 American Society of Tissue Banks
- 1996 **First birth after using frozen-thawed testicular sperm for ICSI**

Indications for testicular sperm freezing

- For fertility treatment
  - → Azoospermia
  - → Preserve sperm for later fertility treatment (ICSI)
  - → Avoid repeated testicular surgery
  - → Avoid problems in coordinating OPU and testicular surgery
  - → Ensure presence of sperm before ovarian stimulation
  - → Select patients for fertility treatment allocation
- For fertility preservation
  - → for boys and adults pre- gonadotoxic treatment

5

When to cryopreserve ?

- Etiology of azoospermia
  - o Excretory (OA)
  - o Secretory (NOA)
- Method of testicular sperm retrieval
  - o Open biopsy
  - o Aspirations
- Occasion of testicular sperm retrieval
  - o Diagnostic
  - o Therapeutic
- Allocation criteria for NOA patients

⇨ **QUALITy**

Etiology of azoospermia

Obstructive azoospermia (OA)	Non-obstructive azoospermia (NOA)
• Mechanical cause • Normal spermatogenesis High sperm numbers • Epididymal or testicular sperm • 100% recovery rate • Causes – Vasectomy – Congenital bilateral absence of the vas deferens (CBAVD) – Post infectious epididymitis – Testicular trauma – Young's syndrome – Retrograde ejaculation	• Biological cause • Severely impaired spermatogenesis Low to absent sperm numbers • Testicular sperm • 50-60% recovery rate • Causes – Chromosomal abnormalities – Yq deletions – Iatrogenic treatment – Cryptorchidism – Testicular torsion – Unknown genetic causes (?)

When to cryopreserve?

- Therapeutic occasion:

  Spermatozoa are frozen **on the day** of oocyte retrieval
  - ICSI with fresh spermatozoa
  - Cryopreservation of supernumerary sperm for later ICSI cycles

- Diagnostic occasion:

  Spermatozoa are frozen **independent** of the day of oocyte retrieval
  - Cryopreservation of spermatozoa for later ICSI cycles
  - ICSI cycles with frozen-thawed spermatozoa

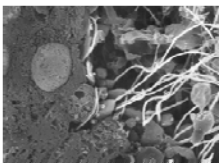
- Pros and cons for both approaches
  - Different for patients with OA or NOA
  - Dependent on sperm retrieval procedure
  - Depending on flexibility of scheduling TESE

Pros and cons of both approaches (NOA)

Approach 1: ICSI-cryo	Approach 2: cryo-ICSI
Loss of sperm quality by freezing is avoided – lower risk of finding only immotile sperm Lower sperm quality limits for ICSI treatment Less restrictive criteria for patient allocation to ICSI	Independent scheduling of sperm and oocyte retrieval Avoid pointless ovarian stimulation of the female partner (if no sperm is retrieved) Less stressful to the couple
Concomitant scheduling of sperm and oocyte retrieval on the same day 50% risk of pointless ovarian stimulation in NOA More stressful to the couple	Risk of not finding motile sperm post-thaw Higher sperm-quality limits for allocation to ICSI treatment (quality loss by freezing-thawing)

OA patients and retrieval method

- Sperm obtained by **TESE / open biopsy**
  - Sperm recovery in 100% of patients, mostly high numbers
  - Both cryo approaches are effective
  - One or two biopsies provide sufficient sperm for several ICSI cycles



OA patients and retrieval method

- Sperm obtained by **TESA / FNA**
  - Sperm recovery in almost 100% of patients
  - Only limited sperm numbers retrieved
  - Easily performed always freshly on the day of oocyte retrieval
  - Freezing not always possible, depending on
    - Sperm number
    - Collection method
      - Droplets under oil
      - Culture medium in dishes
      - Culture medium in tubes

NOA patients and retrieval method

- Sperm obtained by **TESE / open biopsy**
  - Successful in 50-60% of patients: poor numbers and motility, multiple biopsies
  - Cryo and later use for ICSI is possible in many cases
    - Depending on the quality
    - Depending on the criteria for freezing
    - Depending on the allocation criteria for ICSI treatment
  - Scheduling fresh TESE as back up in severe cases
  - Fresh TESE for ICSI is the only option in extremely poor cases

Verheyen et al. 2004, HR 19, 2822

NOA patients and retrieval method

- Sperm obtained by **TESA**
  - Often unsuccessful, no or poor sperm numbers
  - Poor chance to freeze spermatozoa
  - Uncommon procedure in NOA
- Sperm obtained by **micro-TESE**
  - Fair number may be obtained
  - Skilled microsurgeon
  - Reasonable chance to freeze spermatozoa
  - Less common procedure

Schlegel et al. 1999, HR 14, 131
Colpi et al. 2009, RBMOnline 18, 315

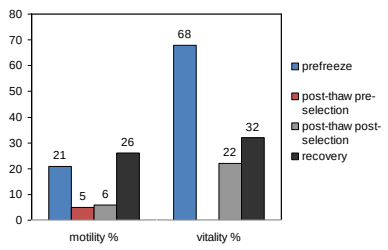
What (quality) to cryopreserve?

- No upper limit
- Lower limit ? In NOA
  - Different from clinic to clinic
  - Depending on patient allocation criteria
  - Possibility to schedule fresh TESE as back-up on day OPU
  - CRG - UZBrussel
    - Number: ≥ 1 spermatozoon
    - Motility: no cut-off, even 0% motility
    - Obtained either after mechanical or enzymatical treatment

Verheyen et al. 1995, HR 10, 2956
Crabbé et al. 1997, 12, 1682

Cryodamage of testicular sperm

- Effect on motility and viability

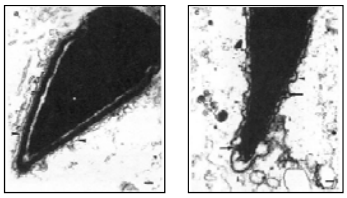


Verheyen et al. 1997, FS 67, 74

Cryodamage of testicular sperm

- Effect on the ultrastructure
  - →Rupture of plasma membranes
  - →Rupture of acrosomal membranes

Nogueira et al. 1999, HR 14, 2041



Pre-freezing | Post-thawing

Cryodamage of testicular sperm

- Effect on DNA fragmentation
  - Comet assay
  - Depending on the freeze- thawing method

    Thompson-Cree et al. 2003, RBM 4, 449
  - Comet assay
  - Increased DNA damage in frozen-thawed testicular sperm (16.5% vs 10.6%)

    Dalzell et al. 2004, FS 82, 1443

How to cryopreserve ?

- Constitution
  - Biopsy
  - Suspension
  - Individual cells
- Preparation
- Cryoprotectant
- Freezing procedure
  - Slow controlled-rate
  - Static vapour
  - Vitrification
  - Freeze-drying
- Carriers

How to cryopreserve? Constitution

- As biopsy or as suspension?
  - Suspension better preserves sperm quality
    - Motility (9% vs 4%)
    - Viability (39% vs 25%)
  - Slower/incomplete penetration of cryoprotectant into a biopsy

Crabbé et al. 1999, Int J Androl 22, 43

How to cryopreserve? Constitution

- Individual spermatozoa (NOA)
  - Time-consuming procedure before freezing
  - Carriers
    - Microcentrifuge tubes
    - Straws
    - Microdroplets under oil
    - Empty zona pellucida
  - Rarely performed
  - European Cell & Tissue Directives

How to cryopreserve? Preparation

- Mechanical procedures
  - Scissors, needles, forceps, glass slides
  - In OA and NOA
  - Rupture of tubules ⇒ sperm release

Verheyen et al. 1995, HR 10, 2956

- Dissection of individual tubules in a biopsy
  - Isolation of most dilated tubules
  - Higher recovery rate in NOA

Kamal et al. 2004, J Androl 25, 123

How to cryopreserve? Preparation

- Enzymatic procedures
  - If mechanical treatment fails
  - Collagenase type I or IV
  - In NOA
  - Degradation of collagen in basement membrane and matrix

Salzbrunn et al. 1996, HR 11, 752
Crabbé et al. 1997, HR 12, 1682
Crabbé et al. 1998, HR 13, 2791

- Erythrocyte-lysing buffer
  - If red blood cells hamper sperm visualization
  - Improved treatment procedure
  - Either before or after enzymatic treatment

Nagy et al. 1997, FS 68, 376

How to cryopreserve? Preparation

- In-vitro culture
  - Disagreement about the effectiveness on **motility**

Liu et al. 1997, HR 12, 1667
- OA: Improved sperm morphology and quality of motility
- NOA: No change in motility

Wu et al. 2005, JARG 22, 389
- Improved motility after 48h up to 72h culture
- Both OA and NOA

  - Negative effect on **DNA fragmentation**

Dalzell et al. 2004, FS 82, 1443
- Increased DNA fragmentation by 4 hour incubation (Comet assay)
- Recommendation to inject without delay

How to cryopreserve? Preparation

- Exposure to motility enhancers
  - Phosphodiesterase inhibitors

Tash and Means 1983, Biol. Reprod. 28, 75
- Increase intracellular cAMP
- Enhance sperm motility

  - Pentoxifylline most widely used
  - Theophylline preparation
    - Commercially available

Ebner et al. 2011, FS 96, 1331
- Improves motility in 98,5% of cases
- Improves ICSI outcome due to better sperm selection

How to cryopreserve? Preparation

- Concentration/dilution
  - OA with high numbers: dilute suspension before freezing
  - NOA with low numbers: concentrate (or dilute) suspension
  - Avoid refreezing

How to cryopreserve? Cryoprotectant

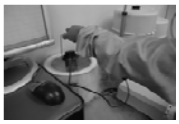
- Glycerol
  - Cryoprotectant of choice for mature spermatozoa
  - Commercially ready-prepared cryomedia
  - Testicular tissue structure is not preserved
  - No survival of germ cells
- DMSO
  - Cryoprotectant of choice for preservation of tubule structure
  - Fertility preservation for prepubertal boys (spermatogonial stem cells)

    Keros et al. 2005, HR 22, 1384; Goossens et al. 2008, FS 89, 725
  - Best maintains tissue capacity to initiate spermatogenesis

    Jahnukainen et al. 2007, HR 22, 1060

How to cryopreserve? Freezing procedure

- Slow controlled-rate vs static vapour freezing
  - No clear evidence from the literature
  - Same methods as for semen cryopreservation
  - Easy-to-use programmable freezers
  - No specific programmes for testicular sperm



How to cryopreserve? Freezing procedure

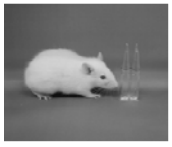
- Vitrification
  - Considered for human sperm cryobanking since 2002
  - Characteristics:
    - Extremely high cooling rates
    - Small volumes (individually aspirated spermatozoa)
    - High concentrations of cryoprotectant
  - Several papers by (only) Isachenko
    - Vitrification?
    - Or ultrarapid freezing?
  - Cryoprotectant-free vitrification?
  - Future for (testicular) sperm vitrification?

How to cryopreserve? Freezing procedure

- Freeze-drying or lyophilization
  - Successful data in animal studies

    Wakayama and Yanagimachi 1998, Nat Biotechnol 16, 639
  - Recent interest in the human

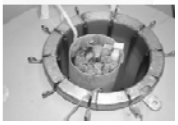
    Gianaroli et al. 2012, FS 97, 1067



Rat derived from fertilized oocytes with freeze-dried sperm stored for 5 years

How to cryopreserve? carriers

- Closed systems preferred
  - High-security sealed straws
  - Sealed cryotubes
  - Correctly, clearly labelled
  - European Cell and Tissue Directives
  - Avoid transmission of pathogens and virusses



How to store?

- Liquid nitrogen LN2 or LN2 vapour
  - Below -132°C
  - Extended storage at -80°C causes degradation over time
  - Day-and-night monitoring of individual tanks
    - Low-level sensors
    - Temperature sensors


ICSI with fresh/frozen testicular sperm

- OA: many reports since 1996

Romero et al. 1996, FS 65, 877

  - Comparable fertilization rate, embryo quality, pregnancy and implantation rate

ICSI with fresh/frozen testicular sperm

- NOA: fewer reports since 1998
  - Mixed OA and NOA
  - ICSI outcome depends on patient selection
    - Allocation criteria for NOA patients to ART
      - One (motile) sperm found
      - Based on observation of motility in preliminary-thawed testicular specimen
    - Limits for testicular sperm freezing of NOA patients

⇩

  - Comparable to decreased fertilization rate and implantation rate

Nicopoullos et al. 2004, FS 82, 691: *Meta-analysis fresh-frozen OA+NOA*

- Similar fertilization, clinical and ongoing pregnancy rate
- Significantly impaired implantation rate

ICSI with fresh/frozen testicular sperm

Human Reproduction Vol.19, No.12 pp. 2822–2830, 2004
Advance Access publication October 18, 2004

Should diagnostic testicular sperm retrieval followed by cryopreservation for later ICSI be the procedure of choice for all patients with non-obstructive azoospermia?

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BACKGROUND: This was a retrospective study to determine if diagnostic testicular biopsy followed by cryopreservation should be the procedure of choice for all patients with testicular failure. **METHODS:** The first part of the study analysed 97 ICSI cycles scheduled with frozen–thawed testicular sperm for 69 non-obstructive azoospermia (NOA) patients. The second part focused on a subgroup of 32 patients who underwent 42 ICSI cycles with frozen and 44 cycles with fresh testicular sperm. Sperm characteristics, fertilization, embryo quality, pregnancy and implantation rates were evaluated. **RESULTS: Part I:** The average time needed to find sperm was 113 min per cycle and 17 min per individual sperm. Fertilization rate, embryo transfer rate, ongoing pregnancy and implantation rates were 58.4%, 83%, 20.8% and 11.3%, respectively. **Part II:** The search time per sperm was higher ($P = 0.016$) in frozen (18 min) than in fresh suspensions (13 min). A higher embryo transfer rate was observed in fresh cycles than in frozen cycles (95.2% vs 76.2%, $P = 0.028$). Fertilization, ongoing pregnancy and implantation rates were comparable for the two groups. **CONCLUSIONS:** Even in a programme with low-restrictive criteria for patient allocation and for sperm cryopreservation, diagnostic testicular biopsy followed by cryopreservation can be the procedure of choice for patients with testicular failure.

ICSI with fresh/frozen testicular sperm

Table V. Comparison of sperm characteristics in the ICSI cycles with fresh (44 cycles) and frozen (42 cycles) testicular sperm of 32 non-obstructive azoospermia (NOA) patients

	Fresh TESE	Frozen TESE	Mann–Whitney
Cycles	44	42	
Search time/cycle (min)	81	110	$P = 0.053$
Search time/sperm (min)	13	18	$P = 0.016$
% oocytes injected with motile sperm	82.3	83.7	NS
Cycles injected with only motile sperm (%)	33/44 (75)	31/42 (74)	NS[a]
Cycles injected with only immotile sperm (%)	3/44 (7)	4/42 (10)	NS[a]
COC/cycle	10.5 ± 6.2	9.3 ± 5.2	NS
Metaphase II/cycle	9.1 ± 5.8	7.6 ± 4.2	NS
% 2PN	58.0 ± 24.2	59.3 ± 25.5	NS
% 1PN	7.0 ± 11.9	7.8 ± 19.2	NS
% ≥ 3PN	3.6 ± 8.3	1.9 ± 4.9	NS

[a]Chi-square test.

Verheyen et al. 2004, HR 19, 2822

ICSI with fresh/frozen testicular sperm

Table VI. Results of embryo transfer, pregnancy and implantation rates after ICSI with fresh (44 cycles) and frozen (42 cycles) testicular sperm of 32 non-obstructive azoospermia (NOA) patients

	Fresh TESE	Frozen TESE	Chi-square
Cycles	44	42	
Transfers (%)	41 (93.2)	32 (76.2)	$P = 0.028$
Embryos/ET	2.6	2.5	NS
Pos hCG/cycle (%)	9/44 (20.4)	8/42 (19.0)	NS
Pos hCG/ET (%)	9/41 (21.9)	8/32 (25.0)	NS
Clinical PR/cycle (%)	7/44 (15.9)	6/42 (14.3)	NS
Clinical PR/ET (%)	7/41 (17.1)	6/32 (18.7)	NS
Implantation rate (%)	8/105 (7.6)	6/81 (7.4)	NS

ET, embryo transfer; Pos, positive; PR, pregnancy rate.

Verheyen et al. 2004, HR 19, 2822

ICSI with fresh/frozen testicular sperm

Should we cryopreserve testicular sperm in NOA patients?

⇓

YES

Similar outcome as fresh after ICSI
But… it works in only 4 out of 5 patients

⇓

Counsel patients for back-up fresh TESE

Freezing for prepubertal boys with cancer

- Only option for fertility preservation
- Before or shortly after initiation of gonadotoxic cancer therapy
- Storage of spermatogonial stem cells
- Future autologous intratesticular transplantation after cure

Brinster et al. 1994, Proc Natl Acad Sci USA 91, 11303
- Spermatogonia are able to colonize the seminiferous tubules
- Induce active spermatogenesis

Schlatt et al. 1999, HR 14, 144
- Multiple injections into rete testis in primates and human is the most promising technique

Freezing for prepubertal boys with cancer

- Protocols and cryoprotectants
  - *Slow programmed freezing*
    - Propanediol-sucrose
      Hovatta et al. 2001, HR Update 7, 378 (60% survival)
    - Ethyleneglycol-sucrose
      Kvist et al. 2006, HR 21, 484 (slow freezing)
    - DMSO
      Keros et al. 2007, HR 22, 1384 (slow freezing)
      Goossens et al. 2008, FS 89, 725 (uncontrolled freezing protocol)
      Wyns et al. 2008, HR 23, 2402 (controlled freezing)

Freezing for prepubertal boys with cancer

- Protocols and cryoprotectants
  - *Vitrification*
    - High concentrations of DMSO-ethyleneglycol-sucrose
      - Well-preserved histology
      - High survival rates
      - High proliferation rates of spermatogonia during long-term culture

      Curaba et al. 2011, FS 95, 1229
    - Re-initiation of spermatogenesis
      - After transplantation in mice
      - In 64% of tubules

      Baert et al. 2012, FS 97, 1152

Freezing for prepubertal boys with cancer

- Future autologous intratesticular transplantation after cure
  - Cell suspension transplantation
  - Tissue grafting

  or In-vitro maturation
- Remaining concerns after 20 years of research
  - Optimize cryosurvival of testicular stem cells
  - Optimize transplantation protocols
  - Increase safety: risk of re-introducing malignant cells
  - Reproductive efficiency ?

Geens et al. 2007, HR 21, 390
Wyns et al. 2010, HR Update16

Freezing for prepubertal boys at UZ Brussel

Overview of indications for testis tissue banking at UZ Brussel

Malignant diseases		Non-malignant diseases	
Leukaemia	8	Drepanocytosis	14
Testicular cancer	1	Klinefelter syndrome	10
Neuroblastoma	1	Thalassemia	3
Osteosarcoma	1	Granulomatous disease	2
B-cell lymphoma	2	Ideopathic medullar	1
Rhabdomyosarcoma	1	aplasia	
Medulloblastoma	1		
Anaplastic ependymoma	1		
Ewing sarcoma	1		
Nasopharynxcarcinoom	1		
Total	18	Total	30

Conclusions

- Testicular sperm freezing is an efficient procedure in order to avoid repeated surgery in obstructive and non-obstructive azoospermia
- In OA, freezing can be performed either on a diagnostic occasion or on the day of OPU
- In NOA, pros and cons should be considered for individual clinics and patients
- In NOA with poor testicular quality, a fresh retrieval is preferably scheduled as back-up on the day of OPU
- Cryodamage is observed at the level of motility, viability, ultrastructure,… comparable to ejaculated sperm

Conclusions

- DNA fragmentation is not affected if adequate freezing procedures are applied
- Testicular spermatozoa are preferably frozen in suspension, obtained after mechanical or enzymatic treatment procedures
- Glycerol is the cryoprotectant of choice for either slow controlled-rate or static vapour freezing of mature testicular sperm
- In case of fertility preservation for prepubertal boys, testicular biopsies are frozen by slow freezing with DMSO as cryoprotectant
- Before fertility restoration is possible, several concerns should be solved and the efficiency should be improved

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Epigenetic events in early embryos
& possible effects of culture conditions

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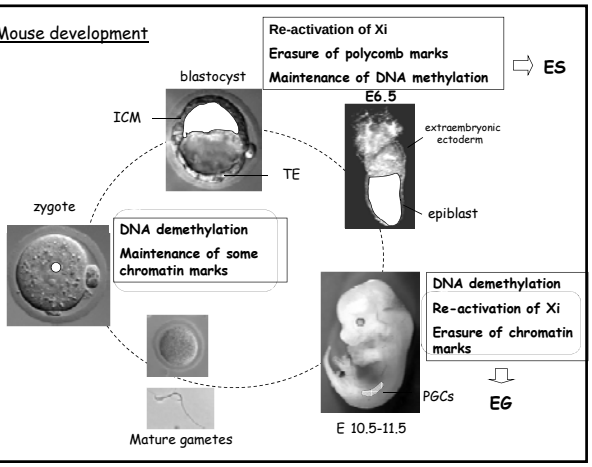
MRC Clinical Sciences Centre
London UK

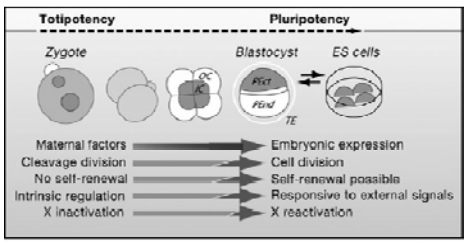
Imperial College London

MRC Clinical Sciences Centre

Mouse development



Preimplantation development



Major metabolic changes

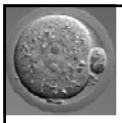
Transcriptional networks ⟺ Epigenetic regulation

Surani, Hayashi and Hajkova,2007

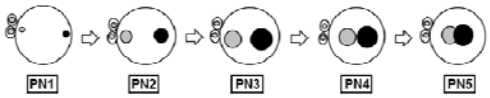
Zygotic epigenetic events

Protamine to histone exchange

DNA demethylation & chromatin assymetry of parental genomes



Schematic representation of pronuclear stages in mouse zygote



(3hpf) (4hpf) (5hpf) (6-7hpf) (8-10hpf)

♂ protamines

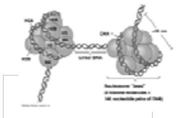
histones

Remodelling sperm genome

Removal of protamines

Deposition of new histones…. Histone chaperones (Hira)

Histone variants:



- Incorporated into chromatin outside S phase
- Contain introns, UTRs
- Outside the the “histone cluster” in the genome

Histones	Features
Archaeal histones	Ancestral histone fold proteins without tails found in singly wrapped tetrameric units that comprise nucleosome particles.
H2A, H2B H2AZ	Canonical core histones encoded by replication-coupled genes. H2A variant found in nearly all eukaryotes that has a diverged self interaction domain.
macroH2A	Vertebrate-specific H2A variant with a C-terminal globular domain. Enriched on the mammalian inactive X chromosome.
H2A Bbd	Vertebrate specific H2A variant that is widely distributed. Relatively deficient on the inactive X-chromosome.
H2AX	H2A form with an SQ[E/D] Φ (Φ = hydrophobic) C-terminal motif that becomes serine phosphorylated at sites of double-stranded breaks.
H3, H4	Canonical core histones encoded by replication-coupled genes.
H3.3 (H3.2 in plants)	H3 variant that replaces H3 and differs at position 31 and at a few residues on helix 2 that allow deposition outside of replication.
Packaging histones	Core and linker histone variants adapted for tight packaging of DNA in sperm and pollen in some organisms.

Henikoff and Ahmad, 2005

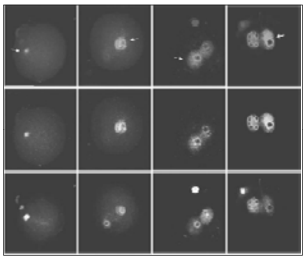
Assymmetric distribution of histone variant H3.3 in zygotes

PN0-1 PN2 PN3-4 PN5

merged

H3.3-HA

DNA



Protamines in the paternal genome are replaced by H3.3

Torres-Padilla et al, 2006

<u>Histone based epigenetic inheritance?</u>

5-15% of histones retained in mature spermatozoa

Presence of canonical histones and testes specific histone variants

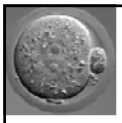
Histones retained over promoters of some developmentally regulated genes (B.Cairns & A.Peters labs)

Potential for inheritance of epigenetic marks (do these histones persist through zygotic reprogramming?)

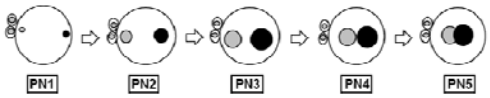
Zygotic epigenetic events

Protamine to histone exchange

DNA demethylation & chromatin assymetry of parental genomes



Schematic representation of pronuclear stages in mouse zygote

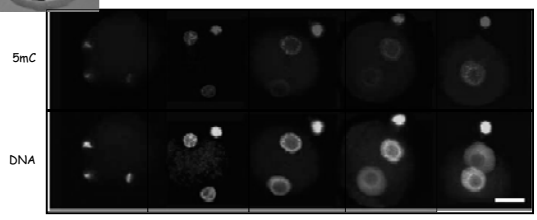


(3hpf) (4hpf) (5hpf) (6-7hpf) (8-10hpf)

♂ protamines

histones

Kinetics of DNA demethylation in mouse zygote



PN0 PN1 PN2 PN3 PN4-5

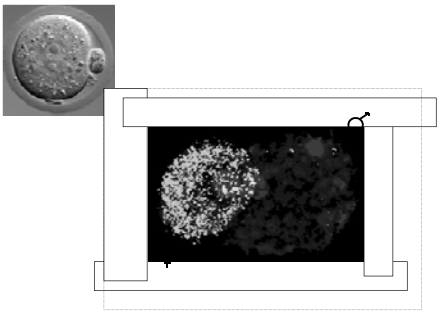
(3hpf) (4hpf) (5hpf) (6-7hpf) (8-10hpf)

♂ protamines

histones

Santos and Dean, 2004

Zygotic reprogramming : DNA demethylation



Barton *et al*, 2001

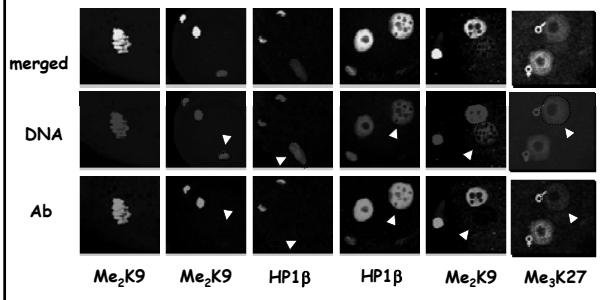
Regulation of epigenetic reprogramming

template or **activity**

Chromatin template

Presence of (de)modification enzymes

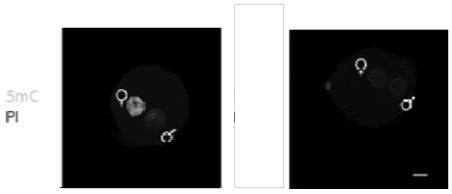
Zygotic reprogramming – chromatin assymmetry



Arney *et al*, 2002, Erhardt *et al*, 2003

Molecular "readers" of the chromatin asymmetry?

Stella / PGC7 : maternally inherited protein
localised in both pronuclei
loss of maternally inherited protein leads to developmental failure during cleavage stages
binds to H3K9me2 (present in the maternal pronucleus)



CTRL Stella $^{-/-}$ X wt

Nakamura et al, NCB 2007
Nakamura et al, Nature 2012

What is the importance of the zygotic (paternal) DNA demethylation?

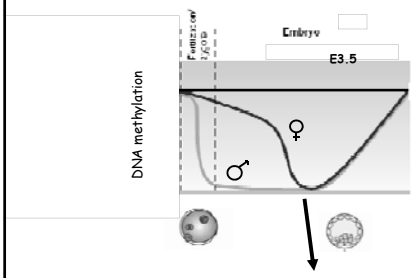
Species specific differences (mouse, bovine, human X sheep, rabbit)

Aberrant (does not occur) in ROSI (development proceeds normally)

Demethylation of maternal genome (Stella maternal ko) leads to preimplanation lethality

What about the maternal genome?

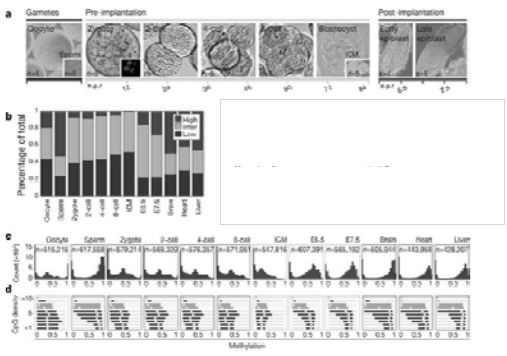
Dynamic changes of DNA methylation in early mouse embryos



Passive DNA demethylation of maternal genome by exclusion of Dnmt1

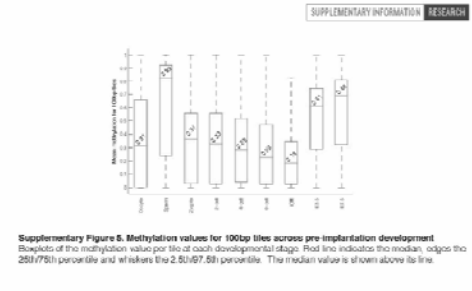
Reik and Walter, 2001

DNA methylation dynamics in mouse preimplantation embryos



Smith et al, Nature 2012

DNA methylation dynamics in mouse preimplantation embryos



Supplementary Figure 5. Methylation values for 100bp tiles across pre-implantation development
Boxplots of the methylation value per tile at each developmental stage. Red line indicates the median, edges the 25th/75th percentile and whiskers the 2.5th/97.5th percentile. The median value is shown above its line.

Smith et al, Nature 2012

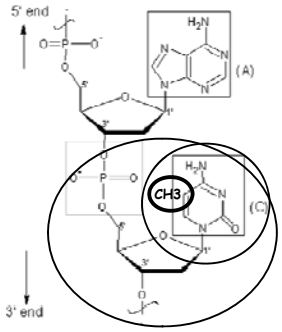
Mechanism of DNA demethylation?

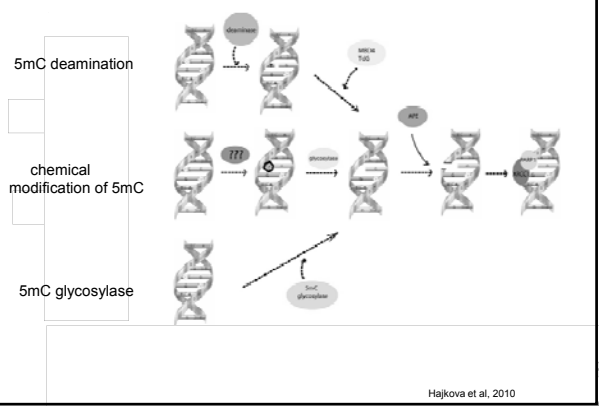
Active DNA demethylation - models….

Direct removal of methyl group

Removal of modified base (BER)

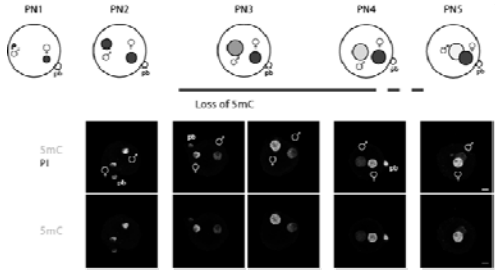
Removal of nucleotide(s) - NER



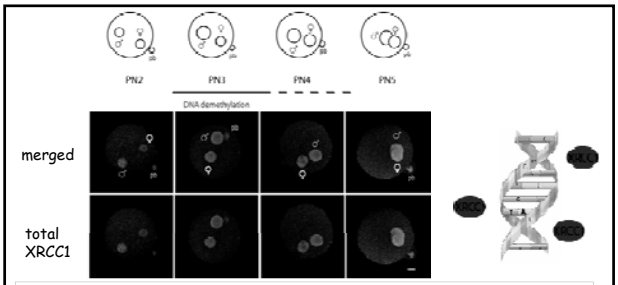


Hajkova et al, 2010

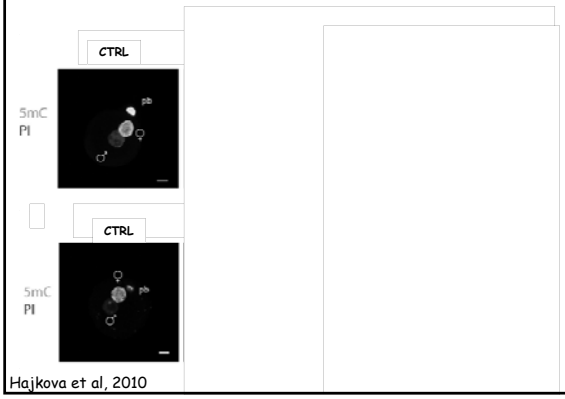
Kinetics of DNA demethylation in mouse zygotes



Hajkova et al, 2010

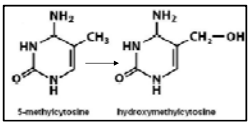


Inhibition of BER pathway perturbes DNA demethylation

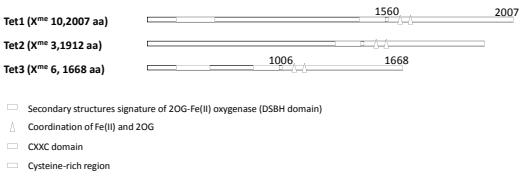


Hajkova et al, 2010

5-hydroxy-methylcytosine

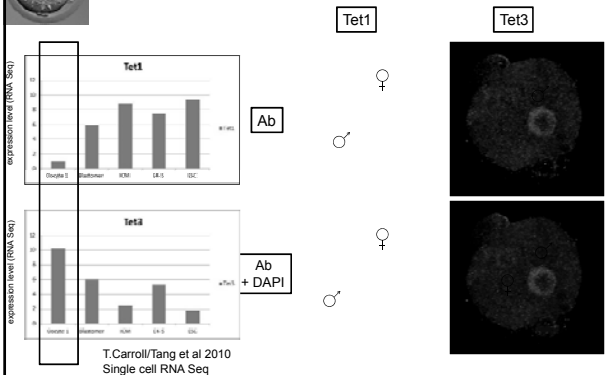


human proteins paralogous to JBP1&JBP2 (Trypanosomes) : **TET1**, **TET2** and **TET3**.



Kriaucionis et al, Science 2009, Tahiliani *et al.*, Science 2009

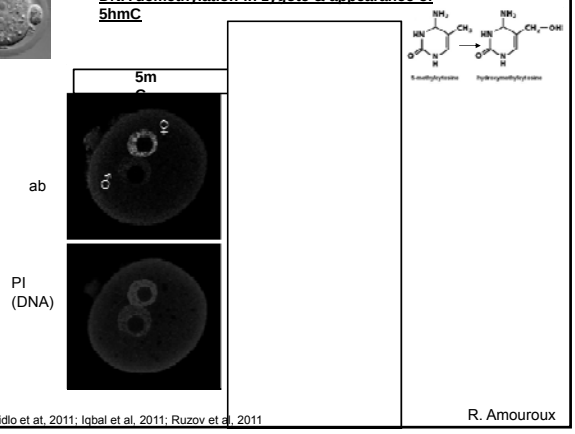
DNA demethylation in zygote & Tet1/Tet3 expression



T.Carroll/Tang et al 2010
Single cell RNA Seq

R.Amouroux, A.Sankar

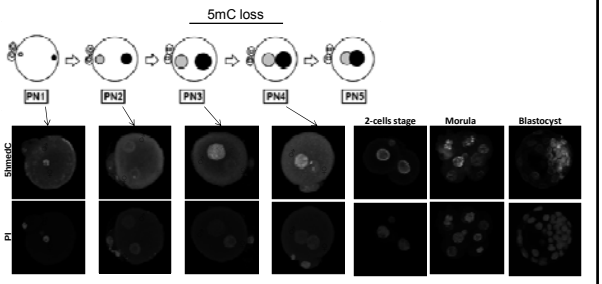
DNA demethylation in zygote & appearance of 5hmC



Wossidlo et at, 2011; Iqbal et al, 2011; Ruzov et al, 2011

R. Amouroux

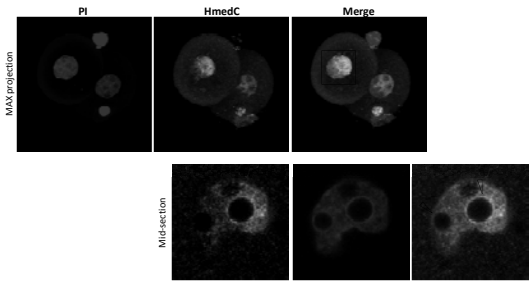
Dynamics of 5hmC during preimplantation development



R.Amouroux

Wossidlo et at, 2011; Iqbal et al, 2011; Ruzov et al, 2011

5hmC asymmetry is maintained until 2-cell stage



HmedC asymmetry between maternal and paternal genome is maintained in 2-cell stage embryos.

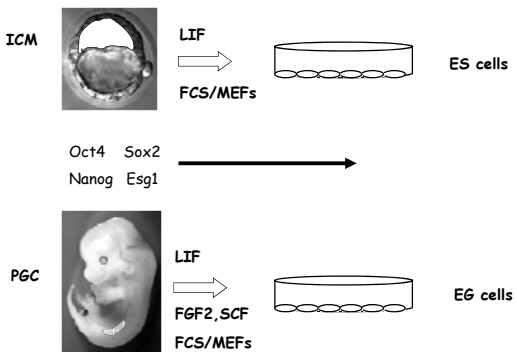
R.Amouroux

Summary

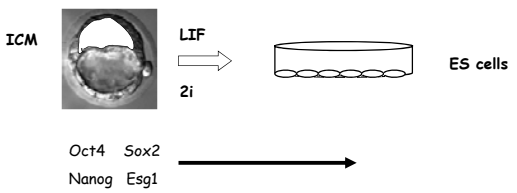
- Chromatin asymmetry between parental genomes (new chromatin assembled on the pat DNA following protamine removal)
- Asymmetry “read” by further molecular players (stella, regulation of transcription of satellite DNA & heterochromatin assembly) -> crucial for successful development
- Asymmetry in DNA methylation (5mC &5hmC)
- Persistence of epigenetic differences during early preimplantation development (genomic imprints)

Impact of culture environment on the regulation of 5mC/5hmC dynamics

Stem cell derivation – „classic" protocol

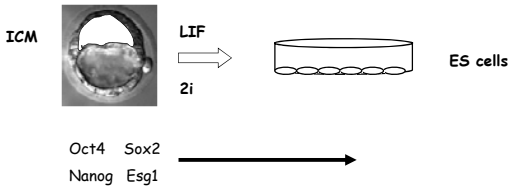


Stem cell derivation – „2i" protocol
(reaching the pluripotent ground state)

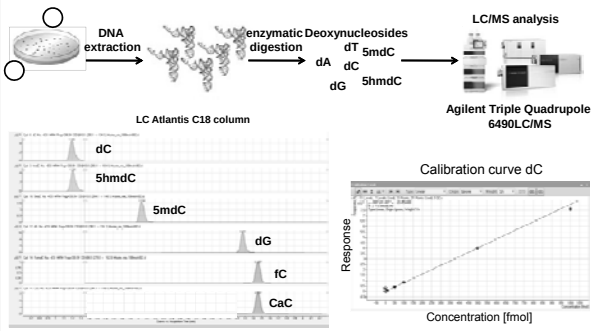


Ying et al, 2008

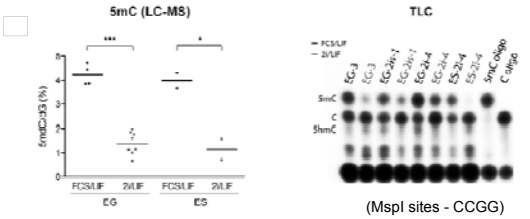
Stem cell derivation – „2i" protocol
(reaching the pluripotent ground state)



Measuring DNA methylation by LC-MS
- method -

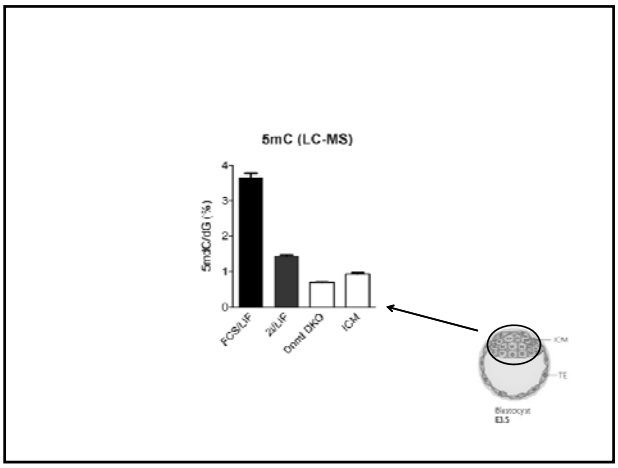


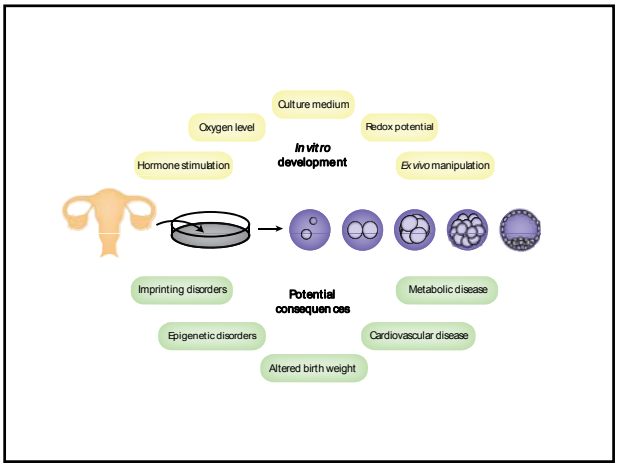
Global DNA hypomethylation in 2i



(MspI sites - CCGG)

male





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Freeze-dried sperm preservation: are we looking at the future?

Luca Gianaroli, M. Cristina Magli, Ilaria Stanghellini, Anna P. Ferraretti

SISMER, Reproductive Medicine Unit, Bologna, Italy

iiarg www.iiarg.com www.sismer.it SISMER

DISCLOSURE

Nothing to disclose

iiarg SISMER

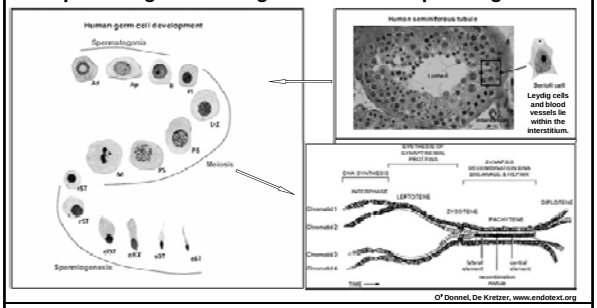
Learning objectives

1. **To describe the main features of sperm cells' structure and functions.**
2. **To provide an overview of sperm cryopreservation.**
3. **To introduce freeze-drying as an alternative sperm preservation technique.**
4. **To discuss about possible clinical applications of sperm freeze-drying.**

iiarg SISMER

Main features of sperm cells' structure and functions

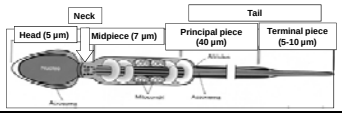
The process of spermatogenesis from immature spermatogonia through meiosis and spermiogenesis

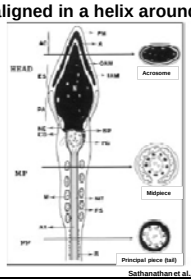


O' Donnel, De Kretzer, www.endotext.org

Sperm structure

- the nucleus contained within the head that, for most mammals, has a flattened, oval shape
- a midpiece, containing mitochondria aligned in a helix around the first part of the tail
- a tail or flagellum

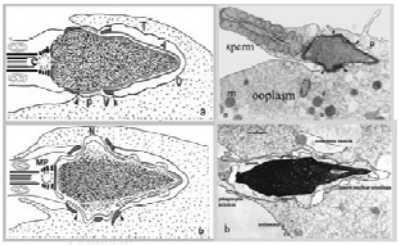




Sathanathan et al., 1996

Sperm structure

The acrosome is a gigantic lysosome that forms around the anterior portion of the nucleus and that controls sperm-oocyte membrane fusion.

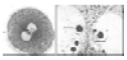


Sathanathan et al.,1996

Sperm contribution to the oocyte

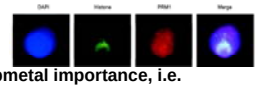
- PLC Zeta1 → Oocyte activation → Meiosis resumption →

- DNA → Fertilization →



- Centrosome → Duplication at pronuclear stage → migration to opposite poles

- Resident histones → enriched at loci of developmetal importance, i.e. imprinted genes, HOX genes, miRNA clusters



- RNA → >3'000 different mRNA coding for proteins needed for early embryo development, and ~ sncRNA species

Li et al., 2008; Yoon et al., 2008; Schatten and Sun, 2009; Hammoud et al., 2011; Kashir et al., 2011 Krawetz et al., 2011

Sperm cryopreservation

Sperm cryopreservation

Spermatozoa present an extremely compact cell organization with highly fluid plasma membrane and low water content (~50%) due to a reduced cytoplasmic component.

⇨ less sensitive to cryoinjury

⇩

minimized by the use of crioprotectants ⇦

Low-molecular weight and highly permeable chemicals that decrease the freezing point of a substance by reducing the amount of salts and solutes present in the liquid phase
→ reduced ice formation

Sperm cryopreservation

Introduced in the 1960's, now forms integral part of ART programs

- Fertility preservation
- Testicular sperm extraction
- Back-up sample for insemination
- Sperm donor program

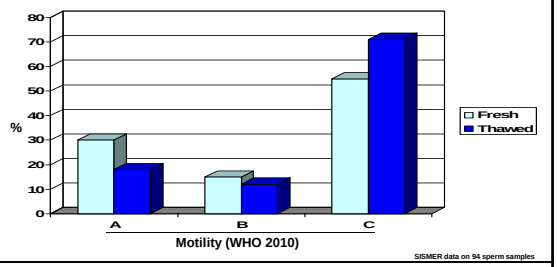
- Slow freezing → progressive sperm cooling over a period of 2-4 h using a programmable freezer

- Rapid freezing → straws are let in liquid nitrogen (LN_2) vapors for ~10min and then plunged LN_2

Sperm cryopreservation

After thawing:

- Decrease in sperm motility



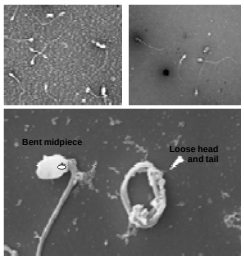
Motility (WHO 2010)

SISMER data on 94 sperm samples

Sperm cryopreservation

After thawing:

- **Decrease in vitality**
- **Increase in tail defects**
- **Increase in acrosomal defects**

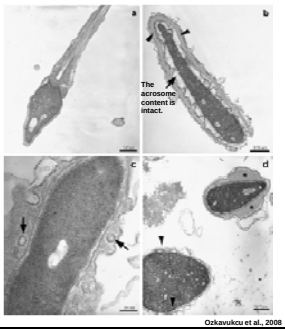


Ozkavukcu et al., 2008

Sperm cryopreservation

After thawing:

- **Increase in acrosomal defects**



a. Normal spermatozoon

b. Subacrosomal swelling. Detachment of the inner acrosomal membrane from the nuclear envelope and filamentous widening of the subacrosomal space (*arrow*).

c Destruction of cell membranes and vesiculations in an acrosome (*arrows*).

d Acrosomal change defect (*lower*): Unaffected equatorial acrosomal content (*arrow heads*), and altered apical acrosomal region. Acrosomal swelling (*upper*): Diffuse and nodular accumulation inside the acrosome (*asterisk*)

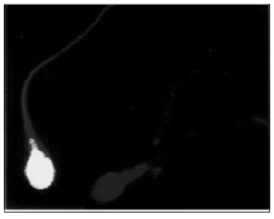
Ozkavukcu et al., 2008

Sperm cryopreservation

After thawing:

- **Increase in sperm DNA damage**

Even if the opinions on the issue of cryopreservation and DNA damage are still controversial, the majority of published data support the hypothesis of decreased DNA integrity in thawed samples.



TUNEL test

Duru et al., 2001; Thomson et al., 2009; Zribi et al., 2010; Riel et al., 2011

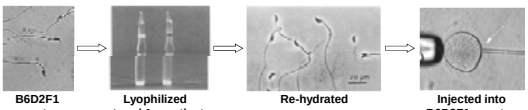
Sperm lyophilization

Sperm lyophilization

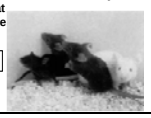
RESEARCH

Development of normal mice from oocytes injected with freeze-dried spermatozoa

Teruhiko Wakayama[1,2] and Ryuzo Yanagimachi[1,3]



B6D2F1 spermatozoa | Lyophilized stored 1 month at room temperature | Re-hydrated | Injected into B6D2F1 oocytes



Three normal mice born

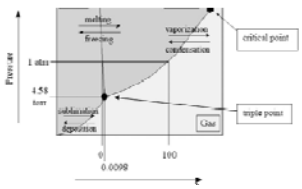
Embryos transferred into a foster mother (CD-1 albino)

Wakayama and Yanagimachi, 1998

Lyophilization or Freeze-drying

It is a dehydration process used to:

- **preserve a perishable material**
- **make the material more suitable for transport**



It works by:

- **freezing the material**
- **reducing the surrounding pressure**
- **making the frozen water in the material to sublimate (directly from the solid phase to the gas phase)**

http://www.scribd.com/doc/377244/Lyophilization-details

Sperm lyophilization

Mice | Rats | Rabbits | Horses




Proven fertility for 2 generations

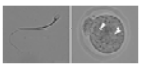
Microsatellite instability analysis performed on these mice and on the two subsequent generations has ruled out genomic instability

Liu et al., 2004; Hirabayashi et al., 2005; Li et al., 2009; Choi et al., 2011

Sperm lyophilization

Bovine

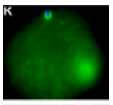
Canine

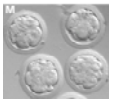


Mouse oocytes fertilized with canine freeze-dried spermatozoa

Pig

Macaque





Embryo development to the 8-16 cell stage

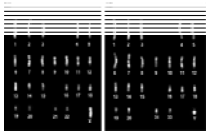
Kwon et al., 2004; Lee K, Niwa K. wt al., 2006; Sanchez-Partida et al., 2008; Watanabe et al., 2009

Sperm lyophilization
Human

Sperm lyophilization - Human

Rehydrated spermatozoa from 2 donors were injected into enucleated mouse oocytes

Sperm chromosomes were examined at the first zygote cleavage. The majority had a normal costitution

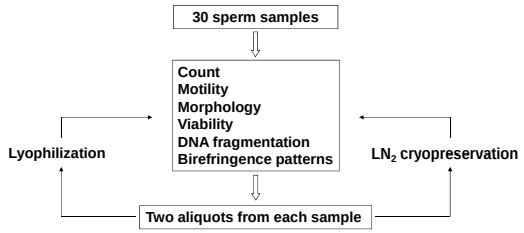


Q-banded chromosome spreads did not show numerical or structural aberrations

The process of lyophilization does not cause damage to sperm chromosomes

Kusakabe et al., 2008

Sperm lyophilization vs. cryopreservation



The aim of the study was to have a basis of comparison with an established technique such as LN_2 sperm freezing

Gianaroli et al., 2012

Sperm lyophilization vs. cryopreservation

Protocol

Lyophilization

Purified sperm sample aliquots of 100 mL each were placed in glass ampules containing 400 mL of buffer solution (10 mM Tris-HCl and 1 mM of EDTA) and frozen at 20° C for 6 hours.

Ampules were put inside a freeze-drying machine (Alpha 1-4 LSC,GmbH) near the condenser, and vacuumed overnight at $220x10^{-3}$ Mbar pressure.

Lyophilized samples were stored at 4° C.

Rehydration

Lyophilized samples were resuspended 1 week later in 100 mL of distilled water.

LN_2 cryopreservation

The HSPM (cryoprotectant human semen preservation was added to the sperm suspension in an equal volume.

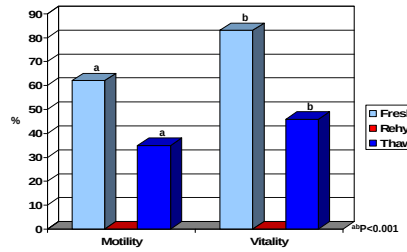
After equilibration at 37° C for 5 minutes, the homogenate was loaded into 0.25 mL freezing straws which were first exposed to LN_2 vapors for 15 minutes and then plunged into LN_2.

Thawing

The suspension was washed twice in T6 medium to remove the cryoprotectant, and the resulting pellet was resuspended in T6 medium

Gianaroli et al., 2012

Sperm lyophilization vs. cryopreservation

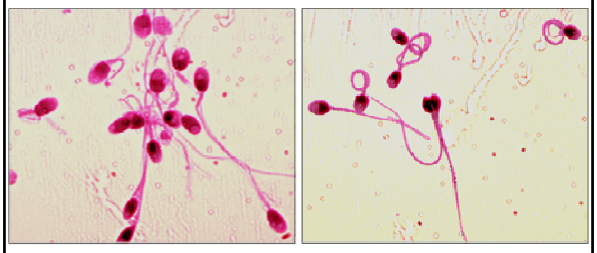


Count / ml
62±44 x 10^6
48±29 x 10^6
55±31 x 10^6

No significant changes in sperm count
Significant decrease in sperm motility and vitality after thawing
No motility, no vitality after rehydration

Gianaroli et al., 2012

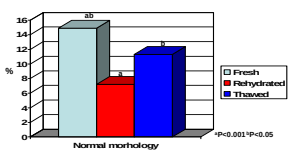
Sperm lyophilization vs. cryopreservation



Lyophilization damages sperm membranes as assessed by the penetration of the vital dye eosin, indicating lack of vitality.

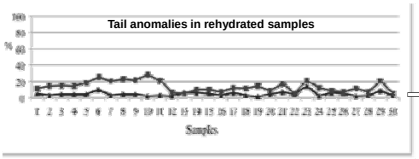
Gianaroli et al., 2012

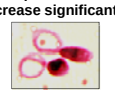
Sperm lyophilization vs. cryopreservation



There is a decrease in the proportion of morphologically normal cells after both preservation techniques

In the majority of cells:
- head morphology is preserved
- tail anomalies increase significantly

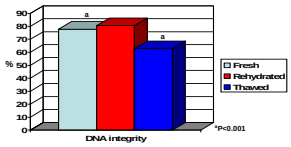




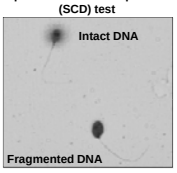
13.5±6.5%
after lyophilization
4.5±2.6%
fresh sample P<0.001

Gianaroli et al., 2012

Sperm lyophilization vs. cryopreservation



Sperm Chromatin Dispersion (SCD) test



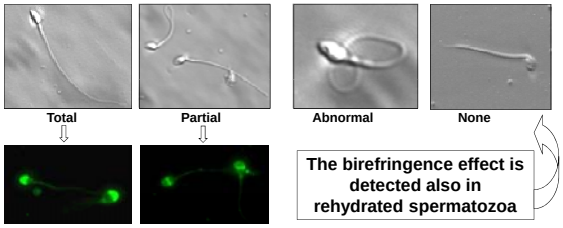
The proportion of spermatozoa with intact DNA:
- is significantly lower after LN2 cryopreservation
- does not change after lyophilization

Gianaroli et al., 2012

Sperm lyophilization vs. cryopreservation

Birefringence is a property that human sperm cells naturally possess due to the presence of longitudinally oriented protein filaments, especially in the acrosomal and nuclear region. It is visible by polarizing microscopy.

Four patterns of birefringence

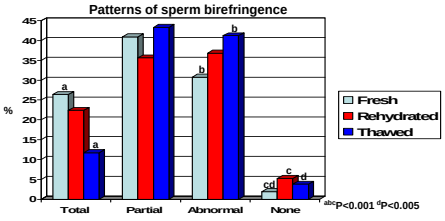


The birefringence effect is detected also in rehydrated spermatozoa

Gianaroli et al., 2010; Gianaroli et al., 2012; Magli et al., 2012

Sperm lyophilization vs. cryopreservation

Patterns of sperm birefringence



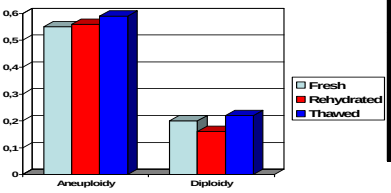
Compared with the fresh samples, the patterns of birefringence
- changed significantly after cryopreservation (total, abnormal, none)
- had a modest increase after lyophilization only in the proportion of spermatozoa devoid of birefringence

Gianaroli et al., 2012

Sperm lyophilization vs. cryopreservation

FISH analysis for 9 chromosomes performed on 4800 cells from 6 samples demonstrated that it is possible to investigate the numerical chromosome status even in freeze-dried samples

Chromosomal integrity is preserved at least in the loci targeted by the specific probes that were used



XY 13 15 16 17 18 21 22

Gianaroli et al., 2012

Possible clinical applications of sperm freeze-drying

Sperm lyophilization: possible clinical applications

Some considerations:

- **ICSI would be necessary**
- **Artificial oocyte activation could be necessary**
- **Results on animal data are reassuring**

Main advantages related to the use of sperm lyophilization:

- **Simplified storing system**
- **Simplified shipping system**
- **By inducing enveloped and non enveloped virus inactivation, it would be especially advantageous for the treatment of infectious samples**

Conclusions

- Although sperm viability and motility are totally compromised after freeze-drying, the sperm chromatin structure is not altered in comparison with fresh samples. Unlike with liquid nitrogen preservation, the procedure does not affect DNA integrity.

- Birefringence characteristics were also mostly preserved in rehydrated spermatozoa, suggesting that the sperm-head inner protoplasmic structures are not altered. Conversely, the proportion of sperm cells with abnormal head birefringence increase meaningfully after thawing.

- The preservation of DNA integrity, which is superior in comparison with LN2 cryopreservation, supports the idea of considering a controlled clinical application.

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