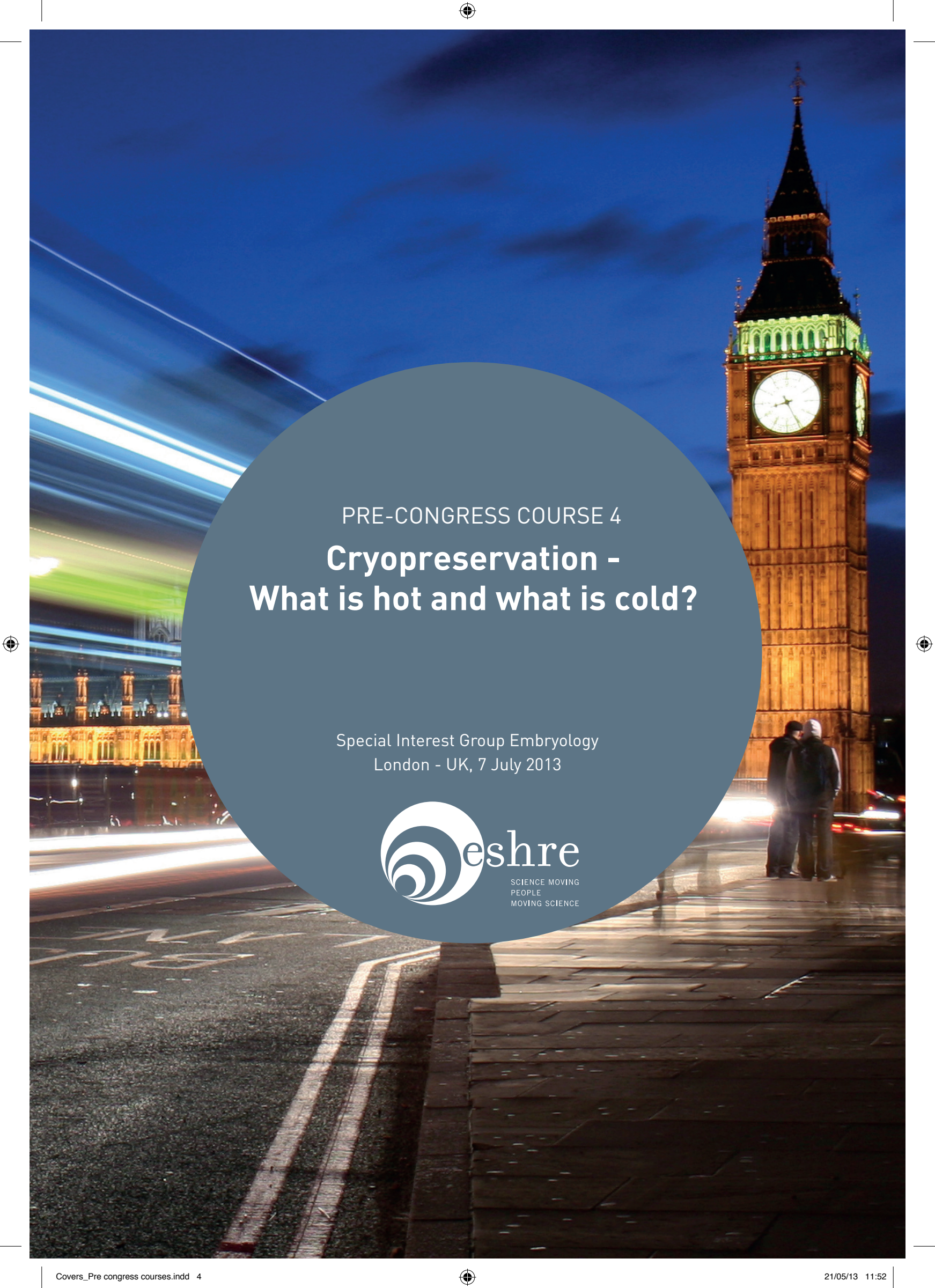




PRE-CONGRESS COURSE 4

# Cryopreservation - What is hot and what is cold?

Special Interest Group Embryology  
London - UK, 7 July 2013







# **What is hot and what is cold?**

**London, United Kingdom  
7 July 2013**

**Organised by  
The ESHRE Special Interest Group Embryology**



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# **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.



Annual Meeting  
LONDON, United Kingdom 7-10 July 2013

**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

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**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.

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

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IVI)

Ice formation

0°C

-4°C

20°C

-10°C

Leibo et al Theriogenology 2007

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

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Biophysical aspects: binary phase diagram

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

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IVI)

Vitrification

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

Saegert and Arav, Reproduction 2011

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

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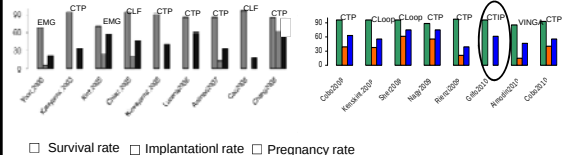
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☐ Survival rate   ☐ Implantation rate   ☐ Pregnancy rate

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

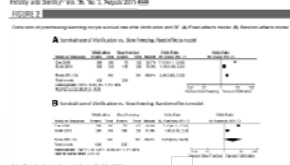


FIGURE 3



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

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

**5-years experience of Egg-banking in IVI Valencia**

|                                 | Egg-bank   | Fresh      |
|---------------------------------|------------|------------|
| Ongoing pregnancy rate/ITI      | 131 (43.7) | 125 (41.7) |
| Ongoing pregnancy rate/cycle    | 131 (44.6) | 125 (43.3) |
| Ongoing pregnancy rate/transfer | 131 (49.1) | 125 (48.3) |

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

OR=0.921  
(0.667-1.274)

|                                                         | 95% CI                 |
|---------------------------------------------------------|------------------------|
| Number of cycles                                        | 3.137                  |
| Number of vitrified oocytes donated                     | 33.096                 |
| Number of embryo transfers/donation                     | 2.729 (91.2)           |
| Number of embryos replaced (Mean $\pm$ SD)              | 5.118 (1.87 $\pm$ 1.8) |
| Number of embryo cryopreservation cycles/donation cycle | 65.7 $\pm$ 69.7        |
| Mean number of re-vitrified embryos (Mean $\pm$ SD)     | 6.412 (2.1 $\pm$ 1.9)  |
| Implantation rate                                       | 1.989 (38.8)           |
| Clinical pregnancy /cycle                               | 1.485 (47.3)           |
| Clinical pregnancy /embryo transfer                     | 1.485 (54.4)           |
| Ongoing pregnancy /cycle                                | 1.222 (38.8)           |
| Ongoing pregnancy /embryo transfer                      | 1.222 (44.7)           |

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



ivi)

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. Fertilite® indicates that oocyte vitrification and warming should no longer be considered experimental. This document replaces the document last published in 2004 titled, "Oocyte Thawing and Oocyte Cryopreservation," Fertil Steril 2009;92:2041-6. (Fertil Steril® 2013;92:217-42, ©2013 by American Society for Reproductive Medicine.)  
For online CME credit related to this document at [www.asrm.org/ivf](http://www.asrm.org/ivf)

**Discuss:** You can discuss this article with its authors and with other ASRM members at [http://fertilityforum.com/guidelines/mature\\_oocyte\\_cryopreservation\\_guideline/](http://fertilityforum.com/guidelines/mature_oocyte_cryopreservation_guideline/)

Use your smartphone to scan this QR code and connect to the document online for this article now.

\* Guidelines for IVF with regard to maximum age for oocyte donation: 12 years after previous age 12 or 13.

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

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ivi)

human reproduction ESHRE PAGES

Oocyte cryopreservation  
for age-related fertility loss†

ESHRE Task Force on Ethics and Law, including, W. Dondorp<sup>1,4</sup>,  
G. de Wert<sup>1</sup>, G. Pennings<sup>2</sup>, F. Shenfield<sup>3</sup>, P. Devroey<sup>5</sup>, B. Tarlatzis<sup>5</sup>,  
P. Barri<sup>4</sup>, and K. Diedrich<sup>7</sup>  
Human Reproduction, Vol.17, No.2 pp. 1131–1137, 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?

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HOWEVER.....

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IVI)

Potential risk of cross contamination

LN<sub>2</sub> mediated infections

- Papova virus,in dermatology, swabs in LN<sub>2</sub> (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)

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

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IVI)

Potential risk of cross contamination

LN<sub>2</sub> mediated infections

- Papova virus,in dermatology, swabs in LN<sub>2</sub> (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

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

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IVI)

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 LN<sub>2</sub> mediated transmission.  
(None of these studies used "open" methods for cryopreservation!)

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

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IVI)

Why don't we have more infections if:

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- 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<sub>2</sub> is not sterile
- Many pathogens survive storage in -196° C
- Cryoprotectants protect also pathogens
- Dewars aren't decontaminated regularly

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

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IVI)

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 pathogen.

Pomeroy et al. Fert. Steril. 2010

❖ Pathogens do not attach to the zona

↓

Zona is an efficient barrier

Fact:

No LN<sub>2</sub> mediated disease transmission in ART

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

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IVI)

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

N=6

HCV

N=11

HBV

N=6

HCV + HBV

N=1

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

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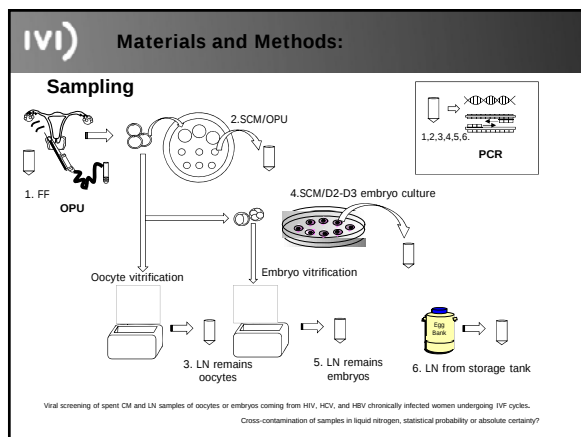
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**IVI) RESULTS**

**TABLE II**

Viral screening of the chronically infected patients according to different types of samples and RT-PCR results

| Patient | Type of sample | No. of assays | Type of assay | RT-PCR results | RT-PCR analysis |
|---------|----------------|---------------|---------------|----------------|-----------------|
| 1       | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 2       | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 3       | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 4       | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 5       | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 6       | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 7       | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 8       | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 9       | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 10      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 11      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 12      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 13      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 14      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 15      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 16      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 17      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 18      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 19      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 20      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 21      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 22      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 23      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 24      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 25      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 26      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 27      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 28      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 29      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 30      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 31      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 32      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 33      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 34      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 35      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 36      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 37      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 38      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 39      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 40      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 41      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 42      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 43      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 44      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 45      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 46      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 47      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 48      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 49      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 50      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 51      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 52      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 53      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 54      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 55      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 56      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 57      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 58      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 59      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 60      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 61      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 62      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 63      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 64      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 65      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 66      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 67      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 68      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 69      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 70      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 71      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 72      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 73      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 74      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 75      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 76      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 77      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 78      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 79      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 80      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 81      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 82      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 83      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 84      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 85      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 86      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 87      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 88      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 89      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 90      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 91      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 92      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 93      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 94      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 95      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 96      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 97      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 98      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 99      | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |
| 100     | FF             | 1             | RT-PCR        | RT-PCR         | Negative        |

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

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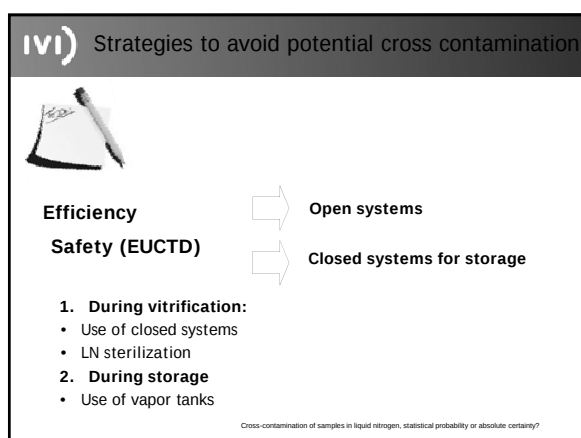
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#### Infertile patients autologous cycles

Table 3 CryoTip versus CryoTop groups: embryological data

[illegible]

Values are mean  $\pm$  SD or n/total (%) unless indicated otherwise.

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

**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 (20.0)     |
| Moderate quality                  | 16 (13.0)     |
| Poor quality                      | 11 (13.8)     |
| Embryos transferred               | 36            |
| Re-cipients with embryos transfer | 20 (10.0)     |
| Embryos transferred               | 1.8 $\pm$ 0.4 |
| Excellent quality                 | 22 (61.1)     |
| Good quality                      | 11 (20.6)     |
| Moderate quality                  | 3 (8.3)       |

Values are n, n (%) or mean  $\pm$  SD

Table 2 Clinical outcomes

| Outcome                       | Wise used<br>oocyte<br>embryo<br>transfer | Wise used oocyte<br>frozen embryo<br>transfer | All<br>transfer |
|-------------------------------|-------------------------------------------|-----------------------------------------------|-----------------|
| Warming<br>cycles             | 20                                        | 3                                             | 23              |
| Transfers                     | 20                                        | 3                                             | 23              |
| Clinical<br>pregnancy<br>rate | 10/20 (50.0)                              | 1/3 (33.3)                                    | 11/23<br>(47.8) |
| Ongoing<br>pregnancy<br>rate  | 9/20 (45.0)                               | 1/3 (33.3)                                    | 10/23<br>(43.5) |
| Implantation<br>rate          | 12/36 (33.3)                              | 2/5 (40)                                      | 14/41<br>(34.1) |

Values are in or n/total (%).

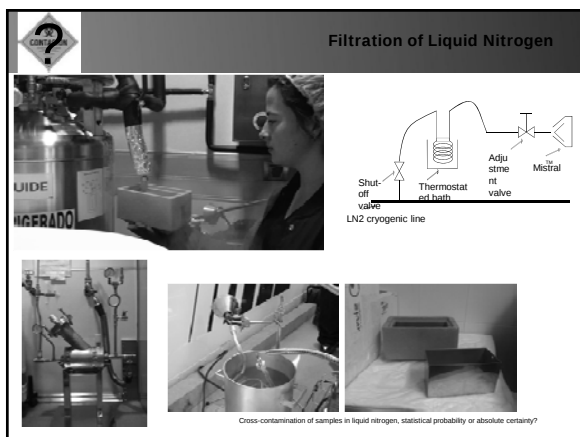
Cross-contamination of samples in liquid nitrogen: statistical probability or absolute certainty?

### RCT evaluating a semi-close device

Clinical trials.gov identifier:NCT01745563

|                             | SC device<br>(N=83) | Conventional device<br>(N=84) | OR (CI95%)          | P     |
|-----------------------------|---------------------|-------------------------------|---------------------|-------|
| N° vitrified/warmed oocytes | 1019                | 1007                          |                     |       |
| Survival rate N (%)         | 920 (90.3)          | 928 (92.2)                    | 0.757 (0.283-1.878) | 0.240 |
| N° 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?




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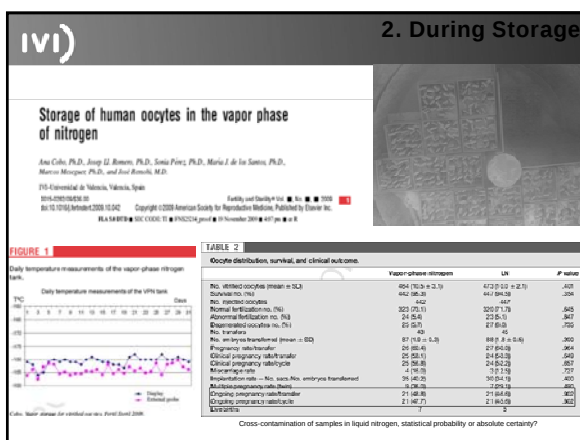
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SIGE pregress course: Cryopreservation - What is hot and what is cold?

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?

David Edgar PhD

*Scientific Director, Reproductive Services/ Melbourne IVF, Royal Women's Hospital  
and  
Senior Lecturer, Department of Obstetrics & Gynaecology, University of Melbourne,  
Victoria, Australia*

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No commercial relationships or conflict of interest to declare

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### 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

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### 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)

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### Slow cooling or vitrification?

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### Slow cooling and vitrification ...

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

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### 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)

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### However, vitrification ...

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

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### But, of course, ...

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

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### The clinical efficiency of cryopreservation

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

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### 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

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### 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

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Cryopreservation of oocytes – slow cooling

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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)

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Optimal dehydration ?

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### Variation in Membrane Hydraulic Permeability of Human Oocytes

Membrane hydraulic permeability  $L_p$  ( $\mu\text{m}/\text{atm}/\text{min}$ )  
measured in individual oocytes at  $20^\circ\text{C}$

| Oocyte | 1    | 2   | 3    | 4    | 5    | 6    | 7    | 8   |
|--------|------|-----|------|------|------|------|------|-----|
| $L_p$  | 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)

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### 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)

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

|                                        | Survival | Fertilisation | Implantation (day 2) |
|----------------------------------------|----------|---------------|----------------------|
| Slow cooled oocytes (women $\leq 38$ ) | 75.1%    | 77.3%         | 16.7%                |
| Fresh oocytes (women $\leq 38$ )       | —        | 79.7%         | 17.3%                |

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

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

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

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

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Therefore

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

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Cryopreservation of oocytes – vitrification

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| Oocyte Vitrification<br>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)       |               |                   |

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| Oocyte Vitrification<br>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)            |                |                    |

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| Multicentre study<br>Unselected infertility cycles (mean age 36) |                                           |  |
|------------------------------------------------------------------|-------------------------------------------|--|
|                                                                  | 486 warming cycles<br>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)                       |                                           |  |

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### 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

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### Cryopreservation of embryos in ART

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When should we transfer and cryopreserve embryos?

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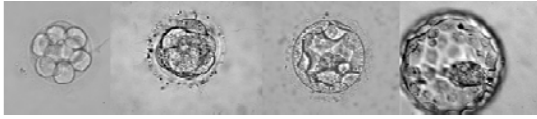
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### Periimplantation stage versus early cleavage stage



#### Pros

Selection of embryos with high implantation potential

"Culling" of suboptimal embryos

#### Cons

?? Loss of potentially viable embryos

?? Increased potential for epigenetic modification

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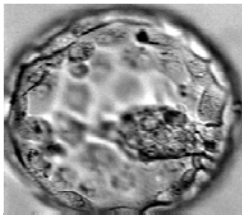
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### Cryopreservation at the blastocyst stage



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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

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Cryopreservation of blastocysts

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

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

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Comparison of blastocyst slow cooling and vitrification

|              | Slow Cooling<br>(9% Glycerol/ 0.2M sucrose) | Vitrification<br>(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

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Cryopreservation at cleavage stages



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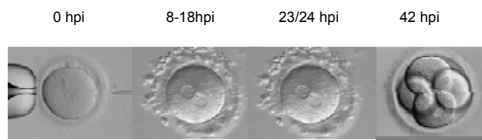
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## Transfer of a single embryo on day 2



Under 36 IMPLANTATION RATE = 28.6%

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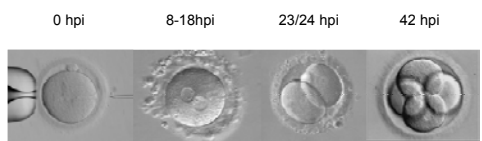
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## Transfer of a single embryo on day 2



Under 36 IMPLANTATION RATE = 42.5%

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

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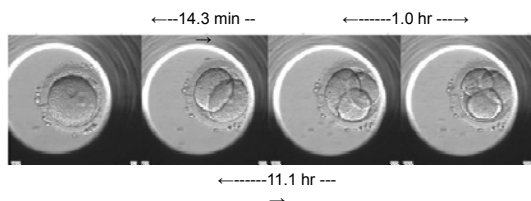
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## Non-invasive imaging during culture of human frozen thawed pronucleate stages predicts blastocyst formation



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

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- 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)

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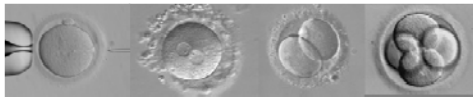
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0 hpi      8-18hpi      23/24 hpi      42 hpi



|               | Implantation rate |
|---------------|-------------------|
| Fresh         | 42.5%             |
| Cryopreserved | 45.2%             |

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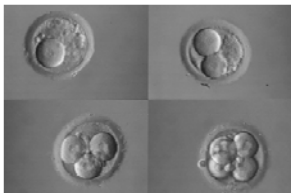
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#### Significance of blastomere loss: Single cryopreserved embryo transfers



|     | 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)

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Confident adoption of early cleavage stage  
SET requires highly effective cryopreservation

Slow cooling or vitrification?

#### Cryopreservation of cleavage stage embryos

|                            | Slow cooling<br>(1.5M PROH + 0.1M sucrose<br>multistep) | Vitrification<br>(15% EG +15%DMSO<br>+0.5M sucrose) |
|----------------------------|---------------------------------------------------------|-----------------------------------------------------|
| Survival<br>(≥ 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 <sup>a</sup> | Slow cooling <sup>b</sup> | P-value |
|-------------------------------------------|----------------------------|---------------------------|---------|
| Cryosurvival                              | 222/234<br>(94.8%)         | 206/232<br>(88.7%)        | 0.02    |
| Embryos with all<br>blastomeres surviving | 173/234<br>(73.9%)         | 106/232<br>(45.7%)        | <0.01   |

<sup>a</sup> EG + PROH + Ficoll + Sucrose

<sup>b</sup> PROH + 0.1M sucrose (multistep)

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

### Vitrification of embryos - most recent data

- Survival  $\approx$  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)

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? Optimal slow cooling

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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)

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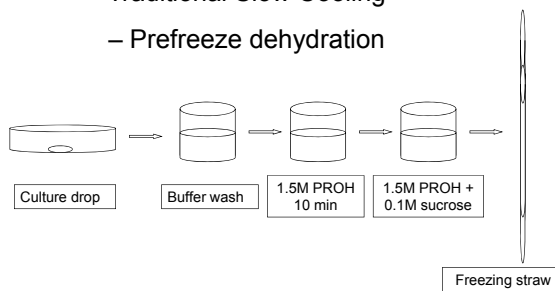
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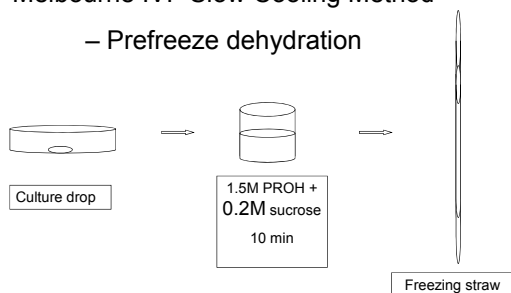
## The Melbourne IVF Slow Cooling Method for Cleavage Stage Embryos

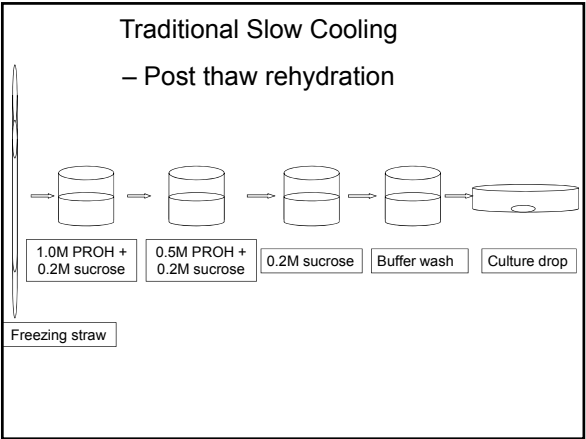
Edgar, Karani & Gook, Rep BioMed Online 19, 521-5 (2009)

### Traditional Slow Cooling – Prefreeze dehydration



### Melbourne IVF Slow Cooling Method – Prefreeze dehydration






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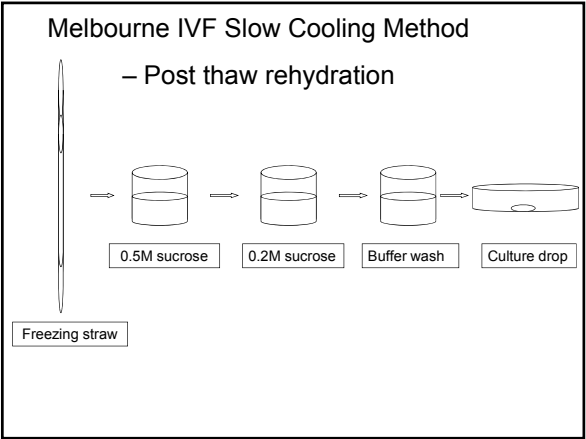
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### Novel slow freezing methodology – key elements

| <u>Conventional</u>                             | <u>MIVF</u>                                                    |
|-------------------------------------------------|----------------------------------------------------------------|
| 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         |

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| 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) |                                                               |                                          |

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| 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%      |

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| 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%        |

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### Summary of outcomes from modified method

Embryos for transfer           ↑ 18%  
Fully intact embryos           ↑ 47%  
Blastomere survival           ↑ 23%  
  
Implantations per embryo thawed   ↑26%

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### Clinical outcomes

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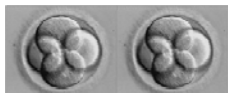
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What is the outcome when we transfer 2  
fresh 4 cell stage embryos (day 2)



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

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#### 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

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#### 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

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### Cumulative FH pregnancy rate in first cycle

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

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### 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)

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### Cryopreservation in ART

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

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IVI)

# Fresh versus frozen oocytes , are we reaching the optimum?

Dr. Maria J. de los Santos, PhD  
Clinical Embryology Laboratory  
IVI Valencia



Annual Meeting  
LONDON, United Kingdom 7 - 10 July 2013

mariajose.delossantos@ivi.es

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IVI)

# Fresh versus frozen oocytes , are we reaching the optimum?

I declare no conflict of interest



Annual Meeting  
LONDON, United Kingdom 7 - 10 July 2013

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IVI)

# Fresh versus frozen oocytes , are we reaching the optimum?

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



Annual Meeting  
LONDON, United Kingdom 7 - 10 July 2013

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Oocyte related Factors

Size (~ 150  $\mu$ m)

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 Ann Reprod Sci 2000

Cochran RB Monovul 2000

Geoff and Susan Hill Fertil Steril 2001

Boyd et al 2002 Expert Rev Med Devices 2002

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Subcellular structures

• 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

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IVI)

Fresh versus frozen oocytes , are we reaching the optimum?

✓ Preserve cytoplasmic structure

✓ Cellular integrity

✓ Subcellular integrity

✓ Mitochondria

✓ Cytoskeletal stability

✓ Meiotic spindle

✓ Actin filaments

✓ Preserve gamete physiology

✓ Metabolic activity

✓ Gene expression profile

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IVI)

Fresh versus frozen oocytes... are we reaching the optimum?



✓ Good clinical outcome

✓ Fertilization

✓ Preimplantational embryo development

✓ Pregnancy rates

✓ Delivery rates

✓ Healthy new borns

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IVI)

Fresh versus frozen oocytes... are we reaching the optimum?



Slow freezing

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IVI)

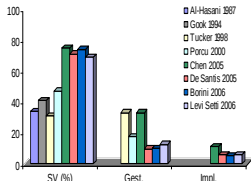
Chen 1986Freezing of human oocytes

Oocyte cryopreservation using slow freezing protocols had important problems:

✓ Highly inefficient

✓ Low consistency

✓ Suboptimal outcome



| Study          | SV (%) | Gest. | Impl. |
|----------------|--------|-------|-------|
| Al-Hazani 1987 | ~40    | ~35   | ~10   |
| Gook 1994      | ~45    | ~30   | ~15   |
| Tucker 1998    | ~50    | ~35   | ~10   |
| Potou 2000     | ~55    | ~30   | ~15   |
| Chen 2005      | ~60    | ~35   | ~10   |
| De Santis 2005 | ~65    | ~30   | ~15   |
| Barini 2005    | ~70    | ~35   | ~10   |
| Levi Seth 2006 | ~75    | ~30   | ~15   |

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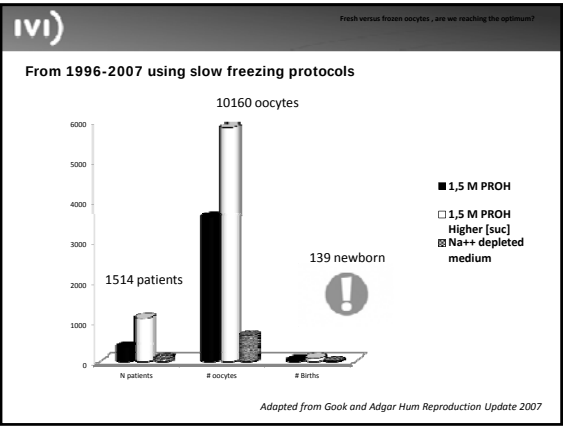
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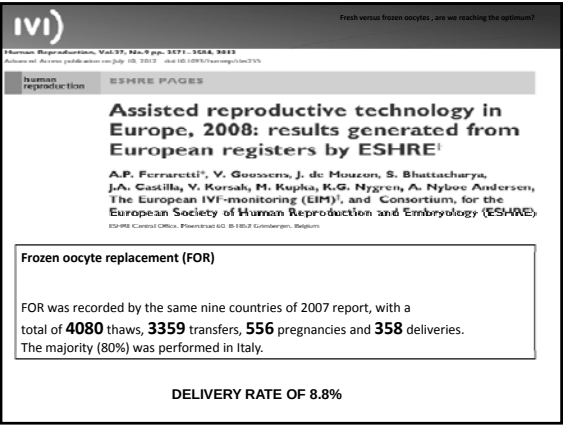
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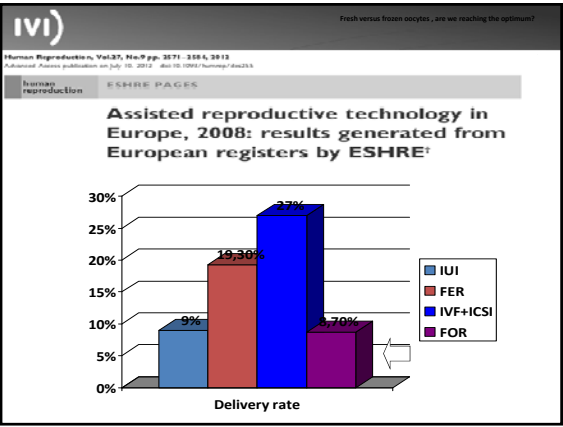
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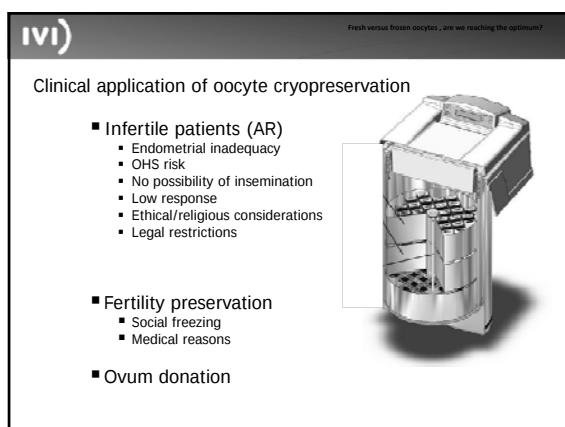
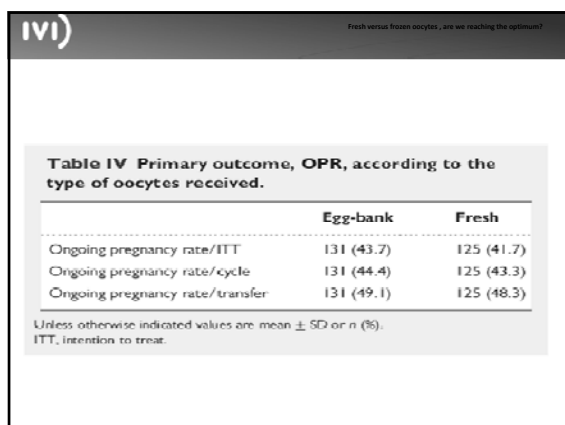
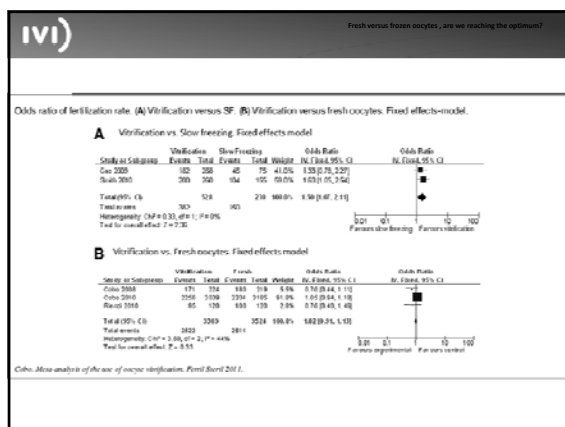
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| IVI) Fresh versus frozen oocytes, are we reaching the optimum? |                  |                       |                  |
|----------------------------------------------------------------|------------------|-----------------------|------------------|
| 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)        |

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| IVI) Fresh versus frozen oocytes, are we reaching the optimum?                                                                              |                                  |                                  |  |
|---------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------|----------------------------------|--|
| Accumulation of oocytes: a new strategy for managing low-responder patients                                                                 |                                  |                                  |  |
| A Cobo <sup>a,*</sup> , Nicolás Garrido <sup>b</sup> , Juana Crespo <sup>c</sup> , Remohi José <sup>c</sup> , Antonio Pellicer <sup>c</sup> |                                  |                                  |  |
| Reproductive Biomedicine Online (2012) 24, 429–432                                                                                          |                                  |                                  |  |
| Table 2 Live birth rate per patient and per embryo transfer.                                                                                |                                  |                                  |  |
|                                                                                                                                             | LR-Acc-Vit                       | LR-fresh                         |  |
| Embryo transfer (n)                                                                                                                         | 220                              | 318                              |  |
| Transfer cancellations/patient (%; 95% CI)                                                                                                  | 9.1<br>(6.5–11.4) <sup>a</sup>   | 24.0<br>(20.8–28.2) <sup>a</sup> |  |
| Implantation rate                                                                                                                           |                                  |                                  |  |
| n/total                                                                                                                                     | 119/440                          | 139/540                          |  |
| % (95% CI)                                                                                                                                  | 25.0<br>(20.7–30.0)              | 25.6<br>(21.9–29.3)              |  |
| Embryo transferred (mean; 95% CI)                                                                                                           | 2.8<br>(1.7–4.1) <sup>b</sup>    | 1.7<br>(1.0–1.8) <sup>b</sup>    |  |
| Live birth rate/embryo transfer                                                                                                             |                                  |                                  |  |
| n/total                                                                                                                                     | 73/209                           | 106/318                          |  |
| % (95% CI)                                                                                                                                  | 31.7<br>(25.7–38.0)              | 34.0<br>(28.7–39.1)              |  |
| Live birth rate/patient                                                                                                                     |                                  |                                  |  |
| n/total                                                                                                                                     | 73/242                           | 106/482                          |  |
| % (95% CI)                                                                                                                                  | 30.2<br>(24.3–36.0)              | 22.4<br>(18.7–26.1)              |  |
| Cumulative live birth rate/patient <sup>c</sup>                                                                                             |                                  |                                  |  |
| n/total                                                                                                                                     | 80/242                           | 114/482                          |  |
| % (95% CI)                                                                                                                                  | 34.4<br>(28.7–40.4) <sup>c</sup> | 23.7<br>(19.9–27.0) <sup>c</sup> |  |

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| IVI) Fresh versus frozen oocytes, are we reaching the optimum?                                                                 |               |                   |                                            |
|--------------------------------------------------------------------------------------------------------------------------------|---------------|-------------------|--------------------------------------------|
| Table 3 Effect of oocyte accumulation by vitrification in the clinical outcome of low-responder PGS patients aged 41–44 years. |               |                   |                                            |
| Outcome                                                                                                                        | ≤5 MI oocytes | ≥6 MI oocytes     | Accumulated vitrified oocytes <sup>a</sup> |
| Mean age (SD)                                                                                                                  | 42.1 (1.1)    | 41.7 (0.9)        | 42.1 (1.0)                                 |
| No. of patients                                                                                                                | 87            | 155               | 105                                        |
| Mean no. of MI oocytes retrieved (SD)                                                                                          | 3.6 (1.2)     | 10.5 (4.5)        | 10.1 (3.3)                                 |
| Transfers (k)                                                                                                                  | 21.8          | 51.6 <sup>b</sup> | 59.2 <sup>c</sup>                          |
| 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          | 19.0              | 19.5                                       |
| Ongoing PR/oocyte retrieval                                                                                                    | 5.8           | 11.6              | 15.2 <sup>d</sup>                          |
| Ongoing IR                                                                                                                     | 26.3          | 19.8              | 24.4                                       |

Values are percentages unless otherwise indicated. IR = implantation rate; MI = metaphase II; PR = pregnancy rate.  
<sup>a</sup>Patients with low ovarian response accumulating ≥6 MI oocytes in several stimulation cycles using vitrification technology.  
<sup>b</sup>P < 0.0001 versus ≤5 MI oocytes.  
<sup>c</sup>P < 0.0001 versus ≤5 MI oocytes.  
<sup>d</sup>P = 0.0299 versus ≤5 MI oocytes.

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IVI)

Fresh versus frozen oocytes...are we reaching the optimum?

Commentary

Obstetric and perinatal outcome in 200 infants conceived from vitrified oocytes

Ri-Cheng Chen<sup>1\*</sup>, Jack Y.J Huang<sup>2</sup>, Seena Lin Tin<sup>1</sup>, Elkin Lucena<sup>2</sup>, Angela Saei<sup>2</sup>, Alejandro Rivas<sup>2</sup>, 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".

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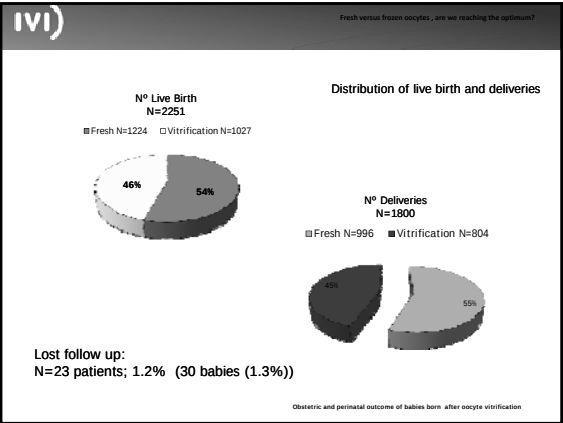
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IVI)

Fresh versus frozen oocytes...are we reaching the optimum?

| 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 twinning)             | 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      |
| 2 <sup>nd</sup> /3 <sup>rd</sup> trim bleeding | 6.3% (5.0-8.1)    | 8.5% (6.7-10.6)   | 1.37 (0.95-1.97) | ns      |
| Membrane rupture <37 <sup>th</sup> week        | 6.5% (5.1-8.2)    | 5.5% (4.1-7.3)    | 0.84 (0.56-1.23) | ns      |
| Preterm del. risk <37 <sup>th</sup> week       | 8.1% (6.5-10.0)   | 8.3% (6.6-10.5)   | 1.03 (0.73-1.46) | ns      |

Obstetric and perinatal outcome of babies born after oocyte vitrification

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IVI)

Fresh versus frozen oocytes , are we reaching the optimum?

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      |

Obstetric and perinatal outcome of babies born after oocyte vitrification

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IVI)

Perinatal outcome

Fresh versus frozen oocytes , are we reaching the optimum?

|                     | 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%              |                  |         |

Obstetric and perinatal outcomes of babies born after oocyte vitrification

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IVI)

Fresh versus frozen oocytes , are we reaching the optimum?



## Improvements

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

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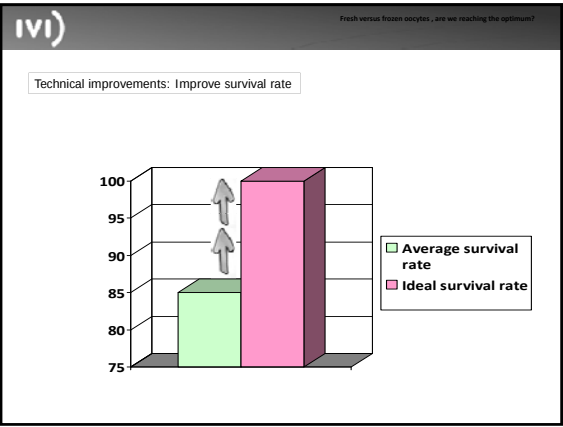
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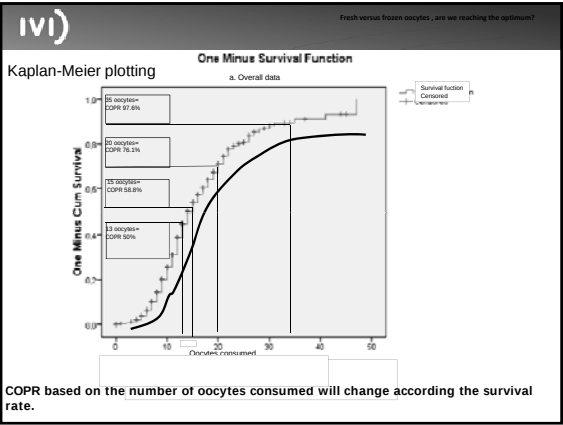
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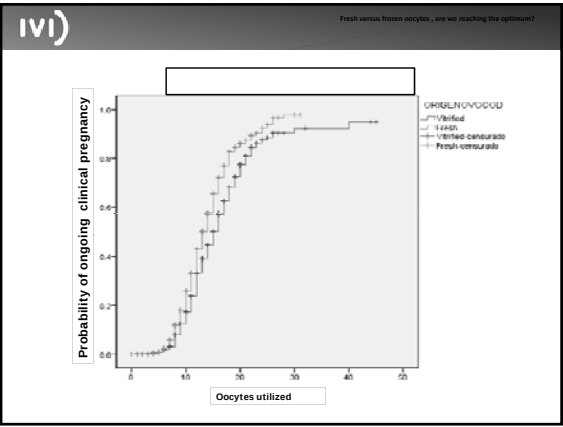
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ivi)

Fresh versus frozen oocytes , are we reaching the optimum?

Factors affecting survival rate

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

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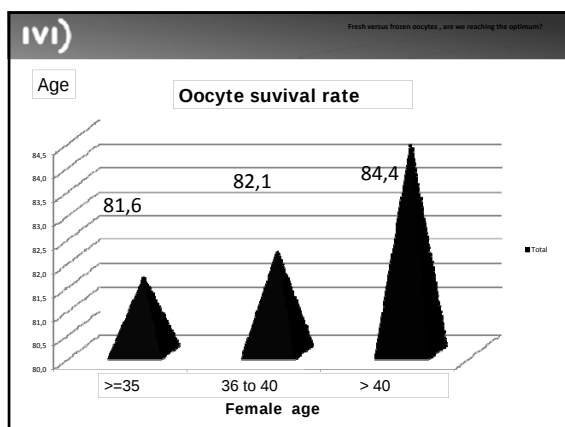
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ivi)

Fresh versus frozen oocytes , are we reaching the optimum?

Ovarian stimulation protocols/response

Forward logistic regression analysys 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 |

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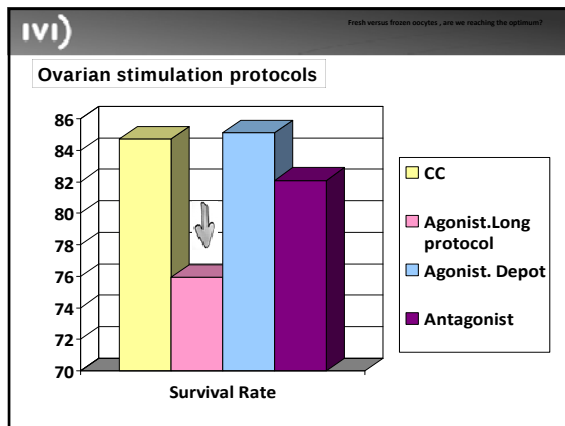
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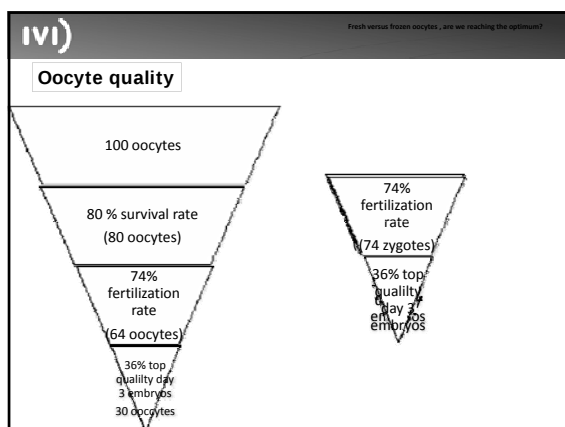
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IVI)

Fresh versus frozen oocytes... are we reaching the optimum?

### 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*

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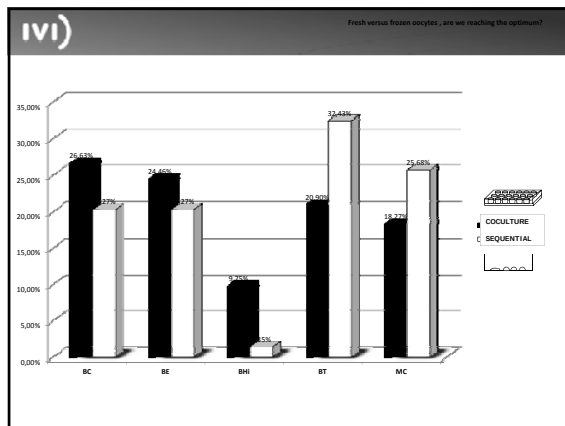
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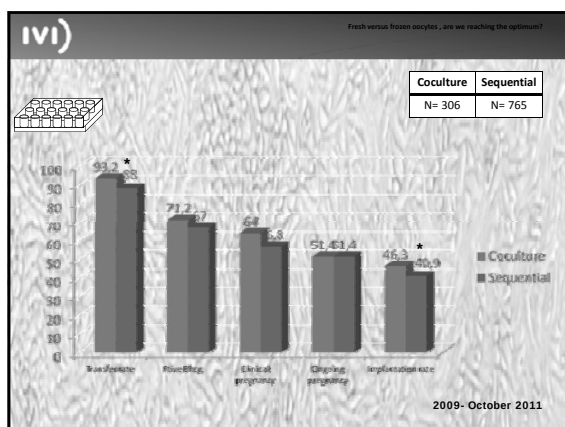
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IVI)

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

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### 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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### Special thanks to:

Dr. Ana Cobo  
Dr. Mar Nohales  
Dr. Giovanna Di Emidio  
Dr. Carla Tatone  
Dr. Remie Dumollard  
IVF team of IVI Valencia

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*What we know is a drop, what we ignore is the ocean*

Isaac Newton

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**IVI)** Fresh versus frozen oocytes...are we reaching the optimum?

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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

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## Declaration

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

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## 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

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When should we cryopreserve ovarian tissue?

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Options for preserving fertility before starting cancer treatment

| Cryopreservation | Prepuber | Immediate chemotherapy/<br>Risk of ovarian stimulation | Partner/<br>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

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Gonadotoxicity: factors

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

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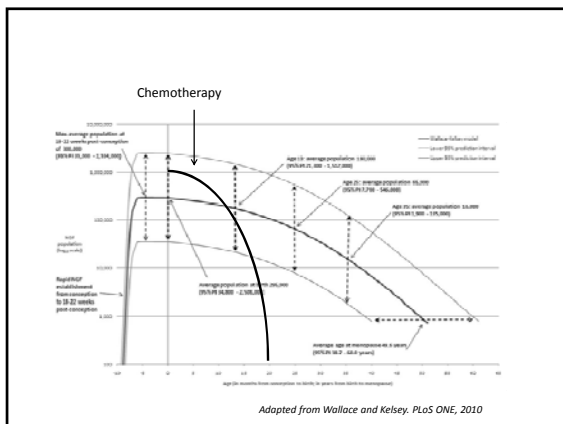
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### Risk of infertility after the treatment

| 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

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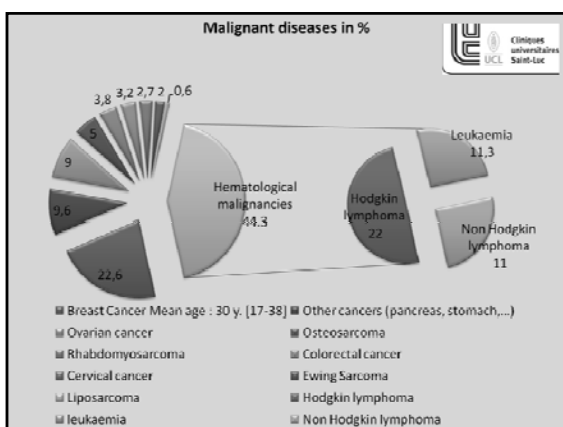
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### Indications: benign diseases

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

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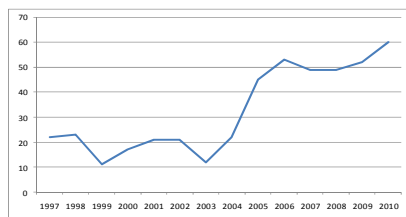
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### Number of ovarian tissue cryopreservation procedures by year in our department



Dolmans et al. J Assist Reprod Genet, 2005

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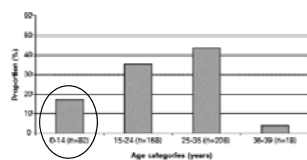
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### Percentage of patients undergoing ovarian tissue cryopreservation (n = 476) by age group



Dolmans et al. J Assist Reprod Genet, 2005

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How should we cryopreserve  
ovarian tissue?

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Conventional freezing – St Luc Hospital UCL

Ovarian biopsy:

Operation theatre (harvesting)



Sterile room (preparation and exposure to CPA)



Cryobank (freezing and storage)

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Operation theatre  
(biopsy harvesting)

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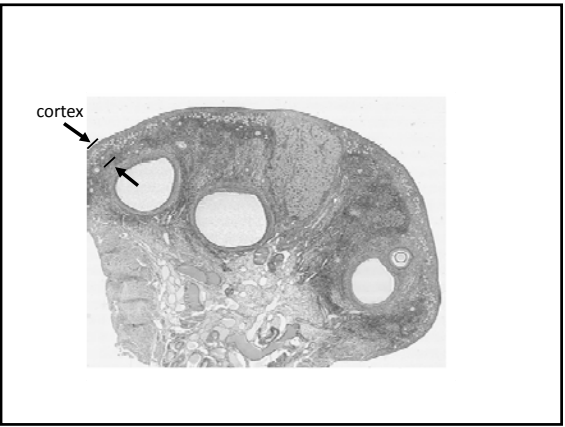
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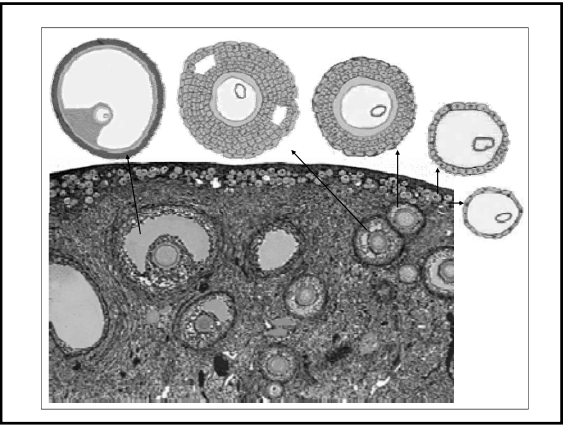
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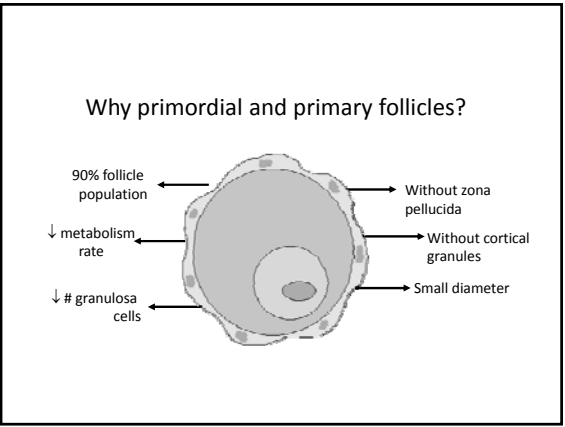
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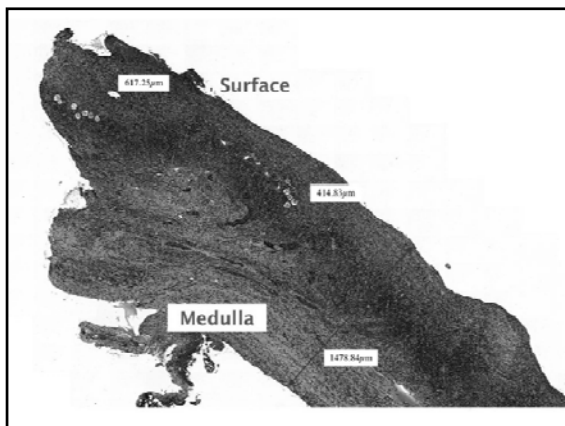
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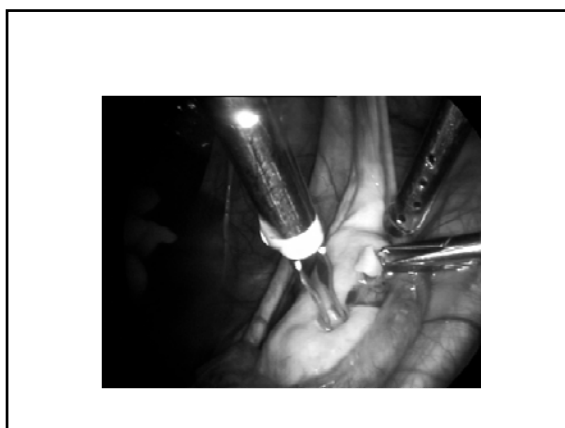
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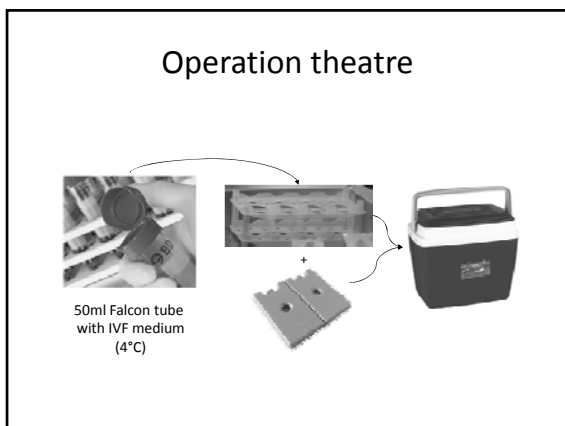
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Sterile Room  
(preparation and exposure to CPA)

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Sterile room – preparation



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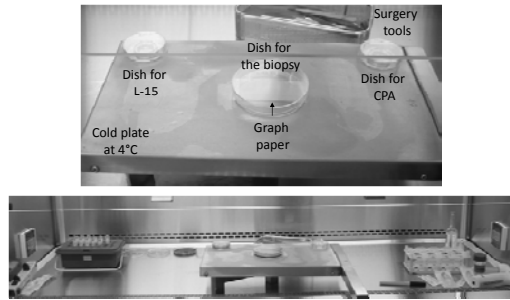
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### Sterile Room (exposure to CPA)



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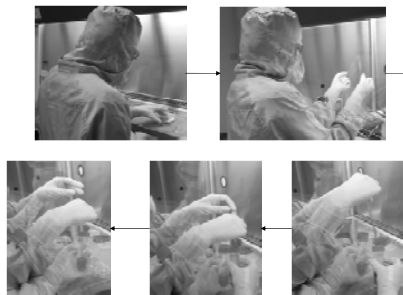
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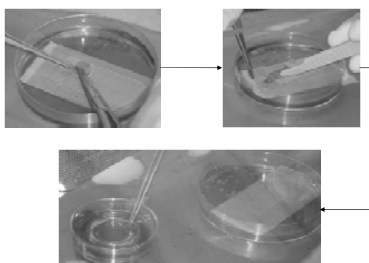
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### Biopsy preparation



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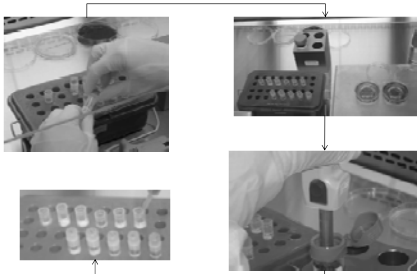
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### Preparation of the cryovials




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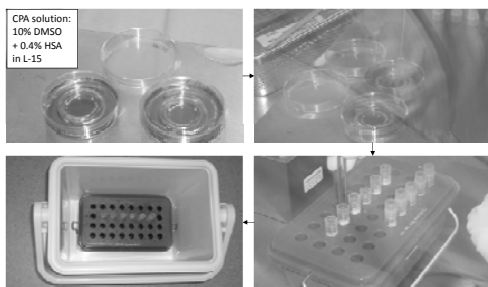
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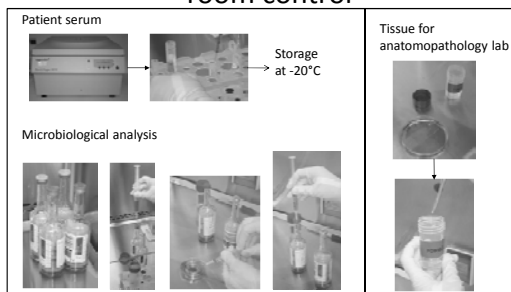
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### Serum, remaining biopsy and sterile room control




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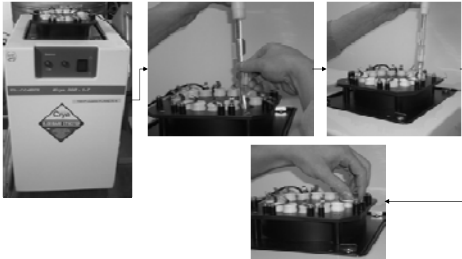
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Cryobank: freezing procedure

Loading the Planner:



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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

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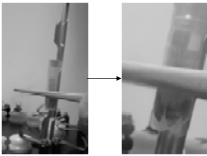
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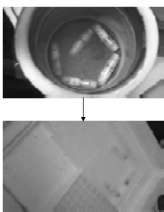
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Cryobank: freezing procedure

Seeding:



Frozen tissue:



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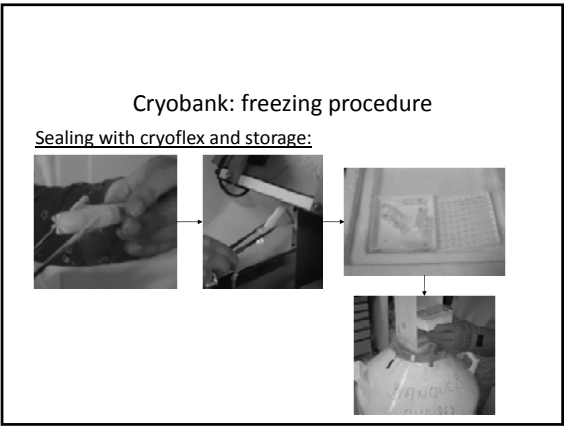
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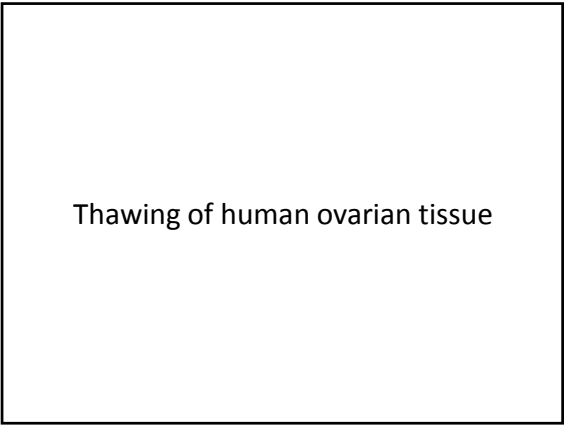
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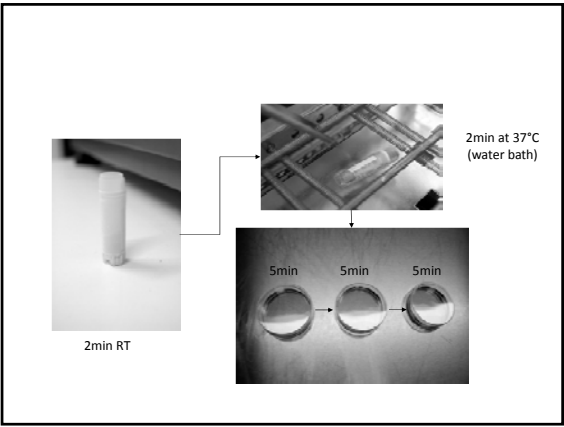
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What about vitrification  
of ovarian tissue?

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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)

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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



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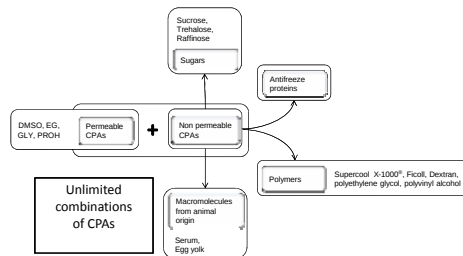
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- High [CPAs]: ↑ Toxicity
  - To decrease CPA toxicity: CPA cocktails




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## Vitrification – High [CPA]

- CPA cocktails
    - Stepwise addition
    - Addition at low temperatures
- } ↓ CPA toxicity

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## 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

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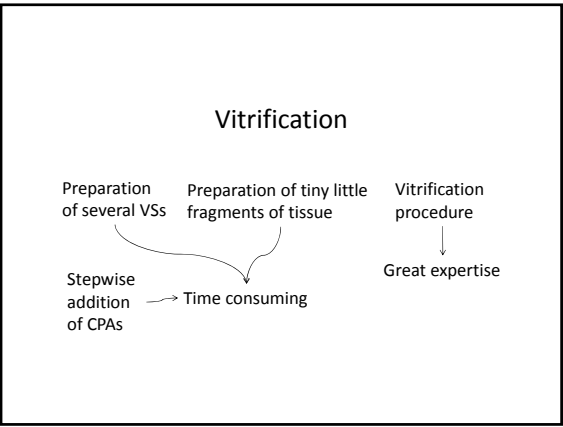
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How vitrification has been working  
for human ovarian tissue?

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**Studies using human ovarian tissue**

| Parameter                                  | Vitrification → warming → fixation<br>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                        |                          |

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| Studies using human ovarian tissue                                 |                                                                                   |                            |                            |
|--------------------------------------------------------------------|-----------------------------------------------------------------------------------|----------------------------|----------------------------|
| Parameter                                                          | Vitricification → warming → IVC → fixation<br>Freezing → thawing → IVC → fixation |                            |                            |
|                                                                    | Vitricification > Freezing                                                        | Vitricification = Freezing | Vitricification < 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                          |

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| Studies using human ovarian tissue                            |                                                                                               |                            |                            |
|---------------------------------------------------------------|-----------------------------------------------------------------------------------------------|----------------------------|----------------------------|
| Parameter                                                     | Vitricification → warming → xenograft → fixation<br>Freezing → thawing → xenograft → fixation |                            |                            |
|                                                               | Vitricification > Freezing                                                                    | Vitricification = Freezing | Vitricification < 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                                                                                             |                            |                            |

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Vitrification: take home message

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Vitrification is not a simple and fast procedure

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So far, vitrification does not seem to be more efficient than freezing to cryopreserve human ovarian tissue

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Where should we use cryopreserved ovarian tissue?

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Transplantation procedure  
orthotopic vs heterotopic site

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| Advantages and disadvantages of heterotopic and orthotopic sites for ovarian tissue transplantation |                                                                                                                                                                                                                         |                                                                                                                                                                                                 |
|-----------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|                                                                                                     | Heterotopic site (subcutaneous)                                                                                                                                                                                         | Orthotopic site                                                                                                                                                                                 |
| Advantages                                                                                          | <ul style="list-style-type: none"><li>• No limitation of the number of fragments transplanted</li><li>• Easy transplantation procedure</li><li>• Easy access for follicular monitoring and oocytes collection</li></ul> | <ul style="list-style-type: none"><li>• Possibility of natural conception</li><li>• Restoration of fertility demonstrated</li><li>• Favourable environment for follicular development</li></ul> |
| Disadvantages                                                                                       | <ul style="list-style-type: none"><li>• Restoration of fertility not yet demonstrated</li><li>• IVF procedure required</li><li>• Effect of the local environment on the oocyte quality is unknown</li></ul>             | <ul style="list-style-type: none"><li>• Number of fragments transplanted limited by the ovarian size</li><li>• Invasive transplantation procedure</li></ul>                                     |

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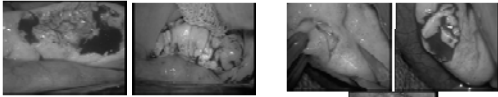
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### Orthotopic transplantation: goal of surgery

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)



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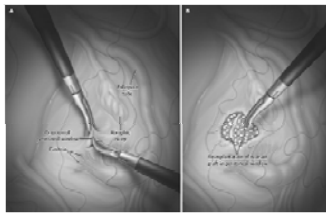
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### Livebirth after orthotopic transplantation of cryopreserved ovarian tissue

Vol 354 October 15, 2004

J Donnez, MM Dolmans, D Derynck, P Jukool, C Pirard, J Squiffet, D Martinez-Madrid, A Van Langendonck



#### 1<sup>st</sup> Laparoscopy

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

#### 2<sup>nd</sup> Laparoscopy

- Transplantation of fragments

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Final considerations:  
results

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## 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??

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### Study on the quality of the eggs

### IVF outcome in patients with orthotopically transplanted ovarian tissue

Maria-Madeleine Dolmans, Jacques Donceel, Alexandra Combastel, Dominique Drenth, Christian Amselem, Anne Van Langendonck, 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




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## What could be affecting follicle development?

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

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### Effect of the transplantation?

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

*Anna-Sophie Van Eyck, M.D.,<sup>1</sup> Brigidette Francine Jordan, Pharm.D., Ph.D.,<sup>2</sup> Bernard Gallot, Pharm.D., Ph.D.,<sup>3</sup> Jean-François Herlitz, Pharm.D., Ph.D.,<sup>4</sup> Anne Van Langendonck, M.Sc., Ph.D.,<sup>5</sup> and Jacques Denayer, M.D., Ph.D.<sup>6</sup>*

*Annals of the New York Academy of Sciences, 1162: 103–110, 2009. © 2009 Wiley-Liss, Inc.*

**FIGURE 2**

Monitoring of pO<sub>2</sub> values (mean ± SEM, n = 6 patients). The pO<sub>2</sub> levels significantly increased from day 6 to day 10. Reoxygenation kinetics were characterized by reoxygenation speed (slope = 2.4 ± 0.4 mmHg/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<sub>2</sub> values were stable until day 21 and remained higher than the value observed on day 3.

**Negative controls** (day 21 post-euthanasia)  
pO<sub>2</sub> (mmHg): 10.5 ± 1.9 mmHg  
// hypoxic value

| Day | pO <sub>2</sub> (mmHg) |
|-----|------------------------|
| 3   | ~12                    |
| 5   | ~22                    |
| 7   | ~28                    |
| 10  | ~32                    |
| 14  | ~31                    |
| 17  | ~25                    |
| 21  | ~26                    |

Van Eyck, EPR to assess ovarian graft reoxygenation, Fertil Steril 2009.

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### Effect of transplantation?

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

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### Effect of the cryopreservation?

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

*Christiane A. Amann, M.D.,<sup>1</sup> Anne Denard,<sup>2</sup> Marie-Nathalie Dolmans, M.D.,<sup>3</sup> Alexandra Carlier,<sup>4</sup> Jacques Denayer, M.D.,<sup>5</sup> Anne Van Langendonck, M.Sc., Ph.D.,<sup>6</sup> and Jacques Denayer, M.D., Ph.D.<sup>7</sup>*

*Human Reproduction, 24(11): 2811–2818, 2009. © 2009 Oxford University Press*

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

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## Conclusion

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

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## Final considerations

Transplantation of cryopreserved ovarian tissue: safety aspects

When transplantation of cryopreserved tissue is not advised

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## 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                        |
| Risk of reimplantation of malignant cells! |                                  | Hodgkin's lymphoma                            |
|                                            |                                  | Nongenital rhabdomyosarcoma                   |
|                                            |                                  | Osteogenic sarcoma                            |
|                                            |                                  | Squamous cell carcinoma of the uterine cervix |
|                                            |                                  | Ewing's sarcoma                               |

Oktaç, Hum Reprod Update, 2001

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Alternatives

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

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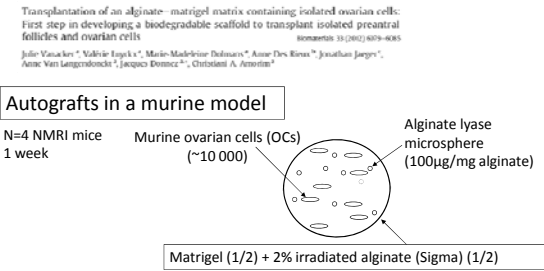
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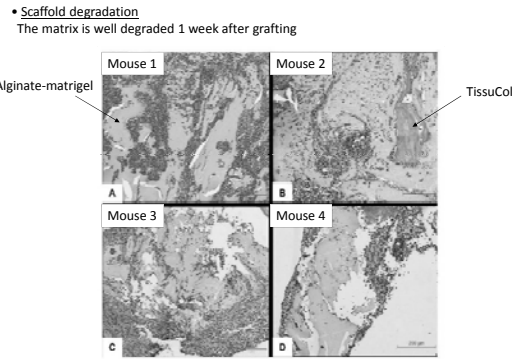
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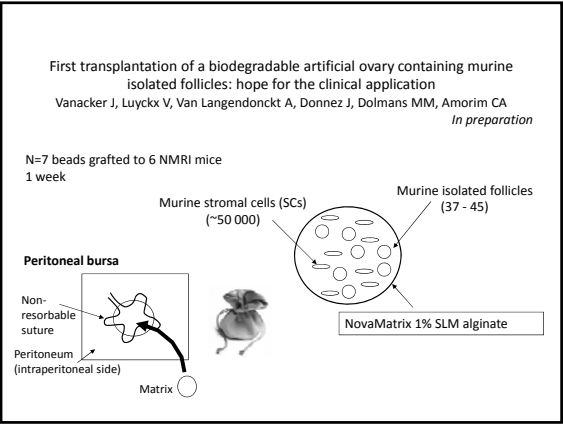
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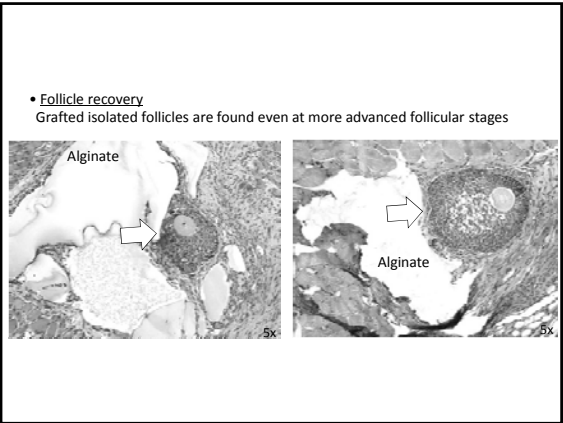
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**Acknowledgments**

Marie-Madeleine Dolmans, MD, PhD  
Jacques Donnez, MD, PhD  
Anne Van Langendonck, PhD  
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Dolores Gonzalez  
Olivier Van Kerk  
Anne Lépage  
Mira Hryniuk, BA

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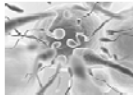
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## Cryopreservation of testicular tissue: when, where and how ?

Greta Verheyen, PhD  
Centre for Reproductive Medicine - UZ Brussel - Belgium  
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

## History of sperm freezing

- 1776 Spallanzani → low  $t^{\circ}$  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**

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## 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

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## When to cryopreserve ?

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



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## Etiology of azoospermia

| Obstructive azoospermia (OA)                                                                                                                                                                                                                                                                                                                                                                                                                                                                  | Non-obstructive azoospermia (NOA)                                                                                                                                                                                                                                                                                                                                                                                                                                    |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <ul style="list-style-type: none"> <li>• Mechanical cause</li> <li>• Normal spermatogenesis<br/>High sperm numbers</li> <li>• Epididymal or testicular sperm</li> <li>• 100% recovery rate</li> <li>• Causes <ul style="list-style-type: none"> <li>– Vasectomy</li> <li>– Congenital bilateral absence of the vas deferens (CBAVD)</li> <li>– Post infectious epididymitis</li> <li>– Testicular trauma</li> <li>– Young's syndrome</li> <li>– Retrograde ejaculation</li> </ul> </li> </ul> | <ul style="list-style-type: none"> <li>• Biological cause</li> <li>• Severely impaired spermatogenesis<br/>Low to absent sperm numbers</li> <li>• Testicular sperm</li> <li>• 50-60% recovery rate</li> <li>• Causes <ul style="list-style-type: none"> <li>– Chromosomal abnormalities</li> <li>– Yq deletions</li> <li>– Iatrogenic treatment</li> <li>– Cryptorchidism</li> <li>– Testicular torsion</li> <li>– Unknown genetic causes (?)</li> </ul> </li> </ul> |

## When to cryopreserve?

- Therapeutic occasion:

Spermatozoa are frozen **on the day** of oocyte retrieval

  - o ICSI with fresh spermatozoa
  - o Cryopreservation of supernumerary sperm for later ICSI cycles
- Diagnostic occasion:

Spermatozoa are frozen **independent** of the day of oocyte retrieval

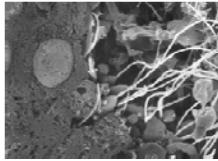
  - o Cryopreservation of spermatozoa for later ICSI cycles
  - o ICSI cycles with frozen-thawed spermatozoa
- Pros and cons for both approaches
  - o Different for patients with OA or NOA
  - o Dependent on sperm retrieval procedure
  - o Depending on flexibility of scheduling TESE

## Pros and cons of both approaches (NOA)

| Approach 1: ICSI-cryo                                                                                                                                                                                                                                                         | Approach 2: cryo-ICSI                                                                                                                                                                                                                            |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <ul style="list-style-type: none"> <li>• Loss of sperm quality by freezing is avoided<br/>– lower risk of finding only immotile sperm</li> <li>• Lower sperm quality limits for ICSI treatment</li> <li>• Less restrictive criteria for patient allocation to ICSI</li> </ul> | <ul style="list-style-type: none"> <li>• Independent scheduling of sperm and oocyte retrieval</li> <li>• Avoid pointless ovarian stimulation of the female partner (if no sperm is retrieved)</li> <li>• Less stressful to the couple</li> </ul> |
| <ul style="list-style-type: none"> <li>• Concomitant scheduling of sperm and oocyte retrieval on the same day</li> <li>• 50% risk of pointless ovarian stimulation in NOA</li> <li>• More stressful to the couple</li> </ul>                                                  | <ul style="list-style-type: none"> <li>• Risk of not finding motile sperm post-thaw</li> <li>• Higher sperm-quality limits for allocation to ICSI treatment (quality loss by freezing-thawing)</li> </ul>                                        |

## 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



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## 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

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## 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*

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## 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*

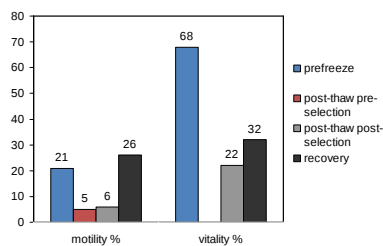
## What (quality) to cryopreserve?

- 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:  $\geq 1$  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

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## 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*

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## How to cryopreserve ?

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



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## How to cryopreserve? Constitution

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

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

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## How to cryopreserve? Constitution

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

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## How to cryopreserve? Preparation

- Mechanical procedures
  - o Scissors, needles, forceps, glass slides
  - o In OA and NOA
  - o Rupture of tubules  $\Rightarrow$  sperm release
- Dissection of individual tubules in a biopsy
  - o Isolation of most dilated tubules
  - o Higher recovery rate in NOA

*Verheyen et al. 1995, HR 10, 2956*

*Kamal et al. 2004, J Androl 25, 123*

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## 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*  
*Crabbe et al. 1997, HR 12, 1682*  
*Crabbe 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*

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## 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

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## 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

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## How to cryopreserve? Preparation

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

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## How to cryopreserve? Cryoprotectant

- Glycerol
  - o Cryoprotectant of choice for mature spermatozoa
  - o Commercially ready-prepared cryomedia
  - o Testicular tissue structure is not preserved
  - o No survival of germ cells
- DMSO
  - o Cryoprotectant of choice for preservation of tubule structure
  - o Fertility preservation for prepubertal boys (spermatogonial stem cells)  
*Keros et al. 2005, HR 22, 1384; Goossens et al. 2008, FS 89, 725*
  - o Best maintains tissue capacity to initiate spermatogenesis  
*Jahnukainen et al. 2007, HR 22, 1060*



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## How to cryopreserve? Freezing procedure

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



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## How to cryopreserve? Freezing procedure

- Vitrification

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

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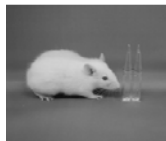
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## How to cryopreserve? Freezing procedure

- Freeze-drying or lyophilization

- o Successful data in animal studies  
*Wakayama and Yanagimachi 1998, Nat Biotechnol 16, 639*
- o 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

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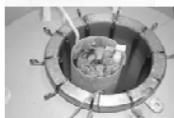
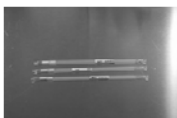
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## How to cryopreserve? carriers

- Closed systems preferred

- o High-security sealed straws
- o Sealed cryotubes
- o Correctly, clearly labelled
- o European Cell and Tissue Directives
- o Avoid transmission of pathogens and viruses



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## 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



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## 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

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## 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

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## ICSI with fresh/frozen testicular sperm

Human Reproduction Vol.25, No.12, pp. 2822–2826, 2004  
Advance Access publications October 15, 2004

doi:10.1093/humrep/deh490

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

G.Verheyen<sup>1</sup>, V.Vermee, L.Van Landuyt, H.Tourmaye, P.Devroey and A.Van Steirteghem

Centre for Reproductive Medicine, University Hospital of the Dutch-speaking Brussels Free University, Laarbeeklaan 101, B-1090 Brussels, Belgium

<sup>1</sup>To whom correspondence should be addressed. E-mail: greta.verheyen@az.vub.ac.be

**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 88.4%, 85%, 20.8% and 11.3%, respectively. Part II: The search time per sperm was higher ( $P = 0.016$ ) in frozen (11 min) than in fresh suspensions (13 min). A higher embryo transfer rate was observed in fresh cycles than in frozen cycles (92.5% vs 76.2%,  $P = 0.026$ ). Fertilization, ongoing pregnancy and implantation rates were comparable for the two groups. **CONCLUSIONS:** Even in a programme with less 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         | $P = 0.083$     |
| 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 <sup>a</sup> |
| Cycles injected with only immotile sperm (%) | 3/44 (7)    | 4/42 (10)   | NS <sup>a</sup> |
| 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 ± 11.0  | 7.8 ± 19.2  | NS              |
| % ≥ 3PN                                      | 3.6 ± 8.3   | 1.9 ± 4.9   | NS              |

<sup>a</sup>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  |                          |    |
| Nasopharynxcarcinom   | 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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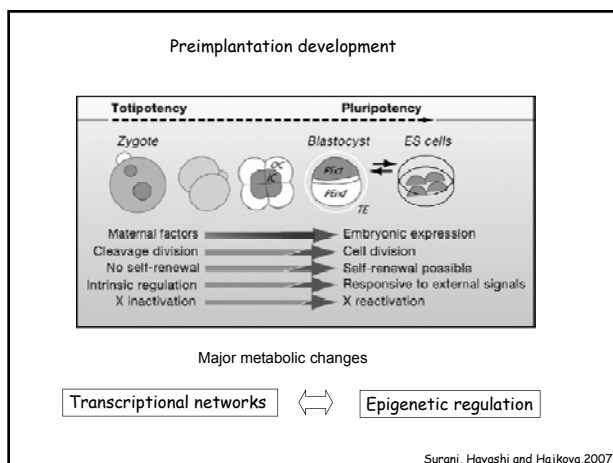
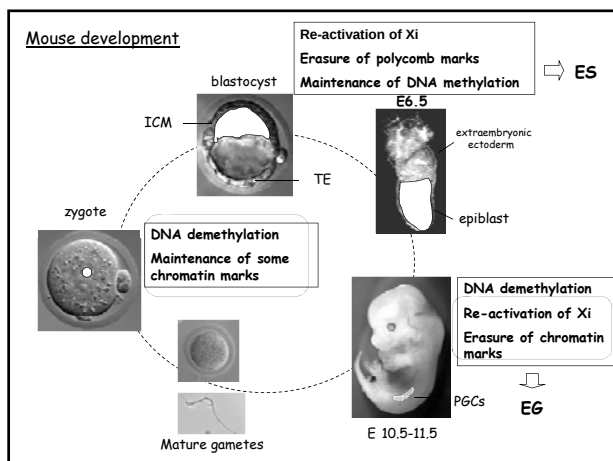
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## Epigenetic events in early embryos & possible effects of culture conditions

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Imperial College  
London

MRC  
Clinical  
Sciences  
Centre



## Zygotic epigenetic events

Protamine to histone exchange

DNA demethylation & chromatin asymmetry of parental genomes

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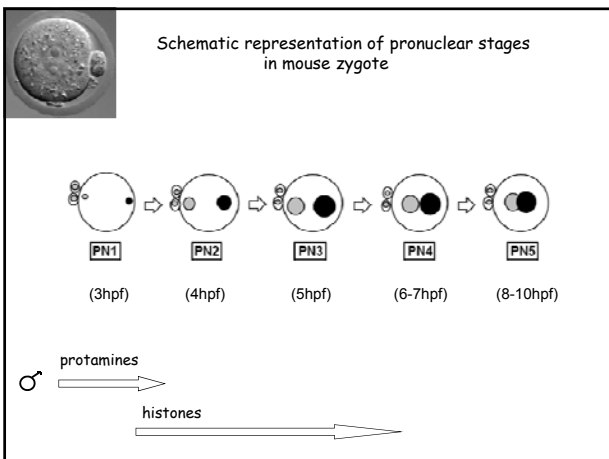
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## Remodelling sperm genome

Removal of protamines

Deposition of new histones.... Histone chaperones (Hira)

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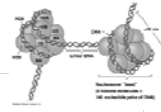
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### Histone variants:

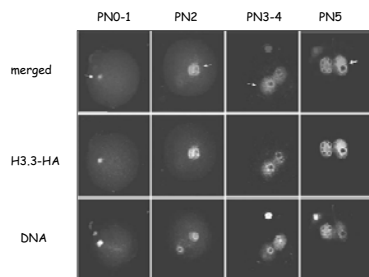
- Incorporated into chromatin outside S phase
- Contain introns, UTRs
- Outside the "histone cluster" in the genome



| Histones          | Features                                                                                                                               |
|-------------------|----------------------------------------------------------------------------------------------------------------------------------------|
| Archival histones | Associated histone that prevents whole cells from instantly wrapped genomic units that comprise nucleosome particles.                  |
| H2A, H2B          | Canonical core histones encoded by replication-coupled genes.                                                                          |
| H2AZ              | H2A variant, found in nearly all eukaryotes that has a disrupted self-interaction domain.                                              |
| macroH2A          | Vertebrate-specific H2A variant with a C-terminal globular domain. Associated with heterochromatin in mouse N. chromatin.              |
| H2A, H2B          | Vertebrate-specific H2A variant that is widely distributed. Homologous to the inactive X-chromosome.                                   |
| H2AX              | H2A form with an SQ(E/T) Q (S) • hydrophobic C-terminal motif that becomes serine phosphorylated at sites of DNA double-strand breaks. |
| H3, H4            | Canonical core histones encoded by replication-coupled genes.                                                                          |
| H3.3, H4.1, H4.2  | H3 variant that replaces H3 and H4 at positions 91 and at a few residues on histone 3 that allow deposition outside of replication.    |
| H3.3, H4.1, H4.2  | C-terminal histone 3 variant, which is highly phosphorylated in spermatocytes and pollen in some organisms.                            |

Henikoff and Ahmad, 2005

### Asymmetric distribution of histone variant H3.3 in zygotes



Protamines in the paternal genome are replaced by H3.3

Torres-Padilla et al, 2006

### Histone based epigenetic inheritance?

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 asymmetry of parental genomes

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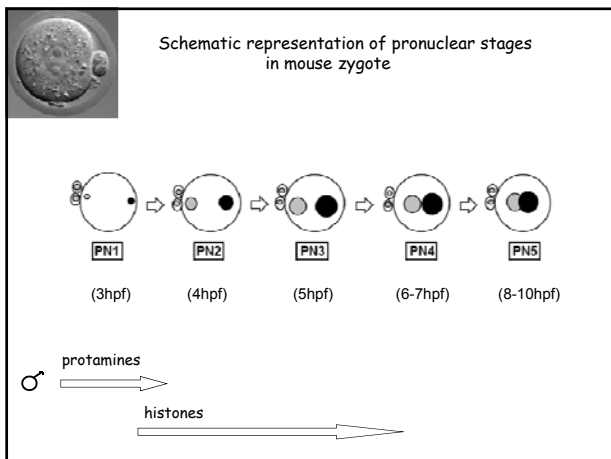
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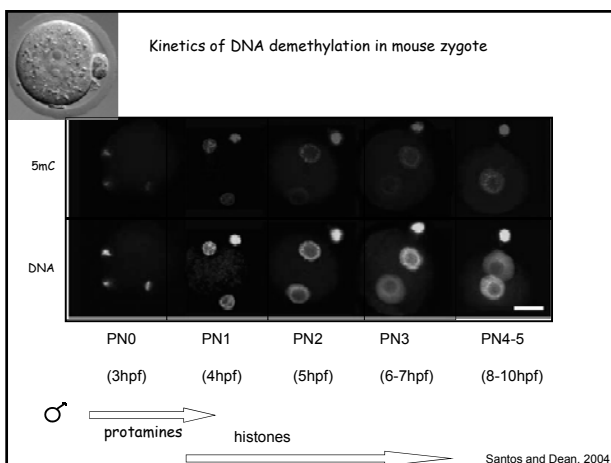
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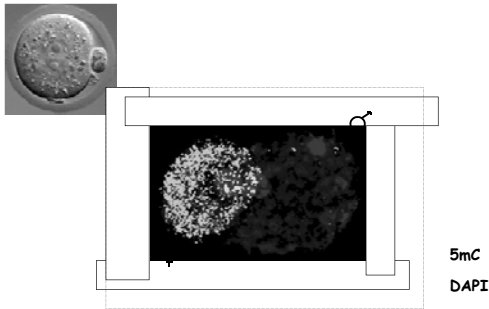
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### Zygotic reprogramming : DNA demethylation



Barton *et al*, 2001

### Regulation of epigenetic reprogramming

template

or

activity

Chromatin template

Presence of (de)modification  
enzymes

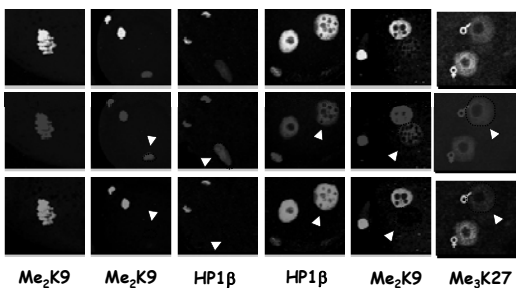
### Zygotic reprogramming - chromatin asymmetry



merged

DNA

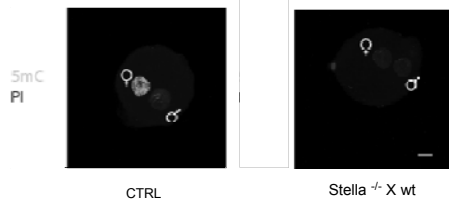
Ab



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)



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

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

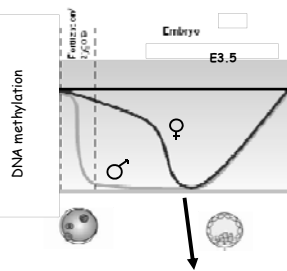
Species specific differences (mouse, bovine, human X sheep, rabbit)

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

Demethylation of maternal genome (Stella maternal ko) leads to  
preimplantation 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

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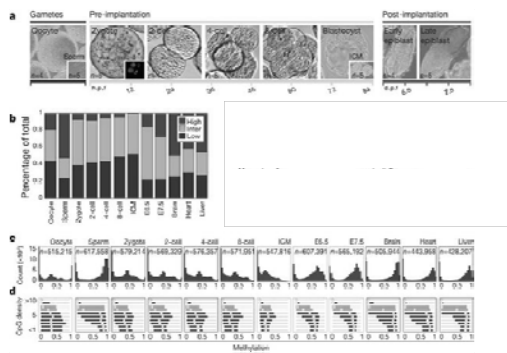
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DNA methylation dynamics in mouse preimplantation embryos



Smith et al, Nature 2012

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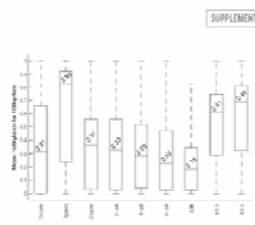
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DNA methylation dynamics in mouse preimplantation embryos



Supplementary Figure 5. Methylation values for 100bp sites across pre-implantation development. Boxplots of the methylation value per 100bp site 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 the line.

Smith et al, Nature 2012

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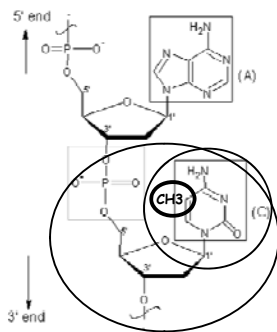
Mechanism of DNA demethylation?

### Active DNA demethylation - models....

Direct removal of methyl group

Removal of modified base (BER)

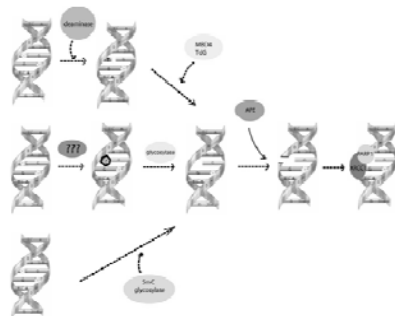
Removal of nucleotide(s) - NER



5mC deamination

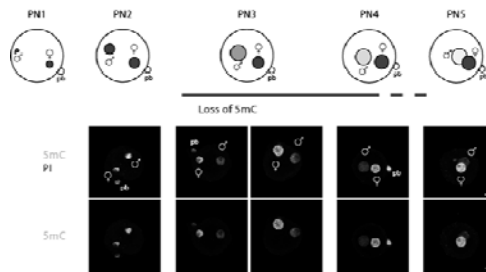
chemical modification of 5mC

5mC glycosylase



Hajkova et al. 2010

### Kinetics of DNA demethylation in mouse zygotes



Hajkova et al, 2010

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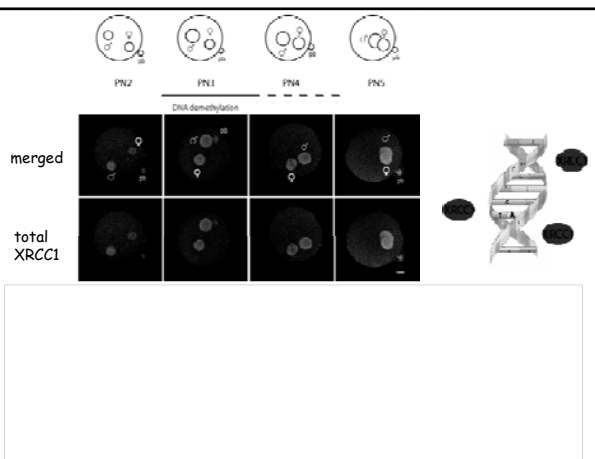
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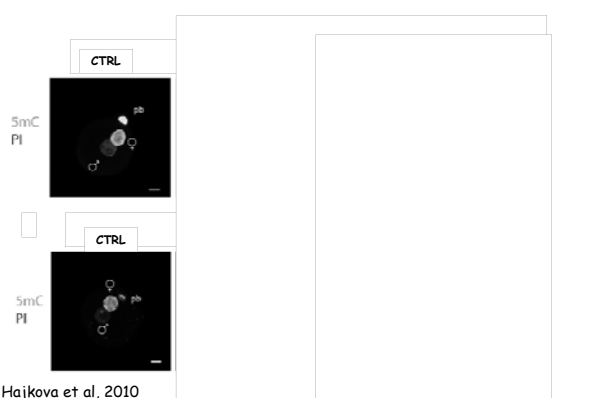
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### Inhibition of BER pathway perturbs DNA demethylation



Hajkova et al, 2010

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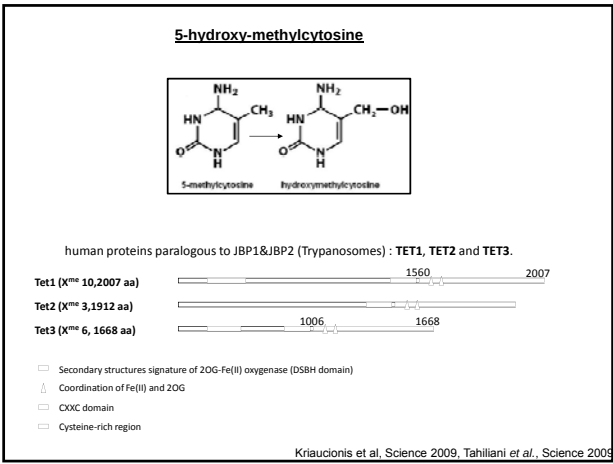
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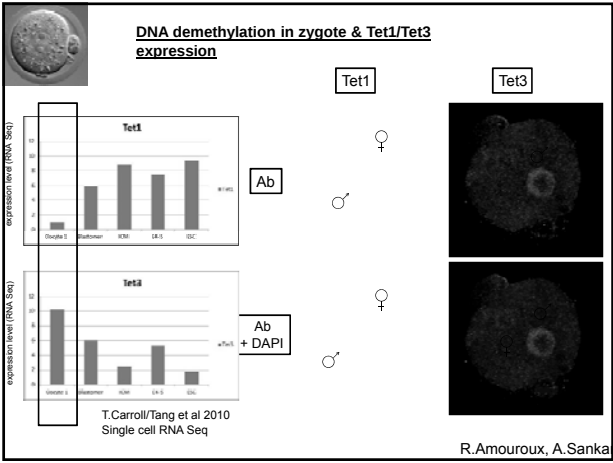
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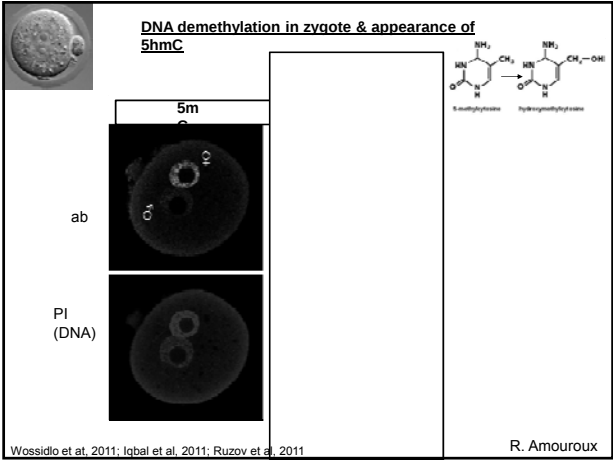
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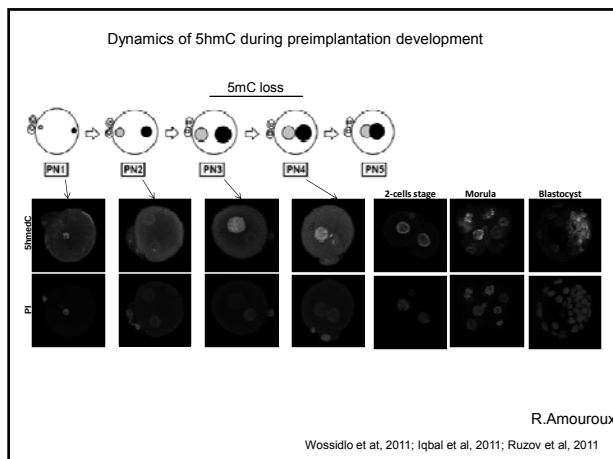
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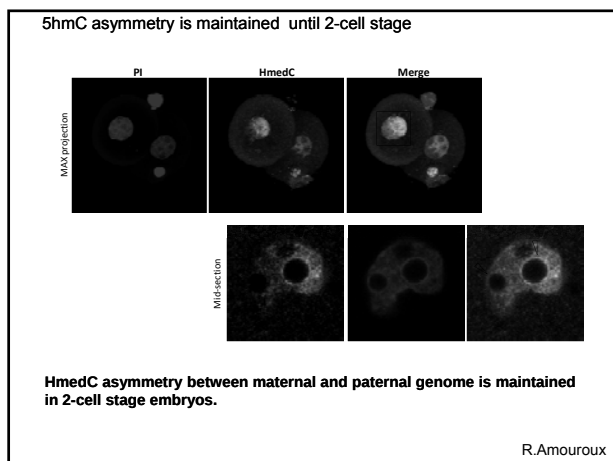
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### Summary

- 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)

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

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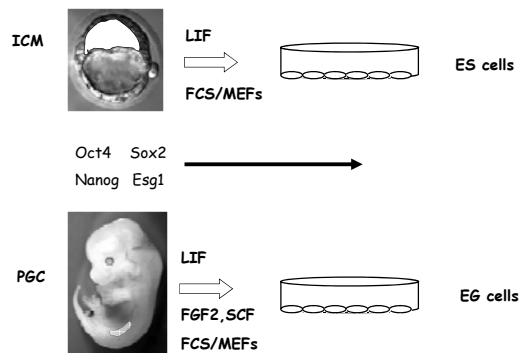
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Stem cell derivation - „classic“ protocol




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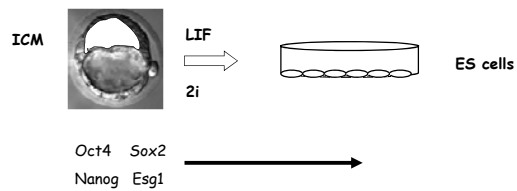
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Stem cell derivation - „2i“ protocol  
(reaching the pluripotent ground state)



Ying et al, 2008

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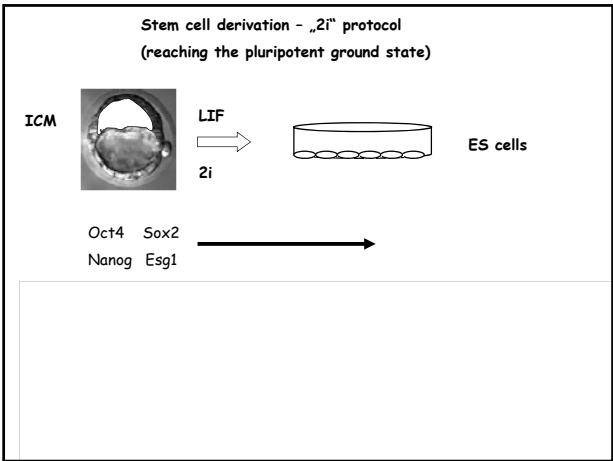
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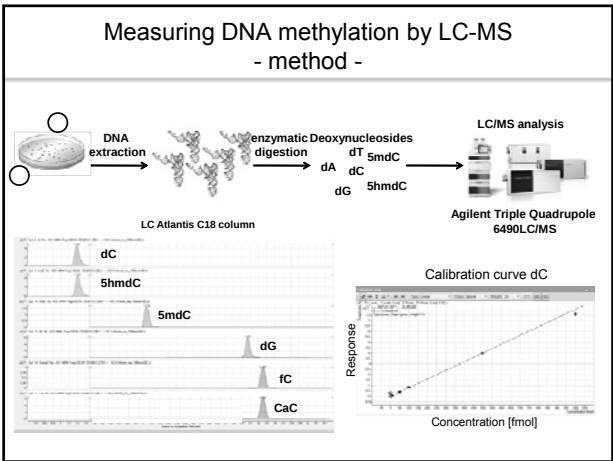
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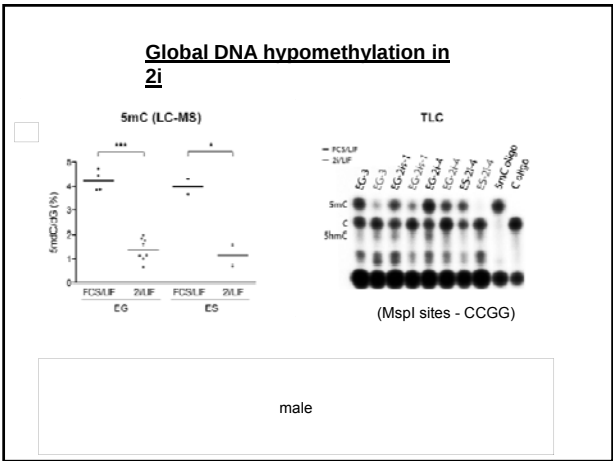
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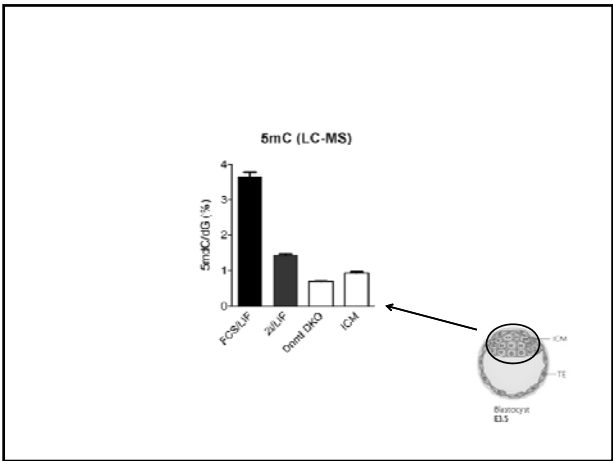
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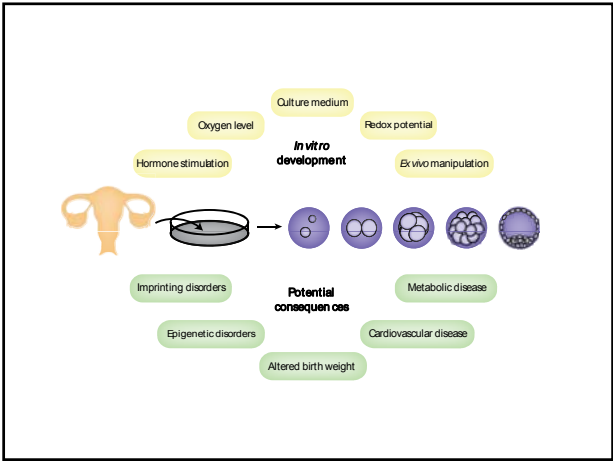
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### Acknowledgment

**Lab members**

|                 |                                                                       |
|-----------------|-----------------------------------------------------------------------|
| Kirsten McEwen  | <u>Azim Surani</u> (Gurdon Institute, Cambridge)                      |
| Aleksandra Turp | Nils Grabole                                                          |
| Buhe Nashun     |                                                                       |
| Rachel Amouroux | Austin Smith (Wellcome Trust Centre for Stem Cell Biology, Cambridge) |
| Peter Hill      | Harry Leitch                                                          |
| TienChi Huang   | Billy Mansfield                                                       |
| Sarah Linnett   |                                                                       |

**Bioinformatics**

|                   |                            |
|-------------------|----------------------------|
| Tom Carroll       | <u>Anne Ferguson-Smith</u> |
| Gopu Dharmalingam | Agilent Technologies       |

**CSC Mass spec facility**

Vesela Encheva

**CSC genomics laboratory**

Laurence Game

**Epigenesys**

**MRC Medical Research Council**

**EuroSystem**

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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


[www.ilarg.com](http://www.ilarg.com)
[www.sismer.it](http://www.sismer.it)


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**DISCLOSURE**

**Nothing to disclose**




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**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.




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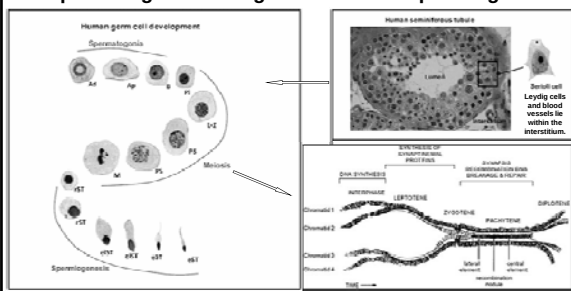
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## Main features of sperm cells' structure and functions

iliarg

5.5.2023

## The process of spermatogenesis from immature spermatogonia through meiosis and spermiogenesis

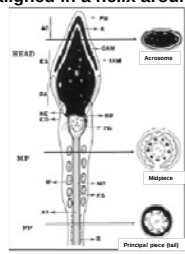
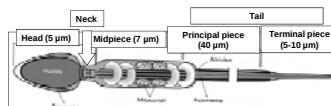


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5.5.2023

## 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

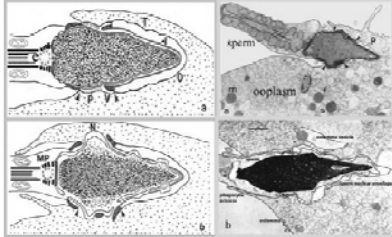


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### Sperm structure

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



Sethuraman et al., 1996

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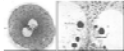
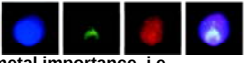
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### Sperm contribution to the oocyte

- PLC Zeta1 → Oocyte activation → Meiosis resumption → 
- DNA → Fertilization → 
- Centrosome → Duplication at pronuclear stage → migration to opposite poles 
- Resident histones → enriched at loci of developmental 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

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### Sperm cryopreservation

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### 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

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

minimized by the use of crioprotectants

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### 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<sub>2</sub>) vapors for ~10min and then plunged LN<sub>2</sub>

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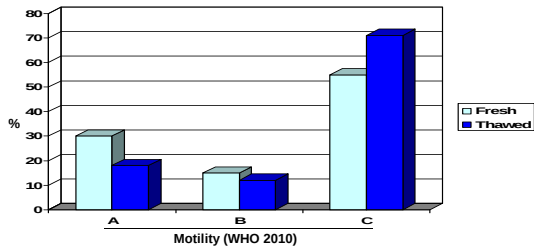
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### Sperm cryopreservation

After thawing:

- Decrease in sperm motility



| Category | Fresh (%) | Thawed (%) |
|----------|-----------|------------|
| A        | ~30       | ~20        |
| B        | ~18       | ~15        |
| C        | ~55       | ~75        |

Motility (WHO 2010)

SEMER data on 94 sperm samples

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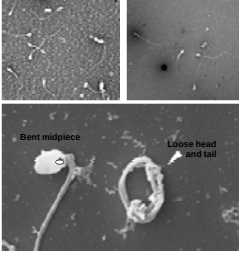
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**Sperm cryopreservation**

After thawing:

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



Bent midpiece  
Loose head and tail

Oskavskiy et al., 2008

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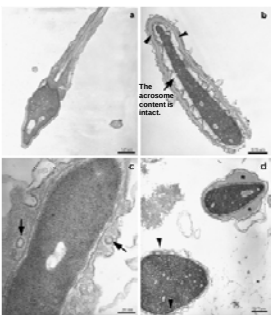
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**Sperm cryopreservation**

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 (asterisks)

Oskavskiy et al., 2008

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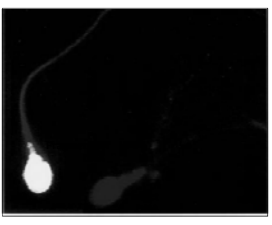
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**Sperm cryopreservation**

After thawing:

- Increase in sperm DNA damage



TUNEL test

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.

Duru et al., 2003; Thomson et al., 2009; Zhib et al., 2010; Riel et al., 2011

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## Sperm lyophilization

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Section 2

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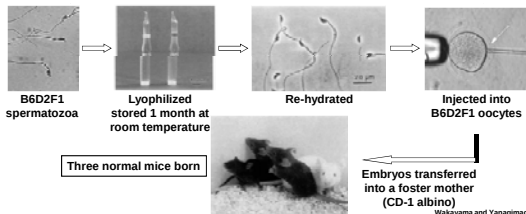
## Sperm lyophilization

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RESEARCH

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

"Teruhiko Wakayama" and "Ryuzo Yanagimachi"



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Section 2

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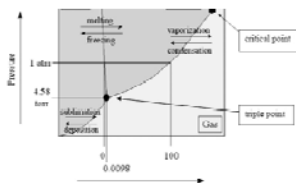
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## Lyophilization or Freeze-drying

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)

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Section 2

<http://www.scribd.com/doc/3772446/lyophilization-details>

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**Sperm lyophilization**

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**Mice**



↓

Proven fertility  
for 2 generations

↓

**Rats**



↓

**Rabbits**



↓

**Horses**



→

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




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**Sperm lyophilization**

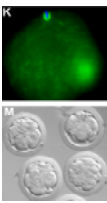
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**Bovine**

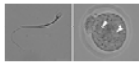


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**Macaque**



**Canine**



Mouse oocytes  
fertilized with  
canine freeze-dried  
spermatozoa

**Pig**



Embryo  
development to  
the 8-16 cell  
stage

Kwon et al., 2004; Lee K, Nawa K. et al., 2006; Sanchez-Perdida et al., 2009; Watanabe et al., 2009




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**Sperm lyophilization**

**Human**




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### 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 constitution

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

The process of lyophilization does not cause damage to sperm chromosomes

Kurokabe et al., 2006

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### Sperm lyophilization vs. cryopreservation

30 sperm samples

Count  
Motility  
Morphology  
Viability  
DNA fragmentation  
Birefringence patterns

Lyophilization

LN<sub>2</sub> cryopreservation

Two aliquots from each sample

The aim of the study was to have a basis of comparison with an established technique such as LN<sub>2</sub> sperm freezing

Gianaroli et al., 2012

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### Sperm lyophilization vs. cryopreservation

#### Protocol

| Lyophilization                                                                                                                                                                     | LN <sub>2</sub> cryopreservation                                                                                                                                                                             |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Purified sperm sample aliquots of 100 µL each were placed in glass ampules containing 400 µL of buffer solution (10 mM Tris-HCl and 1 mM of EDTA) and frozen at 20° C for 6 hours. | The HSPM (cryoprotectant human semen preservation) was added to the sperm suspension in an equal volume.                                                                                                     |
| Ampules were put inside a freeze-drying machine (Alpha 1-4 LSC, GmbH) near the condenser, and vacuumed overnight at 220x10 <sup>-3</sup> Mbar pressure.                            | After equilibration at 37° C for 5 minutes, the homogenate was loaded into 0.25 mL freezing straws which were first exposed to LN <sub>2</sub> vapors for 15 minutes and then plunged into LN <sub>2</sub> . |
| Lyophilized samples were stored at 4° C.                                                                                                                                           | <b>Thawing</b>                                                                                                                                                                                               |
| <b>Rehydration</b>                                                                                                                                                                 | The suspension was washed twice in T6 medium to remove the cryoprotectant, and the resulting pellet was resuspended in T6 medium                                                                             |
| Lyophilized samples were resuspended 1 week later in 100 µL of distilled water.                                                                                                    |                                                                                                                                                                                                              |

Gianaroli et al., 2012

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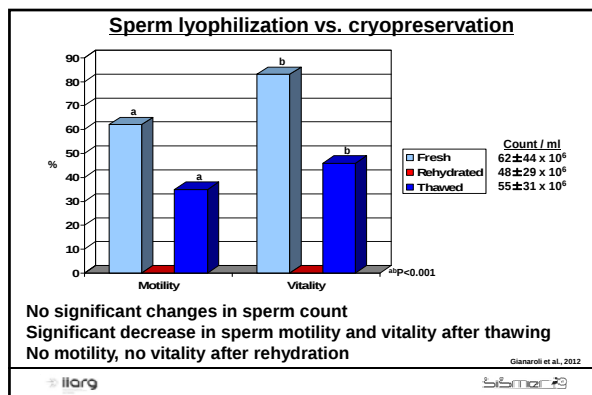
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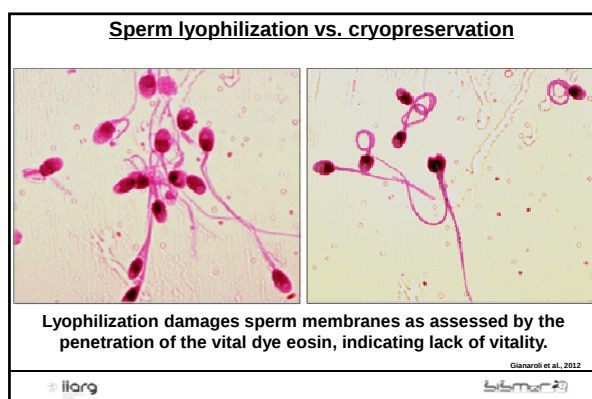
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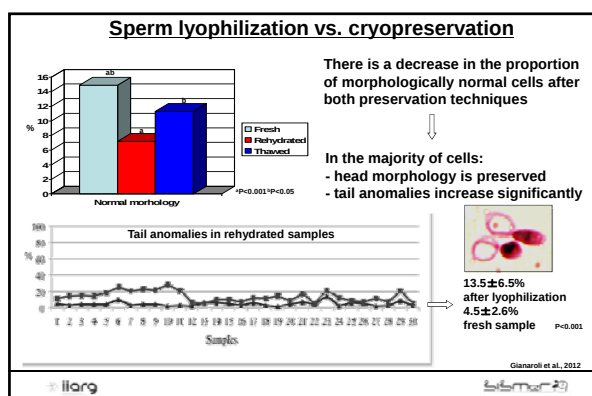
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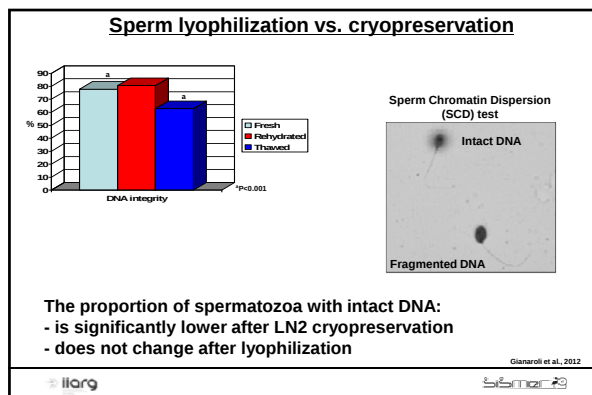
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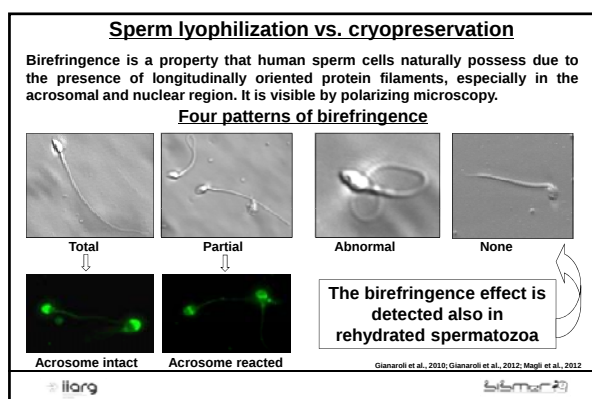
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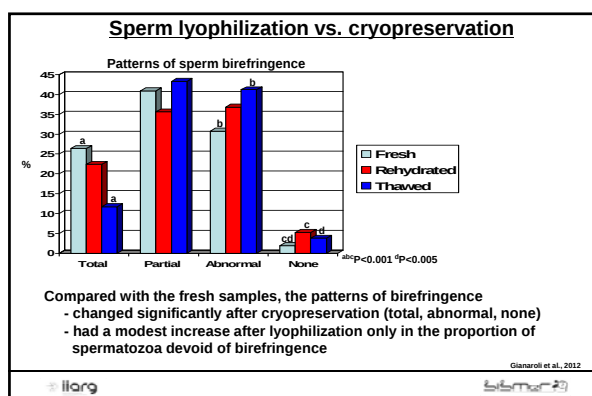
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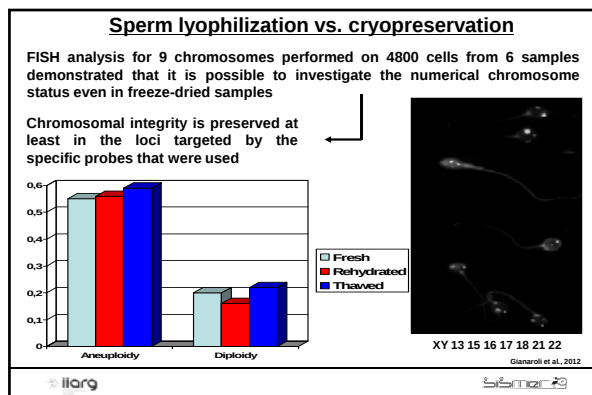
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### Possible clinical applications of sperm freeze-drying

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### 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

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### 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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