Other laboratory technologies: Fertility preservation (oocyte freezing etc...) PGD, PGS...

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LEARNING OBJECTIVES

1) Embryo / 2pn cryopreservation

2) Oocyte cryopreservation

3) PGD

4) PGS

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Cryopreservation
EMBRYOS / 2 pn

- Reasons for cryopreservation of human embryos

- To increase efficiency of ART
- To reduce multiple pregnancies
- To transfer in natural cycle
- Fertility preservation

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Cryopreservation
EMBRYOS / 2 pn

- Cryopreservation policy

1) cryopreserving before morphology becomes a substantial factor: two pronucleate stage

2) optimizing fresh transfer by selecting the morphologically best embryos to be transferred; cryopreservation of spare cleavage stage embryos or blastocysts

Which embryos to cryopreserve?

Which technique to use?

Cryopreservation
Embryos

There is a significant difference in the clinical outcome from fresh and frozen cycles.

Embryos	No of SET's	Implantation Rate
Fresh	2524	31.1% *
Cryopreserved	3020	24.1%

*P<0.001

Is the technique itself detrimental?

www.eshre.eu>specialty groups>special interest group Embryology>archive>Athens2009>Edgar

Cryopreservation
Embryos

Factors impacting could be:

- Quality of embryos prior to cryopreservation
- Biological consequences of freezing/thawing
- Efficiency of methodology

Cryopreservation

Quality of embryos prior to cryopreservation

	Embryos transferred	FH's	Implantation Rate
4 cells* Fresh	1567	260	16.6%
4 cells* Thawed Intact	794	134	16.9%
2 cells* Fresh	899	58	6.5%
2 cells* Thawed Intact	401	29	7.2%

* 40 – 42 hpi

Edgar et al (2000) Human Reproduction 15, 175

Fresh vs. equivalent cryo embryos → NO DIFFERENCE

iiarg

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Cryopreservation

Biological consequences of freezing/thawing

Possible biological consequences of embryo cryopreservation

- Cell loss ! !

Prefreeze blastomeres	Post thaw blastomeres	SCETs	FHs	Implantation rate
4	4	722	179	24.8%
4	3	146	40	27.4%
4	2	92	8	8.7%

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

Cell loss tends to reduce the implantation potential

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Cryopreservation

Biological consequences of freezing/thawing

Possible biological consequences of embryo cryopreservation

! ! - Cell loss ! !

- Arrested/compromised development

Blastomere survival	Resumption of mitosis	SCETs	FHs	Implantation rate
4 of 4	YES	641	165	25.7%
4 of 4	NO	81	14	17.3%
3 of 4	YES	113	34	30.1%
3 of 4	NO	33	6	18.2%
2 of 4	YES	68	7	10.3%
2 of 4	NO	24	1	4.2%

No meiosis resumption tends to reduce the implantation potential

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Cryopreservation

Efficiency of methodology

Slow-freezing or Vitrification ?

Day 3 embryos	Vitrification	Slow freezing	*P*-value
Cryosurvival (%)	222/234 (94.8)	206/232 (88.7)	0.02
Embryos with 100% blastomere survival (%)	173/234 (73.9)	106/232 (45.7)	<0.01

Balaban et al., 2008

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Cryopreservation

Efficiency of methodology

Loutradi et al (Fertil Steril 90, 186-193, 2008) Systematic review and meta analysis on vitrification versus slow freezing of human embryos

Kolibianakis et al (Current opinion in OB/GYN 21, 270-274, 2009) Cryopreservation of human embryos by vitrification or slow freezing: which one is better?

- Vitrification as compared with slow freezing, appears to have higher post-thawing survival rates both for cleavage-stage embryos and for blastocysts
- Post-thawing blastocyst development of embryos cryopreserved in the cleavage stage is significantly higher with vitrification as compared with slow freezing
- No significant difference in clinical pregnancy rates per transfer could be detected between the two cryo methods

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Cryopreservation

Efficiency of methodology

Optimizing slow-freezing → increased dehydration

- Embryos dehydrated in a single step using 1.5M PROH plus 0.2M sucrose prior to slow cooling (10 min)

- Thawing with three steps (0.5M PROH → 0.2M sucrose → medium

Edgar et al., 2009

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Cryopreservation
Efficiency of methodology

	0.1 M Sucrose	0.2 M Sucrose
Embryos Thawed	474	471
Fully Intact (100%)	259 (54.6%)[a]	379 (80.4%)[a]
50%-99% Intact cells	113 (23.8%)	57 (12.1%)
<50% intact	102 (21.5%)[b]	35 (7.4%)[b]

a : p<0.001 b: p<0.001

Edgar et al., 2009

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Cryopreservation
Efficiency of methodology

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

Edgar et al., 2009

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Cryopreservation
Embryos

Conclusions

1. Embryo quality before freezing is strongly associated with post thaw implantation potential
2. Thawed embryos have similar implantation potential to EQUIVALENT fresh embryos fresh embryos
3. Blastomere loss reduces implantation potential
4. Optimal procedures can minimize blastomere loss
5. No data supporting vitrification as a more efficient procedure

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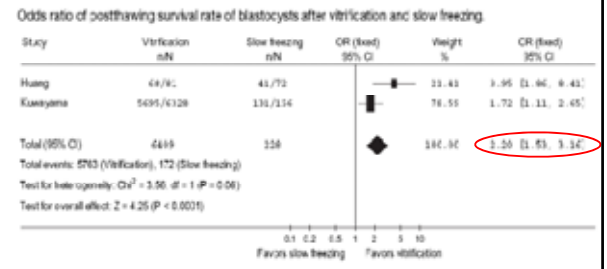
Cryopreservation
Blastocysts



Loutradi et al, Fertil Steril, 2008

Cryopreservation
Blastocysts

Conclusions

1. **RCTs indicate higher survival rates with vitrification as compared with slow freezing**

2. **but similar pregnancy rates**

Cryopreservation
2 pn

- **Satisfies legal requirements in several countries (Germany, Switzerland)**
- **Not viewed as an embryo → less moral and ethical conflict associated with handling and discard**
- **Lack of spindle apparatus**
- **Liquid phase transition temperature is lower than for oocytes rendering the membranes more resistant to chilling injury**

- **High post-thaw survival rates**
- **Little impact on viability - Implantation rates are similar to those reported for transfer of fresh sibling zygotes**
- **Survival is easy to diagnose (all or nothing) by progression to first cleavage**

Cryopreservation
2 pn

Timing

- S Phase in the 2 pn oocyte begins at 9-10 h post-insemination and ceases 3-5 h later
- Duration of G2 Phase is 4-6 h
- Duration of M Phase is 3-3.5 h

Balakier et al., 1993

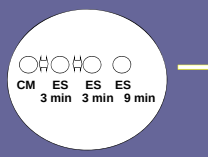
- Optimal time for cryopreservation is 16-20h post-insemination when majority of 2 pn oocytes are in the G2 phase of cell cycle

Cryopreservation
2 pn

- Time restriction
- Cryopreservation of many pre-embryos with unknown developmental potential
- Little opportunity to select the one or two most viable pre-embryos from a cohort for transfer
- Potentially increases the number of cycles before achievement of pregnancy
- Time-consuming and laborious

Slow-freezing or Vitrification ?

Vitrification (Kuwayama method)



Equilibration Solution (ES):
7,5% DMSO
7,5% ETHYLENE GLYCOL
10% HSA

Vitrification Solution (VS)
15% DMSO
15% ETHYLENE GLYCOL
10% HSA
0.5 M Sucrose

Re-warming

Thawing Solution (TS): 1 M Sucrose + 10 % HSA at 37°C for 1 min

Dilution Solution 1 (DS1): 0.5 M Sucrose + 10 % HSA for 3 min

Dilution Solution 2 (DS2): 0.25 M Sucrose + 10 % HSA for 3 min

Cryopreservation
2 pn

Conclusions

1. The 2 pronuclear stage can be successfully cryopreserved with high post-thaw survival
2. There is a significant workload for the laboratory
3. There is a potential delay to achieving pregnancy
4. No data supporting vitrification as a more efficient procedure

Cryopreservation
Oocytes

- It is a less ethically disputable alternative to embryo cryopreservation
- It could solve the dilemma of abandoned frozen embryos in the IVF laboratory
- It gives an opportunity for fertility preservation to women at risk of premature ovarian failure

Slow-freezing or Vitrification ?

Cryopreservation
Oocytes

Slow freezing	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

www.eshre.eu>specialty groups>special interest group Embryology>archive>Bologna2009>Edgar

Cryopreservation
Oocytes

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

	Fresh ICSI	Vitrified/Warmed ICSI (%)	Absolute difference (%) (95% CI)	OR (95% CI)	P
Fertilization (2PN) per sibling oocyte	100/120 (83.3)[a]	95/124 (76.6)[a]	−6.73 (−16.6 to 3.39)	0.65 (0.33 to 1.29)	0.20
Fertilization (2PN) per injected oocyte	100/120 (83.3)[b]	95/120 (79.2)[b]	−4.17 (−14.0 to 5.7)	0.76 (0.37 to 1.53)	0.50
Normal 2PN morphology	96/100 (96.0)[c]	86/95 (90.5)[c]	−5.47 (−13.4 to 1.84)	0.39 (0.08 to 1.49)	0.16
1PN oocytes	3/120 (2.5)[b]	6/120 (5.0)[b]	2.5 (−2.82 to 8.22)	2.05 (0.42 to 12.9)	0.50
3PN	1/120 (0.83)[b]	2/120 (1.66)[b]	0.83 (−3.09 to 5.1)	2.01 (0.10 to 119.9)	1
Degenerated oocytes post-ICSI	1/120 (0.83)[b]	4/120 (3.34)[b]	2.51 (−1.75 to 7.47)	4.08 (0.39 to 203.5)	0.37
Day 2 embryo development	100/100 (100)[c]	93/95 (97.9)[c]	−2.11 (−7.3 to 1.9)	0.0 (0.00 to 0.23)	0.24
Excellent quality embryos	52/100 (52.0)[d]	49/95 (51.6)[d]	−0.43 (−14.2 to 13.3)	0.98 (0.53 to 1.79)	0.90
Good quality embryos	38/100 (38.0)[d]	41/95 (43.2)[d]	5.16 (−8.49 to 18.6)	1.24 (0.67 to 2.28)	0.47
Fair/poor quality embryos	10/100 (10.0)[d]	3/95 (3.16)[d]	−6.84 (−14.6 to 0.42)	0.29 (0.05 to 1.19)	0.10

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

Fresh vs vitrified oocytes → No differences

Rienzi et al., (2010) Embryo development of fresh 'versus'vitrified metaphase II oocytes after ICSI: a prospective randomized sibling-oocyte study. Hum.Rep., 25: 66–73

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Cryopreservation
Oocytes

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

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

Rienzi et al., (2010) Embryo development of fresh 'versus'vitrified metaphase II oocytes after ICSI: a prospective randomized sibling-oocyte study. Hum.Rep., 25: 66–73

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Cryopreservation
Oocytes

TABLE 2
Oocyte distribution, survival, and fertilization.

	Vitrified	Fresh	P value
MII oocytes No. (%)	231 (87.2)	219 (89.7)	.383
MI oocytes No. (%)	19 (7.2)	11 (4.5)	.203
GV oocytes No. (%)	15 (5.7)	14 (5.7)	.974
Survival No. (%)	224/231 (96.9)	—	
No. of injected oocytes	224	219	
Normal fertilization No. (%)	171 (76.3)	180 (82.2)	.126
Abnormal fertilization No. (%)	9 (4.0)	12 (5.4)	.469
Degenerated oocytes No. (%)	7 (3.1)	6 (2.7)	.809

TABLE 3
Embryo

Fresh vs vitrified oocytes → No differences

	Vitrified	Fresh	P value
Cleavage rate day 2 embryos (%)	161/171 (94.2)	176/180 (97.8)	.083
No. of cell day 2 embryos (mean ± SD)	3.8 ± 1.1	3.9 ± 1.5	.567
Good quality day 2 embryos (%)	136/161 (84.4)	126/176 (71.5)	.005
Cleavage rate day 3 embryos (%)	125/161 (77.6)	149/176 (84.6)	.096
No. of cell day 3 embryos (mean ± SD)	6.9 ± 2.3	6.9 ± 2.7	.558
Good quality day 3 embryos (%)	101/125 (80.8)	120/149 (80.5)	.956
No. of embryo undergoing extended culture	78	143	
Blastocyst rate No. (%)	38/78 (48.7)	68/143 (47.5)	.869
Good quality blastocysts No. (%)	24/32 (81.1)	42/60 (70)	.612

Cobo et al., (2008) Clinical outcome of oocyte vitrification. Fertil. Steril. 89:1657-1664

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Cryopreservation
Oocytes

TABLE 4

Clinical results.

	Vitrified	Fresh	Mixed
No. of transfers	23	1	4
No. of embryos transferred (mean ± SD)	49 (2.1 ± 1.2)	2 (2 ± 0)	8 (2.1 ± 0.1)
Pregnancy rate per transfer	15/23 (65.2)	1 (100)	2 (50)
Implantation rate (No. of sacs/ No. of embryos transferred)	20/49 (40.8)	2/2 (100)	2/8 (25)
Multiple pregnancy rate (twin)	5/15 (23.8)	1 (100)	0
Miscarriage rate	3/15 (20)	0	0
Biochemical pregnancy rate	1/15 (6.6)	0	0
Ongoing pregnancy rate	11/23 (47.8)	1 (100)	2 (100)

Note: Numbers in parentheses are percentages.

Cobo et al., (2008) Clinical outcome of oocyte vitrification. Fertil. Steril. 89:1657-1664

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Cryopreservation
Oocytes

Conclusions

1- Oocyte cryopreservation is a promising method especially using vitrification procedures

2- To determine efficacy and safety of oocyte cryopreservation there is still the need to verify the performance on infertile patients in all age categories (both young and old patients

3- Pregnancy Follow up (5% malformation rate according to Cobo at "Updates in infertility treatment" – January 2010 – Seville, Spain)

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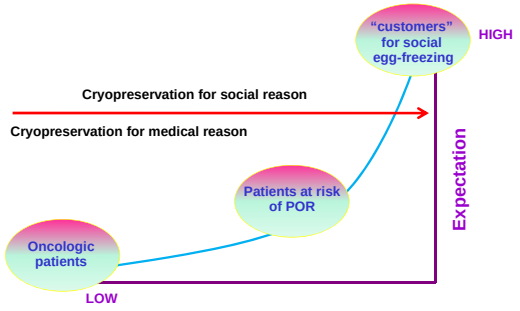
Oocyte cryopreservation – Patients' expectation



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Ovarian tissue cryopreservation

Candidate diseases for ovarian cryopreservation

Cancer patients
- Breast cancer
- Cervical cancer
- Hodgkin's lymphoma
- Non-Hodgkin's lymphoma
- Osteosarcoma
- Ewing's sarcoma
- Wilm's tumor

Bone marrow transplant patients
- Leukemia (?)
- Aplastic anemia
- Sickle cell anemia

Adjunctive oophorectomy
- Endometriosis

Autoimmune diseases
- Collagen vacular diseases (SLE)
- Acute Glomerulonephritis
- Behcet's disease

Ovarian diseases
- BRCA-1 and -2 mutations
- Turner's Syndrome

Ovarian tissue cryopreservation

Preparation of human ovarian tissue for cryopreservation

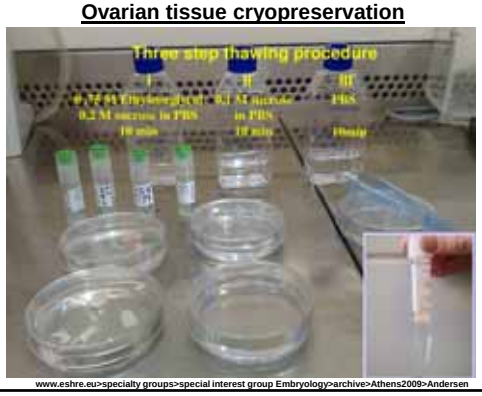
Ovarian tissue cryopreservation

CRYOPRESERVATION PROTOCOL

(A.T. Schmidt et al., Hum Reprod, 2003)

- Cryoprotectant: 1.5 mol/l Ethyleneglycol
  0.1 mol/l Sucrose
  10 mg/ml HSA
- Temperature profile:
  1. Equilibration - rotation (1-2 °C in 30 min)
  2. - 2 °C/min indtil – 9 °C.
  3. Manuel seeding
  4. – 0,3 °C/min until – 40 °C
  5. –10 °C/min until – 140 °C
  6. Liquid nitrogen (– 196 °C)

Ovarian tissue cryopreservation



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Ovarian tissue cryopreservation
Vitrification versus controlled-rate freezing

Based on tissue from 20 women and using morphological characteristics evaluated by light and electron microscopy

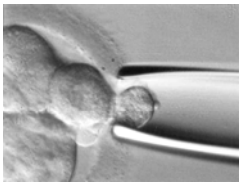
- Vitrification was comparable to slow freezing in terms of preserving follicles in human ovarian tissue
- It appears that the ovarian stroma retained a better morphological integrity after vitrification
- Clinical implication: vitrification is not yet applied in a clinical setting

Keros et al., Hum Reprod, 2009;24:1670

PGD / PGS

Diagnosis → PGD

implies looking for:
- **specific disease mutation**
- **(including X-linked diseases)**
- **chromosome aberration**



Screening → PGS

implies looking for a genetic defect in all members of a population at risk being the risk dependent on the incidence and severity of the defect

PGD / PGS

WHY TO GO FOR IT?

Fertile / infertile couples whose children might inherit
- a severe disease
- a predisposition to a pathology

Fertile / infertile couples in which one partner is carrier of a translocation

Infertile couples aiming at deselecting aneuploid embryos

Fertile couples who wish to save a sibling's life (HLA-typing)

PGD

WHY TO GO FOR IT?

"IVF aims at having a child, PGD aims at having a healthy child and PGD/HLA testing aims at having a healthy and helpful child".

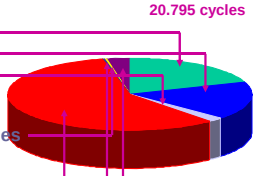
UNESCO's report on preimplantation genetic diagnosis (PGD) and Germ-Line Intervention, 2003.

PGD / PGS

MAIN INDICATIONS

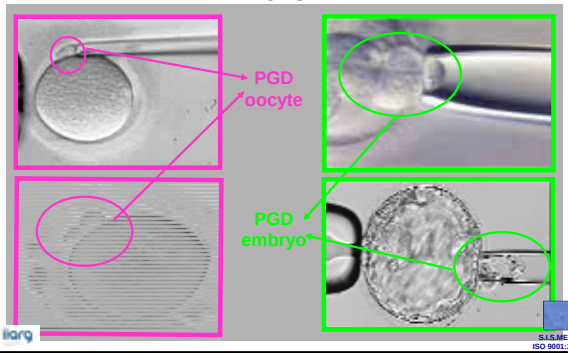
ESHRE PGD consortium data collection 1997-2006

- High risk of genetic disease
  - Single gene disorders
  - Chromosomal abnormalities
  - Late onset diseases (i.e. Huntington Chorea)
- Predisposition to late onset diseases (i.e. cancer)
- HLA typing
- Aneuploidy
- Sex selection for family balancing



Goossens et al. (2009) ESHRE PGD Consortium data collection VIII: cycles from January to December 2006 with pregnancy follow-up to October 2007. Hum Reprod 24,1786-1810.

PGD / PGS
BIOPSY

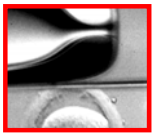


PGD / PGS
BIOPSY

PB	Blastomere	Blastocyst
Pros - Meiosis by-product - Several days for analysis	Pros - For maternal and paternal defects - For mitotic defects	Pros - For maternal and paternal defects - For mitotic defects - Several cells available
Cons - Only for maternal defects - Diagnosis on oocyte counterpart	Cons - Reduces embryonic mass - Mosaicism	Cons - A few hours available - Are TE cells representative of ICM cells?

PGD / PGS
BIOPSY TECHNIQUES

- mechanical



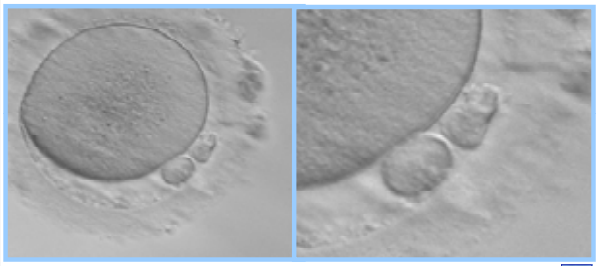
- laser

- chemical

Gianaroli et al., 2002

PGD / PGS
BIOPSY TECHNIQUES

laser



PGD / PGS
BIOPSY TECHNIQUES

The embryo

- mechanical

- laser

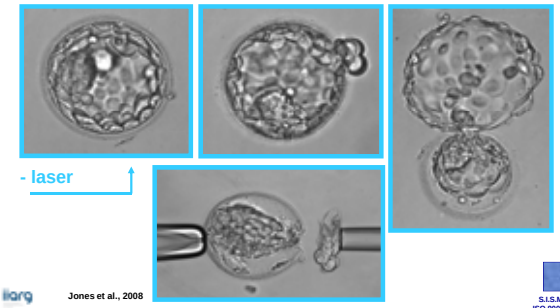
- chemical

Gianaroli et al., 2002

PGD / PGS

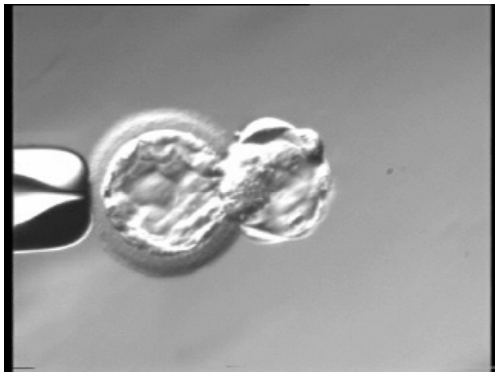
BIOPSY TECHNIQUES

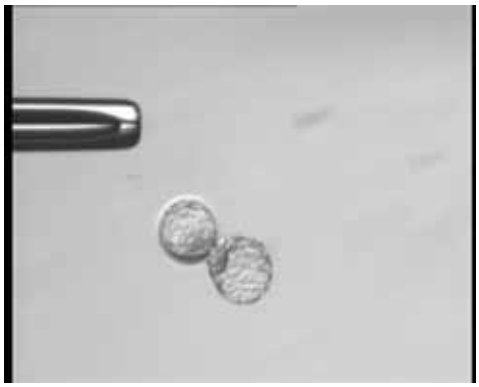
The blastocyst



- laser

Jones et al., 2008





PGD / PGS

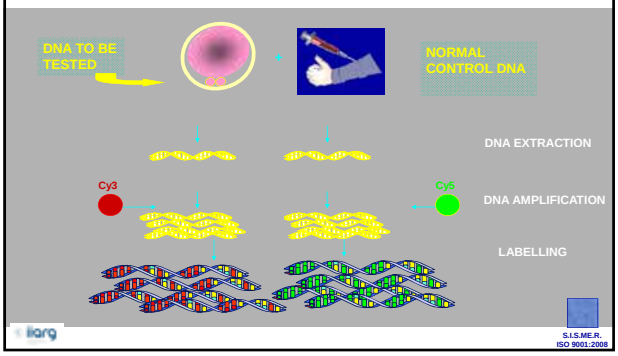
BIOPSY TECHNIQUES

Mechanical	Chemical	Laser
Pros - Avoids the use of heat or acidic solutions	**Pros** - It was the most commonly used method for a long time	**Pros** - Very easy to use
Cons - Requires a double holder - Requires a skilled operator - It is time consuming	**Cons** - Requires a double holder - Requires a skilled operator - Requires the use of acidic solution	**Cons** - Requires the release of heat - Training and skill underestimated?

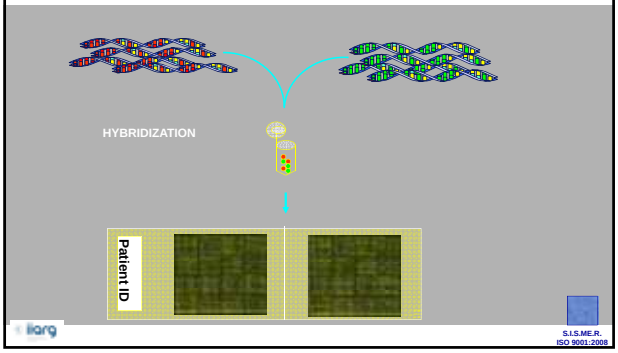
PGS by microarrays CGH

Single cell comparative genomic hybridization (CGH) is an emerging form of preimplantation genetic screening (PGS) that is used after whole genome amplification to detect abnormalities in the number of chromosomes in an oocyte or an embryo.

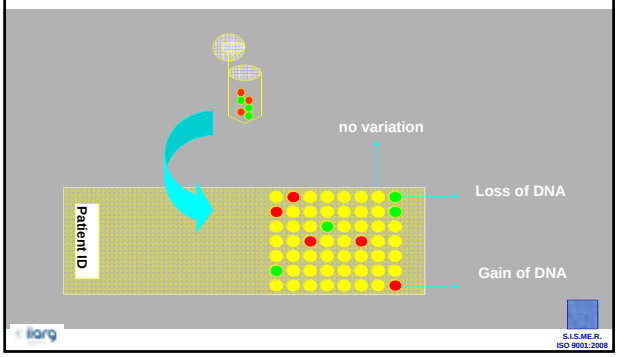
PGS by microarrays CGH



PGS by microarrays CGH



PGS by microarrays CGH



PGS by microarrays CGH

Oocyte polar bodies or single blastomeres are compared to a normal control DNA and the chromosomal status is evaluated using fluorescent dyes on microarrays.

PGS by microarrays CGH

Promising results are coming, but …….

- Is the technique efficient?

To what extent?

- Is the technique reliable?

PGS BY MICROARRAYS CGH

DEBATE

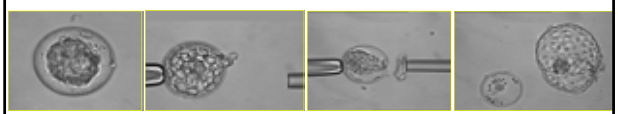
What next for preimplantation genetic screening? A polar body approach!

Joep Geraedts[1,11], John Collins[2], Luca Gianaroli[3], Veerle Goossens[4], Alan Handyside[5], Joyce Harper[6], Markus Montag[7], Sjoerd Repping[8,9], and Andreas Schmutzler[10]

BIOPSY ON BLASTOCYSTS

- More chances of high viability
- Diagnosis more robust and accurate
  Biopsy of several cells allows:
  - Less risk of misdiagnosis due to mosaicism or ADO
  - Reduced impact of embryo biopsy (embryonic cells are not removed)?
  - 100% of chromosome errors detected
  - DNA-based: no need for cell fixation
- **Is it possible to identify competent blastocysts?**

BIOPSY ON BLASTOCYSTS

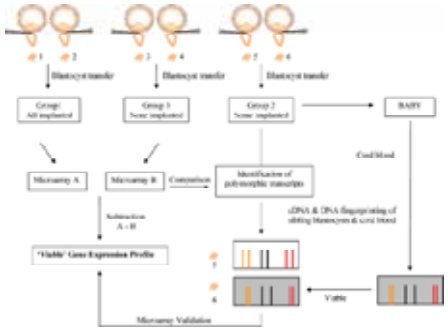


Blastocyst biopsy and DNA fingerprinting to link developmental competence with gene expression patterns

Transfer to the uterus of a single genetically tested normal blastocyst with demonstrated high implantation potential

Jones G, Cram DS, Song B, Kokkali G, Pantos K, Trounson AO 2008 Novel strategy with potential to identify developmentally competent IVF blastocysts. Hum Reprod 23, 1748-1759.

IDENTIFICATION OF COMPETENT BLASTOCYSTS



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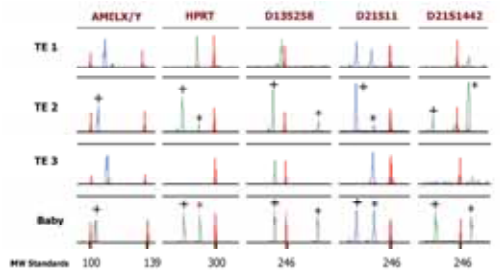
S.I.S.ME.R. ISO 9001:2008

IDENTIFICATION OF COMPETENT BLASTOCYSTS



Over 7000 transcripts expressed exclusively in 'viable' blastocysts

Jones G, Cram DS, Song B, Kokkali G, Pantos K, Trounson AO 2008 Novel strategy with potential to identify developmentally competent IVF blastocysts. Hum Reprod 23, 1748-1759.

S.I.S.ME.R. ISO 9001:2008

PGD / PGS

Conclusions

1. There is a wide range of indications to PGD / PGS

2. The technical procedures are becoming more reliable and efficient

3. The technical approach can be extended to the research for embryo competence

S.I.S.ME.R. ISO 9001:2008

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