Ovarian tissue freezing: slow freezing versus vitrification

Claus Yding Andersen, Professor, MSc, DMSc

Laboratory of Reproductive Biology

University Hospital Copenhagen,
University of Copenhagen
Copenhagen, Denmark

E-mail: yding@rh.dk

"Cryobiology and cryopreservation of human gametes and embryos"
ESHRE Campus workshop, Athens, Greece, September 25 -26th 2009

Rationale and perspectives of cryopreserving and transplanting ovarian tissue

- Fertility can be maintained after curing disease where treatment may harm ovarian function
- And will lead to menstrual cycles and an endogenous hormone production in contrast to other fertility preserving methods

Chemotherapy and gonadotoxicity

Risk of inducing detrimental effects on the gonad

- The specific chemotherapeutic drug used
- Dose of chemotherapy
- Duration of chemotherapy
- Age of woman

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

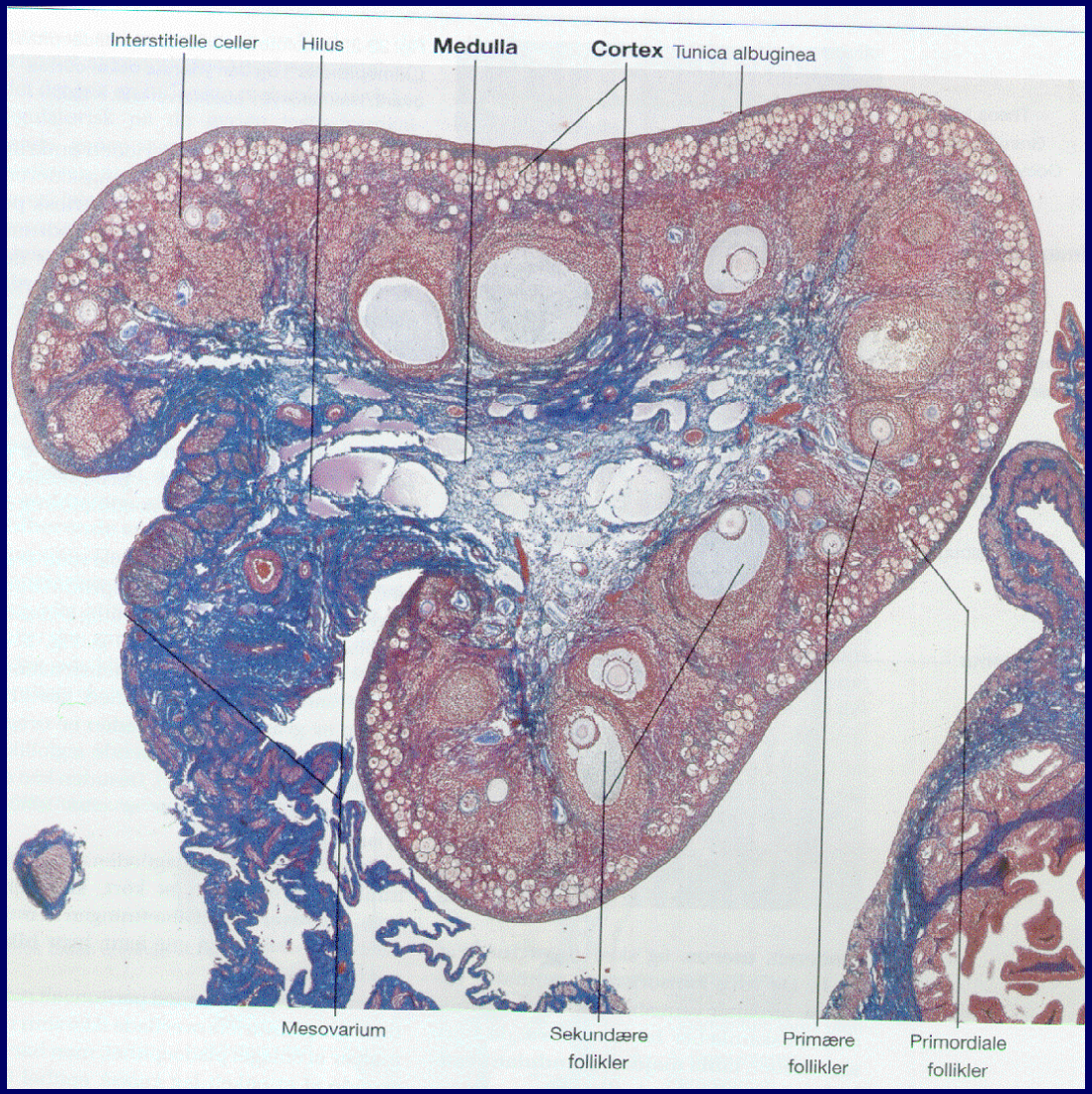
Autoimmune diseases

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

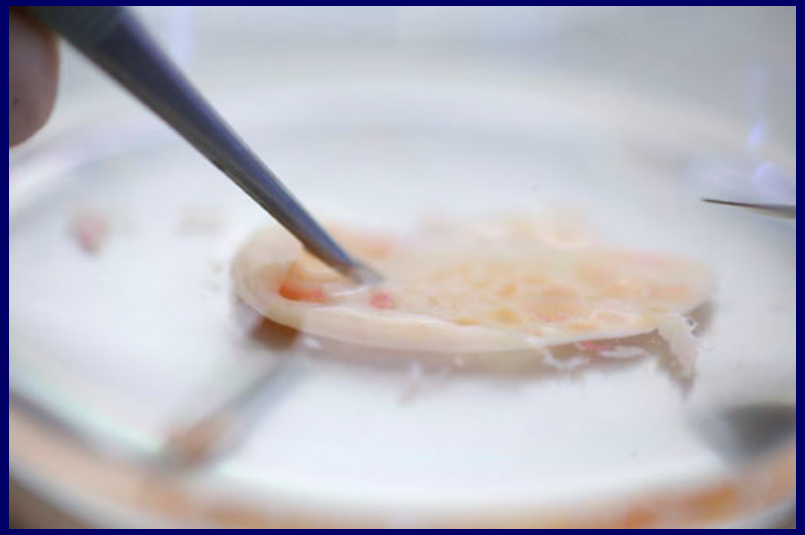
Ovarian diseases

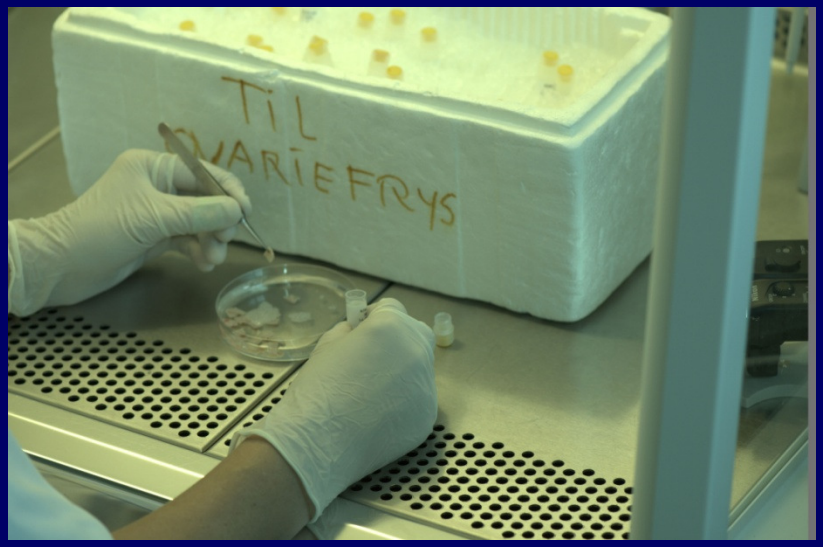
- BRCA-1 and -2 mutations
- Turner's Syndrome

Only the ovarian cortex is cryopreserved



Preparation of human ovarian tissue for cryopreservation





CRYOPRESERVATION PROTOCOL

(KLT. 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 ^{0}C in 30 min)**
2. **- 2 ^{0}C/min indtil – 9 ^{0}C.**
3. **Manuel seeding**
4. **– 0,3 ^{0}C/min until – 40 ^{0}C**
5. **–10 ^{0}C/min until – 140 ^{0}C**
6. **Liquid nitrogen (– 196 ^{0}C)**

Percentage of morphological healthy follicles before and after cryopreservation

6 ovaries in each group

Cryopreservation media:

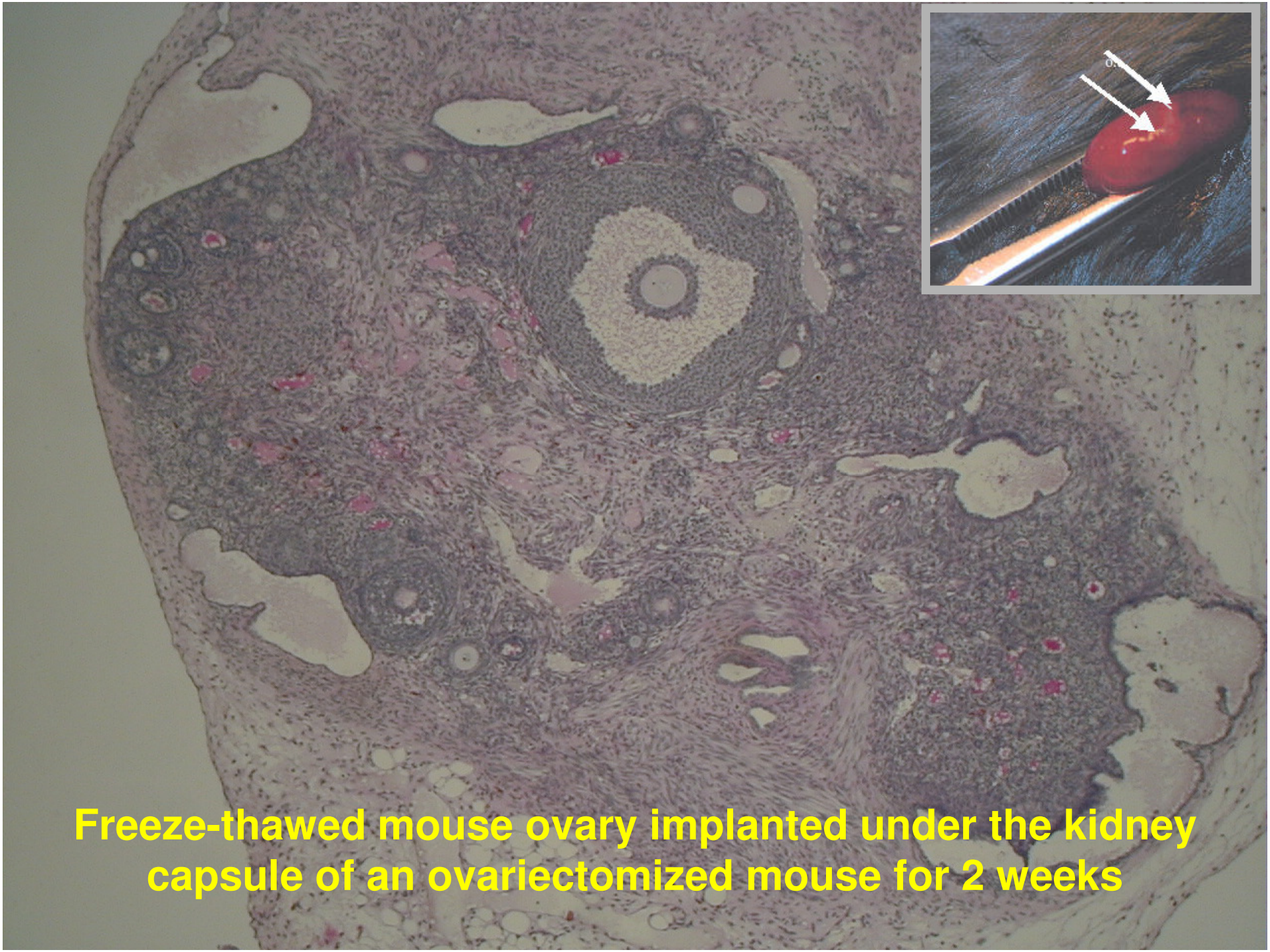
I : Leibowitz medium, 10 % FCS & 1.5 M DMSO

II : Leibowitz medium, 10 % FCS & 1.5 M Ethyleneglycol

III : PBS, 0.1 M sucrose & 1.5 M DMSO

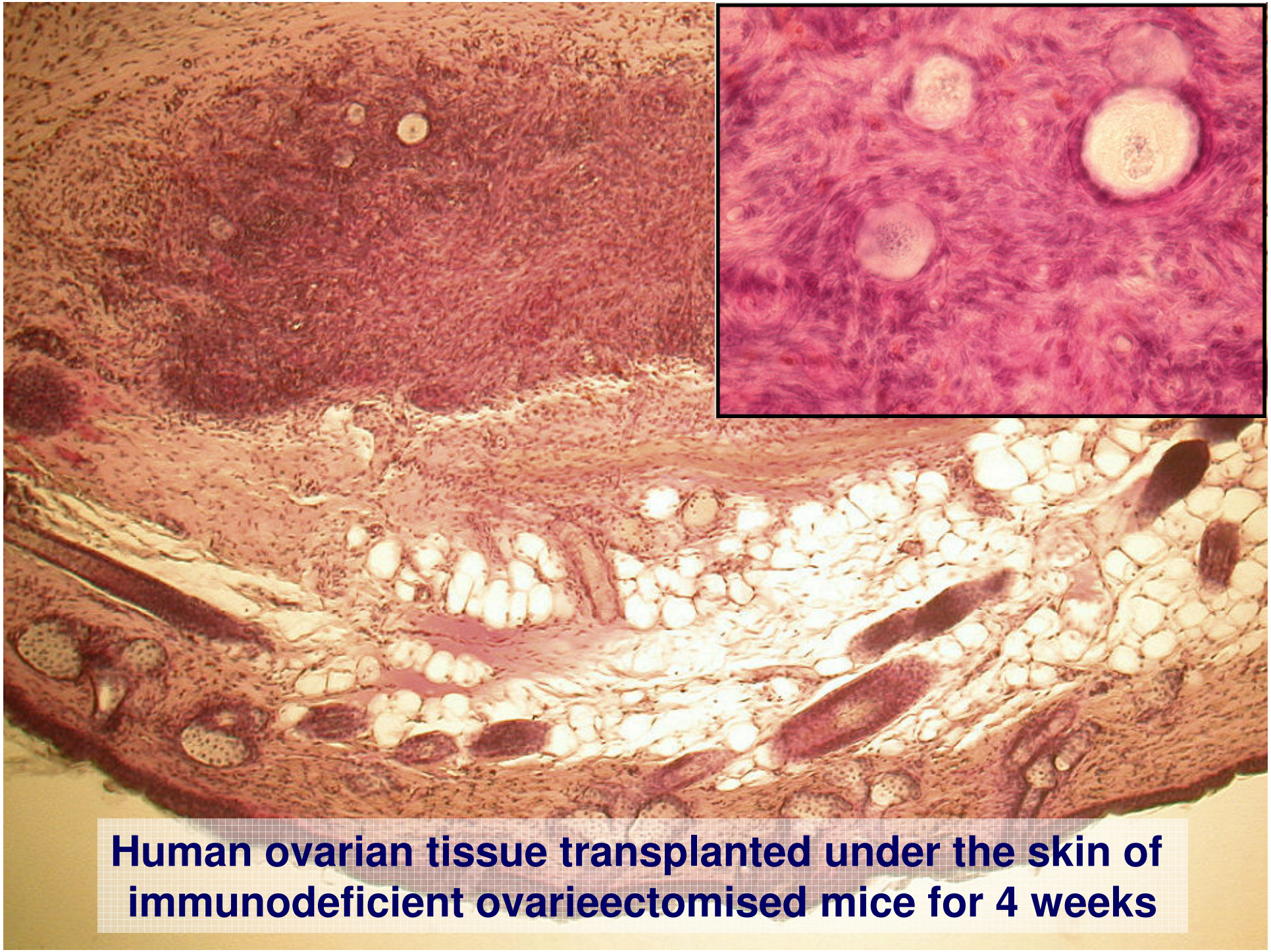
IV: **PBS, 0.1 M sucrose & 1.5 M Ethyleneglycol**

	I	II	III	IV
BEFORE	97 ± 0.5	97 ± 0.7	97 ± 0.8	**97 ± 1.3**
AFTER	32 ± 11	31 ± 11	47 ± 11	**63 ± 8**



Freeze-thawed mouse ovary implanted under the kidney capsule of an ovariectomized mouse for 2 weeks

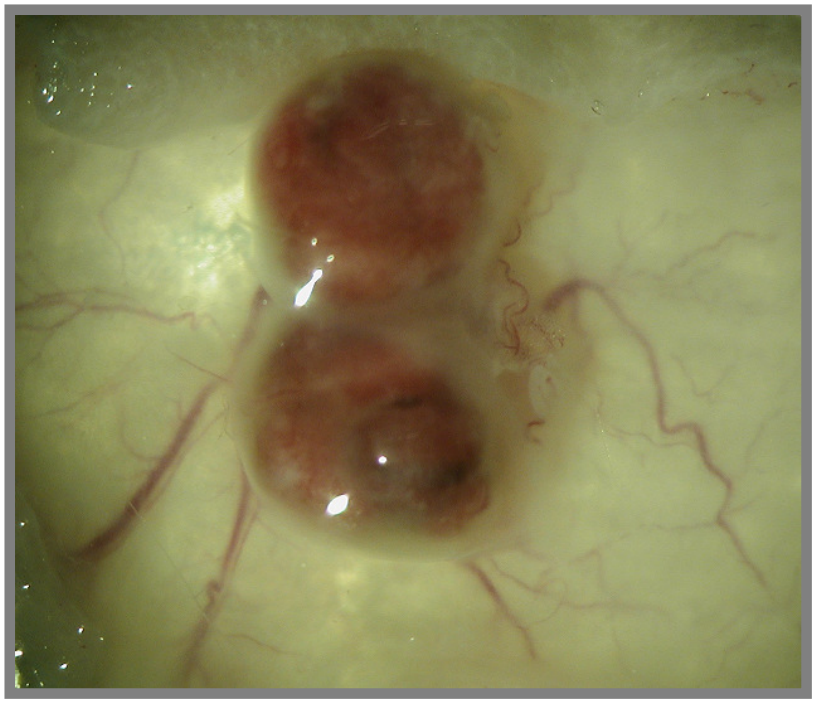

Primordial follicles in the ovarian cortex
from a 12 year old girl



Human ovarian tissue transplanted under the skin of immunodeficient ovarieectomised mice for 4 weeks

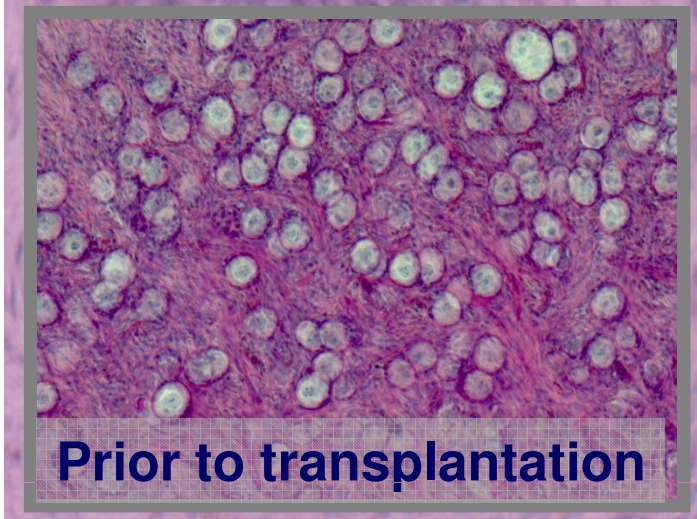
Transplanting ovarian cortical tissue to ovariectomised immunodeficient nude mice for four weeks

Tissue from 42 women (49 transplantations) showed surviving follicles and resulted in no apparent disease development in all cases



Frozen thawed human ovarian cortex implanted under the skin of ovariectomized SCID mice for 4 weeks

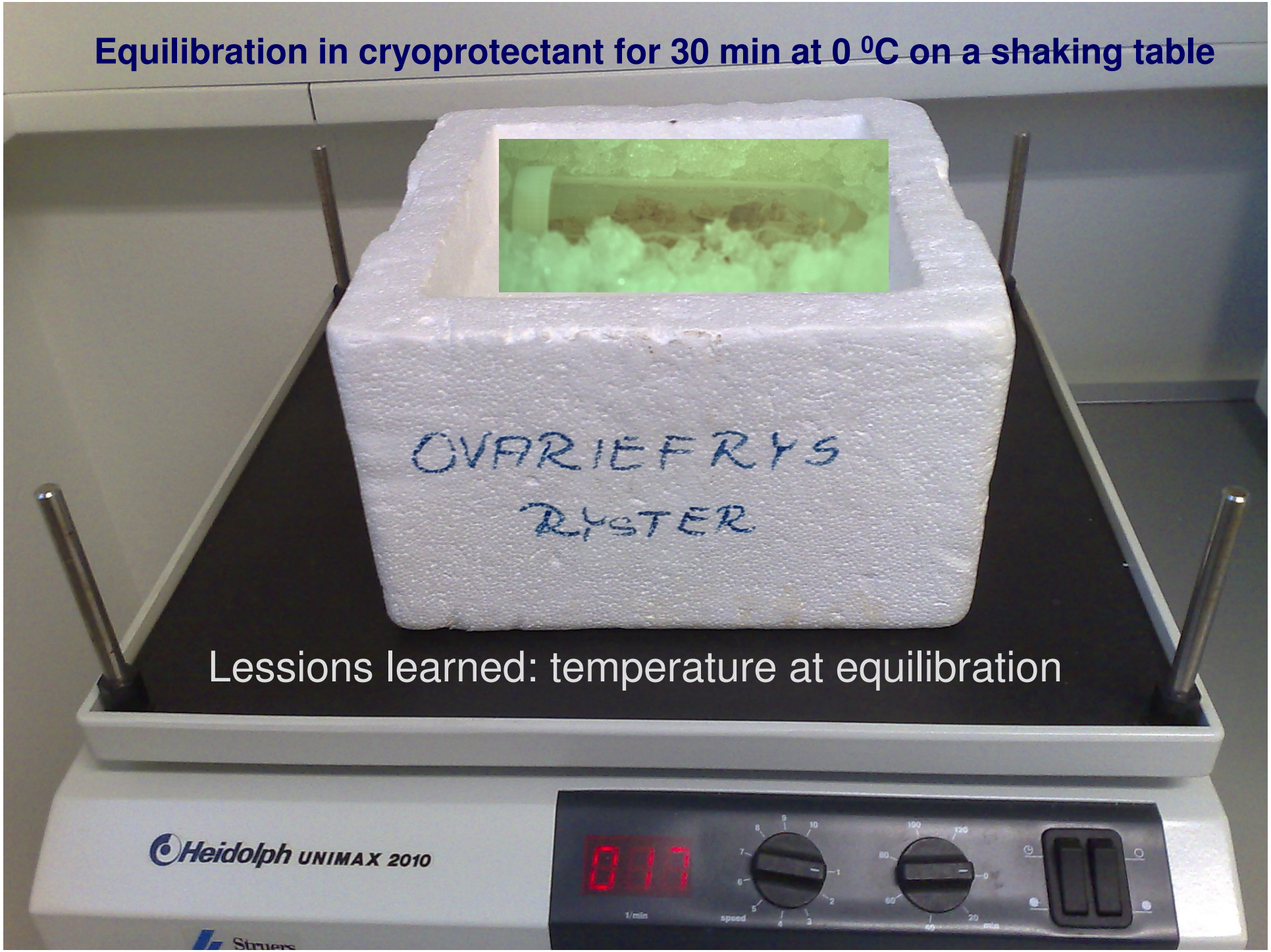
Transport of ovarian tissue – 6 year old girl 20 hours on ice prior to cryopreservation



Prior to transplantation

Human ovarian tissue transplanted under the skin of ovariectomised SCID mice for 4 weeks

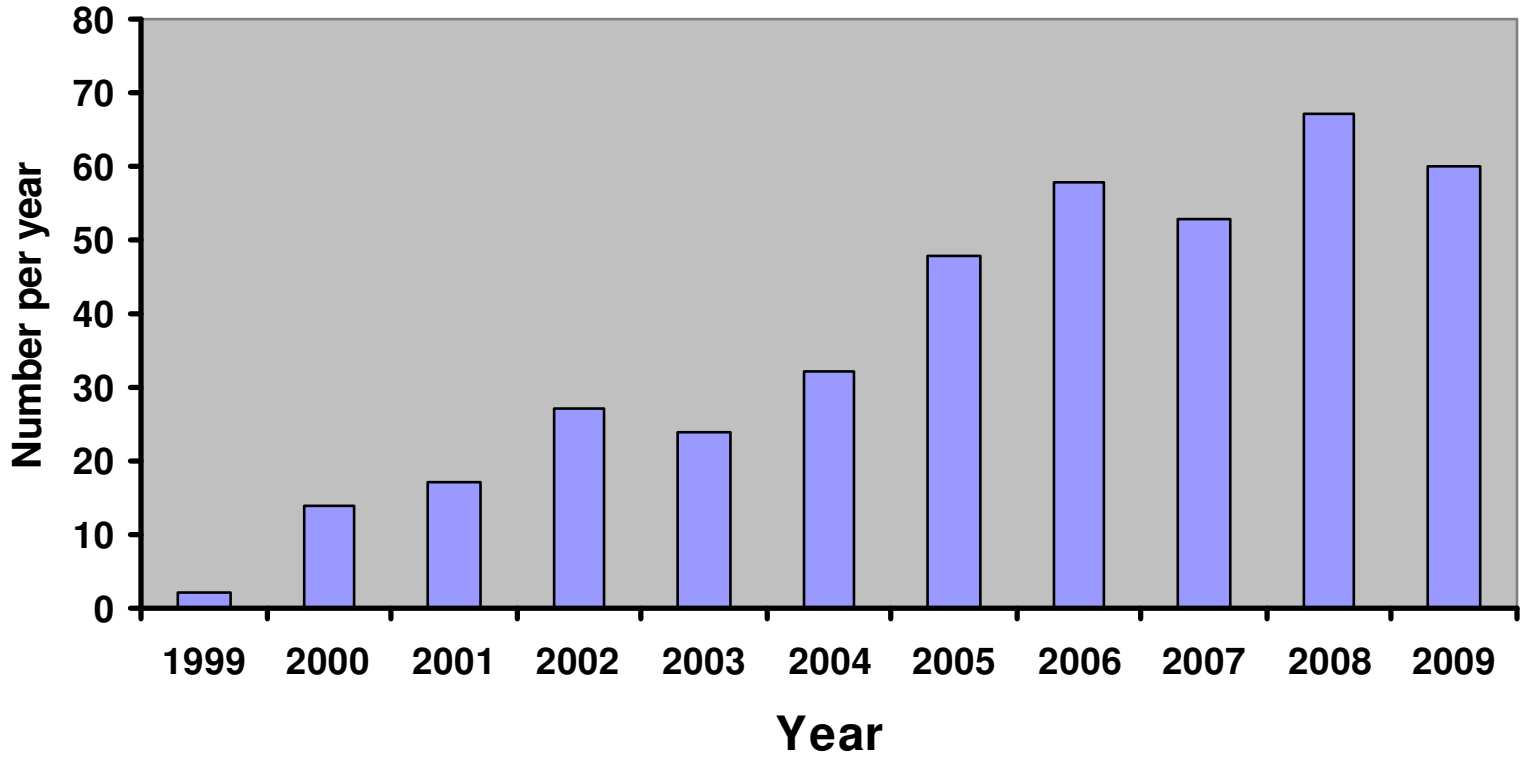
Equilibration in cryoprotectant for 30 min at 0 ^{0}C on a shaking table



Lessions learned: temperature at equilibration

Number of patients with cryopreserved ovarian tissue at University Hospital of Copenhagen

(Sep 2009: 385 patients)

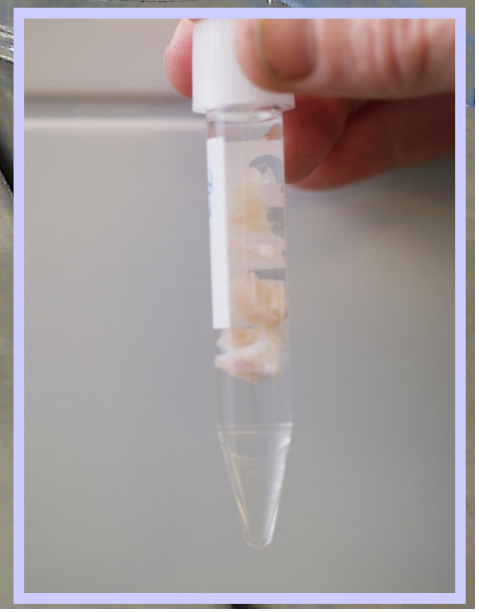


Three step thawing procedure

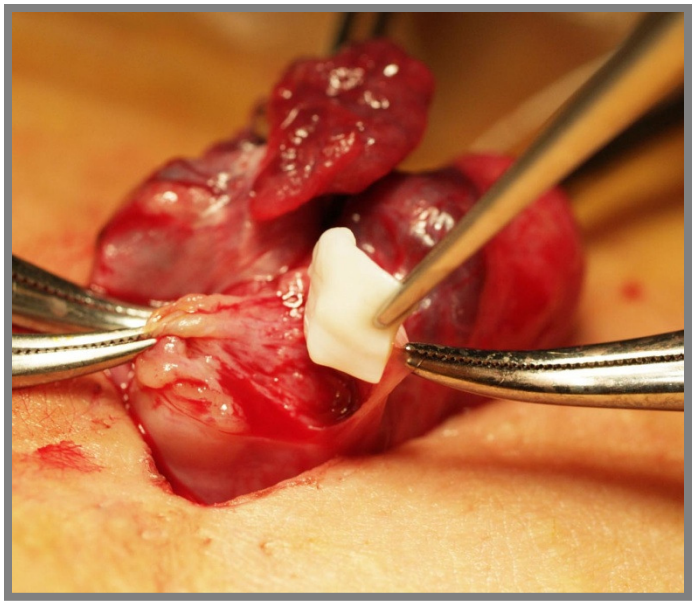
I
0 ,75 M Ethyleneglycol
0,2 M sucrose in PBS
10 min

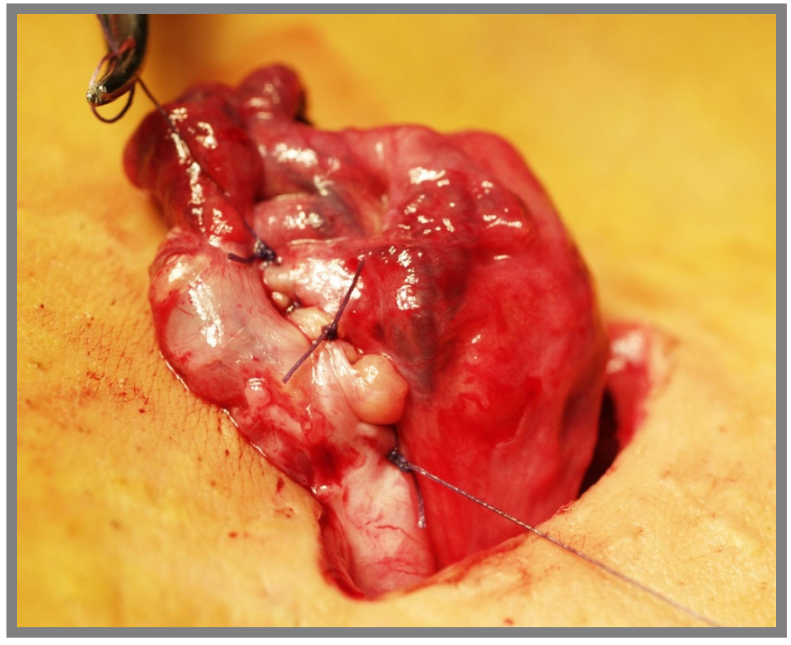
II
0,1 M sucrose
in PBS
10 min

III
PBS
10min



Orthotopic transplantation of ovarian tissue





Transplantation of frozen/thawed ovarian tissue: successful pregnancies

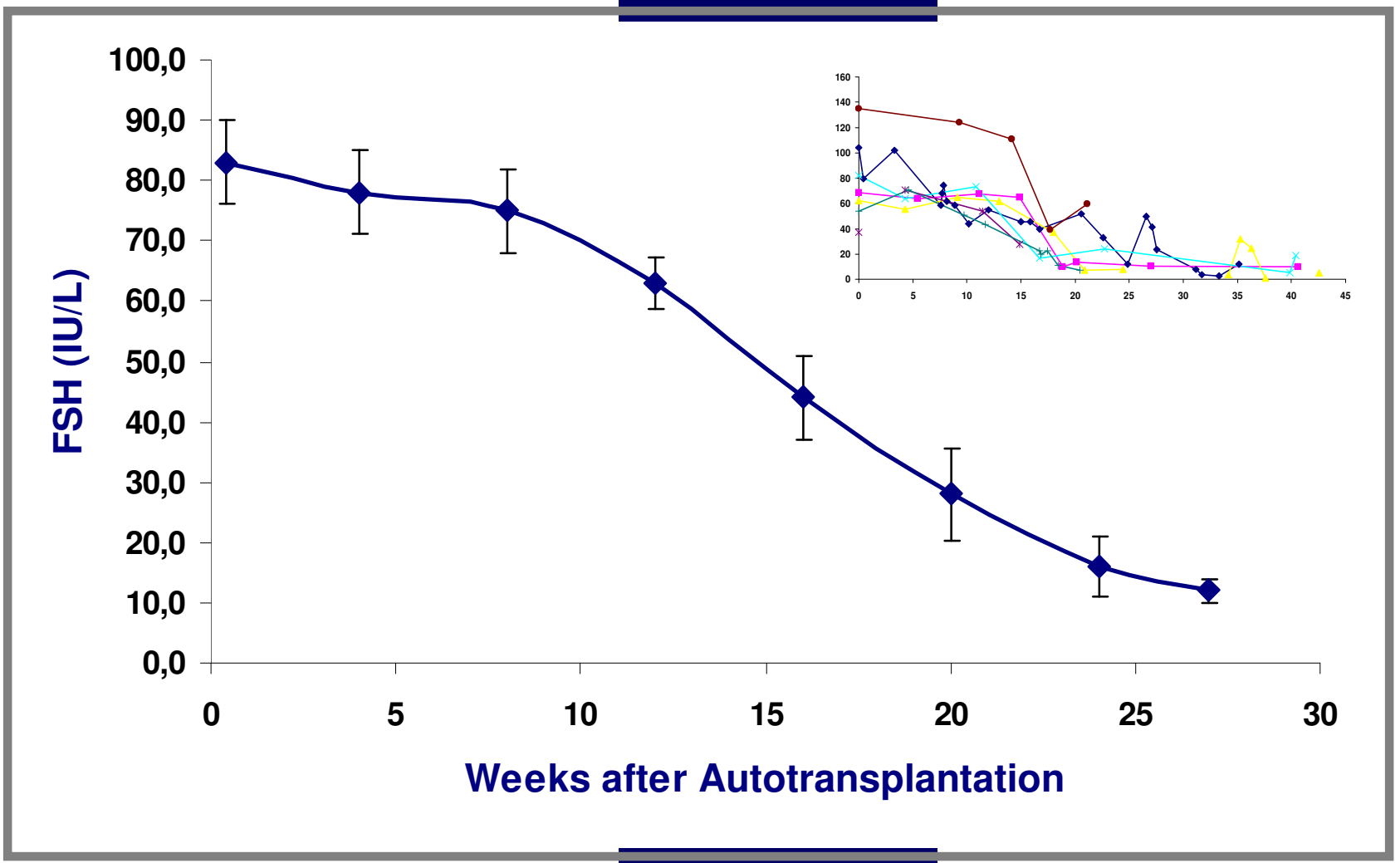
- Belgium 2004 – ♀
- Israel 2005 – ♀
- Denmark 2007 – ♀
- Belgium 2007 – ♀
- Denmark 2008 – ♂
- Denmark 2008 – ♀

All healthy babies

Transplantation of frozen/thawed ovarian cortex with cryopreservation immediately after recovery

Patient	Diagnosis	Age (years)	Proportion of tissue transplanted (%)	Lifespan (months)	No. remaining cryopreserved cortex
1	Non-Hodgkin's Lymphoma	32	A: 20 B: 35	A: 45 B: 21 →	15
2	Hodgkin's Lymphoma	28	A: 40 B: 30	A: 54 B: 6 →	7
3	Hodgkin's Lymphoma	25	A: 60 B: 40	A: 26 B: 37 →	0
4	Paroxymal Nocturnal haemogloburi	19	33	17 →	24
5	Aplastic anaemia	25	40	12 →	10

Mean and individual FSH concentrations following ovarian autotransplantation in 12 women



Overall results of assisted reproduction after transplantation of frozen/thawed ovarian tissue

(September 2009)

	Total
No. cycles	
Follicles asp.	
No. oocytes	
No. Fertilized	
No. transferred	
Pos. hCG	
Children born	

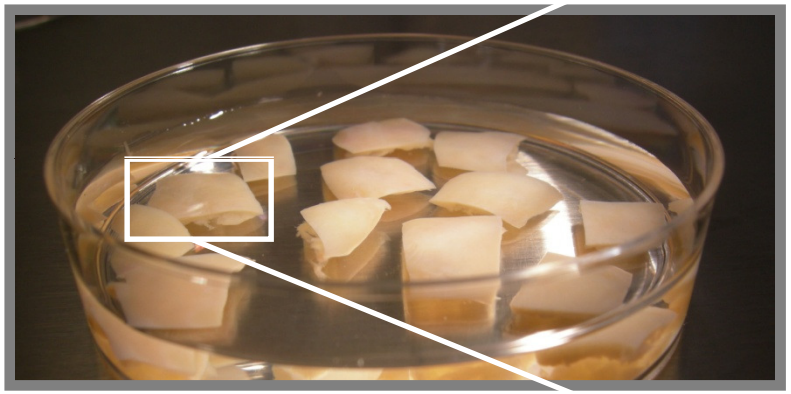
Vitrification of human ovarian cortical tissue

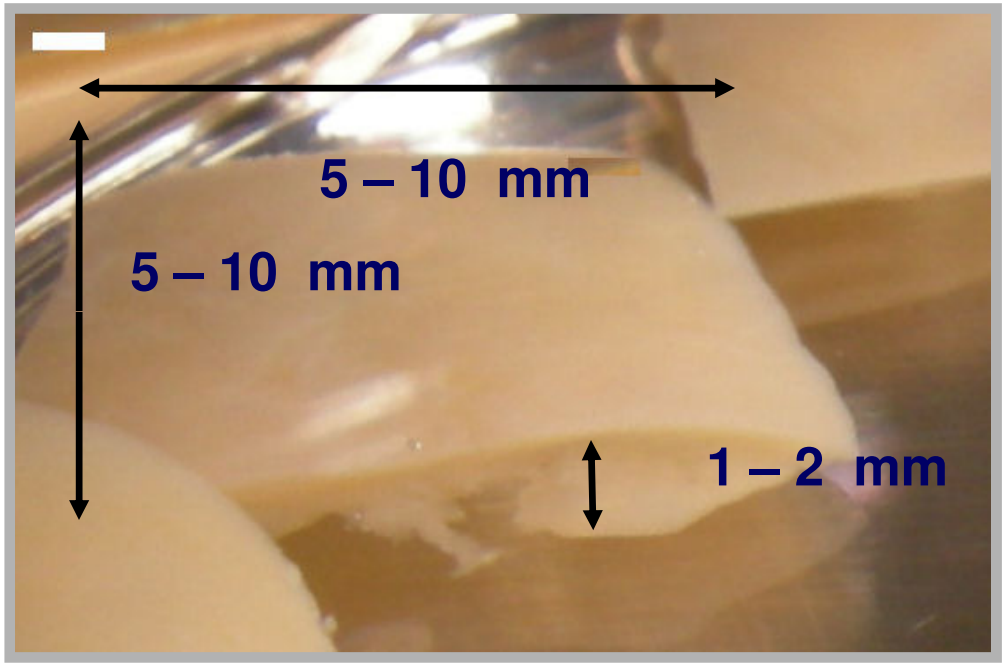
General considerations:

- The type and mixture of the cryoprotectants
- The size of pieces of tissue to be cryopreserved
- The speed of cooling (direct emission in liquid N_2)
- The need of clinical verification – time period

Diffusion distances of cryoprotectants in ovarian tissue

Very different from conditions in oocytes and embryos





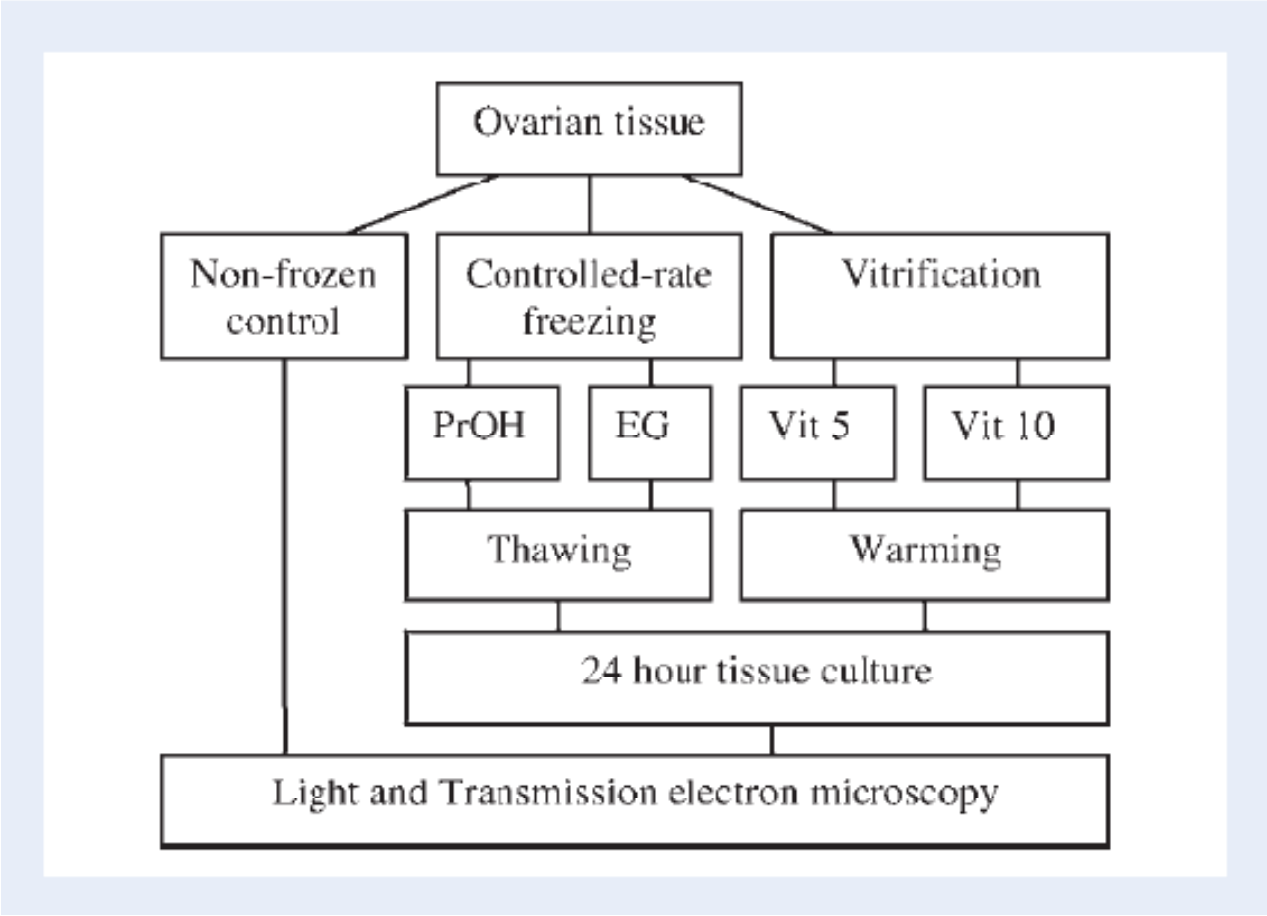
Slow freezing versus vitrification

Slow freezing and vitrification of human ovarian cortical tissue

General considerations:

- The texture of human cortical tissue is very different to most animal tissue
- which may affect the penetration rate of the cryoprotectants and optimal conditions
- and models developed by the use of animal tissue may not be used clinically in connection with human tissue and *vice versa*

Vitrification versus controlled-rate freezing in cryopreservation of human ovarian tissue
Experimental set-up

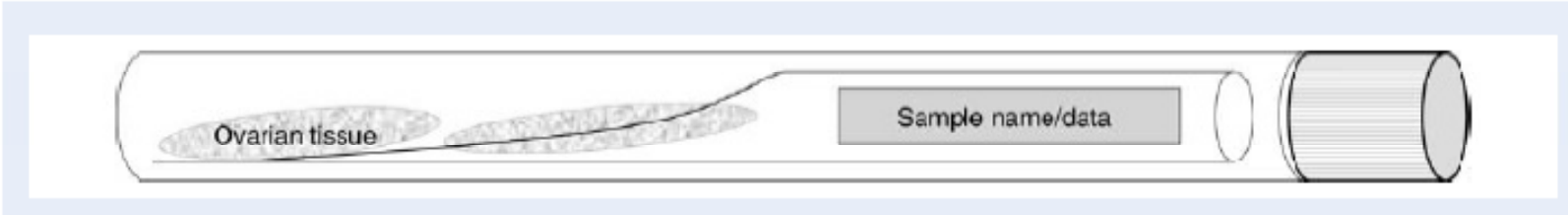


Keros V *et al.*, *Hum Reprod*, 2009;**24**:1670

Vitrification versus controlled-rate freezing in cryopreservation of human ovarian tissue

Step	DMSO (mol/l)	PrOH (mol/l)	Ethyleneglycol (mol/l)	PVP	Temp (^{0}C)
1	0.35	0.35	0.35	-	Room
2	0,7	0,75	0,75	-	Room
3	1,4	1,5	1,5	10 %	+ 4

Duration of each step was either 5 or 10 min



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

Controlled-rate freezing in cryopreservation of human ovarian tissue

- PrOH-protocol
  1,5 mol/l propanediol, 0,1 mol/l sucrose, 25 mg/ml HSA in PBS

  Equilibration at room temperature

- Ethyleneglycol-protocol
  1,5 mol/l ethyleneglycol, 0,1 mol/l sucrose, 10 mg/ml HSA in PBS

  Equilibration at +4 ^{0}C

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

Vitrification versus controlled-rate freezing in cryopreservation of human ovarian tissue

Results:

- Based on tissue from 20 women and using morphological characteristics evaluated by light and electron microscopy
- The study concluded that vitrification was comparable to slow freezing in terms of preserving follicles in human ovarian tissue
- However, 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 V *et al., Hum Reprod*, 2009;**24**:1670

Human ovarian tissue vitrification versus conventional freezing: morphological, endocrinological, and molecular biological evaluation

Step	DMSO (M)	PrOH (M)	Ethylene-glycol (M)	Acet-amide (M)	Temp (^{0}C)	Time (min.)
1	12,5 % of final concentration				Room	5
2	25 %				Room	5
3	50 %				+ 4	15
4	2,62	1,31	0,0075	2,60	+ 4	15

The cortical tissue is plunged directly into liquid nitrogen

Isachenko V *et al., Reproduction*, 2009;**138:**319

Controlled-rate freezing in cryopreservation of human ovarian tissue

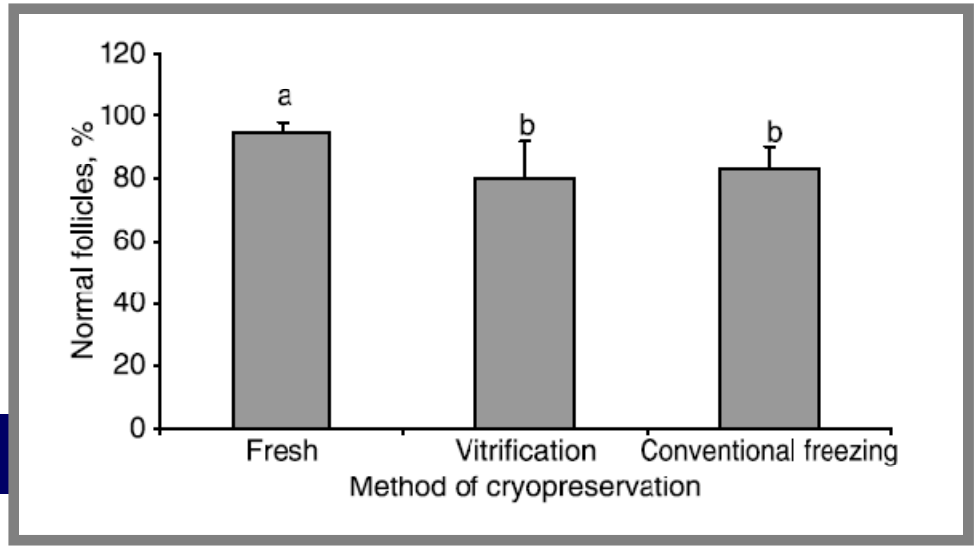
- DMSO-protocol
  1,5 mol/l, 0,1 mol/l sucrose, 10 % SSS in Leibovitz medium

  Equilibration ice cold

Isachenko V *et al., Reproduction*, 2009;**138:**319

Human ovarian tissue vitrification versus conventionalfreezing: morphological, endocrinological, and molecular biological evaluation

- During a 16 day long culture period there were no difference in oestradiol and progesterone secretion
- PCR detection of GAPDH mRNA showed a significant reduced expression in the vitrified cortical tissue
- Morphology:



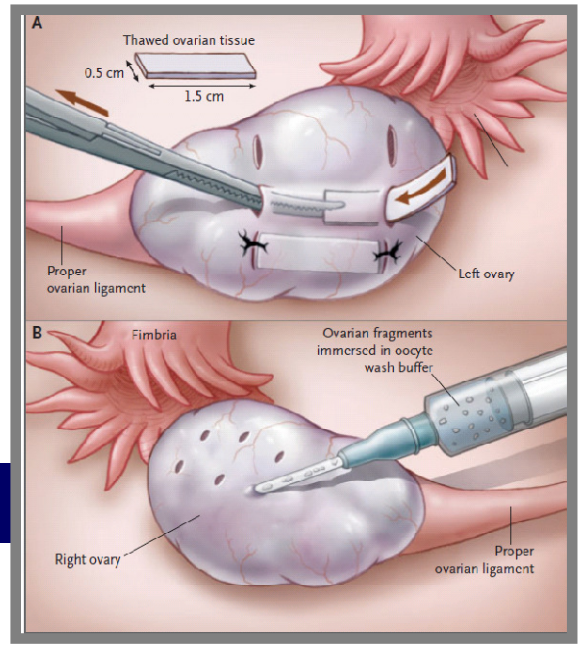
Isachenko V *et al., Reproduction*, 2009;**138:**319

A number of other studies also suggest that there is not too big a difference between vitrification and slow-freezing

- The primordial follicle is pretty resistant to freezing (as is the mature oocyte and testicular tissue)
- The oocyte and the granulosa cells are metabolically relative inactive at the early resting stage
- Easy penetrable cryoprotectants is required at conditions which minimize toxicity (low temperature)

The size of the frozen/thawed cortical pieces used for transplantation

- Most vitrification studies use pieces of cortical tissue considerably smaller than those employed clinically
- Keros V et al. 2009 used pieces of: 1 x 1-2 x 5-8 mm
- Isachenko V et al. 2009 used pieces of 0,3 – 1 x 1-1,5 x 0,7 – 1 mm
- Small pieces of cortical tissue facilitate quick penetration of cryoprotectant and build on experience from oocytes and embryos, but



Vitrification of ovarian tissue:

factors that needs clarification before implimentation in a clinical setting

- The importance of the size of the cortical fragments for subsequent functioning of the tissue
- How to obtain sufficient cooling rates in a clinical setting

Survival of primordial follicles after grafting fresh or frozen-thawed cortical tissue from sheep ovaries to SCID mice

Slow-freezing protocol: 1,5 M DMSO, 10% FSC, Leibowitz medium

Graft type (group no.)	Primordial follicles	
	No. ± SEM	% of control
1) Control	192 ± 47	
2) Fresh	68 ± 11	35
3) Frozen-thawed	54 ± 12	28

The vast majority of follicles are lost following transplantation

Baird D *et al*. *Endocrinology*, 1999;**140**:462

Conclusions

- Ovarian cryopreservation including transport of tissue prior to cryopreservation is now a clinical option
- In combination with ART results from Denmark suggests that ovarian cryopreservation do present a clinical relevant way of preserving fertility
- Efficacy of vitrification of ovarian tissue in a clinical setting still requires development
- Research to enhance transplantation efficiency is warranted

Collaborators

***University Hospital of Copenhagen*:**
Anders Nyboe Andersen
Anne Loft
The Fertility Clinic
Lene Lundwald
Christian Ottesen
Department of Gynaecology

***University Hospital of Aarhus*:**
Erik Ernst
Margit Dueholm
Ditte Trolle
The Fertility Clinic
Department of Gynaecology

***University Hospital of Odense*:**
Lars Westergaard †
Per Emil Rasmussen
The Fertility Clinic
Department of Gynaecology

Laboratory of Reproductive Biology:
Anne Grete Byskov
Mikkel Rosendahl
Kirsten Tryde Schmidt
Tiny Roed
Inga Husum
Marjo Westerdahl
Stine Gry Kristensen
Claus Yding Andersen

The Danish Medical Research Council

The Danish Cancer Foundation