Cryopreservation: Whole ovary and ovarian tissue
Options for fertility preservation

- **Ovarian tissue**
  - Fragments
  - Isolated follicles
  - Isolation
  - Avascular transplantation
    - 17 live births
    - Majority of studies
    - To avoid transmission of malignant cells
  - In vitro culture
  - Avascular transplantation

- **Entire ovary**
  - Vascular transplantation
    - To avoid follicular loss due to ischemia
    - To increase life-span of the graft
Ovarian tissue and whole ovary

**Ovarian tissue:** Slow-rate freezing or vitrification?

**Whole ovary:** Technological challenge and research limitations
Ovarian tissue: Slow freezing

Procedure:

Standard method: Slow-programmed freezing, using medium containing serum albumin, PROH, DMSO or EG combined or not with sucrose.
Not special technical skills: can be repeated in the same conditions and implemented in any lab.
Large strips (8-10x5x1mm) or small cubes (2x2x1mm): Both effective.
Orthotopic transplantation (peritoneal window and ovarian medulla) better than heterotopic position.
Risk of metastasis: screening methods

Results:

After thawing: Good survival and function, reviewed by Hovatta: RBM Online. 2005, 10
But the procedure does not appear in the guidelines of ASRM.
Ovarian tissue: Freezing or vitrification?

1. Better viability/functionality preservation?

2. All vitrification protocols vitrify? Feasible to standardize?

3. Optimal sample size: Storage space? Time-consuming?

Ovarian tissue is not the same as mature oocytes, so benefits obtained with oocyte vitrification cannot be directly transposed to ovarian tissue (and even less to whole ovaries)
Ovarian tissue - Vitrification

1. Viability/functionality preservation better than slow freezing?

YES: Keros et al HR 2009; 24 (better preservation of stroma)

Description of a Clinical grade closed system protocol: Sheikhi et al. HR 2011:26

- Fragment size: 1-1.5 mm³
- 3 steps: VS1 5 min RT, VS2 10 min RT, VS3 10 min 4°C
- One 1.5 mm³ fragment per cryovial
- Tissue never in contact with LN2. Compatible with European tissue directive.

NOT: Isahenko et al Reproduction 2009;138; Oktem et al FS 2011 inpress
2. All vitrification protocols vitrify?

**VITRIFICATION:** A solution/specimen solidifies to form a glass-like (or vitreous) state without any crystal formation during cooling and remains at this state throughout the warming step (Shaw and Jones, HRUpdate 2003; 9)

**How to achieve vitrification and to avoid devitrification?**

- **High viscosity/osmolality of the sample:** Depends on:
  - Concentrations of CPA.
  - Effective CPA penetration into the tissue (compromise with citotoxicity): Exposure steps with increasing CPA concentrations, incubation time, temperature

- **High cooling rate:** Depends on:
  - Small volume (Fragment size + surrounding medium)
  - Carrier material/thickness and conductivity: open carriers (OPS, droplet, solid-surface), close (straw, cryotube).
3. Optimal sample size: Storage space? Time-consuming?

Vitrification is less time-consuming and cheaper for oocytes, but is it for ovarian tissue?

Sample size: Ovarian tissue is not the same as oocytes:
- More volume per sample (1.5mm$^3$ or larger vs oocyte)
- More samples per patient (cortex of 1 ovary vs 10-15 oocytes)

Is it possible to vitrify fragments larger than 1.5mm$^3$?
Amorin et al. FS 2011; 95: Study of different vitrification protocols.
100 microL + 5 fragments 1mm$^3$ able to vitrify at cooling but devitrify at warming

Storage space and time-consuming:
- **Time** for slicing an ovary in 1mm$^3$ pc and placing individually in cryovials?
- If fragments **stored** individually in cryovials, **how many space per patient**?
Whole ovary: technical challenge and research limitations

Difficulties to improve protocols. Why so few studies?

1. Scarcity of whole premenopausal human ovaries for research.
   Sheep ovaries as animal model but:
   - Smaller organ volume (1/6)
   - Anatomical differences between donor and recipient vessels
   Human postmenopausal ovaries: similar vascular pedicle? No follicles

2. Microvascular skills: Ovariectomy, dissection, cannulation, perfusion, microvascular anastomosis. Difficult to implement in every hospital

3. Clinical trials in human patients: There is only one chance for every step to go optimally:
   1. Ovariectomy
   2. Cannulation/perfusion
   3. Cryopreservation/thawing
   4. Vascular transplantation
Lessons learned from whole human ovary studies

Ovariectomy
Pedicle dissection
Cannulation/perfusion
Ideal recipient vessels
Cryopreservation protocols
Vascular transplantation of fresh ovaries
Laparoscopic ovariectomy (Jadoul et al. FS 2007; 87)

Description of laparoscopic ovariectomy for whole human ovary.

1. **Ovarian pedicle long enough (5-6 cm)** If shorter: a narrow artery and a plexus of very thin veins. Uncomfortable/impossible in vitro dissection, cannulation and perfusion.

2. **Short ischemia interval** between application of the clip to the ovarian pedicle and perfusion with heparinized solution.

| A. Ureter. B. Ovarian pedicle. C. Fallopian tube | A. Psoas muscle. B. Clipped ovarian pedicle |

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[Image of anatomical structures labeled A, B, C]
Cannulation and perfusion
(Jadoul et al. FS 2007; 87)

3. Cannulation, perfusion (during cryopreservation and thawing):
A microsurgeon and a biologist.
Cold surface, microsurgical instruments and stereomicroscope.

Whole procedure demands higher technical skills compare to ovarian cortical tissue.
Ideal donor and recipient vessels
(Ploteau et al. FS 2011; 95)

Study of donor and recipient vessels in 10 postmenopausal female cadavers

1. Confirmation of optimal ovarian vascular pedicle length (5cm):

At 5 cm from the ovary, the numerous vessels converge into at least 1 large artery and 1 dominant vein with both an average diameter of 1 mm.
Ideal donor and recipient vessels
(Ploteau et al. FS 2011; 95)

2. Choice of anastomosis technique:
End-to-end anastomosis: Highest patency rate of vascularized free grafts and flaps. Risk of thrombosis is strongly reduced.

3. Choice of recipient pedicle: to fulfill
- Easy surgical accessibility
- Feasible orthotopic transplantation
- Easy puncture of the transplant for IVF
- Optimal size match between the recipient pedicle and the gonadic vessels. A sudden change of caliber may cause turbulence in the blow flow and predispose to arterial or vein thrombosis.
Ideal donor and recipient vessels
(Ploteau et al. FS 2011; 95)

Recipient vessels?

- Deep inferior epigastric (DIE) vessels
- Deep circumflex iliac (DCI) vessels
Ideal donor and recipient vessels
(Ploteau et al. FS 2011; 95)

- Deep circumflex iliac (DCI) pedicle shows the **best size match** for the ovarian vessels to perform an end-to-end microvascular anastomosis.

- DCI pedicle is **accessible** for surgery and further ultrasonic monitoring and oocyte retrieval.

- The grafted ovary in its new pedicle (5cm+5cm) can be transposed to restore a **functional infundibulo-ovarian unit**

*Menopausal status probably reduces the size of the vessels*
Ovarian in vitro perfusion system

In vitro perfusion of postmenopausal ovaries as a model to study functionality of cryopreserved whole ovaries.

Technical challenge: Cannulation of 2/10 ovaries could not be achieved (experimented microvascular surgical team)
Few studies on whole human ovary cryopreservation protocols

Martinez-Madrid et al. FS 2004; 82; FS 2007; 87
3 ovaries (29, 31, 36 y). CPA: 10%DMSO, 0.4%HSA in L15, perfusion at 4°C, for 5min (2.5mL/min) + equilibrium10min. **Passive cooling device**, from 4°C to -80°C (1°C/min). Thawing at 60°C until melted. Washing 3 steps 0.1, 0.05 and 0 M sucrose.
Analysis: After thawing: Histology, viability test, apoptosis, TEM

Milenkovic et al J Assist Reprod Genet 2011 Feb 25
3+4 postmenop. ovaries. CPA:1.5M DMSO, 2%HSA in L15, perfusion at 4°C, for 30 min (1-2mL/min). **Passive cooling device**, from 4°C to -80°C (1°C/min). Thawing at 37°C for 10 min. Washing 3 steps 0.1, 0.05 and 0M sucrose in L15.

Patrizio et al. FS 2007; 88 (suppl 1) Abstract ASRM meeting
4 ovaries (age?). CPA: 10%EG in UW, perfusion at 4°C for 3min. **Multigradient freezing device** from 4°C to -40°C (0.3°C/min). Thawing at 68°C for 20 sec and 37°C 2 min.
Analysis: After thawing: Histology.

Whole human ovary cryopreservation
(Martinez-Madrid et al. FS 2004; 82; FS 2007; 87)

Cryopreservation of a whole organ: Technical challenge:
- Heterogeneous cellular composition
- Large size/volume (6 x than sheep ovary)
- Compromise between penetration CPA/heat diffusion and toxicity
- Cryovial, cryochambers (not feasible in standard biofreezers with programmed cooling rate and seeding)
Whole human ovary cryopreservation
(Martinez-Madrid et al. FS 2004; 82; FS 2007; 87)

**Ovarian artery perfusion + bath:**
- Heparinized physiologic solution
- L15 10% DMSO (1.4M) + HSA
- 5 min at 4 °C, flow rate 2.5 mL/min

Cryovial in **preequilibration** bath: 10 min at 4 °C

**Passive cooling:** Nalgene 5100 Cryo 1 °C Freezing Co

- 4 °C
- -80 °C
- LN₂
- -1 °C/min
Whole human ovary cryopreservation
(Martinez-Madrid et al. FS 2004; 82; FS 2007; 87)

**Thawing:** Water bath at 60 °C until ice melts

Ovarian perfusion + bath in 3 steps:
L-15 + 0.25 M sucrose
L-15 + 0.10 M sucrose
L-15

10 min each, RT
Analysis post-thawing

- Fresh ovary
- Fresh ovary after DMSO perfusion
- Frozen-thawed ovary

Viability
Histology
Apoptosis:
- TUNEL
- Caspase 3
TEM
Outcomes: Viability

High survival rates after thawing:

- follicles (75%)
- vessels
- stroma

Ethidium homodimer 1
Calcein AM
Outcomes: Apoptosis TUNEL

- Antral follicle x200
- Vessels x100 x100
- Antral follicle x200
Outcomes: Apoptosis Caspase 3

A few stromal cells and endothelial cells (less than 1%) showed positive but weak staining

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<thead>
<tr>
<th></th>
<th>Fresh ovary</th>
<th>After DMSO</th>
<th>Thawed ovary</th>
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<td>121</td>
<td>182</td>
<td>244</td>
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Venule x200

Arteriole x200
TEM: healthy primordial and primary follicles

Oocyte

Interdigitations

Follicular cell
TEM: healthy vessels
Conclusions: Cryopreservation study

Description of an accessible cryopreservation protocol for intact human ovary with its vascular pedicle

- High survival rate
- Normal histological structure
- No induction of apoptosis
- Almost no ultrastructural alterations
Multigradient freezing device

Patrizio et al. FS 2007; 88(suppl 1)

Allows controlled cooling rate (0.3°C/min) of a big cryotube and unidirectional solidification:

Video
Vascular transplantation: Fresh human ovaries

Long-term patency (1-16 years).


Hilders et al. Cancer 2004; 101
29 years, cervical carcinoma. Autotransplantation, heterotopic (upper arm). Recipient: Brachial art. and basilic veins. End-to-side

17 years, Turner’s syndrome. Heterotransplantation (two sisters), orthotopic. Inferior epigastric art (end-to-side), Extern iliac vein (end –to-end).

One live birth

Whole-Ovary Transplantation between Monozygotic Twins.


Technical challenge: Vascular transplantation (compare to ovarian cortical grafting)

A. shows various structures in the ovarian vasculature that are key during transplantation of an ovary.

B. the donor’s ovarian artery (0.5 mm in diameter) is prepared for transplantation. T

C.D. The donor’s ovarian artery and ovarian vein are then anastomosed to the recipient’s ovarian artery and vein.
Lessons learned from animals: Cryopreservation and vascular transplantation

**Rat:** Yin et al. (Hum Reprod 2003), Wang et al (Nature 2002;415:385)
LIVE-BIRTH

**Rabbit:** Chen et al. (Human Reprod 05)
LIVE-BIRTH

**Sheep:**
Bedaiwy et al. (Fertil Steril 2003; 79)
Arav et al. (Hum Reprod 2005; 20; RBMonline 2010:20)
Imhof et al. (Fertil Steril 2006; 85) LIVE-BIRTH
Courbiere et al. (Fertil Steril 2009; )
Onion et al. (Hum Reprod 2009; 24)
Cryopreservation and vascular transplantation: Frozen sheep ovaries

**Bedaiwy et al.** (Fertil Steril 2003; 79) SLOW FREEZING

- 11 transplants (all immediate vascular patency)
- 8/11 lost due to thrombotic events (10 days)
- 3/11 restoration of ovarian function

**Arav et al.** Hum Reprod 2005; 20: RBM Online 2010:20 SLOW FREEZING

- 8 transplants (5/8 immediate vascular patency)
- 3 years after: 3/8 Progesterone activity and intact blood vessels (MRI)
- 6 oocytes, one parthenogenetic embryo (8-cells)
- 6 years after: superovulation treatment: CL in 2 ovaries, 1 atrophic ovary. 36 antral follicles, 4 GV oocytes, IMV to MII. 1/3 Progesterone activity, intact blood vessels and follicles at various stages.

**Imhof et al.** Fertil Steril 2006; 85 SLOW FREEZING

- 8 transplants, 6/8 vascular patency (19 months)
- major vessels free of thrombosis
- areas of histological damage (30-50%) due to local thrombosis and capillary fibrosis

LIFEBIRTH
Cryopreservation and vascular transplantation: Frozen sheep and vitrified ovaries

Onion et al. (Hum Reprod 2009; 24). SLOW FREEZING
To the neck (aorta). Immediate vascular patency, maintained in (7/7 cryo, 3/4 control). Functional CL (2/7 cryo, 1/4 control). Castrate gonadotrophin levels (5/7 cryo, 2/4 control). Primordial follicle density was reduced in both cryo and control.

The inability to restore full ovarian function was related to loss of primordial follicles rather than vascular patency in both frozen and fresh tissue, suggesting factors associated with cannulation and perfusion may contribute to depletion.

Fresh ovaries: 4/5 recovered endocrine ovarian function (2.5 months after graft). 2 livebirths (12 and 25 months after transplant). 6% follicle survival
Vitrified ovaries: 4/5 recovered endocrine ovarian function (6 months after graft). Total follicle lost
Summary

Cortical tissue:

Slow freezing:
Standar method, well established
17 live births

Vitrification:
Contradictory results
Not standard method: fragment size, Vitri medium and carrier.

Whole ovary:
Few studies, research limitations
Only one chance for every step to go optimally. Critical points:
  Cannulation (technical skills)
  Cryopreservation protocol,
  Vascular transplantation (technical skill, risk of thrombosis)