Epigenetic marks in offspring of cryopreserved immature ovary of mouse

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Cancer Incidence in 2008

Major advances in oncological treatments and diagnosis have resulted in a marked improvement in the survival of children and young adults with cancer over the last decade.

- 182,000 women with breast cancer in USA
- 16,000 women under the age of 45

Options to preserve fertility have been explored before therapy.
Incidence of Malignant Childhood Cancers

122 / million in USA or 1700 new cases / year in France (half of cases before 5)

- 7% sarcoma
- 29% leukaemia
- 5% bone tumor
- 4% germinal malignant tumor
- 4% retinoblastoma
- 2% liver
- 2% epithelial tumor
- 19% nervous central system tumors
- 10% lymphoma
- 9% NBL
- 8% Wilm’s tumor
- 1% other

80% of children and teenagers become long term survivors
-> 1/600 adult between 20 and 39
Infertility Risk after Chemotherapy

**Girl**

- **Alkylating agents**: chlorambucil, cyclophosphamide, ifosfamid, melphalan, busulphan and procarbazine

  → Direct ovarian lesions: follicular apoptosis both in growing follicles and in the dormant primordial follicle population

- **Myeloablative chemotherapy used as preparation for stem cell transplantation:**
  - busulphan-melphalan
  - cyclophosphamide + busulphan

**Risks**: - no spontaneous puberty
- sterility
- acute ovarian failure increasing with age of patient
Infertility Risk after Radiation Therapy

**Girl**

- **Ovary:**
  - Doses (10-20 Gy) act on dividing and non-dividing cells, block cellular division associated with permanent ovarian failure
  - Total body irradiation 14.4 Gy: 90% infertility

- **Uterus:**
  - Risk on foetal development: premature birth and hypotrophy
  - Miscarriage

The chance of spontaneous pregnancy in women treated after 25 years of age has been estimated to be only 5%

*Lobo RA, NEJM 2005*
Embryo freezing or oocyte freezing: impossible before puberty

Ovary cryopreservation is a promising experimental technology:
- Whole ovary in child
- During abdominal tumor surgery, can be rapidly performed
- Small fragments of ovarian cortex (histology)
- Slow freezing protocol (DMSO)
Ovarian Cryopreservation in France 1995-2007

n=510

Poirot C, GRECOT 2008
Ovarian Cryopreservation in France (GRECOT)

Mean age: 17.8 year old

Solid tumors and hematologic malignancies represent the most frequent part in pediatric surgery

Poirot C, GRECOT 2008
Cryopreservation before Puberty

- Survival improvement after cancer treatment during childhood
- Ovarian function impairment: fertility / puberty

Ovarian cryopreservation as only therapeutic option

In France: 100 children under 12 years, since 1998

⇒ Fertility ?
⇒ Puberty induction ?
Graft of Ovarian Cortex

**Etiologies:**
- POF after chemotherapy (11)
- Homozygous twins, POF unknown etiology (7)
- Bilateral ovariectomy (5)

**Graft:**
- fresh (10) / frozen (13)

**Site:**
- heterotopic (7) / orthotopic (12) / combined (4)

Bedaiwy MA, Human Reprod 2008
Results of Adult Ovarian Cryopreserved Grafting

To date, 43 women who underwent cryopreserved or fresh ovarian transplantation have been reported, leading to the restoration of spontaneous cycles for several months

Bedaiwy MA et al, Hum Reprod 2008
Results of Adult Ovarian Cryopreserved Grafting

- Heterotopic reimplantation : 0
- Orthotopic reimplantation : 5 births

Pregnancies after Heterologous Cortex Ovarian Graft

• Fresh cortex and one from frozen tissue: births
  
  *Silber SJ, NEJM 2005 and 2007*
  
  *Silber SJ, Hum Reprod 2008*

Concept of ovarian cortical grafting is based on the fact that all follicles containing eggs are located in the outer 1 mm of the ovary which can be sutured to the recipient’s medulla

Limitation: loss of two thirds of follicles due to ischemia during revascularization

Data from studies in animal suggest that microvascular whole-ovary transplantation could avoid this problem!
Pregnancy after Microsurgical Transplantation of an Intact Ovary

Pair of 38-year-old *monozygotic twins*

Donor: 2 children, normal ovarian function
Recipient: POF at 15 years (FSH 81 mIU/mL)

Donor’s ovarian veins were anastomosed to the recipient’s ovarian arteries

Immediate normal blood flow after an ischemic period of 100 min…. First cycle day 101 after transplantation, 11 regular menstrual cycles Then, pregnancy!

*Silber SJ et al. NEJM 2008*
No report using pre pubertal ovaries!

Jeruss JS, NEJM 2009
Ovarian Cryopreservation in Adult Mice

- Use of similar protocol of cryopreservation as in humans
- Spontaneous gestation
- Follicle loss > 50%
- No study with pre-pubertal grafting

We performed orthotopic transplantation using fresh and/or cryopreserved whole ovaries to restore both endocrine function and fertility
Animal Models

N = non pubescent (Day 18)

A = Adult

NNF or NNC
n = 27  n = 11

NAF or NAC
n = 16  n = 9

AAF or AAC
n = 15  n = 8

Total of 86 females
Cyclic Hormonal Activity

Spontaneous puberty 15 days after grafting

Cyclic activity (cycle: 5 days)
Ovaries after one Month post Grafting

Neovascularization
Ovarian Graft after one Month

Proliferation study: antibody anti-PCNA
**Follicular Density**

Follicle loss about 50%

No influence of cryopreservation
Fertility

Implantation site (IS)  Corpus Luteum (CL)

Anatomic problem Implantation defect
**Litter Size**

![Bar chart showing litter size comparison across different groups.](image)

- **AAC**
- **AAF**
- **NAC**
- **NAF**
- **NNC**
- **NNF**
- **Ovx**
- **Control**

### Number of pups/litter

- **AAC**
- **AAF**
- **NAC**
- **NAF**
- **NNC**
- **NNF**
- **Ovx**
- **Control**

**p<0.001**

**Normal ovulation rate**

**Anatomical mislocation**

**Implantation failure**

**Embryonic loss**

**Unilateral grafting**
Depletion of Functional Follicle

NNF

NCC

NAF

NAC

3 months after the ultimate gestation
Conclusion (1)

- Possibility of spontaneous puberty
- Follicle loss unrelated to cryopreservation process
- Gestation in all groups
- Decreased litter size
- Embryonic loss
- Premature ovarian failure
Is ovarian grafting safe for progeny?

Is genomic imprinting correctly set?
Both maternal and paternal genomes required in mammals

Imprinting is due to epigenetic marks and leads to monoallelic expression according to the parental allele

Characteristics:
- during gametogenesis
- clusters
- DNA methylation

Control of embryonic growth
Epigenetic Modifications

- DNA methylation (CG rich regions)

- Acetylation/methylation of histones associated to DNA

\[ \text{Ac} \quad \text{Me} \quad \text{Ub} \quad \text{Su} \quad \text{P} \]

- acetylation
- methylation
- ubiquination
- sumoylation
- phosphorylation
Characteristics of Imprinted Genes

Organization in domains

CG rich regions (DMR) and repeated sequences

Existence of Imprinting Center

(ICR, acquisition methylation on specific allele during gametogenesis)

Antisense RNA not translated

Asynchronous replication
Life Cycle of Methylation Imprints

Zygote

Blastocyst

Embryo

Maintenance

Somatic development

Mature gametes

Imprinting control elements (IC1)

Establishment

Erasure

Primordial germ cells

From Reik et al. Nat Rev Genet. 2001
Methylation Reprogramming in the Germ Line and Embryo

From Reik et al. Nat Rev Genet. 2001
Genomic Imprinting

~ 100 identified genes, cluster
Genomic Imprinting

- Neonatal Diabetes Mellitus
- Silver-Russell
- Silver-Russell Wiedemann-Beckwith
- Uniparental disomy 14 (UPD14)
- Angelman Willi-Prader
- PHPA, B
Assisted Reproductive Technologies (ART) and Diseases Related to Imprinting

Sperm cells: fresh or frozen

ICSI vs IVF

Frozen embryo

Ovarian stimulation

blastocyst transfert/ culture medium

No identification of specific procedure

Gicquel et al Am J Hum Genet 2003
Chang et al Fertil Steril 2005
Ludwig et al J Med Genet 2005
Assisted Reproduction Technologies (ART) and Genomic Imprinting Disorders

**Beckwith-Wiedemann syndrome: 4 series**

*Loss of methylation at maternal locus ICR2/KCNQ1OT1*

**Angelman syndrome: 5 cases (4 ICSI)**

*Loss of methylation at maternal locus SNRPN*

**Large Offspring Syndrome (Sheep)**
- Young et al. Nat Genet 2001

*Loss of methylation at maternal locus DMR2 of IGF2R gene*

**Russell Silver Syndrome (RSS)**
- Prevalence (6%) of ART in a series of patients RSS (Trousseau)

*Loss of methylation at paternal locus ICR1*
Does ART interfere with establishment or maintenance of methylation marks in the imprinted regions?
Genomic Imprinting

Both maternal and paternal genomes required in mammals

Differentially methylated region (DMR)

Methylation of DMR

BWS: genomic region of interest is 11p15.5 which contains two DMRs:

- H19 and IGF2 genes (H19 ICR)
- CDKN1C gene (KvDMR1)
Animal Models

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AAF or AAC
n=15   n=8

Total of 86 females

N=non pubere (Day 18)

A=Adult
Methylation Status of H19 ICR and Lit1 KvDMR1 in Pups from Grafted Mice

Aberrant genomic imprinting induces numerous genetic disorders (BWS) linked to ch 11p15.5

Maher E.R. Hum Mol Genet 2005
Methylation of \textit{H19-Igf2} DMR

\begin{itemize}
\item non methylated maternal allele 0.4kb
\item methylated paternal allele 4kb
\end{itemize}

\textit{stœchiometric epigenotype}

\begin{itemize}
\item 4 kb \textit{pat}
\item 0.4kb \textit{mat}
\end{itemize}

\textit{methylation defect on paternal allele}

\begin{itemize}
\item 4 kb \textit{pat}
\item 0.4kb \textit{mat}
\end{itemize}

\textbf{Methylation index} = 4 kb intensity / (4+0.4kb intensity)
**H19-Igf2 DMR Methylation Status**

**Manipulated versus Controls**

**Fresh versus Cryopreserved**

**No difference**
No difference between groups

Sauvat F et al PLoS ONE 2008
Conclusion (2)

- No significant imprinting alterations of pups from grafted mice as compared to control

- No effect of cryopreservation on imprinting mechanisms

- **BUT** reduced litter size maybe due to spontaneous abortion linked to malformations: Imprinting?
Alternatives to cryoconservation

Transplantation of a whole cryopreserved ovary cannot be proposed for all patients, due to the risk of tumour cell transmission during the procedure.

Research programme is needed to develop alternatives such as:

- Isolate follicles from frozen ovarian cortex
- *in vitro* follicular culture to generate oocytes

*Yet to be tested*
Conclusions

• Because all data indicate that the reproductive lifespan of grafted ovarian tissue is limited, the main target of ovarian grafting is restoration of fertility

• This model supports the legitimacy to propose cryopreservation in young girls before gonadotoxic treatment as a tool to restore fertility, as has been done in adult women

• Regarding our current knowledge concerning this procedure, one should remain cautious when delivering information to patients and their family at the time of cryopreservation, in terms of puberty induction and potential risks for children
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