Fertility restoration in prepubertal boys: perspectives with testicular tissue

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Disclosure information: Nothing to declare related to the topic
Introduction
Fertility preservation for prepubertal boys
Preserving the spermatogonial stem cell

Cryopreservation of the SSC
Thawing
In vitro maturation
Transplantation

Sperm Production

Introduction
Fertility preservation for prepubertal boys
Who is candidate?

<table>
<thead>
<tr>
<th>Oncological</th>
<th>Non-oncological</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before gonatotoxic therapies</td>
<td>Debatable</td>
</tr>
<tr>
<td>• Low risk of infertility</td>
<td>• Risk of testicular degeneration:</td>
</tr>
<tr>
<td></td>
<td>— Cryptorchidy</td>
</tr>
<tr>
<td></td>
<td>— Klinefelter</td>
</tr>
<tr>
<td></td>
<td>— Y-microdeletions</td>
</tr>
</tbody>
</table>

BUT ... No complications following testicular biopsy

Wyns et al., 2010, 2011; Picton et al., 2015; Uijldert et al., 2017
Introduction

Fertility preservation for prepubertal boys

Cryopreserving the SSC in its niche

• Maintenance of somatic cells needed for subsequent SSC maturation
• Modification of epigenetic patterns of germ cells in case of SSC niche disruption (Goosens et al., 2011)
• Possibility of later cell isolation

The fertility restoration approach depends on the disease:

• Risk of testicular tissue contamination with cancer cells
• Competent niche
• Competent SSCs
Fertility restoration options with prepubertal TT (animals)
Where are we now?

Autografting of
- Tissue pieces
- Cell aggregates

Transplantation of cell suspensions

Preimplantation embryo development
NH primates (Hermann et al., 2012)

Offspring
- Mouse
- Rat
- Goat
- Chicken
- Lamb
- Zebrafish

In vitro maturation of germ cells

Offspring
- Mouse (organ culture method)
  Sato et al., 2011
- Monkey (Liu et al., 2016)

Autografting of
- Mouse (tissue and cell aggregates)
- Rabbit (tissue)
- Pig (tissue xenografts)
- Quail

Monaramooz et al., 2002

Fertility restoration with prepubertal tissue (humans)
Where are we now?

SSC Niche

D I S S O C I A T E D

- SD culture
  - Embryo culture
  - Tissue transplantation
  - ND

P R E S E R V E D

- Organotypic culture
  - Tissue transplantation
  - ND

- Tissue xenotransplantation

R E C O N S T I T U T E D

- Organs
  - ND

De Michele, Vermeulen & Wyns, 2017
### Tissue transplantation

**XenoTransplants of fresh/slow-frozen /vitrified human ITT**

**Spermatogonia: MAGE-A4**

<table>
<thead>
<tr>
<th>Control non-grafted</th>
<th>Fresh grafted</th>
<th>Slow-frozen grafted</th>
<th>Vitrified grafted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-grafted tissue</td>
<td>Fresh tissue</td>
<td>Slow-frozen tissue</td>
<td>Vitrified tissue</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mage A4 positive cells/ST</th>
<th>Control</th>
<th>Fresh</th>
<th>Slow-Frozen</th>
<th>Vitrified</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6.71 ± 7.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.23 ± 0.27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.28 ± 0.52&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.49 ± 1.14&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>% recovery</td>
<td>100%</td>
<td>3.4%</td>
<td>4.1%</td>
<td>7.3%</td>
</tr>
</tbody>
</table>

- Recovery of spermatogonia
- No difference for fresh or cryopreserved grafts

<sup>a</sup> and <sup>b</sup> differ significantly (p < 0.001)

Poels et al., 2013

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**Spermatogonial differentiation to pachytene stage**

Poels et al., 2013
Transplantation of cryopreserved ITT: challenges ...

Spermatogonial recovery rates after xenografting of human ITT

- **5 days**: 53% to 67%
- **3 weeks**: 14.5%
- **6 months**: 4.1%

> Most of spermatogonial loss occurs before 3 weeks

Intact seminiferous tubules after xenografting of human ITT

- **5 days**: 18.6% to 21%
- **3 weeks**: 82.2±16.5%
- **6 months**: 89.7±17.9%

> ST integrity did not worsen over time concomitantly with SG loss
> ST partially recover from initial insult → only empty SSCs niches remain

Tissue transplantation

Why spermatogonial loss?

- Spermatogonial proliferation capacity maintained

% of MAGE-A4 (spermatagonia) and Ki67 (proliferation) positive cells

> Proliferative activity of spermatogonia is higher after grafting
> No difference between F, SF and V grafts

Poels et al., 2013
**Tissue transplantation**

**Why spermatogonial loss?**

- Inadequate recipient environment or ischemic stress before revascularization responsible for increased apoptosis/necrosis?

3 days

3 weeks

<table>
<thead>
<tr>
<th></th>
<th>Fresh n=3</th>
<th>Grafts (3 days) n=3; G=5</th>
<th>Fresh n=9</th>
<th>Grafts (3 weeks) n=9; G=9</th>
</tr>
</thead>
<tbody>
<tr>
<td>CASPASE-3</td>
<td>0.1 ± 0.163</td>
<td>0.063 ± 0.096</td>
<td>0.096 ± 0.135</td>
<td>0.014 ± 0.022</td>
</tr>
<tr>
<td>TUNEL</td>
<td>0.004 ± 0.007</td>
<td>+++</td>
<td>0.001* ± 0.003</td>
<td>0.032 * ± 0.040</td>
</tr>
</tbody>
</table>

*p=0.044

Wyns (personal data)

**Tissue transplantation**

**Why spermatogonial loss?**

- Negative impact of the grafting procedure
  - Early ischemia due to avascular grafting?

Tissue culture with VEGF: increased number of tubules with elongating spermatids in bovine grafts (Schmidt et al, 2006)

Need for improvement in early graft revascularization
Perspectives with transplantation of cryopreserved ITT

- Safety issue

  • As few as 20 leukemic cells injected into a testis can induce disease relapse (Jahnukainen et al., 2001)
  
  • Leukemic cells can survive cryopreservation/xenotransplantation and increase generalized leukemia in the nude mouse host (Hou et al., 2007)

⇒ Procedure not applicable if risk of cancer cells’ contamination

Transplantation of tissue pieces: the cryopreservation procedure is only partially responsible for spermatogonial stem cell loss and/or impairment

Cryopreservation procedure

- Cooling rate CPAC
- Thawing protocol

Grafting procedure

- Revascularisation
- Recipient environment

⇒ Need for studies on graft revascularisation
⇒ Need for a preclinical model
**Perspectives with transplantation of cyropreserved ITT**

- Reducing tissue damage due to ischemic stress before revascularization

![Diagram of N-Acetyl Cysteine and Testosterone]

- Protective effect in germ cell cultures by apoptosis inhibition (Erkkila et al., 1998)
- Protective effect on tissues in case of testicular torsion/distortion (Turkm en et al., 2012)

![Diagram of spermatogonia cells]

- Supression of apoptosis in human GC and crucial role in GC survival (Erkkila et al., 1997)
- Testo withdrawal → progressive ↑ apoptosis and DNA fragmentation (Tawar k et al., 2002)

**Spermatogonia: MAGE A4**

<table>
<thead>
<tr>
<th></th>
<th>Control non-grafted</th>
<th>Thawed</th>
<th>Thawed+NAC</th>
<th>Thawed+Testo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-grafted</td>
<td>Mage A4 positive cells/ST</td>
<td>3.02 ± 2.09</td>
<td>2.22 ± 1.67</td>
<td>1.91 ± 1.47</td>
</tr>
<tr>
<td>% Recovery</td>
<td>100%</td>
<td>67%</td>
<td>63%</td>
<td>53%</td>
</tr>
</tbody>
</table>

- Presence of spermatogonial cells in all grafting groups
- Number of SG/ST not statistically different between groups (Poels et al., 2014)
Perspectives with transplantation of cryopreserved ITT

- Use of VEGF nanoparticles to promote early graft revascularisation

Drug attached to nanoparticles in hydrogels

Constant and controlled concentration, reduced dispersion

Perspectives with transplantation of cryopreserved ITT

- Development of appropriate embedding matrices for tissue engraftment

Compromise between solidity and tissue integration into the host
Perspectives with transplantation of cryopreserved ITT

- VEGF nanoparticles loaded in two different matrices to optimize graft outcome

**Day 5**

![Graph showing vascular density and VEGF expression](image)

Poels et al., 2016

Perspectives with transplantation of cryopreserved ITT

- VEGF nanoparticles loaded in two different matrices to optimize graft outcome

**Day 21**

Higher preservation of undifferentiated spermatogonia (x 2)

![Images of tissue samples and bar graph](image)

Poels et al., 2016
Perspectives with cryopreserved ITT

- VEGF nanoparticles loaded in two different matrices to optimize graft outcome

* p ≤ 0.05 compared to non-grafted control

- Insufficient stabilization of the vasculature by day 21?
  (new blood vessels could be leaky and rupture easily)

- Suboptimal exposure to VEGF?

Transplantation of isolated SSCs: challenges ...

- Safety issue: cancer cell decontamination with cell sorting

<table>
<thead>
<tr>
<th>References</th>
<th>Species</th>
<th>Cell-sorting technique</th>
<th>Markers</th>
<th>Evaluation after cell sorting</th>
<th>Outcome (% of residual contamination)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fujita et al. [43]</td>
<td>Mouse</td>
<td>FACS</td>
<td>H2B/CD45- (MHC d)</td>
<td>Cell transplantation histology: testis, bone marrow, perfused vessels of recipient site</td>
<td>No tumor</td>
</tr>
<tr>
<td>Fujita et al. [49]</td>
<td>Human</td>
<td>FACS</td>
<td>MHC d (CD45-), CD11b</td>
<td>RT-PCR for germ cell markers (DAZ, HIF, VASA, NANOG, STELLAR, OCTA)</td>
<td>1.45% K562 cells (CD45), 0% K562 cells (DAZ) (for induction of MHC d)</td>
</tr>
<tr>
<td>Devery et al. [50]</td>
<td>Mouse</td>
<td>MACS + FACS</td>
<td>H2B/CD45- (MHC d)</td>
<td>FACs, orthotopic cell transplantation</td>
<td>0.93% HNK1+ cells, 5.1% (1/52) contaminating/orphan</td>
</tr>
<tr>
<td>Human</td>
<td>FACS</td>
<td>H2B/CD45- (MHC d)</td>
<td>FACs, intravital microscopy, PCR for B cell receptor</td>
<td>0.58% 58+ cells/1/11 contaminating/orphan</td>
<td></td>
</tr>
<tr>
<td>Horns et al. [51]</td>
<td>Norbmus primates</td>
<td>FACS</td>
<td>CD19-CD45</td>
<td>Xenograft in vivo + allogeneic microscopy</td>
<td>No tumor</td>
</tr>
<tr>
<td>Cormier et al. [52]</td>
<td>Mouse</td>
<td>BSYCAM-CD45-FLICA</td>
<td>Xenograft in vivo</td>
<td>0% contamination, (≤ 0.05% for cancer cell detection)</td>
<td>0.0–0.8%</td>
</tr>
</tbody>
</table>

(CDA, colony-forming unit of adherent cells; CD45, cell integrin marker of hematopoietic cells; CD19, B-cell receptor marker; CD34, colony-forming unit of megakaryocytes/erythrocyte progenitors; DAZ, deleted in azoospermia-like gene; HIF, hypoxia-inducible factor; H2B, histone H2B; HNk1, high-mannose antigen; K562, malignant K562 cells; mice, Balb/c mice; MM, major histocompatibility complex; SSCs, side-population cells; VASA, variably amplified sequence; VASA, vasculature-associated gene; Wt, wild type; Xeno, xenograft transplantation)
Transplantation of isolated SSCs: challenges...

- Low number of SSCs contained in a small testicular biopsy

**Propagation of Human Spermatogonial Stem Cells In Vitro**

*Conclusion* Long-term culture and propagation of human spermatogonial stem cells in vitro is achievable.

- Technique further applied to propagate prepubertal SSCs (Sadri-Ardekani et al., 2011)
- Technique useful to eliminate cancer cells? (Sadri-Ardekani et al., 2011, 2014)
- Damage to the SSC niche due to chemotherapeutic and/or radiotherapy (Bar-Shira Maymon et al., 2004)
- No valid study model for human SSC transplantation

Perspectives using reconstituted SSC niches

- Development of organoids

After cancer treatment

**In Vivo** Transplantation and spermatogenesis restoration

**Cells' self-organization**
Decellularized tissue scaffold
Manufactured matrices

**ICSI**

**In Vivo** spermatogenesis

Testicular biopsy before cancer treatment

Cryopreservation
Thawing/warming

Elimination of neoplastic contamination

Culture

Cell sorting (FACSMACS)

Decontaminated testicular cells
Perspectives using reconstituted SSC niches
Organoids using decellularized prepuberal testicular tissue

- Importance of the ECM

- In search of an optimal and accessible scaffold

- Best compromise between DNA elimination and ECM preservation
Perspectives using reconstituted SSC niches
Organoids using decellularized prepuberal testicular tissue

Perspectives using intact SSC niches: in vitro maturation of ITT
In vitro maturation in humans: state of the art
In vitro maturation of prepubertal testicular tissue: challenges ...
The SSC niche maturation process

- Sertoli cells:
  - Leydig cells:
  - Testosterone production

The SSC niche maturation process

Caires et al., 2010; Sharpe et al., 2003

In vitro maturation of human prepubertal testicular tissue

- Long term organotypic culture
  - Tubules’ integrity

Score 0 (bad) to 4 (very good)
- absence of cell-cell detachment
- absence of cell detachment from the BM
- no more than 5% of nuclear pyknosis
- identification of Sertoli and germ cells.

→ STs are well preserved (score 3-4) during the culture without significant difference
→ No difference between the two culture media

De Michele et al., 2017
In vitro maturation of human prepubertal testicular tissue

- Long term organotypic culture

**Spermatogonial cells survival and proliferation capacity**

![Graph showing Spermatogonial cells survival and proliferation capacity with bars for Testosterone and hCG over days.]

De Michele et al., 2017

Cliniques universitaires Saint-Luc–WVS Christine

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In vitro maturation of human prepubertal testicular tissue

- Long term organotypic culture

**Sertoli cell maturation: Sertoli cell proliferation rate**

![Graph showing Sertoli cell maturation with bars for Testosterone and hCG over days.]

De Michele et al., 2017

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In vitro maturation of human prepubertal testicular tissue

- Long term organotypic culture

  Sertoli cell maturation: evolution of AMH secretion

  

  Statistically significant decrease in AMH secretion

De Michele et al., 2017

In vitro maturation of human prepubertal testicular tissue

- Long term organotypic culture

  Androgen receptor expression
  2 year-old boy

De Michele et al., 2017
In vitro maturation of human prepubertal testicular tissue

- Long term organotypic culture

Leydig cells survival and functionality

Conclusions

- **Animals**: encouraging results with offspring in non-human primates (tissue transplantation)

- **Humans**:
  - Transplantation of ITT only for patients with benign diseases
    - further improvement of avascular grafting technique
  - Transplantation of SSCs: need for preclinical models to evaluate the SSCs’ differentiation potential
    - perspectives with organoid development
  - IVM of ITT: research limited due to the scarcity of tissue

BUT achievement of the pubertal transition of the maturation phase of the SSC niche
THANK YOU FOR YOUR ATTENTION

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