Testicular tissue: When and how should it be cryopreserved?

Greta Verheyen
Centre for Reproductive Medicine
UZ Brussels, Belgium
ESHRE Campus Granada, 25-26 March 2010

Outline
- History of sperm freezing
- Indications for testicular sperm freezing
- When to cryopreserve?
  - Two approaches: Pros and cons
    - OA and NOA
  - Retrieval methods
- Which quality to cryopreserve?
- Cryodamage to testicular sperm
- How to cryopreserve?
- ICSI with fresh/frozen testicular sperm
- Freezing for prepubertal boys
- Conclusions

History of sperm freezing
- 1776 Spallanzani - low t° effects on human spermatozoa
- 1866 Montegazza suggested cryobanks for animal semen
- 1949 Polge used glycerol as cryoprotectant for mammalian spermatozoa
- 1950 successful use of extenders
- 1953 Sherman observed fertilization and embryo development with human sperm frozen on dry ice
- 1964 First birth after glycerol freezing of human sperm in liquid nitrogen
- 1973 First association of sperm banks (CECOS - France)
- 1976 American Society of Tissue Banks
- 1996 First birth after using frozen testicular sperm for ICSI
Indications for testicular sperm freezing

- Fertility treatment
  - Azoospermia
  - Preserve sperm for later fertility treatment (ICSI)
  - Avoid repeated testicular surgery
  - Avoid problems in coordinating OPU and testicular surgery
  - Ensure presence of sperm before ovarian stimulation
  - Select patients for fertility treatment allocation

- Fertility preservation
  - for boys and adults before starting gonadotoxic treatment

Testicular sperm freezing is dependent on

- Etiology of azoospermia
  - Excretory (OA)
  - Secretory (NOA)

- Method of testicular sperm retrieval
  - Open biopsy
  - Aspirations

- Allocation criteria for NOA patients

- Occasion of testicular sperm retrieval
  - Diagnostic
  - Therapeutic

Etiology of azoospermia

**Obstructive azoospermia (OA)**

- Mechanical cause
- Normal spermatogenesis
- High sperm numbers
- Epididymal or testicular sperm
- 100% recovery rate
- Causes
  - Vasectomy
  - Congenital bilateral absence of the vas deferens (CBVAD)
  - Post infectious epididymitis
  - Testicular trauma
  - Young’s syndrome
  - Retrograde ejaculation

**Non-obstructive azoospermia (NOA)**

- Biological cause
- Severely impaired spermatogenesis
- Low to absent sperm numbers
- Testicular sperm
- 50-60% recovery rate
- Causes
  - Chromosomal abnormalities
  - Yq deletions
  - Iatrogenic treatment
  - Cryptorchidism
  - Testicular torsion
  - Unknown genetic causes (?)
When to cryopreserve?

- Factor Time – Occasion
  - Diagnostic occasion
  - Therapeutic occasion

- Factor Quality
  - High
  - Fair
  - Low
  - Extremely low

Two cryo approaches:

1. Spermatozoa can be frozen on the day of oocyte retrieval
   - First ICSI with fresh spermatozoa
   - Cryopreservation of supernumerary sperm for later ICSI cycles

2. Spermatozoa can be frozen on a day independent of oocyte retrieval
   - Cryopreservation of spermatozoa for later ICSI cycles
   - ICSI cycles with frozen-thawed spermatozoa

Pros and cons for both approaches

- Different for patients with OA or NOA
- Dependent on sperm retrieval procedure
- Depending on flexibility of scheduling TESE

Pros and cons of both approaches

**Approach 1: ICSI- cryo**
- Pro: Loss of sperm quality by freezing is avoided – lower risk of finding only immotile sperm
- Pro: Lower sperm quality limits for ICSI treatment
- Pro: Less restrictive criteria for patient allocation to ICSI
- Con: Concomitant scheduling of sperm and oocyte retrieval on the same day
- Con: 50% risk of pointless ovarian stimulation in NOA
- Con: More stressful to the couple

**Approach 2: cryo- ICSI**
- Pro: Independent scheduling of sperm and oocyte retrieval
- Pro: Avoid pointless ovarian stimulation of the female partner (if no sperm is retrieved)
- Pro: Less stressful to the couple
- Con: Risk of not finding mobile sperm post-thaw
- Con: Higher sperm quality limits for allocation to ICSI treatment (quality loss by freezing-thawing)
**OA patients and retrieval method**

- Sperm obtained by **TESE or open biopsy**
  - Sperm recovery in 100% of patients, mostly high numbers
  - Both cryo approaches are effective
  - One or two biopsies provide sufficient sperm for several ICSI cycles

**OA patients and retrieval method**

- Sperm obtained by **TESA or FNA**
  - Sperm recovery in almost 100% of patients
  - Only low sperm numbers retrieved
  - Easily performed always freshly on the day of oocyte retrieval
  - Freezing not always possible, depending on:
    - Sperm number
    - Collection method
      - Droplets under oil
      - Culture medium in dishes
      - Culture medium in tubes

**NOA patients and retrieval method**

- Sperm obtained by **TESE or open biopsy**
  - Successful in 50-60% of patients: poor numbers and motility, multiple biopsies
  - Cryo and later use for ICSI is possible in many cases
  - Depending on the quality
  - Depending on the criteria for freezing
  - Depending on the allocation criteria for ICSI treatment
  - Scheduling fresh TESE as back up in severe cases
  - Fresh TESE for ICSI is the only option in extremely poor cases

Verheyen et al. 2004, HR 19, 2822
NOA patients and retrieval method

- **Sperm obtained by TESA**
  - Often unsuccessful, no sperm or poor numbers obtained
  - Poor chance to freeze spermatozoa
  - Uncommon procedure in NOA

- **Sperm obtained by micro-TESE**
  - Fair number may be obtained
  - Skilled microsurgeon
  - Reasonable chance to freeze spermatozoa
  - Less common procedure

Schlegel et al. 1999, HR 14, 131
Colpi et al. 2009, RBMOnline 18, 315

Which quality to cryopreserve?

- **No upper limit**
- **Lower limit? In NOA**
  - Different from clinic to clinic
  - Depending on patient allocation criteria
  - Possibility to schedule fresh TESE as back-up at OPU
  - UZBrussel - CRM
    - Number: ≥ 1 spermatozoon
    - Motility: no cut-off, even 0% motility
    - Obtained either after mechanical or enzymatical treatment

Verheyen et al. 1995, HR 10, 2956
Crabbé et al. 1997, 12, 1682

Cryodamage of testicular sperm

- **Effect on motility and viability**

Verheyen et al. 1997, FS 67, 74
**Cryodamage of testicular sperm**

- **Effect on the ultrastructure**
  - Rupture of plasma membranes
  - Rupture of acrosomal membranes
  
  Nogueira et al. 1999, HR 14, 2041

- **Effect on DNA fragmentation**
  - Comet assay
  - Depending on the freeze-thawing method
  
  Thompson- Cree et al. 2003, RBMOnline 4, 449
  - Comet assay
  - Increased DNA damage in frozen-thawed testicular sperm (16.5% vs 10.6%)
  
  Dalzell et al. 2004, FS 82, 1443

---

**How to cryopreserve?**

- **Constitution**
  - Biopsy
  - Suspension
  - Individual cells

- **Preparation**

- **Cryoprotectant**

- **Freezing procedure**
  - Slow controlled rate
  - Rapid vapour
  - Vitrification

- **Carriers**
How to cryopreserve? Constitution

- Biopsy or suspension?
  - Suspension better preserves
    - Motility (9% vs 4%)
    - Viability (39% vs 25%)
  - Slower/incomplete penetration of cryoprotectant into the cells of a biopsy
  
  Crabbé et al. 1999, Int J Androl 22, 43

How to cryopreserve? Constitution

- Individual spermatozoa (NOA)
  - Time-consuming procedure before freezing
  - Carriers
    - Microcentrifuge tubes
    - Straws
    - Empty zona pellucida
    - Microdroplets under oil
  - Rarely performed
  - European Cell & Tissue Directives

How to cryopreserve? Preparation

- Mechanical procedures
  - Scissors, needles, forceps, glass slides
  - In OA and NOA
  - Rupture of tubules - sperm release
  
  Verheyen et al. 1995, HR 10, 2956

- Dissection of biopsy
  - Isolation of most dilated tubules
  - Higher recovery rate in NOA
  
  Kamal et al. 2004, J Androl 25, 123
How to cryopreserve? Preparation

**Enzymatic procedures**
- If mechanical treatment fails
- Collagenase type I or IV
- In NGA
- Degradation of collagen in basement membrane and matrix
  
  Salzbrunn et al. 1996, HR 11, 752  
  Crabbé et al. 1997, HR 12, 1682  
  Crabbé et al. 1998, HR 13, 2791

**Erythrocyte-lysing buffer**
- If red blood cells hamper sperm visualization
- Improved treatment procedure
- Either before or after enzymatic treatment
  
  Nagy et al. 1997, FS 68, 376

---

**In-vitro culture**
- OA: Improve sperm morphology and quality of motility
- NOA: No change in motility
  
  Liu et al. 1997, HR 12, 1667

- Recommendation to inject without delay/incubation
- Increase of DNA fragmentation by 4 hour incubation
- Comet assay
  
  Dalzell et al. 2004, PS 82, 1443

---

Dalzell et al. FS 2004

**Relationships between fragmented DNA in fresh, frozen-thawed, and post-cryopreservation incubated testicular sperm.**

<table>
<thead>
<tr>
<th>Time point of analysis</th>
<th>Mean ± S.E.</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh</td>
<td>10.6 ± 1.81</td>
<td>.892</td>
</tr>
<tr>
<td>4 hour</td>
<td>22.1 ± 3.49</td>
<td>.026</td>
</tr>
<tr>
<td>24 hour</td>
<td>19.1 ± 2.33</td>
<td>.017</td>
</tr>
<tr>
<td>Freeze thawed</td>
<td>16.5 ± 1.60</td>
<td>.0004</td>
</tr>
<tr>
<td>4 hour post thaw</td>
<td>20.5 ± 1.45</td>
<td>.00004</td>
</tr>
<tr>
<td>24 hour post thaw</td>
<td>30.4 ± 1.71</td>
<td>&lt;.00000</td>
</tr>
</tbody>
</table>

Note: P values are comparisons to fresh data. n = 34.

How to cryopreserve? Preparation

- **Concentration/dilution**
  - OA with high numbers: dilute suspension before freezing
  - NOA with low numbers: concentrate (or dilute) suspension
  - Avoid refreezing

How to cryopreserve? Cryoprotectant

- **Glycerol**
  - Cryoprotectant of choice for mature spermatozoa
  - Addition of extenders (commercially available media)
  - Testicular tissue structure is not preserved
  - Germ cells do not survive
- **DMSO**
  - Cryoprotectant of choice for preservation of tubule structure
  - Fertility preservation for prepubertal boys
  - Best maintains tissue capacity to initiate spermatogenesis
  - Keros et al. 2005, HR 22, 1384; Goossens et al. 2008, PS 89, 725
  - Jahnukainen et al. 2007, HR 22, 1060

How to cryopreserve? Freezing procedure

- **Slow controlled-rate vs rapid vapour**
  - No evidence from the literature
  - Same methods as semen freezing
  - Vapour freezing = procedure of choice
  - Rapid freezing necessitates rapid thawing
    - Vanheusden et al. 1993
    - Thompson-Cree et al. 2003
- **Vitrification**
  - Extremely high cooling rates
  - Small volumes (individually aspirated spermatozoa)
  - High concentrations of cryoprotectant
  - Low efficiency
How to cryopreserve? carriers

- Closed systems
  - High-security sealed straws
  - No ampoules, no cryotubes
  - Correctly, clearly identified
  - European Cell and Tissue Directives
  - Avoid transmission of pathogens and viruses

- Liquid nitrogen or vapour
  - Store safely
  - Day and night monitoring

ICSI with fresh/frozen testicular sperm

- OA: Many reports since 1996
  - Romero et al. 1996, FS 65, 877
    - Comparable fertilization rate, embryo quality, pregnancy and implantation rate

- NOA: Few reports since 1998
  - Criteria for testicular sperm freezing
  - Comparable to impaired fertilization rate and implantation rate
  - Nicopoulos et al. 2004, FS 82, 691: Meta-analysis fresh-frozen OA+NOA
    - Similar fertilization, clinical and ongoing pregnancy rate
    - Significantly impaired implantation rate

ICSI with fresh/frozen testicular sperm

Should diagnostic testicular sperm retrieval followed by cryopreservation for later ICSI be the procedure of choice for all patients with non-obstructive azoospermia?

G. Verheyen*, J. Van Hoogstraten, B. Van Den Broecke, P. Devroey and A. Van Steirteghem

Clinic for Reproductive Medicine, University Hospital of the Catholic University of Leuven, Laboratory 1A, G. 300, B-8000, Brusel, Belgium.

*Correspondence should be addressed to G. Verheyen, g.verheyen@uku.be

BACKGROUND: This was a retrospective study in 46 patients with non-obstructive azoospermia (NOA) to determine whether the protocol followed by cryopreservation should be the procedure of choice for all patients with indicated fertility. METHOD: The first part of the study evaluated ICSI cycle initiated with frozen-thawed testicular sperm for 46 non-obstructive azoospermia (NOA) patients. The second part focused on a subgroup of 23 patients who underwent ICSI cycles with fresh testicular sperm for NOA patients (ICSI: 2001-2005) and fresh and frozen-thawed sperm for NOA patients (ICSI: 2006-2009). RESULTS: Table 1. The fertilization rate was 72% (28/39) in fresh group compared to 63% (20/32) in frozen-thawed sperm group. A higher fertilization rate in fresh group was statistically significant (P < 0.05). The overall implantation rate was 32% (16/50) in fresh group and 22% (9/41) in frozen-thawed sperm group. A higher implantation rate in fresh group was statistically significant (P < 0.05). CONCLUSION: Even in a situation with non-obstructive azoospermia, diagnostic testicular sperm followed by cryopreservation can be an alternative procedure to ICSI with fresh testicular sperm.
ICSI with fresh/frozen testicular sperm

Table V. Comparison of sperm characteristics in the ICSI cycles with fresh (44 cycles) and frozen (42 cycles) testicular sperm at 32 non-obstructive azoospermia (NOA) patients.

<table>
<thead>
<tr>
<th>Sperm characteristic</th>
<th>Fresh TESE</th>
<th>Frozen TESE</th>
<th>Mann-Whitney</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycles (N)</td>
<td>44</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>Sperm-free cycles (%)</td>
<td>81 (18.8)</td>
<td>71 (17.1)</td>
<td>0.882</td>
</tr>
<tr>
<td>Cycles injected with only sperm (%)</td>
<td>30 (30.0)</td>
<td>29 (30.0)</td>
<td>NS</td>
</tr>
<tr>
<td>Cycles injected with only testicular sperm (%)</td>
<td>24 (30.0)</td>
<td>25 (30.0)</td>
<td>NS</td>
</tr>
<tr>
<td>COC cycle (mean)</td>
<td>11.5 ± 6.2</td>
<td>11.3 ± 5.2</td>
<td>NS</td>
</tr>
<tr>
<td>Male sperm (%)</td>
<td>50.0 ± 21.2</td>
<td>50.0 ± 21.2</td>
<td>NS</td>
</tr>
<tr>
<td>% of &gt;=50 sperm (%)</td>
<td>8.6 ± 8.3</td>
<td>10.8 ± 6.9</td>
<td>NS</td>
</tr>
</tbody>
</table>

ET, embryo transfer; Pos, positive; PR, pregnancy rate.

ICSI with fresh/frozen testicular sperm

Table VI. Results of embryo transfer, pregnancy and implantation rates after ICSI with fresh (44 cycles) and frozen (42 cycles) testicular sperm of 32 non-obstructive azoospermia (NOA) patients.

<table>
<thead>
<tr>
<th>Embryo transfer (%)</th>
<th>44 (93.2)</th>
<th>42 (95.6)</th>
<th>0.038</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pos 6CGE cycle (%)</td>
<td>0/4 (20.0)</td>
<td>0/4 (20.0)</td>
<td>NS</td>
</tr>
<tr>
<td>Pos 6CGE+ET (%)</td>
<td>0/4 (20.0)</td>
<td>0/4 (20.0)</td>
<td>NS</td>
</tr>
<tr>
<td>Clinical PR/ET (%)</td>
<td>7/4 (17.5)</td>
<td>6/4 (15.9)</td>
<td>NS</td>
</tr>
<tr>
<td>Implantation rate (%)</td>
<td>8/10 (7.6)</td>
<td>6/9 (17.4)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Should we cryopreserve testicular sperm in NOA patients?

- **YES**
  - Similar outcome as fresh after ICSI
  - But... works in 4 out of 5 patients
  - Counsel patients for back-up fresh TESE
Freezing for prepubertal boys with cancer

- Only option for fertility preservation
- Before initiation of gonadotoxic cancer therapy
- Storage of spermatogonial stem cells
- Future autologous intratesticular transplantation after cure

Brinster et al. 1994, Proc Natl Acad Sci USA 91, 11303
- Spermatogonia are able to colonize the seminiferous tubules
- Induce active spermatogenesis
Schlatt et al. 1999, HR 14, 144
- Multiple injection into rete testis in primates and human most promising technique

Freezing for prepubertal boys with cancer

- Future autologous intratesticular transplantation after cure
  - Cell suspension transplantation
  - Tissue grafting
  - In-vitro maturation

Concerns
- Technical feasibility? Transplantation protocol and storage
- Safety? Risk of re-introducing malignant cells
- Reproductive efficiency?

Geens et al. 2007, HR 21, 390
Wyns et al. 2010, HR Update 16

Freezing for prepubertal boys with cancer

Clinical application UZ Brussels
Requests: n=23

Accepted: n=15
  - Sickle cell anemia (8)
  - Thalassemia (1)
  - Leukemia (3)
  - Idiopathic aplastic anemia (1)
  - Granulomatosis (1)

Refused: n=1
Parents declined after counselling: n=7
  - Leukemia (4)
  - Sickle cell anemia (2)
  - Idiopathic aplastic anemia (1)
Conclusions

- Testicular sperm freezing is an efficient procedure in order to avoid repeated surgery in obstructive and non-obstructive azoospermia
- In OA, freezing can be performed either on a diagnostic occasion or on the day of OPU
- In NOA, pros and cons should be considered for individual clinics or patients
- In NOA with poor testicular quality, a fresh retrieval is preferably scheduled as back-up on the day of OPU
- Cryodamage is observed at the level of motility, viability, ultrastructure… comparable to ejaculated sperm
- DNA fragmentation is not affected if adequate freezing procedures are applied
- Testicular spermatozoa are preferably frozen in suspension, obtained after mechanical or enzymatic treatment procedures
- The suspension should be diluted or concentrated in order to optimize the number of treatments
- Glycerol is the cryoprotectant of choice for either slow controlled-rate or rapid vapour freezing of mature testicular sperm
- Testicular sperm should be stored in closed straws in liquid nitrogen or vapour

Conclusions

- In case of fertility preservation for prepubertal boys, testicular biopsies are frozen by slow freezing with DMSO as cryoprotectant
- Before fertility restoration is possible, concerns should be solved and the efficiency should be improved
Thank you!