Testicular tissue: When and how should it be cryopreserved?

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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

QUALITY

Etiology of azoospermia

Obstructive azoospermia (OA)	Non-obstructive azoospermia (NOA)
• Mechanical cause	• Biological cause
• Normal spermatogenesis High sperm numbers	• Severely impaired spermatogenesis Low to absent sperm numbers
• Epididymal or testicular sperm	• Testicular sperm
• 100% recovery rate	• 50-60% recovery rate
• Causes	• Causes
- Vasectomy	- Chromosomal abnormalities
- Congenital bilateral absence of the vas deferens (CBAVD)	- Yq deletions
- Post infectious epididymitis	- Iatrogenic treatment
- Testicular trauma	- Cryptorchidism
- Young's syndrome	- Testicular torsion
- Retrograde ejaculation	- Unknown genetic causes (?)

When to cryopreserve?

- Factor Time – Occasion
  - → Diagnostic occasion
  - → Therapeutic occasion
- Factor Quality
  - → High
  - → Fair
  - → Low
  - → Extremely low

When to cryopreserve?

- 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 motile 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?

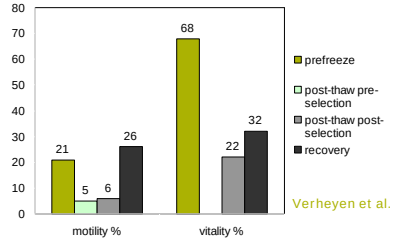
- No upper limit
- Lower limit ? In NOA
  - → Different from clinic to clinic
  - → Depending on patient allocation criteria
  - → Possibility to schedule fresh TESE as back-up at OPU
  - → UZBrussel - CRM
    - Number: ≥ 1 spermatozoon
    - Motility: no cut-off, even 0% motility

    ⇒ 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

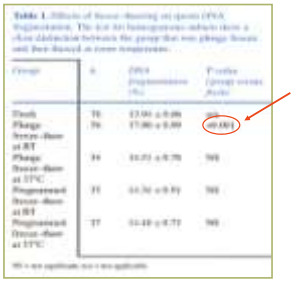
Cryodamage of testicular sperm

- 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

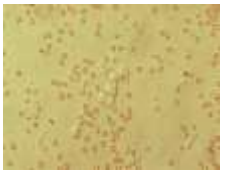
How to cryopreserve? Constitution

- 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 NOA
  - → 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

How to cryopreserve? Preparation

- In-vitro culture
  - → OA: Improval 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, FS 82, 1443

How to cryopreserve? Preparation

Dalzell et al. FS 2004

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

	DNA fragmentation (%)	
Time point of analysis	Mean ± S.E.	*P* value
Fresh	10.6 ± 1.03	—
4-hour	22.1 ± 3.49	.052
24-hour	19.1 ± 2.33	.017
Frozen–thawed	16.5 ± 1.00	.0001
4-hour post-thaw	29.5 ± 1.45	.00004
24-hour post-thaw	30.4 ± 1.71	<.00001

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

Dalzell, Incubation of testicular sperm. Fertil Steril 2004.

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

  Keros et al. 2005, HR 22, 1384; Goossens et al. 2008, FS 89, 725
  - → Best maintains tissue capacity to initiate spermatogenesis

  Jahnukainen et al. 2007, HR 22, 1060

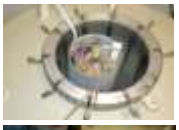
How to cryopreserve? Freezing procedure

- 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

  Verheyen 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 ampoulles, no cryotubes
  - → Correctly, clearly identified
  - → European Cell and Tissue Directives
  - → Avoid transmission of pathogens and virusses
- 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

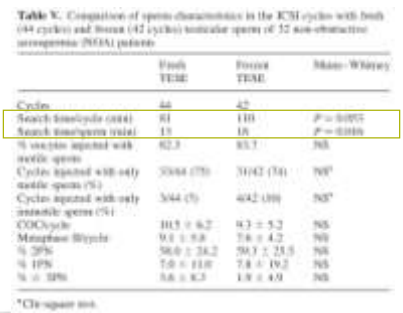
  Nicopoulos et al. 2004, FS 82, 691: *Meta-analysis fresh-frozen OA+NOA*
  - → Similar fertilization, clinical and ongoing pregnancy rate
  - → Significantly impaired implantation rate

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[1], V.Vernaeve, L.Van Landuyt, H.Tournaye, P.Devroey and A.Van Steirteghem

ICSI with fresh/frozen testicular sperm



	Fresh TESE	Frozen TESE	Mann–Whitney
Cycles	44	42	
Search time/cycle (min)	81	110	$P = 0.055$
Search time/sperm (min)	15	18	$P = 0.016$
% oocytes injected with motile sperm	82.3	83.7	NS
Cycles injected with only motile sperm (%)	33/44 (75)	31/42 (74)	NS*
Cycles injected with only immotile sperm (%)	3/44 (7)	4/42 (10)	NS*
COCs/cycle	10.5 ± 6.2	9.3 ± 5.2	NS
Metaphase II/cycle	9.1 ± 5.8	7.6 ± 4.2	NS
% 2PN	56.0 ± 24.2	59.1 ± 25.5	NS
% 1PN	7.0 ± 11.0	7.8 ± 19.2	NS
% ≥ 3PN	3.6 ± 8.3	1.9 ± 4.9	NS

*Chi-square test.

Verheyen et al. 2004, HR 19, 2822

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

	Fresh TESE	Frozen TESE	Chi-square
Cycles	44	42	
Transfers (%)	41 (93.2)	32 (76.2)	$P = 0.028$
Embryos/ET	2.6	2.5	NS
Pos. hCG/cycle (%)	9/44 (20.4)	8/42 (19.0)	NS
Pos. hCG/ET (%)	9/41 (21.9)	8/32 (25.0)	NS
Clinical PR/cycle (%)	7/44 (15.9)	6/42 (14.3)	NS
Clinical PR/ET (%)	7/41 (17.1)	6/32 (18.7)	NS
Implantation rate (%)	8/105 (7.6)	6/81 (7.4)	NS

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

Verheyen et al. 2004, HR 19, 2822

ICSI with fresh/frozen testicular sperm

- 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

Conclusions

- 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 !