Sperm Cryopreservation: which sperm sample and which method

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The History of Semen Cryopreservation

- 1776: Spallanzani
- 1866: Montegazza- soldier could beget a legal heir with stored semen
- 1930-1940: some sperm survived after freezing
- 1949: first cryoprotectant- glycerol (Polge *et al* , 1949)
- 1950: glycerol- egg yolk- citrate → ↑ animal semen use
- 1963: Freezing in liquid Nitrogen at –196^{0}C
- Optimization of protocols (reviewed by Brotherton, 1990; Storey et al, 1998)
- Last 30 years - ↑ types of sperm frozen
- Little improvement in success or our knowledge of cryo-physiology

The Milestones of Cryopreservation

1953- first birth from frozen ejaculated sperm

Bunge and Sherman, 1953

1995- first birth from frozen epididymal sperm

Devroey et al, 1995

1996- first birth from frozen testicular sperm

Gil- Salmon et al, 1996

Uses of Semen Cryopreservation

Freezing provides people with future fertility potential

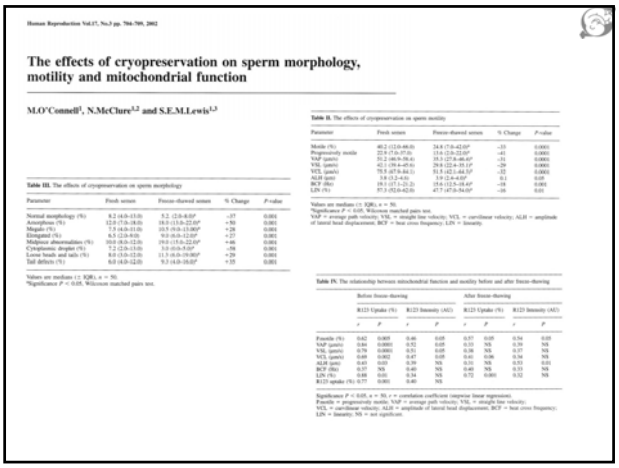
- Cancer and Multiple Sclerosis
- ART - patient
- - donor
- Pre- operative insurance
- Vasectomy insurance
- Post-mortem sperm retrieval

Procedures for Cryopreservation

- Consent forms
- Costs of storage
- Fate of gametes in event of man's death
- Screening for Hepatitis, Syphilis, Chlamydia, CMV and HIV
- Separation in tanks after quarantine 6 mths
- Straws or vials- what material?
- Labelling systems
- Monitoring of stored samples

British Fertility Society Lab Practice, Lewis et al, 2005

The effects of cryopreservation on sperm quality



Human Reproduction Vol.17, No.3 pp. 704–709, 2002

The effects of cryopreservation on sperm morphology, motility and mitochondrial function

M.O'Connell[1], N.McClure[1,2] and S.E.M.Lewis[1,3]

Table III. The effects of cryopreservation on sperm morphology

Parameter	Fresh semen	Freeze-thawed semen	% Change	*P*-value
Normal morphology (%)	8.2 (4.0–13.0)	5.2 (2.0–8.0)*	–37	0.001
Amorphous (%)	12.0 (7.0–18.0)	18.0 (13.0–22.0)*	+50	0.001
Megalo (%)	7.5 (4.0–11.0)	10.5 (9.0–13.00)*	+28	0.001
Elongated (%)	6.5 (2.0–9.0)	9.0 (6.0–12.0)*	+27	0.001
Midpiece abnormalities (%)	10.0 (8.0–12.0)	19.0 (15.0–22.0)*	+46	0.001
Cytoplasmic droplet (%)	7.2 (2.0–13.0)	3.0 (0.0–5.0)*	–58	0.001
Loose heads and tails (%)	8.0 (3.0–12.0)	11.5 (6.0–19.00)*	+29	0.001
Tail defects (%)	8.0 (4.0–12.0)	9.3 (4.0–18.0)*	+35	0.001

Values are medians (± IQR). $n = 50$.
*Significance $P < 0.05$, Wilcoxon matched pairs test.

Table II. The effects of cryopreservation on sperm motility

Parameter	Fresh semen	Freeze-thawed semen	% Change	*P*-value
Motile (%)	60.2 (52.0–68.0)	24.8 (7.0–42.0)*	–33	0.0001
Progressively motile	22.9 (7.0–37.0)	13.6 (2.0–22.0)*	–41	0.0001
VAP (μm/s)	51.2 (46.9–58.4)	35.3 (27.8–46.4)*	–31	0.0001
VSL (μm/s)	42.1 (39.4–45.6)	29.8 (22.6–35.1)*	–29	0.0001
VCL (μm/s)	75.5 (67.0–84.1)	51.5 (42.1–64.3)*	–32	0.0001
ALH (μm)	3.6 (3.2–4.6)	3.9 (2.6–4.0)*	0.1	0.05
BCF (Hz)	19.3 (17.1–21.2)	15.6 (12.5–18.4)*	–18	0.001
LIN (%)	87.3 (82.0–92.0)	47.7 (47.0–54.0)*	–46	0.01

Values are medians (± IQR). $n = 50$.
*Significance $P < 0.05$, Wilcoxon matched pairs test.
VAP = average path velocity; VSL = straight line velocity; VCL = curvilinear velocity; ALH = amplitude of lateral head displacement; BCF = beat cross frequency; LIN = linearity.

Table IV. The relationship between mitochondrial function and motility before and after freeze-thawing

	Before freeze-thawing				After freeze-thawing			
	R123 Uptake (%)		R123 Intensity (AU)		R123 Uptake (%)		R123 Intensity (AU)	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*
Fractile (%)	0.42	0.005	0.46	0.05	0.57	0.05	0.54	0.05
VAP (μm/s)	0.79	0.0001	0.51	0.05	0.38	NS	0.37	NS
VSL (μm/s)	0.79	0.0001	0.52	0.05	0.38	NS	0.35	NS
VCL (μm/s)	0.69	0.002	0.47	0.05	0.41	0.06	0.34	NS
ALH (μm)	0.43	0.03	0.39	NS	0.31	NS	0.53	0.01
BCF (Hz)	0.37	NS	0.40	NS	0.40	NS	0.33	NS
LIN (%)	0.88	0.01	0.34	NS	0.72	0.001	0.52	NS
R123 uptake (%)	0.77	0.001	0.40	NS				

Significance $P < 0.05$, $n = 50$, r = correlation coefficient (stepwise linear regression).
Fractile = progressively motile; VAP = average path velocity; VSL = straight line velocity; VCL = curvilinear velocity; ALH = amplitude of lateral head displacement; BCF = beat cross frequency; LIN = linearity; NS = not significant.

Cryo-injury is expressed as

Structural Damage-

- **loss of membrane integrity**
- **↓intact acrosomes**
- **cytoplasmic vacuolation**
- **mitochondrial distortion**
  (reviewed by Nijs and Ombelet, 2001)
- **↓ normal morphology**
  (~40%, ↑ amorphous heads and midpiece abnormalities)
  (Verheyen et al,1997; Hammadeh et al, 1999; O'Connell et al, 2002)
- **Alterations in permeability and conformation of phospholipid bilayers**
  (Mazur et al, 1984; Parks and Graham, 1992)

Cryo-injury is expressed as

Loss of viability

(Nijs and Ombelet, 2001)

Functional Damage to the surviving population-

- **Loss of Motility (25-75%), VSL, VCL, VAP**

(Hammadeh et al, 1999; Esteves et al, 2000; Nijs et al, 2000; Donnelly et al, 2001)

- **Infertile mens' sperm more susceptible to cryoinjury**

(Holden et al, 1997; Nijs et al, 2000, Donnelly et al, 2001; de Paula et al, 2006)

Alterations in plasma and mitochondrial membrane potentials

- ↓ $[Ca2^{+}]_i$ and ↓ response to Progesterone
- → $[Ca^{2+}]_e$ entry → Capacitative motility
- ↓ intact acrosomal caps ↓ acrosin activity
- ↓ Rhodamine 123 uptake -36%
- ↓ Rhodamine 123 activity -47%
- ↓ Progressive motility - 41% (greater in infertile sperm)

(Mack and Zanevald, 1987;Alvarez and Storey, 1993; Rossato et al, 2000; O' Connell et al, 2002)

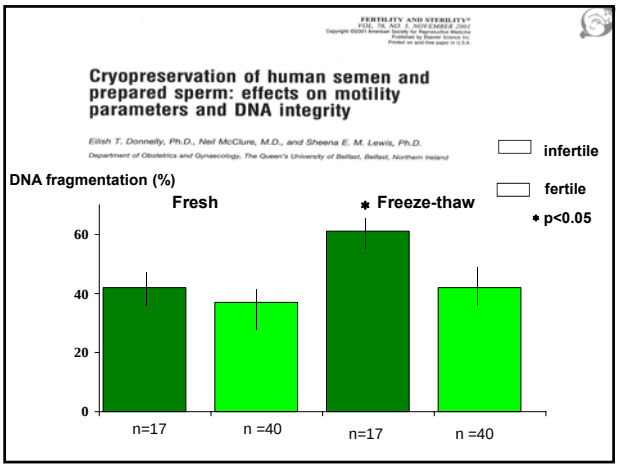
Cryo-injury is expressed as

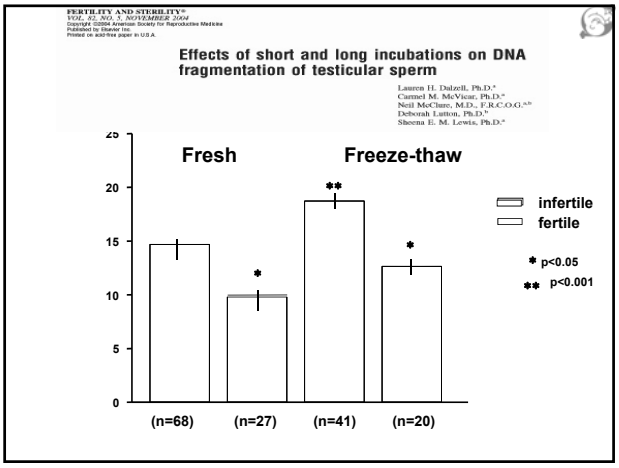
- No chromosomal damage
  (Li, Overstreet et al, 2007)
- ↑ Abnormal DNA condensation→ ↓ fertilization
  (Hammadeh et al, 2000, 2001)
- ↑Chromatin structure alterations (DNA – Protamine relationships) → ↓ fertilization
  (Royere et al, 1991)
- ↑ DNA fragmentation
  (Donnelly et al, 2001; Dalzell et al, 2001; de Paula et al, 2006)

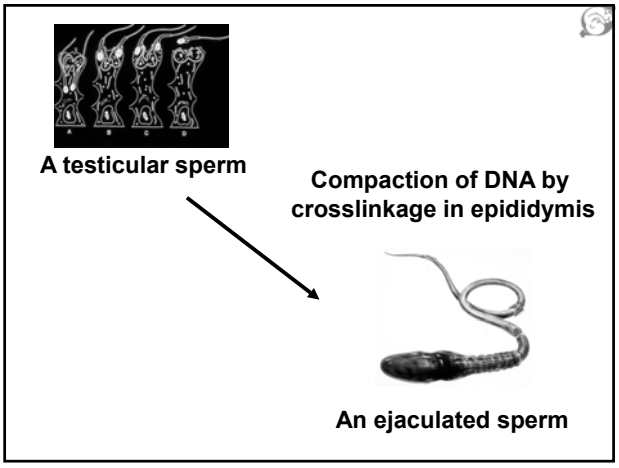
Can DNA integrity predict ART success?

Nuclear DNA anomalies lead to:-

- Failure of fertilization in IVF
  Bianchi et al, 1993; Sun et al, 1997
- Failure to implant in ICSI
  Sakkas et al, 1996; Lopes et al, 1998
- Increased time to conception
- Increased miscarriage rate
  Evenson et al, 1999; Carrell et al, 2003
- Poor embryo development
  Morris et al, 2002; Tomsu et al, 2002
- Post-implantation loss and malformations
  Robaire et al, 1985
- Childhood cancers
  Knight and Marrett, 1997


FERTILITY AND STERILITY®
Cryopreservation of human semen and prepared sperm: effects on motility parameters and DNA integrity
Eilish T. Donnelly, Ph.D., Neil McClure, M.D., and Sheena E. M. Lewis, Ph.D.
Department of Obstetrics and Gynaecology, The Queen's University of Belfast, Belfast, Northern Ireland
infertile
fertile
* p<0.05
DNA fragmentation (%)
Fresh
* Freeze-thaw
60
40
20
0
n=17
n =40
n=17
n =40


FERTILITY AND STERILITY®
Effects of short and long incubations on DNA fragmentation of testicular sperm
Lauren H. Dalzell, Ph.D.
Carmel M. McVicar, Ph.D.
Neil McClure, M.D., F.R.C.O.G.
Deborah Lutton, Ph.D.
Sheena E. M. Lewis, Ph.D.
Fresh
Freeze-thaw
infertile
fertile
* p<0.05
** p<0.001
20
15
10
5
0
(n=68)
(n=27)
(n=41)
(n=20)


A testicular sperm
Compaction of DNA by crosslinkage in epididymis
An ejaculated sperm

When does Cryo-injury Occur?

During freezing

- intracellular ice formation
- Osmotic stresses

(Muldrew and McGann, 1988; Watson, 1995; Devireddy et al, 2000, Morris et al, 2007)

During thawing

- Rapid warming prevents recrystallization

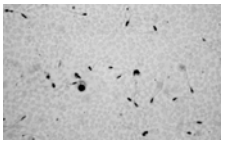
(Watson, 1995)

Not during storage

- Cryoprotectants are crucial for protection...?

(Mortimer, 1994; Yildiz et al, 2007)

The effects of cryopreservation on ART outcomes with ejaculated and epididymal sperm



Cryo-injury leads to reduced success in ART

- IUI by husband- ↓ CPR

  (*Sherman, 1973)*

- IUI by donor - ↓ CPR

  (Richter et al, 1984)

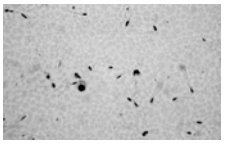
- IVF and ICSI - ↓ FR, IR, CPR

  (Critser et al, 1987; Crabbe et al, 1999; Hammadeh et al, 1999)

- ICSI/MESA- ↑ CPR with F-T sperm

  (Devroey et al, 1995, Wood, Lewis-Jones et al, 2002)

The effects of cryopreservation on ART outcomes with testicular sperm



Comparison of the effects of two methods of cryopreservation on testicular sperm DNA

No difference in sperm DNA fragmentation when f-t in suspension or in biopsy

E. Kristine Steele, B.Sc., M.R.C.O.G.,* Neil McClure, M.D., M.R.C.O.G.,*† and Sheena E. M. Lewis, E.M., Ph.D.*

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Article

Effects of cryopreservation on testicular sperm nuclear DNA fragmentation and its relationship with assisted conception outcome following ICSI with testicular spermatozoa

Current success rates of fresh v frozen testicular (OA) cycles

- **FR, IR and CPR significantly reduced**

  (Nicopoullos et al, 2003,Dr Croo et al, 1998; Wood et al, 2002)

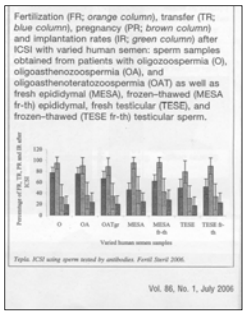
- **IR significantly impaired**

(RR1.75,95% CI 1.10-2.80, p=0.02: meta-analysis-of 1476 cycles Nicopoullos et al, 2004)

- **No impairment in outcome- FR, IR, CPR**

  (Friedler et al, 1998;Ben- Yosef et al, 1999; Tournaye et al, 1999 ;Habermann et al, 2002 ; Thompson- Cree et al, 2003)

Immunological evaluation of sperm potentiality


Fertilization (FR; *orange column*), transfer (TR; *blue column*), pregnancy (PR; *brown column*) and implantation rates (IR; *green column*) after ICSI with varied human semen: sperm samples obtained from patients with oligozoospermia (O), oligoasthenozoospermia (OA), and oligoasthenoteratozoospermia (OAT) as well as fresh epididymal (MESA), frozen–thawed (MESA fr-th) epididymal, fresh testicular (TESE), and frozen–thawed (TESE fr-th) testicular sperm.

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Tepla, 2006

Success rates depend on aetiology of azoospermia

- **FR are higher and miscarriage rates are lower for men with acquired azoospermia but CPR and IR are similar**
  (meta- analysis Nicopoullos et al, 2004)
- **↓ CPR with time post vasectomy**
  (Abdelmassih et al. 2002; Borges et al., 2003; McVicar et al, 2004)
- **↓ CPR with NOA (usually fresh)**

A danger of incubating post-thaw testicular sperm to acquire motility

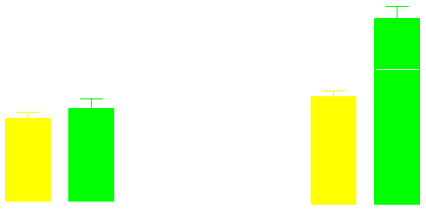
Overnight incubation of post-thaw testicular sperm

- routine clinical practice
- reasons: convenient
  non invasive
  quick viability test

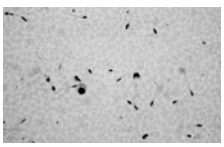
Effects of 24-hour incubation after freeze-thawing on DNA fragmentation of testicular sperm from infertile and fertile men

Fertile

Infertile

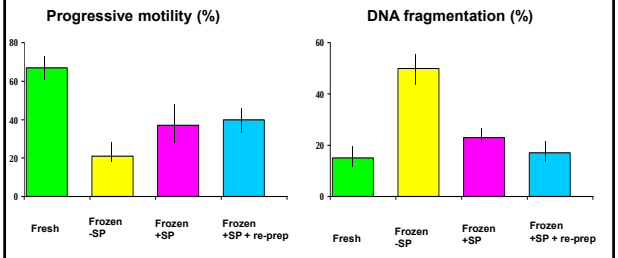


A better way to freeze sperm



Comparison of individual antioxidants of sperm and seminal plasma in fertile and infertile men

Cryopreservation of human semen and prepared sperm: effects on motility parameters and DNA integrity



Challenges of Cryopreservation

Reduction of cryoinjury from-

- **Ice crystals- reduction or prevention**
  (*Mudrew and McGann, 1988; Devireddy et al, 2000)*

Combat with

- **programmable freezing**
- **cryoprotectants**

(Mortimer, 1994; Watson, 1995)

Challenges of Cryopreservation

Reduction of cryoinjury from-

- **Reactive oxygen species generation**

(Alvarez and Storey, 1992; ell et al, 1993, Kumar and Das, 2005; Peris et al, 2007)

Depletion of antioxidants

- **GSH ↓78%, SOD ↓50%**

(Bilodeau, Gagnon et al, 2000; Peris et al, 2007)

Vitrification or Ultra Rapid Freezing

•Process involves solidifying liquids without crystallization

•Blastocysts and births from slow rate frozen embryos from vitrified oocytes with rapid plunge (1700^0C) frozen sperm

Merlo et al, 2008; Wang et al, 2008; Chen et al, 2008

•Embryo cryopreservation by vitrification

Shaw et al, 1991; Stehlik et al, 2005; Zhou et al, 2005, Libermann and Tucker, 2004;

in liquid nitrogen slush

Yavin et al, 2008

• Semen Vitrification > 30 000^0C/min

v 3-600^0C/min ultra-rapid freezing

v <100^0C/min $LiqN_2$ plunge

Vitrification of Sperm

DNA integrity and motility of human sperm after standard slow freezing versus cryoprotectant-free vitrification

Isachenko et al, 2004

Rapidly cooled human sperm: no evidence of intracellular ice formation

GJ Morris, 2006

Acromosomal status and mitochondrial activity of human sperm vitrified with sucrose

Isachenko et al, 2008

Freeze drying Sperm for ICSI Use only

Mouse sperm freeze dried

Ward, Whittingham, Yanagimachi et al, Biol Reprod 2003

- sperm plunged into $LiqN_2$ for 20s then freeze- dried for 4h
- stored at 4^0C
- ↓ zygotes with normal karyotypes (96% v 75%)
- ↓embryos developing into fetuses (58% v 35%)
- no further deterioration with time
- normal live offspring were born after 1.5yr

Freezing without a Cryoprotectant for ICSI Use Only

Mouse sperm frozen without cryoprotectant

Ward, Whittingham, Yanagimachi et al, Biol Reprod 2003

- sperm kept on surface of $LiqN_2$ for 10 min then plunged into $LiqN_2$, thawed at RT for 5 min
- ↓ zygotes with normal karyotypes (87% v 75%)
- ↓embryos developing into fetuses (45% v 35%)
- no further deterioration with time
- normal live offspring were born after 1.5yr

ROS, antioxidants and sperm freezing

- Freezing caused ↓ GSH (-78%) and SOD (-50%) activity *Bilodeau, Gagnon et al, 2000*
- Post-thaw addition of thiols (GSH, Cysteine, NAC) prevent H_2O_2 mediated↓ motility *Bilodeau, Gagnon et al, 2001*
- Pyruvate, metal chelators or oviductal catalase also prevented H_2O_2 mediated↓ motility *Bilodeau et al, 2002*
- Controversy- membrane damage due to LP *Mossad et al, 1994*
- No ↑ increase in post thaw LP, nor did post thaw addition of H_2O_2 did not ↑LP *Peris, Bilodeau et al, 2007*

Benefits of antioxidant addition during cryopreservation

- **Catalase maintains motility**
  (Foote, 1967, Bilodeau et al, 1999)
- **∝- tocopherol and ascorbate → ↑ viability**
- **SOD and Catalase → ↑ embryo numbers**
  (Roca et al, 2005)
- **Ascorbate → ↑ hamster egg penetration**
  → ↑ implantation in cows

(Beconi et al, 1993, Kumar et al, 2003)

Latest Advances and Challenges

- **Cryobanking testicular tissue for prepubertal boys**
- **Testicular stem cell transplantation**
- **Clinical application and safety**

(Schlatt et al, 2000-7; reviewed by Tournaye et al, 2004)