Sperm vitrification

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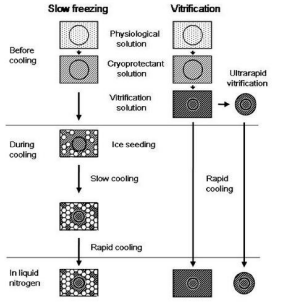

Figure 1. Schematic presentation of an embryo (circle) before cooling, during cooling and in liquid nitrogen in slow freezing, conventional straw vitrification, and ultrarapid vitrification. White hexagons represent ice crystals.

With permission from V. Isachenko

Caracteristics of vitrification

- Direct contact between of cells / tissue with liquid nitrogen
- Vitrificacion eliminates crystallization totally
- Fast methods: vitrification / thaw
- Using small volumes for freezing it is improve the rate of freezing
- Freezing rate of approx. -15 000 to -30 000 ° C / min
- Minimizes osmotic injury
- Reduces procedure time for cryopreservation (2-10 minutes)
- Simple Protocols
- Eliminates the cost of expensive programmable cooling equipment

Variables that may influence the effectiveness of vitrification

- Type and concentration of cryoprotectants (generally all cryoprotectants are toxic)
- Medium used for the maintenance of the cells
- Temperature of vitrification solutions
- The Time the cells / tissue are expose to the cryoprotectors prior to the inmersion into liquid nitrogen
- Volume of crioprotectant used on cell / tissue
- Equipment used for vitrification
- Experience of the operator
- Quality and developmental stage of cells or tissue
- Direct contact of liquid nitrogen and biological matherial can be a source of contamination during vitrification. To reduce this risk is essential to use sterile nitrogen for freezing and storage

Problems with to the present freezing methods

- Loss of motility and vitality
- Increased membrane damage
- Non-physiological acrosome reaction
- Induction of apoptosis

Vitrification of spermatozoa

Principles

- Ultra quick freezing of a small sample volume
- Direct contact with N2 → prevents ice formation (Lieberman et. Al., 2002; Isachenko et. Al., 2003)
- Physical process of solidification → no crystallizes, becomes viscous, it passes from liquid to solid state, similar to glass

Human sperm vitrification

↓

Highly effective in preserving the function and sperm motility(Isachenko et. al., *Reproduction*, 2008)

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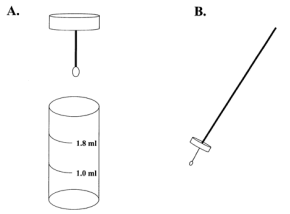
Reduce:
Osmotic damage
Time and cost

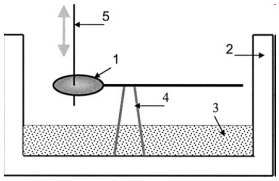
Vitrification Methods

Cryoloop used for ultrafast freezing



Schuster et al., Human Reprod 18:788, 2003.

Method in grid

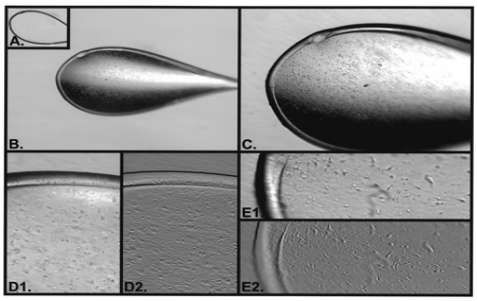


1: Copper loop with film of spermatozoa suspension, **2:** foam box,

3: liquid nitrogen, **4:** foot for loop, **5:** needle

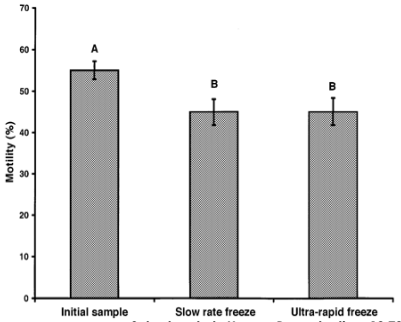
Isachenko et al., Biol Reprod 71:1167, 2004.

Sperm in Cryoloop



Schuster et al., Human Reprod 18:788, 2003.

Changes in sperm motility in old methods



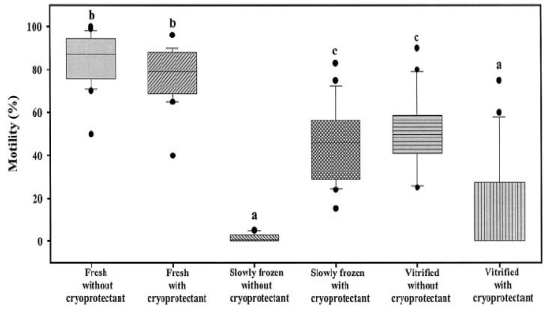
Schuster et al., Human Reproduction 18:788, 2003.

Phosphatidylserine Externalisation after cryopreservation of sperm

	Patients (n=10)			Donors (n=5)		
	Pre-Cryo	Post-Cryo	P	Pre-Cryo	Post-Cryo	P
Normal Cells	85±4	59±2	0.0001	73±5	61±3	0.0005
Annexin V positive	**11±3**	**24±2**	**0.002**	**21±4**	**30±3**	**0.001**
TUNEL (%)	13±2	14±2	0.4	5±1	5±1	0.6

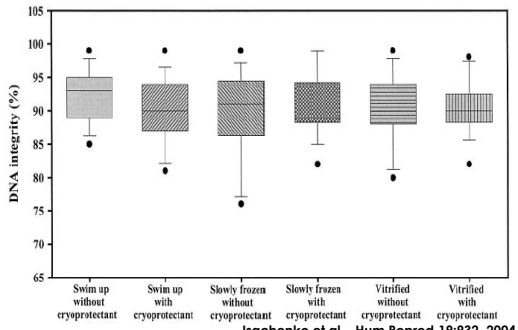
Duru et al., J Androl 22:646, 2001.

Vitrification / Slowly Freezen



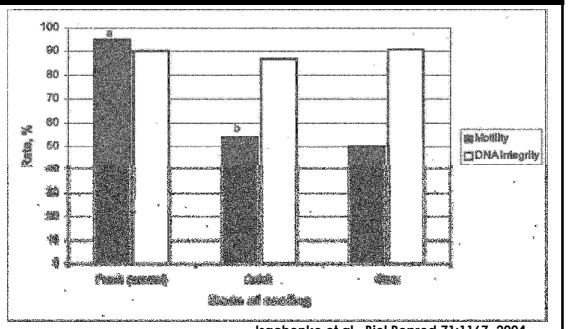
Isachenko et al., Hum Reprod 19: 932, 2004

Vitrification / Slowly Freezen



Isachenko et al., Hum Reprod 19:932, 2004

Vitrification without cryoprotectants



Isachenko et al., Biol Reprod 71:1167, 2004

Vitrification of spermatozoa and IVF

Total oocytes, n	After fresh spermatozoa (control)				After vitrification with rapid cooling				After freezing with slow cooling			
	Oocytes, n	16 h, 2PN and 3PN, n	48 h, 4-6 blastomeres, n	56 h, EB and BL, n	Oocytes, n	16 h, 2PN and 3PN, n	48 h, 4-6 blastomeres, n	56 h, EB and BL, n	Oocytes, n	16 h, 2PN and 3PN, n	48 h, 4-6 blastomeres, n	56 h, EB and BL, n
4	2	2	2 transfer	-	1	1	1	1	1	1	1	1
6	3	2	2 transfer	-	2	2	2	1	1	1	1	0
8	4	3	3 transfer	0	2	2	1	0	2	2	2	2
10	5	4	3 transfer	1	2	1	1	1	3	2	1	1
12	5	5	3 transfer	1	3	2	2	2	4	4	3	1
12	6	4	3 transfer	0	3	3	1	1	3	3	2	1
13	6	4	3 transfer	0	3	2	2	1	4	3	2	1
15	8	7	3 transfer	2	3	2	2	2	4	3	2	2

(2PN) oocytes with two pronuclei; (3PN) oocytes with three pronuclei; (EB) early blastocysts; (BL) blastocysts.
No differences between spermatozoa vitrified using quick and slow cooling (P>0.01).

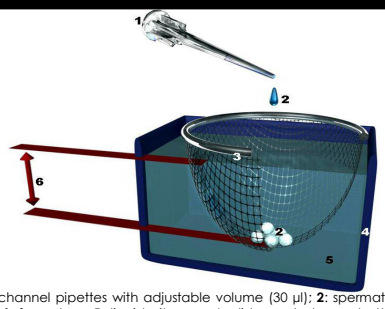
Isachenko et al., Biol Reprod 71:1167, 2004

Vitrification not aseptic of sperm

Materials and methods

- Make sperm selection by swim-up technique, migration-sedimentation or gradient centrifugation by Isolate ®. After obtaining the sperm selected to perform counting and resuspended in 150 to 200 ul medium HTF-HSA 1%.
- Add an equal volume of 0.5 M sucrose dissolved in water and maintain the sperm suspensions at 37 ° C in atmosphere of 5% CO2 for 5 minutes.
- Aliquots of 30 ml suspension of sperm are deposited directly and rapidly into the liquid N2. They form solid spheres that float and then sink to the bottom of the liquid when they have vitrified completely.
- Simultaneously place the cryotubes, and labeled with name and number of sample in contact with liquid N2, so that when they are cold storage areas.

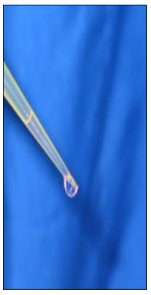
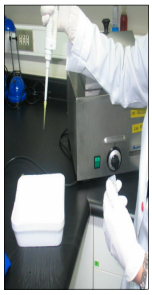
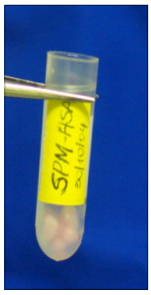
Vitrification procedure



1:single channel pipettes with adjustable volume (30 µl); **2**: spermatozoa suspension; **3**: strainer; **4**: foam box; **5**: liquid nitrogen; **6**: distance between bottom of strainer and surface of LN (minimum 3 cm).

Isachenko et al., Reproduction 136:167, 2008.

Vitrification not aseptic

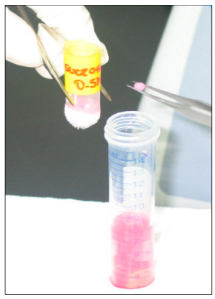
Storage

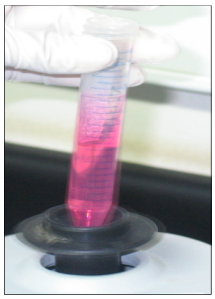
- Place the vitrified sphere in cryotubes, which must contain approximately 300 ul of liquid LN_2 inside. Stored for at least 24 h in LN_2 before to the evaluation

Devitrification

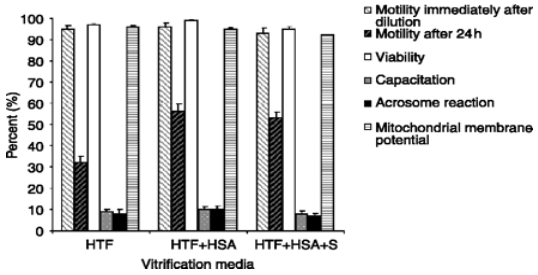
- The thawing is carried out by quickly submerging spheres one by one (not more than five spheres) into 5 ml HTF with 1% HSA pre-warmed to 37 °C accompanied by gentle vortexing for 5–10 s.
- The post-thaw sperm suspension was maintained at 37 °C/5% CO2 for 10 min and then concentrate by centrifugation at 380 *g* for 5 min. The cell pellet is finally resuspended in HTF-HSA.

Devitrification



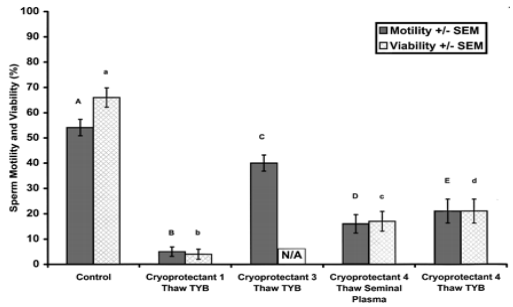


Influence of HTF alone or in combination with HSA or HSA + sucrose on sperm parameters before cryopreservation

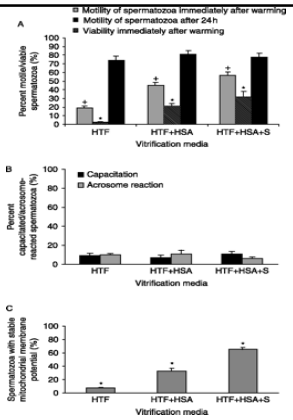


Isachenko et al., Reproduction 136:167, 2008.

Cryoprotectants in relation to motility and vitality after ultra-rapid freezing on cryoloops



Schuster et al., Human Reproduction 18:788, 2003



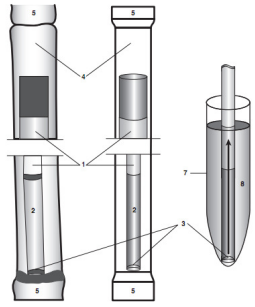
Isachenko et al., Reproduction 136:167, 2008.

Aseptic vitrification of sperm

Materials and methods

- Make sperm selection by swim-up technique, migration-sedimentation or gradient centrifugation by Isolate ®. After obtaining the sperm selected to perform counting and resuspended in 150 to 200 ul medium HTF-HSA 1%.
- Add an equal volume of 0.5 M sucrose and maintain the sperm suspensions at 37 ° C in atmosphere of 5% CO2 for balance for 5 minutes.
- Sperm are vitrified in 0.25 cc straws (0.5 x 10^6 sperm).
- Straws are sealed and are deposited directly and immediately in liquid N_2.

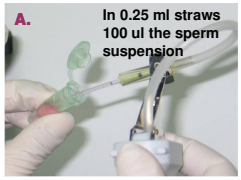
Vitrification procedure

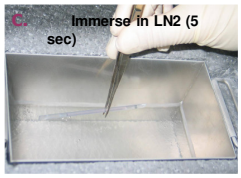


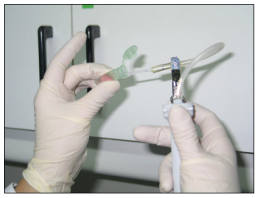
1. Internal 0.25 ml open-pulled straw, filled with 0.01 ml
2. Suspension of spermatozoa
3. Meniscus of suspension
4. 0.5 ml straws
5. Heat Sealed
6. Marked end of open-pulled straw
7. Tube for warming
8. Warming medium

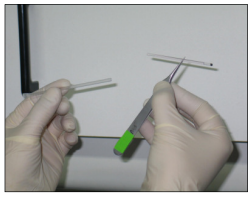
Isachenko et al., Reprod Biomed Online 10:350, 2005.

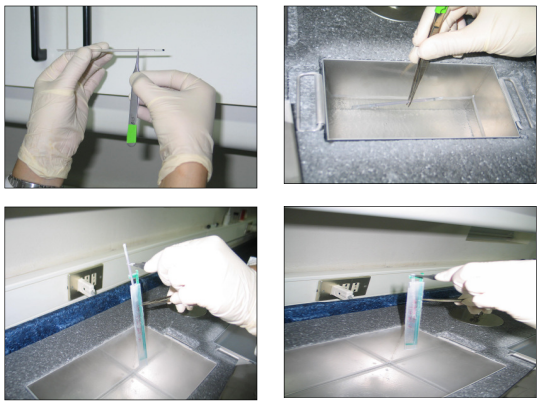
Aseptic vitrification



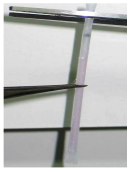








Devitrification

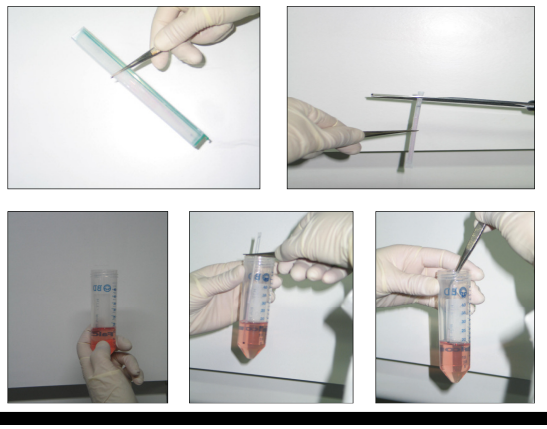


6 mL HTF-BSA 1% at 37 ° C
(tube of 15 mL)

Insert straw
0.25 cc

Centrifuge at 1800 rpm
5 minutes

Evaluation



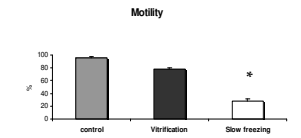
Cryopreservation of human sperm

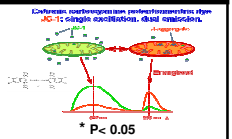
Comparison between aseptic vitrification and conventional freezing on the sperm function

Materials and methods

% Mobility Sperm	**Progressive Mobility (A) WHO**	FLOW CYTOMETRY (FACSCalibur, Becton Dickinson)
Pot.Membrane Mitochondrial	**JC-1** **Mit-E-Mitochondrial** Permeability Detection Kit, Biomol	
Plasma Membrane Integrity	**SYBR-14** LIVE/DEAD® Sperm Viability Kit Molecular Probes	
Acrosome Membrane Integrity	**PSA-FITC** Sigma L-2857	
Phosphatidyl serine Translocation	**ANEXINA V-FITC** APOPTEST™. FITC Nexins Research, The Netherlands	
DNA Fragmentation	**TUNEL** In Situ Cell Death Detecction Kit ROCHE	

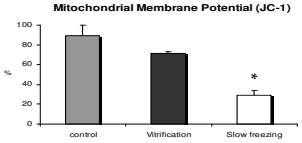
Results

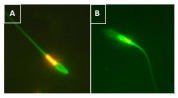




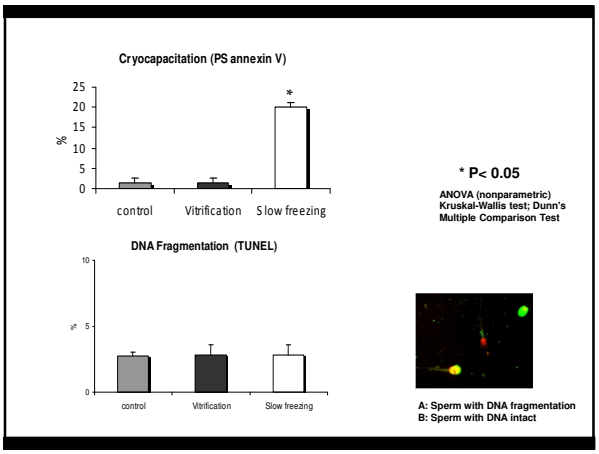
* P< 0.05

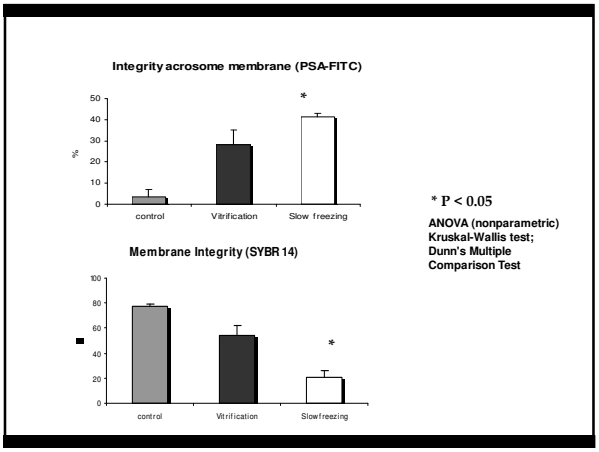
ANOVA (nonparametric) Kruskal-Wallis test; Dunn's Multiple Comparison Test

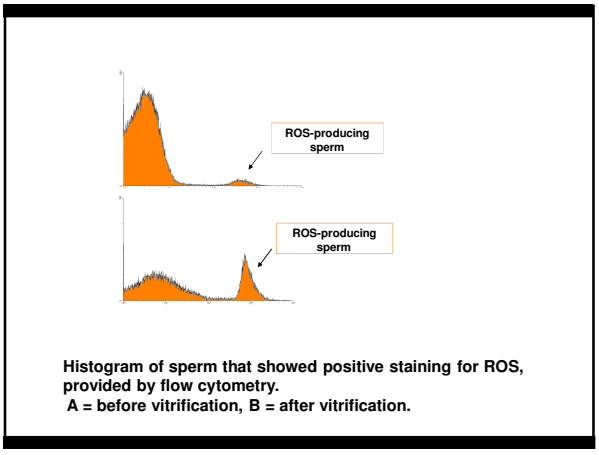




A: Sperm with ΔΨMMit intact
B: Sperm with ΔΨMMit decreased







Histogram of sperm that showed positive staining for ROS, provided by flow cytometry.
A = before vitrification, B = after vitrification.

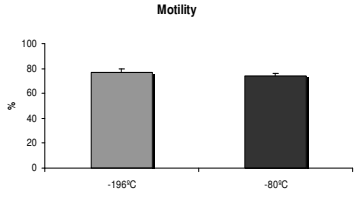
Conclusions

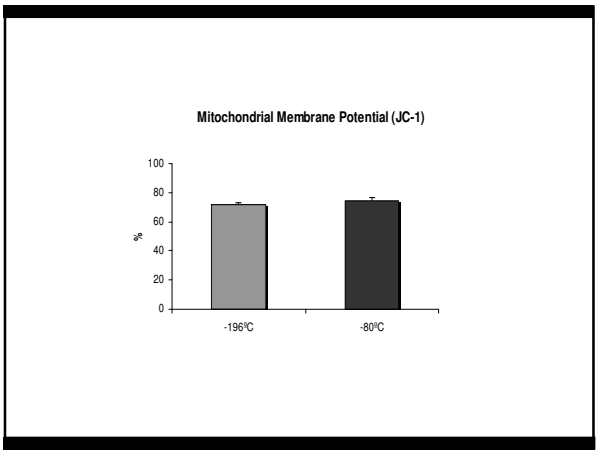
- **Aseptic vitrification can be used in the laboratory of reproductive medicine**
- **The vitrification technique using media supplemented with albumin and sucrose allow cryopreservation of sperm of different species of mammals**
- **This technique is simple, fast and low cost can be an efficient alternative for cryobanks**
- **The use of antioxidants may be beneficial for the reduction of ROS**
- **To use the sample immediately reduces the risk of DNA fragmentation that occurs more frequently in cryopreserved sperm (testicular biopsy)**

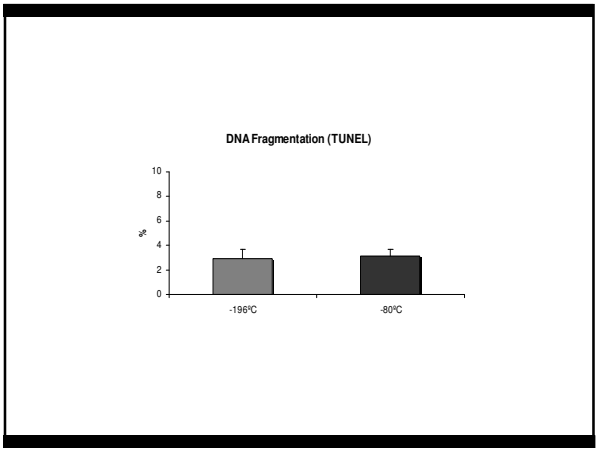
Vitrification of human spermatozoa:

Comparisons between storage -196 °C and -80 °C

Results







Conclusions

- **Aseptic vitrified sperm can be preserved - 86 º C**
- **This will simplify the storage of samples, especially in individuals who want to preserve their future fertility**
- **This will simplify the storage of samples, reduced storage space, less time and effort to find stored samples and safer for the operator.(burns, spills of liquid nitrogen when refilling, proper airing)**
- **It generate a significant decrease in the cost of storage of samples, especially in patients that allows them OAT with IUI cycles prior to ICSI**

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- Supported by FONCECYT 1070594, CONICYT, Chile.