Cryoprotectant, freezing/thawing, packing, storage and transportation of frozen samples

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Disclosures of commercial and/or financial relationships

- I have no commercial and/or financial relationships with manufacturers of pharmaceuticals, laboratory supplies and/or medical devices scrutinized in this lecture.
- I am the supervisor of Emma Holmes, who is employed by Nidacon.

Disclosures of interest 2

- I do have concern for both the children and the grandchildren to be.

Learning objectives 1

- That cryoprotectant agents means addition of particles
- That addition of particles increase the osmolarity surrounding the spermatozoa
- That increased osmolarity results in water transport out from the spermatozoa
- That optimal freezing means that (1) ice-lakes of pure water are formed and (2) that particles & spermatozoa are localized in " veins" of cryoprotectant and with high osmolarity.

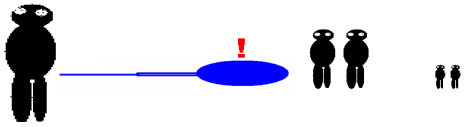
Learning objectives 2

- That osmotic shock (hyper-or hypo-) and uncontrolled crystallization can damage membranes and organelles
- That thawing means that ice-lakes melt, osmolarity in veins decrease and water flux into the spermatozoon.

Learning objectives 3

- That the post-thaw results are influenced by the prevailing osmolarity in semen.
- That semen osmolarity varies between samples from the same man
- That osmolarity is a function of "todays" relative relation between fluid from the prostate, the seminal vesicles and time after ejaculation.
- That spermatozoa are ejaculated with prostatic fluid
- That the water content of the sperm nucleus may be influenced by " todays" sequence of ejaculation.

The spermatozoon is a messenger cell carrying messages for healthy grand-children.



And, a hit in a spermatozoon in the epididymis or the test tube today…

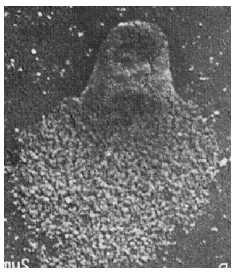
May result in a grand-child with an unbalanced translocation with 

impaired psychomotor development and malformations

dual roles

RESIST-Protect

DELIVER



FREEZING –
preservation something fresh without keeping its biological function

CRYOBIOLOGY – study of life at low temperature

CRYOPRESERVATION – stabilizing cells at cryogenic temperatures

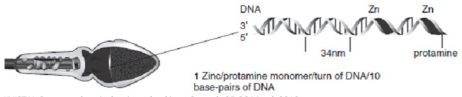
Cryopreservation

- **OBJECTIVE of CRYOPRESERVATION**

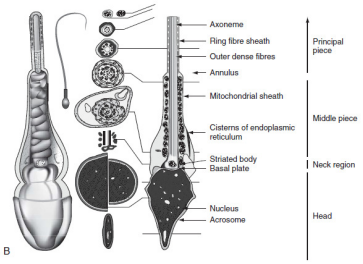
  to minimize damage to biological materials during freezing and low temperature storage

- **BASIC PRINCIPLE of CRYOBIOLOGY**

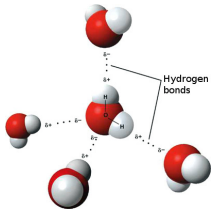
  the extent of freezing damage depends on amount of free water in the system and the ability of the water to crystallize during freezing

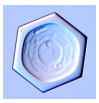


The amount of free water may vary in different compartments, between different spermatozoa in the same sample, between samples from the same man, and between men.



Courtesy by AF Holstein



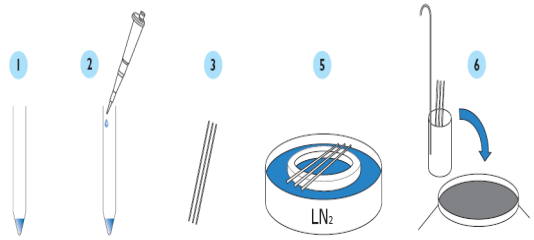


Hexagonal prism

Free water molecules bind to each other ….

ice is formed

The process



CBS™ High Security sperm straws

System for sealing CBS™ High Security straws

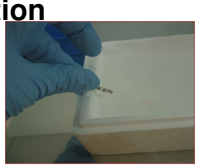
http://www.cryobiosystem-imv.com

Freezing methods
Slow programmed cooling & Vitrification

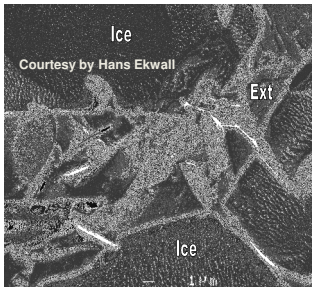
http://www.cryologic.com/

FREEZE CONTROL®

CryoPreservation Systems

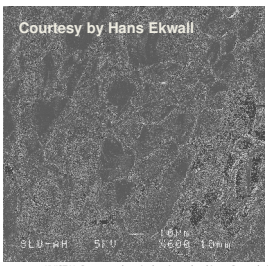


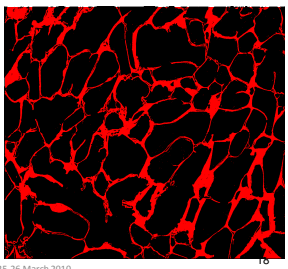
Higher magnification of the sample showing lakes, veins and spermatozoa



- Lakes:
  - extracellularly frozen free-water
- Veins:
  - containing frozen extender, frozen bound-water, frozen solutes, and frozen spermatozoa

Image analysis, to quantify the relative distribution of lakes and veins





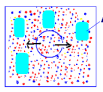
Cryoprotectant solutions

- **Cryoprotective agents (CPA):**
permeable - Glycerol, Propane diol, DMSO, EG …
non-permeable: sugars - Sucrose, Trehalose;
polymers- PVP, PEG; PEO …
- **Other components:** proteins - human serum, HSA, FCS, egg-yolk,
vitamins, antibiotics, antioxidants, vitamins …
- **Basic medium:** DMEM/F12, PBS, RPMI, IVF medium,
Earle's medium, Leibovitz medium …

Courtesy by Victoria Keros

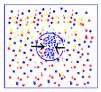
Freezing problems Cell damage

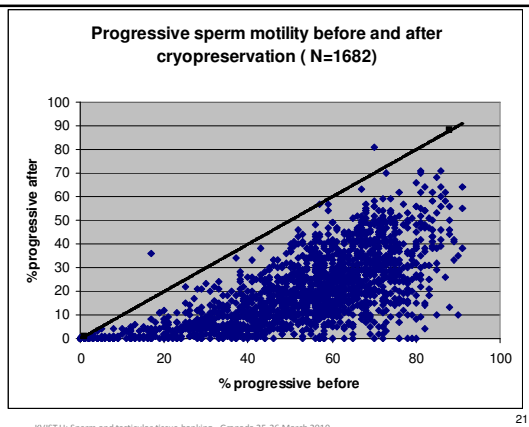
Freezing:
Crystal formation
Osmotic shock (dehydration-shrinking)



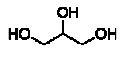
- physical destruction of membranes
- organelle disruption

Thawing:
Osmotic shock (rehydration-swelling)
Recrystallization



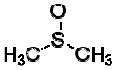


Different Cryoprotectants



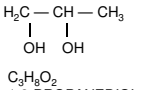
$C_3H_5(OH)_3$
Glycerol
(mol wt . 92.09)

$(C_6H_9NO)_n$
Polyvinylpyrrolidone (PVP)

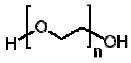


C_2H_6OS
DMSO
(mol wt 78.13)
Dimethylsulfoxide

$HOCH_2CH_2OH$
Ethyleneglycol (EG)
mol wt 62.07

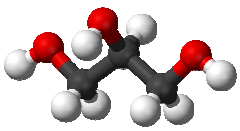


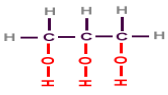
$C_3H_8O_2$
1,2-PROPANEDIOL
mol wt 76.10
(=Propylene glycol (PG))



$(-CH_2CH_2O-)_n$
Polyethylene oxide (PEO)

Glycerol - Most commonly used for semen cryopreservation



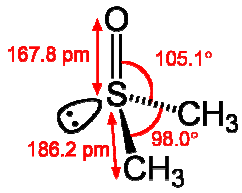


Glycerol
propane1,2,3 triol

Molar mass 92.09382 g/mol

Used by us here:
Glycerol 0,97M (=8,9 g%)
Glukos 2,2 mM
Penicillin G (20 000 IE/mL)
in PBS

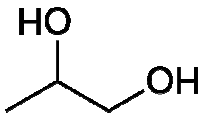
Dimethylsulfoxide



SO(CH3)2

Melting point: 18,5 °C

Propylene glycol (PG)



C3H6(OH)2

Meltingpoint: -59 °C

1,2-Propandiol

ethyleneglycol,

$C_2H_4(OH)_2$

Meltingpoint: -13 °C

1,1132 g/cm^3

1,2-etandiol, **Etyleneglycol, Dihydroxietane, Monoetyleneglycol**

Cooling rates

- **Slow rate cooling**
- **- un-programmed: - holding on/in ice, refrigerator**
  → frozen in nitrogen vapour
  ↓ liquid nitrogen
- **- programmed rate -controlled freezing: Planer Kryo, Cryologic**

- **Rapid cooling rate** - **plunging directly into liquid nitrogen – re-crystallization effect**

- **Vitrification – rapid cooling + high concentrations of CPA**

Storage

The lower the temperature - the longer the viable storage period

+ 4℃ 6-48 hour
- - 70℃ months – years
- -130℃ *"glass transition of water"*
  (for spermatozoa <-130℃)
- -196℃ LN2 - *eternity*

- **Liquid Phase Storage** offers a uniform temperature (-196°C)
  - liquid nitrogen enters the package
  - sample contamination
- **Vapour Phase Storage** offers a temperature gradient (-70 °C to -100°C)

From a theoretical perspective though, it is likely that relatively long-term storage should be possible

At temperatures below –130℃ (the glassy transformation temperature),

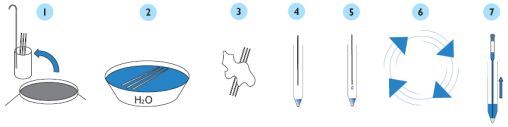
beyond which ice-crystal growth and hence recrystallization is apparently impossible .

Water bound to proteins stop moving.(Meryman, 1956)

Thawing / warming

Slow or rapid?

Rapid is preferable!
- water bath 37-40°C to avoid recrystallization



GMP quality for clinical samples

- Sterile environment
- Sterile materials and substances
- No animal derived products
- Long-term storage in a closed system

Transport of samples

- 1) A formal request from the treating clinic
- a certified tissue bank (ICSI, IVF) or
- treating clinic (insemination).
  Identity of the man, amount of samples, date of treatment
- The request is answered.

- 2) Patients agreement, witnessed (not partner).
- (a) out-take (b) transporter (c) the receiver.

Transport of samples

- 3) Verification from the transporter purpose of LN2 dry shipper transport, latest date for delivery
- 4) Verification from the treating clinic arrival, and in acceptable condition.
- Acceptable conditions? (days?; inspection ok?; measurements on arrival ?, monitoring of temperature?

Transport in dry-shipper loaded with absored LN2 –

never dry ice!!

"glass transition of water"

Dry shipper .

	Cryo Shipper Mini
Performance	
Liquid Nitrogen capacity (liters)	5.9
Static Evaporation Rate (liters/day)	0.84
Static Holding Time (days)	7
Unit Dimensions	
Neck Opening (in/mm)	8.5
Overall Height (in/mm)	20
Outside Diameter (in/mm)	11.63
Weight Empty (lbs/Kg)	16.5
Weight Full (lbs/Kg)	34.25
Cavity Size	216 x 51 x 254
Plastic Shipping Container Weight	10.91
Plastic Shipping Container Dimensions (d x h mm)	216 x 610

10.90 Kg

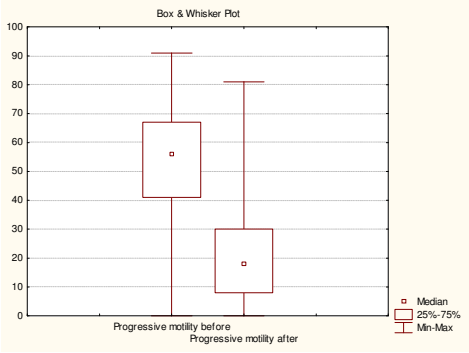
http://shop.planer.co.uk/product

Features	VOYAGEUR 2
Effective capacity (l)	1,75
Absorbed capacity (l)	1,35
Diameter of neck (mm))	30
Weight empty (kg)	2,4
Weight full (kg)	3,5
External diameter (mm)	174
Total height (mm)	395
Daily evaporation (l/D)	0,1
Dynamic holding time (d)**	8
Number of canisters	2
Diameter of canisters (mm)	26
Height of canisters (mm)	120
Number of level of goblets	1
Total capacity 0.25ml straws	220
Total capacity 0.5ml straws	100

Dry shipper

http://www.dmc.airliquide.com/file/otherelement/pj/voyageur-gb11864.pdf

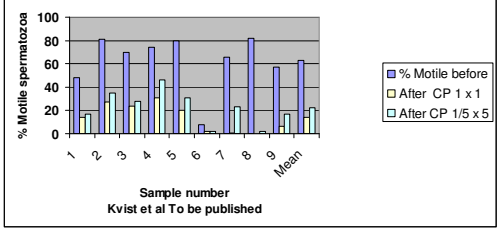
Known Problems: Drop in Motility & Vitality



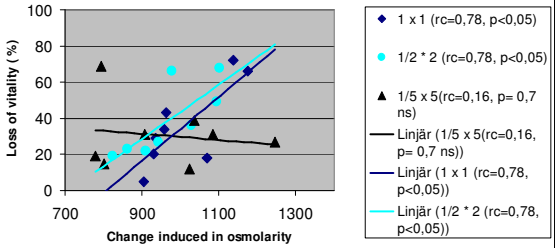
Osmolarity

- Adding particles (cryo-protectant) affects semen osmolarity and may result in an hyperosmotic shock.
- The osmolarity in semen.

Step-wise addition of CP counteract loss of motility after Freeze-thawing (p<0,01).

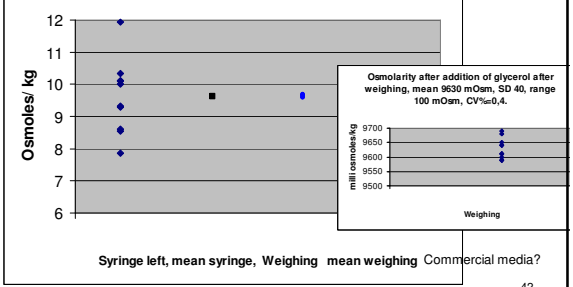


Loss in vitality in relation to change in osmolarity induced by addition of CP.



Kvist et al to be published 2010

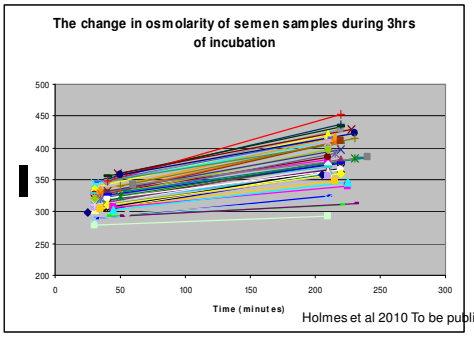
Osmolarity after addition of glycerol by volume (syringe CV 12%, range 4000 mOsm) or weighing (CV 0,4%, range 100 mOsm).



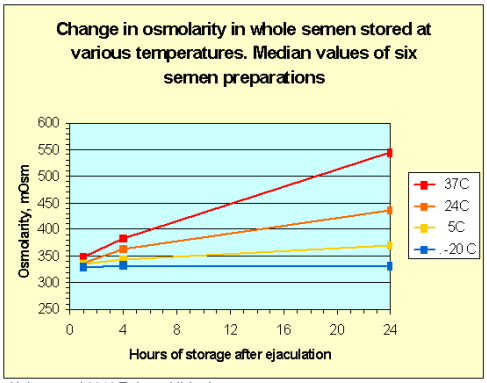
Commercial media?

Does the osmolarity of semen play a role for cryo-preservation?

Osmolarity increases after ejaculation – and the increment varies between patients!

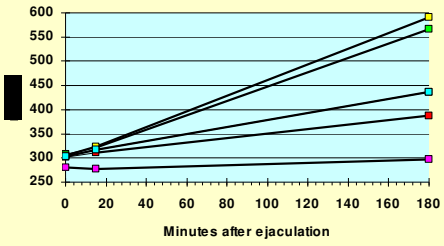


Holmes et al 2010 To be published



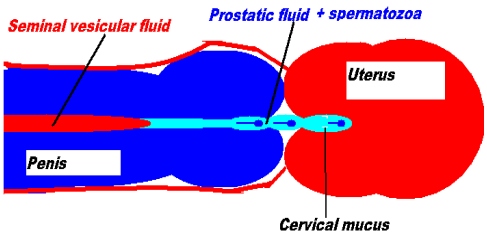
Holmes et al 2010 To be published

Change in osmolarity in first split ejaculate fraction from 5 donors. Samples incubated at 37C from < 5 min after ejaculation (281-308) until 3 hours.



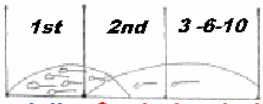
Holmes et al 2010 To be published

Man offers the woman spermatozoa in prostatic fluid Physiology of ejaculation



Ejaculatory sequence

1949



Prostatic fluid ***Seminal vesicular fluid***

No gel
Vitality +
Motility +

Chromatin stability +
Chromatin zinc +

Gel
Motility-
Vitality-

Chromatin zinc -
Chromatin stability -- ++ (SS)

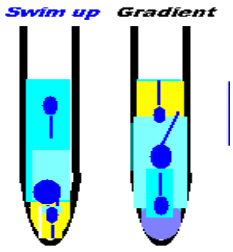
On osmolarity

- Spermatozoa are expelled in prostatic fluid at ejaculation in an environment that appears to be isotonic, 290 mOsm/l.
- Spermatozoa in whole liquefying semen are trapped in an unphysiological artifact - created by the laboratory.
- After ejaculation osmolarity increases rapidly.

More on semen osmolarity

- The increment is an individual property of every given semen sample and is related to its contribution of prostatic fluid and to storage temperature.
- Individually increased osmolarity, affects the response to cryo-preservation procedure.

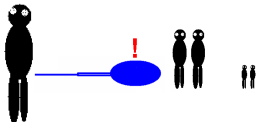
And procedures involving hypotonic shock as ……



What more than Motility and Vitality may be affected?

- Motility and Vitality are functions of the sperm as a messenger!

- Healthy grandchildren are the result of intact messages.
- Can these be harmed?



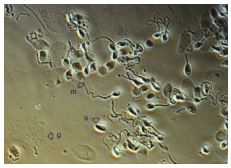
The Messages are **security locked**

- **The intact DNA – the genome**
- Structural defects
- Numerical defects
- DNA- strand – breaks, DNA-adducts..
- **The "normal" epigenetics**
- Protamines in place protecting and silencing > 95% of the genome
- "The normal" Methylation of paternal DNA
- "The normal" Acetylation and Methylation of Sperm Histones
- The sperm RNA
- The sperm nuclear Proteins
- The paternal centrosome
- The Factors initiating the placenta

The nucleus: dual roles resist and deliver zinc lock

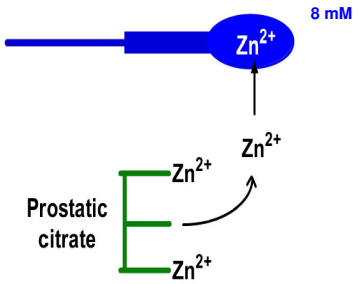
- 1878 Miescher Sulfonuklein
- In contrast the sperm rapidly decondenses in the oocyte
- **< 5min after ejaculation**

90% decondensed If exposed to zinc-chelating EDTA

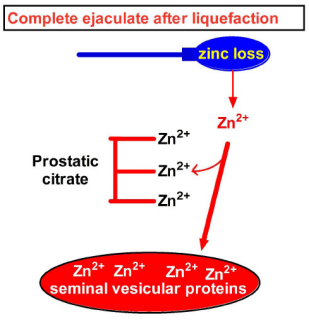


- **Paternal effect on genomic activation, clinical pregnancy and live birth rate after ICSI with cryopreserved epididymal versus testicular spermatozoaReprod Biol**
- **Endocrinol. 2009; 7: 142. Published online 2009 December 3. doi: 10.1186/1477-7827-7-142.**
- Nina Desai,1 Faten AbdelHafez,1 Edmund Sabanegh,2 and James Goldfarb1
- For cryopreservation, ES and TESE samples were diluted 1:1 with test **yolk** buffer-glycerol cryoprotectant (Irvine) and aliquotted into cryovials. Vials were vapor frozen for 30 minutes prior to immersion in liquid nitrogen.

Chromatin zinc is retained by prostatic fluid



Chromatin zinc is depleted by Seminal vesicular fluid



- Loss of zinc means a destabilization
- that may increase the amount of free water within the nucleus and
- create additional problems for cryopreservation.

Vesicular fluid chelates chromatin zinc

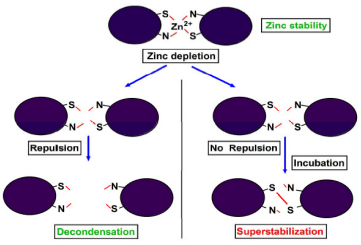
- Spermatozoa expelled in vesicular fluid at ejaculation reveal lower zinc content in the chromatin (Björndahl, 1990).
- Spermatozoa incubated in seminal vesicular fluid loose zinc

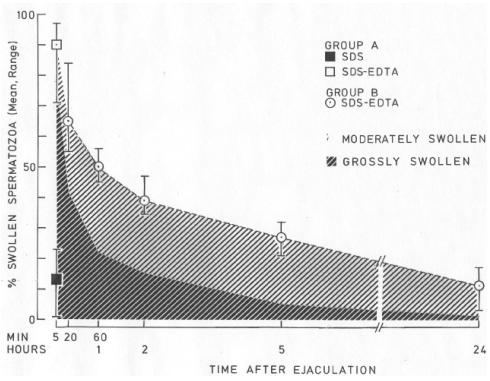
Dual actions by Zinc:
(1) stabilizes the structure and (2) prevents oxidation
Removal of zinc gives two possibilities!

immediate **decondensation** otherwise **superstabilization**

CAVE!

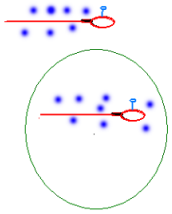
This is just a brain model!





With ICSI, a new era of man-induced evolution started 1991 - all messenger functions were by-passed by the injecting embryologist

Intra cytoplasmic sperm injection
- means that the sperm membrane + medium enters the ooplasm



Calls for controlled and traceable conditions excluding factors modulating genetics

Freezing Medium - TYB with Glycerol & Gentamicin, Catalog #90128
for cryoprotection when freezing human sperm
- 20% egg yolk - from USDA certified SPF (Virus Free) laying flocks, heat inactivated at 56°C for 30 minutes
- 12% v/v Glycerol
- 10 µg/mL Gentamicin Sulfate
- Available in 20 x 5 mL and 100 mL configurations
- Store below -10°C
- Shelf life: 2 years from date of manufacture

Sperm Maintenance Medium*, Catalog #99176
for cryoprotection when freezing human sperm
- 28% v/v Glycerol without egg yolk
- Available in 100 mL configuration
- without antibiotics
- Store below -10°C
- 20 mg/ml (2%) Human Serum Albumin
- Shelf life: 2 years from date of manufacture

FROM:
www.irvinesci.com

Sperm Freezing Medium

Sperm Freezing Medium is for the cryopreservation of human spermatozoa and tissue from testicular biopsies.

Background
Cryopreservation of spermatozoa serves to augment the success of assisted conception treatments for infertile couples.

The use of biologically derived components in freezing media may give rise to batch to batch variation and the potential for inconsistent product performance.

Sperm Freezing Medium is ready-to-use and free of undefined biological material, and therefore exhibits uniform product performance. The product is a modification and further optimisation of the formulation described by Mahadevan & Trounson 1983.

Sperm Freezing Medium is CE marked.

MediCult
Innovation with Care

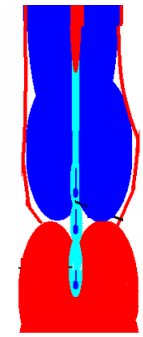
Package Info

From www.nidacon.com

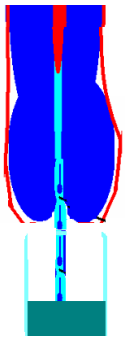
- **Sperm CryoProtec™ II** **3+1**
- Sodium chloride
  - Magnesium sulphate
  - Potassium dihydrogen phosphate
  - Glycerol
  - Sodium bicarbonate
  - H2O
  - Potassium chloride
  - Glucose
  - Lactate
  - Pyruvate
  - EDTA
  - HEPES

- ***From www.cryosinternational.com***
- **The SpermCryo™** medium has been manufactured and used by Cryos since 1987 for the freezing of more than 150,000 ejaculates from donors as well as depositor (cancer patients, etc.). More than 12,000 DI-pregnancies and thousands of AIH-pregnancies have been achieved by use of sperm frozen with this low dilution medium without proteins or antibiotics.
- **SpermCryo™ All-round**: Gives a 30% better survival rate and can be used for freezing of all kinds of semen whether it is oligospermia, teratospermia, athenospermia, normospermia, raw semen or spermatozoa that has been purified with a swim-up or a gradient centrifugation and testicular aspirated spermatozoa.
  Delivered in sterile bottles with teflon-coated rubber-membrane.
  Shelf life: 1 years from production date at 2-8°C.

? Composed of?



Collected and selected directly at ejaculation



Why not collect and select directly at ejaculation?