Cryopreservation of mammalian spermatozoa

허용수

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History of sperm cryopreservation

Luyet (1937) : sperm + fructose → −70 ℃ (dry ice)

Philips & Lardy (1940) : sperm + egg yolk

Polge (1949) : sperm + glycerol → revival spermatozoa

Bunge & Sherman (1953) : fertility capacity

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Reasons for improved freezing technique of sperm

- Testicular sperm, epididymal sperm, small number of sperm → need high recovery rate
- Poor quality sperm donor ↑ → reducing sperm donor
- Cancer patients → cytotoxic chemotherapy, radiotherapy → lead to testicular failure, ejaculatory dysfunction, unreconstructable obstruction
- Non-malignant diseases → kidney disorders, diabetes, ulcerative colitis → immunosuppressive or cytotoxic therapy
- Animal AI → high dose of cryopreserved sperm → fertility rates similar to fresh sperm

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Particularities of the sperm cell

- ✓ Haploid cell
- ✓ Highly condensed chromosome
- ✓ Devoid of cytoplasm and other cellular organelles (except nucleus, acrosome, mitochondria)
- ✓ Very little endoplasmic reticulum and golgi apparatus

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Unique structure of the sperm cell

✓ Surface area : volume ratio is very large (120μm^2: 28,5~35.0 μm^3)

✓ Cytoplasmic volume is small and has a high protein composition and relatively low water content

✓ Cellular permeability to water and cryoprotectant is high

✓ Heterogeneous mixture (shape, maturation status, functional potential)

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Cell damages after freezing and thawing

✓ Temperature reduction → cold shock, dehydration, ice crystal → physical damage of cellular membrane

✓ Cryoprotectant agent (CPA) → osmotic shock → negative influence on the genetic material

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Cryoprotectant agent (CPA)

- Permeable CPA

Glycerol, ethylene glycol, dimethyle sulphoxide, propylene glycol.

- Impermeable CPA

Sucrose, trehalose, fructose, dextran
Ficoll, polyvinylpyrrolidone, polyethylene glycol

- Cytotoxicity, Glass transition temperature (T_g), Viscosity

(saha et al., 1996)

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Egg yolk

Protective effect is not entirely clear

Complex mixture (Cholesterol, phospholipids, antioxidants)

Reduce deleterious effects of hyperosmotic salt solutions on membrane structures during cooling

Fortification of the cell membrane by the lipid components of the egg yolk → Some of these components (low-density lipoprotein, glycolipids, cholesterol) may become incorporated into the membranes, reducing their tendency to gel during cooling

Yolk-tris-glycerol → efficient radical scavenger

(Ostashko et al., 1978)

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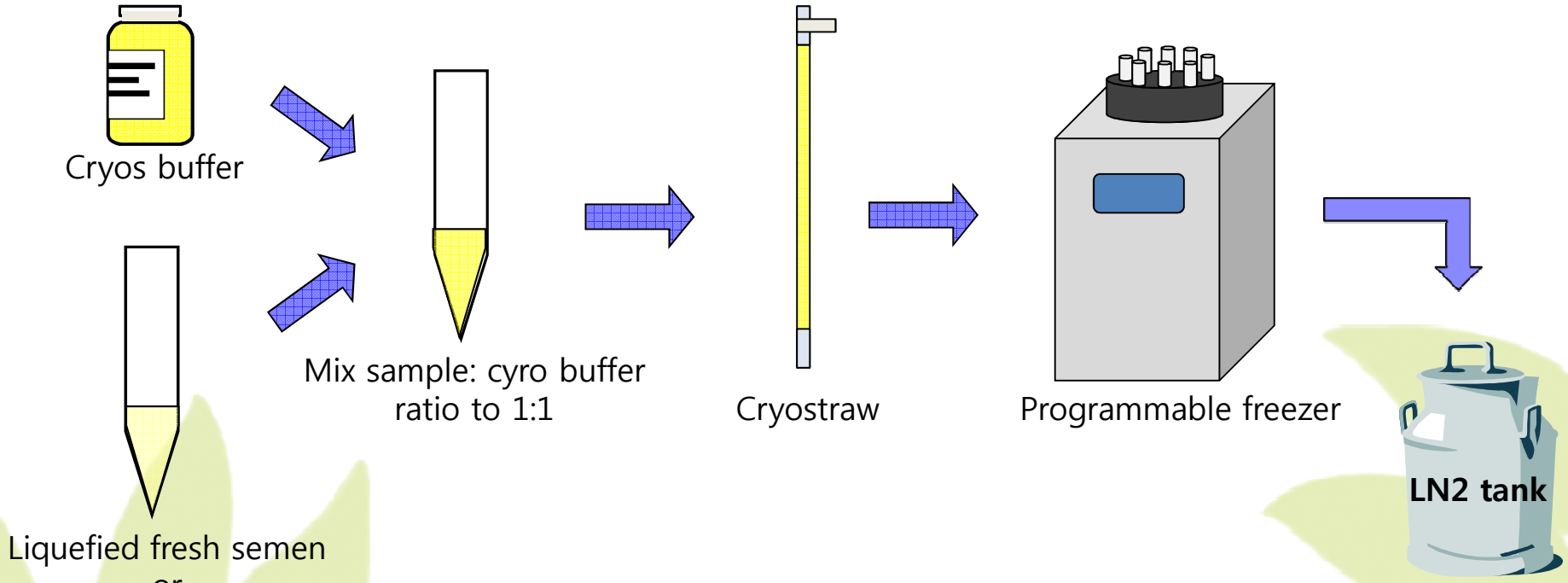
Slow freezing and rapid freezing I

Slow freezing

(slow cooling rate, low concentration CPA 5~7%)

R.T 1~2 ℃ /min → -7 ℃ nucleation → 10 ℃ /min → -100 ℃ plunge into LN_2



IrvineScientific manual

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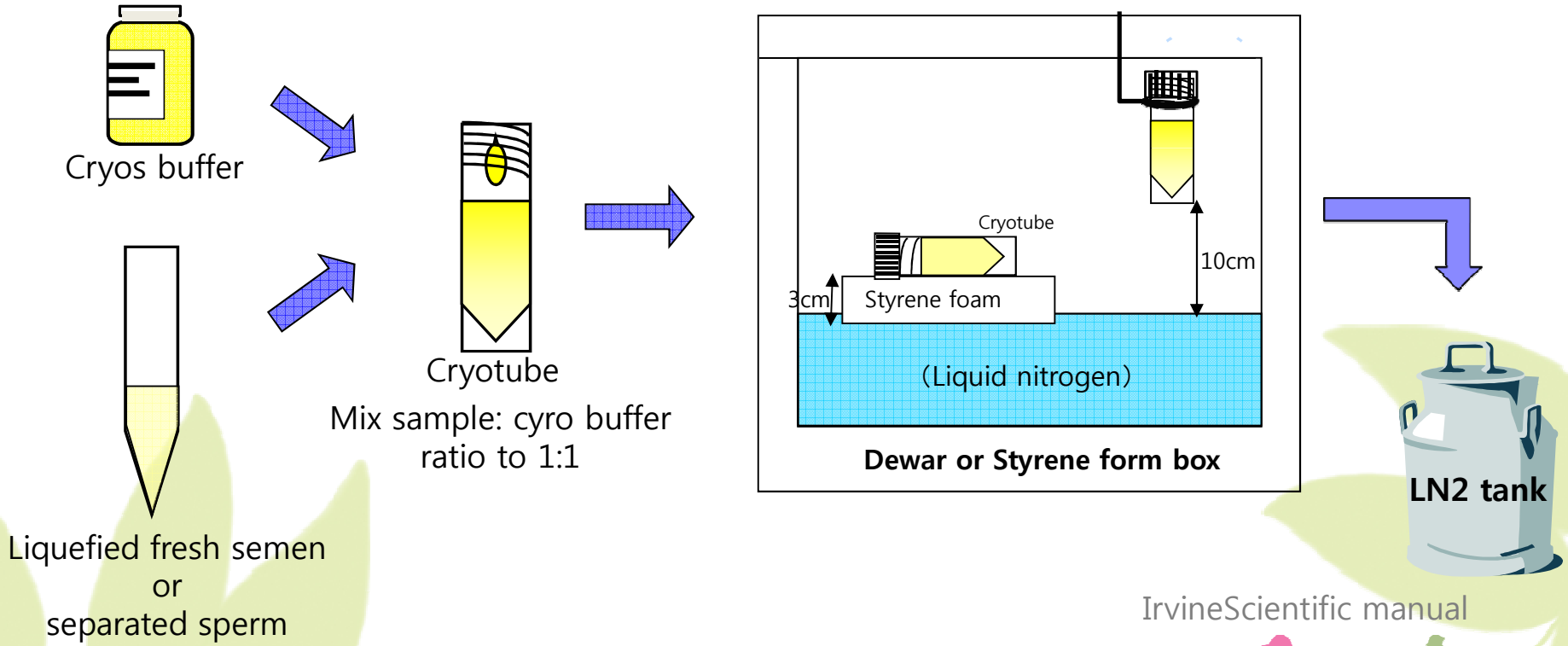
Slow freezing and rapid freezing II

Rapid freezing

(high cooling rate >100 ℃/min, high concentration CPA 30~50%)

10cm above LN_2 or −80 ℃ for 15min → plunge into LN_2



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Optimum cooling rate I

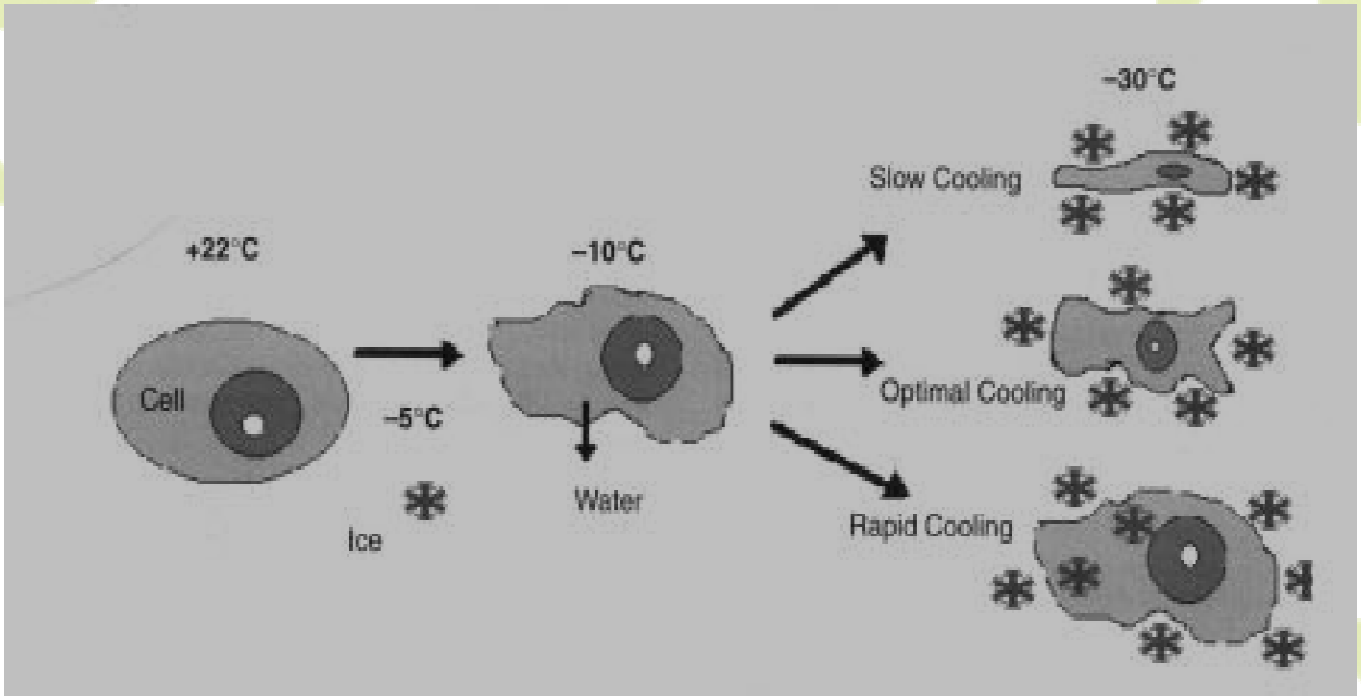

Figure Schematic drawing of physical events in cells during freezing
(Gao et al., 2000)

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Optimum cooling rate II

❖ The optimal rate of cooling is cell-type specific, depending on CPA and water permeability

(Sperm cooling → 10,000 ℃/min → intracellular ice crystal → survival rate ↓; Gao et al., 1997)

❖ There are very few reports on the effect of cooling rate on sperm survival

❖ A broad response curve exists with little difference in survival observed following cooling at 1 ℃/min up to 100 ℃/min (Henry et al., 1993)

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Sperm cooling

Sperm freezing → hyperosmotic stress →

high water permeability → loss water very rapidly →

cellular shrinkage is not severe →

intracellular ice formation maybe restricted

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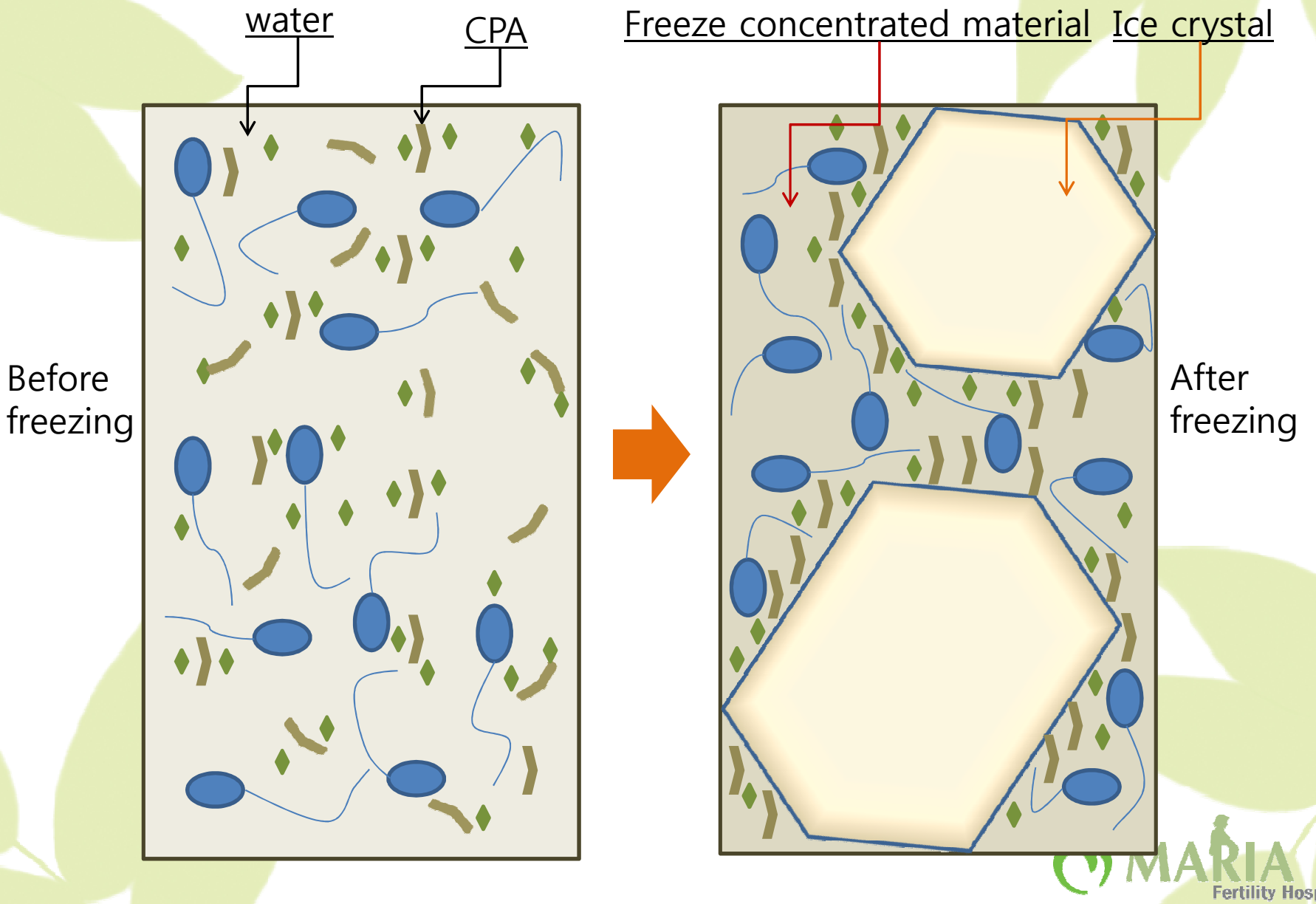
ice crystal formation



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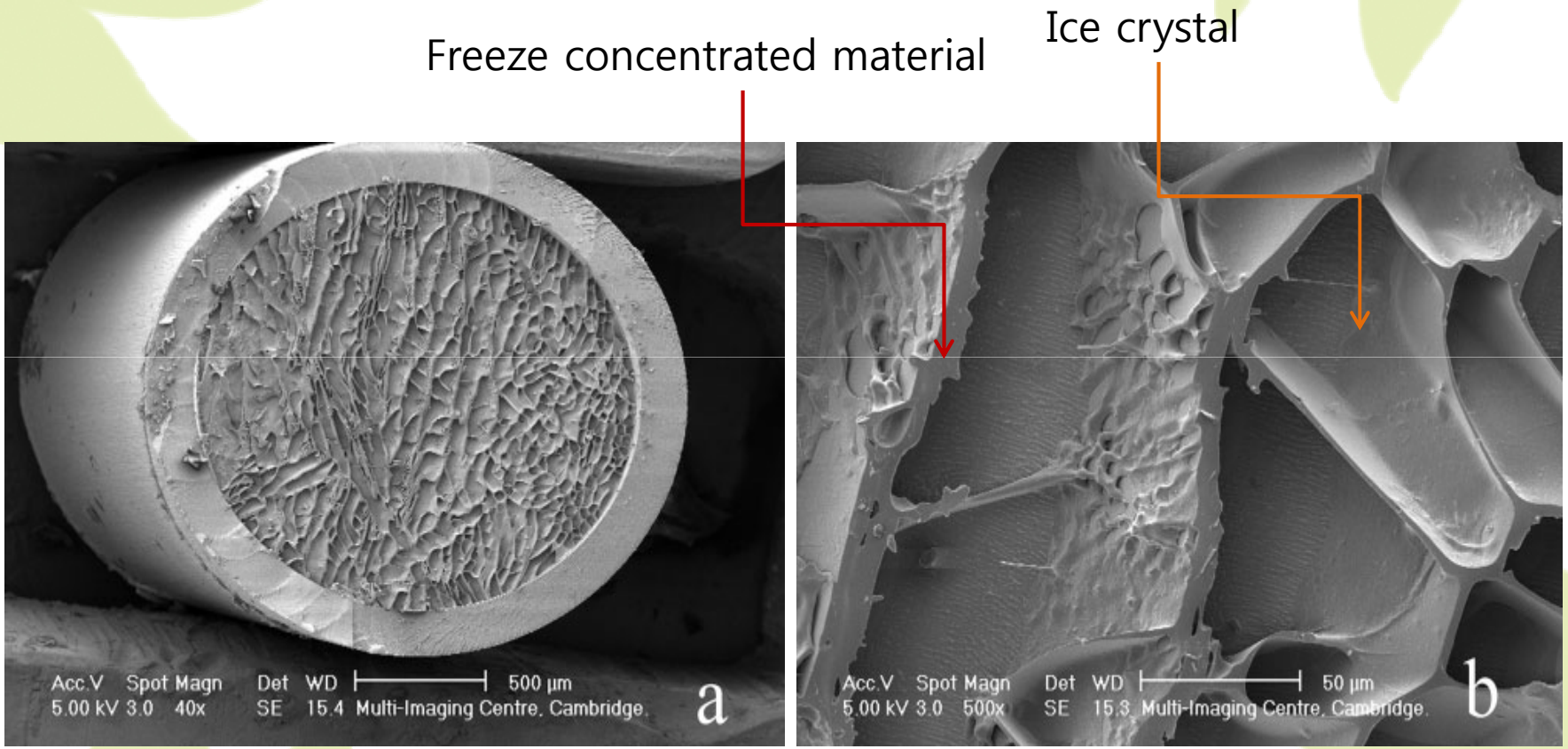
Cryo-Scanning electron microscopy (SEM)



(Morris 2006)

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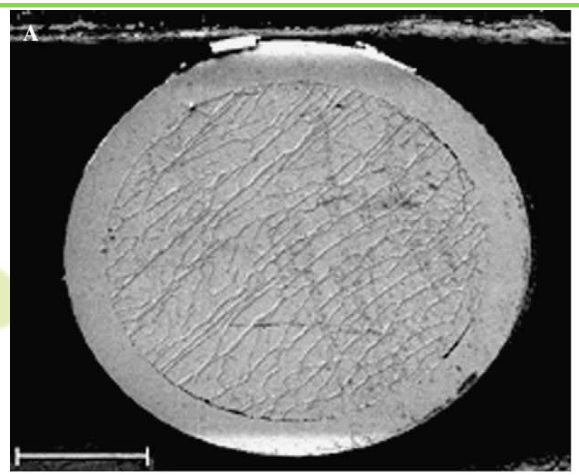
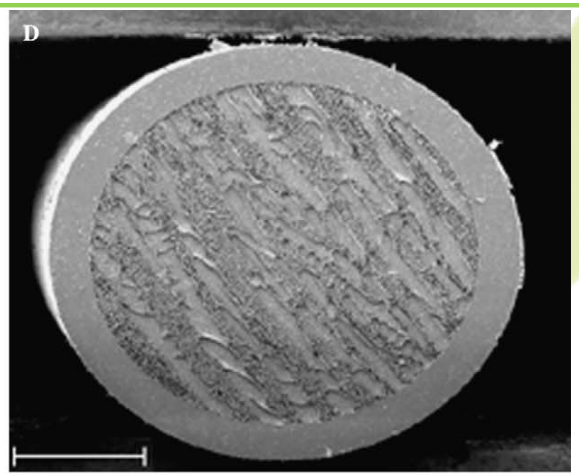
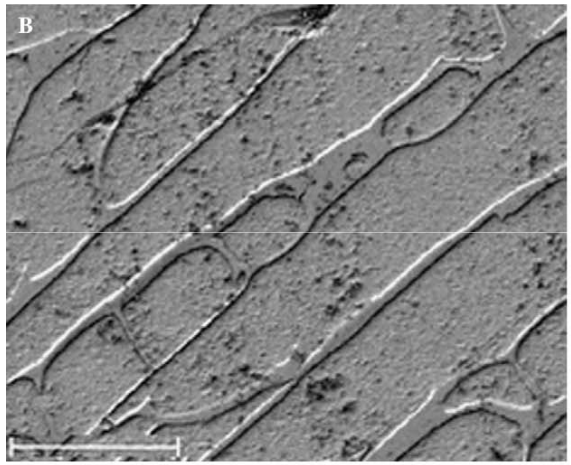
Slow cooling rate (10 ℃ /min)

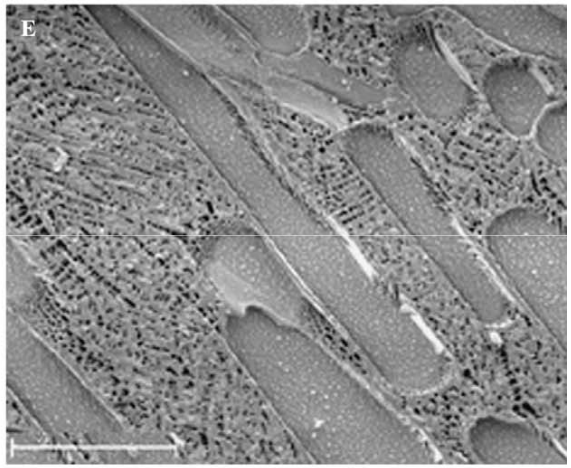




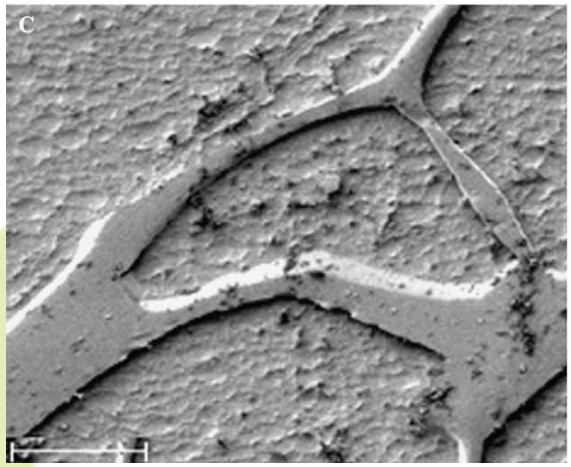
Rapid cooling rate (1000 ℃ /min)

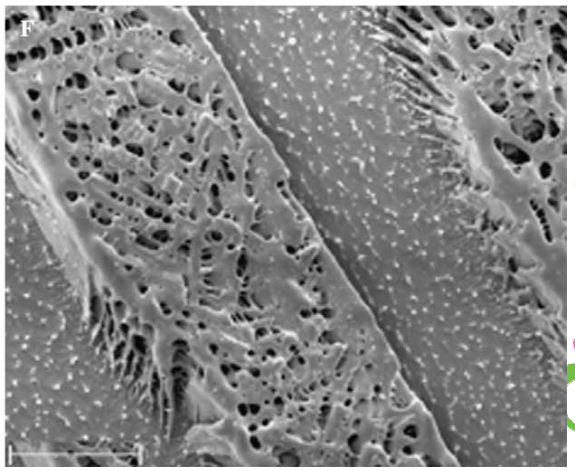
Ice vol. : 85%





Ice vol. : 50%





(Morris 2006)

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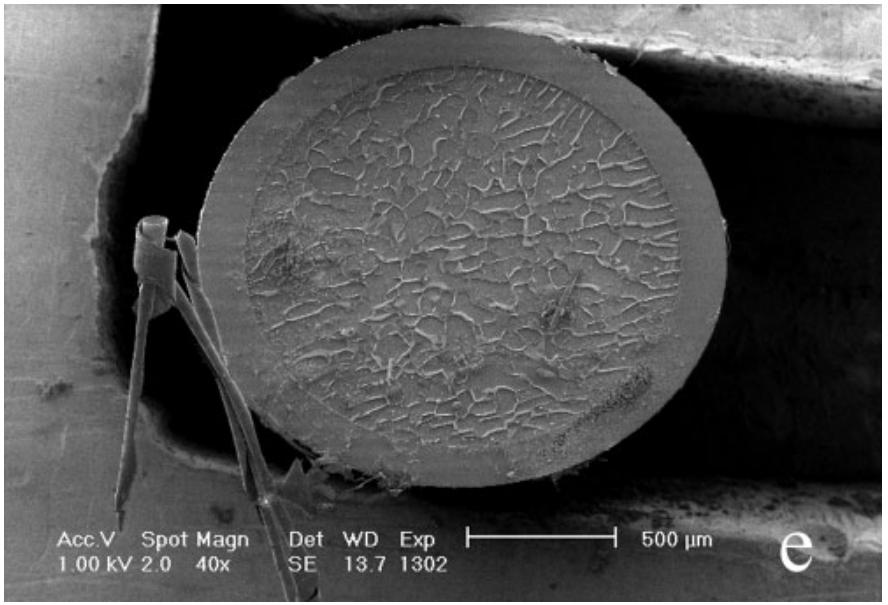
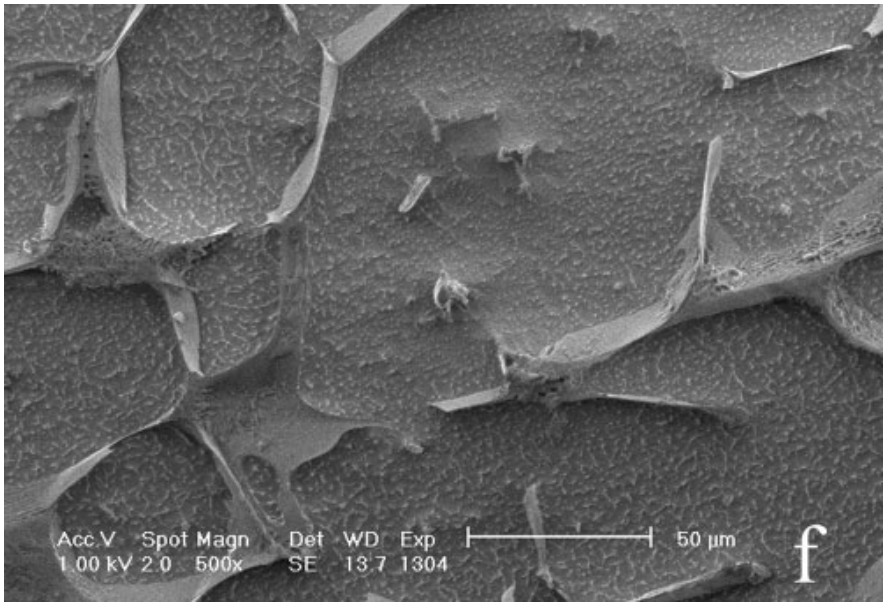
CPA (X)
Slow cooling rate
(10 ℃ /min)





(Morris 2006)

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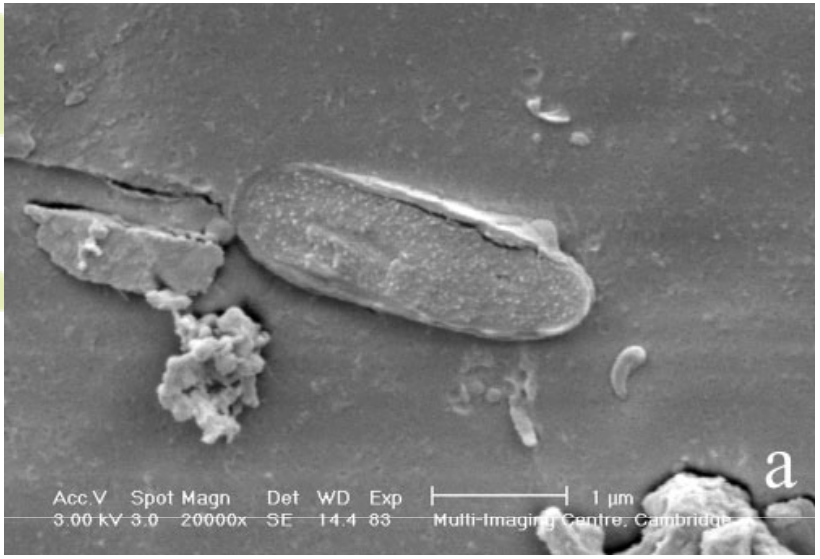
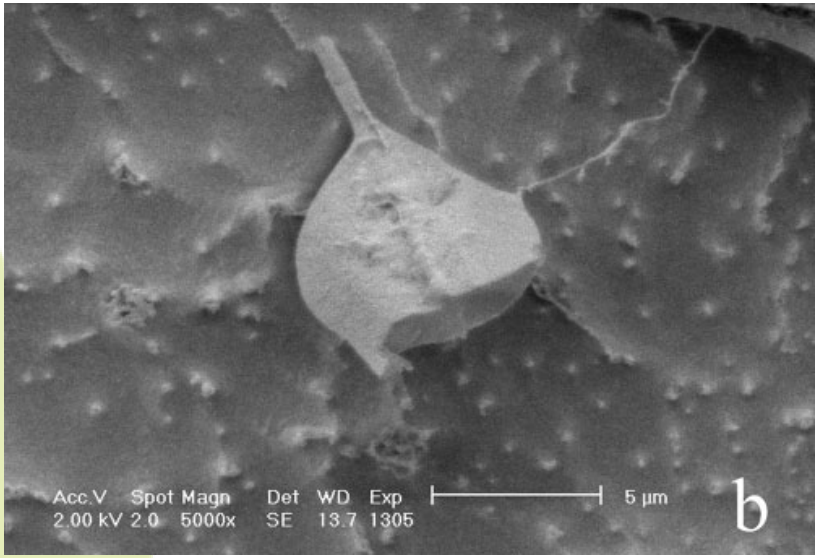
Cryo-SEM image of human spermatozoa



Human spermatozoa
3000 ℃ /min
CPA : glycerol



Human spermatozoa
3000 ℃ /min
CPA : (X)

(Morris 2006)

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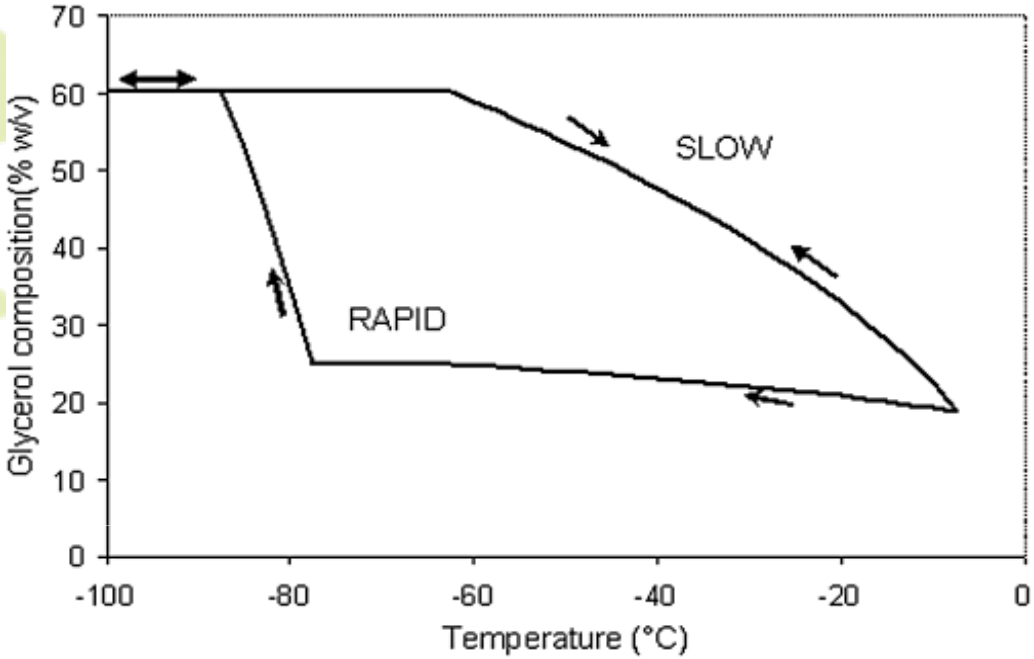
Viscosity of CPA & osmotic damage



(Morris 2006)

Figure A schematic of the glycerol concentration encountered by human spermatozoa after nucleation at –7.5°C and slow cooling (below 10°C/min) and rapid cooling (3000°C/min) to –100°C in the presence of glycerol.

Glycerol (-50°C) viscosity : similar viscosity → 1.2-propanediol.
much lower viscosity →Dimethylsulphoxide (DMSO)
higher viscosity → sugars (sucrose, glucose, fructose),
polymers (maltodextrin, polyvinylpyrrolidone)

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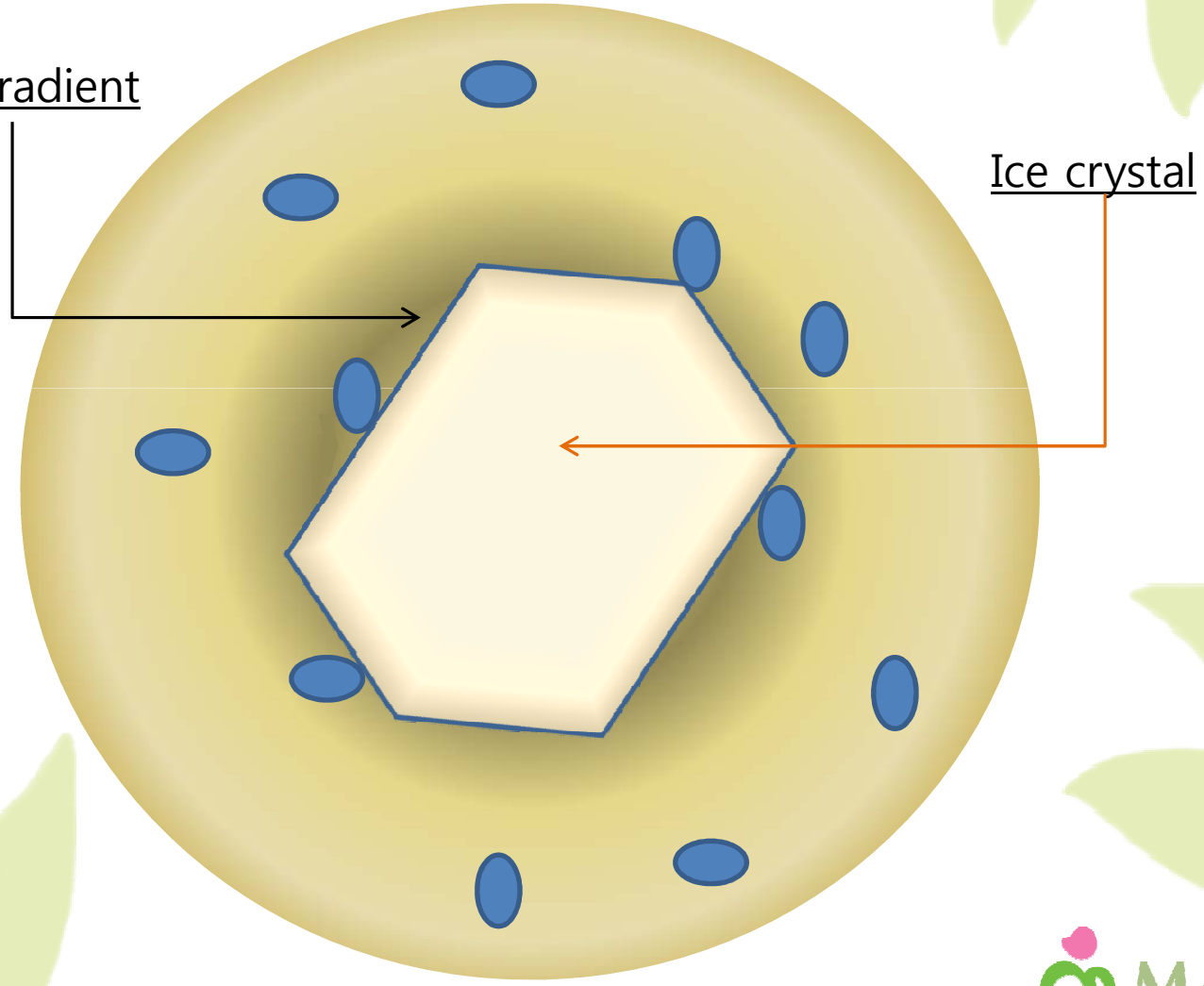
CPA gradient



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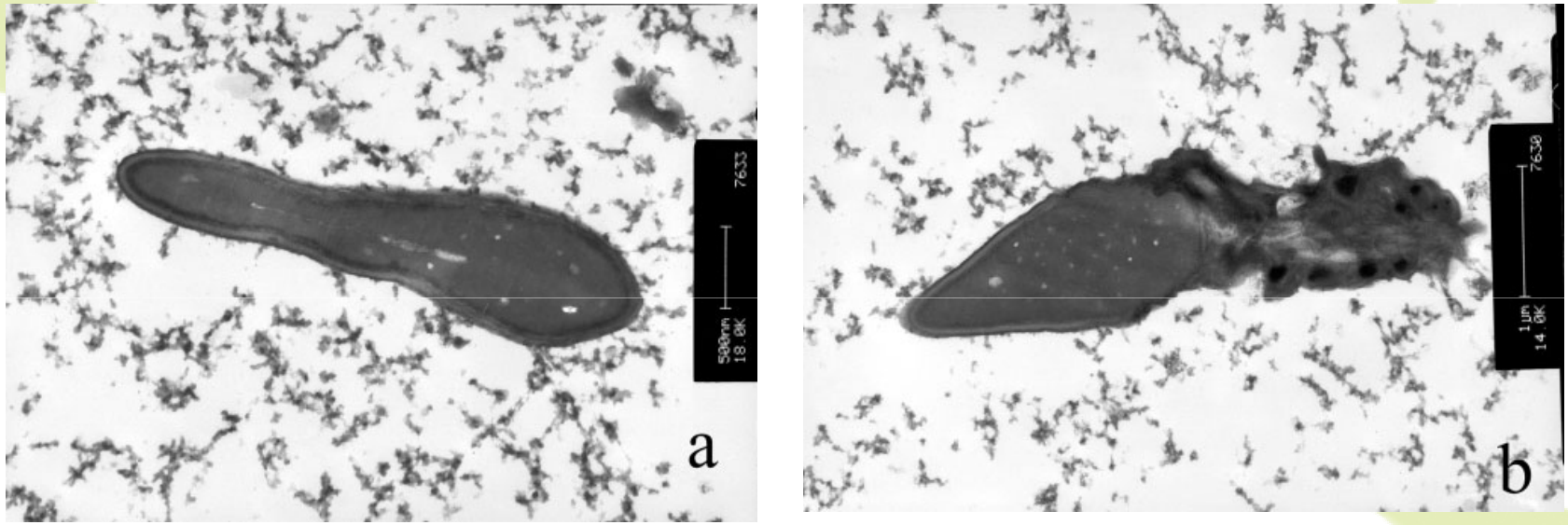
Electron microscopy of freeze-substituted images



Human spermatozoa, 10 ℃ /min, CPA : glycerol

(Morris 2006)

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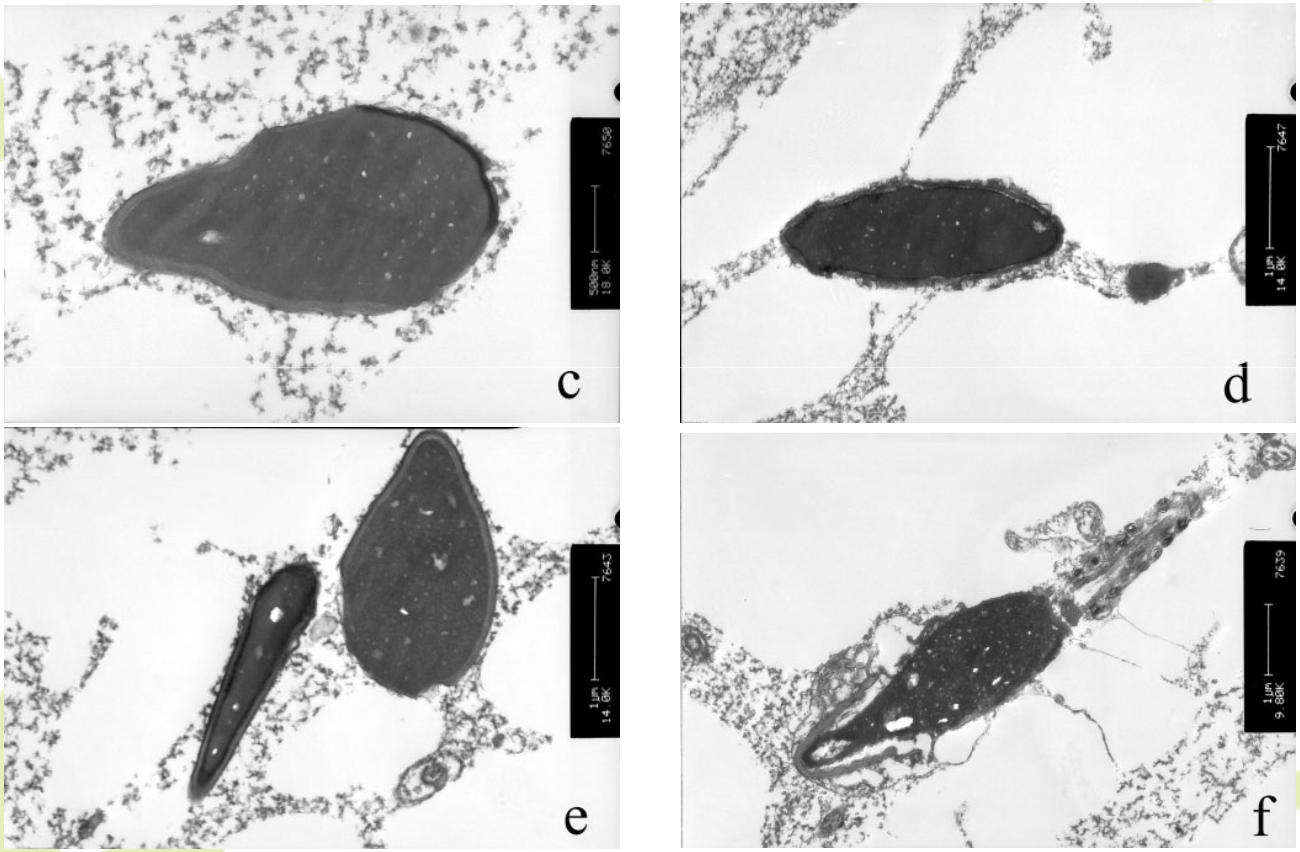
Electron microscopy of freeze-substituted images II



Human spermatozoa, 3000 ℃ /min, CPA : glycerol (Morris 2006)

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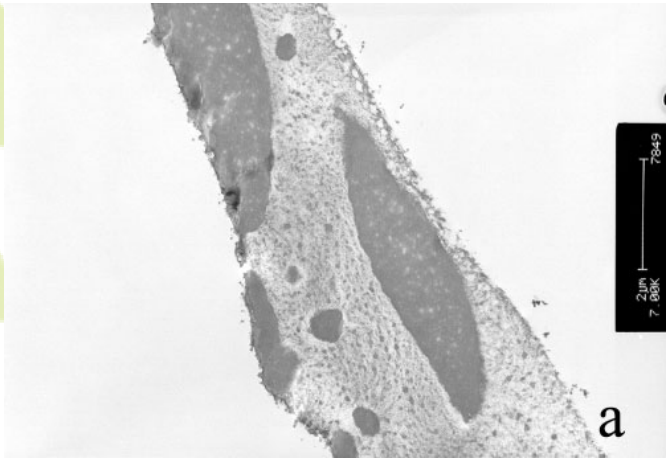
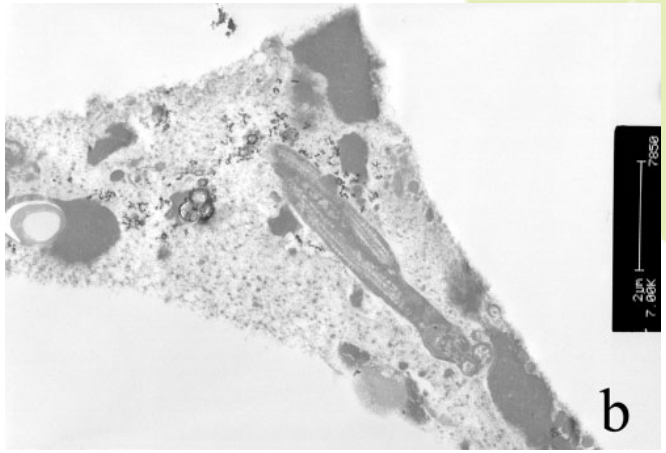
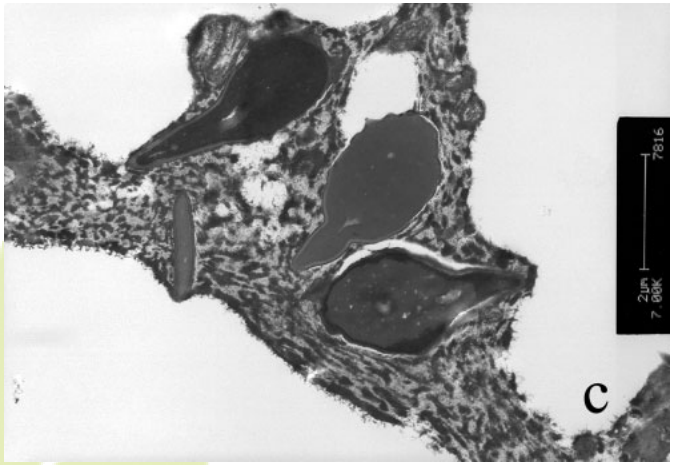
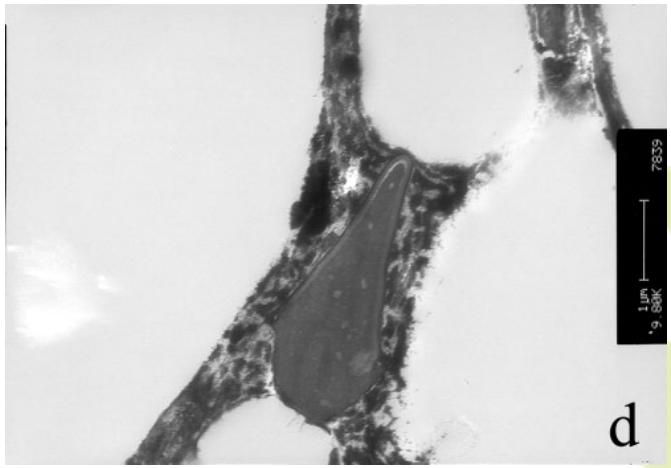
freeze-substitution of cryopreserved human sperm





Human spermatozoa, 10 ℃ /min, CPA : (X)





Human spermatozoa, 3000 ℃ /min, CPA : (X)

(Morris 2006)

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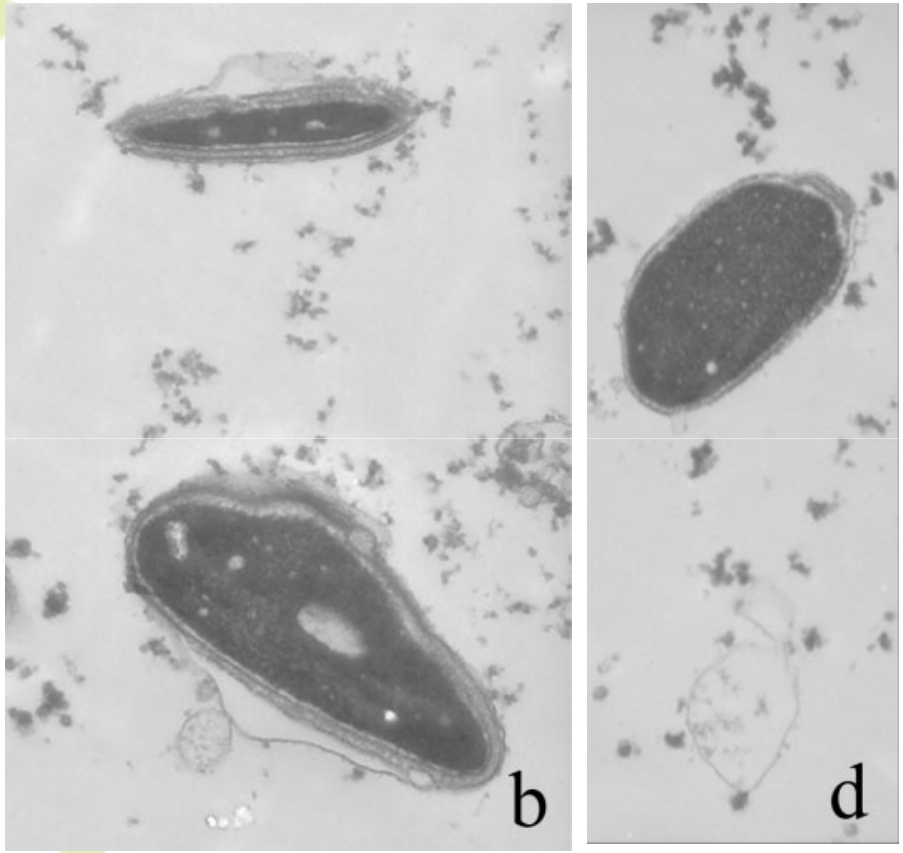
freeze-substitution of cryopreserved human sperm



(Morris 2006)

Human spermatozoa, 3000 ℃ /min → 1 ℃ /min to -40 ℃, CPA : glycerol

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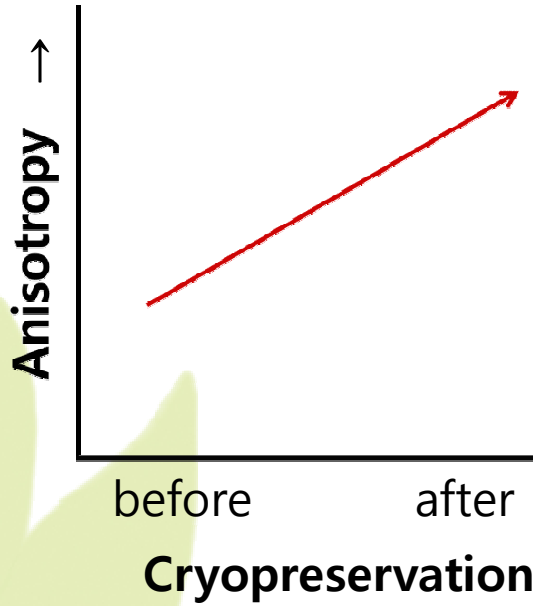
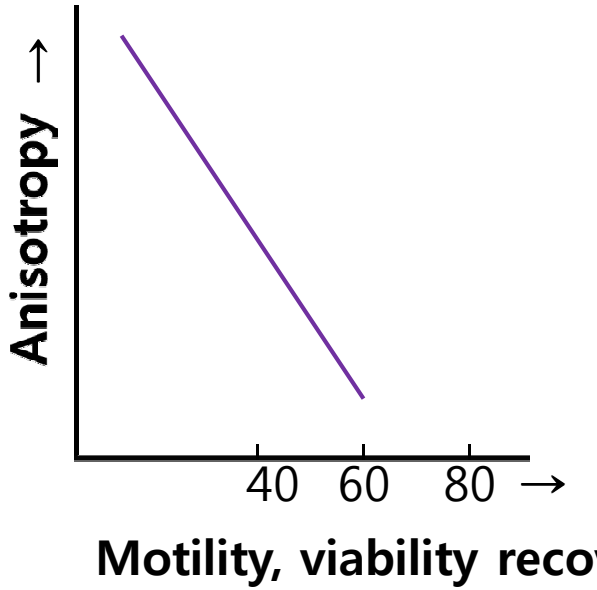
Sperm membrane fluidity

Cryopreservation processes result in a loss of membrane fluidity

Membrane fluidity assessed by measuring the fluorescence polarization anisotropy

(Giraud et al., 2000)





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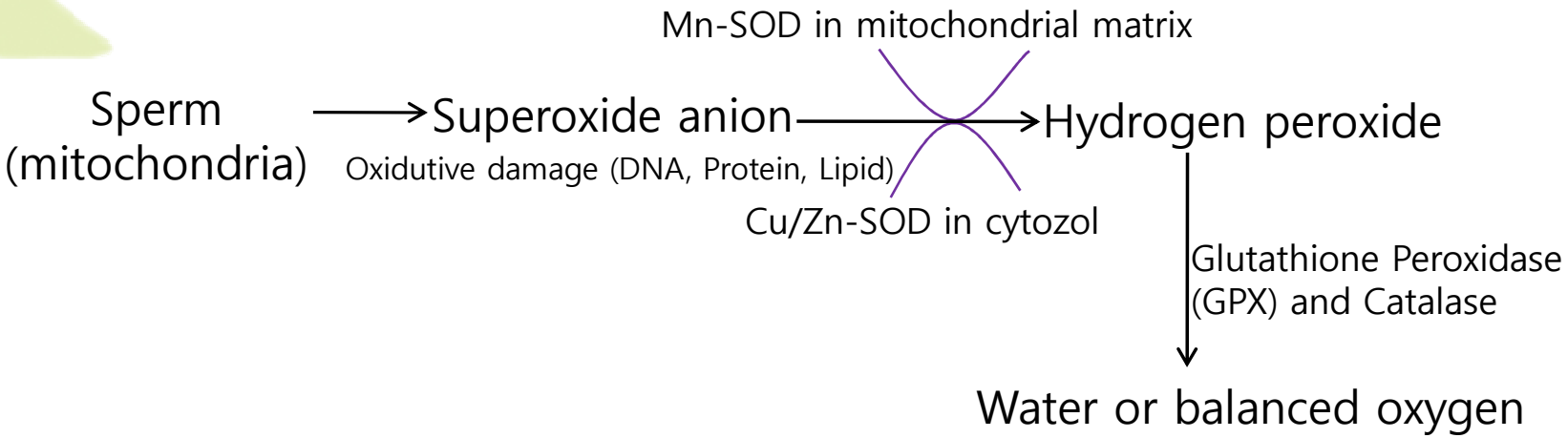
Superoxide dismutase (SOD)and motility after thawing I



Plasma membrane (polyunsaturated fatty acid)

↓ ROS

Increase membrane permeability, loss of ATP

↓

Motility loss

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Superoxide dismutase (SOD)and motility after thawing II

Mature sperm (higher SOD content) → easy to avoid cell damage

Immature sperm produce high levels of superoxide anion → oxgen radical induced cell damage → motility loss

Ejaculated sperm comprised discrete subsets of spermatozoa, wi th different degrees of maturation- density gradient fractionation

Both SOD and GPX activities should be coupled and they play a central role in protecting mammalian sperm against oxygen radical-induced damage leading to motility loss

(Calamera et al., 2003; Buffone et al.,2012)

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Capacitation – related events

Removal or inactivation of decapacitation factors on the sperm surface → by washing sperm in simple salt sol'n

Chages of Cholesterol/phospholipid ratio → membrane fluidity, permeability

Reactive oxygen species (ROS) → inducing hyper activation and capacitation

(Medeiros et al., 2002)

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Apoptosis – like phenomenon

Cryopreservation induces apoptotic sperm DNA

Apoptotic DNA fragmentation
(Normozoospermic men < Oligozoospermic men)

(Paula et al., 2006)

Membrane permeability ↑
Bax (pro-apoptotic factor) – detected
Bcl-2 (anti-apoptotic factor) – not detected
Cytochrome c – detected
AIF (apoptosis inducing factor) – detected
Pro-caspase-9, activated caspase-9 – detected
(bovine-Martin et al., 2007)
Caspase-3 and caspase-8 – detected
(human- Paasch et al., 2004a,b, 2005)

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Long term effects of sperm cryopreservation

Total loss of average recovery rate after long term storage was only 28% -a significant decrease with time of about 20%.

The concentration of motile spermatozoa, especially in patients with testicular cancer was so much reduced

- Kolodziej et al., 1990 -

sperm motility and viability are well preserved up to 5 years after cryopreservation (cancer patients)

- Rofeim et al., 2005-

Decrease of fertilizing ability of thawed sperm → P34H, α-tubulin marker

- Desrosiers et al., 2006-

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Repeated freezing and thawing I

3 cycles of cryopreservation and thawing of spermatozoa

Wash and dilute with new CPA : unwashed and recryo

Wash and density grandient : unwashed and recryo

(Thomson et al., 2010)

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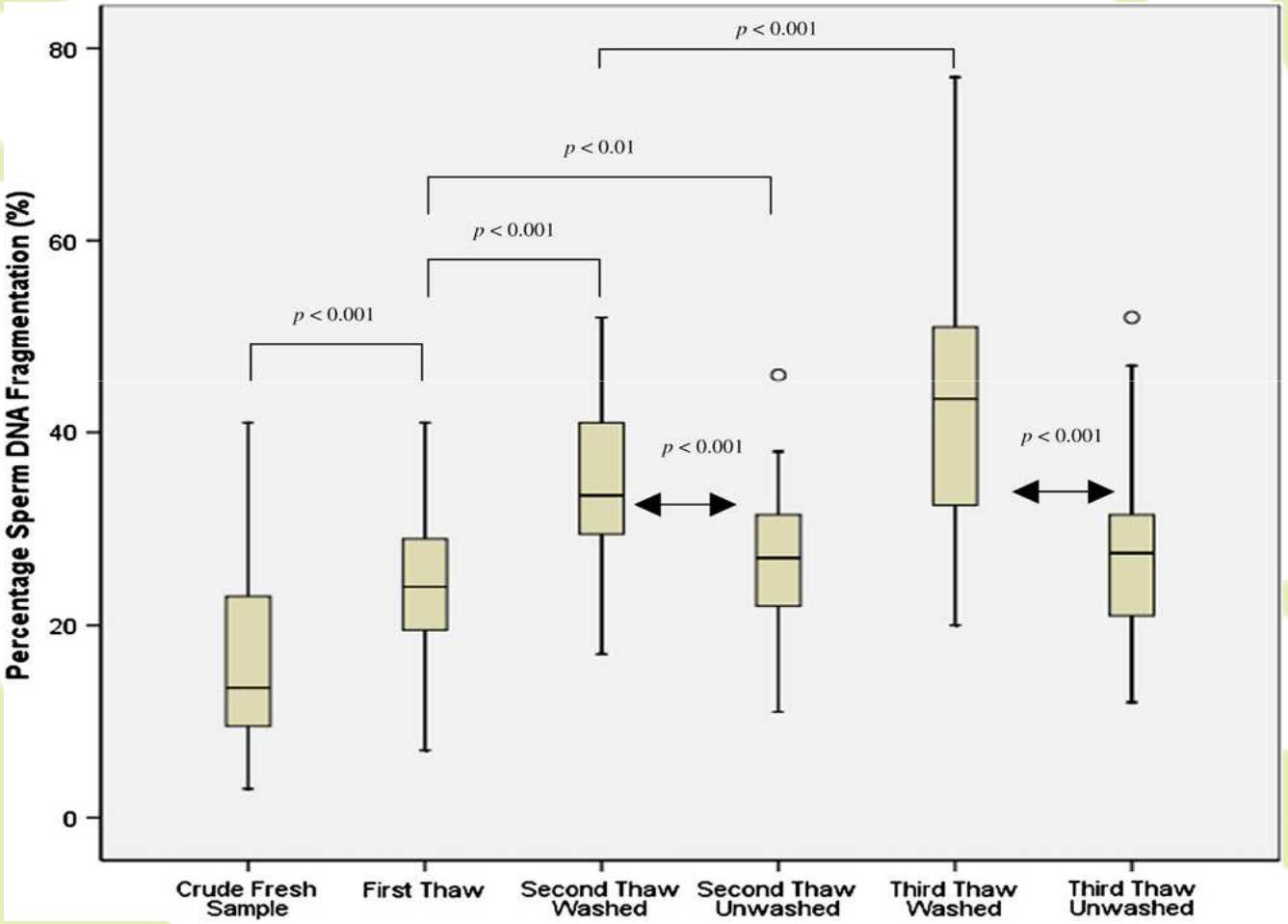
Repeated freezing and thawing II

(DNA fragmentation of nonseparated semen)



(Thomson et al., 2010)

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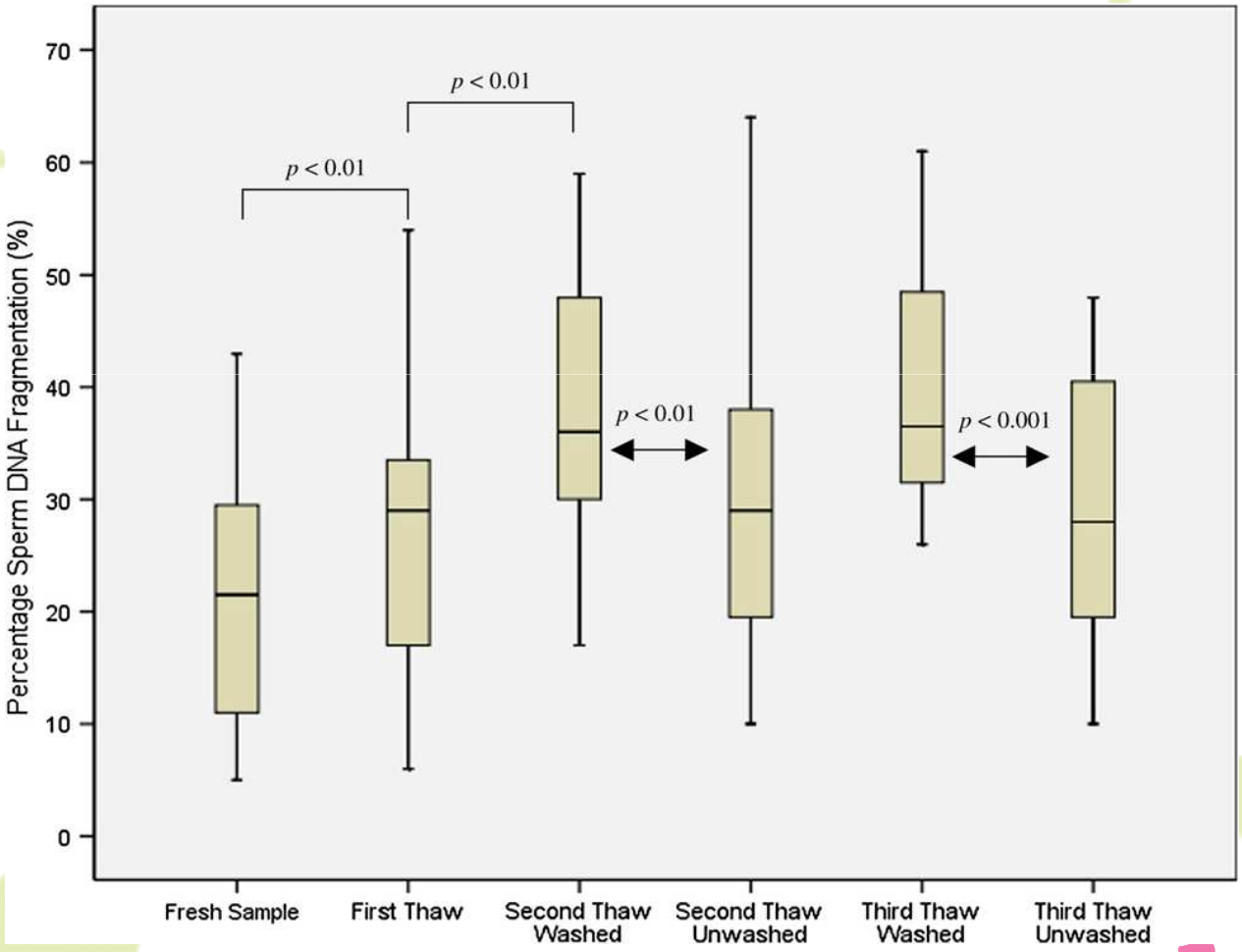
Repeated freezing and thawing III

(DNA fragmentation of density gradient separated semen)



(Thomson et al., 2010)

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Very low numbers of sperm cryopreservation

Conventional cryopreservation techniques are inadequate for preserving individually selected sperm (oligozoospermia, cryptozoospermia, microsurgically obtained sperm–MESA, TESE, TESA, PESA)

→ Loss caused by adherence to the vessel wall

Other vessel

-Hamster zonae (Cohen et al., 1997)
-Human zonae (Hsieh et al., 2000)
-Microdrops in culture dishes
-ICSI pipettes
-Alginate beads (Herrler et al., 2006)
-Cryoloop (Schuster et al., 2003; Desai et al., 2004)

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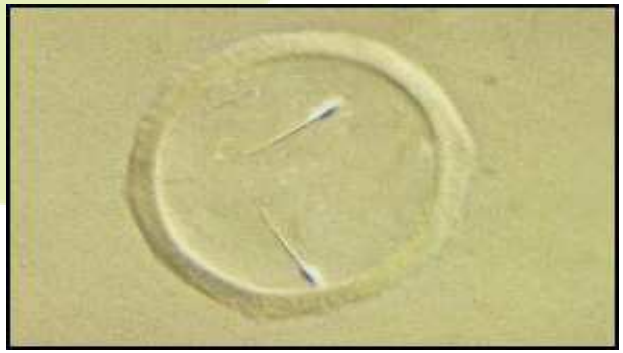
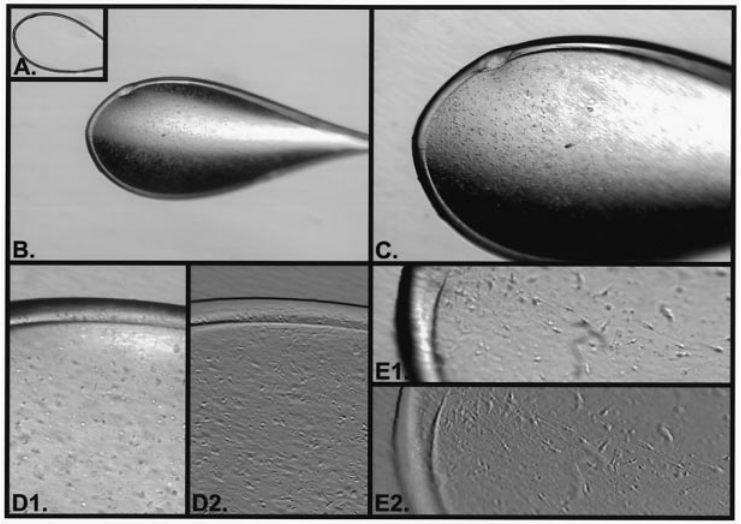
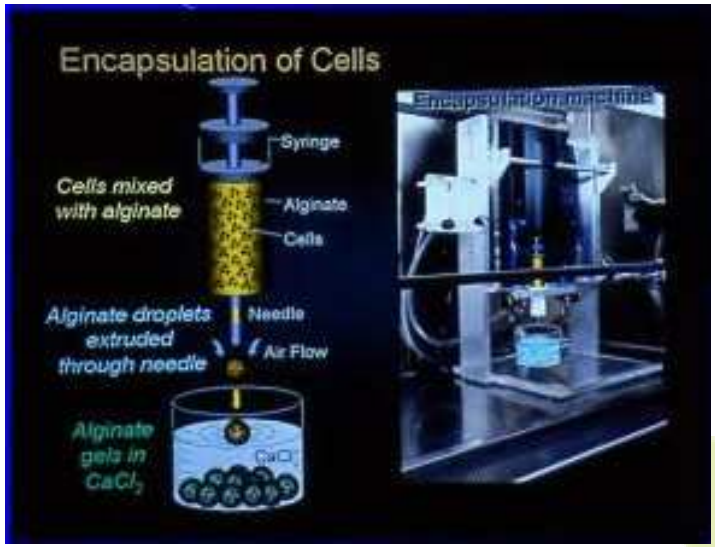
Very low numbers of sperm cryopreservation



Cohen et al., 1997



Herrler et al., 2006



from McMaster university

Schuster et al., 2003
Desai et al., 2004

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Freeze-drying of spermatozoa

Fresh sperm → gradient separation → sperm aliquot + buffer sol'n → freeze-drying

◆ Advantage : dry ice, LN_2 free, no contamination, store refrigerator (-4 ℃) or maybe ambient temperature, easy move and shipment

◆ Disadvantage : lipid-peroxidation, total loss of motility and viability

DNA integrity maintained → no chromosome aberration (mouse, rabbit, rats, human)

Kusakabe et al., 2008, Gianaroli et al., 2012

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Sperm preservation

Drying without freezing

Mouse sperm + trehalose-EGTA sol'n → evaporatively dried under nitrogen gas → store 4 ℃ and 22 ℃ for 1 wee k and 5 months → ICSI → assess development → liveborn offspring

(McGinnis et al., 2005; Li et al., 2007)

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New approach

No requirement a high concentration of potentially toxic CPA

Cryoloop + small drop

Very rapid cooling and warming rates and a very small drop size

(Isachenko et al., 2004)

Use cold CPA (sucrose→ trehalse, reduce concentration of glycerol, add HSA)

Use aluminum block as a cooling device

(Vutyavanich et al., 2010)

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Conclusion

Cell damage to sperm is not caused by intracellular ice formation.

The most important factor in the reduction of sperm viability was osmotic shock

Use very rapid cooling and warming rates and a very small drop size

Cryo swim-up or density gradient treated sperm

Spermatozoa have unusual cryobiological behavior and improvements in their survival have not been amenable to conventional approaches of cryobiology

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