

Emerging Biobanking Strategies: Cryopreservation at Elevated Subzero Temperatures and Dry Storage Approaches

Wim Wolkers

Biostabilization laboratory - Lower Saxony Centre for Biomedical Engineering, Implant Research and Development;
Unit for Reproductive Medicine, University of Veterinary Medicine Hannover, Germany



Outline

From cryopreservation to freeze-drying for storage of cells and tissues

- > Cryopreservation of cells (sperm, blood cells) and tissues (heart valves)
- > Cryogenic storage at elevated subzero temperatures
- > Freeze-drying for storage at room temperature

Preservation methodologies for cells and tissues

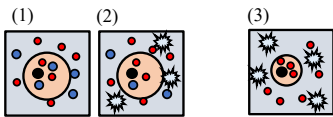
Hypothermic storage, cryopreservation, vitrification, freeze-drying emerging technologies: supercooling, partial freezing, isochoric freezing

	hypothermic storage	cryopreservation	vitrification	freeze-drying
process	isothermal	slow cooling	rapid cooling	- slow cooling - sublimation - desorption
protective agents (examples)	chilling-protective solutions	glycerol, dimethyl sulfoxide (low concentration)	dimethyl sulfoxide, ethylene glycol (high conc.)	sucrose, trehalose, proteins
storage temperature/ state	~4°C In aqueous solution	below -150°C; in frozen/glassy state (T _g <-100°C)	below -150°C; glassy state (T _g <-100°C)	room temperature; in glassy state (T _g >22°C)
storage duration	short term	long term		

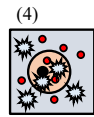


Slow freezing cryopreservation

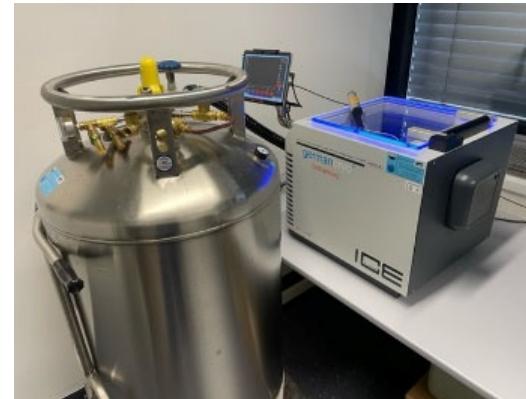
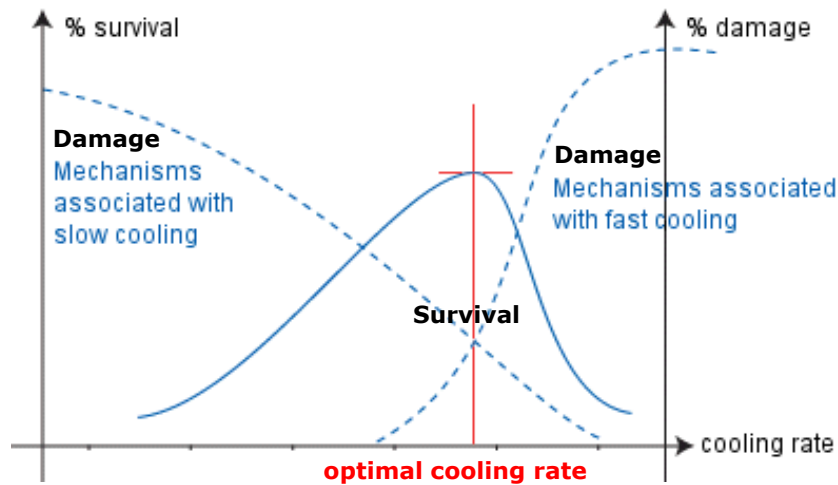
Two factor hypothesis of cryodamage cell-specific optimal cooling rate



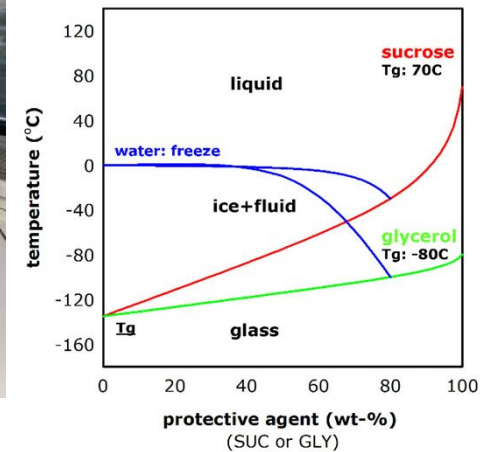
Slow freezing:
Cell death through dehydration
and solution effects



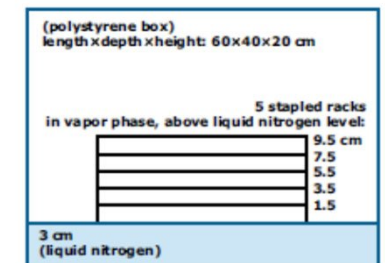
Rapid freezing:
Cell death through
intracellular ice building



Controlled rate freezer

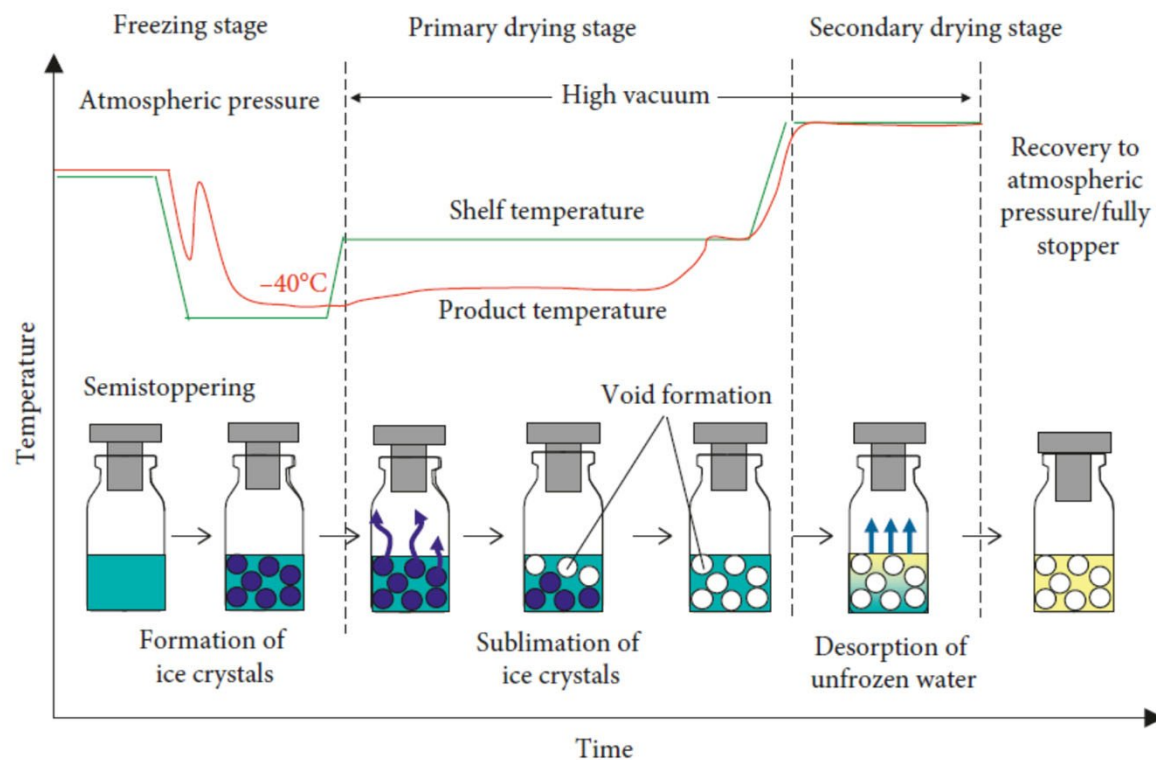


Controlled rate freezing above LN2 vapor



Freeze-drying for storage at room temperature

Programmable freeze-dryer: freezing, primary and secondary drying



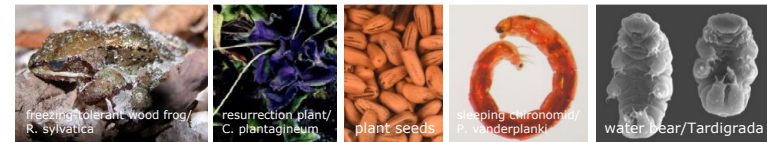
Pilot scale freeze-dryer

Protective agents for preservation at higher temperatures

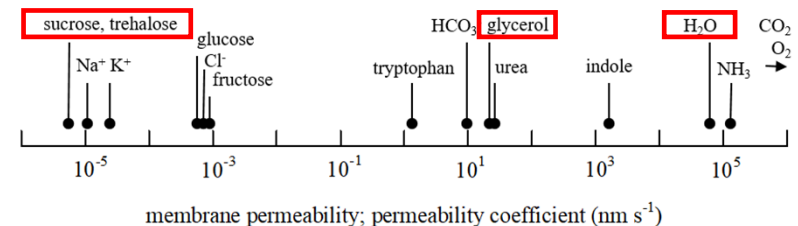
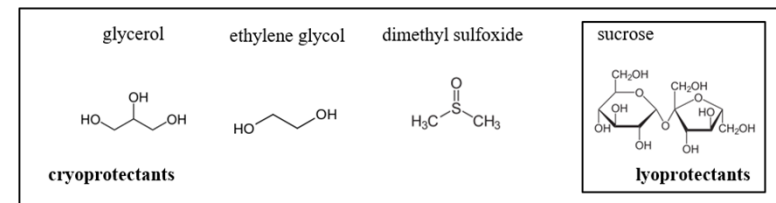
Permeating and non-permeating cryoprotective agents (CPAs)

Use glass-forming sugars and polymers, that may also serve as lyoprotectants

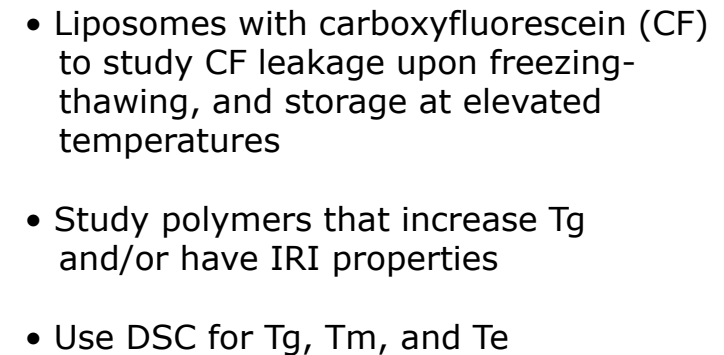
- permeating cryoprotective agents (e.g., glycerol, DMSO) can move across cellular membranes, for providing intracellular protection, depress the temperature at which ice is formed, and slow cellular dehydration during freezing vitrification upon solute concentration
- non-permeating sugars (e.g., sucrose, trehalose) normally membranes are impermeable for sugars facilitate (extracellular) glass formation, and water replacement upon dehydration
- osmotically inactive polymers (e.g., PVP, HES, BSA) facilitate glass formation (i.e., increase T_g) and spacing
- ice recrystallization inhibitors
- AFPs, HSPs, LEA and CAHS proteins
- antioxidants, scavengers



lessons from nature

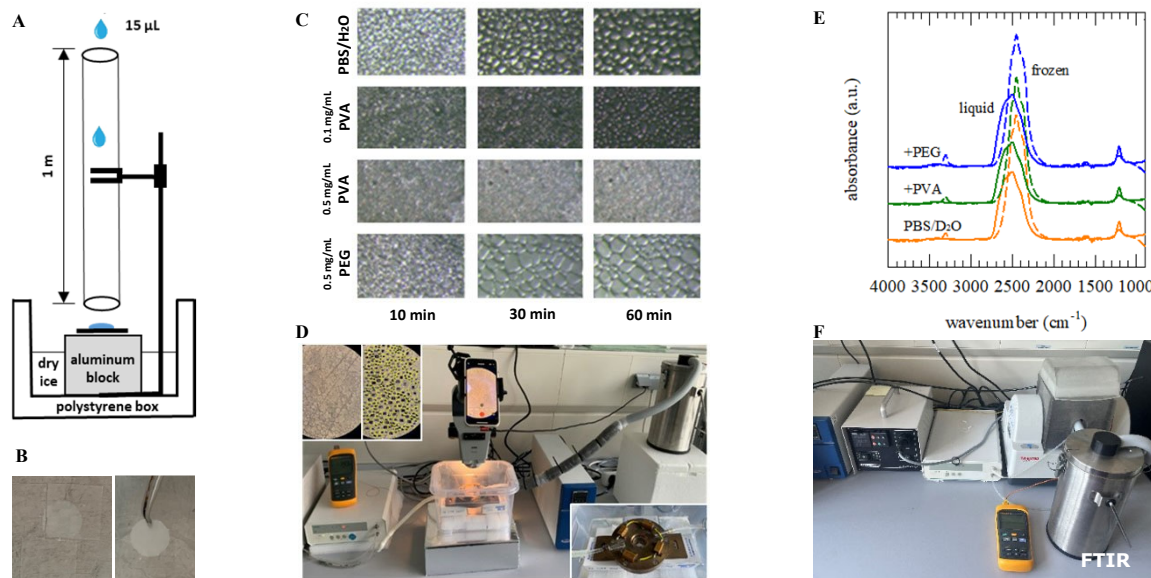


Test combinations of CPAs, biopolymers, and ice recrystallization inhibitors during storage of biospecimens at various temperatures



Custom designed cryomicroscopy and FTIR setup

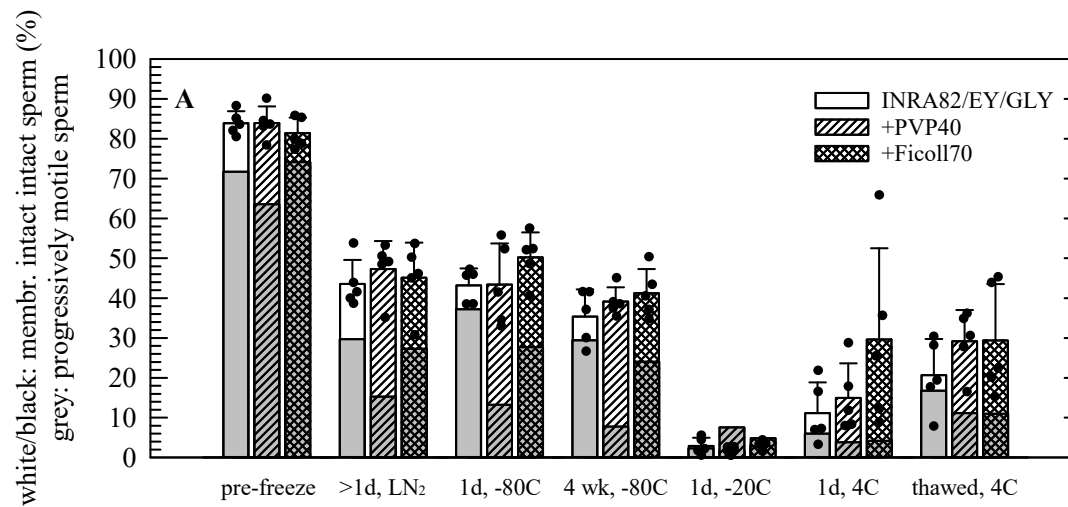
Cryomicroscopy to study ice recrystallization (inhibition) kinetics FTIR to study hydrogen bonding interactions during ice crystal growth



- Left: splat assay to make thin frozen films for custom designed cryomicroscopy setup (PVA inhibits ice recrystallization)
- Right: Temperature controlled FTIR to assess hydrogen bonding interactions

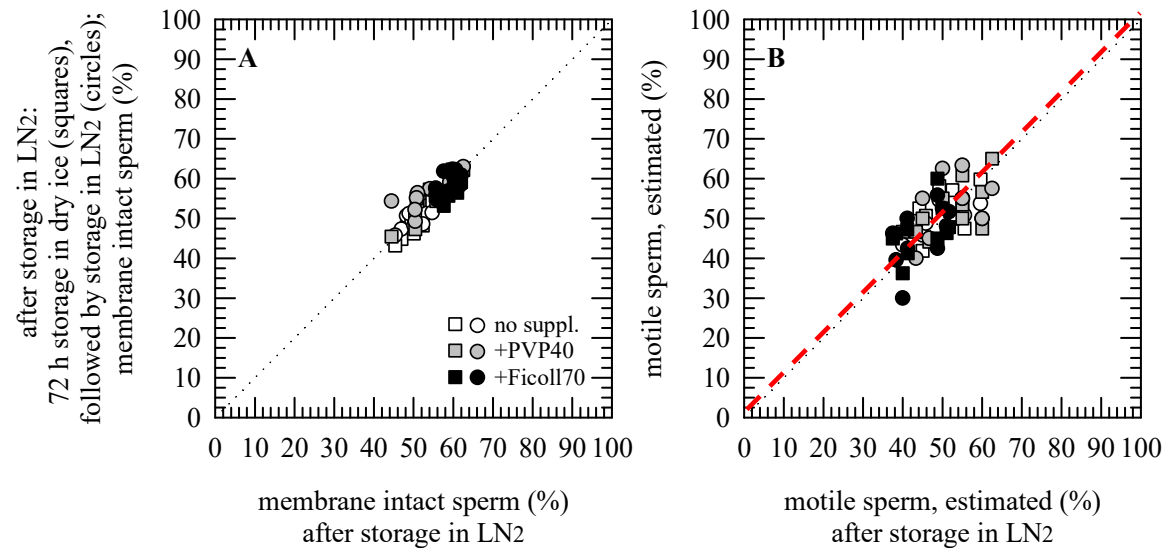
Cryostorage of stallion sperm at elevated temperatures

Storage at -80C appears safe, conditions provoking ice recrystallization are damaging, PVP/Ficoll addition may have beneficial effects



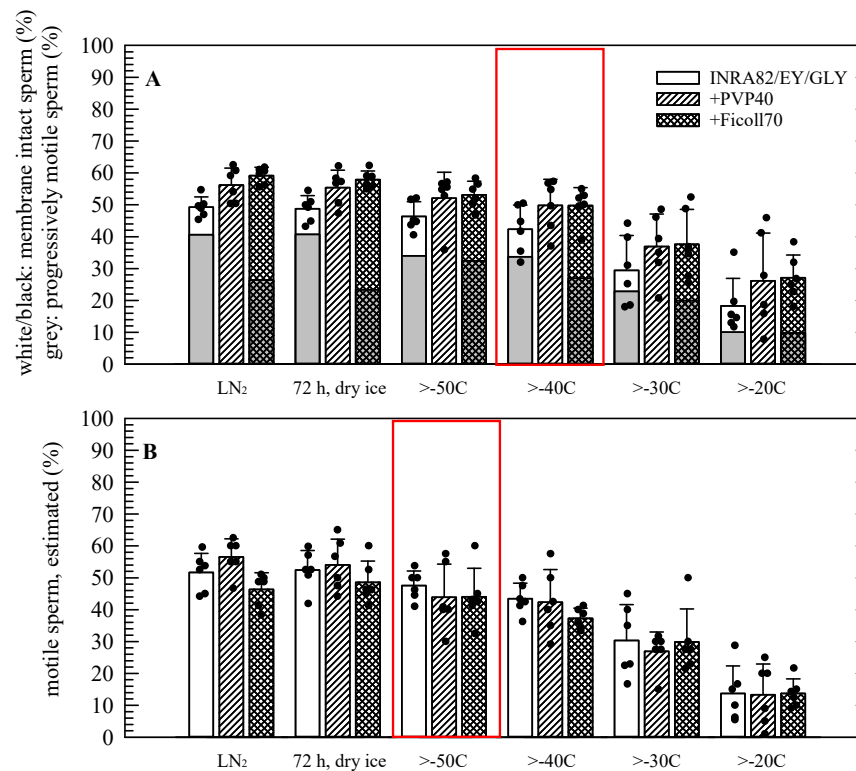
Relocation of sperm; LN2 -> dry ice -> LN2

**Sperm viability is preserved during storage (3 d) in dry ice (-70C)
no losses upon relocation to LN2 after dry ice storage (input = output)**



Critical temperature during dry ice evaporation

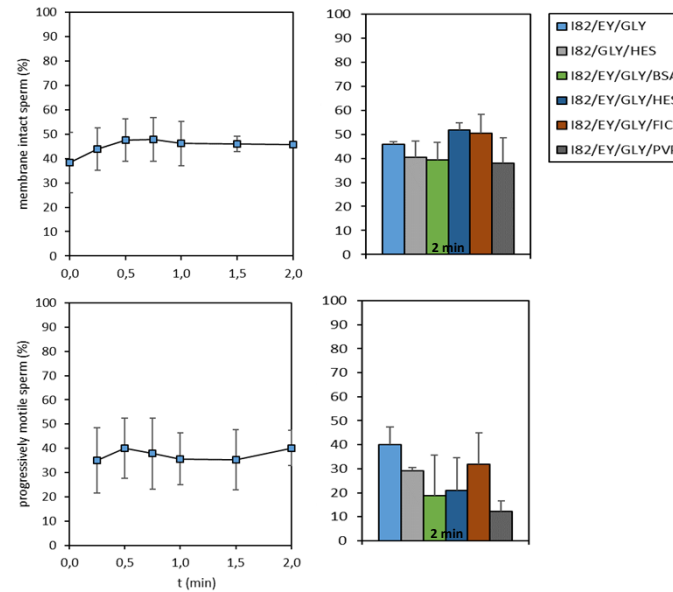
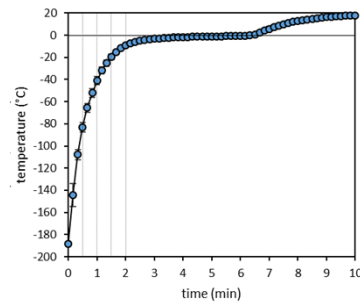
Sperm should not warm above -40C, motility is compromised earlier than membrane intactness; PVP/Ficoll appear to stabilize



- > Microscopic and nucleocounter assessments reveal similar values
- > Note: addition of PVP increases the medium viscosity and particles interfere with CASA
-> estimated motility with microscope

Stability of sperm in frozen straws outside storage tank

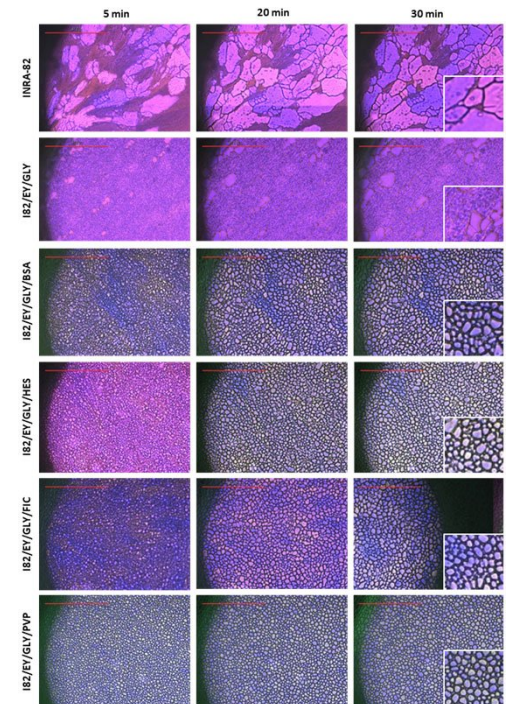
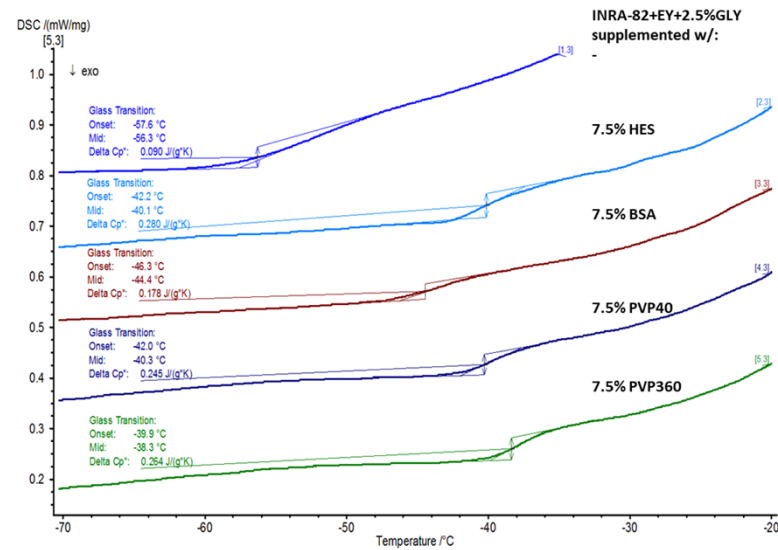
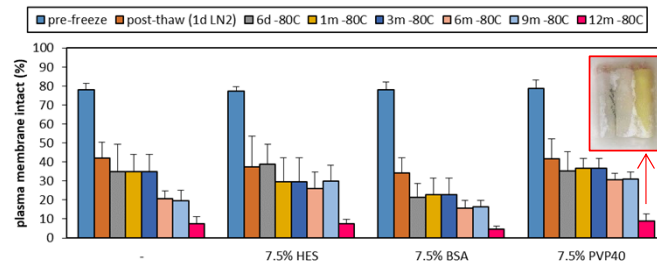
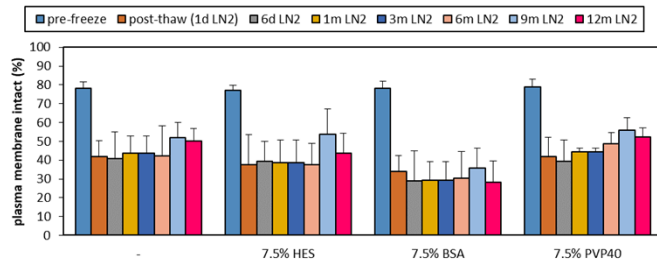
Straws can be kept 2 min at room temperature: no beneficial effect of polymers



- > straws were taken from LN2 held in air for up to 2 min followed by return to storage in LN2
- > sperm analysis after >1d in LN2, ordinary thawing: 30 s at 37°C

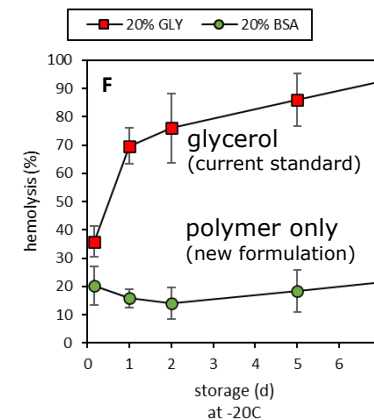
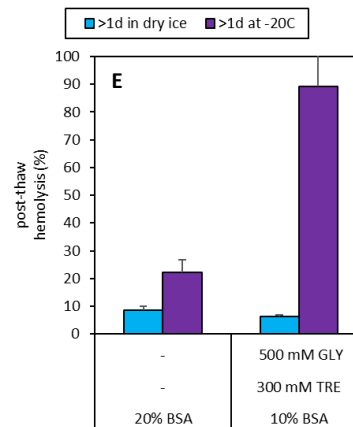
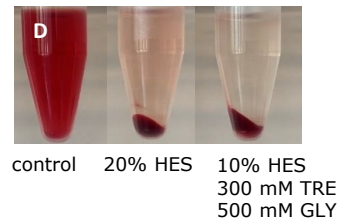
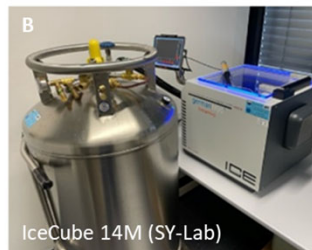
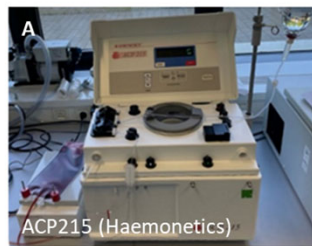
Cryostorage of stallion sperm LN2 vs -80C

Cryosurvival at -80C during storage for 12 months, in freezing extender supplemented with HES, BSA or PVP



Storage of frozen red blood cells at elevated temperatures

Red blood cells can be stabilized at -20C



New process

- Stable at -20C
- Easy processing for clinical use

Dry biobanking of sperm by freeze-drying

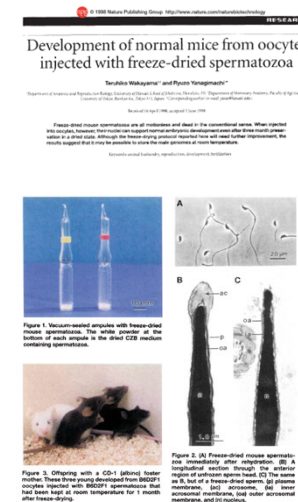
History and state of the art

landmark: Wakayama and Yanagimachi, 1998

- mouse oocytes can be fertilized with freeze-dried sperm i.e., with using ICSI
 - here no specific lyoprotectants were used
- successful fertilization with freeze-dried sperm is described for a variety of species,
 - including rat, cattle, pig and horse (see review by Keskinetepe and Eroglu 2021)
- less success with freeze-drying of somatic cells, and GV-oocytes, i.e., embryonic development after nuclear transfer

improved success

- by adding a chelating agent (EDTA), alkaline conditions (pH 8), adding antioxidants, and use of lyoprotectants (trehalose)



Chapter 39

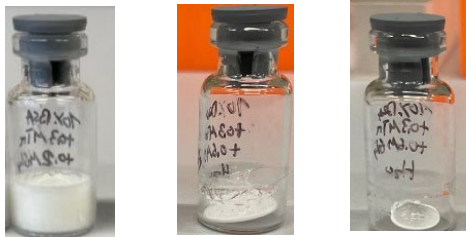
Preservation of Mammalian Sperm by Freeze-Drying

Levent Keskinetepe and Ali Eroglu

Species	Reference	Storage temperature (°C)	Storage duration	Fertilization (%)	Blastocyst formation (%)	Live offspring (%)
Mouse	[20]	FC		ND	ND	ND
		4		92	92	28
		25		98	76	18
	[36]	FC	3 months	100	86	ND
		4		84	72	ND
	[51]	FC	3-4 weeks	ND	ND	58
		4		ND	ND	21
	[46]	FC	1.5 years	95	59	ND
		4		94	21	ND
		4	3 months	91	13	ND
	[37]	FC	6 months	ND	ND	ND
		4		96	ND	65
	[52]	FC	1 year	98	50	21
		4	6 months	100	ND	39
Rat	[53]	FC		95	ND	33
		4		ND	ND	ND
	[50]	FC	3 years	78	ND	28
		25		94	ND	58
	[54]	FC		94	ND	17
		25		77	ND	
	[22]	FC		99	27	35
		4	2-6 weeks	94	30	14
	[55]	CC	1 year	79	ND	36
		4		70	ND	7
Rabbit		25	1 year	75	ND	0
	[56]	FC		97	50	ND
		4	1 year	86	ND	16
	[23]	FC		ND	ND	ND
		4	5 years	ND	ND	11
	[25]	FC		97	30	ND
		4	2 years	70	24	0.4

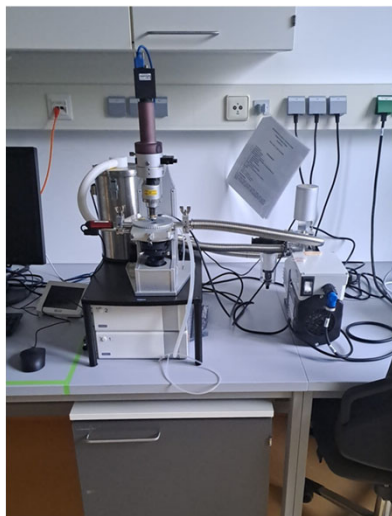
Formulation testing by freeze-drying cryomicroscopy

Formulations of trehalose and biopolymers for freeze-drying of stallion sperm

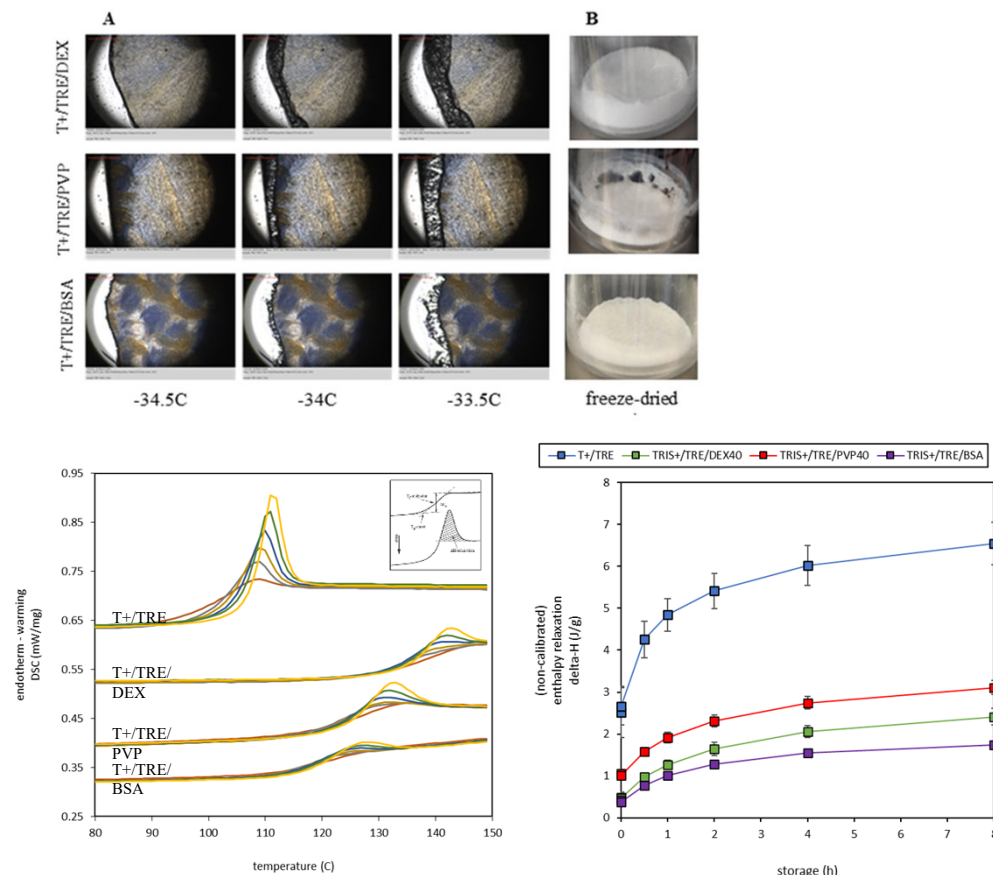


Porous cake

collapse

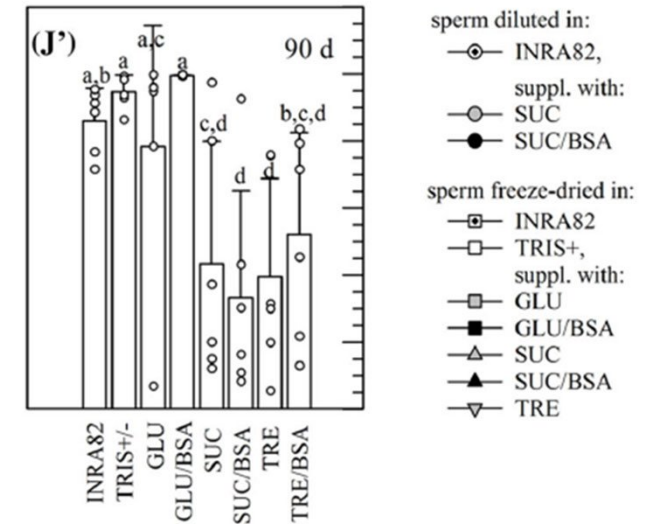
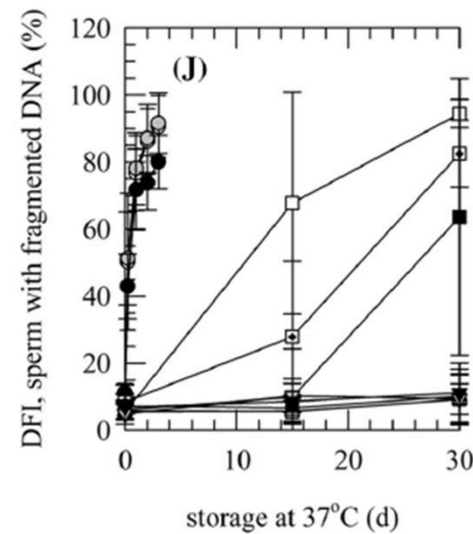
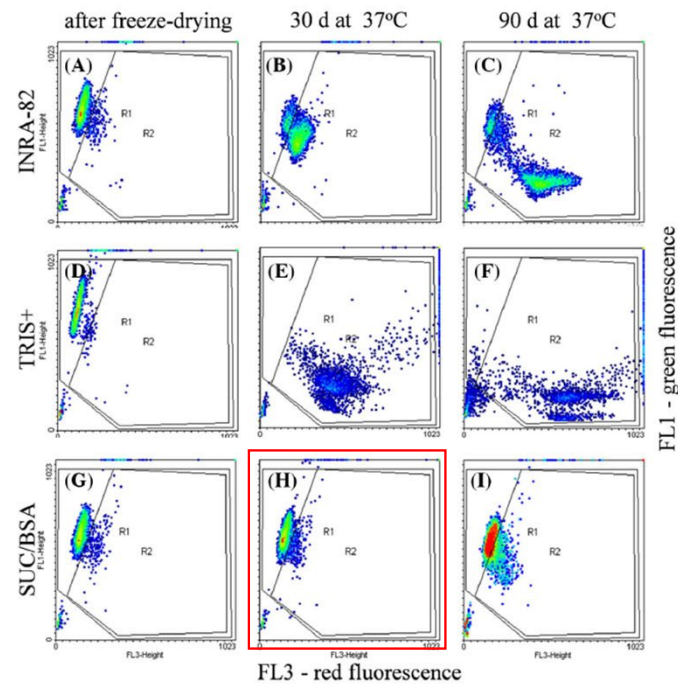


Freeze-drying cryomicroscope



Chromatin stability in freeze-dried stallion sperm

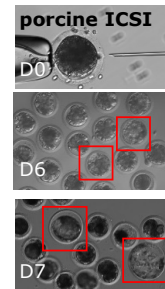
Chromatin stable for 3 months at 37C in disaccharide/albumin formulations
presence of non-reducing glucose has detrimental effects



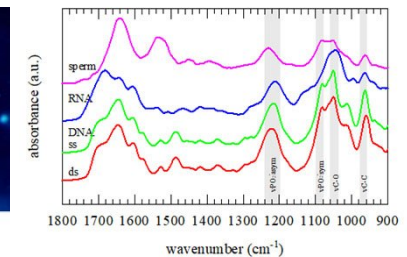
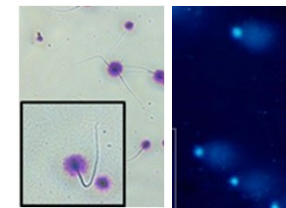
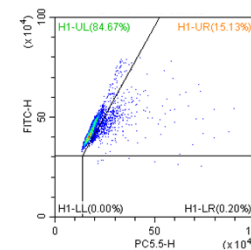
Dried sperm, with intact nuclei, can be used for fertilization

Intracytoplasmic sperm injection, assessment of sperm chromatin structure

- intracytoplasmic sperm injection (ICSI)
 - in vitro fertilization, embryo development
- **assessment of sperm chromatin structure** (i.e., susceptibility to induced damage)
 - sperm chromatin structure assay (SCSA)
 - sperm chromatin dispersion (SCD) or halo-test
 - single cell gel electrophoresis, or comet-assay
- non-invasive spectral fingerprinting (FTIR, Raman)

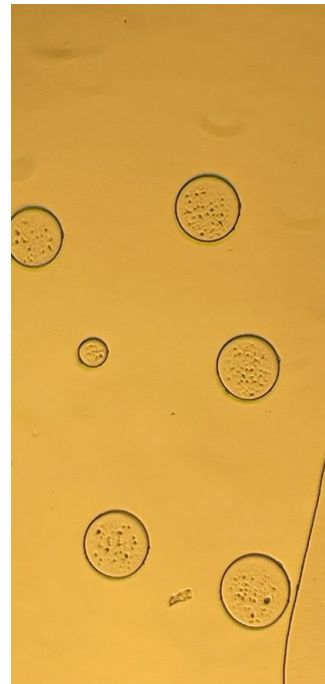
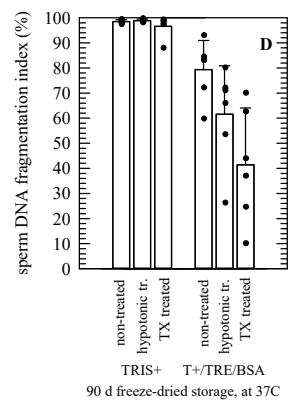
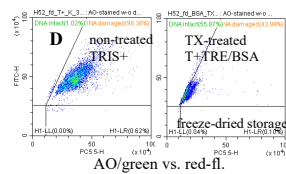
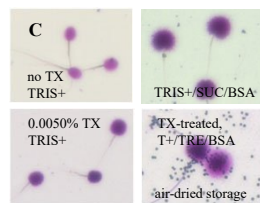
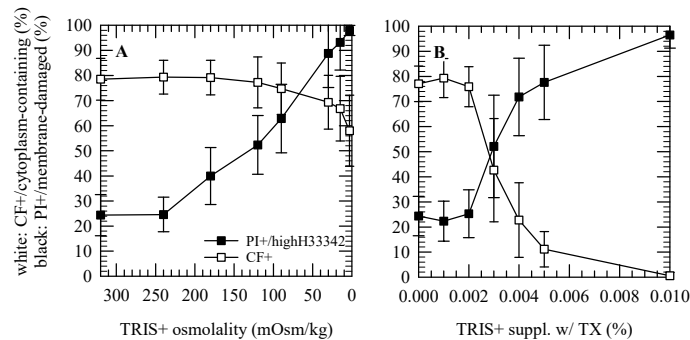


developmental stage	ICSI with	
	freeze-dried sperm	air-dried sperm
total oocyte number	140 (5×20–30)	140 (5×20–30)
matured	94 (67±2%)	102 (73±5%)
fertilized	56 (60±19%)	62 (61±6%)
cleaved	42 (46±11%)	49 (48±5%)
blastocysts	7 (8±8%)	9 (8±5%)
blast. nuclei number	27 ±11	29 ±18



ARTiCELL: Encapsulation of genetic resources in artificial cell-like structures

Controlled membrane permeabilization to remove ROS packing sperm nuclei in giant unilamellar vesicles for dry storage

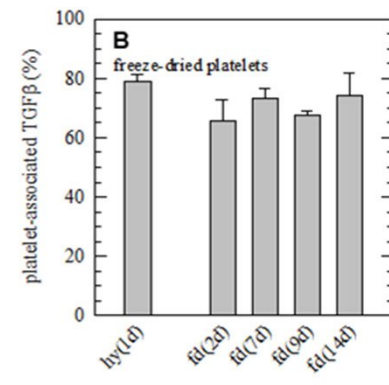
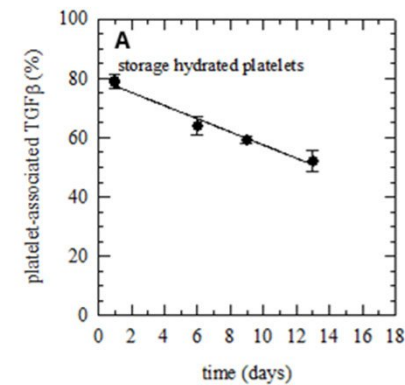
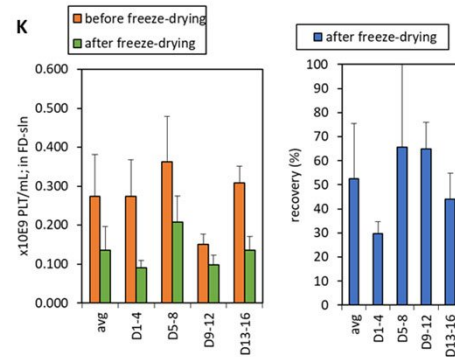
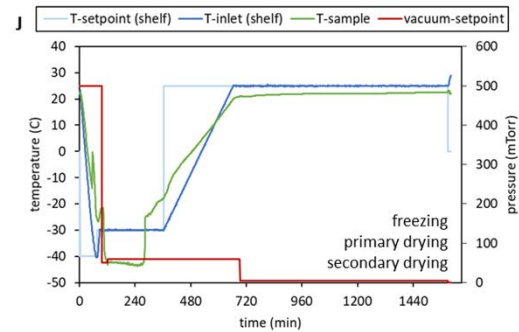
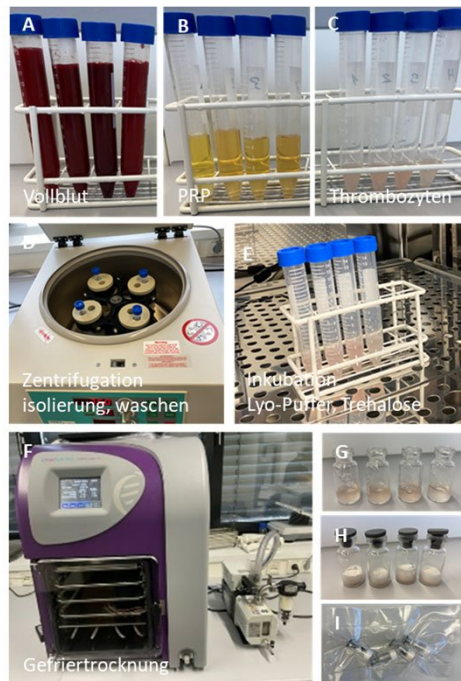


> Left: Chromatin of Triton-treated sperm is more stable during accelerated aging

> Right: Giant unilamellar vesicles (GUVs)

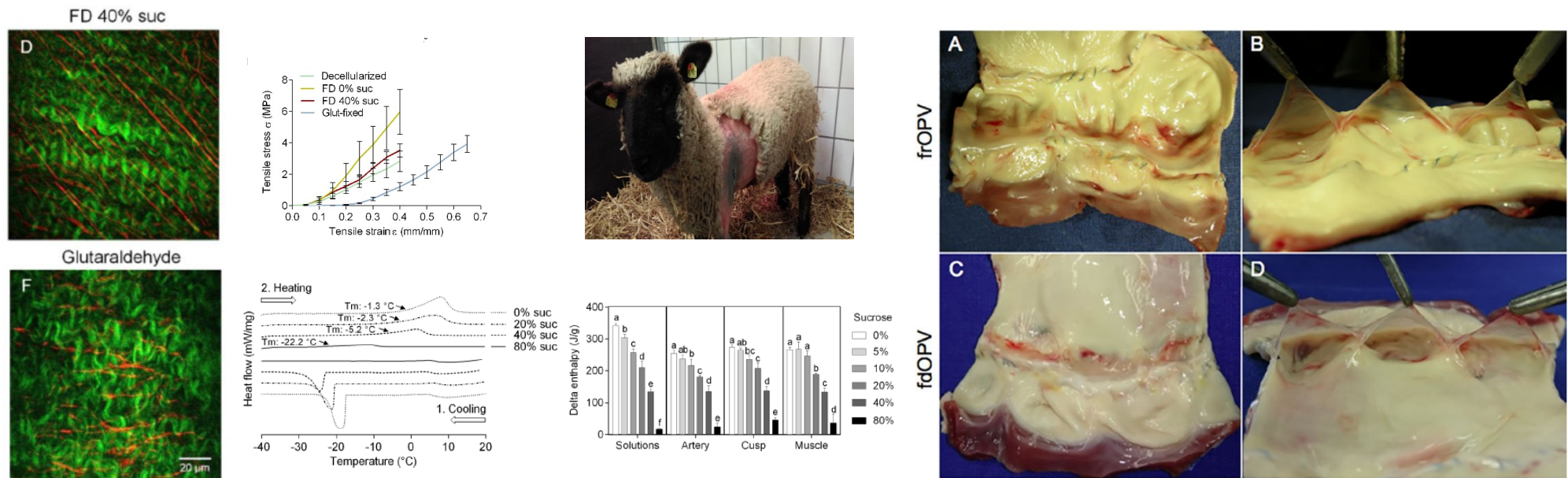
Freeze-dried platelets

For arthritis therapy and wound healing



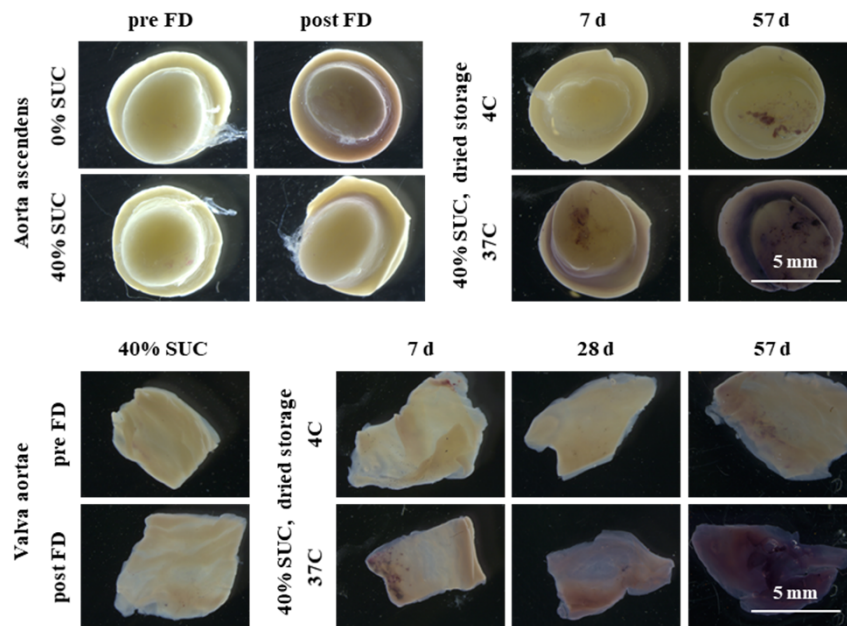
Freeze-dried valves perform equally good in vivo as control valves

Matrix architecture, thermal, and biomechanical properties preserved
explanted tissue shows cell repopulation and no signs of degeneration/calcification



Storage stability of freeze-dried heart valves: NBT detection

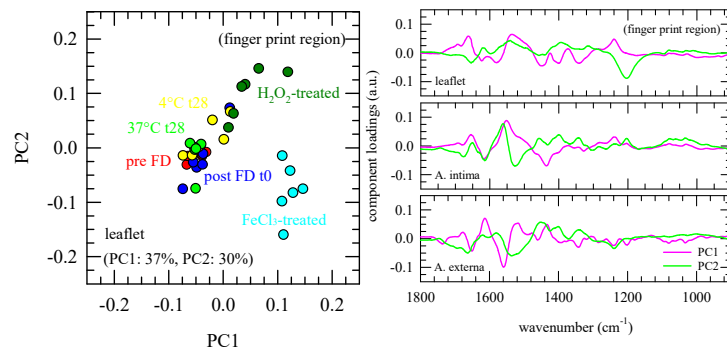
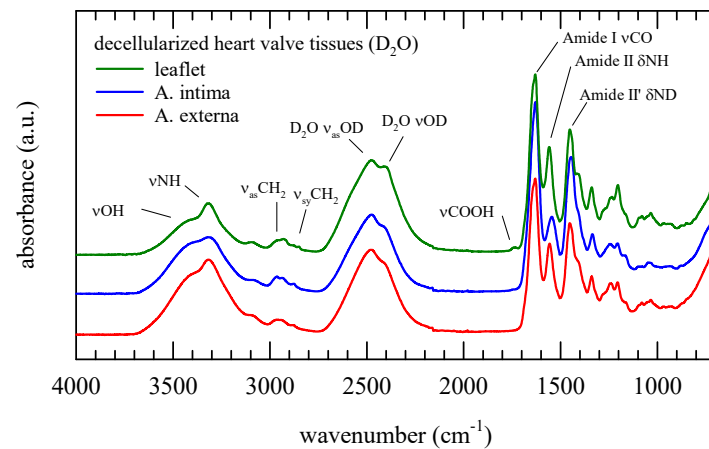
Oxidative damage (NBT-formazan formation) in the absence of protectants effects of sucrose and different storage conditions



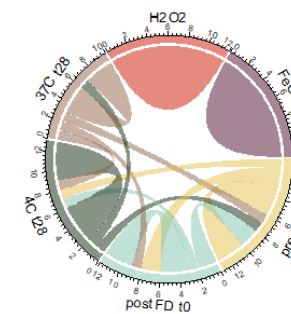
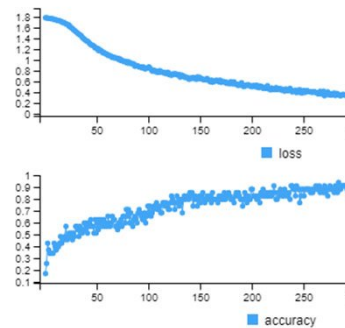
- Accumulation of reactive oxygen species in decellularized and freeze-dried heart valve tissues, results in NBT-formazan production and blue staining
- Both artery and leaflet tissues were analyzed, before and after freeze-drying (FD) with(out) 40% sucrose (SUC) during 4 weeks dried storage at 4/37°C

Storage damage in freeze-dried tissue: Machine learning empowered FTIR

Storage does not alter the tissue's biochemical fingerprint (analysis using PCA and supervised machine learning)



predicted label	true label					
	pre FD	post FD	4C, 28d	37C, 28d	H ₂ O ₂ -tr.	FeCl ₃ -tr.
pre FD	3	2	1	1	0	0
post FD	2	3	0	1	0	0
4C, 28d	1	1	4	1	0	0
37C, 28d	0	0	1	3	0	0
H ₂ O ₂ -tr.	0	0	0	0	6	0
FeCl ₃ -tr.	0	0	0	0	0	6



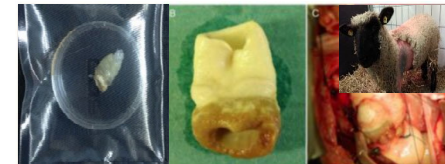
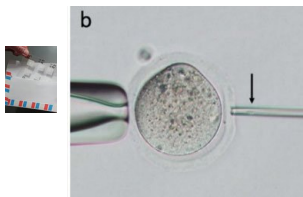
Summary and concluding remarks

Cryostorage at elevated temperatures in high T_g formulations

- Energy saving, cell shipment, remote locations
- Custom designed freezing formulation
- Intrinsic cell properties (membrane permeability barrier)

Dry biobanking

- Opportunities: gene reserves, artificial reproduction technologies, transfusion medicine, cell therapeutics, tissue replacement therapies
- Challenges: combat oxidation damage, intracellular delivery of lyoprotectants, homogeneous tissue permeation



Acknowledgements

Thank you for your attention

Christoph Stoll (graduated 2012)



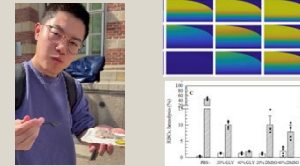
Miao Zhang (graduated 2017)



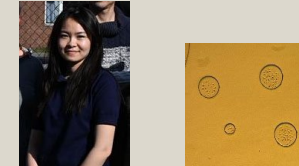
Raffaele Brogna (graduated 2021)



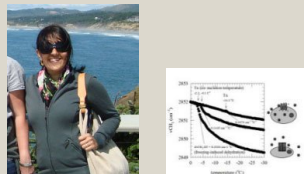
Dejia Liu (graduated 2026)



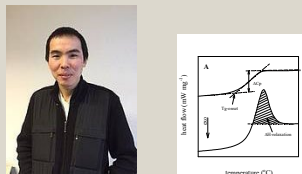
Jingyao Zhu (started 2026)



Maryam Akhoondi (grad. 2013)



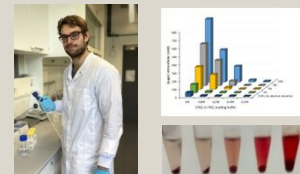
Bulat Sydykov (graduated 2018)



David Pruß (graduated 2022)



Tobias Braun (started 2022)



You?

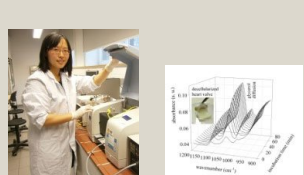
funding:

- DFG:
- ARTiCELL
- Bundeswehr:
- HEMOFORCE

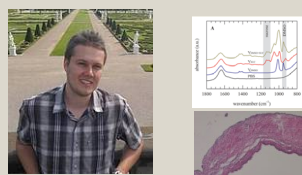
correspondence:

Wim Wolkers
willem.wolkers@tiho-hannover.de

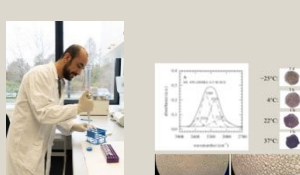
Shangping Wang (grad. 2015)



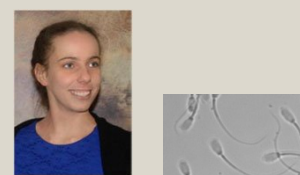
Andrés Vásquez Rivera (gr. 2019)



Sükrü Caliskan (graduated 2023)



Charlotte Sielaff (started 2026)



Harriette Oldenhof

