Principles of cell cryopreservation

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The structure of the lecture:

Block I. Principles of cryopreservation:

- What happens during freezing – cryodamaging factors;
- Cryoprotectants
- Main stages of cryopreservation
- Cooling rates and initiation of ice formation
- Cryoprotectant toxicity
- Thawing
- Determination of cell viability

Block II. Working examples in the field of stem cell cryopreservation for clinical use

- Alternative approaches for stem cell cryopreservation with reduced DMSO concentration
- Alternative quality control methods for stem cell cryopreservation
- Hypothemic storage (storage at 4°C) of stem cell suspensions for clinical applications
- Lyophilization of stem cell-derived secretome

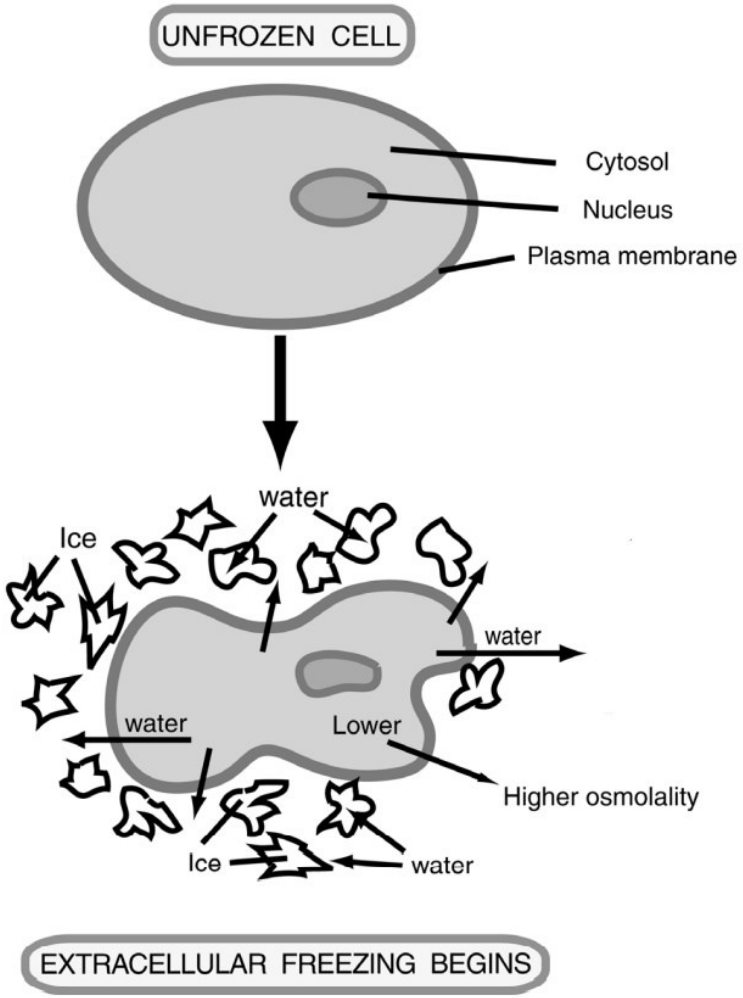
Cryobiology – the field of biology that studies the effects of low temperatures on biological objects

(From Greek κρύος — cold, bios — life u logos — science)

Storage duration:

4°C → Several hours;
-40°C → Several days;
-80°C → Several weeks;
-196°C → centuries!

What happens to cells during freezing?

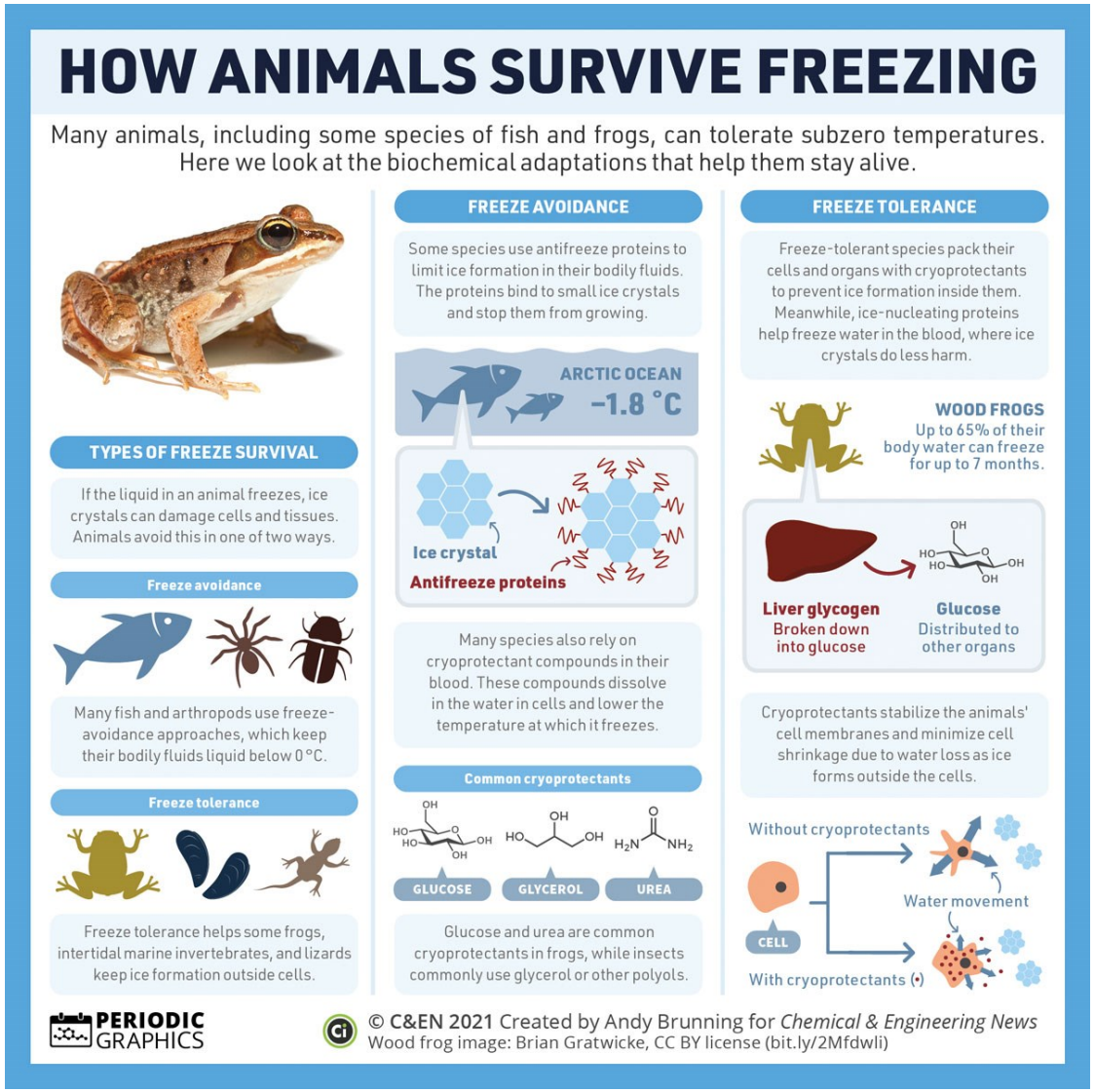


- The water outside cells freezes
- The concentration of salts increases, driving the water outflux from the cell
- Leads to the acute dehydration
- Remaining intracellular water crystallizes
- Cell death

We need to find a balance:

- reduce the intracellular water to avoid intracellular ice formation
- prevent excessive dehydration

What have we learned from nature?

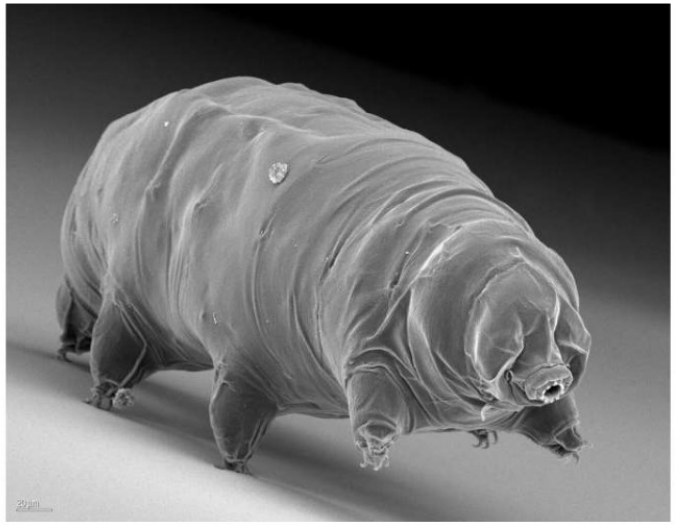


https://cen.acs.org/biological-chemistry/biochemistry/Periodic-Graphics-animals-survive-freezing/99/i4

OCTOBER 7, 2022

How tardigrades survive freezing temperatures

by Andrea Mayer-Grenu, University of Stuttgart



It is only under the microscope that the similarity of its namesake becomes apparent: the plump, round …

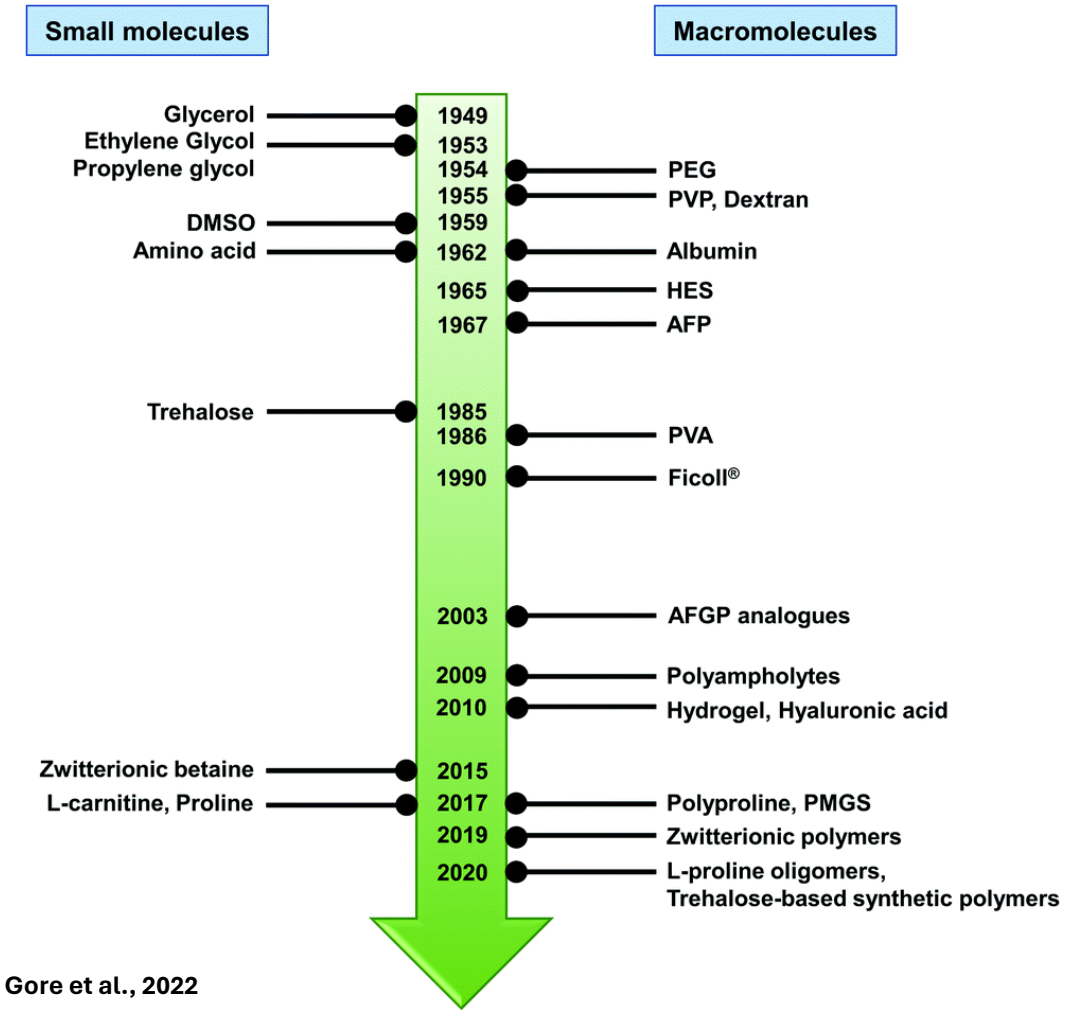
Cryptobiosis / cryobiosis

- Stop metabolism
- Produce osmolytes and sugars to reduce and control ice formation
- Controllable cellular dehydration (almost complete)

Cryoprotectants or cryoprotective agents (CPA)

Two most critical factors of cryodamage:

- *excessive dehydration*
- *intracellular crystallization*



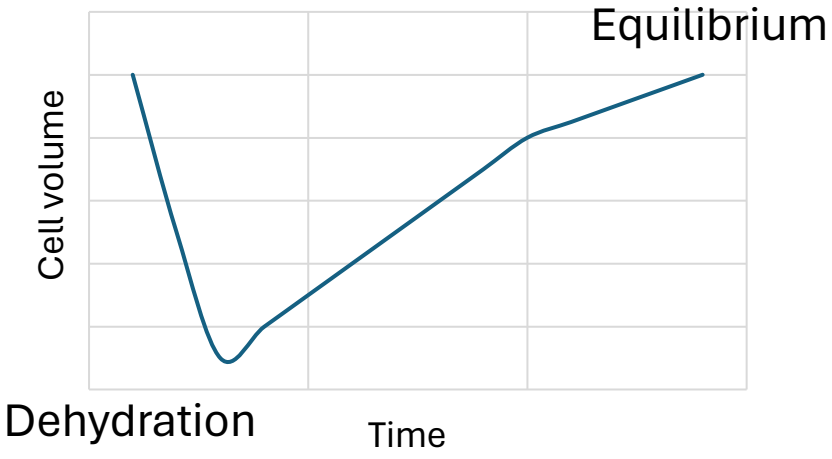
Gore et al., 2022

Type of CPA	Name	Molecular weight
Permeating	Dimethylsulfoxide (DMSO)	78,13
	Glycerol	92,09
	Ethylene glycol (EG)	62,07
	Propylene glycol	76,09
Non-permeating with low molecular weight	Glucose	180,12
	Sucrose	342,30
	Trehalose	378,33
	Raffinose	594,52
	Hydroxyethyl starch (HES)	130-200 kDa
	Albumins	67 kDa
Non-permeating with high molecular weight	Polyvinylpyrrolidone (PVP)	$3\text{-}36 \times 10^4$
	Polyvinyl alcohol (PVA)	$2\text{-}12 \times 10^4$
	Polyethylene glycol (PEG)	$2\text{-}400 \times 10^2$
	Dextran	$1\text{-}200 \times 10^4$

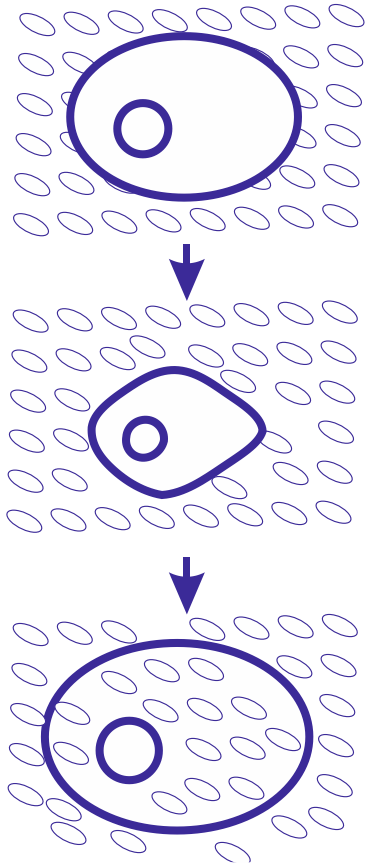
What are the main steps of cryopreservation?

Stages:	Damaging factors:
• Addition of cryoprotective agens (CPA);	→ • Osmotic injury / CPA toxicity *(rate / temperature matters*)
• Cooling/freezing of samples (Usually to -80°C… -196°C);	→ • Overcooling and freezing injury
• Storage;	→ • Ice growth during temperature fluctuations
• Thawing (Usually at 37°C – 40°C);	→ • Thawing injury
• Removal of cryoprotectant (washing);	→ • Osmotic injury again / CPA toxicity

Addition of CPA



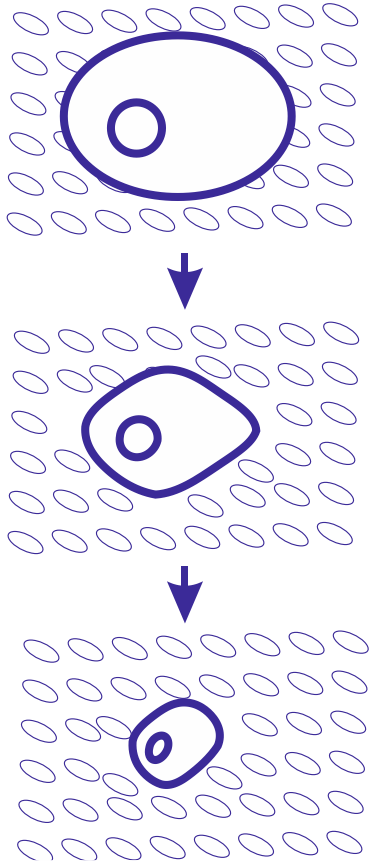
Permeating CPA
(DMSO, glycerol, ethylene glycol)



Volume recovery, the CPA penetrates into cell

Dehydration

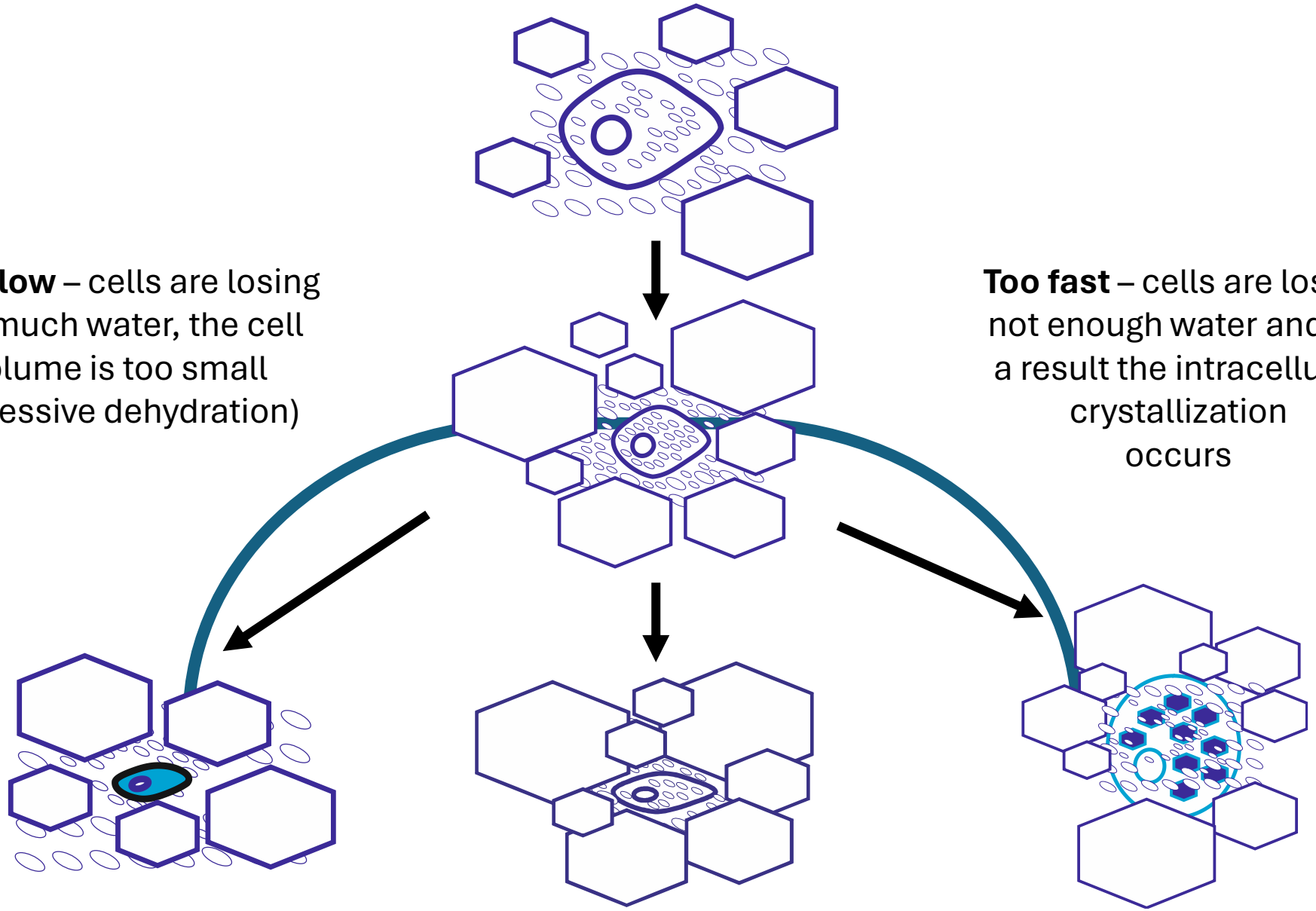
Non-permeating CPA
(Sugars, PEG, PVP, HES)



More dehydration, CPA does not penetrate the cell

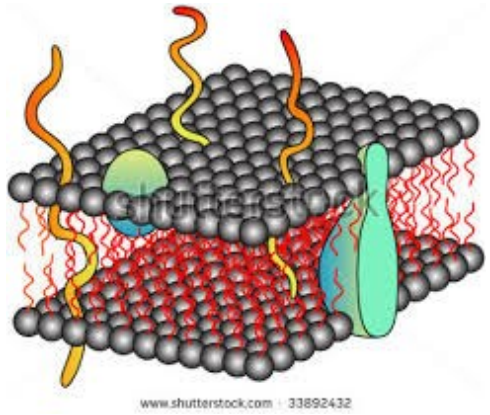
Optimal cooling rate

Too slow – cells are losing too much water, the cell volume is too small (excessive dehydration)



Too fast – cells are losing not enough water and as a result the intracellular crystallization occurs

Optimal cooling rate



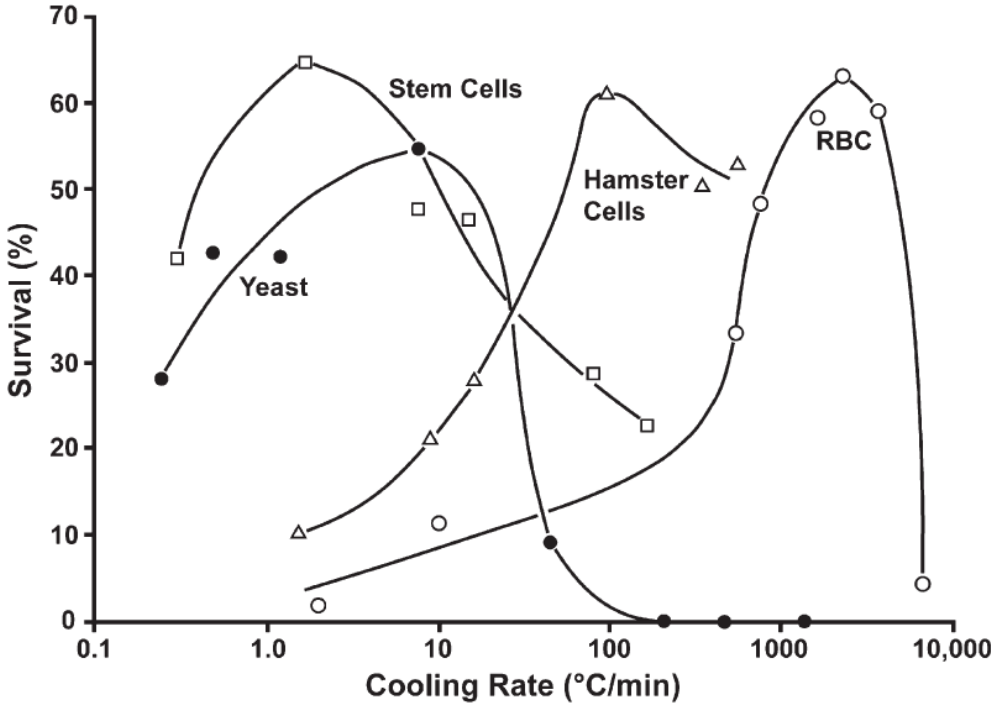
Different cells have different membranes

↓

Different membranes have different water transport characteristics

↓

The control of water transport and a cooling rate (crystal formation) is a basis for the development of “optimal” cooling rate



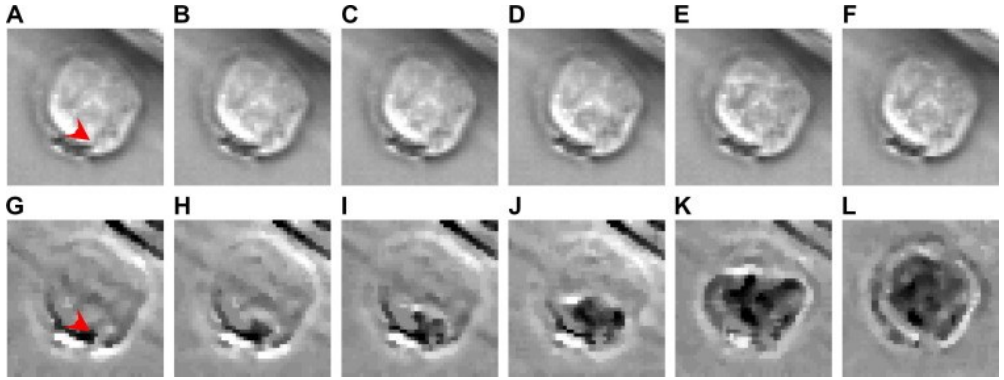
How may we find the best cooling rate?

Most common (let's try) approach

We just try different ways and maybe find the most appropriate

Scientific approach

We will study the intracellular ice formation using cryomicroscopy



Stott et al., 2009

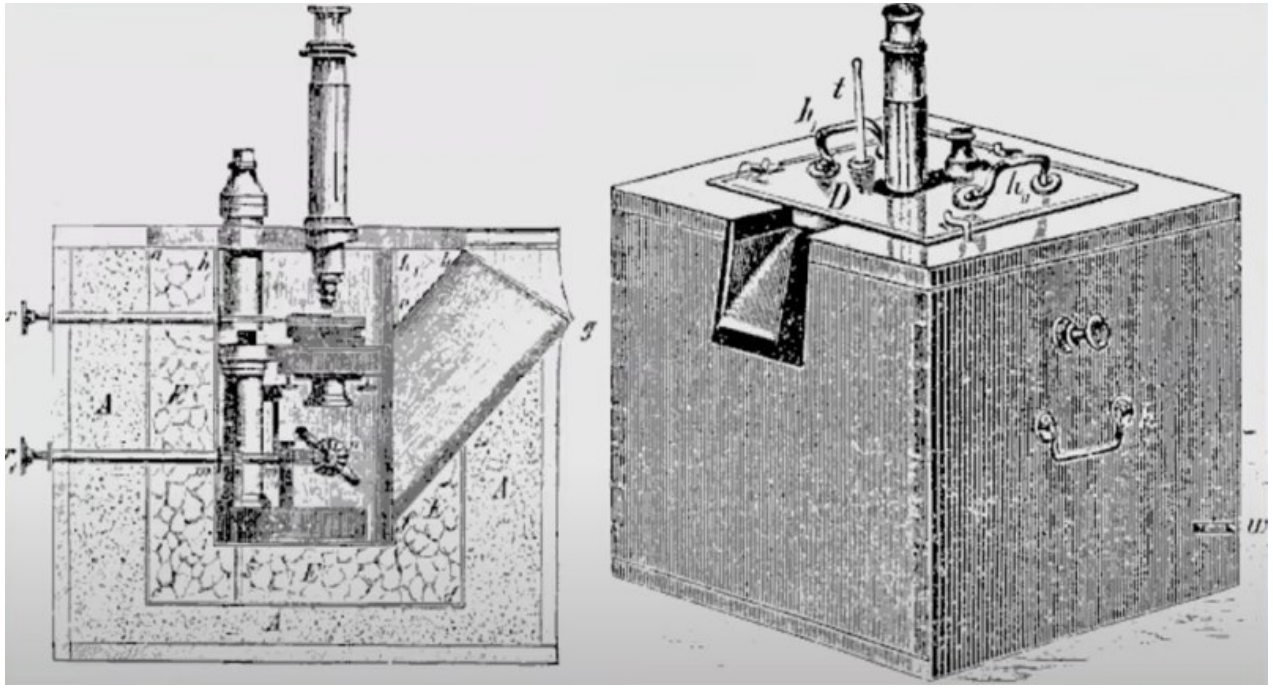
News | Published: 12 October 2017

Cryo-electron microscopy wins chemistry Nobel

Daniel Cressey & Ewen Callaway

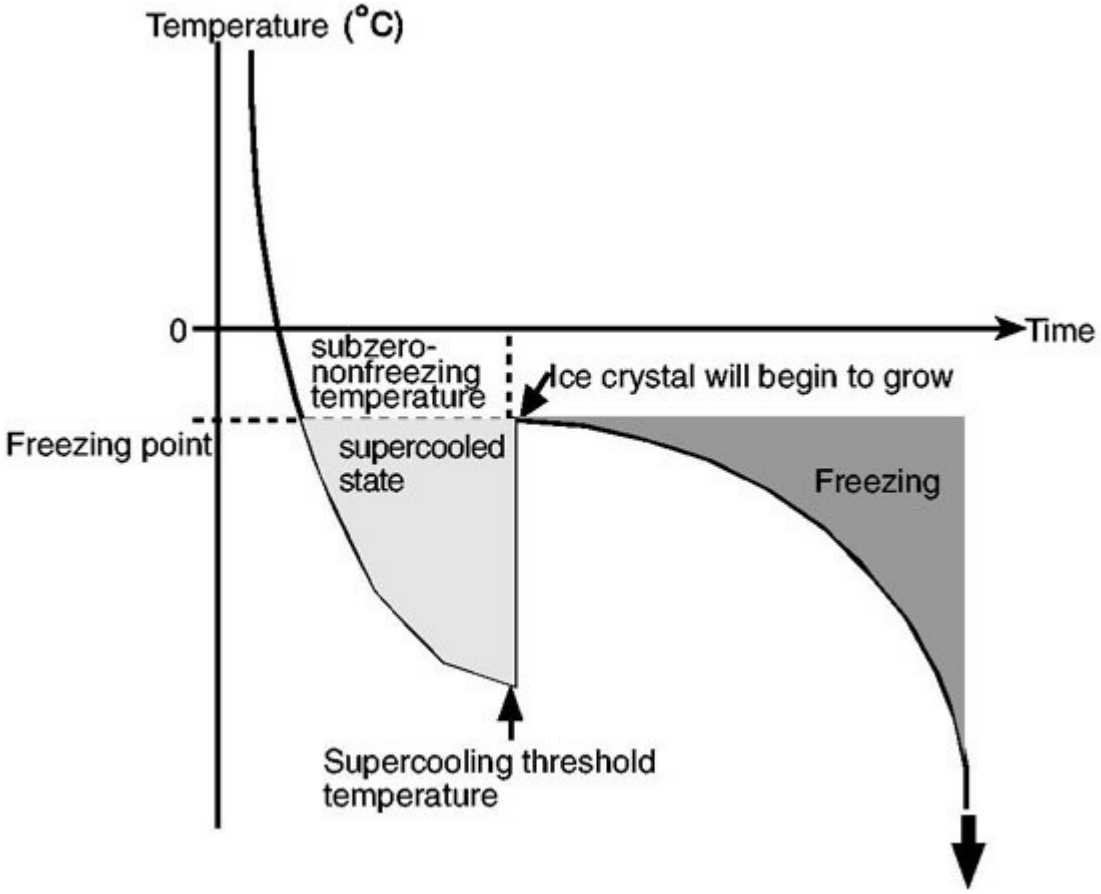
Nature **550**, 167 (2017) | Cite this article

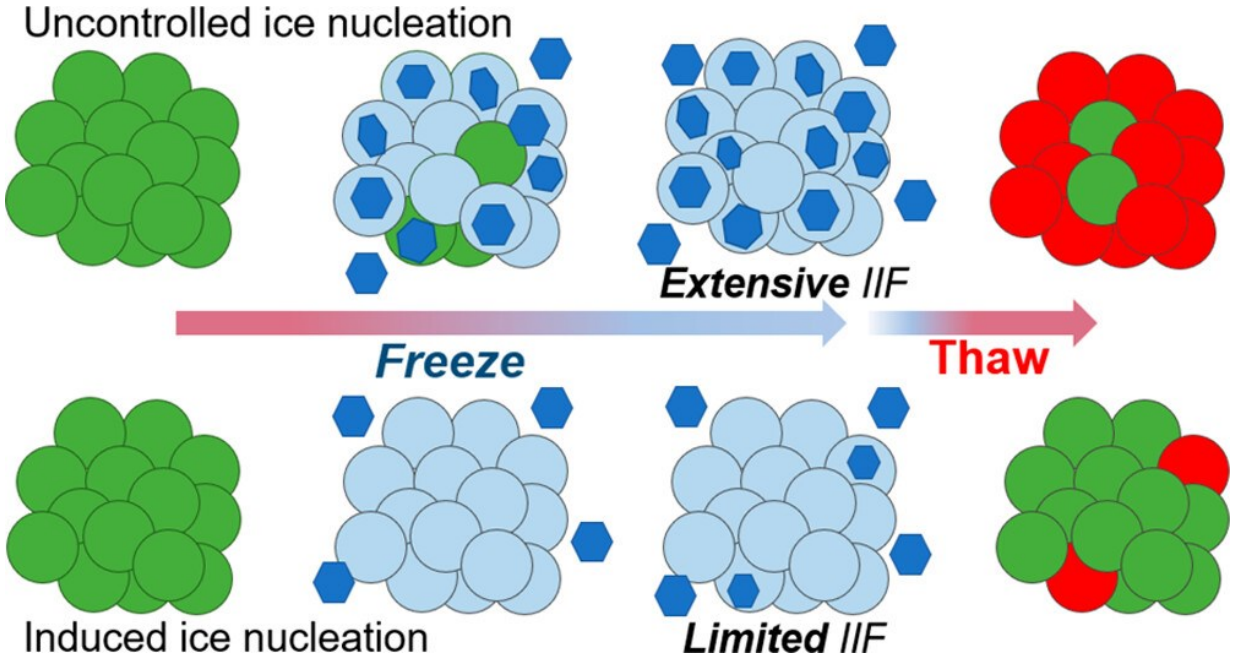
The first cryo-microscope



What are the other risks?

Overcooling (or supercooling)





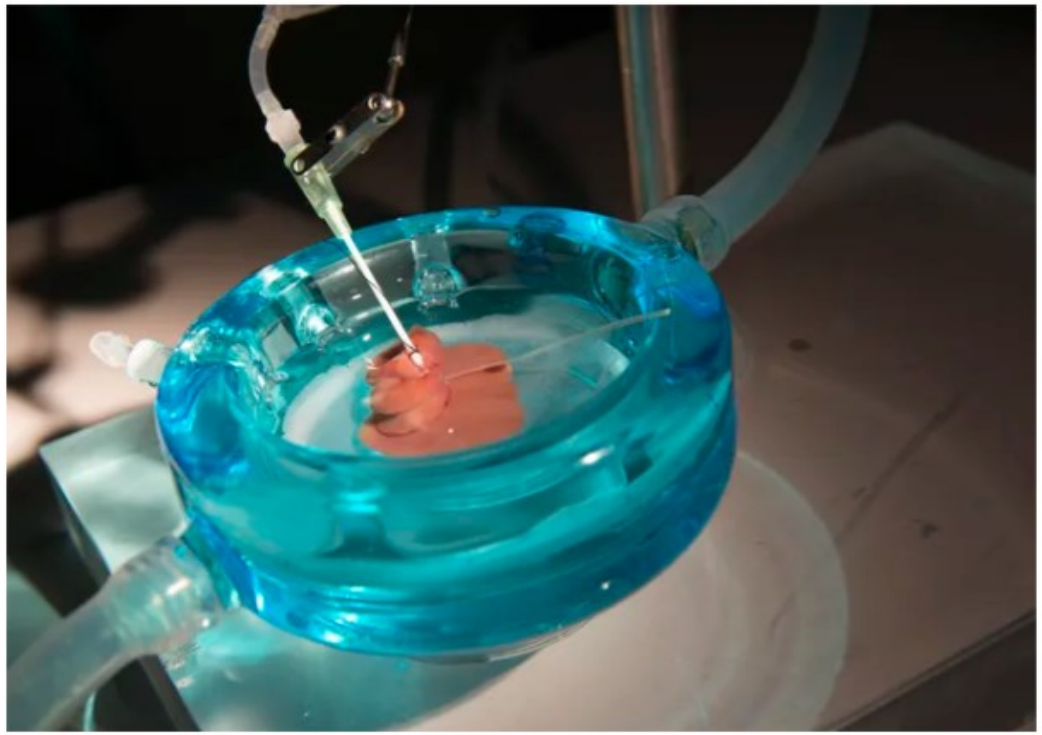
Murray et al., 2023

Supercooling can be also good!

New 'Supercooling' Technique Helps Preserve Organs

News

By Charles Choi published June 30, 2014



A supercooled rat liver sits in the preservation solution in the machine perfusion system. (Image credit: Wally Reeves, Korkut Uygun, Martin Yarmush, Harvard University)

REVIEW article

Front. Transplant., 23 October 2023
Sec. Vascularized Composite Allotransplantation
Volume 2 - 2023 | https://doi.org/10.3389/frtra.2023.1269706

This article is part of the Research Topic
Editors' Showcase: Vascularized Composite Allotransplantation
View all Articles >

Supercooling: a promising technique for prolonged preservation in solid organ transplantation, and early perspectives in vascularized composite allografts

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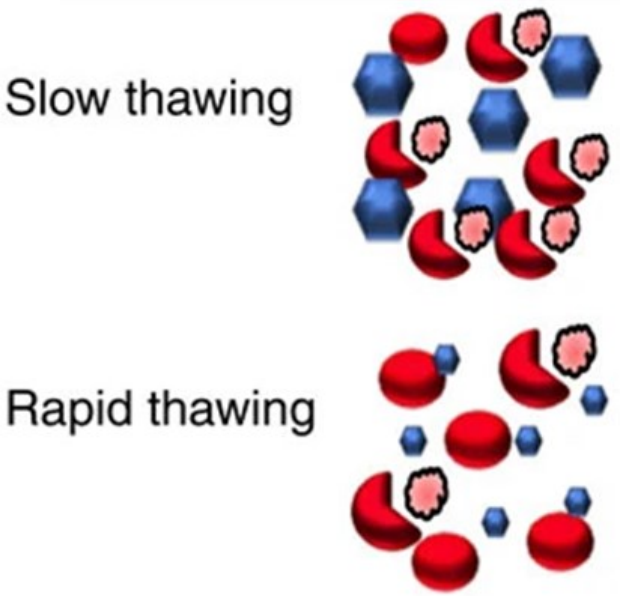
What happens at thawing? Is it also damaging?



- **Recrystallization** (small ice crystals can uncontrollably grow during slow thawing)
- **Osmotic stress**
- **Overheating** the sample can result in the increased **toxicity of CPA** (leaving the ice crystal approach)

-196°C

+37-40°C

Toxicity of cryoprotectants (DMSO as an example)

REGENERATIVE MEDICINE, VOL. 15, NO. 3 | REVIEW

Open Access

Dimethyl sulfoxide: a central player since the dawn of cryobiology, is efficacy balanced by toxicity?

Maooz Awan, Iryna Buriak, Roland Fleck, Barry Fuller, Anatoliy Goltsev, Julie Kerby, Mark Lowdell, Pavel Mericka, Alexander Petrenko, Yuri Petrenko, Olena Rogulska, Alexandra Stolzing & Glyn N Stacey

Published Online: 28 Apr 2020 | https://doi.org/10.2217/rme-2019-0145

Stem cells

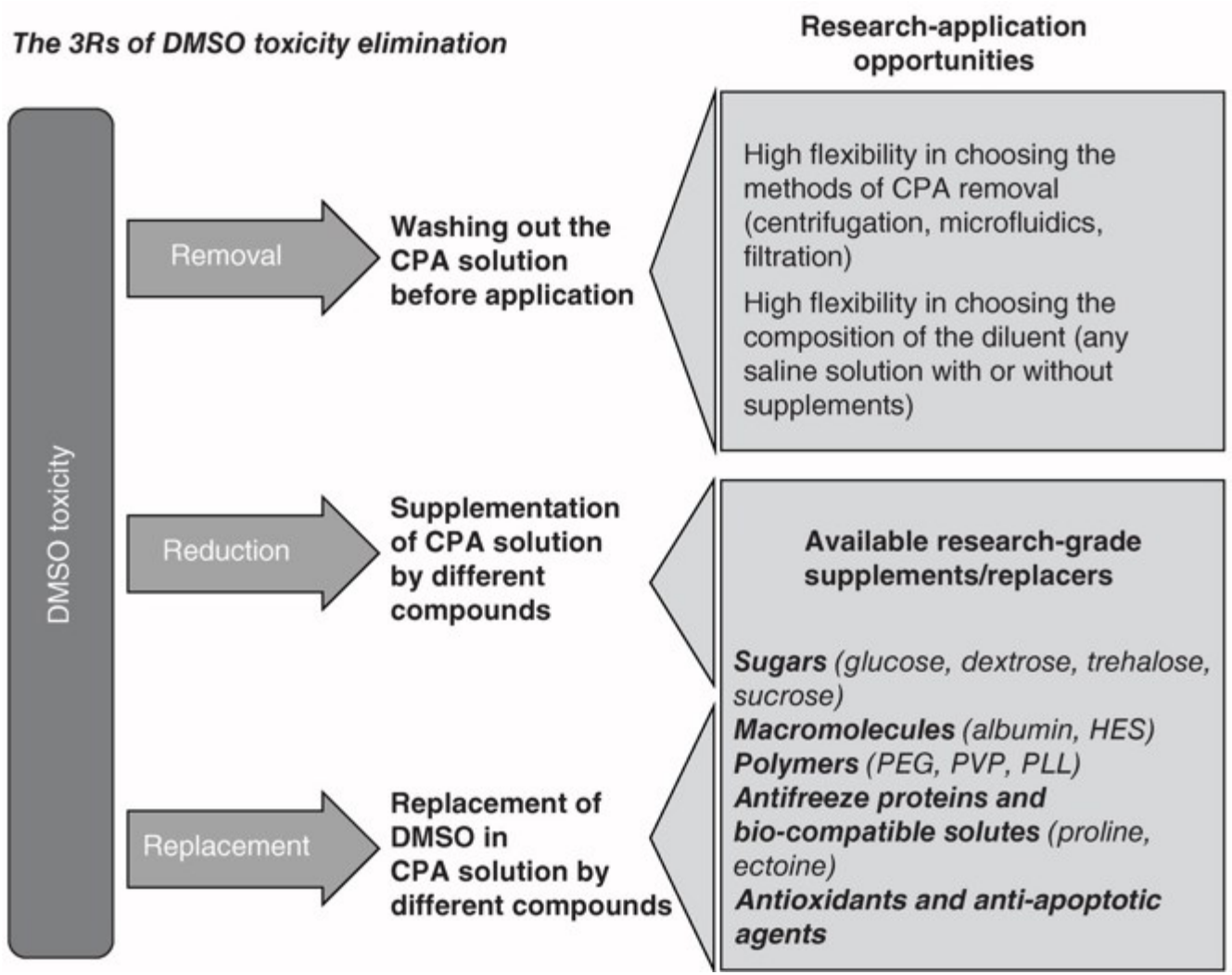
Human embryonic stem cells HUES-7, foreskin-derived mesenchymal stem cells	0.01, 0.1, 1.0% (v/v)	RT°	– Dose-dependent changes in cell viability, morphology, adhesion and gene expression: inhibition of embryoid bodies formation, decrease in adhesion, cell death – Low and medium DMSO doses upregulate mesodermal markers – Higher DMSO doses downregulate ectodermal differentiation	[11]
Human umbilical cord blood stem cells	40%, 10% and DMSO removal	RT° and post thaw	– 40% DMSO lethal. 10% DMSO no viability reduction after 1 h. DMSO washout improved viability Optimum 7.5–10%	[12]

Adverse reactions for patient

Adverse events	DMSO-depleted (19 patients)	Unmanipulated (34 patients)	p Value
Gastrointestinal symptoms (nausea, vomiting, abdominal cramps)	0	7	
Vasovagal syncope	0	1	
Angina pectoris	0	1	
Other cardiovascular symptoms (bradycardia, tachycardia, hypotension, hypertension)	3	16	
Headache	1	2	
Pressure on breast/ neck	1	3	
Total number of adverse events	5	30	
Total number of patients with adverse effects	3	16	0.024

Akkok et al., 2008

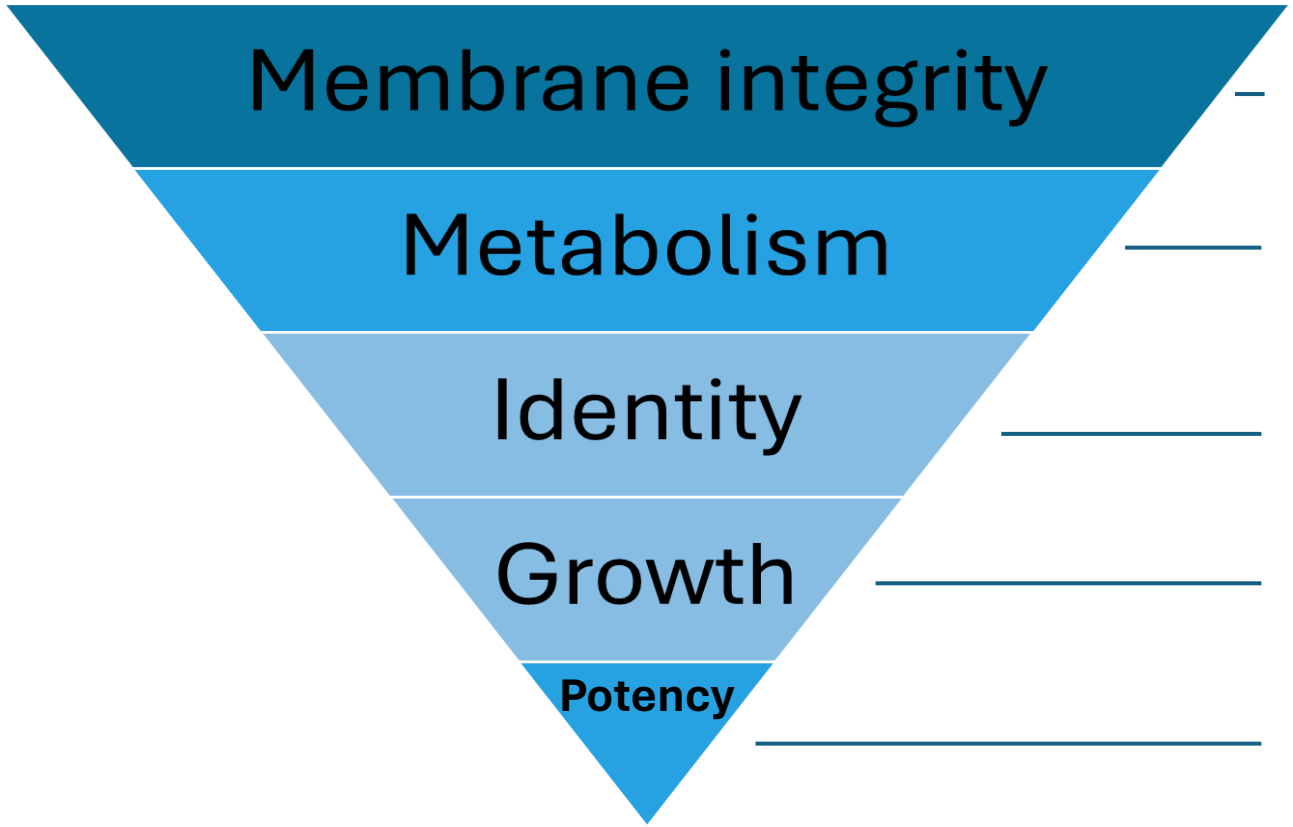
Can we reduce the toxicity of DMSO?



So, we cryopreserved the cells, even thawed and removed the cryoprotectant… work done?

Should we check anything?

Determination of the viability of cells



Check if the membranes are ok and cell number

Deeper check, if the general metabolism is not altered, should be done after some time

We should check if there are no abnormalities in cell phenotype, transformations etc.

We should check how cells behave in culture – can they attach and grow. Are they different from the fresh cells?

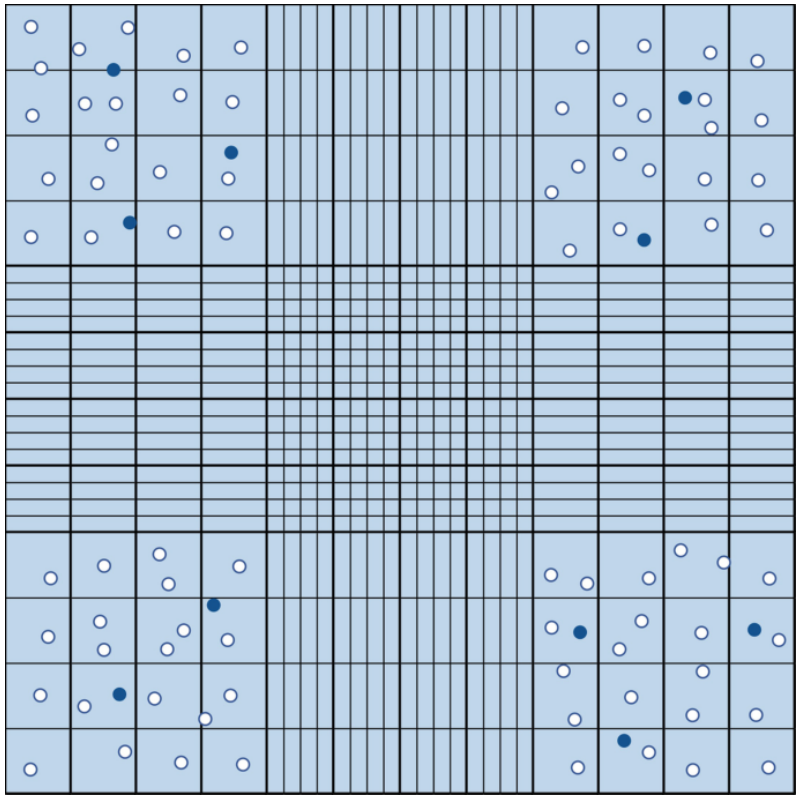
We should check their specific functional properties *in vitro*

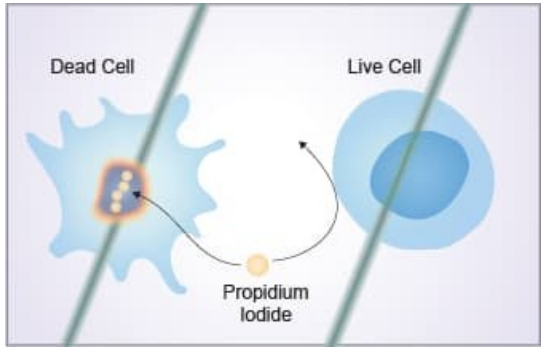
Transplantation

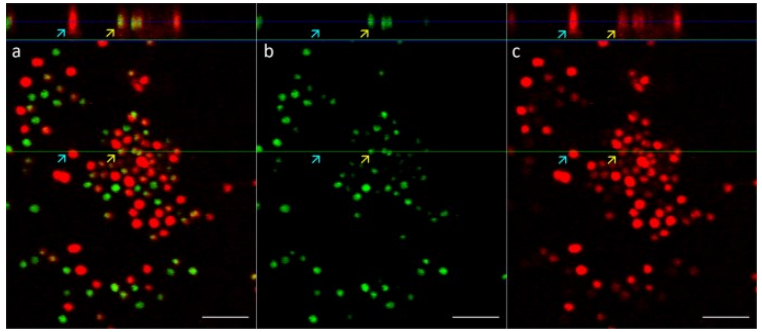
If the cells are aimed to be used for clinical applications – ideally, we should check how they behave in vivo after transplantation

Membrane integrity

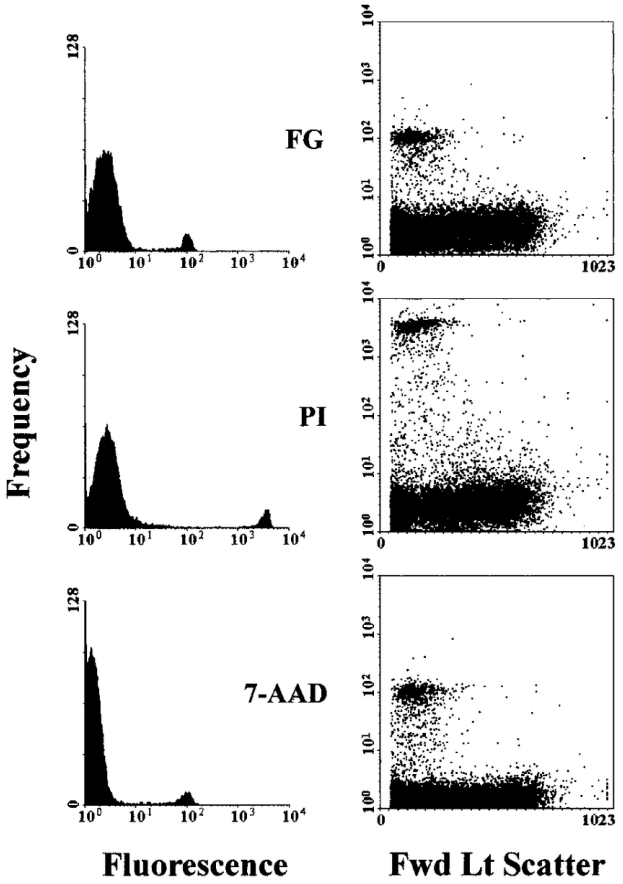
Trypan blue staining





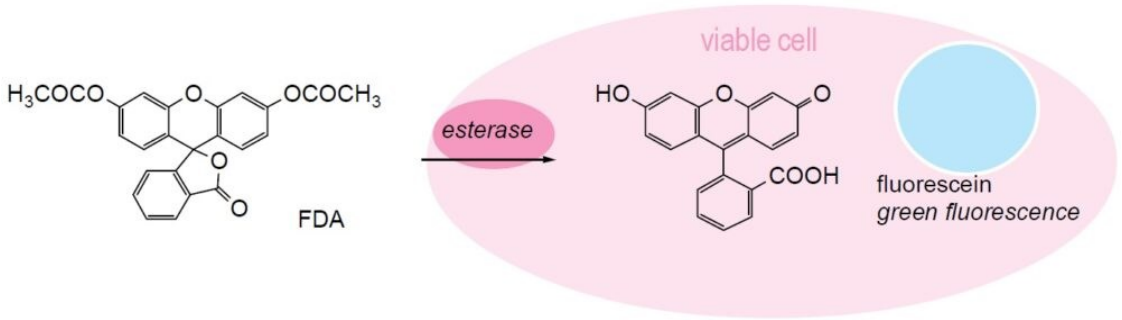


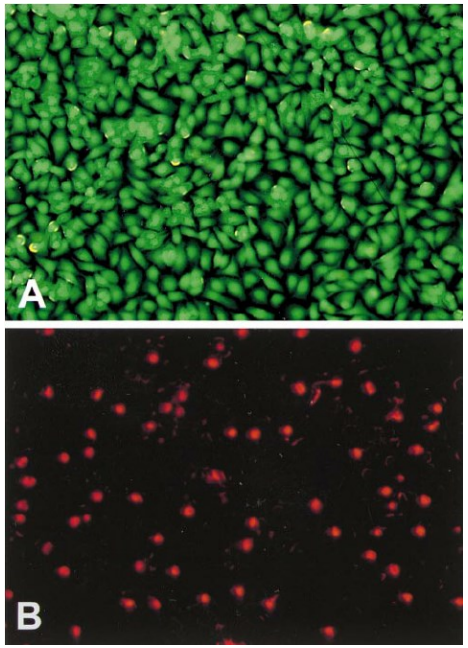
Propidium iodide staining



Metabolic activity determination

Fluorescein diacetate

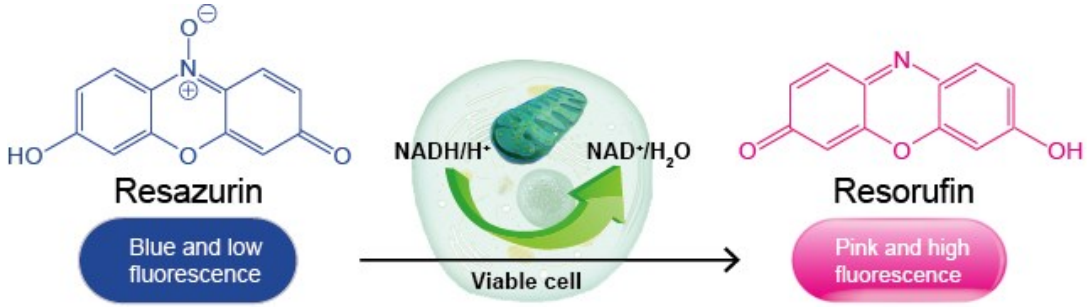




Live

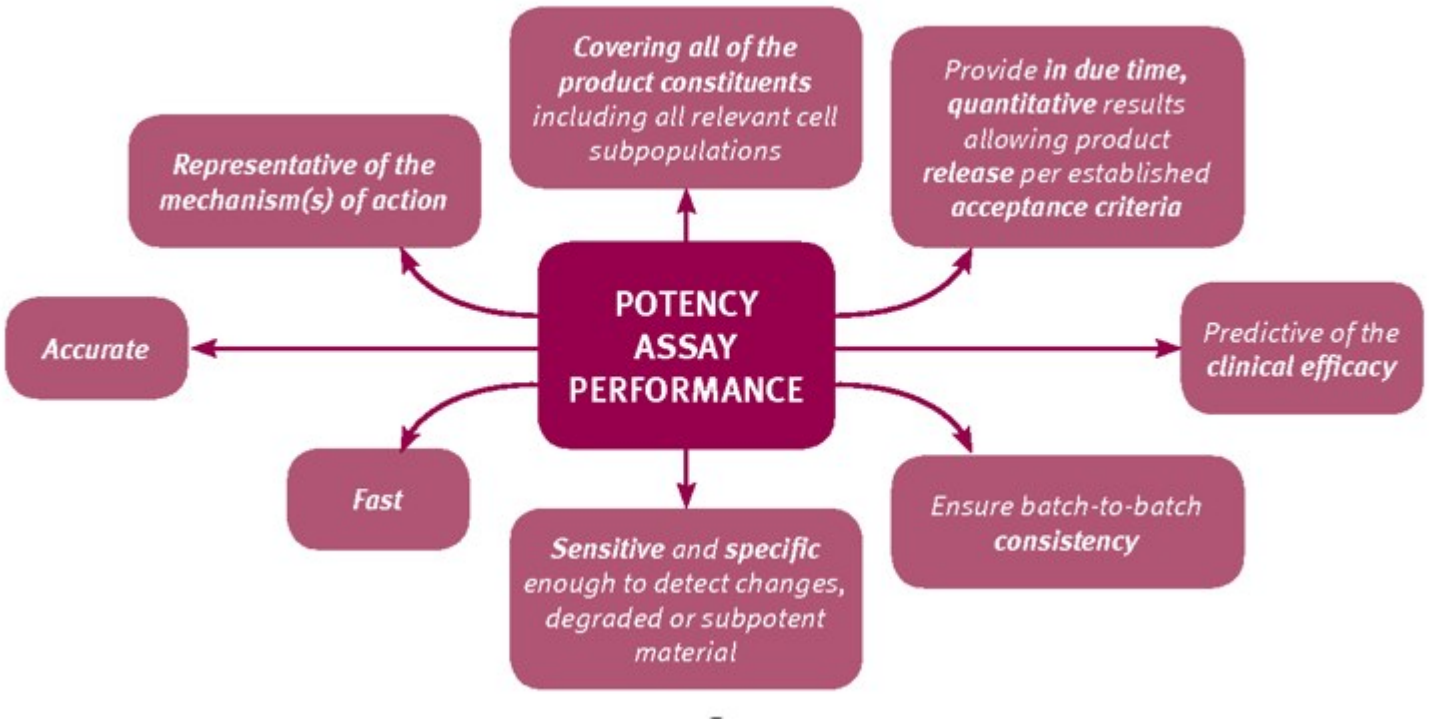
Dead

Alamar blue / resazurin



Proliferation and specific functional activity – potency assays

Each cell type has specific functional properties, so adequate methods should be used to confirm the potency



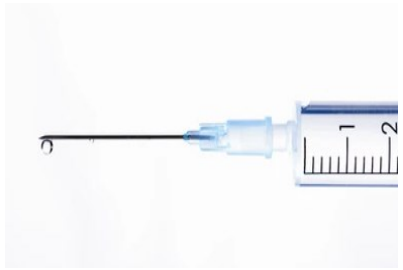
Mesenchymal stem cells: differentiation, paracrine / immunomodulatory properties;

Hematopoietic stem cells: colony-forming capacity;

Pancreatic islet cells: insulin production

General cryopreservation protocol:

Addition of CPA	Controlled freezing	Storage	Thawing	CPA removal	Testing
Drop-wise 2-3 min	-1°C per min down to -80°C Fast transfer to -196°C (120 – 180 min)	at -150-196°C (years)	Fast, 3-5 min	Drop-wise addition of diluent, centrifugation 15 min	72 hrs to weeks



Questions?

Take-home message part one:

Cryoprotectants: there are various type of cryoprotectants. Some of them (glycerol, DMSO) may permeate the cells and protect its' intracellular content avoiding intracellular crystallization. Others (sugars, polymers, starches) cannot permeate, but help to provide controlled dehydration.

Cooling rates: It is important to use the optimal cooling rate during the cryopreservation to avoid the excessive dehydration (too slow cooling) or intracellular crystallization (too fast cooling).

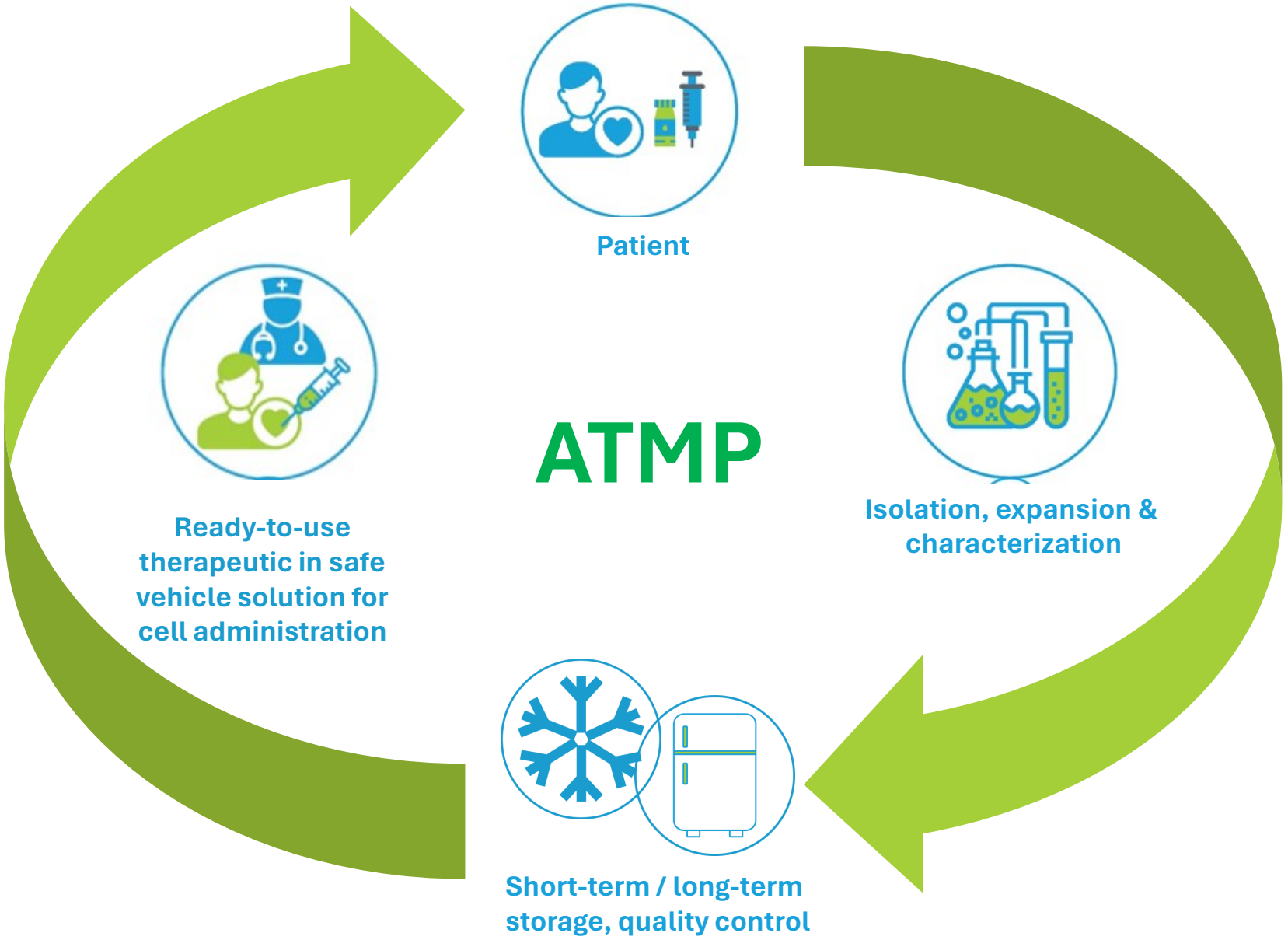
Storage: It is important to keep the constant storage temperatures without fluctuations to avoid the damage.

Thawing: The rapid thawing rates are preferable since they allow to avoid the recrystallization process

Quality control: It is important to use the panel of tests, which will show the overall viability/recovery of cells and their functional activity. It may include studying the membrane integrity, metabolic activity, growth in vitro and implementation of specific function.

Block II. Working examples in the field of stem cell cryopreservation for clinical use

- Alternative approaches for stem cell cryopreservation with reduced DMSO concentration
- Alternative quality control methods for stem cell cryopreservation
- Hypothemic storage (storage at 4°C) of stem cell suspensions for clinical applications


Patient
Isolation, expansion & characterization
Short-term / long-term storage, quality control
Ready-to-use therapeutic in safe vehicle solution for cell administration
ATMP

Multipotent mesenchymal stromal cells

Multipotent mesenchymal stromal cells

DIFFERENTIATION

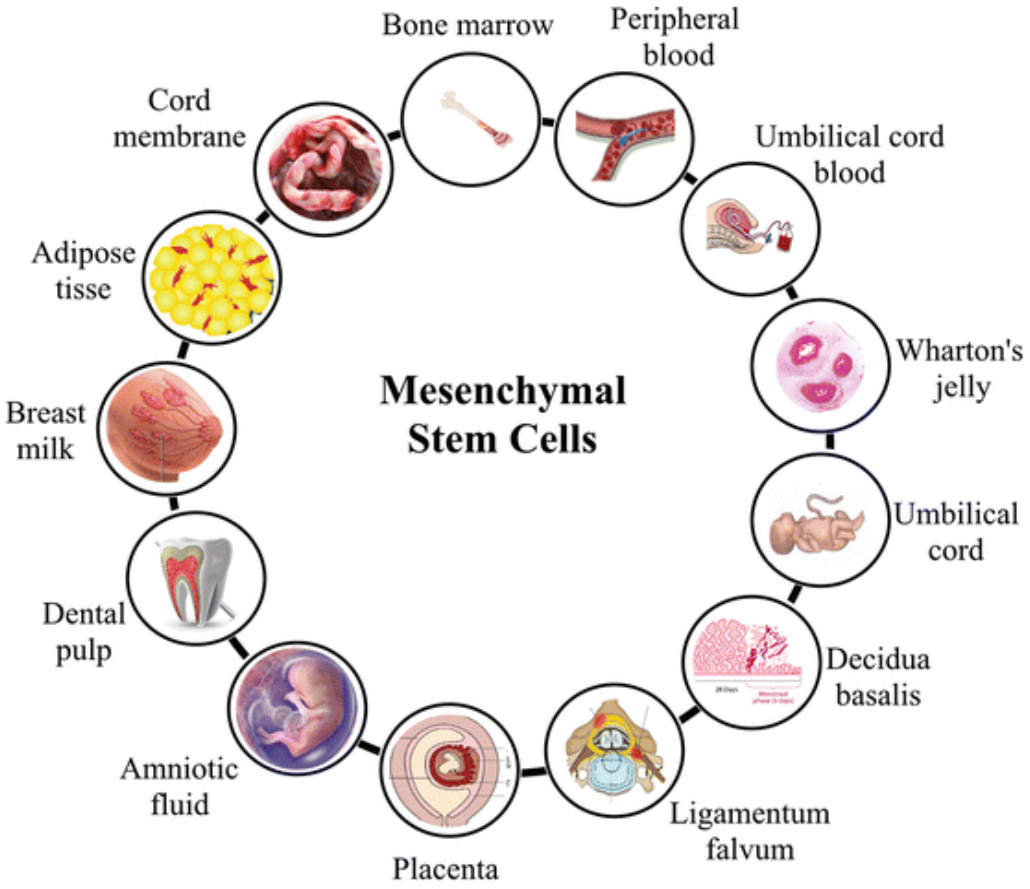
Can differentiate into at least 3 lineages (adipogenic, osteogenic, chondrogenic)

IMMUNOMODULATION

Modulate immune response *in vitro* and *in vivo*. Provide anti-inflammatory action

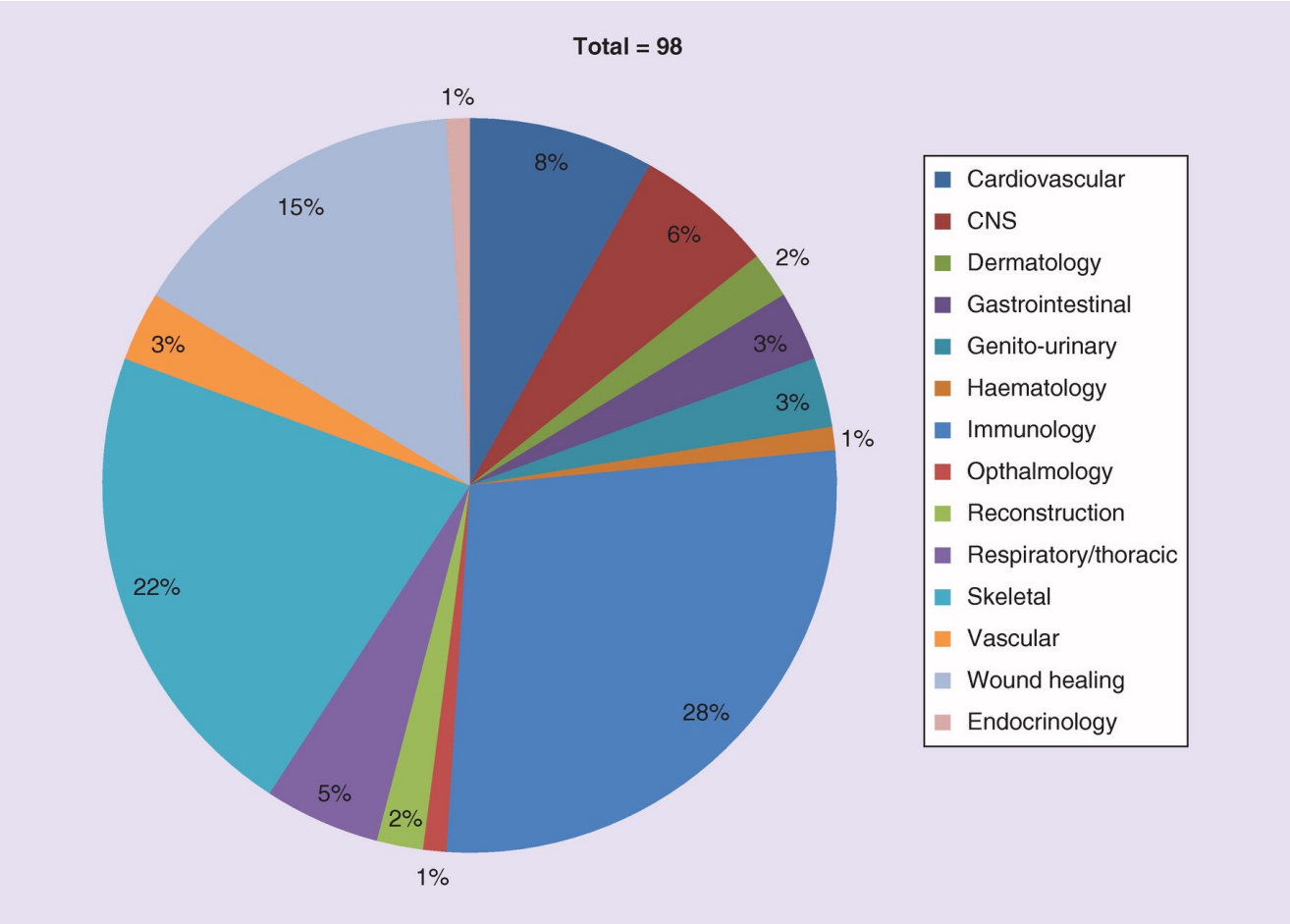
PARACRINE ACTIVITY

Secrete growth factors, cytokines, extracellular vesicles



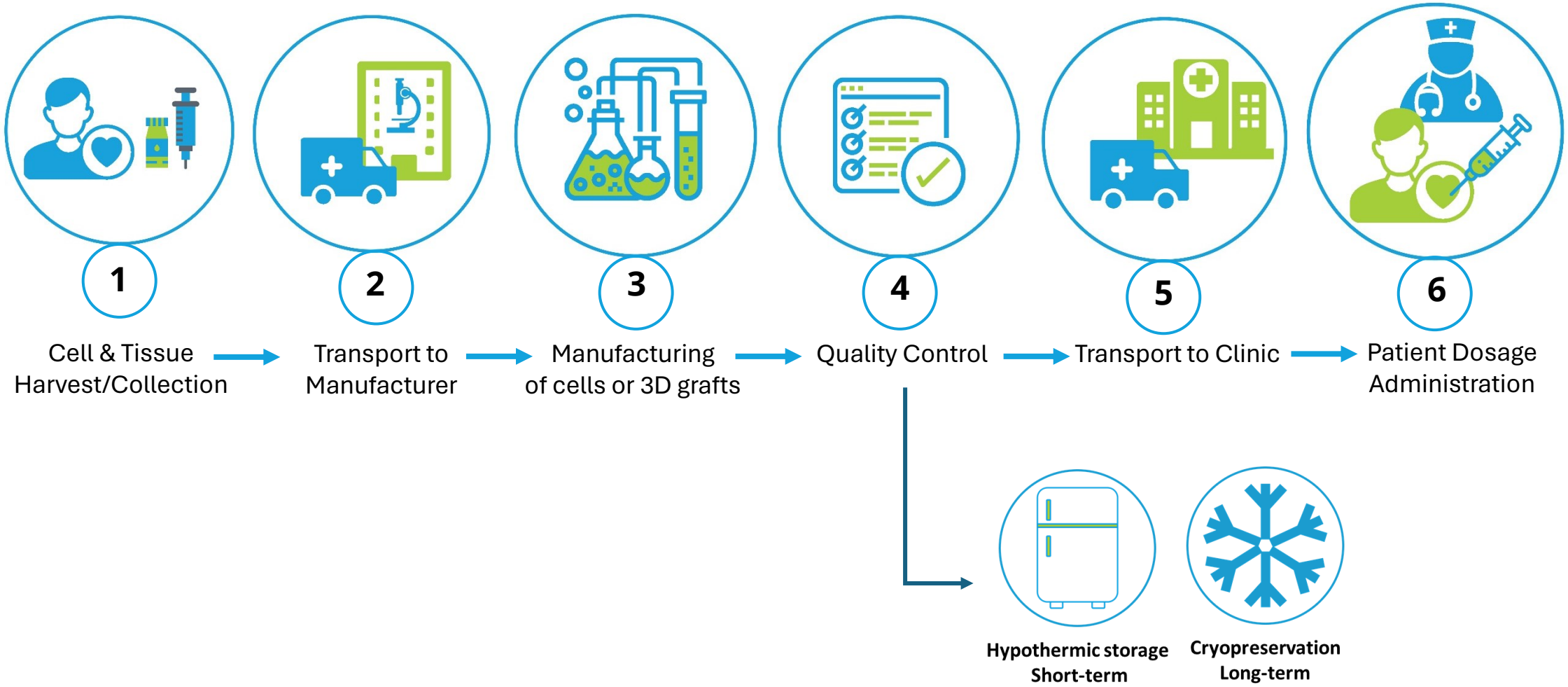
Identity, purity, potency = Quality control

EU clinical trials involving ‘mesenchymal stem cell’.

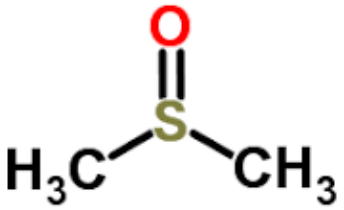


Wilson et al., 2019

The processing



Challenge



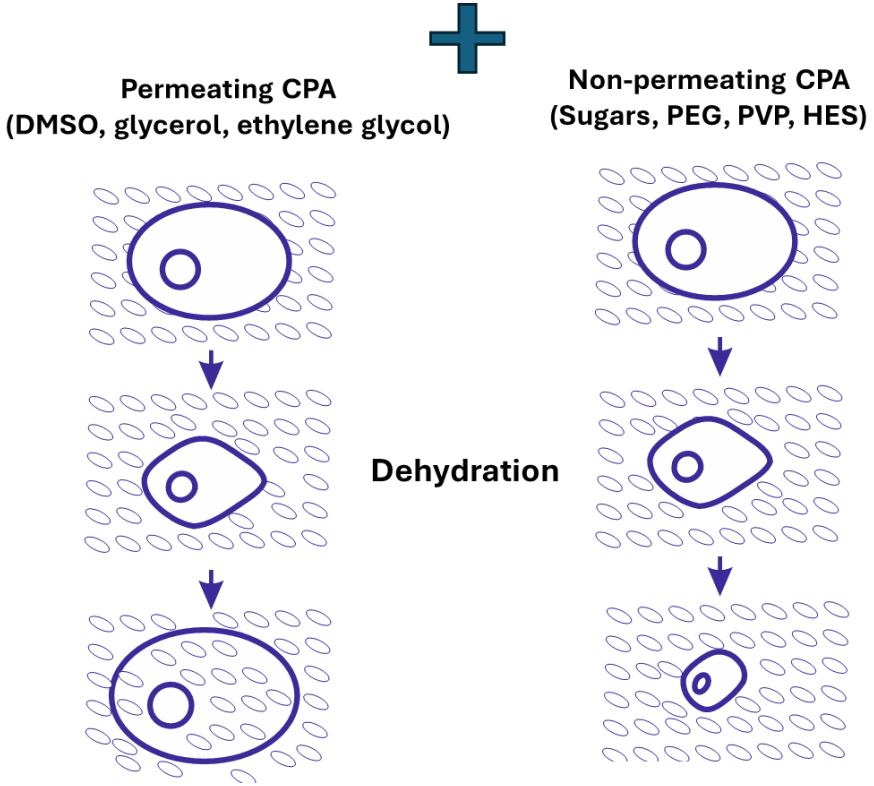
DMSO → toxic, should be removed

Replacements, additives to reduce or remove DMSO

- Sugars (glucose, dextrose, trehalose, sucrose)
- Macromolecules (albumin, HES)
- Polymers (PEG, PVP, PLL)
- Antifreeze proteins and biocompatible solutes (proline, ectoine)
- Antioxidants and anti-apoptotic agents

Non-toxic cryopreservation of adipose tissue MSCs

Non-toxic cryopreservation of adipose tissue MSCs



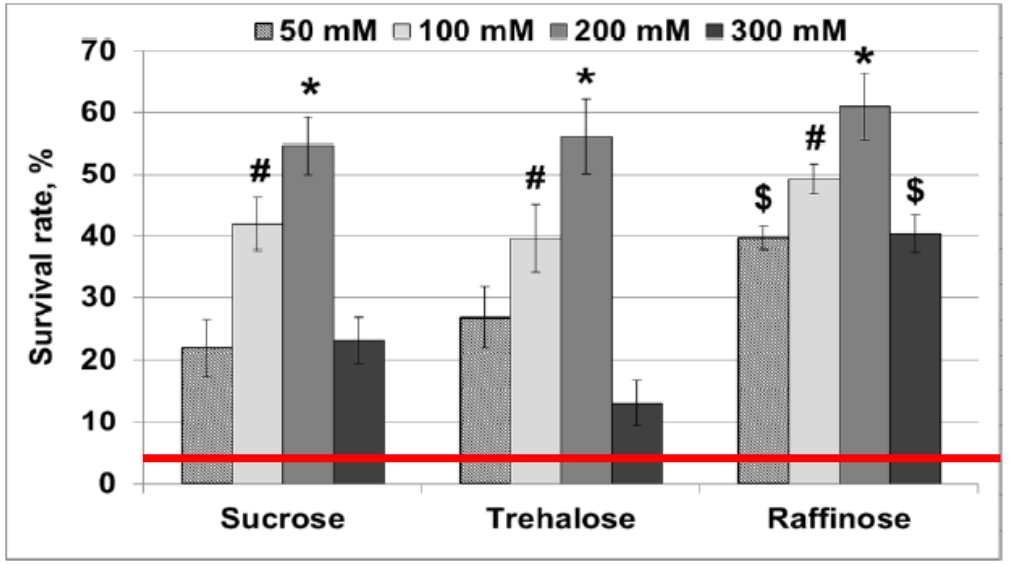
Loading non-permeable CPA into cells

If we culture cells in the presence of sugar – it will go inside the cells by endocytosis

CryoLetters 35 (3), 239-246 (2014)

A SUGAR PRETREATMENT AS A NEW APPROACH TO THE Me_2SO- AND XENO-FREE CRYOPRESERVATION OF HUMAN MESENCHYMAL STROMAL CELLS

Yuri A. Petrenko*, Olena Y. Rogulska, Vitalii V. Mutsenko and Alexander Y. Petrenko



Cytotechnology
April 2017, Volume 69, Issue 2, pp 265–276 | Cite as

DMSO-free cryopreservation of adipose-derived mesenchymal stromal cells: expansion medium affects post-thaw survival

Authors Authors and affiliations
Olena Rogulska, Yuri Petrenko, Alexander Petrenko

Searching for best methods for the assessment of viability and functional activity of MSCs (QC – potency)

MSCs should be characterized:

Safety
- Sterility, Endotoxin, Mycoplasma
- Tests for opportunistic viruses

Identity
- Specific test to distinguish it from others

Purity
- Free of extraneous materials

Potency
- Assay for biological function

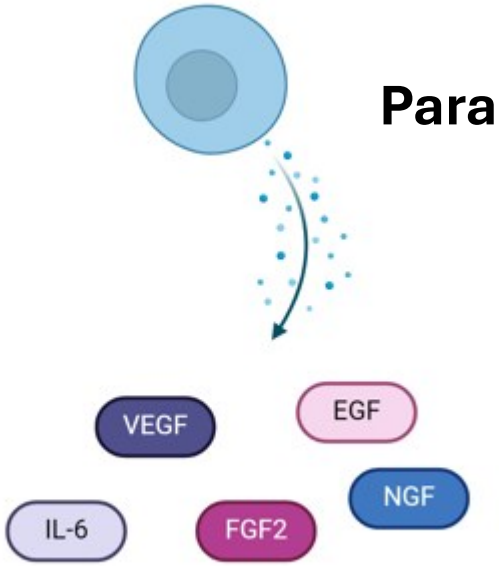
Usual methods for MSCs:

- Phenotype / viability
- Ability to grow (>3 days)
- Differentiation capacity (>3 weeks study) - recovery

Desirable methods for MSCs:

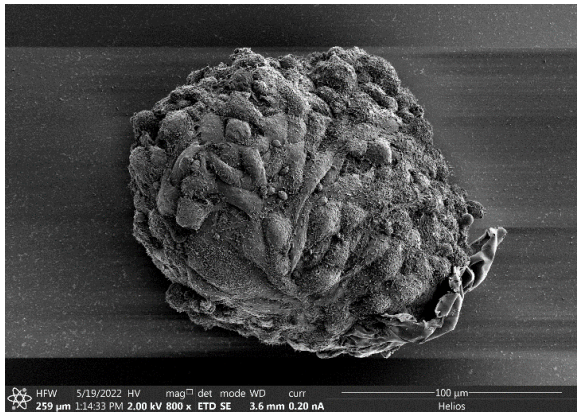
- Fast (72 hrs) & informative
- Show functional state of the cells
- Mimic natural conditions

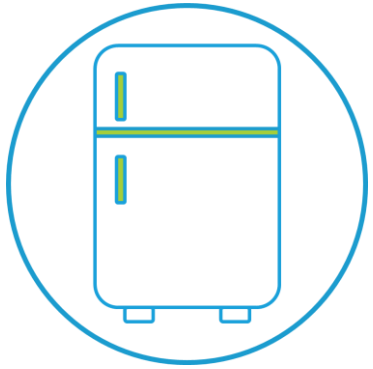
Searching for best methods for the assessment of viability and functional activity of MSCs (QC – potency)



Paracrine activity – capacity to secrete growth factors and cytokines

3D culture – capacity to build cell-cell contacts

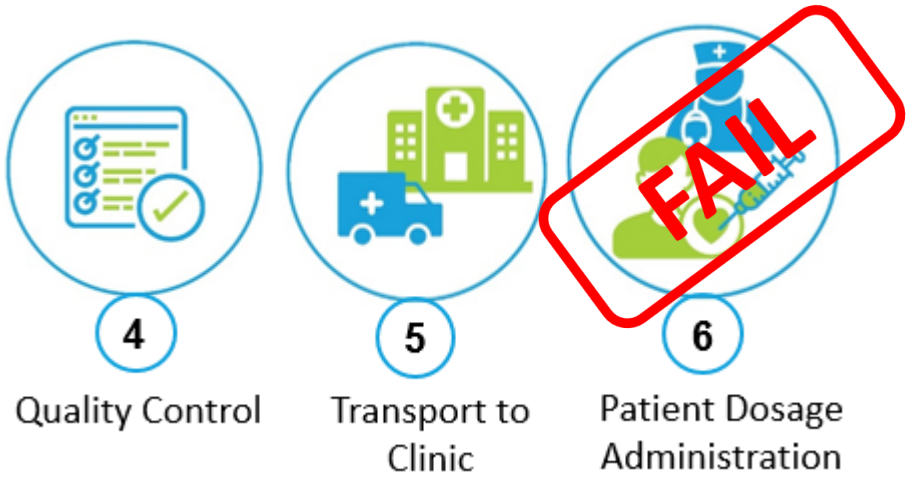




Hypothermic storage
Short-term

Non-frozen storage of cells, tissues or organs at positive temperatures, below physiological (37°C)

Hypothermic storage in stem cell therapy



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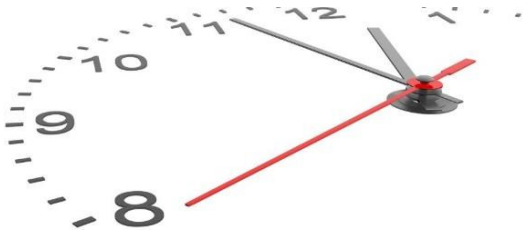


Minimally 72 hours are needed to conduct QC tests and deliver the MSCs to bedside

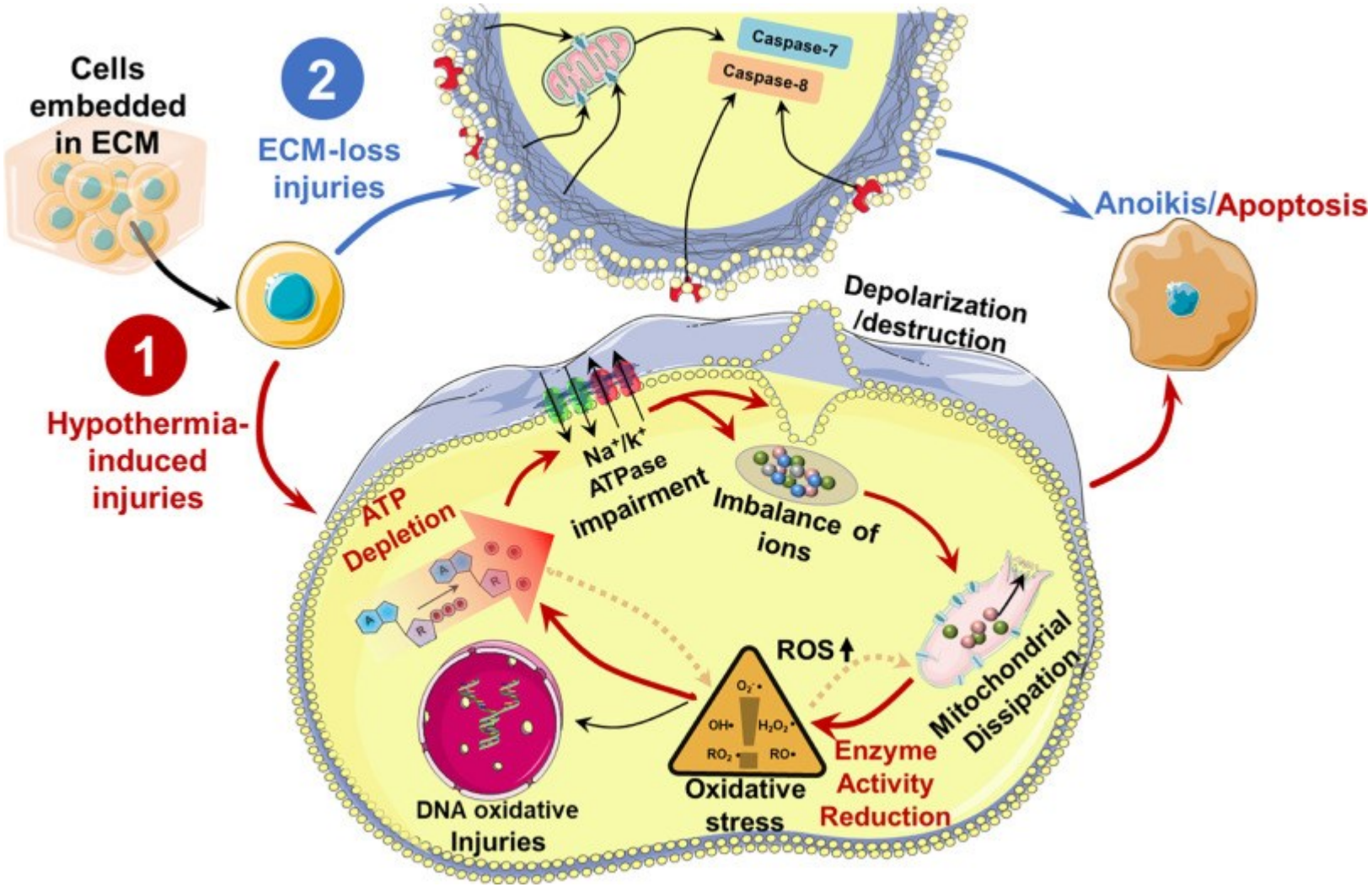
Challenge

MSCs should be administered to patient in clinically safe vehicle solution, which should preserve viability of cells during **at least 72 hours**

The answer

We should develop the hypothermic storage conditions or non-toxic cryopreservation protocol, when the solution can be used as vehicle for cell delivery

Damaging factors during hypothermic storage

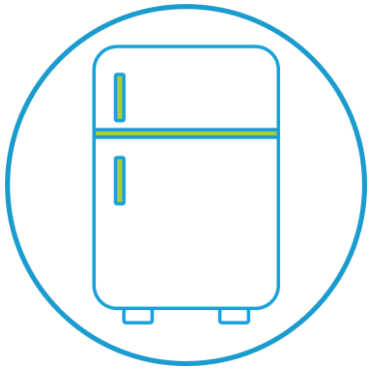


What should we do?

- Stabilize the membrane
- Provide pH support
- Reduce ROS by antioxidants
- Provide ECM

Ma et al., 2021

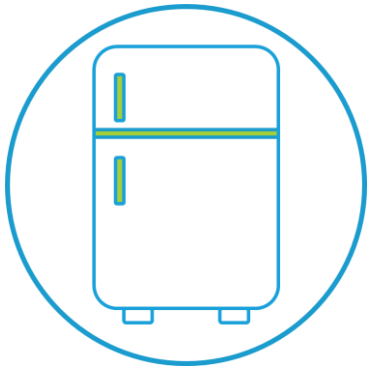
Clinical-grade solution for hypothermic storage



Hypothermic storage

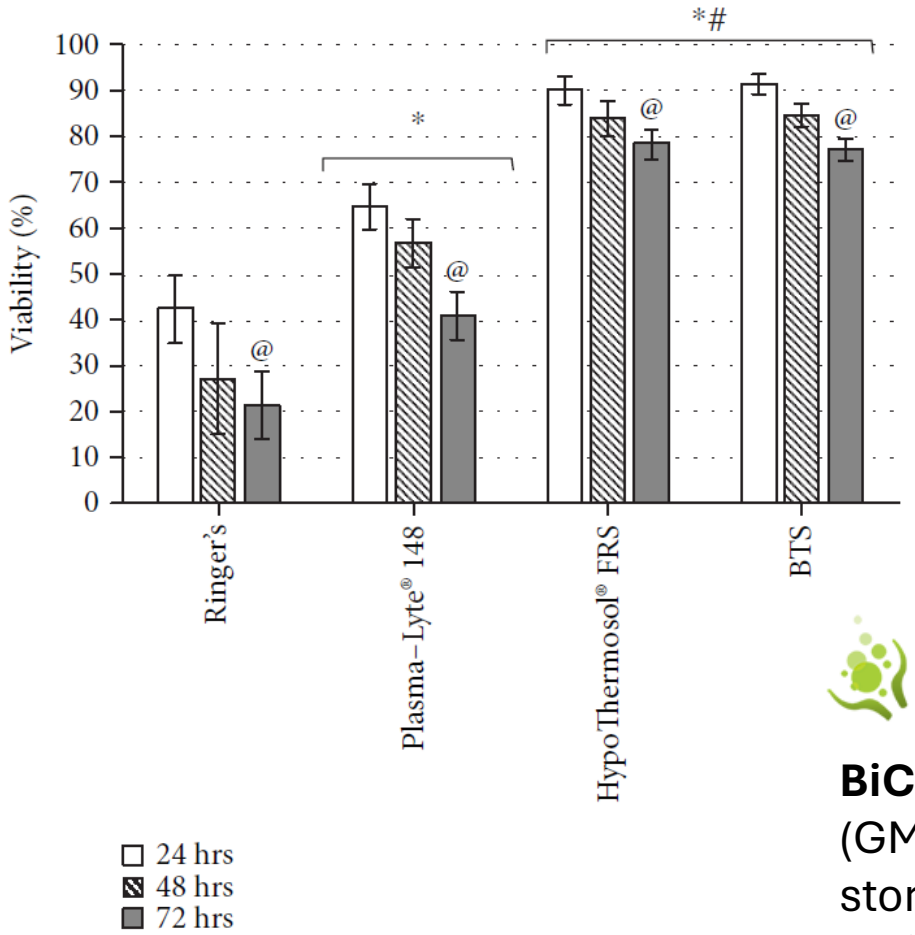
- Animal component-free
- Simple, minimal essential composition
- Ensure sufficient viability of cells, preserve functional properties
- Consists of clinically-acceptable compounds to ensure the use of the solution as a vehicle for the administration of cells to patient

Petrenko Y., Chudickova M, Vackova I., Groh T., Kosnarova E., Cejkova J., Turnovcova K., Petrenko A., Sykova E., Kubinova S. Stem cells international, 2019, Article ID 5909524

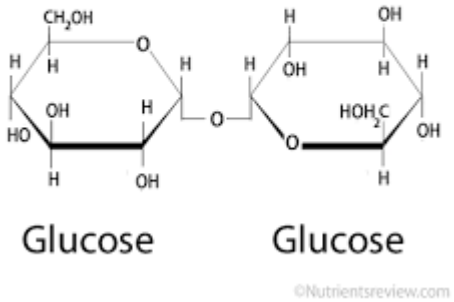


Hypothermic storage

We developed Buffered trehalose solution (BTS)



Trehalose



We substitute Na^+ for impermeable molecule

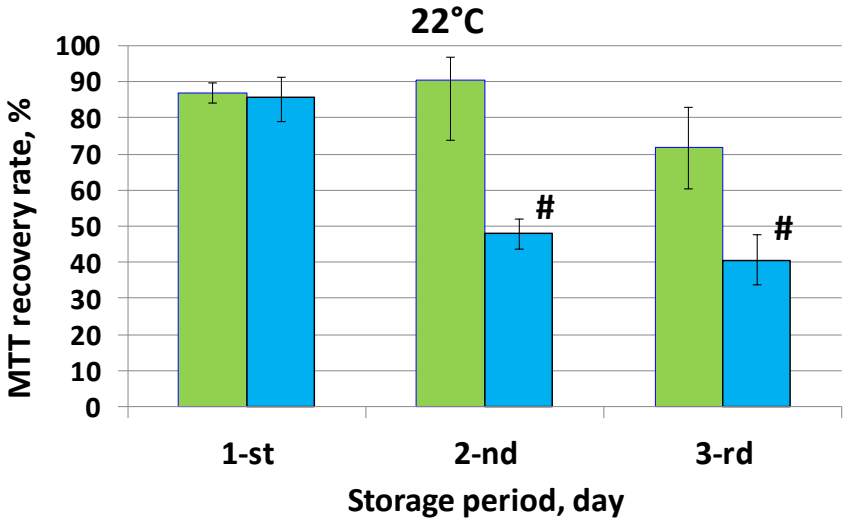
Bioinova

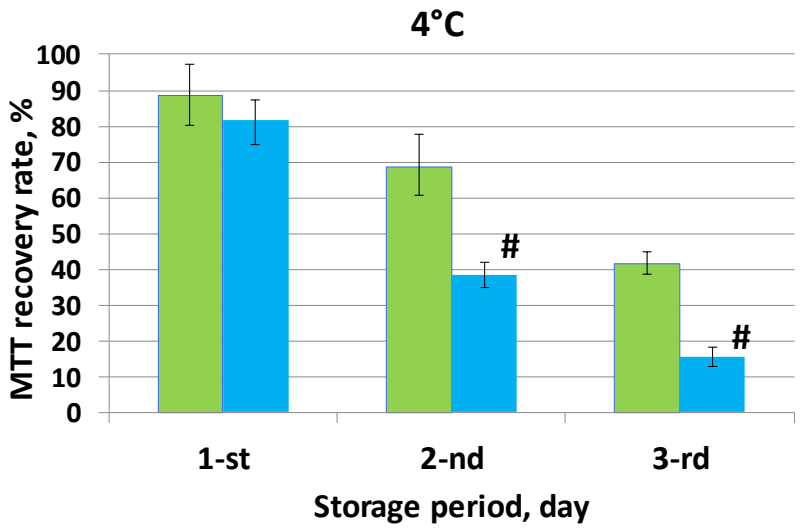
BiCureSol® - Good Manufacturing Procedure (GMP)-compliant solution of excipients for storage and transportation of cells maintaining **cell viability above 92% for at least 72 hours** at 2-8 °C.

3D culture to improve the viability (alginate beads)

MSCs encapsulated into alginate beads were stored in sealed vials at **ambient** (22˚C) and **hypothermic** (4˚C) temperatures

- MSCs in AMS
- MSCs in suspension





 # - P<0.05 with respect to cells in AMS

Take-home message second part:

Clinically-relevant approaches: compared to research grade approaches, the technologies associated with clinical-grade cryopreservation require using specific acceptable substances, reproducible techniques, closed systems and automation.

Reduction of DMSO concentration: it is possible to pre-treat cells with sugars in culture to remarkably increase their survival after cryopreservation

Importance of timing in hypothermic storage: In clinical cell manufacturing, the timing is important. The usual minimal quality control tests last around 72 hrs (sterility), so it is important to preserve the cells in non-frozen state for at least 3 days. During the hypothermic storage it is important to stabilize the membrane of the cells to avoid excessive cell swelling.

QC control: It is necessary to develop the alternative potency assays to confirm the preservation of functional properties. These assays should be adequate to the expected clinical application