Cryopreservation of hematopoietic stem cells: how did we get here and where are we going?

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Clinical context for HSC cryopreservation

Cryopreservation is typically used for
- Umbilical cord blood
- Autologous HSC transplantation

*(Short term liquid storage is used for other applications)*

Cryopreservation permits
• Coordination with patient care regimes
• Completion of typing and safety testing
• Genetic diversity of cells available
Standard protocol #1

- Cryoprotectant: 10% DMSO
- Cooling rate: 1 C/min
- Controlled rate freezing
- Storage on LN$_2$

Standard protocol #2

- Cryoprotectant: 5% DMSO+6% HES.
- Cooling rate: approximately 1 C/min
- Cooling in a mechanical freezing
- Storage: LN$_2$ or -80 C

Components of a Cryopreservation Protocol

- Liquid storage/prefreeze processing
- Introduction of solution
- Cooling protocol
- Storage conditions
- Warming protocol
- Post thaw assessment

Additional factors:
- Cell density
- Cell source

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Formulation and introduction of a cryopreservation solution
CPAs influence post thaw viability

![Graph showing the influence of cooling rate on viability with different CPAs](image)

- **1.25 M Glycerol**
- **0.8 M Glycerol**
- **0.4 M Glycerol**
- **Buffered Saline**
Mechanisms of action for CPAs

Colligative Effect

Stabilization of Cell Membrane

Change in crystal growth and structure
  - vitrification
  - growth inhibitors

*These changes can occur where the protective agent is found (intracellular and/or extracellular space)*
Partitioning of CPAs

High Molecular Weight Agents
- Polymers (HES, PVP)
- Sugars (trehalose, raffinose)
- Antifreeze Proteins

Low Molecular Weight Agents
- DMSO
- Propylene Glycol
- Glycerol
- Methanol

How did we get here: DMSO is a low molecular weight CPA that penetrates the cell membrane. This property is largely why we use it.
Introduction of CPA solutions

Method #1: Combining equal volumes of cell suspension and 20% v/v DMSO solution.

Method #2: Using a syringe pump to infuse slowly high concentrations of DMSO.

DMSO and cells are typically cooled on ice prior to addition of the CPA solution.
Introduction of CPA solutions, cont

*Cryopreservation solutions are not physiological.*

**Mechanisms of Damage**

- **Osmotic Stresses**
  - Volumetric excursions resulting from efflux of water followed by influx of CPA.

- **Biochemical Toxicity**
  - cytoskeletal reorganization
  - suppression of normal metabolism
  - shift in membrane composition
Strategies to reduce cell losses from introduction

**Protocol Modifications**

- Step-wise or gradual introduction.
- Introduction at reduced temperatures.
  - Acts on both mechanisms of toxicity.
Evolutionary changes

Base of cryopreservation solution
- Then: tissue culture medium
- Now: licensed electrolyte solutions (Normosol R, Plasmalyte A)

Protein additive
- Then: fetal calf serum
- Now: human serum albumin, polymers

These changes have improved the safety of the product without damaging viability or transplantation potential.
Importance of cooling rate and its selection
Cooling rate and freezing response: cryomicroscopy of hepatocytes

- Isolated rat hepatocytes were frozen at 100 C/min in isotonic saline solution.
- Dehydration of intracellular ice formation were observed.
Survival as a Function of Cooling Rate

Diagram showing survival as a function of cooling rate for bone marrow, yeast, hamster ovaries, and red blood cells (RBC). The x-axis represents cooling rate (°C/min) and the y-axis represents survival (%) with values ranging from 0.1 to 10,000 on the x-axis and 0 to 70 on the y-axis. The graph demonstrates different survival curves for each category, with bone marrow and yeast showing higher survival rates at lower cooling rates, while hamster ovaries and red blood cells exhibit higher survival at higher cooling rates.

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Cooling protocol

Protocol elements

- Initial equilibration phase
- Initial cooling
- Seeding Step
- Secondary cooling
- Cooling to final temperature

Optimal cooling protocols are influenced strongly by solution composition
Segments of cooling protocol

Segment One:

• Typically the chamber is pre-cooled. This segment permits the sample to achieve a uniform initial temperature.

• Insufficient time for equilibration is harmful.
Cooling protocol, cont.

- **Segment two:**
  - The cooling rate for high subzero temperatures.
  - When a protocol specifies a cooling rate (i.e. 1 C/min), that means the cooling rate at segment two.
Cooling Protocol, cont.

• Segment 2a:
  – Also known as the seeding step.
  – Sharp drop in temperature followed by the increase is intended to force nucleation of the extracellular solution.
Methods of seeding sample

• “Automatic seeding”
  – Clinical protocols.

• Manual seeding
  – IVF protocols.

• Uncontrolled seeding.
  – Both clinical and laboratory settings.
“Delayed” Latent heat

Nucleation and growth of ice releases the latent heat of fusion.

The temperature at which this occurs, $T_{nuc}$ is important.

Material Science
- Concentration of extracellular solution
- Ice crystal growth characteristics

Biology:
- Permeability of cell to water
- Membrane phase characteristics

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Relationship between $T_{\text{nuc}}$ and IIF

- IIF is an accepted mechanism of damage.
- Cooling rate, $T_{\text{nuc}}$ and cell type influence fraction of cells with IIF.
Low temperature storage

- Convention:
  - LN$_2$

- Emerging interest in the use of -80 C freezers for storage of products.
Relevant biophysics during storage

• Material science
  – Extracellular solution is only partially frozen until the eutectic temperature.
  – $T_{\text{eut}}$ for 10% DMSO solution $\sim -70$ C.

• Cell biology
  – Complete suppression of biochemical reactions $< -150$ C.
  – Storage at -80 C: biochemical function still present.
Storage comparison

• LN2 storage
  – Requires capital equipment and access to LN2
  – Stability of products: 10,000-30,000 years.

• Mechanical freezer
  – Reduced capital expenditures
  – Need for back up power supply
  – Stability of products:
    • Increasing the temperature of storage decreases the shelf life

*Product is not replaceable. Improper storage can result in product loss.*
Warming Protocol

• The same temperature range must be traversed during warming.
  – Recrystallization damage
  – Long-term exposure to high concentrations can also be damaging

• Cooling protocol influences warming response.
Cooling and warming interaction

- Dotted line: intracellular thermodynamic state upon cooling.
- Solid lines: intracellular thermodynamic state during warming.
- Different cooling protocol will shift dotted line and influence intracellular states upon warming.

Thawing protocols

• Conventional freezing: rapid thawing.
• Typically, thawing is performed in a warm water bath.
• Hints:
  – Swirling sample in the bath increases heat transfer and increases warming rate.
  – Increasing bath temperature increases warming rate.
Thawing baths, cont.

• Bath temperature:
  – Convention: 37 °C.
  – Higher bath temperatures can be used.
    • 42 °C is the lower threshold for hyperthermic damage
    • >45 °C is the upper threshold for hyperthermic damage. You will experience cell losses from high temperatures
    • Between the two thresholds, there are specific kinetics of damage.
Post thaw assessment: HSC

Convention:
• TNC, CD34+, ‘viability’, CFU
• Assessment is performed prefreeze but not always post thaw.
• Material to be assayed: integral segments, vials, bag, etc.
Post thaw assessment, cont.

Complexity intrinsic to cryopreservation:

- DMSO can cleave surface receptors
- Residual DMSO can influence colony formation.
- Freezing environment changes with volume frozen.
- Membrane integrity assays are not reliable.
Post thaw viability assessment

- Lymphocytes were frozen using conventional methods.
- Cell counts and viability were performed at different times post thaw.
Post thaw apoptosis

- Post thaw cell death
  - Measured starting in the 1990s
  - Observed in various cell types
- Mediated by the mitochondria and activation of Caspase-3 (Stroh, 2002)
- Caspase inhibition has been shown to reduce post thaw apoptosis losses.
Caspase Inhibition

- Commercial solutions exist which contain caspase inhibitors (cryopreservation and hypothermic storage).
- Exposure to caspase inhibitors can result in reduced/delayed cell proliferation.
- Further work is needed to understand and optimize use of caspase inhibitors in preservation solutions.
Issues unique to HSCs

CD34+ cell/colony forming cell

• Finding a needle-in-a-haystack.
  – Measurement variances are high and vary with operator.

• Surface marker/cell biology paradox
  – Ex vivo culture
  – Cryopreservation?
Emerging science and technology
New sources of HSCs

- Fetal liver-based hematopoietic progenitor cells
  - Gestational age influences post thaw recovery.
  - Osmotic characteristics have been determined.
- Blood cells from hESC.

Freezing response for different cell types may suggest that different freezing strategies may be needed.
Alternatives to DMSO as a CPA
Preservation in nature
What's the common theme?

<table>
<thead>
<tr>
<th>Osmolyte system (occurrences)</th>
<th>Principal osmolytes</th>
</tr>
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<tbody>
<tr>
<td>A. Polyhydric alcohol-polyols</td>
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<tr>
<td>Cyanobacteria</td>
<td>Glucosylglycerol</td>
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<tr>
<td>Synechococcus sp.</td>
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<tr>
<td>Fungi</td>
<td>Arabitol</td>
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<tr>
<td>Saccharomyces rouxii</td>
<td>Arabitol, glycerol, mannotol</td>
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<tr>
<td>Aspergillus niger</td>
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<tr>
<td>Lichens</td>
<td>Mannotol</td>
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<td>Lichina pygmaea</td>
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<td>Unicellular algae</td>
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<td>Danzickia spp.</td>
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<tr>
<td>Chloroglastris peronnesioides</td>
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<tr>
<td>Osmorhizus multicarinatus</td>
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<tr>
<td>Multicellular algae</td>
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<tr>
<td>Fucus spp.</td>
<td>Mannotol</td>
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<td>Vascular plants</td>
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<tr>
<td>Gossypium hirsutum L.</td>
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<td>Insects (freeze-tolerant or -resistant)</td>
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<tr>
<td>Euaoria solidaecea (Diptera)</td>
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<td>Bacillus cephi (Hymenoptera)</td>
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<td>Crustaceans</td>
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<tr>
<td>Artemia salina (emerging larvae)</td>
<td>Glycerol, trehalose</td>
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<tr>
<td>Vertebrates</td>
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<tr>
<td>Hyla versicolor</td>
<td>Glycerol</td>
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<tr>
<td>B. Amino acids and amino acid derivatives</td>
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<tr>
<td>Eubacteria</td>
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<tr>
<td>Klebsiella aerogenes</td>
<td>Glutamic acid, proline</td>
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<tr>
<td>Salmonella enteritidis</td>
<td>Glutamic acid, proline</td>
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<tr>
<td>Streptococcus faecalis</td>
<td>γ-Aminobutyric acid, proline</td>
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<td>Prokaryotes</td>
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<tr>
<td>Microcystis audax</td>
<td>Gymic acid, alanine, proline</td>
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<td>Vascular plants</td>
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<tr>
<td>Sphagnum sp.</td>
<td>Betaine</td>
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<td>Arctopax spongiosa</td>
<td>Betaine</td>
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<td>Actin sp.</td>
<td>Betaine</td>
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<tr>
<td>Mesobryandlerium nodiferum</td>
<td>Betaine</td>
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<tr>
<td>Invertebrates</td>
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<tr>
<td>All phyts of marine invertebrates (see examples in Table 1)</td>
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<tr>
<td>Cyclostomes</td>
<td>Various amino acids</td>
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<tr>
<td>Myxine glutinosa (jugfish)</td>
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<tr>
<td>Amphibians</td>
<td>Various amino acids</td>
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<tr>
<td>Bufo marinus</td>
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<tr>
<td>C. Urea and methylamines</td>
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<tr>
<td>Cartilaginous fishes (elasmobranchs and holoccephalians marine and estuarine):</td>
<td></td>
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<tr>
<td>see examples in Table 1</td>
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<tr>
<td>Conulus (Lutiberria chadroniae)</td>
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<tr>
<td>D. Urea: estivating forms</td>
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<tr>
<td>Molluscs</td>
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<td>Bivalves desilatiss</td>
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<td>Langnines: African and South American</td>
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<tr>
<td>Amphibians</td>
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<td>Scaphopodes sp. (spadefoot tetd)</td>
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<td>E. Inorganic ions</td>
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<tr>
<td>Archaeobacteria</td>
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<tr>
<td>Halobacterium sp.</td>
<td>K⁺</td>
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<tr>
<td>Yancey, P.H., et al., 1982, Science, 217, p1214</td>
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</tbody>
</table>

“Natural osmolytes affect the structure of water”

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Sugars for HSC preservation

- Mammalian cells are stabilized by sugars/polyols
  - Glucose/sucrose
  - Trehalose
  - 3MOG
- Sugars act synergistically with glycerol
- Trehalose added to conventional freezing protocols improves post thaw recovery
Sugars, cont.

- Cells have been frozen with low levels of trehalose (200-300 mM) with high levels of recovery.
- Principal challenge: ‘delivery’
- Secondary challenge: selecting sugars.
Methods of introducing sugars

• Many methods have been used.
• Very few would be appropriate for the clinic
  – Cell numbers involved
  – Regulatory concerns
Sugar uptake: Glut receptor

• Hepatocytes were incubated with 3-O-methyl-glucose.
• Sugar uptake via Glut- II receptor.

Sugimachi, Tissue Engineering, 2006
Sugar uptake: purinergic receptor

• Mouse macrophage cells were incubated with trehalose solutions containing ATP.
• Uptake via purinergic receptor was determined.

Elliot, Cryobiology, 2006
Cytoprotective agents

Observations:
- A significant number of cells are lost from post thaw apoptosis.
- Oxygen free-radical generation is observed during freezing.

Outcome
- Supplement media with cytoprotective additives to mitigate these effects.
  - Catalase, α-tocopherol
  - Caspase inhibitors
Post thaw processing

**PBSC or Bone Marrow**
- Thaw at bedside
- Immediate infusion

**Umbilical cord blood**
- Wash with abumin/dextran solution
- Reinfuse
Issues in post thaw processing

• Frozen and thawed HSCs are reinfused directly into adult patients.
  – DMSO present in the cell suspension is responsible for adverse reactions.

• Umbilical cord blood is routinely washed and cell losses >25%.

Therein lies the conundrum: direct infusion of cryopreserved hematopoietic stem cells results in significant adverse reactions but washing cells to remove DMSO results in significant cell losses that, in turn, adversely affect transplant outcome.

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Microfluidics

• Microfluidics: flow of fluids in a microscale channel (laminar flow).
• Advantages: low power requirements, manufacturability, control over cell motion.
• Microfluidics will grow to play an increasing role in the clinical laboratory.
Microfluidics, cont.

Cytonome
Cell sorting of $10^9$ cells/hr

Micronics
CBC and Differential

This technology will permeate the clinical lab/doctor’s office in the next 5 years.
More clinical laboratory services will move from the laboratory to the point of use.
Microfluidic processing of cells

- Remove DMSO from frozen and thawed cell suspension using a microscale device.
- Volume throughput of 2-3 ml/min.
- Removal of 95% DMSO concentration (1.4M to 0.07 M).
- Device size should permit bench top or bedside processing.
Device prototype

• Prototype was developed and performance measured.
• Excellent correlation between theory and experiment.
• Cell recovery > 90%.
HSC preservation: the future

• Cell processing for freezing
  – Closed microfluidic devices (no centrifugation).
  – Improvement in bags and devices.

• Non-toxic protective agents for cell preservation
  – Low level (~300 mM concentrations).
  – No or low adverse effects.
Interested in learning more?

- Professional short course
  http://www.me.umn.edu/education/shortcourses/preservation/index.htm
- Society for Cryobiology
  http://www.societyforcryobiology.org/
- International society for cell therapy
  http://celltherapysociety.org/
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  – National Blood Foundation
  – National Institutes of Health
Evolution cont: cell concentration

- Freezing at high concentration reduces
  - Total dosage of DMSO infused.
  - Storage space required.
- ‘Normal’ concentration: 30-50 \( \times 10^6 \) cells/ml.
- “High” concentration: \(~200\times10^6\) cells/ml.
- Increasing cell concentration beyond approximately 20% cytocrit, results in reduced post thaw recovery.
Cell concentration, cont.

Relationship between cell density and cytocrit:

\[ C = \frac{V_{\text{total cell}}}{V_{\text{total}}} \]

Sample calculation: Estimate the maximum cell concentration for a cell with diameter of 20 \( \mu\text{m} \)

\[
V_{\text{cell}} = \frac{4}{3} \pi r^3 = \frac{4}{3} \pi (10 \times 10^{-6} \text{ m})^3 = 4.2 \times 10^{-15} \text{ m}^3 = 4.2 \times 10^{-9} \text{ ml}
\]

\[
0.2 = CV_{\text{cell}} \quad C = 48 \times 10^6 \text{ cells / ml}
\]

Freezing response is altered when the water content of the cells becomes a significant fraction of the water present.

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