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Basic Cell Processing
Cryopreservation
CD34 Enrichment
RBC Depletion

Carolyn A Keever-Taylor
Deborah Griffin
Cryopreservation - Goals

• Long term (weeks-years) storage of cellular therapy products with preservation of function

• Allows for:
  - Banking of products (e.g. HPC, Cord Blood)
  - Storage while patients undergo addition disease treatment or conditioning for transplant
  - Collection of allogeneic donors in advance of infusion (several reasons)
  - Storage for potential or planned future use (DLI, serial infusions, etc)
  - Completion of product release testing
Cryopreservation- Basic Requirements

• Preparation of cells for freezing
• Selection and use of cryoprotectants. Mitigate freezing-induced membrane damage due to hyperosmolality, ice crystals and heat generated during the transition from liquid to solid (heat of fusion)
• A controlled slow rate of freezing to allow water to leave the cytoplasm
  - Targeted reduction of the “heat of fusion” during phase transition (good but not essential)
• Storage at cold temperatures, <-80°C a minimum, Colder is better
Cell Preparation Considerations

- **Cell density** - Lower may be better, but too low results in:
  - More bags to store
  - Increased toxicity from DMSO infusion (limit to 10 mL of a 10% solution/kg/day)

- **Cellular composition** - Product affected by:
  - RBC content - Excessive hemolysis if RBC not reduced (marrow and blood)
  - Granulocyte content - pH changes and toxicity from granule contents (do not survive freezing process as well as mononuclear cells)

- **CD34 content, planned # of infusions**
Pre-Processing

• HPC, Marrow or HPC, Cord Blood
  - RBC reduced using:
    • Buffy coat preparation
    • HES sedimentation Density gradient separation
  - Cell concentration- 1-2 x 10^8 TNC/mL marrow

• HPC, Apheresis
  - Cell concentration- commonly 4.0 x 10^8 TNC/mL.
    up to 5.6 x 10^8 shown to have acceptable recovery
  - Granulocyte content- Aim for a MNC content ≥ 70% at collection

• In general low concentrations better, less clumping and lower viscosity
Effect of HPC, Apheresis MNC content and Viability Post Thaw
Effect of Retraining on Apheresis Product MNC-MCW Experience

2005 Problem Recognized For Autologous Products & Discussed

2006 Discussed again & Retaining Recommended

2007 Effect of Retraining in Nov 2006
Cryopreservation Medium

• **Cryoprotectant**
  - DMSO at 5-10% optimal. Most often 10%
  - Hydroxyethyl Starch (HES) (6%), + 5%DMSO
  - Pentastarch (available ?) + Dextrose + DMSO

• **Protein Source**
  - Plasma or serum—Commonly 10%, up to 90% for some TC (May not be optimal from some patients)
  - HSA- 4 to 5% from 25% source

• **Diluent**
  - Isotonic electrolyte solution (Normosol R or Plasmalyte A)
  - 0.9% NaCL for infusion
  - Tissue culture medium (MEM, RPMI) (not recommended)
DMSO Sources

- **Cryoserve** - Bioniche Pharma
- **Stemsol** - Protide Pharmaceuticals
- **CryoSure** - WAK-Chemie Medical GmbH

Consider validation of more than one source
Cryopreservation Medium Other Considerations

• Rate of addition
  - Slow to minimize effects on cell volume

• Temperature at use
  - Toxic effects of DMSO increase with temperature

• Daily preparation vs in advance
  - Quality issues for each lot

Not all labs do the same. Variations have similar outcomes. Must validate your procedure.
“Dump Freezing”- Products transferred to a constant -80°C for 24 h then transfer to LN2, results in 1-3°C freeze rate/minute to -80°C
- Does not mitigate heat of fusion
- Harder to document rate
- Freezer should not be entered, problem if multiple products over the day
Cryopreservation
Controlled Rate Freezing

- Computer controlled chambers-Staged freezing process controlled with LN2
  - Seed freezing point and super-cool to reduce transition heat
  - Documented freezing record (paper chart or computer record)
  - Multiple runs in a day
Effect of Freezing Rate

For bone marrow viable cell recovery and CFUc growth are best with freezing rate between 1-3 degrees C/minute.
Computer Controlled Freezing

Let product = Chamber
Before start

Need to determine during validation

Region I
1°/min

Latent Heat of fusion

Region II
Supercooling
Then resume
1°/min (some 2-3°)

Product Temperature

Region III
Increase Rate to
5-10°/min

Chamber Temperature

End temp may vary
from -60 to -100

Graph showing temperature and time with labeled regions and notes.
Variables Affecting Freezing Programs

• Freezer manufacturer
• Cryopreservation medium composition (e.g. %DMSO)
• Bag size, shape and material
• Product Volume
• Probe type & what is monitored
  - Ribbon probe
    • Advantage: Monitors actual product
    • Disadvantage: Readings influence by chamber & press
  - Bag probe
    • Advantage: More accurate readings
    • Disadvantage: Must use dummy bag without product
  - Probe in vial
    • Advantage: No dummy bag preparation
    • Disadvantage: Not same materials, volume or geometry
Probe Placement Tips

**Bag Probe**
- Probe Tip in bag
- No overwrap on probe bag
- Probe bag same size and volume as product

**Ribbon Probe**
- Label Pocket
- Probe Tip on exterior of bag
- Cassette
- Bag
- Ribbon-probe
- Shelf
- Additional bag material
Cryopreservation Storage Issues

• Mechanical Freezers
  - -80°C may be acceptable for short term (months).
  - -120°C allows for longer storage, upper limit unclear.
  - Fluctuations due to entry an issue.

• LN2 Storage Freezers
  - Vapor Phase ≤-150°C
    • Minimize temp gradient with racking system (aluminum)
    • Jacketed freezers
  - Liquid phase -196°C
    • Risks associated with potential viral contamination
    • Higher risk of bag breakage (?)
Thawing

- Direct Thaw
- Dextran/Albumin Wash
- Dextran/Albumin Dilution in Bag
## Thawing-Compared

<table>
<thead>
<tr>
<th></th>
<th>Direct Thaw</th>
<th>Dex/Alb Dilute</th>
<th>Dex/Alb Wash</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Location</strong></td>
<td>Bedside</td>
<td>Laboratory</td>
<td>Laboratory</td>
</tr>
<tr>
<td><strong>Product monitoring</strong></td>
<td>Surrogate QC vials or segments</td>
<td>Surrogate QC vials or segments</td>
<td>Each product for recovery &amp; viability</td>
</tr>
<tr>
<td><strong>Recovery</strong></td>
<td>Death from Osmotic stress</td>
<td>Improved due to less osmotic stress</td>
<td>Improved due to less osmotic stress</td>
</tr>
<tr>
<td><strong>Infusion Rx</strong></td>
<td>Common</td>
<td>Common</td>
<td>Rare</td>
</tr>
<tr>
<td><strong>Processing Cell loss</strong></td>
<td>None</td>
<td>None</td>
<td>Potential, though usually low</td>
</tr>
<tr>
<td><strong>Trouble shooting</strong></td>
<td>Difficult</td>
<td>Difficult</td>
<td>Easier</td>
</tr>
<tr>
<td><strong>Time</strong></td>
<td>Preparation, Transport, clean-up</td>
<td>Approx 20 min/bag in Lab</td>
<td>Approx 45 min/bag in Lab</td>
</tr>
</tbody>
</table>
MCW Experience 2007
Dextran/Albumin Wash

% Recovery Post Thaw
MCW Experience QC Vial Thaw vs Product Thaw Viability

Viability at Thaw

Thaw Viability

$ r^2 = 0.1033 $
Effect of Time Post Dextran/Albumin Wash on Viability

Cells From EBV-specific T cell line stored at 1-6°C, N=3
CD34+ HPC Enrichment - Why?

- To remove unwanted populations that may contain:
  - Tumor cells (not common)
  - Autoimmune populations of T or B cells
  - Allogeneic T cells that can cause GvHD
- Allows for engineering of graft by add-back of selected populations
Cell Enrichment (CD34)

- Two systems in current use. Both use a principle of cell targeting by CD34 antibodies and capture through magnetic interactions. Unwanted cells are washed through, and captured cells are collected.
Cell Enrichment (CD34)

- Isolex System-Baxter
  - Approved for Clinical Use
  - Highly automated
  - Cells are released from capture beads
  - CD34+ cell recoveries approx 50-60%
  - CD3 log depletion approx 4.0

MCW Experience

<table>
<thead>
<tr>
<th>N</th>
<th>CD34 Purity</th>
<th>CD34 Recovery</th>
<th>CD3 Log Dep</th>
</tr>
</thead>
<tbody>
<tr>
<td>92</td>
<td>96.6% ± 3.7%</td>
<td>52.8% ± 13.9%</td>
<td>4.31 ± 0.30</td>
</tr>
</tbody>
</table>
Isolex

- Add IvIG to block FC receptors
- Set up and load onto device
- Platelet wash using spinning membrane
Isolex

CD34 Ab binding to CD34+ cells

Bead-conjugated anti-mouse Ab binding

Captured cells & excess Ab bind to magnet. Non-binding cells wash through

PR34 peptide releases captured CD34+ cells

Released cells collected
• Antibody added based on TNC and target CD34%
  - At maximum loading TNC, should not exceed 1% CD34
• Single Column holds up to: 8.0 x 10^{10} TNC
  - Multiple selections common for allo-donors for adult patients
  - Caution collection program to seek high MNC content
Cell Enrichment (CD34)

• **CliniMACS- Miltenyi**
  - Currently requires IND or IDE
  - Automated only for selection steps
  - Cells retain ferrous iron particles
  - CD34+ cell recoveries 60-70%
  - CD3 log depletion 4.5-5.0 logs

**MCW Experience**

<table>
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<th>CD34 Recovery</th>
<th>CD3 Log Dep</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>95.2%</td>
<td>±11.0%</td>
<td>±10.3%</td>
</tr>
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</table>
CliniMACS

- **Platelet wash**
  - Bag method or
  - Cell washer (Cobe 2991)
- **Antibody incubation**
- **Antibody Wash**
- **Filter**
- **Set up and load cells onto column**
CliniMACS

Ferrous iron particles (biodegradable)
Cell Enrichment (CD34)

Starting Cells

Post Enrichment

Post enrichment measurement of CD34 is simple
Cell Enrichment (CD34)

Accurate detection of CD3+ cells more difficult, especially for products enriched on the CliniMACS
CD34-Enrichment Issues

- CD34 doses larger than for other HPC products to ensure engraftment
  - Multiple collections for a single transplant
- Limit in number of cells/column for optimal recovery and purity
  - Multiple columns for a single collection
- Device failures may be difficult to recover from
  - Clotted products
  - Air locks
Cells Loaded vs Recovery - Isolex

Best not to exceed column capacity
CD34-Enrichment Issues

• Products not selected on day of collection should be:
  - Diluted to $\leq 2.0 \times 10^8$ cells/mL
    • Concurrent plasma - Must request
    • 5% HSA- Expensive, supply concerns
    • Selection buffy- Requires preparation
  - Stored cold or warmed prior to processing
• Products with low cellular content may be pooled for selection to minimize use of columns, reagents, and time
• High granulocyte content can cause clumping and reduce purity and recovery
CD34-Enrichment Sampling Issues

- Excess sampling may result in significant cell loss that could compromise patient.
- Flow must come from infused product. Need adequate numbers to measure rare populations.
  - Multi-color analysis to minimize number of tubes
  - Include viability dye to reduce background from dead cells
- Sterility may come from CD34-depleted fraction
- Endotoxin if required, should come from enriched product. Requires assessment per mL of product infused
- Archive vials recommended. Take minimum amount (2 x 10^6 cells).