Peripheral blood derived hematopoietic progenitor cells (HPC): An overview of a successful application of cryobiology

A. Sputtek¹, E. Klyuchnikov², N. Kröger², P. Peine¹, A.W. Rowe³

¹ Department of Transfusion Medicine,
² Clinic for Stem Cell Transplantation,
University Medical Center Hamburg-Eppendorf,
Hamburg (Germany)

³ New York University Medical School,
New York, NY (USA)
Hematopoiesis

Bone marrow

- Myeloic progenitor
- Multipotential hematopoietic stem cell
- Lymphoid progenitor
- Megakaryocyte
- Erythroblast
- Myeloic precursor cell
- pre T-cell
- pre B-cell

Peripheral blood

- Thrombocytes
- Erythrocytes
- Granulocytes
- Monocytes
- T-Lymphocytes
- B-Lymphocytes

Modified from N. Kröger 2005
Blood stem cell transplantation in combination with high dose chemotherapy (+ whole body irradiation)

Autologous therapies require cryopreservation.

Modified from N. Kröger 2005
## Indications for autologous 1st time stem cell transplantations in Germany in 2010

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>N (trend)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Leukemia</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AML 1&lt;sup&gt;st&lt;/sup&gt; CR</td>
<td>12</td>
<td>0.4</td>
</tr>
<tr>
<td>Multiple myeloma</td>
<td>1297</td>
<td>47.5</td>
</tr>
<tr>
<td>NHL</td>
<td>924</td>
<td>33.8</td>
</tr>
<tr>
<td>M. Hodgkin</td>
<td>149</td>
<td>5.5</td>
</tr>
<tr>
<td>Germ cell tumors</td>
<td>132</td>
<td>4.8</td>
</tr>
<tr>
<td>Neuroblastoma</td>
<td>47</td>
<td>1.7</td>
</tr>
<tr>
<td>Ewing sarcoma</td>
<td>44</td>
<td>1.6</td>
</tr>
<tr>
<td>Soft-tissue tumors</td>
<td>11</td>
<td>0.4</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Other solid tumors</td>
<td>41</td>
<td>1.5</td>
</tr>
<tr>
<td>Others</td>
<td>73</td>
<td>2.8</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>2730</strong></td>
<td><strong>100</strong></td>
</tr>
</tbody>
</table>

*Annual Report 2010 German Registry for Stem Cell Transplantation 2010 (http://www.drst.de)*
Hematopoietic stem cell sources (CD34+ cells)

- Bone marrow
- Peripheral blood
- Cord blood

Modified from N. Kröger 2005
### European Perspective:
**Autologous blood stem cell sources 2009**

<table>
<thead>
<tr>
<th>Country</th>
<th>Bone Marrow</th>
<th>PBSC</th>
<th>PBSC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Italy</td>
<td>30</td>
<td>2343</td>
<td>98.7</td>
</tr>
<tr>
<td>United Kingdom</td>
<td>17</td>
<td>1586</td>
<td>98.9</td>
</tr>
<tr>
<td>The Netherlands</td>
<td>5</td>
<td>535</td>
<td>99.1</td>
</tr>
<tr>
<td>Sweden</td>
<td>3</td>
<td>341</td>
<td>99.1</td>
</tr>
<tr>
<td>Spain</td>
<td>8</td>
<td>1314</td>
<td>99.4</td>
</tr>
<tr>
<td>France</td>
<td>7</td>
<td>2522</td>
<td>99.7</td>
</tr>
<tr>
<td>Germany</td>
<td>3</td>
<td>2592</td>
<td>99.9</td>
</tr>
<tr>
<td>Switzerland</td>
<td>0</td>
<td>279</td>
<td>100</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>73</strong> ←</td>
<td><strong>11512</strong> ↑</td>
<td><strong>99.4</strong> ↑</td>
</tr>
</tbody>
</table>

*Annual Report 2010 German Registry for Stem Cell Transplantation 2010 (http://www.drst.de)*
Hematopoietic stem cell sources
(\(\text{CD}34^+\) cells)

- Bone marrow
- Peripheral blood
- Cord blood

+ Growth factors
  (e.g. G-CSF)

Modified from N. Kröger 2005
HUMAN BLOOD STEM CELLS
DO NOT X-RAY!
Sterile connection to top-and-bottom bag
Transfer in top-and-bottom bag
Preparation for centrifugation
Centrifugation for 15 min at 200 g
Removal of plasma
Closed system processing set
Final \( \text{Me}_2\text{SO} \) concentration: 9 - 10% (v/v)
Aliquotation in 1 – 4 units
Final volume: 105 +/- 5 ml (v/v)
Flat sample, thickness: 6 – 7 mm
Controlled cooling at 1.5 K/min down to −90 °C
Two Factor Hypothesis

intracellular ice formation

osmotic dehydration

resultant survival

P. Mazur 1970
Survival CFU<sub>spleen</sub> (%) vs. cooling rate (K/min)

- 1.25 M glycerol
- 0.8 M glycerol
- 0.4 M glycerol
- 0 M glycerol

Mouse BM Stem Cells

S. Leibo et al. 1970
Aim of study #1 with human peripheral blood stem cells:

Investigation of the effect of cooling rates and cryoprotective mixtures (Me$_2$SO/HES) on the numerical MNC recovery, membrane integrity, and clonogenicity of human PBSC.
Cooling rates*

- 1.4 K/min: controlled cooling (KRYO 10-16/II; 10-22, Planer)
- 5 K/min: uncontrolled cooling (-80 °C mechanical refrigerator)
- 10 K/min: controlled cooling (KRYO 10-16/II; 10-22, Planer)
- 15 K/min: controlled cooling (KRYO 10-16/II; 10-22, Planer)
- 20 K/min: uncontrolled cooling (vapor phase over LN₂)
- 160 K/min: uncontrolled cooling (LN₂)

* = determined between freezing temperature and –45 °C
Cryoprotective Mixtures

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Me₂SO</th>
<th>HES 70/0.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.5%</td>
<td>2.5%</td>
<td>HES 70/0.5</td>
</tr>
<tr>
<td>5%</td>
<td>6%</td>
<td>HES 70/0.5</td>
</tr>
<tr>
<td>2.5%</td>
<td>7.5%</td>
<td>HES 70/0.5</td>
</tr>
<tr>
<td>0%</td>
<td>10%</td>
<td>HES 70/0.5</td>
</tr>
<tr>
<td>10%</td>
<td>0%</td>
<td>HES 70/0.5</td>
</tr>
</tbody>
</table>
MNC after thawing before colony assay (Coulter counter)

![Graph showing MNC recovery vs. cooling rate. Different concentrations of Me$_2$SO and HES are tested, with varying recovery rates at different cooling rates.]

- 0% Me$_2$SO / 10% HES
- 2.5% Me$_2$SO / 7.5% HES
- 5% Me$_2$SO / 6% HES
- 7.5% Me$_2$SO / 2.5% HES
- 10% Me$_2$SO / 0% HES

Recovery (%) vs. Cooling rate (K/min)
Membrane integrity (Trypan blue exclusion) after thawing

Recovery (%)

Cooling rate (K/min)

- 0% Me$_2$SO / 10% HES
- 2.5% Me$_2$SO / 7.5% HES
- 5% Me$_2$SO / 6% HES
- 7.5% Me$_2$SO / 2.5% HES
- 10% Me$_2$SO / 0% HES
Conclusions

• Numerical MNC recovery and membrane integrity of PBSC after thawing are less influenced by suboptimal cooling protocols than hematopoietic cell assays.

• Hematopoietic cell assays clearly suggest that the optimum cooling rate for human PBPC is not critical in the range from 1 to 5 K/min as long as 5% Me₂SO and 6% HES, 7.5% Me₂SO and 2.5% HES or 10% Me₂SO in the absence of HES are present in the suspension to be frozen.

• To generate optimum cooling rates, a –80 °C mechanical refrigerator is as effective as a liquid nitrogen operated controlled rate freezer.
Storage in the vapor phase over liquid nitrogen
Highest temperature in the frozen unit after 2 h with open lid: -172 °C
Aim of study #2:

Investigation of the effect of 3 and 6 months storage at

\(< - 170 \, ^\circ C\) (vapor phase over liquid nitrogen)

\(- 80 \, ^\circ C\) (mechanical refrigerator)

on the numerical MNC recovery, membrane integrity, and clonogenicity after cryopreservation in the presence of 10% Me$_2$SO.
Materials & Methods

- 52 reference samples of PBSC concentrates obtained by apheresis from 13 patients (7 multiple myeloma, 6 lymphoma)

- Volume/cryoprotectant: ca. 1.5 ml/10% Me₂SO

- Cooling: controlled rate freezer
  - 0 °C to –45 °C at 1 °C/min
  - -45 °C to –90 °C at 10 °C/min
  - transfer to vapor phase over liquid nitrogen
  - or –80 °C mechanical refrigerator

- Frozen storage: (n = 14 for each group)
  - group 1: below –170 °C for 3 months
  - group 2: at –80 °C for 3 months
  - group 3: below –170° for 6 months
  - group 4: at –80 °C for 6 months

- Thawing: shaking water bath (37 °C) for 3 min; t = 15 ± 3 °C
Clonogenicity (after thawing and washing)

-170 °C, 3 months
-80 °C, 3 months
-170 °C, 6 months
-80 °C, 6 months

p < 0.05
n.s.
Aim of study #3:

Investigation of the effect of 16 months storage at

a) < -170 °C (vapor phase over liquid nitrogen)
b) -150 °C (mechanical refrigerator)

on the numerical WBC recovery, membrane integrity, CD34+ content and clonogenicity of human PBPC cryopreserved in the presence of 10% Me₂SO.
Materials & Methods

- **12 pairs** (= 24 units, 3 pairs CD34+ enriched) autologous PBSC concentrates obtained by apheresis from 11 patients (5 lymphoma, 2 sarkoma, 1 neuroblastoma, 1 multiple myeloma, 1 germ cell tumor, 1 hepatoblastoma) which were no longer needed for treatment.

- **Volume/cryoprotectant**: 105 ± 5 ml/10% Me₂SO (WAK-Chemie)

- **Cooling**: controlled rate freezer (Kryo 10, Series III, Planer)
  - 0 °C to −45 °C at 1 °C/min
  - -45 °C to −90 °C at 10 °C/min
  - transfer to vapor phase over liquid nitrogen

- **Frozen storage**: Vapor phase over liquid nitrogen for 73 (range 4-127) months, then transfer of one unit of each pair into a −150 °C refrigerator, continuation of storage at −150 °C (MDF-1156, SANYO) of boths units for 16.4 (range 15.7-16.7 months). Temperature recorded using a data logger (ELPRO-BUCHS) with 4 thermocouples.

- **Thawing**: shaking water bath (37 °C, GFL Type 1086) for 3 min
Materials & Methods #2

- **Directly after thawing**
  - WBC concentration (FACS: CD45 FITC)
  - Membrane integrity (FACS: 7-AAD exclusion)
  - CD34+ (FACS: CD34 PE)

- **After removal of the cryoprotectant**
  (stepwise dilution, centrifugation, removal of supernatant)

  - WBC concentration (counter) (Cell-Dyn Ruby, Abbott)
  - Membrane integrity (trypan blue exclusion)
  - Colony forming potential (14 days semi-solid cell culture, colony > 50 cells)

- **Statistics**
  - (Student´s t-Test; significantly different if p < 0.05)
WBC $[10^8/ml]$, FACS

$y = 0.90 \times$

$r = 0.99$

$p = 0.07$ (n.s.)
7-AAD Exclusion [%]

\[ y = 1.00 \times \]
\[ r = 0.95 \]
\[ p = 1.00 \text{ (n.s.)} \]
CD34$^+$ $[10^6 \text{ kg/b.w.}]$

$y = 1.05x$

$r = 0.90$

$p = 0.61 \text{ (n.s.)}$
CFU $[10^5 / \text{kg b.w.}]$

$y = 0.99x$

$r = 0.90$

$p = 0.96 \text{ (n.s.)}$

datapoint 20.3/10.6 not considered (outlier)
Summary

The data support the conclusion that a reliable –150 °C freezer is as suitable for long-term storage of PBSC in the presence of 10% Me₂SO as storage in the vapor phase over LN₂. There was no systematic decline in viability measured in terms of 6 different parameters after more than 16 months when the –150 °C freezer was used.

Conflict of interest: The study was supported by Ewald Innovationstechnik GmbH, Bad Nenndorf, Germany.
Transportation in liquid nitrogen „dryshipper“
Thawing in shaking water bath (37 °C, 3 min) at patient’s bedside
Administration complete in 5 – 10 min after thawing
Directly after thawing
5 h after thawing
Thanks for experimental work to

- Sylvie Jetter (cryopreservation)
- Cathrin Schwarz (frozen storage)
- Claudia Benndorf (postthaw storage)