Freeze dry and automated cryopreservation technology

Amir Arav
Cryopreservation of Oocytes, Sperm, Embryos, Ovarian Tissue, Testicular tissue, Stem Cells.

Vitrification has to be standardized
Need of Automation

Storage in LN2 is very demanding, takes a lot of space, is **costly**
Need of Dry storage
What is Required from an Automatic Vitrification Device

1. Cooling and warming rates should be $>20,000^\circ\text{C/min}$.
2. Volume on cryo-carrier should be $<0.1\mu\text{l (MDS)}$.
3. Easy to operate and to store at LN.
4. Automate all vitrification (including insertion to LN) and warming steps.
5. Mechanical damage should be avoided.
6. **Short time** procedure.
7. Should operate as an **open or closed** system.
Loading The Capsules (E.Vit)

- 50 microns square holes
- Bovine oocytes
- Straw with capsule & bovine oocytes
Solution Moves Up While Embryos Moves Down
SARAH – A Simple and Safe Automated Vitrification Device
1. Sarah can vitrify up to 30 oocytes/embryos at once (in 6 Mini straws with 5 oocytes in each).
2. Sarah can vitrify up to 18 ovarian tissue slices (in 6 Maxi straws with 3 samples (10 X 5mm) in each).
Mice Oocytes and Embryos Results

→ 95% survival (19/20) with MII mice oocytes.

→ 100% survival (20/20) with 8 cell stage embryos (80% (16/20) blastocyst rate).

→ 100% survival (35/35) with blastocysts stage embryos (80% hatching).
## Bovine Oocytes Results

<table>
<thead>
<tr>
<th>Bovine Results</th>
<th>% Cleavage</th>
<th>% Blastocysts</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vitrified Zygotes</strong></td>
<td>54% (19/35)</td>
<td>9% (3/35)</td>
</tr>
<tr>
<td><strong>Fresh Zygotes</strong></td>
<td>65% (13/20)</td>
<td>20% (4/20)</td>
</tr>
<tr>
<td><strong>Vitrified Oocytes</strong></td>
<td>73% (61/84)</td>
<td>7% (6/84)</td>
</tr>
<tr>
<td><strong>Fresh Oocytes</strong></td>
<td>83% (125/150)</td>
<td>11% (17/150)</td>
</tr>
</tbody>
</table>

Table 3: Bovine zygotes and MII oocytes development after vitrification with the Sarah system compared to the fresh controls. (p, NS)

Arav et al., JARG May 26, 2018
Successful Vitrification of IVP Ovine Embryos at the Blastocyst Stage

ES: 7.5% DMSO + 7.5% EG + 20% FCS in TCM-199
VS: 18% DMSO + 18% EG + 0.5M Trehalose + BSA in TCM-199

Ledda et al., JASB 2019
Survival rates after 24 h post warming culture

Survival rates after 24 h

* **

***

Survival rates

% survival

6 dd 7 dd 6 dd 7 dd
Multistep Two step

56,25 95,35 56,55 86,25

*** & ** p<0.01; * p<0.05

Ledda et al., JASB 2019
Tunel assay: apoptosis after 24 h post warming culture

- **Dinamic System**
  - Early Blastocysts
  - Blastocysts Expanded

- **Two-Step**

- **Control**

Ledda et al., JASB 2019
Direct Transfer of Vitrified cattle Embryo
Direct transfer of vitrified horse embryo
Direct Transfer of Vitrified Human Embryo
The new robotic Sarah inside incubator

24-well dish
Gene expression analysis of ovine prepubertal testicular tissue vitrified with a novel cryodevice (E.Vit)

Daniela Bebbere¹ • Sara Pinna¹ • Stefano Nieddu¹ • Dity Natan² • Amir Arav² • Sergio Ledda¹

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<table>
<thead>
<tr>
<th>Treatment</th>
<th>Plasma membrane integrity (mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 0 h</td>
<td>89.67% ± 1.45 a</td>
</tr>
<tr>
<td>Control 2 h</td>
<td>86.67% ± 1.33 a</td>
</tr>
<tr>
<td>Control 24 h</td>
<td>81.00% ± 4.58 a</td>
</tr>
<tr>
<td>Vitrified 0 h</td>
<td>66.00% ± 4.73 b</td>
</tr>
<tr>
<td>Vitrified 2 h</td>
<td>59.67% ± 4.18 b</td>
</tr>
<tr>
<td>Vitrified 24 h</td>
<td>31.00% ± 3.46 c</td>
</tr>
</tbody>
</table>
E. Sep straw for direct IUI

Load sperm medium
Load sperm

Semen

fertileSAFE
Swim into syringe
Other automatic vitrification Products

ESCO AuVis

Genea Gavi
Summary

➢ E.vit is a 1/4cc straw and 1mm Capsule.
➢ Vitrifies oocytes, embryos & tissue slices.
➢ Operates as a safe open system or a semi-closed system.
➢ Cooling and warming rates of $>20,000^\circ\text{C}/\text{min}$.
➢ Can vitrify 30 oocytes simultaneously in 17 min.
➢ Automatic insertion into LN.
➢ Automatic warming, washing and culture
Imagine a world where cells are stored at room temperature with no need for liquid nitrogen...
Ancient Freeze Drying

The Incas stored their potatoes (*Chuño*) and other food crops on the mountain heights of Peru.
Israeli researchers have germinated a date palm seed from 2,000-year-old which was found in Masada (Judean Desert).
Today's Biologics Industry

More than 55% of the biologics product manufactured today are Lyophilized

40% are delivered in liquid form

Only 5% are delivered frozen
Freeze drying of mouse sperm

“Mice From Dust: Just Add Water”

Table 4. Postimplantation development of oocytes fertilized by ICSI with fresh, frozen, or freeze-dried spermatozoa

<table>
<thead>
<tr>
<th>Sperm treatment</th>
<th>Medium for sperm dispersion and storage</th>
<th>Mouse strain</th>
<th>Sperm storage time, days</th>
<th>No. zygotes transferred (in vivo, exp.)</th>
<th>No. recipients*</th>
<th>No. (%) normal embryos</th>
<th>No. (%) abnormal embryos</th>
<th>Examination on day 14 postcoitum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freshs</td>
<td>Hepes-CZB</td>
<td>C57BL/6J</td>
<td>8-10</td>
<td>75.0 (5)</td>
<td>54 (89)</td>
<td>5.1 (0.8)</td>
<td>3.9 (0.7)</td>
<td>0.1 (0.1)</td>
</tr>
<tr>
<td>Frozen and kept at -7°C</td>
<td>EGTA</td>
<td>C57BL/6J</td>
<td>5-7</td>
<td>70.0 (5)</td>
<td>40 (80)</td>
<td>2.5 (0.5)</td>
<td>3.5 (0.7)</td>
<td>0.1 (0.1)</td>
</tr>
<tr>
<td>Freeze-dried and kept at 4°C</td>
<td>EGTA</td>
<td>C57BL/6J</td>
<td>5-7</td>
<td>75.0 (5)</td>
<td>70 (85)</td>
<td>2.5 (0.5)</td>
<td>3.5 (0.7)</td>
<td>0.1 (0.1)</td>
</tr>
</tbody>
</table>

*Each recipient had 1-2 oocytes fertilized.

PNAS | November 20, 2001 | vol. 98 | no. 24 | 13501-13506
Freeze-Dried Sperm Fertilization Leads to Full-Term Development in Rabbits\(^1\)

Ji-Long Liu,\(^2,3\) Hirokazu Kusakabe,\(^4,6\) Ching-Chien Chang,\(^2\) Hiroyuki Suzuki,\(^7\) David W. Schmidt,\(^3\) Marina Julian,\(^5\) Robert Pfieffer,\(^3\) Charles L. Bormann,\(^5\) X. Cindy Tian,\(^3\) Ryuzo Yanagimachi,\(^6\) and Xiangzhong Yang\(^2,3\)

Department of Animal Science/Center for Regenerative Biology,\(^5\) University of Connecticut, Storrs, Connecticut 06269
Institute for Biogenesis Research,\(^6\) University of Hawaii Medical School, Honolulu, Hawaii 96822
Faculty of Agriculture and Life Sciences,\(^7\) Hirosaki University, Hirosaki 036-8561, Japan

Live Rats Resulting From Injection of Oocytes With Spermatozoa Freeze-Dried and Stored for One Year

SHINICHI HOCHI,\(^1\) KAORI WATANABE,\(^1\) MEGUMI KATO,\(^2\) AND MASUMI HIRABAYASHI\(^2,3\)

Full-term development of hamster embryos produced by injecting freeze-dried spermatozoa into oocytes.

Muneto T, Horiuchi T.
Production of live foals via intracytoplasmic injection of lyophilized sperm and sperm extract in the horse

Y H Choi¹, D D Varner², C C Love², D L Hartman³ and K Hinrichs¹,²

Figure 1 Foals produced from (A) a lyophilized sperm, 11 days old and (B) a sperm from sperm extract, 16 days old.
Dry Human Sperm

Dry Sperm Pellet
RESULTS OF DNA INTEGRITY OF DRIED SPERM WITH DARYA

Fresh=81.06%±9.2%
Dried=81.3%±3.5%

RESULTS OF DNA INTEGRITY OF FREEZE DRIED SPERM USING DARYA AFTER 3 WEEKS STORAGE AT DIFFERENT TEMPERATURES

<table>
<thead>
<tr>
<th>Freeze-Drying human sperm</th>
<th>Intact DNA %</th>
<th>Cells con. x 10^6cells/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh</td>
<td>81.06 ± 9.2</td>
<td>10</td>
</tr>
<tr>
<td>Immediate rehydration with Medium</td>
<td>71.19 ± 7.7</td>
<td>5.375</td>
</tr>
<tr>
<td>Immediate rehydration with Lyo solution</td>
<td>81.31 ± 3.5</td>
<td>8.25</td>
</tr>
<tr>
<td>Rehydration after 20 days storage at 30°C</td>
<td>1 ± 0</td>
<td>6.25</td>
</tr>
<tr>
<td>Rehydration after 20 days storage at 4°C</td>
<td>80.84 ± 7.1</td>
<td>8.75</td>
</tr>
<tr>
<td>Rehydration after 20 days storage -20°C</td>
<td>87.76 ± 0.8</td>
<td>10.25</td>
</tr>
</tbody>
</table>

ICSI of freeze dried human sperm into donor oocytes

7 embryos out of 9 oocytes injected
High post-thaw survival of ram sperm after partial freeze-drying

Amir Arav\textsuperscript{1} · Antonella Idda\textsuperscript{2} · Stefano Mario Nieddu\textsuperscript{2} · Yehudit Natan\textsuperscript{1} · Sergio Ledda\textsuperscript{2}

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Table 2 Post-thaw sperm motility: (A) after freezing and holding for 1 h at two different temperatures using two different solutions (Lyo A and Lyo B) and (B) after partial freeze-drying (PFD) at two different temperatures using two different solutions (Lyo A and Lyo B)

<table>
<thead>
<tr>
<th>Temperature</th>
<th>A. Post-thaw sperm motility after holding 1 h</th>
<th>B. Sperm motility after PFD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lyo A</td>
<td>Lyo B</td>
</tr>
<tr>
<td>10 °C</td>
<td>3% ± 1.4% a</td>
<td>3% ± 2% a</td>
</tr>
<tr>
<td>25 °C</td>
<td>3.3% ± 2.8% a</td>
<td>1.2% ± 2.5% a</td>
</tr>
</tbody>
</table>

Results are shown as means ± SD. Different letters (a, b) indicate significant difference in sperm motility between rows and between columns ($P < 0.001$)
Dried STEM CELLS
Determination of CD34+/CD45+ cell number within the MNC population (FACS)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total WBC (10^6/ml)</th>
<th>%CD34</th>
<th>CD34 Total (cell number/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh UCB</td>
<td>5.8</td>
<td>0.21</td>
<td>12400</td>
</tr>
<tr>
<td>Fresh MNC</td>
<td>2.3</td>
<td>0.68</td>
<td>15600</td>
</tr>
<tr>
<td>Lyo 1</td>
<td>2.5</td>
<td>0.69</td>
<td>17300</td>
</tr>
<tr>
<td>Lyo 2</td>
<td>2.5</td>
<td>0.70</td>
<td>17600</td>
</tr>
</tbody>
</table>

MNC survival after freeze drying with solution A

Natan and Arav PLOS one 2009
CFU assay of fresh and rehydrated MNC derived from human UCB

<table>
<thead>
<tr>
<th>Sample</th>
<th>Well 1</th>
<th>Well 2</th>
<th>Total colonies</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lyo 4</td>
<td>1 –E; 20-GM</td>
<td>1 –E; 10-GM 2 –Mix</td>
<td>2 –E, 30-GM, 3-Mix =35</td>
<td>CFU-E/GM/Mix</td>
</tr>
<tr>
<td>Lyo 5</td>
<td>6-GM, 4 Mix</td>
<td>16-GM, 2 Mix</td>
<td>22 –GM, 6-Mix =28</td>
<td>CFU-GM/Mix</td>
</tr>
</tbody>
</table>

CFU-E = Growth of erythrocyte colonies
CFU-GM = Growth of granulocytes and macrophage colonies
CFU-Mix = mixture of CFU-GM and CFU-GM colonies

Natan and Arav PLOS one 2009
Preservation of Differentiation and Clonogenic Potential of Human Hematopoietic Stem and Progenitor Cells during Lyophilization and Ambient Storage

Representative CFU groups enumerated following freeze-thawing and freeze drying
Freeze dried lymphocyte for nuclear transfer
Freeze drying of MII ovine oocytes

Delivered to Prof. Loi in Italy in regular post

Loi and Arav unpublished data 2010
Spindle transfer from lyophilized oocytes into enucleated oocytes

Loi and Arav unpublished data 2010
First two cell embryo obtained from injection of a metaphase plate from a freeze-dried oocyte into fresh enucleated oocyte

Loi and Arav unpublished data 2010
VitDrying bovine oocytes

Loi and Arav unpublished data 2010
Results

For group A, 70% of the oocytes were recovered after rehydration but only 1/7 stained as viable (14% ; green fluorescent); for group B, 71% were recovered and 10/17 stained as viable (59%). For group C (Vit drying), 88% were recovered and 23/30 stained as viable (77%) (p < 0.05).
Vit-Drying and Rehydration Human Oocytes (50% survived)

Arav et al., UNPUBLISHED
Ovarian Tissue Freeze drying

Arav et al., UNPUBLISHED