Human Oocyte And Embryo Cryopreservation


Summary of Syllabus:

- Brief overview of human egg and embryo freezing.
- Discussion of merits of currently utilized cryopreservation protocols.
- Practical issues of cryopreservation with reference to egg or embryo selection, and thaw replacement protocols.
- Alternative cryopreservation technologies.

Cryostorage of the female gamete

The last few years have seen a significant resurgence of interest in the potential benefits of human egg freezing. Essentially, these benefits are:

1. Formation of donor “egg banks” to facilitate and lessen the cost of oocyte donation for those unable to produce their own oocytes.
2. Provision of egg cryostorage for women wishing to delay their reproductive choices.
3. Convenient cryopreservation of ovarian tissue taken from women about to undergo therapy deleterious to such tissue, which may threaten their reproductive health.

The technology so far applied clinically has been based directly on traditional human embryo cryopreservation protocols, and has produced relatively few offspring. Fortunately to date, no abnormalities have been reported from these pregnancies, regardless of the persistent concerns that freezing and thawing of mature oocytes may disrupt the meiotic spindle and thus increase the potential for aneuploidy in the embryos arising from such eggs. With respect to cryostorage of donated oocytes there have been several reports that have shown some success with this approach (Polak de Fried et al, 1998; Tucker et al, 1998a; Yang et al, 1998 & personal communication). Six pregnancies have generated 10 babies Appendix 1.

Cryopreservation of the preimplantation human embryo:

While human embryo cryopreservation has become a well-established technology in assisted human reproduction, it has yet to become fully clear as to which stage preimplantation embryos are best cryostored. Indeed on the face of it, the superiority of blastocyst stage freezing over 1-cell pronucleate stage freezing in terms of implantation per thawed embryo transferred, is countered by the loss of embryos that lack the wherewithal to grow for five to six days in vitro (Mandelbaum et al, 1998). Countering the benefits of freezing cleavage stage embryos is the partial survival of multi-cellular embryos (Van den Abbeel & Van Steirteghem 2000), where “partial” embryos may give rise to live births even from one surviving cell, but viability is reduced (Tucker et al. 1995). Ultimately there seems to remain some degree of clinic choice of philosophy of approach over when to freeze (Tucker et al. 1995). If one were to assume, however, that the majority of in vitro culture of human embryos might eventually be carried out to the blastocyst stage, then it would seem redundant to freeze embryos at an earlier stage. Not to belabor the point, but selection is the central essence of extended culture, enabling poorer viability embryos to arrest in development so “selecting” themselves as non-candidates for fresh transfer or cryopreservation. Although to some this may seem wasteful of embryos, the net result is probably that chances of pregnancy Table 1a).

Routine Freezing of Blastocysts
Revisiting the original blastocyst cryopreservation protocol, Menezo & Veiga (1997) modified the protocol such that it became extremely convenient and at least as successful as the earlier protocol (1992). Differing clinics, however, have struggled with inconsistent results with the latter protocol, and started research variants to improve on consistency. In fairness, much of this has probably been due to inexperience on the part of many embryologists, both with selecting blastocysts of sufficient quality to freeze, and also understanding the subtleties of cryopreservation and the impact that even the slightest variation, no matter how unintentional, might have on consistency. The most common practice to attempt improved consistency has been to reintroduce one or two glycerol concentration steps in the thaw, with one or two extra sucrose dilutions (for two examples of modified protocols, refer to Appendices 2&3). Not a major change, and not too great an increase in time commitment. A typical example of a shift in outcomes within a cryoprogram following a change to a modified protocol would be the results from Boston IVF, where Jeannine Witmyer reports that in their first 13 thaws using the “1997 Menezo/Veiga” protocol, they achieved one ongoing pregnancy. After the introduction of a modified thaw approach similar to that used at Shady Grove (Appendix 2), then they achieved six pregnancies from 19 thaws. Small numbers, but they changed nothing else in their approach.

More complicated has been the experience at Shady Grove Reproductive Science Center, Rockville, MD, where initially we undertook eight thaws as per Menezo & Veiga (1997) and with only a 13.5% cryosurvival rate (7/52 blastocysts survived thawing), we did achieve one healthy pregnancy. Appendix 2, we got 80% cryosurvival (35/44), with five ongoing pregnancies from 13 thaws. Many factors clearly have an impression on these experiences, not the least of which are the differences in the “holding media”, freezing in straws, vials, or ampoules and possibly even the type of programmable freezer. Subsequent to that time, we have experienced other fluctuations in outcome regardless of the quality of the blastocysts at the time of thaw, with respect to the replacement protocol. Specifically, changes in the progesterone supplementation have seemed to have had a profound impact, such that with the use of Crinone we achieved only 3 ongoing pregnancies post-thaw in 25 cycles with an implantation rate of 6% (4/70). Moving to the use of intra-muscular progesterone, this returned results to a rate of 6 clinical pregnancies, with 4 ongoing/viable from 8 thaws, with an implantation rate of 25% (5/20). Again numbers here are small, and seemingly contradictory to some reports in the literature with the use of vaginal progesterone gels (Warren et al. 1999), or suppositories (Lightman et al. 1999). This serves not to stimulate lack of credibility in other's results, but to underscore the multi-factorial nature of assisted reproduction in general, and how the least variation in approaches clinic to clinic may have a significant effect on outcomes.

Melanie Freeman (Table 1b) reports that her clinic’s results in Nashville have become more successful with a shift away from the “multi-step” protocol (Menezo et al., 1992) to her own variant of the modified protocol for blastocyst cryopreservation (Appendix 3). Different freezing protocols can be thawed in the modified as can be seen in the second column of Table 1b, with reasonable outcomes. This has been our experience also, suggesting that thawing, at least in these types of protocols, can seem to be more critical than the freezing.

The impact of assisted hatching on thawed embryo implantation at Shady Grove RSC can be seen in Table 2. Numbers as yet are low. Interestingly, the cleavage/pronucleate thawed embryos appeared to gain no advantage from the assisted hatching procedure. Though due to the small numbers and lack of real discrimination of embryo quality at the earlier stages of embryo transfer, it is probable that it would take much higher numbers than with the thawed blastocysts to discern any real significance. It is logical that the hatching of the blastocysts should be beneficial for thawed blastocysts (Figure 1), given that many of them have been frozen on day-six at which stage it appears that assisted hatching is beneficial for fresh blastocysts (Tucker 1999). Secondly, the zona pellucida is thought to undergo problematic hardening during the freeze/thaw procedure (Tucker et al., 1991). In some cases zona fractures can be caused routinely (Van den Abbeel & Van Steirteghem 2000) depending on the means of cryopreservation. Embryos with holes already present in their zonae can successfully survive cryopreservation and give rise to pregnancies (Magli et al. 1999). Generally the cryopreservation protocols discussed above can be well-defined and controlled from the laboratory perspective, so if fluctuations in pregnancy outcomes continue regardless of good cryosurvival, then clinical management problems outside of the lab are probably at fault. An example of this is given above from the management of the “artificial” cycles with vaginal progesterone gel instead of intra-muscular progesterone during thawed blastocyst replacements. This was completely unanticipated. Many simple errors are possible, including, for example, calculation of the day of transfer. The easiest way to consider this is to calculate the “day of
“ovulation” (whether in a “natural” or “artificial” transfer) cycle then thaw and transfer all blastocysts on the fifth day of development, counting “ovulation” day as day-zero. This mirrors what would happen normally in an IVF cycle, but where some manipulation of timing may be needed for whatever practical reasons, then it is better to err on the “early” side when thawing the embryos.

The Future of Egg & Embryo Cryopreservation

Firstly, it is hoped that more clinics will become increasingly comfortable with blastocyst freezing as it currently exists. This will only be possible if extended culture is perceived to become sufficiently consistent. With production of good quality mid to fully expanded blastocysts with well-defined ICM and trophoderm on day-five/six, it is possible to settle on consistently successful cryoprotocol for such embryos using the present technology. Even so, as Menezo & Veiga proved (1997), protocols can always be made simpler and more convenient. To this end, it has to be noted that vitrification protocols are starting to enter the mainstream of human ART. Protocols successfully applied for bovine oocytes and embryos have been used initially with human oocytes (Kuleshova et al., 1999), and initial trials been undertaken with human blastocysts (Lane et al., 1999). Vitrification is very simple, requires no expensive programmable freezing equipment, and relies especially on the placement of the embryo in a very small volume of vitrification medium that must be cooled at extreme rates not obtainable in regular enclosed cryostraws and vials. Whatever the approach to cryostorage, the aim of blastocyst cryopreservation will be to maximize the potential viability of each embryo thawed and replaced, such that the numbers of embryos thawed and transferred may be kept to a minimum. Oocyte cryopreservation will slowly enter the mainstream of techniques in ART, in remains important that we research the consequences of these therapies carefully to ensure that we truly do no harm (Wennerholm et al. 1998; Dulioust et al. 1999).

Table 1a. Blastocyst Cryopreservation outcomes: Center for Assisted Reproduction, Texas Del Marek et al.

<table>
<thead>
<tr>
<th>Year</th>
<th>1998</th>
<th>1999</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thaws</td>
<td>110</td>
<td>106</td>
</tr>
<tr>
<td>Transfers</td>
<td>101</td>
<td>101</td>
</tr>
<tr>
<td>Del. /Ongoing Preg.</td>
<td>24%</td>
<td>33%</td>
</tr>
<tr>
<td>Average # embryos ET</td>
<td>2.3</td>
<td>2.1</td>
</tr>
</tbody>
</table>

Table 1b. Blastocyst Cryopreservation outcomes: Nashville, Tennessee. Melanie Freeman et al.

<table>
<thead>
<tr>
<th>Period</th>
<th>1993-8</th>
<th>“Multi-step Freeze/Mod. Thaw”*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thaws</td>
<td>720 embryos</td>
<td>73</td>
</tr>
<tr>
<td>Survived</td>
<td>533(74%)</td>
<td>59(81%)</td>
</tr>
<tr>
<td>#/thaw</td>
<td>4.2</td>
<td>2.9</td>
</tr>
<tr>
<td>#/ET</td>
<td>3.1</td>
<td>2.4</td>
</tr>
<tr>
<td>Del. P.R.</td>
<td>38/173(22%)</td>
<td>7/25(28%)</td>
</tr>
<tr>
<td>Emb. Imp.</td>
<td>67 sacs(12.6%)</td>
<td>14 sacs(23.7%)</td>
</tr>
</tbody>
</table>

* 1999 onward, protocol used as in Appendix 2; more stringent selection criteria for cryopreservation including only mid- to fully expanded blastocysts on day-5/6. Also PZD Assisted Hatching used prior to transfer.
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Grove Fertility RSC, Maryland.

<table>
<thead>
<tr>
<th>Assisted Hatching</th>
<th>No Intervention Post-Thaw</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pregnancy</strong></td>
<td><strong>Emb. Imp.</strong></td>
</tr>
<tr>
<td><strong>BLASTOCYST</strong></td>
<td></td>
</tr>
<tr>
<td>(ET’s = 33)</td>
<td>5/15(33%)</td>
</tr>
<tr>
<td><strong>CLEAVAGE /PRONUCLEATE</strong></td>
<td></td>
</tr>
<tr>
<td>(ET’s = 18)</td>
<td>3/9(33%)</td>
</tr>
</tbody>
</table>

Figure 1. Hatching Blastocyst Post-Thaw.

Appendix 1.
HUMAN EGG CRYOPRESERVATION.
Freezing Human eggs is very similar in principle to early embryo freezing but with several key differences adopted to make outcomes more consistent.

FREEZE:

1. After egg collection, maintain all eggs in culture for >5 hours before attempting cryopreservation.
2. Strip all eggs in hyaluronidase at some point in the 5hrs pre-incubation period.
3. Place all eggs (GV through MII) to be frozen into warm modified HTF and then place them on to the bench at room temperature for 10mins to down (approx. 22degC).
4. Expose to 1.5M 1,2-propanediol (propylene glycol) in modified HTF with 15% HSA for 20mins.
5. Place into 1.5M PROH + 0.2M sucrose for a further 10mins.
6. Rinse cryovial with PROH + Sucrose medium and then fill with 0.3ml of this medium ready to receive the oocytes.
7. Freeze in the following manner:- 22degC down to -5.0degC at a rate of 2.0 degC/min. Hold 15 mins @ -5.0degC, “seed” after 5mins, ensure that the “seed” has been established afterwards (*this is a rather “high” temperature compared to -7.0degC for embryos). Cool further @ -0.3degC/min to -38degC then plunge into liquid nitrogen for storage.

THAW:
1. Place cryovial at room temp for 1min, then place in 30degC water bath till ice crystals have gone.

2. Remove contents into room temp drop of 1.5M PROH + 0.3M Sucrose in modified HTF with 10% HSA, then place into subsequent PROH dilutions for 8mins each of 1.0M, 0.75M, 0.5M, 0.25M, 0.0M all + 0.3M Sucrose.

3. Dilute slowly the final 0.3M Sucrose drop with modified HTF + 10% HSA, then wash eggs through 4-5 drops modified HTF, then 6-8 drops plain HTF + 10% HSA, and place in the incubator.

4. Undertake ICSI on all mature thawed oocytes only after four hours in culture, after which any cytoskeletal damage that may have occurred during freezing will have had an opportunity to repair itself.

mt. 10.19.99

Appendix 2.

BLASTOCYST CRYOPRESERVATION: Shady Grove RSC Protocol.

FREEZING

1. Holding Medium: modified HTF + 10% HSA.
2. Freeze good expanded / hatched or hatching blastocysts on Day-5/6 (unless fertilization delayed, e.g., because of FICSI).
3. Embryos into modified HTF + HSA @ 37deg.C, then move onto cool bench (22degC), and wash through several droplets for about 1 to 2min.
4. Move into 5% glycerol for 8mins.
5. 10% glycerol + 0.2M sucrose for 8mins (including loading time). Load straws / cryovials. (SGRSC uses 1.2ml Nunc cryovials containing 0.3ml medium).
6. Cool @ –2degC/min to –7.0degC; hold for 15min; “seed” after 5min; -0.3degC/min to –38degC, then plunge into liquid nitrogen for storage.

THAWING

1. Room temperature for 1min. Waterbath @ 30degC till ice gone.
2. Locate blastocyst in 10% glycerol + 0.4M sucrose for 30-40sec.
3. 5% glycerol + 0.4M Sucrose for 3mins.
4. 2.5% glycerol + 0.4M Sucrose for 3mins.
5. 0.4M Sucrose alone for 2mins.
6. 0.2M Sucrose for 2mins.
7. 0.1M Sucrose for 1min; move dish to warm scope / bench.
8. Modified HTF + 10% HSA @ 37degC for three washes, then into culture of HTF + 10% HSA.
10. Culture for >4 hours, even overnight to observe re-expansion.

mt. 5/99.

Appendix 3.

BLASTOCYST FREEZING - SHORT PROTOCOL
Melanie R. Freeman, MSTS Nashville Fertility Center, Tennessee

REAGENTS
Glycerol (Sigma, G-2025)  MATERIALS & EQUIPMENT:
Sucrose (Sigma, S-1888) Nunc 4-well multidish
Dulbecco’s Phosphate Buffered Saline (PBS) (GIBCO)  1.8ml cryovials (COSTAR)
Human Serum Albumin (HSA)(IVC)  Kryo-10, 1.7 cell freezer (T/S Scientific)
Isolet (Hoffman IVF Chamber)

BLASTOCYST FREEZE/THAW MEDIA: (make at least one day in advance to de-gas)
Cryo solutions: Filter solutions through 0.2m filters and store in the refrigerator for 2 months.

- Cryo solution #0: (PBS + 5mg/ml HSA). Combine 5 ml of HSA with 100 ml of PBS.
- Cryo solution #1: (PBS + 5mg/ml HSA + 5% glycerol). Add 2.0 ml Glycerol to 38.0 ml Cryo solution #1.
• **Cryo solution #2:** (PBS + 5mg/ml HSA + 10% glycerol + 0.2 M sucrose). Add 5 ml of Glycerol to 45 ml of Cryo solution #4.

• **Cryo solution #3:** (PBS + 5mg/ml HSA + 5% glycerol + 0.2 M sucrose). Add 6 ml of Cryo solution #2 to 6 ml of Cryo solution #4.

• **Cryo solution #4:** (PBS + 5mg/ml HSA + 0.2 M sucrose). Add 3.425 g Sucrose to 50 ml of Cryo solution #1.

**BLASTOCYST FREEZING PROCEDURE:**

1. Prior to freezing, fill Nunc multi-dish wells #1 and #2 with 0.6 ml **Cryo solution #0**. Fill well #3 with 0.6 ml **Cryo solution #1**, and well #4 with 0.6 ml **Cryo solution #2**. Fill each cryovial with 0.3 ml **Cryo solution #2**.

2. Allow the dish and freezing vials to warm to 37°C for 10 minutes.

3. Freezing Program for Kryo-10: Start temp: ambient \(-2°C/min to -7°C \(-0.3°C/min to -37°C\) \(\text{Seeding: manual \(\text{Seeding Temp: -7°C \(\text{soaking (before seeding): 10 min \(\text{Hold (after seeding): for 10 min \(\text{End of program)}\}}\}}\)

4. **Move dish to room temperature**, place all embryos to be frozen in well #1 of the Nunc dish to wash out all culture media. Transfer to well #2 and incubate for 2 minutes.

5. Transfer the embryos to well #3 and incubate for 10 minutes.

6. Transfer the embryos to well #4 and set timer for 10 minutes. When embryos settle to bottom of dish (1-5 minutes) load into vials.

7. Once all vials are loaded, place in the Kryo-10 at the same time. Press the “Run” button when the timer rings (end of 10 minutes). The Kryo-10 will proceed to the seeding temp. Seed each vial carefully using ring-forceps dipped in LN₂.

8. At the end of the freezing program, fill the portable dewar with LN₂. Quickly, remove freezer canes from the Kryo-10 and submerse in the LN₂ in the portable dewar. Lift out and place each vial on a precooled storage cane and submerse storage cane in LN₂ storage dewar.

**BLASTOCYST THAWING PROCEDURE:**

1. Allow at least 4 hours between thaw and embryo transfer.

2. Prior to thawing, fill Nunc multi-dish well #1 with 0.6 ml **Cryo solution #2**, well #2 with 0.6 ml **Cryo solution #3**, well #3 with 0.6 ml **Cryo solution #4**, and well #4 with 0.6 ml **Cryo solution #0**. Allow Nunc dish to warm to room temp for 20 minutes.

3. Prepare a 30°C waterbath. Remove freezing vial(s) from LN₂ and leave at room temp approximately 1-2 minutes until surface frost appears, and seal is easily broken. Tighten vial top and immerse the bottom of the vial(s) in a 30°C water bath for 2 minutes until completely melted. Gently agitate the vials in the waterbath. After 2 minutes no crystals should remain.

4. At room temp, transfer the contents of each vial to the center area of a Nunc 4-well multidish and locate each embryo. Transfer them to well #1 as you find them. When all are in well #1, move the dish to 37°C (in isoletr with no CO2). Allow the embryos to equilibrate for 30-45 seconds. Move the embryos to well #2 and incubate for 3 min.

5. Move the embryos to well #3 and let them remain for 2 minutes. Rinse in well #4, then rinse again several times with growth media (GM) and place in GM until Assisted Hatching (PZD) and transfer.

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