Human Oocyte And Embryo Cryopreservation

October 10, 2011
By Mark Perloe, MD [1]


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 from cryopreserved donor oocytes in these reports. Use of frozen donor oocytes post-thaw not for whole egg donation, but for ooplasmic transfer has been reported with a successful delivery of a twin following thawed ooplasmic donation (Lanzendorf et al., 1999).

Cryostorage of women's own oocytes was originally reported in the case of three births over a decade ago by two centers (Chen, 1988; Van Uem et al, 1987). More recently these successes have been reproduced by others (Porcu et al, 1998; Tucker et al, 1998b; Yang et al, 1998 & personal communication; Kuleshova et al., 1999), giving rise to 10 babies. One other baby has arisen from a clinical circumstance that is not completely unfamiliar to IVF clinics: oocytes had been collected but no sperm were retrievable for insemination. In this case, the oocytes were frozen, and donor semen was selected for future use. Ultimately both sets of gametes were thawed and used in a subsequent IVF attempt, which achieved a health delivery (Moody & San Roman, personal communication).

All of these pregnancies were from frozen-thawed mature oocytes, but for one notable exception, where a pregnancy arose from an immature germinal vesicle (GV) stage egg (Tucker et al, 1998b). Interestingly, this stage of egg development might prove to be a more successful approach for cryopreservation because its oolemma is more permeable to cryoprotectant, and its chromatin is more conveniently and safely packaged in the nucleus (Van Blerkom & Davis, 1994). Such eggs, however, still have to undergo GV breakdown and maturation to the MII stage before fertilization, and therefore their developmental competency is not so clearly established as with fully mature...
oocytes that are frozen. Source of the GV eggs and whether they have been exposed to any exogenous gonadotropins may play a key role in the competency of these eggs (Cortvrindt et al, 1998).

Whether mature or not, standard cryopreservation technologies appear to have their ultimate limitations in not only cryosurvival, but also more importantly in their lack of consistency. 50% cryosurvival is an adequate overall outcome, but not if it is a statistic that is arrived at by 90-100% survival in one case, and 0-10% in the next. Consequently, radically different types of protocol may provide the answer to increased consistent success. One approach has been to replace sodium as the principal cation in the cryoprotectant with choline in an attempt to shut down the sodium ion pumps in the oocyte membrane during cryoprotectant exposure, thus minimizing potentially deleterious “solution effects” during cooling (Stachecki et al, 1998). This has provided significant improvements in murine egg freezing, though it has yet to be applied clinically in the human. Alternatively, traditional slow cooling/rapid thaw protocols might be replaced with vitrification. Which again has been successfully applied in the mouse (O’Neil et al, 1997), bovine (Vajta et al., 1998), and very recently in the human (Kuleshova et al., 1999). While the mouse can be a useful model, it must be remembered that the murine oocyte is only just over half the volume of a human oocyte; this can have a major impact on permeability and perfusion if the two types of egg (Paynter et al. 1999). ICSI has become the accepted norm for insemination of oocytes post-thaw, to avoid any reduction in sperm penetration of the zona with premature cortical granule release (Gook & Edgar 1999).

The most plentiful source of oocytes potentially is ovarian tissue itself, containing as it does many thousands of primordial follicles in healthy cortical tissue. Earlier successful work with cryopreservation of rodent ovarian tissue has led the way to successful cryostorage of both sheep and human tissue (Gosden et al, 1998; Gook & Edgar 1999). Up to 80% survival of follicles has been reported, but the issue is how to handle this tissue following its thaw. Tissue that has been removed, for example, from a about to undergo cancer therapy may contain malignant cells, and therefore may not be safely used for auto-grafting in to such a woman if she were to survive. The tissue might be screened before or after thawing for the presence of malignant cells to enable some assessment of the safety of such an approach, or it may be grown in a host animal (e.g., SCID mouse) until such time as in vitro maturation could be undertaken more effectively. Extended culture of primordial follicles to full oocyte maturity, with subsequent embryonic development and birth has only been recorded in the mouse, and this was not from cryopreserved tissue (Eppig & O’Brien, 1996). Early studies are being undertaken in the human (Abir et al. 1999) with much to be done. Fertility has been restored in sheep, in a good model for the human ovary, following cryostorage of ovarian cortex and auto-grafting (Gosden et al, 1994), and this seems the most likely successful clinical model for restoration of fertility of women who are at risk of losing their ovarian function. This may include not only women about to undergo cancer therapy, but also women who have a family history of early menopause, and those with non-malignant diseases such as thalassemia or certain auto-immune conditions which may be treated by high-dose chemotherapy. Recently it was reported that ovarian function was restored by such means in the human, in a 29 year old patient suffering from hypothalamic amenorrhea subsequent to removal of both her ovaries at age 17 (Oktay et al., 1999).

The myriad routes for cryostorage of the female gamete makes for a confusing vision of where clinical applications may occur. However, different clinical needs may actually be met by differing technological approaches, whether they incorporate whole tissue freezing, separate follicle storage, or cryopreservation of mature oocytes themselves. For our current most consistent protocol for cryopreservation of oocytes retrieved following ovarian stimulation refer to 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 are more clearly defined and potentially improved, whilst the risks of higher order multiple implantation is reduced. Additionally, fewer embryos are frozen as blastocysts, reducing storage requirements, and expectations of pregnancy from those embryos that are frozen will be improved. Therefore, overall efficiency will be increased.

Nevertheless, given the consistently high rates of cryosurvival of cryopreserved early stage embryos, there will probably continue to be certain clinical circumstances where early stage freezing is justified. If a clinic wishes to move its cryopreservation program to blastocyst stage principally, a key question would be what to do with the early stage embryos already cryostored? One progressive approach may be to thaw all embryos at these earlier stages and grow them to blastocysts if possible. In this way, fewer embryos will be kept cryostored, and if an excess of embryos for transfer do reach the blastocyst stage, then they may be re-frozen for later use.

The first successful reports of human blastocyst cryopreservation came from culture in a simple salt solution (Cohen et al. 1985; Fehilly et al. 1985). More recently most cryopreserved blastocysts arose from extended culture of supernumerary embryos not transferred fresh on day-two or -three, usually using co-culture (Kaufman et al, 1995; Freeman, 1998). However, with increasing in growth stage-sequenced culture media, blastocyst culture for fresh transfer has become increasingly common. More convenient cryopreservation protocols for blastocysts (Menezo & Veiga, 1997) have also improved the ease with which this adjustment in a clinic's protocols may be made. Consistently high cryosurvival rates (approximately 90%), and good post-thaw pregnancy rates (38%) are now being achieved by certain clinics with judicious selection of blastocysts for freezing (Marek & Langley, personal communication). The key is how to select potentially viable blastocysts. As culture is extended over a longer period, the rate of development becomes an increasingly important parameter for blastocyst selection (Shoukir et al, 1998). However a range of selection criteria need to be applied to optimize the choice of the blastocysts with the best potential for successful cryopreservation (see below).

**Selection Criteria for Human Blastocysts for Cryopreservation:**

- Expanded blastocyst growth rate: day-5 > day-6 > day-7
- Overall cell number ≥ 60 cells (depending on day of development)
- Relative cell allocation to trophectoderm / inner cell mass
- Original quality of early stage embryo: PN formation, blastomere regularity, mono-nucleation, fragmentation

Issues such as how “early” a blastocyst can be frozen, or if blastocysts that are partially or totally hatched can be consistently cryopreserved, have yet to be adequately answered. Much data may exist from mouse and bovine models, for example, however cell number and levels of lipidation may have a profound differential impact thus minimizing the usefulness of such comparative studies. Hence data will be collected, as has often been the case with human ART, prospectively and used to fine tune future protocols from clinical hindsight. Most embryo cryopreservation protocols currently use a slow freeze / rapid thaw approach. Roughly speaking, slow freeze protocols utilize lower concentrations of cryoprotectants (approx. 1.5M) to avoid the toxicity of such agents during the initial exposure and slow cooling; higher concentrations of cryoprotectant (approx. 4.0M) allow shorter exposure times to the cryoprotectant and “rapid freezing”. Vitrification, using concentrations as high as 6.0M allow extremely high rates of cooling and thawing (>22,000degC/min) without the formation of ice. However, these more convenient protocols of ultra-rapid freezing and vitrification, that eliminate the use of expensive controlled rate freezers, await cross over from use in other species, or validation from more extensive experimental study in humans (Vatja et al, 1998; Lai et al, 1996; Hsieh et al. 1999).

Regardless of the uncertainties of which protocols for cryopreservation will prevail, the future seems to point to increasing success and consistency with embryo cryopreservation. The preparation of the uterus into which the thawed embryos will ultimately be placed seems to be an area of study that is better resolved, with both natural and replacement cycles providing comparable levels of receptivity in naturally cycling women, though differing levels of convenience (Tucker et al, 1995). Indeed, artificially prepared cycles may even effectively dispense with the use of gonadotropin releasing hormone agonists to lessen cost and improve convenience without loss of success (Simon et al,
Why freeze embryos at the blastocyst stage?
The very first report of successful cryopreservation of the human embryo was in 1983 (Trounson & Mohr, 1983) with a pregnancy arising from the freezing in DMSO, thawing and transfer of an eight-cell embryo. Within a year or so appeared the first successes from the use of glycerol to cryopreserve human blastocysts (Cohen et al., 1985; Fehilly et al. 1985). In the same year Lassalle et al. published a simple but consistent protocol using propanediol plus sucrose (1985) that has become probably the most commonly used approach for freezing both early cleavage stage and pronucleate one-cell embryos. Attempts to improve on the consistency and convenience of cryopreserving blastocysts reappeared when, using Vero cell co-culture to enhance extended culture, Menezo et al. (1992) explored the use of a combination of glycerol and sucrose as cryoprotectants to freeze “spare” expanded blastocysts. Essentially all of the above protocols employed a “slow freeze/rapid thaw” approach, requiring the use of a programmable freezer for the controlled rate cooling to temperatures between minus 35degC to minus 150degC. Variants of these protocols remain the current standards for human embryo cryopreservation.

With an increase in IVF-ET cycles being extended to incorporate the fresh transfer of blastocysts, blastocyst cryopreservation is no longer being considered as a last option for “left over” embryos that develop to this stage. Increasingly it is being considered as the sole or at least principal stage at which to freeze. The reason for this is that if selection of blastocysts is to be optimized, then freezing embryos at an earlier stage would reduce the pool from which to choose fresh blastocysts for transfer. Concerns that embryos are in some way being “lost” due to extended culture, because fewer embryos are being used overall compared with previous approaches adopting day-two and three transfer, will be allayed by increasing consistency of extended culture. The central emphasis of blastocyst transfer in any event is to reduce the number of embryos at transfer while maintaining good pregnancy outcomes (see 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. Oddly many of the blastocysts appeared to survive initially, but upon subsequent culture for several hours the cells became increasingly degenerate. Subsequently, with no change other than to thaw into a protocol as per 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 trophectoderm 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, most likely in the area of oocyte donation. Here information, in terms of clinical success of protocols, is generated within months not years, as would be the case with freezing of eggs for single concerned with their future reproductive choices. In accepting that cryopreservation of human eggs and embryos seems here to stay, 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.

<p>| Year | 1998 | 1999 |</p>
<table>
<thead>
<tr>
<th>Period</th>
<th>1993-8</th>
<th>“Multi-step”</th>
<th>“Multi-step Freeze/Mod. Thaw”*</th>
<th>“Mod. Freeze/Thaw”*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thaws</td>
<td>720 embryos</td>
<td>73</td>
<td>54</td>
<td>54</td>
</tr>
<tr>
<td>Survived</td>
<td>533 (74%)</td>
<td>59 (81%)</td>
<td>46 (85%)</td>
<td></td>
</tr>
<tr>
<td>#/thaw</td>
<td>4.2</td>
<td>2.9</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td>#/ET</td>
<td>3.1</td>
<td>2.4</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td>Del. P.R.</td>
<td>38/173 (22%)</td>
<td>7/25 (28%)</td>
<td>9/18 (50%)</td>
<td></td>
</tr>
<tr>
<td>Emb. Imp.</td>
<td>67 sacs (12.6%)</td>
<td>14 sacs (23.7%)</td>
<td>18 sacs (39%)</td>
<td></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.

Table 2. Assisted Hatching and its impact on the cryopreservation program at Shady Grove Fertility RSC, Maryland.

<table>
<thead>
<tr>
<th>Assisted Hatching</th>
<th>No Intervention Post-Thaw</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLASTOCYST</td>
<td></td>
</tr>
<tr>
<td>(ET's = 33)</td>
<td>5/15 (33%)</td>
</tr>
<tr>
<td>CLEAVAGE /PRONUCLEATE</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

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

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 6ml of Cryo solution #2 to 6ml 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 ↓ Seeding: manual ↓ Seeding Temp: -7°C ↓ soaking (before seeding): 10 min ↓ Hold (after seeding) for 10 min ↓ 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 submerge storage cane in LN₂ in portable dewar while the other vials are unloaded. Repeat for all other vials and place 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 isoloet 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.

**References:**


Hsieh YY et al. (1999): Ultrarapid cryopreservation of human embryos: experience with 1,582
Lassalle B et al. (1985): Human embryo features that influence the success of cryopreservation with the use of 1,2 propanediol. Fertil Steril 44:645-51.


Source URL:

Links: