Blastocyst Vitrification: A Review

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Background:

The only method of stable and long-term (practically infinite) preservation and storage of any perishable biological materials, particularly cells, is to keep them in the glassy (vitreous) state. This was apparent to Father Luyet when he titled his pioneering work "The *vitrification* of organic colloids and of protoplasm" and "Revival of frog's spermatozoa *vitrified* in liquid air" (1, 2). He and other *"pioneers of the cryobiological frontiers"* including Lovelock, Meryman, Mazur, Polge, Smith, Levitt, Farrant, and Willadsen, clearly understood some 40-70 years ago that only a glassy state would insure stable and non-lethal preservation of cells. With time, we saw the development of a variety of biopreservation methods, such as slow-cooling (which is just a way of achieving glassy state inside the cell) (3). It was Luyet's work, which would make cryopreservation a science. From the outset, he recognized that ice damage must be avoided and vitrification could be a method for long-term preservation of cell viability (2).

IVF laboratories have been cryopreserving spare embryos for more than 30 years. More than 50,000 babies have been born worldwide from thawed embryos. There is no doubt that the technique can work and has great value in the IVF industry. Today the field has changed quite markedly towards a quicker methodology; vitrification. However, from a basic cryobiological standpoint nothing has really changed. The slow-cooling methods we are familiar with are actually a method of vitrification. Therefore we have been using vitrification for over 30 years, we just did not call it that.

In order to understand rapid-cooling or modern vitrification techniques, let us compare them to the slow-cooling method. During slow-cooling, using a programmable freezer, embryos are exposed to relatively low concentrations of cryoprotectants (1.5M PrOH and usually some sucrose, around 0.2M) equilibrated for 10-25 min at room temperature, loaded into a straw or vial, sealed and placed into a controlled-rate freezer. Ice formation is initially induced extracellularly by seeding at a temperature whereby ice can perpetuate (around -5.5°C or lower) and, as a result of the solute gradient created, freezable water flows out of the cells, minimizing the chance of intracellular ice formation during cooling. As the temperature is gradually lowered, the concentration of cryoprotectant in the liquid phase, which includes the intracellular fluid, increases correspondingly until a level is reached at which additional formation and growth of ice crystals, although possible, are unlikely, even if the temperature drops further (4). Rather, the liquid phase turns into a glassy substance that solidifies without further crystal formation as the temperature continues to decrease. The unfrozen liquid phase remaining within the cells when they are plunged into LN_2 should ideally consist of this glassy substance with all the original cell solutes remaining in solution (4). This suggests that when we slow-cool cells using a penetrating cryoprotectant such as PrOH, and standard slow-cooling protocols, we are actually vitrifying the cells. Indeed, when we slow-cool human embryos, typical survival rates range between 80% and 100%, for many IVF centers. These survival rates would not be possible, at least according to Mazur and company, if intracellular ice formation were occurring (5). This correlates well with the theory that slow-cooling is vitrification. Of course this does not mean that IIF does not or cannot occur, it simply suggests that in conventional embryo

freezing protocols, IIF is not a major source of cell damage. However, if water eflux is inhibited and does not occur in equilibrium, as suggested, and despite a slow cooling rate, IIF could pose a real problem (6).

Vitrification vs. Slow-Cooling:

Slow-cooling regimes have been very successful in clinical IVF, simply based on the fact that, conservatively, over 50,000 children have been born from previously frozen embryos. However, there is room for improvement, especially when it comes to storing blastocysts and oocytes. Blastocyst storage, the focus of this chapter, using slow-cooling procedures has met with little success for most. Studies by Fehilly et al. (7); Hartshorne et al. (8); Menezo et al. (9); and Kaufman et al. (10) demonstrated that human blastocyst cryostorage was possible, however, there is little in the literature regarding this subject. One reason is that IVF clinics at that time were just learning how to culture embryos out to the blastocyst stage, and transferring 8-cell embryos on Day 3 of culture was considered optimal. Therefore, with the relative lack of culture experience to the blastocyst stage and the fact that Day 3 storage was relatively good (around 80% survival), the need did not exist in the late 1980's. One common problem that existed among IVF clinics, and still exists today, is that of multiple offspring being produced from the replacement of more than one embryo on Day 3. Multiples dramatically increase the risks and costs of a pregnancy, and thus replacing only a single embryo would be beneficial. The only problem is that pregnancy rates tend to be lower with only one embryo replaced and this is not conducive to a successful clinic. Replacing blastocysts however, leads to very good implantation and pregnancy rates, and therefore a clinic could maintain relatively good pregnancy rates and reduce multiples at the same time. As embryo culture progressed and more labs were culturing out to the blastocyst stage, to reduce the number of embryos replaced as well as the number of excess embryos that would have to be stored, blastocyst cryopreservation gained importance.

Much of the work on blastocyst cryopreservation by the 1990's had been done in domestic species, and these studies laid the groundwork for human blastocyst storage (11-19). One of the first studies done was by Whittingham with mouse blastocysts (11). Even so, many clinics still struggle with obtaining acceptable success rates by slow-cooling blastocysts (20-22). By the 1990's vitrification or, more accurately, rapid and ultra rapid-cooling research in animal models was prevalent. The main reason was that several commercially valuable species, including bovine and porcine, were sensitive to chilling injury and conventional slow-cooling regimes did not work (23-27). Therefore, the only way to store these cells was to rapidly cool/freeze them from elevated temperatures by direct immersion in LN2, thus avoiding chilling injury.

Blastocyst Vitrification:

The basic principles of removing water from the cell, adding a cryoprotectant to bind the remaining water to prevent ice crystal formation, and cooling to liquid nitrogen temperatures is the same as it has been in the past. Since one could vitrify an embryo by slow-cooling early studies calculated the intracellular cryoprotectant concentration that occurred after cooling to minus -30°C and exposed embryos to these concentrated solutions at room temperature. A variety of cryoprotectants including DMSO, proplyene

glycol, glycerol, etc. were used at various concentrations. Early studies in the cow, pig, sheep, rabbit, and mouse led to a better understanding of what was possible (19, 25, 28-38). Once again, studies in animal models led the way for work to be done with human blastocysts. During the late 1990's quite a bit of research was being done, now mainly focused upon vitrification of oocytes and embryos, including blastocysts from a variety of species. Numerous births from species sensitive to chilling injury during slow-cooling occurred. However, despite these successes, most of the solutions being used were more or less toxic to the cells and although offspring could be produced, the technique overall, did not work very well. The main reason was that for equilibrium vitrification to occur the cells needed to be exposed to high cryoprotectant concentrations at or above room temperature, and for a reasonable amount of time to dehydrate the cells and load the cell with cryoprotectant. Dimethylsulfoxide (DMSO) was now being used as the primary or one of the primary cryoprotectants because of its rapid passage through cell membranes, despite its toxic potential (39-42). The elevated cryoprotectant concentrations (6-8 Molar), temperature (23°C to 39°C), and duration, all increase toxicity. So the challenge was to reduce the concentration enough to allow for survival without allowing intracellular ice to form and kill the cell. It is likely that the cells did vitrify, however, many died or were dead upon rewarming.

Modifications in media composition and reduction of cryoprotectant concentration led to some improvement of survival but the big breakthrough came when very rapid cooling rates were used (25). Vajta found that by using minute volumes of 1ul or less along with direct submersion into LN_2, using an open container allowed for extremely rapid cooling rates on the order of >10,000°C/min. They found that despite using high cryoprotectant concentrations and relatively high temperatures of 23°C or higher, the extremely rapid cooling rate did not allow enough time for ice to form, even if it could. Longer times in the solutions and slower cooling rates proved detrimental to survival, possibly because of cytotoxicity (42). This novel approach worked well for animal and human embryos and led to a flurry of new vitrification research based on Vajta's model.

From 1998 until the present there have been hundreds of publications on human blastocyst, embryo and oocyte vitrification. Despite posing a greater risk from the potential toxicity of the highly concentrated cryoprotectants and the relatively high exposure temperature, rapid-cooling, in most instances, has met with greater success (42, 43). The cryoprotectant solutions most often used consist of an equilibration solution of 7.5% DMSO and 7.5% Ethylene Glycol and a final vitrification solution of twice that concentration (44). To combat the potentially cytotoxic effects, exposure to the final vitrification solution is usually limited to around 45-90 seconds or less before plunging (45-50). Also, as mentioned above, in order to obtain a rapid cooling rate in the order of >10,000°C/min it is necessary to use very small volumes of media, usually 1ul or less and direct exposure to LN_2. Since most clinics used 0.25cc plastic straws for slow-cooling, new storage devices that could hold very small volumes of media and that allowed for direct contact with LN_2 needed to be developed. Alternative storage containers including electron microscope grids, ultra small nylon loops, hemi-straws, and open pulled straws were tested (25, 51-59). In addition to these, over 15 new devices were developed including the hemi-straw, closed-pulled straw, cryo-top, cryo-

tip, cryo-pette, cryo-loc, cryo-leaf, rapid-i, HSV straw, etc. These devices generally fall into one of two categories; 1) micro-sized straws that could be heat-sealed, or a thin flat plastic blade with a handle. With the cryo-top device, for example, the cell(s) are placed on the end of the device and excess media is removed, leaving the cell(s) covered in a very thin film of media, before plunging directly into liquid nitrogen (44, 60). This allows for an extremely rapid cooling rate of over 20,000°C/min as shown in Table 1.

Table 1. Cooling rates for modern vitrification devices.

Device	Media (ul)	Freezing Rate
0.25cc straw	25ul	4460°C/min
Open-pulled straw	1.5ul	16,340°C/min
Cryo-Top	0.1ul	22,800°C/min
Cryo-Tip	<2ul	12,000°C/min

From Kuwayama et al. (60).

Similar vitrification devices to those in Table 1 allow cooling rates of >15,000°C/min and have resulted in high survival rates (44, 47, 49, 52, 54, 58, 61-67). In fact, the combination of cryoprotectants used in conjunction with very rapid cooling rates has allowed for these results, whereas slower cooling rates have yielded poor survival rates (68, 69). All of these devices, despite claims of "novel method for vitrification", all are based upon the same principle of ultra rapid cooling and warming rates. Some investigators have even gone as far as to use LN2 in a vacuum to create a "slush", increasing the cooling rate even more (65, 70, 71). All of these devices/methods are simply a modification of what Vajta described in 1998.

Potential Problems:

Despite the increase in survival and pregnancy rates, and the relative abundance of recent reports on vitrification, there are numerous potential shortcomings associated with these protocols that have prevented its widespread application and acceptance (72). To start with, viral contamination from direct contact to liquid nitrogen is a concern despite reports indicating that no such contamination has occurred to date (73-75) Other devices that are closed can be more appealing to use as they avoid direct contact with LN2. The cryo-tip, cryo-pette, closed pulled straw, and micro-secure are examples of closed devices (44, 76). Bielanski and Vajta discussed current concerns about the safety of using open containers for vitrification and reviewed the confirmed and theoretical hazards of these procedures in their 2009 manuscript (77). They also suggest methods to avoid these dangers when using current vitrification techniques/devices. Of primary importance is to use a vitrification method that is successful in your particular practice. Secondarily would be to use a secure closed system that would avoid potential contamination problems and that would follow good tissue practice regulations so that foreseeable changes in laboratory regulations would not prevent you from continuing to use that system.

Another drawback is that the technique of placing cells into a highly concentrated vitrification solution, loading them onto a minute container, and plunging into liquid nitrogen, all in less than 45-90 seconds remains technically challenging; and more

importantly, leaves little or no room for error. In a recent paper, (67) reported an impressive >98% oocyte survival rate after thawing, however, they also mentioned that it took their lab over 5 months training to obtain such rates and that operator skill was crucial to guarantee the proficiency of the procedure. Because results are often based upon the technical skill of the person doing the vitrification procedure adaptability and consistency can be poor. Failed experiments or studies with low success rates are rarely, if ever published, thus giving a false impression of overall success rates. Despite these problems, vitrification has led to a marked improvement in blastocyst survival and higher pregnancy rates for many clinics (22, 78). Edgar and Gook recently published an extensive review of the literature comparing overall success rates with slow-cooling vs. rapid-cooling/vitrification (78).

Large-volume Vitrification: A Different Method:

As mentioned above, current vitrification methods use a combination of ethylene glycol and DMSO as cryoprotectants and a small container or device to store the cells. Although there are 20 or more vitrification devices available (hemi-straw, cryo-top, cryo-tip, cryo-loc, cryo-loop, etc...), they all basically function the same; they allow for ultra-rapid cooling and warming of the sample. Besides this approach there is another, very different vitrification system that has met with success and is becoming more popular. This is the large-volume vitrification system.

Large volume vitrification is a somewhat different technique based on the basic cryopreservation principles described above. The use of a large container, a 0.25cc straw for example, that is sterile, that can be loaded and sealed easily in a timely manner, and that uses a significantly slower cooling rate of <2000°C/min, contradicts the idea that a faster cooling rate is better for vitrification. However, slow-cooling is also vitrification, and the cooling rate is very slow, relative to the rates achieved with current rapid-cooling protocols using micro-volume devices. Hence, we have known for decades that you do not need a fast cooling rate to achieve vitrification.

Almost 10 years ago a different method of vitrification using conventional 0.25cc or 0.5cc straws with relatively slow cooling rates was developed (79). This method formerly called S^3 vitrification allows the use of large volumes of media, no DMSO, and sterile, sealable straws to vitrify eggs and embryos (79, 80). This system falls somewhere between slow-cooling (equilibrium freezing) and rapid-cooling (kinetic vitrification) methodology. This method may represent an intermediate closer to equilibrium vitrification; whereby the cell equilibrates with the surrounding solution so that ice cannot form no matter how slow the cooling rate; than kinetic vitrification; whereby the cooling rate is quick enough that ice does not form crystals even though it could, as described above.

The idea of using a larger container for vitrification is not new. Indeed, others have tried to vitrify blastocysts in 0.25cc straws using the conventional EG/DMSO media. Vanderwalzerman acheived 59% survival for all blastocysts vitrified, with only 34% that cleaved further. (68). Escriba et al. (69), had a bit more success with a 62% survival rate. These are among the few studies in the literature where investigators have tried and had, at least, some success vitrifying human embryos from room temperature, using a sealed 0.25cc straw. The paucity of reports on vitrification prior to the mid 1990's suggest that either few investigators were studying vitrification and/or that

success rates were low; only 34 reports were found in the PubMed database up to 1995. By contrast, 33 reports were cited in the PubMed database the 2 years following Vajta's 1998 OPS manuscript, and over 100 from 2000 to 2005.

Based upon the work of Luyet, Whittingham, Mazur, and company, Stachecki realized that slow-cooling allowed for vitrification, if the conditions were correct. Therefore, he concluded that plunging from higher temperatures was possible and ultra-rapid cooling rates were not necessary as long as the vitrification media used would allow for not only the cells to be vitrified, but for their survival upon rewarming. In order to reduce the potential toxicity of the solutions, Stachecki chose to avoid using DMSO and used ethylene glycol and glycerol instead as the major permeable cryoprotectants. This allowed for longer exposure times, which were needed to sufficiently dehydrate and load the cells with cryoprotectants. The time in the final vitrification solution ranged from 90 sec to 2 min and allowed for around 90% survival and >40% pregnancy rates among five different clinics (80). They further showed that even after 4 minutes in the final vitrification solution there was only a 33% decrease in cell survival, and that the solutions were not toxic enough to kill all the cells, despite the long exposure times. In his initial study Stachecki et al. (79) demonstrated further that rapid-cooling rates were not necessary for blastocyst vitrification by cooling them slowly at -100°C for 2 min prior to plunging and storage in LN2. Direct plunging from room temperature also worked well yet still acheived a cooling rate of near 2000°C/min, almost ten-fold slower than Vajta's OPS and other micro-volume devices (See Table 1). These studies demonstrated that despite what other investigators wrote and still write about what is necessary or beneficial to achieve high success rates of vitrified human blastocysts, this new media did not follow convention and other methods of vitrification are possible. This large-volume vitrification system allowed for 1) extended time for embryo equilibration, 2) a large volume container could be used because rapid cooling rates were not necessary, and 3) similar success rates to other rapid-cooling vitrification systems. Stachecki hypothesized that because the large-volume vitrification system did not rely on cooling rate, either rapid or slow cooling would work. This would mean that any micro-volume device such as the cryo-top, cryo-loc, rapid-i, cryo-pette, micro-secure, etc. could also work using this media. Schiewe confirmed this by using the micro-secure device to obtain high survival and pregnancy rates with Stachecki's vitrification media (76). Their current 2011 success rates for vitrifying blastocysts are over 96% with a 57% ongoing/delivery rate (Schiewe, personal communication). Currently there are several clinics successfully using micro-volume devices including the rapid-i, cryo-loc, micro-secure, and cryo-top with this media (unpublished results), demonstrating the versatility of this vitrification media.

Blastocoel Collapse:

As more and more people have used vitrification for storing blastocysts, more information became available along with trends on improving success with the technique. Among these was the problem with storing large expanded blastocysts. Stage-dependent success has also been shown with mouse embryos (34, 81-83), bovine embryos (84), sheep embryos (85) and equine embryos, (35).

Because of the larger amount of blastocoel fluid and the fact that the cryoprotectants had to penetrate through the trophectoderm cells to gain access to the inner cell mass

cells, and penetrate them as well, in a large enough concentration to ensure their survival, many investigators had poor success with these fully-expanded, later stage blastocysts (51, 55, 58, 68, 86). Vanderzwalmen in 2002 suggested that damage to expanded blastocysts seemed to result from ice in the blastocoel fluid and if removed, by collapsing the blastocyst, may result in greater survival. This seems logical as the blastocoel consists mainly of water and salts, similar to the extracellular solution. Therefore, a sufficient amount of cryoprotectant needs to permeate through the trophectoderm cells in order to protect the inner cell mass cells from freezing. All this would need to occur in the limited time frame for the procedure. Whether the damage is from ice forming in the blastocoel and then damaging the cells, which is possible, but unlikely, as ice forms extracellularly during slow-cooling and this does not seem to cause direct damage; or from a failure to provide enough cryoprotectants to the ICM is unknown. However, by collapsing the blastocoel by poking a hole with a needle or renting one by pipetting through a fine bore pipet, the cryoprotectants could gain direct access to inner cell mass cells through this hole. This hole would then reseal/heal in due time without significant negative effect to the blastocyst. Vanderzwalman first demonstrated a stage-dependent effect on human blastocyst survival, with the larger expanded blastocysts failing to survive well. And then showed a marked improvement in survival, by collapsing the blastocoel prior to vitrification (68). This proved a relatively simple method to improve the survival and viability of larger blastocysts. By contrast, Stachecki showed that, using his vitrification media, there was no need to collapse the blastocoel, as most all cells were protected from lysis (79). To demonstrate this they vitrified both human and bovine expanded blastocysts and used a vital (live/dead) stain following warming. Bovine blastocysts are a magnitude larger than human blastocysts with significantly more cells both in the trophectoderm and ICM. Therefore, Stachecki proposed that it would be even more difficult for the ICM's of bovine expanded blastocysts to be protected, at least compared to human blastocysts. They found that the majority of the cells were intact after rewarming in both the human and bovine blastocysts. In numerous instances, all of the cells were intact in the bovine blastocysts. This, together with recent unpublished results by Stachecki that demonstrate high survival rates of hatched human blastocysts, help reinforce the theory that not all vitrification media works the same, and that at least for expanded blastocysts, collapsing the blastocoel is not necessary depending on what vitrification media is used (79, 80).

Day 5 vs. Day 6 Vitrification:

On a similar topic, there are numerous reports that Day 5 blastocysts survive and/or implant and develop in vivo better than Day 6 blastocysts (58, 87-89). There are a number of possible reasons for this. First, is that D6 blastocysts are larger and thus do not vitrify as well unless collapsed (see above). The other has to do with the definition of what is a D6 blastocyst. A normally developing D6 blastocyst should be fully expanded and hatching or hatched, but a slowly developing blastocyst that is very small on D5 would only be expanding by D6. These slowly developing blastocysts may not freeze as well as a "normal" blastocyst. Liebermann, et al. (88) found no differences between similarly graded D5 and D6 blastocysts, with both obtaining over 95% survival. Hiraoka et al. (64) also found excellent survival and pregnancy rates between D5 and

D6 blastocysts when they were collapsed. It is possible that synchronization of the endometrium is better when transferring on D5 vs. D6 (90) and this could be a confounding reason why slower developing D6 blastocysts, in some clinics, do not do as well as D5. In Stachecki's large volume vitrification media, D6 hatching or fully hatched blastocysts survive vitrification as well or better than D5 blastocysts (unpublished observations). With the different media formulation, it seems that the more well-developed blastocysts with nicely defined trophoctoderm and ICM cells survive at a slightly higher rate. Whether this difference is significant is yet to be determined, although the trend among clinics utilizing this system agree that the more expanded blastocysts seem to survive and implant at a higher rate.

Embryo Biopsy and Vitrification:

The first birth after human blastocyst vitrification of biopsied embryos for PGD was reported by Parriego et al. (91). Since then several studies have demonstrated the success of storing biopsied embryos developing to the blastocyst stage. Van Landuyt et al reviewed the success of embryos biopsied on D3 and then vitrified at the blastocyst stage (92). They concluded that in numerous investigations the results were positive and that vitrification was a feasible option. Zhang et al. (93) found that biopsied blastocysts actually survived and developed better than control non-biopsied embryos. They concluded that vitrification of biopsied blastocysts was successful. Keskintepe et al. (94)showed that D3 biopsied blastocysts survived and implanted at a higher rate when vitrified versus being slow-cooled. They show that vitrified biopsied embryos had a pregnancy rate similar to non-biopsied vitrified blastocysts.

Biopsy at the blastocyst stage is becoming more popular as more genetic information is afforded (95). Agca et al. (96) determined post-thaw survival and pregnancy rates of IVP bovine blastocyst that had previously undergone blastomere biopsy for gender determination. They compared post-thaw survival of blastocyst following slow freezing and vitrification and found that survival and pregnancy rates were higher in the vitrified group and that the pregnancy rate was acceptable. Several studies in the human have also showed very good survival and pregnancy outcomes with biopsied blastocysts that were vitrified (95, 97-100).

Conclusion:

Blastocyst culture has become more routine because it has been shown to increase overall pregnancy rates while allowing for improved selection of potentially viable embryos. Furthermore, a reduced number of embryos need to be transferred, resulting in a decrease in high-order multiple pregnancies. With these advances it has become important to develop a successful method of blastocyst storage. Blastocyst cryopreservation has seen a switch from slow-cooling to rapid-cooling or vitrification because of its simplicity and efficiency. Several techniques for blastocyst vitrification have been recently developed and are in use gloablly. Most use DMSO as a permeable cryoprotectant and rely on rapid cooling and warming rates for success. These systems utilize micro-volume containers (cryo-top, cryo-loc, cryo-loop, etc.) to achieve these high cooling rates. The other system, a large-volume vitrification system, does not rely on rapid cooling rates for success, and therefore larger, closed containers, such as a conventional 0.25cc straw can be used. Both systems, although very different, have

reported high survival and pregnancy rates. With the onset of these recent advances in blastocyst cryopreservation, many clinics have demonstrated increased implantation and pregnancy rates per thawed embryo transferred. Being more developed and having a higher cell number, blastocysts, can obtain a greater overall viability following cryopreservation (88). Genetic testing at the blastocyst stage can afford more information by providing more cells for analysis, resulting in better embryo selection potential. Even though, on average, fewer embryos per patient are available to be stored at this late stage, the blastocysts that are cryopreserved, show greater potential for survival and implantation after being thawed, as compared to earlier stage embryos. For all these reasons, a successful blastocyst cryopreservation program has become increasingly relevant for the modern fertility clinic.

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