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Vitrification of Oocytes and Embryos

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1. Introduction

Currently, controlled ovarian hyperstimulation protocols commonly provide embryos in excess of those needed for fresh transfer. Therefore, techniques have been developed to store these surplus embryos in liquid nitrogen (referred to as cryopreservation) for an indefinite period of time without significant compromise of their quality. Based on data from the Centers for Disease Control and Prevention (CDC) from 2001 to 2004, about 18% of all IVF cycles in the USA used frozen embryos for transfer. In addition, data from the same registry compared live births per transfer using frozen and fresh embryos (25% versus 34% respectively) clearly showing that cryopreservation is an important adjunct to maximize the efficiency of every single patient's oocyte retrieval. The fundamental objectives for successful cryostorage of cells in liquid nitrogen at -196°C can be summarized as follows: **1)** arresting the metabolism reversibly, **2)** maintaining structural and genetic integrity, **3)** achieving acceptable survival rates after thawing, **4)** maintain of developmental competence post thaw and, **5)** the technique has to be reliable and repeatable.

Furthermore, all methods and protocols for cryopreservation should be developed such that ice crystals formation and growth inside the cells or tissues must either be eliminated or massively suppressed. One recent hotly debated topic in the area of reproductive cryobiology is whether slow-cooling or rapid-cooling protocols both satisfy the fundamental cryo-biological principles for reduction of damage by ice crystal formation during cooling and warming, and which approach is better. It is the case nonetheless, that both methods of cryopreservation of biological material include six principal steps: **1)** initial exposure to the cryoprotectant (intracellular water has to be removed by gradual dehydration, **2)** cooling (slow/rapid) to subzero temperatures (-196°C), **3)** storage at low temperature, **4)** thawing/warming by gradual rehydration, **5)** dilution and removal of the cryoprotectant agents and replacement of the cellular and intracellular fluid at precise rate and, **6)** recovery and return to a physiological environment.

Although initially reported in 1985 as a successful cryopreservation approach for mouse embryos, vitrification has taken a backseat in human assisted reproduction. However, the practical advantages of this cryopreservation method have more recently caught the attention of many ART laboratories as a feasible alternative to traditional slow freezing methods. Since 1985 more than 2,100 publications can be found referring to the topic of "vitrification", which is further evidence of the burgeoning growth of interest in this cryopreservation technology. One "drawback" considered by embryologists who are not

familiar with the vitrification technique, is the use of higher concentration of cryoprotectants, which does potentially mean that the vitrification solutions are more toxic than their counterpart solutions used for conventional slow freezing. However, with better understanding of the physical and biological principles of vitrification this has lead to numerous successful clinical applications of this technique within the field of assisted reproduction. As of today, all developmental stages of human embryos cultured in vitro have been successfully vitrified and warmed, with resulting offspring. Today, slow freezing technology still has the longest clinical track record, and greater 'comfort level' amongst embryologists. Nevertheless, vitrification with its increasing clinical application is showing a trend of greater consistency and better outcomes when compared to slow freezing technology. Therefore, when (not if) IVF programs overcome the fear of the 'unknown', and take on the challenge of the short learning curve with vitrification, then at that point vitrification will become the clinical standard for human embryo cryopreservation.

Cryopreservation at low temperature slows or totally prevents unwanted physical and chemical change. The major disadvantage to using low temperature cryostorage is that it can lead to the crystallization of water, and thereby this approach can create new and unwanted physical and chemical events that may injure the cells that are being preserved. Although the results achieved by slow freezing in many cases seem quite successful (Gardner *et al*., 2003; Van den Abbeel *et al*., 2005), ice crystal formation still renders traditional slow-freezing programs generally less consistent in their clinical outcomes. Another downside to the slow freezing approach is the time to complete such freezing procedures for human embryos, which can range from 1.5 to 5hrs. This is due to the fact that the slow rate of cooling attempts to maintain a very delicate balance between multiple factors that may result in cellular damage by ice crystallization and osmotic toxicity. Traditionally slow-freeze embryo cryopreservation has been a positive contributor to cumulative patient pregnancy rates, but ultimately the limitations of current slow-rate freezing methods in ART have become more evident in the shootout with vitrification-based cryostorage.

Vitrification is one of the more exciting developments in ART in recent years that attempts to avoid ice formation altogether during the cooling process by establishing a glassy or vitreous state rather than an ice crystalline state, wherein molecular translational motions are arrested without structural reorganisation of the liquid in which the reproductive cells are suspended. To achieve this glass-like solidification of living cells for cryostorage, high cooling rates in combination with high concentrations of cryoprotectants are used. A primary strategy for vitrifying cells and tissue is to increase the speed of thermal conductivity, while decreasing the concentration of the vitrificants to reduce their potential toxicity. There are two main ways to achieve the vitrification of water inside cells efficiently: **a)** to increase the cooling rate by using special carriers that allow very small volume sizes containing the cells to be very rapidly cooled; and **b)** to find materials with rapid heat transfer. However, one has to take into account that every cell seems to require its own optimal cooling rate, e.g., mature unfertilized oocytes are much more sensitive to chilling injury than any of the cell stages of the pre-implantation embryo.The earliest attempts using vitrification as an ice-free cryopreservation method for embryos were first reported in 1985 (Rall & Fahy, 1985). In 1993 successful vitrification of mouse embryos was demonstrated (Ali & Shelton, 1993). Furthermore, bovine oocytes and cleavage-stages were vitrified and

warmed successfully a few years later (Vajta *et al*. 1998). In 1999 and 2000 successful pregnancies and deliveries after vitrification and warming of human oocytes were reported (Kuleshova *et al*., 1999; Yoon *et al*., 2000). Since that time, and because it seems to be that both entities appear to be especially chill-sensitive cells in ART, oocytes and blastocysts seem to receive a potentially significant boost in survival rates by avoiding ice-crystallization using vitrification (Walker *et al*., 2004). In general, vitrification solutions are aqueous cryoprotectant solutions that do not freeze when cooled at high cooling rates to very low temperature. Interest in vitrification has clearly risen as evinced by the almost exponential growth of scientific publications about vitrification. 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 (refered also as "minimal volume approach") that must be cooled at extreme rates not obtainable in traditional enclosed cryo-storage devices such as straws and vials. The importance of the use of a small volume, also referred to „minimal volume approach" was described and published in 2005 (Kuwayama *et al.,* 2005; Kuwayama, 2007). In general, the rate of cooling/warming and the concentration of the cryoprotectant required to achieve vitrification are in inversely related. In addition, recent publications have shown the dominance of warming rate over cooling rates in the survival of oocytes subjected to a vitrification procedure (Serki & Mazur, 2009; Mazur & Seki, 2011).

During vitrification, by using a cooling rate in the range of 2,500 to 30,000^{o}C/min or greater, water is transformed directly from the liquid phase to a glassy vitrified state. The physical definition of vitrification is the solidification of a solution at low temperature, not by ice-crystallization but by extreme elevation in viscosity during cooling (Fahy *et al*., 1984; Fahy 1986). Vitrification of the aqueous solution inside cells can be achieved by increasing the speed of temperature change, and by increasing the concentration of the cryoprotectant used. However, a major potential drawback of vitrification is the use of high concentration of cryoprotectant, and an unintentional negative impact of these cryoprotectants in turn can be their toxicity, which may affect the embryo and subsequent development in utero. It is therefore essential to achieve a fine balance between the speed of cooling and the concentration of the vitrifying cryoprotectants. This is necessitated by the practical limit for the rate of cooling, and the biological limit of tolerance of the cells for the concentration of toxic cryoprotectants being used to achieve the cryopreserved state. It is important to note that recently published papers (Takahashi *et al*., 2005; Liebermann & Tucker, 2006; Liebermann, 2009, 2011) have shown that the use of relatively high concentration of cryoprotectants such as 15% (vol/vol) ethylene glycol (EG) used in an equimolar mixture with dimethyl sulphoxide (DMSO) had no negative effect on the perinatal outcomes from blastocyst transfers following vitrification when compared with those from fresh blastocyst transfers.

Vitrification in principle is a simple technology, that is potentially faster to apply, and relatively inexpensive; furthermore, it is becoming clinically established, and is seemingly more reliable and consistent than conventional cryopreservation when carried out appropriately (Tucker *et al*., 2003; Liebermann & Tucker, 2004).

Cryoprotectant agents are essential for the cryopreservation of cells. Basically two groups of cryoprotectants exist: **1)** permeating (*glycerol, ethylene glycol, dimethyl sulphoxide*); and **2)** non-permeating (*saccharides, protein, polymers*) agents. The essential component of a vitrification

solution is the permeating agent. These compounds are hydrophilic non-electrolytes with a strong dehydrating effect. Furthermore, these CPAs are able to depress the "freezing point" of the solution. Regarding the high concentration of cryoprotectant used for vitrification, and in view of the known biological and physiochemical effects of cryoprotectants, it is suggested that the toxicity of these agents is a key limiting factor in cryobiology. Not only does this toxicity prevent the use of fully protective levels of these additives, but it may also be manifested in the form of cryo-injury above and beyond that seen occurring due to classical causes of cell damage (osmotic toxicity and ice formation) during cryopreservation. In spite of this, the permeating CPA should be chosen firstly by their permeating property, and secondly on the basis of their potential toxicity. Because the permeating CPA is responsible for the toxicity (*the key limiting factor in cryobiology*), different cryoprotectants have been tested for their relative toxicity, and the results indicate that ethylene glycol (EG; MW 62.02) is the least toxic followed by glycerol. Additionally, these highly permeating cryoprotectants are also more likely to diffuse out of the cells rapidly and the cells regained their original volume more quickly upon warming, thus preventing osmotic injury. Therefore, the most common and accepted cryoprotectant for vitrification procedures is ethylene glycol (EG). Today EG is more commonly used in an equimolar mixture with DMSO. Often additives are added to the vitrification solution such as disaccharides. Disaccharides, for example sucrose, do not penetrate the cell membrane, but they help to draw out more water from cells by osmosis, and therefore lessen the exposure time of the cells to the toxic effects of the cryoprotectants. The non-permeating sucrose also acts as an osmotic buffer to reduce the osmotic shock that might otherwise result from the dilution of the cryoprotectant after cryostorage. In addition, permeating agents are able to compound with intracellular water and therefore water is very slowly removed from the cell. Hence the critical intracellular salt concentration is reached at a lower temperature. Removal of the cryoprotectant agent during warming can present a very real problem in terms of trying to reduce toxicity to the cells. Firstly, because of the toxicity of the vitrification solutions, quick dilution of them after warming is necessary; and secondly, during dilution water permeates more rapidly in to the cell than the cryoprotective additive diffuses out. As a consequence of the excess water inflow the cells are threatened by injury from osmotic swelling. In this situation the non-permeating sucrose acts as an osmotic buffer to reduce the osmotic shock. During warming using a high extracellular concentration of sucrose (e.g., 1.0M) counterbalances the high concentration of the cryoprotectant agents in the cell, as it reduces the difference in osmolarity between the intra- and extracellular compartments. The high sucrose concentration cannot totally prevent the cell from swelling, but it can reduce the speed and magnitude of swelling (Liebermann and Tucker, 2002; Liebermann *et al*., 2002a; 2003).

2. Oocytes

The cryopreservation of human oocytes constitutes a important step forward in Assisted Reproductive Technology (ART) despite the fact that for more than 2 decades oocyte cryopreservation has long been the focus of unsuccessful efforts to perfect its clinical application. More recently, vitrification as an alternative to traditional slow freezing prootcols has been shown to provide high degrees of success in vitrified metaphase-II

human oocytes. Although oocyte cryopreservation historically has low efficiency mainly because of low rates of survival, fertilization, and cleavage, data on ~2000 "frozen oocyte" babies born worldwide since 1986 exists. The question arises as to what makes oocytes so unique compared to embryos, besides differences in cell size and membrane permeability? Oocytes have a low volume-to-surface ratio; hence they are less efficient at taking up cryoprotectant and at loosing water. Other differences to be considered are **a)** that the maternal DNA is held suspended in the cytoplasm on the meiotic spindle & not within the protective confines of the nuclear membrane, therefore damage in the DNA and microtubules could explain the limited success of oocytes, **b)** the oocyte is arrested in a state primed for activation, and **c)** the changes in its environment can cause parthenogenetic activation. What are the applications then for oocyte cryopreservation in the US? One application would be to preserve fertility in women with malignant/premalignant conditions who would have to undergo treatment that might negatively impact their future ability to have children (50,000 per year <40 yr old), also in women who may want to delay childbearing ('clock-tickers') because of their careers, partnership status or psychological/ emotional reasons. A very interesting approach is donor oocyte banking, which makes the donor-recipient cycle more convenient by facilitating the "egg donation" and allows quarantining of the oocytes, which provides a unique advantage in economy as well as feasibility. Other applications are if a male is unable to produce a semen sample on the day of egg retrieval and or it could also eliminate ethical/moral questions of producing extra embryos. Overall, oocyte cryostorage offers an opportunity to reduce number of embryos generated per IVF cycle, and therefore lessening the pressure on the patient to increase the number of fresh embryos transferred. In addition, while also reducing embryo cryostorage it has the benefit of helping women "retain ownership" of their ability to be genetic parents at a time of their choosing, a time of greater convenience & health. The live born babies from cryopreserved oocytes have shown no apparent increase in congenital anomalies. Although 13 years later after the first slow-freeze birth, the number of reported babies born as a result of vitrified oocytes is now approaching that of slow-frozen oocytes without any increasing risk in congenital abnormalities (Noyes *et al.,* 2009). Vitrification of oocytes does not appear to increase risks of abnormal imprinting or disturbances in spindle formation or chromosome segregation (Trapphoff *et al.,* 2010). It has the greatest potential for successful oocyte cryopreservation and with its increased clinical application is showing a trend to greater consistency and better outcomes (similar to outcomes between fresh or warmed oocytes). Vitrification of oocytes, when applied to properly screened patients, will be a useful technology in reproductive medicine practice and will constitute a major step forward in ART.

Fortunately to date, no significant increase in abnormalities has been reported from these cryostored oocyte pregnancies (Chian *et al.,* 2009), regardless of the historical concerns that cryopreservation of mature oocytes might disrupt the meiotic spindle and thus increase the potential for aneuploidy in the embryos arising from such eggs. These concerns have mostly been allayed by publications that show no abnormal or stray chromosomes from previously frozen oocytes (Gook & Edgar, 1999), and FISH comparison of embryos from fresh and thawed oocytes show no increase in anomalies (Cobo *et al*., 2001). There also appears to be adequate recovery of the meiotic spindle post-cryopreservation whether using conventional

or vitrification technology (Chen *et al*., 2004; Bianchi *et al.,* 2005; Larman *et al*., 2007). The scientific literature on oocyte cryopreservation grows daily it seems. Most reports focus on clinical pregnancy rates (Boldt *et al*., 2003; Boldt *et al*., 2006), and as such while this data is helpful to increase our confidence in the technology, it does little to research new directions for oocyte cryopreservation.

3. Zygotes

Conventional cryopreservation of pronuclear zygotes (2PN) is well established in countries such as Germany where freezing of later stage human embryos is by law or by ethical reasons not allowed. The time to complete the conventional protocol to cryopreserved zygotes is 98min. In Germany the clinical pregnancy outcomes arising from the frozen/thawed 2PN cycles is about 18%, with an implantation of around 10% per embryo transferred. The time to complete vitrification of zygotes requires approximately 12min. Recently successful vitrification of 2PN with high survival (~ 90%), cleavage rates on day-2 (>80%), and blastocyst formation of 31% and pregnancies were reported (Park *et al*., 2000; Jelinkova *et al*., 2002; Liebermann *et al*., 2002b; Al-Hasani *et. al*., 2007). Zygote vitrification implemented as a clinical setting can provide a clinical pregnancy rate of close to 30%, with an implantation rate of 17% (Al-Hasani *et al.,* 2007). The pronuclear stage appears well-able to withstand the vitrification and warming conditions, which is probably due to the significant membrane permeability changes that occur post-fertilization; such changes to the oolemma may also make it more stable and able to cope with the vagaries of the cold-shock and striking osmotic fluctuations that occur during the vitrification process.

4. Cleavage stage embryo

Reports of human embryo vitrification have been more frequent. Liebermann and Tucker (2002) using either the cryoloop or the hemi-straw system (HSS) showed post-warming survival rates (after 2 hours of culture of day-3 embryos where more than half of their blastomeres were intact) from 84 to 90% which was dependent on the carrier system used. There was a reasonable further cleavage and compaction rate of 34%. This finding supports previous reports in which high survival rates of eight-cell human embryos using 40% EG were documented (Mukaida *et al*., 1998). In comparison to traditional slow-rate cryopreservation, a survival rate of cleavage stage embryos of 76% was reported with vitrification (Jericho *et al*., 2003). Recently reported successful pregnancies and deliveries after vitrification of day-3 human embryos using the OPS have been reported (El-Danasouri and Selman, 2001; Selman and El-Danasouri, 2002). Their results showed a negative correlation between stage of development and survival, eight-cell embryos showed a higher survival rate (79.2%; 62/78) than did embryos with fewer than six cells (21.1%; 11/53) after vitrification (El-Danasouri and Selman, 2001). Despite the fact, that Liebermann and Tucker (2002) achieved a promising post-warming survival rate, overall only about 34% of the surviving embryos had the developmental potential to reach the compaction stage. Recently publications on cleavage stage vitrification provided good outcome data. Loutradi *et al.* (2008) were performing a meta-analysis and systematic review by comparing traditional and vitrification protocols for cleavage stage embryos, and found a survival rate of 84.0% versus 97.0%. In addition, clinical pregnancy rates between 35 and 48%, with implantation rates between 15 to 39% have been reported (Rama Raju *et al*., 2005; Desai *et al*., 2007; Li *et al*.,

2007; Balaban *et al*., 2008). So clearly vitrification appears to have a positive impact on overall embryo utilization. A study on the neonatal outcome of 907 vitrified/warmed cleavage stage embryos found no significant increase in the congenital birth defect rate when compared with pregnancies using fresh cleavage stage embryos (Rama Raju *et al*., 2009).

5. Blastocyst stage

Vitrification of human blastocysts using different carriers shows survival rates of 70% to 90%, with clinical pregnancy rates of 37% to 53% and implantation rates of 20% to 30% ((Yokota *et al*., 2000, 2001; Reed *et al*., 2002; Mukaida *et al*., 2001; 2003; Hiraoka *et al*., 2004; Vanderzwalmen *et al*., 2002; 2003; Huang *et al*., 2005; Liebermann & Tucker, 2006; Liebermann, 2009, 2011).

6. The advantage of blastocyst cryopreservation

Activation of the embryonic genome occurs after the 8-cell stage (3 days postoocyte retrieval) is reached (Braude *et al*., 1988). If the activation does not occur, the embryo will not survive further. Therefore, the improvement of human IVF outcomes requires identification of embryos that will progress beyond the 8-cell stage. Blastocyst culture (5 days postoocyte retrieval) allows for the transfer of embryos that clearly have an activated embryonic genome. This requires that the elimination of embryos in extended culture from day 3 to day 5 should depend solely on their inherited survival potential and not be a consequence of an adverse effect exerted by the sequential media used for culture beyond day 3. Additional advantages in cryopreserving at the blastocyst stage are: 1) At this stage a lower numbers of embryos can be transferred in fresh cycles, resulting in less high order multiple pregnancies, 2) The same is true for cryopreserved blastocysts showing higher pregnancy rates and implantation per thawed embryo transferred, 3) Approximately 120 hours (day five) into development the healthy human embryo should be at the blastocyst stage comprised of some 50 to 150 cells, of which about 20 to 30% make up the inner cell mass (ICM), the remainder making up the trophectoderm (TE), 4) the higher cell number allows better compensation for cryo-injuries, which results in greater viability and faster recovery, 5) the cytoplasmatic volume of the cells is lower, thus the surface-volume ratio is higher, and that in turn makes the penetration of the cryoprotectant faster, and 6) on average fewer embryos per patient were frozen-stored, but each one when thawed has a greater potential for implantation.

Both natural and hormone replacement cycles seem to provide comparable levels of receptivity in naturally cycling women, though they differ in level of convenience. Regardless of the day of cryopreservation of the embryo (whether day 5, 6 or 7), at thawing/warming blastocysts should be treated as if they had been frozen on the fifth day of development. Vitrification of blastocysts has been undertaken utilizing an "open system" (Cryotop; Kitazato Bio Pharma Co. Ltd., Fuji-shi, Japan), and since 2007 on a "closed system" (HSV [High Security Vitrification Kit]; CryoBio System, L'Aigle, France) after a two-step loading with cryoprotectant agents at 24°C. Briefly, blastocysts were placed in equilibration solution, which is the base medium (Hepes-buffered HTF with 20% Serum Supplement Substition (SSS) containing 7.5% (v/v) ethylene glycol (EG) and 7.5% (v/v) dimethyl sulfoxide (DMSO). After 5-7 min, the blastocysts were washed quickly in vitrification solution, which is the base medium containing 15% (v/v) DMSO, 15% (v/v) EG, and 0.5M sucrose, for 45-60sec and transferred onto the Cryotop or HSV using a micropipette. Immediately after the loading of

not more than two blastocysts in a 1µl drop on the Cryotop, the carrier was plunged into fresh clean liquid nitrogen (LN2). After loading the embryos, the Cryotop was capped under the LN2 to seal and protect the vitrified material prior to cryo storage. In contrast, after loading the HSV, the straw was heat sealed and then plunged in LN2, and stored the same way as the cryotop (Liebermann & Tucker, 2006; Liebermann, 2009, 2011).

To remove the cryoprotectants, blastocysts were warmed and diluted in a two step process. With the Cryotop or HSV submerged in LN2, the protective cap (Cryotop) or inner straw (HSV) were removed, and then both carriers with the blastocysts were removed from the LN2 and placed directly into a pre-warmed (~35-37°C) organ culture dish containing 1ml of 1.0M sucrose. Blastocysts were picked up directly from the Cryotop and placed in a fresh drop of 1.0M sucrose at 24°C. After 5min blastocysts were transferred to 0.5M sucrose solution. After an additional 5min, blastocysts were washed in the base medium and returned to the culture medium (SAGE Blastocyst Medium, Trumbull, CT, USA) until transfer.

Between January 2004 and July 2011 the *Fertility Centers of Illinois* "IVF Laboratory River North"(Chicago) has vitrified 13,568 blastocysts *without artificial shrinkage* before the cryopreservation procedure (Table 1). After 2562 frozen embryo transfers (FET) including day 5 and day 6 blastocysts with a mean age of the patients of 34.9 ± 5.1 years, to date we have seen a survival rate, implantation, and clinical pregnancy rate per transfer (cPR) of 97.3%, 30.2%, and 42.5%, respectively (Table 2). After 7 1/2years of vitrifying blastocysts the perinatal outcome is as follow: from 687 deliveries with vitrified blastocysts, 852 babies (422 boys and 430 girls) were born (Table 2). No abnormalities were recorded. The singleton, twin and triplet pregnancy rates were 71%, 27%, and 2%, respectively.

Day of Development	**Day 5**	**Day 6**	**Day 7**	**Total**
Number of Blastocysts vitrified (%)	6220 (46%)	6988 (51%)	360 (3%)	13568

Table 1. Retrospective data from 3,712 patients (average age 33.8±4.9) with blastocyst cryopreservation by vitrification from January 2004 till July 2011.

Technique	**VIT**
Patient's age (y)	34.9 ± 5.1
No. of warmed cycles	2580
No. of transfers	2562
No. of blastocysts warmed	4965
No. of blastocysts survived (%)	4829 (97.3)
No. of blastocysts transferred	4752
Mean no. of blastocysts transferred	1.8
No. of implantations (%)	1432 (30.2)
No. of positive pregnancy/warm (%)	1255 (48.6)
No. of positive pregnancy/VET (%)	1255 (49.0)
No. of clinical pregnancy/warm (%)	1089 (42.2)
No. of clinical pregnancy/VET (%)	1089 (42.5)
Ongoing pregnancies/VET (%)	875 (34.2)
No. of livebirths	852 (422 boys & 430 girls)

Table 2. Retrospective data from the blastocyst cryopreservation program (Fertility Centers of Illinois, Chicago) where vitrification (VIT) technology was applied from January 2004 till July 2011.

When the vitrified-warmed blastocysts were divided into day 5 and day 6 groups, the following data was gather (Table 3 & 4). In 1265 FETs transferring day 5 blastocysts, the survival, implantation, and cPR were 97.6%, 34.8%, and 48.3% compared to 97.2%, 25.3%, and 36.5% of in 1204 day 6 FETs.

Patient's Age	< 35	35-37	38-40	> 40	Donor	Total
Ø Age	30.8±2.6	35.8±0.8	38.8±0.8	42.8±2.0	43.5±4.7	34.6±5.2
Day 5 Cycles	678	248	157	74	112	1269
Day 5 Transfers	677	247	155	74	112	1265
Embryos survived (%)	97.5%	96.9%	98.7%	96.0%	97.7%	97.6
Embryos transferred (MEAN)	1.9	1.8	1.9	1.9	1.8	1.9
Positive Pregnancies/Transfer	56.1%	56.7%	56.1%	51.4%	54.5%	55.8%
Clinical Pregnancies/Transfer	50%	49%	43%	43%	50%	48.3%
Ongoing Pregnancies/Transfer	43%	37%	30%	30%	39%	38.8%
# Sacs	478	152	87	42	69	828
Implantation Rate	37.3%	33.3%	29.7%	29.2%	33.7%	34.8%

Table 3. Retrospective outcome data at FCI from vitrified day 5 blastocysts in regards to the patients age between June 2007 till July 2011.

Patient's Age	< 35	35-37	38-40	> 40	Donor	Total
Ø Age	31.0±2.4	36.0±0.8	38.8±0.8	42.5±1.8	43.9±4.9	35.1±5.0
Day 5 Cycles	586	271	177	103	83	1220
Day 5 Transfers	579	266	176	101	82	1204
Embryos survived (%)	96.7%	97.8%	97.9%	95.2%	99.4%	97.2%
Embryos transferred (MEAN)	1.8	1.8	1.8	1.7	1.8	1.8
Positive Pregnancies/Transfer	43.0%	43.6%	39.2%	31.1%	50.0%	42.1%
Clinical Pregnancies/Transfer	37%	38%	35%	29%	42%	36.5%
Ongoing Pregnancies/Transfer	31%	30%	27%	20%	32%	29.2%
# Sacs	276	128	80	34	41	559
Implantation Rate	25.5%	26.5%	24.8%	19.0%	28.1%	25.3%

Table 4. Retrospective outcome data at FCI from vitrified day 6 blastocysts blastocyst in regards to the patients age between June 2007 till July 2011.

In addition, in 1128 FET using aseptic vitrification, 2041 blastocysts were transferred with a survival, implantation, and cPR of 98.4%, 31.8%, and 44.2%, respectively (Table 5). After 4 1/2 years of vitrifying blastocysts using a closed system the perinatal outcome is as follow: 313 babies (165 boys and 148 girls) were born (Table 5). No abnormalities were recorded.

Technique	**aVIT**
Patient's age (y)	34.5 ± 5.0
No. of warmed cycles	1132
No. of transfers	1128
No. of blastocysts warmed	2102
No. of blastocysts survived (%)	2069 (98.4)
No. of blastocysts transferred	2041
Mean no. of blastocysts transferred	1.8
No. of implantations (%)	650 (31.8)
No. of positive pregnancy/warm (%)	572 (50.7)
No. of positive pregnancy/VET (%)	572 (53.9)
No. of clinical pregnancy/warm (%)	499 (44.1)
No. of clinical pregnancy/VET (%)	499 (44.2)
Ongoing pregnancies/VET (%)	423 (37.5)
No. of livebirths	313 (165 boys & 148 girls)

Table 5. Retrospective data from the blastocyst cryopreservation program (*Fertility Centers of Illinois, Chicago*) where aseptic vitrification (aVIT) technology was applied from June 2007 till July 2011.

Our data has shown that freezing at the blastocyst stage provides excellent survival, implantation and clinical pregnancy (Liebermann & Tucker, 2006; Liebermann, 2009, 2011). To achieve this data the following points should be considered: a) without a successful blastocyst vitrification storage program, extended culture should never be attempted, b) the blastocyst is composed of more cells and therefore better able to compensate for cryo-injury, c) the cells are smaller thus making cryoprotectant penetration faster, and d) on average fewer embryos per patient are cryo-stored, but each one when thawed, has a greater potential for implantation, often with an opportunity for an ET with a single blastocyst.

Furthermore, a vitrification solution with a mixture of 7.5% EG/DMSO, followed by a 15% EG/DMSO with 0.5M sucrose step is safe for clinical use, giving rise to healthy babies without abnormalities. Vitrification of blastocysts using and open or closed system (Cryotop or HSV) is effective for achieving high implantation and pregnancy rates as seen in fresh embryo transfers. Although the outcome in terms of implantation and clinical pregnancy is significantly different when comparing day 5 blastocyst to day 6 blastocysts, our data should encourage cryopreservation of day 6 blastocysts as well. Based on the data presented, it is clear that the vitrification of Day 6 blastocysts is of clinical value since it can result in live births. This observation is confirmed by Saphiro *et al.* (2001) and Levens *et al*. (2008); they found that blastocyst development rate impacts outcome in slow cryopreserved blastocyst transfer cycles.

In conclusion, vitrification of human blastocysts is a viable and feasible alternative to traditional slow freezing methods. The key to this success lies in the more optimal timing of embryo cryopreservation, e.g. individual blastocysts may be cryopreserved at their optimal stage of development and expansion. In addition, the repeatedly discussed topic of using open systems (direct contact between cells and LN2) and the possible danger of contamination by bacteria, fungus or different strains of virus from LN2, can be avoided by moving forward to a closed system providing lower cooling rates, but without a negative impact on the outcome.

7. Contamination of LN2: Open versus closed systems

There are many potential advantages of vitrification in that it is an easy, cheap, fast and an apparently successful cryopreservation method; however, there is one issue that is still up for debate. It has been shown that fungi, bacteria and viruses are able to survive in liquid nitrogen (LN2) (Tedder *et al*, 1995; Fountain *et al,* 1997; Bielanski *et al,* 2000; 2003; Kyuwa *et al*, 2003; Letur-Konirsch *et al*, 2003). Given the direct exposure of the human cells as they are directly plunged into LN2 during the vitrification process, this therefore raises the question as to whether the LN2 has to be sterilized, as it may be a possible source of contamination for those cells. To this point there has been no fungal, viral or bacterial contamination that has been described from about 400 publications related to vitrification since the first report in 1985. Bielanski and colleagues (2000) demonstrated a viral transmission rate of 21 % to human embryos stored in open freezing containers under experimental conditions of extremely elevated viral presence; while in contrast all embryos stored in sealed freezing containers were free from contamination. Based on this observation they proposed that the sealing of freezing containers appears to prevent exposure to potential contaminants. Commercial systems to purify LN2 by filtration have been developed, however this technology to date has received little practical application in IVF laboratories that have active cryopreservation programs. While it is not totally clear that contamination is a real risk in everyday use of LN2, nevertheless it may be prudent to consider routine sterilization of LN2 when open carrier systems are used for vitrification, followed by a sealing of that system for cryo-storage. Further there are currently at least three 'closed' sealed vitrification systems that are commercially available, with FDA clearance, that represent successful alternatives to open systems for embryo vitrification (Liebermann, 2009, 2011)

8. Conclusions and future directions

Vitrification is a very promising cryopreservation method with many advantages, and an ever increasing clinical track record. A standardized vitrification protocol applicable to all stages of the pre-implantation embryo may not be realistic because of: **a)** different surface-to-volume ratios; **b)** differing cooling rate requirements between oocytes, zygotes, cleavage stage embryos and blastocysts; and **c)** variable chill-sensitivity between these different developmental stages. Currently however, the most widely used protocol applied to any embryo stage is the two-step equilibration in an equi-molar combination of the cryoprotectants ethylene glycol and DMSO, at a concentration of 15% each (v/v) supplemented with 0.5 mol/l sucrose.

For the adoption of vitrification in ART, as with all new technologies, there has been initial resistance; but as clinical data has been accrued, this technology is becoming more commonly adopted as standard procedure in many IVF programs worldwide. With this increased use in human assisted reproduction will come evolution of the vitrification process as it is fine tuned to clinical needs, so pushing forward its development to higher levels of clinical efficiency, utilization and universal acceptance.

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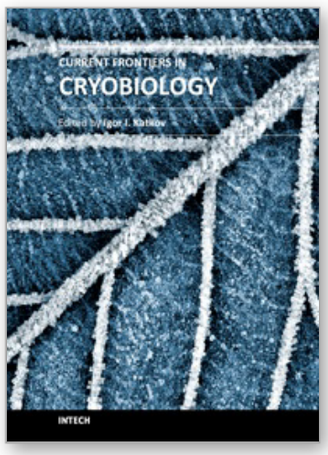

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Almost a decade has passed since the last textbook on the science of cryobiology, Life in the Frozen State, was published. Recently, there have been some serious tectonic shifts in cryobiology which were perhaps not seen on the surface but will have a profound effect on both the future of cryobiology and the development of new cryopreservation methods. We feel that it is time to revise the previous paradigms and dogmas, discuss the conceptually new cryobiological ideas, and introduce the recently emerged practical protocols for cryopreservation. The present books, "Current Frontiers in Cryobiology" and "Current Frontiers in Cryopreservation" will serve the purpose. This is a global effort by scientists from 27 countries from all continents and we hope it will be interesting to a wide audience.

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