S³ Vitrifcation System: A Novel Approach To Blastocyst Freezing

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Reducing multiple pregnancies is a concern of IVF clinics everywhere. Blastocyst transfer is the method of choice for the replacement of just a single embryo. As more clinics become proficient at culturing embryos to the blastocyst stage there is an increasing need to store extra blastocysts. Slow-cooling regimes have been around for over 30 years, and although thousands of babies have been produced throughout the world from this technique, there is room for improvement, especially when it comes to storing blastocysts. Modern, faster methods of cryopreservation, namely vitrification, are enticing because of their apparent ease, reduced procedure time, and published success rates. Rapid freezing has been the focus of research in recent years and, in several laboratories, is now the preferred method for storing human embryos.

The concept of vitrification, or achieving a glass-like state, is not new and was first described in 1860. Rall and Fahy showed that vitrification is a potential alternative to slow-cooling of embryos over 100 years later (Rall and Fahy, 1985). Since then it has been the topic of numerous publications in the IVF field (Ali, 2001; Antinori et al., 2007; Chen et al., 2000; Chung et al., 2000; Cremades et al., 2004; Hiraoka et al., 2004; Kasai and Mukaida, 2004; Kuleshova and Lopata, 2002; Liebermann and Tucker, 2002; Liebermann et al., 2003; Vanderzwalmen et al., 2003; Vanderzwalmen et al., 2002; Wininger and Kort, 2002; Wu et al., 2001; Yoon et al., 2003). Vitrification by rapid cooling proved the only effective method to store the cold-sensitive oocytes and embryos of pigs, cows, and sheep (Beeb et al., 2002; Fahning and Garcia, 1992; Szell et al., 1990; Vajta et al., 1998). Soon after these reports were published, the method of vitrification by rapid cooling was applied to human embryo storage. Many of the recent manuscripts clearly show improved results in terms of survival and clinical pregnancy rates, when using vitrification. However, current vitrification methods have potential problems.

In order to understand rapid-cooling or 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 permeable and non-permeable cryoprotectants (such as 1.5M Propanediol (PrOH) in conjunction with 0.2M sucrose), 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 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 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 (Luyet, 1970). Rather, this remaining liquid phase turns immediately into a glassy substance upon plunging into liquid nitrogen and solidifies without further crystal formation. The unfrozen liquid phase remaining within the cells should now ideally consist of this glassy substance with all original cell solutes remaining in solution (Luyet, 1970). 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 (Mazur, 1963), if intracellular ice formation were occurring. This correlates well with the theory that slow-cooling is vitrification. Now let's examine modern day

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rapid-cooling (vitrification). All of the rapid-cooling forms of vitrification procedures for human embryos described in the recent literature are, in principle, the same. They all involve exposure of oocytes or embryos to high concentrations of cryoprotectant(s) for brief periods of time at or near room temperature followed by loading onto or into a tiny container (cryo-loop, cryo-top, cryo-leaf, cryo-tip, etc.) that may or may not be sealed, then submerged directly into liquid nitrogen and stored. The high osmolarity of the vitrification solution rapidly dehydrates the cell, some of the cryoprotectant enters the cell and binds the remaining water, and submersion into liquid nitrogen quickly solidifies or vitrifies the cell so that any remaining intracellular water not bound by cryoprotectants, does not have time to form a lethal amount of ice crystals. The embryo is effectively vitrified without intracellular ice, similar to slow-cooling. From these descriptions, both techniques, although seemingly very different, have the same outcome of vitrifying the cell. Therefore, the term vitrification can be used to describe both slow-cooling or rapid-cooling, as long as the outcome is the formation of an amorphous glass-like solid.

Compared with slow-cooling, rapid cooling vitrification has allowed for improved survival and pregnancy rates. Reasons for this are numerous, despite the fact that both procedures vitrify the cell. The methods are different enough that, despite posing a greater risk from the potential toxicity of the highly concentrated cryoprotectants used and the relatively high exposure temperature, rapid-cooling has met with greater success in most instances (Hotamisligil et al., 1996; Mukaida et al., 1998). To combat the toxic effects of elevated cryoprotectant levels, exposure to the final vitrification solution is usually limited to around 45-90 seconds or less before plunging in liquid nitrogen (Chung et al., 2000; Hong et al., 1999; Hunter et al., 1995; Shaw et al., 1992; Wu et al., 2001; Yoon et al., 2003). Also, to promote faster solidification, minute amounts of vitrification media are used, usually under 2ul. For example, when using the cryo-top device, the cell(s) are placed on the tip and excess media is removed leaving the cell(s) covered in a very thin film of media before plunging directly into liquid nitrogen. 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
0.25cc straw (S^3 vit)[1]	10ul to 250ul	<100°C/min

From Kuwayama et al., (2005b).
[1]This study.

Similar vitrification devices to those in Table 1 allow cooling rates of >15,000°C/min and have resulted in high survival rates (Antinori et al., 2007; Cremades et al., 2004; Hiraoka et al., 2004; Hong et al., 1999; Huang et al., 2005; Isachenko et al., 2005; Kuwayama et al., 2005a; Lane and Gardner, 2001; Liebermann et al., 2003; Martino et al., 1996; Mukaida et al., 2003; Son et al., 2003; Wu et al., 2001). In fact, the combination of cryoprotectants used in conjunction with very rapid cooling rates has allowed for these results (Table 2), whereas slower cooling rates have yielded poor survival rates (Escriba et al., 2006; Vanderzwalmen et al., 2002).

Table 2. Published survival and pregnancy rates of vitrified human cells.

Study	Cell Type	n	Survival Rate (%)	Pregnancy Rate (%)
(Lane et al., 1999)	Blastocyst	18	83%	N/A
(Choi et al., 2000)	Blastocyst	93	51.6%	25%
(Cho et al., 2002)	Blastocyst	120	84.2%	34.1%
(Reed et al., 2002)	Blastocyst	15	100%	25%
(Vanderzwalmen et al., 2002)	Blastocyst	75	70.6%	22.9%
(Vanderzwalmen et al., 2003)	Blastocyst	186	78.5%	32.4%
(Mukaida et al., 2003)	Blastocyst	725	80.4%	37%
(Son et al., 2003)	Blastocyst	90	90	48%
(Cremades et al., 2004)	Blastocyst	33	82%	N/A
Teramoto et al., 2004 (abs)	Blastocyst	197	100	57.7%
(Hiraoka et al., 2004)	Blastocyst	49	98%	50%
(Huang et al., 2005)	Blastocyst	96	77.1%	53.8%
(Takahashi et al., 2005)	Blastocyst	1129	85.7%	44.1%
(Stehlik et al., 2005)	Blastocyst	41	100	50%
(Kuwayama et al., 2005a)	Blastocyst	6328	90	53%
(Liebermann and Tucker, 2006)	Blastocyst	547	96.5%	46.1%
(Stachecki et al., 2008)	Blastocyst	93	89	65%
(Isachenko et al., 2003)	Embryo	59	71	N/A
(Kuwayama et al., 2005a)	Embryo	5881	100%	44%
(Kuwayama et al., 2005a)	Embryo	897	98	32
(Sher et al., 2008)	Embryo	78	96%	63%
(Balaban et al., 2008)	Embryo	234	94.8%	49%
(Katayama et al., 2003)	Oocyte	46	94%	33.3%
(Kuwayama et al., 2005b)	Oocyte	64	91%	45.4%
(Selman et al., 2006)	Oocyte	24	75%	33%
(Lucena et al., 2006)	Oocyte	159	96.7%	56.5%
(Antinori et al., 2007)	Oocyte	330	99%	32.5%
(Cobo et al., 2008)	Oocyte	27	86.9%	N/A
Cao et al., 2008	Oocyte	292	91.8%	N/A

Despite the increase in survival and pregnancy rates, and relative abundance of recent reports on rapid-cooling vitrification (Table 2), there are numerous potential shortcomings associated with these protocols that have prevented its widespread application and acceptance (Kuleshova and Lopata, 2002). Viral contamination from direct contact to liquid nitrogen is a concern despite reports indicating that no such contamination has occurred to date (Bielanski et al., 2003; Bielanski et al., 2000; Kuleshova and Shaw, 2000). DMSO is often used in these procedures due to its low molecular weight and rapid transport through cellular membrane. However, at least some investigators would rather not use DMSO as it is also known to be a rather toxic cryoprotectant. All

the same, there have been numerous babies produced from embryos vitrified in this cryoprotectant (refer to Table 2). Another drawback for rapid-rate vitrification is that the standard technique of placing cells into a highly concentrated vitrification solution, loading them onto a minute container, and plunging into liquid nitrogen, all in an allotted time frame of 45-90 seconds, remains technically challenging; and perhaps more importantly, leaves little or no room for correcting errors. In a recent paper, Antinori et al., 2007) reported an impressive >98% oocyte survival rate after thawing, however, they also mentioned that it took their lab more than 5 months of 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 personnel performing the vitrification procedure; adaptability and consistency can be poor. Furthermore, failed experiments or studies with low success rates are rarely published, thus potentially giving a false impression of overall success rates for these procedures. Despite these problems, vitrification has led to a marked improvement in survival and higher pregnancy rates for some clinics.

Blastocyst Vitrification

Because the blastocyst is morphologically very different than a cleavage-stage embryo, different challenges need to be addressed when trying to cryo-store these cells. The main problem incurred when freezing blastocysts is that the blastocoel contains mostly water that can form ice when the temperature is lowered, and thus damage the embryo. To overcome this problem, some investigators have tried collapsing the blastocoel either by pipeting the blastocyst in and out of a fine bore pipette or rupturing it using an ICSI needle or similar device (Hiraoka et al., 2004; Son et al., 2003; Vanderzwalmen et al., 2002). Although survival can be increased using these methods, the obvious drawback is that there is an additional step involved that is potentially damaging to the embryo.

In this study we describe a new method to vitrify human blastocysts that is safe, successful, and relatively easy to learn and use. This method and media can be used to vitrify blastocysts of all stages (from cavitating to fully hatched) without reduction of the blastocoel or using DMSO in the media. Our technique uses a standard 0.25cc sterile straw, up to 3 times longer cryoprotectant exposure, ample loading time, and heat-sealing protection. These factors should permit adequate recovery time in cases of operator-error. This alternative method to slow-cooling, entitled ***S³-vitrification***, has been described in a recent study of ours (Stachecki et al., 2008) and we report here updated clinical outcomes from several clinics using this technique.

Materials and Methods

Collection of Blastocysts

Luteal phase GnRH agonist regimes, also called luteal phase lupron protocols, were used for all patient hormonal stimulations and oocytes were collected by standard means with fertilization occurring using ICSI or IVF. Only high quality blastocysts that had a well formed blastocoel, trophectoderm with many cells, and a well-formed visible ICM were chosen for clinical vitrification. The Gardner scale was used to grade blastocysts, where the ICM or trophectoderm was either an A or B (Gardner *et al*., 2000). None of the patients had HCV.

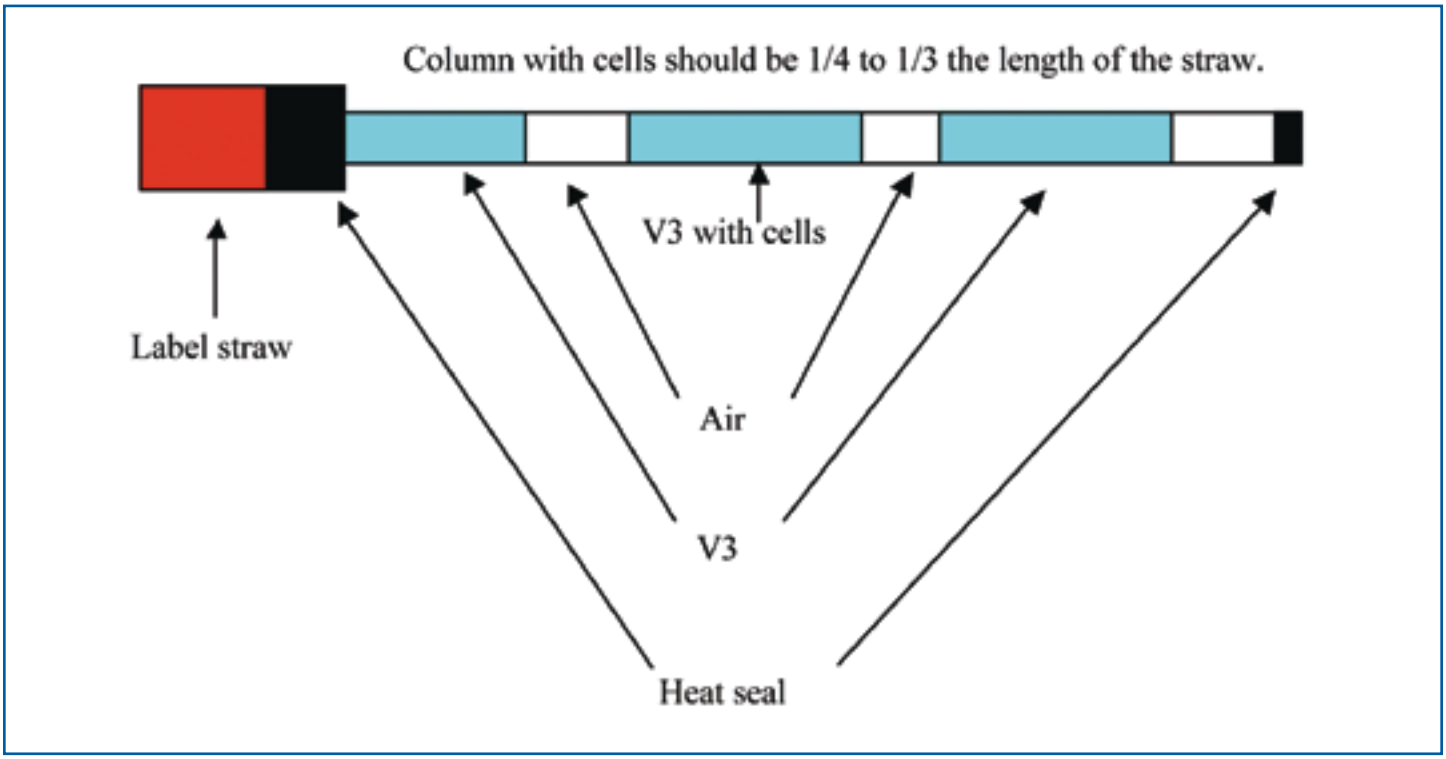
Training

Several embryologists at 5 different IVF clinics in North and South America were trained in the S3 Vitrification procedure over a period of 1 to 2 days. After training, the embryologists practiced vitrifying spare blastocysts consented for research. Upon reaching a level of comfort with the technique and their results, which varied in time from 1 week to 2 months (depending on how much material was available to practice with) they began clinical use.

Vitrification

A series of 3 solutions (V1, V2, V3) were used to vitrify blastocysts according to (Stachecki et al., 2008). Blastocysts were exposed to V1 for 5 min at room temperature (RT), transferred to V2 for 5 min at RT, and then to V3. Once in V3, the cells were immediately loaded into a standard, sterile 0.25cc cryopreservation straw Figure 1. The straws were then heat-sealed at both ends. A 0.5cc straw with patient information was heat-sealed to one end of the 0.25cc straw. The total time it took to load a straw and seal it was under 120 seconds. Straws were then vitrified by pre-cooling in liquid nitrogen vapors (-95°C to -105°C) before being stored in liquid nitrogen. This method of loading and cooling was simple and easily accomplished, within the given time frame, and in most cases there was time to spare prior to cooling. Either 1 or 2 blastocysts were frozen per straw.

Figure 1. Straw Loading Diagram



Thawing and Embryo Replacement

Straws were thawed by holding them in room temperature air before immersion in a water bath (Stachecki et al., 2008). After thawing, the cryoprotectants were removed by dilution at room temperature through a series of five media (T1-T5) at 5 min per step. The blastocysts were warmed on a heated surface (37°C) before being placed in culture at 37°C and then analyzed before being transferred. Embryos deemed to have survived thawing were selected for replacement on an individual patient basis, per the clinic's guidelines.

Results

Table 3 shows each clinic's initial results with S^3 vitrification along with the totals for all five clinics combined. Note that these results are from the first set of blastocysts that the clinics had vitrified and extensive training and practice was not necessary. Additionally, there were multiple embryologists performing the vitrification and thaw procedures at each clinic. A total of 884 blastocysts were thawed. Overall survival rates ranged between 83% and 100% with the average being 88.7%. Fetal heart beat (FHB) rates ranged between 32.8% and 58.7%. Pregnancy rates per transfer ranged between 48-71% with the average being 56.4%. All babies born to date were born healthy with no reported abnormalities.

Table 3. Clinical results of S3 Vitrification on blastocysts.

Clinic	Thawed	Intact	Transfers	Replaced	FHB	Preg/Transfer
A	104	86 (83%)	45	80	47 (58.7%)	32/45 (71.1%)
B	160	141 (88.1%)	77	131	43 (32.8%)	37/77 (48.0%)
C	41	35 (85.4%)	19	35	16 (45.7%)	12/19 (63.1%)
D	566	509 (89.9%)	209	509	N/A	116/209 (55.5%)
E	13	13 (100%)	8	13	5 (38.5%)	5/8 (62.5%)
Total	884	784 (88.7%)	358	768	111 (42.8%)	202/358 (56.4%)

Discussion

Previous reports show that fast-rate vitrification of blastocysts offers a feasible and often better approach to storage than slow-cooling techniques (Table 2). Refinement of earlier methods has led to the use of tiny containers and rapid cooling rates that coincide with a marked increase in blastocyst survival. However, these procedures are not as easy to use for some individuals and have other potential problems as described above. Concerns regarding sterility and ease of use will continue to grow as more and more regulations are placed upon IVF clinics by outside inspecting bodies such as CAP and the FDA. An alternative methodology that avoids these problems, conforms to potential future regulations, and provides for high survival and pregnancy rates, would be very useful. The new technique, ***S³-vitrification***, described here and elsewhere (Stachecki et al., 2008), has been used effectively and reproducibly, at least based upon the few clinics that have used this method. The relatively high survival and pregnancy rates obtained from five clinics in North and South America were collected from their initial attempts at using the procedure and, with continued use, could improve further.

The results varied between clinics, but overall, the rates were similar to other reports in the scientific literature. Although the slow-cool vitrification procedure was similar between the clinics, differences in patient selection, stimulation, patient age, methods of grading embryo survival, etc. could all be expected to differ among embryologists and clinics. For example, Clinic A had the lowest survival rate with 83%, yet had the highest clinical pregnancy rate with 71.1%, over a reasonable amount of transfers (n=45). They appear to have a much more stringent grading system, and even though survival seemed to be lower, the blastocysts that they deemed to have survived led to the highest pregnancy rate among the clinics. We cannot, and did not want to control for individual differences within and between clinics. We feel that this strategy will lead to a more accurate picture of overall success. Likewise, not every clinic will have the same success rate for embryo culture, despite the use of the same culture medium. The data from Table 2 also

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show a high variability of survival (51.6% to 100%) and pregnancy rates (22.9% to 55.6%) for blastocyst storage, despite the fact that the techniques are essentially the same. A storage technique that is highly effective and robust should produce results that, despite the myriad differences within and between labs, will ultimately produce reasonably high pregnancy rates. Although there are higher survival rates reported (see Table 2) collectively, our results are similar to those reported. Rather than spending time making extensive comparisons to other studies, the technique of S^3 vitrification simply represents an alternative method of storing embryos (and oocytes, data not shown), that can potentially improve outcomes and has collectively yielded over 200 pregnancies to date.

What makes S^3 vitrification unique is that it uses a relatively large 0.25cc straw, that can be loaded and sealed easily in a timely manner, and that uses a significantly slower cooling rate of <200°C/min, thus contradicting the concept that a faster cooling rate is better. However, as mentioned in the introduction, slow-cooling can also be viewed as vitrification, even when the cooling rate is very slow, relative to the rates achieved with current vitrification protocols (Table 1). Hence, we have known for decades that you do not need a fast cooling rate for successful vitrification, although we may not have realized it. Due to their design, other vitrification methods, need to use very rapid rates of cooling in order to attain high success rates.

When using S^3 blastocyst vitrification, blastocoel reduction or collapse is not necessary, although it may also work well. It should be noted that in the majority of our testing the blastocoel did not collapse completely and tended to remain about half of its original size. Despite not being fully collapsed, apparently enough water is removed and enough cryoprotectant is around to prevent damage to the trophectoderm and ICM cells based on the survival and pregnancy rates obtained. After thawing and removal of cryoprotectants, blastocysts could easily be graded, and were replaced anytime from 30 minutes to 2 hours after thawing.

The results from the various clinics presented here demonstrate that blastocysts can be vitrified using a simple easy-to-use protocol, in a relatively large, sterile, sealable container without the need for DMSO, and in a manner that allows time for equilibration and loading. So far, S^3 vitrification seems to be easy to learn, effective, and reproducible, yielding high survival and pregnancy rates in a number of reproductive clinics using the procedure for the first time.

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