Article

A new safe, simple and successful vitrification method for bovine and human blastocysts

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Abstract

This study examined a new method for vitrification of blastocysts that is safe, simple and easy to learn and use. Current vitrification techniques have shortcomings that include the use of dimethylsulphoxide, one of the more toxic cryoprotectants, and minute containers that are difficult to handle and are usually open to contamination. Cell handling and loading times are very short, which allows no room for user-associated errors and increases the difficulty of the procedure. This study describes a method of vitrification without these shortcomings. Human and bovine blastocysts were exposed to a series of three cryoprotectant solutions and loaded into a 0.25 ml sterile straw, heat sealed at both ends and vitrified. This technique allowed sufficient time for cryoprotectant exposure, loading, sealing and vitrification. Research blastocysts were thawed, cultured for 24 h, and stained for cell viability. The majority survived and on average had few lysed cells. In clinical studies from three different centres, 81.4% of vitrified blastocysts were intact after thawing. Out of 43 transfers with 76 blastocysts replaced, 44.7% implanted, 43.4% yielded a fetal heart beat, and a total of 32 babies have been delivered or are ongoing. The overall clinical pregnancy per transfer rate was 60.4%. The high survival rates and clinical pregnancy rates obtained with this new, safe and easy-to-use vitrification procedure are encouraging.

Keywords: *cryopreservation, human, vitrification*

Introduction

There is a need, for a quicker, simpler, safer and more successful technique for oocyte and embryo storage. Vitrification, via rapid cooling, seems to be one of the methods of choice and it may replace conventional cryopreservation in the clinical field. It is frequently quoted as being inexpensive, fast and simple. However, these are not important reasons to perform or adapt a technique. The only reasons that should matter are improved performance resulting in higher survival and an overall increase in birth rates, as well as enhanced safety so that the chances of long-term effects and short-term technical concerns are reduced. Nonetheless, rapid freezing or vitrification has been the focus of research in recent years, based upon a growing number of reports in the literature. In many instances it is now the preferred method of choice.

The idea of vitrification or achieving a glass-like state was first described in 1860, and then again in 1937 by Luyet (1937). It was not until nearly 50 ears later in 1985 that Rall and Fahy (1985) described vitrification as a potential alternative to slow-cooling. There have been numerous recent publications on human oocyte and embryo vitrification (Chen *et al*., 2000; Chung *et al*., 2000; Ali, 2001; Wu *et al*., 2001; Kuleshova and Lopata, 2002; Liebermann and Tucker, 2002; Wininger and Kort, 2002; Vanderzwalmen *et al*., 2002, 2003; Liebermann *et al*., 2003; Yoon *et al*., 2003; Cremades *et al*., 2004; Hiraoka *et al*., 2004; Kasai and Mukaida, 2004; Antinori *et al*., 2007).

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Some of these manuscripts clearly show improved results in terms of survival and clinical pregnancy rates with the use of vitrification.

Current vitrification procedures involve exposure of cells suspended in very small volumes to high concentrations of cryoprotectant(s) for brief periods of time, followed by rapid cooling in liquid nitrogen. The high osmolarity of the vitrification solution rapidly dehydrates the cell, and submersion into liquid nitrogen quickly solidifies the cell so that the remaining intracellular water does not have time to form damaging ice crystals. A similar situation occurs during slow cooling; however, the cells are dehydrated over a longer period of time and then plunged into liquid nitrogen at much lower temperatures.

Compared with slow cooling, vitrification has allowed improved survival and pregnancy rates, despite posing a potentially greater risk because of the possible toxicity of the highly concentrated cryoprotectants used and the relatively high exposure temperature (Hotamisligil *et al*., 1996; Mukaida *et al*., 1998). Several new theories or ideas associated with vitrification have since become prevalent in the literature. These include: (i) high concentrations of cryoprotectants are toxic, and exposure to the final solution with the highest concentration should be reduced to 60 s or less (Shaw *et al*., 1992; Hunter *et al*., 1995; Hong *et al*., 1999; Chung *et al*., 2000; Wu *et al*., 2001; Yoon *et al*., 2003); and (ii) the faster the cooling rate, the better the survival.

In order to freeze faster, several new products have been developed based on the open-pulled straw (Vajta *et al*., 1998), which reduces the diameter of a conventional 0.25 ml straw, thus reducing the amount of liquid that needs to be loaded and increasing the speed of vitrification. Kuwayama *et al*. (2005b) measured the cooling rate for a 0.25 ml straw containing 25 µl vitrification solution plunged directly into liquid nitrogen at 4460°C/min, an open-pulled straw containing 1.5 µl vitrification solution at 16,340°C/min, and a cryotop with 0.1 µl vitrification solution at 22,800°C/min. In an earlier study, Vandzerwalmen *et al*. (2002) measured the cooling rate for a 0.25 ml straw near 2,000°C/min. In 2003, Vandzerwalmen *et al*. (2003) reported that using an open hemi-straw with direct plunging into liquid nitrogen, which allowed for a faster cooling rate than with a 0.25 ml straw, increased the embryo survival rates. Similar products, including electron microscope grids, cryo-top, cryo-tip, cryo-leaf and nylon loops, that allow direct contact with liquid nitrogen and minute volumes, have been shown to increase the vitrification speed considerably, in the order of >15,000°C/min, and high survival rates have been reported with their use (Martino *et al*., 1996; Hong *et al*., 1999; Lane and Gardner, 2001; Wu *et al*., 2001; Liebermann *et al*., 2003; Mukaida *et al*., 2003; Son *et al*., 2003; Cremades *et al*., 2004; Hiraoka *et al*., 2004; Huang *et al*., 2005; Isachenko *et al*., 2005; Kuwayama *et al*., 2005a; Antinori *et al*., 2007).

Despite the increase in survival rates and the relative abundance of recent reports on vitrification, the numerous shortcomings associated with these conventional vitrification protocols may have prevented its widespread application and acceptance (Kuleshova and Lopata, 2002). First, several reports of viral contamination in liquid nitrogen have appeared in the literature and are cause for concern when vitrification is not carried out in a sealed container (Bielanski *et al*., 2000, 2003; Kuleshova and Shaw, 2000). Although there are other reports indicating that no such contamination has occurred to date, the safety concern remains (Kuwayama *et al*., 2005a). Second, dimethylsulphoxide (DMSO) is often used in these procedures, although it has been known for years to be one of the more toxic cryoprotectants. Third, the common 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 60 s, remains technically challenging; more importantly, it leaves little or no room for error. 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 laboratory over 5 months of training to obtain such rates, and that operator skill was crucial to guarantee the proficiency of the procedure. A new term 'technical signature' is becoming popular when describing vitrification techniques. This simply means that the results are often based upon the technical skill of the person doing the vitrification procedure. Therefore, the adaptability and consistency of vitrification protocols can be poor, even though only success stories are reported in the literature. Failed experiments or studies with low success rates are probably rarely published, thus giving a false impression of overall success rates. Despite all of these potential shortcomings, vitrification has led to a marked improvement in survival as compared with slow-cooling procedures, and clinics have enjoyed higher pregnancy rates.

As reducing multiple pregnancies becomes a greater focus for assisted reproduction treatment clinics, the transfer of only a single embryo is warranted. In many cases, blastocyst transfer is the method of choice in order to achieve a high pregnancy rate with the transfer of only one embryo. As more clinics become proficient at culturing embryos to the blastocyst stage, there is an increasing need to store the surplus blastocysts. Therefore, numerous recent reports of blastocyst vitrification have been published. Because blastocysts are morphologically very different from a non-cavitating cleavage-stage embryo, their freezing has presented different challenges. The main problem is that the blastocoel is made up mainly of water that can form ice crystals when the temperature is lowered, and thus cause damage to the inner cell mass (ICM) and trophectoderm. To overcome this problem some investigators have tried collapsing the blastocoel either by pipetting the blastocyst in and out of a fine bore pipette or by rupturing it using an intracytoplasmic sperm injection (ICSI) needle or similar device (Vanderzwalmen *et al*., 2002; Son *et al*., 2003; Hiraoka *et al*., 2004). Although these papers report increased survival rates using these methods, the obvious drawback is that an additional procedural step is involved that is potentially damaging to the embryo. An optimal protocol would avoid such manipulations.

The most obvious method of storing embryos safely and easily is to use a conventional 0.25 ml sterile cryo straw. This device is easy to handle, load, and can be heat-sealed at both ends, providing a safe environment to store the cells. This approach has been tried numerous times in the past with only moderate success, as reported by: Yokota *et al*. (2001), 36/45 (80%) survival; Vanderzwalmen *et al*., (2002), 20.3% and 31% survival without collapsing the blastocoel, and 60% and 70.6% after blastocoel collapse; and Escriba *et al*., (2006), 10/16 (62%) survival. In general, the more successful techniques have used micro-sized open containers: electron microscope grids have

been used by Choi *et al*. (2000), Cho *et al*. (2002), and Son *et al*. (2003); open-pulled straws have been used by Cremades *et al*. (2004); CryoTop has been used by Hiraoka *et al*. (2004), Kuwayama *et al*. (2005a), and Liebermann and Tucker (2006); CryoTip has been used by Kuwayama *et al*. (2005a); CryoLoop has been used by Mukaida *et al*. (2001, 2003), and Huang *et al*. (2005). The overall survival rate with these techniques has been around 90% or better.

In this study a new method that can be used to vitrify human and bovine blastocysts is described. The authors have tried to create a safe, simple, and successful technique that is easy to learn and use. In developing the method a 'ground up' approach was taken, rather than simply modifying a current protocol. In the end, a safe and easy method of vitrification was successfully devised for blastocysts of all stages, from cavitating to fully hatched, without reduction of the blastocoel or use of DMSO. The method described uses a large, sterile, sealable container, and allows sufficient time for cryoprotectant exposure, loading of the embryos, heat sealing, and also allows for recovery time should operator error occur. The results of this new and alternative method to slow-cooling, entitled S^3 vitrification, are described herein.

Materials and methods

Collection of blastocysts

All human embryos used in the pre-clinical study were from material disacarded by patients 25–41 years of age, and donated to research with written informed consent in accordance with each internal review board protocol. For all patients, ovarian stimulation was carried with the use of luteal phase gonadotrophin releasing hormone (GnRH) agonist regimes, luteal phase lupron protocols; oocytes were collected under ultrasound guidance by standard means and were fertilized using intracytoplasmic sperm injection or IVF. For initial testing, human blastocysts donated for research on day 5 or 6 that had a visible blastocoel irrespective or whether they had many trophectoderm cells or a visible ICM were vitrified. After the first set of studies, a clinical trial was initiated in three IVF clinics: Pro Criar, The Institute for Reproductive Medicine and Science, and Northwest Centre for Reproductive Sciences. Preliminary data from their first vitrification trials are presented here. For these trials, 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, indicating whether the ICM or trophectoderm was either A or B (Gardner *et al*., 2000). All patients tested seronegative for hepatitis C virus.

Bovine oocytes were purchased from BoMed (Madison, WI, USA) and shipped overnight in a portable heated incubator. Oocytes were cultured for several hours in order to fully mature (24 h from start of maturation) before being inseminated with bull spermatozoa. Oocytes were inseminated in IVF-Talp-containing spermatozoa, and the oocytes were washed and cultured in complete synthetic oviduct fluid (cSOF) that was supplemented with essential and non-essential amino acids (Invitrogen, Carlsbad, CA, USA). After 5 days of culture, good-quality embryos (16-cell to Morulae) were transferred to fresh cSOF medium containing 10% fetal bovine serum, and allowed to develop for an additional 2–3 days. On days 7 and 8, expanded blastocysts with a visible blastocoel and well-defined ICM were selected for vitrification.

Vitrification

Pilot studies were performed to determine optimal conditions for vitrification and subsequent development. A series of three solutions (V1, V2 and V3) were used to vitrify blastocysts. These consisted of a phosphate-buffered medium supplemented with 20% human serum albumin, and glycerol and/or ethylene glycol in increasing concentrations. The respective cell types were exposed to V1 for 5 min at room temperature, transferred to V2 for 5 min at room temperature, and then to V3, making sure that the amount of medium carried over was minimized once in V3. The cells were immediately loaded into a standard 0.25 ml cryopreservation straw (AgTech, Manhattan, KS, USA). The straws were then heat sealed at both ends. The time taken to load a straw and seal it ranged from 60 to 120 s. For cooling, the straws were placed above liquid nitrogen, in the vapour phase (range –95 to –105°C) for 2 min, 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. For the pilot research test, 1–10 blastocysts were frozen per straw. For the clinical trials either 1 or 2 blastocysts were frozen per straw.

Thawing

Straws were thawed by holding them at room temperature in air for 5 s before immersion in a 20°C water bath for an additional 10 s. After thawing, the cryoprotectants were removed by dilution through reduced concentrations of sucrose ranging from 0.85 to 0 mol/l, in a series of five 5 min steps, at room temperature. The blastocysts were then placed into cSOF with amino acids and 10% fetal bovine serum at 39°C (bovine), or potassium-simplex optimized medium (KSOM) with amino acids and 10% human serum albumin at 37°C (human), and allowed to culture overnight. Blastocysts used clinically were washed in an appropriate culture medium (Global plus 10% HSA) and incubated at 37°C for 0–4 h prior to transfer.

Staining

Following 24 h of culture, research bovine and human blastocysts were scored for re-expansion of the blastocoel, and then stained to determine the number of living and dead blastomeres. Blastocysts were incubated in 10 µl/ml propidium iodide in Hepes-buffered KSOM with amino acids at 37°C (human) or 39°C (bovine) for 15 min. The blastocysts were then fixed in 70% ethanol at 5°C for 5 min, and incubated in 70% ethanol containing 10 µg/ml Hoechst (H33342) at room temperature for 5 min. The cells were then placed in a small drop of mounting medium on a slide and gently covered with a coverslip. Care was taken to flatten the blastocysts without rupturing them, in order to count the cells. The number of blastomeres with a pink (dead) nucleus and those with a blue (alive, membrane intact) nucleus were counted. Observations were made using a Nikon Diaphot microscope with epifluorescence capabilities (Opti-Quip, Highland Mills, NY, USA).

Results

Bovine blastocysts from oocytes matured *in vitro*, and inseminated and cultured for 7 or 8 days, were used for the initial laboratory experiments. Blastocysts selected for vitrification were either expanded, fully expanded and/or hatching. All had a well-defined ICM, and all cells were assumed to be intact and alive at the time of vitrification. Human blastocysts donated for research were day 5 or 6 blastocysts that had been rejected for transfer or cryopreservation because of their poor quality. These human blastocysts chosen for vitrification had a poor or no discernable ICM and/or a low number of trophectoderm cells. Basically, anything with a visible cavity was frozen because this material was difficult to obtain. The good-quality bovine blastocysts served as an additional control and represented an embryo with many cells (usually >100) with a well-defined ICM. After developing and testing the S^3 vitrification technique on bovine and human research-quality blastocysts and obtaining consistent survival rates of between 80 and 100% (**Table 1**, **Figures 1** and **2**), clinical trials were then initiated. These were an immediate success.

Surplus human blastocysts donated for research survived at a rate of 83.3% and had 86.4% of their cells intact (**Figures 1** and **3a–d**). The cells that did not survive were in most cases spread randomly over the entire blastocyst and were not localized to one particular area (i.e. the ICM). High-quality, expanded bovine blastocysts survived at a rate of 96.1% and had 93.6% of their cells intact (**Figures 2** and **3e–h**). Again, cellular demise was not localized, but was generally randomly dispersed throughout the embryo.

An additional study was carried out to determine the optimal exposure time for the final vitrification solution and to verify that lengthy exposures would result in cell death. Day 7 bovine blastocysts were pooled prior to vitrification and randomly separated into three groups of 1, 2 and 4 min exposure to V3 prior to cooling. **Table 2** shows that toxicity of the solution was only apparent at the longest exposure period to V3, i.e. 4 min (63% cell survival). In addition two of the three blastocysts in this group (4 min V3 exposure) were classified as having survived, and one re-expanded nicely after 24 h in culture). The groups with 1 and 2 min V3 exposures had higher survival rates of 89.6% and 93.2%, respectively.

Embryologists at three clinics in North and South America who had no previous experience of vitrification were taught S^3 vitrification within a 2-day period. Some clinics performed initial trials on surplus blastocysts that failed to meet their criteria for transfer and were approved for research. Other clinics started using the method clinically immediately. **Table 3** shows the initial S^3 vitrification results for each clinic, and the totals for all three clinics combined. Note that these results are from the first set of blastocysts that the clinics had vitrified; extensive training and practice was not necessary. In the case of clinic B, multiple technicians performed the vitrification and thaw procedures. Overall survival rates ranged between 85% and 95%, with the average being 89.2%. Implantation and fetal heart beat rates ranged between 36% and 71%. Pregnancy rates per transfer ranged between 59 and 88%, with an average of 65.9%. Of the 38 implanted embryos, only one has been spontaneously aborted to date. All babies born to date were born healthy with no reported abnormalities.

Table 1. Vitrification of research-grade embryos.

Species	n	*No. of embryos intact (%)*	*No. of cells alive (%)*
Human[a]	24	20 (83.3)	721/835 (86.4)
Bovine	102	98 (96.1)	6775/7238 (93.6)

[a]Blastocysts donated for research, not suitable for transfer. Blastocysts had a poor quality trophectoderm and/or inner cell mass.

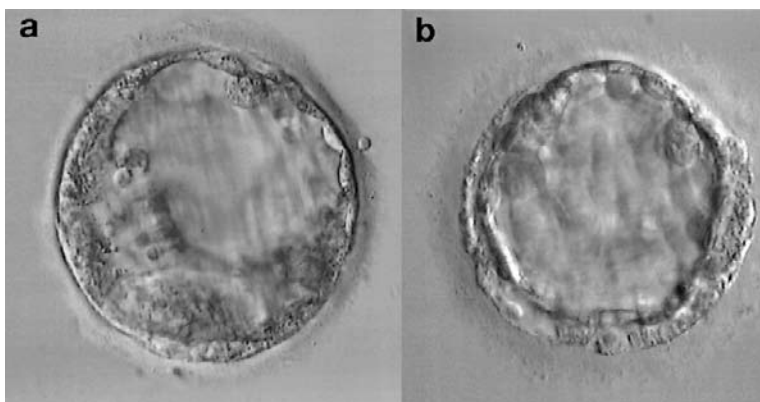

Figure 1. (**a**) Non-frozen day 5 human blastocyst. (**b**) The same blastocyst immediately after thawing and cryoprotectant removal. Note that the blastocyst is not collapsed and the embryo can be easily graded for quality. (**c**) The same blastocyst 24 h after thawing and culture. Note that the embryo has re-expanded and most of the cells appear intact.

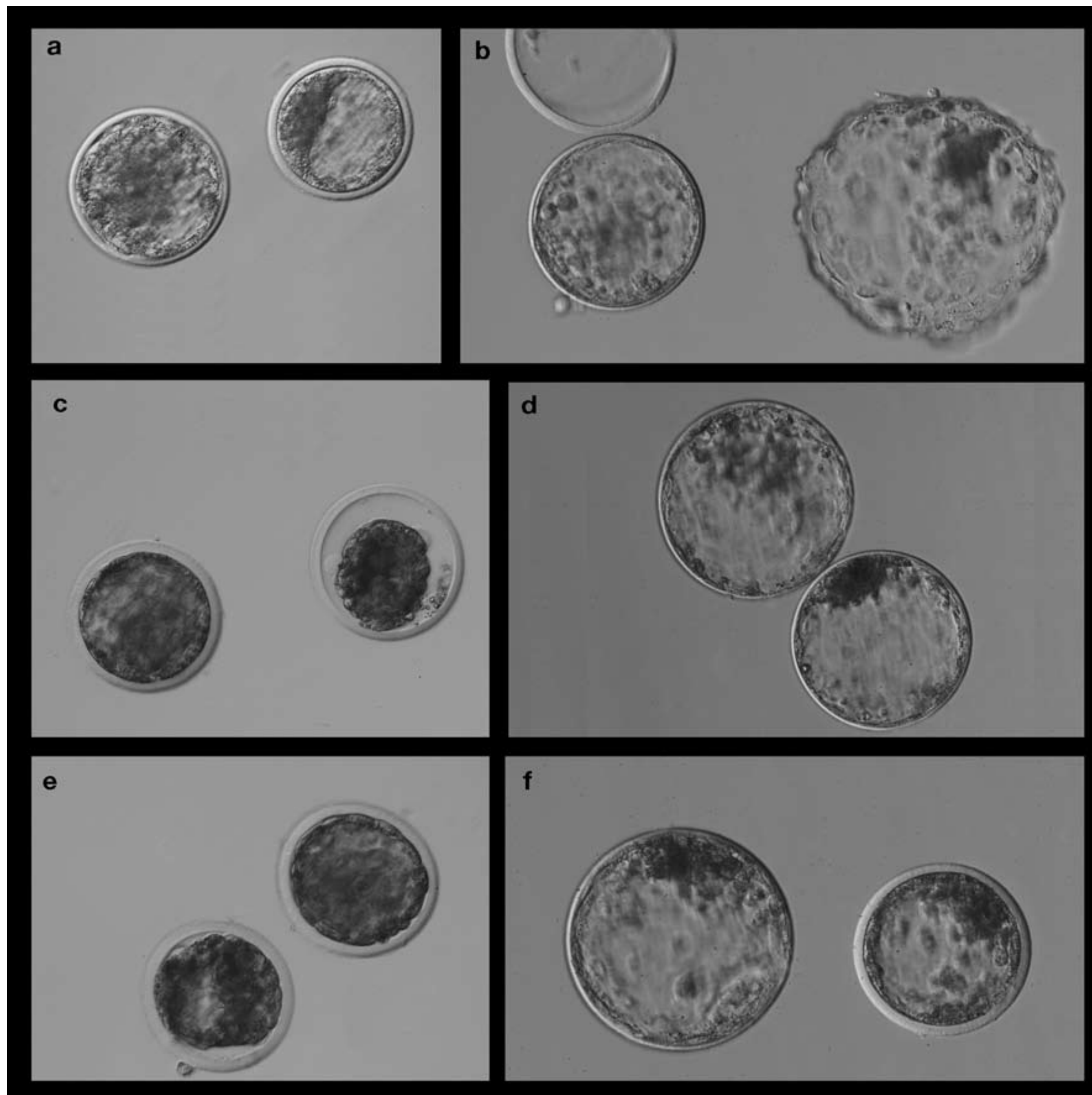

Figure 2. (**a**), (**c**) and (**e**) Vitrified bovine blastocysts (day 7 or 8) immediately after thawing and cryoprotectant removal. Note that many of the blastocysts are not or are only partially collapsed after the thaw process. (**b**), (**d**) and (**f**) The same embryos 24 h after thawing and culture. Note that the blastocysts have expanded and one has fully hatched (**b**).

Discussion

Previous reports show that vitrification of blastocysts offers a feasible and often better approach to storage than slow-cooling techniques. Refinement of earlier methods has led to the use of minute containers and rapid cooling rates that coincide with a marked increase in blastocyst survival. However, these procedures have numerous shortcomings. The results presented here suggest an alternative methodology that avoids these shortcomings and also provides high survival rates of 90% or better in the bovine, and >89% survival in the human, with >65% pregnancy rates. This new technique, entitled S^3 vitrification, has the benefits of being easy to learn and use effectively and reproducibly. Survival and pregnancy data from three clinics in North and South America were collected from their initial attempts at using the procedure, and with continued use these results could improve further.

S^3 vitrification is a different technique based on basic cryopreservation principles. A relatively large container and a sterile 0.25 ml straw can be loaded and sealed easily without rushing the procedure, and the use of a significantly slower cooling rate of >2000°C/min, compared with the rate obtained with a tiny open container (>15,000°C/min), contradicts the idea in current literature that a faster cooling rate for oocyte and embryo vitrification is better. Furthermore, although a longer exposure period was used (12 min prior to cooling) than in the methods described in current reports, there was little if any evidence of toxicity. To address this issue further, bovine blastocysts were exposed for longer periods of time to V3. Despite the small numbers, the data (**Table 2**) confirm that V3, the highly concentrated final vitrification solution, is potentially toxic and detrimental to embryo survival when exposure is prolonged for more than 2 min. However, in the 4 min exposure group, two out of the three blastocysts survived vitrification

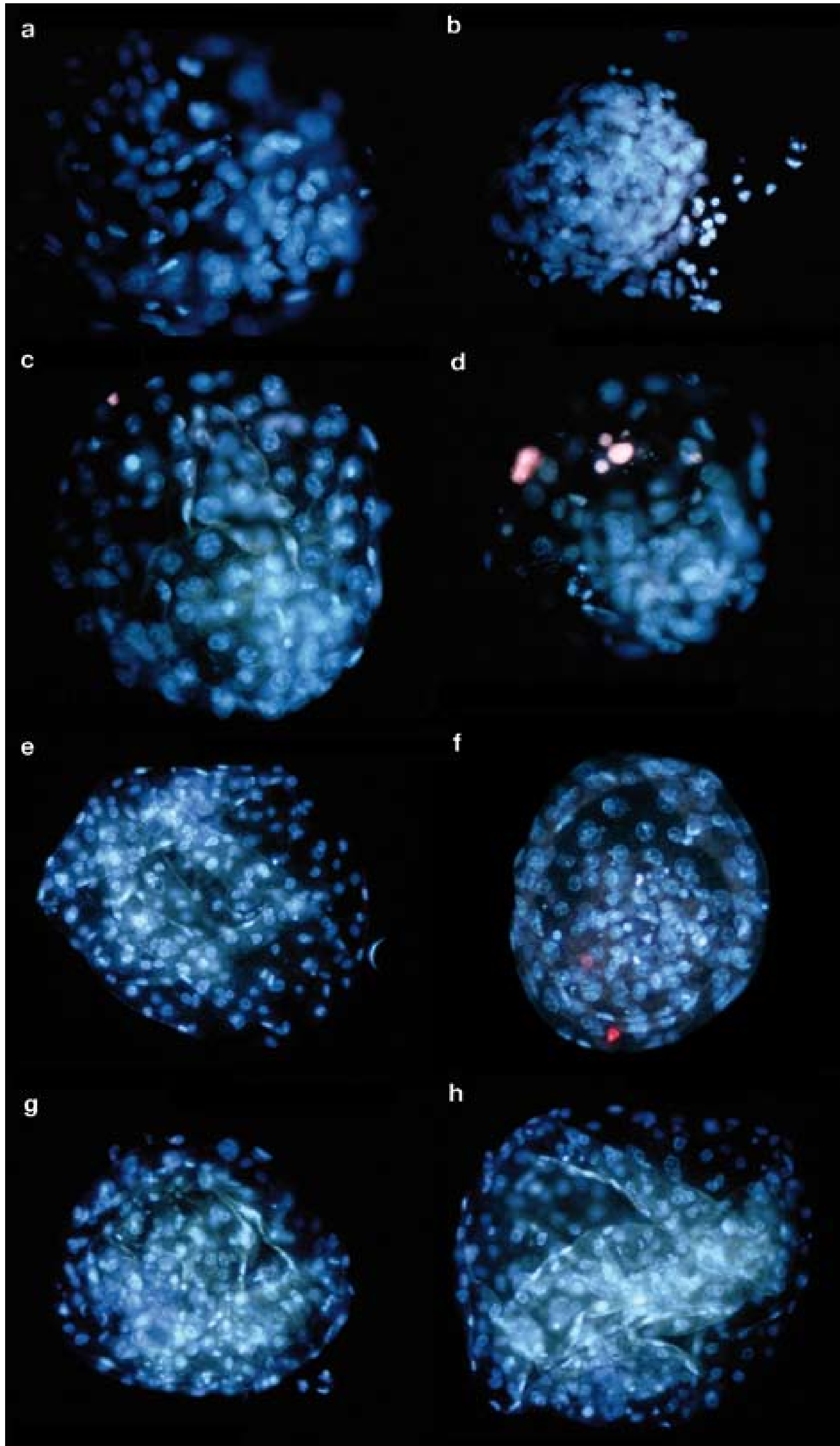

Figure 3. Fluorescent live/dead staining of human (**a**–**d**) and bovine (**e**–**h**) blastocysts 24 h after thawing and culture. Cells with blue nuclei are intact and cells with pink/red nuclei are lysed. Blastocysts with all cells intact (**a**, **b**, **e**, **g** and **h**). The human blastocyst in (**c**) and (**d**) has one and several lysed cells, respectively, but the dead cells do not appear to be in the area of the inner cell mass, which is assumed to be near 5 o'clock in both images.

Table 2. Bovine blastocyst vitrification: exposure time in final cryoprotectant.

No. of blastocysts	*V3 exposure time (s)*	*No. of intact cells*	*No. of lysed cells*	*No. of surviving cells (%)*
2	60	130	15	2/2 (89.6)
3	120	358	26	3/3 (93.2)
3	240	160	94	2/3 (63.0)

Table 3. S^3 Blastocyst vitrification: clinical data.

Clinic	*No. of blastocysts thawed*	*No. of blastocysts intact (%)*	*No. of transfers*	*No. of blastocysts replaced*	*No. of blastocysts implanted (%)*	*No. of blastocysts yielding an FHB (%)*	*No. of ongoing and delivered babies*	*Pregnancy/ transfer (%)*
A	18	17 (94.4)	8	17	12 (70.6)	12 (70.6)	12	7/8 (87.5)
B	33	30 (90.0)	17	27	13 (48.1)	12 (44.4)	12	11/17 (64.7)
C	42	36 (85.7)	22	36	13 (36.1)	13 (36.1)	13	13/22 (59.1)
Total	93	83 (89.2)	47	80	38 (47.5)	37 (46.2)	37	31/47 (65.9)

FHB = fetal heart beat.

and one re-expanded despite having approximately 30% lysed blastomeres. This suggests that although the duration of 4 min was clearly the most detrimental, blastocysts can survive and re-expand, and this prolonged treatment is, surprisingly, not lethal to all cells. Clearly, many different vitrification methods may be successful, and work potentially even better and easier than the one presented here. S^3 vitrification is simply a different technique from those described in the recent literature, with its own reasons and theories regarding why and how it works. The relatively large 0.25 ml straws work well with the vitrification solutions and methods described here, just as tiny open containers work with a different set of solutions and protocols. Because the procedures are different, the methods from one procedure may not succeed with the solutions or containers from another.

Human and bovine blastocysts vitrified using S^3 did not require blastocoel collapse prior to freezing in order to obtain high rates of embryo and cell survival. Because the blastocoel consists mainly of water, ice crystals will form when it freezes, presenting a potentially lethal problem during vitrification. This has led to the artificial reduction or collapse of the blastocoel prior to vitrification, an invasive technique described above. When using S^3 blastocyst vitrification, although blastocoel reduction or collapse may work well, it is not necessary. During vitrification and thawing, in the majority of cases the blastocoel does not collapse entirely and remains about half of its original size (for examples of thawed blastocysts see **Figures 1b**, and **2a**, **c** and **e**). The human blastocyst can often be described as 'folding up' instead of collapsing while moving through the vitrification solutions. Despite being not fully collapsed, apparently sufficient water is removed and there is sufficient cryoprotectant to prevent damage to the trophectoderm and ICM cells (**Figure 3**). After thawing and removal of cryoprotectants the blastocyst can easily be graded, and transferred immediately without the need for culture until re-expansion occurs to confirm its survival (**Figures 1b**, and **2a**, **c** and **e**). This is different from many of the conventional vitrification procedures, where the blastocyst is collapsed or collapses during thawing and cryoprotectant removal (Cho *et al*., 2002; Vanderzwalmen *et al*., 2003). It is difficult to grade the embryo or determine if it has survived after blastocoel collapse, and culture for several hours or overnight prior to transfer has therefore become common practice (Vanderzwalmen *et al*., 2003; Huang *et al*., 2005; Kuwayama *et al*., 2005a; Liebermann and Tucker, 2006) in order to visualize re-expansion and determine whether the embryo and blastomeres are intact. The majority of blastocysts that were cultured overnight re-expanded fully and continued to expand and sometimes hatch (**Figures 1c**, and **2b**, **d** and **f**). Avoiding full collapse of the blastocyst during thawing and cryoprotectant removal is another benefit of the S^3 vitrification procedure, because it allows for instant verification of survival; decisions regarding how many embryos should be thawed are more easily made, without waiting for re-expansion.

These results show 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 adequate time for equilibration and loading without having to rush the procedure. In addition to the simplicity this procedure offers, more importantly, it is effective and reproducible, yielding high survival and pregnancy rates in a number of reproductive clinics that used the procedure for the first time.

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