

Article

Vitrification demonstrates significant improvement versus slow freezing of human blastocysts



Ed Stehlik is now in his 20th year as the IVF lab director for the Advanced Institute of Fertility under Dr K Paul Katayama. Throughout these years Ed has co-authored several papers and lectured internationally on topics covering all aspects of human embryology, including test yolk buffer sperm treatment, biopsy techniques and vitrification. The laboratory's clinical work has resulted in many of the 'firsts' in Midwest USA, including embryo cryopreservation, PZD, ICSI and assisted hatching. Among its most notable accomplishments is co-author Joni Stehlik's success in producing the first two US babies delivered following vitrification of human oocytes as documented on CBS's 48-h programme.

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Abstract

Blastocyst culture has reduced the number of embryos transferred per cycle, whilst simultaneously creating new quandaries regarding supernumerary blastocyst cryopreservation. This retrospective study was undertaken to compare a slow freezing protocol to a vitrification protocol for cryopreservation of day 5 and day 6 human blastocysts. To demonstrate this, the survival, implantation rate and pregnancy rates were compared after thawing, assessment and embryo transfer of 86 consecutive day 5 and day 6 thawed blastocyst transfer cycles from January 1, 2002 to December 31, 2003. Seventy-one day 5 slow-frozen (SF) blastocysts were thawed and 59 embryos survived the thawing (83.1%). An average of 2.5 SF blastocysts was replaced per embryo transfer, resulting in a pregnancy rate of 16.7% (4/24). Concurrently, 41 vitrified (VIT) blastocysts were thawed and all 41 survived the thawing process (100%). An average of 2.0 VIT blastocysts was replaced per embryo transfer, resulting in a pregnancy rate of 50% (10/20). Survival, pregnancy and implantation rates of day 5 VIT blastocysts have significantly increased ($P < 0.01$, $P < 0.02$ and $P < 0.01$ respectively) over day 5 SF blastocysts. A similar trend was observed with day 6 blastocysts.

Keywords: cryopreservation, human blastocysts, implantation rate, pregnancy rate, survival rate, vitrification

Introduction

Blastocyst culture is now common in most IVF centres around the world. (Summers and Biggers, 2003) Whilst this selection process has simultaneously reduced the number of embryos transferred per cycle and increased implantation rates (Gardner *et al.*, 1998; Jones *et al.*, 1998), it has created new challenges for supernumerary blastocyst cryopreservation. Although 10–15% of a successful IVF programme's pregnancies result from frozen–thawed embryos (Lundin, 2003), advances in technique sometimes lag behind. Many scientists have been unable to achieve comparable blastocyst cryopreservation results when utilizing the conventional slow-freezing techniques resembling those that were achieved with earlier stage embryos (Nakayama *et al.*, 1995; Alper *et al.*, 2001;

Garcia-Velasco and Simon, 2001; Pantos *et al.*, 2001) while others have achieved comparable results (Veeck, 2003).

Blastocyst cryopreservation can be accomplished by one of two methods. The first, slow freezing (SF), led to the first successful human cryopreservation pregnancies (Zeilmaker *et al.*, 1984). This technique attempts to minimize intercellular ice crystal formation by utilizing the equilibrium process to drive cellular dehydration while slowly decreasing the temperature. The utilization of sucrose and cryoprotectants for this purpose has been described (Wright *et al.*, 2004). The second, vitrification (VIT), is a very quick freeze utilizing high concentrations of cryoprotectants that solidify without ice crystal formation. Although vitrification was first described in the late 1800s (Tammann, 1898), substantial research with

animal models was not reported until the mid-1980s (Rall and Fahy, 1985; Rall *et al.*, 1987; Trounson *et al.*, 1988). Familiarity with the technique is still relatively rare, but a few clinics are now reporting pregnancies from vitrified blastocysts (Vanderzwalmen *et al.*, 1997; Lane *et al.*, 1999; Yokota *et al.*, 2001). The goal of both techniques is to minimize intra- and extracellular damage that can occur during the freezing process (for review, see Smith and Silva, 2004). While both techniques are viable, a recent side-by-side cryopreservation study described higher survival and development rates of murine embryos when vitrification was compared with slow-freezing (Walker *et al.*, 2004).

This clinic retrospective study compares results utilizing the slow freezing method versus the minimum volume vitrification method for surplus day 5 and day 6 human blastocysts. The study includes data from 86 consecutive qualifying blastocyst thaw-transfer cycles from January 1, 2002 through December 31, 2003 regardless of age or indication. In order to form clearly defined, comparable data groups, no mixed transfers of both slow-frozen and vitrified blastocysts or mixed transfers of both day 5 and day 6 blastocysts were included in the study.

Materials and methods

Supernumerary embryos having achieved at least the early blastocyst stage (with distinguishable inner cell mass) by day 5, were either slow frozen or vitrified on day 5. Culture of any surplus embryos was sustained until day 6, with SF or VIT repeated for any ensuing day 6 blastocysts. All blastocysts were thawed and transferred in a similar manner to day 5 blastocysts (i.e. in synchronization with day 5 embryo transfer to the recipient uterus). Regardless of the cryopreservation method, embryos were assessed for viability based on membrane integrity with transfers occurring within 30 min of thawing.

Slow freezing

Slow freezing was undertaken by conventional methods (Ménézo *et al.*, 1992). A 10-min equilibration in a 5% glycerol solution in HEPES-buffered HTF media (SAGE, BioPharma, Bedminister, NJ, USA) supplemented with 20% Synthetic Serum Substitute (SSS) (Irvine Scientific, Santa Ana, CA, USA) is followed by a 10-min equilibration in the same media with final concentrations of 9% glycerol and 0.2 mol/l sucrose. A Planer controlled rate freezer (Planer Kryo; T.S. Scientific, Perkasi, PA, USA) and plugged 0.25 ml straws were used. The blastocysts were cooled from room temperature to -7°C at $-2^{\circ}\text{C}/\text{min}$. Manual seeding was performed, and this seeding temperature was maintained for 3 min. The freezer was subsequently cooled from -7°C to -30°C at $-0.3^{\circ}\text{C}/\text{min}$. At this point, the temperature was dropped at $-50^{\circ}\text{C}/\text{min}$ to -180°C , followed by immediately plunging the straw containing the blastocyst into liquid nitrogen for storage. This entire process required approximately 2.5 h to finish.

Thawing was accomplished by a two-step removal of the cryoprotectants as previously described (Virant-Klun *et al.*, 2003). Succinctly, the blastocysts were thawed quickly at room temperature with a 10-min equilibration in HEPES-buffered HTF medium supplemented with 20% SSS and 0.5M sucrose.

This step was followed by an additional 10-min equilibration in HEPES-buffered HTF medium supplemented with 20% SSS and 0.2 mol/l sucrose prior to transferring the blastocysts to culture media in preparation for transfer. Blastocysts were considered surviving when greater than 50% of the inner cell mass and trophectoderm cells were undamaged by light microscopy observation.

Vitrification

Vitrification was accomplished in two main phases, equilibration and vitrification. The equilibration phase consisted of a three-step introduction and equilibration in the cryoprotectants. This was accomplished by running the blastocysts through culture media/equilibration solution mixtures of 1:1, 1:2 and 0:1. The equilibration solution consisted of 7.5% ethylene glycol and 7.5% dimethylsulphoxide (DMSO) in HEPES-buffered medium 199 with Earle's salts containing 20% SSS. The timing of each introduction and equilibration step was based on the appearance and/or reaction speed of the individual blastocyst in lieu of a set time period. The vitrification step consisted of a rapid rinse through four drops of vitrification solution containing 15% ethylene glycol, 15% DMSO and 0.5 mol/l sucrose in the same HEPES-buffered medium 199 with Earle's salts supplemented with 20% SSS. The rinses were followed by the transfer of blastocysts onto the Cryotop device (Kitazato, Fuji-shi, Japan) in as small a volume as possible (i.e. approximately 2–5 μl) and instantaneous direct contact with liquid nitrogen. The rinsing, loading and plunging steps were completed in less than 1 min. The entire process from culture to frozen only took approximately 5–10 min.

The thawing of VIT blastocysts consisted of an ultra-quick warming in a 37°C thaw solution comprising HEPES-buffered medium 199 with Earle's salts supplemented with 20% SSS and 1.0 mol/l sucrose, followed by a six-step removal of the cryoprotectants. Again, this was facilitated by a series of solution mixtures. Following the quick thaw in thaw solution, the blastocysts were moved through 1:1, 1:2, 1:3 and 0:1 mixtures of thaw solution/dilution solution. Dilution solution consisted of HEPES-buffered medium 199 with Earle's salts supplemented with 20% SSS and 0.5 mol/l sucrose. Upon equilibration, the blastocysts were exposed to a 1:1 mixture of dilution solution/wash solution followed by one final equilibration in wash solution alone. Wash solution consisted of HEPES-buffered medium 199 with Earle's salts supplemented with 20% SSS. Once more, timing of each step was based on individual reaction speed of morphological changes observed under light microscopy. The same standard of membrane integrity/survival percentage to determine viability was applied to the VIT blastocysts. Because of the risk of premature thawing and/or evaporation of this small volume of medium containing the blastocysts, great care was taken to maintain the Cryotop submergence in liquid nitrogen during storage and thaw preparation steps.

Embryo replacement cycle

Patients were primed for embryo replacement by either monitoring their natural cycle or undergoing hormone replacement therapy (Estrace/progesterone) replacement cycle.

For patients utilizing their natural cycle, daily serum oestradiol and LH concentrations were monitored commencing on day 10. Ovulation (embryo day 0) was measured to be 36 h after the initial LH surge (a surge was defined as at least a doubling of the LH concentration over 24 h). Thawing and transfer occurred 5 days later (embryo day 5), regardless of whether the blastocysts were cryopreserved on day 5 or day 6.

Patients initiating an Estrace/progesterone support cycle started mid-luteal Lupron (0.1 ml per day; TAP Pharmaceuticals, Lake Forest, IL, USA). On day 3 of the replacement cycle, Lupron was reduced to 0.05 ml per day and the patient started 2 mg Estrace tablets per day. Estrace (Warner Chilcott, Rockaway, NJ, USA) was increased to 4 mg on day 7 and 6 mg on day 11. Ultrasound on day 13 measured the endometrium. When the endometrium reached 8 mm, 50 mg of progesterone (injectable progesterone in sesame oil; Watson Pharmaceuticals, Morristown, NJ, USA) was given in the afternoon. The following day was considered embryo day 0 and the patient received 50 mg of progesterone in the morning and 50 mg progesterone in the evening. As before, thawing and embryo transfer were carried out 5 days later. In either case, at least 50 mg of progesterone was given daily to support the luteal phase.

Results

Eighty-six consecutive blastocyst transfers were included in the study. Forty-four cycles with day 5 blastocysts and 42 with

day 6 blastocysts (transferred on embryo day 5 of the patient's cycle). Seventy-one day 5 SF blastocysts were thawed, resulting in the survival of 59 blastocysts (83.1%). An average of 2.5 SF surviving blastocysts were replaced per embryo transfer. Four singleton pregnancies were achieved from 24 transfers (16.7%).

Concurrently, 41 day 6 VIT blastocysts were thawed and all 41 survived the thawing process (100%). An average of 2.0 VIT blastocysts were replaced per embryo transfer. Four twin and six singleton pregnancies were achieved from 20 transfers (50.0%). A comparison is shown in **Table 1**.

Seventy-six day 6 SF blastocysts were thawed and 68 survived the thaw (89.5%). An average of 2.5 SF blastocysts were replaced per embryo transfer. Five singleton pregnancies were achieved from 27 transfers (18.5%).

During the same period of time, only 36 day 6 VIT blastocysts were thawed, and all 36 survived (100%). An average of 2.4 day 6 VIT blastocysts was replaced per embryo transfer. Two twin and three singleton pregnancies were obtained from 15 transfers (33.3%). A comparison is shown in **Table 2**.

Upon thawing, a clear difference was seen between the SF and VIT blastocysts. In less than 5 min of thawing, most VIT blastocysts appeared fully expanded (**Figure 1a**); in contrast, the SF blastocysts were seldom re-expanded at the time of embryo transfer (**Figure 1b**).

Table 1. Day 5 cryopreserved blastocyst results.

	<i>Slow freezing</i>	<i>Vitrification</i>	<i>P-value</i>
No. embryo transfers	24	20	
Survival rate (%)	83.1 (59/71)	100 (41/41)	<0.01
Mean no. embryos/embryo transfer	2.5	2.0	
Pregnancy rate/embryo transfer (%)	16.7 (4/24)	50.0 (10/20)	<0.02
Implantation rate (%)	6.8 (4/59)	34.1 (14/41)	<0.01

Table 2. Day 6 cryopreserved blastocyst results.

	<i>Slow freezing</i>	<i>Vitrification</i>	<i>P-value</i>
No. embryo transfers	27	15	
Survival rate (%)	89.5 (68/76)	100 (36/36)	<0.04
Mean no. embryos/embryo transfer	2.5	2.4	
Pregnancy rate/embryo transfer (%)	18.5 (5/27)	33.3 (5/15)	
Implantation rate (%)	7.4 (5/68)	19.4 (7/36)	<0.07



Figure 1. (a) A vitrified, human day-5 blastocyst immediately post-thaw. (b) A slow-frozen, human day-5 blastocyst immediately post-thaw.

Discussion

The difference seen between the SF and VIT blastocysts upon thawing is thought to be due to the lack of blastocole collapse during the vitrification process. During slow freezing, dehydration occurs over a long period of time as ice crystal formation in the medium concentrates the extracellular sucrose. While this process facilitates cellular dehydration, it allows time for blastocole shrinkage/collapse as well. The rapid dehydration and freezing (occurring in under 1 min) of vitrification does not allow time for a complete collapse of the blastocole. Therefore, it appears much easier for the vitrified blastocyst to return to its original expanded state. Another observation noted a perceptible difference in the blastomere survival between the two groups whilst specifically comparing the viable embryos chosen for transfer. Individual VIT blastocysts lost an average of <2% of their cell mass, based on individual membrane integrity, while SF blastocysts lost an average of 12% of their individual blastomeres. Due to the belief that the most effective recovery will occur *in vivo*, it was decided to return surviving blastocysts to the uterus within 30 min of thawing, based on percentage of blastomere survival rather than re-expansion. Presumably, these two phenomena, lack of complete blastocole collapse with the ability to re-expand quickly and the higher retention of membrane integrity, are indicators of the viability and/or elucidate the observed SF blastocyst implantation reduction.

The reduced implantation rate of blastocysts frozen on day 6 was noted regardless of the fact that transfer took place 5 days after ovulation, in an effort to improve synchronization with the endometrium. It is thought that this is a reflection of the overall potential of slower developing embryos compared with

embryos reaching the blastocyst stage by day 5, rather than an effect of either cryopreservation method. This is demonstrated by the fresh blastocyst transfer rates, which consistently show higher implantation of day 5 blastocysts over day 6 blastocysts.

As well as the increase in survival, pregnancy and implantation rates, vitrification offers several other benefits. The first major advantage is its versatility. Vitrification, as well as SF, has been used to cryopreserve fully hatched blastocysts within this study and by other researchers (Quintans *et al.*, 2003; Hiraoka *et al.*, 2004). Two fully hatched blastocysts included in this study were vitrified without changing the protocol (one for each of two patients) and thawing resulted in 100% survival of both the blastocysts and the individual trophectoderm and inner cell mass cell, as observed via light microscopy. One of these two transfers resulted in a pregnancy and both transfers also included a zona intact blastocyst, therefore it was not possible to determine the actual outcome of the fully hatched blastocyst.

Vitrification has also been used to successfully cryopreserve human oocytes (Kuleshova *et al.*, 1999; Yoon *et al.*, 2000; Katayama *et al.*, 2003). Only slight modifications were made to the blastocyst vitrification protocol based upon the individual oocyte reaction times (see explanation below) and the data (Katayama *et al.*, 2003) showed 94 and 91% respectively for survival and fertilization rates of vitrified-thawed human oocytes.

In addition to the rewards for patients in terms of success rates, vitrification also benefits the embryology laboratory. The most obvious is the reduction in time spent cryopreserving blastocysts. Specifically, the SF process requires

approximately 3 h to complete, whereas VIT takes approximately 5–10 min. The VIT time range is given due to the fact that there are no standardized times for each step; instead, the process progresses based on reactions seen in the individual blastocysts. Furthermore, a second benefit is foregoing dependence on controlled rate freezers. The VIT process is relatively simple, and therefore requires no expensive, specialized equipment to purchase and maintain.

Paramount to the VIT process is the ability to freeze the blastocysts as quickly as possible once liquid nitrogen is introduced. In addition to the Cryotops used in this study, several other methods to accomplish this have been described. These include the Cryoloop (Mukaida et al., 2003), an open-pulled straw (Isachekno et al., 2003), and the use of an electron microscope grid (Cho et al., 2002). All are designed to provide storage of the blastocysts in the smallest attainable volume in order to facilitate the highest possible freezing rate.

The vitrification of day 5 and day 6 blastocysts has been exceptionally successful for patients at the clinic. Survival, pregnancy and implantation rates of the day 5 VIT blastocysts have significantly improved as compared with the day 5 SF blastocysts. Since fewer average numbers of VIT blastocysts were transferred per cycle and 40% of pregnancies resulted in twins, the aim is towards single embryo transfer of day 5 VIT blastocysts in the future. Although the results from day 6 blastocysts that were transferred on embryo day 5 of the patient's cycle are promising, the numbers were too small to achieve statistical significance. Moreover, the data would indicate a trend towards utilizing vitrification for all blastocysts cryopreserved in the laboratory to further maximize cryopreservation efficacy for the patients.

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