Clinical outcome of day 5 and day 6 blastocyst vitrification

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Abstract

Objective:
to compare survival, clinical pregnancy and ongoing pregnancy rates of blastocysts vitrified on day 5 and those which had one day delay and vitrified on day 6.

Materials & Methods:
The study included 210 vitrified warmed cycles, 135 patients underwent vitrifivation at day 5 (group I) and 75 patients at day 6 (group II). Blastocyst survival and clinical pregnancy/embryo transfer were primary outcomes. Ongoing pregnancy/embryo transfer was the secondary outcome.

Result(s)
blastocyst post-warming survival rates were comparable between both groups (92.9% (263/283) of day 5 versus 94.9% (166/175) of day 6 blastocysts). There was no statistically significant difference between the 2 groups regarding the mean number of transferred blastocysts. Clinical pregnancy rates were 40.6% (52/128) & 43.7% (31/71) in women who undergone vitrification at day 5and day 6 respectively with no significant differences. Similarly, ongoing pregnancy rate was comparable between the 2 groups, 37.5% (48/128) versus 39.4% (28/71) in groups I & II.

Conclusion(s):
blastocysts vitrified on day 5 have the same survival, clinical and ongoing pregnancy rates of blastocysts which had one day delay and vitrified on day 6.

Key words:
Blastocyst transfer, clinical pregnancy, vitrification.

Introduction

Cryopreservation has become an increasingly important therapeutic strategy in reproductive medicine, with the birth of many infants after use of this procedure. It is important for cryopreservation in general to establish consistent outcomes, especially in terms of embryo cryosurvival to allow high chances of success in performing a frozen embryo transfer (FET). However, standard cryopreservation technologies appear to illustrate their ultimate limitations in their lack of consistency in cryo-survival. Actually, interest has shifted to vitrification as an attractive alternative to slow-freezing methodology (1) and vitrification is now the preferred method of cryopreservation in many centers (2, 3).

It is as an ultra-rapid cooling technique that is simple, potentially faster, starting to become clinically established and seems to have the potential to be more reliable and consistent than conventional cryopreservation when carried out properly (4, 5). Further, the need for controlled-rate freezing equipment, which requires routine calibration and maintenance, is eliminated. The cells are placed into the cryoprotectant, then the cells are placed in a very small volume of cryoprotectant on a special carrier, and then they are cooled at extreme rates by plunging them directly into LN2. With this method, no ice crystals form with avoidance of damage to the cells or the tissues. Actually, Lack of ice crystallization and convenience of the procedure itself are two major advantages which changed entire cryopreservation program of many centers from conventional freezing to vitrification only (2, 3).

With the introduction of sequential culture media in ART, and driven by the large increase in the rate of multiple pregnancies arising from earlier-stage ET, extended culture to the blastocyst stage has become more common. The best available evidence suggests that the probability of pregnancy, implantation and live birth rates after fresh IVF is significantly higher after blastocyst-stage embryo transfer as compared to cleavage-stage embryo transfer (6, 7). However, possibility of some embryos not developing into blastocysts in vitro and as a result cancellation of embryo transfer should be considered. So, blastocyst transfer policy should be applied in good prognosis patients (6, 7). With this concept, many centers have shifted to blastocyst transfer. Consequently, the need to cryopreserve human blastocysts is also increasing. Although the results achieved by conventional slow freezing seem successful (8-10), clinical results with blastocyst cryopreservation have not necessarily been consistent, owing to the higher potential for damaging ice crystal formation in traditional slow-freezing protocols. So, there have been an increasing number of reports of successful human blastocyst vitrification (11-15).

Generally, if there is failure in achieving pregnancy after initial transfer of fresh blastocysts, surplus vitrified blastocysts would be transferred in a subsequent cycle.

Moreover, there have been suggestions that, fresh BT cycles might be canceled for patients who have exhibited poor endometrial receptivity or ovarian hyperstimulation syndrome. Under such circumstances, all available fresh blastocysts would be vitrified for transfer in a subsequent cycle (16). Importantly, previous investigators have found superior implantation rates with fresh transfers occurring at day 5 as compared with day 6. They reported an almost doubled clinical pregnancy and implantation for fresh day 5 blastocyst compared with fresh day 6 blastocysts (17). The one-day delay in expansion was considered in itself an indication of inferior viability.

A pertinent question is whether extra blastocysts which were vitrified on day 5 or the ones which had required 6 days to reach expanded blastocyst and vitrified on day 6 have the same or different embryonic developmental potential upon warming. So, the objective of the current study is to compare survival, clinical pregnancy and ongoing pregnancy rates of blastocysts vitrified on day 5 and those which had one day delay and vitrified on day 6.

Materials & Methods

From October 2007 to November 2010, 210 vitrified-warmed BET cycles were evaluated. 135 women had undergone blastocyst vitrification on day 5 and 75 on day 6. All patients included used standard long protocol for controlled ovarian stimulation (COS) and underwent ICSI. In our program, women who have ≥ 4 grade one embryos (i.e. regular symmetrical blastomeres with no fragmentation) on day 3 after retrieval (18) are counseled for extended culture and BET. Ovarian stimulation was performed as previously reported (19).

Embryo Scoring

Embryos reaching the blastocyst stage, whether on day 5 or day 6, were graded by using the system of Gardner and Schoolcraft (20). Blastocysts were given a number based on the degree of expansion and hatching status (from 1 to 6): 1 = early blastocyst: the blastocele accounts for less than one-half of the volume of the embryo; 2 = blastocyst: the blastocoel occupies more than one-half of the volume of the embryo; 3 = full blastocyst: the blastocoel fills the embryo completely; 4 = expanded blastocyst: the blastocoel is now larger than the early embryo, and the zona pellucida has begun to thin; 5 = hatching blastocyst: trophectoderm (TE) cells have begun to herniate through the zona pellucida; and 6 = hatched blastocyst: the blastocyst has completely escaped the zona pellucida. For blastocysts regarded to be full blastocysts and onward (grades 3–6), a second scoring step was performed under an inverted microscope to assess the inner cell mass (ICM) and the TE. For the ICM, the following descriptions are used: A = tightly packed with many cells; B = loosely grouped with several cells; and C = very few cells. For the TE, the following grading is used: A = many cells forming a cohesive epithelium; B = few cells forming a loose epithelium; and C = very few large cells. Extra blastocysts were only considered for vitrification if they were regarded to be full blastocysts and onward (grades 3–6), Inner cell mass (ICM) scored A-B and trophoectoderm (TE) scored A-B.

Protocol for Vitrification and Warming

Vitrification of blastocysts was undertaken using the Cryoloop carrier system (Vitrolife, Sweeden) 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 solution with 20% serum supplement; Irvine Scientific, USA) containing 7.5% Ethylene glycol (EG) and 7.5% DMSO. After 8–13 minutes, the blastocysts were washed quickly in vitrification solution, which is the base medium containing 15% DMSO, 15% EG, and 0.5 mol/L sucrose. These 2 solutions were to be used in sequence according to the step-wise microdrop vitrification protocol. Importantly, blastocysts were exposed to the vitrification solution ≤30 seconds. From last microdrop, 1-3 blastocysts in < 1uL media was loaded into the Cryoloop carrier, capped under the LN2 with the cryovial immerged in the LN2 till final storage.

Patients not achieving a clinical pregnancy returned for a frozen blastocyst transfer cycle. All women received letrozole (Femara, Novartis), one tablet (2.5 mg)/day, starting from day 3 of the cycle for 5 days. When dominant follicle reached ≥18mm and endometrium thickness≥8mm, 10000 IU of HCG were given (day 0). Vaginal administration of progesterone (cyclogest, Florham Park, NJ) was initiated on day HCG+3 (usually 4 days before the frozen blastocyst transfer was scheduled).

On day of vitrified blastocyst transfer, to remove the cryoprotectants, blastocysts were warmed and diluted in a two-step process. With the Cryoloop submerged in LN2, the protective cap was removed and placed directly into a pre-warmed (approximately 30°C) organ culture dish containing thawing solution (HEPES buffered solution containing gentamycin sulphate, 1.0 mol/L sucrose and 20% serum supplement).After 1 minute, blastocysts were transferred to dilution solution (HEPES buffered solution containing gentamycin sulphate, 0.5 mol/L sucrose and 20% serum supplement) for 4 minutes. Then, blastocysts were transferred to the washing solution (HEPES buffered solution containing gentamycin sulphate and 20% serum supplement) for 9 minutes and then returned to the culture medium (Sage Blastocyst Medium) until transfer. Whether vitrification was performed on day 5 or day 6, one to three blastocysts were transferred into the patient's uterus on day HCG+7.

Serum β-hCG tests were performed two weeks after ET and transvaginal ultrasound (US) were scheduled three weeks later to confirm a clinical pregnancy. Spontaneous abortion was defined as the spontaneous loss of a clinical pregnancy before 20 completed weeks of gestational age (21). Clinical pregnancy rate was defined as the number of clinical pregnancies expressed per 100 embryo transfer cycles (21). On-going pregnancy rate was defined as the number of clinical pregnancies, continuing beyond 20 weeks of gestation and expressed per 100 initiated embryo transfer cycles.

Outcome measures

Blastocyst survival and clinical pregnancy/embryo transfer were primary outcomes. Ongoing pregnancy/embryo transfer was the secondary outcome.

Data were statistically described in terms of mean ± standard deviation (SD), frequencies (number of cases) and relative frequencies (percentages) when appropriate. Analysis was carried out by means of a X2 test using computer programs Excel version 7 (Microsoft Corporation, NY, USA). Statistical significance was defined as $P<0.05$.

Results

The study included 210 vitrified warmed cycles, 135 patients underwent vitrification at day 5 (group I) and 75 patients at day 6 (group II).Table 1 shows the mean age and clinical outcome of patients who completed the vitrified blastocyst transfer program. No significant differences could be observed regarding age in the two groups. Of 135 women who had vitrification at day 5, 128 women underwent warmed BET (94.8%, 128/135). Meanwhile, 71 of the 75 women who had vitrification at day 6 had undergone warmed BET (94.7%, 71/75) with no significant differences between the

2 groups. Regarding the blastocyst post-warming survival rates, 92.9% (263/283) of day 5 blastocysts and 94.9% (166/175) of day 6 blastocysts survived after warming and this difference was not significant. 260 blastocysts were transferred in first group, while 145 blastocysts were transferred in second group with no statistically significant difference between the 2 groups regarding the mean number of transferred blastocysts. Clinical pregnancy rate was 40.6% (52/128) in women who undergone vitrification at day 5 and was 43.7% (31/71) among those who had vitrification at day 6 with no significant differences. Similarly, ongoing pregnancy rate was comparable between the 2 groups, 37.5% (48/128) versus 39.4% (28/71) respectively in groups I & II.

Discussion

Data from the present study suggest that, blastocysts which had shown one day delay and vitrified on day 6, results in similar survival, clinical and ongoing pregnancy rates when transferred in subsequent cycles compared to transfer of blastocysts vitrified on day 5.

Previous studies have demonstrated that fresh embryos reaching the blastocyst stage and transferred on day 5 had a significantly higher pregnancy rate than those blastocyst embryos transferred on day 6 (17). We recently performed a study (submitted for publication) upon174 patients who had undergone BET on day 5 and 22 participants who did not have expanded blastocysts on day 5 and were left for one day, and all developed expanded blastocysts and had undergone BET on day 6. Blastocysts transferred on day 5 implanted at nearly twice the rate of blastocysts transferred on day 6 (40% vs. 19%, $P < 0.05$). Pregnancy rates were also almost twice as high in day 5BET {106/174 (60.9%)} than those undergoing day 6BET {7/22(31.8%)}. Similarly, ongoing pregnancy/ live-birth rates were also higher in first group{91/174(52.3%)} than in those undergoing day 6BETgroup{6/22(27.3%)} Actually, Shapiro et al. present provocative retrospective data suggesting that synchrony of embryo and endometrial development may be an important factor in pregnancy rates following blastocyst transfer (17).

So, the transfer of blastocysts which had shown one day delay in expansion and transferred on day 6 might result in embryo-endometrial dyssynchrony. Moreover, it might be suggested that, the more slowly developing blastocysts could be innately compromised to some extent. Importantly, Embryos that were vitrified on day 6 were required to be expanded blastocysts and, before they were transferred, must have survived the warming process. These requirements may have selected better-quality embryos than day 6 blastocysts transferred in the fresh cycle. It appears that, there is profound clinical value in knowing they can be vitrified as late as day 6, successfully warmed and result in ongoing pregnancy. Additionally, it is plausible that a more synchronous transfer of these warmed blastocysts contributed to the good outcome.

In accordance with current study findings, Richter et al, suggested that blastocysts cryopreserved on day 6 resulted in similar pregnancy rates when transferred to artificially prepared endometrium in cryopreserved cycles or in donor egg cycles, compared to transfer of blastocysts cryopreserved on day 5 (22). So, with the reported high clinical and ongoing pregnancy rates following vitrified-warmed transfer of day 6 blastocysts, it might be a good policy to encourage vitrification of supernumerary embryos reaching the blastocyst stage beyond day 5. In the meantime, this study should stimulate further investigation in this field in the ongoing quest to improve outcomes from in vitro fertilization and ICSI.

There are other issues with vitrification that need further discussion. Concerns about introduction of high concentrations of cryoprotectant, which are necessary to prevent mechanical damage from ice, exist with vitrification. The problem of cryoprotectant toxicity is an immediate and practical one, just as it is to a lesser extent in classic slow- cooling procedures. Extremely rapid cooling allows a decrease to be made in the concentration of the cryoprotectant and thereby a reduction in potential toxicity (23).

The greatest advantages of vitrification have been seen in chill-sensitive cells such as oocytes and blastocysts (24). The main characteristic of the blastocyst is its fluid-filled cavity, the blastocele. It has been reported that, with increasing volume of the blastocelic cavity, the survival rate drops with vitrification. This is thought to be due to insufficient permeation of cryoprotectant into the blastocelic cavity, such that residual water may promote ice crystallization during the vitrification process. Several articles report that survival rates in cryopreserved expanded blastocysts could be improved by artificial reduction of the blastocelic cavity (12-14, 25-26). In our protocol and others (5, 16), we proceeded without any opening in the zona pellucida before vitrification independent of the size of the blastocelic cavity. The previous concern appears theoretical rather than practical and proceeding without blastocele collapse spares extra-procedure with a comparable survival and PR (5, 16).

Another concern has been made that fungi, bacteria, and viruses are able to survive in LN2 (27-29). Given that with vitrification the cells are directly plunged into LN2, they therefore have direct contact with LN2 and so the question arises as to whether the LN2 has to be sterilized because it may be a possible source of contamination. Use of clean LN2 for the initial vitrification step, followed by sealing of the carrier, seems to address the concern of potential contamination during cryostorage. To further reduce fears of contamination, it is possible to store material from potentially infectious patients separately from seemingly noninfectious samples. Therefore, it is important to perform routine screening tests for viral infections, including hepatitis B and C, on all couples undergoing infertility treatment. In the event that a couple screens positive, we offer vitrification of blastocysts. Even though we consider the risk of cross contamination during storage to be almost infinitesimal, in such cases we nevertheless recommend placing embryos in specially designated tanks, or shipping them off-site. It is worth noting that to date no viral, fungal, or bacterial contamination event has been described from many publications related to vitrification since 1985.

So, concerns about vitrification are well defined, limited in number, and easily surmountable. In general, with much shorter protocols, vitrification [1] is able to be undertaken on a more flexible basis by laboratory staff, [2] allows for the potential reduction in personnel time needed during the entire vitrification process, [3] simplifies laboratory techniques for cryopreservation in human ART, and [4] may enable more optimal timing of embryo cryopreservation, e.g., individual blastocysts may be cryopreserved at their optimal stage of development and expansion. Interest levels will inevitably rise, given the potential benefits of vitrification. This in turn will drive its development to higher levels of clinical efficiency and utilization (1, 31-32).

In conclusion, blastocysts vitrified on day 5 have the same survival, clinical and ongoing pregnancy rates of blastocysts which had one day delay and vitrified on day 6.

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Table I: Vitrification/warming data of blastocysts vitrified on day 5 and day 6.

	Day 5(n=135)	Day 6(n=75)	P
Age (years)	32 ± 3.1	31.8 ± 3.9	0.84
Transfer cycles	128 (94.8%)	71 (94.7%)	1
Survived blastocysts	263/283 (92.9%)	166/175 (94.9%)	0.41
Blastocysts transfer	2.03 ± 0.5	2.04 ± 0.4	0.88
CP/ET	52(40.6%)	31(43.7%)	0.67
OP/ET	48(37.5)	28(39.4%)	0.78

CP=clinical pregnancy, ET=embryo transfer, OP= ongoing pregnancy. Data presented as mean ± SD unless otherwise specified. P > 0.05 non-significant