Comparison of clinical outcomes following vitrified warmed day 5/6 blastocyst transfers using solid surface methodology with fresh blastocyst transfers

K Muthukumar, Mohan S Kamath, Ann M Mangalaraj, TK Aleyamma, Achamma Chandy, Korula George
Reproductive Medicine Unit, Christian Medical College, Vellore, Tamil Nadu, India

Address for correspondence:
Dr. Mohan S Kamath, Reproductive Medicine Unit, Christian Medical College Hospital, Vellore - 632 004, Tamil Nadu, India.
E-mail: dockamz@gmail.com

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ABSTRACT

OBJECTIVES: The literature regarding clinical outcomes following day 5/6 vitrified warmed blastocysts transfer has been conflicting. We decided to evaluate and compare the clinical outcomes following vitrified warmed day 5/6 blastocyst transfer using a solid surface vitrification protocol with fresh blastocyst transfers. **SETTINGS:** University teaching hospital. **STUDY DESIGN:** A total of 249 women were retrospectively analyzed: 146 fresh day 5 blastocyst (group 1), 57 day 5 vitrified warmed blastocyst (group 2), and 46 vitrified warmed day 6 blastocyst (group 3) transfer cycles. Vitrification was done using solid surface methodology (non immersion protocol). The main outcomes were implantation rates, clinical pregnancy, and live birth rate per embryo transfer. **RESULTS:** The baseline clinical characteristics were similar among all three groups. The implantation and clinical pregnancy rates following vitrified warmed day 6 blastocyst transfers (20.9% and 32.6%) were significantly lower as compared to day 5 fresh and vitrified warmed day 5 blastocyst transfers (40.3% and 56.1%, 36.3%, and 52.6%). However, there was no significant difference in the live birth rates across the three groups (group 1: 37.6%, group 2: 40.3%, and group 3: 28.2%). **CONCLUSION:** No statistically significant difference was observed in live birth rates between fresh day 5 blastocyst transfers and vitrified warmed day 5/6 blastocyst transfers. Vitrification of blastocysts using solid surface methodology is an efficient method of cryopreservation.

KEY WORDS: Blastocyst, solid surface methodology, vitrification

INTRODUCTION

Vitrification is a well-established method of cryopreservation. In addition to the advantage of lack of ice formation, it has a better cryosurvival rate and does not require expensive equipments when compared to slow freezing.[1-3] However, toxicity due to the use of high concentration of cryoprotectants and the possible risk of viral contamination are some of the concerns associated with vitrification.[2,3] Over the years, different carriers and loading devices have been introduced in both the open and closed methods of vitrification.[1,3-6] Kader *et al*. has reviewed the efficacy and safety of the different carrier systems used during blastocyst vitrification.[4]

Blastocyst transfer is associated with higher pregnancy and live birth rates as compared to cleavage stage transfer.[7-9] With an increasing emphasis on single embryo transfer, a blastocyst stage embryo transfer would be the preferred option.[10] An efficient cryopreservation program to store supernumerary embryos would further maximize the pregnancy rates.

A few studies have compared the laboratory and clinical outcomes following vitrified day 5 and day 6 blastocyst transfer cycles, but the findings have been conflicting.[2,11,12] Liebermann *et al*. concluded similar clinical outcomes following vitrified day 5 and day 6 blastocyst transfers as opposed to Levens *et al*., who found lower implantation and pregnancy rates following vitrified day 6 blastocyst transfers.[2,11] A recent systematic review, which looked at frozen day 5 and day 6 blastocyst transfer outcomes concluded that slower developing blastocysts,

cryopreserved on day 6, at the same development stage as those preserved on day 5, have similar clinical outcomes, although the majority of studies included used slow-freeze methodology.[13]

When introduced initially, vitrification was done using an open method, whereby a droplet of media containing the blastocyct was immersed directly into liquid nitrogen. Fearing viral transmission, measures such as the use of sterilized or clean liquid nitrogen during the procedure and subsequent storage in hermetically sealed straws were introduced to decrease the risk.[3,14] In the closed system, the embryos do not come in direct contact with liquid nitrogen, a process desirable in terms of decreasing the risk of transmission. Although the cooling rates achieved are slower with the closed system, the clinical outcomes are promising.[3,4] In the solid surface vitrification (SSV) system, the droplet containing the blastocyst is brought in contact with a pre-cooled metal block, thus avoiding direct contact with liquid nitrogen. SSV has been found to be a highly efficient method for cryopreserving embryos and oocytes.[15,16] We used the SSV that is comparable to the closed system since the embryos do not come in direct contact with liquid nitrogen during the procedure.

We decided to evaluate and compare the clinical outcomes of fresh day 5 blastocyst transfer and day 5/6 blastocyst vitrification transfer cycles using SSV.

MATERIALS AND METHODS

All women undergoing either a fresh or a vitrified warmed blastocyst transfer between 2009 and 2011 were analyzed.

ART cycles were carried out using either the standard long protocol or the antagonist protocol. Controlled ovarian hyper-stimulation (COH) was initiated using recombinant gonadotrophins. Oocyte retrieval was planned 35 h after hCG administration. Retrieved oocytes were incubated for 3-4 h in fertilization medium (SAGE fertilization medium, Trumbull, Connecticut, USA). Group culture and short incubation (2 h) was followed in *in vitro* fertilization (IVF). ICSI was performed after denudation of oocytes. Fertilized oocytes were transferred into cleavage medium (SAGE cleavage medium, Trumbull, Connecticut, USA), incubated in bench top incubators (MINC, Cook IVF, Eight Miles Plains, Australia) with triple gas mixture (6% carbon dioxide, 5% oxygen, and 89% nitrogen) and observed for cleavage on day 3. On day 3, if less than four grade 1 embryos were obtained, embryo transfer was performed and supernumerary embryos were cultured till day 5/6.

On day 3, if four or more grade 1 embryos were obtained, they were transferred into blastocyst medium (SAGE blastocyst medium, Trumbull, Connecticut, USA) and cultured in until day 5. Embryo selection and transfer was carried out on day 5. The numbers selected for transfer depended on the clinical situation and embryo quality, but were never more than 3.

On day 5, if the supernumerary embryos were at the morula stage, they were further cultured until day 6. On day 5 or day 6, each embryo, which had developed to the blastocyst stage, was scored according to Gardner grading system based on the degree of expansion, hatching status, and development of inner cell mass and trophoectoderm.[17] Embryos with a score of 3AA or more were considered good quality and those with score less than 3AA were considered poor quality. Only good quality embryos were chosen for vitrification.

Supernumerary blastocysts were vitrified using solid surface methodology.[15] Blastocysts were placed in equilibrium solution containing 8% ethylene glycol (EG) and 8% dimethyl suphoxide (DMSO) for 1 min and 50 s during which time blastocoel collapsing was achieved by mechanical pipetting. Collapsed blastocysts were then placed in vitrification solution containing 16% EG, 16% DMSO, and 0.68 M Trehalose for 30 s, loaded in a drop (3 μl) onto a sterile fiber plug carrier (Cryologic, Victoria, Australia) and brought in contact with sterile surface of pre-cooled metal block (Cryologic, Victoria, Australia), causing glassy bead formation. The fiber plug loaded with the vitrified blastocysts were put in a pre-cooled sleeve and kept in the cryobank.

Warming was done in a step-wise manner by removing the cryoprotectant using in-house filter sterilized trehalose (0.33 M and 0.22 M) and blastocyst medium.[15] After an hour, survival was assessed under an inverted microscope (×400) by evaluating the number of viable trophoectodermal, inner cell mass cells, and the degree of re-expansion of the blastocoel cavity. Laser hatching was then carried out and the blastocysts incubated for a further 2-3 h prior to transfer. Assisted hatching was performed only on vitrified-warmed blastocysts.

Women who were planned for transfer of vitrified blastocysts were started on estrogen valerate (Progynova, Schering AG, Germany) 2 mg, daily once, from the first day of the periods and the dose was increased to 4 mg (days 6-9) and subsequently 6 mg daily (days 10-15). Transvaginal ultrasound scan was done on day 15 for assessment of endometrial thickness. Vaginal progesterone pessaries (Orgagest, Schering AG, Germany) 800 mg daily, were initiated once an endometrial thickness ≥7 mm was documented. Transfer of one to three blastocysts that survived was done on the 6th day following initiation of progesterone therapy, after pre transfer counseling.

Pregnancy was detected by doing a serum beta hCG on day 12 after embryo transfer.

Women with a positive β-hCG (>5 mIU/ml) were advised to continue both estrogen and progesterone supplementation, and a transvaginal ultrasound was carried out 2 weeks later to confirm clinical pregnancy (documented intrauterine gestational sac) and fetal viability (presence of a fetal cardiac activity). If confirmed, antenatal care was provided and the women were followed until delivery. Hormone supplementation was stopped after 12 weeks.

Women who delivered elsewhere were contacted and information regarding the pregnancy outcome was obtained.

Vitrified warmed cycles in which both day 5 and day 6 vitrified blastocysts were transferred together were excluded from the analysis to maintain homogeneity. Institutional review board approval was not taken due to retrospective nature of the study.

Patients were divided into three groups: Group 1 (fresh day 5 blastocyst transfer), group 2 (day 5 vitrified warmed blastocyst transfer), and group 3 (day 6 vitrified warmed blastocyst transfer).

Data was analyzed using SPSS 14 software (SPSS Inc, Chicago, IL, USA). Student's *t* test and Chi-square analysis with Fisher's exact test (one-tailed) was used for parametric and non-parametric data to measure the difference between the groups, respectively, and $P < 0.05$ was considered as significant.

RESULTS

Table 1 shows the mean age and body mass index (BMI) of the patients who underwent fresh day 5 blastocyst transfer and vitrified-warmed day 5/6 blastocyst transfer. There was no significant difference in mean age (group 1: 30.64 ± 3.89 years, group 2: 29.75 ± 4.46 years, and group 3: 30.92 ± 3.85 years) or BMI (group 1: 31.73 ± 8.6, group 2: 25.34 ± 4.73, and group 3: 24.17 ± 4.17) among the three groups. The distribution of patients according to the indication for ART was also similar among the three groups as shown in Table 2.

The cryosurvival rate (85.4% vs. 79.59%), re-expansion (68.7% vs. 56.41%), and hatching rates (20.4% vs. 25.64%) were similar in both vitrified groups (days 5 and 6) as shown in Table 3.

The number of good quality embryos available for transfer was significantly higher in the fresh blastocyst group when compared to vitrified day 5/6 groups. Good quality embryos were significantly lower in number in group 3 when compared to that in group 2.

As shown in Table 4, the clinical pregnancy rates between fresh day 5 blastocyst and vitrified warmed day 5 blastocysts transfer were similar (56.16% vs. 52.63%). The clinical

Table 1: Patient characteristics and laboratory parameters

Parameters	Fresh blastocyst transfer (*n*=146) group 1	Vitrified day 5 blastocyst transfer (*n*=57) group 2	Vitrified day 6 blastocyst transfer (*n*=46) group 3
Female age mean (SD)	30.64 (3.89)	29.75 (4.46)	30.92 (3.85)
BMI mean (SD)	31.73 (8.6)	25.34 (4.73)	24.17 (4.17)
Embryo number transferred Mean (SD)	2.42 (0.57)	2.32 (0.63)	1.87 (0.70) #*P* 0.001 ‡*P* 0.003
Transfered blastocyst Good quality (%) (≥3AA)	286/315 (90.79)	98/120 (81.66) **P* 0.0118	57/84 (67.85) #*P* 0.0001 ‡*P* 0.03
Transfered blastocyst Poor quality (%) (<3AA)	29/315 (9.2)	22/120 (18.33) **P* 0.01	27/84 (32.14) #*P* 0.0001 ‡*P* 0.03

**P* value when comparing fresh day 5 and vitrified warmed day 5 blastocyst transfers groups, #*P* value when comparing fresh day 5 and vitrified warmed day 6 blastocyst transfers groups, ‡*P* value when comparing vitrified warmed day 5 and vitrified warmed day 6 blastocyst transfer groups, Significant *P*<0.05

Table 2: Distribution of cycles according to indication for ART

Indication (%)	Fresh blastocyst transfer (*n*=146) group 1 (%)	Vitrified day 5 blastocyst transfer (*n*=57) group 2 (%)	Vitrified day 6 blastocyst transfer (*n*=46) group 3 (%)
Male factor	49/146 (33.56)	23/57 (40.35)	16/46 (31.57)
Tubal factor	41/146 (28.08)	13/57 (22.80)	17/46 (42.10)
PCOS	26/146 (17.80)	10/57 (17.54)	4/46 (5.2)
Combination	16/146 (10.95)	7/57 (12.28)	5/46 (10.52)
Endometriosis	8/146 (5.4)	2/57 (3.5)	3/46 (7.89)
Unexplained	6/146 (4.1)	2/57 (3.5)	1/46 (2.6)

Significant *P*<0.05; PCOS: Polycystic ovarian syndrome

pregnancy rates were significantly lower in vitrified warmed day 6 blastocyst transfer group (32.60%) when compared to fresh/vitrified day 5 transfers. The miscarriage rates were higher following fresh day 5 transfers (32.92%) as compared to vitrified warmed day 5/day 6 transfers (23.3% and 13.33%), although the difference did not reach statistical significance.

The implantation rates were significantly lower in vitrified warmed day 6 blastocyst transfer group as compared to fresh/vitrified warmed day 5 blastocyst groups (group 3: 20.93% vs. group 1: 40.39%, $P = 0.001$; and group 2: 36.36%, $P = 0.02$).

The preterm delivery rate was significantly higher in vitrified warmed day 6 group when compared to vitrified warmed day 5 blastocyst transfer (38.46% vs. 4.3%, $P = 0.01$). No significant difference was observed when preterm delivery rates were compared between fresh day 5 and vitrified warmed day 5/6 transfers. The multiple pregnancy rates were similar in all three groups.

The live birth rates were similar with no significant difference observed among the three groups (group 1: 37.67%, group 2: 40.35%, and group 3: 28.26%) [Table 4].

DISCUSSION

Over the years, vitrification protocols have been standardized, and, due to its inherent advantages over traditional slow freeze, vitrification has found worldwide acceptance as an alternative cryopreservation method.[3,4] However, concerns regarding toxicity of cryoprotectants and the risk of viral transmission remain.[18,19] Some studies have looked at the neonatal outcome following vitrified blastocyst transfers in order to address concerns regarding safety issues with the use of ethylene glycol as a cryoprotectant and found it safe.[18,20] Classic cryoprotectant DMSO is considered more toxic than other cryoprotectants.[21] However, with widespread usage of vitrification, more information regarding neonatal outcome is available now, and the overall results have been reassuring.[18,20,22]

We obtained similar cryosurvival rates following warming in both day 5 and day 6 blastocyst group (85.46% vs. 79.59%). Earlier studies have found significantly higher cryosurvival in the day 5 blastocyst group as compared to day 6 blastocyst group.[1,23] However, Liebermann *et al*. obtained similar survival rates following warming in the day 5 and day 6 vitrified blastocyst groups.[2]

Shapiro *et al*. found higher implantation and pregnancy rates with transfer of fresh day 5 blastocyst as compared to fresh day 6 blastocyst transfer.[24] However, the results of frozen-thawed day 5 and day 6 transfers have been inconclusive with many studies showing similar outcomes, especially when morphological quality was similar at the time of freezing.[13]

Although Liebermann *et al*. found significant higher implantation rates (33.4 vs. 25.9, $P < 0.01$) for vitrified warmed day 5 blastocyst transfer as compared to day 6 warmed transfers, the live birth/ongoing pregnancy rates were similar.[2] Stehlik *et al*. found significantly lower

Table 3: Comparison of post warming blastocyst parameters between days 5 and 6 vitrified cycles

Laboratory outcome	Vitrified warmed day 5 cycle (%)	Vitrified warmed day 6 cycle (%)
Cryo-survival	147/172 (85.46)	78/98 (79.59)
Re-expansion	101/147 (68.70)	44/78 (56.41)
Hatching	30/147 (20.40)	20/78 (25.64)

Significant $P<0.05$

Table 4: Clinical outcomes

Clinical outcome	Fresh blastocyst transfer (n=146) group 1 (%)	Vitrified day 5 blastocyst transfer (n=57) group 2 (%)	Vitrified day 6 blastocyst transfer (n=46) group 3 (%)
Clinical pregnancy	82/146 (56.16)	30/57 (52.63)	15/46 (32.60) #P 0.006 ‡P 0.04
Singleton	30/82 (36.58)	18/30 (60) *P 0.03	7/15 (46.66)
Multiple pregnancy rate	49/82 (59.75)	12/30 (40)	8/15 (53.33)
Twins	38/82 (46.34)	9/30 (30)	6/15 (40)
Triplets	11/82 (13.41)	3/30 (10)	2/15 (13.33)
Implantation rate	143/354 (40.39)	48/132 (36.36)	18/86 (20.93) #P 0.001 ‡P 0.02
Miscarriage rate	27/82 (32.92)	7/30 (23.33)	2/15 (13.33)
Preterm	9/55 (16.36)	1/23 (4.3)	5/13 (38.46) ‡P 0.01
Livebirth rate	55/146 (37.67)	23/57 (40.35)	13/46 (28.26)

*P value when comparing fresh day 5 and vitrified warmed day 5 blastocyst transfers groups, #P value when comparing fresh day 5 and vitrified warmed day 6 blastocyst transfers groups, ‡P value when comparing vitrified warmed day 5 and vitrified warmed day 6 blastocyst transfer groups, Significant $P<0.05$

pregnancy rates (33% vs. 50%) with vitrified-warmed day 6 blastocyst transfer when compared to vitrified-warmed day 5 transfers.[25] In our study, we found significantly higher implantation rates (36.3% vs. 20.9%, $P < 0.02$) following vitrified warmed day 5 blastocyst transfers as compared to day 6 vitrified warmed transfers, but there was no significant difference in live birth rates (40.3 vs. 28.2%, $P = 0.28$) in both the groups, which was in concurrence with the findings of Liebermann *et al*.[2]

In a recent study, Zhu *et al*. observed statistically significant improvement in implantation rates (37.0% vs. 25.2, $P < 0.05$) and clinical pregnancy rates (55.1% vs. 36.4%, $P < 0.05$) following vitrified warmed blastocyst transfer as compared to fresh blastocyst transfer.[26] The authors have suggested that the reason for higher success rates in vitrified cycles is better endometrial receptivity and synchronization during natural cycles as compared to stimulated cycles, where the estradiol levels are supra physiological. In light of these findings, vitrifying all fresh blastocysts and transferring them in subsequent cycles has been advocated.[12,26]

While our study demonstrated no significant difference in clinical pregnancy rates (56.1% vs. 52.6, $P = 0.32$) between fresh day 5 blastocyst transfer and vitrified warmed day 5 blastocyst transfer, there was a significant difference in clinical pregnancy rate when the vitrified warmed day 6 blastocyst transfer group were compared to fresh blastocyst transfer (32.6 vs. 56.1%, $P = 0.006$) and vitrified day 5 transfer (32.6 vs. 52.63%, $P = 0.04$). The difference might be attributed to the significantly lower mean number of embryos per transfer in vitrified-warmed day 6 blastocyst group as compared to mean number of embryos per transfer in fresh/vitrified-warmed day 5 blastocyst groups. Furthermore, availability of good quality embryos was significantly lower in day 6 vitrified group, which explains the lower implantation rates in vitrified day 6 group. However, more importantly, the live birth rates were similar across the three groups. Even though implantation rates were significantly lower in the vitrified warmed day 6 blastocyst group as compared to the other groups, the lower miscarriage rate (fresh day 5 blastocyst: 32.92%, vitrified day 5 blastocyst: 23.33%, and vitrified day 6 blastocyst: 13.33%) resulted in similar live births among three groups.

In an earlier study focusing on closed system vitrification, higher concentrations of ethylene glycol/DMSO (20%) were used.[19] In contrast, we used lesser concentration of 16% ethylene glycol and 16% DMSO in the vitrification solution in order to minimize the cryoprotectants toxicity. The loading volume of 3 μl is more as compared to other investigators who used 1 μl.[4] Use of larger loading volume in our protocol did not affect the pregnancy rates and made it technically easier for the operator to load the embryos, thereby facilitating learning as well.

Important limitations of our study are the smaller numbers and retrospective nature of the study. Also, the final clinical outcomes need to be carefully interpreted in light of the fact that the embryological parameters like embryo quality and numbers were significantly better in the fresh blastocyst transfer group when compared to vitrified warmed day 6 group. However, the results suggest satisfactory clinical outcomes with vitrified warmed day 6 transfers.

To our knowledge, this is the first study that has reported comparative live birth rates following vitrified warmed day 5/6 blastocyst transfer using SSV (non-immersion protocol). We found similar live birth rates following transfer of vitrified-warmed day 5/6 blastocysts and fresh day 5 blastocysts transfers. SSV is a safe, simple, and effective method of blastocyst cryopreservation.

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