Blastocyst cryopreservation using solid surface vitrification: A preliminary study

ABSTRACT

OBJECTIVE: The objective was to evaluate the effectiveness of a blastocyst cryopreservation program using solid surface vitrification. SETTING: This study took place in a university teaching hospital. STUDY DESIGN: Retrospective observational study. MATERIALS AND METHODS: Women undergoing frozen embryo transfer cycles over a 4-year period between 2006 and 2010 were studied. The cryopreservation policy followed was a vitrification protocol performed at the blastocyst stage, using a solid surface (nonimmersion) method. The post-thaw survival rate, implantation rate, clinical pregnancy rate, live birth rate, and neonatal outcome were recorded. RESULTS: Eighty-one women underwent 86 frozen embryo transfer cycles. Of the 240 blastocysts warmed, 204 survived giving a cryosurvival rate of 85% (204/240). The clinical pregnancy, implantation, miscarriage, ongoing pregnancy, and live birth rates per transfer were 47%, 29%, 12%, 16%, and 23% respectively. Of the 20 live births, there were 16 singletons and 4 twins. Eleven boys and 13 girls were delivered with no major or minor abnormality detected. CONCLUSION(S): The blastocyst vitrification protocol using the solid surface method is effective with results comparable to fresh blastocyst transfers. While retaining the rapid cooling effect, the nonimmersion technique eliminates the risk of contamination and disease transmission. Larger studies with long-term follow-up data would further confirm the efficacy and safety of this method of vitrification.

KEY WORDS: Blastocyst, solid surface method, vitrification

INTRODUCTION

More than 3.5 million babies have been born worldwide using the assisted reproductive technology (ART) with the numbers likely to increase through the widespread use of in vitro fertilization (IVF).[1] Multiple pregnancies, the result of multiple embryo transfers, have been identified as one of the major complications in the field of ART and clearly merit close scrutiny.[2] Apart from increasing maternal and fetal morbidity and mortality, it also increases the total cost incurred per birth, in terms of the prolonged hospital stay and use of intensive care facilities.[3–5] The recognition of this problem together with an improvement in implantation rates has resulted in fewer embryos being transferred. A single embryo transfer would be the ideal way of dealing with this situation even though practical considerations remain while implementing this policy.[6]

In comparison to cleavage stage embryo transfer, blastocyst transfer is associated with higher implantation and pregnancy rates.[7] Clinicians should now be able to counsel couples toward choosing lesser numbers or even opting for single blastocyst transfers, with greater confidence. With fewer blastocysts being transferred, more are likely to be available for cryopreservation.

An efficient cryopreservation program is required to optimize the blastocyst transfer outcomes. Vitrification, currently a well-accepted method of cryopreservation, has the advantage of the lack of intracellular ice formation in comparison to slow cooling methods.[8] The drawback of vitrification is the exposure of embryos to a relatively high concentration of cryoprotectants, raising concerns regarding toxicity and osmotic damage.[9] The transmission of disease due to exposure to contaminated liquid nitrogen
and long-term storage is another issue that needs to be addressed.[10]

An assortment of carriers and loading devices has been introduced in both the open and closed methods of vitrification. Many centers have further modified the techniques and obtained favorable clinical outcomes. We decided to evaluate the efficacy and safety of our vitrification protocol using the solid surface method (nonimmersion).

MATERIALS AND METHODS

Women undergoing IVF–ICSI cycles at a university-level teaching hospital between Oct 2006 and Oct 2010 were included in the study.

We performed embryo transfer on either day 3 or 5 in the fresh IVF cycle depending upon clinical parameters and the number and grade of embryos. Irrespective of the day of transfer, all supernumerary embryos were cultured till the blastocyst stage and good quality blastocysts were cryopreserved using the vitrification protocol.

IVF/ICSI protocol

All patients underwent downregulation using either the long, short, or antagonist protocols with GnRH analogs and controlled ovarian hyperstimulation (COH) with gonadotrophins. Oocytes were retrieved 35 hours after hCG administration. Retrieved oocytes were incubated for 3–4 hours in the fertilization medium (SAGE fertilization medium; USA) and depending upon the clinical situation (indication, number of oocytes, and previous fertilization), a decision for IVF or ICSI was taken. Group culture and short incubation (2 hours) was followed for IVF. The denudation of oocytes (mechanical and enzymatic) was done before ICSI was carried out. Oocytes were incubated overnight in benchtop incubators (MINC; Cook IVF, Australia) with a triple gas mixture (6% carbon dioxide, 5% oxygen, and 89% nitrogen) and observed for fertilization after 16–18 hours. The fertilized oocytes were transferred into the cleavage medium (SAGE cleavage medium; USA), incubated, and observed for cleavage on day 3.

On day 3, if less than 4 grade I embryos (8-cell stage with no or minimal fragmentation) were obtained, embryo transfer was carried out and surplus embryos were transferred into the blastocyst medium (SAGE blastocyst medium; USA) and cultured till day 5/6.

If 4 or more grade I embryos were observed on day 3, they were likewise transferred into the blastocyst medium (SAGE blastocyst medium; USA) and cultured till day 5/6. Blastocyst transfer was performed on day 5; the numbers transferred depended upon the clinical situation and grading of the blastocysts, but never exceeded 3 on any occasion.

Surplus blastocysts obtained after day 3/5 transfer were evaluated on day 5 and good quality blastocysts (grade I/II) were chosen for cryopreservation [Figure 1a]. If development was delayed, embryos were further cultured until day 6 and selected for cryopreservation if the grade was met. Blastocysts were graded using the criteria of Gardner and Schoolcraft.[11]

Vitrification protocol

Supernumerary, moderate, or fully expanded blastocysts with cohesive trophoectoderm (day 5 or 6) were vitrified by using the solid surface vitrification method.[12] We used a precooled metal block (Cryologic, Victoria, Australia), fiber plug (Cryologic, Victoria, Australia), and an in-house prepared equilibrium and vitrification solution which consisted of ethylene glycol (EG; cat no. 10, 246-6; Sigma-Aldrich, Germany), dimethyl sulf oxide (DMSO; cat no. D-2650; Sigma-Aldrich, UK), trehalose (cat no. T0167; Sigma-Aldrich, USA), and a base-buffer solution (HEPES HTF medium, cryobase; Cook IVF, Australia). The equilibrium solution was prepared by dissolving 8% EG and 8% DMSO in a cryobase buffer solution where as the vitrification solution was prepared by dissolving 16% EG, 16% DMSO, and 0.68 M trehalose in a cryobase buffer solution. Using a four-well Nunc dish, the blastocysts (one at a time) were placed initially in the equilibrium solution at 37°C for 2 min.

During this period, using a dissecting microscope, the blastocoel cavity was collapsed mechanically with a small diameter bore pulled pipette, in order to facilitate better permeation of cryoprotectants and prevent ice crystal formation during vitrification.[13] The blastocysts were then transferred to the well containing the vitrification solution for less than 30 seconds, loaded in a 3 µl drop onto a sterile fiber plug, and brought in contact with the sterile surface of a precooled metal block (dry heat sterilized), causing glassy bead formation. The metal block was precooled by placing it in a container containing liquid nitrogen (−196°C) and care was taken to ensure that the liquid nitrogen

Figure 1: (a) Expanded day 5 blastocysts before vitrification. (b) Vitrified day 5 blastocysts after warming and incubating for 3 hours, showing re-expansion and hatching.
level was maintained at a predetermined level and did not come in contact with the upper surface. Touching the upper surface of the metal block with the fiber plug ensured avoidance of direct contact with liquid nitrogen. The loaded fiber plug was positioned in a sterile precooled close-ended sleeve which was locked into place and then transferred into goblets submerged in liquid nitrogen, for storage [Figure 2].

**Warming and assessment of survival**

Warming of vitrified blastocysts was done using an in-house prepared filter sterilized trehalose solution (0.33 and 0.22 M) and blastocyst medium (SAGE blastocyst medium; USA). The warming solution and media were equilibrated overnight in the incubator, transferred into a four-well dish and then placed for at least 15 min in the bench top incubator (MINC; Cook IVF, Australia) prior to utilization. The first and second wells contained 0.5 ml of 0.33 M trehalose while the third well contained 0.5 ml of the 0.22 M trehalose solution. The blastocyst medium, 0.5 ml, was aliquoted in the fourth well.

The Nunc dish was placed on the heated stage of a dissecting microscope. The fiber plug was removed gently from the sleeve and the glassy bead containing the blastocysts was placed into the first well of 0.33 M trehalose, under vision. The blastocysts were immediately transferred into the next well with 0.33 M trehalose solution and washed to prevent the carry-over of cryoprotectants and incubated for 5 min. Next, they were incubated for 5 min in 0.22 M trehalose and finally incubated in the blastocyst medium for another 5 min. The blastocysts were then transferred, washed in 0.7 ml of the blastocyst medium and placed in a 10 µl drop of the blastocyst medium covered with 0.7 ml of mineral oil (SAGE, USA) in a Nunc four-well dish. Survival was assessed under an inverted microscope (×400) by the percentage of the viable trophoectodermal cell and inner cell mass together with the degree of re-expansion of the blastocoel cavity [Figure 1b].

Warmed blastocysts were scored based on the degree of expansion and the hatching status: 1: early blastocyst with a blastocoel <50% of the volume of the embryo; 2: A blastocyst with a blastocoel that is >50% of the volume of the embryo; 3: a blastocyst with a blastocoel completely filling the embryo; 4: a blastocyst with a blastocoel volume larger than that of the early embryo with a thin zone; 5: a hatching blastocyst with the trophoectoderm starting to herniate through the zona; 6: a hatched blastocyst, in which the blastocyst has completely escaped from the zona.[11] Laser hatching (15 µm drill) was done and the blastocysts were incubated for 2 h for re-expansion prior to the transfer.

**Transfer of blastocysts and assessment of the outcome**

All women planned for a frozen embryo transfer cycle were started on an increasing dose of estrogen valerate (Progynova, Schering AG, Germany) from the first day of the period for endometrial preparation. On day 15, transvaginal ultrasound evaluation was done and if the endometrial thickness was more than 8 mm, micronized progesterone (Orgagest vaginal pessaries; Schering AG, Germany) was administered and the transfer planned after 5 days of progesterone therapy. One to three surviving blastocysts were transferred following pretransfer counselling. A serum β-hCG was done on day 12 following the transfer to detect pregnancy.

Women with a positive β-hCG (>25 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 till delivery. Hormone supplementation was stopped after 12 weeks.

Neonatal parameters like birth weight, sex, and major and minor anomalies were recorded. Women who delivered elsewhere were contacted and information regarding the pregnancy outcome was obtained.

The implantation rate was defined as the number of gestational sacs as determined by ultrasound by the number of embryos transferred.

The clinical pregnancy rate was defined as clinical pregnancy (ultrasound visualization of the gestational sac) per embryo transfer.
Live birth rate was defined as the number of deliveries with at least one liveborn baby per embryo transfer.

Data were analyzed using SPSS software (SPSS Inc., Chicago, IL, USA).

RESULTS

Over a 4-year period from October 2006 to October 2010, a total of 957 IVF/ICSI cycles were carried out and a total of 732 blastocysts were cryopreserved. During the same period, 88 cycles of vitrified blastocyst transfer were planned on 83 women and 240 blastocysts were thawed.

In two women, a transfer was not possible as none of the blastocysts survived. A total of 81 women underwent 86 vitrified blastocyst transfer cycles.

The mean female age was 30.3 ± 4.4 years (range 20–39) and the mean body mass index was 24.6 ± 4.4 as shown in Table 1.

The cryosurvival rate for vitrified blastocysts was 85% (204/240). A total of 181 blastocysts were transferred in 86 cycles with the mean number of embryos transferred being 2.2 ± 0.7 [Table 1].

An implantation rate of 29% (53/181) was obtained.

Forty-seven woman had a positive pregnancy test (β-hCG >25 mIU/ml) out of 86 cycles (55%). Forty clinical pregnancies were recorded giving a clinical pregnancy rate of 47% (40/86) per transfer. Among 40 clinical pregnancies, 5 had a missed abortion and 1 woman had an ectopic pregnancy.

Among the clinical pregnancies, there were 22 singletons, 15 twins, and 2 triplets. Of the 15 twin pregnancies, 4 had early single fetal demise (vanishing twin). Both the triplets underwent fetal reduction. The multiple pregnancy rate was 42% (17/40).

Out of total of 20 deliveries, 24 babies were born (11 boys and 13 girls). A live birth rate of 23% (20/86) per transfer was obtained.

8 babies (7 following twin pregnancies) were of low birth weight (<2.5 kg) and no major or minor anomalies were recorded among the newborns.

Presently, 14 pregnancies are ongoing (14/86, 16%; Table 2).

We performed 44 fresh blastocyst transfers in a period of 1 year (2009). The mean number of blastocyst transferred was 2.6 ± 0.6. An implantation rate of 36% (42/118) and clinical pregnancy rate/transfer of 45% (20/44) was obtained.

DISCUSSION

The development of a sequential medium for blastocyst culture and transfer has resulted in better implantation and pregnancy rates. Consequently, an effort to reduce the multiple pregnancy rates has received a fresh impetus. A reduction in the number of embryos transferred without a compromise on the pregnancy rates is an important issue and this has been made possible by the better implantation rates obtained after blastocyst transfers. Limiting the number of embryos in a fresh transfer cycle will result in more good quality embryos being available for cryostorage, necessitating an efficient cryopreservation program. Results with slow freeze protocols have been inconsistent. In addition to being time consuming and laborious, the slow freeze methodology does not completely eliminate the ice crystal injury, which is an important cause of embryo damage.

Vitrification for human embryos was introduced in the late nineties and has been shown to be effective in cryopreserving cleavage and blastocyst stage embryos. Essentially, the vitrification process is carried out by loading

| Table 1: Clinical and laboratory characteristics |
|-----------------------------------------------|
| Parameters                                       | Mean ± SD |
| Female age (years)                              | 30.3 ± 4.4 |
| Body mass index (kg/m²)                         | 24.6 ± 4.4 |
| Number of frozen/thaw cycles planned            | 88        |
| Number of actual transfer cycles                | 86        |
| Number of embryos thawed                       | 240       |
| Number of embryos survived                     | 204       |
| Cryosurvival rate (%)                           | 204/240 (85) |
| Mean number of embryos transferred              | 2.2 ± 0.7 |
| Implantation rate (%)                           | 53/181 (29) |
| Grading of surviving embryos (%)                |           |
| Grade I                                         | 50/204 (24) |
| Grade II                                        | 108/204 (53) |
| Grade III                                       | 46/204 (23) |

| Table 2: Clinical outcomes following frozen blastocyst transfers |
|---------------------------------------------------------------|
| Parameters                                       | Percentage |
| Positive pregnancy/transfer (%)                    | 47/86 (55) |
| Clinical pregnancy/transfer (%)                   | 40/86 (47) |
| Live birth/transfer (%)                           | 20/86 (23) |
| Ongoing pregnancy/transfer (%)                    | 14/86 (16) |
| Miscarriage rate (%)                              | 5/40 (12)  |
| Ectopic rate (%)                                  | 1/40 (2)   |
| Multiple pregnancy rate (%)                       | 17/40 (42) |
| Singleton                                        | 22/40 (55) |
| Twin                                            | 22/40 (55) |
| Triplet                                          | 22/40 (55) |
| Total number of babies (boys:girls) *            | 24 (11:13) |
| Major/minor anomalies                             | Nil        |
| Low birth weight (<2500 g)                        | 8          |

*Values in parenthesis are expressed in ratio
the droplet of media containing the blastocyst(s) onto a carrier system, which is then brought in contact with liquid nitrogen by either an open or closed method. In the open method, vitrification is achieved by directly plunging the droplet containing the blastocyst into liquid nitrogen, thus achieving a very rapid cooling effect.

The introduction of any new technology raises apprehensions regarding safety, and long-term effects and concerns regarding cryoprotectant toxicity and viral contamination have been raised. With time, the vitrification protocol has been refined to make it more efficacious. The efficacy and safety of different carrier systems or loading devices used during blastocyst vitrification have been reviewed recently. Several carriers have been tried in order to refine and improve the cooling rates and post-thaw outcomes like nylon mesh, electron microscopic grids, cryoloop, cryotop, and cryotip. Other methods to improve the cooling rate include the use of slush nitrogen (VitMaster) and the minimal volume cooling (MVC) technique which increase the cost and require more expertise. The potential benefits of an open system of vitrification in terms of higher cooling rates obtained by a direct contact with liquid nitrogen are at least to an extent offset because of the risk of viral transmission. The issue of the risk of contamination from liquid nitrogen by direct contact during cryopreservation and long-term storage has been debated and options of moving toward closed system and sterilizing the liquid nitrogen have been explored. The sterilization of liquid nitrogen using filtration (0.2 μm filters) or ultraviolet (UV) irradiation makes the process more time consuming and laborious, besides increasing the cost.

These concerns lead to the development of the closed method, wherein a direct contact with liquid nitrogen is avoided by covering the carrier system. Although the rapidity of cooling may be compromised and will depend upon factors like heat transfer and the material used to cover the loading system, the closed system minimizes the risk of disease transmission, and results comparable to the open system of vitrification have been demonstrated using cryotip, high-security vitrification (HSV kit; CryoBiosystems, France) and Vitrisafe method. The choice of the storage container depends on the vitrification tool. For safe loading, commercially available kits are available (VitSet, Minitübe, Tiefenbach, Germany). The recently introduced HSV kit ensures a leak-proof seal and is resistant to high pressure.

The solid surface method, which may be considered to be a type of closed method, involves a direct contact of the loaded embryos with a precooled metal block achieving high cooling rates and avoiding the need of plunging embryos into liquid nitrogen. Solid surface vitrification (nonimmersion protocol) was evaluated originally in embryos of different animal species and was found to be effective. A recent study has further confirmed the efficiency of solid surface vitrification in preventing cross-contamination during the procedure and subsequently during storage (using a sealed outer container). The clinical efficacy of this vitrification protocol over slow freeze in human embryos has been evaluated in previous studies.

Vitrification is a demanding process requiring the ability to handle blastocysts gently but with speed. As with all new procedures, a learning curve is inevitable. Nevertheless, a skillful embryologist will be able to achieve competence within a reasonable period of time.

In our study, we have used the fiber plug as a carrier method, and using the closed system of vitrification with the solid surface methodology, we have achieved clinical outcomes comparable to other open and closed systems of vitrification. We were thus able to circumvent the drawbacks of the open method while still utilizing the advantages of rapid freezing. We achieved a cryosurvival rate of 85% which is similar to other studies. Out of 204 surviving blastocysts, 181 (89%) were transferred achieving a clinical pregnancy rate of 47% per transfer, a live birth rate of 23% per transfer, and an ongoing pregnancy rate transfer of 16%. For comparison, we looked at our fresh blastocyst transfer results. An implantation rate of 36% (42/118) and a clinical pregnancy rate/transfer of 45% (20/44) were obtained following fresh blastocyst transfers. These comparable implantation and clinical pregnancy rates of vitrified and fresh blastocyst transfers highlight the efficiency and robustness of the vitrification program.

Our multiple pregnancy rate was 42% with the mean number of blastocysts transferred being 2.2. Although high, this may be attributed to the desire to maintain good clinical outcomes when introducing a new laboratory procedure. The only two recorded triplet pregnancies were in the initial year of the introduction of the program. Having established the efficacy of the solid surface vitrification protocol, we have now evolved new guidelines for vitrified blastocyst transfers restricting the number of blastocysts transferred to 2, similar to our fresh blastocyst transfer policy. For some patients, we have performed elective single blastocyst transfers after counseling. An effective vitrification program makes it easier for clinicians to promote and for couples to accept the concept of limiting the number of embryos transferred.

Few studies have looked at neonatal outcomes following the transfer of vitrified cleavage stage or blastocyst stage embryos. Takahashi et al. in his study looked at the...
neonatal outcome following vitrified blastocyst transfers. He confirmed the safety of the vitrification protocol for routine clinical use and also established the safety of EG as a cryoprotectant.[34] Rama Raju et al. established the efficacy of vitrification as a cryopreservation methodology for cleavage stage embryos.[35] In our study, 24 babies (11 boys and 13 girls) were delivered following a vitrified blastocyst transfer and none were found to have any major or minor congenital anomaly. Though the numbers are small, it is still reassuring and the data obtained are useful in counseling couples who are understandably worried about neonatal outcomes.

The main drawback of the study is the smaller number of cycles evaluated, which makes it difficult to draw any firmer conclusions from it. Though DMSO and EG are commonly used cryoprotectants worldwide, safety concerns remain. However, the neonatal outcome of babies born following vitrification programs using DMSO and EG have been reassuring.[17,36] The concentration of trehalose used (0.68 M) in the vitrification solution in our study was on the higher side. Recent protocols have started using lower concentrations of trehalose/sucrose in the vitrification solution in order to minimize the risks associated with the use of potentially toxic cryoprotectants.[37]

In conclusion, we would like to lay emphasis on the safety and efficacy of using the solid surface nonimmersion protocol. It has the advantage of a rapid cooling effect without a direct contact with liquid nitrogen, thus assuaging fears regarding disease contamination. Good clinical outcomes following a successful vitrification program allows one to consider limiting the number of embryos transferred in both fresh and frozen transfer cycles. However, the numbers studied are few and we would recommend a larger series with longer follow-up to establish safety.

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