Neonatal outcome after vitrified day 3 embryo transfers: a preliminary study

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Objective: To compare and evaluate the neonatal outcome in infants born after vitrified day 3 embryo transfers with that of fresh day 3 embryo transfers.
Design: Retrospective analysis.
Setting: Private assisted reproductive center.
Patient(s): Sixty-nine women, who delivered 89 babies.
Intervention(s): Surplus day 3 cleavage stage embryos were vitrified using ethylene glycol-based solution by cryoloop carrier system and transferred after warming.
Main Outcome Measure(s): Post-thaw survival of embryos, implantation and pregnancy rates (PR), neonatal outcome, and congenital birth defects.
Result(s): A total of 907 vitrified day 3 embryos from 285 cycles were warmed and 817 survived (90.37%), which were used for embryo transfer. The pregnancy, implantation, miscarriage, and live birth rates achieved were 36.84%, 18.11%, 7.71%, and 24.21%, respectively. Of the 69 deliveries, singleton, twins, and triplets comprised of 72.46%, 26.08%, 1.44%, respectively, and 8 (11.59%) were preterm deliveries. The mean Apgar scores were 8.3, 9.2, 9.4 at 1, 5, and 10 minutes. The congenital birth defect rate was 1.18%. These results were comparable with pregnancies using fresh embryo transfers.
Conclusion(s): This preliminary study shows that vitrification is an efficient method of cryopreservation of human day 3 embryos with neonatal outcome comparable to fresh embryo transfers. (Fertil Steril® 2009;92:143–8. ©2009 by American Society for Reproductive Medicine.)

Key Words: Day 3 embryos, vitrification, cryoloop, neonatal outcome, congenital birth defects

Vitrification has shown great promise for cryopreservation of human embryos and is now regarded as a potential alternative to the conventional slow freezing method because of its consistent cryosurvival and successful pregnancy outcomes. In the recent years, as vitrification is being widely used in clinical practice worldwide, concerns regarding its safety have been raised. The major concerns are exposure of the embryos to a high concentration of cryoprotectants (1–3) and possible viral contamination as a result of direct contact of embryos with liquid nitrogen (4, 5). At present, only a few studies addressed the issue of perinatal outcome of pregnancies achieved using vitrified embryo transfers (6, 7). The retrospective study by Takahashi et al. (6) on outcome of pregnancies using vitrified blastocysts showed no adverse effect on the perinatal outcome in comparison with fresh blastocyst transfers. Liebermann and Tucker (7) also reported no congenital birth defects in pregnancies achieved by transferring vitrified blastocysts. However, at present to our knowledge no clinical study has reported the neonatal outcome of infants born using vitrified day 3 embryo transfers. In the present study, we compared and analyzed, retrospectively, the obstetric and neonatal outcomes of infants born after transfer of vitrified day 3 embryos with fresh day 3 embryos.

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MATERIALS AND METHODS

All chemicals were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO) unless stated otherwise.

Patients

Krishna IVF Clinic is a private outpatient facility for infertility management. The practice of vitrification of day 3 surplus embryos was started in the clinic on November 2004. The policy of the clinic is to perform day 3 embryo transfers to patients who undergo IVF/intracytoplasmic sperm injection (ICSI) treatment and the surplus good grade I and II embryos after transfer are vitrified on the same day. Vitrified embryos were warmed and transferred to patients who failed to conceive after fresh embryo transfer. A total of 285 patients (of these, 78 were IVF and 207 were ICSI) underwent vitrified day 3 embryos transfers between May 2005 to June 2007. During the same period, fresh day 3 embryo transfers were performed in 598 patients (of these, 155 were IVF and 443 were ICSI). These patients underwent infertility treatment for polycystic ovary disease (PCOD), endometriosis, tubal factor, low ovarian reserve, and male factor infertility problems. None of these patients had a family history of genetic

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disorders. This retrospective study was approved by Institutional Review Board (IRB), Krishna IVF Clinic.

Stimulation Protocol, Embryo Culture and Grading

Ovarian stimulation was achieved using GnRH agonist (GnRH-a) and recombinant FSH (Recagon; Organon, Hyderabad, India). Human chorionic gonodotropin at a dose of 10,000 IU was given after two follicles of 18 mm or more were visualized in the ultrasound scan. Oocyte retrieval was scheduled 36 hours later by transvaginal ultrasonography (TVS)-guided aspiration. Oocytes were fertilized using either conventional IVF or ICSI and incubated in fertilization media (Sage Biopharma, Chennai, India). After 16–18 hours oocytes were examined for fertilization. Oocytes with two pronuclei (2PN) and having a second polar body were considered as normally fertilized. The fertilized oocytes were washed twice and cultured in cleavage media (Sage Biopharma) for 48 hours. Before transfer, embryos were graded based on the morphological condition using criteria outlined by Veeck (8). The grading system of cleavage stage embryos is as follows: grade 1: embryo with blastomeres of equal size, no cytoplasmic fragments; grade 2: embryo with blastomeres of equal size, minor cytoplasmic fragments or blebs; grade 3: embryo with blastomeres of distinctly unequal size, few cytoplasmic fragments or none; grade 4: embryo with blastomeres of equal or unequal size, significant cytoplasmic fragmentation; grade 5: embryo with few blastomeres of any size, severe or complete fragmentation. Embryo transfer was done on the third day after retrieval and the surplus grade I and II embryos were vitrified.

Vitrification of Day 3 Embryos

The protocol for the vitrification used was a modified version of the cryoloop vitrification protocol reported by Danasouri and Selman (9) and described previously (10). Cryoprotectant solutions and media were warmed briefly in an incubator at 37°C. Both vitrification and warming procedures were performed on a heating stage maintained at temperature of 37°C. After rinsing in HEPES-buffered medium, the embryos were placed in 10% ethylene glycol solution and incubated for 5 minutes. Embryos were then transferred to 40% ethylene glycol in 0.6 M sucrose solution for 30 seconds. Cryoloop was dipped into the same media to create a thin film of vitrification solution (3–5 μL) on the nylon loop. One to three embryos were then immediately loaded onto the nylon loop and directly plunged into a cryovial containing liquid nitrogen. These cryovials were closed tightly and stored in liquid nitrogen tanks.

Warming of Embryos and Assessment of Survival

The embryos were warmed by passing them through sucrose solutions of decreasing osmolality to remove the cryoprotectant and rehydrate the embryos. Warming solutions and media were equilibrated in an incubator at 37°C. With the cryovial submerged in liquid nitrogen, the vial was opened and the loop containing the embryos was removed from the liquid nitrogen and plunged directly into 1.0 M sucrose solution for 2.5 minutes at 37°C. Embryos were then transferred through different concentrations of sucrose solution (i.e., 0.5 M, 0.25 M, and 0.125 M) for 2.5 minutes at each step at 37°C. After warming, the embryos were immediately observed under stereo microscope and assessed for morphological survival. Embryos with $\geq$50% intact blastomeres and no signs of fracture of zona pellucida (ZP) were considered as survived (11, 12). The survived embryos were washed twice in G1 media (Vitrolife, Mumbai, India) and cultured in equilibrated fresh G1 media for 2–3 hours before embryo transfer. At the time of transfer, the thawed embryos were examined again, those still fulfilling the survival criteria were considered for transfer.

Embryo Transfer and Pregnancy Confirmation

For vitrified-warmed embryo transfers, patients were subjected to a controlled cycle using GnRH analogue (Lupride; Sun Pharmaceuticals India Ltd., Vadodara, India) and estrogen replacement (Progynova; Cadila Healthcare Ltd., Ahmedabad, India) for the preparation of the endometrium. On average, 2.87 embryos were transferred to the patient. Luteal support was given by P vaginal suppositories (Uterogestan; Laboratories Besins International, Paris, France). Two weeks after embryo transfer, serum hCG was measured for confirmation of pregnancy, and a diagnosis of clinical pregnancy was made after visualization of fetal heart pulsation 4 weeks later by TVS.

Neonatal Assessment

All the infants delivered in both groups were evaluated for birth weight, Apgar score, and need for admission into the neonatal intensive care unit. Similarly, they were all screened for congenital birth defects and karyotyping to rule out genetic abnormalities.

Statistical Analysis

Retrospective data for the present study were obtained from the patient data base system (File maker pro; FileMaker Inc, Santa Clara, CA) from May 2005 to June 2007. Eighty-nine neonates born from the 69 pregnancies using vitrified embryos were evaluated for congenital birth defects and karyotypic abnormalities. The results were compared with 216 neonates conceived after fresh day 3 embryo transfers resulting from similar stimulation, culture, and transfer protocols during the same period. The data were examined for any differences by χ^2 analysis and Student's *t*-test. A P value of $<.05$ was considered as statistically significant.

RESULTS

In the vitrified embryo transfer group, the mean maternal age was 31.44 $\pm$ 4.75 years. The survival rate of vitrified day 3 embryos after warming was 90.37% (817/904) and there was no decrease in the survival rate after incubation before embryo transfer. The mean number of embryos transferred was 2.87 in 285 cycles. One hundred five (36.84%) pregnancies were achieved, with an implantation rate of 18.11%. Of

TABLE 1

Clinical parameters of fresh and vitrified day 3 embryos transfers.

Parameters	Fresh cycle	Vitrification
Patients' age (y)	32.11 ± 4.61	31.44 ± 4.75
No. of total cycles	604	312
No. of cycles transferred	598	285
No. of embryos transferred	1,576	817
Mean no. of embryos transferred	2.63	2.87
Implantation rate (%)	371 (23.54)	148 (18.11)[a]
No. of clinical pregnancies (% per embryo transfer)	251 (41.97)	105 (36.84)
No. of deliveries (% per embryo transfer)	166 (27.75)	69 (24.21)
No. of miscarriages (% per embryo transfer)	48 (8.02)	22 (7.71)
No. of ongoing pregnancies (% per embryo transfer)	28 (11.15)	11 (10.47)
No. lost follow-up (% per embryo transfer)	9	3

Note: Values are expressed as mean ± SD.
[a] $P < .05$, when compared to fresh cycle.

Rama Raju. Neonatal outcome of vitrified embryos. Fertil Steril 2009.

those pregnancies, the total number of deliveries were 69 (24.21%), 11 were ongoing pregnancies (10.4%) and 22 resulted in miscarriage (7.71%). Three patients (2.85%) were lost to follow-up. In the fresh embryo transfer group, the mean maternal age was 32.11 ± 4.61 years. The mean number of embryos transferred was 2.63 in 598 cycles. Two hundred fifty-one (41.97%) pregnancies were achieved with an implantation rate of 23.54%. Of those pregnancies, the total number of deliveries were 166 (27.25%), 28 were ongoing pregnancies (11.15%) and 48 resulted in miscarriage (8.02%). Nine patients (3.58%) were lost to follow-up. There was no statistical difference in terms of maternal age and pregnancy rates (PR) between the two groups (Table 1). There was also no significant difference with respect to cause of infertility between the two groups.

Table 2 shows the obstetric parameters of patients in both groups. The mean gestational age was 37.0 ± 2.2 weeks and 8 (11.59%) were preterm deliveries in the vitrification group, whereas in the fresh embryo transfer group the mean gestational age was 36.8 ± 2.8 weeks and 24 (14.45%) were preterm deliveries. There was no statistically significant difference in the mean gestational age, preterm deliveries, and mode of delivery between the two groups. There were also no statistically significant difference in the rate of pregnancy-related complications such as gestational diabetes, pregnancy-induced hypertension, and antenatal bleeding between the two study groups.

Table 3 shows the perinatal outcome of the children born in this follow-up study. Of the 69 deliveries achieved using vitrified embryo transfers, a total of 89 babies were born (50 males and 39 females). There were 50 singletons (72.46%), 18 sets of twins (26.08%), and 1 set of triplets (1.44%). The mean birth weight was 2,516 ± 600 g and the mean Apgar score was 8.3, 9.2, and 9.4 at 1, 5, and 10 minutes. Nine

TABLE 2

Comparison of pregnancy-induced complications and mode of delivery between day 3 fresh and vitrified embryo transfers.

Parameters	Fresh embryo transfer	Percentage (%)	Vitrified embryo transfer	Percentage (%)
Spontaneous vaginal delivery	40	24.09	13	18.84
Cesarean delivery	125	75.3	56	81.15
Vacuum or forceps extraction	1	0.6	0	—
Mean gestational age (wk)	36.8 ± 2.8	—	37.0 ± 2.2	—
Preterm (<37 wk)	24	14.45	8	11.59
Antenatal bleeding	9	5.42	3	4.34
Gestational diabetes	16	9.63	5	7.24
Pregnancy-induced hypertension	12	7.22	3	4.34

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TABLE 3

Neonatal parameters in pregnancies with fresh and vitrified day 3 embryos.

Parameters	Fresh embryo transfer	Percentage (%)	Vitrified embryo transfer	Percentage (%)
Live born	216	—	89	—
Male	119	55.09	50	57.47
Female	97	44.90	39	43.82
Mean birth weight (g)	2,623 ± 770	—	2,516 ± 600	—
<1,500 g	15	6.94	8	8.98
1,500–2,500 g	69	31.94	30	33.7
>2,500 g	132	61.12	51	57.3
Singleton	118	71.68	50	72.46
Twins	46	27.71	18	26.08
Triplets	2	1.20	1	1.44
Mean Apgar score				
1 min	8.2 ± 1.6		8.3 ± 1.8	
5 min	9.3 ± 1.2		9.2 ± 1.5	
10 min	9.6 ± 1.0		9.4 ± 1.1	
Admission to NICU	24	14.45	9	13.04

Note: NICU = neonatal intensive care unit.

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(13.04%) neonates needed admission into neonatal intensive care unit for observation. In the fresh embryo transfer group, of 166 deliveries achieved using fresh embryo transfers, a total of 216 babies were born (119 male children and 97 female children). There were 118 singletons (71.68%), 46 sets of twins (27.71%), and 2 sets of triplets (1.20%). The mean birth weight was 2,623 ± 770 g and the mean Apgar score was 8.2, 9.3, and 9.6 at 1, 5, and 10 minutes. Twenty-four (14.45%) neonates needed admission in the neonatal intensive care unit for observation. No statistical difference was observed in terms of mean birth weight, mean Apgar score, rate of singleton, twins, and triplets between the two study groups. The mean gestational age and birth weights were compared for singleton pregnancies in both groups to avoid the bias in these parameters due to multiple pregnancies. The results show no statistical difference between the two groups (Table 4). Fetal reduction was not performed in the patients included in this study as the patients refused to undergo this procedure.

Congenital birth defects are presented in Table 5. A single minor congenital malformation was observed in the vitrification group (1.18%), and four malformations comprising of a single major and three minor malformations (2.78%) were observed in the fresh embryo transfer group. In the vitrification group, a child was observed to have a supernumerary digit. In the fresh embryo transfer single cases of

TABLE 4

Comparison of mean gestational age and birth weights for singleton and multiple pregnancies between fresh and vitrified embryo transfer groups.

Pregnancy	No. of patients	Mean gestational age (wk)	Mean birth weight (g)	*P* value
Singleton Fresh embryo transfer	118	37.24 ± 2.09	3.06 ± 672	NS
Vitrified embryo transfer	50	37.53 ± 1.98	2.92 ± 808	
Twin Fresh embryo transfer	46	36.04 ± 1.86	2.19 ± 481	NS
Vitrified embryo transfer	18	35.91 ± 1.72	2.03 ± 534	
Triplet Fresh embryo transfer	02	34.10 ± 0.14	1.53 ± 308	NS
Vitrified embryo transfer	01	34.2 ± 0.00	1.46 ± 207	

Note: Values are expressed as mean ± SD.
NS = not significant.

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TABLE 5

Incidence of major and minor malformations in neonates.

Birth defects	Fresh embryo transfer	Vitrified embryo transfer
Minor	3	1 (supernumerary digit)
	1 (cleft lip)	
	1 (talipes)	
	1 (supernumerary digit)	
Major	1	0
	1 (diaphragmatic hernia)	
Total (%)	4 (2.78)	1 (1.18)

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diaphragmatic hernia, cleft lip, talipes, and supernumerary digit each were documented. No statistically significant difference was observed between the two groups.

DISCUSSION

With improvement in stimulation protocols and embryo culture methods, more developmentally competent embryos are produced, resulting in a surplus of good quality embryos available for cryopreservation. Furthermore, with a shift in policy by most of the IVF clinics worldwide to transfer less embryos, cryopreservation of surplus embryos has become an absolute necessity. Hence the development of a robust cryopreservation method has always been a priority for most of the assisted reproductive technology (ART) clinics. The first reported pregnancy from transfer of cryopreserved human cleavage-stage embryo dates back to 1983 by Trounson and Mohr (13) using the slow freezing protocol. Since then conventional slow freezing has been widely used to freeze the embryos. Subsequent to these initial breakthroughs, the PRs achieved using this protocol, however, remained low in comparison to fresh cycles, despite many modifications of this protocol. In addition, the slow freezing protocol requires expensive equipment and a substantial amount of time. These shortfalls have led to a search for better alternatives to overcome these problems. In 1985, Rall and Fahy (14) reported the first successful vitrification of mammalian embryos. Since then several investigators reported successful vitrification rates of human embryos and better efficiency and reliability of this technique in comparison with slow freezing. Vitrification is now regarded as a promising alternative to conventional slow freezing (15–19).

Several publications have described protocols for vitrification of human oocytes, day 3 cleavage-stage embryos and blastocysts using different combination of cryoprotectants and carrier systems resulting in successful live births (15–21). These studies assessed the vitrification protocols for embryo freezing with respect to embryo survival and PRs and concluded that vitrification is an acceptable procedure for freezing of embryos.

However, in spite of the good consistent results with vitrification technique certain issues, like chemical toxicity and viral contamination, are still a concern that has been raised by several investigators (3–6) and recently, attention has focused on the long-term effects of the procedure. Although this technique has been in clinical use for the past decade, only a few studies have addressed the long-term effects on the neonatal outcome in infants born after vitrified embryo transfers and most of the studies were done on pregnancies achieved using vitrified blastocysts (6, 7). Takahashi et al. (6) recently reported that there was no significant difference in the perinatal outcome between neonates born using fresh blastocysts and vitrified blastocysts. Ethylene glycol and dimethyl sulfoxide (DMSO) were used as cryoprotectants and cryoloop as a carrier system in the present study. The findings reported by Takahashi et al. (6) confirm the safety of the vitrification procedure for routine clinical use and also establish ethylene glycol as a safe and effective cryoprotectant for vitrification of human embryos. Liebermann and Tucker (7), in 2006, compared vitrification with conventional slow freezing of day 5 and day 6 blastocysts using ethylene glycol as a cryoprotectant and reported normal deliveries with no congenital abnormalities. However, because culturing of embryos to blastocysts before transfer is not done in most clinics and where day 3 embryo transfer is done routinely, vitrification of excess embryos on the day of transfer is practical. Hence, a study on the neonatal outcome of transfer of vitrified day 3 embryos is justified.

In the vitrification procedure, the possibility of viral contamination through liquid nitrogen is a concern raised by several investigators because of the direct contact of embryos with liquid nitrogen (4, 5). Preventive measures to avoid viral contamination, such as sterilization of liquid nitrogen by filtration and vitrification methods using sealed containers like straw in straw (19, 22), may be adapted, although no effects attributable to possible viral infection have been observed in these studies.

In the present study, the perinatal outcome of vitrified day 3 embryo transfers was compared with that of fresh embryo transfers. No significant difference was observed in terms of mean gestational age, birth weight, male and female sex ratio, congenital birth defect rate, and karyotypic abnormalities. These results support that vitrification of day 3 embryos using an ethylene glycol-based solution and a cryoloop carrier system is an effective, practical, and safe method of cryopreservation for routine clinical use.

In conclusion, vitrification is a simple, inexpensive method with proven efficiency for cryopreservation of human embryos. However, very few studies have addressed the effects of vitrification on fetal outcome. To our knowledge this is the first study that has addressed the perinatal outcome of neonates born using vitrified day 3 cleavage-stage embryos,

although a few studies have addressed the safety of this procedure for vitrification of blastocysts. The present study confirms the efficacy of vitrification for use as a method of cryopreservation of human day 3 embryos for clinical application. However, additional long-term follow-up studies with more participants and evaluation of cognitive functions is needed to confirm the results and safety of this procedure.

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