Outcomes of day 3 embryo transfer with vitrification using Cryoleaf: a 3-year follow-up study

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Received: 18 March 2012 / Accepted: 28 May 2012 / Published online: 10 June 2012
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Abstract

Objective To compare success rates of vitrified-warmed with fresh and frozen-thawed ETs

Design Retrospective.

Setting Public fertility center.

Patient(s) Cryopreserved- thawed/warmed ETs were included in this study. Fresh cycles, in which supernumerary embryos were cryopreserved, were set as the fresh control group. **Intervention(s)** Supernumerary day 3 embryos were cryopreserved by slow-freezing or vitrification and transferred after thawing or warming.

Main Outcome Measure(s) Comparison of two cryopreservation techniques with respect to post-thaw survival of embryos, implantation and pregnancy rates, neonatal outcome, and congenital birth defects.

Results A total of 962 fresh, 151 freezing-thawed and 300 vitrified-warmed cycles were included in this study. The survival and intact cell rates in the vitrification group were

significantly higher compared with those in the slow freezing group (88.5 % vs 74.5 % and 86.6 % vs 64.0 %). The implantation, clinical pregnancy and live birth rates of the vitrification group were similar to the fresh and significant higher than slow freezing group. There were no significant differences in mean gestational age, birth weight, stillbirth, birth defects and the prevalence of neonatal diseases among three groups.

Conclusion Vitrified-warmed ETs yield comparable outcomes with fresh ETs and is superior to frozen-thawed ETs regarding the survival rate and clinical outcomes.

Keywords Vitrification · Slow-freezing · Embryo transfer · In vitro Fertilization · Neonatal outcomes

Capsule Two embryo cryopreservation techniques were compared regarding survival and clinical outcomes. The vitrified-warmed ET yields comparable outcomes with the fresh ET and is superior to the frozen-thawed ET.

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Introduction

Following the first reports of human pregnancies after the transfer of cryopreserved embryos [1], cryopreservation has become an increasingly important therapeutic strategy in reproductive medicine. Since then, slow freezing has become the preferred method of embryo cryopreservation in most IVF units. Slow freezing of the cell and its surrounding environment at a controlled rate, in combination with the use of cryoprotectants at low concentrations, avoids the growth of intracellular ice crystals. The application of slow freezing in IVF not only increases the cumulative pregnancy rate, but also allows for IVF treatment to be more flexible.

However, worldwide applications of slow freezing technology have exposed their limitations in the lack of consistency with a wide range of survival rate and clinical pregnancy rates, e.g. 6–54 % of embryo survival rates have been reported, depending on cell stage, methodology used and replacement protocols [2–7]. As a result, numerous

protocols have been developed in an attempt to improve cryopreservation performance. Vitrification has emerged as an attractive alternative to slow-freezing methodology [8–10]. The major difference between vitrification and conventional cryopreservation procedures is to promote extremely rapid cooling in order to achieve crystal free vitrification status.

Vitrification has shown great promise for cryopreservation of human embryos, however, Vitrification is a relatively new method for embryo cryopreservation. Whether embryo vitrification is superior to slow freezing is still a matter of controversy [11–15]. In addition, safety concerns remain regarding the high concentration of cryoprotectants used in vitrification, which can potentially be cytotoxic [13, 16]. So far, only a limited number of studies addressed the issue of perinatal outcome of pregnancies [17, 18]. Further randomized controlled trials that examine neonatal outcomes and congenital anomalies are necessary to adequately judge the efficacy and safety of vitrification in comparison to the established slow freezing method [19].

As a result, this comparative clinical study was designed to evaluate the results of the Cryoleaf system of vitrification and slow freezing for the cryopreservation of human cleavage stage embryos on day 3. Parameters assessed included post-warming survival rate, post-warming embryo morphology and clinical outcomes. In addition, the obstetric and neonatal outcomes of infants born as a result of cryopreserved embryos were analyzed to investigate the safety and efficacy of this embryo cryopreservation method.

Methods

Patients

This study was approved by the ethical committee. Cryopreserved- thawed/warmed embryo transfers from July 2007 to May 2010 were included in this study. Only fresh cycles in which supernumery embryos were available for cryopreservation were considered as the fresh control group. This was decided so as to avoid the bias from differences of success potentials.

Ovarian stimulation

All patients used standard long or short stimulation protocols using GnRH analog (Diphreline, IPSEN) and gonadotropins (HMG, Li Zhu China and GONAL-F, Merck Serono, Swiss) for controlled ovarian hyperstimulation. Human chorionic gonadotropin (HCG, Lizhu China) at a dose of 6500 IU~10000 IU was administered after two follicles of 20 mm or more were visualized in the ultrasound scan. Oocyte retrieval was scheduled 34 h~36 h later by transvaginal ultrasonography (TVS)-guided aspiration. The

embryos were cultured in the culture medium drops covered with mineral oil in Falcon tissue culture dishes (353001; Becton Dickinson, Franklin Lakes, USA). In detail, 4 h after follicle retrieval, the oocytes were inseminated by IVF or intracytoplasmic sperm injection (ICSI) and cultured in G-IVF medium (0.1 ml) (Vitrolife, Sweden) at 37°C in an atmosphere of 5 % CO₂. At 15–18 h after insemination, fertilization was checked and then continuously cultured in G-1 medium (0.1 ml) (Vitrolife, Sweden). After warming, embryos were placed in G-2 medium (0.1 ml) (Vitrolife, Sweden) under mineral oil at 37°C in a humanized atmosphere of 5 % CO₂ in air for 30 min of incubation before embryo assessment. Embryos with more than 50 % intact blastomeres were considered viable.

Each embryo was scored based on the following: grade I: number of cells ≥ 4 on day 2, or number of cells ≥ 6 on day 3, with equal size blastomeres, regular morphology, integrated zona pellucida, no vacuoles, and fragments <10 %; grade II: number of cells <6 on day 3, with cells of equal size or roughly equal size, particles in cytoplasm, fragments between 11 %~30 %; grade III: embryo with blastomeres of distinctly unequal size, irregular morphology, distinct particles in cytoplasm, fragments between 31 %~50 %; grade IV: abnormal rate of embryo development, severely unequal cell size, significant cytoplasmic particles, a great quantity of vacuoles, fragments >50 %. The embryos of Grade I to III are defined as utilizable embryos, and will be transferred or cryopreserved.

Endometrial preparation

In cryopreserved embryo replacement cycle, all patients received hormone replacement therapy. Estradiol valerate (Progynova, Schering AG, Berlin, Germany) was initiated as follows: from day 1 to 6 of the cycle 4 mg per day was prescribed, and increased to 6 mg/d from day 6 to day 9 and to 8 mg/d from day 9 to day 12. Transvaginal ultrasound, serum estradiol and progesterone levels were used for monitoring the endometrial preparation. Vaginal progesterone, 90 mg per day (Crinone, Merck Serono Switzerland) was administered, after the thickness of endometrial reached 8 mm. The estradiol and progesterone treatment was continued until week 10 of gestation.

Protocol for slow freezing and thawing procedure

Embryos for freezing were transferred to 0.5 M propanediol (slow freezing kit, SAGE) for 10 min at room temperature, 1.0 M propanediol for 5 min, and 1.5 M propanediol solution for 10 min. Embryos were then transferred to 1 ml of 1.5 M propanediol+0.1 M sucrose and loaded into 0.25 ml (IMV, France) straws containing this same solution and sealed. The straw was loaded into an automated biological vertical freezer (Kryo-360, Planer Product Ltd, UK). The temperature was

slowly reduced to -7°C at a rate of $2^{\circ}\text{C}/\text{min}$. Ice nucleation was induced manually at -6°C . After a holding time of 10 min at -6°C , the straws were slowly cooled to -30°C at a rate of $-0.3^{\circ}\text{C}/\text{min}$ and then rapidly to -150°C at a rate of $-50^{\circ}\text{C}/\text{min}$. The straws were then transferred into liquid nitrogen tanks and stored until thawing.

On the day of thawing, embryo straws were removed from liquid nitrogen, exposed to room temperature for 30 s and then immersed in a water bath at 30°C for 30 s. The contents of the straw were expelled into a dry dish. Subsequently, the embryo was picked up and transferred into 1.5 mol/l propanediol plus 0.2 mol/l sucrose (Thaw kit, SAGE) for 5 min, and then transferred to 0.2 mol/l sucrose for 10 min. Finally embryos were washed through seven drops of the dilute solution (Thaw kit, SAGE) before moving into the culture dish where embryos were held for up to 3 h at 37°C before transfer.

Protocol for vitrification and warming procedure

The solution medium for the cryoprotectants was phosphate buffered saline (PBS, SIGMA, St Louis, MO, USA) supplemented with 20 % serum substitution supplement (SSS, Irvine Scientific, USA). A two-step cryoprotectant loading process was used. Embryos were transferred into equilibration medium, containing 7.5 % (v/v) ethylene glycol (EG, Sigma, Steinheim, Germany) + 7.5 % (v/v) dimethyl sulphoxide (DMSO, Sigma, Steinheim, Germany) in the solution medium for 5–10 min at room temperature. After initial shrinkage, embryos regained their original volume, and were transferred into vitrification medium (VM) consisting of 15 % (v/v) EG, 15 % (v/v) DMSO and 0.5 mol/l sucrose (Sigma, Steinheim, Germany) in the solution medium. After 20 s of suspension in VM, embryos were loaded into McGill Cryoleaf (Medicult, Jyllinge, Denmark) and plunged into liquid nitrogen for storage. For the warming process, the solution for dilution after thawing was made of 1, 0.5, 0.25 and 0 mol/l sucrose in PBS with 20 % SSS. Cryoleaf was directly inserted into warming medium (WM, 37°C) that contained 1 M sucrose for 1 min. The warmed embryos were then transferred through a sequence dilution media DM1 (0.5 M sucrose), DM2 (0.25 M sucrose) and DM3 (0 M sucrose) for 3 min each at room temperature. The criteria for successful embryo recovery was $\geq 50\%$ of blastomeres to survive.

Embryo transfer

Embryo warming was scheduled on the afternoon of day 4 after initiation of progesterone treatment. Embryo transfer was performed the same day. A Maximum of 3 vitrified-warmed embryos were transferred under ultrasound guidance, and luteal-phase support was achieved with

progesterone and estradiol valerate, which was continued daily for at least 2 weeks after embryo transfer. Serum hCG concentrations were measured 14 days after embryo transfer. Clinical pregnancy and implantation rates were determined by the detection of sacs by ultrasound at 6 weeks after embryo transfer. If the pregnancy test was positive, patients were followed with serial ultrasound to determine fetal viability and a prescription of estradiol valerate (6 mg/d) and progesterone (60 mg/d) was continued until 10 weeks of gestation.

Follow-up

All pregnant women were followed up until 1–2 months after parturition. All of the infants delivered were evaluated for complications during pregnancy or at delivery, gestational age, gender, birth weight and defects.

Statistical analysis

Statistical analysis was carried out using SPSS17.0 statistics software. The results were expressed as means $\pm$ SD or percentages of control. One-way ANOVA was utilized for comparisons among various treatments. The chi-square test was used for analysis of differences in the case of percentage comparison, such as the survival rate. Significant difference was defined as $p < 0.05$.

Result

A total of 962 fresh (843 patients), 151 freezing-thawed (106 patients) and 300 vitrified-warmed cycles (229 patients) were included in this study from 2007 to 2010. No-body was lost to follow-up. The various patient parameters were highlighted in Table 1. No significant differences were identified among the fresh, slow freezing and vitrification groups.

The clinical outcomes of the cryopreserved embryos were summarized in Table 2. The results showed that the mean number of cryopreserved embryos per patient was similar between the two groups. A significantly higher number of embryos thawed per cycle was shown in the slow freezing group (2.9 vs 2.6, $P = 0.001$). While the survival and intact cell rates after warming in the vitrification group were significantly higher compared with the slow freezing group (88.5 % vs 74.6 % and 86.6 % vs 64.0 %, respectively, $p < 0.001$).

Clinical results from fresh, frozen-thawed and vitrified-warmed cycles were showed in Table 3. The implantation, clinical pregnancy and live birth rates of the slow freezing group were significantly lower in comparison with those of the vitrification and fresh groups. Except for the lower spontaneous vaginal delivery rate in the vitrification group (7.1 % vs. 17.9 %, $p < 0.05$), all other clinical results were

Table 1 Clinical parameters

Parameters	Fresh cycle	Slow freezing	Vitrification
Patients' age (y)	30.4±4.3	30.9±4.0	30.8±4.1
Basal FSH level	6.5±2.8	6.8±2.1	6.6±2.6
Female factor infertility (%)	447/843(53.0)	58/106(54.7)	128/229(55.9)
Male factor infertility (%)	160/843(19.0)	17/106(16.0)	50/229(21.8)
Mixed infertility (%)	221/843(26.2)	26/106(24.5)	43/229(18.8)
Unexplained infertility (%)	15/843(1.8)	5/106(4.7)	8/229(3.5)
Fertilization via IVF (%)	440/843(52.2)	55/106(51.9)	131/229(57.3)
Fertilization via ICSI (%)	159/843(18.9)	20/106(18.9)	50/229(21.8)
Fertilization with both IVF and ICSI (%)	244/843(28.9)	31/106(29.3)	48/229(21.0)
Retrieved oocytes per patient	13.2±5.6	12.6±5.2	13.4±5.6

not significantly different between the vitrification and the fresh group. On average, a live birth could be achieved from every 7.8 vitrified-warmed embryos in the vitrification group, as opposed to every 13.1 frozen-thawed embryos in the slow-freezing group.

Table 4 reveals the perinatal outcome of the children born in this follow-up study. A total of 106 babies were born (59 males and 47 females) from 85 deliveries as the result of vitrified-warmed embryo transfers. The multiple-birth rate in the vitrification group was significant higher than the slow freezing group (24.7 % vs. 6.3 %, $p<0.05$). There were no statistically significant differences in mean gestational age, birth weight, stillbirth, neonatal death, birth defects and the prevalence of neonatal diseases among three groups. Details of the fetus and neonatal defects are summarized in Table 5.

Discussion

Embryo cryopreservation is now an integral part of IVF treatment. The transfer of cryopreserved–thawed/warmed embryos constitute about 20 % of all embryo transfers worldwide [20]. Slow freezing of the cell and its surrounding environment at a controlled rate, in combination with the use of low concentrations of cryoprotectants, avoids the formation of damaging intracellular ice crystals. In 1985, an alternative method of cryopreservation called vitrification was first reported [21]. In vitrification the embryo and surrounding solution are cooled very rapidly so that they solidify to a glasslike (vitreous) state without forming ice crystals. Vitrification uses high concentrations of cryoprotectants and rapid

cooling rates (15,000 °C–30,000 °C/min) as the embryos are plunged directly into liquid nitrogen, eliminating both intracellular and extracellular ice crystal formation. Vitrification is also simpler and more robust as it does not require expensive equipment (and therefore avoids the potential problems of equipment malfunction). It also requires smaller amounts of liquid nitrogen (LN) and is less time consuming to perform.

Compared to slow freezing, vitrification has been found to result in better survival rates following embryo thawing and in higher pregnancy rates following subsequent embryo transfer [12, 22, 23]. Vitrification protocols, choice of CPA, stage of embryo development and type of carrier have all contributed to the wide variability in clinical outcomes in the published literature [12, 24–30]. To date, there are two meta-analysis studies comparing human embryo cryopreservation by slow programmed freezing with vitrification [8, 19]. These indicate that vitrification appears to be associated with a significantly higher post-thawing survival rate than slow freezing. However, concrete conclusions regarding its value are not easy to be drawn, because most of the available studies are indirect, noncomparative, and/or not adequately powered. Further clinical studies are still required to confirm the previous studies and, in addition, allow the assessment of the two cryopreservation methods, slow-rate freezing and vitrification, regarding pregnancy achievement and safety issues.

Our study has confirmed earlier reports that vitrification can be achieved by fast cooling and minimum liquid volume. We implemented the Cryoleaf system for our embryo vitrification program. In total, 733 of 825(88.5 %) embryos

Table 2 The clinical outcome of slow freezing and vitrification

Parameters	Slow freezing	Vitrification	<i>P</i> -value
Cryopreserved embryos per patient	5.5±3.2	6.1±3.6	0.351
No. of embryos thawed per cycle	2.9±0.9	2.6±0.7	0.001
Embryo survival rate (%)	343/460(74.6)	733/825(88.5)	<0.001
Intact embryos rate (%)	294/460(63.9)	714/825(86.6)	<0.001

Table 3 Assisted reproduction outcomes of fresh cycles, slow freezing and vitrification

Parameters	Fresh cycle	Slow freezing	Vitrification	P-value (Fresh vs. Slow)	P-value (Fresh vs. Vitrification)	P-value (Slow vs. Vitrification)
General clinical data						
Embryos Transferred	2.2±0.9	2.9±0.90	2.6±0.7	0.07	0.23	0.45
Implantation rate (%)	298/1448(20.6)	48/334(14.4)	142/719(19.8)	0.010 ^a	0.651	0.035
Clinical pregnancy rate (%)	239/657(36.4)	39/151(25.8)	109/300(36.3)	0.014 ^a	0.989	0.025
Multiple pregnancy rate(%)	58/657(8.8)	9/151(6.0)	29/300(9.7)	0.249	0.675	0.181
Live birth (LB) rate (%)	225/657(34.3)	35/151(23.2)	106/300(35.3)	0.009 ^a	0.743	0.009 ^a
Loss of pregnancy						
Early abortion rate(%)	23/239(9.6)	3/39(7.7)	18/109(16.5)	0.701	0.064	0.175
Late abortion rate(%)	15/239(6.3)	3/39(7.7)	2/109(1.8)	0.739	0.075	0.082
Induced abortion rate(%)	2/239(0.8)	1/39(2.6)	0	0.895	1.000	0.264
Ectopic pregnancy rate(%)	9/239(3.8)	0	4/109(3.7)	0.619	1.000	0.573
Mode of delivery						
Spontaneous vaginal delivery rate(%)	34/190(17.9)	5/32(15.6)	6/85(7.1)	0.755	0.019	0.157

survived after vitrification. The survival rate and intact cell rate after warming in the vitrification group were significantly higher when compared with the slow freezing group (88.5 % vs. 74.6 % and 86.6 % vs. 64.0 %). This is similar to other vitrification systems [12, 22, 23]. This suggests that embryo vitrification is more efficient than the slow freezing in terms of retaining the morphologic integrity and therefore the viability of embryos. In addition, this higher survival rate with vitrification coincided with an increase in clinical and implantation pregnancy rates, although a significantly higher number of

embryos thawed per cycle was observed in the slow freezing group as a result of more embryos being thawed due to the lower survival rate. These findings were in agreement with other reports [23, 31]. However these studies failed to deliver complete data of live births. At the time of writing, all pregnant women in this study had been followed up until 1~2 months after parturition. A significantly higher live birth rate combined with higher multiple pregnancy rate were revealed in the vitrification group than in the slow freezing group (35.4 vs. 23.2 and 24.7 vs. 6.3 respectively). On

Table 4 Neonatal parameters in pregnancies with fresh cycle, slow freezing and vitrification

Parameters	Fresh cycle	Slow freezing	Vitrification	P-value (Fresh vs. Slow)	P-value (Fresh vs. Vitrification)	P-value (Slow vs. Vitrification)
Gestational age(wk)	38.66±2.51	38.47±2.06	38.16±2.73	0.686	0.124	0.563
Birth weight(g)	3126.53±674.37	3205.71±779.50	3062.07±790.11	0.545	0.448	0.307
Include: <1500 g	1	3	5			
1500~2500 g	24	0	15			
2500~4000 g	186	31	77			
>4000 g	14	1	9			
Male rate(%)	128/225(56.89)	19/35(54.29)	59/106(55.66)	0.773	0.833	0.887
Female rate(%)	97/225(43.11)	16/35(45.71)	47/106(44.33)			
Multiple births rate(%)	35/190(18.42)	2/32(6.25)	21/85(24.71)	0.087	0.232	0.025
Singleton	155	30	64			
Twins	35	1	21			
Triplets	0	1	0			
Birth defects rate(%)	4/225(1.78)	1/35(2.86)	0	0.673	0.312	0.254
Stillbirth and neonatal death rate (%)	1/225(0.44)	0	3/106(2.83)	1.000	0.189	0.574
The prevalence of PROM, preterm delivery, and neonatal morbidity(%)	2/225(0.89)	2/35(5.71)	2/106(1.89)	0.089	0.596	0.257

Table 5 The prevalence of PROM, preterm delivery, and neonatal morbidity

Parameters	Fresh cycle	Slow freezing	Vitrification
Induced abortion because of malformations	1 (Congenital heart disease) 1 (Fetal lymphoid hygroma)	1 (Talipes)	0
Malformations	1 (Laryngeal cartilage hypoplasia) 1 (supernumerary digit)	0	0
Stillbirth and neonatal death	1 (Fetal distress)	0	2 (Very low birth weight children of twin) 1 (Dysplasia)
Neonatal diseases	2 (Premature delivery, Infection)	1 (Cerebral hemorrhage, Hypoxia) 1 (Premature rupture of membrane)	2 (Respiratory distress)

average, every 7.8 vitrified-thawed embryos can achieve a live birth in comparison with every 13.1 frozen-thawed embryos in the slow-freezing group.

Furthermore, outcomes of embryo vitrification were comparable with those of fresh cycles. There was not significant difference in implantation, clinical pregnancy, and live birth rates between the vitrification and fresh groups. This indicates, on the one hand, that vitrification not only retains morphologic but also functional integrity of the embryos. On the other hand, the vitrified-warmed cycle may benefit from better endometrial receptivity and enhanced synchronization between embryo and endometrial development. Several studies have demonstrated that endometrial receptivity can be adversely affected by controlled ovarian stimulation [32–35]. With comparable results with fresh cycles, transferring fewer embryos in vitrified-thawed cycles is being considered in our center in an attempt to reduce multiple pregnancy and increase accumulative success rates.

When considering the vitrification procedures, there has been debate about the possible risk of cross-contamination via liquid nitrogen contact in this “open” system [36, 37]. Viral contamination was found in zona pellucida of bovine embryos in unsealed containers stored in LN with high concentration of bovine virus equivalent viremic stage. However, the concentration of virus in LN from repeatedly washed contaminated embryo can be expected to be far lower than reported previously [18]. We perform routine screening tests for viral infections, including HIV and hepatitis B and C, on all couples undergoing IVF and frozen embryo transfer. Patients with negative virus screening results or those who tested positive for Hepatitis B surface antigen (HBsAg) positive only were accepted for embryo cryopreservation. There were no new cases of viral infection identified by transfer of cryopreserved embryo. However, studies involving large data from multiple centers are still needed to investigate this potential issue of contamination using an open system.

In the present study, the perinatal outcome of vitrified day 3 embryos was compared with that of fresh embryos. No significant differences were observed in the mean gestational

age, birth weight, sex ratio, congenital birth defects, and abnormalities. These results support other studies both on cleavage and blastocyst vitrification [17, 18].

To our knowledge, this study has shown for the first time consistent embryology, pregnancy, and neonatal outcomes for embryo vitrification in large scale by direct comparison with slow freezing and controlled fresh cycles. Detailed comparison of live birth, delivery information and incidence of congenital anomalies will be practically helpful for IVF clinics to shift embryo slow-freezing to vitrification.

In conclusion, vitrification of day 3 embryos using an ethylene glycol-based solution and Cryoleaf carrier system is an effective, practical, and safe method of embryo cryopreservation. With comparable results for fresh cycles, embryo vitrification will help us to reduce the number of embryos transferred without compromising accumulative success rates.

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