Slow freezing should not be totally substituted by vitrification when applied to day 3 embryo cryopreservation: an analysis of 5613 frozen cycles

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Abstract

Purpose This study aimed to compare slow freezing (SF) and vitrification (VT) techniques for day 3 embryo cryopreservation in infertile couples.

Methods This retrospective cohort study enrolled 5613 infertile patients, with 7862 frozen-thawed day 3 embryos and 3845 vitrified-warmed day 3 embryos, from 2010 to 2014, at a single center. The rates of embryo survival, pregnancy, implantation, miscarriage, live birth, and live birth weight were compared between the two groups.

Results A total of 5613 cycles with 5520 transfers were analyzed. Using SF, the rates of overall embryo survival and fully intact blastomeres were lower than those in VT (91.5 vs. 97.4 % and 68.7 vs. 92.3 %, respectively). The rate of good quality embryos after thawing/warming was lower in SF than in VT. In single frozen embryo transfer cycles (FETs), the pregnancy and implantation rates were similar between the two groups (35.0 vs. 40.8 % and 34.6 vs. 35.9 %, respectively). In double FETs, the pregnancy rate per cycle was also similar between the groups (58.8 vs. 58.4 %). The implantation rate per embryo transfer was significantly higher with SF than with VT (38.8 vs. 34.6 %). With adjustment for maternal

age and the number of good quality embryos, differences in implantation rate remained significant (adjusted *P* value, SF vs. VT *P*<0.05). No independent effect was found for the method of cryopreservation on the pregnancy rate. No significant differences in the rates of miscarriage, live birth, and live birth weight were observed between the two techniques.

Conclusions Despite the significantly low embryo survival rate, fully intact blastomere rate, and good quality embryo rate in SF, the pregnancy and implantation rates were not adversely affected in single and double FETs. SF yielded an equivalent miscarriage rate, live birth rate, and live birth weight compared with VT. The SF protocol to cryopreserve day 3 embryos still should be considered.

Keywords Slow freezing · Vitrification · Cryopreservation · Implantation rate · Pregnancy outcome

Introduction

Day 3 embryo cryopreservation is a routine procedure in assisted reproductive technology. Cryopreservation allows the storage of supernumerary embryos for future use and thus enhances the cumulative pregnancy rate in in vitro fertilization (IVF). In addition, cryopreservation avoids the incidence of late ovarian hyperstimulation syndrome in high-risk patients, while it maintains the probability of pregnancy in future embryo transfer cycles [1, 2]. Interestingly, an increasing amount of studies have shown that the success rates after frozen-thawed embryo transfer (FET) are now similar to or better than the success rates of fresh embryo transfer [3]. Therefore, there is an increasing appeal for FET policy. However, while we have attempted to perform the FET strategy, an alternative of the cryopreservation protocol has become a major issue.

Capsule The comparable clinical pregnancy and live birth rate between slow frozen and vitrified embryos in this retrospective study provides further evidence for the continued use of slow freezing of day 3 embryos.

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The main problem during embryo cryopreservation is the formation of intracellular ice, which can lead to cell damage and development arrest [4, 5]. To overcome these problems, cryopreservation methods and cryoprotective solutions are constantly improving and developing. Since the first publication of frozen embryo transfer pregnancy [6], slow freezing has become the predominant cryopreservation protocol in most IVF centers. During slow freezing, at a controlled rate, embryos are exposed to relatively low concentration of cryoprotectants and dehydration without excessive shrinkage [5]. Although slow freezing is likely still the most widely used cryopreservation method for human embryos, its lower survival rate has forced IVF laboratories to switch from slow freezing to vitrification in the recent years [7]. Vitrification obtains a higher embryo survival rate and fully intact blastomere rate compared with slow freezing [8]. However, vitrification uses high concentration of cryoprotectants [9] and there are still concerns regarding its safety. The effect of vitrification on embryo development potential and birth outcomes after vitrification still needs to be determined.

Vitrification was introduced in our assisted reproductive technology (ART) center in 2012 for day 3 embryos. The decision to switch the cryopreservation protocol was based on the reported high embryo survival rate, low risk of losing the chance of embryo transfer [10, 11], and higher blastulation rates [12] by vitrification. However, some previous studies have found no difference in the pregnancy rate between vitrification and slow freezing [13, 14]. Therefore, there is an obvious need to analyze the results of two cryopreservation protocols before totally abandoning slow freezing.

In the present study, we compared the rates of embryo survival, pregnancy, implantation, miscarriage, and live birth as well as live birth weight of day 3 embryo transfer after cryopreservation using either slow freezing or vitrification.

Materials and methods

Study design

This study was approved by the institutional review board of our hospital. In this retrospective cohort study, 7862 frozen-thawed (slow freezing) day 3 embryos of all IVF/intracytoplasmic sperm injection (ICSI) thawing cycles that were carried out in the assisted reproductive unit of our hospital between January 2010 and December 2013 were included for analysis. The same analysis was performed for 3845 vitrified-warmed day 3 embryos of warming cycles between January 2012 and June 2014. Analysis was limited to only one or two day 3 frozen embryo-transferred cycles. We excluded those cycles without accurate medical record numbers and missing data.

Morphological survival was analyzed according to the cell stage before cryopreservation. The pregnancy and implantation rates were analyzed after one or two frozen embryos were transferred in patients for 3657 slow freezing cycles and 1956 vitrification cycles.

Ovarian stimulation and IVF/ICSI treatment

Patients were stimulated as previously described [15]. Briefly, two ovarian stimulation protocols for IVF treatment (long and short protocols) and two types of recombinant follicle-stimulating hormone (rFSH: Gonal-F; Serono Laboratories, Aubonne, Switzerland or Puregon; NV Organon, Oss, The Netherlands) were used. Follicular development was monitored by ultrasound scanning, and final oocyte maturation was induced by injection of human chorionic gonadotropin (hCG) as soon as three follicles of 18 mm in diameter were observed. Oocyte retrieval was performed 36 h after hCG administration.

Embryo selection for cryopreservation and evaluation after thawing/warming

Day 3 embryos with at least four blastomeres and ≤ 35 % fragmentation were selected for cryopreservation. Embryo morphology was scored as described by Veeck as follows [16]: grade 1, embryos had blastomeres of equal size and no cytoplasmic fragmentation; grade 2, embryos had blastomeres of equal or unequal size and cytoplasmic fragmentation to ≤ 20 % of the embryo surface; grade 3, embryos had blastomeres of equal or unequal size and 21–49 % overall cytoplasmic fragmentation; and grade 4, embryos had blastomeres of equal or unequal size and cytoplasmic fragmentation to ≥ 50 % of the embryo surface. An embryo with 6–12 blastomeres and grades 1 and 2 was defined as good quality. Embryos were evaluated for morphological thaw survival immediately after thawing/warming. Embryos with at least 50 % of their cells intact were considered as survived and able to be transferred.

Cryopreservation protocols

Slow freezing of day 3 embryos was performed as described by Edgar et al. [17], which involved a slow controlled freezing and thawing protocol with propanediol/sucrose (Freezing Kit; Irvine Scientific, Santa Ana, CA, USA) as the cryoprotectant, as described previously [18]. For vitrification of embryos, an open system was selected based on previous studies for its safety [19, 20]. The open vitrification procedure was performed using the Cryotop strip in combination with ethylene glycol-dimethylsulfoxide-sucrose (Kitazato Supply Co., Fujinomiya, Japan) as the cryoprotectant.

Preparation of the FET cycle

In our center, three main types of clinical protocols were used for endometrial preparation: the natural cycle, hormone replacement cycle, or human menopausal gonadotropin (HMG)-stimulated cycles [18]. For patients with regular menstrual cycles, the natural cycle was proposed and embryo transfer was performed 3 days after ovulation, or 4.5 days after the luteinizing hormone peak. In patients with an irregular ovulation or anovulatory cycle, a hormone replacement treatment or HMG-stimulation protocol was used.

Outcome measures

Maternal age (years) was registered at the time of oocyte retrieval. The thaw survival rate was calculated as embryos that survived per the total number of warmed embryos. Biochemical pregnancy was assessed by detecting serum β -hCG levels at 12 days after transfer, and 50 IU/L or greater β -hCG levels were considered as positive. The pregnancy rate was calculated by the number of β -hCG-positive pregnancies per patient. Five weeks after transfer, the number of gestational sacs and presence of fetal heart motion were evaluated by ultrasound. The thawing transfer implantation rate was calculated using the number of gestational sacs per number of embryo transferred.

Statistical analysis

Data analysis was performed using the Statistical Package for the Social Sciences Version 16.0 (IBM Corp., New York, NY, USA). A P value <0.05 indicated significance. Comparison of continuous data was performed using the independent t test. Categorical variables were compared by Pearson's chi-square test. For analyzing the rate of pregnancy and implantation, logistic regression analysis was used to adjust for potential confounding factors (maternal age and the number of good quality embryos).

Results

Analysis of patients' clinical parameters

During the 4-year study period, our ART center performed a total of 5613 thawing cycles, of which 3657 (65.2 %) used slow freezing/thawing and 1956 (34.8 %) used vitrification/warming (Table 1). Maternal age was an average of 0.4 years older in the vitrified group than in the slow frozen group ($P<0.01$). The number of good quality embryos after thawing/warming was lower with slow freezing than with vitrification. The overall embryo survival rate after vitrification was 97.4 % (3744/3845) and the proportion of embryos with

fully intact blastomeres was 92.3 %, which were significantly higher compared with slow frozen/thawed embryo transfer (91.5 and 68.7 %, respectively, $P<0.001$).

Analysis of pregnancy and implantation rates in single FETs

In total, 526 cycles of frozen-thawed single FET and 485 cycles of single FET vitrified day 3 embryos were analyzed (Table 2). The analyses included only single transfer of surviving embryos (≥ 50 % of intact cells). Maternal age was comparable between the two groups ($P>0.05$). The number of good quality embryos was significantly higher in the vitrified group than in the slow frozen group [(400; 82.5 %) vs. (366; 69.6 %), $P<0.001$]. Furthermore, the pregnancy and implantation rates were similar between the groups regarding overall and good quality embryo transfer ($P>0.05$).

Analysis of pregnancy and implantation rates in double FETs

The pregnancy and implantation rates after double FETs are shown in Table 3. A total of 2645 pregnancies were obtained, with 1795 (67.9 %) from slow freezing and 850 (32.1 %) from vitrification. The pregnancy rate of overall embryos was similar between the groups (58.8 vs. 58.4 %, $P>0.05$), but the implantation rate per embryo transfer was significantly higher in the slow frozen group than in the vitrified group (38.8 vs. 34.6 %, $P<0.001$). With regard to the transfer of good quality embryos, the pregnancy and implantation rates after double good quality embryo transfer were significantly higher in the slow frozen group compared with the vitrified group (67.7 vs. 63.6 %, $P<0.05$; 46.5 vs. 38.5 %, $P<0.001$, respectively). When adjustment was made for maternal age and the number of good quality embryos, the difference in implantation rate remained significant (adjusted P value, slow frozen vs. vitrified $P<0.05$). No independent effect was found for the method of cryopreservation on the pregnancy rate ($P>0.05$).

Ongoing pregnancy and neonatal outcomes

The number of ongoing pregnancies at the time of analysis was 1343 in the slow frozen group and 189 in the vitrified group (Table 4). The live birth rate was similar between the two groups ($P>0.05$). No significant difference was found in spontaneous miscarriages before 12 weeks or after 12 weeks ($P>0.05$). Moreover, the live birth weight of neonates from singleton and twin pregnancies was slightly lower with slow freezing than with vitrification, but this difference was not significant ($P>0.05$).

Table 1 Comparison of patients' clinical parameters using slow frozen and vitrified day 3 embryo cryopreservation

	Slow freeze	Vitrified	<i>P</i> value
No. of cycles	3657	1956	
Maternal age (years)	31.4±4.3	31.8±4.5	<0.01 ^a
Cause of infertility			
Male factor	622 (17.0 %)	342 (17.5 %)	
Tubar factor	2523 (69.0 %)	1230 (62.9 %)	
Endometriosis	139 (3.8 %)	111 (5.7 %)	
Ovarian factor	205 (5.6 %)	131 (6.7 %)	
Unexplained	168 (4.6 %)	142 (7.3 %)	
Natural cycles	153 (4.2 %)	27 (1.4 %)	
HRT cycles	3241 (88.6 %)	1803 (92.2 %)	
HMG-stimulated cycles	263 (7.2 %)	126 (6.4 %)	
Insemination methods			
IVF	2518 (68.9 %)	1289 (65.9 %)	
ICSI	934 (25.5 %)	624 (31.9 %)	
Half-ICSI	205 (5.6 %)	43 (2.2 %)	
No. of thawed/warmed embryos	7862	3845	
No. of thawed/warmed embryos per cycle	2.15±0.716	1.97±0.632	<0.001 ^a
No. of good quality embryos before cryopreservation	5860 (74.5 %)	2940 (76.5 %)	<0.05 ^b
No. of good quality embryos after thawing/warming	4889 (62.2 %)	2801 (72.8 %)	<0.001 ^b
Embryo survival rates	7190 (91.5 %)	3744 (97.4 %)	<0.001 ^b
Intact blastomere rates	5401 (68.7 %)	3549 (92.3 %)	<0.001 ^b
Endometrial thickness (mm)	9.3±1.7	9.5±1.9	>0.05 ^a

^aIndependent *t* test^bPearson's chi-square test

Discussion

It is imperative to consider that the cryopreservation method employed in ART centers has some impact on embryo quality. There are a handful of studies suggesting that vitrification obtains higher embryo survival rate and blastulation rate [12, 21]. In 2012, our ART center gradually shifted from slow freezing to vitrification. Therefore, data on both cryopreservation protocols

were available for comparison. The decision to shift protocols was based on the quantity of literature reporting higher embryo survival rate and fully intact blastomere rate in vitrified embryos [5, 10]. The current study compared the pregnancy rate and neonatal outcomes between slow frozen and vitrified day 3 embryo techniques and evaluated their effect on immediate and future development of embryos. In accordance with most previous reports, the current study indicated that the embryo

Table 2 Comparison between pregnancy and implantation rates of single FETs using slow frozen or vitrified day 3 embryos

Variable	Slow freeze	Vitrified	<i>P</i> value
No. of cycles	526	485	
Maternal age (years)	32.3±4.8	32.3±4.7	>0.05 ^a
No. of transferred embryos	526	485	
No. of good quality embryos transferred	366 (69.6 %)	400 (82.5 %)	<0.001 ^b
Pregnancy rates			
Overall embryos	184 (35.0 %)	198 (40.8 %)	>0.05 ^b
Good quality embryos	167 (45.6 %)	186 (46.6 %)	>0.05 ^b
Implantation rates			
Overall embryos	182 (34.6 %)	174 (35.9 %)	0.671 ^b
Good quality embryos	165 (45.1 %)	162 (40.5 %)	0.200 ^b

^aIndependent *t* test^bPearson's chi-square test

Table 3 Comparison of pregnancy and implantation rates per transfer between slow frozen and vitrified day 3 embryos using double FETs

Variables	Slow freeze	Vitrified	<i>P</i> value
No. of cycles	3054	1455	
Maternal age (years)	31.1±4.1	31.5±4.3	<0.05 ^a
No. of transferred embryos	6108	2910	
No. of good quality embryos transferred	4365 (71.5 %)	2275 (78.2 %)	<0.001 ^b
Pregnancy rates per cycle			
Overall embryos	1795 (58.8 %)	850 (58.4 %)	>0.05 ^b
Good quality embryos	1185/1751 (67.7 %)	609/957 (63.6 %)	<0.05 ^b
Implantation rates			
Overall embryos per embryo transfer	2371 (38.8 %)	1008 (34.6 %)	<0.001 ^b
Good quality embryos	1629/3052 (46.5 %)	737/1914 (38.5 %)	<0.001 ^b

^a Independent *t* test^b Pearson's chi-squared test

survival rate and fully intact blastomere rate were significantly lower in slow freezing than in vitrification. The pregnancy rate was similar between the two groups in single and double FETs. However, notably, the implantation rate per embryo transfer in double FETs was significantly higher with the frozen-thawed method than with vitrification in the present study. Furthermore, the rates of pregnancy and implantation with transfer of good quality embryos were significantly higher with slow freezing than with vitrification. These data indicate that, although the slow freezing technique resulted in more damaged embryos and lost more embryos, it did not affect the embryo implantation potential and pregnancy outcomes. Clinical outcomes may actually be better with slow freezing because more good quality embryos were obtained with vitrification.

The design of the current study and the different periods for slow freezing and vitrification may have resulted in some biases, which might have affected our results. However, because this study was retrospective, we were able to enroll a large number of cases, providing valuable information on the rate of embryo survival, pregnancy, and implantation. Moreover, neither the embryologist evaluating the embryos and the embryo selection criteria for cryopreservation nor the culture

medium and environment have changed over time at our center. Thorough evaluation and taking into consideration many parameters, such as safety and workload, when choosing the best embryo cryopreservation method, are important. Vitrification requires a minimal setup time and is a quick procedure, but it requires more hands-on time per cryopreserved embryo [22]. Slow frozen embryos, which predominantly survive intact after thawing, appear to retain their pre-freeze developmental potential, and optimal dehydration prior to slow freezing can result in high survival rates (90 %) [23–25]. One lysed blastomere of a slow frozen embryo has no significant effect on implantation potential because similar implantation rates are observed when three out of four and four out of four blastomeres survive thawing [26].

A retrospective study by Van Landuyt et al. [8] showed that vitrified embryos had a higher survival rate, better overnight development, and allowed a higher transfer rate but had similar implantation potential, compared with slow frozen embryos. The authors concluded that the number of single-embryo transfers with vitrified embryos was limited, and the results did not prove that vitrified embryos were more viable than slow frozen embryos. In the current study, we found that slow

Table 4 Comparison of ongoing pregnancies and neonatal outcomes after transfer of slow frozen or vitrified day 3 embryos

Variable	Slow freeze	Vitrified	<i>P</i> value
No. of pregnancies	1343	189	
No. of ongoing pregnancies after 12 weeks	1132	152	
Spontaneous miscarriages (% per pregnancy, before 12 weeks)	12.10 %	15.30 %	>0.05 ^a
Spontaneous miscarriages (% per pregnancy, after 12 weeks)	2.10 %	0.70 %	>0.05 ^a
Live birth rate per embryo transfer	1119/3783 (29.6 %)	115/399 (28.8 %)	>0.05 ^a
Live birth weight of singleton pregnancies (g)	3286.5±570.4	3327.5±491.9	>0.05 ^b
Live birth weight of twin pregnancies (g)	2499.6±477.0	2619.6±499.9	>0.05 ^b

^a Pearson's chi-square test^b Independent *t* test

freezing resulted in a 5 % lower survival rate and 23 % lower intact blastomere rate than vitrification but yielded similar pregnancy and implantation rates in single FETs. More interestingly, the implantation rate per embryo transfer was significantly higher with slow freezing than with vitrification in double FETs, despite a decreased rate of survival, blastomeres, and good quality embryos. These results suggested that slow freezing may have a minimal impact on development and implantation potential of embryos.

Vitrification uses high concentrations of cryoprotectants [9] and may have higher potential cell toxicity, which may be one of the major factors affecting development of embryos. Several studies have shown similar pregnancy and implantation rates between slow freezing and vitrification [13, 27]. Vitrified embryo transfer is associated with lower hCG concentrations on day 12 post-embryo transfer compared with slow freezing embryo transfer [18]. In the current study, a higher implantation rate in slow freezing than in vitrification was found. To the best of our knowledge, our study is the first report to suggest a better embryo implantation potential in slow freezing cycles. We had a large number of embryos used for slow freezing and vitrification, as well as a relatively higher thaw survival rate (91.5 %) for slow freezing in our ART center compared with results reported by other ART centers. Importantly, the current study used a traditional method for slow freezing that has been shown to be significantly inferior to a modified method produced by Edgar et al. [24]. This modified method showed a 47 % increase (from 54.6 to 80.4 %) in the proportion of all embryos that survive fully intact [24]. The intact blastomere rate was 68.7 % in the current study. Considering the close association between loss of blastomeres and loss of implantation potential [17], a significant improvement will be obtained if we adopt this modified method.

When appraising the efficacy of two cryopreservation protocols, embryo survival is an important issue but far enough to determine which one is actually better [23]. The rates of pregnancy, implantation, miscarriage, and live birth were used as the most important end-points. In the current study, no significant differences were found in the rates of miscarriage and live birth in slow freezing compared with vitrification. These findings suggested that no method was superior regarding embryo development after implantation. Moreover, there was no difference in live birth weight between the two methods, although a slightly lower birth weight was observed in slow freezing. The birth outcomes from slow frozen embryos where propanediol was used as the permeating cryoprotectant are reassuring [23]. However, the birth outcomes of vitrification need to be further investigated for the use of the more potentially toxic, permeating cryoprotectants (dimethyl sulfoxide and ethylene glycol) [23].

In conclusion, day 3 embryos can be cryopreserved with comparable clinical pregnancy and live birth rates using

traditional slow freezing and vitrification. The comparative better implantation potential associated with slow frozen embryos in this retrospective study provided further evidence for the continued use of slow freezing of day 3 embryos. Optimal slow freezing methods would also need to be established because they may alleviate concerns on safety issues relating to exposure to high concentrations of cryoprotectants used in vitrification.

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Conflict of interest The authors declare that they have no conflict of interest.

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