Human embryo cryopreservation: one-step slow freezing does it all?

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Abstract

Purpose Previous studies have shown that a modified one-step slow freezing method with higher sucrose concentration (0.2 M) can achieve higher embryo and blastomere survival rates that are comparable to vitrification. However, no study has evaluated the efficacy of a one-step method using commercial slow freezing kit without altering its composition. This retrospective study examines the effects of using 1.5 M PROH with 0.1 M sucrose (F2 medium) alone in a one-step slow freezing method compared to the conventional two-step method.

Methods Cleavage stage embryos from 526 thaw cycles previously cryopreserved by either the conventional two-step slow freezing method or the modified one-step method were studied. The embryo and blastomere survival rates, cleavage rate, clinical pregnancy rate and live birth rate were compared between the two groups.

Results The results showed that the embryo survival rate was significantly higher in the modified one-step method compared to the conventional two-step method (86.9 % and 83.1 %, respectively; $p=0.04$). Total blastomere survival rate was also significantly increased as a result of the modification

(81.0 % versus 76.5 %; $p<0.001$). However, there was no statistical difference in the cleavage rates, clinical pregnancy rates (CPR/ET) and live birth rates between the two methods. **Conclusions** Slow freezing using the one-step method is superior to the conventional two-step method in terms of embryo and blastomere survival rates without affecting cleavage rate and clinical outcomes. It can be routinely applied to cleavage stage embryo cryopreservation in IVF centres for greater workflow efficiency.

Keywords Cryopreservation · Slow freezing · Sucrose · Embryo

Introduction

In a single ovarian stimulation cycle, multiple oocytes are retrieved resulting in multiple embryos suitable for implantation. In Singapore, a maximum of two embryos per transfer is mandated unless there are extenuating clinical circumstances, which allow for the transfer of three embryos as described in the Singapore Ministry of Health (MOH) Licensing Terms and Conditions on Assisted Reproduction Services dated 26th April 2011 [1]. Furthermore, single embryo transfer (SET) is increasingly encouraged in most developed countries to reduce the risk of perinatal mortality due to multiple pregnancy resulting from IVF treatment [2]. Some countries such as those in Scandinavia make SET a legal requirement. This trend has resulted in an excess of usable embryos per fresh cycle after embryo transfer. Cryopreservation of human cleavage stage embryos is therefore an essential procedure in clinical in-vitro fertilization (IVF) programmes and has been so for almost three decades.

A change in ART legislation in 2008 [3] resulted in a sharp increase in our centre's workload. In order to cope with the increase, we streamlined some of our procedures to ensure the workload remained manageable by increasing manpower and

Capsule The modified one-step slow freezing method for human embryo cryopreservation is comparable to the conventional two-step method.

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equipment and refining our work processes. Cryopreservation of excess embryos was one of the main work processes involved. To ensure that exposure time of the embryos to cryoprotectant was constantly maintained across the numerous cases, the slow freezer had to perform two runs daily which is inefficient as it required more manpower. Therefore, we aimed to modify this work process without compromising clinical outcomes.

There are two approaches being widely applied worldwide to cryopreserve human cleavage stage embryos, namely slow freezing and vitrification. Over the past few years, vitrification has been shown to be superior to slow freezing by increasing the proportion of fully intact cleavage stage embryos [4]. However, recent studies have suggested that the modified slow freezing method using sucrose of higher osmolarity is as good as vitrification in achieving more than 90 % embryo survival rates [5].

Modification of the standard two-step slow freezing cryopreservation by exposure to higher sucrose concentration has been found to improve human oocyte survivability [6]. This modified method has been extrapolated for use on human cleavage stage embryos [7, 8] and on biopsied cleavage stage embryos [9]. Recently, it has been postulated that a simplified one-step slow freezing method with higher concentration of sucrose can result in improved cryosurvival [5].

To our knowledge, no study has been done to assess the efficacy of using a modified one-step slow freezing method with existing commercial embryo freezing kits. In this retrospective study, we compared clinical outcomes of thaw cycle embryos employing a one-step method versus the conventional two-step method of slow freezing using a commercial human embryo freezing kit with no modification to the sucrose concentration.

Materials and methods

The study involved the analysis of data from 526 frozen cycles cryopreserved either via conventional two-step (295 cycles; 891 embryos) or one-step slow freezing (231 cycles; 693 embryos). The mean age of patients was similar in both groups (two-step method: 35.9 years $\pm$ 3.8; one-step method: 35.1 years $\pm$ 3.9). Cases were spread among four reproductive medicine specialists of various seniorities. Although the distribution of cases varied according to the seniority and popularity of the clinicians, the proportion of cases performed by each clinician in each group was more or less the same.

Stimulation regime

In our centre, IVF treatment cycles between 2007 and 2012 were performed predominantly using the long protocol

stimulation regime. An estimated 90 % of the patients underwent down regulation with GnRH agonist, followed by stimulation with recombinant FSH (Puregon, MSD, USA). The rest of the patients underwent short cycle with GnRH antagonist (Cetrorelix: Cetrotide, Merck-Serono, Switzerland). Oocyte maturation was triggered with 10,000 IU urinary human chorionic gonadotrophin (hCG; Pregnyl, MSD, USA). Oocyte retrieval was performed 36 h later under ultrasound guidance.

Embryo assessment

All cases were evenly distributed among three types of culture media (HTF, P1 or SSM: Irvine Scientific Inc., USA). Using our centre's scoring system, embryos were graded on Day 2 or 3 based on a scale from 0 to 5, with 5 being the best grade. Grading is based on the observation of cell number, size, shape and granularity of blastomeres as well as the degree of fragmentation seen in the embryo. Only embryos graded 3 or better were considered suitable for transfer. Two of the best embryos were selected for transfer routinely while surplus good quality embryos were cryopreserved either by the conventional two-step method or the modified one-step method.

Embryo cryopreservation

Conventional two-step method

Our centre uses the commercial embryo freezing kit manufactured by Irvine Scientific Inc., USA. This consists of two solutions: F1 medium (1.5 M PROH) and F2 medium (1.5 M PROH with 0.1 M sucrose). Embryos were equilibrated at room temperature first in F1 medium for 10 min before transferring to F2 medium and loaded into cryogenic straws.

Modified one-step method

Embryos were dehydrated in F2 medium only at room temperature for 10 min before being loaded into cryogenic straws.

Cryogenic straws were then transferred to a programmable freezer (Kryo 10 Series II, Planer Products, UK) at start temperature of 23 °C before reducing to -7 °C at -2 °C/min. Seeding was performed at this point where ice nucleation was induced manually against the straws for a few seconds with a cotton swab pre-soaked in liquid nitrogen. Cooling was then continued at -0.3 °C/min to reach -30 °C and then cooled rapidly to -150 °C at -50 °C/min before plunging the frozen cryogenic straws into liquid nitrogen for storage.

Thawing

Embryos were thawed using a commercial embryo thawing kit (Irvine Scientific Inc., USA) either on the day of embryo transfer or the day before embryo transfer after an overnight culture for approximately 24 h. Frozen straws were removed from the cryostorage tank and embryos were thawed rapidly by exposure to ambient temperature regardless of the freezing method. Embryos were then rehydrated by sequential incubation in T1 medium (1.0 M propanediol solution containing 0.2 M sucrose), T2 medium (0.5 M propanediol solution containing 0.2 M sucrose) and T3 medium (0.2 M sucrose), for 5 min each at room temperature. Embryos were then transferred to equilibrated culture medium at 37 °C and blastomere survival was assessed. Embryo replacement was carried out using a soft catheter (Sure View® Wallace®, Smiths, UK) under ultrasound guidance.

Criteria for transferrable post-thaw embryos

In our centre, an embryo is considered to have survived and suitable for transfer if at least 50 % of the original number of blastomeres remain intact. Embryos with less than 50 % of intact blastomeres are normally not transferred.

Analysis of results

A clinical pregnancy is achieved when at least one gestational sac is detected by ultrasound examination at the 6th week after implantation per embryo transfer. Patients were followed up by their respective clinicians throughout their pregnancies until delivery. A live birth is defined as a successful delivery per transfer regardless of the number of babies delivered. Statistical analysis was performed using the chi-squared test with $p < 0.05$ being statistically significant.

This retrospective study was approved by the SingHealth Centralised Institutional Review Board.

Results

Embryo and blastomere survival rates

Table 1 illustrates the post-thaw embryo survival rates using the one-step and the two-step slow freezing methods. Embryo survival rate of the embryos cryopreserved using the one-step method was significantly higher than that of embryos cryopreserved using the two-step method with 86.9 % of thawed embryos surviving from the modified one-step group compared to 83.1 % from the conventional group. A significant difference was therefore expected and seen in the blastomere survival rate between the one-step method (81.0 %) and the two-step method (76.5 %).

Table 1 Comparison of embryo and blastomere survival rates between the modified one-step and the conventional two-step slow freezing method

	One-step (F2 only)	Two-step (F1 + F2)	Significance (<i>p</i> value) ^a
No. of thaw cycles	231	295	
Mean age	35.1±3.9	35.9±3.8	
No. of embryos thawed	693	891	
No. of embryos survived	602	740	
Embryo survival rate (%)	86.9	83.1	0.04
No. of blastomeres thawed	3,177	3,925	
No. of blastomeres survived	2,572	3,004	
Blastomere survival rate (%)	81.0	76.5	<0.001

^a $p < 0.05$ are considered significant

Cleavage rate

13.8 % of the thaw cycles had embryos thawed on the day of transfer (14.9 % for the one-step method and 13.0 % for the two-step method) while the remaining cohort of embryos were thawed a day before embryo transfer and cultured overnight. About 69.0 % of embryos in both groups resumed mitotic cell division after a 24-h post-thaw culture incubation (69.2 % for the one-step method versus 68.6 % for the two-step method). Cleavage rates for embryos thawed the day before did not differ significantly regardless of the cryopreservation method used (Table 2).

Clinical pregnancy and live birth rates

Of a total of 526 thaw cycles, 97.3 % (512/526) resulted in embryo transfer procedures (ET) with at least one embryo transferred (98.3 % with a mean of 1.9 embryos in the one-step method versus 96.6 % with a mean of 1.8 embryos in the two-step method). Fourteen thaw cycles were cancelled because there were no remaining viable embryos suitable for transfer. The proportion of cancelled thaw cycles in the two-

Table 2 Comparison of overnight thawed embryo cleavage rates between the modified one-step and the conventional two-step slow freezing method

	One-step (F2 only)	Two-step (F1 + F2)	Significance (<i>p</i> value) ^a
Total no. of embryos thawed	693	891	
No. of embryos thawed on the day of transfer	103	116	
No. of embryos thawed overnight	590	775	
No. of embryos thawed overnight cleaved	408	532	
Overnight cleavage rate (%)	69.2	68.6	0.89

^a $p < 0.05$ are considered significant

step method (3.4 %) was double that of the one-step method (1.7 %), but the ET rate was not statistically different between both methods.

The higher clinical pregnancy rate (CPR) of 34.0 % for the two-step method versus 29.5 % for the one-step method was not statistically significant and neither were the live birth rates of 21.8 % and 20.7 % respectively. Miscarriage rate was higher in the two-step method (36.1 %) compared to the one-step method (29.9 %) though not statistically significant (Table 3).

Discussion

Embryo cryopreservation is an important aspect of ART. It allows for the storage of excess embryos for future use should a fresh cycle fail or if the patient wishes to have a subsequent child. Optimizing frozen embryo survival depends heavily upon good cryopreservation techniques which can ensure maximal frozen embryo viability and minimal risk of cryodamage. Studies have shown that embryo viability is affected by the loss of blastomeres during freezing and thawing processes resulting in reduced developmental potential [10], lower implantation rates [11–14] and subsequent live birth rates [11, 14].

Based on an early publication, most centres that currently perform slow freezing have been using PROH and 0.1 M sucrose for almost three decades. In recent years, various researchers have studied the impact of higher sucrose concentration on clinical outcomes. In a study of biopsied cleavage stage embryos, an elevated 0.2 M sucrose was found to improve cryosurvival [9]. Another study on frozen human embryos using 0.3 M sucrose improved early embryo survival but this did not translate into increased birth rates [15].

Table 3 Comparison of clinical pregnancy and live birth rates per ET between the modified one-step and the conventional two-step slow freezing method

	One-step (F2 only)	Two-step (F1 + F2)	Significance (<i>p</i> value) ^a
No. of thaw cycles	231	295	
No. of embryo transfers (ET)	227	285	
Mean no. of embryos transferred	1.9	1.8	
ET rate (%)	98.3	96.6	0.37
No. of clinical pregnancies	67	97	
Clinical pregnancy rate per ET (%)	29.5	34.0	0.32
No. of miscarriages	20	35	
Miscarriage rate (%)	29.9	36.1	0.51
No. of live births	47	62	
Live birth rate (%)	20.7	21.8	0.86

^a *p* < 0.05 are considered significant

Previous studies comparing the survival rates of slow cooled and vitrified embryos showed that the latter had a higher survival rate [4, 16, 17]. A modified one-step slow freezing method using twice the concentration of sucrose, that is 0.2 M, also resulted in increased cryosurvival. This method was found to be as good as vitrification [5]. It would appear theoretically that there are two equally efficacious cryopreservation methods to choose from. However, there are inherent risks in the vitrification method. Contamination with infectious disease pathogens may occur during or after vitrification particularly with the use of open systems, which are known to incur safety concerns [18–20]. Transportation of vitrified embryos may cause inadvertent thawing which may affect the survivability of the embryos [21]. Being a relatively new procedure, there is little data on the potential toxicity of cryoprotectants used in vitrification [22, 23] and the long term birth outcomes from vitrified embryos remain unknown.

As the modified one-step slow freezing can achieve results as good as vitrification without the disadvantages of the latter, our centre performs slow freezing almost exclusively on cleavage stage embryos.

We reported our embryo and blastomere survival rates to be better using the modified method compared to the conventional method (Table 1) where the clinical pregnancy rates and live birth rates remain unaffected. However, these survival rates were slightly lower than a study reported by Edgar et al. in 2009 where 0.2 M sucrose concentration was used [5]. Despite the latter study giving better results, our centre adhered to the use of the commercial slow freezing kit without altering the sucrose concentration to avoid the potential problems associated with inaccuracy and inconsistency during preparation of freezing medium. The intrinsic problems associated with the use of laboratory-prepared solutions are shown in a recent study by Fasano et al. who reported a lower embryo survival rate of 63.8 % using the same cryoprotectant concentration [24].

We recognise that this retrospective study lacks the scientific power and rigour of a double-blind randomised controlled trial. However, we are encouraged by the results of our study and remain convinced that the use of 1.5 M PROH with 0.1 M sucrose solution alone is as efficacious as sequential dehydration of cleavage stage embryos according to the protocol recommended by the manufacturer of the commercial kit in terms of clinical outcomes. With this modified protocol, we are able to cryopreserve more cases with the programmable cryofreezer in a single run and hence reduce the manpower operational time by half. The modified one-step method can therefore be applied routinely to cleavage stage embryo cryopreservation in IVF centres to increase efficiency in terms of time, manpower and logistics, without compromising clinical outcomes.

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