ORIGINAL ARTICLE

Phasing-in of vitrification into routine practice: why, how, and what

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Objective To evaluate and compare the laboratory and clinical outcomes of vitrification with slow-freezing method for cryopreservation of embryos and blastocysts in an in-vitro fertilisation programme.

Design Retrospective analysis of all the 104 cycles of frozen embryo and blastocyst replacements from 2003 to 2008 and all the 149 cycles with embryos or blastocysts for vitrification from 2006 to 2009.

Setting Hospital-based Licensed Assisted Reproduction Treatment Centre in Hong Kong.

Participants All participants having frozen embryos or blastocysts transfer from 2003 to 2008.

Interventions Surplus embryos and blastocysts after fresh transfer were cryopreserved by vitrification method.

Main outcome measures Cryosurvival rate after freeze-thawing of early cleavage embryos and blastocysts by the two cryopreservation methods of slow-freezing and vitrification, and the pregnancy rate, implantation rate, delivery rate and live-birth rate achieved.

Results Cryosurvival rates of both vitrified blastocysts (79%) and early cleavage–stage embryos (88%) were significantly higher, as compared with the slow-freezing groups (57% and 72% respectively, both $P<0.05$). Pregnancy rates, delivery rates, and implantation rates were all significantly higher with vitrification regardless of the embryo types. Both implantation and live-birth rates were higher (31%, odds ratio=14 and 27%, odds ratio=11, respectively) per vitrified blastocyst transferred as compared with slow-freezing (both 3%).

Conclusion Vitrification improved clinical outcomes of both frozen embryos and especially blastocyst transfers. It conferred upon both blastocysts and embryos better developmental potential after the vitrify-thaw procedure.

Introduction

Application of vitrification to single cell revival was first introduced some 60 years ago by Polge et al[1] on spermatozoa dating back to 1949. In 1984, Fahy et al[2] elaborated on the idea of vitrification as an alternative approach to cryopreservation of mammalian embryos. However, after the world's first successful pregnancy from a frozen human embryo in Australia in 1983,[3] the slow-freezing method with controlled-rate freezers dominated cryopreservation of human embryos in the field of assisted reproduction. Since then slow-freezing became the routine method of cryopreservation of human embryos.[4,5] In 1990, the first successful human delivery from vitrified cleavage-stage embryos was reported by Gordts et al.[6] Ever since, vitrification has aroused interest in the field of cryopreservation of human embryos and gametes and has gained popularity among embryologists and clinicians, despite apprehension regarding exposure to high concentrations of cryoprotectants during the process.[7-12] In recent years, numerous studies have been reported on technical refinements to enhance embryo safety and survival.[13-15]

The slow-freezing protocol has been the routine cryopreservation method used for surplus embryos on day 2 or day 3 (D2/3) in the Dr Stephen Chow Chun-kay Assisted Reproduction Centre, Hong Kong since its establishment in 1998. The centre's first babies (a triplet delivery of two boys and a girl), by means of frozen embryo transfer (FET), were

Key words
Blastocyst; Cryopreservation; Embryo implantation; Freezing; Pregnancy rate

Hong Kong Med J 2011;17:119-26

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玻璃化凍存法逐步進入臨床常規：應用的原因、方法、進展及成效

目的 在人工受孕計劃中，評估及比較玻璃化冷凍和慢速凍存兩種方法在保存胚胎及胚泡組織的實驗室及臨床結果。

設計 回顧分析於2003至2008年期間所有冷凍胚胎或胚泡共104個移植周期，以及於2006至2009年期間所有接受玻璃化凍存胚胎或胚泡共149個周期。

安排 香港一所駐院的持牌輔助生育中心。

參與者 2003至2008年期間所有接受冷凍胚胎或胚泡移植的人。

主要結果測量 經慢速凍存及玻璃化凍存兩種方法處理後，早期卵裂的凍融胚胎和凍融胚泡的存活率，以及其妊娠率、植入率、分娩率和嬰兒出生存活率。

結果 經玻璃化凍存的胚泡和早期卵裂的胚胎，其存活率分別為79%及88%，高於經慢速凍存處理的存活率（分別為57%及72%，P<0.05）。無論是胚胎或胚泡，玻璃化凍存組都明顯有較高的妊娠率、分娩率及植入率。至於胚泡移植方面，玻璃化凍存組的植入率（31%，比數比=14）及嬰兒出生存活率（27%，比數比=11）都高於慢速凍存組（植入率及嬰兒出生存活率皆為3%）。

結論 玻璃化凍存可以改善冷藏胚胎（尤其是胚泡移植）的臨床結果，並賦予凍融胚胎及胚泡較佳的發展潛力。

born in August 1999. In May 2002, for a selected group of patients the provision of blastocyst transfers was introduced by sequential culture and the first baby by this means was born in May 2003. However, the cryosurvival rate of blastocysts after thawing was low (57%). This might have been due to cryo-injury by intracellular ice-crystal formation, which was a major obstacle. Other than extracellular ice-crystals, cryopreservation may compromise living cells and give rise to osmotic shock and solute effect owing to a large blastocoelic cavity mainly containing water. In January 2006, the first baby from a frozen blastocyst was born. In the same year vitrification replaced slow-freezing as the routine protocol for blastocysts and D2/3 cleavage-stage embryos in May and October, respectively. Encouragingly the first pregnancy from vitrification of blastocysts took place in the same year with subsequent delivery of a healthy boy (in June 2007). For vitrification of D2/3 cleavage-stage embryos, the first pregnancy occurred in May 2007, and was followed by delivery of a boy in February 2008. From the data, the cryosurvival rate of vitrified blastocysts after thawing improved (up to 79%). Therefore, since 2006 vitrification has been adopted as the routine cryopreservation method for all embryo stages.

The principle of vitrification aims to eliminate the formation of intra- and extra-cellular ice-crystals as well as osmotic shock from ultra-rapid cooling, and thereby reduce damage to living cells which are vitrified in a glass-like amorphous solid state free of any crystallised structure. The small size of embryos yields a large surface-to-volume ratio and an ultra-small holding volume of cryoprotectant solution for embedding the embryos. These features make such types of specimen extremely suitable for revival by vitrification, because ultra-rapid cooling and thawing rates can be achieved more easily than for tissues and organs.[16-19] In addition, the reported high post-thaw cryosurvival rate of vitrified embryos[7,12] confines the number of embryos in each vitrification procedure and enhances low-capacity holding devices, which also facilitates ultra-rapid freezing.[20]

The aims of this study were to review the progress of vitrification in the authors' centre and compare the results of slow-freezing and vitrification. All the frozen embryo replacement (FER) cycles involving thawing of frozen embryos and blastocysts for transfer from 2003 to 2008 were included in this analysis, and entailed FET and frozen blastocyst transfer (FBT) cycles. The primary outcome measure was the cryosurvival rate after thawing. The secondary outcome measures were pregnancy, implantation, delivery, and live-birth rates.

Methods

Slow-freezing and thawing of day 2/3 embryos

Embryo Freeze Media Kit (Irvine Scientific, Santa Ana [CA], US) and Embryo Thaw Media Kit (Irvine Scientific, Santa Ana [CA], US) were employed for slow-freezing and thawing of surplus morphologically good embryos after fresh embryo transfers. Morphological criteria for cryopreservation were based on a scoring system (1-6), which depended on the rate of division judged by the number of blastomeres with respect to day of culture, regularity of blastomeric shape, equability of blastomeric size, and degree of fragmentation. For cryopreservation, embryos had to have achieved a score of 5 or 6 and were at least 2-cell on D2 and 6-cell on D3 and had less than 20% fragmentation.[21] The freezing method was based on the published standard propanediol and sucrose two-step freezing protocol and the three-step thawing protocol.[4,5] The basal medium consisted of modified human tubal fluid (mHTF) with HEPES buffer to maintain proper pH during cryopreservation. Details of media composition and protocols are shown in Box 1. After treatment with the freezing media, embryos were loaded in 0.25 mL straws and placed in the chamber of the controlled-rate freezer (Kryo 360-1.7, MRV controller; Planar Co, UK). Then they underwent seven freezing steps at different rates, from room temperature to -150°C and

were finally plunged into liquid nitrogen at -196°C. Seeding was performed at the eutectic point of the solution (-7°C). The whole freezing cycle and the thawing cycle lasted for about 3 hours and about 40 minutes, respectively.

Thawing of the embryos was carried out in the afternoon 1 day before transfer with the Embryo Thaw Media Kit (Irvine Scientific). Thawed embryos were considered cryosurvived when morphologically intact blastomere(s) were present after thawing. The latter were allowed to be cultured for about 18 to 20 hours in equilibrated Early Cleavage Medium (Irvine Scientific, Santa Ana [CA], US) with 10% Serum Substitute Supplement (SSS; Irvine Scientific, Santa Ana [CA], US) at 37°C in a 5% CO_2 incubator before transfer. No more than three embryos were replaced in any transfer cycle.

BOX 1. Reagents and protocol for slow freezing and thawing of day 2/3 embryos*

Embryo Freeze Media Kit • Embryo Freeze Medium 1 (EF1)—1.5 M propanediol + 12 mg/mL HSA in mHTF • Embryo Freeze Medium 2 (EF2)—1.5 M propanediol + 0.1 M sucrose + 12 mg/mL HSA in mHTF
Embryo Thaw Media Kit • Embryo Thaw Medium 1 (ET1)—1.0 M propanediol + 0.2 M sucrose + 12 mg/mL HSA in mHTF • Embryo Thaw Medium 2 (ET2)—0.5 M propanediol + 0.2 M sucrose + 12 mg/mL HSA in mHTF • Embryo Thaw Medium 3 (ET3)—0.2 M sucrose + 12 mg/mL HSA in mHTF
Freezing protocol (1) Immerse in EF1 for 10 minutes (2) Transfer to EF2 for 30 seconds (3) Load into 0.25 mL straws and proceed with multi-step controlled-rate freezing programme for embryos (2.5 hours) in the controlled-rate freezer (Planer, UK). Manual seeding needed
Thawing protocol (1) Rapid thaw straw with plug to warm plate at 37°C for about 30 seconds until ice disappears (2) Expel contents as a droplet into a sterile Petri dish (3) Transfer into ET1 for 5 minutes (4) Transfer into ET2 for 5 minutes (5) Transfer into ET3 for 10 minutes (6) Transfer to mHTF with 12 mg/mL HSA for 10 minutes (7) Repeat step 6 for 10 minutes at 37°C (8) Transfer to ECM with 10% SSS and culture at 37°C in 5% CO_2 incubator until transfer

* HSA denotes human serum albumin, mHTF modified human tubal fluid medium, ECM Early Cleavage Medium, and SSS Serum Substitute Supplement

Slow-freezing and thawing of blastocysts

The Blastocyst Freeze Media Kit (Irvine Scientific, Santa Ana [CA], US) and Blastocyst Thaw Media Kit (Irvine Scientific, Santa Ana [CA], US) were employed for slow-freezing of surplus blastocysts with an intact inner cell mass (ICM) and trophectoderm (TE) on day 5 (D5), day 6 (D6), and day 7 (D7). The Blastocyst Freeze Media Kit was composed of two media, based on a modification of Ménézo et al's two-step glycerol and sucrose protocol,[22-24] and the Blastocyst Thaw Media Kit was a modification of Menezo et al's thawing protocol.[25] The basal medium consisted of mHTF with HEPES buffer to maintain a proper pH during cryopreservation. Detailed media composition and the protocol adopted are shown in Box 2. After treatment with the freezing media, blastocysts were loaded in 0.25 mL straws and placed in the chamber of the controlled-rate freezer. They then underwent four freezing steps at different rates from room temperature to -150°C and were finally plunged into liquid nitrogen at -196°C. Seeding was performed at the eutectic point of the solution (-7°C). The whole freezing cycle and the thawing cycle lasted for about 3 hours and about 30 minutes, respectively.

Thawing of blastocysts with the thawing kit was carried out early in the morning on the day of transfer. Thawed blastocysts were considered cryosurvived when more than 50% of ICM and TE cells were morphologically intact after thawing.[26] They were cultured for about 3 to 4 hours in equilibrated MultiBlast Medium (Irvine Scientific, Santa Ana [CA], US) with 10% SSS at 37°C in a 5% CO_2 incubator for re-expansion of the ICM before transfer. No more than two blastocysts were replaced in any transfer.

BOX 2. Reagents and protocol for slow freezing and thawing of blastocysts*

Blastocyst Freeze Media Kit • Blastocyst Freeze Medium 1 (BF1)—5% glycerol solution + 12 mg/mL HSA in mHTF • Blastocyst Freeze Medium 2 (BF2)—9% glycerol solution + 0.2 M sucrose + 12 mg/mL HSA in mHTF
Blastocyst Thaw Media Kit • Blastocyst Thaw Medium 1 (BT1)—0.5 M sucrose + 12 mg/mL HSA in mHTF • Blastocyst Thaw Medium 2 (BT2)—0.2 M sucrose + 12 mg/mL HSA in mHTF
Freezing protocol (1) Immerse in BF1 for 10 minutes (2) Transfer to BF2 for 10 minutes (3) Load in 0.25 mL straws and proceed with multi-step controlled-rate freezing programme for blastocysts (2 hours) in the controlled-rate freezer (Planer, UK). Manual seeding needed
Thawing protocol (1) Rapid thaw straw with plug to warm plate at 37°C for 30 seconds until ice disappears (2) Cut off seal, connect a 1-mL syringe and expel contents as a droplet into a sterile Petri dish (3) Transfer into BT1 for 10 minutes (4) Transfer into BT2 for 10 minutes (5) Transfer to MBM with 10% SSS and culture at 37°C in 5% CO_2 incubator for 3 to 4 hours for re-expansion prior to transfer

* HSA denotes human serum albumin, mHTF modified human tubal fluid medium, MBM MultiBlast Medium, and SSS Serum Substitute Supplement

Vitrification and thawing of blastocysts and day 2/3 embryos

The vitrification protocol was the same for both cleavage-stage embryos and blastocysts. Morphological criteria for cryopreservation were the same as those for slow-freezing. The closed system was applied to hold the embryos. To eliminate contact

BOX 3. Reagents and protocol for vitrification and thawing of day 2/3 embryos and blastocysts*

Vitrification Freeze Kit • Equilibrium Solution (ES)—7.5% (v/v) DMSO + 7.5% (v/v) ethylene glycol + 20% (v/v) Dextran Serum Supplement in HEPES M199 • Vitrification Solution (VS)—15% (v/v) DMSO + 15% (v/v) ethylene glycol + 20% (v/v) Dextran Serum Supplement in HEPES M199
Vitrification Thaw Kit • Thawing Solution (TS)—1.0 M sucrose + 20% (v/v) Dextran Serum Supplement in HEPES M199 • Dilution Solution (DS)—0.5 M sucrose + 20% (v/v) Dextran Serum Supplement in HEPES M199 • Washing Solution (WS)—20% (v/v) Dextran Serum Supplement in HEPES M199
Vitrification protocol (1) Transfer embryo(s) [3 maximum] or blastocyst(s) [2 maximum], to the top (T) of the ES (20 μL) for 5-15 minutes (2) Transfer to the centre (C) of VS1 for 5 seconds, VS2 (C) for 5 seconds, then VS3 (C) for 10 seconds (3) Transfer to VS4 (T), load (<10 μL) into 0.25 mL straws, seal and plunge into liquid nitrogen within 90 seconds
Thawing protocol (1) Rapid thaw straw with plug to warm plate at 37°C for about 3 seconds (2) Expel contents as a droplet onto a sterile Petri dish (3) Rinse straws by aspirating an equal volume (<10 μL) of TS, merge drops and allow spontaneous mixing for 1 minute and then into the bottom (B) of another drop of TS for 1 minute (4) Transfer to DS1 (B), then DS2 (B) for 2 minutes each (5) Transfer for 3 minutes' exposure to each WS1 (B), WS2 (T) and WS3 (T) (6) Transfer D2/3 embryos to pre-equilibrated ECM + 10% SSS at 37°C for overnight culture in 5% CO_2 incubator prior to transfer (7) Transfer blastocysts to pre-equilibrated MBM + 10% SSS at 37°C for 3-4 hours' culture in 5% CO_2 incubator for re-expansion prior to transfer

* DMSO denotes dimethyl sulfoxide, ECM Early Cleavage Medium, SSS Serum Substitute Supplement, and MBM MultiBlast Medium

contamination, the embryos did not have direct contact with liquid nitrogen.[13,27-30] Cryostraws with the volume of 0.25 mL were employed for holding the embryos after treatment with cryoprotectant solutions in less than 10 μL, before sealing and being plunged into liquid nitrogen. Vitrification Freeze Kit (Irvine Scientific, Santa Ana [CA], US) and Vitrification Thaw Kit (Irvine Scientific, Santa Ana [CA], US) were selected as the reagents. The composition of these solutions was based on published methods[7,11,20] utilising a combination of ethylene glycol and dimethylsulfoxide as the permeating cryoprotectant. These were reported as successful methods for vitrification and recovery of human embryos[20] and capable of achieving comparable implantation and pregnancy rates as FET cycles of slow-freezing embryos.[11] The basal medium consisted of M199 with HEPES buffer to maintain proper pH during cryopreservation. The procedures were performed at room temperature of 22 to 27°C with two solutions of increasing concentration of cryoprotectants in the vitrification kit. The latter was used in sequence according to the stepwise microdrop vitrification protocol. The thawing kit consisted of three solutions of decreasing concentration of sucrose, a non-permeating cryoprotectant used in sequence according to the stepwise microdrop protocol. Media composition and procedures used are summarised in Box 3 and diagrammatically shown by flowcharts in the Figure.

The FER cycle outcomes (cryosurvival, pregnancy, implantation, and live-birth rates) following vitrification and slow-freezing were compared with respect to the embryo stages (D2/3 embryos and blastocysts). Primary outcome measures of differences in cryosurvival rate were evaluated using Student's *t* tests. Significance was set at a P value of less than 0.05. Differences in rates between groups (pregnancy, implantation, and live-birth rates) were evaluated by odds ratios (ORs). An OR of greater than 1 implied a positive statistical relationship with the first group. Demographic data of patients were also analysed for any significant differences between groups.

Results

Experience and overview in vitrification freeze cycles

Up to 2009, a total of 149 vitrification cycles had been performed for cryopreservation of surplus embryos since the phasing-in of the procedure into the programme in May 2006. All the freezing and thawing procedures were carried out by a single embryologist. In all, 75 cycles had vitrification on D2 or D3 embryos, and 74 on D5, D6, or D7 blastocysts. There were 419 cleavage-stage embryos, of which 170 (41%) embryos were vitrified on D2 and 249 (59%) on D3. There were 181 blastocysts, of which 128 (71%) were vitrified on D5, 50 (28%) on D6, and 3 (2%) on D7.

Result from frozen embryo replacement cycles

From 2003 to 2008, there were 104 FER cycles, of which 75 were FET and 29 were FBT cycles. All the cycles were with post-thaw viable embryos or blastocysts for transfer. All the cycles were within 3 years of the cryopreservation period. There were no significant differences in the ages of patients both at cryopreservation and FER, endometrial thickness prior to FER, and numbers of previous failed replacement cycles.

Frozen embryo transfer cycles

The cryosurvival rate and other clinical outcome measures for FET cycles of the two cryopreservation methods are summarised in Table 1. In the vitrification group, 50 embryos survived after thawing of 57 vitrified D2/3 embryos, giving a cryosurvival rate of 88%, whereas 249 of 346 slow-freezing embryos survived after thawing, giving a cryosurvival rate of 72%. Thus, vitrification resulted in a significantly higher cryosurvival rate than slow-freezing for FET cycles (P<0.05).

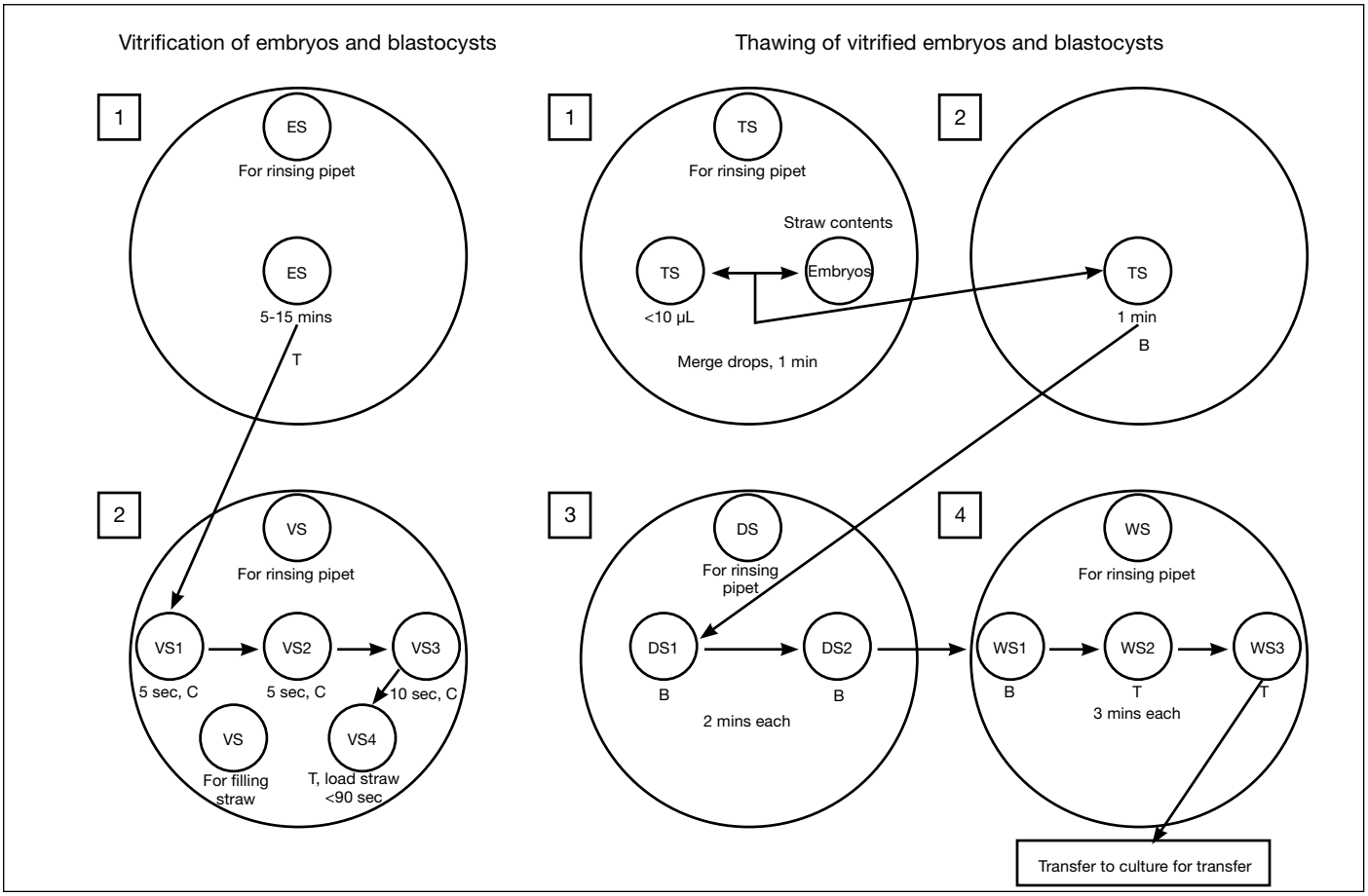

FIG. Flowchart of vitrification and thawing of embryos and blastocysts (by stepwise microdrop [20 µL] method)

⟶ denotes transfer specimen to next drop, B bottom of drop, C centre of drop, DS Dilution Solution, ES Equilibrium Solution, T top of drop, TS Thawing Solution, VS Vitrification Solution, and WS Washing Solution

There were four pregnancies in the vitrification group resulting in a pregnancy rate of 31% per transfer cycle. All the pregnancies ended in normal singleton deliveries (2 boys and 2 girls), therefore delivery and live-birth rates per transfer cycle were both 31%. The implantation rate, delivery rate, and live-birth rate per embryo transferred were all 11%.

There were nine pregnancies in the slow-freezing group, resulting in a pregnancy rate of 15%. One pregnancy ended in miscarriage and one was biochemical only. The remaining seven resulted in normal deliveries (5 boys and 3 girls) of which one was a twin delivery. Delivery and live-birth rates per transfer cycle were 11% and 13%, respectively. Implantation rate, delivery rate, and live-birth rate per embryo transferred were 5%, 4%, and 5%, respectively.

A positive statistical relationship was observed between the pregnancy rate and the vitrification group when compared with the slow-freezing group, as manifested by the pregnancy rate per cycle (31% and 15%, respectively; OR=2.62), delivery rate per cycle (31% and 11% respectively; OR=3.49), and the live-birth rate per cycle (31% and 13% respectively; OR=3.00).

Frozen blastocyst transfer cycles

The cryosurvival rate and other clinical outcome measures for FBT cycles of the two cryopreservation methods are summarised in Table 2. In the vitrification group, 30 of 38 blastocysts survived after thawing, giving a cryosurvival rate of 79%, whereas in the slow-freezing group, 35 of 61 blastocysts survived after thawing, giving a cryosurvival rate of 57%. Thus, vitrification resulted in a significantly higher cryosurvival rate than slow-freezing for the FBT cycles ($P<0.05$).

There were nine pregnancies in the vitrification group resulting in a pregnancy rate of 64% per transfer cycle. Two pregnancies ended in miscarriage and one was ectopic. The remaining six resulted in normal deliveries (3 boys and 4 girls), of which one was a twin delivery. Delivery and live-birth rates per transfer cycle were 43% and 50%, respectively. The implantation rate, delivery rate, and live-birth rate per blastocyst transferred were 31%, 23%, and 27%, respectively.

TABLE 1. Summary results of frozen embryo transfer (FET) cycles carried out in 2003 to 2008

	FET cycles (n=75)*		P value or odds ratio (OR)
	Vitrification	Slow-freezing	
No. of cycles	13	62	
Patient's age (years) at cryopreservation	34.00 ± 2.80	34.05 ± 2.84	P>0.4
Patient's age (years) at FET	34.46 ± 2.85	35.53 ± 3.49	P>0.1
Endometrial thickness (mm)	7.65 ± 1.42	8.09 ± 1.92	P>0.2
Previous failed replacement cycles	2.08 ± 1.38	2.03 ± 1.44	P>0.5
Embryos thawed	57	346	–
Cryosurvival rate	50/57 (88%)	249/346 (72%)	P<0.05
Embryo transferred/cycle	2.92 ± 0.28 (38/13)	2.69 ± 0.64 (167/62)	P>0.05
Pregnancy rate/cycle	4/13 (31%)	9/62 (15%)	OR=2.62
Delivery rate/cycle	4/13 (31%)	7/62 (11%)	OR=3.49
Live-birth rate/cycle	4/13 (31%)	8/62 (13%)	OR=3.00
Implantation rate/embryo transferred	4/38 (11%)	9/167 (5%)	OR=2.07
Delivery rate/embryo transferred	4/38 (11%)	7/167 (4%)	OR=2.68
Live-birth rate/embryo transferred	4/38 (11%)	8/167 (5%)	OR=2.33

* Data are shown as No. or mean ± standard deviation

TABLE 2. Summary results of frozen blastocyst transfer (FBT) cycles carried out in 2003 to 2008

	FBT cycles (n=29)*		P value or odds ratio (OR)
	Vitrification	Slow-freezing	
No. of cycles	14	15	
Patient's age (years) at cryopreservation	33.00 ± 3.23	33.40 ± 3.52	P>0.3
Patient's age (years) at FBT	33.43 ± 3.18	34.13 ± 4.05	P>0.2
Endometrial thickness (mm)	8.42 ± 2.62	8.49 ± 1.83	P>0.5
Previous failed replacement cycles	1.71 ± 0.91	1.60 ± 0.74	P>0.4
Blastocysts thawed	38	61	–
Cryosurvival rate	30/38 (79%)	35/61 (57%)	P<0.05
Blastocyst transferred/cycle	1.86 ± 0.54 (26/14)	2.13 ± 0.83 (32/15)	P>0.15
Pregnancy rate/cycle	9/14 (64%)	1/15 (7%)	OR=25.20
Delivery rate/cycle	6/14 (43%)	1/15 (7%)	OR=10.50
Live-birth rate/cycle	7/14 (50%)	1/15 (7%)	OR=14.00
Implantation rate/blastocyst transferred	8/26 (31%)	1/32 (3%)	OR=13.78
Delivery rate/blastocyst transferred	6/26 (23%)	1/32 (3%)	OR=9.30
Live-birth rate/blastocyst transferred	7/26 (27%)	1/32 (3%)	OR=11.42

* Data are shown as No. or mean ± standard deviation

There was one pregnancy in the slow-freezing group that resulted in a normal delivery of boy, giving a pregnancy rate of 7% per transfer cycle. The delivery rate and live-birth rate per transfer cycle were both 7%. The implantation rate, delivery rate, and live-birth rate per blastocyst transferred were all 3%.

A positive statistical relationship was also observed in FBT cycles between the pregnancy rate and the vitrification group in contrast to the slow-freezing group. This was manifested by the pregnancy rate per cycle (64% and 7%, respectively; OR=25.2), delivery rate per cycle (43% and 7%, respectively; OR=10.5), and live-birth rate per cycle (50% and 7%; OR=14.0).

Our results correlated well with the benefit of vitrification over slow-freezing on revival of blastocysts, as manifested by the higher cryosurvival rates (79% vs 57%, P<0.05) and live-birth rates (50% vs 7% per cycle; OR=14.0, and 27% vs 3% per blastocyst transferred; OR=11.4).[7,11,12]

Discussion

Vitrification seems to be a more promising means of improving blastocyst cryosurvival than slow-freezing, which results in improved pregnancy and delivery rates. Vitrified embryos and blastocysts had a higher potential to implant; the implantation rate per embryo (11%) and blastocyst (31%) being higher than those encountered with slow-freezing (5% and 3%, respectively).

Cryopreservation of embryos generally consists of three steps. The first is the preparation of embryos with varying concentrations of different kinds of cryoprotectant solutions. The second is the loading of embryos into the chosen holding-and-storage devices. These can be open or enclosed, with holding volumes of less than 1 µL to 1 mL, and include straws,[31] grids,[32] cryoloops,[33,34] cryotops,[14] cryoleaf,[35] plastic blade,[36] etc. Third, such devices are prepared for the final step of freezing either by a controlled-rate freezer, direct plunge into liquid nitrogen, or contact with a super-cooled surface. The idea of vitrification or "eliminating the formation of ice-crystal" is theoretically ideal but the mutual combination of the three steps listed above should be judiciously scrutinised to provide an optimal match and minimise cryo-injuries to embryos, that should not be limited to cryosurvival.[15] Each step is of vital importance to embryos to ensure a high survival rate and ultimately a high live-birth rate of healthy offspring. The use of high concentrations of cryoprotectants has to be restricted to a short period of time (in terms of seconds). To achieve the ultra-rapid cooling rate of over -30 000°C/minute, a minimal volume of less than 10 µL must be considered. In

the long run, the vitrification must be refined to be compatible with the chosen cryoprotectants, the holding volume, the holding-and-storage device, and the way to ensure a rapid-freezing rate.

In addition to significant improvements in clinical outcomes, vitrification offers a more cost-effective alternative by relying on the expertise of cryopreservation specialist rather than expensive controlled-rate freezers, which are also costly to install and maintain. There are, however, increased technical difficulties associated with vitrification. These include manipulation of embryos or blastocysts in extremely small volumes of solution and in very brief periods (seconds), and techniques requiring high levels of concentration and experience. Moreover, the procedures are neither simple nor fast and can obviously burden a busy laboratory, since the embryos and blastocysts are usually vitrified in groups limiting to three and two in each holding device, respectively. Thus, it may not be time-efficient to vitrify a large number of embryos. The pros of vitrification, however, outweigh the cons, especially in terms of outcomes. Thus, the trend towards vitrification as a means of embryo and blastocyst cryopreservation seems inexorable.

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