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## ARTICLE

# Slow freezing, vitrification and ultra-rapid freezing of human embryos: a systematic review and meta-analysis

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**Abstract** Embryo cryopreservation is an important aspect of assisted reproduction. Many methods have been described, but they have been poorly investigated in randomized trials, highlighting the need for a systematic review of the literature. Meticulous electronic/hand searches were performed to locate randomized trials (RCT) comparing embryo cryopreservation methods. Primary outcomes were clinical pregnancy rate (CPR) and incidence of congenital abnormalities. Secondary outcomes included live-birth (LBR), ongoing pregnancy (OPR), implantation (IR), and miscarriage (MR) rates. Data were extracted to allow for an intention-to-treat analysis and analysed using a random-effects model. Literature search revealed 11 RCT, of which five were excluded. The quality of the included studies was variable, but generally poor. There was a significantly higher CPR, OPR and IR with vitrification compared with slow freezing (odds ratio (OR) = 1.55, 95% confidence interval (CI) = 1.03–2.32, OR = 1.82, 95% CI = 1.04–3.20 and OR = 1.49, 95% CI = 1.03–2.15, respectively). In addition, there was a significantly lower CPR and OPR with embryo ultra-rapid freezing compared with slow freezing (OR = 0.35, 95% CI = 0.16–0.76 and OR = 0.37, 95% CI = 0.17–0.81, respectively). Vitrification is superior to slow freezing, which in turn is superior to ultra-rapid freezing. However, more well-designed and powered studies are needed to further corroborate these findings. 

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**KEYWORDS:** cryopreservation, embryo, slow freezing, ultra-rapid freezing, vitrification

## Introduction

Embryo cryopreservation is now considered a vital part of a successful IVF programme. The transfer of cryopreserved–thawed/warmed embryos constitute about 20% of all embryo transfers worldwide (Liebermann et al., 2003). Prior to the availability of cryopreservation technology, women producing excess oocytes had to choose between inseminating only a small portion of retrieved oocytes or having to discard excess un-transferred embryos (Veeck, 2003).

Embryo cryopreservation prevents the wastage of super-numerary embryos. It encourages transfer of fewer embryos per cycle and thus can lower multiple pregnancy rates. With the establishment of an effective cryopreservation programme, the overall chance of conception and the cumulative pregnancy rate in an IVF programme can be enhanced without transferring excessive numbers of embryos. Furthermore, embryo cryopreservation can be used to postpone the embryo transfer altogether in patients at risk of ovarian hyperstimulation syndrome or patients preparing to undergo radiation or chemotherapy. Moreover, amongst fertility preservation methods, embryo cryopreservation appears to be the one with the most satisfactory results (Shamoni and Oktay, 2005).

The development of and improvements in embryo cryopreservation technology over the last decade have helped to increase overall clinical pregnancy rates from a single oocyte retrieval cycle (Schroder et al., 2003). Embryos have been successfully cryopreserved at all stages: pronuclear (Al-Hasani et al., 2007; Barg et al., 1990; Senn et al., 2000; Van den Abbeel et al., 1997a), cleavage (Desai et al., 2007; Kuwayama et al., 2005; Mauri et al., 2001; Van der Elst et al., 1997), morula (Tao et al., 2001) and blastocyst (Clifford et al., 2007; Kuwayama et al., 2005; Liebermann and Tucker, 2006; Menezo, 2004) stages. There are three main categories of embryo cryopreservation techniques: slow freezing, ultra-rapid freezing and vitrification.

Slow freezing involves step-wise programmed decrease in temperature. The procedure is lengthy and requires the use of expensive instrumentation. Ice crystal formation within the cell being frozen can have extremely deleterious effects (Pegg, 2005).

In recent years, researchers have explored other more simple techniques requiring less expensive technology, such as ultra-rapid freezing and vitrification. Ultra-rapid freezing can be considered a midway technique between slow freezing and vitrification. It is quicker than the slow-freezing technique, does not involve the use of programmable machines and requires lower concentrations of cryoprotectant agents (CPA) than those used in vitrification (Trounson and Sjoblom, 1988; Trounson et al., 1987; Van den Abbeel et al., 1997b).

Vitrification solidifies the sample into a glass-like state, thus avoiding the formation of both intra- and extracellular ice (Vajta and Kuwayama, 2006). This is usually accomplished through the use of relatively high concentrations of CPA and/or very high cooling rates (15,000–30,000°C/min) (Liebermann et al., 2002). Vitrification is less expensive as it does not involve expensive instrumentation. Moreover, this technique is far more time-efficient, requiring only several minutes as compared with 1–2 h with controlled-rate slow freezing.

Numerous studies investigating the different embryo cryopreservation techniques are available in the literature. However, whether one technique is superior to the others, is still a matter of controversy. To date only one other meta-analysis technique to has compared human embryo cryopreservation by slow programmed freezing (Loutradi et al., 2008) vitrification. The only primary outcome measure examined was the post-thaw survival rate. The aim of the current systematic review and meta-analysis is to evaluate and compare three different methods for embryo cryopreservation, namely slow programmed freezing, ultra-rapid freezing and vitrification, with the clinical pregnancy rate as the primary outcome measure. The research question was defined as ‘Does the method of embryo cryopreservation affect the clinical outcomes in women undergoing embryo transfer using cryopreserved–thawed/warmed embryos?’ Clearly, such an evaluation would be invaluable in addressing the question of which cryopreservation technology would be most efficient in the clinical setting.

## Materials and methods

### Criteria for considering studies for this review

The literature search included published, unpublished and ongoing randomized controlled trials on the embryological and reproductive outcomes after the use of different methods of embryo cryopreservation (slow freezing, ultra-rapid freezing and vitrification) at different developmental stages (pronuclear (PN), cleavage, morula and blastocyst) in humans. For studies to be included in the analyses, data must have been provided on one of the primary outcome measures.

### Types of outcome measures

The primary outcome measures for this systematic review were the clinical pregnancy rate (CPR) per randomized woman and the incidence of congenital abnormalities and gross malformation in children conceived. Secondary outcome measures were the live-birth rate (LBR) ongoing pregnancy rate (OPR), miscarriage rate (MR) and multiple pregnancy rate (MPR) per randomized woman. In addition, the post-thaw embryo recovery rate, embryo survival rate (as defined by the included studies) and embryo implantation rate (IR) were investigated. Finally, subgroup analyses were performed to determine the effect of embryo stage at the time of cryopreservation and transfer on the primary outcomes.

### Search strategy

A meticulous electronic search (last updated November 2008) of MEDLINE (from 1960 to November 2008), EMBASE (from 1980 to November 2008) and Cochrane Central Register of Controlled Trials (CENTRAL) on the Cochrane Library Issue 4, 2008 was performed. Moreover, the National Research Register (NRR), UK Clinical Research Network Study Portal, the metaRegister of Controlled Trials (mRCT), Korea-Med, Iranian Academic Centre for Education, Culture and Research’s Scientific Information Database (SID) and the Latin American and Caribbean Health Sciences Literature

database (LILACS) were searched. All searches were performed without language restrictions. The search strategy used for MEDLINE and EMBASE are presented in [Appendices A and B](#). In addition, a hand-search of conference abstracts (e.g. American Society for Reproductive Medicine, European Society for Human Reproduction and Embryology), grey literature and the citation lists of recent included studies and recent review articles for further trials was carried out. Finally, ongoing and unpublished trials were sought by contacting experts in the field. Two reviewers (FFA, AMAS) independently reviewed the identified reports, with disagreements resolved by consensus.

## Methods of the review

A standardized data extraction form was developed and piloted for consistency and completeness in consultation with the Cochrane Handbook for Systematic Reviews of Interventions version 5.0.1 ([Higgins and Green, 2008](#)). Trials were considered for inclusion and trial data extracted. Data management and statistical analyses were conducted using [Review Manager version 4.3.2 \(RevMan\)](#).

Individual outcome data were included in the analysis if they met the pre-stated criteria. Where possible, data was extracted to allow for an intention-to-treat analysis, defined as including in the denominator all randomized cycles. If data from the trial reports was insufficient or missing, the

investigators of individual trials were contacted via e-mail for additional information, in order to perform analyses on an intention-to-treat basis. Two reviewers (FFA, AMAS) independently extracted data from the included reports with disagreements resolved by consensus.

For the meta-analysis, the number of participants experiencing the event in each group of the trial was recorded. Heterogeneity of the included studies was determined by visual inspection of the outcome tables and by using the chi-squared test for heterogeneity with a  $P$  value  $<0.10$  indicative of possible heterogeneity. In addition, the  $I^2$  test was used in an attempt at quantifying any apparent inconsistency. It describes the percentage of the variability in effect estimates that is due to heterogeneity rather than sampling error (chance) ([Higgins et al., 2003](#)). An  $I^2$  value greater than 50% may be considered to represent substantial heterogeneity. Furthermore, the possibility of publication bias was investigated using the funnel plot analysis.

## Comparison methods

Two comparative methods were used for evaluation: the direct (head-to-head) and the adjusted indirect comparison methods. For the direct comparisons, comparison of the result of group B with the result of group C within a randomized controlled trial gave an estimate of the efficacy of intervention B versus C. Direct meta-analysis of binary data

**Table 1** Characteristics of included studies.

<i>Study</i>	<i>Country</i>	<i>Study period</i>	<i>Interventions</i>	<i>Method of randomization</i>	<i>No. of participants (No. of cycles)</i>	<i>Financial support</i>
Bernal et al. (2008)	USA	January 2007–April 2008	Vitrification versus slow programmed freezing	Alternate randomization	115 women (64:51)	No external financial support
Kim et al. (2000)	Korea	January 1998–July 1999	Vitrification versus slow programmed freezing	Unclear	Unclear (42:216)	None declared
Li et al. (2007)	China	June 2005–March 2007	Vitrification versus slow programmed freezing	Alternate randomization	80 women (40:40)	None declared
Mauri et al. (2001)	Brazil	January 1991–March 2000	Ultra-rapid freezing versus slow programmed freezing	Computer-generated randomization list	160 women (80:80)	No external financial support
Rama Raju et al. (2005)	India	May 2004–June 2005	Vitrification versus slow programmed freezing	Unclear	164 women (84:80)	None declared
Van den Abbeel et al. (1997a)	Belgium	October 1992–March 1993	Ultra-rapid freezing versus slow programmed freezing	Randomization list	Unclear (100:100)	Belgium National Fund for Medical Research

Table 2 Characteristics of cryopreservation cycles.

	<i>Bernal et al. (2008)</i>		<i>Kim et al. (2000)</i>		<i>Li et al. (2007)</i>		<i>Mauri et al. (2001)</i>		<i>Rama Raju et al. (2005)</i>		<i>Van den Abbeel et al. (1997a)</i>	
	Vitrification Slow freezing		Vitrification Slow freezing		Vitrification Slow freezing		Ultra-rapid freezing	Slow freezing	Vitrification Slow freezing		Ultra-rapid freezing	Slow freezing
Type of down-regulation/ ovarian stimulation protocol	GnRH long agonist (leuprolide acetate)		GnRH long agonist (buserelin acetate)		GnRH long agonist		GnRH long agonist (leuprolide acetate)		—		GnRH long agonist (buserelin acetate)	
Type of gonadotrophin used for ovarian stimulation	Recombinant FSH		FSH/HMG		—		FSH		—		HMG	
Type of signal for triggering final oocyte maturation	Recombinant HCG		HCG (10,000 IU)		HCG (8000—10,000 IU)		HCG (10,000 IU)		—		—	
No. of oocyte collection cycles	—	—	—	—	40	40	—	—	84	80	100	100
No. of oocytes retrieved (mean ± SD)	—	—	—	—	—	—	—	—	—	—	13.2 ± 4.8	13.1 ± 3.9
Method of fertilization	IVF/ICSI		IVF		IVF/ICSI		ICSI		—		IVF	
Cryopreserved embryo stage	Blastocyst stage		Blastocyst stage		Cleavage stage		Day-2 cleavage stage		Day-3 cleavage stage		2 PN stage	
Cryopreservation carrier	Cryolock	Plastic straw	Plastic straw	Plastic straw	Cryoloop	—	Straws	Straws	Cryoloop	—	Plastic ministraws	Glass ampoules
Cryoprotectant(s) used	15% EG, 15% DMSO and 0.5 mol/l sucrose	9% glycerol and 0.2 mol/l sucrose (Quinn's Advantage Blastocyst Freeze kit; SAGE, Biopharma, Trumbull, CT, USA)	10% glycerol then 10% glycerol + 20% EG then 25% glycerol + 25% EG	5% glycerol then 9% glycerol + 0.2 mol/l sucrose	1.5 mol/l PROH + 1.0 mol/l sucrose	7.5% EG + 7.5% PROH then 15% EG + 15% PROH + 0.5 mol/l sucrose	3 mol/l DMSO + 0.25 mol/l sucrose	Freeze-kit 1 (IVF Science Scandinavia): PBS, PROH and 1.5 mol/l PROH + 0.1 mol/l sucrose	40% EG + 0.6 mol/l sucrose	Commercial kit (Vitrolife, Sweden)	2.25 mol/l PROH + 2.25 mol/l DMSO + 0.25 mol/l sucrose	1.5 mol/l PROH, 1.5 mol/l PROH + 0.1 mol/l sucrose

Table 2 Continued.

	Bernal et al. (2008)	Kim et al. (2000)	Li et al. (2007)	Mauri et al. (2001)	Rama Raju et al. (2005)	Van den Abbeel et al. (1997a)
	Vitrification Slow freezing	Vitrification Slow freezing	Vitrification Slow freezing	Ultra-rapid freezing	Vitrification Slow freezing	Ultra-rapid freezing
No. of cryopreserved embryos per woman (mean ± SD)	3.16 ± 1.44	2.9 ± 1.98	—	6.6 ± 3.2	6.5 ± 3.2	4.6 ± 3.1
Total no. of cryopreserved embryos	202	148	141	790	80	80
					436	420
						4.5 ± 3.3

DMSO = dimethyl sulphoxide; EG = ethylene glycol; GnRH = gonadotrophin-releasing hormone; HCG = human chorionic gonadotrophin; HMG = human menopausal gonadotrophin; ICSI = intracytoplasmic sperm injection; PBS = phosphate-buffered saline; PROH = 1,2-propanediol.

was performed using the DerSimonian and Laird method utilizing a random-effects model (DerSimonian and Laird, 1986) and the odds ratio (OR) and 95% confidence intervals (95% CI) were evaluated. Direct meta-analysis of continuous data was performed using the mean difference method utilizing a random-effects model.

If direct comparison was not possible due to the lack of available trials comparing group B with group C, then adjusted indirect comparison was performed using the method described by Bucher et al. (Bucher et al., 1997; Song et al., 2003). The indirect comparison of intervention B and C was adjusted by the results of their direct comparisons with a common intervention A. In brief, given two estimated effects  $\theta_{AB}$  and  $\theta_{AC}$  for comparisons of group A versus group B and group A versus group C respectively, then the effect for the comparison of group B versus C is estimated as follows:  $\theta_{BC} = \theta_{AB} - \theta_{AC}$  and its variance is  $\text{var}(\theta_{BC}) = \text{var}(\theta_{AB}) + \text{var}(\theta_{AC})$ . A 95% CI for  $\theta_{BC}$  is obtained as  $\theta_{BC} \pm 1.96 \sqrt{\text{var}(\theta_{BC})}$ . The estimates of effect, denoted by  $\theta$ , relate to the scale on which the data would be analysed; in this case being the log OR or mean difference for binary and continuous data, respectively. The validity of the adjusted indirect comparisons depends on the internal validity and similarity of the included trials.

### Methodological quality of included studies

The methodological characteristics of the included studies, including study design, number of participants (Table 1) and procedure-specific characteristics of the cryopreservation and thawing/warming cycles (Tables 2 and 3) were collected. The methodological quality was assessed using a quality checklist (Table 4). Quality evaluation was also performed by two reviewers independently (FFA, AMAS), with disagreements resolved by consensus.

## Results

### Search results

A highly sensitive search of the literature revealed over 2700 possible relevant publications. Following removal of duplicate publications and detailed evaluation of the titles/abstracts of the citations, 11 prospective randomized controlled trials were identified (Figure 1). Subsequently, five were excluded (one still ongoing with no available data for evaluation and four with no data on primary outcomes of this review) and six trials were included. Four of these included trials compared vitrification with slow freezing, while two trials compared ultra-rapid freezing with slow freezing. It is important to note that none of the included randomized clinical trials directly compared vitrification to ultra-rapid freezing.

### Vitrification versus slow programmed freezing

For the primary outcome measures, there was a statistically significantly higher incidence of clinical pregnancies with embryo vitrification compared with slow freezing (89/230 versus 128/387; OR = 1.55, 95% CI = 1.03–2.32) (Figure 2). With regards to the incidence of congenital anomalies in

**Table 3** Characteristics of thawing/warming cycles.

	<i>Bernal et al. (2008)</i>		<i>Kim et al. (2000)</i>		<i>Li et al. (2007)</i>		<i>Mauri et al. (2001)</i>		<i>Rama Raju et al. (2005)</i>		<i>Van den Abbeel et al. (1997a)</i>	
	<i>Vitrification</i>	<i>Slow freezing</i>	<i>Vitrification</i>	<i>Slow freezing</i>	<i>Vitrification</i>	<i>Slow freezing</i>	<i>Ultra-rapid freezing</i>	<i>Slow freezing</i>	<i>Vitrification</i>	<i>Slow freezing</i>	<i>Ultra-rapid freezing</i>	<i>Slow freezing</i>
No. of thawing/warming cycles	64	51	42	216	40	40	58	45	40	23	78	68
No. of embryos thawed/warmed per woman (mean ± SD)	2.67 ± 0.85	2.84 ± 1.93	—	—	—	—	6.5 ± 2.9	6.2 ± 3	—	—	3.5 ± 1.6	3.7 ± 1.5
Total no. of embryos thawed/warmed	171	145	141	790	80	80	—	—	127	120	—	—
Survival rate definition	Blastocyst without lysed cells		—	—	—	—	Presence of only one intact blastomere after the thawing/warming process		—	—	Percentage of recovered embryos that were morphologically intact after thawing/warming and subsequent dilution of the cryoprotectant	
Embryos surviving cryopreservation	159	110	105	537	71	73	—	—	121	72	—	—
Embryos surviving cryopreservation (100% morphologically intact)	128	55	—	—	—	—	—	—	—	—	—	—
Transferred embryo stage	Blastocyst stage		Blastocyst stage		Cleavage stage		Cleavage stage		Cleavage stage		Cleavage stage	

Table 4 Quality checklist.

	<i>Bernal et al. (2008)</i>	<i>Kim et al. (2000)</i>	<i>Li et al. (2007)</i>	<i>Mauri et al. (2001)</i>	<i>Rama Raju et al. (2005)</i>	<i>Van den Abbeel et al. (1997a)</i>
Was a proper method of randomization described?	–	–	–	+	–	+
Was a proper method of randomization concealment described?	–	–	–	+	–	–
Was there blinding of patients, care-givers, or study coordinators?	–	–	+	+	–	–
Was an a-priori sample size calculation performed prior to the recruitment of patients?	–	–	–	–	–	–
Was an intention-to-treat analysis used for calculating the outcome measures?	–	–	–	–	–	–
Was a clear description of withdrawals and/or dropouts available?	–	–	–	+	–	+

+ = Evident in the manuscript or through evidence provided by the authors.

the newborn children, only one study (Bernal et al., 2008) reported on this outcome measure and no cases were found in either group.

For the secondary outcomes, there were statistically significantly higher ongoing pregnancy (Figure 3) and embryo implantation rates with embryo vitrification compared with slow freezing (66/190 versus 94/347, OR = 1.82, 95% CI = 1.04–3.20 and 133/454 versus 181/749, OR = 1.49, 95% CI = 1.03–2.15, respectively), which is in line with the clinical pregnancy rate. The multiple pregnancy and miscarriage rates were not statistically significantly different between the two groups (26/104 versus 12/91, OR = 2.11, 95% CI = 0.99–4.52 and 4/190 versus 19/347, OR = 0.57, 95% CI = 0.16–2.03, respectively). Similarly, there was no statistically significant difference in the reported live-birth rates between embryo vitrification compared with slow freezing (18/148 versus 17/131; OR = 0.87, 95% CI = 0.36–2.12). It is important to note that only two trials reported on this outcome measure (Bernal et al., 2008; Rama Raju et al., 2005) and in both studies not all patients had reached term.

Demographically, the average numbers of embryos frozen/vitrified and thawed/warmed per randomized woman were similar between the two groups. Also, the average number of embryos being recovered post-thaw/warming was similar in both groups (351/687 versus 255/645; OR = 1.95, 95% CI = 0.92–4.15), but the embryo survival rate was significantly in favour of embryo vitrification (128/171 versus 55/145; OR = 4.87, 95% CI = 3.01–7.88).

### Ultra-rapid freezing versus slow programmed freezing

For the primary outcome measures, there was a statistically significantly lower incidence of clinical pregnancies with embryo ultra-rapid freezing compared with slow freezing (12/112 versus 23/95; OR = 0.35, 95% CI = 0.16–0.76). With regard to the incidence of congenital anomalies in the newborn children, no trials reported this outcome.

For the secondary outcomes, there was a statistically significantly lower ongoing pregnancy rate with ultra-rapid freezing compared with slow freezing (11/112 versus 21/95; OR = 0.37, 95% CI = 0.17–0.81), which is in line with the clinical pregnancy rates. No analysable data was available for the embryo implantation rate. The multiple pregnancy and miscarriage rates were not statistically significant between the two groups (2/112 versus 4/95, OR = 0.44, 95% CI = 0.09–2.18 and 1/112 versus 2/95, OR = 0.38, 95% CI = 0.03–4.30, respectively). Likewise, there was no statistically significant difference in the live-birth rates between embryo ultra-rapid freezing and embryo slow freezing (4/54 versus 8/50, OR = 0.42, 95% CI = 0.12–1.49).

Demographically, the average numbers of embryos frozen and thawed per randomized woman were similar between the two groups (data not presented). Unfortunately, no analysable data were available for the incidences of post-thaw embryo recovery and embryo survival rates.

### Vitrification versus ultra-rapid freezing

Vitrification resulted in significantly higher clinical pregnancy and ongoing pregnancy rates compared with ultra-rapid freezing (OR = 4.43, 95% CI = 1.84–10.66 and OR = 4.92, 95% CI = 1.88–12.87, respectively). Meanwhile, the multiple pregnancy, live birth and miscarriage rates were comparable in both groups (Table 5). However, insufficient data was available to estimate the incidence of congenital anomalies in children born following cryopreserved embryo transfer, the implantation rate and the incidences of post-thaw/warmed embryo recovery and embryo survival rates in both groups.

### Subgroup analyses

Finally, subgroup analyses were performed to determine the effect of embryo stage at the time of cryopreservation and transfer on the primary outcomes (Table 6). One trial cryo-

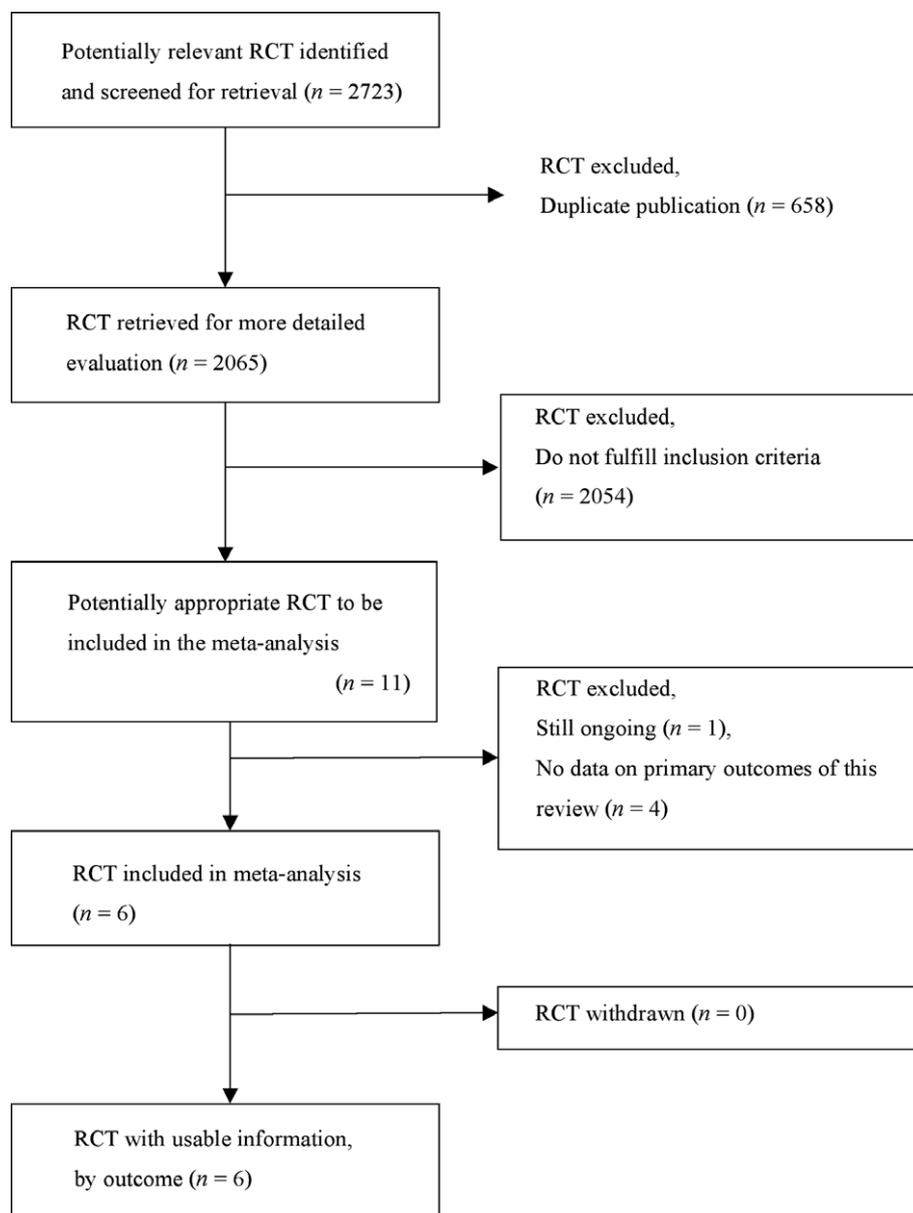


Figure 1 QUOROM chart. RCT, randomized controlled trial.

preserved embryos at the pronuclear stage (Van den Abbeel et al., 1997a), three trials cryopreserved embryos at the cleavage stage (Li et al., 2007; Mauri et al., 2001; Rama Raju et al., 2005), and two trials cryopreserved embryos at the blastocyst stage (Bernal et al., 2008; Kim et al., 2000). All the trials transferred the embryos at the stage in which they were cryopreserved, with the exception of one trial that allowed the embryos to develop to the cleavage stage prior to transfer (Van den Abbeel et al., 1997a). The odds of a clinical pregnancy for embryos cryopreserved and/or transferred at the cleavage stage were noted to be significantly in favour of slow freezing (OR = 0.32, 95% CI = 0.12–0.84 and OR = 0.35, 95% CI = 0.16–0.76 for cryopreservation and transfer, respectively) and vitrification (OR = 0.14, 95% CI = 0.04–0.54 and OR = 0.16, 95% CI = 0.05–0.52 for cryopreservation and transfer, respectively) when compared with ultra-rapid freezing. The re-

sults of the other analyses did not show any significant differences.

## Discussion

This systematic review was conducted to evaluate and compare the efficacy and clinical pregnancy outcomes with three different cryopreservation protocols used for human embryos at pronuclear, cleavage or blastocyst stage. A total of 765 cycles meeting the inclusion criteria were available for study. The current meta-analysis suggests that post-thaw/warming survival, implantation, clinical pregnancy and ongoing pregnancy rates were significantly affected by the method used for human embryo cryopreservation. Moreover, miscarriage rates were similar between the treatment groups.

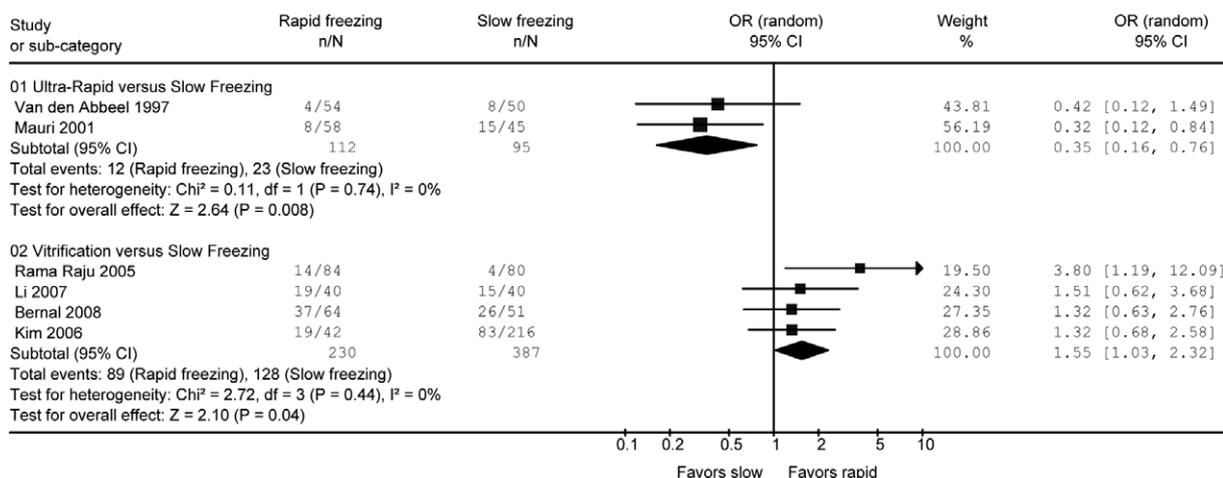


Figure 2 Forest plot for clinical pregnancy rate.

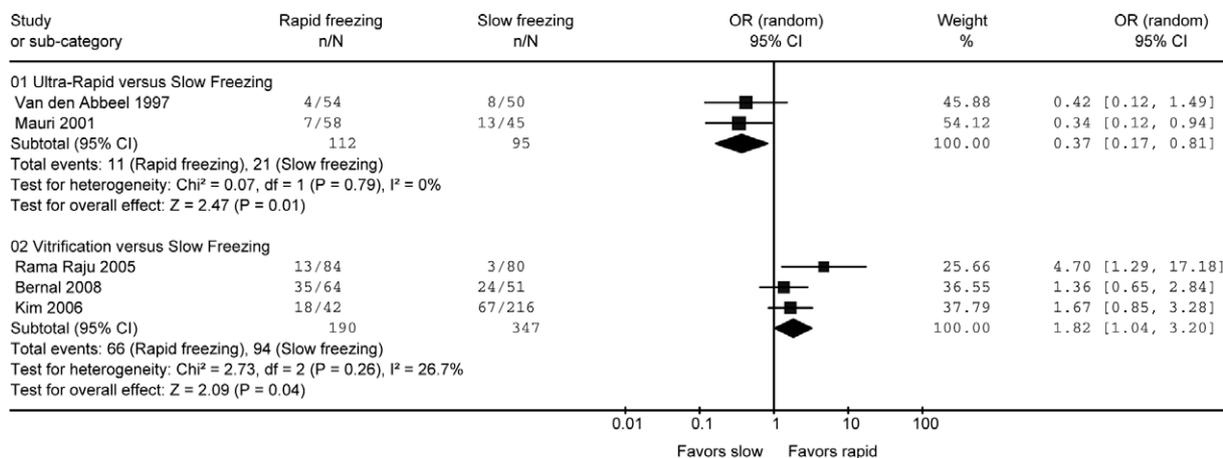


Figure 3 Forest plot for ongoing pregnancy rate.

Table 5 Outcome measures for adjusted indirect comparison of vitrification versus ultra-rapid freezing.

Comparison or outcome	Effect size
Primary outcome measure Clinical pregnancy rate <sup>a</sup>	4.43 (1.84–10.66)
Secondary outcome measure Multiple pregnancy rate	4.80 (0.82–28.02)
Miscarriage rate	1.53 (0.09–24.39)
Ongoing pregnancy rate <sup>a</sup>	4.92 (1.88–12.87)
Live-birth rate	2.07 (0.44–9.66)

Values are odds ratio (95% confidence interval).  
<sup>a</sup>Statistically significant.

To date there has only been one other meta-analysis comparing slow cryopreservation and vitrification (Loutradi et al., 2008). As in the present study, it was concluded that vitrification resulted in significantly higher post-warming survival. Pregnancy outcomes were not compared in the Loutradi study, however. In addition, the aforementioned systematic review included both randomized and non-randomized trials and excluded trials that were presented only as conference proceedings.

The current meta-analysis focuses on clinical outcomes. The data suggests superiority of vitrification technology to controlled-rate slow freezing methods for human embryo cryopreservation. The rate of embryo survival was significantly higher with vitrification versus slow freezing. This also coincided with an increase in implantation and ongoing pregnancy rates with vitrification. Ultra-rapid freezing appeared to be inferior to the other two methodologies. However, the limited number and quality of studies available for analysis did not allow for a definitive conclusion.

Amongst the three cryopreservation techniques surveyed, slow programmed freezing still predominates but re-

**Table 6** Clinical pregnancy rates for subgroup analyses.

Comparison	Method	PN stage	Cleavage stage	Blastocyst stage
<i>Embryo stage at time of cryopreservation</i>				
Vitrification versus slow freezing	Direct analysis	NA	2.22 (0.91–5.43)	1.32 (0.81–2.17)
Ultra-rapid freezing versus slow freezing	Direct analysis	0.42 (0.12–1.49)	0.32 (0.12–0.84) <sup>a</sup>	NA
Vitrification versus ultra-rapid freezing	Adjusted indirect analysis	NA	0.14 (0.04–0.54) <sup>a</sup>	NA
<i>Embryo stage at time of embryo transfer</i>				
Vitrification versus slow freezing	Direct analysis	NA	2.22 (0.91–5.43)	1.32 (0.81–2.17)
Ultra-rapid freezing versus slow freezing	Direct analysis	NA	0.35 (0.16–0.76) <sup>a</sup>	NA
Vitrification versus ultra-rapid freezing	Adjusted indirect analysis	NA	0.16 (0.05–0.52) <sup>a</sup>	NA

Values are odds ratio (95% confidence interval); NA = not applicable; PN = pronuclear.

<sup>a</sup>Statistically significant.

cent literature suggests a trend towards acceptance of vitrification in clinical assisted reproduction technology laboratories. With slow freezing, a wide range of clinical pregnancy rates have been reported, depending on cell stage, methodology used and replacement protocols. Clinical pregnancy rates with pronuclear-stage embryos have ranged from 10% to 44% (Salumets et al., 2003; Schroder et al., 2003; Seelig et al., 2002; Senn et al., 2000; Tummon et al., 2006; Vyjayanthi et al., 2006). Pregnancy rates of 6–54% have been reported with cleavage stage embryos (Chi et al., 2002; Edgar et al., 2007; Kuwayama et al., 2005; Nagy et al., 2005; Rienzi et al., 2005; Salumets et al., 2003, 2006; Van der Elst et al., 1997; Warnes et al., 1997; Wang et al., 2001; Ziebe et al., 1998). Frozen blastocyst studies have reported pregnancy rates of 9–64% (Kaufman et al., 1995; Liebermann and Tucker, 2006; Medved et al., 2006; Shapiro et al., 2008; Van den Abbeel et al., 2005; Veck et al., 2004; Virant-Klun et al., 2003).

It should be noted, however, that considerable improvement in clinical outcomes with slow freezing of blastocysts has been observed in recent years. A better understanding of cryobiological principles, enhanced embryo culture systems and resultant improved blastocyst quality may also have contributed towards this upward shift (Chi et al., 2002; Gardner et al., 2000; Marek et al., 2001; Mauri et al., 2001; Nagy et al., 2005; Senn et al., 2000; Veck et al., 2004; Vyjayanthi et al., 2006).

Ultra-rapid freezing protocols for human embryo cryopreservation were first investigated by researchers in the late 1980s (Barg et al., 1990; Feichtinger et al., 1991; Gordts et al., 1990; Lai et al., 1996; Trounson and Sathananthan, 1990; Trounson and Sjoblom, 1988; Trounson et al., 1987, 1988). Compared with slow freezing, this technique required a shorter duration of embryo exposure to the CPA but at a higher concentration. In comparison with vitri-

fication, ultra-rapid freezing used far lower concentrations of CPA and it was suggested that the relatively lower CPA concentrations may result in less toxicity (Van den Abbeel et al., 1997b). The problem of intra-cellular ice crystal formation in the cell(s) being frozen was still however a problem. This may be a contributing factor to the variable (but generally low) clinical pregnancy rates achieved with the ultra-rapid freezing technique, which have for the most part hindered the widespread adoption of this technology in clinical laboratories.

Vitrification has gained popularity in recent years and outcomes have rivalled those obtained with traditional slow-freezing methods. Unlike the situation in ultra-rapid freezing, CPA concentrations in vitrification solutions are quite high, such that on immersion into liquid nitrogen the solution becomes glass-like. This vitrified state prevents the formation of ice crystals completely (Vajta and Kuwayama, 2006). The downside of this methodology has been the potential cytotoxicity of such high concentrations of CPA to the embryo. Vitrification protocols, choice of CPA, stage of embryo and type of carrier have all contributed to the wide variability in clinical outcomes in the published literature. Al-Hassani et al. used 28% CPR when vitrifying warmed PN-stage embryos (Al-Hasani et al., 2007), while CPR of 16–44% were reported using vitrified/warmed cleavage-stage embryos (Desai et al., 2007; Kuwayama et al., 2005; Saito et al., 2000; Zhu et al., 2005). Success rates with vitrified-warmed blastocysts have ranged from 23% to as high as 62% (Hiraoka et al., 2004; Kuwayama et al., 2005; Lee et al., 2007; Liebermann and Tucker, 2006; Mukaida et al., 2006; Vanderzwalmen et al., 2002).

Paramount to the current success of vitrification has been the evolution of new vessels or carriers for vitrification of cells that allow extremely fast cooling rates, sometimes greater than 20,000°C/min (Shaw and Jones, 2003). Tradi-

tional cryopreservation carriers, including the cryovial and the 0.25 ml insemination straw, have relatively thick walls that limit cooling and warming rates (~2500°C/min) (Vajta and Nagy, 2006) and the sample to be frozen is contained in fluid volumes of 50–1000 µl. In contrast, vitrification carriers are characterized by having very thin walls or completely open like the cryoloop. The cell is frozen in minuscule volumes of fluid to maximize the rate of temperature decrease when immersed in liquid nitrogen.

In this systematic review, both direct and indirect evidence was utilized to evaluate the available data. Since prospective, well-designed randomized, controlled trials provide the most valid evidence of relative efficacy of competing techniques, they were utilized first and foremost in evaluations. One of the limitations of this analysis was the lack of sufficient studies providing direct comparisons between slow freezing, ultra-rapid freezing and vitrification. Although adjusted indirect comparisons can yield useful information and generally agree with results from direct head-to-head comparisons (McAlister et al., 1999), it should be noted that such comparisons are dependent on the similarity and internal validity of the included studies. Further corroboration on the superiority of vitrification over other methods of cryopreservation will require direct comparison in randomized trials.

A second limitation of this meta-analysis was the wide date range it encompassed and the impact of evolving technologies for embryo culture and cryopreservation. Clearly, success of blastocyst-stage cryopreservation has coincided with continued improvements in both culture milieu for in-vitro growth and an understanding of cryobiology. For this reason, comparisons may be skewed against slow cryopreservation which had been the method of choice until only recently when advances in vitrification technology have allowed it to gain acceptance. Also a variety of different cryoprotectants and techniques were utilized in the studies included within this meta-analysis. Finally, the limited data set available for analysis did not allow for detailed comparison of live birth, delivery information or incidence of congenital anomalies.

In the current study, embryo cryopreservation by slow controlled-rate freezing, ultra-rapid freezing and vitrification were compared. Results of the current meta-analysis indicate that embryo vitrification is superior to slow freezing based on direct comparison of embryo survival and clinical pregnancy rates. Ongoing pregnancy and implantation rates were also higher with vitrification as compared with slow freezing. Within the limited data set available, using both direct and indirect evidence, ultra-rapid freezing appeared to be inferior to slow programmed freezing as well as vitrification. Vitrification is a relatively new and promising method for embryo cryopreservation. However, further randomized controlled trials that examine neonatal outcomes and congenital anomalies are necessary to adequately judge the efficacy and safety of vitrification.

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## Appendix A. Search strategy used for MEDLINE (Ovid interface)

randomized controlled trial.pt.  
 randomized controlled trials as topic.sh.  
 random allocation.sh.  
 controlled clinical trial.pt.  
 comparative study.pt.  
 double blind method.sh.  
 single blind method.sh.  
 clinical trial.pt.  
 clinical trials as topic.sh.  
 clinical.mp. and trial\$.tw.  
 crossover studies.sh.  
 (crossover or crossover).tw.  
 cross.mp. and over.tw.  
 (singl\$ or doubl\$ or trebl\$ or tripl\$).tw. and (blind\$.mp. or mask\$.tw.)  
 random\$.tw.  
 research design.sh.  
 1–16  
 animals not (animals and humans).sh.  
 17 not 18  
 reproductive techniques.sh.  
 fertilization in vitro.sh.  
 in vitro fertiliz\$.tw.  
 IVF.tw.  
 sperm injections, intracytoplasmic.sh.  
 ICSI.tw.  
 embryonic structures.sh.  
 embryo.tw.  
 embryo implantation.sh.  
 embryo.mp. and implantat\$.tw.  
 embryo.mp. and transfer\$.tw.  
 PN.mp. and stage.tw.  
 blastocyst.sh.  
 blastocys\$.tw.  
 cleavage stage, ovum.sh.  
 cleavage\$.mp. and stag\$.tw.  
 pregnancy.sh.  
 pregnan\$.tw.  
 pregnancy outcome.sh.  
 pregnancy.mp. and outcome.tw.  
 pregnancy.mp. and rate\$.tw.  
 clinical.mp. and pregn\$.tw.  
 ongoing.mp. and pregn\$.tw.  
 deliv\$.tw.  
 livebirth\$.tw.  
 live-birth\$.tw.  
 20–45  
 19 and 46  
 cryopreservation.sh.  
 cryopreservation.tw.  
 cryo-preservation.tw.

cryo.tw.  
 cryoprotective agents.sh.  
 tissue.mp. and preservation.tw.  
 dimethyl sulfoxide.sh.  
 dimethyl.mp. and sulfoxide.tw.  
 ethylene glycol.sh.  
 ethylene.mp. and glycol.tw.  
 freez\$.tw.  
 froz\$.tw.  
 nitrogen.sh.  
 nitrogen.tw.  
 liquid\$.mp. and nitrogen.tw.  
 propylene glycols.sh.  
 propylene.mp. and glycol\$.tw.  
 slow.mp. and freez\$.tw.  
 ultra and rapid.mp. and freez\$.tw.  
 ultra-rapid.mp. and freez\$.tw.  
 vitrification.tw.  
 48–68  
 47 and 69

## Appendix B Search strategy used for EMBASE (Ovid interface)

randomized controlled trial/  
 randomized controlled trials as topic/  
 random allocation/  
 controlled clinical trial/  
 comparative study/  
 double blind method/  
 single blind method/  
 clinical trial/  
 clinical trials as topic/  
 clinical.mp. and trial\$.tw.  
 crossover studies/  
 crossover.tw.  
 crossover.tw.  
 cross.mp. and over.tw.  
 (singl\$ or doubl\$ or trebl\$.mp. or tripl\$.tw.) and (blind\$ and mask\$).tw.  
 random\$.tw.  
 1–16  
 nonhuman/not nonhuman/and humans/  
 17 not 18  
 exp infertility therapy/  
 fertilization in vitro/  
 in vitro fertiliz\$.tw.  
 IVF.tw.  
 intracytoplasmic sperm injections/  
 ICSI.tw.  
 embryonic structures/  
 embryo.tw.  
 nidation/  
 embryo.mp. and implantat\$.tw.  
 embryo.mp. and transfer\$.tw.  
 PN.mp. and stage.tw.  
 blastocyst/  
 blastocys\$.tw.  
 cleavage stage, ovum/  
 cleavage\$.mp. and stag\$.tw.

pregnancy/  
 pregnan\$.tw.  
 pregnancy outcome/  
 pregnancy.mp. and outcome.tw.  
 pregnancy.mp. and rate\$.tw.  
 clinical.mp. and pregn\$.tw.  
 ongoing.mp. and pregn\$.tw.  
 deliv\$.tw.  
 livebirth\$.tw.  
 live-birth\$.tw.  
 20–46  
 19 and 46  
 cryopreservation/  
 cryopreservation.tw.  
 cryo-preservation.tw.  
 cryo.tw.  
 cryoprotective agents/  
 tissue.mp. and preservation.tw.  
 dimethyl sulfoxide/  
 dimethyl.mp. and sulfoxide.tw.  
 ethylene glycol/  
 ethylene.mp. and glycol.tw.  
 freez\$.tw.  
 froz\$.tw.  
 nitrogen/  
 nitrogen.tw.  
 liquid\$.mp. and nitrogen.tw.  
 propylene glycols/  
 propylene.mp. and glycol\$.tw.  
 slow.mp. and freez\$.tw.  
 ultra and rapid.mp. and freez\$.tw.  
 ultra-rapid.mp. and freez\$.tw.  
 vitrification.tw.  
 48–68  
 47 and 69

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