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ASSISTED REPRODUCTION

Vitrification versus slow freezing gives excellent survival, post warming embryo morphology and pregnancy outcomes for human cleaved embryos

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Abstract
Purpose The objective of this retrospective study was to evaluate the efficacy of vitrification and slow freezing for the cryopreservation of human cleavage stage embryos in terms of post-warming survival rate, post-warming embryo morphology and clinical outcomes.
Methods The embryos of 305 patients at cleavage stages were cryopreserved either with vitrification (153 patients) or slow-freezing (152 patients) methods. After warming; the survival rate, post-warmed embryo morphology, clinical pregnancy and implantation rates were evaluated and compared between the two groups.
Result(s) In the vitrification group versus slow freezing group, the survival rate (96.9% vs. 82.8%) and the post-warmed excellent morphology with all blastomeres intact (91.8% vs. 56.2%) were higher with an odds ratio of 6.607 (95% confidence interval; 4.184–10.434) and 8.769 (95% confidence interval; 6.460–11.904), respectively. In this group, the clinical pregnancy rate (40.5% vs. 21.4%) and the implantation rate (16.6% vs. 6.8%) were also higher with an odds ratio of 2.427 (95%confidence interval; 1.461–4.033) and 2.726 (95% confidence interval; 1.837–4.046), respectively.
Conclusion(s) Vitrification in contrast to slow freezing is an efficient method for cryopreservation of human cleavage stage embryos. Vitrification provides a higher survival rate, minimal deleterious effects on post-warming embryo morphology and it can improve clinical outcomes.

Keywords Vitrification · Slow freezing · Human cleavage stage embryos · Survival rate · Post-warming embryo morphology · Clinical outcomes

Capsule Cryopreservation of human cleavage stage embryos with vitrification versus slow freezing provides better laboratory and clinical outcomes.

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Introduction

Successful cryopreservation of human embryos was first reported in 1983 by Trounson and Mohr [1] with multicellular embryos that had been slow-cooled using dimethyl sulphoxide (DMSO). Subsequent modifications of the technique, introducing 1,2-propanediol and sucrose as cryoprotectants [2] and slow-cooling to −30°C prior to plunging into liquid nitrogen, resulted in the introduction of cryopreservation as a standard method offered by virtually every full-service IVF program world-wide [3].

Slow freezing is known as equilibrium freezing due to the exchange of fluids between the extra- and intracellular spaces and results in safe freezing without serious osmotic and deformation effects to cells [4]. This technique is accepted to be a safe procedure because of the use of relatively low concentration of cryoprotec-

tants that might not cause serious toxic and osmotic damage. However, as low concentrations of cryoprotectants may be insufficient for avoiding ice crystal formation within the cells, the slow freezing is more time-consuming and requires an expensive programmable freezing machine; most of the embryologists are not satisfied with this technique and try to find other cryopreservation protocols such as vitrification [5–8]. The latter technique as first reported by Rall and Fahy in 1985 [9] for the cryopreservation of mammalian embryos, with a later attempt for human cleavage-stage embryo, and followed by a successful delivery in 1990 [10].

Vitrification is a non-equilibrium method and may be regarded as a radical approach in which ice crystal formation is totally eliminated. Nevertheless, it requires an extremely high cooling rate along side much higher concentrations of cryoprotectants when compared with slow freezing [11]. This method does not require expensive equipment and is not time-consuming. Human embryo vitrification has been attempted with a variety of vessels such as electron microscope grids [12, 13], open pulled and hemi-straws [14–16], the Flexipet [17], the Cryotop [18] and the CryoLoop [5, 19–23].

Until now, vitrification has been widely used for the cryopreservation of human oocytes [18, 24, 25], in vitro matured oocytes [26, 27] pronuclear stage [6–28], cleavage stage [5, 7, 14, 18, 29–34], or blastocyst-stage [8, 18, 35–42]. However there are few publications that show clinical data on the basis of vitrification versus slow freezing, especially for the cleavage stage [5, 8]. Therefore this comparative clinical trial study has been designed to evaluate the results of vitrification and slow freezing for the cryopreservation of human cleavage stage embryos on day 2 and day 3 in terms of post-warming survival rate, post-warming embryo morphology and clinical outcomes.

Materials and methods

Patients

This study compared the laboratory and clinical outcome of 152 slow frozen-thawed embryo transfer cycles from January 2005 till January 2007 with 153 vitrified-warmed embryo transfer cycles from January 2007 till March 2008 at Royan Institute (Tehran, Iran). During this study, all conditions and protocols for human embryo culturing were kept constant in our lab.

Ovarian stimulation and fresh embryo transfer

Ovarian stimulation was performed following down regulation as previously described [43]. In brief, suppression of pituitary gonadotropin secretion with the GnRH agonist buserelin acetate (Suprefact, Hoechst AG, Allemagne, Germany) by SC injection (500 mg/d) or by nasal spray (800 mg/d) was commenced in the mid luteal phase of the preceding ovarian cycle (day 21). Once ovarian suppression was confirmed (serum E2 of ≤ 50 pg, FSH of ≤ 12 IU and LH of ≤ 5 IU), ovarian stimulation was initiated by using purified hMG (SC injection, 150 IU/d; Serono, Aubonne, Switzerland). The dose was increased in tandem with ovarian follicular development and monitored by serial vaginal ultrasonography. When at least three follicles reached 18 mm in diameter; GnRH agonist and hMG were discontinued and hCG (10,000 IU; Pregngl; Organon, Oss, the Netherlands) was administered. Oocyte retrieval was performed by ultrasound-guided follicle aspiration, 36–38 h after hCG administration. The oocytes underwent standard IVF and ICSI and were cultured in G-1 (version 3; Vitrolife, Kungsback, Sweden), supplemented with 10% recombinant human serum albumin (rHA, Vitrolife) for 2 to 3 days. On the day of embryo transfer (44–72 h after sperm insemination or injection), the embryos were scored according to the following quality criteria: excellent morphology (2–4 even size blastomeres with $\leq$ 10% fragmentation for day 2, 6–8 even size blastomeres with $\leq$ 10% fragmentation for day 3), good morphology (2–4 even or uneven size blastomeres with 10%–20% fragmentation for day 2, 6–8 even or uneven size blastomeres with 10%–20% fragmentation for day 3), or poor morphology (uneven few blastomeres with > 20% fragmentation). Depending on the patients' embryos; a maximum of 3 to 4 embryos with the best morphology were selected and cultured for 20 min up to 2 h in EmbryoGlue (Vitrolife) prior to embryo transfer. The embryo transfer was then performed with a Labotect catheter (Labotect, Straberg, Germany).

Protocol for slow freezing and thawing procedure

Before January 2007, our routine strategy for human cleaved embryo cryopreservation was a slow freezing protocol, as described elsewhere [44] with some modification. In brief, the suitable surplus embryos were first incubated in equilibration solution comprising 1.5 mol/L 1,2-propanediol (Sigma) in Ham's-F10 medium (Gibco, Invitrogen, Life Technologies, Paisley, Scotland), supplemented with 20% (vol/vol) Albuminal-5 (containing 5% human serum albumin; Blood Research Center, Tehran, Iran) at room temperature for 10 min and then transferred to freezing solution (1.5 mol/L 1,2- propanediol and 0.5 mol/L sucrose; Sigma) in Ham's-F10 medium supplemented with 20% Albuminal-5 for an additional 10 min. Thereafter, three to five embryos were loaded into plastic mini-straws

(Cryostraw 0.2 m; Labotect GmbH, Labor-Technik-Gottingen, Germany) and the freezing program was executed as follows. Embryos were placed in an automatic self-seeding machine (CTE 880; Cryo-Technik-Erlangen, Germany) at 23.0°C; cooled at −1.0°C/min to 0.0°C, at −0.5°C/min to −2.0°C, at −0.3°C/min to −3.0°C, at −0.2°C/min to −5.0°C, at −0.1°C/min to −7.0°C, held for 5 min at −7°C for self seeding (in cases of any poor self seeding, with a cooled soup, mechanical seeding was induced) and at −0.3°C/min to −33°C. Embryos were then held at −33.0°C for 30 min before being plunged into liquid nitrogen. The patient's straw was then stored in liquid nitrogen for at least 2 months.

On the day of frozen–thawed embryo transfer the patient's straw was removed from liquid nitrogen, exposed to room temperature (30 s) and immersed in a water bath at 30°C (30 s). The embryos were removed from the straw and then incubated in a series of decreasing 1, 2-propanediol concentrations (1.0 mol/L for 5 min and 0.5 mol/L for 5 min) in the thawing solution (0.5 mol/l sucrose and 20% [vol/vol] Albuminar-5 in Ham's-F10) for 5 min and finally in sucrose-free thawing solution for 5 min before being transferred to G-1 (version 3). The frozen–thawed embryos were then classified as an excellent morphology (100% of cells survived with < 10% fragmentation), good morphology (100% of cells survived with 10–20% fragmentation), poor morphology (≥50% cells survived with or without any fragmentation) or as degenerated embryos (<50% of cells survived). Depending on the patient's embryos, a maximum of 3 to 4 embryos with the best morphology were selected and cultured for 20 min up to 2 h in EmbryoGlue. Embryo transfer was then performed with a Labotect catheter.

Protocol for vitrification and warming procedure

From January 2007 onward, our routine strategy for human cleaved embryo cryopreservation was vitrification. The vitrification/warming protocol was performed according to the method described previously [86]. Suitable surplus embryos were first incubated in equilibration solution comprising 7.5% ethylene glycol (EG) (Sigma-Aldrich, Steinheim, Germany) and 7.5% dimethyl sulphoxide (DMSO) (Sigma-Aldrich) in Ham's F-10 media supplemented with 20% Albuminal-5 for 5–15 min (depending on the time needed for re-expansion of the cell) at room temperature. After an initial shrinkage and recovery, they were aspirated and placed into the vitrification solution (15% EG, 15% DMSO, 0.5 M sucrose) in Ham's F-10 medium supplemented with 20% Albuminal-5 for 50–60 s at room temperature. After having observed cellular shrinkage, embryos were aspirated and placed on the tip of the Cryotop (Kitazato, Japan). No more than four embryos were placed on each Cryotop. Cooling of the embryos was done by direct contact with liquid nitrogen. The Cryotops were stored in liquid nitrogen for at least 2 months. On the day of frozen–thawed embryo transfer the patient's Cryotop was removed from liquid nitrogen. The embryos were exposed to thawing solution (1 M sucrose in Ham's F-10 medium supplemented with 20% Albuminal-5) for 50–60 s at 37.0°C temperature and then transferred into dilution solution of 0.5 M sucrose for 3 min, followed by another dilution solution of 0.25 M sucrose for 3 min, both at room temperature. The warmed embryos were placed 4–5 times into washing solution (Ham's F-10 medium supplemented with 20% Albuminal-5) before being transferred to G-1 (version 3). The frozen–thawed embryos were then morphologically classified as described for the slow freeze protocol. A maximum of three to four embryos with 50% of blastomeres intact were selected for intrauterine transfer. The selected embryos were transferred into EmbryoGlue and the embryo transfer was then performed with a Labotect catheter

Endometrial preparation in patients with frozen–thawed embryo transfer

Endometrial preparation in patients who were supposed to receive frozen–thawed embryos either with a slow freeze or with vitrification protocol was first performed as described elsewhere [45]. In brief, buserelin, a GnRH agonist (Suprefact, 500 mg SC; Hoechst AG), was started in the mid luteal phase (day 21) of the menstrual cycle or approximately 10 days before the expected onset of the next period. Two to three days after subsequent menstruation, serum E2 and LH levels were measured to confirm down regulation. If E2 levels were ≤ 50 pg and LH levels were ≤ 5 IU/ml, endometrial preparation was started (day 1 of treatment). Estradiol valerate (Aburaihan Co., Tehran, Iran) was given by mouth; at 4 mg/d from day 1 to day 6, at 6 mg/d from day 6 to day 9 and at 8 mg/d from day 9 to day 12 When endometrial thickness reached 8 mm buserelin injections were discontinued and micronized pessaries (Progesterone 50; Aburaihan Co.) were commenced at 100 mg/d. Estradiol valerate was reduced to 4 or 6 mg/d. Both progesterone and estradiol valerate were given up to the day of the pregnancy test or onset of the next spontaneous period.

Three days after commencement of progesterone (about day 15 of treatment) patients received their frozen–thawed embryos and 15 days later they were assayed for serum β-hCG. If the pregnancy test was positive, patients were followed with serial ultrasound to determine fetal viability and a prescription of estradiol valerate (4 or 6 mg/d) and progesterone (100 mg/d) was continued until 10 weeks of gestation.

Statistical analysis

Differences amongst variables of the vitrification and slow freezing groups were analyzed using the chi-squared and Fisher's exact tests for categorical variables or the Student's t-test for continuous variables, as appropriate A P value $<$ 0.05 was considered to be statistically significant. Comparisons of the survival rate, morphology of post-warmed embryo, pregnancy and implantation rates between the two groups were also presented as odds ratios with corresponding 95% confidence intervals.

Results

The patients' characteristics in the last fresh ART cycles for slow freezing and vitrification groups are summarized in Table 1. The results indicate that the number of ICSI and ICSI/IVF cycles in both groups were similar; whereas the IVF cycle alone was significantly higher in the slow freezing group (5.3% vs. 0%; P=0.003). Moreover, the mean female age, mean male age, the numbers of: patients with each infertility indication, previous attempts for ART cycles, retrieved oocytes, of cleaved embryos, cancelled embryo transfer cycles due to the ovarian hyperstimulation syndrome (OHSS) and the rate of pregnancy were all similar between the two groups. However, after observing numerous multiple pregnancies with transfer of fresh embryos in our lab, we decided to change the "number of transferred fresh embryos policy" from January 2007. This change of policy coincided with the data collection of the vitrification group and resulted in a significantly lower number of transferred fresh embryos in the vitrification group as compared with that of the slow freezing group (2.22±0.58 vs. 3.11±0.77; P=0.000).

The clinical outcome of the frozen-warmed embryo transfer for the vitrification and slow freezing groups have been summarized in Table 2. The results indicated that the mean number of cryopreserved embryos per patient was higher in the slow freezing group (8.18±4.0 vs. 6.57±3.50; P=0.000) whereas the percentages of different morphology embryos before cryopreservation were similar between the two groups. The mean duration of embryo storage per day was significantly greater in the slow freezing group (239.80±200.30 vs. 155.20±54.60; P=0.000).

After warming, in the vitrification group, the mean number of warmed embryos was significantly lower (4.70±1 vs. 6.19±2; P=0,000); whereas the survival rate per warmed embryo was significantly higher as compared with the slow freezing group (699/721, 96.9% vs. 779/941, 82.8%.; P=0.000). In the vitrification group, the percentage of the embryos with excellent morphology and all intact blastomeres was significantly higher (642/699, 91.8% vs. 438/779, 56.2%; P=0.000). In contrast, the slow freezing group had more embryos with good morphology, poor morphology and with some degenerated blastomeres (294/779, 37.7% vs. 58/699, 8.2%, and 47/779, 6% vs. 0/699, 0%, respectively; P=0.000). In the latter group 7 out of 152 patients, because of degenerated embryos, did not receive embryo replacement (4.60% vs. 0%; P=0.000).

Moreover, in spite of the low mean number of selected embryos for transfer in the vitrification group (3.43±0.60 vs. 3.87±1.37; P=0.000), the clinical pregnancy and implantation rates were significantly higher as compared with those of the slow freezing group (40.5% vs. 21.4%, and 16.6% vs. 6.8% respectively; P=0.000). Nevertheless,

Table 1 Patients' characteristics in the last fresh ART cycle

Parameter	Vitrification	Slow freezing	P-value
ART cycles	153	152	–
IVF cycles (%)	0/153 (0)	8/152 (5.3)	0.003 [a]
ICSI cycles (%)	115/153 (75.2)	109/152 (71.7)	0.495
IVF/ICSI cycles	38/153 (24.8)	35/152 (23)	0.711
Female ages (mean ± SD)	29.8±5	30.7±5.4	0.132
Male ages (mean ± SD)	35.2±4.8	35.8±6.6	0.376
Male factor infertility (%)	88/153 (57.5)	88/152 (57.9)	0.947
Female factor infertility (%)	1/153 (0.7)	2/152 (1.3)	0.558
Mixed infertility (%)	63/153 (41.2)	60/152 (39.5)	0.762
Unexplained infertility (%)	1/153 (0.7)	2/152 (1.3)	0.558
Previous ART cycles (mean ± SD)	1.46±0.99	1.49±0.62	0.757
Retrieved oocytes (mean ± SD)	18.88±8.63	18.95±7.50	0.933
Cleaved embryos (mean ± SD)	11.7±5.8	12.1±4.6	0.477
Transferred embryos per cycle (mean ± SD)	2.22±0.58	3.11±0.77	0.000 [a]
Cancelled embryo transfer due to OHSS (%)	9/153 (5.9)	15/152 (9.9)	0.196
Pregnancy rate (%)	0/153 (0)	0/152 (0)	1.000

OHSS ovarian hyper stimulation syndrome

[a] statistically significant differences were found between the two groups

Table 2 Clinical outcome of human cleavage stage embryo transfer after cryopreservation with vitrification and slow-rate freezing methods

Parameter	Vitrification	Slow freezing	*P*-value
Cycles with cryopreserved embryos	153	152	–
Cryopreserved embryos per patient (mean ± SD)	6.57±3.5	8.18±4.0	0.000 [a]
Cycles with day 2 embryo cryopreservation (%)	100/153 (65.4)	112/152 (73.7)	0.114
Cycles with day 3 embryo cryopreservation (%)	53/153 (34.6)	40/152 (26.3)	0.114
Morphology of embryo before cryopreservation per cryopreserved embryo (%)			
Excellent	965/1024(94.2)	1162/1246(93.3)	0.339
Good	59/1024(5.8)	82/1246(6.6)	0.421
Poor	0/1024(0)	2/1246(0.2)	0.505
Duration of embryo storage (days) (mean ± SD)	155.2±54.6	239.8±200.3	0.000 [a]
Warmed embryos per patient (mean ± SD)	4.7±1	6.19±2	0.000 [a]
Survival rate per warmed embryo (%)	699/721(96.9)	779/941(82.8)	0.000 [a]
Morphology of embryo after warming per survived embryo (%)			
Excellent	642/699(91.8)	438/779(56.2)	0.000 [a]
Good	58/699(8.2)	294/779(37.7)	0.000 [a]
Poor	0/699(0)	47/779(6)	0.000 [a]
Cycles with all degenerated embryos (%)	0/153	7/152 (4.6)	0.007 [a]
Frozen warmed-embryos transferred per cycle (mean ± SD)	3.43±0.6	3.87±1.37	0.000 [a]
Clinical pregnancy rate per transfer cycle (%)	62/153 (40.5)	31/145 (21.4)	0.000 [a]
Implantation rate per embryo transfer (%)	87/525 (16.6)	40/589 (6.8)	0.000 [a]
Multiple pregnancies per pregnant patient (%)	19/62 (30.6)	7/31 (22.6)	0.414
Single (%)	44/62 (71)	25/31 (80.6)	0.315
Twin (%)	15/52 (24.2)	6/31 (19.4)	0.599
Triplet (%)	3/62 (4.8)	1/31 (3.2)	1.000
Quadruplet (%)	1/62 (1.6)	0/31 (0)	1.000

[a] statistically significant differences were found between the two groups

the rates of: multiple pregnancies, singles, twins, triplets and quadruplets were similar in both groups.

The feasibility of using vitrification or slow freezing methods for cryopreservation of human cleavage stage embryos has also been determined by the odds ratio test with a 95% confidence interval. In the vitrification group versus slow freezing group:, the survival rate per warmed embryo (96.9% vs. 82.8%), the post-warmed excellent morphology per survived embryo (91.8% vs. 56.2%), clinical pregnancy rate per embryo transfer cycle (40.5% vs. 21.4%) and the implantation rate per transferred embryo (16.6% vs. 6.8%) were all higher with an odds ratio of 6.607 (95% confidence interval: 4.184–10.434), 8.769 (95% confidence interval: 6.460 – 11.904), 2.427 (95% confidence interval: 1.461–4.033) and 2.726 (95% confidence interval: 1.837–4.046), respectively. Whereas in the slow freezing group, the post-thawed good and poor morphology per survived embryo (37.7%, 6% respectively) were higher than those of the vitrification group (8.20%, 0% respectively) with an odds ratio of 6.828 (95% confidence interval: 5.022–9.283) and 44.817 (95% confidence interval: 6.167–325.709), respectively.

Discussion

Vitrification of human cleavage stage embryos versus slow-rate freezing was evaluated in recent years by several investigators [5, 7, 8, 32]. Zheng et al. [32], in an experimental study, used abnormal biopsied 8-cell stage embryos and demonstrated significant improvement of embryo survival following vitrification (94%) compared with slow freezing (15%). However, these investigators observed no statistical difference between biopsied vitrified and biopsied slow-frozen embryos regarding blastocyst development (19.6% and 12.5%, respectively) or blastocyst hatching rates (77.8% and 100%, respectively). In a larger clinical study, Kuwayama et al. [8] cryopreserved 4-cell stage embryos and demonstrated significant improvement of embryo survival following cryotop vitrification (98%) compared with slow freezing (91%). Nevertheless, these investigators reported similar pregnancy rates per transfer for vitrification (27%) and slow freezing (32%). In another clinical study, Rama Raju et al. [5] cryopreserved 8-cell stage embryos and observed significant improvement of embryo survival following cryoloop vitrification (95%)

versus slow freezing (60%). In contrast to Kuwayama et al. [8], the latter group achieved significantly better implantation and pregnancy rates in the vitrification group (14.9% and 35%, respectively) as compared with those of the slow freezing group (4.2% and 17.4%, respectively). In a more recent pre-laboratory study by Balaban et al. [7], the overall survival rate was significantly higher with vitrification of human 8-cell stage than with slow freezing (94.8% vs. 88.7%). Furthermore, the proportion of embryos with 100% intact blastomeres was significantly higher after vitrification when compared with slow freezing (77.9% vs. 51.4%). Progression rate to the blastocyst stage was also significantly higher after vitrification than after slow freezing (60.3% vs.49.5%). There were non-significant trends for better blastocyst quality (52.2% vs. 42.1%) and for higher hatching rates (31.3 % vs. 21.5%) in the vitrification group compared with slow freezing. Our results also revealed that in the vitrification group; the survival rate per total warmed embryos (96.9%) and the post-warmed excellent morphology per total survived embryos (91.80%) were both higher than those of the slow freezing group (82.8%, 56.2%). Whereas in the slow freezing group, instead of the post-warmed excellent morphology, the post-thawed good and poor morphology per total survived embryos (37.7%, 6%) increased as compared with those of in the vitrification group (8.2%, 0%). These findings were in agreement with those authors who reported better survival rate after vitrification of human cleavage stage embryos versus slow freezing and also were in agreement with Balaban et al. [7] who were the first authors to report post-warmed embryo morphology. However, the latter investigators did not show data related to either good or poor post-thawed embryo morphology in both the vitrification and slow freezing groups. The low survival rate and the presence of highly poor post-thawed embryos during the slow freezing procedure could be related to either ice crystal formation that usually happens because of the low concentration of cryoprotectant in the slow protocols or to the low metabolism of embryo during the procedure of cryopreservation. Several investigators [46–49], in animal models, have shown that slow freezing induces significant cellular trauma, including altered metabolism and a reduction in viability. This was also observed by Balaban et al. [7] who compared human embryo metabolism following cryopreservation by slow freezing or vitrification and observed pyruvate uptake by the embryos was significantly reduced following slow freezing when compared with embryos that underwent vitrification.

However, patients with vitrified-warmed embryos versus those with slow frozen-thawed embryos; because of the higher survival rate per total warmed embryos (96.9% vs. 82.8%) and no cancelled embryo transfer cycle due to the degeneration of all post-warmed embryos (0% vs. 4.60%), have more opportunity to receive all post-warmed embryos. Their embryos, because of the higher excellent morphology (91.8% vs. 56.2%), have a minimal risk to lose their morphology after warming. In our slow freezing group, in order to compensate for the poor morphology of the slow frozen-thawed embryos and to increase the chances of successful pregnancy, we were compelled to increase the number of embryos for the transfer. The results, however, reversely indicated that the clinical outcomes including implantation and pregnancy rates tended to increase significantly in the vitrification group (16.6% and 40.5%, respectively) rather than in the slow freezing group (6.8% and 21.4%, respectively). These finding were in agreement with Rama Raju et al. [5] who indicated better implantation and pregnancy rates for the cleavage stage of human vitrified-warmed embryos versus slow frozen-thawed embryos, whereas they were in disagreement with Kuwayama et al. [8] who revealed similar clinical outcomes for the both vitrification and slow freezing groups. This controversy may have been related to the different day of the embryo transfer that for Kuwayama et al. [8] was 2 days after embryo warming and exactly at the blastocyst stage, whereas for the present study and Rama Raju et al. [8] was immediately after embryo warming. These finding were also comparable to the results of those investigators who cryopreserved human cleavage stage embryos with only the vitrification technique [7, 14, 18, 30, 33, 34]. However in a more recent study Balaban et al. [7], in a cohort of 73 patients who had their supernumerary embryos cyropreserved with vitrification, reported a 30% implantation rate and 49% clinical pregnancy rate which were higher than those of the other investigators. These highly successful clinical outcomes could be related to the type of cryoprotectant (Propandiol; PROH) that was used instead of DMSO in Balaban's study. These authors explained the disadvantages of DMSO in the cryoprotectant solution: 1) a very potent solvent that could be easily introduced into the embryo, 2) it has some untoward effects on intracellular physiology and may increase intracellular calcium, most likely through disruption of intracellular organelles [50], and 3) it has been shown to cause cellular differentiation by effecting DNA methylation in other cell types [51, 52]. DMSO should be replaced by another cryoprotectant such as PROH which has fewer side effects. However, this idea needs to be researched further.

Moreover, in our study, for the first time the feasibility of either vitrification or slow freezing was evaluated for the cryopreservation of human cleavage stage embryos by an odds radio test. As a result, in the vitrification versus slow freezing; the survival rate was higher [odds ratio: 6.607 (95% confidence interval: 4.184–10.434)]. This was in agreement with Loutradi et al. [53] who on the basis of prospective comparative trials,, the current systemic review

and meta-analysis suggested that post-warming survival rate of cleavage stage embryos was significantly higher after vitrification as compared with slow freezing (odds ratio: 15.57, 95% confidence interval: 3.68–65.82). However the latter investigators, because of insufficient data, failed to show any statistical significant differences between vitrification and slow freezing in terms of implantation and pregnancy rates. Whereas in our study, not only the implantation and pregnancy rates were higher in the vitrification than those of the slow freezing [odds ratio: 2.726 (95% confidence interval: 1.837–4.046) and 2.427 (95% confidence interval: 1.461–4.033) respectively], the excellent morphology of post warming embryos was also higher in vitrification as compared with slow freezing [odds ratio: 8.769 (95% confidence interval: 6.460–11.904)].

The higher odds ratio for the survival rate, excellent morphology of post warming embryos, implantation and pregnancy rates after vitrification will encourage us to cryopreserve at least the human cleavage stage embryos by vitrification rather than slow freezing. On the other hand, because of higher multiple pregnancies in the present study, it is more feasible to warm a lower number of embryos for each transfer in order to increase the possible number of frozen embryo transfer cycles.

In conclusion vitrification, in contrast to slow freezing, is an efficient method for cryopreservation of human cleavage stage embryos. With providing higher survival rates and minimal deleterious effects on post warming embryo morphology it can improve clinical outcomes.

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