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Research Article

Comparison of vitrification and slow freezing for cryopreservation of cleavage stage embryos (Day 3) and its impact on clinical outcome

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ABSTRACT

Background: The objective of this retrospective study was to compare the efficacy of slow freezing and Vitrification for the cryopreservation of supernumerary cleavage stage embryos on day 3 after IVF and its impact on clinical outcome.
Methods: 485 supernumerary embryos of IVF cycles (from Oct 2011 to Dec 2012) were cryopreserved by slow freezing method while 502 embryos (from Jan 2013 to April 2014) by Vitrification method. 362/485 and 230/502 embryos were thawed for FET cycles (65 patients in each group).After warming the survival rate, post warmed embryo morphology, clinical pregnancy and implantation rates were evaluated and compared between the two groups.
Results: There were 65 frozen thawed cycles in each group. The percentage of excellent and good morphology embryos before cryopreservation were same in both the groups, but after thawing the results were significantly in favour of Vitrification as compared to Slow freezing. In Vitrification group versus Slow freezing group, the different outcomes were survival rate (96.95% vs. 69.06%, p-0.000), post warmed excellent morphology embryos (94.17% vs. 60.8%, p-0.000) clinical pregnancy rate (41.53% vs. 21.53%, p-0.043) and the implantation rate (14.41% vs. 7.01%, p-0.024).
Conclusions: Vitrification is a promising alternate to the conventional slow freezing method in terms of not only excellent survival and post warmed excellent morphology embryo rate but also higher clinical pregnancy and implantation rate.

Keywords: Vitrification, Slow freezing, Human cleavage stage embryos, Survival rate, Clinical pregnancy rate, Implantation rate

INTRODUCTION

Cryopreservation of supernumerary embryos produced during human IVF provides an opportunity for patients to have repeated attempts following a single drug stimulation cycle and improving cumulative pregnancy rate. The transfer of cryopreserved embryos constitutes about 20% of all embryo transfers worldwide (Liebermann et al. 2003).[1] It prevents the wastage of supernumerary embryos and also encourages transfer of fewer embryos per cycle and thus lowers multiple pregnancy rates.

Successful cryopreservation of human embryos was first reported in 1983 by Trounson and Mohr with multi cellular embryos that had been slow cooled using Dimethyl sulphoxide (DMSO).[2] Now a days two important methods of cryopreservation are slow freezing

and vitrification. Slow freezing is known as equilibrium freezing due to the exchange of fluids between the extra and intra cellular spaces and thus avoid serious osmotic and deformation effects to it.[3] It requires low concentration of cryoprotectants which is less toxic but may be insufficient for avoiding ice crystal formation within the cells. It is more time consuming and requires an expensive programmable freezing machine, so Vitrification is now regarded as a potential alternative to the conventional slow freezing method.

Vitrification was first reported by Rall and Fahy in 1985 for mammalian embryos with a later attempt for human cleavage stage embryos and followed by a successful delivery in 1990.[4,5] Vitrification is a non-equilibrium ultra-rapid method of cryopreservation where by the embryo is transitioned from 37^0c to -196^0c in <1minute resulting in extremely fast rates of cooling (>10,0000c/min). It has the advantage of preventing ice crystal formation by a short exposure to high concentrations of cryoprotectants with low water content and eliminating the use of expensive equipments.[4,6] The main drawback is exposure of the embryos to a high concentration of cryoprotectants which may have a detrimental effect.[4] This can be minimized by allowing a very short exposure i.e. 30-40 seconds, combination of cryoprotectants and using a less toxic cryoprotectant.[8,9] Some of the cryoprotectants are DMSO glycerol, ethylene glycol, propanediol and sugars.

To facilitate rapid heat transfer minimal volumes are used in Vitrification by the use of minute tools or carriers like pulled and hemistraws.[10-15]

Embryos have been successfully cryopreserved at all stages like pronuclear, cleavage, morula and blastocyst stage.[15-20]

The aim of our study is to evaluate the results of Vitrification and Slow freezing for the cryopreservation of human cleavage stage embryos on Day 3 in terms of post warming survival rate, embryo morphology and clinical outcome.

METHODS

The study was performed in the Assisted Reproductive unit of Mahatma Gandhi University of Medical Sciences and Technology.

This retrospective study compared the laboratory and clinical outcome of 65 patients having slow frozen thawed embryo transfer cycles (362 embryos) from Jan 2011 to March 2012 with 65 patients having vitrified-warmed embryo transfer cycles (230 embryos) from Jan 2013 to April 2014. During this study all conditions and protocols for human embryo culturing were kept constant in our lab. All cycles were analyzed retrospectively as all embryos after Jan 2013 were cryopreserved by Vitrification & before that mostly by slow freezing.

Only long protocol cases were selected in which ovarian stimulation was performed following down regulation by GnRH agonist (Inj Iupride 1mg, Sun Pharma) from Day 21 of previous cycle. Ovarian stimulation was done by using r- FSH (Inj Gonal-F, Merck Serono S. A. Switzerland) or HMG (Inj Menogon, Ferring, Germany). The dose was increased according to follicular study and serum hormones. When at least 3 follicles reached ≥ 18mm in diameter, HCG 10,000 I.U. (Inj. Sifasi, Serum Institute) was given and oocyte retrieval was performed 36-38 hrs. after HCG. The oocytes underwent standard IVF and / or ICSI were cultured in lab for 3 days. The Day 3 embryos were scored as: Excellent morphology (6-8 even size blastomeres with ≤10% fragmentation), good morphology (6-8 even or uneven size blastomeres with 10-20% fragmentation) & poor morphology (uneven few blastomeres with > 20% fragmentation).

After transfer of 2-3 excellent morphology embryos on Day 3 in fresh cycle, the supernumerary embryos (excellent and good morphology) were cryopreserved.

Protocol for Slow freezing and thawing procedure – It was done by using commercial kit (Origio, Medicult medium, Denmark) for freezing and thawing. The instructions written in the manual were applied in the same way. After thawing embryos were observed for 2-3hrs before transfer.

Protocol for Vitrification and thawing procedure- This was done by using commercial Kit (Origio, Medicult medium, Denmark). In brief, embryos were first placed in equilibration media containing 1,2 propanediol, ethylene glycol and sucrose for 5-15 min, at room temperature and then transferred to minimum volume of Vitrification medium after having observed cellular dehydration and rehydration. Embryos were then placed on the tip of cryoleaf and directly plunged into liquid nitrogen in less than 1 minute (open method).

On the day of FET, the cryoloop was removed from liquid nitrogen tank. The embryos were kept for 3 min. in warming media at 37^o c and then transferred to dilution media 1 and 2 at room temperature for 3 min. in each. Finally the embryos were washed twice in washing media before shifting to incubator for 2-3 hrs. before transfer.

The frozen-thawed embryos were classified as excellent morphology (100% of cells survived with <10% fragmentation), good morphology (100% of cell survived with 10-20% fragmentation) poor morphology (≥50% cells survived with or without any fragmentation) or as degenerated embryos (<50% of cells survived). 3-4 embryos of best morphology were selected for FET.

For endometrial preparation Estradiol Valerate 6-8mg was started from day 3 of period after down regulation with GnRH agonist. It took 14-20 days of therapy for endometrium to reach ≥7mm and E2 levels ≥300pg/ml. After that natural micronized progesterone was started for

3 days by vaginal (600 mg/day) or I.M. route (100 mg/day). (Cap/Inj Gestone, Ferring). After FET both hormones were continued for 15 days till serum β– HCG test. In positive cases, clinical pregnancy was confirmed by Trans vaginal Sonography (TVS) 15 days after β – HCG test and hormones continued till 14 weeks in confirmed cases. The implantation rate was derived from the number of foetuses with a heartbeat divided by the total number of embryos transferred. For statistical analysis chi-square test was used to evaluate the strength of association with p <0.05 taken as statistically significant.

RESULTS

Table-1 shows the patients characteristics in the fresh ART cycle for slow freezing and Vitrification group. The mean female age, numbers of retrieved oocytes, number of cleaving embryos, number of transferred embryos per cycle, number of supernumerary embryos and clinical pregnancy rate in fresh cycle were all similar between the two groups.

Table-2 shows the laboratory outcome of frozen-thawed embryos for the Vitrification and Slow freezing groups. The results indicated that the percentage of excellent and good morphology embryos before cryopreservation were similar between the two groups. The number of embryos warmed per cycle was less in Vitrification group as compared to slow freezing group. So the total number of the embryos thawed for similar number of cycles (65) was less in vitrification group (45.81% vs. 74.63%, p-0.000).

The survival rate was significantly high in Vitrification group as compared to Slow freezing group (96.95% vs. 69.06%, p-0.000). Similarly, after thawing the percentage of excellent morphology embryos was significantly high in Vitrification group as compared to Slow freezing (94.17% Vs. 60.8%, p-0.000). In contrast the number of good and poor morphology embryos were high in Slow freezing group (30.4% vs. 5.38%, p- 0.000) and (8.8% Vs.44%. p-0.000) respectively.

Table -3 shows the clinical outcome of frozen-thawed embryo transfer in two groups. The clinical pregnancy rate (41.53% vs. 21.53%, p-0.043) and implantation rate (14.41% vs. 7.01%, p-0.024) were significantly high in Vitrification group as compared to Slow freezing group. There was no significant difference in miscarriage rate (14.28% vs. 8%, p-0.938) and multiple pregnancy rates (14.28% vs. 25.92%, p-0.644) in the two groups. This comparative study also included odds ratio test with a 95% confidence interval.

Table 1: Characteristics of fresh cycles.

Sr. No.	Parameters	Slow freezing	Vitrification	P- Value
1.	Total Cycles	100	100	-
2	IVF cycle	63%	58%	0.563
3.	IVF-ICSI Cycles	37%	42%	0.563
4.	Female age (Mean ± S.D.)	30.56 ± 3.65	30.55 ± 3.95	0.464
5.	Oocytes retrieved (Mean ± S.D.)	16.60 ± 4.50	16.50 ± 4.40	0.874
6.	M II Oocytes (Mean ± S.D.)	10.48 ± 1.80	10.80 ± 1.70	0.198
7.	Cleaving Embryos (Day 3) (Mean ± S.D.)	8.92 ± 1.25	9.20 ± 1.10	0.094
8.	Transferred Embryos per cycle (Mean ±) S.D.	2.5 ± 0.513	2.6 ± 0.502	0.160
9.	Supernumerary Embryos (Mean ± S.D.)	6.8 ± 0.767	7.0 ± 0.740	0.062
10.	Selected Embryos for cryopreservation per cycle (Mean ± S.D.)	4.8 ± 0.600	5.0 ± 0.590	0.149
11.	Clinical Pregnancy rate in Fresh cycle	25%	28%	0.749
12.	Total number of embryos frozen	485	502	

Table 2: Laboratory outcome in FET cycles.

Sr. No.	Parameters	Slow freezing	Vitrification	CI	P-value
1.	Total Number of embryos frozen	485	502	-	-
2.	Morphology of embryos before cryopreservation Excellent Good	 92.4% 7.6%	 93.8% 6.2%	 0.0456-0.0176 0.0476-0.0456	 0.458 0.458
3.	No. of FET Cycles	65	65	-	-
4.	Warmed embryos per Cycle (Mean ± S.D.)	5-6	3-4	-	-
5.	Total no. of embryos Thawed	362 (74.63%)	230 (45.81%)	0.2307-0.3533	0.000
	Morphology of thawed embryos Excellent Good Poor	 152/250 (60.8%) 76/250 (30.4%) 22/250 (8.8%)	 210/223 (94.17%) 12/223 (5.38%) 1/223 (.44%)	 0.0332-0.1892 0.2113-0.3591 0.0447-0.1224	 0.000 0.000 0.000
6.	No. of embryos Surviving	69.06%	96.95%	0.3451-0.2127	0.000
7.	Frozen-warmed embryos transferred per cycle	3-4	3-4	-	-

Table 3: Clinical outcome in FET cycles.

Sr.No.	Parameters	Slow Freezing	Vitrification	CI	P-value
1.	Clinical pregnancy rate per transfer cycle	14/65 (21.53%)	27/65 (41.53%)	0.3620-0.0405	0.043
2.	Implantation rate per embryo transfer	16/228 (7.01%)	32/222 (14.41%)	0.1680-0.0132	0.024
3.	Miscarriage rate	2/14 (14.28%)	2/27 (8%)	0.1327-0.2567	0.938
4.	Multiple pregnancy rate	2/14 (14.28%)	7/27 (25.92%)	0.3842-0.1498	0.644

DISCUSSION

Cryopreservation of embryos is an effective way of increasing the cumulative pregnancy rate of IVF cycles. Clinical success with cryopreservation depends on many factors like patient age, stimulation protocol, quality of embryos and developmental stage at freezing, type of cryopreservation parameters of cooling and warning etc.

In our study the mean age of patient, stimulation protocol, quality of embryos selected for freezing were same. To avoid variation we used commercially available kits for cooling and thawing in both groups. For Vitrification we used Ethylene Glycol (EG) based media (Medicult cooling and thawing media, Origio, Denmark). Ethylene Glycol has proven to be a stable cryoprotectant, with less toxicity and high permeation ability suggested that Ethylene Glycol diffuses into and leaves the embryos very rapidly due to its low molecular weight, hence embryos do not undergo osmotic shock during freezing and thawing procedures.[21,22] The embryo survival rate of 96.95% with 94.17% having excellent morphology in our study, further supports this concept. Mukaida et al (1998) reported a survival rate of 81% with EG based media.[23] Danasouri and Selmen (2001) reported a low survival rate of 79.2% but higher pregnancy rates of 30.5% with EG.[10] We used cryoloop for storage in Vitrification. One issue with Vitrification on cryoloops is that it involves direct exposure to liquid nitrogen. The risk of cross contamination in liquid nitrogen storage containers even at -1960c has been widely debated (Bielanski et al 2003), but no studies have demonstrated unintentional uptake by a human embryo of any pathogen during Vitrification even with the use of open systems.[24]

We select cleavage stage (Day 3) embryos for Vitrification in our study. Though there are few studies on day 3 embryo vitrification.[10,15-19,23,25] The survival rate in vitrification group (96.95%) in our study is comparable to Kuwayama et al 2005 (98%),[17] Ram Raju et al 2005 (95.3%),[15] Valojerdi et al 2009 (96.9%)[19] and Balaban et al 2008 (94.8%).[18] This is higher than Desai et al (85%).[16]

Because of comparatively poor survival rate, we thawed more embryos in slow freezing group as compared to Vitrification (5-6 vs. 3-4). The frozen warmed embryos with excellent and good morphology transferred per cycle were kept same in both groups i.e. 3-4.

The clinical pregnancy rate and implantation rate in our study was significantly high in Vitrification group (41.53% and 14.41%). This is comparable to Rama Raju et al (35% and 14.9%),[15] Valojerdi et al (40.50% and

16.6%)[19] and Desai et al (44% and 20%),[16] though the survival rate was low in the study of Desai et al (85%).[15] The survival rate was high (98%) but clinical pregnancy rate was similar to Slow freezing group (27% Vs. 32%) in the study of Kuwayama et al.[17] This controversy may have been related to the different day of embryo transfer (2 Days after embryo warming at the blastocyst stage) in the study of Kuwayama et al.[17] While in our study it was done 2-3 hours after warming.

These findings were also comparable to the results of those investigators who cryopreserved human cleavage stage embryos with only the vitrification technique.[10,13,25] The Balaban et al (2008) reported 49.3% clinical pregnancy rate and 29.7% implantation rate, higher than our study.[18] This could be related to type of cryoprotectant (Propandiol, PROH) used by Balaban et al.[18]

Vitrification is a relatively new technique. So far the single largest study by Takahashi et al (2005) shows no significant difference in mean gestational age, birth weight or congenital birth defect rate in vitrified-warmed blastocyst transfer and fresh blastocyst transfer groups.[26]

In conclusion, vitrification in contrast to slow freezing is a simple inexpensive and efficient method of freezing human cleavage stage embryos with higher survival rate and excellent morphology of warmed embryos. The vitrification method also improves the clinical pregnancy and implantation rates. Vitrification at the cleavage stage allows more embryos to be vitrified per patient and alleviated the need for extended culture of large numbers of spare embryos. The patient is also benefitted by having embryos frozen at both early and late stages.

Superiority of one cryoprotectant over the others and their appropriate combination need further larger studies to make conclusion.

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