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Effect of extended embryo culture after thawing on clinical pregnancy rate

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ABSTRACT

Background: Embryos cryopreservation is an essential service in assisted reproductive techniques (ART) centers and each center should have its own protocol to maximize the outcome of frozen-thawed cycles. After thawing, embryo selection can either be according to morphology or after assuring viability by extending culture and waiting for resumption of mitosis.
Objective: of this randomized controlled trial was to compare clinical pregnancy rate of immediate versus delayed post thawing embryo transfer in frozen embryo transfer (FET) cycles.
Patients and Methods: The study was conducted on 150 FET cycles in which embryos were vitrified on day 2 or 3. After thawing, cases with more than 50% blastomere loss were excluded as well as, cases with recurrent implantation. Endometrium was prepared by programmed estrogen/ progesterone method. After thawing cases were randomized for either immediate embryo transfer or extended culture for 18–72 hours before transfer.
Results: Number of cryopreserved embryos and survival rates were comparable among both groups. Survival rates in both groups was above 95% and in the extended culture group 72.1 % of embryos resumed miosis. Clinical pregnancy rate was 50.7% in immediate transfer group I, versus 41.3% in the extended culture group but the difference was statistically nonsignificant.
Conclusion: Extended culture after thawing helps in embryo selection but does not increase clinical pregnancy rate.

Keywords: ICSI; Frozen embryo transfer; Thawing; extended culture; blastocyst transfer.

SOMMARIO

Background: la crioconservazione degli embrioni è un servizio essenziale nei centri di tecniche di riproduzione assistita (ART) e ogni centro dovrebbe avere il proprio protocollo per massimizzare l'esito dei cicli congelati e scongelati. Dopo lo scongelamento, la selezione dell'embrione può essere in base alla morfologia o dopo aver assicurato la vitalità estendendo la cultura e aspettando la ripresa della mitosi.
Obiettivo: di questo studio randomizzato controllato è stato quello di confrontare il tasso di gravidanza clinica tra trasferimento di embrioni post-scongelamento immediato e ritardato nei cicli di trasferimento di embrioni congelati (FET).
Pazienti e metodi: lo studio è stato condotto su 150 cicli FET in cui gli embrioni sono stati vetrificati il giorno 2 o 3. Dopo lo scongelamento, sono stati esclusi i casi con perdita di blastomeri superiore al 50%, nonché i casi con impianto ricorrente. L'endometrio è stato preparato con metodo estrogeni / progesterone programmato. Dopo lo scongelamento, i casi sono stati randomizzati per il trasferimento immediato dell'embrione o per la coltura estesa per 18-72 ore prima del trasferimento.
Risultati: il numero di embrioni crioconservati e i tassi di sopravvivenza erano comparabili tra i due gruppi. I tassi di sopravvivenza in entrambi i gruppi erano superiori al 95% e nel gruppo di coltura estesa il 72,1% degli embrioni ha ripreso la miosi. Il tasso di gravidanza è stato del 50,7% nel gruppo II di trasferimento immediato, rispetto al 41,3% nel gruppo con coltura estesa, ma la differenza non era statisticamente significativa.
Conclusione: la coltura estesa dopo lo scongelamento aiuta nella selezione degli embrioni ma non aumenta il tasso di gravidanza.

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INTRODUCTION

Most controlled ovarian stimulation protocols used in assisted reproductive techniques (ART) cycles leads to multifollicular response and hence surplus embryos. Competent embryo cryopreservation program will improve cumulative pregnancy rate following a single oocyte retrieval. In addition, the availability of cryopreservation is essential step toward decreasing the number of transferred embryos per cycle and hence decrease the incidence of multiple gestation[1].

Moreover, there is a growing trend now toward decreasing fresh embryo transfer and freezing all embryos due various indications as PGS cycles, prophylaxis against OHSS, abnormal endometrium and even the freeze all for all policy[2].

Trounson et al in 1983 reported the first human pregnancy after frozen embryo transfer (FET). Since then, there has been great improvements in the freezing, thawing and endometrial preparation techniques[3]. Now various stages from zygote to blastocyst can be vitrified successfully. Recently most ART centers extend embryo culture till the blastocyst stage for better embryo selection and higher implantation and pregnancy rates. On the other hand, extended embryo culture may decrease the surplus embryos available for cryopreservation[4,5].

AIM OF THE STUDY

The aim of this study is to compare clinical pregnancy rate of immediate versus delayed post thawing embryo transfer in frozen embryo transfer (FET) cycles.

SETTING

Randomized controlled trial that was carried out at a governmental ART center (El Shatby maternity hospital, Alexandria university, Egypt)

PATIENTS

The study was conducted on 150 FET cycles. Inclusion criteria were cases whose embryos were vitrified at day 2 or 3 and with at least 3 embryos per cryotop. Exclusion criteria was the loss of more than half of the blastomeres in more than one embryo during the thawing procedure. Moreover, cases with recurrent implantation failure were excluded from the study.

METHODS

The study had received approval from the medical ethical committee of the Faculty of Medicine, Alexandria University (number 00007555- FWA No: 00018699). An informed written consent was obtained from all participants before enrollment in the study. The study has been carried out in accordance with the code of Ethics of the World Medical Association (1964 Declaration of Helsinki and its later amendments.

Endometrial preparation was done by programmed cycle in which oral estradiol valerate 8mg/day (Cycloprogynova 2mg-Bayer®) was started on cycle day 3. When endometrium exceeded 8 mm with triple line morphology vaginal progesterone suppository 400mg and intramuscular 100mg (Prontogest, IBSA®) were started. Day 3 embryos were transferred on day 4 of progesterone administration while day 5 embryos were transferred on day 6 progesterone. Serum pregnancy test was done after 14 days of ET. Clinical pregnancy was defined as the observation of a gestational sac with fetal heart pulsation 2-3 weeks after the positive pregnancy test.

VITRIFICATION AND WARMING PROCEDURE

Embryos were vitrified and thawed using open system Kitazato ® cryotop system. Vitrification kit (Kitazato ®, KOVT 8010, Japan) were used. At room temperature embryos were first put in equilibration solution comprising 7.5% ethylene glycol (EG) and 7.5% dimethyl sulphoxide (DMSO) in Human serum with 20% Albumin for 5–15 minutes (depending on the time needed for re-expansion of the cell). Then embryos were moved to the vitrification solution (15% EG, 15% DMSO, 0.5 M sucrose) in Ham's F-10 medium supplemented with 20% Albumin for 50–60 seconds. After observing cellular shrinkage, embryos were aspirated and placed on the tip of the Cryotop. Ultra- rapid cooling of the embryos was done by direct plunging into liquid nitrogen.

Thawing was also done using Kitazato ® thawing kit (Kitazato KO VT 8020, Japan). The embryos were put into thawing solution (1 M sucrose in Ham's F-10 medium supplemented with 20% Albumin) for 50–60 seconds at 37.0°C temperature and then transferred into dilution solution of 0.5 M sucrose for 3 minutes, followed by another dilution solution of 0.25 M sucrose for another 3 minutes, both at room temperature.

Thawed embryos were then classified as an excellent (100% of cells survived with < 10% fragmentation), good morphology (100% of cells survived with 10–20% fragmentation), poor morphology (< 100 and more than 50% cells survived with or without any fragmentation) or as degenerated embryos (<50% of cells survived).

After thawing cases fulfilling the inclusion and exclusion criteria were randomized using computer generated tables for two groups (75 patients in each group):

Group I (immediate transfer): Embryos were transferred 1/2–5 hours after thawing and embryos are selected morphologically.

Group II (extended culture): Embryos were cultured after thawing for 18–72 hours. In this group selecting embryo for transfer was according to resumption of mitosis after thawing and progressing to blastocyst.

RESULTS

The study was conducted on 150 patients who were randomized into two groups (75 each) after thawing of their embryos. Both groups had comparable demographic and clinical data (**Table 1**).

Table 1 . *Demographic and clinical data*

	Group I (n = 75)		Group II (n = 75)		P	
Age (years) Min. – Max. Mean ± SD.	.0 – 37.0 27.71 ± 5.03		19.0 – 36.0 28.44 ± 5.22		0.382	
Duration of infertility Min.-Max Mean S.D.	1.0-13 5.44 2.608		1.0-9.0 4.71 2.304		3.06 0.08	
Cause of infertility Male factor Endometriosis Polycystic ovary Tubal factor Unexplained	38 10 5 18 4	52.9% 12.9% 5.7% 24.3% 4.3%	34 13 12 12 4	47.1% 17.1% 15.7% 15.7% 4.3%	5.35 0.253	
Induction protocol Agonist Antagonist	35 40	46.7 53.3	34 41	45.3 54.7	$\chi 2$ 0.027	P 0.870

There was no statistically significant difference between the immediate transfer and the extended culture group regarding the number of cryopreserved embryos (3.69 ± 0.75 Vs 4.0 ± 0.97) respectively (**Table 2**). In addition, the grading of embryos was also comparable. After thawing both groups had survival rate above 95% (**Table 3**).

Table 2. *Vitrification data*

CRYO	Group I	Group II	Test of sig.	P
Embryo per cryotop: Min. – Max. Mean ± SD. **Total**	3.0 – 6.0 3.69 ± 0.75 **277**	3.0 – 7.0 4.0 ± 0.97 **300**	U= 2351.5	0.064
Grade A embryos Min. – Max. Mean ± SD. **Total**	2.0 – 6.0 3.27 ± 0.92 **245**	3.0 – 6.0 3.77 ± 0.76 **283**	U= 2278.0	0.378
Grade B embryos Min. – Max. Mean ± SD. **Total**	1.0 – 3.0 1.63 ± 0.68 **32**	1.0 – 2.0 1.42 ± 0.51 **17**	U= 96.5	0.424

Table 3. *Survival rate after thawing in both groups*

	Group I	Group II	$\chi 2$	P
	No	No		
Survival Frozen Thawed Survival rate	277 265 95.7%	300 290 96.7%	0.037	0.847

As shown on **Table 4**. most of group II cases (65%) were day 3 embryos and after thawing were cultured till day 5. While 25% of the cases were day 2 embryos that was just cultured for 1 day. Three cases were day 2 and was transferred on the morula stage after culturing for only 2 days due to logistics and we cultured four cases for 72 hours (from day 2 till day 5). We could not find any statistically significant difference in the rate of resumption of miosis among all these groups and on average 72.1 % of our embryos resumed miosis after thawing. The embryos of 53 cases were cultured till day 5 after thawing, 83% of them had at least one blastocyst (more than half of them were expanded), while the embryos of another 6 patients were arrested at the morula stage and ET was done after counselling the patients. Unfortunately, 3 cases had arrested embryos before the morula stage and ET was cancelled.

According to our embryo transfer policy, we

on average transfer 3 cleavage stage embryos or 2 blastocysts on day 5. So, the number of transferred embryos was significantly higher on group I (3.24 ± 0.73) than group II (2.29 ± 0.90) (p <0.001).

Table 4. *Distribution of cases in group II according to the day of thawing and day of transfer*

Thawing day	Transfer day	No of cases	No. of embryos	Resumption of mitosis	%
2	3	19	59	42	71.2
2	4	3	12	10	83.3
2	5	4	18	13	72.2
3	5	49	201	144	71.6
Total		**75**	**290**	**209**	**72.1**

Table 5. *Clinical pregnancy rate per started cycle*

Pregnancy	Group I (n = 75)		Group II (n = 75)		χ^2	MCp
	No.	%	No.	%		
Pregnant	38	50.7	31	41.3		
Cancelled	2	2.7	3	4.0		

Clinical pregnancy rate per started cycle:

Our results demonstrated that pregnancy rate was 50.7% in group I, and 41.3% in group II. This difference was statistically nonsignificant. Among group I cases day 2 and day 3 had comparable pregnancy rates (50% Vs 52.2%). However, in group II, as shown on **Table 6**, we found significant lower pregnancy rates when embryos are transferred on day 3 or day 4 versus day 5 blastocysts. Embryo transfer was cancelled for 2 cases in the first group and 3 cases in the second group. Cases who agreed to transfer retarded embryos at the morula stage 50% of them got pregnant (3/6).

Table 6. *Pregnancy rate in group II according to day of thawing and day of transfer*

Thawing- transfer	Pregnancy rate
Day 2 embryos	
2 – 3	6/19 (31.5%)
2 – 4	1/3 (33.3%)
2 – 5	2/4 (50%)
Day 3 embryos	
3 – 5	22/49 (44.9%)

DISCUSSION

Timing of embryo transfer is a crucial decision to take in ART cycles. The rational behind day 5 transfer is that physiologically, blastocysts, not cleavage stage embryos, are present in the uterine cavity. Moreover, uterine contractility is less in day 5 as compared to day 3. In addition, culturing embryos till day 5 carries the advantage of natural embryo selection[6,7].

In fresh cycles, the value of blastocyst transfer has been proved by Papanikolaou et al, meta-analysis and Glujovsky et al, Cochrane study however, the evidence in thawed cycles is not that clear[5,8].

Carvalho et al performed an elegant retrospective study comparing day 3 and day 5 embryo transfer in both fresh and thawed cycles. They were able to demonstrate the improved outcome of blastocyst transfer in fresh cycles only. In FET cycles both day 3 and day 5 transfer had a comparable implantation, pregnancy and live birth rates. Their explanation was that in fresh cycles the supraphysiologic estradiol levels resulting from controlled ovarian stimulation is negatively affecting day 3 endometrium but not day 5 endometrium[9].

However, data from the Society for Assisted Reproductive Technology for 10 years (2004-2013) from almost 250,000 cycles indicated that for FET odds of live birth increases 49% following blastocyst transfer as compared to cleavage stage embryos[10].

At El Shatby ART center, our survival rate for vitrifying cleavage stage embryos is better than that of blastocysts, so we prefer to vitrify embryos at day 2/3. Our mean survival rate after thawing of cleavage stage embryos is 96.2%. We vitrify embryos using the Cryotop open system. The reported survival rates of cleavage stage embryos using Cryotop ranged from 92.8% (Chi et al) to 96.3% (Lin et al)[11,12].

After thawing, selecting embryos for transfer can either be according to morphology (intact blastomeres) or after assuring viability by extending culture and waiting for resumption of mitosis[13,14].

The study was done in a governmental ART center and due to tight resources, we usually have to vitrify 3-4 embryos per Cryotop and sometimes even more. So, we are always trying to improve our embryo selection criteria. The rational of our study was to extend embryo culture after thawing to ensure embryo viability via resumption of miosis to allow better embryo selection. Moreover, extending the culture till the blastocyst stage will permit natural embryo selection.

Rentao Jin et al performed a randomized control trial in which post thawing embryos were randomly divided to extended culture for 16-18 hours or immediate transfer within 2 hours. The extended culture group had a nonsignificant increase in clinical

pregnancy rate (42.4% Vs 40.9) and they concluded that extended culture does not improve pregnancy rates in FET cycles. Lei Guo et al. also had the same conclusion after studying the data of more than 130 FET cycles[15,16].

However, a recent study by Haas et al found a significant increase in pregnancy rate in FET cycles after extended culture for 20-22 hours after thawing. According to their data if fair quality embryos progressed to better quality embryos pregnancy rates increases from 9% to 25.4%[17].

We had a comparable clinical pregnancy rates in the immediate transfer and the extended culture group (50.7% Vs 41.3% respectively). An earlier study by Eftekhar et al, also studied the outcome of post-thawing culturing cleavage stage embryos till the blastocyst stage. Both groups had a comparable clinical pregnancy rates, but they found a significant increase in ongoing pregnancy rate in the blastocyst group (42.9 vs. 24.6 %). Unfortunately, in our study the end point was the clinical pregnancy[18].

From our study we can conclude that extended embryo culture after thawing helps in embryo selection, but it does not increase clinical pregnancy rate. Extended embryo culture can be used for cases inwhich more than 2 embryos are vitrified in the same cryotop to allow natural embryo selection. Moreover, it may be used in those labs that prefer to vitrify cleavage stage embryos without compromising clinical pregnancy rate.

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