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Original Research

Effect of extended embryo culture after thawing on clinical pregnancy rate

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Summary

Purpose: This randomized controlled trial is aiming to compare the clinical pregnancy rate of immediate versus delayed post thawing embryo transfer in frozen embryo transfer (FET) cycles. *Materials 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. The endometrium was prepared by programmed estrogen/progesterone method. After thawing, cases were randomized to immediate embryo transfer or extended culture for 18-72 hours before transfer. *Results:* Number of cryopreserved embryos and survival rates were similar in both groups. Survival rates was above 95% in both groups and in the extended culture group 72.1% of embryos resumed miosis. Clinical pregnancy rate was 50.7% in immediate transfer group and 41.3% in the extended culture group. *Conclusion:* Extended culture after thawing helps in embryo selection but does not increase clinical pregnancy rate.

Key words: ICSI; Frozen embryo transfer; Thawing; Extended culture; Blastocyst transfer.

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 an essential step toward decreasing the number of transferred embryos per cycle lowering the incidence of multiple gestation [1]. Moreover, there is a growing trend favoring decreasing fresh embryo transfer and freezing all embryos due to various indications as preimplantation genetic screening (PGS) cycles, prophylaxis against ovarian hyperstimulation syndrome (OHSS), suboptimal 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 successfully vitrified. Recently, most ART centers extend embryo culture until the blastocyst stage for better embryo selection and higher implantation and pregnancy rates. On the other hand, extended embryo culture may decrease the surplus of embryos available for cryopreservation [4, 5].

The aim of this study is to compare clinical pregnancy rates of immediate versus delayed post thawing embryo transfer in FET cycles. This randomized controlled trial that was conducted at a governmental ART center (El Shatby Maternity Hospital, Alexandria University, Egypt).

Materials and Methods

The study was conducted on 150 FET cycles. Cases whose embryos were vitrified at day 2 or 3 and with at least three embryos per cryotop were eligible for inclusion. Cases with loss of more than half of the blastomeres in more than one embryo during the thawing procedure as well as cases with recurrent implantation failure were excluded from the study.

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

Endometrial preparation was performed by programmed cycle with oral estradiol valerate 8 mg/day (Cyclo progynova 2 mg) started on cycle day 3. When endometrium exceeded 8 mm with triple line morphology, vaginal progesterone suppository 400 mg and intramuscular 100 mg were started. Day 3 embryos were transferred on day 4 of progesterone administration while day 5 embryos were transferred on day 6 of progesterone administration. Serum beta human gonadotrophin level were measured after 14 days of ET. Clinical pregnancy was defined as the observation of a gestational sac with fetal heart activity 2-3 weeks after a positive serum pregnancy test.

Embryos were vitrified and thawed using cryotop open system. Vitrification kit was also used. At room temperature embryos were first placed in equilibration solution

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Table 1. —*Demographics and clinical data.*

	Group I (n = 75)		Group II (n = 75)		*p*	
Age (years)						
Min. - Max.	0.0 - 37.0		19.0 - 36.0		0.382	
Mean ± SD.	27.71 ± 5.03		28.44 ± 5.22			
Duration of infertility						
Min. - Max.	1.0-13		1.0-9.0		3.06	
Mean	5.44		4.71		0.08	
S.D.	2.608		2.304			
Cause of infertility						
Male factor	38	52.90%	34	47.10%		
Endometriosis	10	12.90%	13	17.10%	5.35	
Polycystic ovary	5	5.70%	12	15.70%	0.253	
Tubal factor	18	24.30%	12	15.70%		
Unexplained	4	4.30%	4	4.30%		
Induction protocol						
Agonist	35	46.7	34	45.3	χ^2	*p*
Antagonist	40	53.3	41	54.7	0.027	0.870

Table 2. — *Vitrification data.*

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

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

	Group I No	Group II No	χ^2	*p*
Survival				
Frozen	277	300		
Thawed	265	290	0.037	0.847
Survival rate	95.70%	96.70%		

comprising 7.5% ethylene glycol (EG) and 7.5% dimethyl sulphoxide (DMSO) in human serum with 20% Albumin for 5 to 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 performed by direct plunging into liquid nitrogen.

Thawing was also done using a thawing kit. The embryos were placed 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 three minutes, followed by another dilution solution of 0.25 M sucrose for three more minutes, both at room temperature.

Thawed embryos were then classified as having excellent morphology (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 were randomized 1 : 1 using computer generated tables into two groups (75 patients in each group):

Group I (immediate transfer) embryos morphologically selected were transferred 1/2-5 hours after thawing. Group

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

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.

Table 5. — *Clinical pregnancy rate per started cycle.*

Pregnancy	Group I (n = 75)		Group II (n = 75)		χ^2	^{MC}p
	No.	%	No.	%		
Pregnant	38	50.7	31	41.3		
Cancelled	2	2.7	3	4		

χ^2, *p*: χ^2 and *p* values for Chi square test for comparing between the two groups; ^{MC}p: *p* value for Monte Carlo for Chi square test for comparing between the two groups.

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). 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).

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

According to the authors' embryo transfer policy, they on average transfer three cleavage stage embryos or two blastocysts on day 5. Therefore the number of transferred embryos was significantly higher on group I (3.24 ± 0.73) than group II (2.29 ± 0.90) ($p < 0.001$).

The present results demonstrated that pregnancy rate was 50.7% in group I, and 41.3% in group II (Table 5). This difference was statistically nonsignificant. Among group I cases, day 2 and 3 had comparable pregnancy rates (50% *vs.* 52.2%). However, in group II, as shown on Table 6, the authors found significant lower pregnancy rates when embryos are transferred on days 3 or 4 *vs.* day 5 blastocysts. ET was cancelled for two cases in the first group and three cases in the second group. Cases who agreed to transfer retarded embryos at the morula stage and 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

The preferred timing to perform ET is a crucial decision to make 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 which favor implantation. In addition, culturing embryos untill day 5 has the advantage of natural embryo selection [6, 7].

In fresh cycles, the value of blastocyst transfer has been proved by Papanikolaou *et al.*, in a meta-analysis and by Glujovsky *et al.*, in a Cochrane review however, the evidence regarding thawed cycles is still unclear [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 demonstrated an improved outcome of blastocyst transfer in fresh cycles only. In FET cycles, both day 3 and day 5 transfer, had comparable implan-

tation, pregnancy, and live birth rates. They concluded that in fresh cycles, the supraphysiologic estradiol levels resulting from controlled ovarian stimulation is negatively affecting the endometrium at day 3 but not at day 5 [9]. However, data from the Society for Assisted Reproductive Technology over ten years (2004-2013) including almost 250,000 cycles indicated that for FET the odds of live birth increases 49% following blastocyst transfer as compared to cleavage stage embryos [10].

At El Shatby ART center, the survival rate for vitrifying cleavage stage embryos is better than survival of blastocysts, so the authors prefer to vitrify embryos at day 2/3 using the Cryotop open system. Our mean survival rate after thawing of cleavage stage embryos is 96.2%. The reported survival rates of cleavage stage embryos using Cryotop ranged from 92.8% to 96.3% [11, 12]. After thawing, embryo selection for transfer can be performed according to morphology (intact blastomeres) or after, ensuring viability by extending culture and waiting for the resumption of mitosis [13, 14].

The current study was performed in a governmental ART center with tight resources, requiring to vitrify 3 or 4 embryos per Cryotop. Improving embryo selection criteria is a priority. The rational of this study was to extend embryo culture after thawing to ensure embryo viability via resumption of miosis allowing better embryo selection. Moreover, extending the culture until the blastocyst stage allows natural embryo selection.

Jin *et al.* performed a randomized controlled trial in which post thawing embryos were randomly assigned to extended culture for 16-18 hours or immediate transfer within two hours. The clinical pregnancy rate similar in both groups (42.4% *vs.* 40.9%) concluding that extended culture does not improve pregnancy rates in FET cycles. Guo *et al.* had the same results 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. In their study, when fair quality embryos progressed to better quality embryos, pregnancy rates increased from 9% to 25.4% [17].

This study had a similar 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 reported the outcome of post-thawing culturing cleavage stage embryos till the blastocyst stage. Both groups had 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 the present study, the end point was the clinical pregnancy rate [18].

From this study we concluded 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 in which more than two embryos are vitrified in the same cryotop to allow natural embryo selection. It may be used in labs that prefer to vitrify cleavage stage embryos without compromising clinical pregnancy rate.

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Conflict of Interest

The authors declare no conflict of interest.

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References

[1] Hoffman B.L., Schorge J.O., Schaffer J.I., Halvorson L.M., Bradshaw K.D., Cunningham F.G.: "Treatment of infertile couple". *In: Sajid M.* (ed). *Williams Gynecology*. China: The McGraw-Hill Companies, 2012, *20*, 540.

[2] D'Angelo A., Amso N.: "Embryo freezing for preventing ovarian hyperstimulation syndrome". *Cochrane Database Syst. Rev.,* 2002, *2*, CD002806.

[3] Trounson A., Mohr L.: "Human pregnancy following cryopreservation: thawing and transfer of an eight-cell embryo". *Nature,* 1983, *305*, 707.

[4] Lin Y.P., Cassidenti D.L., Chacon R.R., Soubra S.S., Rosen G.F., Yee B.: "Successful implantation of frozen sibling embryos is influenced by the outcome of the cycle from which they were derived". *Fertil. Steril.,* 1995, *63*, 262.

[5] Glujovsky D., Blake D., Farquhar C., Bardach A.: "Cleavage stage versus blastocyst stage embryo transfer in assisted reproductive technology". *Cochrane Database Syst, Rev*., 2012, *7*, CD002118.

[6] Fanchin R., Ayoubi J.M., Righini C., Olivennes F., Schönauer L.M., Frydman R.: "Uterine contractility decreases at the time of blastocyst transfers". *Hum. Reprod*., 2001, *16*, 1115.

[7] Gardner D.K., Lane M., Calderon I., Leeton J.: "Environment of the preimplantation human embryo in vivo: metabolite analysis of oviduct and uterine fluids and metabolism of cumulus cells". *Fertil. Steril.,* 1996, *65*, 349.

[8] Papanikolaou E.G., Kolibianakis E.M., Tournaye H., Venetis C.A., Fatemi H., Tarlatzis B., Devroey P.: "Live birth rates after transfer of equal number of blastocysts or cleavage-stage embryos in IVF. A systematic review and meta-analysis". *Hum. Reprod.,* 2008, *23*, 91.

[9] Carvalho B.R., Barbosa M.W., Bonesi H., Gomes D.B., Sobrinho, Cabral Í.O., Barbosa A.C., *et al.*: "Embryo stage of development is not decisive for reproductive outcomes in frozen-thawed embryo transfer cycles". *JBRA Assist. Reprod*., 2017, *21*, 23.

[10] Holden E.C., Kashani B.N., Morelli S.S., Alderson D., Jindal S.K., Ohman-Strickland P.A., McGovern P.G.: "Improved outcomes after blastocyst-stage frozen-thawed embryo transfers compared with cleavage stage: A Society for Assisted Reproductive Technologies Clinical Outcomes Reporting System study". *Fertil. Steril.*, 2018, *110*, 89.

[11] Chi F., Luo C., Yin P., Hong L., Ruan J., Huang M., *et al.*: "Vitrification of day 3 cleavage-stage embryos yields better clinical outcome in comparison with vitrification of day 2 cleavage-stage embryos". *Zygote*, 2015, *23*, 169.

[12] Lin T.K., Su J.T., Lee F.K., Lin Y.R., Lo H.C.: "Cryotop vitrification as compared to conventional slow freezing for human embryos at the cleavage stage: survival and outcomes". *Taiwan J. Obstet. Gynecol*., 2010, *49*, 272.

[13] Guerif F., Cadoret V., Poindron J., Lansac J., Royere D.: "Overnight incubation improves selection of frozen-thawed blastocysts for transfer: preliminary study using supernumerary embryos". *Theriogenology,* 2003, *60*, 1457.

[14] Al-Azawia T., Tavukcuoglua S., Afshin A., Al Hasani K.S.: "Cryopreservation of human oocytes, zygotes, embryos and blastocysts:

A comparison study between slow freezing and ultra rapid (vitrification) methods". *Middle East. Fertil Soc. J.,* 2013, *18*, 223.
[15] Jin R., Tong X., Wu L., Luo L., Luan H., Zhou G., *et al.*: "Extended culture of vitrified-warmed embryos in day-3 embryo transfer cycles: a randomized controlled pilot study". *Reprod. BioMed. Online,* 2013, *26*, 384.
[16] Guo L., Luo C., Quan S., Chen L., Li H., Guo Y., *et al.*: "The outcome of different post-thawed culture period in frozen-thawed embryo transfer cycle". *J. Assist. Reprod. Genet.,* 2013, *30*, 1589.
[17] Haas J., Meriano J., Bassil R., Barzilay E., Casper R.F.: "Prolonged culture of blastocysts after thawing as a tool for improving prediction of success". *J. Assist. Reprod. Genet*., 2018, *35*, 2195.
[18] Eftekhar M., Aflatoonian A., Mohammadian F., Tabibnejad N.: "Transfer of blastocysts derived from frozen-thawed cleavage stage embryos improved ongoing pregnancy". *Arch. Gynecol. Obstet*., 2012, *286*, 511.

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