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Optimizing Embryo Transfer Timing: Day 4 Versus Day 3 And Day 5 In Fresh ICSI Cycles, A Randomized Controlled Trial

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Abstract

Background: Most assisted reproductive technology (ART) cycles are transferred on day 3 or day 5. Day 4 transfer is very uncommon practice in most ART centers. Day 4 transfer to the patients and clinicians will increase the flexibility of the embryo transfer (ET) timing and decrease the in vitro culture duration. Study objectives: This study aimed at comparing the outcome of day 3, day 4, and day 5ET regarding implantation rate and chemical, clinical and ongoing pregnancy rates.

Patients and Methods: a randomized controlled trial on 384 ICSI cycles. Patients were under 40 years complaining of primary infertility who at least had 10 retrieved oocytes were randomized to three groups (128 each) on the day of oocyte retrieval for either day 3, day 4 or day 5 ET. Severe male factor, PGT-A and freeze all cycles were excluded. The primary outcome measure was the clinical pregnancy rate while, the secondary outcomes included implantation rates, chemical pregnancy and ongoing pregnancy rates.

Results: No significant differences were observed among the 3 groups regarding demographic data, semen parameters and number of cases with history of recurrent implantation failure. Moreover, the number of retrieved oocytes, number of matured oocytes and fertilization rate were comparable. Day 4 and day 5 ET had comparable clinical pregnancy rates(60.2% and 62.5% respectively). However, the clinical pregnancy rate was significantly lower in day 3 group (46.9%). The same finding was also found in the chemical and ongoing pregnancy rates in which, day 4 and 5 ET had comparable outcomes while day 3 ET was significantly inferior to them.

Conclusion: Day 4 (morula) transfer is as good as day 5 (blastocyst) transfer regarding clinical and ongoing pregnancy rates. Offering day 4 ET will increase the flexibility of the embryo transfer policy in the ART clinics without compromising the cycle outcomes.

Keywords: ICSI, Embryo transfer, Day 4 transfer, Day 5 transfer, Morula, Blastocyst.

Introduction

In assisted reproductive technology (ART) cycles, choosing embryos with the best implantation potential for transfer is a crucial step towards maximizing the pregnancy outcomes (1). Even with the use of the most effective culture media, day 5

embryo transfer (ET) at the blastocyst stage still include some risks, such as developmental arrest (2, 3). In spite of the continuous advances in laboratory equiments and culture media almost 50-60%% of human embryos develop into blastocysts in vitro (4).

Extended culture of human embryos, potentially exposes them to multiple stresses which might affect the blastulation rate and hence their implantation potentials (5). It is postulated that reducing the in vitro culture time, reduces the exposure to various stresses which might have epigenetic effects on the developing embryos (6, 7). Furthermore, compared to cleavage stage embryos, both morulae and blastocysts are better synchronized with the endometrium and possess activated embryonic genomes (8).

In most ART laboratories, usually embryo transfer (ET) is done on day 3 or day 5 while day 4 ET is not a common practice. It was always argued that there is no reliable method to grade day 4 morula. However,various researchers used compaction as a marker of the morula quality (9, 10).

In vivo, human embryos are known to enter the uterus on the fourth day four after fertilization (11). So, in ART cycles embryo transfer on day 4 may be a more physiologic option. Day 4 ET may also be done for logistic reasons either in the ART laboratory or concerning the patient's or the clinician's schedule. Interestingly, trial of day 4 ET dated back to 1994 by Huisman et al, who reported encouraging results (12).

Therefore, the aim of this study was to evaluate the clinical outcomes of Day 4 ET and compare them to Day 3 and day 5 ET. The outcomes included: implantation rates, chemical, clinical pregnancy rates, and ongoing pregnancy rates.

Materials and Methods

Patients:

This randomized control trial was conducted at the ART center of El Madina women hospital, Alexandria, Egypt between October 2021 and December 2023. 384 primary infertile patients indicated for ICSI were randomized using computer generated tables on the day of ovum pick up for either day 3, day 4 or day 5 embryo transfer (ET). Our inclusion criteria were, basal FSH less than 12 mIU/mL, female age less than 40 years and at least 10 retrieved oocytes. Poor responders and cases with severe male factor (defined as count less than 5 million per ml, motility less than 10% or normal morphology less than 4%) were excluded. Moreover, PGT-A cycles as well as freeze all cycles were excluded too.

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Ovarian stimulation protocols:

All patients were stimulated using the fixed GnRH antagonist protocol. Starting on cycle day 2/3 recombinant FSH (Gonal-F; Merck Serono.) was administrated. The initial daily dose ranged between 150 and 225 IU according to the patient's age, ovarian reserve, and response to prior ovarian stimulation. The dosage of recombinant FSH was then modified according to the ovarian response. Patients were followed up using serial transvaginal ultrasound scans and serum estradiol levels.

On the 6th day of the stimulation, patients received daily subcutaneous injection of Cetrorelix (Cetrotide; Merck Serono.) 0.25 mg along with the recombinant FSH. When three or more of the biggest follicles reached a diameter of at least 18 mm, the patients were injected with 6500 IU of recombinant HCG (Ovitril, Serono, Switzerland). The transvaginal oocyte retrieval was carried out 35–36 hours following HCG Injection.

IVF laboratory steps and embryo culture:

After ovum pick up, oocytes were denuded by the chemical and mechanical techniques to be ready for injection. ICSI was done for metaphase II oocytes. Injected oocytes were cultured in Total Global® single step media and were incubated in triple gas box incubators (Labotect®,Germany) at 37 °C, 6.4% CO2, and 5.0% O2. Fertilization was confirmed 17-19 hours after insemination by the presence of two pronuclei, then embryo transfers were performed on day three, four or day five according to the patient's group.

Embryo grading

Day3 embryos were graded according Kamardi et al. (2021)(13). Grade A embryo has eight or more equal sized blastomeres with a fragmentation of less than 10% and no multinucleated blastomeres. while Grade B embryo has an unequal blastomeres and/or fragmentation ranging from 10% to 20%. Blastocysts are graded according to Gardner grading system, which takes into consideration the expansion, quality of the inner cell mass (ICM) and quality of the trophectoderm (TE) (14). Grade A blastocyst (4AA) shows full expansion with tightly packed cells ICM and TE is formed of cohesive layer. While blastocysts of lower quality have smaller blastocele, loose ICM and/or incomplete layer of TE as described by Gardner. Day 4 morula was graded according to Tao et al. (2002)(15); grade A morula (complete compact) embryos are described as having achieved total compaction on day four, meaning they have lost all the blastomere borders. Grade B morula, more than 50% of their cells show compaction (partial compact). While grade C, are noncompact or less than 50% compact.

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Embryo transfer criteria:

Top- quality embryos were transferred on either day 3/4/5 according to patients' group. 1-3 embryos were transferred according to our embryo transfer (ET) policy guided by the patient's age and if there is a history of recurrent implantation failure (RIF). ET was performed under transabdominal ultrasound guidance by the same clinician.

Luteal phase support and clinical outcomes:

Luteal phase was supported by vaginal progesterone 800 mg daily (Prontogest® suppository, IBSA) in addition to daily intramuscular 50 mg progesterone (Prontogest® ampoule, IBSA).

Biochemical pregnancy was defined as serum HCG levels ≥10 IU/l on day 14 after oocyte retrieval. Clinical pregnancy was determined by ultrasound detection of one or more gestational sac at 6 weeks of gestation and ongoing pregnancies were defined by presence of at least a viable fetus by the end of the first trimester. Implantation rate was calculated by dividing the number of gestational sacs divided by the number of transferred embryos, as described by the ICMART committee 2009 (16).

Statistical analysis of the data:

Data was fed to the computer and analyzed using IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp) Qualitative data were described using number and percent. The Kolmogorov-Smirnov test was used to verify the normality of distribution Quantitative data were described using range (minimum and maximum), mean, standard deviation, median and interquartile range (IQR).Chi-square test for categorical variables, to compare between different groups. Monte Carlo correction for chi-square when more than 20% of the cells have expected count less than 5.Kruskal Wallis test for abnormally distributed quantitative variables, to compare between more than two studied groups, and Post Hoc (Dunn's multiple comparisons test) for pairwise comparisons. The significance of the obtained results was judged at the 5% level.

Results

The study was conducted on 384 infertile patients indicated for ICSI (Figure 1).

The patients were randomized into 3 equal groups, 128 patients in each group.

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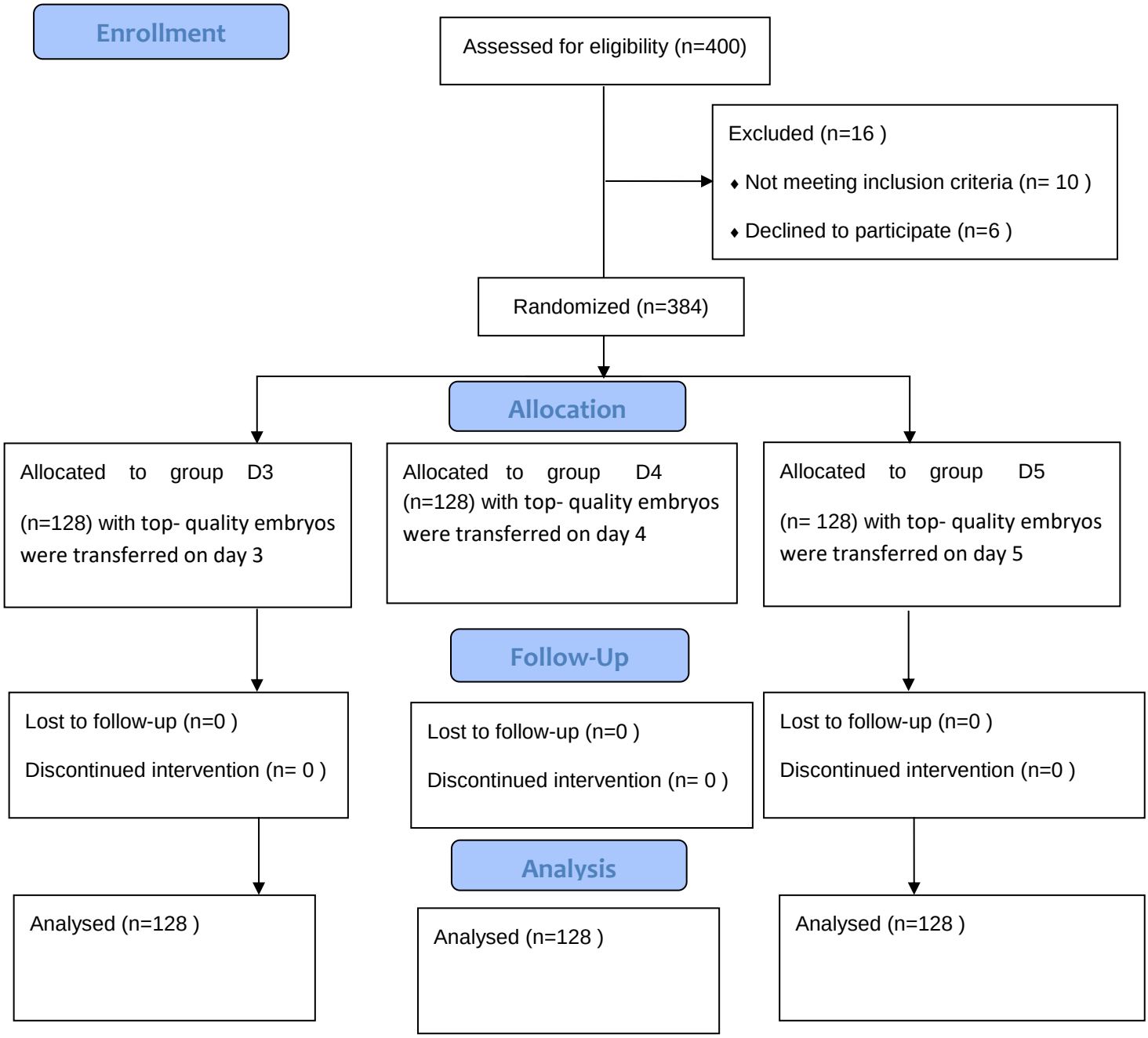
Figure 1 – Flow-chart of the Participants in the RCT



The three studied groups had comparable demographic data and semen parameters as shown in table (1).

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Table (1): Demographic characteristics of patients in the studied groups

	Day 3 (n = 128) Median (range)	Day 4 (n=128) Median (range)	Day 5 (n = 128) Median (range)	Test of sig.	p
Duration of infertility (years)	7.0 (3.0 – 10.0)	6.0 (3.0 – 10.0)	7.0 (4.0 – 10.0)	H= 0.962	0.618
Age (years)	34.84 ± 3.07	34.38 ± 3.29	34.16 ± 3.14	F= 1.535	0.217
Sperm Count	36.0 (11.0 – 73.0)	34.0 (6.50 – 62.0)	29.0 (7.50 – 54.0)	H=5.293	0.071
SP abnormality	96.0 (94.0 – 97.0)	95.0 (90.0 – 97.0)	96.0 (93.0– 97.50)	H=5.450	0.066
Motility	41.4 (14.0–67.0)	36.3 (12.0–56.0)	37.50 (17.0–64.0)	H=0.674	0.714
RIF (>2 failed trials)	12 (9.4%)	7 (5.5%)	9 (7.0%)		
Sig. bet. grps		p_1=0.233, p_2=0.6606		0.481	

RIF: recurrent implantation failure. Sig. bet. grps: significancy between groups. H: H for **Kruskal Wallis test**, ◎²: **Chi square test, F**: **F for One way ANOVA test -** p: p value for comparing between **the studied groups** p_1: p value for comparing between **Day 3** and **Day 4** p_2: p value for comparing between **Day 4** and **Day 5** Normal data were expressed using Mean ± SD. Abnormal data were expressed using Median (IQR)

Table (2): Comparison of laboratory data of the studied groups:

	Day 3 (n = 128) Median (range)	Day 4 (n=128) Median (range)	Day 5 (n = 128) Median (range)	H	p
Number of retrieved oocytes	16.0 (13.0 – 20.0)	16.0 (13.0 – 19.0)	16.0 (13.0 – 18.0)	0.644	0.725
Number of M2	12.0 (9.0 – 14.50)	12.0 (9.0 – 15.0)	12.0 (10.0 – 15.0)	1.071	0.585
Fertilization rate (%)	73.51 (62.77 – 86.7)	78.17 (60 – 90.45)	78.2 (65.38 – 93.3)	3.699	0.157
Number of transferred embryos	3.0 (2.0 –3.0)	3.0 (2.0 –3.0)	3.0 (2.0 – 3.0)	1.912	0.384

H: H for **Kruskal Wallis test.** p: p value for comparing between **the studied groups**

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There was also no significant difference in the distribution of cases with history of recurrent implantation failure, defined as more than 2 previously failed trials, among day 3, day 4 and day 5 ET groups (9.4%, 5.5% and 7% respectively). Regarding the laboratory data, there was no significant difference in the number of retrieved oocytes, mature (metaphase II) oocytes, fertilization rate or the number of transferred embryos (table 2).

As demonstrated in table 3, there was no significant difference in the chemical, clinical and ongoing pregnancy rates between day 4 (62.5%, 60.2% and 39.1% respectively) and day 5 ET (73.4, 62.5% and 46% respectively). However, compared to day 4 and 5 ET, day 3 ET had significantly lower pregnancy rates (49.2, 46.9 and 23.4% respectively).

There was a trend towards a higher abortion rate in day 4 group compared to day 5 group (35.1% Vs 25% respectively). However, this difference was statistically insignificant ($p= 0.169$). This was also reflected on the ongoing pregnancy rate that was 39.1% in the day 4 group versus 46% in the day 5 group, but again this difference was statistically insignificant ($p=0.207$).

As shown in table 4 almost two thirds of the day 4 ET group had at least one completely compact morula and, the clinical pregnancy rate of these subgroup of patients was 71.3%. On the other hand, day 4 cases who transferred lower quality morula had a significantly low clinical pregnancy rate of only 36.6%. The same result was also found in day 5 group, as cases with at least one class A blastocyst had a clinical pregnancy rate of 73.8% versus only 16% in cases who had poor quality blastocysts.

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Table (3): Pregnancy outcomes the studied groups:

	Day 3 (n = 128)	Day 4 (n=128)	Day 5 (n = 128)	Test of sig.	p
Implantation rate (%)					
Mean ± SD.	61.98 ±25.44	67.06 ±23.98	72.01 ± 23.47	H=10.008*	0.007*
Sig. bet. groups		p_1=0.079, p_2=0.160			
Number of sacs					
Median (range)	1.0 (1.0 –2.0)	2.0 (1.0 –2.0)	2.0 (1.0 –3.0)	H= 7.948*	0.019*
Chemical pregnancy rate					
Number (%)	63 (49.2%)	80 (62.5%)	94 (73.4%)		<0.001*
Sig. bet. groups		p_1=0.032*, p_2=0.061			
Clinical pregnancy rate					
Number (%)	60 (46.9%)	77 (60.2%)	80 (62.5%)		0.025*
Sig. bet. groups		p_1=0.033*, p_2=0.497			
Abortion rate					
Number (%)	30 (50%)	27 (35.1%)	20 (25%)		0.009*
Sig. bet. groups		p_1<0.001*, p_2= 0.169			
Ongoing pregnancy					
Number (%)	30 (23.4%)	50 (39.1%)	60 (46%)	9.370*	0.009*
Sig. bet. groups		p_1<0.001*, p_2=0.207			

χ^2: **Chi square test.** H: H for **Kruskal Wallis test,** pairwise comparison bet. each 2 groups were done using **Post Hoc Test (Dunn's for multiple comparisons test).** p: p value for comparing between **the studied groups** p_1: p value for comparing between **Day 3** and **Day 4** p_2: p value for comparing between **Day 4** and **Day 5.** *: Statistically significant at $p \leq 0.05$

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Table (4): Comparison of the quality of embryos transferred among the studied groups and the pregnancy rates.

Grading	Total	Clinical Pregnancy	On-going pregnancy
	No. (%)	No. (%)	No. (%)
D3	**(n = 128)**	**(n = 60)**	**(n = 30)**
Grade A (yes) *	104 (81.3%)	50 (48.1%)	25 (24%)
Grade B (no) *	24 (18.8%)	10 (41.7%)	5 (20.8%)
D4	**(n=128)**	**(n = 77)**	**(n = 50)**
Complete compact / morula (yes) *	87 (68.0%)	62 (71.3%)	40 (46%)
Partial compaction / early morula (no) *	41 (32.0%)	15 (36.6%)	10 (24.4%)
D5	**(n=128)**	**(n = 80)**	**(n = 60)**
Good blastocyst (yes) *	103 (80.5%)	76 (73.8%)	56 (54.4%)
Poor blastocyst (no) *	25 (19.5%)	4 (16%)	4 (16%)

***Note:** Yes, refers to at least one top-quality (class A) embryos transferred No, refers to absence of top-quality (class A) embryos transferred

Discussion

Currently, most ART centers perform embryo transfer either at the blastocyst stage (days 5/6) or the cleavage stage (days 2/3). Day 4 (morula stage) ET, is still uncommon practice. The lack of a standard grading system for the morula stage may at least partially explain this practice (17). In the presented study we randomized cases for either day 3, day 4 or Day 5 transfer and compared various cycle outcomes including; implantation rate, chemical and clinical pregnancy rates,

abortion rates and ongoing pregnancy rate.
Our data showed that day 4 transfer led to a significant improvement in chemical, clinical and ongoing pregnancy rates when compared to day 3 embryo transfer. Grifo et al. (1998)(18) and Pantos et al. (2008)(19) also showed that transfer of day 4 morula had better clinical pregnancy rate as compared to day 3 cleavage stage embryos. It was found that large number of embryonic genes are expressed in the morula stage compared to the cleavage stage embryo. So, embryos reaching this stage have higher future survival rate (20-22). It was even found that that compaction on day 4 is a sign of embryonic genome activation (17).

The present RCT found that day 4 and day 5 transfers had comparable clinical and ongoing pregnancy rates. Previous studies had also the same conclusions as Li (2018)(17) did not find significant difference between day 5 blastocyst transfer and the morula transfer on day 4 regarding implantation rate (36.3% vs. 39.6%), and clinical pregnancy rate (49.5% vs. 51.9%). In addition, a cohort study of 599 fresh transfer cycles study by Holschbach *et al.* (2017)(23), compared day 4 to day 5 ET could not also find any significant difference in clinical and ongoing pregnancy rates. Moreover, Feil et al. (2008)(24) showed that for single embryo transfers (SET), there was no statistically significant difference between day 4 and day 5 ET.

Additionally, Skorupski *et al.*, (2007) (25) have investigated day 4 transfers in non-donor cycles. They reported an overall live-birth rate of 54.4% and observed that implantation rate was higher in younger age groups. Importantly, pregnancy and live-birth rates remained comparable through age groups up to 40 years old, underscoring the feasibility and efficacy of day 4 transfer across varying maternal ages.

Simopoulou *et al.* (2019)(26) meta-analysis evaluated the validity of the Day 4 embryo transfer as an option in ART laboratories. The review included 6 prospective studies and 9 retrospective cohort studies Surprisingly they could not find any significant difference in clinical pregnancy rates and ongoing pregnancy/live birth rates when comparing day 4 with day 2, day 3, and day 5 embryo transfers. Furthermore, no significant variations were found in the cancellation and miscarriage rates between D4 and D3 or D4 and D5 ET. Moreover, regarding neonatal outcome there was a lower incidence of preterm birth in the day 4 group.

Two more recent studies by Zhang et al (2021)(27) and Sun et al (2024)(28) comparing day 4 and day 5 ET also found no significant difference regarding clinical pregnancy and live birth rates between the 2 timings. Both studies were retrospective studies while our study was a randomized control trial.

A more flexible ET timing strategy that provides the ART centers the option of day 4 in addition to the standard day 5 transfer may help to solve some of the time constraints issues for the clinicians, embryologists and even for the patients. This

policy is completely justified if all the data proves that both day 4 and day 5 transfer have comparable outcomes. In addition to the logistical advantages, day 4 transfer reduces the in vitro exposure of embryos to any unfavorable environmental, culture or laboratory conditions as well as maximizing the implantation potential due to minimal uterine contractility at this time (29, 30). Shorter *in vitro* culture (4 days compared to 5 days) may be associated with a lower risk of monozygotic twinning, preterm birth, fetal deformities, and genetic/epigenetic changes which have been linked to blastocyst culture (31).

Day 4 ET may be considered more physiologic as in vivo embryos usually reach the uterus 4 days after fertilization (32). In addition to the better synchronization, the uterus provides a different nutritional environment to the embryo from that in the fallopian tube (33). Moreover, Sang et al., (2021)(34) described a morula-endometrial cross talk in which morulae show expression of receptor genes and corresponding ligand genes were identified in the endometrium. So, transferring the embryo on day 4 may potentially improve implantation.

One of the main reasons that discourages ART laboratories to apply a day 4 transfer policy is the lack of a standardized morula grading system. Tao *et al.* (2002)(15) established grading criteria for day 4 morula depending on the presence of compaction and its degree. We used their grading system in this study. When we transferred at least one completely compact morula we had a clinical pregnancy rate of 71.3% as compared to almost half this percentage (36.6%) if the compaction is less than 50% of the morula or if it is completely non compact. This finding highlights the validity and importance of the compaction in this embryonic stage and its utilization as a grading criterion.

However, our study has some limitation including the limited sample size and that our end point was the ongoing pregnancy rate not the live birth rate. Moreover, the number of transferred embryos was not fixed among study groups. We have an ET policy which takes into consideration the patient's age and if there is a history of recurrent implantation failure. In addition, we excluded PGT-A cycles from the study population. In our center, and in Egypt in general, due to financial issues the utilization of PGT-A is very limited, so the ploidy of the transferred embryos was unknown. Studies in which only euploid embryos are transferred excludes an important cofounding factor that affects pregnancy rate and miscourage rate. We also did not report the neonatal outcome of the cycles.

Conclusion

Day 4 (morula) transfer can result in comparable clinical outcomes to day 5 (blastocyst) transfer. This conclusion will increase the flexibility of the timing of ET in fresh ICSI cycles. Moreover, this strategy will help to decrease in vitro culture duration which will decrease the work load on the incubators and allow easier scheduling of the ET for the patients, clinicians and embryologist.

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Conflict of interest: None.

Ethics Approval: Approval was obtained from the ethics committee of Faculty of medicine, Alexandria University in 2024. Approval number was 0108525.

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