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Original Research Article

Stage matters: comparing day 4 versus day 5 versus day 3 frozen embryo transfers

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ABSTRACT

Background: Morphological grading of embryo transfer remains as key determinant for *in vitro* fertilization (IVF) success. Though day 5 embryo transfer has more implantation potential, day-4 transfer is considered in cases where we suspect a risk of "no embryo to transfer." Aim was to compare the pregnancy outcomes of day-4 versus day 3 versus day-5 frozen embryo transfer.
Methods: This is a retrospective comparative study. 672 sub-fertile women who underwent intracytoplasmic sperm injection (ICSI) during a period of 6 years (2019-2025) were included in the study. These women totally underwent 947 embryo transfers. Fresh embryo transfers were excluded from the study. Patients were followed up till delivery and their pregnancy outcomes were studied.
Results: The clinical pregnancy rate of day 4 embryo transfer was not statistically significant when compared with day 3 and day 5 groups (p=0.13). The live birth rate and miscarriage rate in day 4 embryo transfers were comparable with other two groups and was approaching clinical significance (p=0.06). The preterm delivery rate was lower in day 4 group when compared to other two groups and was statistically significant (p=0.006). The number of ectopic pregnancies was also less in day 4 group when compared to others (only two in day 4 group, five in day 3 group and three ectopic pregnancy in day 5 group).
Conclusions: In this study, day 4 embryo transfer gives comparable results when compared with blastocysts in terms of clinical pregnancy rate, reduced miscarriages, preterm deliveries and ectopic pregnancies.

Keywords: Embryo transfer, Subfertility, Day 4, Pregnancy outcomes, Blastocyst

INTRODUCTION

The selection of good quality and viable embryos plays a key role in the success of an embryo transfer cycle.[1] The timing of embryo transfer remains a crucial determinant of success in assisted reproductive technology (ART). While cleavage-stage (day 2/3) and blastocyst-stage (day 5/6) transfers are widely practiced, day 4 embryo transfer at the morula or compaction stage represents an intermediate strategy that has gained renewed interest. Advances in sequential culture media and improved laboratory conditions have enabled extended embryo culture beyond the cleavage stage, allowing better embryo selection after embryonic genome activation, which occurs between the 4-8 cell stage.[2] Embryo genome activation is the shift of control of early embryonic development from maternal mRNA to embryo's own genome. Despite with the advent of extended culture system, it is questionable whether day 5 transfer is suitable for all patients.[3]

Biological rationale for day 4 embryo transfer

At this stage, intercellular junctions are established, and embryos with intrinsic developmental competence are

more likely to progress further. Unlike blastocyst transfer, day 4 transfer reduces prolonged in-vitro exposure and the risk of cycle cancellation due to developmental arrest before day 5. The rationale behind day 4 frozen embryo transfer is, that in nature, embryos enter the uterine cavity as morulae, being more physiological, thereby providing a better embryo-endometrium synchronization making them developmentally more advanced than cleavage-stage embryos.[4] Uterine contractility is reduced around day 4-5, potentially favouring implantation.

Day 4 embryo transfer may be particularly useful in patients with an intermediate number of good-quality embryos, cycles at risk of blastocyst culture failure, laboratory or scheduling constraints (e.g., avoiding day 3 or day 5 transfer on non-working days), strategies aiming to reduce multiple pregnancy rates via single embryo transfer and good-prognosis patients where extended culture may not offer additional benefit.

Morula transfer has not gained wide spread support from many fertility centers due to the lack of standard grading or classification, when compared with cleavage stage embryos or blastocysts.[5] But recently, few studies have classified day 4 embryos based on compaction and blastulation.[6]

Aim of the study

Aim of the study was to compare the pregnancy outcomes of day-4 versus day-5 versus day 3 frozen embryo transfers.

METHODS

Study type, place and duration

This was a retrospective comparative study conducted among 672 subfertile women who underwent ICSI at the Institute of Reproductive Medicine, The Madras Medical Mission Hospital, Chennai. The study duration was 6 years (2019-2025). Data collection was done retrospectively to analyse the pregnancy outcomes of day 4 versus day 5 versus day 3 frozen embryo transfers.

Inclusion and exclusion criteria

All the subfertile women who underwent ICSI during this period were included in the study. These 672 women underwent 947 embryo transfer cycles. Fresh embryo transfers were excluded from the study.

Procedure

Of the subfertile women attending our hospital, after initial evaluation those who needed IVF, were stimulated following an antagonist protocol. Those who required treatment with IVF were stimulated from second day of cycle using HMG, recombinant FSH or highly purified HMG. The starting dose ranged from 225 to 300 IU, depending on patient's age and ovarian function, as well as their responses to previous ovarian stimulation. Standard fixed antagonist protocol was started from day 5 of stimulation.

They were followed up with serial folliculometry and hormone assays. Once the follicles were ready, appropriate trigger injection was given and oocyte retrieval was done 35 hours after trigger. ICSI was done within 3 to 4 hours of oocyte retrieval. Fertilization was observed 18±2 hours after based on the appearance of 2PN. Embryos were cultured and the fertilized embryos were frozen on day 3 using standard vitrification protocol.

Patients came in the next subsequent cycles for endometrial preparation. After reaching satisfactory endometrial thickness >8 mm, progesterone was started and accordingly frozen embryos were thawed, cultured and transferred.

They were divided into 3 groups: day 3 embryo transfer, day 4 embryo transfer and day 5 embryo transfer. Luteal phase support was given and patients were followed up till delivery and their pregnancy outcomes were studied. The primary endpoint was clinical pregnancy rate and secondary endpoints were livebirth rate and miscarriage rate. In our study we graded the day 3 and 5 embryos using Gardners and Schoolgrafts grading system and day 4 embryos were graded using the latest ESHRE consensus 2024.[7,8]

Ethical approval

This study is approved by the ethics committee of The Madras Medical Mission Hospital [EC Reg No. ECR/140/Inst/TN/2013/RR-20].

Statistical analysis

Data were entered in Microsoft excel software and analyzed using SPSS software windows V17. Age, type and cause of subfertility and day of embryo transfer were summarized as frequency with proportion. Chi square test is applied as appropriate, $p<0.05$ is taken as significant.

RESULTS

In our study, 690 subfertile women underwent 935 ICSI cycles during the study period. Out of 935 frozen embryo transfers, there were 388 (41.5%) day 3 embryo transfers, 367 (39.2%) day 4 frozen embryo transfers and 180 (19.3%) day 5 embryo transfer (Table 1).

Majority of women in the study, around 68% were between the age group 21-35 (n=636), followed by 30% belonging to the age group 36 to 45 years (n=281) and only 2% belonged to the age group of >45 years (n=18) (Table 2).

In our study, 64.6% of the population had primary subfertility and 35.4% of them had secondary subfertility.

The most common indication for ICSI was male factor (22.9%) as shown in the (Table 3). The other indications for ICSI include uterine factor (14.7%), poor ovarian reserve (14.3%), genetic causes (14.2%), tubal factor (7%), PCOS (5.2%), unexplained infertility (4.2%), combined male and female factor (3.7%), recurrent pregnancy loss (3.2%), uterine anomalies (3.9%), endocrine disorders (3.1%), immunological factors (2.5%) and pelvic tuberculosis (0.8%).

The primary outcomes of the study, clinical pregnancy rate was 43.3% in day 4 embryo transfers, comparable to 39.1% in day 3 embryo transfers and 17.6% among day 5 embryo transfers. The p=0.13 and was not statistically significant (Figure 1).

The livebirth rate among different groups were as follows; among day 4 embryo transfers it was 43.3%, in day 3 transfers, it was 41.9%, and day 5 transfers it was 14.9% (Figure 2). The miscarriage rate of day 4 embryo transfer was 18.9%, among day 3 transfers it was 46.2% and day 5 transfers was 34.9% (Figure 3). The p=0.06 for both live birth rate and miscarriage rate and was approaching clinical significance.

There were five ectopic pregnancies in day 3, three in day 5 transfers and only two in day 4 transfers, The p=0.17 and was not statistically significant. Though the day 4 embryo group had a lesser number of ectopic pregnancies, due to the low number of ectopic events, there is a lack of clinical significance. Another favourable outcome with day 4 transfers was the lesser preterm delivery rate. It was 20.1% in day 4 transfers which was lesser than the other two groups as shown in figure 4. The p=0.006; statistically significant.

The multiple pregnancy rate among day 4 embryo group was 38.3%, among day 3 group it was 45.7% and in day 5 transfer it was 16%. The p=0.6, making the groups comparable.

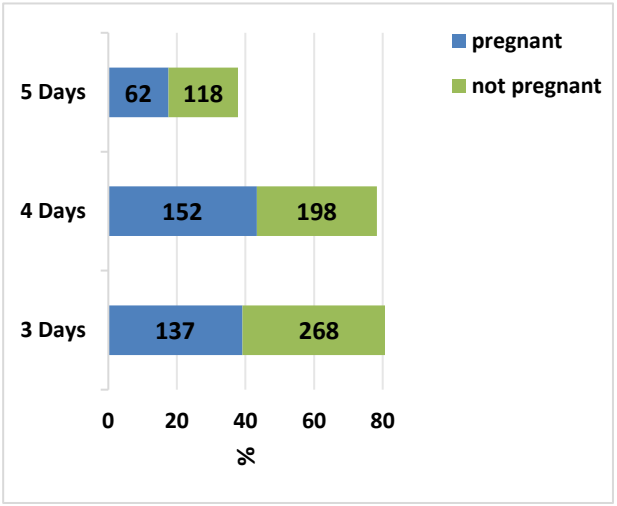

Figure 1: Clinical pregnancy rate.

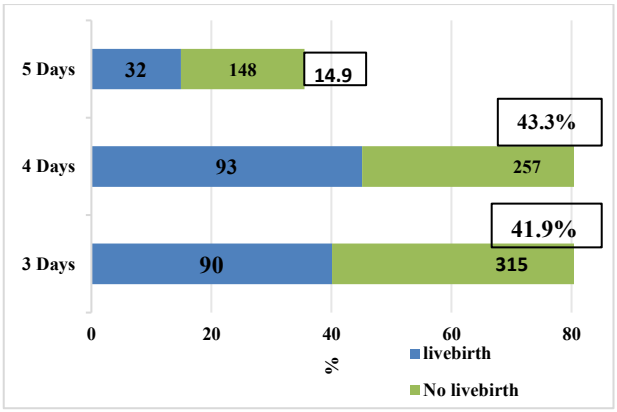

Figure 2: Livebirth rate.

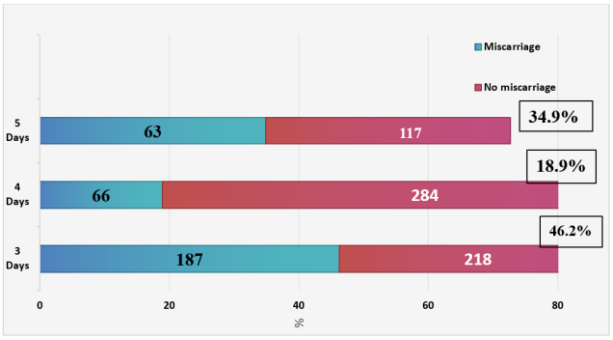

Figure 3: Miscarriage rate.

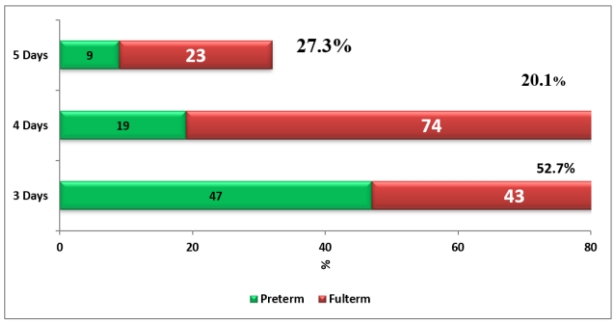

Figure 4: Preterm delivery rate.

Table 1: Day of embryo transfer.

Day of embryo transfer	N	Percentage (%)
Day 3	388	41.5
Day 4	367	39.2
Day 5	180	19.3
Total	935	100

Table 2: Age distribution.

Age (in years)	N	Percentage (%)
21-35	635	68
36-45	281	30
>45	18	2
Total	935(n)	100

Table 3: Indications for ICSI.

Indications	Percentage
Male factor	22.90
Combined male and female	3.70
Uterine	14.70
Tubal	7
POR	14.30
Genetic causes	14.2
PCOS	5.2
Unexplained infertility	4.2
Uterine anomalies	3.9
Recurrent pregnancy loss	3.2
Endocrine disorders	3.1
Immunological factors	2.5
Pelvic tuberculosis	0.8

Table 4: Study outcomes.

Study outcomes, (n=935)		Day of transfer			P value
		Day 3	Day 4	Day 5	
Livebirth rate	%	41.9	43.3	14.9	0.060
	N	90	93	32	
Clinical pregnancy rate	%	39.1	43.3	17.6	0.135
	N	137	152	62	
Preterm deliveries	%	52.7	20.1	27.3	0.006
	N	47	19	9	
Miscarriage rate	%	46.2	34.9	18.9	0.06
	N	49	37	20	

DISCUSSION

Day 4 morula transfer was first documented as a case report of two successful pregnancies by JunTao et al from Arizona in 2001.[9] Ever since worldwide many centers have found day 4 transfers an equally valuable option or as in our study even better option in terms of high livebirths, lesser miscarriages, lesser preterm births and fewer ectopic pregnancies.

During our embryo transfer protocol, we thaw the day 3 frozen embryos and by transferring them the subsequent day after an overnight culture, we are able to ensure the viability of the embryos. Day 4 embryos have completed compaction and undergone embryonic genome activation; this is the self-correction mechanism and the exclusion of aneuploid cells from the compacting embryo. This is the reason for the higher livebirth rate in our study; similar to the study by Hui et al in which day 4 morula transfers achieved higher pregnancies than day 3 transfers.[10]

In our study the clinical pregnancy rate of day 4 embryo transfers is comparable to day 3 and day 5 transfers (p=0.135). The livebirth rate of day 4 embryo transfers was high when compared to other two groups (p=0.06), which shows that it is approaching clinical significance. This is similar to the other study by Zhang et al done in 2021 and study done by Li et al.[11]

There were lesser preterm births in day 4 group (20.1%) when compared to other two groups. In a retrospective study by Li et al comparing day 4 and day 5 embryos, the term birth rate was higher in day 4 transfers than day 5 transfers (100% vs. 78.3%, respectively, p=0.025).[12] This can be well explained due to the advantage of activation of embryonic genome, apoptosis and checkpoints of cell cycles activation in morula which gives less mosaicism in embryos. Hence this natural step of embryo selection leads to lesser miscarriages and more term births. This interesting observation was concluded in the systematic review and network meta-analysis by Simopoulou et al "statistically significant" lower preterm birth rates associated with D4 ET, in contrast with D5 ET (RR=0.19; 95% CI=0.05-0.67; p=0.01).[13]

The miscarriage rate was also lesser in day 4 transfer group when compared to other groups (p=0.06), with a trend towards statistical significance. This may be due to less in vitro time and more time in uterine environment during implantation so that the interruption of epigenetic regulatory mechanism is minimal.[13]

The number of ectopic pregnancies were less in the day 4 group, but the lack of clinical significance (p=0.17) may be due to lesser number of the ectopic events, suggesting that larger studies are required to adequately assess this outcome. As day 4 embryos are in the physiologically appropriate time to enter the uterine cavity, thus favouring intrauterine implantation.

There are certain scenarios in which we have found day 4 transfer useful:

Biological advantages

Genetic selection

Because the human genome activates at the 4-8 cell stage, waiting until day 4 allows clinicians to identify embryos that have successfully "switched on" their DNA. Those with significant abnormalities often stop dividing before reaching this stage.

Natural filtering

Day 4 embryos may have lower rates of chromosomal mosaicism, as the embryo's internal checkpoints and self-correction (apoptosis) systems begin to function after genome activation.

Physiological timing

In a natural pregnancy, the embryo typically enters the uterus 3-4 days after fertilization.[14] A Day 4 transfer aligns perfectly with this biological clock, ensuring a more synchronized uterine environment. Also, the uterus is quiescent at this stage.[15]

Clinical and laboratory benefits

Reduced in-vitro stress

Keeping embryos in the lab until Day 5 (blastocyst stage) can sometimes lead to epigenetic changes, monozygotic twinning, or preterm delivery.[16] Transferring at the morula stage limits this artificial exposure.

Resource management

For busy clinics with high incubator usage, day 4 transfers can prevent "overcrowding." Frequent door openings can destabilize the temperature and pH; moving embryos to the uterus earlier avoids these suboptimal conditions.

Flexibility

It offers a practical alternative for patients or medical teams who cannot perform a transfer on day 5, preventing cycle cancellations.

Better alternative

There have been many studies which reported higher "cancellation rate" in day 5 embryo transfer in scenarios where no embryos progressed to blastocysts stage. This produces more burden on the patient, clinician and the embryologist.[17] Literature emphasizes that around 30% of embryos deviate from the usual developmental velocity and grow slower than expected.[18] Thus, for these kind of embryos, even in the absence of compaction on day 4, we can transfer such embryos on day 4 so as to utilize the implantation window and its potential.[19] Thus, being in the era of reducing multiple pregnancy, transferring single morula offers a better and comparable result as a good quality blastocyst.[20]

Limitations

Embryo selection and grading challenges

One limitation of day 4 transfer is the lack of universally accepted grading criteria. However, several studies have adopted modified grading systems based on compaction percentage and early cavitation, as proposed in the ESHRE Istanbul consensus and further refined by Li et al and Bavishi et al. When standardized grading is applied, morula selection becomes reliable and reproducible, yielding outcomes comparable to blastocyst transfer.

CONCLUSION

Current evidence suggests that day 4 morula embryo transfer offers clinical pregnancy and live birth rates comparable to day 5 blastocyst transfer, provided appropriate embryo selection is employed. Day 4 transfer represents a flexible, physiologically sound, and clinically effective alternative, particularly in settings where blastocyst transfer is not optimal or feasible. With standardized grading systems and careful patient selection, day 4 embryo transfer may play an important role in individualized ART strategies aimed at maximizing singleton live births while minimizing treatment burden and multiple pregnancy risk.

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Ethical approval: The study was approved by the Institutional Ethics Committee the Madras Medical Mission Hospital [EC Reg No. ECR/140/Inst/TN/2013/RR-20]

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