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Original Research Article

Are all day 6 blastocysts inferior to day 5 blastocysts? A retrospective study comparing reproductive outcomes between day 5 and day 6 good quality blastocysts in various clinical scenarios

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ABSTRACT

Background: Day 5 (D5) blastocysts have a better reproductive outcome than Day 6 (D6) blastocysts in fresh cycle transfer. However, conflicting results were observed in frozen embryo transfers (FET), primarily due to presence of multiple confounding factors. The aim of study was to compare reproductive outcomes between D5 and D6 good quality blastocysts in FET in different clinical scenarios causing infertility - female, male, unexplained and combined (male and female) factors.
Methods: This was a retrospective cohort study conducted at multiple chains of a private fertility center. A total of 16,996 D5 and 4,382 D6 good quality blastocysts were included. Clinical Pregnancy Rates (CPR), Implantation Rates (IR), Miscarriage Rates (MR) and Biochemical Pregnancy Rates (BPR) were compared in four different scenarios.
Results: In female factor, D5 blastocysts have better CPR (60.85% vs 47.95%) and IR (47.15% vs 36.13%) compared to D6 blastocysts. Similar outcomes were observed in unexplained factors where D5 blastocysts had better CPR (59.64% vs 45.83%) and IR (45.63% vs 33.08%) than D6 blastocysts. In male factor, CPR (53.61 % vs 49.79%) and IR (40.12% vs 39.96%) were comparable between D5 and D6 blastocysts. Similar comparable rates were found in combined causes - CPR (51.26% vs 48.97%) and IR (39.45% vs 37.78%).
Conclusions: Reproductive outcomes were better in D5 than D6 blastocysts in female and unexplained factors. The results were comparable in male and combined factors indicating that not all D6 blastocysts can be considered inferior to D5 blastocysts.

Keywords: Blastocysts, Frozen embryo transfer, Factors, Infertility

INTRODUCTION

Quality of embryos is one of the most important factors determining the success of In Vitro Fertilisation (IVF). Ideally embryos reach the blastocyst stage on fifth day of culture development (116±2 hours after insemination).[1] However, not all embryos develop at the same rate depending on the quality and inherent genetic potential leading to a cohort of blastocysts on day 5, day 6 and sometimes even day 7 of embryo culture.[2]

It is a well-known fact that Day 5 (D5) blastocysts have better implantation potential compared to Day 6 (D6) blastocysts in fresh cycle transfer, primarily due to displaced window of implantation and endometrial advancement.[3,4] Various studies have shown similar outcomes in frozen embryo transfer (FET) cycles suggesting that besides endometrial asynchronization, various intrinsic and metabolic factors affecting embryo viability might contribute to higher implantation potential of D5 blastocysts compared to slow growing D6 blastocysts.[5-8] However, conflicting results were found in other studies generating some controversy regarding the reduced pregnancy potential of D6 blastocysts.[9,10]

The aim of current study was to compare reproductive outcomes between D5 and D6 good quality blastocysts in FET cycles in four different clinical scenarios causing infertility - female factors, male factors, unexplained and combined factors (male and female).

METHODS

This was a retrospective cohort study conducted at multiple chains of a fertility centre between 2018 to 2023. Data was retrieved from the medical records maintained at each centre. All the patients fulfilling the inclusion criteria who have undergone FET cycles were then included in the study.

Patients' selection

Patients with the following inclusion criteria were then selected.

Female factors

Advanced maternal age (> 35 years), low ovarian reserve (AMH < 1.1ng/ml or Antral Follicle Count < 5-7 follicles), Polycystic ovarian disease (PCOD) not conceiving with multiple (6 or more) cycles of ovulation induction (OI) and 3 or more failed cycles of Intra Uterine Insemination (IUI), moderate to severe endometriosis (based on revised American Society for Reproductive Medicine scoring system) and bilateral tubal blockage.

Male factors

Oligo/astheno/teratozoospermia (sperm count <15 million/ml, progressive motility <32% and morphology <4%) or combination of these, cryptozoopermia (the condition by which spermatozoa cannot be observed in a fresh semen sample, but can be found after centrifugation and microscopic observation of the pellet) and surgically retrieved sperms in males with azoospermia.

Only first FET cycle results were included in the study.

Third party reproduction (donor oocyte/donor sperm/surrogacy), poor quality blastocysts (Grade CC, CB OR BC), suboptimal endometrium (thickness <7mm, absence of triple line pattern, fluid in the cavity, endometrial polyp, severe adenomyosis), fresh transfers and day 3 embryo transfers were excluded from the study to have more uniformity. Among D6 blastocysts, only those patients who did not form any embryos on day 5 of culture were taken into consideration to reduce embryo selection bias.

Stimulation protocols

Patients undergoing IVF were stimulated with either recombinant/highly purified Follicle Stimulation Hormone (FSH) or highly purified Human Menopausal Gonadotropin (HMG) with dose ranging from 150 IU to 300 IU based on age, Body Mass Index (BMI), ovarian reserve, and ovarian response in previous cycles. The dose of injections was then adjusted based on follicle size as monitored by transvaginal ultrasound (TVS) done every 2 to 3 days. Antagonist was added once leading follicle reached 14mm in diameter. When leading follicles attained a mean diameter of 18 to 20mm in size, ovulation was triggered with either human chorionic gonadotropin (hcg) or triptorelin (agonist) injection. Oocyte retrieval was performed under TVS guidance 34 to 36 hours after administration of trigger injection.

Embryo culture

Post oocyte retrieval, cumulus stripping was performed after 2 to 4 hours to assess the number of mature oocytes. Intracytoplasmic Sperm Injection (ICSI) was done in all mature oocytes. Fertilisation was assessed (16 to 18 hours post ICSI) by the appearance of two distinct pronuclei (2PN) and two polar bodies. The embryos were then group cultured in trigas benchtop incubators using single step culture media (Vitromed, Germany). Embryos development was assessed on day 3 and day 5 of culture. Day 5 blastocysts were then graded according to modified Gardner and Schoolcraft criteria[11] and frozen using vitrification technique (Kitazato method). Slow growing embryos which had not reached blastocyst stage on day 5 were transferred to a fresh culture media dish and reassessed on day 6. On day 6 of culture, good quality blastocysts were vitrified whereas growth arrested embryos were discarded.

Embryo grading

Morphological grading of blastocysts was done using modified Gardner and Schoolcraft criteria.[11] Briefly, the Inner Cell Mass (ICM) was graded as A, when numerous tightly packed cells were seen; B, when several and loosely packed cells were observed; C, in case of very few cells; and D, if no cells were seen or more than 50% of cells were degenerated. Also, the Trophectoderm (TE) was graded as A, when many cells were organized in stretch contact forming the TE; B, when several cells were organized in a loose epithelium; C, if few cells with abnormal disposition in the TE were present; or D, if only very few irregular large cells with necrotic aspect constituted the TE. The grade of expansion was classified as BL1, when blastocoel was less than half of the volume of the embryo or BL2, when blastocoel was at least half of the volume of the embryo, and both considered early blastocysts with no cell differentiation between ICM and TE. A full blastocyst was classified as BL3, when the blastocoel filled the embryo completely; BL4, when an expanded blastocyst with a thin zona pellucida (ZP) was seen; and BL5, when cells started to herniate through the ZP or BL6, if the blastocyst had completely escaped from the ZP. Blastocysts were evaluated on D5 of embryo culture and if blastocyst quality was deemed sub-optimal and expansion or number of cells present in the TE were insufficient, embryos were left in culture and re-evaluated on D6.

Good quality blastocysts were graded as AA, AB, BA and BB with an expansion grade of 3 or more. Only these embryos were included in the study.

Vitrification-warming protocol

Blastocysts selected for transfer were warmed and cultured in a blastocyst medium at 37 °C (5% CO2 and 5% O2) for 1–2 hours before transfer. The survival of warmed blastocyst was checked at the timing of FET and was defined as > 80% of cells intact and full re-expansion. The fully expanded blastocysts were then transferred to the patients.

Endometrial preparation and embryo transfer

Two main protocols were used for endometrial preparation. In Artificial hormonal cycles, patients were started on 6-8mg per day of estradiol valerate from second day of menses. Ultrasound was done after 10-12 days of hormonal therapy. Once the endometrium reached more than 7mm of thickness with triple line pattern, patients were started on 400mg of vaginal micronized progesterone and 10mg of oral dydrogesterone twice daily.

Natural cycle FET was performed for patients with regular ovulatory cycles. Ovulation was monitored with frequent transvaginal scans and serum hormonal measurements. Endometrium criteria to do transfer was same as in Artificial hormonal cycle.

The blastocyst transfer was done on the sixth day after confirming ovulation (in natural cycle) or progesterone exposure (in Artificial cycle) using soft tipped catheter under ultrasound guidance. First serum beta - human chorionic gonadotropin (b-hcg) test was done 12 days post FET cycle followed by sac scans at 5th and 6th week of gestation in case the beta hcg value came positive. Luteal support was continued up to 10 weeks of gestation in all the pregnant patients.

Outcome measures

The reproductive outcomes of D5 and D6 blastocysts were divided into four different categories based on the primary cause because of which patients were advised to undergo IVF-female factors, male factors, unexplained and combined factors (male and female).

The primary outcome was Clinical Pregnancy Rate (CPR) defined as pregnancy confirmed by visualization of fetal cardiac activity in gestational sac between 6th and 7th week of gestation on ultrasound.[12] The secondary outcomes were implantation rate (IR), Miscarriage Rate (MR) and Biochemical Pregnancy Rate (BPR). IR was defined as the ratio of total number of intrauterine gestational sacs visible on ultrasound to the number of embryos transferred. MR was defined as miscarriage which occurred before 22 weeks of gestation after confirming intrauterine sac on early ultrasounds. BPR was described by beta-HCG detection in serum which did not result in a clinical pregnancy.[12]

Statistical analysis

Clinical outcomes were expressed as frequencies and percentages. Comparative analyses between D5 and D6 blastocysts, stratified by infertility factors type groups, were performed using chi square test for categorical variables, including CPR, IR, BPR and MR. A two tailed p-value <0.05 was considered statistically significant. All statistical analyses were conducted using R studio (version 4.4.1; R foundation for statistical computing, Vienna, Austria).

RESULTS

Among 16,996 D5 blastocysts 5,311 (31.2%) and 2,582 (15.2%) were of patients who were advised IVF due to female factor and male factor respectively. Majority of the blastocysts i.e. 8120 (47.8%) had combined male and female factors resulting in infertility. Rest 983 (5.8%) blastocysts were of those patients where no obvious cause of infertility could be detected on evaluation.

Among 4,382 D6 blastocysts 1,276 (29.1%) were of female factor infertility and 670 (15.3%) were due to male factor infertility. 2091 (47.7%) blastocysts belonged to combined causes and the remaining 345 (7.9%) blastocysts were of patients with no detectable cause of infertility. The same distribution is shown in Table 1.

Table 1: Comparison of factors causing infertility between day 5 and day 6 blastocysts.

Factors	Day 5 blastocyst (%)	Day 6 blastocyst (%)
Female factor	5,311 (31.2)	1,276 (29.1)
Male factor	2,582 (15.2)	670 (15.3)
Combined factors	8,120 (47.8)	2,091 (47.7)
Unexplained factors	983 (5.8)	345 (7.9)

The baseline parameters were found to be comparable between the two groups as shown in Table 2.

In female factors causing infertility, D5 blastocysts have better CPR (60.85% vs 47.95%) and IR (47.15% vs

36.13%) compared to D6 blastocysts. Similar outcomes were observed in unexplained factors causing infertility where D5 blastocysts had better CPR (59.64% vs 45.83%) and IR (45.63% vs 33.08%) than D6 blastocysts. There was no statistically significant difference in MR and BPR between D5 and D6 blastocysts in the above factors (Table 3).

Table 2: Comparison of baseline parameters between day 5 and day 6 blastocysts.

Baseline parameters	Day 5 blastocysts	Day 6 blastocysts	P value
Age of female (years) (mean +/- SD)	30.97±3.46	31.34±3.67	0.292
Type of infertility			
Primary (%)	69.2	66.9	0.478
Secondary (%)	30.8	33.1	0.516
Endometrial thickness at the time of transfer (mm)	9.32±1.26	9.18±1.45	0.527
Average number of embryos transferred	1.62±0.38	1.55±0.51	0.384
Sperm count (million/ml)	21.25±5.81	18.93±4.15	0.115
Sperm motility (%)	28.45±6.75	25.61±5.39	0.269
Mean oocyte retrieved	14.45±4.12	12.98±3.54	0.486

Table 3: Comparison of various reproductive parameters in different clinical scenarios causing infertility between day 5 and day 6 blastocysts.

Factors causing infertility	Day of blastocyst	Clinical pregnancy rates (%)	Implantation rates (%)	Abortion rates (%)	Biochemical pregnancy rates (%)
Female factor	Day 5	60.85	47.15	14.49	6.78
	Day 6	47.95	36.13	14.98	7.43
	P value	<0.05	<0.05	0.678	0.013
Male factor	Day 5	53.61	40.12	13.43	7.62
	Day 6	49.79	39.96	17.95	8.02
	P value	0.417	0.417	<0.05	0.312
Unexplained factors	Day 5	59.64	45.63	9.47	8.64
	Day 6	45.83	33.08	9.82	8.24
	P value	<0.05	<0.05	0.305	0.417
Combined factors	Day 5	51.26	39.45	13.21	7.99
	Day 6	48.97	37.78	17.99	8.16
	P value	0.545	0.462	<0.05	0.515

In male factor causing infertility, CPR (53.61 % vs 49.79%) and IR (40.12 % vs 39.96%) were comparable between D5 and D6 blastocysts. Even patients with combined causes of infertility, D6 embryos were found to be non-inferior to D5 embryos with respect to CPR (51.26% vs 48.97%) and IR (39.45% vs 37.78%) as shown in Table 3. BPR were also statistically comparable between the two groups. However, MR rates were more in D5 blastocysts compared to D6 in both male and combined factors of infertility which were found to be statistically significant (Table 3).

DISCUSSION

To the best of our knowledge, this is the first study which has compared the reproductive potential of good quality D5 and D6 blastocysts in various clinical factors responsible for causing infertility in patients. Our study demonstrates better reproductive potential of D5 blastocysts compared to D6 blastocysts in patients where the primary reason of infertility is predominantly female factor resulting in compromised oocyte quality. Similar results were observed in patients with no obvious detectable cause of infertility. However, in patients undergoing IVF due to compromised semen parameters (male factor), D6 blastocysts were found to have similar pregnancy rates as compared to D6 blastocysts. Even though the overall pregnancy rates were slightly less in patients where both oocyte and sperms were compromised compared to other factors, there was no statistically significant difference in the CPR and IR between D5 and D6 blastocysts.

The selection of embryo with the highest implantation potential is an ongoing challenge for the embryologist.[13-15] Although the data demonstrating superiority of D5 over D6 blastocysts in fresh transfer cycles is well established, the same outcomes apply in FET cycles is still a matter of debate.

In a meta-analysis by Sunkara et al to assess the influence of delayed blastocyst formation on the outcome of frozen-thawed blastocyst transfer, it was found that slower developing blastocysts cryopreserved on D6 but at the same stage of development as those developing on D5 have similar clinical and ongoing pregnancy/ live birth rates following frozen thawed transfer cycles.[9]

They also proposed that the ability of *in vitro* embryos to develop into blastocyst within a defined time frame post fertilization confers a certain amount of biological competence resulting in favourable implantation and pregnancy rates. This developmental window appears to extend up to Day 6 after fertilization.

Abdala et al conducted a retrospective study to determine the variables most affecting CPR in FET cycles in D5 and D6 euploid blastocysts.[16] The results showed that clinical outcomes were more affected by lower quality of embryos rather than the day of blastocyst biopsy. Also, from the re-analysis performed where only good quality embryos (grade A and/or B for ICM and TE) were considered, clinical outcomes were not statistically significant different between blastocysts biopsied on D5 or D6. However, there was a favourable trend with respect to euploidy rates towards D5 blastocysts over D6. Similar study by Jiang et al also showed that it was not the stage of development (D5/D6), rather the blastocyst quality transferred that played an important role in pregnancy outcomes.[17] Our current study has not considered euploid blastocysts while comparing outcomes as not all patients can be offered genetic testing due to strict government guidelines on the indications of PGT. Sometimes patients opt out of PGT despite being offered due to personal or financial reasons. So, the sample size would have become very less for us to conclude any findings.

Another meta-analysis done by Bourdon et al concluded that D5 blastocysts have higher Live Birth Rates (LBR) and CPR in both fresh and frozen cycle transfers.[18] However, in this metanalysis age of the patient and number of blastocysts were identified as confounders. Also, the oocyte origin (donor or autologous) leading to the transferred embryo, the morphological and ploidy criteria used for selection of embryos for transfer on D5 and D6, were heterogeneously reported which may act as potential bias. In the current study, the age of patients and number of embryos were comparable between the two groups. Also, third party reproduction and poor-quality embryos were excluded to have better interpretation of results.

Many studies have shown that more chromosomal abnormalities are associated with poor egg quality. 10-25% of oocytes of women in their early thirties are aneuploid which increases to more than 50% of eggs in women over 40. This makes aneuploidy in eggs a leading cause of pregnancy loss and birth defects in humans.[19] This might be one of the reasons why in our study D5 blastocysts pregnancy rates were better than D6 in female and unexplained factors causing infertility while there was no difference in the clinical outcomes between D5 and D6 in male factors. In unexplained factors causing infertility, oocyte quality is often found to be a contributing factor, but because it cannot be measured using conventional tests, many cases are incorrectly labelled as unexplained.[20]

Aneuploidy of post-zygotic origin mainly occurs during cleavage divisions. Delayed timing of first and subsequent cleavage division may cause slower blastocyst development. These events may negatively affect the complex transition of embryonic genome activation and gene transcription from maternal to embryonic genome, finally leading to differentiation of ICM and TE, as well as genetic mosaicism or aneuploidy.[21] Also, D5 blastocysts were found containing significant greater mitochondrial DNA (mtDNA) quantity than D6 blastocysts do, and mtDNA quantity could be a key factor, which can affect blastocysts development rate. This might be the contributing factor of better reproductive outcomes of D5 blastocysts in female and unexplained factors.

In clinical practice, obviously D5 blastocysts are preferred over D6 blastocysts for transfer if couple has the option of choosing between the two. However, in cases where patients have got blastocysts only on D6 of culture, this study might help in prognosticating and counselling the success rates after determining the cause of infertility in individual cases. With the advent of new technologies for embryo selection like Artificial Intelligence (AI) in ART, we might have more clarity regarding implantation potential of embryos and choosing the best blastocysts for transfer. However, counselling the patients regarding the entire procedure and expected success rates forms the cornerstone of every IVF treatment.

Most of the studies have not considered the contributing factors responsible for infertility while comparing the outcomes between D5 and D6 embryos. To the best of our knowledge, this is the only study which have analysed reproductive outcomes between good quality D5 and D6 blastocysts in different clinical causes of infertility.

The study is limited by its retrospective nature, unequal sample size and inability to track till live birth rates as most of our patients are referred to the obstetricians after completing 22 weeks of pregnancy. The primary purpose of the study was to evaluate reproductive outcomes between D5 and D6 FET cycles. The confounding factors affecting the result primary includes age of patient, BMI, quality of blastocysts, endometrial thickness and euploidy status of embryos. As per our study, maternal age is comparable between the two groups and we have only included good quality embryos and good endometrial lining to adjust for the potential confounders. Since the data is retrieved from the online medical records of different centres, the information regarding other factors like BMI and euploidy status was not mentioned in many cases. We are working on strengthening our online data entry system to have a more valid data for future studies where we can incorporate logistic regression to further validate our findings. Further well-designed prospective studies assessing till LBR and perinatal outcomes will provide a deeper insight into the reproductive outcomes between D5 and D6 blastocysts.

CONCLUSION

Reproductive outcomes were found to be better in day 5 compared to day 6 blastocysts in female factor and unexplained factors causing infertility. However, the results were comparable in male factor and combined factors causing infertility indicating that not all day 6 blastocysts can be considered inferior to day 5 blastocysts.

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