Original Article

Comparative Embryo Development Outcomes following Extending Embryo Culture to Day 6: A Retrospective Cohort Study

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Abstract

Background: Past studies have shown that culturing slow-growing embryos from day 5 to day 6 may increase vitrification yield. This study aims to evaluate if the proportion of embryos eligible for vitrification increases by growing embryos not vitrified by day 5 to day 6.

Materials and Methods: In this retrospective cohort study, a Canadian tertiary-care clinic-based cohort was identified between August 2019 and December 2020. *In vitro* fertilization (IVF) cycles involving autologous oocytes with at least one viable day 5 embryo were selected for inclusion. We compared embryo developmental outcomes of IVF cycles performed before and after an embryo cryopreservation policy change. Prior to March 2020, good-quality day 5 blastocysts of any stage were eligible for vitrification, and after that date, good-quality expanded blastocysts on either day 5 or day 6 were eligible. The primary outcome is the comparative proportion of embryos eligible for vitrification. The secondary outcome is to identify embryo, maternal and cycle factors that are predictive of day 6 vitrification.

Results: A total of 3,438 viable embryos across 679 consecutive IVF cycles were included in this study. After the policy change, we found similar mean proportions of blastocysts eligible for cryopreservation (46.9% per IVF cycle in group 2 vs. 44.4% in group 1, mean difference 0.025, 95% confidence interval -0.021 to 0.071, P=0.28). The mean number of cryopreserved embryos were significantly higher in group 2 (mean 2.2 vs. 1.7 embryos, P=0.007). Factors that predicated an embryo's progression to day 6 included: younger age of egg provider, presence of an early blastocyst on day 5, and cycles involving surgically-retrieved sperm.

Conclusion: A cryopreservation policy change to include good-quality full and expanded day 6 blastocysts while avoiding to vitrify early blastocysts on day 5 yielded comparable proportions of embryos eligible for vitrification per IVF cycle.

Keywords: Blastocyst, Delayed Blastulation, Embryo Development, Vitrification

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Introduction

Human embryos follow a specific developmental timeline, with morphologic features exemplifying milestones reached in a coordinated sequence of growth. Most embryos reach the blastocyst stage 5 days after fertilization. However, embryos demonstrate varying pace of development. Some embryos will either fail to begin blastulation or begin blastulation but not reach the full blastocyst stage by day 5. In normal circumstances, an embryo should arrive in the uterus by day 5 to 6 after fertilization, and the hatched blastocyst may begin implantation by day 6 to 7 (1).

Under similar laboratory conditions, embryos in extended culture can vary in their developmental rates, reaching the blastocyst stage on day 5, 6, or 7 (2, 3). It is estimated that 30% of embryos are slow-growing, with some that never reach the blastocyst stage. Common causes of slow embryo development may involve advanced maternal age and aneuploidy (4-6). Research has shown that the transfer of fresh, slow-growing embryos result in lower implantation and clinical pregnancy rate in comparison with fully expanded day 5 blastocysts (7). There is also a growing body of evidence suggesting that day 6 embryos are associated with lower implantation rate,

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clinical pregnancy rate, and live birth rate (5, 7, 8). It is yet unclear whether this is due to embryo aneuploidy with delayed blastulation, or due to missing the implantation window (9, 10). There are relatively few studies that have looked specifically at the developmental potential of embryos that have not reached the blastocyst stage by day 5. This is a critical component of patient counselling in situations while only slow growing embryos that haven't yet reached the blastocyst stage are available on day 5. There is little evidence describing the potential of these slower developing embryos to reach the blastocyst stage on day 6, and what specific factors may be associated with day 6 blastulation.

In our clinic, prior to June 2020, embryos were considered for cryopreservation only if they reached blastocyst stage by day 5. Good-quality early (stage 1 and 2), full (stage 3) and expanded (stage 4 and 5) blastocysts would then be vitrified on day 5 and the remaining embryos would be discarded. Past research on vitrified-thawed embryo transfer outcomes reports that culturing slow-growing day 5 embryos to day 6 may improve blastocyst yield, clinical pregnancy, and live birth rates (11-14). Therefore, we changed our cryopreservation strategy to include vitrification of good-quality full and expanded blastocysts (FEBs) on both day 5 and day 6 as of June 1, 2020. If an embryo did not meet vitrification criteria on day 5 it was incubated until day 6. The objective of this study is to evaluate if a change in cryopreservation policy results in a change in the proportion of embryos eligible for cryopreservation.

Materials and Methods

We retrospectively identified a patient cohort in the context of a quality assurance project based on the Ottawa Fertility Centre in Ottawa, Ontario, Canada, between August 2019 and December 2020, inclusively. *In vitro* fertilization/intracytoplasmic sperm injection embryo transfer (IVF/ICSI-ET) cycles involving autologous oocytes with at least one viable day 5 embryo that were selected for inclusion. Donor oocyte, frozen oocyte, preimplantation genetic testing (PGT), and surrogacy cycles were excluded. We divided this population into two groups. Group 1 included IVF cycles performed between September 2019 and March 2020, prior to the change in cryopreservation policy. Hence, group 1 included good-quality early, full, or expanded day 5 blastocysts. Whereas group 2 included IVF cycles performed between June and December 2020, after the policy change. Therefore, group 2 included good-quality FEBs (stage ≥3) that were frozen on either day 5 or day 6. Embryo development outcomes were compared between these two groups. Blastocyst grading was performed by experienced embryologists according to the Gardner morphology criteria (10). Each blastocyst was evaluated based on its expansion stage, its inner cell mass (ICM) development and the appearance of its trophectoderm (TE). The degree of blastocyst expansion was categorized into 5 stages: i. A non expanded embryo with the blastocoel filling <50%, ii. A blastocoel filling >50% of an embryo, iii. A blastocoel filling the entire blastocyst, iv. An expanded blastocyst with a thin zona pellucida, and v. A hatching blastocyst. FEBs with tightly packed or loosely grouped ICM, and a cohesive epithelium (score AA, AB, BA, BB) were considered to be good quality.

The primary outcome was the proportion of embryos eligible for cryopreservation. This is calculated in group 1 as the number of good-quality day 5 blastocysts (any stage), including embryos which were transferred fresh, over the total number of viable day 5 embryos. In group 2, this proportion was calculated as the number of good-quality FEBs (stage ≥3) on either day 5 or day 6 over the total number of viable embryos. The secondary outcome was to identify factors that are predictive of day 6 good-quality blastulation. Embryos were slow-growing if they did not reach at least a stage 3 blastocyst by day 5 of embryo culture, and blastulation was defined as slow-growing embryos that progressed to a day 6 FEB. All embryos derived from eligible cycles were selected for cryopreservation based on their Gardner morphological scoring (15).

We recorded data relating to patients' infertility diagnosis, IVF treatment, and embryo development outcomes. Patient data such as maternal age, gravidity, parity, follicular phase follicle stimulating hormone (FSH), antral follicle count (AFC), anti-mullerian hormone (AMH), body mass index (BMI), cause of infertility, type of IVF protocol, and total FSH dose used were collected. Outcome data such as the number of cumulus oocyte complexes (COCs) and the number of mature oocytes (MIIs) retrieved, also the number of fertilized oocytes, method of fertilization, and number of embryos transferred, were recorded. We evaluated age, method of fertilization, multi/binucleation status, embryo grade on day 5, and sperm origin, in relation to embryo progression to determine which ones are most correlated to development on day 6. Data pertaining to the IVF treatment cycle were retrieved from the Ottawa Fertility Centre laboratory database. This database captures all IVF cycles conducted at the Ottawa Fertility Centre. Since this project falls within the context of a quality assurance program, the study received approval for exemption from ethical review by the Ottawa Health Science Network Research Ethics Board.

Descriptive analysis was performed for the primary outcome comparing the proportion of embryos eligible for cryopreservation between day 5 and day 6. Statistical comparisons for nominal categorical variables were performed by using the Fisher Exact test for non-parametric data or the Chi-squared test for parametric data. Comparisons of continuous variables were done by using the two-sided t test. Data analysis was performed using Stata16.

Results

A total of 3,438 viable day 5 embryos (morulae and blastocysts) across 679 IVF cycles were included in this study. Baseline patient characteristics such as mean patient age, gravidity, parity, ovarian reserve measures

(FSH, AFC, AMH), BMI, and cause of infertility were comparable between the two study groups. Cycle characteristics, such as the type of IVF protocol, total recombinant FSH dose used, number of COCs retrieved, number of MIIs retrieved, number of fertilized oocytes, and method of fertilization were also not significantly different between the two groups (Table 1). Group 1 included a total of 1,667 viable day 5 embryos, whereas group 2 included a total of 1,771 viable day 5 embryos. The average proportion of blastocysts eligible for cryopreservation was comparable before and after the policy change (46.9% in group 2 vs. 44.4% in group 1, mean difference 0.025, 95% confidence interval -0.021 to 0.071, P=0.28, Table 2). The mean number of cryopreserved embryos was significantly higher in group 2 (mean 2.2 vs. 1.7 embryos, P=0.007).

Table 1: Baseline patient and cycle characteristics between patients in groups 1 and 2

Characteristics	Group 1 (n=341)	Group 2 (n=338)	P value
Age at cycle start (Y)*	35.2 (4.1)	35.3 (4.1)	0.73†
Gravidity	0.8 (1.1)	0.9 (1.4)	0.09†
Parity	0.2 (0.03)	0.3 (0.03)	0.30†
FSH (IU/L)	7.5 (2.2)	7.6 (2.2)	0.66†
AFC	24.8 (12.5)	24.9 (12.6)	0.88†
AMH (pmol/L)	24.4 (16.4)	24.2 (20.4)	0.93†
BMI (kg/m^2)	26.5 (5.6)	27.0 (5.5)	0.25†
Cause of infertility (%)			0.36‡
Male factor	144 (31.7)	133 (29.6)	
Decreased ovarian reserve/advanced maternal age	106 (23.3)	109 (24.2)	
Unexplained	52 (11.5)	56 (12.4)	
Endometriosis	41 (9.0)	31 (6.9)	
Tubal factor/peritoneal	41 (9.0)	40 (8.9)	
Ovulatory disorder/PCOS	35 (7.7)	50 (11.1)	
Other	35 (7.7)	31 (7.0)	
Type of IVF Protocol (%):			0.92‡
Antagonist	276 (80.9)	296 (87.5)	
Agonist	22 (6.5)	10 (3.0)	
Microdose flare	43 (12.6)	32 (9.5)	
Total FSH dose (IU)	2743.2 (1395.1)	2749.8 (1423.3)	0.95†
Number of COCs retrieved	12.2 (6.3)	12.2 (7.0)	0.95†
Number of MIIs retrieved	9.6 (5.1)	9.8 (5.5)	0.71†
Number of fertilized oocytes	6.8 (3.8)	7.1 (4.6)	0.31†
Method of fertilization (%)			
Standard IVF	134 (39.3)	157 (46.6)	
ICSI	207 (60.7)	180 (53.4)	

*; Presented as means with standard deviation for continuous variables, or as proportions for categorical variables, †; The two-sided t test was used for comparison of continuous variables, ‡; The Chi-squared test was used for comparison of categorical variables, AFC; Antral follicle count, AMH; Antimullerian hormone, BMI; Body mass index, COC; Cumulus oocyte complex, FSH; Follicle-stimulating hormone, G; Gravidity, ICSI; Intracytoplasmic sperm injection, IVF; *In vitro* fertilization, and PCOS; Polycystic ovarian syndrome.

Table 2: Comparison of embryos between patients in groups 1 and 2

Outcomes	Group 1	Group 2	P value
Proportion of blastocysts eligible for cryopreservation per retrieval	0.44	0.47	0.28†
Total number of viable embryos on day 5	1667	1771	-
Total number of viable embryos on day 6	0	740	-
Mean number of fresh embryos transferred on day 5 per retrieval (SD)*	1.5 (0.6)	1.4 (0.6)	0.25‡
Total number of vitrified blastocysts	598	753	-
Mean number of vitrified blastocysts per retrieval (SD)	1.7 (2.0)	2.1 (2.2)	0.007‡
Mean number of vitrified full/expanded day 5 blastocysts per retrieval (SD)	1.6 (1.9)	2.2 (2.4)	0.0001‡
Mean number of vitrified full/expanded day 6 blastocysts per retrieval	N/A	0.2	-

*; Presented as means with standard deviation for continuous variables, †; The Chi-squared test was used for comparison of categorical variables, ‡; The two-sided t test was used for comparison of continuous variables, and SD; Standard deviation.

Table 3: Impact of various factors on embryo progression on day 6

Factors	Freezable day 5 embryos	Non-freezable day 5 embryos	Freezable day 6 embryos	% progression to day 6 good-quality FEB*
Total	n=772	n=999	n=70	n=7.0
Age (Y)				
<35	426	475	42	8.8
35-37	201	247	16	6.5
38-40	102	167	7	4.2
41-42	23	70	4	5.7
≥43	20	40	1	2.5
Method of fertilization				
Standard IVF	399	529	42	7.9
ICSI	373	470	28	6.0
Multi/binucleation				
Yes	208	323	21	6.5
No	564	676	49	7.2
Embryo progression	NA			
M1		87	1	1.1
M2		148	9	0.6
1		220	21	9.5
2		159	19	11.9
3BC/CB/CC		134	8	6.0
4BC/CB/CC		188	11	5.9
5BC/CB/CC		57	2	3.5
Sperm origin	NA			
PESA/TESE		27	4	14.8
Ejaculate		944	71	7.5

*; Percentage of embryos that reached vitrification criteria by day 6, B; Blastocyst, FEB; Full or expanded blastocyst, ICSI; Intracystoplasmic sperm injection, IVF; *In vitro* fertilization, M; Morula, NA; Not applicable, PESA; Percutaneous epididymal sperm aspiration, and TESE; Testicular sperm extraction.

Amongst the 999 embryos that were not frozen on day 5 of embryo culture, 614 (34.7%) were slow-growing (including morulae and early blastocysts) and the remainder had poorer-quality FEBs (stage 3 to 5 grade BC/CC/CB). Of these slow-growing embryos, 280 (45.6%) progressed to a FEB on day 6, but only 49 (8.0%) were of good quality and could be vitrified. Overall, only 7.0% of 999 embryos which did not meet vitrification criteria on day 5 progressed to good-quality FEBs on day 6 and vitrified (Table 3). Age was significantly associated with the chance of progression to a good-quality FEB. Embryos from women 35 years-old or younger had an 8.8% chance of having non-freezable day 5 embryos progress to good-quality FEBs by day 6, whereas the chance is only 2.6% for those equal or older than 43 years-old. Similarly, we saw a relationship between embryo morphology and embryo progression rate. Day 5 early blastocysts (stage 1 or 2) had a 9.5-11.9% chance of becoming good-quality FEBs on the next day, whereas morulae only had a 0.6-1.1% chance of becoming good-quality FEBs. Sperm origin appeared to have a significant role in the chance of embryo progression to a good-quality FEB on day 6. Indeed, we found that 14.8% of non-freezable day 5 embryos that derived from surgically-retrieved sperm (either by epididymal or testicular extraction) progressed to good-quality FEBs on day 6, compared to only 7.5% for embryos derived from ejaculate sperm.

The method of fertilization did not appear to impact the chance of progression to a good-quality FEB on day 6. Among slow-growing embryos on day 5, we found the progression to a good-quality FEB to be 7.9% for embryos fertilized through standard IVF, and 6.0% for embryos fertilized through ICSI. Similarly, multi/binucleation did not influence the progression rate to good-quality FEB. 6.5% of multi- or binucleated day 5 embryos progressed to become good-quality FEB on day 6, compared to 7.2% of normal embryos. Therefore, factors that predicted an embryo's progression to day 6 included: younger age of egg provider, presence of an early blastocyst on day 5 (stage 1 or 2), and cycles involving surgically retrieved sperm (by either epidydimal or testicular extraction). Despite having embryo-level data at present, the fertility potential of these day 6 vitrified embryos is yet to be determined.

Discussion

Our results suggest that extending embryo culture to day 6 yields a comparable proportion of blastocysts available for cryopreservation. Rates of progression of a slow-growing embryo (morula or early blastocyst on day 5) to a FEB on day 6 was 45.6%, however only 8.0% were of good-quality. The rate at which non-frozen day 5 embryos developed into good-quality FEBs was dependent on factors like age, embryo stage on day 5, and sperm origin.

Although the policy to extend embryo culture to day 6 by itself is not innovative (8), the strength of our study is in its embryo-specific data. It lends itself well to the assessment of the natural history of slow-growing embryos, and also helps us generate hypotheses about predictors of improved blastulation. We believe the

results of our research will contribute to improved patient counseling, physician knowledge, and patient satisfaction.

In the literature, rates of progression to expand blastocyst on day 6 are highly variable. Among their population of 894 embryos, Tannus et al. (11) reported a 72% chance for day 5 morulae to progress to day 6 FEBs (stage 3 to 5), and a 92% chance for day 5 early blastocysts (stage 1 and 2) to progress to day 6 FEBs; giving them an overall day 6 blastulation rate of 87%. Similarly, in a study involving ICSI cycles of patients younger than 37 years old, Ivec et al. reported that 84.4% of their day 5 morulae became blastocysts by day 6. However, the authors reported 'clinically usable Day 6 blastocysts' to be much lower at 35% (16). On the other hand, Kort et al. (17) reported day 6 blastulation rates of 54% from their day 5 morulae. Other studies have described even lower day 6 blastocyst formation rates in the range of 20-40% (18, 19). Based on their high rates of blastulation, some authors supported extending the culture of slow-growing embryos to day 6 until fully expanded blastocysts could be achieved, and then transferring in subsequent freeze-thaw embryo transfer cycles (11). They argued that this process allows for better embryo-endometrium synchronization, and better embryo selection since expanded blastocysts are more predictive of subsequent pregnancy outcomes. The embryo progression rate is generally comparable to what has been reported in the literature. Unlike past studies, we focused on good-quality FEBs, which are more predictive of pregnancy outcomes. This difference could explain the lower rates of blastulation found in our study compared to certain other studies. Laboratory practices and procedures may have been partially responsible for this disparity; differences in baseline patient characteristics may be also contributed. Our data places the argument for universally growing slower developing embryos to day 6 into doubt. Given the lower chance of progressing to a good-quality FEB by day 6 documented in our patient population, those who have slower-growing embryos to start, may be better off transferring day 5 embryos fresh, rather than growing them to day 6 in the hopes of improved chances at cryopreservation and future pregnancy. Slower-growing embryos, irrespective of duration of embryo culture, may ultimately be of poorer prognosis.

Several characteristics have been reported to influence the rates of embryo progression to day 6. For instance, there is significant evidence suggesting that ploidy, developmental and morphological characteristics of the earlier stage embryo are indicators of its potential for blastulation after day 5 (16-20). Optimal morphology of embryos (defined by higher numbers of blastomeres and minimal fragmentation) on day 3 has also been associated with better blastulation rates (21-23). In a comparison of day 5 morula and day 5 cavitating morula (early blastocyst), the subsequent day 6 blastulation rate was significantly higher in the cavitating morula group than in the morula group (18). In a study of morulae biopsied on day 5, Kort et al. (17) also reported significantly higher blastulation rates in euploid embryos, with 83% of day 5 euploid morulae becoming blastocysts on day 6 compared to 49% of aneuploid morulae. Tsai et al. (20) reported a day 6 blastulation rate of 88.9% among morphologically optimal day 5 morula (grade M1 based on SART scoring system). Other findings are less supportive of embryo morphology as a strong predictor of day 6 blastulation, with one study reporting no significant differences in the overall rates of blastulation between M1, M2, and M3 day 5 morulae-however, the clinically usable blastocyst formation rate was significantly higher among M1 and M2 compared to M3 morulae (16). Aside from the characteristics of the embryo itself, there are limited and conflicting reports in the literature as to what other factors might be predictive of day 6 blastulation. Maternal age has been shown by many studies to have no direct impact on day 5 blastulation rates (19, 24-26), but it is a potential moderator of blastocyst formation rates through its association with the number of oocytes retrieved, embryo quality, morphology, and aneuploidy rates (17, 19, 25, 26). When specific to day 6 blastulation rates, the role of maternal age remains unclear; with one study reporting it to be significantly associated with blastulation of day 5 morula (18), and another indicating no such association (20). There is conflicting evidence for the effects of fertilization method on blastulation rates. Some studies have shown that blastocyst formation rates are significantly higher with IVF than ICSI (27-29), whereas others have shown comparable blastulation rates (30, 31), with one study reporting better blastulation rates with ICSI than IVF (26). Etiology of infertility is likely a contributor to these discordant findings, as Hong et al. showed ICSI to be associated with higher blastocyst formation rate in couples with male factor infertility, but not overall (24, 32). Our data also failed to show any difference in blastulation rates between the IVF and ICSI cycles. For ICSI cycles, testicular sperm has been associated with significantly higher day 6 blastulation rates than epididymal or ejaculated sperm (29). This finding is confirmed through our data, where we found 14.8% progression to day 6 full and expanded blastocysts (FEBs) for testicular sperm, compared to 7.5% for ejaculate sperm. More research into the specific semen parameters that leads to higher day 6 blastulation rate should be performed. In the assessment of predictive factors for day 6 FEBs, our findings are concordant with previously published evidence. Other factors that have been studied but have not been shown to have a significant association with day 6 blastulation rates including number of oocytes retrieved, number of fully developed day 5 blastocysts, number of slow developing day 5 embryos, and insemination method (18, 20).

Overall, the results of our study suggest that extending culture to day 6 led to comparable proportions of embryos eligible for cryopreservation. The strength of this study is in its embryo-specific data. It lends itself well to the assessment of the natural history of slow-growing embryos that helps us to generate hypotheses about predictors of improved blastulation. Most importantly, this study presents center-specific data so our physicians can

counsel patients with concrete and relevant information. The external validity of our study may be of concern since the study is based on a single clinic cohort. Embryo ploidy was also not considered in this study. Past research had demonstrated a reduction in the prevalence of euploidy by increasing time to embryo blastulation (10, 33). Due to the exclusion of PGT cycles, we were unable to assess the contribution of ploidy to blastulation. Additionally, this is a retrospective cohort study, therefore several confounders may be at play. Although we found comparable baseline characteristics between the two groups under study, there is certainly possibility of residual confounding from unknown confounders. As this is a chart review, we are also reliant on previously recorded data; there may be a small proportion of incomplete or inaccurate dataset. Given the embryos vitrified under our new policy have, for the most part, not been thawed, our study was limited to embryo developmental outcomes, rather than implantation or pregnancy outcomes.

Patient counselling individualized to maternal factors, embryo morphology, and IVF cycle characteristics should be provided. Physicians should warn patients that the overall chance of progression from slow-growing day 5 embryos to day 6 good quality FEBs (stage 3-5) is low at our center. Recognizing that good prognosis patients such as those who are younger than 35, have euploid embryos, and those used testicular sperm, are more likely to have embryo progression to day 6.

Conclusion

The change in cryopreservation policy to include good-quality day 6 FEBs while eliminating early blastocysts yielded similar proportions of embryos eligible for cryopreservation per retrieval. Embryos' blastulation potential on day 6 was influenced by factors such as maternal age, stage of embryo development on day 5, and use of surgically-retrieved sperm. This study adds to the existing literature on extended embryo culture and ascertains the viability of this approach in our patient population. These results will aid physicians, embryologists, and patients in making more informed decisions concerning the management of slow-growing embryos. Further research into the implantation potential of these day 6 embryos should be performed.

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Authors' Contributions

C.Q.W., M.-C.L.; Contributed to project development, data analysis and manuscript preparation. M.C., S.T.; Contributed to literature search and data collection. D.Sh., J.G.; Contributed to data interpretation, manuscript editing and reviews. All authors approved the final manuscript.

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