Developmental potential of slow-developing embryos: day-5 morulae compared with day-5 cavitating morulae

Jigal Haas, M.D.,[a,b] Jim Meriano, M.A.,[a] Rawad Bassil, M.D.,[a] Eran Barzilay, Ph.D.,[a,b] Eran Zilberberg, M.D.,[a] and Robert F. Casper, M.D.[a]

[a]TRIO Fertility Partners, Division of Reproductive Sciences, University of Toronto, Toronto, Ontario, Canada; [b]IVF Unit, Department of Obstetrics and Gynecology, Chaim Sheba Medical Center, Tel- Hashomer, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel

Objective: To describe and compare the ongoing pregnancy rate between morulae and cavitating morulae (CAVM) transferred on day 5, to describe and compare the blastulation rate between day 5 morulae and CAVM, and to describe the pregnancy rate of these slow-developing blastocysts during a frozen embryo transfer (FET) cycle.
Design: Retrospective cohort study.
Setting: Single tertiary care medical center.
Patient(s): Delayed-development embryos: 3,321 cycles that included 10,304 embryos on day 5 that were cultured until day 6.
Intervention(s): Development of morula and CAVM to the blastocyst stage.
Main Outcome Measure(s): Blastulation rate.
Result(s): The fresh embryo transfers comprised 186 patients with 82 embryos at the morula stage and 104 embryos at the CAVM stage. The pregnancy rate (15.8% vs. 21.1%) and the ongoing pregnancy rate (15.8% vs. 17.3%) were comparable between the groups. The study group included 10,304 day-5 delayed embryos: 5,395 morulae and 4,909 CAVM on day 5. The blastulation rate was statistically significantly higher in the CAVM group compared with the morula group (39.2% vs. 20.4%). We included 201 FET cycles: 77 warmed blastocysts that developed from a morula on day 5 and 124 warmed blastocysts that developed from CAVM on day 5. The clinical pregnancy rate was comparable between the two groups per embryo transfer (21.3% vs. 24.7%).
Conclusion(s): Transferring of fresh, slow-developing embryos seems to improve the cycle outcomes compared with culturing for another day and then vitrifying and thawing later. (Fertil Steril® 2019;111:105–11. ©2018 by American Society for Reproductive Medicine.)
El resumen está disponible en Español al final del artículo.

Key Words: Cavitating morula, morula, slow-developing embryos

The blastocyst stage is the last embryonic stage before implantation and is assumed to be the best stage of embryo selection compared to embryos at cleavage stage. Not every embryo will reach the blastocyst stage even when using the most advanced culture media. There has been a debate for the last few years over whether to prefer embryo transfer at the cleavage stage or to culture the embryos to the blastocyst stage and transfer them at day 5 (1).

The rationale for blastocyst transfer is to improve both uterine and embryonic synchronicity and enable self-selection of viable embryos, thus resulting in better live-birth rates per transfer, and the rationale for cleavage-stage transfer is the possibility that extended culture may in fact harm viable embryos through suboptimal culture conditions, which could be prevented by earlier transfer at day 3. A study published recently compared the cumulative live-birth rates after cleavage stage on day 3 and blastocyst transfers on day 5 and showed that although the number of embryo transfers necessary until the first live birth was statistically significantly lower for blastocyst-stage

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Reprint requests: Jigal Haas, M.D., TRIO Fertility Partners, 655 Bay Street, 11th floor, Toronto, Ontario, Canada M5G 2K4 (E-mail: jigalh@hotmail.com).

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embryos, the cumulative live-birth rates were comparable between the two groups (2). A Cochrane review published in 2016 (1) also concluded that there was no evidence of a difference between the day-3 and day-5 groups in cumulative pregnancy rates derived from fresh and frozen-thawed cycles after a single oocyte retrieval.

Because of the high blastulation rate in our clinic (58%, unpublished data), we routinely culture embryos until day 5 in order to transfer/freeze a fully developed blastocyst. Some of the embryos do not develop to the blastocyst stage on day 5, some of them are not viable, and some of them are slow-developing embryos at the morula or cavitating morula (CAVM) stage. It is normal for an embryo to be at the morula stage on day 4, but the data are limited regarding the etiology, prognosis, and how to proceed when the embryo is still at the morula stage on day 5. Studies have described good pregnancy results in cycles that included fresh transfers of morulae on day 4 (3–5), but to our knowledge there is a lack of evidence in the literature regarding pregnancy outcomes with embryos at morula stage on day 5.

Our study describes and compares ongoing pregnancy rates between morulae and CAVM transfers on day 5. We describe and compare the blastulation rates between morulae and CAVM, and examine the pregnancy rate of slow-developing blastocysts during the subsequent frozen embryo transfer (FET) cycles.

MATERIALS AND METHODS

This was a single center, retrospective cohort study of 3,321 in vitro fertilization (IVF) cycles between January 2012 and November 2016. The study was approved by the research ethics board at Mount Sinai Hospital in Toronto.

Study Group

At our clinic the protocol is to transfer slow-developing embryos—either morulae or cavitating morulae (CAVM)—on day 5 only if there is no blastocyst to transfer. If there is a blastocyst on day 5, the slow-developing embryos are cultured until day 6. At day 6, only expanding or fully expanded blastocysts are vitrified; the rest are discarded. Our study included 3,321 cycles, including 10,304 slow-developing embryos on day 5 that were cultured until day 6, and 184 cycles of 233 slow-developing embryos transferred on day 5. The cycles with combined transfers of slow-developing embryos and blastocysts were excluded from the study. The slow-developing embryos that reached expanding or fully expanded blastocysts on day 6 were vitrified; of this group, 201 embryos were thawed and transferred during natural cycles or hormonally substituted FET cycles.

Embryogenesis

Oocytes were injected with sperm in drops of modified human tubal fluid medium (mHTF; LifeGlobal) supplemented with 10% LGPS (v/v) overlaid with mineral oil (lite oil; LifeGlobal) using Narishige manipulators (Narishige International) and Zeiss microscope equipped with Hoffman optics (Carl Zeiss Microscopy). Once the injection was complete, the oocytes were washed in warm equilibrated Global medium supplemented with 10% (v/v) LGPS. The oocytes were then placed into pre-equilibrated proteinated Global medium. The oocytes were cultured in a 37°C, humidified incubator set to 5.5% CO_2 and 5.0% O_2. Fertilization was assessed at 18 hours for the presence of two distinct pronuclei and two polar bodies, which we considered normally fertilized.

The embryos were assessed on days 2 and 3 for cleavage, nucleation, and fragmentation. Embryos were not assessed on day 4. On days 5 and 6, the embryos were assessed for blastocyst development and graded with the Gardner and Schoolcraft method (6). Those embryos that were not blastocysts were characterized as either cavitating morulae or noncavitating morulae (arrested nondeveloping embryos).

Frozen Cycle Endometrial Preparation

Artificial hormone replacement. Patients started on day 2–3 of the cycle with oral administration of 2 mg of estradiol (Estrace; Allergan Pharmaceuticals) twice daily for endometrial preparation, which was increased by a step-up protocol to 8 mg/d. An ultrasound endometrial assessment performed about 10 days later, assessed the lining as ready for the ET procedure when the endometrial thickness was $\geq$6.5 mm. If the lining was not adequate, endometrial estrogen priming continued, and ultrasound assessment was undertaken to confirm further endometrial thickening. Participants commenced luteal support via vaginal administration of progesterone suppositories 200 mg three times daily according to the proposed day of embryo thawing and transfer. The embryos were thawed on day 6 of progesterone and transferred after 2–4 hours.

Natural cycles. After spontaneous menstruation, patients were monitored by serial ultrasound for endometrial thickness, follicular development, luteinizing hormone (LH) and progesterone levels until a rise in LH level was observed (LH level exceeded 180% of the baseline value) corresponding to a day before oocyte pickup/ovulation. On the following day, progesterone suppositories (200 mg three times daily) were started. The embryos were thawed on day 6 of progesterone and transferred after 2–4 hours.

Morphology. A top-quality embryo (TQE) was defined as an embryo graded $\geq$3BB according to the scale proposed by Gardner and Schoolcraft (6) and >90% expanded after warming. We defined CAVM as a very early blastocyst with a blastocoel less than 10% of blastocyst. Vitrification was performed using the Irvine Scientific "Freeze Kit" (cat. no. 90,133-SO) with high security vitrification straws. Clinical pregnancy was defined as visualization of a gestational sac; ongoing pregnancy necessitated the visualization of fetal cardiac activity up to 12 weeks' gestational age on transvaginal or pelvic ultrasound.

Statistical Analysis

Comparison of continuous variables between the two groups was conducted using Student's *t*-test and Mann-Whitney test, as appropriate. The chi-square test was used for comparison of categorical variables. Logistic regression analysis was

employed for multivariate analysis. Variables used in the regression model included maternal age, estradiol level on the day of triggering, number of eggs retrieved, number of fully developed blastocysts on day 5, and number of slow-developed embryos day 5. $P<.05$ was considered statistically significant. Statistical analyses were conducted using the IBM Statistical Package for the Social Sciences (IBM SPSS v.20; IBM Corporation).

RESULTS

Fresh Transfers of Slow-developing embryos on day 5

One hundred and eighty-six patients had fresh embryo transfers of slow-developing embryos: 82 embryos at the morula stage and 104 embryos at the CAVM stage. The pregnancy rate (15.8% vs. 21.1%, respectively; P=not statistically significant [NS]) and the ongoing pregnancy rate (15.8% vs. 17.3%, respectively; P=NS) were comparable between the two groups (Table 1). Eight patients in the CAVM group (7.6%) and four patients in the morulae group (4.9%) had embryos that developed to the blastocyst stage and were cryopreserved on day 6.

Progression to Blastocyst

The study included 10,304 day-5 slow-developing embryos: 5,395 morulae and 4,909 CAVM on day 5. The blastulation rate was statistically significantly higher in the CAVM group compared with the morula group (39.2% vs. 20.4%; $P<.001$), and the progression to TQE was statistically significantly higher in the CAVM group (35.9% vs. 17.7%; $P<.001$) (Table 2).

Prediction of Progression to Blastocyst

We performed a regression analysis to demonstrate whether different variables predict which slow-developing embryo may develop into a blastocyst. The variables included in the analysis were age, number of eggs retrieved, number of fully developed blastocysts on day 5, and number of slow-developing embryos on day 5. Age was the only variable that was found to predict the development to blastocyst stage (odds ratio 0.97; 95% confidence interval, 0.96–0.98), and the older patients were found to have a lower blastulation rate compared with the younger patients (Table 3).

TABLE 1

Fresh morulae and cavitating morulae transfer.

Parameter	Morula	CAVM	*P* value
N	82	104	NS
Clinical pregnancy rate	15.8%	21.1%	NS
Ongoing pregnancy	15.8%	17.3%	NS

Note: CAVM = cavitating morula; NS = not statistically significant.

Haas. Potential of slow-developing embryos. Fertil Steril 2018.

TABLE 2

Progress of morulae versus cavitating morulae to the blastocyst stage.

Parameter	Morula	CAVM	*P* value
N (embryos)	5,395	4,909	
Developed to blastocysts	1,105 (20.4%)	1,926 (39.2%)	<.001
Developed to TQE	958 (17.7%)	1,766 (35.9%)	<.001

Note: CAVM = cavitating morula; TQE = top-quality embryo.

Haas. Potential of slow-developing embryos. Fertil Steril 2018.

FET Cycles of Slow-developing Embryos that Became a Blastocyst on Day 6

We included only single-embryo transfers of slow developing embryos that became blastocysts on day 6. We included 201 FET cycles, 77 thawed blastocysts that developed from morulae on day 5, and 124 thawed blastocysts that developed from a CAVM on day 5 (Table 4). The age was comparable between the two groups, as was the survival rate of the vitrified-thawed embryos (96% vs. 97.6%; P=NS). The pregnancy rate, clinical pregnancy rate, and ongoing pregnancy rate were comparable between the two groups. The pregnancy loss rate (before week 11 of gestation) was high in the morula group (37.5%), but due to a small sample size it was not statistically significantly different from that of the CAVM group (16.7%; $P=.1$).

Theoretical Calculation

Morula. An average of 20.4% of morula developed to the blastocyst stage on day 6 (Table 2). Only 13.3% of the transferred blastocysts (that developed from the morulae) during FET cycles resulted in an ongoing clinical pregnancy. Therefore, 2.71% of the morulae may be likely to result in an ongoing pregnancy.

CAVM. An average of 39.2% of the CAVM developed to the blastocyst stage on day 6. Only 20.6% of the transferred blastocysts (that developed from the CAVM) during FET cycles resulted in an ongoing clinical pregnancy. Therefore, 8.1% of the CAVM may be likely to result in an ongoing pregnancy.

TABLE 3

Regression analysis/prediction of development of morulae/cavitating morulae to blastocyst.

			95% CI for OR	
Parameter	Significance	OR	Lower	Upper
Age	<.001	0.97	.96	.98
E_2 level on day of triggering	.184	1.000	1.000	1.000
No. of eggs	.491	1.003	.987	1.01
No. of total blastocysts on D5	.527	1.005	.989	1.022

Note: CAVM = cavitating morula; CI = confidence interval; D5 = day 5; E_2 = estradiol; OR = odds ratio.

Haas. Potential of slow-developing embryos. Fertil Steril 2018.

TABLE 4

Frozen embryo transfer cycles: pregnancy outcome of slow-developing blastocysts.

Parameter	Developed from morula	Developed from CAVM	*P* value
Embryos warmed	75	124	
Nonsurviving embryos	3 (4%)	3 (2.4%)	NS
Age	36 ± 4.2	35.5 ± 3.9	NS
TQE (%)	69/75 (92%)	114/124 (92%)	NS
Medical FET	68/75 (90.6%)	111/124 (89.5%)	NS
Natural FET	7/75 (9.4%)	13/124 (10.5%)	NS
Pregnancy	18 (24%)	32 (26.4%)	NS
Clinical pregnancy	16 (21.3%)	30 (24.7%)	NS
Ongoing pregnancy	10 (13.3%)	25 (20.6%)	NS
Missed abortion	6 (37.5%)	5 (16.7%)	NS

Note: CAVM = cavitating morula; FET = frozen embryo transfer; TQE = top-quality embryo.

Haas. Potential of slow-developing embryos. Fertil Steril 2018.

DISCUSSION

Although assisted reproductive technology is four decades old, we believe that insufficient attention has been paid to slow-developing embryos at day 5 after retrieval. We found a reasonable ongoing pregnancy rate in the fresh transfer cycle group, but a low blastulation yield and a low ongoing pregnancy rate in the vitrified-warmed slow-developing embryo group.

Fresh Transfers of Slow-developed Embryos on Day 5

Kovacic et al. (7) classified blastocysts and morulae on day 5 of in vitro culture and established the implantation capacity and developmental ability of various morphologic types of blastocysts and morulas. Based on the different morphologic parameters, eight different categories were defined (Bl to B8) The first category (BI) contained optimal blastocysts, and the B8 category included small blastocysts and morulae. They showed that the birth rate declined from Bl to B8 embryos transferred. Normal-looking early blastocysts and normal compact morulae on day 5 were considered as embryos with delayed development, and they resulted in a pregnancy rate of 26%. In our study we found a lower pregnancy rate (15.8% to 21.1%), but it was difficult to compare the two studies due to lack of important information such as age and number of embryos transferred.

Fresh Transfers of Slow Developing Embryos on Day 6

Previous studies have showed decreased pregnancy rates when transferring slow developing blastocysts on day 6 compared to normally developed blastocysts on day 5 in fresh cycles. Barrenetxea et al. (8) compared the pregnancy rate according to the day of transfer in fresh transfer cycles. They found a significantly increased pregnancy rate when transferring embryos on day 5 compared with slow-developing blastocysts on day 6, and the pregnancy rate was extremely low in the transfer day-6 group (11%). Hashimoto et al. (9) also demonstrated a lower pregnancy rate of slow-growing embryos on day 6 compared with normally developing embryos.

In our clinic we do not perform fresh transfers of slow-developing blastocysts on day 6. As mentioned previously, we transfer the slow-developing embryos (morula or cavitating morula, CAVM) on day 5 if there is no other developed blastocyst to transfer. If there is a blastocyst on day 5, the slow-developing embryos are cultured until day 6. At day 6, only blastocysts are vitrified, and the rest are discarded.

Progression to Blastocyst

Zakharova et al. (10) described the blastocyst yield obtained during normal morula development (from day 4 to day 5) to be 91% in biopsied and nonbiopsied embryos. Kort el al (11). aimed to better understand slower embryo aneuploidy rates and implantation potential. They found that day-5 morulae had a significantly higher aneuploidy rate, even though the majority of day-5 morula progressed to become blastocysts by day 6. Euploid day-5 morulae had a statistically significantly higher rate of making blastocyst on day 6 compared with day 5 morulae that were aneuploid. In their study 54% to 61% of the day-5 morulae progressed to become blastocysts on day 6. Only 296 embryos were included in the study, and there was no differentiation between morulae, cavitating morulae, and unexpanded blastocysts.

Ivec et al. (12) determined the influence of delayed compaction and fragmentation on the developmental capacity of morulae. They found that the measurement of compaction timing and cytoplasmic loss in morulae could assist in predicting their ability to develop into optimal blastocysts.

In our study we included a large number of embryos (10,304), and we analyzed the development of morula and CAVM embryos separately. We also performed a regression analysis to demonstrate whether different variables predict which slow-developing embryo might develop into a blastocyst, and we found that age was the only variable to predict the development to blastocyst stage. Unfortunately, we did not biopsy the embryos on day 5 and therefore could not compare embryo development of euploid versus aneuploid slow-developing embryos. We do not grade morulae in our clinic, so our study was not able to determine whether potential grading of the morulae could predict the development to blastocyst.

FET Cycles of Slow-developing Embryos that Became a Blastocyst on Day 6

Studies involving frozen-thawed blastocyst transfers have reported conflicting results regarding whether the rate of blastocyst formation before cryopreservation affects treatment outcome (13–19). A meta-analysis concluded that there is a statistically significant increase in the clinical pregnancy rate with day-5 frozen-thawed blastocyst transfers compared with day-6 frozen-thawed blastocyst transfers. Our group recently published a study demonstrating lower pregnancy rate with high-quality day-6 frozen-thawed

blastocyst transfers compared with day-5 high-quality frozen-thawed blastocysts (20).

Whether slower-growing blastocysts have a higher rate of aneuploidy is also still debatable. Kroener et al. (21) showed that delayed blastulation was not associated with increased aneuploidy rates, but absence of blastulation was associated with increased aneuploidy. Similarly, Capalbo et al. (22) demonstrated that faster growing embryos (day-5 blastocysts) showed a similar euploidy rate compared with slower growing ones (day-6 blastocysts).

Yang et al. (19) showed that delayed blastulation was not associated with increased aneuploidy rates, but the pregnancy rate with euploid day-5 embryos was statistically significantly higher that with euploid day-6 blastocysts. By contrast, Taylor et al. (23) examined the euploidy rates and outcomes between day-5 and day-6 blastocysts and showed that day-5 blastocysts had a higher chance of being euploid than day-6 blastocysts. He also showed that when only euploid day-5 or euploid day-6 blastocysts were transferred during a cryopreserved embryo transfer, the cycle outcomes were similar.

In our current study we compared the pregnancy rate between vitrified/warmed blastocysts that developed from morula and blastocysts that developed from CAVM. We did not find a statistically significant difference between the two groups (13.3% vs. 20.6%), but the sample size was too small to detect a difference of 7 percent. The pregnancy loss rate (before week 11 of gestation) was very high in the morula group (37.5%), but due to the small sample size the pregnancy loss rate was not statistically significantly higher than that of the CAVM group (16.7%, $P=.1$). We believe that the high rate of pregnancy loss can be explained by an increased aneuploidy rate, as demonstrated by some of the studies. Because preimplantation genetic screening is not routinely performed in our clinic, this hypothesis could not be verified.

Slow-developing embryos are transferred in our clinic during a fresh cycle as a morula/CAVM on day 5 with a reasonable ongoing pregnancy rate (15% to 17%), or they can be cultured another day, vitrified if they develop to a blastocyst, and then transferred in a FET cycle. As we demonstrated previously, an average of 20.4% of morulae developed to the blastocyst stage on day 6, and only 13.3% of the transferred blastocysts (that developed from the morulae) during FET cycles resulted in an ongoing clinical pregnancy. Therefore, 2.71% (20.4% × 13.3%) of the morulae may be likely to result in an ongoing pregnancy.

An average of 39.2% of the CAVM developed to the blastocyst stage on day 6, and only 20.6% of the transferred blastocysts (that developed from the CAVM) during FET cycles resulted in an ongoing clinical pregnancy. Therefore, only 8.1% (39.2% × 20.6%) of the CAVM may be likely to result in an ongoing pregnancy.

Because the theoretical ongoing pregnancy rate per morula is only 2.71%, and only 8.1% of the CAVM will result in an ongoing pregnancy when vitrified on day 6, it is reasonable to transfer a fresh morula/CAVM during the fresh cycle to optimize the chance of the morula/CAVM to develop to an ongoing pregnancy, and to vitrify blastocysts on day 6 without compromising the future capability of developing to an ongoing pregnancy.

There are some limitations in our study. This was a retrospective study, and to conclude that fresh transfers result in better pregnancy outcomes, a prospective randomized controlled study should be performed. In addition, there are many factors that can influence the pregnancy rate such as the experience of the physician or the embryologist performing the transfer, difficulty inserting the transfer catheter, endometrial thickness and pattern, and so on. Those factors were not controlled for in the study.

In conclusion, slow-developing embryos have a reasonable ongoing pregnancy rate in fresh transfers but a low blastulation yield, leading to a low ongoing pregnancy rate in the vitrified-warmed slow-developing embryo group. This is important information that should be taken into consideration on day 5 if only morula or CAVM are available for transfer.

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Potencial de desarrollo de los embriones de desarrollo lento: mórulas en día 5 comparadas con mórulas en cavitación en día 5.

Objetivo: Describir y comparar las tasas de embarazo evolutivo entre las mórulas y las mórulas en cavitación (CAVM) transferidas en día 5, describir y comparar la tasa de blastulación entre las mórulas en día 5 y CAVM y describir la tasa de embarazo de estos blastocistos de desarrollo lento en un ciclo de transferencia de embriones congelados (FET).

Diseño: Estudio retrospectivo de cohortes.

Lugar: Un único centro médico de tercer nivel.

Paciente(s): Embriones de desarrollo lento: 3.321 ciclos que incluyeron 10.304 embriones en día 5 que fueron cultivados hasta día 6.

Intervención(es): Desarrollo de las mórulas y CAVM a estadio de blastocisto.

Medida(s) del resultado principal: Tasa de blastulación.

Resultado(s): Los ciclos de transferencia en fresco incluyeron a 186 pacientes con 82 embriones en estadio de mórula y 104 embriones en estadio de CAVM. Las tasas de embarazo (15,8% vs 21,1%) y las tasas de embarazo evolutivo (15,8% vs 17,3%) fueron comparables entre los grupos. El grupo de estudio incluyó 10.304 embriones lentos en día 5: 5.395 mórulas y 4.909 CAVM en día 5. La tasa de blastulación fue significativamente mayor estadísticamente en el grupo de CAVM comparado con el grupo de mórula (39,2% vs 20,4%). Incluimos 201 ciclos de FET: 77 blastocistos descongelados que se desarrollaron a partir de mórula en día 5 y 124 blastocistos descongelados que se desarrollaron a partir de CAVM en día 5. La tasa de embarazo clínico por transferencia embrionaria fue comparable entre los dos grupos (21,3% vs 24,7%).

Conclusión(es): La transferencia de embriones de desarrollo lento en fresco parece mejorar los resultados del ciclo comparado con el cultivo durante un día más, su vitrificación y posterior transferencia en diferido.