Accepted Manuscript

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PII: S1472-6483(17)30374-7
DOI: http://dx.doi.org/doi: 10.1016/j.rbmo.2017.07.017
Reference: RBMO 1803

To appear in: *Reproductive BioMedicine Online*

Received date: 18-2-2017
Revised date: 26-7-2017
Accepted date: 27-7-2017

Please cite this article as: Samer Tannus, Yoni Cohen, Weon-Young Son, Tal Shavit, Michael-Haim Dahan, Cumulative live birth rate following elective single blastocyst transfer compared with double blastocyst transfer in women aged 40 years and over, *Reproductive BioMedicine Online* (2017), http://dx.doi.org/doi: 10.1016/j.rbmo.2017.07.017.

Short title: Cumulative live birth rate after blastocyst transfer in advanced maternal age

Cumulative live birth rate following elective single blastocyst transfer compared with double blastocyst transfer in women aged 40 years and over

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Key message

Blastocyst quality and transferring two blastocysts were found to be the most significant independent predictors for live birth in women aged ≥40 years. Single blastocyst transfer resulted in lower live birth rates and lower multiple birth rates; however, the cumulative live birth rate was similar to double blastocyst transfer.

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Abstract

This retrospective cohort study aimed to identify predictive factors for live birth following blastocyst transfer in women aged 40–43, and to compare the cumulative live birth rate (LBR) following elective single blastocyst (eSBT) and double blastocyst (DBT) transfer. The study included 411 women who had fresh blastocyst transfers on day 5. In stepwise logistic regression, independent predictive factors for live birth were: transferring fully expanded blastocysts (Gardner stage ≥3) (odds ratio [OR] 3.5, 95% confidence interval [CI] 1.59–9.71) and transferring two blastocysts compared with a single blastocyst (OR 1.7, 95% CI 1.08–2.9). Maternal age was not found to be significant (OR 0.78, 95% CI 0.62–1.1). When comparing eSBT (n = 150) with DBT (n = 151), the DBT group achieved higher LBRs (26.5 versus 19.3%, P = 0.017) and higher multiple births (0 versus 17.5%, P = 0.02). However, the cumulative LBR was similar (28.0 versus 31.1%), with significantly lower multiple births in the eSBT group (0 versus 14.9%, P = 0.03). These results indicate that in women aged 40–43, when fully expanded blastocysts are achieved, maternal age is not a predictor for live birth, and eSBT can be performed without compromising cumulative LBRs.

Keywords*: advanced maternal age, blastocyst, cumulative live birth rate, multiple birth, predictive factors*

Introduction

Extending embryo culture to the blastocyst stage has the advantage of enabling self-selection of the most viable embryos. A recent Cochrane meta-analysis reported higher live birth rates (LBRs) following blastocyst transfer compared with cleavage embryo transfer (Glujovsky *et al.*, 2016), and a large retrospective study showed a reduced number of cycles needed, both fresh and vitrified-warmed, to have a live birth with blastocyst transfer compared with cleavage embryo transfer (De Vos *et al.*, 2016). However, there is still ongoing debate over whether extended embryo culture should be offered to the entire IVF population. In unselected IVF patients, it can result in an increased cycle cancellation rate (Van der Auwera *et al.*, 2002) and in a reduced number of surplus embryos available for cryopreservation (Glujovsky, 2016). This can potentially decrease the cumulative LBRs. Moreover, adverse perinatal outcomes are increased following blastocyst transfer, as a recent meta-analysis showed increased preterm birth rate, large for gestational age newborns and increased perinatal mortality, when compared with cleavage embryo transfer (Martins *et al.*, 2016). Extending embryo culture can potentially improve embryo selection in older women. Oocyte aneuploidy rate is age-dependent, and in women aged 40 years and over, it is reported to be >80% (Gutierrez-Mateo *et al.*, 2004). Aneuploid embryos are less likely to continue development to the blastocyst stage. A study that implemented PGS showed a lower likelihood of aneuploid cleavage embryos developing to blastocysts compared with euploid cleavage embryos (18% versus 56%) (Vega *et al.*, 2014). Improved

embryo selection in older women can enable practices that aim to optimize maternal and neonatal outcomes such as single embryo transfer (SET), to reduce the incidence of multiple births. Recently, the American Society for Reproductive Medicine (ASRM) issued new guidelines about the number of embryos to transfer. It was recommended that in women aged 40 and over, no more than three blastocysts should be transferred (ASRM, 2017). Furthermore, when euploid blastocysts are available after PGS, SET is recommended. Overall, there are few studies that have assessed the outcomes after blastocyst transfer (Tannus *et al.*, 2017; Ubaldi *et al.*, 2015), and no study has reported the cumulative LBR in this age group. With the increasing trend of extending embryo culture, it is important to identify prognostic factors for live birth in advanced maternal age and to report the cumulative LBRs following elective blastocyst transfer. To the best of our knowledge, this analysis has not been performed before. Hence, the aim of this study was to identify predictive factors for live births following blastocyst transfer in women aged 40 years and over, and to compare the cumulative live births between the group who had eSBT with the group who had double blastocyst transfer (DBT).

Materials and methods

This was a retrospective cohort study, conducted at a university-affiliated reproductive centre. The clinic guideline is to treat women with autologous oocytes up to the age of 43 years. The patients' demographics, indication for treatment, stimulation cycle characteristics and outcomes are all recorded prospectively in computerized medical charts. All the IVF cycles that were performed in women aged ≥40, in which embryo culture was extended to the blastocyst stage, were reviewed for possible inclusion. The embryology laboratory strategy is to extend embryo culture to the blastocyst stage when at least three good-quality, cleavage stage, day 3 embryos are present in culture irrespective of maternal age. Inclusion criteria included the following: maternal age ≥40 years at the beginning of the ovarian stimulation cycle, embryo culture that was extended to the blastocyst stage and fresh embryo transfer was performed on day 5. Exclusion criteria included the following: oocyte donation cycles, PGD/PGS cycles and cycles with freeze-all embryos. Each subject was included once during the study period. Based on provincial guidelines, up to two blastocysts can be transferred in this age group. The decision regarding the number of blastocysts to transfer (single or double), was made by the treating physician. The fertility unit includes several clinicians that manage patients independently, and with the lack of local clinic guidelines, the decision on the number of transferred embryos reflected the physician clinical approach, along with other clinical factors such as previous patient history, outcome of previous embryo transfer cycles and the number of available blastocysts.

Ovarian stimulation and embryo procedures

Ovarian stimulation was performed under pituitary suppression using one of the three following protocols: gonadotrophin-releasing hormone (GnRH) agonist micro-dose flare protocol, mid-luteal GnRH agonist long protocol or GnRH antagonist short protocol. Final oocyte maturation was induced

with 10,000 IU of human chorionic gonadotrophin (HCG), when at least two follicles were >17 mm in diameter. Oocyte collection was performed 36–38 h after HCG triggering. Insemination of retrieved oocytes was performed by conventional IVF or intracytoplasmic sperm injection (ICSI). The zygotes were cultured in cleavage medium (COOK Medical, Sydney, Australia). Embryonic development was assessed daily. If there were at least three good-quality embryos on day 3 (8 cells and <20% fragmentation), embryos were cultured to the blastocyst stage in the blastocyst medium (COOK Medical) and transferred on day 5. Luteal support in fresh cycles included oestradiol valerate 6 mg and micronized vaginal progesterone 300 mg daily. Luteal support was continued until 10 weeks of gestation.

Blastocyst assessment

Blastocysts were graded according to Gardner criteria (Gardner *et al.*, 1998) and the score was dependent on blastocyst expansion, inner cell mass (ICM) development and trophectoderm (TE) appearance. Depending on their expansion score, blastocysts were considered as early blastocysts (stage 1 and 2) and fully expanded blastocysts (FEB) (stage ≥3). Quality of the blastocysts was dependent on ICM and TE scoring: top quality blastocysts (grade I) (AA), good quality (grade II) (AB and BA), average quality (grade III) (AC, CA, BB) and poor quality (grade IV) (BC, CB, CC). The laboratory policy is not to transfer poor-quality embryos, and generally these embryos are discarded. Remaining supernumerary blastocysts of at least average quality (Gardner stage ≥3bb) were cryopreserved by vitrification on day 5 or 6 (Son *et al.*, 2003).

Comment [SH1]: Author: please check the use of 'stage'/'grade' – I think it should be as here, stage when talking about Gardner criteria (1, 2, 3), but grade when talking about ICM and TE scoring (I, II, III)? Could you confirm?

Endometrial preparation for vitrified-warming cycles

Endometrial priming was achieved with daily oral oestradiol valerate (6 mg) beginning on cycle day 2–3 for 11 days. Endometrial thickness was assessed by transvaginal echography. Once the endometrium thickness reaches 8 mm with a trilaminar appearance, micronized vaginal progesterone (300 mg) was started daily. Embryo transfer was performed on day 6 of progesterone treatment. Luteal support was continued until 10 weeks of pregnancy.

Statistical analysis

Continuous data and categorical variables are expressed as mean ± standard deviation (SD) or as a percentage. For predictor analysis, univariate analysis using Student's t-test and chi-squared test as appropriate was performed to compare baseline characteristics and cycle outcomes parameters between cycles with live birth and cycles which did not achieve live birth. Variables that were significant in univariate analysis and those with *P*-values <0.1 were further analysed in a stepwise multiple logistic regression model. Odds ratios (OR) and 95% confidence intervals (CI) were calculated. To compare eSBT and DBT, Student's t-test and chi-squared test were applied as appropriate. LBRs and cumulative LBRs were assessed with logistic regression to control for confounders, which included age, number of previous cycles and embryo expansion score.

Statistical analysis was performed using SAS software (SAS Institute Inc., USA). $P \leq 0.05$ was considered statistically significant.

The main outcome measure was the LBR. Live birth was defined as delivery of a live fetus at gestational age ≥24 weeks. Clinical pregnancy was defined as a gestational sac seen by vaginal ultrasound scan by 6 weeks of gestation. The study was performed according to the guidelines of the Research Ethics Board, which approved the study on 16 May 2013 (reference no. 13–053-SDR).

Results

During the study period, 411 women met the inclusion criteria and were included in the study. The mean age in the whole group was 40.9 ± 0.9 years and the LBR in the whole cohort was 20% (n = 83). Description of the embryo transfer cycles is shown in **Table 1**. It is evident from this table that there is a wide heterogenicity in the number of available blastocysts, some having only one blastocyst and others having two blastocysts or more.

Identifying independent factors for live birth

When comparing baseline and clinical characteristics of the treatment cycles, women who had a live birth required a lower dose of gonadotrophins per oocyte retrieved, had a higher number of oocytes retrieved, more zygotes formed, and had a higher proportion of embryos that were transferred at the fully expanded stage (**Table 2**). There was no difference in maternal age, number of transferred embryos, the proportion of cycles with DBT and in cycles in which supernumerary embryos were available for cryopreservation (**Table 2**). A stepwise logistic regression model for predictors of live birth is shown in **Table 3**. Variables that were statistically significant in **Table 2** or with P-values <0.1 were included to build the model. The morphology score of the blastocysts was not included in the model because it was dependent on the expansion score (FEB, Gardner stage ≥3 were of better quality than early blastocysts, Gardner stage 1 and 2), therefore blastocyst expansion was used as a morphological predictor in this model. This model shows that the most significant predictors for live birth were: transferring FEB and transferring two blastocysts compared with a single blastocyst (**Table 3**).

Cumulative LBR following eSBT compared with DBT

The multiple birth rate in the cohort was 8.4% and all cases occurred following DBT. The next step was to assess the feasibility of eSBT, as a method to reduce the incidence of multiple births in this age group. For this we compared the LBR and the cumulative LBR in the group of women who had eSBT (n = 150) with the subgroup of good prognosis DBT, who had FEB to transfer (including all the elective DBT cycles, n = 79, and non-elective DBT cycles in which at least one of the two blastocysts transferred was fully expanded, n = 72). Results of the fresh embryo transfers are shown in **Table 4**. After controlling for the number of previous cycles and blastocyst expansion score, the DBT group achieved

higher LBRs, OR 1.8 (95% CI 1.17–3.2), *P* = 0.017, and a higher multiple birth rate (0 versus 17.5%), *P* = 0.02 (**Table 4**). The analysis of the subsequent FBT cycles is presented in **Table 5**. One hundred and nine women in the eSBT group underwent 138 FBT cycles and 50 women in the DBT group underwent 51 FBT cycles (women with previous live birth following fresh transfer were excluded from FBT cycle analysis, *n* = 2). The number of transferred embryos per cycle was similar, and the LBR was similar in each group. No multiple births resulted from FBT cycles. The cumulative LBR when combining both the fresh and cryopreserved cycles was similar between the two groups (28.0 versus 31.1%), OR 1.7 (95% CI, 0.88–3.40).

Comment [SH2]: Author: Please define FBT.

Characteristics of women who had multiple births

To further characterize which women who received double blastocysts are at increased risk of multiple birth we performed two comparisons. The first is based on the number of available blastocysts and results are shown in **Table 6**. Women who had eDBT had higher multiple births (25.0% vs 5.8%) and a non-significant trend toward higher LBR compared with non-eDBT. The second comparison was between women who had grade II DBT to women who had DBT in which one or both blastocysts were grade III. In this comparison, there was no statistical difference between the LBR and the multiple births between the two groups (**Table 7**).

Discussion

Maternal age is considered the most significant factor that affects fertility potential (DeCherney and Berkowitz, 1982). In recent years, there has been an increase in women seeking pregnancy after the age of 35 years (Mills *et al.*, 2011). A recent registry from the USA showed that the number of live births per woman in their early 40s has increased from 7.4 per 1000 women in 1999 to 10.3 per 1000 in 2011 (Hamilton *et al.*, 2013). Despite their age, a subset of these women that undergo fertility treatments can still have excellent ovarian response to stimulation and can benefit from practices such as SET and embryo cryopreservation. The cohort in this study consisted of good responder patients (average of 11 oocytes retrieved) who had extended embryo culture. We found that the most important predictors for live birth were blastocyst expansion score and transferring two blastocysts. Interestingly, maternal age, the gonadotrophin dose and the number of retrieved oocytes were not found to be significant predictors. These results differ from previous studies that aimed to identify predictors for live birth in younger women. Steinberg *et al.* (2013) found in a large retrospective study that the strongest predictors for live birth following SET were: transferring blastocysts compared with cleavage embryos, maternal age younger than 38 years, having >3 embryos available for cryopreservation and retrieving >10 oocytes. In another recent retrospective study, predictors for double embryo implantation were blastocyst transfer, retrieving >10 oocytes and age <35 years (Martin *et al.*, 2016). Another smaller study showed that embryo quality and age are the strongest predictors of good outcomes (Hunault *et al.*, 2002). In our study, the number of retrieved oocytes in the multivariate analysis was not a significant predictor for live birth. This

finding could be explained by the high oocyte aneuploidy rates, and thus in limited blastocyst development. This can diminish the effect of the number of collected oocytes. The availability of supernumerary embryos for cryopreservation, contrary to previous studies, was not found to be a predictive factor for live birth. This can be explained by the fact that in part of these cycles, the blastocyst was transferred at the early stage (Gardner stage I and 2), which contributed to lower LBR, while the remaining blastocysts were kept in culture and vitrified on day 6 when full expansion was reached. There is now a mounting body of evidence in favour of deferring the transfer of slow-growing blastocysts and keeping them in culture until full expansion is achieved and transferring them in subsequent warming cycle (Shapiro *et al.*, 2016). This practice can possibly optimize endometrium–embryo synchrony and increase the implantation rate; however, it may result in no embryos being available for fresh transfer. Interestingly in this age group (40–43 years), maternal age was not found to be a significant predictor for live birth. It can be concluded that the competence of the oocytes to result in the formation of good-quality blastocysts is more important than age *per se*. This observation was demonstrated in a study that included 300 patients aged 18–45 years. The study showed that the proportion of embryos that reached FEB decreases with increasing maternal age. However, when FEB is achieved, the pregnancy rates were similar, irrespective of maternal age (Shapiro *et al.*, 2002). This finding can be explained by the higher proportion of euploidy found in FEB compared with cleavage stage embryos (Demko *et al.*, 2016). Another potential explanation for this finding is the narrow maternal age (40–43 years) that was included in this study. Transferring two blastocysts was associated with higher LBRs, however this was associated with higher multiple births. Limiting the incidence of multiple births is of paramount importance in advanced maternal age because of the increased risk of adverse maternal and neonatal outcomes such as pre-eclampsia and preterm birth (Lamminpaa *et al.*, 2012; Waldenstrom *et al.*, 2016). Similar to our results, a recent Cochrane meta-analysis found a higher LBR when two embryos were transferred compared with SET, but with higher multiple births (Pandian *et al.*, 2013). To further identify the role of eSBT in good prognosis older women, we compared the outcomes following eSBT compared with DBT and found similar cumulative live birth and significantly reduced multiple birth rates. These results imply that in older women, when embryo culture results in good-quality blastocysts, SET can be performed without compromising the cumulative LBR. One of the intriguing findings in this study is the large variation in the number, developmental stage and quality of the blastocysts by day 5 (**Table 1**), despite the similarity in the number of retrieved oocytes. Based on the results of this study, patient counselling should depend on the number and quality of the available blastocysts; in patients who have three or more FEB, transferring two fresh blastocysts will result in higher LBRs compared with single blastocyst transfer, however, this will be associated with significant risk of multiple births (**Table 6**). This subgroup of patients can benefit the most from the practice of SET. In cases in which two blastocysts are available, DBT can result in a small increase in the risk of multiple birth. In this case, transferring two blastocysts should be considered after appropriate counselling and considering the previous history of the patient. Our study

is the first to investigate predictors for live birth and to report the cumulative LBR following blastocyst transfer in advanced maternal age, but it has some limitations that should be recognized. The retrospective nature is a major limitation. Women who had eSBT compared with DBT could have favourable prognoses, as evident by having a lower number of previous IVF cycles (**Table 4**) and this can be a source of bias. However, even with this bias in favour of eSBT, we could show that there is an increase in the incidence of multiple births when DBT was performed. This emphasizes that older good prognosis women can benefit from the practice of SET. Another limitation of the study is that it was not powered to detect differences in the cumulative LBR, and the 3% difference between the groups (**Table 5**) could be significant when comparing larger populations.

In conclusion, our aim in this study was to assess predictive factors for live birth in good prognosis women who had embryos that were cultured to the blastocyst stage. We found that blastocyst quality and the number of transferred blastocysts are the most important prognostic factors. We also found that the practice of eSBT is feasible and results in reduced multiple birth rates, without compromising the cumulative LBRs.

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Declaration: The authors report no financial or commercial conflicts of interest.

Table 1. Description of the study group.

Maternal age (years)	40.9 ± 0.9
Blastocyst transfer cycles, *n* (%):	
Mandatory SBT	86 (21)
eSBT	150 (36.5)
Non-eDBT	96 (23.4)
eDBT	79 (19.2)
Live birth rate, %	20.2 (83)
Twin delivery rate, %	8.4 (7)

Mandatory SBT = single blastocyst transfer with no remaining embryos to cryopreserve; eSBT = elective single blastocyst transfer, with remaining embryo to cryopreserve; Non-eDBT = double blastocyst transfer, no remaining embryos to cryopreserve; eDBT = elective double blastocyst transfer with remaining embryos to cryopreserve.

Table 2. Comparison between cycle characteristics that ended in live birth with cycles that did not end in live birth.

	Live birth (n = *83)*	*No live birth* (n = *328)*	P-*value*
Age	40.8 ± 0.84	41.0 ± 0.70	NS
BMI (kg/m^2)	24.6 ± 5.3	24.9 ± 5.4	NS
Primary infertility, %	63	61	NS
AFC	15.8 ± 9.6	14.0 ± 9.6	NS
Basal FSH (IU/l)	7.44 ± 2.43	7.9 ± 4.1	NS
Previous number of IVF cycles	0.80 ± 0.98	0.89 ± 1.13	NS
Previous parity, %	25	27	NS
Duration of infertility (years)	3.2 ± 2.5	3.3 ± 2.0	NS
Infertility diagnosis:			
Unexplained, %	41	36	NS
Male factors, %	25	23	NS
Female factors, %	25	30	NS
Combined factors, %	9	11	NS
FSH (IU) per oocyte retrieved	298 ± 246	413 ± 390	0.008
Peak oestradiol levels (pmol/l)	7534 ± 3294	7325 ± 3671	NS
Oocytes retrieved	12.35 ± 5.23	11.00 ± 4.60	0.02
Fertilization rate	77%	78%	NS
No. of zygotes	7.83 ± 3.75	6.73 ± 2.72	0.003
No. of embryos transferred	1.47 ± 0.50	1.39 ± 0.48	NS
Cycles with double embryo transfer, *n* (%)	41 (49.4)	134 (40.9)	NS
Blastocyst parameters			
FEB, *n* (%)	103 (83.1)	300 (64.5)	<0.001
Blastocyst grade, *n* (%):			
Grade 1 and 2, *n* (%)	110 (88.7)	361 (77.6)	<0.01
Grade 3, *n* (%)	14 (11.3)	104 (22.4)	<0.01
Cycles with cryopreserved embryos, *n* (%)	53 (66.3)	176 (53.7)	NS

124 blastocysts were transferred in the live birth group and 465 blastocysts in the no live birth group.
AFC = antral follicle count; BMI = body mass index; FEB = fully expanded blastocyst; NS = not statistically significant.

Table 3. Logistic regression model for live births.

	OR	*95% CI*	*Adjusted* P-*value*
Embryo expansion (Gardner ≥3 versus 1 and 2)	3.5	1.59–9.71	0.001
No of embryos transferred (2 versus 1)	1.77	1.08–2.90	0.039
Maternal age (years)	0.78	0.62–1.10	NS
FSH (IU) per oocyte retrieved	0.99	0.99–1.00	NS
No. of oocytes collected	1.03	0.99–1.08	NS
No. of 2pn embryos	1.05	0.93–1.20	NS
Cycles with remaining embryos for cryopreservation	1.36	0.77–2.40	NS

Variables that were significant in univariate analysis or with *P*-values <0.1 were used to construct this logistic regression model.
OR = odds ratio; CI = confidence interval; NS = not statistically significant.

Table 4. Comparison between fresh eSBT and DBT[a] cycles.

	eSBT (n = *150)*	*DBT* (n = *151)*	P-*value*
Maternal age	40.73 ± 0.83	40.90 ± 0.91	NS
No. of previous cycles	0.5 ± 0.8	1.1 ± 1.0	<0.01
Total gonadotrophin dose (IU)	3250 ± 1695	3596 ± 1706	NS
No. of oocytes retrieved	11.70 ± 4.7	11.74 ± 4.9	NS
No of zygotes	7.36 ± 3.14	7.35 ± 3.00	NS
Blastocyst parameters:			
FEB, *n* (%)	130 (86.7)	218 (72.2)	0.0006
Blastocyst grade:			
Grade 1+2	139 (92.7)	254 (84.1)	0.005
Grade 3	11 (7.3)	48 (15.9)	0.001
Clinical pregnancy, *n* (%)	49 (32.7)	66 (43.7)	0.048
Live birth rate, *n* (%)	29 (19.3)	40 (26.5)	0.017
Multiple births, *n* (%)	0	7 (17.5)	0.02

Other baseline parameters such as AFC, BMI, infertility diagnoses, infertility duration and previous parity were similar between the groups.
DBT = double blastocyst transfer; FEB = fully expanded blastocyst; NS = not statistically significant; eSBT = elective single blastocyst transfer.
[a] DBT group included women with elective DBT *n* = 79, and women with at least one embryo transferred as fully expanded blastocyst *n* = 72.

Table 5. Characteristics of cryopreserved blastocyst cycles and cumulative live birth rate.

	FBT cycles in eSBT (*n*= 138)	FBT cycles in DBT (*n* = 51)	*P*-value
No. of embryos warmed per cycle	1.47 ± 0.66	1.76 ± 0.75	0.014
No. of blastocysts transferred per cycle	1.24 ± 0.53	1.27 ± 0.70	NS
Live birth rate, *n* (%)	13/138 (9.4)	7/51 (13.7)	NS
Cumulative live birth rate, *n* (%)	42/150 (28.0)	47/151 (31.1)	NS
Cumulative multiple birth rate, *n* (%)	0	7 (14.9)	0.03

341 blastocysts were vitrified in the eSBT group (*n* = 150) and 172 blastocysts in the subgroup who had elective DBT (*n* = 79).
NS = not statistically significant.

Table 6. Comparison of the live birth rate and the multiple birth rate between patients who had elective double blastocyst transfer compared with non-elective double blastocyst transfer.

	eDBT (n = *79)*	*Non-eDBT* (n = *96)*	*P-value*
FEB, *n* (%)	113 (71.5)	105 (54.7)	<0.01
LBR, *n* (%)	24 (30.4)	17 (17.7)	NS
Multiple birth rate, *n* (%)	6 (25.0)	1 (5.9)	<0.01

FEB = fully expanded blastocyst; LBR = live birth rate; NS = not statistically significant.

Table 7. Comparison of the live birth rate and the multiple birth rate between patients who had grade II double blastocyst transfer to patients who had grade III double blastocyst transfer[a].

	Grade II (n = *120)*	*Grade III*[a] (n = *55)*	*P-value*
FEB, *n* (%)	163 (67.8)	55 (50)	<0.01
LBR, *n* (%)	30 (25)	11 (20)	NS
Multiple birth rate, *n* (%)	6 (20)	1 (9)	NS

FEB = fully expanded blastocyst; LBR = live birth rate; NS = not statistically significant.
[a] One or both blastocysts were grade III.