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ASSISTED REPRODUCTION TECHNOLOGIES

Prognostic value of growth of 4-cell embryos on the day of transfer in fresh IVF-ET cycles

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Abstract
Purpose To investigate the prognostic value of growth of 4-cell embryos on the day of transfer in determining clinical pregnancy and live birth rates after fresh in vitro fertilization (IVF)-embryo transfer (ET) cycles.
Methods Retrospective cohort study of all patients between January 2008 and January 2013 initiating fresh IVF-ET cycles resulting in embryos that were not more than 4 cells 72 h after oocyte retrieval in the morning of their transfer. Patients were stratified into 2 groups based on whether embryos did or did not grow more than the 4-cell stage on the afternoon of ET. The odds of clinical pregnancy and live birth were considered as primary outcomes. Student's *t*-tests and Chi-square ($\chi 2$) tests were used as inidicated, with logistic regression controlling for maternal age and number of embryos transferred.
Results Three hundred forty three patients were identified for inclusion: 165 and 178 patients had 4-cell embryos with and without growth on the afternoon of ET, respectively. The demographic and baseline IVF cycle characteristics of the study cohort were comparable. Patients with embryo growth had higher clinical pregnancy (13.9 % vs. 4.49 %) and live birth (10.9 % vs. 3.37 %) rates compared to patients without embryo growth. This represented an overall increased odds of clinical pregnancy [Odds ratio (OR)=3.44; 95 % Confidence Intervals (CI) 1.49–7.93; P=0.004)] and live birth (OR=3.51; 95 % CI 1.36–9.07; P=0.01). The increased odds remained unchanged after adjusting for maternal age and number of embryos transferred.
Conclusions Transfer of 4-cell embryos 3 days after oocyte retrieval can result in clinical pregnancies and live births, albeit at a low rate. Growth of an embryo more than the 4-cell stage on the afternoon of ET may serve as a positive prognostic factor for IVF-ET cycle outcome.

Keywords 4-cell embryo · Embryo transfer · IVF · Pregnancy outcomes

Capsule Growth of a day-3 embryo beyond the 4-cell stage on the afternoon of transfer may serve as a positive prognostic factor for IVF-ET cycle outcome.

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Introduction

In the search for the best embryo, various aspects of embryo development and its effects on pregnancy rate after in vitro fertilization (IVF) have been studied. While parameters such as morphology, degree of fragmentation, and number of embryos transferred are important in predicting implantation success, several studies have proposed that cleavage state and day-3 cell number have the most significant effect on the chances of implantation and subsequent pregnancy [1–3]. In current practice, most embryos transferred 3 days after oocyte retrieval and insemination are considered optimal when reaching the 6-to 8-cell stage [1]. Cummings et al. [4] reported that slowly or rapidly growing embryos implanted less frequently than their normal embryo counterparts, which reached the 4-cell stage by 45.4 h after insemination. Similarly, Trounson et al. [5] observed that the mean time to reach the 4-cell stage is 45.7 h after insemination, suggesting that day 3 (72 h) 4-cell embryos have a slower rate of cleavage, and therefore, a lower likelihood of successful implantation and pregnancy.

Previous studies have shown that pregnancy rates remain low when embryos transferred on day 3 consist of fewer than four blastomeres [3–6]. Many embryos, however, reach only the 4-cell stage on day 3 and are transferred as such. Yet, there are very few studies comparing the pregnancy outcomes of 4-cell embryos to developing counterparts after embryo transfer (ET) on day 3. The primary objective of this study is to investigate the prognostic value of growth of 4-cell embryos on the day of transfer in determining clinical pregnancy and live birth rates after fresh IVF-ET cycles.

Materials and methods

Cycle inclusion criteria

The Weill Cornell Medical College institutional review board approved our retrospective cohort study protocol. All couples initiating fresh IVF-ET cycles at the Ronald O. Perelman and Claudia Cohen Center for Reproductive Medicine between January 2008 and January 2013 resulting in embryo transfer were analyzed for potential inclusion. Cycles utilizing donor oocytes, donor sperm, surgically retrieved sperm, or those with incomplete records were excluded. Patients were stratified into 2 groups based on embryo growth on the afternoon of ET: the first group consisted of patients with ≥1 embryo that grew more than the 4-cell stage, while the second group had patients with ≥1 embryo that remained at the 4-cell stage. Finally, patients having embryos with>4 cells on the morning of ET were excluded from the analysis. If a patient had a 4-cell embryo on the afternoon of ET along with >1 4-cell embryo that had grown from the morning to the afternoon of ET, then only the embryos that showed growth were transferred.

Clinical and laboratory protocols

Ovarian stimulation, human chorionic gonadotropin (hCG) trigger, oocyte retrieval, embryo culture, and ET were performed per standard protocols [7]. Patients were down regulated in the preceding luteal phase with either oral contraceptive pills (Ortho-Novum, Janssen Pharmaceuticals) or 0.1 mg (Estradiol) E_2 patches (Climara, Bayer Healthcare Pharmaceuticals). Ovarian stimulation was carried out to maximize follicular response while minimizing the risk of ovarian hyperstimulation syndrome (OHSS). Patients were stimulated with gonadotropins followed by pituitary suppression with a GnRH antagonist (Ganirelix Acetate, 0.25 mg [Organon]; or Cetrotide, 0.25 mg [EMD-Serono]) based on a flexible protocol as previously described [7]. Gonadotropin doses were based on factors such as patient age, weight, antral follicle count, antimüllerian hormone and previous response to stimulation.

hCG was used as the ovulatory trigger. Ovidrel (EMDSerono), Novarel (Ferring Pharmaceuticals) or Pregnyl (Schering-Plough) was administered according to a sliding scale (10,000 IU for E_2<1,500 pg/mL, 5,000 IU for E_2 1, 501–2,500 pg/mL, 4,000 IU for E_2 2,501–3,000 pg/mL, and 3,300 IU for E_2>3,001 pg/mL). In most cycles, the hCG trigger was given when the two lead follicles reached a mean diameter≥17 mm. Oocyte retrieval was performed under conscious sedation anesthesia using transvaginal ultrasound guidance approximately 35–37 h after hCG administration. Luteal support with 50 mg of intramuscular progesterone daily was begun the day after retrieval. IVF was carried out using conventional insemination or intracytoplasmic sperm injection (ICSI) based on the male partner's semen analysis or the couple's history. All embryos were cultured using an in-house culture media. Cleavage embryos were assessed on day 2 (44–46 h after insemination or sperm injection) and on day 3 (66–72 h insemination or sperm injection). Embryos were graded based on criteria described by Veeck [8]. On the afternoon of ET, the number of blastomeres in each embryo was assessed once again. All patients underwent ET on day 3. Transfers were performed with Wallace catheters at approximately 1 cm less than the uterine depth identified at prior trial transfer (Marlow/Cooper Surgical). Ultrasound guidance was utilized only when the transfers were deemed difficult based on the prior trial transfer.

Study variables

The following demographic and baseline characteristics were extracted from patient charts: age (years), gravidity, parity, body mass index (kg/m^2), prior IVF attempts, follicle stimulating hormone (FSH) level at cycle start (mIU/mL), total days of ovarian stimulation, total dosage of gonadotropins administered (IU), peak endometrial stripe (mm), peak estradiol (E_2) level (pg/mL), total number of oocytes retrieved, total number of mature oocytes, and utilization of ICSI. For IVF-ET outcomes, the number of embryos transferred, implantation rate, positive pregnancy rate, clinical pregnancy rate, biochemical pregnancy rate, spontaneous miscarriage rate, and live birth rates were recorded. Positive pregnancy rate was defined as positive serum bhCG levels 10–12 days after embryo transfer. Implantation rate was defined as number of gestational sacs seen via transvaginal ultrasonography out of the total number of embryos transferred. Clinical pregnancy rate was defined as the number of intrauterine gestations with fetal cardiac activity per IVF-ET cycle. A biochemical pregnancy was defined as positive hCG without a gestational sac. Any pregnancy loss after visualization of an intrauterine gestation was considered a spontaneous miscarriage. Any birth after 24 weeks gestation age was considered a live birth.

Statistical analysis

Categorical variables were expressed as number of cases (n) and percentage of occurrence (%). Continuous variables were checked for normality and expressed as mean ±standard deviation (SD). When indicated, nominal variables were expressed as median [interquartile range (IQR)]. Chi-square ($\chi 2$) with Mantzel-Hansel correction and Fisher's exact test were used for categorical variables. Student's *t*-test was utilized for continuous variables. The odds of clinical pregnancy and live birth were considered as primary outcomes. These odds were adjusted with logistic regression for maternal age and number of embryos transferred. Statistical significance was set at $P<0.05$. Statistical analyses were performed using STATA version 13 (College Station, TX: StataCorp LP).

Results

Three hundred forty three patients with known IVF-ET outcomes met inclusion criteria. Of these, 165 (48.1 %) and 178 (51.9 %) patients had 4-cell embryos with and without growth on the afternoon of ET, respectively. For embryos replaced, the mean embryo cell number±SD in the >4-cell group on the afternoon of ET was 5.8±0.88. Table 1 compares the demographic and baseline IVF characteristics of patients with and without embryo growth. Overall, there were no differences in mean age, gravidity, parity, body mass index, number of previous IVF attempts, basal FSH levels, total days of stimulation, total gonadotropins administered, peak endometrial stripe, or peak E_2 level. The use of ICSI was also comparable between the groups. However, the median (IQR) number of oocytes retrieved in the >4-cell embryo group [7 (IQR 5–10)] was higher compared to the 4-cell embryo group [5 (IQR 3–7)]; $P<0.001$. Similarly, more mature oocytes were obtained in the former group compared to the latter [5 (IQR 3–6) vs. 3 (IQR 2–5); $P<0.001$).

Table 2 compares the IVF-ET outcomes between the two groups. The median (IQR) number of embryos transferred in the >4-cell embryo group was 1 (IQR 1–2), which was comparable to 1 (IQR 1–2) embryos in the 4-cell group ($P=0.99$). There was no difference in the implantation (8.62 % vs. 3.14 %), biochemical pregnancy (7.27 % vs. 6.74 %) and spontaneous miscarriage rates (3.03 % vs. 1.12 %) between the two groups. Patients in the >4-cell embryo group, however, had higher positive pregnancy odds [Odds ratio (OR)=2.01; 95 % Confidence Intervals (CI) 1.13–3.63; $P=0.03$)] compared to the 4-cell embryo group. The clinical pregnancy and live birth rates were 13.9 % and 10.9 %, respectively in the >4-cell embryo group, which were significantly higher than the 4-cell embryo group. This represented an overall increased odds of clinical pregnancy (OR=3.44; 95 % CI 1.49–7.93; $P=0.004$) and live birth (OR=3.51; 95 % CI 1.36–9.07; $P=0.01$) in patients with embryo growth beyond than the 4-cell stage on the afternoon of ET. The increased odds for clinical pregnancy remained unchanged when adjusting for maternal age (aOR=3.05; 95 % CI=1.33–7.01; $P=0.007$) as well as number of embryos transferred (aOR=3.23; 95 % CI=1.45–7.70; $P=0.003$). Similarly, the odds for live birth were unchanged after adjusting for maternal age (aOR=2.41; 95 % CI=1.02–5.71; $P=0.04$) and the of embryos transferred (aOR=2.49; 95 % CI=1.04–5.96; $P=0.03$).

Table 1 Comparison of baseline characteristics of study cohort ($n=343$)

Parameter	>4 Cells at ET ($n=165$)	4 Cells at ET ($n=178$)	*P*
Age (years)	38.2 (±4.63)	38.9 (±4.29)	0.15
Gravidity	1.36 (±0.69)	1.30 (±0.41)	0.32
Parity	0.49 (±0.29)	0.45 (±0.27)	0.19
BMI (kg/m^2)	22.9 (±6.36)	22.3 (±6.46)	0.39
Previous IVF attempts	2.61 (±1.16)	2.63 (±1.32)	0.88
Basal FSH level (mIU/mL)	5.21 (±3.61)	5.19 (±3.68)	0.96
Total stimulation days	10.3 (±2.18)	10.6 (±2.48)	0.24
Total gonadotropins administered (IU)	4554.1 (±2060.8)	4730.8 (±2146.1)	0.44
Peak endometrial stripe (mm)	11.6 (±6.57)	11.2 (±2.29)	0.45
E_2 level on day of hCG trigger (pg/mL)	1375.3 (±764.8)	1249.4 (±626.4)	0.09
Total oocytes retrieved	7 (IQR 5–10)	5 (IQR 3–7)	<0.001
Total mature oocytes	5 (IQR 3–6)	3 (IQR 2–5)	<0.001
ICSI utilized	132 (80 %)	153 (86 %)	0.26

Data are presented as mean±standard deviation, median [interquartile range (IQR)], and n (%)

BMI Body Mass Index, *IVF* In Vitro Fertilization, *E_2* Estradiol, *FSH* Follicle Stimulating Hormone, *hCG* Human Chorionic Gonadotropin, *ICSI* Intracytoplasmic Sperm Injection

Table 2 Comparison of fresh IVF-ET outcomes of study cohort (n=343)

Parameter	> 4 Cells at ET (n=165)	4 Cells at ET (n=178)	P
Embryos transferred	1 (IQR 1–2)	1 (IQR 1–2)	0.99
Implantation rate	8.62 %	3.14 %	0.10
Positive Pregnancy rate	35 (21.2 %)	21 (11.8 %)	0.03
Clinical Pregnancy rate	23 (13.9 %)	8 (4.49 %)	0.004
Biochemical pregnancy rate	12 (7.27 %)	12 (6.74 %)	0.98
Spontaneous miscarriage rate	5 (3.03 %)	2 (1.12 %)	0.39
Live birth rate	18 (10.9 %)	6 (3.37 %)	0.01

Data are presented as mean±standard deviation, median [interquartile range (IQR)], and n (%)

Discussion

Our study shows that transfer of developmentally lagging embryos i.e., 4-cell embryos 3 days after oocyte retrieval can result in clinical pregnancies and live births, albeit at a low rate. If 4-cell embryos are considered for transfer, then growth of such embryos beyond the 4-cell stage on the afternoon of ET may serve as a positive prognostic factor for IVF-ET cycle outcome.

The choice of the best embryo continues to be a matter of considerable research and debate since the inception IVF [9]. The relationship between good embryo quality, specifically cleavage state and cell number, and pregnancy rate in IVF-ET cycles is well established. For example, in cleavage stage embryos, pregnancy rates are negatively correlated with slowly cleaving embryos [9]. Outcomes of 4-cell embryos transferred on day 2 have been well studied. Giorgetti et al. [10] found that among 858 single embryo transfers, embryos transferred at the 4-cell stage implanted twice as often as did 2-cell embryos. Similarly, Ziebe et al. [11] found that the transfer of 4 cell-embryos on day 2 resulted in significantly higher implantation and pregnancy rate compared with 2-cell embryos. Thus, a day 2, 4-cell embryo appears to have the ideal cleavage rate.

Cell number on day 3 has been correlated with developmental potential [9], with different grading systems showing a correlation with implantation and pregnancy potential [4, 11–14]. A positive association has been found between the number of cells in day 3 embryos (up to 8) with implantation and pregnancy rates when compared to embryos with less than 8 cells [12–14]. In their retrospective study of 389 patients, Ertzeid et al. [3] compared the outcomes of IVF cycles after ET with control 8-cell versus 4-cell embryos on day 3. The authors reported a 45 % pregnancy rate per embryo transferred and a 34 % live birth rate per embryo transferred in the 8-cell group. In contrast, the pregnancy and live birth rate per embryo transferred in the 4-cell group was 19 % and 5 %, respectively. Consistent with these findings, our data showed 3 times higher odds of clinical pregnancy and live birth in the >4-cell group compared with the 4-cell group. Although our findings, in terms of trends, are similar to Ertzeid et al.'s findings, the absolute difference between clinical pregnancy and live birth rates between the two studies are due to two main reasons. First, the control group in Ertzeid et al. study consisted of 263 patients with 8-cell embryos, which is the normally expected cleavage stage and cell number on day 3. However, the control group in our study comprised of patients with 4-cell embryos on day 3 that showed some growth beyond the 4-cell stage on the afternoon of ET. Thus, our control group had developmentally lagging embryos as opposed to normal 8-cell embryos. Second, the demographics and clinical response to ovarian stimulation differed between the two studies i.e., our study had more poor responders. While the mean age±SD of the study and control population in Ertzeid et al. study was 32.7±3.6 and 32.3±3.5, respectively, the corresponding ages in our study were 38.2±4.63 and 38.9±4.29, respectively. Furthermore, patients in that study responded better to ovarian stimulation than ours, as highlighted by the lower total doses of gonadotropins±SD used (2439±1091 vs. 4730.8±2146.1 IU 4-cell group; 1971±743 vs. 4554.1±2060.8 IU control group) and more number of oocytes±SD retrieved (6.9±3.9 vs. 5.55 ±3.97 4-cell group; 10.1±5.2 vs. 7.27±3.74 control group).

Major strengths of this study are the inclusion of poor responder patients and the use of developmentally lagging embryos showing some growth beyond the 4-cell stage on the afternoon of ET as opposed to normal 8-cell embryos. However, we also acknowledge several limitations. Our study does not evaluate the reasons for slow cleavage or low cell numbers in day 3, 4-cell embryos. Previous studies have indicated that maternal age can impact embryo cleavage rates. For example, Janny and Menezo showed a clear declination in the quality of embryos derived from aging oocytes [15]. These findings are consistent with the mean age of our study cohort. Some studies have also postulated that poor embryo quality may be, in part, due to deficiencies in sperm-derived cytoplasmic factors or oocyte activating substances [16, 17]. Our study also did not assess the development potential of developmentally lagging 4-cell embryos in extended embryo culture. In a recent study by Zhao et al. [1], 764 patients undergoing blastocyst culture with 1522 surplus 4-cell embryos on day 3 were compared with 2391 patients with 5934>4-cell embryos also undergoing extended embryo culture. The authors showed that

4-cell embryos on day 3 displayed lower blastulation rates (31.7 % vs. 55.4 %); however, once blastocysts were obtained, its implantation, clinical pregnancy and ongoing pregnancy rates were similar to the control group. Finally, due to retrospective nature of our study, we remain uncertain whether our findings would be true in a prospective setting, particularly in a larger cohort of poor prognosis patients.

In conclusion, our study highlights that transfer of developmentally lagging 4-cell embryos on day 3 can result in clinical pregnancies and live births, but at a low rate. Progression of an embryo beyond the 4-cell stage on the afternoon of ET (day 3) can serve as a positive prognostic factor for clinical pregnancy and live birth. While these findings may be important for poor prognosis patients, recent evidence also suggests that extended culture until the blastocyst stage followed by blastocyst biopsy to assess euploidy may help optimize implantation and pregnancy rates in these patients [18].

Conflict of Interest The authors declare that they have no conflict of interest

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