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Original Article

Early blastulation of day 4 embryo correlates with the increased euploid rate of preimplantation genetic screening cycles

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ABSTRACT

Objective: It is known that embryos with faster growing potential, especially in blastocyst development, correlate with the increased euploid rate. Our study investigated the preimplantation genetic screening cycle to analyze the correlation between early blastulation (EB) on day 4 embryo and the euploid rate.
Materials and methods: This is a retrospective study examining 273 biopsied blastocysts after preimplantation genetic screening obtained from 54 patients from March 2013 to March 2017. Of the 273 biopsied embryos, 81 had early blastulation on day 4 and were classified as the EB (+) group, while the other 192 had no early blastulation and were classified as the EB (−) group. Euploid rates were compared between the two groups. A total of 34 single euploid embryos were transferred, with 14 from the EB (+) group and 20 from the EB (−) group. Clinical pregnancy was compared between the groups.
Results: There is a statistically significant increase in the euploid rate in the EB (+) group (49.4% vs. 34.4%, $p = 0.02$). The clinical pregnancy rate was also increased in the single euploid embryo transfer group with early blastulation, but did not reach statistical significance (71.4% vs. 50.0%, $p = 0.211$).
Conclusions: Early blastulation of day 4 embryo correlates significantly with the euploid rate. Early blastulation of day 4 embryo may serve as a potential aid for embryo selection for transfer in preimplantation genetic screening cycles.

Introduction

Embryo assessment is one of the fundamental steps in assisted reproductive technology. Several methods can be performed, including conventional static morphologic assessment [1–3], morphokinetic morphologic assessment [4–7] or preimplantation genetic screening (PGS) [8]. Various research has been conducted to compare the efficacy of different embryo selection strategies [9,10]. Despite the controversy towards routine use of PGS and lack of validation on different testing platforms, it still remains as the most promising tool for embryo selection. The technique is also known as preimplantation genetic testing for aneuploidy (PGT-A), aiming at analysis of the 24-chromosome copy number of the embryo and selecting the euploidy to transfer. The molecular technology of fluorescence in situ hybridization (FISH) for chromosome assessment is gradually replaced by comparative genomic hybridization (CGH), quantitative PCR (qPCR) or next-generation sequencing (NGS). After different time to complete the test result, patients need to receive frozen embryo transfer in most situations.

On the other hand, with the aid of time-lapse morphokinetic technology, embryos with faster growing pace have been found to be correlated to euploid status. Mumusoglu, S. et al. further compared different time-lapse morphokinetic parameters and found that time start to blastulation (Tsb) with 96.6 h, which is approximately day 4 post ovum pick-up, had significant predictive value of euploid embryos [6]. In spite of the above technologies, static morphology observation was by far the most widely used method for embryo selection. We tried to investigate the relationship between day 4 early blastulation and the euploid rate,

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hoping to add a parameter of conventional static morphology to aid in embryo selection with the goal of increasing the clinical pregnancy rate.

Material and methods

Study design

This is a retrospective study conducted in the Infertility Division of Mackay Memorial Hospital from March 2013 to March 2017. We included the patients who received preimplantation-genetic screening and their embryos were underwent morphology observation on day 4 to see if having EB or not. The included patients were then divided into two groups, one of them were with EB on day 4, and the other were without EB on day 4. The ploidy status and the clinical pregnancy rate of single euploid embryo transfer were also compared between the two groups.

The indication of PGS were due to one of the following reasons: a) advanced maternal age defined as more than 38-years-old, b) recurrent (more than two times) pregnancy loss, c) repeated (more than two times) IVF failure, and d) elective reasons.

The study was approved by Institutional Review Board (IRB) of Mackay memorial hospital (17MMHIS182e).

Ovarian stimulation protocols

All patients began ovarian stimulation with a flexible starting dosage of recombinant follicular-stimulation hormone (FSH) (Gonal-F; Merck SeronoS.p.A.) dosing from 150 to 300 IU on the third day of menstruation, for three days. The starting dosage was determined by the patient's age, ovarian function, and the response to previous ovarian stimulation. The recombinant FSH dosage was then adjusted according to follicular growth, monitored by serial transvaginal ultrasound. After at least two follicles measured larger than 14 mm in diameter, patients started to receive daily subcutaneous injections of Cetrorelix (Cetrotide; Merck Serono, Baxter Oncology GmbH) 0.25 mg along with the recombinant FSH. When at least two of the largest follicles had extended to at least 18 mm in diameter, final oocyte maturation was dual triggered by 250 mcg of recombinant human chorionic gonadotropin (hCG) (Ovidrel; Merck SeronoS.p.A.), which was equivalent to approximately 6500 IU hCG according to the manufacturer's data, plus 0.2 mg of Triptorelin (Decapeptyl; Ferring GmbH). Transvaginal ovum retrieval (TVOR) was undertaken 34–36 h after administration of hCG.

Ovum retrieval and embryo culture

The oocytes retrieved were washed with Flushing Medium, product of Origio ®. IVF was induced with intracytoplasmic sperm injection (ICSI) to prevent from the contamination of semen fluids or cumulus cells for PGS exams. Within 16–18 h after fertilization, the oocyte with two pronuclei (2PN) was considered as normally fertilized. The fertilized embryos were then cultured with global® medium, manufactured by LifeGlobal ®, in Thermo® CO2 incubator (FORMA/Heracell 240i, CO_2 6%, O_2, 5%). The morphologic grading of blastocysts was based on Gardner and Schoolcraft, 1999, and was routinely performed on day 5 and 6 post ovum pick-up.

Observation of EB

Occurrence of EB was recorded at 17:00 on day 4, approximately 96–100 h post-insemination. Since we performed insemination 40 h after hCG trigger, the time of insemination ranged from 13:00 to 17:00 on ovum retrieval day in all cases.

Embryo biopsy and preimplantation-genetic screening

The embryos were cultured to day 5 or day 6 for trophectoderm biopsy. All biopsy procedures were conducted on a heated stage in a dish prepared with two 5 μl droplets of global® medium, manufactured by LifeGlobal ®, overlaid with pre-equilibrated mineral oil. A diode laser was used to assist the opening of a 10–20 μm hole in the zona pellucida on day 3. Average of five trophectoderm cells were then aspirated into the biopsy aspiration pipette (Origio ®) and transferred to a separate PCR tube. All biopsied blastocysts were cryopreserved by a vitrification protocol. Comprehensive chromosome analysis and interpretation were conducted by Array CGH or Next Generation Sequencing (GGA Corp.®, Taipei, Taiwan).

Thawed single euploid embryo transfer

After the results of the PGS were finalized, the patient began the thawed cycle with endometrial preparation by Estrade and Crinone gel. All patients who had planned for embryo transfer underwent sonography for survey of uterine lesion and proceeded to hysteroscopy polypectomy if a suspicious lesion was found. None of the patients included in the study had adenomyosis or submucosal myomas. Single euploid embryo selected for embryo transfer was thawed on the sixth day of progesterone administration. Embryo transfer was performed with the aid of transabdominal ultrasound. Guardia™ AccessET Embryo Transfer Catheter (COOK©) was used for embryo transfer.

Luteal phase support and confirmation of pregnancy

The luteal phase was supported by vaginal gel (Crinone gel 8%, Merck SeronoS.p.A) twice daily and Estrade 8 mg daily. Serum β-hCG was measured 14 days after embryo transfer, and a value above 5 IU/mL was considered to be a positive pregnancy. Clinical pregnancy was defined as a pregnancy confirmed by ultrasound visualization of the gestational sac. The luteal support was then continued until the 12th week of gestation after the establishment of luteal-placental shift for all pregnancies.

Statistical analyses

Statistical analysis was performed using SPSS version 22.0 software. Continuous variables were presented as mean with standard deviation (SD). For categorical variables, the values were presented as raw frequencies with corresponding percentages, and the between-group differences were assessed either by a chi-square test or by the Fisher exact test. $P < 0.05$ was considered statistically significant.

Results

273 embryos obtained from 54 patients undergoing 58 PGS cycles were included in our study. The indications for PGS cycles in our study were advanced maternal age (33.3%), recurrent pregnancy loss (29.6%), elective reasons (25.9%), and repeated IVF failure (11.1%). The average age of the patients was 38.6 years (Table 1). Average estradiol (E2) level on trigger day was 1692 pg/ml. MII oocyte rate was 81.2%. Fertilization rate was 71.2%. Fig. 1 lists the incidence of euploid embryos in the EB group and the non-EB group. There is a trend of a higher euploid rate in the EB group regardless of the embryo quality and this reached statistical significance when all of the embryos were accounted for (EB vs. non-EB: 40/81 (49.4%) vs. 66/192 (34.4%), $p < 0.05$). A trend of higher clinical pregnancy rate was also observed in the EB group which underwent single euploid transfer (EB vs. non-EB: 10/14 (71.4%) vs.

Table 1
Patients characteristics.

	Mean ± SD
Patient numbers	54
Age	38.6 ± 3.87
AMH(ng/mL)	4.26 ± 2.44
E2 on trigger day (pg/ml)	1692.7 ± 1285.6
P4 on trigger day (ng/ml)	1.13 ± 1.00
No. of oocytes retrieved/per cycle	17.4
MII oocytes (%)	753 (81.2%)
Fertilization rate	71.2%

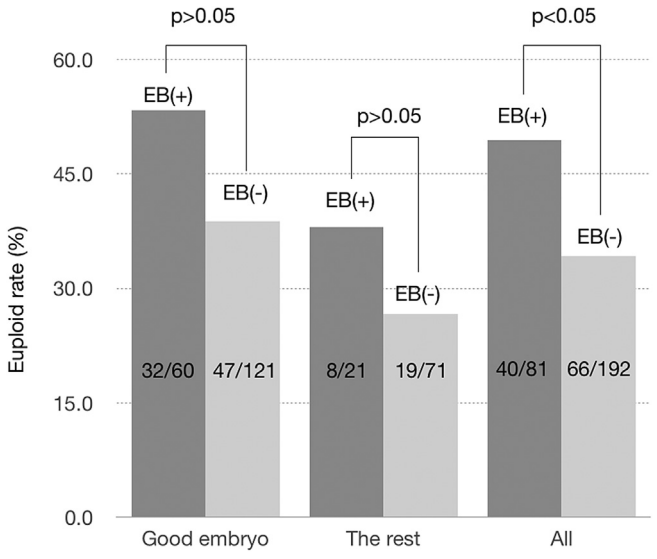

Fig. 1. Incidence of euploid blastocytes was compared between EB (+) and EB (−) groups. Good embryos are defined as day 5 morphology grading better than 3BB.

10/20 (50.0%), p > 0.05) (Fig. 2). Singleton gestational sac was noted during all follow-up periods. Among 54 patients, 17 of them (31.5%) had no embryo transfer due to no euploidy available, and 6 of them received double embryo transfer, while 4 of them with euploid embryo had delayed transfer for personal reasons.

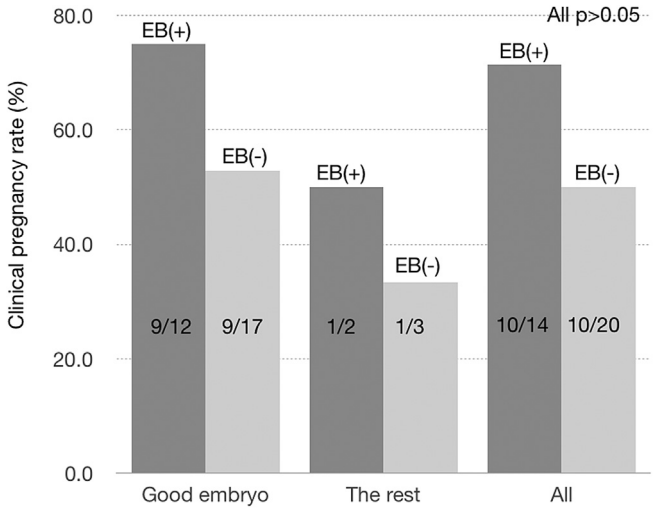

Fig. 2. Clinical pregnancy rate was compared between EB (+) and EB (−) euploid embryo transfer group. Good embryos defined as day 5 morphology grading better than 3BB. Increased clnical pregnancy rate is noted in EB(+) groups but does not reach statistically significance (p>0.05).

Discussion

Our data demonstrates that embryos with EB shown on day 4 post ovum-pickup were more likely to be euploidy. This checkpoint was designed based on several studies by time-lapse morphokinetic or static morphologic assessment that pointed out fast growing pace in the blastocyst stage was correlated to euploid status [5,11]. Earlier start of blastulation on 96 h post-insemination is found to be a significant marker that correlates with euploid rate [6]. In our study, we extended the observation time to 96–100 h post-insemination and still yielded similar conclusions. Although time-lapse morphokinetic assessment has the advantage of real-time recording of the morphology changes on the embryo and improves live birth rate compared to the conventional embryology selection [9], it has some drawbacks such as high cost and low availability. By incorporating the observation of the time-lapse assessment into static morphologic evaluation, we added an extra observation point on day 4 afternoon to see if the embryo showed blastulation or not. The results suggest that EB on day 4 may serve as an alternative method to predict the ploidy status of the embryo if PGS examination is not available. However, this “EB of day 4 embryo“ method requires the embryo to be taken out of the culture system one more time compared to the conventional method. Whether this extra maneuver will have negative impact on the embryos needs further investigation.

Much of the research has been focused on the late stage of embryo progression and concluded that this period is a much better predictor of clinical pregnancy than early cleavage stage of embryos [12] and that shorter duration of blastulation correlated positively with ongoing pregnancy rate [13]. Meanwhile, there have been other studies using “early blastulation” to predict the clinical pregnancy outcome [3,14,15]. The concept of using timing of blastulation to imply the growth pace of the embryo is the same as our concept. However, it has a slightly different definition of EB from our study as it compares blastulation on day 5 or day 6, and concludes that the former one with faster embryo development rate, termed “early blastulation” by their definition has better euploid rate and clinical pregnancy rate. Our method is to observe the embryo with EB on day 4, which may be more coincident with the faster growing potential of the embryo.

PGS performed in woman older than 37 years old improves clinical pregnancy rate, implantation rate and live birth rate per embryo transfer and decreases pregnancy loss in women with history of recurrent miscarriage [16–19]. In the strategy of single embryo transfer, PGS could make accurate selection of euploid embryo, result in similar ongoing pregnancy rate but dramatically decrease the rate of multiple pregnancies compared with non-PGS cycles [20–22]. However, the benefits and limitations of its application on different patient population need to be clarified by further randomized controlled trials. In our data, the euploid embryo with EB on day 4 achieved higher clinical pregnancy rate in the single euploid embryo transfer which suggests that this group may have better embryo viability compared to the euploid but non-EB group. Thus, EB on day 4 may be incorporated into the selection process of euploid embryos when performing the single embryo transfer. However, in our results of single euploid embryo transfer, there were 14 without clinical pregnancy among 34 embryo transfers. Some research has shown that elevated mitochondria DNA (mtDNA) levels in blastocyst biopsy specimens correlates with implantation failure in single euploid blastocyst transfers [23,24], which highlights the need for further research to investigate other aspects of embryo viability.

Small sample size and retrospective design are limitations of our study. We do not classify the participants into different age groups since there is research suggesting diminished maternal age effect on pregnancy rate of euploid embryo transfer [18]. As with the

accuracy of PGS, some argue that the biopsy technique itself would affect the result. Our embryo biopsy was conducted by three embryologists who were highly skilled in the procedure. Although the average five cell numbers biopsied were slightly lower than the recommended of six cells according to a recent review of PGS [25], our "no signal" rate was 3.2%. Two different platforms used for PGS analysis (aCGH had conducted before 2014, and shifted to NGS until now) might pose bias, too. More investigation should be done to verify the clinical application of "EB of day 4 embryo" on embryo selection technologies.

Conclusion

Early blastulation of day 4 embryo correlates significantly on the euploid rate. It may serves as a potential aid for embryo selection for transfer in preimplantation genetic screening cycles.

Conflicts of interest statement

H.TY. has nothing to disclose L.KK. has nothing to disclose. H.YM. has nothing to disclose. L.MH. has nothing to disclose. L.RS. has nothing to disclose. W.YW has nothing to disclose.

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