Earlier day of blastocyst development is predictive of embryonic euploidy across all ages: essential data for physician decision-making and counseling patients

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Abstract

Purpose The purpose of this study is to evaluate whether day of blastocyst development is associated with embryo chromosomal status as determined by high-density oligonucleotide microarray comparative genomic hybridization (aCGH).

Methods This is a retrospective cohort analysis, including women who underwent in vitro fertilization (IVF) with trophoctoderm biopsy at a single private fertility center from January 2014 to December 2014. Repeat cycles were excluded. Cycles were assessed for percentage of blastocysts biopsied on days 5, 6, or 7 and rate of euploid embryos per cycle. Cycles were stratified by Society for Assisted Reproductive Technology (SART) age groups (< 35, 35–37, 38–40, 41–42, > 42) and by donor status.

Results A total of 388 IVF cycles and 2132 biopsied blastocysts were evaluated. The percentages of blastocysts biopsied on days 5, 6, and 7 were 62.5, 35.8, and 1.7%, respectively. Blastocyst euploid rates on days 5, 6, and 7 were 49.5, 36.5, and 32.9%, respectively. Earlier blastocyst development was associated with a significantly increased euploid rate ($p < 0.0001$). Younger maternal age ($p < 0.0001$) and higher number of blastocysts biopsied per patient ($p = 0.0063$) were both independently associated with greater percentage of euploidy.

Conclusions Earlier blastocyst development is independently associated with a higher likelihood of embryonic euploidy in both autologous and donor embryos. In non-biopsied embryos, these data support selection of day 5 blastocysts for transfer over later-developing embryos. These results can assist with patient counseling regarding expectations and outcomes. To our knowledge, this is the first study to examine embryonic euploidy as stratified by both day of blastocyst development and SART age group.

Keywords Blastocyst development · SART age group · Trophoctoderm biopsy · Euploid blastocyst · PGS

Introduction

Human oocytes and embryos are well established to have high rates of aneuploidy that increase with advancing maternal age [1, 2]. High oocyte and embryo aneuploidy translate to decreased pregnancy rates and higher spontaneous abortion rates as maternal age increases [3]. Recent studies using comprehensive chromosomal screening (CCS) of trophoctoderm (TE) biopsies report embryo aneuploid rates ranging from as low as 21% to as high as 85% depending on maternal age at the time of oocyte retrieval [4, 5]. Even patients 25 years of age or younger are reported to have significant aneuploid rates of up to 44% [4–6]. While a multitude of factors may contribute to the inefficiency of in vitro fertilization (IVF), the ability to select genetically competent embryos is of paramount importance.

The most promising methodology to improve embryo selection to date is preimplantation genetic screening (PGS). The transition to TE biopsy and advancement of technology for genetic analysis with array comparative genomic hybridization (aCGH) and next-generation sequencing [7] has led to

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improved test accuracy and greater routine use of PGS. However, there continues to be controversy surrounding broad application of PGS [8]. Thus far, two randomized controlled trials (RCTs) have shown significantly improved ongoing pregnancy rates following single embryo transfers (SET) of embryos selected by PGS after TE biopsy compared to morphologic assessment alone [9, 10]. A third RCT by Scott et al. found that utilization of PGS can significantly improve efficiency of IVF with improved live birth rate per embryo transfer [4]. Furthermore, the transfer of single euploid blastocysts identified using PGS has significantly decreased multiple gestation rates as compared with transfer of multiple non-tested embryos of equivalent morphology [10, 11]. Limiting the generalizability of these studies is inclusion of a predominately young, good prognosis patient population [8, 12]. Several retrospective and prospective cohort studies evaluating outcomes in poor prognosis patients, including those with recurrent pregnancy loss, advanced maternal age, or repeated implantation failure, have also shown improved implantation or live birth rates with use of PGS compared to morphologic assessment [13–16]. The lack of randomization and variation in methodology (cleavage, polar body, blastomere, or TE biopsy) in these cohort studies, however, restricts the ability to draw clinically significant conclusions. Thus, currently PGS is often not routinely used and embryo development and morphology remain important factors in embryo selection.

A large body of literature has examined alternative ways to identify the most promising embryos. Many studies have evaluated the relationship between embryo morphology and embryo developmental rate with primary outcomes including embryonic euploidy, pregnancy, and live birth rates. Overall, studies on embryo morphology have consistently shown a relationship between better embryo morphology and improved pregnancy and live birth rates [17–19]. However, while use of morphology does improve the likelihood of choosing a euploid embryo above the chances in random selection, morphology alone cannot accurately or reliably reveal embryo ploidy status [10, 20].

Additional studies have attempted to guide embryo selection based on rate of embryo development, some through selecting by the day of blastocyst formation and others through performing morphokinetic assessment using time-lapsed imaging [21, 22]. The relationship between day of blastocyst formation and embryo chromosomal status is inconsistent, with some studies finding a significantly higher euploid rate with blastocyst formation by day 5 [21], and others finding no difference [23]. There are also variable data on whether pregnancy rates are improved [24, 25] or equivalent [26, 27] after transfer of day 5 blastocysts as compared with later developing day 6 blastocysts. Notably, these data all look specifically at frozen embryo transfers where endometrial synchrony should not be a factor in outcomes.

Despite the vast amount of research on these different methodologies for embryo selection, there are limited data specifically examining euploid embryo selection by day of blastocyst formation and biopsy. The present study evaluates the relationship between day of TE biopsy and embryonic ploidy status as determined by high-density oligonucleotide aCGH, after controlling for maternal age and donor status.

Materials and methods

All patients who underwent IVF with TE biopsy for CCS with high-density oligonucleotide aCGH between January 2014 and December 2014 at Fertility and Surgical Associates of California were screened for inclusion in the study. A total of 452 cycles and 2683 biopsied blastocysts were initially assessed. After exclusion of repeat cycles from the same patient, a total of 388 cycles and 2132 biopsied blastocysts from each patient's first cycle were analyzed. A wide range of infertility diagnoses including diminished ovarian reserve (57%), male factor (11%), tubal/uterine factor (6%), ovulatory dysfunction (4%), unexplained infertility (4%), and multiple factors/other (20%) comprised the study population. At the study center, approximately 90% of patients choose to have TE biopsy, thus providing a broad representation of the infertility population presenting for treatment. Both donor and autologous cycles were included. For the purpose of this study, embryos with a CCS result that was non-diagnostic (< 2%) were considered aneuploid. Equivalent numbers of non-diagnostic outcomes resulted from day 5 and day 6 biopsies. All reported segmental deletions and duplications were also considered aneuploid. Mosaic results were considered aneuploid by the testing laboratory if $\geq 40\%$ mosaicism was present, and euploid if < 40% mosaicism was present. Re-biopsy results were not included.

Controlled ovarian hyperstimulation was performed using standard long GnRH agonist or GnRH antagonist protocols utilizing a combination of recombinant FSH (Follistim; Merck, Kenilworth, NJ, USA) and human menopausal gonadotropin (Menopur; Ferring Pharmaceuticals, Parsippany, NJ, USA). When the two lead follicles reached ≥ 18 mm in mean diameter, final oocyte maturation was triggered with either subcutaneous hCG (5000–10,000 units) or a combination of subcutaneous GnRH agonist (leuprolide acetate 1 mg) and hCG 1000iu. Oocyte retrieval was performed 36 h after trigger injection. Cycles using both conventional insemination and intracytoplasmic sperm injection (ICSI) were included in the study. For oocytes undergoing conventional insemination, motile sperm at a concentration of 150,000–200,000/mL were co-incubated overnight in Quinn's Fertilization media with 5% HSA. Following confirmation of fertilization, all embryos were then transferred into Quinn's Advantage Plus Cleavage media. For oocytes undergoing ICSI, mature oocytes were

injected 5–6 h post retrieval and subsequently cultured in Quinn's Advantage Plus Cleavage media until day 3. All cleavage stage embryos were then transferred into 15–30 μ l of Quinn's Advantage Plus Blastocyst media for group culture at 5% oxygen concentration and routinely incubated until days 5, 6, or 7. Each day, embryos were checked in the morning between 7 and 9 a.m. to determine readiness for biopsy and again at the time of biopsy, which is typically performed between 12 and 2 p.m. Embryos were graded based upon criteria set by Schoolcraft et al. [28] and determined to be ready for biopsy at the expanding blastocyst stage when a clear distinction between the inner cell mass and TE can be observed. The expanding blastocyst stage determined day of TE biopsy and was thus considered the day of blastocyst development. Blastocysts were stabilized with a holding pipette and 20- μ m biopsy pipette was then used to remove ~ 3–10 TE cells for biopsy with assisted cutting by the laser. Biopsied cells were washed with a washing buffer, placed in tubes with cell lysis buffer, and cryopreserved at -20°C before being sent off for testing. Biopsied TE cells were analyzed for all 24 chromosomes using standard high-density oligonucleotide aCGH (Agilent Technologies) by the testing laboratory (PacGenomics, Agoura Hills, CA). Oligonucleotide aCGH used by the testing laboratory has been previously validated and has been shown to detect deletions as small as 1.8–2.4 Mb (Agilent) [29, 30].

Cycles were assessed for the percentage of blastocysts biopsied on each day (days 5, 6, or 7) as well as the rate of euploid embryos per cycle. Additional clinical data for all cycles were collected, including infertility diagnosis, partner age, donor status, stimulation protocol, patient age, and number of blastocysts. Cycles were stratified by SART age groups (< 35, 35–37, 38–40, 41–42, > 42) as well as by donor status.

The main outcome measure was the association between rate of blastocyst development (days 5, 6, or 7) and euploid rate. Statistical analysis was performed in SAS 9.4 using a mixed model test of fixed effects. The mixed model was used to control for known confounders of chromosomal status including oocyte age, partner age, donor status, infertility diagnosis, and total number of blastocysts biopsied. Furthermore, the mixed model test of fixed effects was used to examine the relationship between rate of blastocyst development and euploid rate in order to adjust for within patient correlation and control for possible confounders. Analysis by cycle, as opposed to by embryo, was performed to control for non-independence of embryos from the same patient. An additional subgroup analysis was performed analyzing only cycles with both day 5 and day 6 blastocysts available for biopsy. A generalized linear mixed model of random effect was used to evaluate the relationship between euploid rate and day of blastocyst development in this subgroup of patients, controlling for SART age group and total number of blastocysts

biopsied. For all comparisons, statistical significance was set at p value of < 0.05 .

Due to the retrospective nature, the Institutional Board Review at University of California Los Angeles deemed this study exempt.

Results

A total of 388 IVF cycles and 2132 blastocysts were included for analysis. Fertilization was performed using only ICSI in 35% of cycles, only conventional insemination in 8%, and split ICSI conventional insemination in 54% of cycles. The mean patient age was 35 years at the time of retrieval. For initial analysis and patient counseling purposes, descriptive studies were performed with the donor cycles as a separate group from the autologous cycles that were segregated by SART age categories. Oocyte donors were significantly younger than the < 35-year-old autologous oocyte group (25.8 vs. 31.6 years) and had both a higher average number of total blastocysts (13.8 vs. 7.64) and a higher average number of biopsied blastocysts per patient (8.6 vs. 6.4). For autologous cycles, the percentage of euploid blastocysts per cycle (Table 1) and the percentage of cycles with ≥ 1 euploid blastocyst (Table 2) increased with decreasing age. Patients < 35 years old had 65.7% euploid blastocysts per cycle (Table 1) and 94% of their cycles had at least 1 euploid blastocyst (Table 2).

TE biopsy was performed on day 5 in 62.5% of embryos, on day 6 in 35.8% of embryos, and on day 7 in 1.7% of embryos, with an absolute number of only 36 blastocysts biopsied on day 7 during the study period. Results from PGS demonstrated that the average blastocyst euploid rates across all age groups on days 5, 6, and 7 were 49.5, 36.5, and 32.9%, respectively.

Percent euploidy was subsequently assessed by day of blastocyst biopsy (days 5, 6, or 7) for each SART age category (Fig. 1). Earlier blastocyst development ($p < 0.0001$), younger female age ($p < 0.0001$), and higher number of blastocysts biopsied per patient ($p = 0.0063$) were significantly associated with increased euploidy. Although the overall trend for increased euploidy with earlier blastocyst maturity is significant, the main effect comes from higher euploid rates in day 5 blastocysts. The significant association between earlier blastocyst development and embryo euploid rate was shown across all SART age groups. Additional subgroup analysis of the 207 cycles with both day 5 and day 6 blastocyst biopsies was performed to ensure the findings in the initial analysis were not skewed by patient variability resulting from better prognosis patients with only day 5 embryo biopsies and worse prognosis patients with only day 6 embryo biopsies. In this subgroup, after controlling for SART age group and number of blastocysts biopsied, euploid rates were still significantly

Table 1 Baseline patient and embryo characteristics stratified by patient age

SART age group	Total cycles	Average age (years)	Cycles (n)	Average oocytes retrieved/cycle	Fertilization rate ^a (%)	Blastulation rate ^b (%)	Blastocysts biopsied (n)	Euploid blastocysts (n)	Euploid blastocysts per cycle ^c (%)
Donor	77	25.8	77	33.3	66.4 ± 3.3	62.8 ± 4.3	666	507	76.1 ± 4.3
< 35	84	31.6	84	18.6	66.8 ± 3.9	64.2 ± 4.5	534	351	65.7 ± 5.8
35–37	58	35.8	58	15.3	62.9 ± 5.1	63.2 ± 5.7	298	148	49.7 ± 7.7
38–40	91	39.1	91	13.7	65.1 ± 3.8	60.3 ± 4.4	437	187	42.8 ± 6.0
41–42	47	41.4	47	7.6	71.4 ± 5.9	52.6 ± 6.8	117	33	28.2 ± 9.7
> 42	31	43.9	31	8.0	76.9 ± 6.5	45.0 ± 7.8	80	8	10.0 ± 4.6

^a Number of oocytes fertilized/total oocytes retrieved^b Number of blastocysts/total oocyte fertilized^c Average percent of euploid blastocyst per cycle ± CI

higher in day 5 blastocysts compared to day 6 blastocysts (OR 1.64, 95% CI (1.30, 2.08)) (Table 3). Donor status was not significantly associated with blastocyst euploidy after controlling for female age and number of blastocysts biopsied per patient ($p = 0.235$). Infertility diagnosis, male age, and stimulation protocol were not significantly associated with euploidy after controlling for covariates.

Discussion

Deciding which embryo to choose for transfer can often present a challenge, as morphology alone does not always predict embryo ploidy. This study examined a large cohort of biopsied blastocysts that underwent CCS and demonstrates that earlier day of blastocyst development is independently associated with greater euploidy. The significant relationship between rate of blastocyst development and euploid rate persisted in the subgroup of patients with both day 5 and day 6 blastocysts available for biopsy, demonstrating that these findings are not

just due to patient variability. Furthermore, the finding of improved euploid rate in earlier developing blastocysts persisted in both autologous and donor embryos.

Prior studies evaluating embryo ploidy status and rate of blastocyst development have found inconsistent results [26, 29]. Our results are unique in that they stem from a larger and more diverse population than those of previous studies. Additionally, to the best of our knowledge, this is the first study to examine the relationship between embryo chromosomal status determined by TE biopsy and day of blastocyst development while stratifying by age. These results demonstrate that an embryo that matures more rapidly and is ready for biopsy on day 5 is more likely to be chromosomally normal than an embryo that is not ready until days 6 or 7. In those patients who choose not to pursue PGS, these data can be extrapolated to encourage transfer of a day 5 blastocyst over a later-developing one to maximize the chance of euploid embryo selection.

These results are also encouraging for patients who do not have euploid day 5 blastocysts available for transfer. Our

Table 2 Average number of blastocysts and percentage of euploid blastocysts stratified by day of blastocyst biopsy and SART age groups

SART age group	Total cycles	Ave D5 ^a (n)	Ave D6 ^b (n)	Ave D7 ^c (n)	Euploid D5 ^d (%)	Euploid D6 ^e (%)	Euploid D7 ^f (%)	Cycles with ≥ 1 euploid blastocyst (%)
Donor	77	8.34 (6.9)	5.26 ± 3.1	0.23 ± 0.7	78.3 ± 4.5	63.8 ± 6.7	63.9 ± 9.5	100.0
< 35	84	4.57 (4.3)	2.94 ± 2.5	0.13 ± 0.4	71.7 ± 5.6	58.1 ± 7.8	50.0 ± 9.6	94.0
35–37	58	3.12 (3.3)	2.41 ± 2.1	0.03 ± 0.2	57.9 ± 9.0	41.5 ± 10.1	50.0 ± 18.2	86.2
38–40	91	2.97 (3.0)	1.96 ± 2.0	0.09 ± 0.4	46.2 ± 6.9	33.0 ± 8.0	33.3 ± 10.6	78.0
41–42	47	1.28 (1.5)	1.15 ± 1.1	0.06 ± 0.2	34.2 ± 11.3	21.1 ± 10.6	0.0	44.7
> 42	31	1.13 (2.7)	1.42 ± 1.3	0.03 ± 0.2	8.8 ± 6.9	1.4 ± 2.4	0.0	9.7

^a Average number of blastocysts that developed on day 5 ± SD^b Average number of blastocysts that developed on day 6 ± SD^c Average number of blastocysts that developed on day 7 ± SD^d Average percent of day 5 biopsied blastocysts found to be euploid ± CI^e Average percent of day 6 biopsied blastocysts found to be euploid ± CI^f Average percent of day 7 biopsied blastocysts found to be euploid ± CI

Fig. 1 Blastocyst euploid rate stratified by day of embryo biopsy and patient age

findings demonstrate that a significant percentage of day 7 blastocysts are euploid particularly in the younger patient groups. This is consistent with prior studies demonstrating that viable pregnancies can result from transfer of day 7 blastocysts, although less frequently than with transfer of day 5 or 6 blastocysts, and these embryos should therefore not be discarded [25, 31]. Kovalesky et al. demonstrated that continued embryo culture and biopsy on day 7 resulted in a 26.7% euploid rate, which is consistent with the 32.9% euploid rate found in our study. Notably, the number of day 7 biopsies was low in our study, with no euploid day 7 blastocysts found in women over the age of 38. A larger population of patients from a broader age range would make these conclusions about day 7 embryos more generalizable.

We also demonstrate that donor status does not independently predict embryo ploidy after controlling for other covariates, namely, female patient age and day of embryo biopsy. While it appears from the raw data that aneuploid rates are consistently lower for the donor group when compared with the < 35-year-old autologous patients, this difference is no longer significant when controlling for the age difference between the two groups.

Our study has a number of important strengths. The data come from a single, high-volume center experienced in TE biopsy and consist of a diverse cohort of patients choosing to have PGS. The patients were high, normal, and low responders who had a broad array of indications for IVF with

PGS, making these results highly generalizable. Additionally, all blastocysts were sampled by TE biopsy and results were from a single calendar year, a relatively short period of time, thus minimizing bias associated with change in laboratory personnel and technique as possible confounders. All PGS was performed in the same genetic screening laboratory using one approach, ensuring uniformity of technique. Additionally, there were no repeat patient cycles that would potentially add repeat cycle bias.

There are also weaknesses. A combination of conventional IVF and ICSI fertilization techniques was used in the study population, with the majority of cycles splitting oocytes between the two techniques. Given the analysis was done on per cycle, rather than per embryo basis, we were unable to control for use of ICSI. Of note, prior studies are mixed on whether use of ICSI results in a difference in rate of blastocyst formation rate [32–35]. Moreover, while day of embryo biopsy may be considered a surrogate endpoint for day of blastocyst development, these designations are clearly distinct and depend on both the stage of blastocyst development used to designate blastocyst formation and the time at which each individual lab protocol dictates that TE biopsy takes place [36]. Additionally, while aCGH is commonly used and a well-validated methodology for PGS, newer techniques such as NGS are becoming more widely employed. Notably, however, the high-density oligonucleotide aCGH methodology used for PGS in this study has been shown to detect deletions as small as 1.8–

Table 3 Euploid rate for cycles with both day 5 and day 6 blastocysts available for biopsy, controlling for SART age group and total number of blastocysts biopsied

	Total cycles	Total D5 ^a blastocysts	Percent euploid D5 ^a (% ± SD)	Total D6 ^b blastocysts	Percent euploid D6 ^b (% ± SD)	Odds ratio	95% CI	<i>p</i> value
Blastocysts available on both day 5 and day 6	207	828	60.4 ± 33.9	717	47.9 ± 38.9	1.64	1.30–2.08	<i>p</i> < 0.001

^a Biopsied blastocysts on day 5

^b Biopsied blastocysts on day 6

2.4 Mb [29, 30], proving to be significantly more sensitive than the 10 Mb detection limit of lower density bacterial artificial chromosome aCGH [37]. Furthermore, initial validation studies comparing high-density oligonucleotide aCGH and NGS have shown similar detection limits of 1.8 Mb [38]. Given comparable sensitivity and specificity between aCGH and NGS, there is no reason to believe that outcomes would differ significantly. However, a similar study using NGS would have to be performed to validate these results with other genetic testing platforms. Lastly, although these data support an increased probability of embryonic euploidy with earlier blastocyst development, in the absence of PGS, the day of blastocyst development, even in conjunction with morphologic assessment, is by no means diagnostic of euploid status.

Our study demonstrates a clear segregation of embryo euploid rates by maternal age and day of blastocyst development, using very sensitive high-density oligonucleotide aCGH. These data can be used to assist in counseling patients undergoing IVF with or without PGS. These statistics help provide realistic expectations for the likelihood of having euploid embryos available for transfer, while also taking into account the rate of blastocyst formation and female patient age. For those patients who forgo PGS, these data show that more rapidly developing embryos that reach the blastocyst stage by day 5 are more likely to be euploid.

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Compliance with ethical standards

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

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