A greater number of euploid blastocysts in a given cohort predicts excellent outcomes in single embryo transfer cycles

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Received: 22 January 2014 / Accepted: 10 March 2014 / Published online: 23 March 2014
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Abstract

Purpose This multicentered retrospective study analyzed whether the quantity of euploid blastocysts in a given cohort after comprehensive chromosomal screening can be used to identify candidates for single embryo transfer.

Methods Blastocysts from 437 patients underwent trophectoderm biopsy followed by array comparative genomic hybridization. Embryos were then selected for single or double embryo transfer. The number of euploid blastocysts produced and transferred for each patient was recorded, as was clinical pregnancy rate and multiple gestation rate.

Results In patients with ≤ 3 euploid blastocysts, clinical pregnancy rate was higher in double, compared to single embryo transfers. However, in patients with ≥ 4 euploid blastocysts, clinical pregnancy rate was not reduced with single embryo transfer was performed, whereas the multiple gestation rate was greatly reduced.

Conclusions Size of the euploid embryo cohort is a marker for success in single embryo transfer cycles. Patients who

produce at least four euploid blastocysts are outstanding candidates for single embryo transfer.

Keywords Single embryo transfer (SET) · Preimplantation Genetic Screening (PGS) · Trophectoderm biopsy · Array comparative genomic hybridization (array-CGH) · Assisted reproductive technology (ART)

Introduction

The ultimate goal of assisted reproductive technology (ART) is to provide one healthy child at a time. All advances in the field over the past 30 years have aimed towards maximizing the probability of pregnancy, while limiting the risk of multiple gestations. Despite this aim, 47.3 % of all infants born following ART in 2009 were part of multiple births [1], and 28.7 % of cycles in the United States resulting in live birth in 2011 were twins or higher order pregnancies [2]. The risks associated with multiple gestation have been well documented and include preeclampsia [3], gestational diabetes [4], preterm delivery, low birth weight [5], cerebral palsy [6], and perinatal mortality [7]. Single embryo transfer (SET) has been proposed as a means for limiting multiple gestations in ART. However, prospective studies comparing SET to double embryo transfer (DET) have returned mixed results [8, 9, 10]. As a result, elective SET (eSET) is still infrequently practiced in the United States, accounting for only 4.2 % of embryo transfers in 2011 [2], while in Europe is more common due to laws mandating it [11, 12].

Currently, methods for optimizing embryo selection are still mostly based on morphological assessment. However, morphology scores have only loosely been correlated with the chromosomal competence of embryos. Multiple studies

Capsule This multicentered study demonstrates that patients who produce ≥ 4 euploid embryos have equivalent clinical pregnancy rates whether one or two embryos is replaced.

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have shown that highly graded embryos can be chromosomally abnormal [13, 14, 15, 16] and aneuploidy is the leading cause of failed implantation and miscarriage [17, 18, 19, 20, 21, 22, 23]. While initial attempts at preimplantation genetic screening (PGS) in cleavage stage embryos failed to demonstrate improved pregnancy rates, trophectoderm biopsy coupled with comprehensive chromosomal screening (CCS) [24, 25] have yielded encouraging results [26, 27, 28, 29, 30].

The promising results of CCS have reinvigorated the discussion regarding more routine implementation of SET. Improved pregnancy rates and decreased miscarriage rates have been reported in SET cycles following CCS compared with SET using standard morphologic criteria [31, 28]. Recent data from a randomized controlled trial even demonstrated that a SET of a chromosomally normal embryo has similar pregnancy rates to a DET of untested embryos [32]. However, studies examining CCS have not reported specifically on the characteristics of the biopsied cohort. In particular, no study to our knowledge has addressed whether the size of the euploid embryo cohort following TE biopsy was predictive of improved pregnancy outcomes. The presence of supernumerary embryos has been suggested as an indirect marker for embryo quality [33, 34, 35] and aneuploidy rate has been demonstrated to be unrelated to the number of embryos produced [36]. Thus, it stands to reason that achieving a larger cohort of confirmed euploid blastocysts would be similarly associated with higher pregnancy rates. In Forman et al. [32] for example, the average number of confirmed euploid blastocysts remaining following SET was 3.2. This suggests a surplus of euploid embryos may have been a marker for the excellent outcomes seen following SET in this study. However, no studies have examined whether size of the euploid blastocyst cohort can be used to predict improved outcomes in SET cycles.

Thus, this investigation retrospectively examined whether the quantity of euploid blastocysts in a given cohort was useful in identifying the best candidates for SET while maintaining a high pregnancy rate and limiting multiple gestations.

Materials and methods

This is a retrospective analysis of CCS by array-comparative genomic hybridization (aCGH) of human embryos referred to Reprogenetics for aneuploidy screening. Blastocysts from 437 patients from 16 referral centers that provided follow up information were evaluated between June 2010 and August 2012. At least one center was included from the following locations: California, Nevada, Oregon, Illinois, Florida, Georgia, Pennsylvania, New York, Massachusetts, and Canada. Of note, this database has been utilized to previously report PGS data from Reprogenetics [36, 22].

This study was determined to be exempt from institutional review board (IRB) approval. According to the Western IRB

in Olympia, Washington, under common rule 45 CFR 46.101(b) [20], exemptions include “research, involving the collection or study of existing data, documents, records, pathological specimens, if these sources are publicly available or if the information is recorded by the investigator in such manner that subjects cannot be identified, directly or through identifiers linked to subjects.”

Inclusion criteria

The study population comprised women who underwent PGS with aCGH for aneuploidy screening (generally due to a history of recurrent pregnancy loss, infertility, or advanced maternal age). Extensive details regarding prior infertility workup, such as karyotype analysis or thrombophilia labs were not available. Embryos generated from oocyte donation cycles were included. Oocyte donors were all ≤ 35 years of age. PGS was performed in oocyte donor cycles as per patient preference. Patients who failed to produce at least one euploid embryo were excluded from the analysis. In addition, patients who elected not to undergo an embryo transfer at the time of the analysis (either due to poor embryo morphology or patient preference) were not included. There were 18 patients who had 3 or more embryos replaced. Sixteen became pregnant and 7 of those had multiple sacs. We chose to limit our investigation to cases with only 1 or 2 embryos transferred due to the high incidence of pregnancy and multiple pregnancy observed in these cases. Finally, women who underwent PGD for diagnosis of balanced translocations or single gene defects were excluded. Thus, in total, 297 patients who underwent SET or DET after confirmation of at least one euploid embryo by PGS with aCGH were included in the analysis. The age range of the subjects was 20 to 45 years, with an average of 34.7.

Embryo biopsy

On day 3 of incubation, a hole was made in the zona pellucida of viable embryos using a laser to allow hatching. Resultant embryos were cultured to day 5 and any embryos not suitable for biopsy on day 5 were subsequently cultured to day 6. On day 5 or 6, suction was subsequently applied to the hatched trophectoderm to isolate and retrieve a small clump of trophectoderm cells using the laser. The biopsied cells were placed in Eppendorf tubes, frozen in dry ice, and then transported to Reprogenetics for PGS analysis.

Array-CGH

Some embryos were vitrified while awaiting results of CCS by aCGH following the method described by Gutierrez et al. [25]. Briefly, cells were washed and collected into sterile PCR tubes. The samples and reference male DNA were lysed,

fragmented and SurePlex amplification reagents were added (BlueGnome, Cambridge, UK). Labeling was performed according to manufacturer's instructions with Cy3 dye for test DNA and Cy5 dye for reference male DNA. Labeled samples and reference male DNAs were mixed and applied to a 24Sure (BlueGnome) microarray and co-hybridized. They were then washed in $\times 2$ saline sodium citrate (SSC)/0.05 % Tween 20. Microarray slides were then centrifuged and scanned (InnoScan 710AL; Innopsys, Carbonne, France). Images were analyzed using BlueFuse Multi software (BlueGnome). Once a specific amplification was observed, autosomes were analyzed for the presence or absence of whole or partial chromosome ratios using a 3SD assessment, $\geq 0.3\log$, ratio call or both. Hybridization quality control was performed with sex mismatched female samples, which were required to show a consistent gain on chromosome X and a consistent loss on chromosome Y. Sex-matched male samples were required to consistently show no change on either chromosome X or Y.

Embryo replacement

The decision for timing of embryo replacement was made by individual clinicians and patients at each referral center. If information regarding the chromosomal status of the embryo was available in time for fresh transfer, some centers opted to transfer the embryos on day 6 of the stimulated or egg donor cycle. In other cases, clinicians opted to vitrify the embryos either as part of their PGS protocol or while awaiting results of aneuploidy screening.

Outcomes and statistical analysis

The number of euploid blastocysts in each cohort was recorded for each patient. The number of embryos transferred was also recorded. The decisions regarding the number of embryos to transfer were made collaboratively by embryologist, physician, and patient in accordance with American Society of Reproductive Medicine guidelines. The authors of this investigation were not privy to the details of these conversations. The primary outcome collected was clinical pregnancy (CPR) defined as the presence of a gestational sac in the uterus. Multiple gestation rate (MGR), defined as the presence of more than one gestational sac in the uterus, was also collected.

In order to test the hypothesis that the cohort size of euploid blastocysts affected the outcome in question (CPR), a multiple logistic regression model was constructed. This model was designed to assess associations of the following independent variables: 1) number of euploid blastocysts in a given cohort, 2) number of euploid blastocysts transferred in an individual patient, 3) individual clinic-specific offset and 4) female patient age. Due to a correlation between patient age and number of euploid embryos, multicollinearity between these two variables was also considered.

Fitting was performed by using an iterative routine that adjusted coefficients for the parameters and maximized the log (likelihood). Final "best fit" was determined using the minimum Akaike Information Criterion (AIC). All permutations between independent variables were tested. The relative association of individual parameters in determining the outcome was assessed in two ways: 1). The improvement in the maximum log (likelihood) when the parameter included in the fitting was compared with the maximum log (likelihood) with the parameter not included in the fitting procedure, and 2) the normalized coefficients in the regression equation were determined indicating the effect of a 2 standard deviation change of the independent parameter in the regression equation while holding the other fitting parameters constant at their mean values. The relationships between the independent variables and the outcome variables determined by the multiple logistic regression analysis were subsequently used to examine whether the size of the euploid blastocyst cohort and the number of blastocysts transferred could be used to identify ideal candidates for single embryo transfer.

After establishment of the association of euploid blastocyst cohort size and CPR, patients were then separated by the number of euploid blastocysts produced and the number transferred. This separate analysis was used in order to present readers with numbers that can aid in clinical decision making and assist with patient counseling regarding number of embryos to transfer based on size of the euploid blastocyst cohort. The data was also analyzed after separating fresh blastocyst transfers and frozen blastocyst transfers.

Results

Fitting the incidence of pregnancy was performed with all permutations of free parameters, using the three different independent parameters (each of the parameters individually, all three pairs of the three parameters and all three parameters together). For each permutation, the offset was an additional free parameter with a fitted value for each of the 16 clinics. Calculation of the AIC from the maximum log likelihood for each trial revealed that the best fit was attained when only the number of euploid embryos available and the number of embryos transferred were included in the regression equation:

$$\ln(\text{OR CP}) = 0.17 \times \#EE + 0.46 \times \#ET - 1.29$$

where OR CP is the odds ratio for clinical pregnancy, #EE is the number of euploid embryos available for transfer for each patient, and #ET is the number of embryos transferred. While age was strongly negatively correlated with number of euploid blastocysts produced ($R = -0.46$), adding age as a parameter did not significantly improve the fit. Ninety-five percent confidence limits for the regression coefficients were 0.05 to 0.29 for the

#EE coefficient and -0.09 to 1.03 for the #ET coefficient. The arithmetic average of the clinic-specific offset was -1.29 ± 2.2 (mean $\pm$ std. dev.). While the clinic-specific offset had a different value for each of the 16 clinics, no further consideration was given to the strength of association between clinic-specific offset and outcomes since clinic-to-clinic variability was not the focus of this study.

Stepwise inclusion of the number of euploid embryos and the number of embryos transferred improved the likelihood that the data were generated from the model by 54-fold and 3.9-fold, respectively. A change of the number of euploid embryos available from one standard deviation below the mean to one standard deviation above the mean (1.13 to 6.19) increased the probability of pregnancy by 20.5% . A change of the number of embryos transferred from one standard deviation below the mean to one standard deviation above the mean (0.94 to 1.93) increased the probability of pregnancy by 11.3% . Therefore, the probability of clinical pregnancy exhibited a strength of association in the following order:

Number of euploid embryos > Number of embryos transferred

As a next step, we wished to choose a threshold to select patients with an excellent prognosis for single embryo transfer. An odds ratio of 1 (pregnant = not pregnant) for clinical pregnancy with single embryo transfer using the regression equation occurred when the number of euploid embryos available was 2.1 and the weighted average of the clinic-specific offset (-0.81) was used. The odds ratios for patients with 3 and 4 available euploid embryos were 1.15 and 1.36 yielding probabilities of clinical pregnancy of 0.54 and 0.58. In order to maintain a live birth rate of roughly 50% (accounting for roughly 10% rate of pregnancy loss from euploid transfers [29, 30] from a 58% clinical pregnancy rate) we chose to set the threshold at 4 euploid embryos. Patients were stratified by the number of euploid blastocysts produced (≤ 3 vs ≥ 4), as well as the number of embryos transferred (1 vs 2).

Table 1 presents the data after separation of the patient population based on a threshold of 4 or more euploid

blastocysts. 168 patients produced ≤ 3 euploid blastocysts within their cohort. These patients had a significantly higher CPR when two embryos were transferred compared to only one (64.9% vs. 42.3% , $p < 0.01$). Of the pregnancies in the DET group, 38% were multiples, while none of the pregnancies in the SET group were multiple. Notably, one quarter of all DET in this group resulted in a multiple gestation. Also presented in Table 1, 129 patients produced ≥ 4 euploid blastocysts within their cohort. In this group, however, no significant difference in CPR was noted between the DET and SET groups (78.4% vs. 74.5% , $p = 0.6$), while multiple gestations were five times more likely in the DET group (52% vs. 10% , $p < 0.001$).

Table 2 demonstrates clinical pregnancy rate following SET after stratification by number of euploid blastocysts produced and evaluation by chi-squared analysis. CPR was significantly higher when patients produce at least 4 euploid blastocysts, when compared to patients who produce three or fewer blastocysts (74.5% vs. 43.5% , $p < 0.001$). Tables 3 and 4 display pregnancy outcomes after stratification of the data into SART age group criteria. Consistent with the findings in the multiple logistic regression analysis, age was not associated with differences in outcomes in either the ≤ 3 euploid blastocyst or ≥ 4 euploid blastocyst group.

Of the 297 patients, 117 had fresh blastocyst transfers and 180 had frozen blastocyst transfers. The CPR was no different between the two groups (fresh: 64% [65/117] vs. frozen: 60% [108/180], $p = 0.47$). Fresh and frozen transfers were evenly distributed among SET cycles (39.8% fresh transfers [66/166] and DET cycles (38.9% fresh transfers [51/131]).

Of the patients in the ≤ 3 euploid blastocyst group, 13.7% (23/168) were from oocyte donation cycles, versus 22.4% (29/129) in the ≥ 4 euploid blastocyst group. Oocyte donor recipients were less likely to receive an SET than autologous cycle patients (27% [14/52] vs. 53.5% [145/263]). Of the 55 SETs in the ≥ 4 euploid blastocyst group, 8 were from oocyte donors. The CPR following SET in this group was no different between oocyte donor recipients and autologous cycle patients (75% [6/8] vs. 74.5% [41/55]).

Table 1 Pregnancy outcomes by number of euploid blastocysts and number of embryos transferred

	# ET	CPR (%)	Mult gest (n)	MGR/pregnancy
≤ 3 euploid blastocysts ^b	1	42.3 % (47/111)	0	0 %
	2	64.9 % (37/57)	14	38 %
	<i>p</i> -value ^a	<0.01	<0.001	<0.001
≥ 4 euploid embryos ^c	1	74.5 % (41/55)	4	10 %
	2	78.4 % (58/74)	30	52 %
	<i>p</i> -value ^a	0.6	<0.0001	<0.001

^a Chi-squared test performed

^b Average age: SET: 36.9 years, DET: 35.7 years

^c Average age: SET: 32.8 years, DET 32.0 years

Table 2 SET results by number of euploid embryos in a given cohort

No. Euploid Embryos	CPR
1	41.7 % (25/60)
2	43.3 % (13/30)
3	42.9 % (9/21)
4	84.2 % (16/19)
5	72.7 % (8/11)
6	66.7 % (6/9)
≥7	68.9 % (11/16)

^a CPR for ≤3 vs. ≥4 Euploid blastocysts = 43.5 % vs. 74.5 % ($p < 0.001$)

Discussion

These results demonstrate that the number of euploid blastocysts produced in a given cohort following CCS is predictive of improved clinical pregnancy rate. After adjustment for individual clinic differences, the size of the euploid blastocyst cohort was more strongly associated with clinical pregnancy rate in the multiple logistic regression than both number of euploid blastocysts transferred and female patient age. Furthermore, meeting a threshold of 4 euploid blastocysts resulted in an equivalent clinical pregnancy rate whether one or two blastocysts were replaced. When clinical pregnancy is achieved in these patients, SET results in a five-fold reduction in multiple gestation. These findings suggest that patients who produce a greater number of euploid blastocysts are well suited for SET.

Our hypothesis is that the improved CPR in patients with a surplus of euploid blastocysts is likely due to the fact that a greater number of evaluable blastocysts allows the embryologist to select the embryo(s) for transfer with the most favorable morphology. When multiple euploid blastocysts are produced, clinics are afforded the ability to perform both genetic and morphologic selection prior to embryo transfer. While we acknowledge that a majority of patients undergoing IVF with CCS by TE biopsy fail to achieve a threshold of four euploid blastocysts, we maintain that this is an important observation. In our database, 31.8 % (139/437) of patients who sent samples to Reprogenetics for CCS met this criteria. Furthermore, in the randomized controlled trial of SET following CCS

Table 4 Age stratification of CPR in >4 euploid embryo group

Age (y)	CPR: 1 embryo replaced	CPR: 2 embryos replaced	Total CPR
<35	74.1 % (20/27)	72.3 % (34/47)	73 % (54/74)
35–37	78.6 % (11/14)	87 % (13/15)	63.9 % (24/29)
38–40	71.4 % (10/14)	90 % (9/10)	79.2 % (19/24)
41–42	–	100 % (2/2)	100 % (2/2)
>42	–	–	–

^a Mean age: 32.1 years

^b No significant difference in outcomes between different age groups

versus DET of unbiopsied embryos by Forman et al. [32] the average number of surplus euploid embryos following a SET was 3.2. Thus, a significant number of patients meet this criteria. Identification and education of these optimal candidates for SET could serve to improve upon the 4.2 % eSET currently reported in the United States. This approach may help to decrease the incidence of multiple gestation following ART that is currently burdening our healthcare system.

The population with three or fewer euploid blastocysts represents a greater counseling challenge for clinicians. While many of these patients only produced one normal embryo, thus necessitating an SET, a significant number were presented with a choice between eSET or DET. While a DET in these patients demonstrated an improved CPR, the additional morbidity and cost associated with a 38 % multiple gestation rate is of great concern. While many patients may not be willing to diminish their chances at an ongoing pregnancy, they should be well aware of the consequences of multiple gestation. Further studies would be well served to highlight the cumulative pregnancy rate of sequential SET in such patients.

Previously published data from the same database presented here demonstrated that the overall cohort size was unrelated to aneuploidy rate [36]. Thus, the odds of having at least one euploid blastocyst increased with every additional blastocyst available for PGS. The implications of this finding in concert with the data presented here are vast. In patients motivated for a SET, meeting a threshold number of euploid blastocysts increases the chance of pregnancy with SET. While aggressive stimulation has been suggested to be detrimental to the overall euploidy rate [37], the data presented by Ata et al. [36] demonstrated that size of the embryo cohort was unrelated to euploidy rate. This study did not examine stimulation protocols when analyzing how size of the embryo cohort affected euploidy rates, however. The relationship between stimulation, cohort size, and euploidy rate should be better studied in future trials.

Additionally, analysis of these data indicates that the number of euploid blastocysts is a better predictor of success in SET cycles than age. This finding is consistent with recently

Table 3 Age stratification of CPR in <3 euploid embryo group

Age (y)	CPR: 1 embryo replaced	CPR: 2 embryos replaced	Total CPR
<35	38.5 % (10/26)	72.2 % (13/18)	52.3 % (23/44)
35–37	40 % (10/25)	60 % (9/15)	47.5 % (19/40)
38–40	48.4 % (15/31)	63.2 % (12/19)	54 % (27/50)
41–42	55.6 % (10/18)	33.3 % (1/3)	52.3 % (11/21)
>42	18.2 % (2/11)	100 % (2/2)	30.8 % (4/13)

^a Mean age: 36.5 years

^b No significant difference in outcomes between different age groups

reported data by Harton et al. [30] that confirmation of euploidy diminishes the effect of age on implantation rate. However, this conclusion should be tempered with the knowledge that age, despite its insignificant association with clinical pregnancy, was significantly associated with the number of euploid embryos. While age decreases the proportion of euploid embryos, confirmation of euploidy, in addition to the production of surplus euploid blastocysts allows for SET in patients that otherwise would rarely be offered SET. While PGS with CCS is not the only useful tool in identifying these patients, few advances have demonstrated as much promise at such an early stage of study.

The four sets of monozygotic twins out of 88 clinical pregnancies in the SET group is notable and should not be ignored. Both blastocyst transfer [38] and manipulation of the zona pellucida have been proposed as a risk factor for monozygotic twinning [39, 40], but data has been conflicting [41, 42]. The only data to our knowledge examining monozygotic twinning rates in PGD patients failed to show an association, but these biopsies were done in cleavage stage embryos [43]. While the rate of monozygotic twinning here is concerning, it is notable that the four cases in our cohort were from clinics with smaller numbers of cases submitted to our genetics laboratory. Is the rate of monozygotic twinning seen here related to biopsy technique, or biopsy experience? Larger studies of SET following TE biopsy will be needed to address this question.

This study has several limitations. While we suggest here that a greater number of evaluable euploid blastocysts allows the embryologist to include morphology as a criterion in choosing embryos for transfer, we did not analyze whether there was any correlation between morphology and outcomes. This information, as well as data regarding stimulation protocols were unavailable for our investigation and would be valuable in future studies. Furthermore, we did not analyze the effect of the individual clinic-specific offset on clinical outcomes because this was not the focus of our study. We did note some variation between clinics. However, the majority of clinics contributed fewer than 15 patients to the study, and the individual variation by clinic should be interpreted with caution. We chose to lump the clinic effect together in order to make useful clinical recommendations. As more data accumulates, individual clinic-specific offsets can be calculated and used by each center to guide their transfer practices.

As momentum continues to build for SET with the movement away from cleavage-stage biopsy based PGS systems toward blastocyst biopsy and comprehensive chromosomal screening via microarray, array-CGH, or next-generation sequencing, identification of ideal candidates for SET using PGS is essential. There remains a significant untapped pool of patients who would benefit from SET. Until these patients are educated and counseled as such, multiple gestation will remain a common complication of ART.

Acknowledgments We would like to thank our referral centers for their assistance with the collection of data. The referral centers who contributed to this work are ART Reproductive Center (Beverly Hills, CA), San Diego Fertility Center (San Diego, CA), La Jolla IVF (La Jolla, CA), Stanford Fertility Clinic (Stanford, CA), Acacio Fertility Center (Laguna Niguel, CA), OriginElle Fertility Clinic (Montreal, Quebec), NewLIFE – New Leaders in Fertility and Endocrinology (Pensacola, FL), Reproductive Biology Associates (Atlanta, GA), Georgia Reproductive Specialists (Atlanta, GA), Fertility Centers of Illinois (Highland Park, IL), Boston IVF (Waltham, MA), Fertility Center of Las Vegas (Las Vegas, NV), NYU Fertility Center (New York, NY), Oregon Reproductive Medicine (Portland, OR), and Main Line Fertility and Reproductive Medicine (Bryn Mawr, PA).

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