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ORIGINAL ARTICLE *Infertility*

What is the reproductive potential of day 7 euploid embryos?

Carlos Hernandez-Nieto[1,*], Joseph A. Lee[1], Richard Slifkin[1], Benjamin Sandler[1,2], Alan B. Copperman[1,2], and Eric Flisser[1,2]

[1]Reproductive Medicine Associates of New York, New York, NY 10022, USA. [2]Obstetrics, Gynecology and Reproductive Science, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA.

*Correspondence address. Reproductive Medicine Associates of New York, LLP 635 Madison Avenue, 10th Floor, New York, NY 10022, USA. E-mail: chernandez@rmany.com

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STUDY QUESTION: What is the rate of euploidy and the reproductive potential of embryos biopsied after 6 days of development?

SUMMARY ANSWER: Embryos biopsied after 6 days of development have higher rates of aneuploidy; however, day 7 euploid embryos selected at transfer can achieve acceptable pregnancy rates and live birth (LB) outcomes.

WHAT IS KNOWN ALREADY: Recent publications have shown promising treatment results after euploid day 7 embryo transfers (ETs), albeit these studies were limited by small sample sizes. Whereas the current clinical standard has been to discard embryos that do not reach expansion by day 6 of development, the lack of robust data surrounding the clinical utility of day 7 embryos warrants further evaluation.

STUDY DESIGN, SIZE, DURATION: Retrospective cohort analysis in a single, academic *in vitro* fertilization (IVF) center from January 2012 to March 2018. A total of 25 775 embryos underwent trophectoderm (TE) biopsy and preimplantation genetic testing for aneuploidy (PGT-A). Additionally, the clinical IVF outcomes of 3824 single, euploid frozen embryo transfer (FET) cycles were evaluated.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Cohorts were segregated by day of TE biopsy following oocyte retrieval (day 5, day 6 or day 7). PGT-A was performed to identify embryonic ploidy rates. Secondly, IVF and LB outcomes after single, euploid FET were evaluated for each cohort.

MAIN RESULTS AND THE ROLE OF CHANCE: A total of day 5 ($n = 12\,535$), day 6 ($n = 11\,939$) and day 7 ($n = 1298$) embryos were included in the study analysis. The rate of embryo euploidy was significantly lower in day 7 blastocysts compared to day 5 or day 6 cohorts (day 7 = 40.5%; day 5 = 54.7%; day 6 = 52.9%; ($P < 0.0001$)). After adjusting for age, anti-Müllerian hormone, BMI, embryo quality and number of embryos biopsied, there was a significant association between aneuploidy and embryos biopsied on day 7 when compared to day 5 biopsied embryos (OR = 1.34, CI 95% 1.09–1.45, $P = 0.001$) and day 6 biopsied embryos (OR = 1.26, CI95% 1.07–1.16, $P < 0.001$).
A sub-analysis of subsequent 3824 single, euploid FET cycles (day 5: $n = 2321$ cycles; day 6: $n = 1381$ cycles; and day 7: $n = 116$ cycles) showed significant differences among cohorts in implantation, clinical pregnancy, LB and clinical loss rates. There was a significant decrease in the odds of implantation, clinical pregnancy and LB, but no association with clinical loss or multiple pregnancy rates in patients who utilized day 7-biopsied embryos during treatment.

LIMITATIONS, REASONS FOR CAUTION: The retrospective nature of the study and potential variability in the study center's laboratory protocol(s) compared to other reproductive treatment centers may limit the external validity of our findings. Additionally, patients who transferred euploid embryos, biopsied on day 7 of development due to an absence of day 5 or day 6 counterparts, may have introduced selection bias in this study.

WIDER IMPLICATIONS OF THE FINDINGS: Embryonic developmental stage, morphological grade and ploidy status are paramount factors affecting ET selection and implantation potential. This study reveals that embryos ineligible for TE biopsy on day 5 or day 6 of development may benefit from extended culture to day 7. Our study demonstrates patient benefit when extended culture to day 7 of development is routinely performed for embryos failing to meet biopsy criteria by day 5 or 6.

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TRIAL REGISTRATION NUMBER: This retrospective analysis was approved by an Institutional Review Board (WIRB PRO NUM: 20161791; Study Number: 1167398).

Key words: embryo culture / extended culture / preimplantation genetic testing / aneuploidy / pregnancy rate / trophectoderm biopsy / blastocyst / embryo cryopreservation / single embryo transfer

Introduction

Embryo selection is one of the most important factors influencing the success of ART treatment. A major advancement in the practice of *in vitro* fertilization (IVF) has been the development of advanced embryo culture media (Sunde *et al.*, 2016), which has extended embryo culture and enabled reproductive specialists to pivot away from cleavage-stage (day 3) (Taylor *et al.*, 2014; Minasi *et al.*, 2016) to blastocyst (day 5/6) stage embryo transfer (ET) (Gardner *et al.*, 1998; Lane and Gardner, 2007; Smith *et al.*, 2012). Notably, blastocyst transfer during ART treatment has improved embryo selection and endometrial-embryo synchrony, resulting in higher implantation and delivery rates while decreasing multiple pregnancy rates in fresh and frozen-thawed embryo transfer (FET) cycles (Glujovsky *et al.*, 2016).

More recently, improved cryopreservation techniques and molecular screening platforms have allowed embryo trophectoderm (TE) biopsy for preimplantation genetic testing for aneuploidy (PGT-A). The use of embryonic chromosome analysis as a screening tool has allowed reproductive specialists to select euploid embryos for transfer, which has enhanced IVF cycle outcomes (Scott *et al.*, 2012; Kang *et al.*, 2016; Treff and Zimmerman, 2017). Improvements in vitrification techniques have insured the reliability of successful embryo cryopreservation and rewarming, further favoring the patient outcome (Lawrenz and Fatemi, 2017). Along with PGT-A to screen embryos, the ability to vitrify blastocysts and plan for subsequent transfer after endometrial preparation avoids the potential detrimental effects of an altered supra-physiological hormonal milieu coming from the preceding ovarian stimulation. This strategy has increased clinical pregnancy rates (CPRs) and live birth (LB) outcomes (Al-Azemi, *et al.*, 2012; Capalbo *et al.*, 2016a; Wang *et al.*, 2017)

As reproductive specialists, we continue to investigate the complexities of embryo development during extended culture to establish optimal tools to aid clinical treatment. Delayed embryo development has been associated with poor early blastocyst quality and reduced viability (Lelaidier *et al.*, 1995; Shoukir *et al.*, 1998; Wirleitner *et al.*, 2016). Yet there is growing interest in learning whether this concept is evidence-based. Simply, can embryos cultured to day 7 of development be safely biopsied for genetic screening, and if interpreted to be euploid, can these embryos be selected for transfer to add to potential clinical success?

The first reports to evaluate patient outcome after the transfer of unscreened day 7 blastocysts showed lower pregnancy and LB rates compared to patients who utilized day 5 or day 6 blastocysts (Shoukir *et al.*, 1998; Utsunomiya *et al.*, 2004). These early studies appear to have negatively influenced clinical perception, biasing many embryology laboratories and ART practices against routinely culturing embryo beyond day 6 of development.

Recent studies evaluating the reproductive potential of day 7 embryos have served as a basis for further exploration of the clinical outcomes of embryos cultured past the current day 6 standard (Hiraoka *et al.*, 2008; Wirleitner *et al.*, 2016; Tiegs *et al.*, 2018; Hammond *et al.*, 2018; Du *et al.*, 2018). Most recently, Whitney *et al.* found a subset of biopsied day 7 embryos that were euploid (35.9%), therefore eligible for transfer (Whitney *et al.*, 2019). Moreover, that study observed viable pregnancies and LBs with day 7 embryos, extending an opportunity for patients who do not have day 5 or 6 embryos available during their IVF cycles to become pregnant.

The purpose of our study is to confirm the reproductive potential of day 7 blastocysts. The study both investigates the genetic composition of day 7 embryos biopsied for PGT-A and evaluates patient cycle outcomes following the largest cohort of single, day 7 euploid FET cycles by a single ART center. The study design includes the most modernized approach to current ART treatment standards by incorporating a full spectrum of genetic screening, cryopreservation techniques and transfer selection strategies (Penzias *et al.*, 2018). Finally, this analysis of day 7 embryos aims to promote an additional treatment strategy for patients who otherwise would not have the chance to continue care or achieve a successful pregnancy.

Materials and Methods

Study design and patient populations

Main analysis

A retrospective study was performed at a single center on infertility patients who completed an IVF cycle with PGT-A from January 2012 through March 2018. During IVF cycles, all embryos were cultured through day 7 of development. Cohorts were established based on the day an embryo achieved blastocoel expansion (Modified Gardner's $\geq$4), with TE cells herniating through the zona pellucida, at which time the embryo was biopsied for PGT-A and subsequently cryopreserved by vitrification (day 5, day 6 or day 7) as described previously (Nazem *et al.*, 2019). PGT-A screened embryos received a chromosome copy number analysis and were assigned to the following categories: euploid, aneuploid or inconclusive.

Cases involving multiple blastocyst biopsy, patients using donor oocytes, severe male factor infertility utilizing testicular sperm extraction and patients or partners harboring chromosomal rearrangements, such as balanced translocations or heterochromatic polymorphisms on a peripheral karyotype, were excluded from analysis.

Sub-analysis

The study's sub-analysis included infertility patients who completed an IVF cycle with PGT-A followed by synthetic endometrial preparation and a single, euploid FET cycle from January 2012 through March 2018. Cohorts were established based on the day of embryo biopsy and cryopreservation after oocyte retrieval following controlled ovarian hyperstimulation (COH) (day 5, day 6 or day 7).

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Recipients of donor oocytes and patients with a diagnosis of uterine factor infertility, recurrent pregnancy loss (RPL), recurrent implantation failure (RIF), active hydrosalpinges or severe male factor infertility requiring testicular sperm extraction were excluded from the analysis.

Stimulation protocols

Patients underwent COH for IVF as previously described (Sekhon *et al.*, 2018). Follicular development was monitored using transvaginal ultrasonography. When at least two follicles reached 18 mm in diameter, final oocyte maturation was induced with either hCG (5000–10 000 IU, Novarel, Ferring Pharmaceuticals, Parsippany, NJ, USA), recombinant human chorionic gonadotropin (250–500 µg, Ovidrel, EMD Serono, Rockland, MA, USA) or, in high responders at risk of Ovarian Hyperstimulation Syndrome, a dual trigger with 2 mg of leuprolide acetate (Lupron, AbbVie Laboratories, Chicago, IL, USA) and 1000 IU of hCG. Thereafter, patients underwent vaginal oocyte retrieval under transvaginal ultrasound guidance 36 h after oocyte maturation was triggered.

Laboratory procedures

Embryo culture and TE biopsy techniques

Within 4–6 h following retrieval, oocytes that reached the metaphase II stage (MII) underwent ICSI. From day 0–3 of development, embryo(s) were cultured in Sage Quinn's Advantage® Cleavage Medium (Cooper Surgical, Trumbull, CT, USA) and supplementation was administered periodically (i.e. 5% human serum albumin (100 mg/ml, HSA-Solution™, Vitrolife, Göteborg, Sweden) on day 0, and 10% serum substitute supplement (SSS™ Irvine Scientific, Santa Ana, CA, USA) with 6% protein components consisting of 84% pharmaceutical grade HSA (50 mg/m SSS™, Irvine Scientific, Santa Ana, CA, USA) from day 1 to day 7)). Low-oxygen conditions were provided by mini-incubators (Panasonic Sterisonic GxP incubator, Sanyo North America, Wood Dale, IL, USA) throughout culture (5% oxygen, 5.8% carbon dioxide, 89.5% nitrogen (from day 1 to 3); 5% oxygen, 5.8% carbon dioxide, 89% nitrogen (from day 3 to 7)). Embryo culture was sustained using Nunclon 60-mm dishes with 50 µL microdrops under 100% paraffin oil (Ovoil™, Vitrolife, Göteborg, Sweden). Embryos reaching day 4 in culture were transferred from Sage Quinn's Advantage® Cleavage Medium (zero glucose, pyruvate-dominant) to glucose-rich G-2™ (Vitrolife Blastocyst Media, Vitrolife, Göteborg, Sweden) with 10% supplemental protein (SSS™, Irvine Scientific, Santa Ana, CA, USA). Assisted zona drilling was performed using 2 or 3, 200–300 µs laser pulses (ZILOS-tk Laser, Hamilton Thorne Biosciences, MA, USA) to facilitate TE herniation. Blastocyst TE biopsies were performed on day 5, day 6 or day 7 of embryo development and are contingent on when an embryo reaches a morphologic grade of at least 4CC (modified Gardner score). TE biopsy was carried out under oil in Falcon 1006 Petri dishes (Becton Dickinson, Franklin Lakes, NJ, USA) in 10 µL drops of G-2 media (Vitrolife) supplemented with 10% SSS™. Using Olympus IX70 and IX71 microscopes with Narishige micromanipulators (Nashirige International, Inc. Amityville, NY, USA), the blastocyst was secured with a thick-walled, blunt glass-holding pipette (internal diameter 20–30 µm) for stabilizing the TE. Four to seven TE cells were drawn into the lumen of a thin-walled biopsy pipette (internal diameter 30 µm) and removed from the blastocyst via the use of 300 µs of near-infrared pulsations and gentle traction. The biopsy samples were cryopreserved in hypotonic wash buffer and submitted for chromosome copy number analysis, which was performed with quantitative real-time PCR, and/or next-generation sequencing-based analysis (Next-Gen seq) on two different off-site genetics laboratories (Good Start Genetics Inc., Framingham, MA, USA; Foundation for Embryonic Competence, Basking Ridge, NJ, USA). Biopsied embryos received a genetic interpretation of euploid, aneuploid or inconclusive result as described previously (Hernandez-Nieto *et al.*, 2017). Embryos with mosaic profiles were reported as aneuploid. No mosaic embryos were electively transferred.

Embryo grading

Blastocysts were graded based on a proprietary, center-specific scoring system (Modified Gardner's System) (Veeck *et al.*, 2004) for blastocysts intended to undergo TE biopsy for PGT. The degree of expansion was defined as follows: 1 = early blastocyst: cavity beginning to form, 2 = early blastocyst: cavity is <50% of the volume of the embryo, 3 = full blastocyst: cavity completely fills the embryo, 4 = expanded blastocyst: cavity volume has exceeded volume of embryo in zona resulting in at least four to five cells herniating out of zona, 5 = hatching blastocyst: 50% or more of TE is herniating through zona and 6 = hatched blastocyst: blastocyst completely escaped from the zona. The inner cell mass (ICM) grading was determined as follows: A = many cells tightly compacted, B = some cells tightly compacted or organizing, C = some cells disorganized and D = few cells disorganized. TE was graded as follows: A = many cells forming a cohesive epithelium, B = moderate cells forming a loose epithelium, C = some cells forming a loose epithelium and D = very few cells.

Cryopreservation and rewarming techniques

Blastocysts were cryopreserved immediately after TE biopsy using the Cryotop™ method (Kitazato Corp., Shizuoka, Japan), and rewarmed utilizing the modified Cryotop technique that has been described previously (Kuwayama, 2007). After embryo warming, survival was assessed by the morphologic grade of the TE, ICM and the ability of the blastocoel to re-expand. Embryos were regraded based on the modified Gardner scoring system as described above. Only embryos that survived rewarming and subsequently re-expanded were included in the study. Embryos that had >50% arrested/necrotic TE or ICM cells were catalogued as not surviving the thaw (Hardarson *et al.*, 2012).

Endometrial preparation and ET

ETs were performed into a synthetically prepared endometrium, as previously described (Nazem *et al.*, 2019). For each patient, the uterine cavity was prepared with micronized oral estradiol (Estrace, Teva Pharmaceuticals, NJ, USA) 2 mg twice daily for 4 days, then 2 mg three times daily. After a minimum of 12 days of estradiol administration, transvaginal ultrasonography was performed to assess endometrial lining. When a maximum thickness of at least 7 mm was achieved, 50 mg of intramuscular progesterone in oil (Progesterone injection, Watson Pharma Inc., Parsippany, NJ, USA) was administered daily. For all clinical cases, thawing and transfer of the embryos was carried out on the sixth day of progesterone supplementation regardless of the day of embryo development at time of cryopreservation. Euploid embryos with the top morphology grade were selected for transfer. In gender

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Table I Demographic characteristics, COH parameters and embryologic data comparisons between cohorts.

	Day 5		Day 6		Day 7		ANOVA	
	n = 12 535		n = 11 939		n = 1298			
	Mean	SD±	Mean	SD±	Mean	SD±	p value	
Age at retrieval (years)	35.32	4.66	35.60	4.72	36.01	4.78	<0.0001	*
Body Mass Index (kg/m2)	23.62	4.24	23.62	4.27	23.92	4.39	0.0501	
Prior IVF stimulation cycles	0.53	0.97	0.64	1.10	0.79	1.47	<0.0001	*
Gravida	1.17	1.40	1.25	1.43	1.02	1.33	<0.0001	*
Para	0.42	0.75	0.47	0.81	0.42	0.75	<0.0001	*
Baseline day 3 FSH (IU/mL)	5.97	3.26	5.90	3.21	6.09	3.39	0.14	
AMH (ng/mL)	4.54	5.22	4.03	4.54	3.59	4.13	<0.0001	*
BAFC	14.53	7.71	13.65	7.43	13.02	7.05	<0.0001	*
Day of surge	14.10	19.28	13.64	16.96	14.81	21.99	0.03	*
Cumulative GND dose (IU)	3273.61	1282.32	3443.36	1315.30	3777.54	1277.82	<0.0001	*
Surge E2 (pg/mL)	2641.22	1247.15	2553.73	1232.89	2455.60	1197.60	<0.0001	*
Surge P4 (ng/mL)	0.92	0.54	0.94	0.51	0.94	0.52	0.001	*
Eggs Retrieved	19.86	11.21	19.18	11.11	17.30	9.21	<0.0001	*
MII eggs	15.61	9.15	14.65	9.25	12.98	7.60	<0.0001	*
Fertilized eggs (2PN)	13.19	8.11	12.30	8.13	10.68	6.56	<0.0001	*
Biopsied Blastocysts	8.19	5.21	7.13	4.96	5.65	4.16	<0.0001	*
Aneuploid embryos count/%	5305/12535	42.30%	5271/11939	44.10%	716/1298	55.10%	<0.0001	*
Euploid embryos count/%	6867/12535	54.70%	6325/11939	52.90%	530/1298	40.50%	<0.0001	*
Inconclusive embryos count/%	363/12535	2.80%	343/11939	2.80%	52/1298	4.00%	0.06	

Note: Data presented as mean ± standard deviation, unless stated otherwise. ANOVA = Analysis of variance test; AMH = anti Mullerian hormone; BAFC = basal antral follicle count; COH = Controlled Ovarian Hyperstimulation; E2 = estradiol; FSH = Follicle stimulating hormone; GND = gonadotropins.

*Statistical significance, $P < 0.05$.

selection for family balancing cases, the highest-graded embryo of the preferred genetic sex was transferred. Embryos biopsied on day 5 were preferentially selected over biopsied day 6 embryos of any grade. Among embryos biopsied on the same day of development, ICM grade was prioritized in embryo selection, followed by expansion grade, and then TE grade.

Outcome measures

The primary outcomes analyzed included implantation rate (IR): the number of intrauterine gestational sacs per embryo transferred, CPR: the proportion of patients with ultrasonographically detectable fetal cardiac activity, clinical pregnancy loss (CPL): pregnancy loss occurring after the presence of a confirmed gestational sac, multiple pregnancy: two or more fetal poles with observable cardiac activity after presumed monozygotic splitting and LB: complete delivery of a product of fertilization after ≥22 completed weeks of gestational age, which breathes or shows evidence of life (Zegers-Hochschild *et al.*, 2017)

Statistical methods

Statistical analysis was performed using SAS version 9.4 (SAS institute Inc., Cary, NC, USA). Descriptive data was compared by ANOVA, Chi-squared and Student's *T*-test when appropriate. The results were expressed as percentages, means and SDs with Clopper–Pearson binomial 95% CI. Adjusted odds ratios (OR) with 95% CI were calculated using univariate and multivariate logistic regression analyses to assess the effect of the day of embryo biopsy and the odds of implantation, clinical pregnancy, pregnancy loss, LB and multiple pregnancy. The logistic regression models were fitted with generalized estimating equations (GEE) to account for patients who underwent multiple FET cycles. All variables that showed significance and were thought to be clinically relevant were included as covariates in the model. All *P*-values are two-sided with a clinical significance level of $P < 0.05$.

Power analysis

For the main analysis, a sample size of 524 embryos per group was calculated to detect a difference of 10% in euploidy rate with 90% power (alpha = 0.05).

For the sub-analysis, to detect a difference in LB rates from a single euploid FET, a sample size of 96 ET's per group was calculated to detect a difference of 20% in LB rates with 80% power (alpha = 0.05).

Regulatory approval

This retrospective analysis was approved by an Institutional Review Board. Patient information was de-identified before data analysis.

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Results

Main analysis

A total of 25 772 blastocysts from 4136 patients were analyzed in the study. Nearly half (48.6%) of the embryos analyzed were biopsied on day 5 ($n = 12\,535$), 46.30% on day 6 ($n = 11\,939$) and 5.0% on day 7 ($n = 1298$). In the present study, 23.5% of 5883 IVF/PGT-A cycles ($n = 1385$) yielded only aneuploid embryos. Patient demographic and cycle characteristics are described in Table I. Significant differences were found in patient age at the time of vaginal oocyte retrieval, gravidity, parity, total cumulative gonadotropin international units (IU) used during COH, serum estradiol and progesterone levels the day of ovulation trigger, serum anti-Müllerian hormone (AMH), basal antral follicle counts, number of eggs retrieved, mature (MII) oocyte counts and number of biopsied embryos among cohorts (Table I). When analyzing the chromosomal composition of the embryos based on the day the biopsy was performed, significant differences were found in the rates of euploid embryos between cohorts: 54.7% of day 5 embryos ($n = 6867$), 52.9% of day 6 embryos ($n = 6325$) and 40.8% of day 7 embryos ($n = 530$) ($P < 0.0001$). The percentage of inconclusive PGT-A results was similar between cohorts: day 5: 2.9% ($n = 363$), day 6: 2.9% ($n = 343$) and day 7: 4% ($n = 52/1298$) ($P = 0.06$).

When parsing data by Society for Assisted Reproductive Technology (SART)-defined maternal age groups (A (<35 years), B (35–37 years), C (38–40 years), D (41–42 years) and E (>42 years)), the proportion of aneuploid embryos increased with advancing maternal age. The percentage of euploid embryos was significantly lower in day 7 biopsies compared to day 5 or day 6 for each age category (SART), with the exception of patients aged 38–40 years old ($P = 0.11$) (Fig. 1). Using an adjusted regression analysis with a GEE model that controlled for potential confounders (patient age at oocyte retrieval, BMI, serum AMH level, embryonic morphologic quality and number of embryos biopsied during the cycle), when comparing against day 5 biopsied embryos, there was a significant association between embryos biopsied on day 7 and the odds of aneuploidy (OR = 1.34, CI 95% 1.09–1.45, $P = 0.001$). Also, when comparing day 7 biopsied embryos against day 6 biopsied embryos, a significant association with the odds of aneuploidy was demonstrated (OR = 1.26, CI 95% 1.07–1.16, $P = <0.001$).

Sub-analysis

A sub-analysis was performed to examine differences in IVF outcomes (i.e. IR, CPR, multiple pregnancy rate, LB rate and clinical loss rate) in patients who underwent single, euploid FET over a synthetically prepared endometrium. A total of 3818 single, euploid FET cycles from 2622 patients were analyzed. Cohorts were defined based on the day of embryonic development on which the TE biopsy was performed: day 5 = 2321 FET cycles; day 6 = 1381 FET cycles; and day 7 = 116 FET cycles. Demographic characteristics of the patients are included in Table II. Significant differences were found among the cohorts in patient age at retrieval and age at ET, BMI, serum AMH levels, gravidity, parity status, previous number of oocyte retrievals and previous number of euploid ETs. No other significant differences were found for other stimulation parameters, such as serum estradiol, serum progesterone levels and endometrial thickness on the day of ET (Table II). Clinical IVF outcomes are depicted on Table III. Implantation, clinical pregnancy and LB rates were significantly lower for embryos biopsied on day 7 when compared to the other cohorts; however, no statistical differences were found for clinical loss rate and multiple pregnancy rates from established pregnancies (Table III). When analyzing morphological embryo quality assessment during the single, euploid FET cycle, replaced day 7 embryos were categorized as 'good quality,' (Modified Gardner score ≥4BB) in 58.6% ($n = 68$) of cases, 'moderate quality,' embryos (4BC or 4CB) represented 24.1% ($n = 28$) of the cases and 'fair quality,' embryos (4CC) 17.2% ($n = 20$). These proportions were significantly different when compared with similarly graded day 5 or day 6 embryos (Table III). The percentage of thawed, but non-surviving embryos on the day of ET, gestational age at delivery and reported birthweights at delivery were comparable among cohorts (Table III). When analyzing the quality of day 7 euploid embryo morphology, 'good quality' embryos yielded better IVF outcomes when compared with, 'moderate' and 'fair quality' embryos (Table IV).

Lastly, a logistic regression analysis fitted with a GEE Model to control for multiple confounders showed that after adjusting for endometrial thickness at ET, serum progesterone level at ET, patient's age at oocyte retrieval, patients age at ET, BMI and embryo quality at ET, there was a negative association between embryos biopsied on day 7 and the odds of implantation (OR, 0.32; CI 95%, 0.21–0.48 ($P < 0.001$)), clinical pregnancy (OR, 0.33; CI 95%, 0.21–0.511 ($P < 0.001$)) and LB rates (OR, 0.28; CI 95%, 0.18–0.44 ($P < 0.001$)). However, this association was not found for the odds of CPL (OR 1.34, CI 95% −0.38 to 2.68 ($P = 0.39$)).

Discussion

The results of this retrospective cohort analysis demonstrate that embryos fully expanded on day 7 can be successfully biopsied and selected for transfer during ART treatment. However, we found that day 7 embryos are 34% more likely to be aneuploid compared to embryos biopsied on day 5, and 26% more likely to be aneuploid compared to embryos biopsied on day 6. Furthermore, after adjusting for multiple confounders, the high rate of aneuploidy remained significant with embryos biopsied on day 7. Despite the day of embryo biopsy, PGT-A results were mostly correlated with maternal age (Fig. 1). In the largest and most comprehensive study to date, we observed an overall euploidy rate of 40.5% in embryos biopsied on day 7. Our study's finding is similar to previously published reports that concluded a 20–43.5% euploidy rate for embryos biopsied on day 7 of development (Capalbo *et al.*, 2014; Su *et al.*, 2016; Portmann *et al.*, 2011; Kaing *et al.*, 2018; Whitney *et al.*, 2019).

It has been theorized that delayed first cleavage division (Barrie *et al.*, 2017), cytoplasmic exclusion during compaction stage (Mizobe *et al.*, 2017), embryonic genome activation (Ivec *et al.*, 2011) and patient-related factors such as age or infertility diagnosis (Kirkegaard *et al.*, 2016) may be associated with an increase in embryonic aneuploidy rates (Minasi *et al.*, 2016). Often, we observed poor morphologic scores for day 7 embryos as compared to day 5 or day 6 embryos. For instance, in FET cycles utilizing day 7 embryos, only 58.6% of our day 7 study cohort was classified as 'good' quality embryos (≥4BB) as compared to 95.8% on day 5 and 85.5% on day 6. However, the association between poor morphologic embryo scores and ploidy status has been demonstrated to be 'weak' and at times a significant proportion of aneuploid embryos have high morphologic grades (Capalbo

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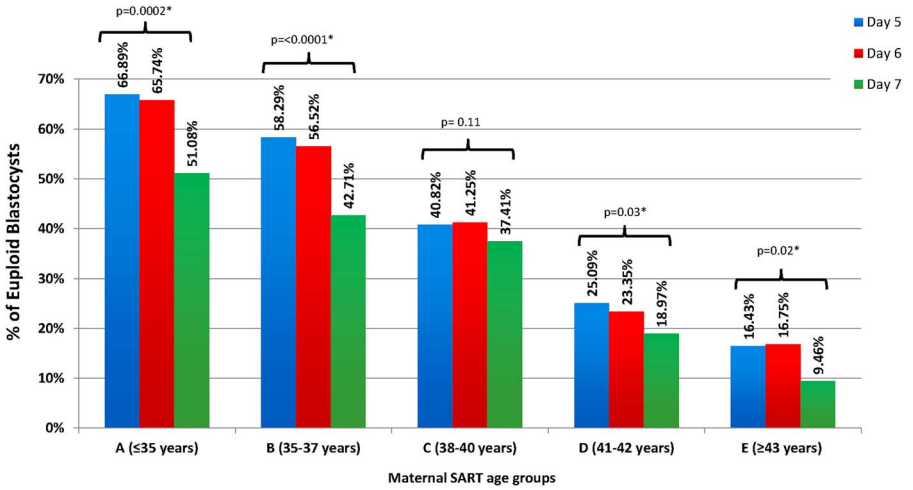

Figure 1 Percentages of euploid embryos classified by SART age group based on embryo biopsy day. Data presented as years (maternal SART age groups) and percentage of euploid blastocysts. Color coding based on day of embryo development: blue: day 5 embryos; red: day 6 embryos; green: day 7 embryos. SART = Society for Assisted Reproductive Technology. * ANOVA, Statistical significance, $P = <0.05$.

Table II Demographic characteristics of patients that underwent a single euploid embryo transfer analyzed by embryo biopsy day.

	Day 5 embryo FET cycles		Day 6 embryo FET cycles		Day 7 embryo FET cycles		ANOVA	
	n = 2321		n = 1381 FET cycles		n = 116 FET cycles			
Characteristic	Mean	SD	Mean	SD	Mean	SD	p-value	sign
Age at Retrieval	35.65	3.93	36.26	4.03	37.00	3.70	<0.0001	*
Age at Transfer	36.01	3.97	36.83	4.06	37.33	3.70	<0.0001	*
BMI (kg/m2)	23.59	4.17	24.00	4.60	24.03	4.48	0.01	*
Gravida	1.01	1.21	1.21	1.29	1.26	1.22	<0.0001	*
Para	0.41	0.73	0.54	0.80	0.55	0.77	<0.0001	*
Peak E2 (pg/mL)	442.28	419.99	421.92	411.39	539.66	604.63	0.22	
Surge P4 (ng/mL)	0.31	0.19	0.32	0.29	0.29	0.12	0.49	
P4 at embryo transfer (ng/mL)	27.40	10.60	28.50	17.50	28.40	8.80	0.07	
AMH (ng/mL)	4.21	4.89	3.16	3.79	2.96	3.87	<0.0001	*
Endometrial Thickness at Transfer (mm)	9.48	2.13	9.42	2.25	9.32	1.96	0.58	
Previous oocyte retrievals	1.44	0.80	1.68	1.27	1.67	1.01	<0.0001	*
Previous Euploid embryo Transfers	0.38	0.62	0.54	0.69	0.47	0.69	<0.0001	*

Note: Data presented as mean ± standard deviation, unless stated otherwise. ANOVA = analysis of variance. E2 = Estradiol; FET = Frozen Embryo Transfer; P4 = Progesterone; AMH = anti-Müllerian hormone.

*Statistical significance, $P < 0.05$.

et al., 2014). Furthermore, we observed that frozen euploid day 7 embryos with better morphologic scores had statistically significant higher IRs (58.8%) when compared with 'moderate' (32.1%) or 'fair' quality embryos (15%). Also when comparing the lower morphological quality day 7 embryos against higher quality day 7 embryo cohorts, we observed that the 'fair' quality group showed very low clinical and LB rates; nevertheless, these differences didn't reach statistical significance, although this data should be interpreted cautiously due to very limited sample sizes (Table IV).

Genomic screening of embryos allows patients to benefit from increased IRs, decreased multiple pregnancy rates and shortened time to pregnancy (Capalbo et al., 2016b; Kang et al., 2016; Treff and Zimmerman, 2017). In our study, 40.5% of day 7 embryos analyzed were found to be euploid and therefore available for transfer. Thus, prior precedent that has established abandoning embryo culture after day 6 merits reassessment. Notably, patients who require embryo culture to day 7 for embryo expansion can be comforted in knowing that some of these embryos may be suitable for transfer.

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Table III Clinical IVF outcomes after a single euploid FET cycle among cohorts based on embryo biopsy day.

	Day 5 embryos		Day 6 embryos		Day 7 embryos			
	N	%	N	%	N	%	p value	sign
Implantation rate	1809/2321	**77.9**	962/1381	**69.6**	52/116	**44.8**	0.0001	*
Clinical pregnancy rate	1520/2321	**65.4**	777/1381	**56.2**	35/116	**30.1**	0.0001	*
Live birth rate	1311/2321	**56.4**	633/1381	**45.8**	25/116	**21.5**	0.0001	*
Multiple pregnancy rate	18/1311	**1.3**	14/633	**2.2**	0/25	**0**	0.44	
Clinical loss rate	209/1809	**11.5**	144/962	**14.9**	10/52	**19.2**	0.34	
Gestational age at delivery (weeks) Mean/SD	38.3	±2.3	38.1	±2.1	38.2	±2.1	0.2	
Delivery birthweight (gr) Mean/SD	3332.69	±587.65	3282.79	±590.89	3195.62	±496.88	0.3	
FET Embryo quality:								
-Good	2224/2321	**95.8**	1181/1381	**85.5**	68/116	**58.6**	0.0001	*
-Moderate	61/2321	**2.6**	131/1381	**9.4**	28/116	**24.1**	0.0001	*
-Fair	36/2321	**1.5**	69/1381	**5.0**	20/116	**17.2**	0.0001	*
Thaw/non survival rate	37	**1.55**	35	**2.5**	4	**3.4**	0.11	

Note: Data presented as proportions and percentages, mean and ± standard deviations, unless stated otherwise.
FET = Frozen embryo transfer;
*Statistical significance, $P < 0.05$.

Table IV Clinical IVF outcomes among euploid Day 7 frozen embryo transfers based on morphologic grading.

Day 7 embryos (Embryo Quality Grade$^{\alpha}$):	Good quality (≥4BB)	Moderate quality (4BC or 4 CB)	Fair quality(4CC)	p-value
Embryos transferred	68	28	20	
Implantation rate (%)	40/68 (58.8)	9/28 (32.1)	3/20 (15)	0.04*
Clinical pregnancy rate (%)	27/68 (39.7)	6/28 (21.4)	2/20 (10)	0.1
Live birth rate (%)	18/68 (26.4)	5/28 (17.8)	2/20 (10)	0.39

α = Modified Gardner Scoring System (Expansion; Inner Cell Mass; Trophectoderm).
*= Statistical significance, $P < 0.05$.

The first studies to analyze the reproductive potential of day 7 embryos did not use many of the present-day genomic screening tools or evidence-based treatment strategies available in the modern IVF setting. First, the lack of embryo genomic screening prior to embryo selection may have misrepresented day 7 ET potential (Shoukir *et al.*, 1998; Utsunomiya *et al.*, 2004; Du *et al.*, 2018). Second, studies that included fresh and/or frozen day 7 ETs did not employ a single, euploid FET cycle model (Hiraoka *et al.*, 2008; Hiraoka *et al.*, 2009; Kovalevsky *et al.*, 2013; Du *et al.*, 2018). These first reports did not take advantage of the single, euploid FET model and therefore may have prematurely drawn conclusions about the reproductive potential of day 7 embryos.

Our study distinguishes itself from previous studies evaluating the clinical utility of day 7 euploid embryos: our data show that day 7 euploid embryos had significantly lower IR (44.8% ($n = 52/116$)), CPR (30.1% ($n = 35/116$)) and LB rate (21.5% ($n = 25/116$)) compared to day 5 and day 6 biopsied embryo cohorts (Table III). Even after adjusting for important clinical factors in a sophisticated regression model, differences in these clinical outcomes remain. Our study's transfer cycle success rate is comparable to pregnancy outcomes published by Whitney *et al.*, who showed an IR of 56.3% and a LB rate of 43.8% utilizing single euploid day 7 embryos (Whitney *et al.*, 2019), although that study only included a small cohort of day 7 euploid ETs ($n = 16$), whereas ours included a more robust data set of day 7 euploid ETs ($n = 116$). Conversely, when compared to the results published by Su *et al.*, our study demonstrates higher LB rates compared to that study's reported 13.3% (Su *et al.*, 2016). Additionally, that study was limited to only 15 day 7 euploid embryo FETs. Our study provides the strongest evidence available to date for the reproductive potential of day 7 embryos.

There are potential clinical conditions that may explain the low IR observed in patients who transfer day 7 euploid embryos. Some researchers have scrutinized the quality of the day 7 euploid embryo (Wirleitner *et al.*, 2016; Morbeck 2017; Whitney *et al.*, 2019). In our study, the high incidence of low morphologic quality (<4BB) day 7 FET embryos (17.2%) as compared to day 5 or day 6 FETs embryos (1.5% and 5.0% respectively) may be associated with a loss of cellular viability, increased TE and ICM cellular degeneration and/or increased ICM/TE cell death rates. All of these clinical factors have been previously correlated with delayed embryo growth and extended culture conditions (Hardy *et al.*, 1989; Whitney *et al.*, 2019). A number

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of considerations such as suboptimal blastocyst mitochondrial DNA copy number, increased sensitivity to mosaicism, genome activation errors and a greater prevalence of variants of unknown significance have been suggested to compromise day 7 embryo implantation potential and pregnancy success rates (Morbeck, 2017; Wells, 2017; Fragouli et al., 2018). Moreover, immunological interactions, uterine acceptability of embryos or highly variable technical conditions such as ET technique, embryo culture conditions and TE biopsy-related processes have been correlated with lower clinical outcomes of day 7 ETs (Su et al., 2016; Van Echten-Arends et al., 2011).

There was no statistically significant difference in non-concurrent or inconclusive PGT-A results of day 5 (2.8%), day 6 (2.8%) or day 7 embryos (4%) ($P = 0.06$) (Table I). The slightly increased incidence of uninterpretable analysis results observed for day 7 embryos may be explained by delayed embryo growth, increased cellular degeneration, poor TE cell quality or technical issues, such as conservative interpretation of genetic data and sample collection techniques (Moschini et al., 2014). Nevertheless, this study was not powered to assess this finding. All of these caveats are worthy of greater attention and further evaluation.

When analyzing clinical loss rate, our study demonstrated that day 5 embryos have a CPL rate of 11.5%, contrasted with 14.9% for day 6 and 19.2% for day 7 embryos ($P = 0.34$). Interestingly, differences in clinical loss rates were not statistically significant, even after adjusting for confounders (OR, 0.90; CI 95%, 0.45–1.8; $P = 0.78$). However, the clinical loss rate should be interpreted cautiously because our study sample size was limited and the power analysis was not strong enough to detect this specific outcome. When analyzing multiple pregnancy rates, no differences were found among cohorts (day 5 = 1.3%, day 6 = 2.2%, day 7 = 0.0%). These findings are consistent with other studies in which day 7 embryos had multiple pregnancy rates ranging from 0–15% (Hiraoka et al., 2009; Su et al., 2016; Du et al., 2018).

Notwithstanding our best efforts to avoid biases in the study, some shortcomings and limitations exist in the analysis. The retrospective nature of our study increases the chance of selection bias. However, we utilized a big-data approach and an adjusted regression analysis that utilized a GEE model to minimize this bias. By utilizing a multi-level GEE model we were able to assess associations between our predictor variables and their effect on ploidy and pregnancy outcomes while accounting for the same patient appearing multiple times on different cycles within the same database.

Another limitation of this study is the day of embryo biopsy, which can be considered to be a surrogate endpoint for blastocyst development, as the day of biopsy is dependent on laboratory protocol. Therefore, potential variability across IVF laboratories could limit the external validity of our findings. Another important limitation is relevant within our subanalysis: not all of the single, euploid FET occurred as the first transfer cycle for every patient. In some cases, patients had day 5 and day 6 embryos for transfer; if unsuccessful, they had the opportunity to use euploid day 7 blastocysts in subsequent FET cycles. Nevertheless, our statistical analysis utilizing a GEE model accounted for these repeated measures, and the correlations between all the important variables related to embryo implantation were also corrected during the statistical analyses.

The data analyzed in this study came from a single, high-volume center experienced in TE biopsy and encompassed a diverse cohort of patients (i.e. normal, low and high responders) who electively had embryos screened by PGT-A for a broad array of indications. Our analysis excluded patients with recognizable factors for failing embryo implantation or poor embryonic development, such as severe male factor infertility cases with testicular extracted sperm, patients or partners harboring chromosomal rearrangements such as balanced translocations or heterochromatic polymorphisms, diagnosis of RPL, RIF, uterine factor infertility and confirmed presence of hydrosalpinges, thus making our findings more generalizable.

Furthermore, our study was appropriately powered for the main outcomes of interest (differences in ploidy rates and LB outcome) and the total number of cycles/embryos analyzed met the required sample sizes based on our power analysis.

Another strength of our study is the use of a clinically validated PGT-A technique, which ensured uniformity within the embryonic genetic results (Fiorentino et al., 2014; Werner et al., 2014; Lee et al., 2015). However, as chromosomal analysis involves the assessment of only a few cells extracted from the embryo, the real incidence of embryo mosaicism and copy number variants was not analyzed or reported for this study.

To our knowledge, this study is the largest to evaluate a cohort of single, day 7 euploid embryos used for transfer by patients who underwent IVF that describes gestational age at delivery and birthweight. Only one other published article has described gestational age and birthweights in patients who utilized day 7 ETs. Du et al. showed no differences in low birth rates, congenital malformations and early deaths on a large cohort of neonates. However, this analysis did not screen embryos for genomic composition prior to transfer in a synthetically prepared environment (Du et al., 2018). When comparing transfer selection of day 5, day 6 or day 7 embryos, we demonstrated no differences in average gestational ages (day 5 = 38.3 ± 2.3 weeks, day 6 = 38.14 ± 2.1 weeks and day 7 = 38.2 ± 2.1 weeks, ($P = 0.2$)) and average birthweights at delivery (day 5 = 3332 ± 587 grams, day 6 = 3282 ± 590 grams and day 7 = 3195 ± 496 grams ($P = 0.3$)).Thus, we add further insight and greatly contribute to the growing body of evidence supporting day 7 embryo culture and use in the modern IVF setting.

Developmental stage, morphological grade and ploidy status remain paramount factors influencing ET selection and affecting blastocyst implantation potential. Our data concurs with prior reports of the clinical utility of day 7 embryos in the modern IVF setting (Kovalevsky et al., 2013; Capalbo et al., 2014; Su et al., 2016; Wirleitner et al., 2016; Du et al., 2018; Haas et al., 2019; Kaing et al., 2018; Whitney et al., 2019). Our robust dataset and sophisticated modeling is encouraging, and we recommend routine extension of embryo culture until day 7 of embryo development.

We advise using high morphologic quality day 5 and day 6 euploid embryos as a first-line approach during ART treatment. However, for patients with embryos requiring extended culture, there is reassurance that a successful pregnancy outcome is possible with the transfer of a day 7 euploid embryo.

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Authors' roles

All the authors of this manuscript have made substantial contributions to the conception or design of the work or the acquisition, analysis or interpretation of data for the work and have contributed to drafting the work or revising it critically for important intellectual content. All authors have approved the final version to be.

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Conflict of interest

Dr Alan Copperman is Advisor or Board Member of Sema 4 (Stake holder in Data), Progyny and Celmatix.

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