Pushing the limits of detection: investigation of cell-free DNA for aneuploidy screening in embryos

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Objective: To determine the accuracy of cell-free DNA (cfDNA) in spent embryo medium (SEM) for ploidy and sex detection at the cleavage and blastocyst stages. To determine if assisted hatching (AH) and morphologic grade influence cfDNA concentration and accuracy.
Design: Prospective cohort.
Setting: Academic fertility center.
Patient(s): Nine patients undergoing IVF; 41 donated two-pronuclei embryos and 20 embryos from patients undergoing preimplantation genetic testing for aneuploidy (PGT-A).
Interventions(s): In a donated embryo arm, SEM was collected on days 3 and 5, with one-half of the embryos undergoing AH before and one-half after. In a clinical arm, SEM was collected on day 5 before trophectoderm (TE) biopsy. Samples underwent PGT-A with the use of next-generation sequencing. cfDNA results were compared with corresponding whole embryos and TE biopsies.
Main Outcome Measure(s): Concordance rates, sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for ploidy and sex detection with the use of cfDNA.
Result(s): Of 141 samples, cfDNA was amplified in 39% and 80.4% of days 3 and 5 SEM, respectively. Concordances for ploidy and sex, respectively, were 56.3% and 81.3% between day 3 cfDNA and whole embryos, and 65% and 70% between day 5 cfDNA and TE biopsies. Day 5 cfDNA sensitivity and specificity for aneuploidy were 0.8 and 0.61, respectively. PPV and NPV were 0.47 and 0.88, respectively. Timing of AH and morphology did not influence cfDNA concentration or accuracy.
Conclusion(s): cfDNA is detectable on days 3 and 5, but more accurate on day 5. Although our data suggest moderate concordance rates, PGT-A with the use of cfDNA must be further optimized before clinical implementation. (Fertil Steril® 2018;110:467–75. ©2018 by American Society for Reproductive Medicine.)
El resumen está disponible en Español al final del artículo.

Key Words: Cell-free DNA, preimplantation genetic testing for aneuploidy, next-generation sequencing

Selecting the best embryos is crucial for improving patients' chance of achieving a live birth with the use of in vitro fertilization (IVF). Blastocyst transfer and preimplantation genetic testing for aneuploidy (PGT-A) have led to improved outcomes (1–3). Although transferring euploid embryos improves implantation rates, this may be a less viable option for older women or those with severely diminished ovarian reserve (4, 5). This patient population may produce fewer or no blastocysts during an IVF cycle, with reported no-blastulation cycle rates ranging from 7.6% to 32.0% (6, 7). For these reasons, there is increasing interest in noninvasive methods for embryo assessment. Well studied tools include time-lapse morphokinetics and metabolomics of spent embryo medium (SEM). However, there is limited evidence that these approaches improve clinical outcomes (8–12).

Studies have investigated nucleic acids in SEM and demonstrated different microRNA and mitochondrial DNA profiles in embryos based on quality and implantation potential (13–15). The

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next logical application for SEM is the use of cell-free DNA (cfDNA) for aneuploidy screening. Recent studies have documented the ability to detect and sequence cfDNA in SEM with the use of cytogenic techniques such as array comparative genomic hybridization (aCGH) and modern sequencing platforms such as single-nucleotide polymorphism (SNP) sequencing and next-generation sequencing (NGS) (16–18). Although results have been encouraging, further studies are needed to assess the validity of this tool on different sequencing and bioinformatics platforms before clinical implementation. In the present study, our primary aim was to investigate the accuracy of cfDNA in SEM with the use of an improved method for DNA capture followed by NGS. We also sought to determine other factors that may influence accuracy of cfDNA, such as the timing of SEM collection, timing of assisted hatching (AH), and morphologic grade of the embryos. We also sought to establish a threshold concentration for which cfDNA could be detected and used to accurately predict the chromosomal status of an embryo. We hypothesized that cfDNA would have >90% concordance with whole embryos and trophectoderm (TE) biopsies. We also hypothesized that AH would increase cfDNA concentration and the accuracy of sequenced cfDNA for aneuploidy screening. We predicted that poor morphologic grade would be associated with higher DNA shedding and higher accuracy of cfDNA for aneuploidy screening.

MATERIALS AND METHODS

We conducted a prospective study comparing the accuracy of aneuploidy screening with the use of cfDNA in SEM compared with TE biopsies and whole embryos by means of NGS. The study was composed of a pilot portion with two separate arms using donated research embryos, as well as a clinical portion using patient samples. Institutional Review Board approval was obtained from the University of Southern California (HS-15-00858).

Pilot Study

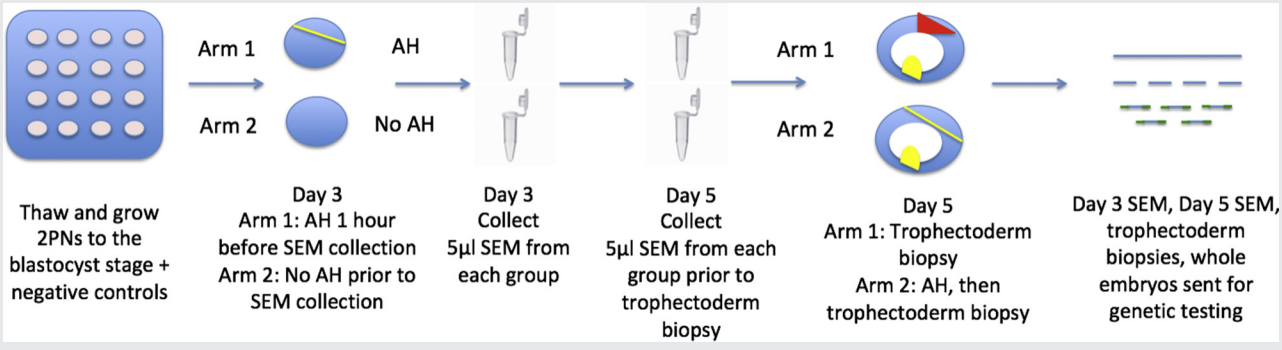
For the pilot study, previously cryopreserved embryos donated to research were used. All embryos were from previous oocyte donation cycles, previously fertilized by means of intracytoplasmic sperm injection (ICSI) and subsequently cryopreserved at the zygote stage by means of the slow-freeze technique with 1.5 mol/L propylene glycol and 0.1 mol/L sucrose (Irvine Scientific). Embryos were thawed with the use of Irvine Scientific embryo thaw kit with serial dilutions of cryoprotectant. After the thaw, embryos were placed in labeled wells corresponding to their study ID. All embryos were cultured in 25 μL continuous single-culture medium (CSC; Irvine Scientific) with the use of a Vitrolife Micro-Droplet dish overlayed with Liteoil (Life Global) at 37°C with 5% O_2 and 8% CO_2. They were removed from the incubator on days 3 and 5 for morphologic grading and for collection of SEM and TE biopsy.

We investigated factors that may potentially influence the concentration and accuracy of cfDNA, including timing of SEM collection and AH. In the first arm, AH was performed on day 3, after which 5 μL SEM was collected on both days 3 and 5. Blastocysts then underwent TE biopsy on day 5. In the second arm, we collected 5 μL SEM on days 3 and 5, before any AH. Embryos then underwent AH on day 5 after SEM collection, followed by TE biopsy. All corresponding whole embryos were saved for sequencing. All samples (days 3 and 5 SEM, TE biopsies, and corresponding whole embryos) were placed in RNase- and DNase-free polymerase chain reaction (PCR) tubes and stored at −30°C until ready for analysis. Negative control samples were culture medium placed in empty wells with the same incubation parameters. Pipette tips were changed between sample collections to avoid contamination. For all TE biopsies, the Lykos laser system (Hamilton Thorne) was used to remove approximately five cells (Fig. 1).

Clinical Study Design

For the clinical arm, we prospectively recruited patients planning to undergo PGT-A as a part of their IVF cycle from March through August 2017. All oocytes underwent stripping of all visible cumulus cells with the use of hyaluronidase before ICSI. Stripped mature oocytes were then fertilized by means of ICSI and cultured per clinical protocol in continuous single-culture medium (as described above). All blastocysts underwent AH on day 5 and TE biopsy on day 5 or 6. Five

FIGURE 1



Experimental diagram for pilot study. 2PN = two pronuclei; AH = assisted hatching; SEM = spent embryo medium.

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μL SEM was collected from wells after embryos were removed for AH and biopsy. All samples (SEM and TE biopsies) were placed in RNase- and DNase-free PCR tubes, and stored at −30°C. All pilot and clinical samples were shipped on dry ice to a genetic laboratory for testing.

Morphologic Grading

On day 3 of embryo development, a cleavage-stage morphologic score was assigned based on a 3-point grading system using features including cell number, fragmentation, symmetry, and shape (19). At the blastocyst stage, morphologic score was based on expansion stage, quality of inner cell mass, and quality of TE (20).

Chromosomal Analysis

TE biopsies and whole embryos were subject to cell lysis followed by whole-genome amplification (WGA) with the use of Picoplex (Rubicon). During the WGA process, DNA was randomly amplified. Day 3 and 5 SEM samples underwent WGA in a similar fashion, except that SEM underwent 20 cycles instead of the standard 14 cycles to ensure that enough DNA would be amplified. WGA products were quantified with the use of Qubit dsDNA HS Assay (Life Technologies) and 300 ng whole-genome DNA was subject to fragmentation and adaptor ligation. The pool of libraries was quantified, and 26 pmol/L was used for emulsion PCR for template enrichment on Ion Sphere using the OT2 200 Kit (Life Technologies). The enriched library was then further purified to remove non-templated spheres, with the use of Ion One Touch ES Enrichment. Samples were individually barcoded, then mixed with a specific complexity to allow at least 200,000 reads per sample. For the library, ~40 samples were loaded onto each Ion 530 chip with a chip capacity of >12 million reads and a chip loading of 80%. The final library was sequenced with the use of the Ion S5 Sequencer (Life Technologies). Each sample had an average of 150,000–200,000 reads and ~200 bp size per amplicon, totaling 30–40 million bp per sample. All reads were filtered for polyclonals and aligned to the human genome database with the use of Torrent Suite Software for Sequencing Data Analysis. Quality reads were scored for aneuploidy with the use of Ion Reporter 5.0 software (Thermo Fisher Scientific). Each sample had a unique study ID, and investigators performing NGS on SEM were blinded regarding which arm of the study design samples derived from, as well as regarding the results of referent samples (whole embryos or TE biopsies).

Statistical Analysis

Concordance rates were calculated for aneuploidy and sex between the following groups: day 3 SEM and corresponding whole embryos, day 5 SEM and whole embryos (for pilot samples), day 5 SEM and TE biopsies, and TE biopsies and whole embryos. Chi-square, Pearson exact, and Kruskal-Wallis tests were used to test for association between concordance and blastulation status, AH (yes vs. no), ploidy (euploid vs. aneuploid), embryo morphology (fair/good vs. poor), and fragmentation index (high ≥20%, medium 10%–20%, and low <10%). Day 3 morphology was used with statistical tests using day 3 cfDNA, and day 5 morphology with statistical tests using day 5 cfDNA. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated for detection of aneuploidy and sex. Given that previous data on cell-free fetal DNA (cffDNA) showed an increased ability to distinguish aneuploid cases with increasing sequencing depth, we also performed a logistic regression to test the relationship between sequence depths of days 3 and 5 cfDNA and predictive value for determining ploidy status (21). Receiver operating characteristic (ROC) curves were created and the areas under the ROC curve (AUCs) were determined. The AUC measures how well a test can distinguish between normal and abnormal groups. A value of 1 represents a perfect test and 0.5 represents inability to distinguish between groups. *P* values <.05 with the use of a two-tailed alpha were considered to be significant. Stata 13 (Statacorp) was used for analyses. Samples yielding results with an indeterminate read for ploidy or sex were resequenced. Samples with any final indeterminate read were excluded from our analysis.

RESULTS

For the pilot study, 45 research embryos, from three different patients, previously cryopreserved at the zygote stage were thawed; 41/45 embryos (91.1%) survived the thaw, and 23/41 (56%) became blastocysts. At day 3, cfDNA was detectable in 40/41 (97.6%) of SEM samples, and only 16/41 samples (39%) generated sufficient sequence reads to obtain accurate chromosome copy status. On day 5, cfDNA was detectable in 40/41 (97.6%) of day 5 SEM samples, and 33/41 (80.4%) generated reads. Median day 3 cfDNA concentration was significantly higher on day 3 (111 ng/μL, interquartile range [IQR] 96.6–120 ng/μL) than on day 5 (105 ng/μL, IQR 41.4–117 ng/μL; *P*<.01). From our clinical arm, the median age at oocyte retrieval was 37 (range 33–43) years. All (20/20, 100%) day 5 SEM samples were sequenced, with the median cfDNA concentration being 88.7 ng/μL (IQR 84.3–92.7 ng/μL). The lowest concentrations of cfDNA leading to accurate detection of ploidy were 76.2 ng/μL on day 3 and 63.2 ng/μL on day 5 (Supplemental Table 1, available online at www.fertstert.org).

The concordance rate between TE biopsies and whole embryos for ploidy and sex was 25/27 (93%) and 26/27 (96.3%), showing a high concordance as previously established (21). Concordances between day 3 cfDNA and corresponding whole embryos were 9/16 (56.3%) for ploidy and 13/16 (81.3%) for sex. Concordances between day 5 cfDNA and whole embryos were 15/33 (45.5%) for ploidy and 26/33 (78.8%) for sex. A total of 33/41 research embryos (80.5%) generated sequence reads, with 20/33 (60.6%) of those progressing to the blastocyst stage and 13/33 (39.4%) arresting before the blastocyst stage. Concordance for ploidy in day 5 SEM and whole embryos was significantly higher in the blastocyst versus arrested embryo group: 13/20 (65.0%) versus 2/13 (18.2%); *P*=.005. However, concordance was not different for sex between blastocysts and arrested embryos: 17/20 (85%) versus 9/13 (69%); *P*=.28. For all blastocysts, including research (n = 20) and clinical (n = 20) samples, concordances for ploidy and sex

between day 5 cfDNA and TE biopsies were 26/40 (65%) and 28/40 (70%). Of blastocysts, there were 8/19 (42.1%) false positive and 3/21 (14.3%) false negative calls for aneuploidy from day 5 cfDNA. In determining embryo sex, male embryos were more likely to be misdiagnosed than female with the use of cfDNA. There were 26 female and 27 male embryos (as determined from sequencing the whole embryo or TE biopsy). However, from day 5 cfDNA, 17/27 male embryos(63%) were misdiagnosed as female whereas 0/26 of female embryos (0%) were misdiagnosed as male (P=.001; Table 1; Supplemental Table 2; Supplemental Figs. 1 and 2 [Supplemental Table 2 and Supplemental Figs. 1 and 2 available online at www.fertstert.org]).

Sensitivity, specificity, PPV, and NPV were calculated for detection of aneuploidy and sex with the use of cfDNA. The reference for day 3 cfDNA was the corresponding whole embryo read and for day 5 cfDNA was the corresponding TE biopsy read. Day 5 cfDNA had a sensitivity of 0.8, specificity of 0.61, PPV of 0.47, and NPV of 0.88 for aneuploidy detection and overall performed better than day 3 cfDNA (Table 2). The odds ratio for determining ploidy based on sequence depth with the use of day 3 cfDNA was 0.97 (95% confidence interval [CI] 0.95–0.99; P=.02). The AUC was 0.82, suggesting that sequence depth discriminated between euploid and aneuploid embryos, with our data showing that aneuploid embryos had a higher number of reads on day 3. The odds ratio for day 5 cfDNA was 0.99 (95% CI 0.98–1.0; P=.12). The AUC was 0.6, indicating that day 5 cfDNA sequencing depth serves as a poor screening tool for distinguishing euploid and aneuploid embryos. Although increasing sequencing depth has been shown to provide better discrimination for targeted trisomies 21 and 18 aneuploidy screening using cffDNA in maternal serum, this is not true of general aneuploidy screening using embryonic cfDNA in SEM (21) (Fig. 2).

AH was not associated with a difference in cfDNA concentrations, either on day 3 (median cfDNA was 112 vs. 110 ng/μL for AH vs. no AH, respectively; P=.83) or on day 5 (median cfDNA was 89.2 vs. 106 ng/μL for AH vs. no AH; P=.17). Concordance rates for ploidy and sex were not significantly different between AH and no AH groups for day 3 and day 5 (Table 1). When comparing euploid versus aneuploid embryos, cfDNA concentration from day 3 SEM was higher in aneuploid versus euploid embryos (115.58 vs. 102.83 ng/μL; P=.08). The number of sequence reads was higher in aneuploid embryos versus euploid embryos from day 3 cfDNA as well (113,388 vs. 70,030; P=.008). This relationship was not significant for cfDNA concentration (84.6 vs. 85.8 ng/μL; P=.9) or number sequencing reads (157,036 vs. 139,500; P=.13) from day 5 SEM.

Morphology and fragmentation were not associated with cfDNA concentration or with concordance rates. Embryos with low, moderate, and high fragmentation had median concentrations of 114, 96.2, and 112 ng/μL (P=.13), respectively, on day 3, and 86.2, 92.8, and 103 ng/μL (P=.36) on day 5. Good/fair versus poor embryos had similar cfDNA concentrations on day 3 (110 vs. 120 ng/μL; P=.2) and day 5 (89.8 vs. 86 ng/μL; P=.7). Concordance rates for ploidy were not significantly different between good/fair versus poor morphology embryos with the use of day 5 cfDNA: 19/28 (67.9%) versus 7/12 (58.3%); P=.56. There were no poor-quality embryos yielding cfDNA on day 3. When comparing low/medium and high fragmentation embryos, there was no difference in concordance for ploidy with the use of day 3 cfDNA: 3/5 (60%) vs. 6/11 (54.5%); P=.84. Concordance was similar in low/medium versus high fragmentation embryos with the use of day 5 cfDNA: 18/29 (62%) vs. 8/11 (72.7%); P=.53.

DISCUSSION

Age-associated aneuploidy contributes to decreased pregnancy rates and higher miscarriage rates (22, 23). To obviate this problem, clinicians have incorporated PGT-A to improve the selection of euploid embryos. TE biopsies from blastocysts have been shown to be safer than cleavage-stage blastomere biopsies and do not have negative effects on implantation rates according to a randomized control trial (24, 25). TE biopsies also provide more accurate reads owing to a higher number of cells sampled and possibly

TABLE 1

Concordance rates between cell-free DNA (cfDNA), trophectoderm biopsy, and whole embryos, n (%).

	Concordance for ploidy				Concordance for sex			
Pair	Total	AH	No AH	*P* value[c]	Total	AH	No AH	*P* value[c]
Day 3 cfDNA vs. whole embryo[a] (n = 16)	9/16 (56.3%)	4/8 (50.0%)	5/8 (62.5%)	.61	13/16 (81.3%)	5/8 (62.5%)	8/8 (100%)	.06
Day 5 cfDNA vs. whole embryo[a] (n = 33)	15/33 (45.5%)	5/16 (31.3%)	10/17 (58.8%)	.11	26/33 (78.7%)	12/16 (75.0%)	14/17 (82.4%)	.61
Day 5 cfDNA vs. trophectoderm biopsy[b] (n = 40)	26/40 (65.0%)	16/28 (57.1%)	10/12 (83.3%)	.16	28/40 (70.0%)	17/28 (60.7%)	11/12 (91.7%)	.07
Day 5 trophectoderm biopsy vs. whole embryo[a] (n = 27)	25/27 (92.6%)	12/14 (85.7%)	13/13 (100%)	.22	26/27 (96.3%)	14/14 (100%)	12/13 (92.3%)	.48

Note: AH = assisted hatching.
[a] Includes research embryos only.
[b] Includes both research embryos and clinical samples.
[c] Chi-square analysis or Fisher exact test used to compare AH vs. no AH groups; P<.05 was considered to be significant.

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TABLE 2

Characteristics of cell-free DNA (cfDNA) as a screening test for aneuploidy and sex.

Test	Sensitivity	Specificity	Positive predictive value	Negative predictive value
Day 3 cfDNA aneuploidy detection (n = 16)	0.33 (−0.2-0.87)	0.69 (0.44–0.94)	0.20 (−0.15-0.55)	0.82 (0.69–1.05)
Day 3 cfDNA sex detection	0.38 (0.12–0.65)	0.8 (0.55–1.05)	0.71 (0.38–1.05)	0.5 (0.26–0.75)
Day 5 cfDNA aneuploidy detection (n = 40)	0.80 (0.55–1.0)	0.61 (0.41–0.81)	0.47 (0.23–0.71)	0.88 (0.71–1.0)
Day 5 cfDNA sex detection	0.34 (0.15–0.54)	1.0 (1.0–1.0)	1.0 (1.0–1.0)	0.57 (0.41–0.71)
Trophectoderm biopsy aneuploidy detection (n = 27)	1.0 (1.0–1.0)	0.90 (0.78–1.03)	0.75 (0.45–1.05)	1.0 (1.0–1.0)
Trophectoderm biopsy sex detection	1.0 (1.0–1.0)	0.94 (0.84–1.05)	0.88 (0.65–1.1)	1.0 (1.0–1.0)

Values depicted as point estimate (95% confidence interval).

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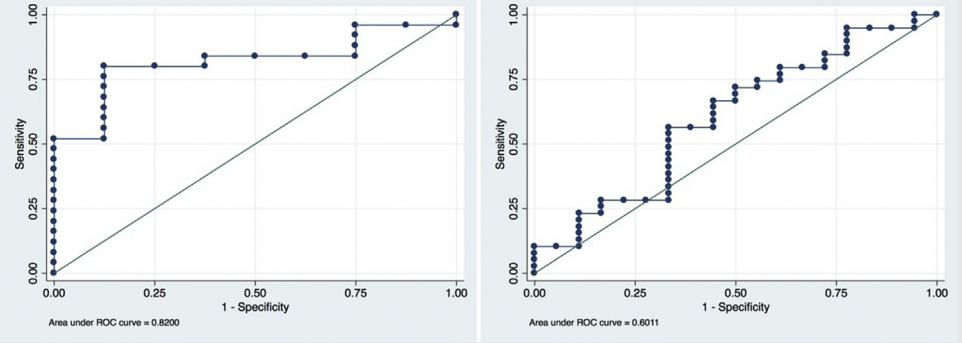
aneuploidy self-correction that occurs during embryo development (26, 27). However, there is still debate regarding the efficiency of PGT-A and whether it improves live birth rates across all age groups (28–30). cfDNA has emerged as a noninvasive strategy for aneuploidy screening. If optimized, it may be an option for those that produce no or poor-quality blastocysts, and it may theoretically mitigate potential adverse effects of TE biopsies on embryos, particularly in laboratories with less experience performing them. Two studies involving aspiration of blastocele fluid (BF) as a potential source of cfDNA have yielded reasonable concordance rates (48%–97%) (31, 32). However, studies of BF have not been replicated with the use of modern sequencing platforms, and techniques for BF collection still involve embryo manipulation. SEM has been more recently investigated, with one study showing a high accuracy for ploidy screening with the use of NGS (86%) (17). Despite this, it is necessary to replicate results in different centers with different sequencing and bioinformatics platforms to assess generalizability of previous findings. In addition, it was unclear whether accurate diagnoses of aneuploidy could be made earlier in the cleavage stage.

cfDNA can be derived from apoptotic or necrotic cells, or from release by actively dividing cells (33). cfDNA usually consists of fragmented segments averaging 70–200 bp in length compared with genomic DNA, which is 3 million bp (34). This brings into question whether random fragments of cfDNA released by the embryo provide sufficient genomic coverage. With TE biopsy, an average of five cells are sampled, and, after cell lysis and amplification, the typical genomic coverage is ∼72% with a depth of 30× reads (35, 36). With the use of the Thermo Fisher Scientific platform for NGS, the coverage for TE biopsies is 50–60 million reads, which leads to ∼2% coverage of the genome. We were able to achieve similar numbers of reads for cfDNA in SEM. Despite the ability to reliably sequence cfDNA on day 5, calculations for specificity and NPV as 0.61 and 0.88 are still not high enough to reassure patients that a euploid embryo is being selected. cffDNA as a screening tool for trisomies 21, 18, and 13, respectively, yields specificity values of 0.99, 0.9, and 1.0 and NPVs of 1.0, 1.0, and 1.0 (37). Meanwhile, PGT-A with TE biopsy with the use of NGS yields a specificity of 0.99–1.0 and NPV of 1.0 for aneuploidy (38, 39). Xu et al. published rates

FIGURE 2



Receiver operating characteristic curves for day 3 and day 5 cell-free DNA (cfDNA) based on sequencing depth. (*Left*) The area under the receiver operating characteristic curve (AUC) for day 3 cfDNA with the use of varying sequencing depth cutoffs was 0.82. This indicates that sequencing depth does discriminate between euploid and aneuploid embryos, with aneuploid embryos having higher amounts of DNA in spent embryo medium. (*Right*) The AUC for cfDNA predictive values with the use of varying sequencing depth cutoffs was 0.60. This indicates that sequencing depth does not discriminate between euploid and aneuploid embryos and does not enhance day 5 cfDNA as a screening tool for aneuploidy.

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of specificity and NPV as 0.84 and 0.91 with cfDNA sequenced on an MALBEC-NGS platform (17). These rates are higher than what we found in our study, possibly owing to differences in methodology. In their study, embryos were previously vitrified on day 3 and thawed and grown to the blastocyst stage. In our study, research embryos were previously cryopreserved and thawed at the zygote stage and clinical embryos were grown in continuous medium after fertilization. This may potentially lead to detection of cfDNA from residual cumulus cells. Although it is theoretically possible that residual cumulus cells not completely stripped may lead to maternal contamination, we attempted to minimize this effect by removing all visible cumulus cells from oocytes before ICSI. Vera-Rodriguez et al. reported a maternal contamination rate of 60.8% in cfDNA from SEM (18). Their study reported a lower concordance rate than Xu et al., with matching aneuploid calls at 30.4% (18). Although our overall concordant rate for ploidy with the use of day 5 cfDNA was 65%, if we exclude matching 46,XX calls (because of an inability to account for maternal contamination in these samples), we have a concordant aneuploid call rate of 8/30 (27%) between day 5 cfDNA and TE biopsies, similarly to Vera-Rodriguez et al. They also reported a sensitivity for Y chromosome detection of ~29.6%, similar to our 34% sensitivity for Y chromosome detection using day 5 cfDNA (18). The misdiagnosis of male embryos as female may reflect a limited ability to amplify and sequence the Y chromosome from low DNA concentrations. However, the similar rate of discordant aneuploid calls and low sensitivity for Y chromosome detection suggest that maternal contamination likely diminishes the ability to detect and sequence pure embryonic DNA. An alternate explanation for misdiagnosis is detection of cfDNA from individual cells that have undergone postmitotic error or amplification error. Another factor to consider with cfDNA is the inability to detect and characterize mosaicism, which carries a higher risk of miscarriage, failed implantation, and lower pregnancy rates (40, 41). We could not assess for mosaicism in our study owing to the nature of the DNA source; this remains to be characterized in future studies.

Despite having higher concentrations of cfDNA on day 3, high-quality cfDNA was more likely to be successfully amplified in day 5 SEM. However, we were still able to achieve accurate reads from some of the day 3 cfDNA samples. Interestingly, there was a significant difference in sequence reads for aneuploid versus euploid embryos on day 3, suggesting that at that stage, abnormal cells may have higher rates of DNA shedding via cell degradation or active secretion of DNA into the medium, which leads to detection of cfDNA not reflective of the rest of the embryo. Compared with the cleavage stage, blastocyst-stage embryos may have a higher proportion of embryonic cfDNA reflective of actual ploidy status in SEM as dividing embryos continue to secrete cfDNA while cfDNA released from abnormal cells is concurrently degraded.

AH is used before TE biopsy, but has also been used with the goal of improving clinical pregnancy rates in poor-prognosis patients (42, 43). We hypothesized that AH would facilitate the release of cfDNA into the culture medium. Though AH is thought to be benign in the human preimplantation embryo, temperatures during AH with the use of the diode laser beam can reach 130–160°C (44). A study on mouse embryos examining the effects of the diode laser on DNA damage found higher DNA damage in AH versus non-AH embryos, and even higher proportions when AH was performed earlier in embryonic development (45). Any potential DNA damage from laser AH could theoretically lead to cross-contamination with actively secreted biologic DNA. In contrast to our hypothesis, our results demonstrated slightly higher cfDNA concentrations and concordance rates for embryos that did not undergo AH before SEM collection. This, however, did not reach statistical significance, possibly owing to a small sample size.

Strengths of the present study include the exploration of several factors influencing accuracy of screening with the use of cfDNA, including timing of AH and SEM collection as early as the cleavage stage. We were also able to include TE biopsies and whole embryos as reference groups in our research cohort. Limitations include a small sample size and inability to distinguish false aneuploid reads as amplification errors versus contamination from aneuploid cells undergoing apoptosis. Protocols for cfDNA included additional six rounds of amplification, which could theoretically lead to a higher error rate due to amplification bias (46). However, bias is typically minimized during targeted NGS, because there is high coverage for consensus sequences, which reduces the noise from random errors (47). We also did not follow patients to invasive prenatal diagnostic screening, which would provide better information regarding the accuracy of cfDNA as a screening tool.

To our knowledge, this is the first study to sequence cfDNA from the cleavage stage, as well as to determine whether AH influences the viability of cfDNA as a screening tool. cfDNA is detectable and can be successfully sequenced at the cleavage and blastocyst stages, with a minimum concentration of 63.2 ng/μL leading to an accurate ploidy diagnosis. In addition, we found that DNA was more likely to be amplified and accurate on day 5. AH does not appear to be necessary or helpful for detection and sequencing of cfDNA for PGT-A. Although sequence depth does not appear to influence accuracy of ploidy discrimination, enhancing embryonic cfDNA isolation protocols may improve the purity of the sample and thus sensitivity and specificity of sequence reads. In conclusion, cfDNA in SEM is not currently optimized for aneuploidy screening in embryos, but with further improvement, it remains a promising tool for noninvasive PGT-A.

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Ampliando los límites de detección: la investigación de ADN celular libre en la detección de aneuploidías en embriones

Objetivo: Determinar la precisión del ADN celular libre (ADNcl) en el medio de cultivo utilizado por los embriones (MUE) para la detección de ploidías y el sexo en los estadíos de división celular y blastocisto. Determinar si la eclosión asistida (EA) y el grado de morfología influyen en la concentración y precisión del ADNcl.

Diseño: Estudio prospectivo.

Lugar: Centro académico de fertilidad.

Paciente(s): Nueve pacientes sometidas a FIV; 41 embriones de dos pronúcleos donados y 20 embriones de pacientes sometidas a la prueba genética preimplantatoria para aneuploidías (PGP-A).

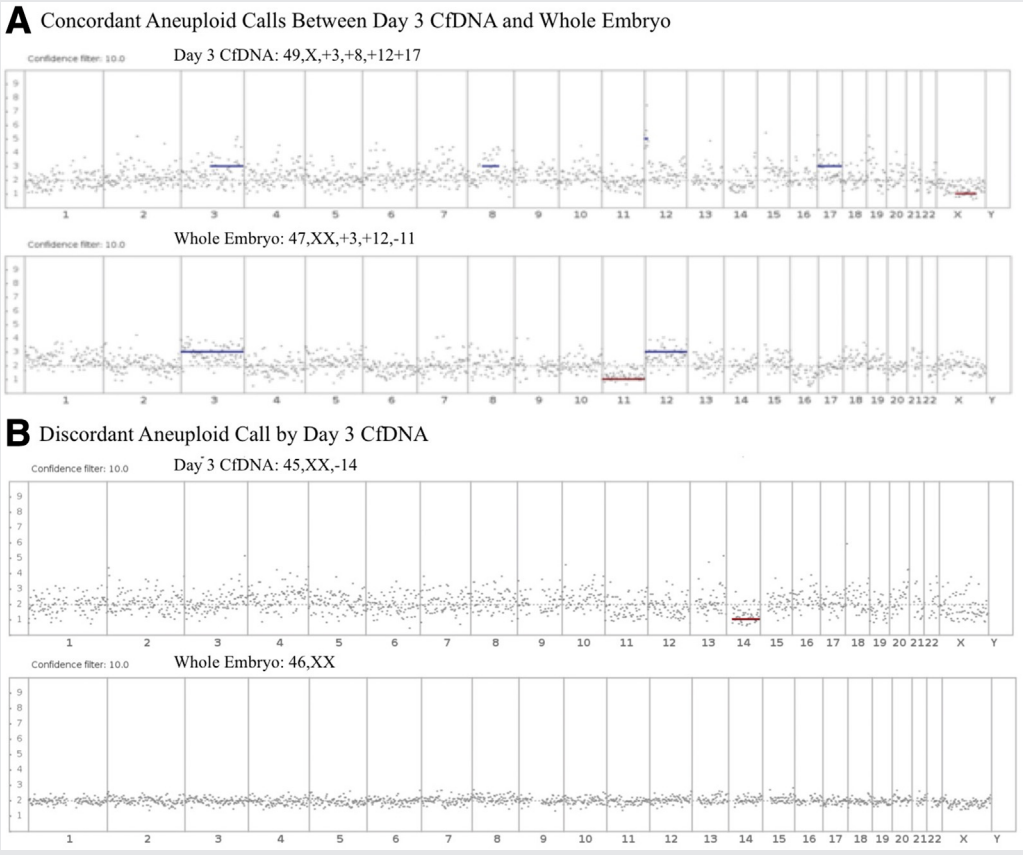
Intervenciones: En la rama de los embriones donados, el MUE fue obtenido en los días 3 y 5, en una mitad de los embriones se obtuvo antes de la EA y en la otra mitad después. En la rama clínica, el MUE fue obtenido en el día 5 antes de la biopsia de trofoectodermo (TE). Las muestras fueron sometidas a PGP-A con el uso secuenciación de última generación. Los resultados de ADNcl fueron comparados con su correspondiente embrión completo y con la biopsia de TE.

Medidas de los resultados principales: Tasas de concordancia, sensibilidad, especificidad, valor predictivo positivo (VPP) y valor predictivo negativo (VPN) para la detección de ploidías y el sexo con el uso de ADNcl.

Resultado(s): En 141 muestras, el ADNcl fue amplificado en el 39% y 80,4% para los MUE de días 3 y 5, respectivamente. Las concordancias entre ploidías y el sexo fueron del 56,3% y 81,3% para el ADNcl de día 3 y embriones completos respectivamente, y el 65% y 70% para el ADNcl de día 5 y las biopsias de TE. La sensibilidad y especificidad del ADNcl para aneuploidías en día 5 fueron 0,8 y 0,61, respectivamente. El VPP y el VPN fueron 0,47 y 0,88 respectivamente. El tiempo en que se realizó la EA y la morfología no influyeron en la concentración ni en la precisión del ADNcl.

Conclusión(es): El ADNcl es detectable en los días 3 y 5, pero es más preciso en el día 5. Aunque nuestros datos sugieren tasas moderadas de concordancia, el PGP-A con el uso de ADNcl debe optimizarse aún más antes de su implementación clínica.

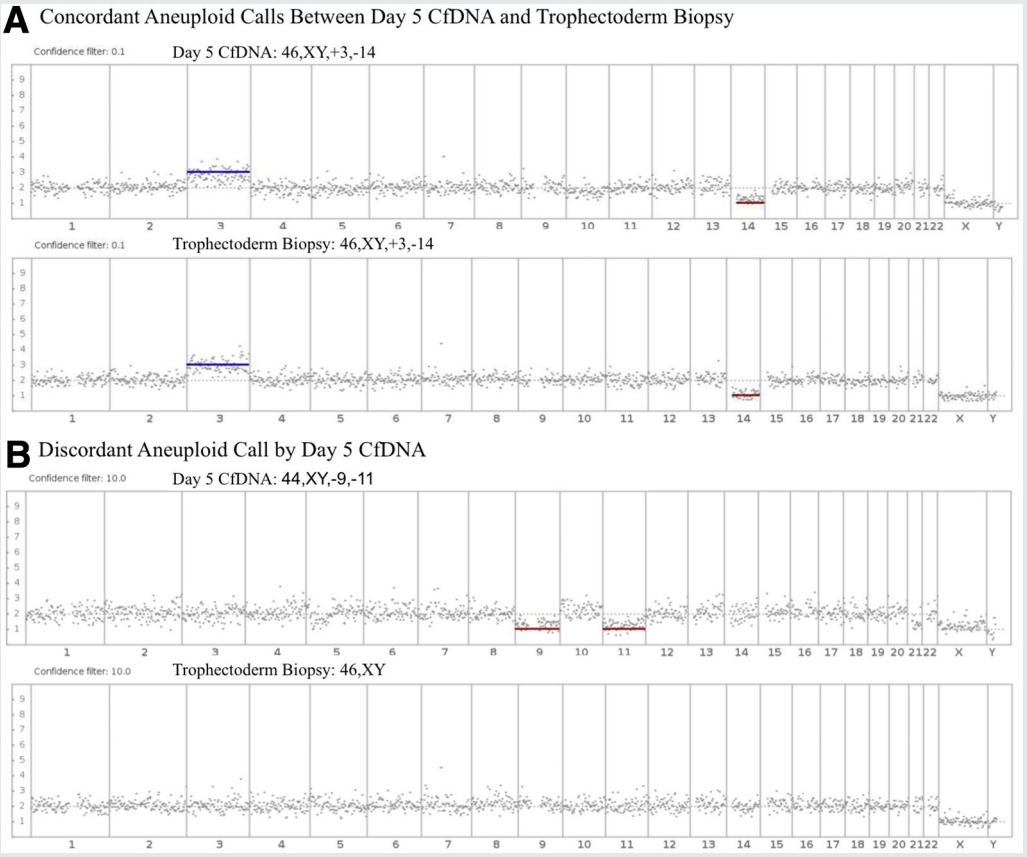
SUPPLEMENTAL FIGURE 1



Visual analysis of day 3 cell-free DNA (cfDNA) and whole embryo sequences. **(A)** Concordant aneuploid calls between day 3 cfDNA and whole embryo. **(B)** Discordant aneuploid calls between day 3 cfDNA and whole embryo (false positive).

Ho. Cell-free DNA for aneuploidy screening.. Fertil Steril 2018.

SUPPLEMENTAL FIGURE 2



Visual analysis of day 5 cell-free DNA (cfDNA) and trophectoderm biopsy sequences. **(A)** Concordant aneuploid calls between day 5 cfDNA and trophectoderm biopsy. **(B)** Discordant aneuploid calls between day 5 cfDNA and trophectoderm biopsy (false positive).

Ho. Cell-free DNA for aneuploidy screening.. Fertil Steril 2018.