BUKTI SUBMIT and ACCEPT

Evaluater	Original comments of the reviewer	Reply by the author(s)
1	This article analyzes the concordance between non-invasive preimplantation genetic testing for aneuploidy (niPGT-A) using spent embryo culture medium (SECM) and PGT-A through trophectoderm biopsy in 28 SECM samples from 21 infertile couples. The aim of this prospective study is to assess the diagnostic potential of cfDNA in SECM compared to the standard trophectoderm biopsy for determining embryo ploidy. The overall concordance between the two methods was 53.8% (14/26), with the interpretability of SECM cfDNA reaching 93% (26/28). Although the interpretability was high, the concordance rate was relatively low. This study is clear, well-structured, and relevant, and warrants publication. However, further improvements might be achieved following the discussion of several issues.	Dear Evaluater 1 We appreciate the time and effort that you have dedicated to providing your valuable feedback on our manuscript. Here are our responses point-to-point to your questions or suggestions.
	Is the trophectoderm biopsy the gold standard for PGT-A in Jakarta ?	Trophectoderm biopsy is the current gold standard to earn embryonic sample for PGT-A procedures in almost all IVF clinics in Indonesia including our clinics in Jakarta.
	The author mentions/describes embryonic mosaiscim; Do you use a threshold of mosaiscim? to decide a non-transfer based on the level of embryonic mosaicism ?	We have added the information in the revised manuscript.
	L53 : The authors should mention the risk of human error during the biospy procedure (destruction of embryo cell during the extraction…)	Done
	The author must specify the different steps after the oocytes retrieval : did they make successive rinsing steps to avoid maternal contamination ? Is it possible that the maternal contamination came from the lab technician?	Explanations have been updated in the revised manuscript. As we did not analyze the original source of cfDNA presence in culture media, we cannot confirm whether it originated from laboratory technicians (embryologists or geneticists) as well. In addition, we have reviewed several papers on cfDNA, and to the best of our knowledge, none of the original investigations are available to confirm this issue. Therefore, in our opinion, this matter remains speculative and requires further clarification.
	Is there bias or an element which could explain the difference of the results of this study when compared to the ones of Rubio et al 2019 ? (in more than 1300 blastocysts?)	Done, thank you for this suggestion.
	L 106 : Is the biopsy procedure made in the same culture dishes in which the embryo remained from D0 to D5-6 and the cfDNA measured is of this SECM (Were any of the embryos transfered into another culture dish before the biopsy with analysis of the « new » SECM ») ? I believe the quantity of DNA in the SECM must be higher in the D0 to D5/6 SCM than an « alternate one » used only for the biopsy? In my opinion this part can be explained more precisely, I found it unclear.	Thank you for pinpointing this to our attention. We have revised the section to enhance clarity and have included a figure as a visual representation of the workflow.
	Is the blastocoel fluid collected during the biopsy procedure ? Is it released in the SCM after the biopsy?	Blastocyst fluid was not collected during the biopsy procedure. Our internal investigation revealed that collecting blastocoel fluid was challenging, particularly in controlling the pressure of the needle for aspiration. Consequently, we opted to explore SCEM cfDNA instead of blastocoel fluid. To clarify our methodology, we have included an additional sentence to highlight our approach
2	This article compares the aneuploidy results from 28 SECM cfDNA samples to 28 TE biopsy samples. It is a well-conducted and well-written study. However, before acceptance, it would be useful to make the following modifications:	Dear Evaluater 2 We express our gratitude for the opportunity to make revision to our manuscript. We found your suggestion very useful and have modified the manuscript accordingly

	Abstract: Method section lines 15 to 18, the sentences are redundant; please simplify	Done
	Line 68: Remove the specificity of the culture medium "G-2."	Done
	Change to be clearer: Studies focusing on the concordance between cfDNA in SECM and TE biopsy have revealed heterogeneous results.	Done. Thank you for your suggestion.
	Line 83: Remove the capitalization from "This."	Done (sentence has been rephrased)
	In the Materials and Methods section, some clarifications need to be made regarding the biopsy method: After hatching, is the embryo rinsed and changed in culture medium? If yes, how? To perform the trophectoderm biopsy, is the embryo moved to a new drop? If yes, what volume of drop and what medium is used? What volume of culture medium is pipetted for analysis? Illustrating the method used with a figure would be beneficial	Thank you for bringing this out. We have clarified the procedures (Figure 1 was added).
	The authors specify in line 101 that the continuous culture medium used is SAGE or GTL. Have you compared the results based on the culture medium used? Because regarding the culture medium, DNA traces have been reported in the culture medium as early as the second day, although it can be assumed that the nuclear genome of viable embryos is minimally or not represented at such early stages (the majority of the genetic material present is probably mitochondrial, derived from cellular fragments). Changing or renewing the medium on day 3 may help eliminate contaminants, but it also means that embryonic DNA entering the medium during the first three days of culture will likely be lost. A culture medium blank would have been rigorous to eliminate any potential contamination source from the medium.	Unfortunately, we did not compare our results based on the type of continuous culture medium used. The G-TL medium predominated (almost 80% of its use in the presented cycle), thus rendering it unsuitable for comparison. This study has addressed the utilization of unexposed-embryo culture media as a control to mitigate potential contamination sources from the medium. We immensely appreciate your comment, and upon reflection, we acknowledge that we only mentioned the use of culture medium blank without elaborating on the results. Therefore, we have included this information in the revised version. Thank you
	Line 156: The concentration after WGA in the TE biopsy samples was higher than that in the SECM sample. Except for 2 embryos, is there an explanation?	Given that all samples underwent similar handling and collection procedures, and the recorded volume of SECM cfDNA collected was consistently similar across all samples, no explanatory factors can be attributed to this matter.
	Line 171: The authors provide results in terms of general concordance. In the introduction, a distinction is made between general and full concordance. Can the authors also make this distinction?	The concept of full concordance, denoting precise agreement between cell-free DNA (cfDNA) in SCEM and TE in terms of genotype outcomes, was explored in our study. Our findings revealed a complete absence of full concordance (table 1). We opted not to include this analysis in our study due to compelling evidence of contamination, chiefly evidenced by the low concordance observed in sex-chromosome agreement. In our opinion, representing full concordance would be inappropriate in our report of results.
	Lines 173-176: Please provide a bit more detail on the method.	Done
	Lines 193-195: The explanation is not very clear.	Done
	Line 182: What is the percentage of good blastocysts or bad blastocysts in the study?	We have included data on the percentage of good blastocysts and fertilization rates. We opted not to include data on moderate to poor quality blastocysts rates, as it can be inferred from the good blastocysts rate.
	It would be interesting for the authors to discuss the limitations of their study: limited cohort, absence of post-hatching rinsing with probable maternal contamination by granulosa cell. Another possibility is to perform hatching on day 4, change the embryo to a culture medium drop with a reduced volume (for example, 10µL instead of 25 µL) to concentrate the DNA	We have addressed the limitations pertaining to this research. Our internal clinic investigation yielded consistent results regardless of changing the medium on either day 3 or day 4 (data pending publication). Consequently, we have chosen not to highlight this as a study limitation. Thank you.

Reply to the editor's and evaluators comments

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Action Links	JOGOH-D-24-00202	Non-Invasive Pre-implantation Genetic Testing's Reliability for Aneuploidy using Cell-free DNA in Embryo Culture Media	Feb 20, 2024	May 30, 2024	Completed - Accept	May 30, 2024	Accept

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Non-Invasive Pre-implantation Genetic Testing's Reliability for Aneuploidy using Cell-free DNA in Embryo Culture Media

Dear Prof Wiweko,

Thank you for submitting your manuscript to Journal of Gynecology Obstetrics and Human Reproduction.

I am pleased to inform you that your manuscript has been accepted for publication.

My comments, and any reviewer comments, are below.
Your accepted manuscript will now be transferred to our production department. We will create a proof which you will be asked to check, and you will also be asked to complete a number of online forms required for publication. If we need additional information from you during the production process, we will contact you directly.

We appreciate and value your contribution to Journal of Gynecology Obstetrics and Human Reproduction. We regularly invite authors of recently published manuscript to participate in the peer review process. If you were not already part of the journal's reviewer pool, you have now been added to it. We look forward to your continued participation in our journal, and we hope you will consider us again for future submissions.

Kind regards,
Anne Mayeur
Associate Editor

Journal of Gynecology Obstetrics and Human Reproduction

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8 dari banyak

Our reference: JOGOH 102808
Article reference: JOGOH_JOGOH-D-24-00202
Article title: Non-Invasive Pre-implantation Genetic Testing's Reliability for Aneuploidy using Cell-free DNA in Embryo Culture Media
To be published in: Journal of Gynecology Obstetrics and Human Reproduction

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BUKTI REVISI

Abstract

Objective:

The presence of embryonic cell-free DNA (cfDNA) in spent embryo culture media (SECM) may offer valuable advantages for non-invasive testing of embryo ploidy or genetic characteristics compared to trophectoderm (TE) biopsy. This study aimed to assess the diagnostic potential of SECM cfDNA as a non-invasive sample for chromosomal copy number testing in blastocysts within the clinical setting of *in-vitro* fertilization.

Method:

This prospective observational study collected 28 SECM cfDNA samples which were matched with TE biopsy samples from 21 infertile couples who underwent IVF-PGT-A cycles. SECM samples were obtained from blastocysts that were cultured for approximately 5/6 days in an uninterrupted time-lapse incubator. Both sets of samples were collected during the biopsy procedure. The Variseq Illumina platform was utilized for ploidy measurement. The study evaluated the informativity and interpretability of SECM cfDNA, concordance of general ploidy status, and sex chromosome agreement between the two sample types.

Results:

SECM cfDNA had a high informativity rate (100%) after double amplification procedure, with a result interpretability of 93%. Two out of the 28 SECM cfDNA samples were uninterpretable and regarded as overall noise samples. The diagnostic potential of

SECM cfDNA, when compared to TE biopsy the standard reference, was relatively low at 53%. Maternal DNA contamination remains the major obstacle that hinders the widespread clinical adoption of SECM cfDNA in the routine practice of pre-implantation genetic testing for aneuploidy within IVF settings.

Conclusion:

A significant modification must be implemented in the IVF laboratory to minimize DNA contamination and this necessitates suggesting adjustments to oocyte denudation, embryo culture media preparation, and sample collection procedures.

Keywords: cell-free DNA, non-invasive PGT-A, PGT-A, ploidy analysis, IVF

INTRODUCTION

The standard practice of pre-implantation genetic testing (PGT) in vitro fertilization (IVF) involves the use of a small number of biopsied embryonic trophoblast cells to screen for chromosomal anomalies such as aneuploidy (PGT-A), structural re-arrangement (PGT-SR), or monogenic analysis (PGT-M). Focusing on PGT-A analysis, the current discussion has addressed some issues regarding the use of trophoblast cells to determine the embryo's chromosomal constitution. Firstly, recent evidence suggests that aneuploid embryonic cells are more likely to be found in the trophectoderm (TE) cells which have a different cell lineage from the inner cell mass (ICM) that develops into the fetus [1,2]. Secondly, a single biopsy of trophectoderm cells may not adequately represent the embryo's ploidy status according to mathematical modeling [3]. Thirdly, the clinical utilization of TE cells may be limited by the potential for false positive and negative results due to embryo mosaicism.[4]

A study has demonstrated that the general concordance of TE with ICM was 87.5% and only 75% for all chromosome concordance

when using 81 donated aneuploid blastocysts [5]. Despite these concerns, TE cells remain widely acknowledged as the standard reference material for genetic analysis. In addition, the primary drawback of the current PGT-A lies in its invasive biopsy procedure, necessitating the removal of several trophoblast cells. This process carries a potential risk for embryo damage depending upon the technical proficiency of the laboratory [6], the susceptibility to human error during the aspiration of embryonic cells, which could compromise embryo viability, and unknown long-term consequences on perinatal outcomes [7]. Therefore, non-invasive methods have been meticulously sought to overcome these limitations.

The discovery of embryonic cell-free DNA (cfDNA) in spent embryo culture media (SECM) represents a potential breakthrough in pre-implantation genetic analysis and is considered to provide a more accurate representation of the embryonic genome, especially in the case of mosaic embryos, when compared to the use of trophectoderm cells for PGT-A.[8] Double-stranded and mitochondrial embryonic cfDNA has been identified in the blastocoel fluid [9] and SECM [10]. However, SECM cfDNA has gained prominence due to its superior DNA integrity and the simplicity of its collection procedure.[11] Notably, embryonic cell-free DNA is detectable in almost all SECM samples, with varying levels that are sufficient for amplification and subsequent ploidy assessment. Such informative value of SECM cfDNA has been confirmed in several studies, highlighting its potential for ploidy assessment.[8,11] Shamonki and Coworkers [12] were the first to demonstrate the presence of cfDNA in SECM, with a detection rate of 55 out of 57 SECM samples collected by changing the culture media from day 3 to day 5/6. However, only 2 out of the 55 media samples were suitable for ploidy analysis through array comparative genome hybridization (aCGH). Other studies have also supported the quantifiability of SECM cfDNA and its potential value for genetic analysis. [13,14]

In contrast to SECM cfDNA informativity, studies focusing on the concordance between cfDNA in SECM and TE biopsy have revealed heterogeneous results. Concordance could be assessed in two ways: general concordance (which considers whether the embryo is euploid or aneuploid) and full concordance (which assesses similarity in genotype results). General concordance has been observed to range between 15.4% to 100% while full concordance has varied from 5.9% to 100%.[8] On the other hands, a significant study conducted by Rubio and Colleagues [5] across 8 IVF clinic centers in 2020, involving over a thousand biopsied blastocysts and their corresponding SECM cfDNA, demonstrated a good average concordance rate of 78.2% with a range of 72.5%-86.3%. The research also highlighted the importance of customizing certain conditions of embryo culture to ensure the reliable utilization of SECM cfDNA. This study aims to assess the clinical utility of SECM cfDNA as an alternative sample for blastocyst ploidy measurement compared to the standard TE biopsy sample within a clinical setting, particularly within a clinical context employing an uninterrupted time-lapse incubator for embryo culture.

MATERIALS AND METHODS

Study participants and embryo culture procedure

This prospective observational single-center study was conducted at Morula IVF Jakarta Clinic, Jakarta, Indonesia. A total of 28 SECM samples were collected from 21 couples who underwent the IVF-PGT-A cycles within a specific period from September 2022 – January 2023. There were no specific pre-selection criteria for the subjects. All the women received the GnRH antagonist protocol for ovarian stimulation as previously described [15].

Each oocyte-cumulus complex (COC) was immersed in 0.5 mL of hyaluronidase solution (Hyase, Vitrolife, Sweeden) for less than 30 seconds and moved to 0.5 mL G-MOPS medium (Vitrolife, Sweeden). Following a 10-minute incubation period, COCs denudation was carried out using 150 µm stripper tips attached to the STRIPPER® micropipette (Cooper Surgical Fertility) to ensure the removal of cumulus cells to minimize maternal contamination in the culture media. In cases where residual cumulus cells persisted, stripper tips with a diameter of 135 µm were used. Prior to sperm injection, all oocytes underwent gentle washing in GMOPS medium to eliminate any remaining cumulus cells. After sperm injection, either through ICSI or IMSI, the sperm-injected oocytes were subjected to standard washing procedures to minimize adverse effects due to extensive pipetting, followed by culture in a MIRI® time-lapse incubator (under culture conditions: 37°C, 6% of CO_2, and 5% of O_2) utilizing a specific petri-dish (Culture-Coin; ESCO Medical Technologies). Each well of the culture dish was loaded with 25 µL of continuous culture media, either G-TL (Vitrolife, Sweeden) or SAGE (Origio, Sweeden), for single embryo culture. No media changes were performed for any of the embryos. All developed embryos underwent laser-assisted hatching procedures (OCTAX Laser Shoot-TM) on day 4 of embryo culture to facilitate the release of several trophoblast cells. The biopsy was conducted on either the 5th or 6th day of embryo culture depending on the blastocyst development.

Biopsy and sample collection

On the day of the biopsy, good-quality blastocyst(s) for PGT-A were transferred to a new petri dish. The dish contained several drops of 15 µL G-MOPS medium, in which the biopsy took place (Figure 1). During the transfer of expanded blastocysts with extruded embryonic cells from the culture coin dish to the biopsy, Eppendorf sterile PCR clean pipette tip of 200 µL was used, resulting in a

reduction of the original volume of culture media. The biopsy process began by disrupting the trophoblast cell junctions using one or two laser pulses followed by the gentle aspiration of the cells into the biopsy needle. The Aspirated trophoblast cells were then rinsed in sterile phosphate--buffered saline (PBS) solution (Cell Signaling Technologies, Inc) supplemented with 1% PVP (Origio) and directly placed in 2.5 µL of PBS in a sterile tube. The process of collecting SECM cfDNA samples from the culture coin petri dish commenced with the careful removal of the mineral oil utilized during embryo culture, minimizing the risk of oil aspiration into the sample. Subsequently, the corresponding spent culture media from the biopsied blastocysts were then aspirated and transferred into a sterile tube, with volumes ranging approximately from 10 to 12 µL. cfDNA samples solely derived from SECM, with no blastocysts undergoing blastocoel fluid aspiration. Both sets of samples were subsequently stored at -20°C and promptly shipped to the genomics laboratory for chromosomal copy number analysis.

DNA extraction, whole genome amplification, and quantification

DNA extraction and amplification were carried out for both sets of samples using SurePlex DNA amplification system kits (Illumina, San Diego CA) following the manufacturer's instructions (SurePlex summary protocol V4). Briefly, 2.5 µL of cell extraction buffer was added into either 2.5 µL SECM, 2.5 µL of PBS containing aspirated trophoblasts cells, or 2.5 µL unexposed-embryo culture media as a control. This was followed by the addition of 5 µL of the cocktail master mix. The reaction was run in a PCR thermal cycle (Biorad) following a specific program as per the instructions. Subsequently, a 5 µL pre-amplification cocktail was loaded and the reaction was run on the thermal cycle. To initiate DNA amplification, a 60 µL amplification cocktail was added to the reaction and then run on a PCR

thermal cycle. This study conducted a double amplification process on the SECM cfDNA samples to improve the DNA yield for sequencing, as previously performed in a previous study.[13] DNA quantification following WGA was performed using the Qubit High Sensitivity dsDNA kit with a Qubit 3 device (Thermo Fisher).

Library preparation, sequencing, and data analysis

Veriseq PGS-MiSeq kit (Illumina, USA) was employed for library preparation including DNA tagmentation, neutralization, PCR clean-up, and DNA normalization. The manufacturer's instructions for PGS Library Preparation version 4 were followed. After the creation of pooled libraries, Sequencing was carried out using a high throughput next-generation sequencing machine (Miseq, Illumina, USA). The sequencing process underwent quality control assessments, which included evaluating cluster density, clusters passing filter, and estimating the yield. Following sequencing, the Fastq files were analyzed using BlueFuse Multi Software V4.5 to determine chromosomal copy numbers. The criteria for deciding the ploidy status of both samples were established as follows: euploid embryo characterized by a complete set of chromosomes with less than 30% heterogeneity in cell genotyping, was defined; mosaicism was deemed present when a mixture of euploid and aneuploid embryonic cells fell between 30% and 50% of the sample, categorized as low-level, and between 51% and 80%, categorized as high-level; aneuploid embryo, indicating the gain or loss of chromosomes, was designed for any value of mixture in cell genotyping exceeding 80%.

Outcome measures

The informativity and interpretability of cfDNA were assessed. Informativity was defined as the success rate of cfDNA amplification, while interpretability referred to the success rate of obtaining readable NGS results. Concordance was defined as a congruent diagnostic interpretation result between SECM cfDNA and TE biopsy samples, including general concordance (similarity of diagnosis) and agreement concerning sex chromosomes. The predictive capability of ploidy status in cfDNA samples compared to the standard reference (TE biopsy) was illustrated using accuracy metrics, particularly the area under the curve (AUC) of the receiver operating characteristic (ROC) curve. Accuracy denotes the capability of SECM cfDNA to correctly classify both true positive (TP) and true negative (TN) of ploidy status against the standard reference. It was calculated as the total count of TP and TN divided by the total number of cases (N). Sensitivity was defined as the ability of SECM cfDNA to diagnose euploid blastocyst, calculated as the number of TP divided by the total number of TP and false negatives (FN). Specificity reflects the capacity to identify aneuploid blastocyst accurately. It was calculated by dividing the number of TN by the total number of TN and false positive (FP). The positive predictive value (PPV) was determined by dividing TP by the total number of TP and FP, while the negative predictive value (NPV) was calculated as TN divided by the total number of TN and FN.

Statistical analysis

Data analysis was performed using SPSS version 20.0 (SPSS, Inc., Chicago, IL, USA). Descriptive data were presented as means ± SD or as medians (min-max), depending on the data distribution, and proportions were expressed as percentages (n (%)). A convolution metric between the two methods was constructed to calculate accuracy, sensitivity, specificity, positive prediction value, and negative

prediction value. Global data presentation was used to assess general and sex chromosome concordance between SECM cfDNA and TE biopsy.

RESULTS

The mean age of the women who participated in the study was relatively young (35 years) with an average infertility duration of 8 years and a normal body mass index (<25 kg/m^2). Primary infertility was reported in 61.9% of the couples, while the rest had secondary infertility. The most common cause of infertility was unexplained infertility (47.7%), followed by recurrent failure of intra-uterine insemination (28.6%) and sperm-related factors (23.8%). Clinical characteristics including basal hormone levels, hormone levels on the day of hCG maturation injection, and total gonadotropin usage were within the normal reference range (Supplemental Table 1). Notably, the mean DNA concentration after WGA in the TE biopsy samples was higher than that in the SECM sample (35.6 ng/µL vs. 19.5 ng/µL, with a mean difference of 16.1 ng/µL (10.85-21.3 ng/µL, 95% CI). This difference persisted even after performing double amplification steps in SECM. The DNA concentration ranged from a minimum of 21.6 ng/µL to a maximum of 70 ng/µL in the TE biopsy samples, whereas, in the SECM samples, it varied from 2.9 ng/µL to 36.6 ng/µL (Supplemental Table 2). The control sample of unexposed embryo culture media exhibited very low DNA concentration (0.32 ng/µL) as determined by Qubit analysis after double WGA. The informativity of SECM cfDNA was 100%, indicating that DNA could be quantified in all the samples.

Quality control (QC) measures were conducted for the sequencing process, prior to interpreting the chromosomal copy number results. The cluster density (1260K/mm^2) was within the desired range (1200-1300K/mm^2). Furthermore, both the clusters passing filter (85.8%)

and the estimated yield (1289.1MB) met the reference standards. The interpretability of SECM cfDNA reached 93%, with only 2 out of 28 samples failing the QC and regarded as noisy results. Genotyping results are presented in Table 1.

The general concordance in relation to ploidy status between the two samples was 53.8% (14/26) and 65.4% (17/26) for the sex chromosome concordance. Interestingly, out of the 10 embryos with XY sex chromosomes in the TE biopsy samples, nine were identified as XX in the SECM samples. The accuracy of SECM in comparison to the standard reference for ploidy measurement was 0.57 (AUC ROC, 95% CI 0.34-0.79) with 100% sensitivity and 13.3% specificity. The Positive and negative predictive values were 45.8% and 100%, respectively (Table 2).

DISCUSSION

The present study has demonstrated that SECM cfDNA exhibits high informativity and interpretability but low concordance with TE biopsy as a standard reference. The origin of cfDNA in SECM could be attributed to abnormal embryonic cell apoptosis, secondary necrosis, or its potential release by in-vitro-developed embryos through extracellular vesicles [16]. This study also highlighted that SECM cfDNA samples from grade I embryos according to the Gardner grading system (which considers factors such as blastocoel expansion rate (5,4,3) and the quality of the ICM and trophectoderm (AA, AB, or BA)) [17] contained a sufficient amount of DNA, allowing for chromosomal copy number analysis without the risk of amplification failure.

This study did not conduct any tests to definitively determine whether the cfDNA originated solely from embryonic DNA, but it suggested the presence of female DNA contamination in the analyzed SECM samples. Evidently, in 9 out of 10 blastocysts with XY

sex chromosomes in TE biopsy results, SECM cfDNA samples were identified as XX. This indicates the presence of maternal DNA contamination in SECM, which masked the Y chromosome. Moreover, very low DNA concentration was detected in the culture media of unexposed embryos as a control. We presumed that this contamination may have originated from the addition of human serum albumin, a protein commonly added to commercial media for embryo culture, as substantiated by another research group.[14] While other potential sources of contamination warrant consideration by embryologists or geneticists, current evidence in support of this notion remains lacking. In addition, this study confirmed that the modified denudation approach utilized herein for oocytes did not fully eliminate potential contamination derived from maternal cumulus cells. During the denudation process employing 135 μm stripper tips, we exercised caution, recognizing that extensive pulling in and out of oocytes to smaller-sized stripper tips could lead to adverse effects, such as mechanical-induce alteration to oocyte [18] and an increased incidence of metaphase II spindle deviation from polar body I [19].

Evidently, there is no consensus yet on the optimal embryo culture duration for SECM to achieve a high concordance with TE biopsy. The collection of SECM from days 3-5/6 or 4-5/6 is theoretically expected to reduce maternal contamination arising from cumulus cells and consequently result in a good agreement with the TE biopsy results. In fact, several studies that have utilized freshly cultured SECM from days 3-5 have reported varying levels of general concordance, ranging from 54.9% (28/51) [13] to 76.3% (29/38) [20]. On the contrary, Liu and Colleagues [21] demonstrated a high concordance between the two sample types (83.87%, 26/31) utilizing a 5-day incubation period from sperm injection to the blastocyst stage. This study employed 5/6-days-cultured SECM to evaluate its practicality in clinical settings, particularly when using an uninterrupted time-lapse incubator. Although the nature of our SECM cfDNA (D0-D5/6 without medium changing) differed from Vera-Rodrigues and colleagues (2 days culture, D3-D5 SECM), we achieved a similar level

of general concordance. This suggests that eliminating potential maternal DNA contamination may be challenging without specific treatments. This presumption is further supported by another research group that assessed the clinical value of SECM cfDNA from either continuous or sequential culture media for PGT-M analysis in the clinical setting [22]. According to TE genotyping results, embryos with homozygote alleles were demonstrated to have mutant alleles in SECM samples, suggesting the presence of extra-embryonic DNA, likely originating from maternal cumulus cells and polar body [22].

Several studies have examined the use of frozen embryos and their corresponding SECM after certain hours or overnight culture following warming for sample analysis. Huang and Colleagues[23] attained remarkably high concordance between SECM cfDNA and whole embryos (93.8%, 45/48) by using SECM from vitrified-warmed donated blastocysts overnight. Similarly, Kuznyetsov and Coworkers[24] also demonstrated a high general concordance between SECM from vitrified-warmed blastocysts SECM and TE DNA biopsy (87.5%, 21/24), as well as with whole DNA embryo (96.4%, 27/28). The nature of SECM in the aforementioned studies may differ from the practice of collecting SECM from fresh embryo culture. Nevertheless, those studies provide substantial evidence that cfDNA in SECM could potentially correspond to embryo DNA characteristics; thus, offering a reliable alternative for non-invasive ploidy assessment or potentially replacing TE biopsy in the future.

It appears that SECM cfDNA collected after the vitrification of embryos attains high concordance.[23–25] Xu et al.[25] conducted extensive research in which 42 vitrified-warmed D3 cleavage and subsequent embryo culture to blastocyst stage displayed high general concordance of SECM cfDNA with whole embryos as the standard reference (85.7%, 36/42). Summarizing the findings of those studies

indicates that an interrupted culture period; for instance, vitrification or a complete vitrified-warming procedure reduces the risk of maternal DNA contamination. During the vitrification process, cleavages or blastocysts are notably exposed to a high osmotic solution to facilitate dehydration and prevent ice crystal formation. SECM collected from certain hours, overnight or 48 hours cultural environment after vitrification results in a high amplification rate, interpretable results, and high concordance with corresponding blastocysts or trophoblast cells.

This study highlights the challenges associated with the clinical utilization of SECM cfDNA, primarily due to the presence of DNA contamination. It is evident that without implementing specific interventions in the IVF laboratory to reduce non-embryonic DNA, the use of SECM cfDNA could compromise the interpretation of genetic analysis. Optimization efforts could be directed toward the unique oocyte denudation technique, embryo culture environment, timing of SECM cfDNA collection, and amplification procedure. In contrast to the largest multi-center research employing 1301 human blastocysts, we noticed several distinct methodological approaches that could account for the variance of results. Firstly, that study executed culture media changes on day 4 by transferring the embryo to a fresh 10 μL culture media drop, subsequently collecting SECM cfDNA samples from that new medium after a minimum culture period of 40 hours. Secondly, no assisted hatching procedure was allowed before collecting SECM cfDNA. These factors likely influenced the disparate outcomes observed in our study.

There is a suggestion that SECM cfDNA could serve as an alternative sample when genetic testing via TE biopsy yields inconclusive results.[26] We also observed the potential clinical application of SECM cfDNA for screening chromosomal copy numbers, especially

when patients have untested vitrified blastocysts. The present study is subject to some limitations including a relatively small sample size, limited cohort, and performing assisted hatching before SECM cfDNA sample collection. In conclusion, while the study provides substantial informativity and interpretability, the general concordance between SECM cfDNA and TE biopsy samples is relatively low and falls short of being acceptable for clinical use, unless the issue of non-embryonic DNA contamination can be effectively diminished. Customized interventions in the IVF laboratory should be implemented to overcome this limitation caused by contamination.

REFERENCES:

[1] Yang M, Rito T, Metzger J, Naftaly J, Soman R, Hu J, et al. Depletion of aneuploid cells in human embryos and gastruloids. Nat Cell Biol. 2021;23(4):314–21.

[2] Griffin DK, Brezina PR, Tobler K, Zhao Y, Silvestri G, Mccoy RC, et al. The human embryonic genome is karyotypically complex, with chromosomally abnormal cells preferentially located away from the developing fetus. Human Reproduction. 2023 Jan 1;38(1):180–8.

[3] Gleicher N, Metzger J, Croft G, Kushnir VA, Albertini DF, Barad DH. A single trophectoderm biopsy at blastocyst stage is mathematically unable to determine embryo ploidy accurately enough for clinical use. Reproductive Biology and Endocrinology. 2017;15(1):33.

[4] Popovic M, Dhaenens L, Boel A, Menten B, Heindryckx B. Chromosomal mosaicism in human blastocysts: The ultimate diagnostic dilemma. Hum Reprod Update. 2020;26(3):313–34.

[5] Rubio C, Navarro-Sánchez L, García-Pascual CM, Ocali O, Cimadomo D, Venier W, et al. Multicenter prospective study of concordance between embryonic cell-free DNA and trophectoderm biopsies from 1301 human blastocysts. Am J Obstet Gynecol. 2020;223(5):751.e1-751.e13.

[6] Homer HA. Preimplantation genetic testing for aneuploidy (PGT-A): The biology, the technology and the clinical outcomes. Australian and New Zealand Journal of Obstetrics and Gynaecology. 2019;59(2):317–24.

[7] Tocci A. The unknown human trophectoderm: implication for biopsy at the blastocyst stage. 1925; Available from: https://doi.org/10.1007/s10815-020-01925-0

[8] Brouillet S, Martinez G, Coutton C, Hamamah S. Is cell-free DNA in spent embryo culture medium an alternative to embryo biopsy for preimplantation genetic testing? A systematic review. Reprod Biomed Online. 2020;40(6):779–96.
[9] Palini S, Galluzzi L, De Stefani S, Bianchi M, Wells D, Magnani M, et al. Genomic DNA in human blastocoele fluid. Reprod Biomed Online. 2013;26(6):603–10.
[10] Stigliani S, Anserini P, Venturini PL, Scaruffi P. Mitochondrial DNA content in embryo culture medium is significantly associated with human embryo fragmentation. Human Reproduction. 2013 Oct 1;28(10):2652–60.
[11] Leaver M, Wells D. Non-invasive preimplantation genetic testing (niPGT): The next revolution in reproductive genetics? Hum Reprod Update. 2020;26(1):16–42.
[12] Shamonki MI, Jin H, Haimowitz Z, Liu L. Proof of concept: preimplantation genetic screening without embryo biopsy through analysis of cell-free DNA in spent embryo culture media. Fertil Steril. 2016;106(6):1312–8.
[13] Vera-Rodriguez M, Diez-Juan A, Jimenez-Almazan J, Martinez S, Navarro R, Peinado V, et al. Origin and composition of cell-free DNA in spent medium from human embryo culture during preimplantation development. Human Reproduction. 2018;33(4):745–56.
[14] Hammond ER, McGillivray BC, Wicker SM, Peek JC, Shelling AN, Stone P, et al. Characterizing nuclear and mitochondrial DNA in spent embryo culture media: genetic contamination identified. Fertil Steril. 2017;107(1):220-228.e5. Available from: http://dx.doi.org/10.1016/j.fertnstert.2016.10.015
[15] Boediono A, Handayani N, Sari HN, Yusup N, Indrasari W, Polim AA, et al. Morphokinetics of embryos after IMSI versus ICSI in couples with sub-optimal sperm quality: A time-lapse study. Andrologia. 2021 May 1;53(4):e14002.
[16] Handayani N, Aubry D, Boediono A, Wiweko B, Sirait B, Sini I, et al. The origin and possible mechanism of embryonic cell-free DNA release in spent embryo culture media: a review. J Assist Reprod Genet. 2023;40(6):1231–42.
[17] Gardner DK, Lane M, Stevens J, Schlenker T, Schoolcraft WB. Blastocyst score affects implantation and pregnancy outcome: towards a single blastocyst transfer. Fertil Steril. 2000;73(6):1155–8.
[18] Rienzi LF, Maggiulli R, Ubaldi FM. Oocyte Denuding. In: In Vitro Fertilization. Springer International Publishing; 2019. p. 133–45.
[19] Rienzi L, Ubaldi F, Martinez F, Iacobelli M, Minasi MG, Ferrero S, et al. Relationship between meiotic spindle location with regard to the polar body position and oocyte developmental potential after ICSI. Human Reproduction. 2003 Jun 1;18(6):1289–93.
[20] Li P, Song Z, Yao Y, Huang T, Mao R, Huang J, et al. Preimplantation Genetic Screening with Spent Culture Medium/Blastocoel Fluid for in Vitro Fertilization. Sci Rep. 2018;8(1):9275.
[21] Liu W, Liu J, Du H, Ling J, Sun X, Chen D. Non-invasive pre-implantation aneuploidy screening and diagnosis of beta thalassemia IVSII654 mutation using spent embryo culture medium. Ann Med. 2017 May 19;49(4):319–28.
[22] Capalbo A, Romanelli V, Patassini C, Poli M, Girardi L, Giancani A, et al. Diagnostic efficacy of blastocoel fluid and spent media as sources of DNA for preimplantation genetic testing in standard clinical conditions. Fertil Steril. 2018;110(5):870-879.e5.

[23] Huang L, Bogale B, Tang Y, Lu S, Xie XS, Racowsky C. Noninvasive preimplantation genetic testing for aneuploidy in spent medium may be more reliable than trophectoderm biopsy. Proc Natl Acad Sci U S A. 2019;116(28):14105–12.
[24] Kuznyetsov V, Madjunkova S, Antes R, Abramov R, Motamedi G, Ibarrientos Z, et al. Evaluation of a novel non-invasive preimplantation genetic screening approach. PLoS One. 2018 May 1;13(5).
[25] Xu J, Fang R, Chen L, Chen D, Xiao JP, Yang W, et al. Noninvasive chromosome screening of human embryos by genome sequencing of embryo culture medium for in vitro fertilization. Proc Natl Acad Sci U S A. 2016;113(42):11907–12.
[26] Rubio C, Rienzi L, Navarro-Sánchez L, Cimadomo D, García-Pascual CM, Albricci L, et al. Embryonic cell-free DNA versus trophectoderm biopsy for aneuploidy testing: concordance rate and clinical implications. Fertil Steril. 2019;112(3):510–9.

Figure legend

Figure 1. Schematic representation of the methodological trajectory of the research

Table Legend:

Table 1. Chromosomal analysis comparison of SECM cfDNA and TE biopsy

Table 2. Performance comparison between SECM cfDNA in comparison to TE biopsy for ploidy status measurement

Supplemental Table Legend:

Supplemental Table 1. Summarized baseline, clinical, and laboratory outcomes of studied subjects

Supplemental Table 2. Comparison of DNA concentration between TE and SECM cfDNA after whole genome amplification.

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