Zygote

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Cite this article: Li H-X *et al*. (2022) An additional medium renewal of D4 embryo culture improves the concordance of noninvasive chromosome screening with trophectoderm biopsy. *Zygote*. **30**: 849–853. doi: 10.1017/S0967199422000363

Received: 15 September 2021
Accepted: 13 June 2022
First published online: 22 September 2022

Keywords:
Blastocyst; Culture medium; Euploidy; Morphology; Noninvasive chromosome screening

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An additional medium renewal of D4 embryo culture improves the concordance of noninvasive chromosome screening with trophectoderm biopsy

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Summary

Our research question was to evaluate the chromosome concordance of trophectoderm (TE) biopsy with noninvasive chromosome screening (NICS) using embryo culture medium renewed twice on Day 3 (D3) and Day 4 (D4). In this study, we evaluated 64 cycles with 223 biopsied blastocysts. These were categorized into two groups based on replacing embryo culture medium on D3 (control group) or on D3 and D4 (experimental group). The fundamental characteristics and main outcomes were compared. The concordance rates of NICS results with TE biopsy were determined according to next generation sequencing results. In total, 103 experimental and 120 control embryo cultures were collected, and the euploid status was analyzed using NICS technology. The overall concordance rates with TE biopsy of the experimental and control groups were 0.86 and 0.75, respectively. Statistically significant difference was found between the two groups. An additional medium renewal of the D4 embryo culture can improve the concordance of NICS with TE biopsy.

Introduction

A major goal of *in vitro* fertilization/intracytoplasmic sperm injection–embryo transfer (IVF/ICSI–ET) is to select embryos with the best potential for transfer and to maximize the clinical pregnancy rate of patients (Zaninovic and Rosenwaks, 2020). Preimplantation genetic testing (PGT) with trophectoderm (TE) biopsy has been widely adopted as a conventional assisted reproductive technology (Takeuchi, 2021). Preimplantation genetic testing for aneuploidies (PGT-A) and embryo biopsies provide a more direct evaluation of chromosome status and lead to increased embryo implantation and pregnancy rates (Dreesen *et al.*, 2014; Marin *et al.*, 2021). Embryo biopsy involves the removal of multiple trophectoderm cells at the blastocyst stage or of an individual cell at the cleavage stage (Schoolcraft *et al.*, 2010).

However, there are still several problems related to the diagnostic false negatives and false positives and safety of this approach. Due to technical roadblocks and concerns regarding the long-term health of subsequent generations, the clinical application of PGT is limited. As shown in animal studies, embryo biopsy results in delayed formation of the blastocyst cavity and leads to an increased risk of neurodegeneration and dysfunction in subsequent generations (Zhao *et al.*, 2013; Wu *et al.*, 2014; Xu *et al.*, 2016). Therefore, a less-invasive technique that uses culture medium or blastocyst cavity fluid of the embryo to analyze genomic DNA is desirable (Tobler *et al.*, 2015; Magli *et al.*, 2016; Hammond *et al.*, 2017; Farra *et al.*, 2018; Kuznyetsov *et al.*, 2018).

Culture medium is likely to be a more reliable source because the results of screening blastocyst cavity fluid are usually inconsistent compared with those of embryo biopsies (Shamonki *et al.*, 2016; Chen *et al.*, 2020; Vagnini *et al.*, 2020). Chromosome aneuploidy can be detected using DNA assay on the fifth or sixth day using embryo culture medium. It is necessary to avoid maternal DNA contamination of granulosa cumulus cells around embryos (Barbash-Hazan *et al.*, 2009). Moreover, preimplantation aneuploid embryos undergo self-correction in correlation with their developmental potential. Self-correction of aneuploid and mosaic embryos occurs probably during development towards the blastocyst stage, especially the embryo compaction stage (Barbash-Hazan *et al.*, 2009; Franco, 2019).

To date, the concordance of TE biopsy and noninvasive chromosome screening (NICS) from culture medium is still a controversial issue. In this study, to further eliminate maternal DNA contamination, embryos were repeatedly pipetted 3–5 times and carefully denudated of surrounding cumulus cells on D3 and cultured in blastocyst medium in the control group. In the experimental group, embryos were pipetted on D3 and cultured in blastocyst medium,

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and then transferred again on D4 to a fresh droplet of the blastocyst culture medium following several repeated flushing. We analyzed the chromosome concordance of TE biopsy with NICS using embryos culture medium renewed twice on Day 3 (D3) and Day 4 (D4).

Materials and methods

This single-centre cohort study used the medical records of 64 infertile patients who underwent PGT-A cycles between September 2016 and January 2020. This study was approved for the noninvasive chromosome screening protocol of embryo culture by the Ethics Research Committee, the First Hospital of Lanzhou University, China.

Study group

Patients who underwent ICSI of the PGT-A cycle were included in this study. The inclusion criteria were as follows: underwent PGT-A due to (1) advanced maternal age (≥38 years), (2) repeated implantation failures (≥3 times) or (3) recurrent abortion (≥2 times) with blastocyst trophoblastic ectoderm biopsy fertilized using ICSI therapy. Embryos were repeatedly pipetted 3–5 times and carefully denudated on D3 and cultured in blastocyst medium in control group. In experimental group, embryos were pipetted on D3 and cultured in blastocyst medium, and then transferred again on D4 to a fresh droplet of the blastocyst culture medium following several repeated flushing. According to Gardner's classification (Ferrick *et al*., 2020), the grades of high-quality blastocysts include AA, AB, BA and BB. To avoid blastocyst waste as much as possible, blastocysts above CC grade can be used for biopsy. In the experimental group, after the culture was renewed on D3, the embryos were transferred to new medium again on D4. In total, 223 samples of blastocyst culture medium were collected on the 5th/6th day after biopsy, and the results of NICS from blastocyst culture medium were compared with those of PGT-A from trophoblast ectoderm. There were 103 samples in the experimental group and 120 samples in the control group. The consistencies of PGT-A and NICS in the two groups were calculated.

Ovarian stimulation

In the long or short protocols, the patients received controlled ovarian stimulation with recombinant follicle-stimulating hormone (FSH) (Puregon®, Organon, the Netherlands) and gonadotropin-releasing hormone agonists (Decapeptyl® Ferring, Germany). FSH doses were changed according to the ovarian response, and the ovarian response was assessed using ultrasound and by evaluating oestrogen and progesterone levels. When there were at least three dominant follicles ≥18 mm in diameter, 5000–10,000 units of human chorionic gonadotropin (Pregnyl®, Saint-Prex, Switzerland) were administered to induce oocyte maturation. At 36 h after induction, follicular aspiration was performed under ultrasound guidance (Li *et al*., 2021).

Embryo culture and medium collection

Egg stripping needles (Cook Medical, America) were used to remove cumulus cells. ICSI was performed to fertilize all embryos in accordance with the manufacturer's instructions. On the first day (16–18 h after insemination), the fertilization status was measured and the appearance of two polar bodies and two prokaryotic cells was observed. Subsequently, the embryos were cultured in an incubator (37°C, 6% CO_2, and 5% O_2) with Vitrolife G-1 plus cleavage medium (Vitrolife, Goteborg, Sweden). The Gardner grading method was used to evaluate embryo development on the third day after insemination (Marin *et al*., 2021). All embryos were cultured for 3 days and repeatedly pipetted 3–5 times using 140-µm stripper tips (CooperSurgical Fertility, USA), then carefully washed and cultured overnight in 30-µl droplets of Vitrolife G-2 plus blastocyst medium (Vitrolife, Goteborg, Sweden). On D4, individual embryos were replaced in 30-µl droplets of G-2 plus medium and cultured for 1–2 days to the blastocyst stage in the incubator. Next, 20 µl of blastocyst medium was taken from each embryo and transferred into RNase–DNase-free PCR tubes with 5 µl of cell lysis buffer. The same amount of blastocyst medium was used as a negative control, but it was not used for embryo culture. All collected samples were maintained at −80°C until NICS testing was performed.

Blastocyst biopsy and vitrification

On the 5th or 6th day after fertilization, the zona pellucida of the fully expanded blastocyst was perforated using a refractive index (RI) laser to form a narrow pore. Duration time of the laser was 0.07–0.1 ms. Laser aperture was 0.14–0.20 micron. The manipulation procedure video can be viewed (Video S1). Immediately after that, 5–10 trophoblast cells were extracted along the zona pellucida pore with a biopsy needle for genetic testing. Vitrification of blastocysts was performed using the VitriFreeze™ Media Kit (Kitazato, Japan) after biopsy within 30 min.

Whole-genome amplification (WGA) and NICS assay

Single–cell WGA was performed using multiple annealing and looping-based amplification cycles (MALBAC) in accordance with the manufacturer's protocol to amplify DNA from the culture medium and embryos (Xu *et al*., 2016) (Yikon Genomics). In brief, D3 embryos obtained using ICSI were placed in blastocyst culture medium, and blastocyst medium was collected for WGA and MALBAC. Genome-wide amplification products from the blastocyst medium were sequenced on an Illumina HiSeq 2500 platform. Trimmomatic-0.30 software was used to trim raw data from the sequencing library; low-quality bases (quality score <20) and adapters were removed, whereas high-quality reads were aligned using Genome Browser sequences (genome.ucsc.edu). Chromosome ploidy information was obtained from the DNA of the embryo culture medium.

Statistical analysis

To evaluate the diagnostic efficiency of the blastocyst culture medium sample, the sensitivity, specificity, false-positive rate, false-negative rate, positive predictive value, and negative predictive value were calculated according to the corresponding TE biopsy results. The formulae were as follows: sensitivity = (true positives)/(true positives + false negatives); specificity = (true negatives)/(true negatives + false positives); false-positive rate = (false positives)/(true negatives); false-negative rate = (false negatives)/(true positives); positive predictive value = (true positives)/(true positives + false positives); negative predictive value = (true negatives)/(true negatives + false negatives) (Chen *et al*., 2020).

IBM SPSS v.22 was used to analyze the data. Student's *t*-test was adopted to compare the continuous variables that were represented by mean values plus or minus standard deviation (SD), and the chi-squared test to compare the categorical variables. When the *P*-value was <0.05, the comparative result was considered to indicate a statistically significant difference.

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Table 1. Demographic characteristics

	Experimental group	Control group	*P*-value
Total number of controlled ovarian hyperstimulation cycles	30	34	
Age	32.97 ± 5.89	33.43 ± 6.38	0.46
Body mass index (kg/m^2)	21.75 ± 3.23	22.10 ± 3.51	0.57
AMH level (ng/ml)	3.79 ± 2.66	3.48 ± 2.92	0.29
E2 level on trigger day (pg/ml)	2476.94 ± 1074.22	2535.56 ± 1190.52	0.10
P4 level on trigger day (ng/ml)	1.03 ± 0.42	0.98 ± 0.44	0.79
Number of oocytes retrieved	12.85 ± 6.23	13.27 ± 5.79	0.24
Number of MII oocytes retrieved	10.61 ± 5.48	10.90 ± 5.87	0.32

Note: Values are presented as numbers or mean ± standard deviation (SD).
AMH, anti-Müllerian hormone; E2, serum estradiol; MII: metaphase II; P4, serum progesterone.

Results

Demographic data and cycle features

In total, 64 controlled ovarian hyperstimulation cycles that were subjected to ICSI fertilization were included in the study. The patient number of advanced maternal age, repeated implantation failures and recurrent abortion was 7, 11, 12 in the experimental group, and 9, 13, 12 in the control group ($P > 0.05$). The average age of the women was 33.17 ± 5.25 years. In total, 692 oocytes in metaphase at the second meiotic division (MII) were obtained (Table 1). ICSI produced 549 two-pronuclei (2PN) zygotes and 543 available embryos (the D3 cleavage stage was more than four cells), of which 223 blastocysts were developed for biopsy. Finally, there were 103 samples in the experimental group and 120 samples in the control group.

No significant differences were found between the experimental group and control group in baseline characteristics and demographic data, including age, body mass index, anti-Müllerian hormone level, serum estradiol and progesterone levels on the trigger day, and in the number of metaphase II oocytes retrieved (Table 1).

Comparison of embryo morphology

In total, 223 biopsied blastocysts were annotated into two groups according to whether an additional medium renewal of D4 embryo culture was carried out. Table S1 shows a comparison of embryo morphology between the two groups. There were no significant differences in embryo morphology characteristics (number of blastomeres, embryo ratings and fragments).

Comparison of the performance of the experimental versus control group

The concordance of PGT-A and NICS in the two groups was calculated. As shown in Table 2, generally the concordance rates with TE biopsy of the experimental and control groups were 0.86 and 0.75, respectively. Statistically significant differences were found between the two groups ($P = 0.03$, $\chi^2 = 4.56$). The sensitivity, specificity, false-positive rate, false-negative rate, positive predictive value, and negative predictive values of the experimental and control groups were calculated based on the corresponding TE biopsy results (Table 3).

Table 2. Comparison of concordance rate between the experimental and control group

	Concordance rate (NICS vs. TE biopsy)	
	Experimental group ($n = 103$)	Control group ($n = 120$)
Overall concordance[a]	0.86 (89/103)	0.75 (90/120)
Euploid	0.88 (44/50)	0.76 (47/62)
Aneuploid	0.85 (45/53)	0.74 (43/58)

Note: Values are represented by numbers.
[a]$P = 0.03$, $\chi^2 = 4.56$.

Table 3. Comparison of the performance of the experimental versus control group

Performance characteristics	Experimental group ($n = 103$)	Control group ($n = 120$)	χ^2	*P*-value
Sensitivity	0.88 (44/50)	0.76 (47/62)	2.70	0.10
Specificity	0.85 (45/53)	0.74 (43/58)	1.95	0.16
False-positive rate	0.18 (8/45)	0.35 (15/43)	3.33	0.06
False-negative rate	0.14 (6/44)	0.32 (15/47)	4.28	0.04
Positive predictive value	0.85 (44/52)	0.76 (47/62)	1.36	0.24
Negative predictive value	0.88 (45/51)	0.74 (43/58)	3.47	0.06

Discussion

It is increasingly believed that selective ET in IVF/ICSI cycles is advocated for maintaining acceptable pregnancy rates and reducing the risk of multiple pregnancies (Takeuchi, 2021; Zaninovic and Rosenwaks, 2020). The embryo culture purpose of IVF is to select the best embryos for transfer to achieve a healthy single pregnancy. Because there is increasing global consensus to avoid multiple pregnancies after IVF/ICSI–ET, the use of selective single–ET, supported by PGT-A screening, has been promoted to maintain acceptable pregnancy rates while reducing the risk of multiple pregnancies (Li *et al.*, 2021). Chromosome abnormality is one of the main reasons for IVF failure. Now it is recognized that PGT can be used to screen embryos by detecting the structure and number of 23 pairs of chromosomes, which can reduce pregnancy failure caused by embryo chromosome abnormalities, significantly improving the clinical pregnancy rate of ET, and reducing the abortion rate. Multiple studies have shown that conventional PGT can reduce the IVF miscarriage rate from 26–33.5% to

https://doi.org/10.1017/S0967199422000363 Published online by Cambridge University Press

6.9–11.1% and can increase the clinical pregnancy rate from 43.9–45.8% to 55–70.9% (Kemper *et al.*, 2019; Marin *et al.*, 2021).

Unfortunately, biopsies of trophoblastic ectodermal cells from blastocysts are usually taken. Due to the limitation of safety factors and technical difficulties, it is difficult to popularize the TE biopsy technology for chromosome screening over a wide range. This method of sampling is invasive to the embryo and may potentially affect the quality and development of the embryo. In this situation, noninvasive chromosome testing for preimplantation embryo using cell-free DNA could be conducted as a promising approach for aneuploidy screening (Huang *et al.*, 2019). Therefore, NICS based on collecting culture medium and MALBAC–next generation sequencing (MALBAC-NGS) was developed. Previous reports have shown that false-positive rates were statistically less common in NICS than in PGT-A when both were compared with total blastocyst chromosome screening (gold standard), respectively, indicating that NICS is a very effective embryo chromosome screening technology (Fang *et al.*, 2019). To date, it has been reported that healthy children have been born through NICS technology for patients carrying balanced translocations and chromosomal inversions (Xu *et al.*, 2016; Jiao *et al.*, 2019).

However, there is a technical problem in that the consistencies of NICS and TE biopsy results have not been completely resolved. The concordance of TE biopsy and NICS from blastocoel fluid or blastocysts culture medium is still a controversial issue. Maternal contamination is the major technological challenge for the embryo medium-based PGT-A. Therefore, minimizing the risk of maternal DNA contamination is a very important problem for NICS technology. In our study, we found that an additional medium renewal of D4 embryo culture could improve the concordance of NICS with TE biopsy. To avoid further granulosa cell contamination, we improved the protocols for blastocysts culture medium collection as follows: (1) all cumulus cells were removed carefully prior to ICSI operation; (2) cleavage embryos were mechanically pipetted using 140-μm stripper tips on D3 and transferred to a fresh droplet; (3) embryos were transferred again on D4 to a fresh droplet of G-2 plus medium (blastocyst culture medium) following several repeated flushings. According to our previous experience, mechanically pipetting on D3 cannot injury the embryos, because there was no significant increase in egg diameter and embryo diameter on D3.

In this study, 223 blastocysts and one-to-one culture medium were selected for the final analysis. Of these, the overall concordance rates with TE biopsy of the experimental and control groups were 0.86 and 0.75, respectively. Statistically significant differences were found between the two groups. In addition, the false-negative rate in the experimental group was significantly lower than that in the control group (0.14 versus 0.32, $P = 0.04$), suggesting that an additional medium renewal of D4 embryo culture can improve the concordance of NICS with TE biopsy.

To the best of our knowledge, this is the first report on the chromosome concordance of TE biopsy with NICS using embryos culture medium renewed twice on D3 and D4; there was a high correlation between NICS samples and embryo samples, with sensitivity and specificity values of 0.92 and 0.89, respectively. The positive predictive value is reasonable and the negative predictive value is high, indicating that this NICS technique can be used to screen blastocysts chromosomes.

However, there were several limitations to our study. Maternal DNA contamination is the major technological challenge for NICS. In this study, each D4 embryo was again carefully denuded of the surrounding granulosa cumulus cells, and then washed thoroughly, then finally cultured individually. Even so, it was hard theoretically to totally eliminate maternal DNA contamination. Furthermore, the experimental groups were not randomized controlled. The TE biopsy was performed by the same experienced embryologist in our reproductive centre. Additional studies are necessary to investigate the transferability of NICS between laboratories with independent subjects and large-scale prospective controlled randomized studies.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/S0967199422000363

Availability of data and material. The datasets used during the current study are available from the corresponding author on reasonable request.

Acknowledgements. The authors would like to thank Shi-Long Xue and Cai-Xia Ma for English language editing and technical assistance.

Declaration statement. We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work; there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled.

Funding. This study was supported by the Gansu Province Science and Technology Foundation for Youths (Nos. 20JR10RA705, 21JR1RA001), Innovation Fund for Higher Education of Gansu Province (No. 2021B-008) and Scientific Research Fund of the First Hospital of Lanzhou University (No. ldyyyn2021-15).

Conflicts of interest statement. There are no conflicts of interest.

Ethics approval. This study was approved by the Ethics Committee of the First Hospital of Lanzhou University (LDYYLL2019–42).

Consent to participate. The authors certify that they have obtained all appropriate patient consent forms. The patients have given the consent for their clinical information to be reported in the journal. The patients understand that name and initials will not be published and due efforts will be made to conceal identity, but anonymity cannot be guaranteed.

Consent for publication. Not applicable.

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