Diagnostic efficiency of blastocyst culture medium in noninvasive preimplantation genetic testing

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Objective: To evaluate the diagnostic efficiency of spent blastocyst culture medium (BCM) in noninvasive preimplantation genetic testing (niPGT) by comparing the karyotype concordance with corresponding inner cell mass (ICM) among initial trophectoderm (TE) biopsy, TE re-biopsy, and BCM sampling.
Design: Re-analysis aneuploid/mosaic blastocysts donated for research by couples.
Setting: Institutional in vitro fertilization center.
Patient(s): A total of 12 couples donated their blastocysts, which had previously been diagnosed as aneuploid or mosaic by initial TE-biopsy preimplantation genetic testing for aneuploidy (PGT-A) for research.
Intervention(s): A total of 26 frozen–thawed blastocysts were re-analyzed by TE re-biopsy, ICM biopsy, and the collection of spent BCM.
Main Outcome Measure(s): Karyotype concordance rates.
Result(s): For 23 embryos diagnosed as aneuploid by initial TE biopsy, 78.3% of initial TE samples, 87.0% of TE re-biopsies samples, and 78.3% of BCM samples were concordant with corresponding ICM samples, and for three mosaic embryos, the concordance rates with ICM of these three groups were 0%, 100%, and 100%, respectively. With the corresponding ICM result as the true result, sensitivity of both niPGT-A and initial TE were 100%; however, the false-positive rate (FPR) of initial TE was higher than that of niPGT-A (100% vs. 0).
Conclusion(s): niPGT-A using BCM had diagnostic efficiency similar to that of TE-biopsy PGT-A. In the case of mosaic embryos, niPGT-A using BCM may be more reliable for predicting karyotypes of ICM than initial TE biopsy. (Fertil Steril Rep® 2021;2:88–94. ©2020 by American Society for Reproductive Medicine.)
Key Words: Noninvasive preimplantation genetic testing, blastocyst culture medium, trophectoderm biopsy, inner cell mass

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Preimplantation genetic testing (PGT) with trophectoderm (TE) biopsy is widely used to identify embryos with chromosomal and/or genetic abnormalities, and it has helped thousands of couples to achieve healthy infants. However, there are still many problems related to the diagnostic accuracy and safety of this approach. Biopsy of approximately 5 to 10 TE cells still cannot be regarded as a perfect representation of the actual chromosomal status of the inner cell mass (ICM). In addition, the diagnostic accuracy of a single TE biopsy could be compromised by the presence of chromosomal mosaicism in blastocyst-stage embryos (1, 2). Chromosomal mosaicism has been defined as the presence of two or more cell lines with different genotypes in an individual sample (3). It has been widely detected in human preimplantation embryos (4) rather than in later placental samples (5). To date, several studies have demonstrated that the increasing reports of chromosomal mosaicism might be related to the higher sensitivity of the next-generation sequencing (NGS) platform (6, 7) and TE biopsy itself (3, 7, 8). Transfer of embryos diagnosed as "abnormal" by TE biopsy in PGT for

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aneuploidy (PGT-A) could still result in healthy live births (9–12), which indicate limits of the diagnostic accuracy of TE biopsy (13). In addition, invasive biopsy techniques are inevitably accompanied by the risk of compromising the viability of embryos (14, 15). In this case, ongoing studies are conducted to verify whether noninvasive preimplantation genetic testing for aneuploidy (niPGT-A) using cell-free DNA (cfDNA) could be promoted as a promising approach for aneuploidy screening.

Cell-free DNA samples in blastocoel fluid (BF) were first amplified successfully to identify embryos with X-linked disorder by Palini et al. (16), which opened a new era of possibilities for niPGT-A. Subsequently, extracellular cfDNA in spent culture medium (17) was found to contain more medical-related genetic information than that in BF. Although the application of cfDNA has been promoted in niPGT, it is still unclear regarding the original source and composition of the cfDNA sample, which is possibly a mixture of embryonic DNA and maternal cell contamination (18). In addition, the concordance of ICM and cfDNA from BF or spent culture medium is still a controversial issue (19–22).

In this study, for the first time, to our knowledge, we re-analyzed blastocysts previously diagnosed as aneuploid or mosaic by TE biopsy and assessed the diagnostic efficiency of blastocyst culture medium (BCM) sampling in niPGT-A. Karyotype concordances with corresponding ICM were compared among initial TE biopsy, TE re-biopsy, and BCM sampling. We further compared the diagnostic accuracy and reliability between niPGT-A using BCM and TE-biopsy PGT-A in aneuploid and mosaic embryos.

MATERIALS AND METHODS

Ethics

The procedures performed in this study and data collection were approved by the ethics committee at the Center for Reproductive Medicine, the Sixth Affiliated Hospital of Sun Yat-Sen University (Program No.2017ZSLYEC-016S). Patients consented to donate their embryos for research, and informed consent was obtained from each couple.

Study Subjects

A total of 26 blastocysts with aneuploid (n = 23) or mosaic (n = 3) diagnosis were donated for research from 12 couples with indications of preimplantation genetic testing for chromosomal structural rearrangements (PGT-SR) or preimplantation genetic testing for aneuploidy (PGT-A). All the PGT cycles were performed at the Center for Reproductive Medicine in the Sixth Affiliated Hospital of Sun Yat-Sen University, from July 2018 to July 2019.

Embryo Culture and Initial TE Biopsy

All collected oocytes were fertilized by intracytoplasmic sperm injection (ICSI). Embryos were cultured individually in sequential medium (Vitrolife Sweden AB) in a 37°C humidified atmosphere of 6% CO_2, 5% O_2 balanced with N_2. On day 5 or day 6, blastocysts were scored based on the Gardner and Schoolcraft system (23). The morphological score of 26 donated blastocysts for re-analysis was at least 4BB. Biopsy of trophectoderm cells was performed as a routine procedure. Briefly, a biopsy pipette (TPC) with an inner diameter of 30 μm was inserted into the hole, and 5 to 10 TE cells were suctioned out. After biopsy, blastocysts were frozen by vitrification according to the manufacturer's protocol (VT101, Kitazato). The TE samples were then transferred into RNase-DNase–free PCR tubes containing 5 μL of cell lysis buffer (XK043, Yikon Genomics) and stored at −80°C until further processing.

TE re-biopsy, ICM, and BCM Sample Collection

After thawing as in our previous report (24), donated embryos were cultured individually for 15 hours to re-expand in 15-μL microdroplets of equilibrated G2 medium. Exactly 6 hours later after artificial shrinkage by laser (ZIOLS-tkTM, Hamilton Thorn Bioscience Inc.), approximately 10 μL of BCM samples was collected. Drops of culture medium only were collected as blank controls. ICM biopsy was performed according to a previously published method (25). TE was re-biopsied (>10 cells) at other sites of the trophectoderm compartment. All of the ICM, TE re-biopsy, and BCM samples were collected in RNase-DNase–free polymerase chain reaction (PCR) tubes containing 5 μL of cell lysis buffer (XK043, Yikon Genomics) and stored at −80°C for the following aneuploidy analysis.

DNA Sequencing and Determination of Aneuploidy by NGS

The MALBAC single-cell whole genome amplification (WGA) method was used to amplify DNA from all cells and BCM samples according to the manufacturer's protocol (YK005 and YK008, Yikon Genomics). Constructions of sequencing cDNA libraries were carried out simultaneously with amplification. The library was successfully sequenced on an Illumina HiSeq 2500 platform, and approximately 2 million raw reads per cell sample were generated. For couples with chromosomal abnormalities, chromosome deletions or duplications for the target chromosomes (typically >10 Mb) were identified in BCM analysis; otherwise, only long and short arms of chromosomes were recognized.

Embryos were diagnosed as euploid, aneuploidy, or mosaic by NGS results. The diagnostic criterion for mosaic embryo in biopsy samples was based on the current guidelines published by Preimplantation Genetic Diagnosis International Society (PGDIS) (3). The mosaicism threshold was set at 70% to identify true aneuploidy. Therefore, embryos were classified as mosaic if the level of mosaicism ranged from 40% to 70%, whereas a level of less than 40% was labeled as euploid and more than 70% as aneuploid. For chr13, chr16, chr18, and chr21 chromosomes, the criterion for mosaicism was set to be 30%.

Data Analysis

The primary outcomes of this study, concordance rates with ICM biopsies, were compared among groups (initial TE biopsy, TE re-biopsy, and BCM samples). The karyotype concordance of each two groups was defined as the presence of the same abnormal chromosome, especially in the target chromosome,

in different testing results compared. The clinical concordance of all four samples was defined as the concordance in embryo diagnosis, such as uniform aneuploid or not. To assess the diagnostic efficiency of the BCM sample, the false-positive rate, false-negative rate, sensitivity, specificity, accuracy, positive predictive value (PPV), and negative predictive value (NPV) were calculated according to the corresponding ICM results. Formulas are as follows: sensitivity = (true positives)/(true positives + false negatives); specificity = (true negatives)/(true negatives + false positives); accuracy = (true positives + true negatives)/samples; false-positive rate = (false positives)/(true negatives); false-negative rate = (false negatives)/(true positives); PPV = (true positives)/(true positives + false positives); NPV = (true negatives)/(true negatives + false negatives).

Statistical Analysis

Categorical variables were presented as frequencies. Statistical analyses were performed with the χ^2 test, Yates' correction, or Fisher's exact test accordingly when comparing frequencies or proportions. All statistical analysis was performed using SPSS 22.0 software (SPSS). Values of $P<.05$ were considered as significant.

RESULTS

Study Subjects

Twelve couples were included, with the maternal age ranging from 24 to 43 years and averaging 30.8 years. Karyotypes of couples are provided in Table 1. A total of 26 donated blastocysts were re-analyzed in this study; 20 were thawed after vitrification on day 5, and six were thawed after vitrification on day 6.

NGS Results

All re-biopsy and BCM samples were amplified successfully, and no DNA was detected in 26 blank culture medium samples. The NGS results of conventional TE biopsy, TE re-biopsy, ICM, and BCM samples are summarized in Table 2, classified by karyotype concordance. We noted that three embryos diagnosed as chromosome mosaicism by initial TE biopsy turned out to be uniformly euploid in corresponding TE re-biopsy, BCM, and ICM results (Table 2, embryo numbers 12, 14, and 16). In addition, the karyotype result of the BCM sample of the number 19 embryo [46,XX,-Xp (×1)] was obviously different from the results of other groups [47,XY,+13(×3)]. This sex discrepancy result indicates possible maternal contamination of the BCM sample.

Karyotype Concordance and Clinical Concordance

In total, 46.2% of the embryos (12/26) had karyotype concordant results among the initial TE biopsy, TE re-biopsy, and BCM samples, whereas others (14/26) had at least two discordant results. Of these karyotype discordances, results were categorized according to whether the clinical decision to transfer would be changed (clinical discordance) or not changed (clinical concordance). For example, 78.5% (11/14) of karyotype discordances (Table 2) that were considered to be discordant in aneuploid diagnosis (chromosomes and/or ploidy) were classified as being of clinical concordance (uniform aneuploid), and 21.4% (3/14) of karyotype discordances were considered to be discordant in clinical diagnosis.

Comparison of Karyotype Concordance with ICM Among Initial TE Biopsy, TE Re-biopsy, and BCM Samples

As shown in Table 3, for 26 donated embryos, generally the concordance rates with ICM of the initial TE biopsy, TE re-biopsy, and BCM were 69.2%, 88.5%, and 80.8%, respectively. No statistically significant difference was found between each of the two groups ($P>.05$). In detail, for 23 embryos diagnosed as aneuploid by initial TE biopsy, 78.3% of initial TE samples, 87.0% of TE re-biopsy and 78.3% of BCM samples were concordant with corresponding ICM samples, although there was no statistically significant difference between each of the two groups. However, for three mosaic embryos, the concordance rates with ICM of these three groups were 0% (0/3), 100% (3/3), and 100% (3/3), which was statistically significant ($P<.05$).

TABLE 1

Karyotypes of couples who donated blastocysts for re-analysis.

Female karyotype	Male karyotype	PGT type	Couples, n	Donated blastocysts, n
46,XX	46,XY,t(7;17)(q11.21;q25)	PGT-SR	1	2
46,XX,t(1;2)(p32;p11.2)	46,XY	PGT-SR	1	2
46,XX	46,XY,t(4;21)(p12;q22),13pstk+	PGT-SR	1	2
46,XX	46,XY,t(1;5)(q36.1;q22)	PGT-SR	1	2
46,XX,t(5;12)(q11.2;p11.2)	46,XY	PGT-SR	1	2
46,XX	46,XY,t(3;11)(p23;q13.3)	PGT-SR	1	1
45,XX,der(13;14)(q10;q10)	46,XY	PGT-SR	1	2
46,XX	45,XY,der(13;14)(q10;q10)	PGT-SR	2	3
45,XX,der(13;14)(q10;q11)	46,XY	PGT-SR	2	8
46,XX,inv(9)(p12q13)	46,XY	PGT-A	1	2
Total			12	26

Note: PGT = preimplantation genetic testing.

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TABLE 2

Results of initial trophectoderm biopsy, trophectoderm re-biopsy, blastocyst culture medium, and inner cell mass.

Couples' karyotype	ID	Initial TE biopsy	TE re-biopsy	BCM	ICM	Clinical concordance
46,XX,t(1;2)(p32;p11.2)	03	−1p(×1),+2p(×4),+2q(×3)[a]	−1p(×1),+2p(×4),+2q(×3)[a]	−1p(×1),+2p(×4),+2q(×3)[a]	−1p(×1),+2p(×4),+2q(×3)	Yes
46,XX,t(1;2)(p32;p11.2)	04	−1p(×1),+2p(×3),+16(×3)[a]	−1p(×1),+2p(×3),+16(×3)[a]	−1p(×1),+2p(×3), +16(×3)[a]	−1p(×1),+2p(×3),+16(×3)	Yes
46,XY,t(4;21)(p12;q22),13pstk+	06	+4q(×3),−21q(×1)[a]	+4q(×3),−21q(×1)[a]	+4q(×3),−21q(×1)[a]	+4q(×3),−21q(×1)	Yes
46,XY,t(1;5)(q36.1;q22)	08	+8(×3)[a]	+8(×3)[a]	+8q(×3)[a]	+8(×3)	Yes
46,XY,t(3;11)(p23;q13.3)	11	−3p(×1),+11q(×3)[a]	−3p(×1),+11q(×3)[a]	−3p(×1),+11q(×3)[a]	−3p(×1),+11q(×3)	Yes
45,XX,der(13;14)(q10;q10)	13	+1(×3)[a]	+1(×3)[a]	+1(×3)[a]	+1(×3)	Yes
45,XY,der(13;14)(q10;q10)	15	−16(×1)[a]	−16(×1)[a]	−16(×1)[a]	−16(×1)	Yes
45,XX,der(13;14)	17	+13(×3)[a]	+13(×3)[a]	+13(×3)[a]	+13(×3)	Yes
(q10;q11)	23	+13(×3),+16(×3)[a]	+13(×3),+16(×3)[a]	+13(×3),+16(×3)[a]	+13(×3),+16(×3)	Yes
	24	−13(×1)[a]	−13(×1)[a]	−13(×1)[a]	−13(×1)	Yes
46,XX,inv(9)	25	+2(×3),−16(×1),+21(×3)[a]	+2(×3),−16(×1),+21(×3)[a]	+2(×3),−16(×1),+21(×3)[a]	+2(×3),−16(×1),+21(×3)	Yes
(p12q13)	26	+10(×3),−22(×1)[a]	+10(×3),−22(×1)[a]	+10(×3),−22(×1)[a]	+10(×3),−22(×1)	Yes
46,XY,t(7;17)	01	+7q(×3),−17q(×1)[a]	+7q(×3)	+7q(×3)	+7q(×3),−17q(×1)	Yes
(q11.21;q25)	02	−7q(×1),+17q(×3)[a]	−7q(×1)	−7q(×1),+17q(×3)[a]	−7q(×1),+17q(×4)	Yes
46,XY,t(4;21)(p12;q22),13pstk+	05	+4(×3),−21q(×1),−Xq(×1)	+4p(×3),−21q(×1)[a]	+4p(×3),−21q(×1)[a]	+4p(×3),−21q(×1)	Yes
46,XY,t(1;5)(q36.1;q22)	07	−1p(×1),+5q(×3)[a]	−1p(×1),+5q(×3)[a]	+5q(×3)	−1p(×1),+5q(×3)	Yes
46,XX,t(5;12)	09	−5q(×1),+12p(×3)	−5q(×1),+12p(×3)	−5q(×1)[a]	−5q(×1)	Yes
(q11.2;p11.2)	10	−2q(×1), −5q(×1), +12p(×3)	−5q(×1),+12p(×3)[a]	−5q(×1),+12p(×3)[a]	−5q(×1),+12p(×3)	Yes
45,XX,der(13;14)	18	−14(×1)[a]	−14(×1)[a]	−14(×1),−X(×1)	−14(×1)	Yes
(q10;q11)	19	47,XY,+13(×3)[a]	47,XY,+13(×3)[a]	46,XX,−Xp (×1)[b]	47,XY,+13(×3)	Yes
	20	+22(×3)[a]	+22(×3)[a]	−1(×1),−2q(×1),+4q(×3),+22(×3)	+22q(×3)	Yes
	21	+10p(×3),−10q(×1)	−10q(×1)[a]	−10(×1)[a]	−10(×1)	Yes
	22	+9p(×3),−13(×1)	−13(×1)[a]	−13(×1)[a]	−13(×1)	Yes
45,XX,der(13;14)(q10;q10)	12	46,XY,−5(×1,mo,~40%)	46,XY[a]	46,XY[a]	46,XY	No
45,XY,der(13;14)(q10;q10)	14	46,XX,−Xq(×1,mo,~30%),+18q(×3,mo,~30%),+21(×3,mo,~30%)	46,XX[a]	46,XX[a]	46,XX	No
	16	46,XX,−Xq(×1,mo,~50%)	46,XX[a]	46,XX[a]	46,XX	No

Note: BCM = blastocyst culture medium; ICM = inner cell mass; TE = trophectoderm.
[a] Karyotype concordance with corresponding ICM, and results that are underlined represent the false-positive results in the initial TE results.
[b] Sex discrepancy; yes = clinical concordance; no = clinical discordance.

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TABLE 3

Comparison of concordance with inner cell mass among initial = trophectoderm biopsy, = trophectoderm re-biopsy, and blastocyst culture medium stratified by initial = trophectoderm results.

	Concordance rate, n (%)			
Initial TE results	**Initial TE biopsy and ICM**	**TE re-biopsy and ICM**	**BCM and ICM**	***P* value**
Aneuploid (n = 23)	18/23 (78.3)	20/23 (87.0)	18/23 (78.3)	.684
Mosaic (n = 3)	0/3 (0)	3/3 (100)	3/3 (100)	.011[a]
Total (n = 26)	18/26 (69.2)	23/26 (88.5)	21/26 (80.8)	.224

Note: BCM = blastocyst culture medium; ICM = inner cell mass; TE = trophectoderm.
[a] $P < .05$.

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Comparison of the Diagnostic Efficiency Between niPGT-A and Initial TE-Biopsy PGT-A

To evaluate the diagnostic accuracy of niPGT-A, we compared the performance between niPGT-A and initial TE-biopsy PGT-A, with the corresponding ICM result as the true result (Table 4). The FPR for niPGT-A was zero, much lower than that for initial TE-biopsy, which was 100% (3/3). Because of the re-analyzed embryos in the present study that were diagnosed as aneuploid or mosaic, the FNR for initial TE was zero. The FNR for niPGT-A was the same as the FNR for initial TE, whereas the NPV for niPGT-A was 100% (3/3). The sensitivity for both niPGT-A and initial TE were 100% (23/23). The specificity for niPGT was 100% (3/3), much higher than that for the initial TE. In general, the accuracy for the initial TE and niPGT-A was 88.5% and 100%, respectively, although there was no statistically significant difference between the two groups ($P>.05$). The PPVs for initial TE and niPGT-A were 88.5% and 100%, respectively ($P>.05$).

DISCUSSION

The findings of this study show that niPGT-A has similar diagnostic efficiency with TE-biopsy PGT-A. We further found that niPGT-A using BCM may be more reliable for predicting the karyotypes of ICM than TE biopsies in mosaic embryos.

Previous studies have been conducted to analyze the concordance between niPGT-A and TE-biopsy PGT-A, or to compare the diagnostic efficiency between niPGT-A and TE-biopsy in ehivh the rest of the embryo or the whole embryo was regarded as the standard reference (13, 19, 26–28). However, increasing evidence has demonstrated that the genetic results from TE biopsy, as well as the rest of the embryo mixed with ICM with TE, may not truly reflect the genetic status of the blastocyst-stage embryo due to mosaicism (9, 13, 29, 30). A recommended, more reasonable way to verify the diagnostic efficiency of niPGT-A would be to compare BCM and multiple biopsy sites of the embryo, particularly the ICM samples (31). To the best of our knowledge, this is the first reported study to systematically evaluate the diagnostic efficiency of niPGT-A by comparing the concordance among ICM, BCM, and TE (i.e., initial TE and TE re-biopsy) samples.

In this study, three partial "mosaic" embryos diagnosed by initial TE biopsies were detected as euploid by the analysis of TE re-biopsy, BCM, and ICM samples, which further indicated that TE biopsy may not accurately predict the true chromosomal status of ICM. This could be explained by a self-correcting mechanism in mosaic embryos (32), whereby mosaic cells in the cleavage-stage embryo may incorporate into TE cells but not ICM cells. Another possible explanation could be the biopsy manipulation itself. It has been reported that mosaicism may result from biopsy manipulation with laser, accompanied by cell damage and loss of cellular DNA (3), which may subsequently lead to bias in library construction. In addition, unlike previous technologies such as array comparative genomic hybridization (aCGH) (10), higher-resolution NGS-based PGT-A demonstrates increased sensitivity to identify lower-level mosaicism (33, 34).

Euploid/aneuploid mosaic embryos are deemed to be abnormal between euploid and fully abnormal embryos; thus the classification of lower-level mosaicism becomes a new challenge in mosaicism diagnosis, and a threshold for mosaicism needs to be set carefully to classify the embryos as euploid, aneuploid, and mosaic. Preimplantation Genetic Diagnosis International Society. (3) recommends considering and reporting the mosaic spectrum ranges from 20% (relatively lower risk) to 80% (higher risk). Actually, because of the lesser or greater detection abilities for mosaicism, different laboratories may have their own cutoff values for reporting mosaic levels, which may affect the accuracy of TE biopsy and spent BCM. Using the 20% to 80% mosaic threshold for NGS results, Maxwell et al. (33) indicated that of the embryos previously diagnosed as euploid but resulting in miscarriage, 31.6% were diagnosed as mosaic with NGS. Another study demonstrated that mosaic embryos with a lower percentage of abnormal cells (<50%) were associated with pregnancy outcomes similar to those achieved with euploid embryos (35). To minimize the FPR and FNR, the lower cutoff value for mosaic in our PGT laboratory was 40%, whereas the upper value was 70%. In the present study, the levels of mosaicism in three "mosaic" embryos were 40% (monosomy 5), 30% (monosomy X, trisomy 18 and 21), and 50% (monosomy X), respectively. But the results of the samples from TE re-biopsies, ICM and BCM have showed that they were all uniform euploid, which indicated that mosaics in initial TE biopsies were related with false positive rather than the inappropriate threshold for mosaicism in our lab.

TABLE 4

Comparison of the diagnostic efficiency between initial trophectoderm and noninvasive preimplantation genetic testing for aneuploidy with the corresponding inner cell mass result as the true result.

Performance characteristic	Initial TE (n = 26), n (%)	niPGT-A (n = 26), n (%)	*P* value
False-positive rate	3/3 (100)	0/3 (0)	.100
False-negative rate	0/23 (0)	0/23 (0)	–
Sensitivity	23/23 (100)	23/23 (100)	–
Specificity	0/3 (0)	3/3 (100)	.100
Accuracy	23/26 (88.5)	26/26 (100)	.235
Positive predictive value	23/26 (88.5)	23/23 (100)	.237
Negative predictive value	–	3/3 (100)	–

Note: ICM = inner cell mass; niPGT-A = noninvasive preimplantation genetic testing for aneuploidy; TE = trophectoderm.

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We should note that there is a tendency to discard embryos diagnosed as having chromosome mosaicism with initial TE biopsy. However, it has been shown that transfer of embryos diagnosed as "aneuploidy" by TE biopsy in PGT-A could still result in healthy live infants (9–12), which indicated the limits in diagnostic accuracy of TE biopsy. Instead, as a noninvasive operation, BCM sampling could lower the bias mentioned above, and therefore could be more reliable in predicting the karyotypes of ICM and could avoid wasting normal embryos (9–12). As our data showed, niPGT-A using BCM sample could achieve similar diagnostic efficiency similar to that of TE biopsy, and turned out to be consistent with ICM results in those three partial "mosaic" cases. Besides, initial TE biopsy combined with niPGT-A using BCM may achieve a more promising diagnostic accuracy. For example, the embryos with identical euploid results in both TE biopsy and niPGT-A have the highest priority for transfer; and for embryos with mosaic results on initial TE biopsy and euploid results on niPGT-A, the lowest priority for transfer would be observed instead of direct disposal. The combination of TE biopsy and niPGT-A needs further study and the cost of tests should be taken into consideration in clinical applications.

Apart from diagnostic capabilities, the efficiency of amplifying adequate cfDNA is also a pivotal concern for clinical application of niPGT-A. In theory, it is possible for cfDNA from both TE and ICM to leak into spent culture medium, but TE contacts the medium directly whereas ICM does not (31). To enhance the concentration of cfDNA with minimal injury, we performed artificial blastocyst collapse by laser to release cfDNA from BF into the spent BCM. In this study, all of 26 donated embryos resulted in an efficient DNA amplification, which referred to a higher efficiency than reported in previous studies (16, 22, 36). In addition, as reducing the volume of the blastocoel cavity in expanded blastocysts by artificial collapse could improve the vitrification outcome (16, 22, 36, 37) rather than causing injury, it is reasonable to believe that this manipulation does little harm to the embryos when applied in sample collection in niPGT. In total, we believed that BCM mixed with BF in this study could be a promising source of cfDNA to achieve a high amplification rate with minimal injury.

Our study does have some limitations. First, because of the preciousness of euploid embryos in patients undergoing PGT, the sample size of this study is limited, and we reanalyzed only the embryos diagnosed as "abnormal" by initial TE biopsy, which limited the generalizability of our findings and the value of PPV and NPV. Moreover, the cohorts of patients in the present study were carriers of balanced translocations or inversions. In our PGT laboratory, only in the embryos diagnosed as having euploidy are the position of breakpoints to distinguish carrier from noncarrier embryos identified. In other words, the embryos included in this case only underwent the PGT-A process. Second, maternal DNA contamination has been a barrier to the accuracy of BCM samples. For example, in the analysis of embryo number 19, whose maternal karyotype was "45, XX, der (13;14)(q10;q11)," the karyotype result of the BCM sample [46,XX,−Xp (×1)] was obviously different from the results of the other three samples [47,XY,+13(×3)].The contradictory sex results were probably due to the maternal DNA contamination of the BCM collection. It is possible that the maternal contamination may come from the cumulus cells rather than two polar bodies, which degenerate later after fertilization. To solve this problem, we had transferred the thawed blastocyst to a fresh separate droplet of the culture medium following rinsing several times in new drops. Besides, the embryos in the present study were previously cryopreserved on day 5 or day 6, which may effectively reduce the risk of maternal DNA contamination (26) by influence the release of the cell-free DNA. Hence, the risk of maternal DNA contamination in real-life conditions may be higher than indicated by our data. To minimize the possibility of such contamination in the fresh cycle, we improved the protocols for BCM collection as follows: [1] all cumulus cells were removed prior to ICSI; [2] if any residual cumulus cells were observed on day 3, embryos would be mechanically denuded again; and [3] embryos were transferred to a fresh droplet of the blastocyst culture medium following repeated flushing.

In conclusion, with improvements in techniques such as DNA collection, amplification, and sequencing methods, niPGT-A using BCM is becoming a reliable way to identify euploid embryos. BCM sample collection in niPGT could be promoted as a noninvasive and cost-effective method with diagnostic efficiency similar to that of TE-biopsy PGT-A.

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