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Chromosomal Mosaicism in Cleavage Stage Embryos vs. Blastocyst Stage Embryos

El-Bisari S*, Alyounis D, Sayed W and Uppal S
Health Plus Genetics Laboratory, Abu Dhabi, United Arab Emirates

Abstract

Introduction: Chromosomal mosaicism is characterized by the presence of more than one chromosomally different cell line in an individual. Preimplantation chromosomal mosaicism is characterized by the presence of a mixture of chromosomally different cell lines in an embryo. Studies show that mosaicism for whole chromosomes (aneuploidies) in one or more cells (blastomeres) occurred in more than 75% of cleavage stage embryos, whilst 3%-24% of blastocyst stage embryos are chromosomally mosaic.

Aim: The purpose of this study was to standardize and validate a Next Generation Sequencing (NGS) method for comprehensive chromosome testing for aneuploidies and to study the level of mosaicism in cleavages stage *vs.* blastocyst stage embryos.

Methods: The validation involved a retrospective blind assessment of whole genome amplification (WGA) products from 14 cleavages stage embryo biopsies (blastomeres), 6 blastocyst stage embryo biopsies (TE), in addition to their 20 discarded blastocyst stage whole embryos. 42.8% of the cleavages stage embryos showed mosaicism, whilst results between the trophectoderm (TE) biopsies (TEB) and their whole embryos at blastocyst stage showed total concordance as no mosaicism was observed. NGS sensitivity and specificity for calling aneuploidy was found to be 100%.

Conclusion: This is the first study reporting preclinical validation and accuracy assessment of the Ion semiconductor sequencing technology in studying the level of mosaicism in cleavage stage and TE biopsies blastocyst stage embryos *vs.* their whole embryos.

The high level of mosaicism in cleavages stage embryos compared to blastocyst stage embryos does not recommend the PGT-A to be performed on cleavage stage embryos. The NGS proved to be a robust methodology in clinical application of PGT-A.

Keywords: Preimplantation Genetic Testing • Next-Generation Sequencing •cleavage stage embryos • blastocyst stage embryos

Introduction

Chromosomal mosaicism is defined as the presence of two or more chromosomally different cell lines aneuploid/aneuploid, or euploid/aneuploid. Chromosomal mosaicism is well known in prenatal samples, mostly in chorionic villus samples CVS, commonly seen as Confined to the Placenta Mosaicism CPM. CPM was first reported in the human placenta by Warburton et al. and it is defined as chromosomal differences between the fetus and placenta. It's found to occur in roughly 1-2% of all placental tissues analyzed. However only 23% of mosaicism detected at CVS is confirmed in the fetus, the remaining 77% is found to be CPM, which depending on the level of mosaicism and the significance of the chromosome involved may result either in a normal live born, or in an abnormal placental function which may lead to Intrauterine growth restriction (IUGR), spontaneous abortion or intrauterine death [1].

Mosaicism in human embryogenesis

Chromosomal aneuploidy is common in human embryos, causing miscarriage, implantation failure, and IVF failure. The aneuploidy rate is found to be increased in infertile patients with the following indications: advanced maternal age (AMA), repeated implantation failure (RIF), severe male factor and repeated miscarriages (RM). The cleavage stage embryo stands out due to the high level of chromosomal abnormalities, especially mosaicism that arises prior to global embryonic genome activation.

***Address for Correspondence:** Dr. Sara El-Bisari. Health Plus Genomics Laboratory, 11th Hazza bin Zayed St, Al Karama area, Abu Dhabi, United Arab Emirates, E-mail: sara.bisari@hplus.ae*

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What we know

Human embryogenesis begins in a relative genomic transcription silence, where the oocyte-to-embryo transition lasts for approximately 3 days. Within this period fertilization takes place and the zygote starts to develop by undergoing through cell divisions and epigenetic reprogramming however, at this stage the embryo is still transcriptionally non-active. On the 3rd day of embryogenesis, the major wave of embryonic genome activation EGA occurs and genomic transcription starts [2].

Studies have shown that the major wave of EGA is independent of cell number, occurring at day-3 even in poor embryos that have arrested in a stage of less than 8 cells. The oocyte-to-embryo transition occurs in the absence of *de novo* genomic transcription, however all the factors required for the process, whether mRNA or protein, are already present within the oocyte.

In a study by Vanneste et al. titled "chromosome instability is common in human cleavage stage embryos" [1]. It was identified that several chromosomal abnormalities occurred in the embryos, especially in cleavage stage embryos where chromosomal aneuploidies in a mosaic state occurred in more than 80% of them. In addition to chromosome segmental deletions, duplications and amplifications that were reciprocal between presumptive sister blastomeres, suggesting that there is frequent chromosome breakage and fusion during early human development, especially in the cleavage divisions. Keeping in mind that apoptosis in response to chromosomal aneuploidies has not been widely observed in human embryos prior to day-5. The exceptionally high incidence of mosaicisms observed in early embryogenesis is the product of several mechanisms [3].

Effect of parental gametes

Paternal effect: Sperm DNA fragmentation, as well as a functionally effected

centrosome, is the first paternal contributors to the early embryogenesis. The centrosome's critical role in mitosis is proven, as a dysfunctional centrosome causes abnormal chromosomal segregation. Considering that up to 25% of the non-dividing eggs are in fact fertilized but submitted to cell division defects.

Maternal effect: The early embryogenesis, until oocyte-to-embryo transition, is under maternal control. Maternal mRNA and protein content of the oocytes, are of major importance.

The negative effect of maternal age in the oocyte's and the embryo's competence is one of the major causes of female infertility. The advanced maternal age reduces the accuracy of the genomic checkpoints, causing erroneous chromosomal segregation and reducing the DNA repair capability of the oocytes. Aneuploidy and polyploidy are increased with age. In oocytes from young women, sperm defects can be repaired to some point and embryogenesis may proceed unhindered.

Cell cycle effects

Chromosome nondisjunction, anaphase lag, and endoreplication are the main mechanisms that could lead to the formation of different cell lines in early embryogenesis.

Non-disjunction: Failure of sister chromatids to separate during mitosis. Instead of separating, the two chromatids are pulled to one cell, resulting in a cell with a monosomy and another cell with a trisomy. The level of mosaicism in an embryo depends on when the non-disjunction occurred. If the non-disjunction occurred prior to cell differentiation (in a cleavage stage embryo), a general mosaic is created. However, if the non-disjunction occurred in the TE compartment after cell differentiation, then only the TE compartment would have a mix of chromosomally different cell lines which is the definition of chromosomal mosaicism, leaving the inner cell mas (ICM) unaffected from mosaicism, therefore euploid. As the embryo grows and evolves, the TE will become the placenta, and the ICM will become the fetus, the early TE mosaicism will become placental mosaicism and because the mosaicism is contained in the placenta (confined placental mosaicism CPM), the embryo compartment would be euploid.

Anaphase lag: Anaphase lagging is the delayed movement of chromosome or sister chromatids due to spindle defects, resulting in the loss of the chromosome or sister chromatids as they fail to incorporate in the nucleus. If this event occurs prior to cell differentiation, then a general mosaicism is created. However, if this event occurs after cell differentiation, in the TE compartment, then only the TE will be mosaic as it contains the chromosomally different cell lines. The mosaic TE will result in a mosaic placenta (CPM), while the ICM compartment would result in a euploid fetus. In a study conducted by Ioannou et al. testing discarded blastocyst stage embryos, showed that monosomy can occur at a 7x greater rate than trisomy. This would implicate anaphase lagging as the main source of mosaicism in human preimplantation development. This observation is supported by Coonen et al. and Capalbo et al. who found anaphase lagging at rates of 5x and 3x that of non-disjunction, respectively.

A trisomic cell may be corrected by anaphase lagging in a process referred to as trisomy rescue, by discarding the extra chromosome leaving two copies of the chromosome and an eventually euploid embryo.

Endoreplication/Endoduplication: Replication of a chromosome without cell division. This would result in a chromosomal trisomy in the former euploid cell. Chromosome gain is believed to derive from two mechanisms, a cell cycle malfunction in which a chromosome is replicated without subsequent cytokinesis or when mitosis is initiated and shortly thereafter shutdown, resulting in a replicated chromosome.

Aneuploidy rescue uses endoreplication as a successful mechanism for monosomy rescue, by replicating the monosomic chromosome, leading to a chromosomal disomy and a euploid cell.

However, depending on the chromosome involved, monosomy rescue may also lead to a UniParental Disomy (UPD), e.g. UPD of chromosomes 6, 7, 11, 14 and 15, which is associated with well-known syndromes.

A well-known fact is that mosaic embryos can correct an aneuploidy using an aneuploidy rescue mechanism. Cleavage stage embryos diagnosed as mosaic by PGT-A are found to correct one or more aneuploidies by the time they reach the blastocyst stage. Several mechanisms have been proposed to explain mosaicism correction:

1. Preferential growth of the euploid cells.
2. Growth disadvantage of aneuploid cells, or elimination of the aneuploid cells by processes such as apoptosis, leading to a decline in their numbers as the embryo develops, ultimately resulting in a normal embryo leading to a normal fetus.
3. Trisomy rescue via anaphase lag or non-disjunction.
4. Monosomy rescue by replicating the missing copy of the chromosome via endoreplication.

Mosaicism is a very common event in early embryogenesis, and its clinical consequences depends on the chromosome involved, the level of mosaicism, and whether the mosaicism is general or CPM. For example, a low level mosaicism of a trisomy of chromosome 2 in a TE biopsy of a blastocyst stage embryo, would either rescue via trisomy rescue resulting in a euploid fetus, or would sustain in the TE compartment leaving the ICM unaffected but leading to a dysfunctional placenta that could lead to a miscarriage or an IUGR, or it could be a general type of mosaicism in the embryo that could lead to a miscarriage. The significance of the chromosome involved is of high importance; a significant chromosome is one that could lead to a live born regardless of the level of mosaicism it if found in, e.g. A general mosaicism of trisomy 21 could lead to a mosaic Down syndrome [4-6].

However, one must always keep in mind that the level of mosaicism found at analysis does not necessarily reflect the tissue distribution in the fetus.

Research Methodology

Experiment design

PGT-A by NGS validation study: This validation is a comparison blinded study to verify the PGT-A procedures, instruments and performance before it's being introduced into routine use.

This procedure describes the genetic analysis of IVF embryos by analyzing embryo biopsies from cleavage stage (day-3), or blastocyst stage (day-5) embryos using NGS, in order to diagnose genetically affected embryos carrying chromosomal aneuploidies and distinguish them from the genetically unaffected/ euploid and chromosomally balanced embryos which are suitable for embryo transfer. More specifically, the validation involved a retrospective blind assessment of WGA products, from 14 cleavage stage embryo biopsies (11 blastomeres collected and frozen within a period of 6 weeks, and 3 fresh blastomeres), 6 blastocyst stage embryo biopsies (6 TE biopsies collected and frozen within a period of 6 weeks), in addition to discarded blastocyst stage embryos that were biopsied on day-3 or on day-5 and the PGT-A result was abnormal/ aneuploid, therefore, the blastocyst stage whole embryos were tubed and analyzed to study aneuploidy rescue from day-3 to day-5 of embryogenesis.

Consent approval

Samples obtained in this study were obtained with patient consent.

Study part I: 14 cleavage stage embryos were biopsied 2 ways. 1 blastomere was sent to a referral lab to be tested by NGS for PGT-A, and a second blastomere was tested in-house by NGS for PGT-A. If the results came as abnormal/aneuploid from the referral lab, the discarded whole embryo at blastocyst stage would be tubed and sent to a 2nd referral lab to be tested by NGS for PGT-A.

Study part II: 6 cleavage stage embryos were biopsied and the blastomeres were sent to a referral lab, if the result came as abnormal/ aneuploid, the discarded blastocyst stage embryos were biopsied on day-

5 and the TE biopsies were analyzed in-house by NGS for PGT-A, and the remaining blastocyst stage whole embryos were tubed and sent to the 2nd referral lab to be tested by NGS for PGT-A.

Standardization and WGA: The 40 embryo biopsies and discarded blastocyst stage whole embryos were amplified using Ion ReproSeq PGS kit (Thermo Fisher Scientific). The WGA products were then quantified with Qubit dsDNA HS (high sensitivity) assay kit as per the manufacturer's manual (Invitrogen). The average DNA concentration of the samples was between 20-40 ng/ml. The barcodes were assigned as per Ion Reproseq PGS kit protocol (Thermo Fisher Scientific).

Library pooling, purification, and quantification: Barcoded whole genome amplification (WGA) products were pooled, purified, quantified, and processed following the Thermo Fisher Scientific Ion ReproSeq PGS library preparation kit protocol. The barcoded samples libraries were pooled in 24 sample multiplexes. The sequencing run was performed using the Ion ReproSeq PGS kits, and samples were loaded on Ion 520 Chips.

Sequencing analysis

The data generated from the Torrent server were analyzed using software version 5.2 for reads filtering, base calling, barcode filtering, and alignment to the human genome hg19 reference. The plug-in software was also used to check the quality of experiment for each sample with respect to a sufficient number of reads and chip loading percentage. For data analysis, the samples were processed through the Ion Reporter Software version 5.2 by using ReproSeq low-pass whole genome aneuploidy workflow that can detect aneuploidies and large partial chromosome aneuploidies (greater then 20Mb) from a single whole-genome sample with low coverage (minimum 0.01·), and the ReproSeq Mosaic PGS w1.1 r. 0 workflow that can detect mosaicism level at 30% and greater. Normalization was performed by using an informatics baseline generated from multiple normal samples. The analysis for aneuploidy detection was performed by using an algorithm based on a hidden Markov model. The algorithm uses read coverage across the genome to predict the copy-number or whole number ploidy status (i.e., 0, 1, 2, 3, etc.). Before copy-number determination, read coverage is corrected for guanine-cytosine (GC) bias and compared with a precontabled baseline obtained from 10 normal male samples that were processed in a manner representative of the method described herein. The use of 10 samples for baseline calculation substantially reduces the sample-to-sample variance in coverage and results in a smaller number of false-positive calls. The analysis visualization can be viewed in integrated genome viewer (IGV) light version 5.0, and scoring of aneuploidy was based on visualization of the IGV profile indicating losses and gains of the whole chromosome coupled with confidence and precision metrics. Confidence is defined as a log ratio between the observed ploidy value and the expected value. Large confidence values indicate that the algorithm is very certain that the ploidy state differs from the expected. Precision is defined as a log ratio between the likelihood of the assigned ploidy state and a next-closest state. Low precision (<10) denotes uncertainty in the absolute ploidy value assignment. It is possible that high ploidy states (e.g., copy number state >5) will have low precision and high confidence, indicating that the algorithm is uncertain about the absolute ploidy value but quite certain about the existence of a ploidy state that is different from the expected (for example, high confidence means copy number increase is present, but uncertainty in whether the exact copy number is 5 or 6). The results are analyzed in the Tables 1, 2, 3 and 4 [7-10].

Table 1. PGT-A validation results of splitting cleavage stage embryo biopsies vs their whole embryos in blastocyst stage.

Sample ID	Referral lab-1 results (cleavage stage embryo biopsies)	In-house results (cleavage stage embryo biopsies)	Referral lab-2 results (Blastocyst stage Whole embryos)
174	XY, +15	47, XY, +15	47, XY, +15
177	Complex abnormal	44, XX, -4, -13, +15, -19, +20, -22	44, XX, -4, -13, +15, -19, +20, -22
182	Y0, -13, -21	46, XY	46, XY
183	Complex abnormal	48, XYY, -20, +9, +18	45, XY, -18
184	X0, -13, -18, -21	46, XX	46, XX
188	XX, -13	47, XX, +15	47, XX, +15
192	XXXX, +13, +13, +18, +18, +21, +21	43, XX, -15, -16, del(17)(p)	45, XX, -15
193	XYY	45, XY, -4	46, XY
194	XX, +21	45, XX, -22	45, XX, -22
195	XYY	46, XY, -16, +19	45, XY, -16
196	XY, -18	45, XY, -5	46, XY
4	XX, +13	48, XX, +5, +7, +13, -15	46, XX, +13, +15
10	X0	46, XX	46, XX
15	XY, -18	46, XY	46, XY

Table 2. PGT-A validation results (cleavage stage embryo biopsies vs TE biopsies vs their whole embryos in blastocyst stage).

Sample ID	Referral lab-1 results (cleavage stage embryo biopsies)	In- house TE biopsies results	Whole embryos results
213	XY, -7, -22	45, XY, -22	45, XY, -22
217	XX, -21	46, XX	46, XX
218	Complex abnormal	46, XY	46, XY
219	XY, -13	46, XY	46, XY
220	Complex abnormal	46, XY	46, XY
221	3N, XXY	42, XXY, -2, -9, -12, -18, -19	42, XXY, -2, -9, -12, -18, -19

Table 3. PGT-A validation interpretation of results.

Sample ID	Cleavage stage embryo biopsies results	Blastocyst stage Whole embryos results	Aneuploidies found to be in a mosaic state	Rescued aneuploidies
174	47, XY, +15	47, XY, +15		
177	44, XX, -4, -13, +15, -19, +20, -22	44, XX, -4, -13, +15, -19, +20, -22	(+10, +16)	
182	46, XY	46, XY		

183	48, XYY, -20, +9, +18	45, XY, -18	-9, -Y	-20
184	46, XX	46, XX		
188	47, XX, +15	47, XX, +15		
192	43, XX, -15, -16, del(17)(p)	45, XX, -15		-16, del(17)(p)
193	45, XY, -4	46, XY		-4
194	45, XX, -22	45, XX, -22		
195	46, XY, -16, +19	45, XY, -16		+19
196	45, XY, -5	46, XY		-5
4	48, XX, +5, +7, +13, -15	46, XX, +13, +15	-7	+5, +7
10	46, XX	46, XX		
15	46, XY	46, XY		

Table 4. PGT-A validation interpretation of results.

Sample ID	TE biopsies results	Blastocyst stage whole embryos results	Aneuploidies found to be in a mosaic state	Rescued aneuploidies
213	45, XY, -22	45, XY, -22		
217	46, XX	46, XX		
218	46, XY	46, XY		
219	46, XY	46, XY		
220	46, XY	46, XY		
221	42, XXY, -2, -9, -12, -18, -19	42, XXY, -2, -9, -12, -18, -19		

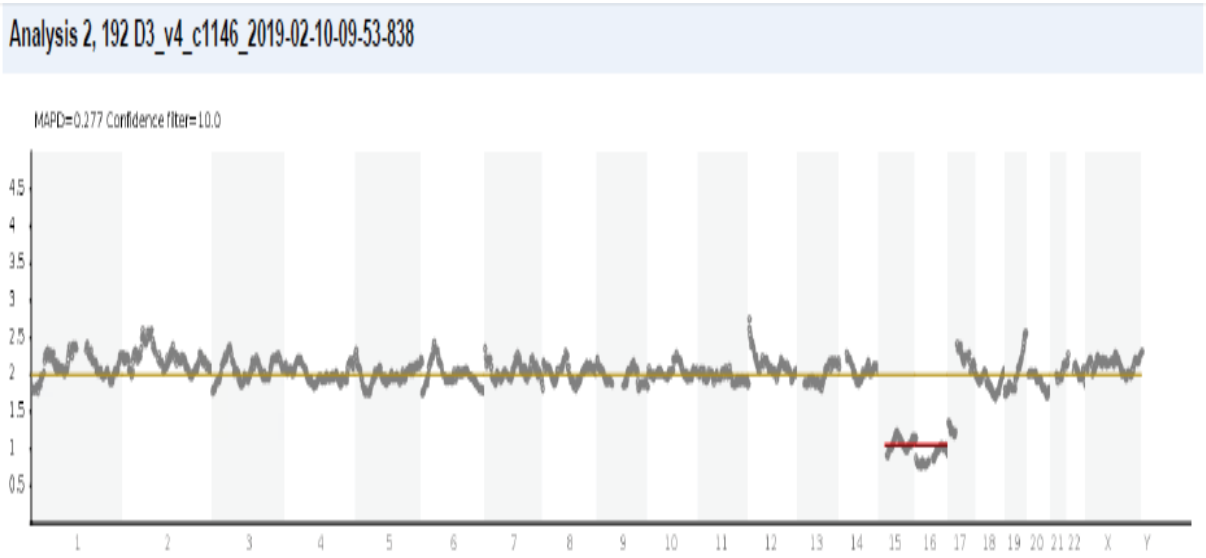

Figure 1. Sample 192: Cleavage stage embryo biopsy NGS results.

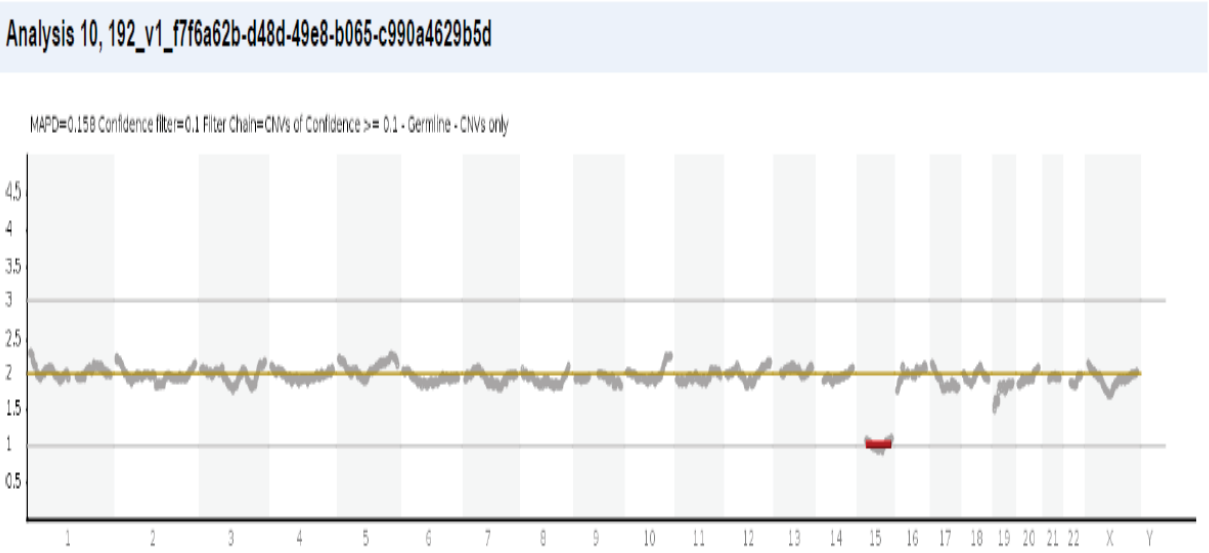

Figure 2. Sample 192: Blastocyst stage whole embryo NGS results.

Results and Discussion

Consistency of NGS PGT-A was evaluated with both previously established cytogenetic karyotypes (control DNAs), and WGA products. Discordant samples were subsequently reevaluated by a 2nd referral laboratory using the same NGS platform.

Results interpretation

The shaded results in Tables 1 and 2 are non-concordant between the 1st referral lab (column 2_shaded) and our lab (column 3). The last column shows the results of the blastocyst stage whole embryos analysis from the 2nd referral lab.

Results interpretation of column 3 (blastomeres) and 4 (whole embryos) revealed the effect of aneuploidy rescue in the embryos from cleavage stage to blastocyst stage of embryogenesis.

E.g. 1. In embryo 192, the blastomere NGS analysis showed monosomies of chromosomes 15, 16 and a deletion of the p arm of chromosome 17, however, the analysis of the blastocyst stage whole embryo showed a monosomy of chromosome 15 only, while monosomy of chromosomes 16 and deletion of 17(p) arm were rescued (not detected in the whole embryo). Analysis of the findings can be explained with aneuploidy rescue from day-3 to day-5 of embryogenesis, where the full and partial aneuploidies of chromosomes 16 and 17 were rescued in the blastocyst stage embryo, leaving only the aneuploidy of chromosome 15.

E.g. 2. In embryo 183, the blastomere NGS analysis showed an additional Y chromosome, monosomy of chromosome 20, a trisomy of chromosome 9 and a trisomy of chromosome 18, the analysis of the blastocyst stage whole embryo showed a monosomy of chromosome 18 only, however monosomies of chromosomes Y and 9 were found in a mosaic state, and the monosomy of chromosome 20 was not detected in the blastocyst stage whole embryo. Analysis of the findings can be explained with aneuploidy rescue from day-3 to day-5 of embryogenesis, were chromosomes Y and 9 are in a mosaic state, aneuploidy of chromosome 20 was rescued in the blastocyst stage embryo, leaving only the aneuploidy of chromosome 18 in a non-mosaic state (Figures 1 and 2).

Conclusion

In conclusion, cleavage stage embryos stand out for the high level of mosaicism and are not recommended to be used for PGT-A, in comparison with blastocyst stage embryos in which the level of mosaicism is remarkably lower. Therefore, the TE biopsy seems to be more representative of the embryo at that stage of embryogenesis and is recommended to be used for PGT-A.

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Author Contributions

S.E: Study design, optimization of laboratory methods, execution of the project work, analysis of data and results, and preparation of the article; D.Y: Execution of the project work; E.M., T.L, M.A, and S.H: Embryo biopsy and tubing; W.S: Support in the enrollment of patients in the study, clinical documentation for the patients and proforma filling, and patient referral for PGT-A studies; S.U: Analysis of data and results and reviewing of the article.

Author Disclosure Statement

No competing financial interests exist.

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