Validation of preimplantation genetic tests for aneuploidy (PGT-A) with DNA from spent culture media (SCM): concordance assessment and implication

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Research

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Abstract

Background: Spent culture medium (SCM) as a source of DNA for preimplantation genetic tests aneuploidy (PGT-A) has been widely discussed.

Methods: Seventy-five blastocysts donated for research provided a unique possibility in which multiple specimens, including trophectoderm (TE) biopsy, SCM, and paired corresponding whole blastocyst (WB) specimens from the same blastocyst source, could be utilized for the purpose of this preclinical validation.

Results: To conduct a validation ploidy concordance assessment, we evaluated the full chromosomal concordance rates between SCM and WB (SCM-to-WB), and between TE and WB (TE-to-WB) as well as sensitivity, specificity and overall diagnostic accuracy. 78.67% (59/75) of NGS results in the SCM group are interpretable, which is significantly lower than their corresponding TE and WB groups. This discrepancy manifests itself in intrinsically low quantity and poor integrity DNA from SCM. Subsequently, remarkable differences in full concordance rates (including mosaicism, and segmental aneuploidies) are seen: 32.2% (SCM-to-WB, 19/59) and 69.33% (TE-to-WB, 52/75), *($p < 0.001$)*. In such cases, full concordance rates were 27.27% (15/55) in SCM-to-WB, and, 76% (57/75) in TE-to-WB *($p < 0.001$)* Collectively, the NGS data from SCM also translated into lower sensitivities, Positive Predictive Value (PPV), Negative Predictive Value (NPV), overall diagnostic accuracies, and higher Negative Likelihood Ratio (NLR).

Conclusions: Our study reveals that DNA is detectable in the majority of SCM samples. Individual chromosomal aberration, such as segmental aneuploidy and mosaicism, can be quantitatively and qualitatively measured. However, TE still provides a more accurate and reliable high-throughput methodology for PGT-A. While cell-free DNA in SCM has the potential to represent a useful strategy in reproductive medicine.

Introduction

A methodology increasingly used to select against embryonic aneuploidy is preimplantation genetic testing of aneuploidies with trophectoderm biopsy [1, 2]. This procedure is based on a direct assessment of the chromosome status of biopsied TE cells to accurately and effectively identify embryos that have the appropriate number of chromosomes. However, because current TE biopsy PGT-A procedures are an inherently invasive challenge, there are fundamental safety and accuracy weaknesses for them. From a micromanipulation standpoint, TE biopsy requires specific equipment and trained personnel with expertise on strict quality assessment of embryo manipulation[3, 4]. Generally, the biopsy procedure involves removing several trophectoderm cells, about 5 to 8 cells, on Day 5 or Day 6 of the embryo at the blastocyst stage[5]. It is a relatively small number of TE cells being biopsied and tested due to the threat of euploid-aneuploid mosaics and chaotic abnormalities. Chaotic abnormalities with multiple aneuploids can occur in two or more cell populations that have a different chromosomal makeup, which makes it difficult to ascertain the causative aneuploids. Thus, where increasing the number of biopsied cells might improve accuracy, it is also likely to reduce implantation rate so that embryologists must also take into account the need to obtain enough trophectoderm cells. Mathematical models demonstrate how TE biopsies cannot provide reliable information about the whole blastocyst. Quantitatively, the sample size (here, the biopsied cell number) is the key factor of PGT-A accuracy and directly relates to embryonic mosaicism and false positive and negative rates [1]. It would take at least a 27-cell biopsy, the minimal level of correct statistical representation, for one meaningful PGT-A TE. Thus, additional concerns arise about the clinical utilization of PGT-A and its accuracy; PGT-A would be invalidated without even having to consider how well TE reflects the ICM, and PGS would not be accurate enough to decide whether or not an embryo should be discarded [6].

Apart from the invasiveness and inaccuracy of the procedure, blastocyst mosaicism also causes TE heterogeneity, which could interfere with both accuracy and precision of the diagnoses [7]. The cell with different chromosomal components may spread randomly throughout the TE [8]. PGT-A with TE biopsy has additionally seen concerns over the long-term health of its offspring. Animal studies suggest that embryo biopsy could delay blastocoel formation and increase the risk of neurodegeneration and dysfunction in the offspring [9]. Additionally, since the execution of biopsy procedure has not been standardized, IVF laboratories use varied techniques in steps such as breaching the zona pellucida, separating the rest of the embryo, and determining the number of cells biopsied from the TE [5].

Given the challenges intrinsic to invasive biopsy procedures, there is increasing interest in developing noninvasive procedures for PGT-A using DNA originate from embryo spent culture media. Despite some promising results demonstrated through successfully amplified DNA and accurately detecting genomic sequences in the majority of PGT-A cases, the PGT-A results are still subject to variation from the aspect of PGT-A full concordance when compared to those obtained from whole embryos or biopsy specimens [10–12]. Nonetheless, this procedure opens a new avenue for quantitative and qualitative evaluation of chromosomal statuses. More importantly, SCM PGT-A would also likely increase access to patients due to the nature of sequencing cell-free DNA in SCM with less invasive[13]., simpler, safer, and lower

PGT-A cost We sought to evaluate whether this DNA in SCM reflects a reliable source of information about the genetic status of the embryo.

Our current goal of preclinical validation is to ensure the SCM for PGT-A consistently achieves expected results with comparable accuracy to current accepted TE biopsy tests. To this end, these testing blastocysts donated for research extend a unique possibility in which multiple specimens, including trophectoderm biopsy, SCM, and paired corresponding whole blastocyst (WB) specimens from the same blastocyst source can serve as the positive control for comparison. Both NGS results from SCM and TE are respectively used against the same positive control which perfectly serves the purpose of this preclinical validation. We are able to measure whether the tested samples are truly representative of the WB derived from the sample. In addition, the full chromosomal concordance assessments between SCM and WB (SCM-to-WB), and between TE and WB (TE-to-WB) as well as sensitivity, specificity and overall diagnostic accuracy were conducted. Furthermore, each individual chromosomal aberration, such as segmental aneuploidy and mosaicism, can be precisely quantitatively and qualitatively measured by comparing each to the positive control. In this analysis, this comparison incorporated the most suitable and accurate validation component for measuring diagnostic reliability and encompassed all chromosomal aberrations. Thus, it is possible to sufficiently identify whether DNA in SCM will deliver a reliable PGT-A test that is consistent with PGT-A with TE sample when respectively compared to the positive control.

Material And Methods

Institutional Review Board Approval

This study was approved by the Ethical Committee of Henan Provincial People's Hospital. Written informed consent was obtained from all study participants. All donated blastocysts had already been diagnosed as abnormal by preimplantation genetic testing for aneuploidy by aCGH with TE biopsy. All blastocysts were donated for research under informed consent by patients undergoing IVF for treatment of infertility at Henan Provincial People's Hospital, China. The donated embryos, related DNA samples, and data were handled anonymously.

Sample preparation

75 blastocysts were warmed and then placed in separate 25-μL droplets of G-2 (Vitrolife, Sweden) with serum protein supplement overlain with mineral oil in Minic-1000 incubators (Cook) at 37 °C in an atmosphere of 5% O2, 6–7% CO2 balanced with N2. Following a 24-hour incubation, the culture drops where blastocysts, called spent culture medium, are incubated were collected and frozen at -20 °C for future cell-free DNA analysis. The corresponding whole blastocyst was then subjected to TE biopsy. Immediately after TE biopsy, biopsied fractions and their remaining corresponding blastocysts were washed twice, transferred into an individual lysis buffer (Yikon Genomics, China), and frozen at -20 °C for WGA. The paired corresponding whole blastocyst served as the positive control for comparison. The culture medium, with no previous contact with the blastocyst, was incubated in the same micro droplet dish and was used as a negative control.

During protocol optimization, at 8 hours (n = 6), the post-thaw incubation time, 20–25 μl spent culture medium was collected from blastocyst cultured drops. This was done in an effort to isolate and amplify DNA. However, results from these were suboptimal, evidenced by low DNA yields and were insufficient to produce conclusive NGS results post whole genome amplification (WGA), evidenced by its noisy profile. These results therefore do not meet necessary quality control scores for interpretation. The results are also similar to previous observations [11] and were not included in the analysis below.

WGA, library preparation, NGS, and data analysis

Whole-genome amplification and library preparation was performed with the use of the ChromInst (Yikon Genomics, EK100100724 NICSInst™ Library Preparation Kit) as described in [14–16]. The sequencing was conducted with Hiseq Rapid SBS Kit v2 on an Illumina Hiseq 2500 platform (Illumina Inc., Santa Clara, CA, USA) generating 1–2 M raw reads for each sample. Initial processing of the sequence reads involved trimming the adapter sequences from the ends of the reads, which were used for sequencing library preparation, followed by filtering software to remove low-quality bases from our reads that did not meet the criteria. This process is especially important when dealing with variant identification. Sequencing data were deposited into the NCBI Sequence Read Archive under accession number PRJNA524206 (PGT-A). The high quality read numbers were aligned and mapped to the human reference genome, hg 19, and counted along the whole genome with a bin size of 1 Mb. They were then normalized by the GC content and a reference dataset. ChromGo™ Analysis Software (EK1001013, Yikon Genomics) was employed to analyze sequencing, determining copy number variations (CNV) and interpreting NGS data. Standard PGT-A results as obtained from SCM, TE, and WB were employed. Chromosomal imbalances ≥ 4 Mb were defined as segmental aneuploidy in this study. When segmental or numerical aneuploidies were detected, the aberrations were always reported. In the case of mosaicism, based on the data obtained by simulating chromosomal mosaicism, we classified 23 pairs

chromosomes into two groups according to their chromosomal trisomy survivability. The NGS pattern detection and classification of aneuploidies was determined by copy number variation (CNV) values.

For survivable trisomy aneuploidy including trisomy error-prone chromosome numbers 13, 16, 18, and 21, CNV values between 1.70 and 2.30 were considered euploid; aneuploidy CNV values between 1.30 and 1.70 or between 2.30 and 2.70 were classified as diploid/aneuploid mosaic; CNV values lower than 1.30 or higher than 2.70 were classified as aneuploidy. This customized cut-off was established based on the reproducibility of our cell line mixtures to set the detection limit of mosaicism based on in-house validation. For the remaining chromosomes, CNV values between 1.60 and 2.40 were considered euploid; aneuploidies with CNV values between 1.40 and 1.60 or between 2.40 and 2.60 were classified as diploid/aneuploid mosaic; CNV values lower than 1.40 or higher than 2.60 were classified as aneuploidies (Fig. 1). Although the resolution of Hiseq 2500 platform NGS is validated to detect segmental (sub-chromosomal) aneuploidies of 20 Mb or larger by the manufacturer, we were able to detect segments as small as 1.0 Mb using our NGS platform and in-house ChromGo™ software. Therefore, the diagnostic limit would certainly be able to reliably detect down to the sub-chromosomal level. Only in the results section of the assessment of subchromosomal instability in blastocysts and reciprocal subchromosomal deletions and duplications, was the segmental CNV taken into account, but not to the exact coordinates of the variation. Similarly, mosaic chromosomes were compared in terms of their presence or absence but not its percentage level. Due to DNA's nature in SCM of intrinsic low quantity/abundance and poor integrity, we expect NGS data in SCM to be relatively noisier than those in TE and WB. To this end, we consider 'aneuploidy' to encompass both whole and segmental chromosome abnormalities for the rest of our study.

The overall diagnostic accuracy [17] of the PGT-A in the testing embryos using the summary of the probabilities are as follows: Overall diagnostic accuracy = $sensitivity * prevalence + specificity * (1\text{-}prevalence)$. Sensitivity and specificity are considered measures of intrinsic diagnostic accuracy based on embryonic cell-free DNA in SCM and DNA from TE biopsied cells related to the paired corresponding blastocyst, respectively. Overall diagnostic accuracy, which is the proportion of correctly classified true-aneuploidy and true-euploidy, can be reported as a global marker of accuracy. [17]

Statistical analysis

Sensitivity, specificity, negative predictive value (NPV) and positive predictive value (PPV) were used to evaluate the detection of chromosome abnormalities. The result of the whole blastocyst was considered as the gold standard. The 95% confidence intervals (CIs) of proportions were calculated by the Wilson score method. Statistical analyses were performed using MedCalc Software version 18.2.1 (MedCalc Software, 2018).

Results

Elimination of zona pellucida as sources of cell-free DNA in SCM

One of the major challenges for cell-free DNA assessment is potential contamination by maternal DNA from granulosa cells[18]. Regarding this challenge we focused on optimizing procedures to ensure that cell-free DNA derives only from the source of developing blastocysts. Through DNA amplification, WGA results will truly reflect the chromosomal status of the corresponding blastocyst and efficiently translate this non-invasive technology into clinical use.

To eliminate the possibility of maternal DNA contamination, we have to rule out the possibility of zona pellucida and associated transzonal projections as potential sources of cell-free DNA in the SCM samples. Thus, the zona pellucida (n = 6) was removed from the blastocyst by repeatedly pipetting with a micropipette and washing the blastocyst thoroughly. The culture media with empty zona pellucida were individually cultured for 24 hours under identical culture conditions. Following NGS, negative control samples were sequenced and generated an amplification-failure pattern in blank culture media samples (Fig. 2a) and culture media with empty zona pellucida (Fig. 2b).

This observation indicates that the non-informative sample present in the control culture media with/without zona, successfully eliminated the possibility of zona pellucida and associated transzonal projections as the source of DNA in SCM.

Overview of DNA amplification and informative NGS results in SCM, TE and WB from the same blastocyst

An overview result of ploidy is shown in Table I. For 75 blastocysts, informative results (successful DNA amplification with interpretable NGS results) were obtained from 100% TE (trophectoderm biopsy) and WB (zona-free whole blastocysts). One of 75 (1.33%) SCM specimen showed maternal contamination. Interpretable NGS result in the SCM group was 78.67% (59/75), significantly lower than their

corresponding TE and WB groups. Of the NGS results per sample, there were no significant differences observed in SCM, TE, or WB aneuploid rates based on their interpretable NGS results: SCM 91.53% (54/59); TE 86.67% (65/75); and WB, 81.33% (61/75).

Table 1
Overview results of NGS results from SCM, TE, and the corresponding blastocyst (WB)

	SCM	TE	WB
Number of Analyzed sample	75	75	75
successful DNA amplification	60 (80%)	75	75
Maternal contamination	1	0	0
Interpretable NGS results	59 (78.67%)	75	75
Total Euploidy	5 (8.47%)	10 (13.33%)	14 (18.67%)
Aneuploidy	54 (91.53%)	65 (86.67%)	61 (81.33%)

The concordance assessment of ploidy between two groups of SCM, TE, and WB

Considering that full chromosomal concordance assessments provide detailed measurements with more precise quantitative and qualitative comparisons of ploidy concordance assessment: first, we define the overall concordance as the sum of the total full concordance and imperfect concordant. The overall concordance rate in SCM (89.83%, 53/59) is similar to that of TE (94.76%, 71/75), ($P = 0.335$). We also define full concordance as all the same chromosomes possessing the same gain or loss between SCM and WB, or between TE and WB, where WB served as the gold standard for this comparison. Figure 3 shows the full chromosomal concordance assessments of ploidy. Remarkable differences in full concordance rates are seen, 32.2% (SCM-to-WB, 19/59) vs 69.33% (TE-to-WB, 52/75), ($p < 0.001$). Furthermore, significant inconsistent anomalies in the full concordance rate were mainly reflected in the aneuploidy, with 23.73% (14/59) in SCM-to-WB, versus 56% (42/75) in TE-to-WB ($p < 0.001$) Aneuploid–aneuploid imperfect concordance shows the degree of dissimilarity of aneuploidies. Imperfect concordance with WB samples indicates that comparing both SCM-to-WB and TE-to-WB gives aneuploid calls, but SCM/TE had fewer or additional chromosomal gains or losses. The imperfect concordant rate in SCM-to-WB is 57.63% (34/59), whereas it is 25.33% (19/75) in TE-to-WB, indicating that the imperfect concordant rate in the SCM-to-WB group is higher than that of TE-to-WB ($p < 0.001$). This difference furthermore demonstrates that interpretable NGS results from TE can better reflect the ploidy status of WB than what can be observed in SCM-to-WB results.

Among aneuploid–aneuploid imperfect concordant cases, a small proportion of reciprocal chromosomal/sub-chromosomal gain-loss complementary pairs were observed in the current study. These aneuploidies were detected in the two sample types among SCM, TE and WB groups, existing as complementary pairs in terms of loss versus gain chromosomally or sub-chromosomally. These complementary pairs were located either on the small (p-) or large (q-) chromosome arm, including their mosaic of their corresponding arms at the affected chromosome.

The reciprocal complementary pairs are observed in 6 blastocysts. These pairs were located on the same or comparable chromosome arm gain and loss positions. The proportion of guanine (G) and cytosine (C) bases in the DNA molecule is usually expressed as GC content. GC base pairs are held together by three hydrogen bonds, while AT and AU base pairs are held together by two hydrogen bonds. This difference is why DNA with low GC-content is less stable than DNA with high GC-content. Among all 22 autosomes, the average of GC-content is 41.62% [19, 20]. 27.27%, 6/22 of autosomes have GC contents below 40%. Eleven total reciprocal chromosomal gain-loss complementary pairs are identified in four chromosomes:3, 5, 6, and 13, coincidently, they all contain low GC content autosomes. The false positive and false negative were classified as discordant. Discordancy is a lack of agreement between ploidy, i.e. a blastocyst is diagnosed as aneuploid in either SCM or TE, meanwhile, their corresponding WB were diagnosed as euploid (false positive) or vice versa (false negative). In discordant cases, there is no remarkable difference in false positive between SCM-to-WB, 10.17% (6/59), and TE-to-WB, 5.33% (4/75), ($p < 0.001$).

In the context of comparative data of full concordances, we conclude that the TE-to-WB group provides a more accurate measurement and representation of the chromosomal constitution of the whole blastocyst.

Table 2
Reciprocal chromosomal arm gain-loss complementary pairs in SCM, TE, and WB: Aneuploid-complementary.

Embryo ID	Samples	NGS results	Subchromosomal arm/mosaic	GC%
5	SEC	+ 3p(pter→p25.3,~11M, × 3)	± 3p	39.67
	TE	-3p(pter→p25.3,~11M, × 1)		
	WB	-3p(pter→p24.3,~19M,×1)		
43	SEC	-5(× 1),-13(× 1),-14(× 1,mos*),+15(× 3,mos*),-Xq(q13.3→qter, ~ 81M, × 1,mos*),...	± 5mos	39.51
	TE	+ 5(× 3,mos*),+14(p11.1→q32.13,~78M,×3,mos*)		
	WB	+ 5(× 3,mos*,~30%)		
46	SEC	(q24.3→qter,~24M,×1),+13q(q21.33→qter,~43M,×3, mos*)	± 13q mos	38.55
	TE	-6q(q24.2→qter,~26M,×1,mos*),-7q(q21.11→qter,~81M,×1,mos*), -13(pter→q31.1,~61M,×1,mos*),-21 (× 1,mos*)		
	WB	-6q(q25.2→qter,~17M,×1),+13q(q21.33→qter,~44M,×3)		
49	SEC	+ 4(pter→q22.1,~90M,×3,mos*),-11q(q13.4→qter,~64M, × 1,mos*),-13(q21.31→qter,~52M,×1,mos*),+19(× 3)	± 13q- mos	38.55
	TE	+ 3(× 3,mos*),+9q(q33.1→qter,~22M,×3,mos*),+13q(q31.1→qter,~33M,×3,mos*),+15(× 3,mos*),+19(× 3),...		
	WB	+ 19(× 3)		
58	SEC	+ 2(p16.1→q22.1,~80M,×3),-3(p11.1→qter,~110M,×1),+6(p21.1→ qter,~125M,×3),+7(pter→q11.23,~75M,×3),8(p23.1→q24.23,~128M,×1),...	± 3p	39.67
	TE	+ 3(p12.3→qter,~122M,×3)		
	WB	+ 3(p12.3→qter,~122M,×3)		
73	SEC	+ 2(× 3),+4(× 3),+6(p22.3→qter,~147M,×3),+7(× 3),+8(× 3),...	± 6p	39.61
	TE	-6p(pter→p22.3,~16M,×1),+10p(pter→p15.1,~8M, × 3)		
	WB	-6p(pter→p22.3,~16M,×1),+10p(pter→p15.1,~8M,×3)		

Sensitivity, specificity and overall diagnostic accuracy

The metrics of sensitivity, specificity, and predictive values are often considered measures of diagnostic accuracy because they provide information on dichotomous tests, which can distinguish between euploidy and aneuploidy blastocysts based on their ploidy. From discordant aspects, both negative predictive values in TE and WB are 100%. Positive predictive values for TE and SCM are 93.85% (61/65) and 88.89% (48/54), respectively. Specificity of DNA in SCM and TE in predicting chromosomal status is based on the incidence of aneuploidy in WB. Specificity of TE (71.43%) is higher than SCM (45.45%) with Sensitivity of 100% for both SCM and TE. Positive likelihood ratio in SCM (1.83) is lower than that of TE (3.50), whereas both negative likelihood ratios in both SCM and TE under current study circumstances are zero. We have to point out the limitation in this study, which is that the majority of the available donated embryos had previously been PGS tested and identified as abnormal. This fact eliminated the feasibility of investigating actual sensitivity, negative predictive value, and negative likelihood ratio. In clinical PGT-A, we expect the negative likelihood ratio to be above zero while sensitivity and negative predictive values should be less than 100%. Overall, diagnostic accuracies in SCM-WB (89.83%) are less accurate than those in TE-WB (94.67%).

In summary, NGS data interpretation for SCM DNA in collectively translated into lower specificity, PPV, PLR, and overall diagnostic accuracies. DNA samples from TE are more stable than those in SCM. SCM is currently suboptimal for aneuploidy screening in blastocysts, but with further improvement, it remains a promising tool for non-invasive PGT-A.

Table 3
Sensitivity, specificity and overall diagnostic accuracy

	SCM		TE	
Statistic	Value	95% CI	Value	95% CI
Sensitivity	100%	92.60–100.00%	100%	94.13–100.00%
Specificity	45.45%	16.75–76.62%	71.43%	41.90–91.61%
Positive Predictive Value (PPV)	88.89%	82.35–93.21%	93.85%	86.95–97.22%
Negative Predictive Value (NPV)	1000%		100.00%	
Positive Likelihood Ratio (PLR)	1.83	1.07 to 3.14	3.50	1.53 to 8.01
Negative Likelihood Ratio (NLR)	0.00		0.00	
Disease prevalence	81.36%	69.09–90.31%	81.33%	70.67–89.40%
Overall diagnostic accuracy	89.83%	79.17–96.18%	94.67%	86.90–98.53%

Discussion

SCM DNA-based PGT-A has emerged as a valuable option that does not have invasive risks and has the capability to detect aneuploidies with excellent sensitivity and reasonable specificity. Although a plethora of validation studies in clinical IVF underline SCM DNA-based PGT-A excellent performance, several reports have resulted in discordancy to known existence of mosaicism in TE, corresponding whole blastocysts, and maternal DNA contamination [21]. It was reported that SCM contributes to maternal DNA contamination [18]. This maternal DNA can cause poor concordance between DNA from SCM and TE biopsies. There are thin cytoplasmic projections called transzonal projections (TZPs) that are located inside zona [22]. TZPs from cumulus cells connect to the oocyte and are crucial for normal oocyte formation. Existing TZPs within zona pellucida could either be a potential source of cell-free DNA, or could interfere with cell-free DNA released from the blastocyst. In our study, culture medium, which was collected from the drop where empty zona pellucida were individually cultured for 24 hours, served as a testing sample to explore if zona pellucida is associated which cell-free DNA; for this reason, zona pellucida were also removed immediately following TE biopsies. The corresponding zona-free blastocysts served as the gold standard of reference in our research cohort. After test results, we eliminated zona pellucida as the source of cell-free DNA in the spent media. Therefore, any further testing of zona pellucida would be an unnecessary testing of background noise added onto NGS and would minimize the possibility of maternal contamination to the testing sample.

As diagnostic tests require both sensitivity and specificity to be as close as possible to 100%, our SCM DNA-based PGT-A results indicated high sensitivity with limited specificity. Therefore SCM DNA-based PGT-A can only be classified as a screening method, rather than a diagnostic test. In comparison to our negative control, the empty culture drop, successful DNA amplification was observed in 100% TE and WB group samples, but only 78.67% in SCM samples. The DNA amplification QC metrics indicate the DNA sample quality from SCM is suboptimal and is likely linked to degraded DNA in nature. According to current diagnostic accuracy studies, any one of the elements in Table 3 may directly or indirectly affect the overall diagnostic accuracy through prevalence of the aneuploidy. All our samples, SCM, TE and WB, were from the same corresponding blastocysts. By using samples from the corresponding blastocysts, we minimized the role of the prevalence of the aneuploidy on overall diagnostic accuracy.

It is noteworthy that overall concordance characterized by dichotomy can only distinguish between euploidy and aneuploidy blastocysts. Our TE-to-WB overall concordance rate is 94.67% which is acceptable and comparable to other studies [10, 11, 23, 24]. Thus, PGT-A with TE biopsied samples for euploidy/aneuploidy detection are accurate. At the same time, specificity rates of TE-to-WB is 71.43%. Overall concordance rates that are high may be attributed to the fact that the blastocysts were donated and had been previously PGT-A tested chromosome aberrations blastocysts. These characteristics increase the risk of positive diagnosis bias, which in turn increases the likelihood of a misled, biased conclusion. Regarding technical limitations, the poor quality of degraded DNA in SCM also increases levels of chromosomal aberrations in SCM. This bias leads to artefactual aneuploidies unless appropriate protocol is applied. NGS data analysis would have to be altered specifically for degraded DNA amplification. The interpretation of results would also have to be based on DNA with intrinsically degraded low quality in SCM. So, both positive-diagnosis biases and DNA sample qualities in SCM associated artefactual aneuploidies with inflated aneuploidy levels in SCM samples. Unlike the overall concordance characterized by dichotomy, full chromosome

concordances not only enhanced assessment of ploidy status, but also offered more detailed measurements with more precise quantitative and qualitative comparisons of chromosomal aberration including mosaicism, segmental aneuploidies, and a considerable number of chaotic NGS results. Our study shows the full concordance rate of TE-to-WB is 69.33%. This rate is similar to 75% found in other studies [11]. Our SCM overall concordance rate (89.83%) is comparable to the rates have been reported [11, 12] and is higher than Ho, et al [10]. However, the full concordance rate of SCM-WB is 32.2%. These full concordance rates are remarkably lower than other study [11], but are similar to this one [12]. These lower rates are likely due to the DNA sample quality in SCM. As stated in other reports, [25–27], this discrepancy is unsurprising given the likely degraded nature of the DNA in SCM.

In theory, it is possible to achieve 100% accurate assay of full chromosome concordance between DNA in SCM/TE and the paired blastocyst. In practice, however, it is unlikely to obtain 'true data' for an embryo if there exists mosaicism and reciprocal aneuploidy. SCM DNA is thought to be released as a consequence of cell apoptosis or necrosis death in the blastocyst.

As we have frequently observed in our daily clinical laboratory, apoptotic cells may be either phagocytosed by neighboring cells, or expelled into the perivitelline space (Fig. 4a) or blastocoel cavity (Fig. 4b)[28, 29]. Evidently, DNA quality in SCM stands out as the most limiting factor for the diagnostic efficiency of genetic data analysis using cell free DNA. Generally, commercial WGA buffers are designed for cell/cell-free samples in small volumes. Yet, the SCM sample from the culture drop has a relatively larger volume, which requires reaction components be scaled up. This requirement not only increases costs, but also necessitates additional validation and optimization. A reduced volume (12 µl) of blastocyst culture media[16] could certainly concentrate DNA in SCM, but it also may deviate from the manufacturer's recommendations. Reducing the volume would therefore require further validation to ensure that the blastocyst's developmental potential is not compromised. In imperfect concordant cases, only a small proportion of reciprocal chromosomal/sub-chromosomal gain-loss complementary pairs were observed in the current study. Biologically, alternative rigorous criteria to classify embryos as mosaic would require the presence of reciprocal aneuploidy in two different cells/cell lines or two different biopsied samples from the same embryo [30].

The frequency of whole chromosome or sub-chromosomal arm gains and losses should be similar. For example, take one biopsy that displayed a monosomy (2n-1) of a specific chromosome and another biopsy from the same embryo displaying trisomy (2n + 1) for the same chromosome [25, 31]. It is expected that at least one embryo should display reciprocal errors for at least one chromosome. Indeed, in our study, we observed 11 reciprocal chromosome arm loss-gain complementary pairs located in four different chromosomes in six blastocysts, which is similar to other observations [8, 18] exhibiting shared complementary aneuploidies. Coincidentally, in current study, all four chromosomes associated with reciprocal gain-loss complementary pairs were low GC-content chromosomes. Studies on human-inherited diseases and cancers also revealed that DNA breakpoints tend to occur in DNA sequences with low GC content [20]. Due to small sample size, we cannot robustly ascertain whether a low GC-content sequence would be more vulnerable to DNA breakpoints than a high GC-content sequence. However, utilizing GC content and reciprocal sub-chromosomal arm gain-loss complementary as a reference may prove a more efficient tool in distinguishing real DNA segments from NGS data noise generated from a sample of DNA in SCM [19, 32] .

Contrarily, our data also shows that the chromosomal aneuploidies were mostly consistent rather than reciprocal. In human and nonhuman primate preimplantation embryos, mis-segregated chromosome was sequestrated into a small nucleus-like structure adjacent to but outside of the primary nucleus [33, 34]. Consequently, chromothripsis may trigger the chromosome inside micronuclei to shatter and the sequential reassembly of fragments through breakage-fusion-bridge cycles, aberrant epigenetic regulation, abortive apoptosis, and other yet unknown mechanisms. Thus, loss and gain reciprocal chromosomal/ chromosome arms may exist as individual chromosomes or segments instead of complementary pairs. This discovery may denote a mechanism that explains why we can only detect small numbers of reciprocal chromosome arm gains and losses. Micronuclei must be regarded as a unique source of unstable genomes, damaged DNA of genetic variations, and possibly, the source of cell-free DNA in the SCM as well.

Conclusion

In this study, the full chromosome concordances of SCM, TE and whole blastocyst contribute experimental evidence to the validation of PGT-A at the blastocyst stage. Due to intrinsically low quantity/abundance and poor integrity associated with DNA samples in SCM, it resulted in diagnosis biases. Subsequently, the results negatively contributed to our full concordance assessment so that the full concordance rate between TE and WB was greater than that of SCM and WB. Because of this bias, we conclude that TE provides a more accurate and reliable high-throughput methodology for PGT-A. While cell-free DNA in SCM has the potential to represent a safe and simple strategy in PGT-A, it still requires further validation. This procedure works as expected, consistently achieves expected results, and is not comparable to currently accepted tests for TE biopsies. Therefore, it is a rule-in test, as opposed to a rule-out test[35], and could be used for optimizing noninvasive embryo prioritization. With further improvement, it remains a promising tool for noninvasive PGT-A. The diagnostic

efficiency of cell-free DNA in SCM may ultimately require custom-tailored design methodology for sample collection (including volume, embryo culture media components), storage, to prevent the length of the DNA in SCM sample from further degradation, and new WGA techniques for the efficient amplification of degraded/short DNA fragment samples.

Abbreviations

PGT-A: preimplantation genetic tests for aneuploidy; SCM: spent culture media; TE: trophectoderm; WB: whole blastocyst; WGA: whole genome amplification; CNV: copy number variation; Cis: confidence intervals; NGS: next generation sequencing; PPV: Positive Predictive Value; NPV; Negative Predictive Value; PLR: Positive Likelihood Ratio; NLR: Negative Likelihood Ratio; TZPs: transzonal projections; G: guanine; C:cytosine.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethical Committee of Henan Provincial People's Hospital. Written informed consent was obtained from all study participants.

Consent for publication

Written informed consent was obtained from each study participant.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interest(s)

The authors have no competing interest to disclose.

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Authors' contributions

B.Y. recruited the patients, designed the experiment, blastocyst biopsy, sample preparation and wrote the article. H.Z. recruited the patients, conducted DNA sample preparation. K.X. recruited the patients, blastocyst biopsy and sample preparation. Y.Y. performed WGA, library preparation, NGS data analysis. Y. W. preformed the data analysis and manuscript preparation. C.Z. conducted conception and design of the study, analysis of data final approval of the version to be published. L.M. conducted conception and design of the study, acquisition of data, analysis and interpretation of data, author of the manuscript.

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Figures

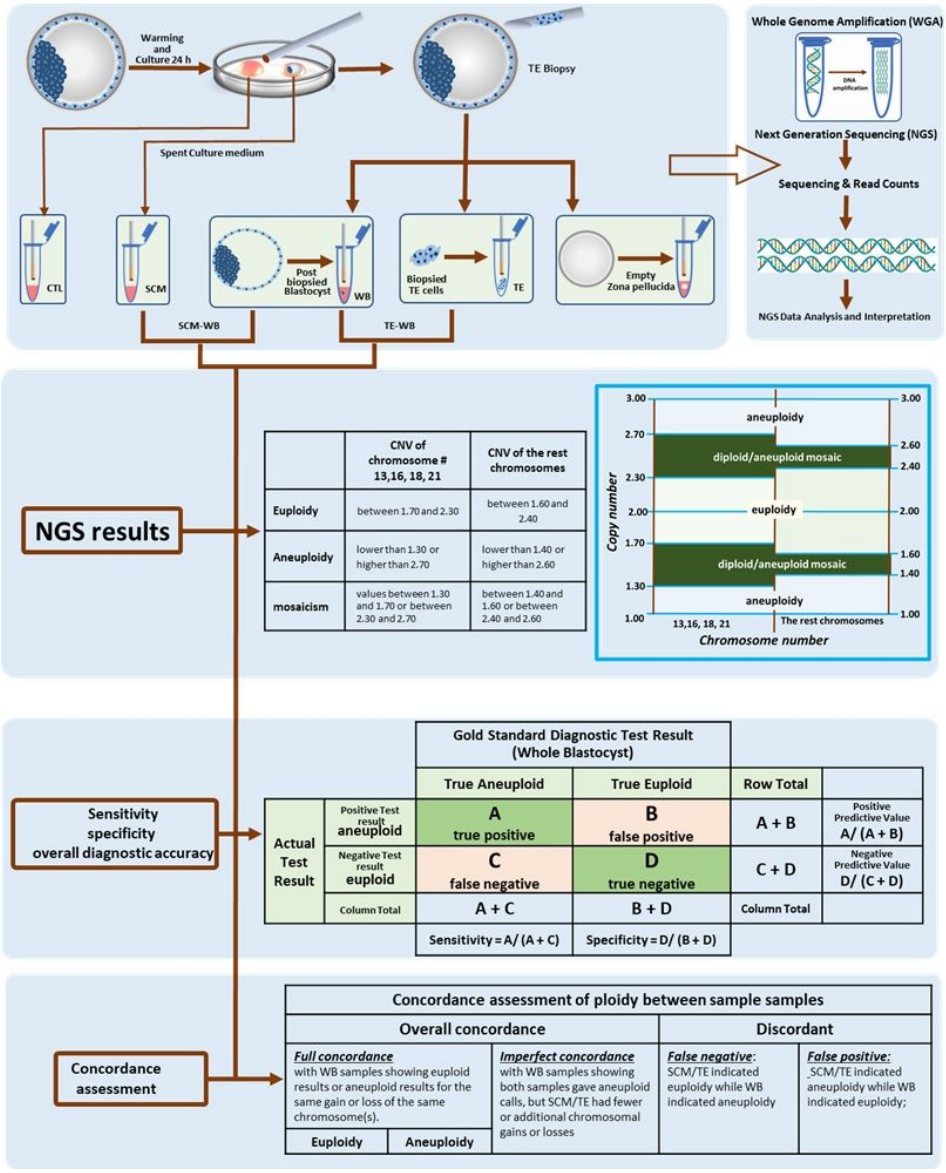
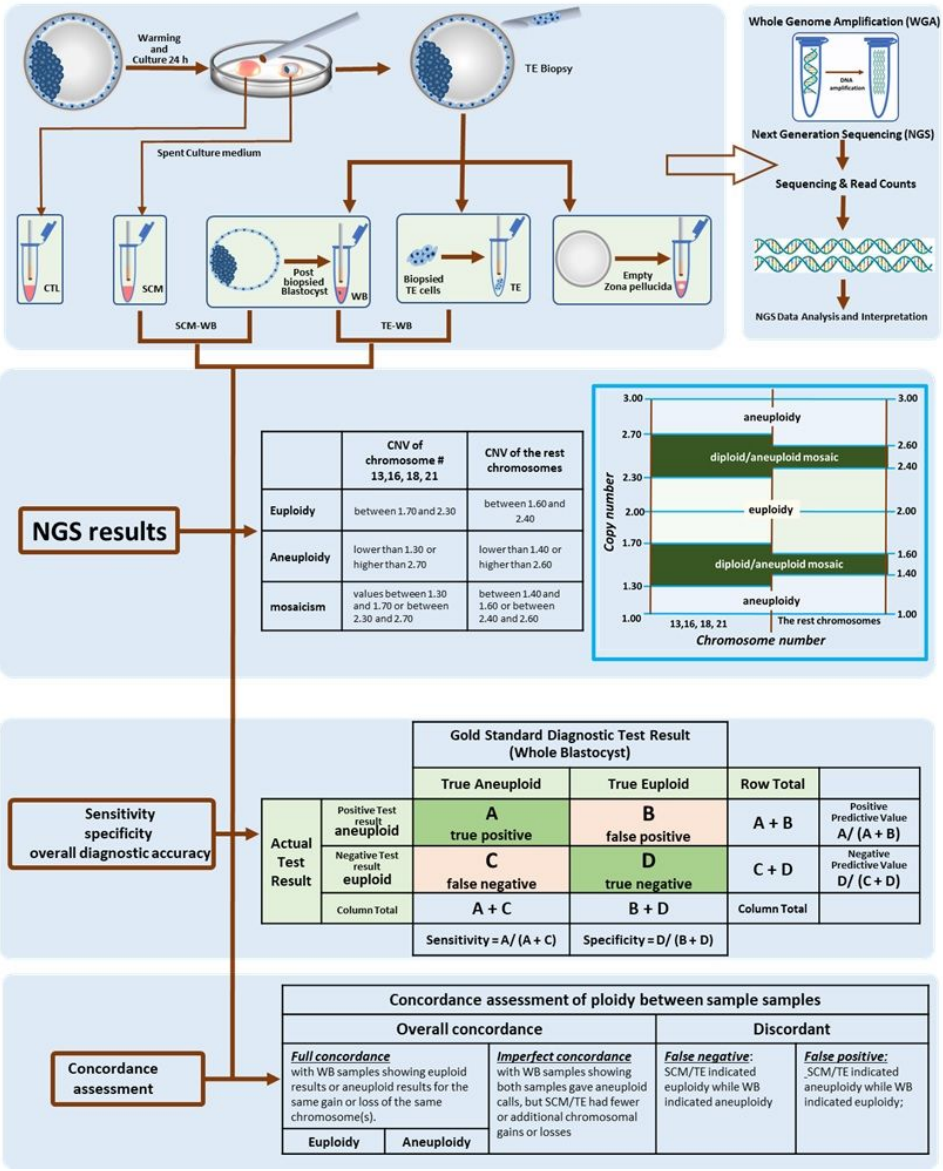


Figure 1

Detailed workflow for obtaining DNA samples from spent culture medium, TE biopsy and their corresponding whole blastocyst. The corresponding whole blastocysts served as the gold standard for this comparison. The samples are lysed and processed using standard clinical workflow for PGT-A. The same Whole Genome Amplified and Next Generation Sequencing from all samples were employed.



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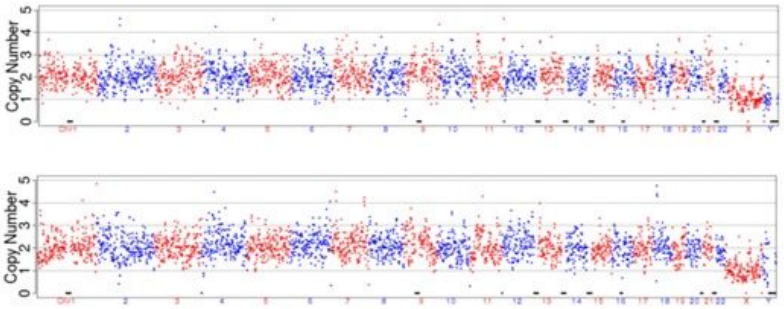
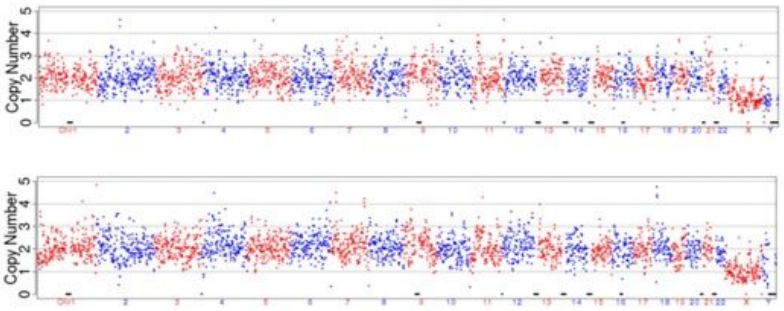


Figure 2

a Negative control I, blank medium negative controls (empty culture drop) associated with each specimen that underwent DNA amplification showed no DNA amplification in all cases. b Negative control II, after 24 hours, cultured medium collected from the culture

drop of empty zona pellucida. Each specimen that underwent DNA amplification showed no DNA amplification in all cases.



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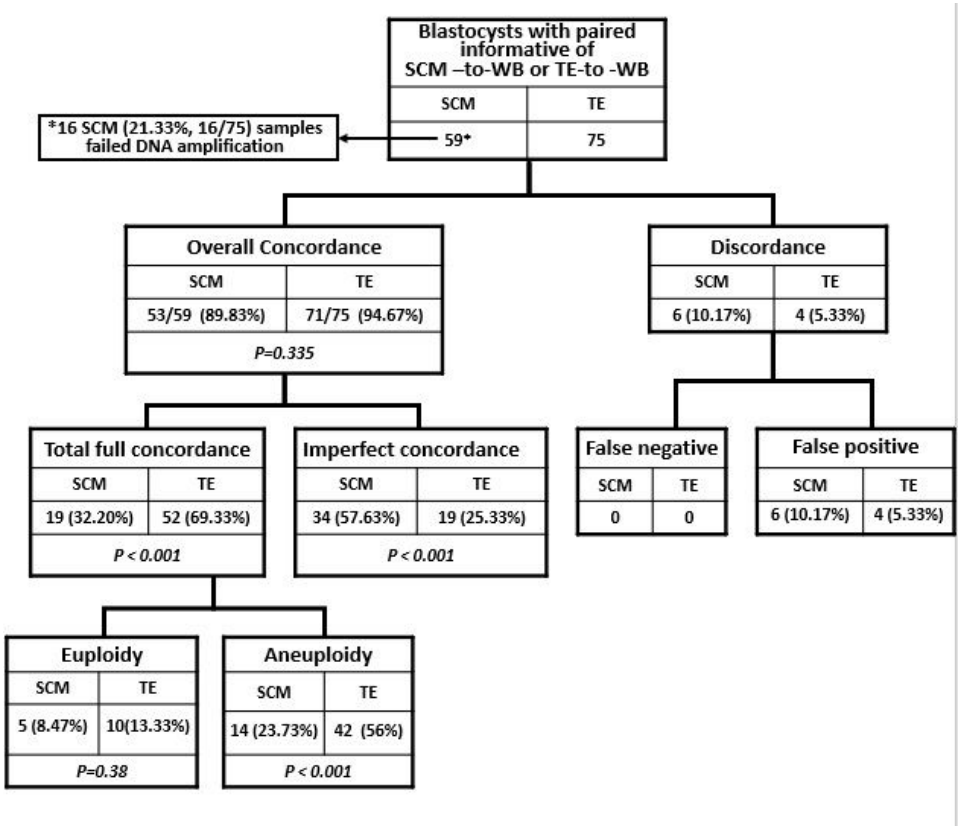
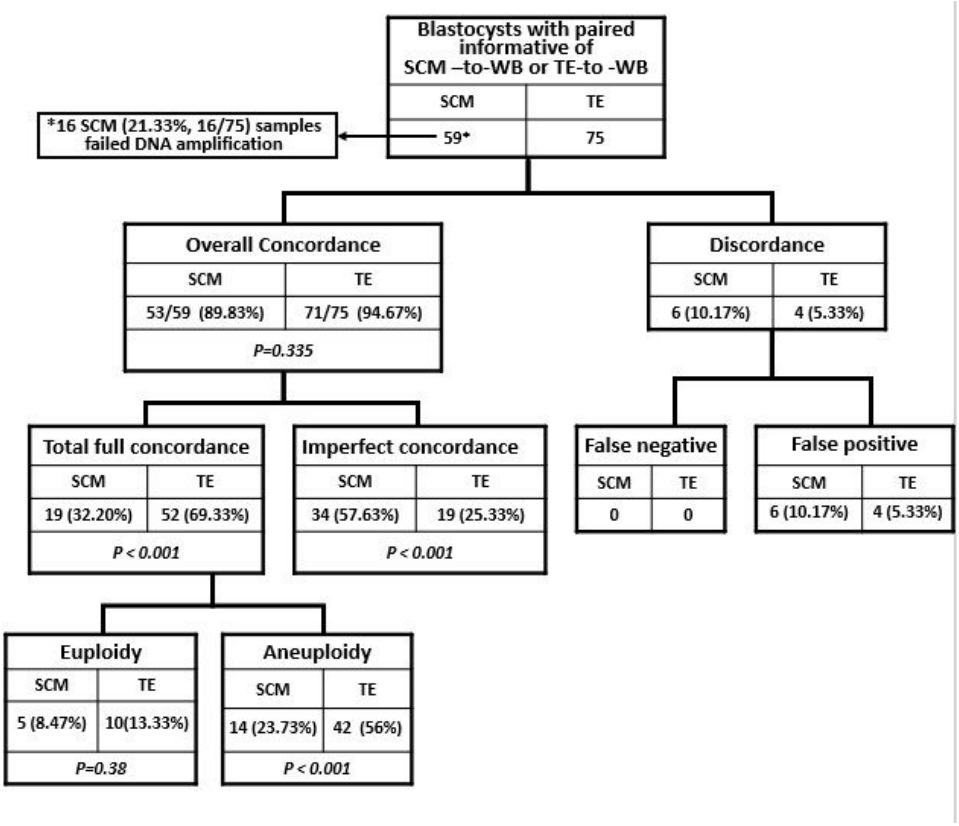


Figure 3

Full chromosomal concordance assessments between SCM and WB (SCM-to-WB), and between TE and WB (TE-to-WB).



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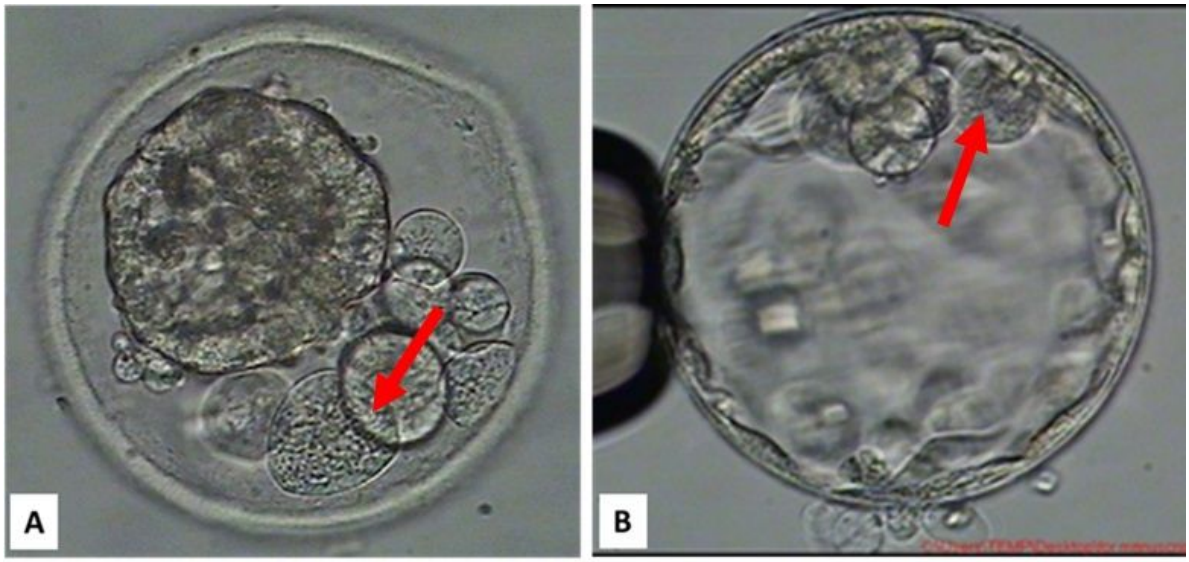
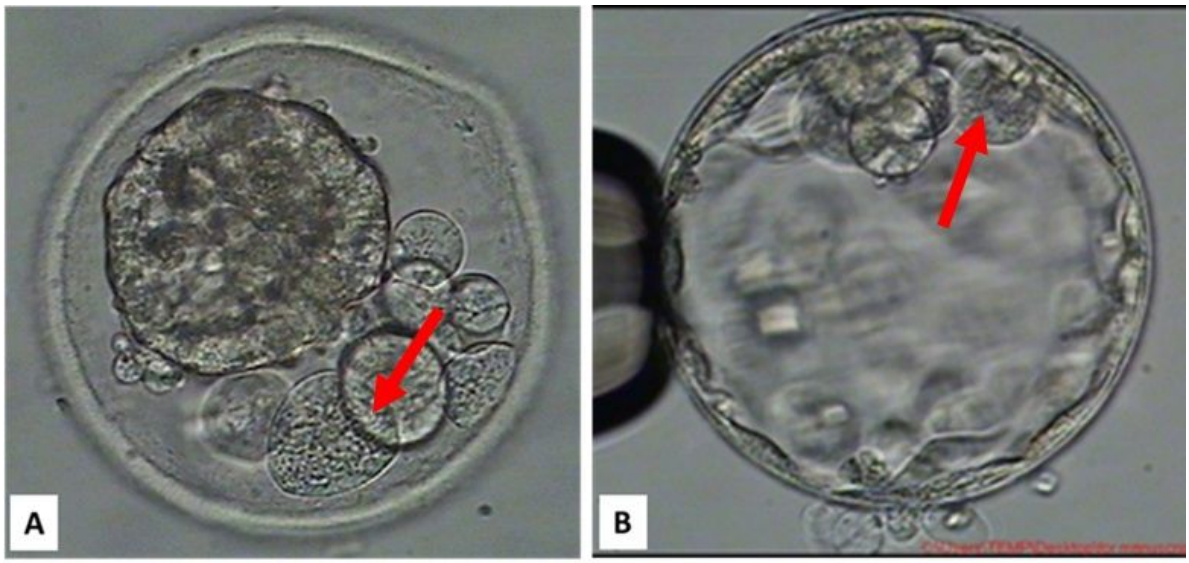


Figure 4

a Embryonic cells phagocytosed by neighboring cells or expelled into the perivitelline space or b which into the blastocoel cavity.



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