

ARTICLE



Non-invasive embryo selection strategy for clinical IVF to avoid wastage of potentially competent embryos

**BIOGRAPHY**

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KEY MESSAGE

Machine learning guided embryo grading system can be used to optimize embryo selection for clinical IVF treatment. This non-invasive embryo selection strategy offers important advantages, such as avoiding invasive biopsy, improving the utilization rate of embryos and decreased cost, potentially increasing accessibility for a wider patient population.

ABSTRACT

Research question: Can a non-invasive embryo transfer strategy provide a reference for embryo selection to be established?

Design: Chromosome sequencing of 345 paired blastocyst culture medium and whole blastocyst samples was carried out and a non-invasive embryo grading system was developed based on the random forest machine learning algorithm to predict blastocyst ploidy. The system was validated in 266 patients, and a blinded prospective observational study was conducted to investigate clinical outcomes between machine learning-guided and traditional non-invasive preimplantation genetic testing for aneuploidy (niPGT-A) analyses. Embryos were graded as A, B or C according to their euploidy probability levels predicted by non-invasive chromosomal screening (NICS).

Results: Higher live birth rate was observed in A- versus C-grade embryos (50.4% versus 27.1%, $P = 0.006$) and B- versus C-grade embryos (45.3% versus 27.1%, $P = 0.022$) and lower miscarriage rate in A- versus C-grade embryos (15.9% versus 33.3%, $P = 0.026$) and B- versus C-grade embryos (14.3% versus 33.3%, $P = 0.021$). The embryo utilization rate was significantly higher through the machine learning strategy than the conventional dichotomic judgment of euploidy or aneuploidy in the niPGT-A analysis (78.8% versus 57.9%, $P < 0.001$). Better outcomes were observed in A- and B-grade embryos versus C-grade embryos and higher embryo utilization rates through the machine learning strategy compared with traditional niPGT-A analysis.

Conclusion: A machine learning guided embryo grading system can be used to optimize embryo selection and avoid wastage of potential embryos.

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KEYWORDS

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INTRODUCTION

Infertility can be treated effectively with IVF and embryo transfer, in which embryo selection is a crucial step. Currently, the most used method to assess the developmental potential of an embryo remains morphological evaluation (*Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011*). Despite its convenience, morphology is a poor indicator of embryo chromosomal composition and is highly subjective. Many embryos are deemed unsuitable for implantation after morphological selection. Multiple embryo transfers increase pregnancy rates while increasing the chance of adverse pregnancy outcomes, which may hamper the health of both the mothers and the infants (*Elster, 2000*). On the other hand, because of the error-prone meiotic and mitotic processes of early embryo development, the clinical outcomes of IVF treatments with single embryo transfer (SET) are often limited by low clinical pregnancy and high spontaneous miscarriage rates (*Vanneste et al., 2009; McCoy et al., 2015*). Therefore, selecting embryos with the best clinical outcome for SET is the key to success in IVF and embryo transfer practice, which poses a great challenge to the evaluation system of embryo implantation potential.

Preimplantation genetic testing for aneuploidy (PGT-A) based on next-generation sequencing (NGS) combined with biopsy trophoctoderm cells from blastocysts has been recently used to select embryos having normal ploidy for transfer. It has been demonstrated to yield acceptable pregnancy outcomes with elective SET (*Scott et al., 2012; Rubio et al., 2017*). Embryo biopsy, however, requires specialized equipment and extensive expertise in embryo treatment, which is difficult to standardize and challenging to undertake in every IVF elective SET treatment. Moreover, the invasive nature of this method poses a potential threat to subsequent development of biopsied embryos and may hamper clinical outcomes as well as increase obstetric or neonatal risks and brings unknown health risks to offspring safety (*Yao et al., 2016; Guzman et al., 2019; Zhang et al., 2019a*). More importantly, the presence of mosaicism has affected the reliability

and accuracy of using a single biopsy of only five to 10 trophoctoderm cells (*Gleicher et al., 2017*). In most cases, the genetic material of trophoctoderm cells can represent inner cell masses (ICM) (*Chuang et al., 2018*); however, biopsies of mosaic trophoctoderm cells may diagnose embryos with normal ICM as abnormal, resulting in the disposal of usable embryos and the wastage of potentially competent embryos. Results from the 'Single Embryo Transfer of Euploid Embryo' randomized clinical trial showed that the indiscriminate use of PGT-A implies a loss of potentially competent embryos owing to incorrect diagnosis, biopsy-related embryo damage, or both (*Pagliardini et al., 2020*).

Stigliani et al. (2013) were the first to examine the genomic DNA content in embryo culture medium; therefore, it is possible to detect blastocyst chromosomal ploidy using embryonic cell-free DNA (*Vera-Rodriguez et al., 2018; Fang et al., 2019; Rubio et al., 2020*). In 2016, we first carried out non-invasive chromosome screening (NICS) depending on sampling genetic material from the clinically spent culture medium (SCM). Five live births from seven couples were achieved with balanced translocation (*Xu et al., 2016*). The NICS approach was then validated in a large-scale study using the whole embryo as a gold standard, and NICS showed promising performance compared with conventional trophoctoderm biopsy preimplantation genetic testing (*Chen et al., 2021*). When used for preimplantation aneuploidy testing, the NICS method, namely a non-invasive version of PGT (niPGT-A), paves the way for the selection of viable embryos while posing minimal risk to the development of the embryos. Studies evaluating the accuracy of niPGT-A, however, found a fluctuating ploidy concordance rate of 33–100% (*Xu et al., 2016; Feichtinger et al., 2017; Ho et al., 2018; Li et al., 2018; Vera-Rodriguez et al., 2018; Fang et al., 2019; Rubio et al., 2019; 2020*). It may be related to amplification methods, potential exogenous DNA contamination or the absence of a uniform threshold for mosaicism. *Huang et al. (2019)* defined mosaicism above 60% as the threshold to distinguish an aneuploid embryo from a euploid one. Given that most aneuploid embryos are not expected to be transferred, this may result in a huge waste of potentially competent embryos

and may increase the cancellation rate of IVF cycles. Additionally, aneuploid cells are more likely to undergo apoptosis for clearance from the embryos than euploid cells, leading to a high mosaic aneuploidy rate during the chromosomal screening of blastocyst culture medium samples (*Bolton et al., 2016*). For clinicians, it is often difficult to know what to do with embryos without normal test results.

Consequently, it is necessary to establish a grading system to prioritize embryos for transfer; in particular, strategies capable of distinguishing actual mosaics and aneuploid embryos from false-positive ones resulting from apoptotic cells are urgently required. The aim of the present study was to establish a non-invasive embryo transfer strategy to provide a reference for embryo selection. To this end, 345 blastocyst embryos were collected, comprehensive chromosome screening was carried out in culture media and whole blastocysts were selected from the same embryos. The machine learning technique was used innovatively for the first time, and euploid and aneuploid embryo-specific copy number variation (CNV) patterns in the culture media were deeply mined. With this technique, different chromosomal profiles from culture media could be assigned different aneuploidy probability predictions based on the gold standard of whole blastocyst embryos. Using the predicted euploidy probability, a prioritized embryo transfer strategy was proposed and a prospective and blinded observational clinical transfer experiment with 266 patients was further designed to validate this strategy. To the best of our knowledge, this is the first large-scale validation of comprehensive chromosome screening in culture medium, with both embryonic validation and blinded prospective clinical confirmation using ploidy information in the culture medium as a predictor for selection of embryos for SET in clinical IVF.

MATERIALS AND METHODS

Study design

First, an embryo selection strategy pattern based on the euploid probability of the SCM was established using 345 pairs of culture medium and whole embryo samples through artificial intelligence algorithms. In this strategy, named machine learning guided NICS grading system, embryos were

divided into three grades (A, B or C) according to euploidy probability using whole embryos as the gold standard. Thereafter, 266 patients who underwent 278 vitrified-warmed single blastocyst transfers were recruited, and the NICS grading system and niPGT-A analysis, were validated using a blinded prospective approach. The primary end point was birth rate, and the secondary end points were miscarriage, clinical pregnancy and ongoing pregnancy rates. The live birth rate was defined as the number of cycles with live births divided by the number of transplant cycles. The clinical pregnancy rate was defined as the number of cycles with gestational sacs visualized on transvaginal ultrasound divided by the total number of transplant cycles at 7–8 weeks of gestation. The miscarriage rate was calculated as the number of pregnancy failures after a gestational sac had been documented by transvaginal ultrasound divided by the total number of clinical pregnancies. Any pregnancy that went beyond 12 weeks of gestation was considered an ongoing pregnancy. Ectopic pregnancy is considered one clinical pregnancy, but it is not one miscarriage.

Embryo and spent culture medium collection

In total, 345 embryos were evaluated at the Reproductive Medicine Center of Nanjing Jinling Hospital (Nanjing, Jiangsu, People's Republic of China), Nanjing Drum Tower Hospital (Nanjing City, Jiangsu Province, People's Republic of China) and Shanghai Changzheng Hospital (Shanghai, People's Republic of China). The ages of the female patients ranged from 20–45 years. This study was approved by the Ethics Committee of Nanjing Jinling Hospital, Drum Tower Hospital and Shanghai Changzheng Hospital (reference number: 2016NJKY-028, 2017-09-02, CZEC [2017-06]); trial registration number: ChiCTR-RRC-17010396), and informed consent from patients was obtained before embryo analysis. The embryos were all derived from intracytoplasmic sperm injection (ICSI) and cultured to the blastocyst stage (day 5/6). Embryos that were not eligible for transfer according to morphological scoring criteria (*Gardner and Schoolcraft, 1999*) were donated and used for subsequent analysis. After the embryos were cultured to day 4, the medium was changed. The volume of the culture medium (SAGE ART-1029) was 25 μ l, and

the embryos were cultured in the form of single droplets in the culture dish (FALCON 353001) at 37°C, 6% CO₂, and 5% O₂. Day-4, 5 and 6 SCM (20–25 μ l) from each embryo was collected and transferred into RNase–DNase-free polymerase chain reaction tubes, each containing 5 μ l of the cell lysis buffer (Yikon Genomics, Suzhou, People's Republic of China) and stored at –80°C until use. Matched whole embryo cells were also transferred into 5 μ l of the lysis buffer. Cell lysis was carried out with 10 μ l each of SCM and whole embryos, according to the manufacturer's instructions (Yikon Genomics). The detailed method has been reported previously (*Huang et al., 2021*).

Patient selection

All patients who underwent ICSI with NICS and had a single frozen–thawed embryo transfer (FET) (using vitrified–warmed embryos) between July 2017 and December 2018 at the Reproductive Medicine Department of Nanjing Jinling Hospital were included in the study. The inclusion criteria were as follows: maternal age younger than 45 years and older than 20 years, embryos derived from ICSI and cultured to the blastocyst stage (day 5/6), blastocyst culture fluid collected in all cycles, and at least one blastocyst resuscitation transfer cycle carried out. The exclusion criteria included patients with abnormal uterine cavity morphology, endometrial lesions, endometrial injury, intrauterine effusion, untreated hydrosalpinx and any circumstances considered unsuitable for participation in this study by the researchers.

Non-invasive chromosomal screening and frozen embryo transfer

All patients underwent ovarian stimulation, oocyte retrieval, ICSI and embryo culture according to our routine protocols. Embryos were evaluated on the morning of day 5/6 using morphological scoring criteria (*Gardner and Schoolcraft, 1999*) and vitrified separately. Additionally, the SCM was collected and subjected to the analysis described above.

Hormone replacement was carried out to prepare the endometrium during the FET cycles. Oestradiol pills were administered orally for 10–18 days, followed by progesterone administration when the endometrial thickness reached 8 mm. A single blastocyst was selected

based on a morphological assessment to transfer to each patient per cycle and was transferred under transabdominal ultrasound guidance.

Whole-genome amplification and next-generation sequencing

Whole-genome amplification was carried out using culture media and whole embryos, followed by library preparation using ChromInst (Yikon Genomics; EK100100724 NICS Inst Library Preparation Kit). Next-generation sequencing was carried out on the MiSeq platform (Illumina, San Diego, CA, USA), which yielded approximately 2 million sequence reads for each sample.

The CNV of each sample was calculated as previously described (*Xu et al., 2016*). R programme was used to plot the copy number of each bin to visualize CNV profiles for all 24 chromosomes. The minimum resolution of CNVs using this approach was 10 Mb. According to Guidelines for the Evaluation of quality control techniques for Preimplantation Chromosomal Aneuploidy Detection reagents (high-throughput Sequencing method) (China), the embryo was classified as 'euploid' when the degree of mosaicism identified was below 30%, 'aneuploid' when the degree of mosaicism was above 70%, and 'mosaic' (euploid–aneuploid mosaics) when the degree of mosaicism was 30–70%. In our ploidy analyses, embryos with five or more abnormal chromosomes were defined as chaotic/abnormal. The data were not sufficient for analysis because the amplification failure was defined as not available. After niPGT-A analysis ($\geq 60\%$ mosaic rates were set as the threshold to distinguish aneuploid from euploid), embryos were divided into euploid, aneuploid and chaotic groups.

Embryo rating and machine learning classifier

The chromosomal euploidy- or aneuploidy-related copy number pattern in the SCM was explored using the machine learning random forest algorithm combined with the standard chromosomal screening results of whole blastocysts. Gold standard labels (aneuploid or euploid) of whole blastocysts were determined based on the 50% mosaicism threshold. Other mosaicism cut-offs, e.g. 30%, 40%, 60% and 70%, were also evaluated; however, the mosaicism threshold of 50% was found to be the best for

obtaining ploidy consistency of SCM and whole blastocysts. The following 11 features were trained by the machine learning methods: 10M-resolution CNV result, 10M-resolution CNV result redefined by 50% mosaicism threshold, arm-resolution CNV result, arm-resolution CNV result redefined by 50% mosaicism threshold, whole chromosome-resolution CNV result, whole chromosome-resolution CNV result redefined by 50% mosaicism threshold, euploidy number with different resolution result, abnormal chromosome number, highest abnormal mosaicism proportion, largest abnormal fragment size corresponding to the highest mosaicism proportion, and presence of sex chromosome abnormality or not. Multiple machine learning methods, i.e. support vector machine (SVM), k-nearest neighbours (KNN), random forest, extreme gradient boosting (XGBoost), and neural network (Nnet), were constructed to train and test the paired SCM whole blastocyst data using R package caret 6.0-86. Embryo rating was measured based on euploidy probability obtained from machine learning algorithms. The detailed running process was as follows: training data were split into subsets randomly by bootstrap resampling; multiple decision trees (500) were generated for each training dataset and feature subsets; euploidy probability predictions from 500 decision trees were averaged to obtain the final prediction by the model; out-of-bag error was used to evaluate the model; and the model was iteratively trained until the optimal one was obtained. The embryos were ranked based on different thresholds of chromosomal euploidy probability obtained from the optimal model of the random forest model.

Statistical analysis

The Shapiro–Wilk test was used to test the normality of continuous variables. Continuous variables were presented as mean with SD (normal distribution) or median with interquartile ranges (non-normal distribution). Categorical variables were described as frequencies and percentages. Continuous variables were compared using one-way analysis of variance when normality and homogeneity of variance assumptions were satisfied. Otherwise, the Kruskal–Wallis H test was applied. For categorical variables, differences between groups were compared using chi-squared or

Fisher's exact tests. Furthermore, a logistic regression model was used to evaluate the effects of each group after adjusting for confounding variables. All *P*-values were two-sided, and results with *P* < 0.05 were considered statistically significant. The sample size was calculated based on comparing three groups A, B and C. The test level was set to 0.05, and the test efficiency was set to 80%. SPSS software programme (version 20.0; IBM, Armonk, NY, USA) was used for analysis.

RESULTS

Prediction of embryo euploidy probability using random forest machine learning

An embryo grading strategy was developed according to the euploidy probability of embryos using 345 paired blastocyst culture medium and whole blastocyst samples with confirmed chromosomal ploidy status. These contained features such as chromosomal mosaicism proportion, segmental aneuploidy size, multiple chromosomal abnormalities and sex chromosome information, which were considered when predicting the euploidy probability of each embryo using a SCM. The SVM, KNN, random forest, XGBoost, and Nnet algorithms were used and compared using 70% training/30% testing split strategy (Supplementary Table 1). In a clinical application scenario, maintaining a low false-negative rate is especially important to best facilitate the implantation of a normal embryo. Hence, the negative predictive value (NPV) is given more consideration than other measures. To this end, combined with the overall performance value and area under the receiver operating characteristic curve (AUC), random forest, which showed an AUC of 0.92 and NPV of 0.93, was selected for further analysis. Consequently, the ensemble learner with multiple decision trees based on a bagging strategy was trained to obtain the optimal tree in which the chromosomal ploidy concordance between samples from culture media and whole blastocysts was the highest (FIGURE 1A). Each culture medium was assigned a euploidy probability at the end of the analysis. As expected, the higher the euploidy probability predicted by SCM, the higher the likelihood of euploidy in the whole blastocyst (FIGURE 1B).

Development of an embryo grading strategy using euploidy probability predicted by non-invasive chromosomal screening

Embryos were graded as A, B or C according to their euploidy probabilities predicted by the CNV pattern, with euploidy probabilities of 0.94 or above, 0.7–0.94 and 0.7 or below for grades A, B and C, respectively. This grading strategy balanced both the euploidy predictive value and specificity, two important indices for embryo selection for implantation (FIGURE 2A).

With a 10-fold cross-validation study of 345 paired training data, each sample could be predicted once. Using the aforementioned grading criterion, 37% of the embryos were categorized as grade A, whereas 29% and 34% of the embryos were categorized as grades B and C, respectively (FIGURE 2B). A-grade embryos (91.4%) from SCM showed consistent chromosome euploidy with corresponding whole blastocysts, with the proportion of B- and C-grade embryos being 76.4 % and 34.6 %, respectively (FIGURE 2C).

Furthermore, the ploidy pattern of different grade-labelled embryos from SCM was explored. The chromosomal copy number pattern of A-grade embryos showed absolute euploidy, low mosaicism or small segmental aneuploidy compared with B- or C-grade embryos (Supplementary Figure 1). As expected, C-grade embryos showed large-scale irregularities in fragment size, such as those at the whole chromosomal level and high mosaicism or 100 % aneuploidy pattern (Supplementary Figure 1).

Non-invasive chromosomal screening grading system as a predictor to reduce the waste of potentially competent embryos

The clinical outcomes of the patients (*n* = 266) who underwent 278 single vitrified-warmed embryo resuscitation transplantation based on morphological assessment were observed (Supplementary Figure 2). The transferred embryos were divided into three groups according to the CNV pattern. Demographics among groups, including women's age, body mass index, serum sex hormone levels, endometrial thickness on transplantation day and men's age and infertility type are shown in Supplementary Table 2.

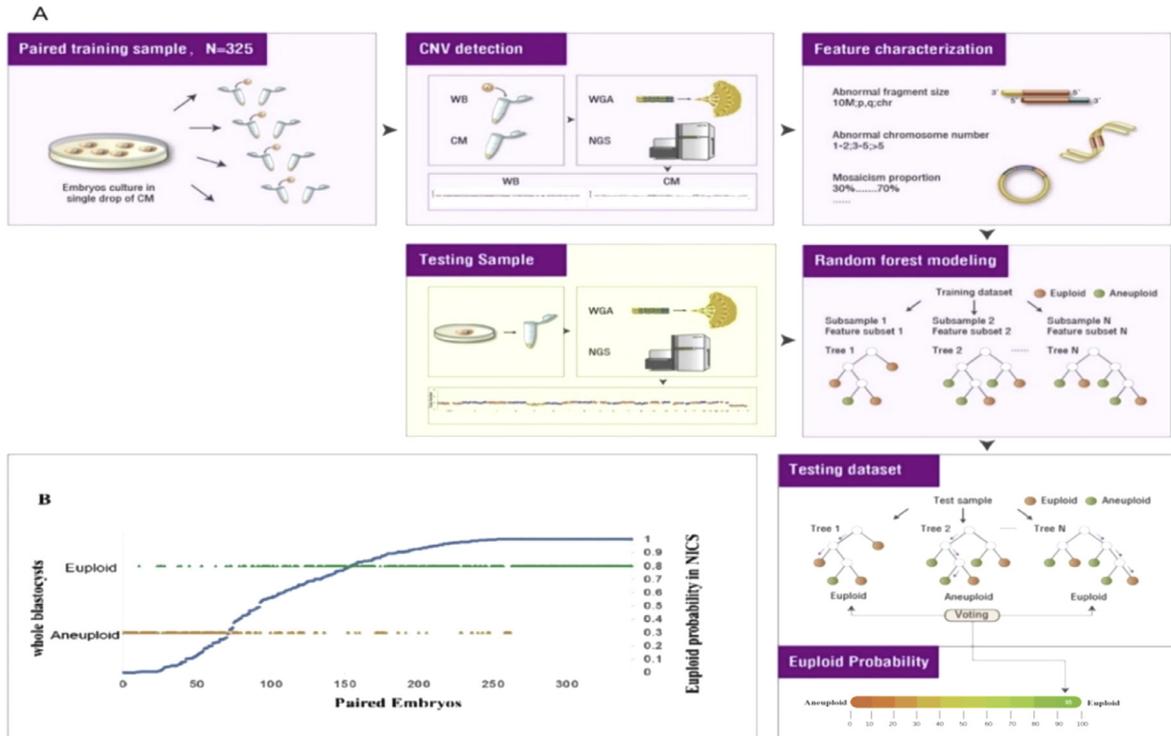


FIGURE 1 Embryo grading system using the machine learning method. (A) Copy number variation (CNV) analysis in a non-invasive chromosomal screening assay. The spent culture medium (SCM) and the whole embryos (WB) were collected separately and used for whole-genome amplification (WGA) using the MiSeq platform and for evaluation of the CNV. Features such as chromosomal mosaicism proportion, segmental aneuploidy size and multiple chromosomal abnormalities were extracted to train the random forest algorithm. Three hundred and 345 pairs of SCM samples and gold standard labels (aneuploid or euploid) of whole blastocysts were used to construct the random forest model. Then, the testing samples were used to run the ensemble learner with multiple decision trees based on a bagging strategy until the optimal one was obtained; (B) consistency comparison of chromosomal ploidy in whole blastocysts and blastocyst SCM using an embryo grading system. CM, congenital malformations; NICS, non-invasive chromosomal screening; NGS, next-generation sequencing.

For each blastocyst transfer, the corresponding culture medium was collected and CNV detected. The investigator was blinded to the CNV results until clinical outcomes were obtained and revealed. Then, a total of 278 FET embryos were assessed using

the NICS grading system, and three grades, A, B or C were assigned. The clinical outcomes were not significantly different between A- and B-grade embryos in pregnancy rate (61.7% versus 57.0%, $P = 0.590$), ongoing pregnancy rate (51.9% versus 48.8%, $P = 0.797$),

live birth rate (50.4% versus 45.3%, $P = 0.728$) and miscarriage rates (15.9% versus 14.3%, $P = 0.655$). The pregnancy rate, ongoing pregnancy and live birth rate in grade C, however, were 40.7%, 27.1% and 27.1%, respectively, significantly lower than those in grades A and B

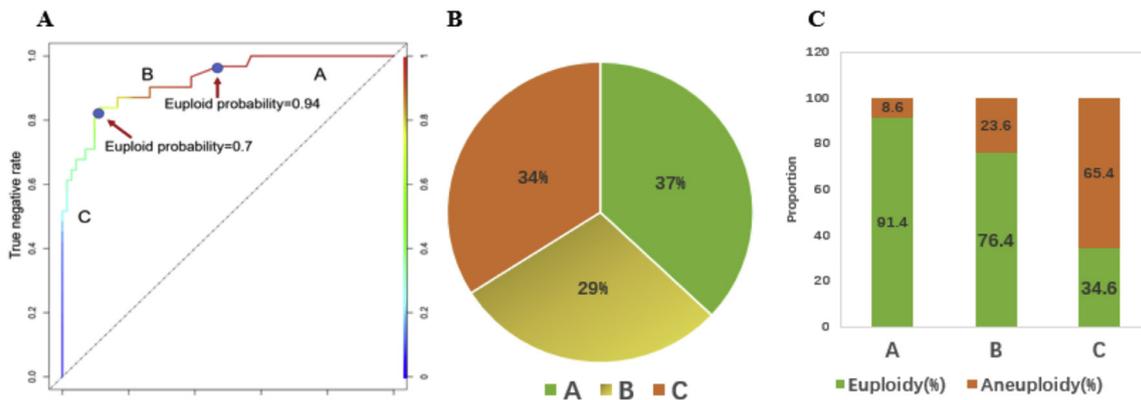


FIGURE 2 Performance of the embryo grading strategy with non-invasive chromosomal screening results. (A) Classification of grades A, B or C by different euploidy probability cut-offs obtained using the random forest method; (B) proportion of each graded embryo in total samples; grade A (37 %); grade B (29 %); grade C (34 %); (C) euploid proportion of each graded embryo based on non-invasive chromosomal screening and aneuploidy results. The euploidy probabilities of embryos in each grade were 91.4%, 76.4% and 34.6%, respectively.

TABLE 1 PREGNANCY OUTCOMES IN PATIENTS WITH DIFFERENT NON-INVASIVE CHROMOSOME SCREENING GRADES

Group	A	B	C	P-value
Pregnancy rate, % (n)	61.7 (82/133)	57.0 (49/86)	40.7 (24/59)	0.010
Pairwise comparisons	B versus C			0.020
	A versus C			0.011
	A versus B			0.590
Miscarriage rate, % (n)	15.9 (13/82)	14.3 (7/49)	33.3 (8/24)	0.068
Pairwise comparisons	B versus C			0.021
	A versus C			0.026
	A versus B			0.655
Ongoing pregnancy rate, % (n)	51.9 (69/133)	48.8 (42/86)	27.1 (16/59)	0.003
Pairwise comparisons	B versus C			0.009
	A versus C			0.001
	A versus B			0.797
Live birth rate, % (n)	50.4 (67/133)	45.3 (39/86)	27.1 (16/59)	0.004
Pairwise comparisons	B versus C			0.022
	A versus C			0.006
	A versus B			0.728

($P = 0.010$, $P = 0.003$ and $P = 0.004$) (TABLE 1).

At the same time, the embryos were assessed using niPGT-A ($\geq 60\%$ mosaic rates were set as the threshold to distinguish aneuploid from euploid) and divided into euploid, aneuploid, and

chaotic groups. Of the 278 embryos with conclusive outcomes, 161 were classified into the euploid group, 76 into the aneuploid group and 41 into the chaotic group. Considering A- and B-grade embryos as transplantable an improved embryo transplantable rate of A- and B-grade embryos was observed

compared with that for euploid embryos (78.8% [219/278] versus 57.9% [161/278], respectively). Similar clinical outcomes were observed with the comparison of A- and B-grade embryos and the euploid group. These were as follows: pregnancy rate (59.8% versus 60.9%), ongoing pregnancy rate (50.7% versus 51.6%), live birth rate (48.4% versus 49.1%) and miscarriage rate (15.3% versus 15.3%) (FIGURE 3). According to the results of the niPGT-A analysis, aneuploid and chaotic embryos were considered non-transplantable embryos. The miscarriage rate of C-grade embryos was higher than that in aneuploid and chaotic groups (33.3% versus 22.8%), whereas clinical pregnancy, ongoing pregnancy and live birth rates of C-grade embryos were lower than those in aneuploid and chaotic groups (40.7% versus 48.7%, 27.1% versus 37.6%, 27.1% versus 36.8%, respectively) (FIGURE 3).

DISCUSSION

At present, the typical method used for embryo selection depends on its morphological score. It is simple, practical, inexpensive and without damage, but morphology evaluation under a microscope is subject to observer bias, and morphology is a poor indicator of embryo chromosomal

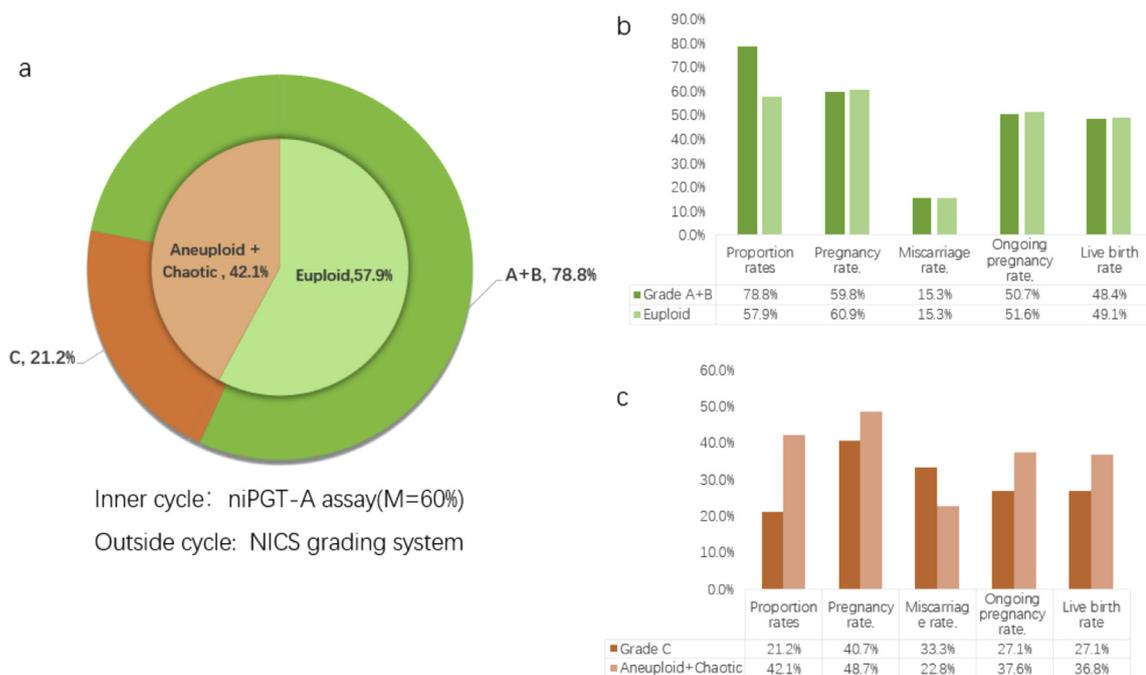


FIGURE 3 Pregnancy outcomes of patients with different non-invasive chromosomal screening grades. (A) The embryo utilization rate of the machine learning strategy and the non-invasive preimplantation genetic testing for aneuploidy (niPGT-A) analysis; (B) pregnancy outcomes between grade A + B and euploid; (C) pregnancy outcomes between grade C and aneuploid.

composition (Capalbo *et al.*, 2014). Trophoctoderm biopsy PGT-A using NGS has been applied extensively to clinical IVF for avoiding the transfer of aneuploid embryos; however, its invasive nature and embryo mosaicism are controversial (Munné *et al.*, 2010, Popovic *et al.*, 2018). A mosaic embryo possesses two or more chromosomally distinct cell lines within the embryo. It is typical to define samples with 20–80% abnormal cells as ‘mosaic’. Next-generation sequencing can reliably detect different patterns of mosaic embryos, including whole chromosomal mosaicism, segmental mosaicism and complex mosaicism, and has been reported to detect mosaicism in about 30% of trophoctoderm specimens (Fragouli *et al.*, 2017; Munné *et al.*, 2017; Nakhuda *et al.*, 2018). Consequently, NGS has generated renewed interest in the phenomenon of embryonic mosaicism, stirring controversy about the accuracy of trophoctoderm biopsies, especially regarding the viability of mosaic embryos. Several studies have reported healthy babies being born after transfer of embryos diagnosed as aneuploid or diploid/aneuploid mosaic (Greco *et al.*, 2015; Zore *et al.*, 2019), with a study reporting a live birth rate of 46.6% (Zhang *et al.*, 2019b). Therefore, labelling embryos as ‘aneuploid’ or ‘euploid’ based on dichotomous screening outcomes may result in the false exclusion of several embryos, leading to an increase in the cancellation rate of IVF cycles.

As a single trophoctoderm biopsy contains approximately five to 10 cells, the extent to which it reflects the chromosomal complement of the entire embryo remains unclear. Bolton *et al.* (2016) showed that the source of cell-free DNA from apoptotic cells in the culture fluid is mainly the ICM, which is more representative of the fetus than the trophoctoderm-biopsied cells. Theoretically, compared with PGT-A for trophoctoderm biopsy, NICS based on SCM avoids embryo damage, reduces the technical platform, and, more importantly, ensures the accuracy of the source of samples. Because of the mosaic nature of human embryos, however, it is likely that DNA in the culture medium is a mixture of DNA obtained from both ICM and apoptotic cells cleared from the embryo, which can result in a false-positive during chromosomal screening and a fluctuating ploidy concordance of 33–100%. Additionally, the definitions of ploidy concordance are different,

especially if all chromosome aberrations (such as fragment aneuploidy and chimerism) are considered. It is unclear whether the segmental or mosaic abnormality is defined as aneuploidy. Moreover, the gold standard samples are different in different studies. In fact, for NICS, no uniform standard is available for setting the threshold to determine euploid versus aneuploid embryos, and mosaicism is defined as the percentage of abnormal cells of 40% or over (Jiao *et al.*, 2019) or 50% or over (Rubio *et al.*, 2019). Huang *et al.* (2019) used whole embryos as a gold standard, and defined mosaicism of over 60% as the threshold to distinguish aneuploid from euploid for the same population. They even observed a higher consistency in culture medium DNA than in trophoctoderm-biopsied samples, indicating that culture medium-based niPGT-A could potentially be more accurate than trophoctoderm-based PGT-A.

Previous PGT-A and NICS studies have defined euploidy based on the percentage of mosaicism, and the embryos with aneuploid, mosaic and chaotic CNV are often not considered for transfer. In the present study, instead of arbitrarily setting a threshold to determine euploid versus aneuploid embryos, an embryo grading strategy was developed according to the euploid probability and implantation potential of the embryos. Features such as chromosomal mosaicism proportion, segmental aneuploid size and multiple chromosomal abnormality information were taken into consideration by the random forest model. To verify the correlation between this scoring system and the clinical outcome, embryos were graded as A, B or C based on different cut-off criteria of their euploidy probability. Different grades of embryos were found to have different characteristics. A-grade embryos showed absolute euploidy, low mosaicism or small segmental aneuploidy; B-grade embryos showed a low rate of mosaicism; C-grade embryos had a high mosaicism proportion or a 100% aneuploid pattern (Supplementary Figure 1). Generally, smaller abnormal fragments and lower mosaic ratios tend to predict higher euploid probabilities.

In the present study, the NICS grading system and 60% mosaicism threshold for identifying aneuploidy method (Huang *et al.*, 2019) were used to evaluate the

embryos simultaneously. The embryos below 60% were regarded as euploid embryos, and the clinical outcomes of A- and B-grade embryos were found to be similar to those of euploid embryos. Surprisingly, the number of euploid and A- and B-grade embryos was found to be 57.9% and 78.8%, respectively. According to the normal probability of the whole embryo and the corresponding clinical outcome, a sequence of embryo transfers with the NICS grading system is, therefore, suggested (FIGURE 4). Embryos in group A have a euploid probability above 90% and should have the highest priority transfer. If the patient does not have a group-A embryo, group-B embryos can be considered because significantly different clinical outcomes were not observed in both group A versus B embryos. If the couple only has C grade embryos, however, they should be informed, and a biopsy or a retake of the culture medium sample should be considered before undergoing another IVF treatment cycle. Such a screening strategy avoids unnecessary overuse of embryo biopsy, which is safer and easier to implement in most IVF cycles. It also avoids high cancellation rates due to the false positives of embryo chromosomal testing. This strategy also avoids early miscarriages caused by chromosomal abnormalities, which is the number one factor of miscarriage, especially in couples with advanced age.

Recently, several studies have used machine learning-guided approaches for the selection of human blastocysts via imaging or video, including morphometric analysis by time-lapse imaging (Curchoe and Bormann, 2019), mathematical and statistical tools (Santos Filho *et al.*, 2012; VerMilyea *et al.*, 2020) and computer-assisted scoring (Alfarawati *et al.*, 2011; Zaninovic *et al.*, 2017). These methods are based on subjective developmental and morphological characteristics. The advantage of our method is that the model predicts the euploidy probability based on the CNV features of each embryo in the SCM. Many machine-learning models such as SVM, KNN, random forest, XGBoost and Nnet were compared, and finally selected random forest for classifier construction based on favourable AUC and NPV performance. Random forest is an ensemble learner with multiple decision trees based on a bagging strategy. It corrects the problem of overfitting that occurs with a decision

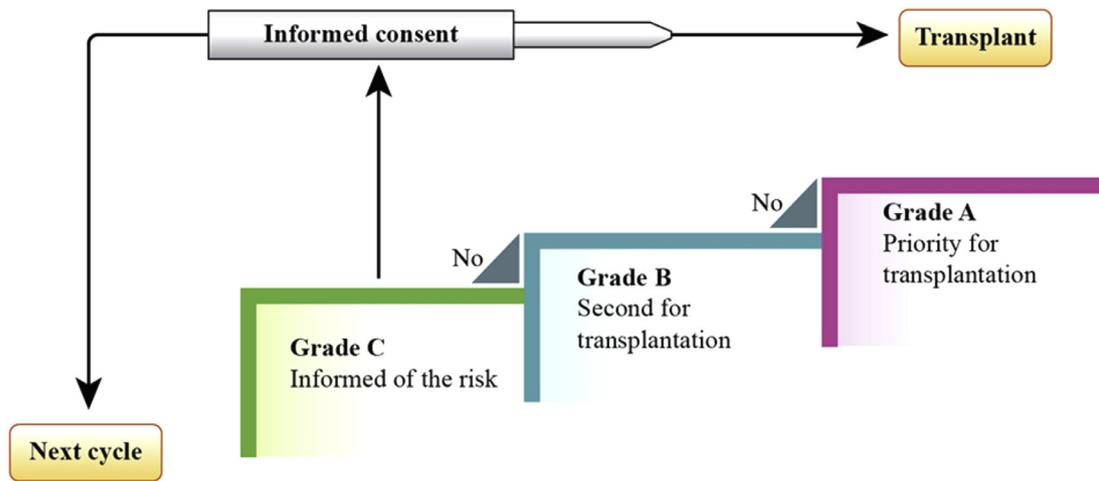


FIGURE 4 Recommended clinical embryo transfer pathways using non-invasive chromosome screening. The embryos in grade A are of first priority for transplantation; embryos in grade B can be considered in the absence of grade A embryo. If only grade C embryos are available, doctors should inform patients about the rating and risk of miscarriage, and patients can choose whether to proceed with the transfer.

tree model and performs better than an individual model. Moreover, the algorithm can be implemented through the client of the ChromGo web version. After logging in, users only need to upload the sequencing results and select the parameters as required. Then the ratings and CNV results were shown. The detailed analysis process is as previously reported (Huang *et al.*, 2021).

In conclusion, the results suggest that this machine learning guided NICS grading system may be a promising approach to optimize the selection of a single embryo for transfer, thus maximizing the chance of live birth and avoiding the wastage of potentially competent embryos. It may, therefore, serve as an effective non-invasive approach. In the future, with the accumulation of comparative studies on niPGT-A and large clinical data, including morphological grade, DNA concentration, aneuploidy screening, mitochondrial copy number, clinical outcome and patient information, an artificial intelligence system based on machine learning may be developed to evaluate the implantation potential of each embryo.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.rbmo.2022.03.006.

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