Human Reproduction, Vol.36, No.4, pp. 1146–1156, 2021
Advance Access Publication on December 30, 2020 doi:10.1093/humrep/deaa352

human reproduction

ORIGINAL ARTICLE *Reproductive genetics*

Lower chromosomal abnormality frequencies in miscarried conceptuses from frozen blastocyst transfers in ART

Jing Li[1,2], Fuli Zhang[1], Bo Sun[1], Shanjun Dai[1], Qingling Yang[1], Linli Hu[1], Hao Shi[1], Jiawei Xu[1], Wenbin Niu[1], and Yihong Guo[1]*

[1]Center for Reproductive Medicine, Henan Province Key Laboratory of Reproduction and Genetics, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan, 450052, China [2]Academy of Medical Science, Zhengzhou University, Zhengzhou, Henan, 450052, China

*Correspondence address. Center for Reproductive Medicine, Henan Province Key Laboratory of Reproduction and Genetics, The First Affiliated Hospital of Zhengzhou University, Henan, China. E-mail: 13613863710@163.com

Submitted on June 7, 2020; resubmitted on November 22, 2020; editorial decision on November 26, 2020

STUDY QUESTION: Are blastocyst culture and cryopreservation in ART associated with chromosomal abnormalities in miscarried products of conception (POC)?

SUMMARY ANSWER: Frozen blastocyst transfer in women aged 35 years or older and frozen embryo transfer (ET) (including both cleavage-stage embryo and blastocyst) in women aged <35 years are associated with decreased frequencies of embryonic chromosomal abnormalities in miscarried POC.

WHAT IS KNOWN ALREADY: Blastocyst culture and embryo cryopreservation have been previously associated with favorable ART treatment outcomes and widely applied in clinical practice. However, the association between these embryo manipulation procedures and embryonic chromosomal abnormalities has not been evaluated to the best of our knowledge.

STUDY DESIGN, SIZE, DURATION: This retrospective study included a total of 720 patients who underwent IVF/ICSI, and the retained POC were obtained. A single-nucleotide polymorphism (SNP)-based chromosomal microarray analysis (CMA) of all miscarried conceptuses was performed.

PARTICIPANTS/MATERIALS, SETTING, METHODS: This study was based on the Clinical Reproductive Medicine Management System/Electronic Medical Record Cohort Database (CCRM/EMRCD) at our center. In total, 720 miscarried POCs were collected from patients undergoing ART (including fresh cleavage-stage ET, fresh blastocyst transfer, frozen cleavage-stage ET and frozen blastocyst transfer), and the incidences and profiles of cytogenetic abnormalities in the miscarried conceptuses were measured via SNP-based CMA.

MAIN RESULTS AND THE ROLE OF CHANCE: The chromosomal abnormality rate in POC varied from 33.7% to 66.7% among the different ET strategies. In the patients aged ≥35 years, frozen blastocyst transfer was significantly associated with a lower incidence of chromosomal aberrations in the POCs (adjusted odds ratio (aOR): 0.171 (95% CI: 0.040–0.738); $P = 0.018$) than fresh blastocyst transfer. In the patients aged <35 years, frozen ET was significantly associated with a lower incidence of chromosomal aberrations than fresh ET in both cleavage-stage ET cycles and blastocyst transfers cycles (aOR: 0.545 (0.338–0.879), $P = 0.013$; and aOR: 0.357 (0.175–0.730), $P = 0.005$, respectively). Trisomy was the most frequent abnormal embryonic karyotype in the different ET strategies, and its frequency significantly differed among strategies ($P < 0.05$).

LIMITATIONS, REASONS FOR CAUTION: This study was retrospectively designed, and we cannot draw any definite conclusions from our results regarding the adequate safety of embryo cryopreservation in ongoing pregnancy.

WIDER IMPLICATIONS OF THE FINDINGS: To our knowledge, this is the first study assessing the associations of ET strategies with the probability of miscarriage associated with embryonic chromosomal abnormalities. However, the underlying mechanism of these associations is unknown; this study may promote research concerning ET strategies and promote comprehensive consultations and recommendations for patients.

STUDY FUNDING/COMPETING INTEREST(S): This work was supported by the Natural Science Foundation of China (Grant No.81571409), Science and Technology Research Project of Henan (Grant No. 172102310009) and Medical Science and Technology Research Project of Henan (Grant No. 201701005). The authors declare no competing interests.

TRIAL REGISTRATION NUMBER: N/A

Key words: chromosomal abnormality / frozen blastocyst transfer / miscarriage / chromosomal microarray analysis / ART

Introduction

Approximately 15% of IVF pregnancies end in first-trimester pregnancy loss (Hipp *et al.*, 2016). Among the factors that may lead to early pregnancy loss, chromosomal abnormalities are the most common, suggesting that they account for ~50% of miscarriages (Pylyp *et al.*, 2018). It has been suggested that maternal age and metabolic disorders may cause or increase the risk of chromosomally abnormal embryos (Meldrum *et al.*, 2016; Li *et al.*, 2019; Ozawa *et al.*, 2019). Analysis of factors that may cause chromosomal abnormalities in embryos is important for assessing the risks of subsequent miscarriages and providing guidance for the selection of subsequent fertility strategies. In addition, among patients who have a lower risk of conceiving chromosomally abnormal fetuses, the treatment should focus on other factors that influence the ongoing pregnancy, such as intrauterine malformations and endocrine diseases.

Because of the lower associated risk of ovarian hyperstimulation syndrome, frozen embryo transfer (ET) has been gradually encouraged instead of fresh ET in IVF practice (Shi *et al.*, 2018; Roque *et al.*, 2019). Previous studies have also suggested that blastocyst transfer results in favorable treatment outcomes compared with cleavage-stage ET (Glujovsky *et al.*, 2016). It is obvious that elective frozen ET and blastocyst-stage transfer have strongly contributed to the improvement of the live birth rate. However, for patients who underwent conception through ART and experience miscarriage, limited research has evaluated the underlying associations between chromosomally abnormal miscarriage and the applications of blastocyst culture and cryopreservation. Single-nucleotide polymorphism (SNP)-based chromosomal microarray analysis (CMA) has advantages over traditional karyotype analysis in determining embryo malformations in pregnancy, as SNP-based CMA is a high-throughput, high-resolution automated process (Shah *et al.*, 2017; Zhang et al., 2018; Daum *et al.*, 2019).

To assess whether ET strategies can affect chromosomal abnormalities in miscarried products of conception (POC), we analyzed the types and frequencies of chromosome abnormalities in first-trimester POC by SNP analysis.

Materials and methods

Patients

This retrospective study was based on the Clinical Reproductive Medicine Management System/Electronic Medical Record Cohort Database (CCRM/EMRCD) at our center. Couples who had genetic diseases and couples who received preimplantation genetic testing were excluded. In total, 720 patients who conceived through IVF/ICSIs and had a first-trimester miscarriage were included. Ethics Committee of the First Affiliated Hospital of Zhengzhou University approved this study, and written informed consent was obtained from all participating subjects during the first consultation.

ART protocols

Ovarian stimulation was performed with an initial dose of gonadotrophin (Gn) ranging from 112.5 to 300 IU according to the BMI and ovarian function of the patients. Follicular development was monitored via a transvaginal ultrasound examination. hCG (Livzon, China) was injected in the muscle when the largest follicle was greater than 20 mm in diameter, and follicles >16 mm accounted for more than 2/3 of all follicles. Oocyte retrieval was performed 37 h after the hCG injection, followed by IVF/ICSI. One to three cleavage-stage embryos or a blastocyst were transferred in fresh ET cycles or thawed cryopreserved ET cycles. Spontaneous miscarriage was diagnosed by ultrasound showing a persistent anembryonic gestational sac or arrest of embryo cardiac activity after the clinically confirmed presence of an intrauterine gestational sac and positive cardiac pulsations.

SNP array analysis

The cytogenetic analysis of the retained POC was carried out by an SNP-based CMA. Chorionic villi were separated and collected from POC that ended in miscarriages. The DNA was purified using an AllPrep DNA Mini Kit (Qiagen, Hilden, Germany), and a Human CytoSNP-12v.21 Array (Illumina, San Diego, CA, USA) was applied to test the SNP array. The SNP array data analysis was performed by using Genome-Studio (Illumina 2011) and Karyo-Studio v1.4. The total signal intensity (log R ratios) and the allelic intensity ratio (B allele frequencies) were examined to determine the copy losses or gains. Calculation of various levels of mosaicism was based on the deviation of allele frequencies from the expected values according to the method used by Conlin *et al.* (2010).

Statistical analysis

SPSS 17.0 (IBM Corp., Chicago, IL, USA) was used for statistical analyses. Z-score normalization was performed during the data preprocessing. Continuous variables are presented as the mean ± SD, and categorical variables are presented as frequencies (percentages). Comparisons among different groups were performed with a one-way ANOVA, chi-square test, Fisher's exact test and Bonferroni-adjusted test. Multiple regression analyses and a single-factor logistic regression were performed to assess the factors that may influence the frequency of chromosome abnormalities. Adjusted odds ratios (aORs) with 95% CIs were estimated. All tests were two-sided, and statistical significance was defined as $P < 0.05$ (adjusted P-value following Bonferroni was 0.008).

Results

General data

As shown in Fig. 1, a total of 720 miscarriage samples were collected, including 642 specimens from IVF cycles and 78 specimens from ICSI cycles. The patients included in the study were diagnosed with male factor infertility (n = 78), tubal factor infertility (n = 303), uterine abnormalities (n = 92), polycystic ovary syndrome (PCOS) (n = 115) and recurrent miscarriage (n = 14). After cytogenetic analysis of chorionic villi by an SNP array, ~59.3% (n = 427) of the subjects showed abnormal karyotypes (Fig. 1).

Variable measurements and identifying their association with chromosomally abnormal miscarriages

We retrospectively compared the baseline characteristics of patients with normal embryonic karyotypes and patients with abnormal embryonic karyotypes, and a single-factor logistic regression was performed to analyze the associations between the clinical variables described in Table I and embryonic aneuploidy in the POC. The results shown in Table I suggest that there were no significant differences between the two groups in terms of the male BMI, levels of thyroid hormones (thyroid-stimulating hormone, TSH; free triiodothyronine, FT3; free thyroxine, FT4), male BMI and rate of some infertility diagnoses, such as male factor infertility, uterine abnormalities and recurrent miscarriage (all $P > 0.05$); additionally, the fertilization method (IVF and ICSI) was comparable in the normal and chromosomal aberration groups (IVF: 89.1% versus 89.2%; $P > 0.05$).

A significant association was observed between embryonic aneuploidy in the POC and the diagnosis of PCOS (OR: 0.465 (0.311–0.697); $P < 0.05$) and tubal factor infertility (OR: 1.406 (1.037–1.905); $P < 0.05$). As expected, both the male and female ages were positively associated with embryonic aneuploidy in the POC (OR: 1.090 (1.061–1.120) and 1.118 (1.084–1.153); all $P < 0.05$).

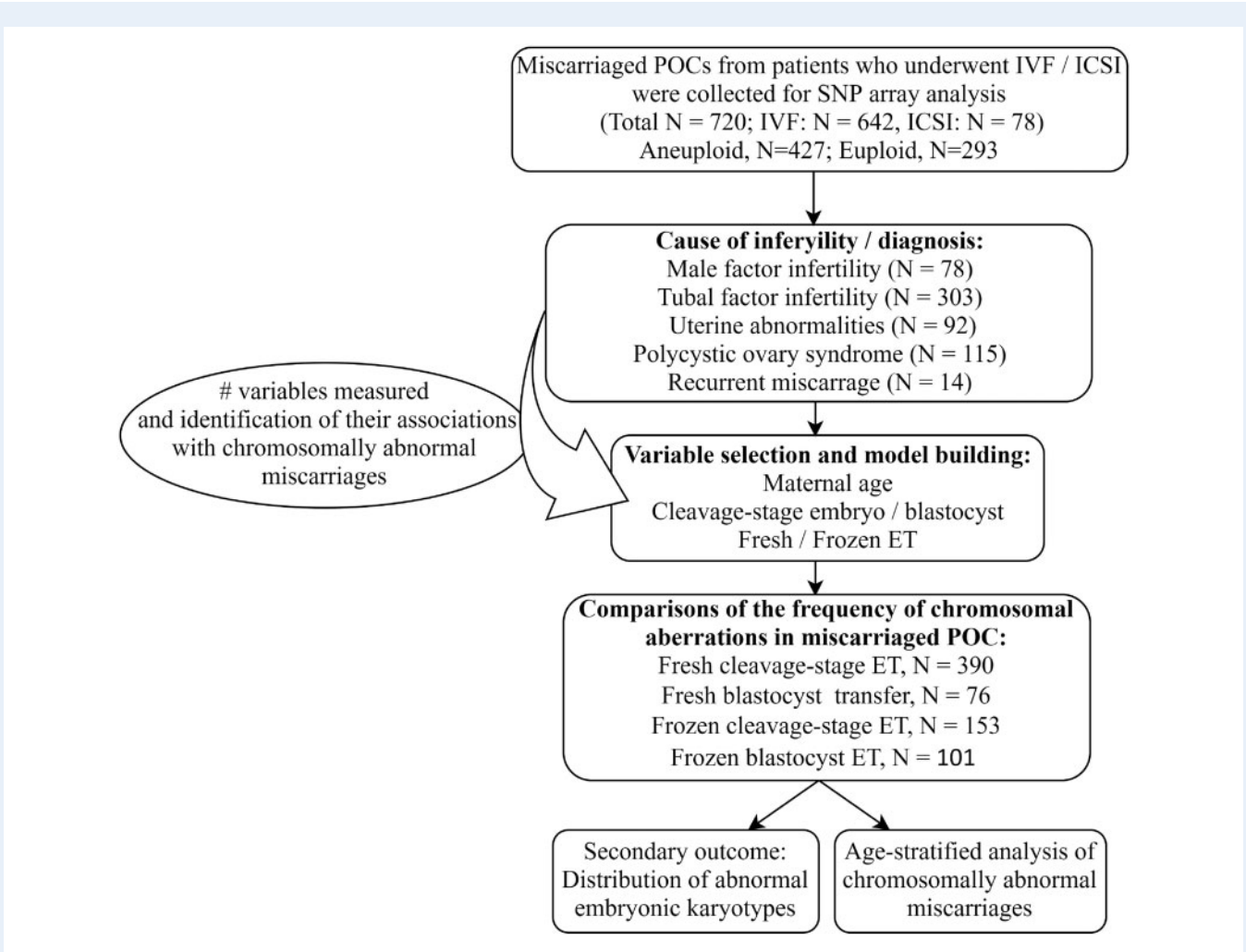

Figure 1. Flow chart of the patients enrolled and the research framework. # Variables associated with chromosomally abnormal miscarriage in a single-factor logistic regression: maternal age, male age, tubal factor infertility, polycystic ovary syndrome (PCOS), gonadotrophin dosage, cycle type (fresh/frozen) and embryo type (cleavage-stage embryo/blastocyst). POC, miscarried products of conception; SNP, single-nucleotide polymorphism; ET, embryo transfer.

Table I Basic clinical characteristics of the patients included in the single-nucleotide polymorphism-based chromosomal microarray analysis of the miscarried products of conception.

Variable	Normal	Chromosomal Aberration	OR [95% CI]	P
Miscarriage cycles (n)	293	427		
Female age (years)	31.06 ± 4.57	33.96 ± 5.40	1.118 [1.084–1.153]	0.000
Female BMI (kg/m^2)	23.20 ± 3.30	22.94 ± 3.07	0.974 [0.930–1.021]	0.282
Male age (years)	31.97 ± 5.11	34.95 ± 6.48	1.090 [1.061–1.120]	0.000
Male BMI (kg/m^2)	24.94 ± 3.81	25.28 ± 3.64	1.026 [0.979–1.074]	0.280
FT3 (pmol/l)	5.12 ± 0.64	5.04 ± 0.82	0.863 [0.666–1.117]	0.262
FT4 (pmol/l)	10.95 ± 2.03	11.07 ± 2.38	1.021 [0.938–1.111]	0.630
TSH (mIU/ml)	2.39 ± 1.10	2.32 ± 1.17	0.945 [0.801–1.114]	0.501
Tubal factor	109 (37.2)	194 (45.4)	1.406 [1.037–1.905]	0.028
Male factor	32 (10.9)	46 (10.8)	1.015 [0.630–1.638]	0.950
Uterine problems	35 (11.9)	57 (13.3)	1.136 [0.724–1.781]	0.580
PCOS	65 (22.2)	50 (11.7)	0.465 [0.311–0.697]	0.000
Recurrent miscarriage	4 (1.4)	10 (2.4)	1.733 [0.538–5.578]	0.357
History of miscarriage	21 (7.2)	32 (7.5)	0.935 [0.538–1.688]	0.869
IVF	261 (89.1)	381 (89.2)	0.985 [0.611–1.588]	0.950
ICSI	32 (10.9)	46 (10.8)	1.015 [0.630–1.638]	0.950
Gn dosage (IU)	2546.71 ± 994.49	2770.91 ± 975.07	1.000 [1.000–1.000]	0.020
Cleavage-stage ET	196 (66.9)	347 (81.3)	2.147 [1.522–3.028]	0.000
Blastocyst transfer	97 (33.1)	80 (18.7)	0.466 [0.330–0.657]	0.000
Fresh ET cycle	160 (54.6)	306 (71.7)	2.102 [1.539–2.871]	0.000
Frozen ET cycle	133 (45.4)	121 (28.3)	0.476 [0.348–0.650]	0.000

SNP, single-nucleotide polymorphism; CMA, chromosomal microarray analysis; ET, embryo transfer; Gn, gonadotrophin; PCOS, polycystic ovary syndrome; TSH, thyroid-stimulating hormone; FT3, free triiodothyronine; FT4, free thyroxine. The data are presented as the mean ± SD. OR, odds ratio, association between the variables and chromosomal aberration.

Moreover, both blastocyst transfer (compared with cleavage-stage ET) and frozen ET (compared with fresh ET) were negatively associated with an abnormal embryonic karyotype in the POC (OR: 0.466 (0.330–0.657) and 0.476 (0.348–0.650); all $P < 0.05$), respectively (Table I).

Key factors affecting the frequency of abnormal embryonic karyotypes

The variables correlated with abnormal embryonic karyotypes in the single-factor logistic regression analysis were subjected to a multiple logistic regression analysis by a stepwise modeling procedure. The female age, blastocyst-stage ET and frozen ET were included in the regression model.

As shown in Fig. 2, advanced maternal age (≥35years) (aOR: 2.747 (1.960–3.850); $P < 0.05$) was significantly correlated with an increase in abnormal embryonic karyotypes in POC. After adjusting for age, the transfer of blastocyst-stage embryos (aOR: 0.619 (0.430–0.893); $P < 0.05$) was significantly correlated with a decrease in abnormal embryonic karyotypes among POC compared with the transfer of cleavage-stage embryos. Similarly, a decrease in abnormal embryonic karyotypes was also observed for frozen ET compared with fresh ET (aOR: 0.561 (0.403–0.781); $P < 0.05$).

Basic clinical characteristics and chromosomally abnormal miscarriage in patients who underwent different ET strategies

The baseline characteristics of the patients who underwent the following ET protocols are presented in Table II: fresh cleavage-stage ET (n = 390), fresh blastocyst transfer (n = 76), frozen cleavage-stage ET (n = 153) and frozen blastocyst transfer (n = 101). The average female age in the group with fresh cleavage-stage ET was significantly higher than those in the group with fresh blastocyst transfer and frozen cleavage-stage ET (33.71 versus 30.84 years, 33.71 vs. 32.07 years; all $P < 0.008$). There were no significant differences among the four groups in terms of the male and female BMI, levels of thyroid hormones (TSH, FT3, FT4), incidence of uterine problems, recurrent miscarriage, tubal factor infertility and fertilization method (all $P > 0.05$) (Table II). In total, 6.2% of the patients in the fresh cleavage-

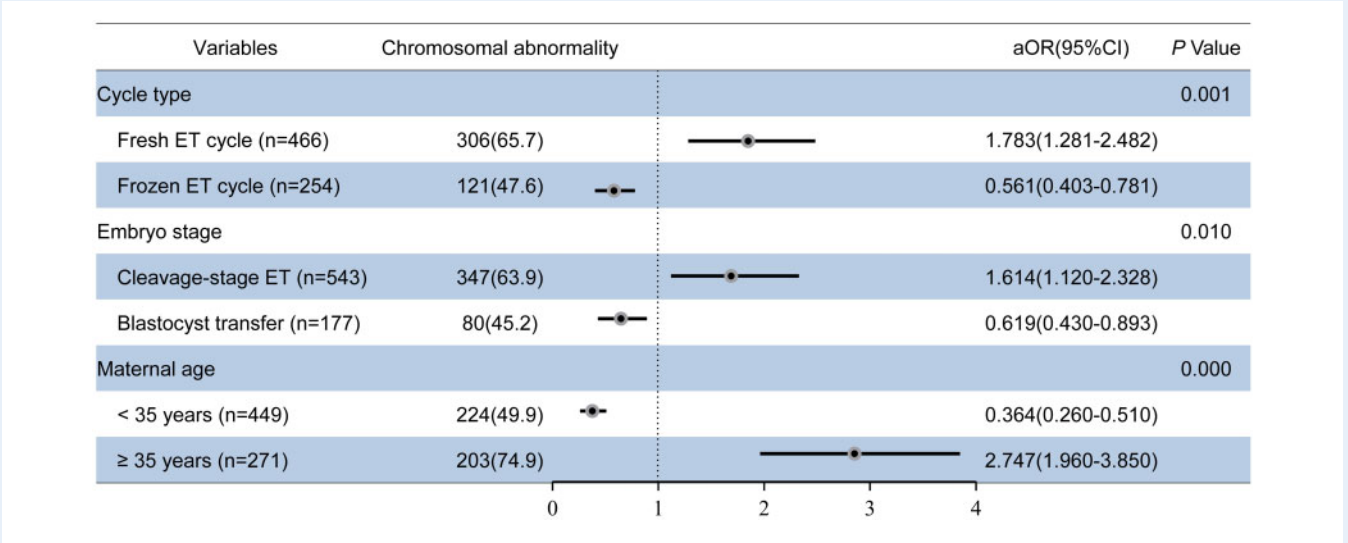

Figure 2. Key factors affecting the frequency of abnormal embryonic karyotypes. 'aOR' indicates that the odds ratio (OR) and 95% CI were adjusted for potential confounding factors.

Table II Basic clinical characteristics of the patients who underwent different embryo transfer strategies.

Variables	Fresh cleavage-stage ET	Fresh blastocyst transfer	Frozen cleavage-stage ET	Frozen blastocyst transfer
Miscarriage cycles (n)	390	76	153	101
Female age (years)	33.71 ± 5.15 [a, c]	30.84 ± 4.33[a]	32.07 ± 5.55[c]	31.74 ± 5.27
Female BMI (kg/m^2)	22.98 ± 3.04	22.90 ± 3.14	23.10 ± 3.38	23.31 ± 33.37
Male age (years)	34.45 ± 6.03[a]	31.62 ± 5.08[a]	33.32 ± 6.22	33.21 ± 6.75
Male BMI (kg/m^2)	25.22 ± 3.35	25.03 ± 4.31	24.58 ± 3.59	25.43 ± 3.94
FT3 (pmol/l)	5.07 ± 0.79	5.01 ± 0.65	5.03 ± 1.38	5.59 ± 0.53
FT4 (pmol/l)	10.97 ± 2.31	11.31 ± 2.05	11.53 ± 0.66	11.82 ± 0.42
TSH (mIU/ml)	2.33 ± 1.14	2.44 ± 1.20	3.06 ± 0.76	2.10 ± 1.37
Tube factor	183 (46.9)	27 (35.5)	59 (38.6)	34 (33.7)
Male factor	63 (16.2) [c]	11 (14.5) [d]	1 (0.7) [c]	3 (3)[d]
Uterine or cervical problems	51 (13.1)	7 (9.2)	16 (10.5)	18 (17.8)
PCOS	24 (6.2) [a, c]	18 (23.7) [a]	44 (28.8) [c, d]	29 (28.7) [d]
Recurrent miscarriage	10 (2.6)	1 (1.3)	2 (1.3)	1 (1)
IVF	327 (83.8) [c]	65 (85.5) [d]	152 (99.3) [c]	98 (97)[d]
ICSI	63 (16.2) [c]	11 (14.5) [d]	1 (0.7) [c]	3 (3)[d]
Gn dosage (IU)	2785.71 ± 976.13[a]	2266.94 ± 929.19[a]	2287.50 ± 901.56	1833.33 ± 864.70
Chromosomal abnormality rate	260 (66.7)	46 (60.5) [d]	87 (56.9) [b]	34 (33.7) [b, d]

Statistical analysis was performed using a one-way ANOVA, Chi-square test, Fisher's exact test and Bonferroni-adjusted test. Statistical significance is defined as $P < 0.008$. The data are presented as the frequency (percentage). 'a', 'b', 'c' and 'd' indicate that significant differences were detected between the two groups.
TSH, thyroid-stimulating hormone; ET, embryo transfer; Gn, gonadotrophin; PCOS, polycystic ovary syndrome; FT3, free triiodothyronine; FT4, free thyroxine.

stage ET group were diagnosed with PCOS, which was significantly lower than that in the fresh blastocyst transfer group (6.2% versus 23.7%, respectively, $P < 0.008$) and frozen cleavage-stage ET group (6.2% versus28.8%, respectively, $P < 0.008$).

The transfer of frozen blastocysts resulted in a significant lower incidence of chromosomally abnormal miscarriages compared with fresh blastocyst transfer and frozen cleavage-stage ET (33.7% versus 56.9%, 33.7% versus 60.5%, respectively; all $P < 0.008$) (Table II).

Table III Distribution of abnormal embryonic karyotypes classified by different embryo transfer strategies.

Variable	Fresh cleavage-stage ET	Fresh blastocyst transfer	Frozen cleavage-stage ET	Frozen blastocyst transfer
N	390	76	153	101
Trisomy	195 (50.0) [b]	32 (42.1) [c]	50 (32.7) [a, b]	18 (17.8) [a, c]
Polysome	15 (3.8)	1 (1.3)	11 (2.0)	3 (1.7)
Triploid	8 (2.1)	3 (3.9)	7 (4.6)	2 (2.0)
Monosomy	13 (3.3)	2 (2.6)	6 (3.9)	4 (4.0)
Mosaicism	12 (3.1)	4 (5.3)	5 (3.3)	2 (2.0)
Structural abnormality	18 (4.6)	4 (5.3)	14 (9.2)	5 (5.0)

Statistical analysis was performed using Chi-square test, Fisher's exact test and Bonferroni-adjusted test. Statistical significance is defined as $P < 0.008$. The data are presented as the frequency (percentage). 'a', 'b' and 'c' indicate that significant differences were detected between the two groups.
ET, embryo transfer.

Age-stratified analysis of chromosomally abnormal miscarriages

The patients were further divided into the following eight groups according to their ET strategies and age: fresh cleavage-stage ET (aged <35 years, n = 215; aged ≥35 years, n = 175), fresh blastocyst transfer (aged <35 years, n = 59; aged ≥35 years, n = 17), frozen cleavage-stage ET (aged <35 years, n = 101; aged ≥35 years, n = 52) and frozen blastocyst transfer (aged <35 years, n = 74; aged ≥35 years, n = 27): among the patients aged <35 years, the incidences of chromosomally abnormal miscarriages among the four ET protocols were 58.6%, 54.2%, 43.6% and 29.7%, respectively, and among the patients aged ≥35 years, these incidences were 76.6%, 82.4%, 82.7% and 44.4%, respectively.

In the comparisons of the frequency of chromosomal aberrations between cleavage-stage and blastocyst transfers, our results showed that there were no significant differences between the fresh cleavage-stage ET and fresh blastocyst ET in both women aged <35 years and women aged ≥35 years ($P = 0.548$ and 0.338, respectively) (Fig. 3A). Among patients aged <35 years who underwent frozen ET, the chromosomal abnormality frequencies were comparable between the cleavage-stage and blastocyst transfers ($P = 0.063$); however, among patients aged ≥35 years, the chromosomal aberration rate of frozen cleavage-stage ET was significantly higher than that of frozen blastocyst transfer (aOR: 6.997 (2.350–20.831); $P = 0.000$) (Fig. 3A).

In terms of the impact of the freeze-thaw process on chromosomal abnormalities, our results suggested that patients aged <35 years had a significantly lower frequency of chromosomal abnormalities in frozen cycles than in fresh cycles for both cleavage-stage ET and blastocyst transfer (aOR: 0.545 (0.338–0.879), $P = 0.013$; aOR: 0.357 (0.175–0.730), $P = 0.005$, respectively) (Fig. 3A). Similarly, patients aged ≥35 years who underwent blastocyst transfers had a significantly lower chromosomal abnormality rate in frozen cycles than in fresh cycles (aOR: 0.171 (0.040–0.738); $P = 0.018$) (Fig. 3A). However, among the patients aged ≥35 years who underwent cleavage-stage ET, the incidence of embryonic chromosomal aberrations was comparable between frozen ET and fresh ET ($P = 0.300$) (Fig. 3A).

Correlations between advancing maternal age and the rate of abnormal embryonic karyotype in POC

As shown in Fig. 3B, we observed constant increases in chromosomal abnormalities with maternal age in fresh cleavage-stage ET (red line, aOR: 1.099 (1.052–1.148); $P = 0.000$), fresh blastocyst transfers (blue line, aOR: 1.149 (1.020–1.294); $P = 0.022$) and frozen cleavage-stage ET (green line, aOR: 1.173 (1.093–1.260); $P = 0.000$). However, no significant association between the risk of chromosomal abnormalities and frozen blastocyst transfer (purple line, aOR: 1.067 (0.985–1.155); $P = 0.115$) was detected, which is consistent with the results indicating that the chromosomal abnormality rates of the patients aged <35 years and those aged ≥35 years who underwent frozen blastocyst transfers were comparable ($P = 0.166$) (Fig. 3A).

Distribution of abnormal embryonic karyotypes classified by different ET strategies

Trisomy was the most frequent abnormal embryonic karyotype in the four groups, and the frequency of trisomy significantly differed among the four groups; however, no significant differences in the frequencies of triploid, monosomy, polysome, mosaicism and structural abnormalities were detected among the four groups ($P > 0.05$) (Table III). In total, 41 cases of miscarriage with structural chromosomal abnormalities were observed; among these cases, duplications had a frequency of 19, 12 cases had deletions and 10 cases had complex structural abnormalities that combined deletions and duplications. Mosaicism was found in 23 cases; among these cases, 17 cases showed trisomy, three cases showed trisomy and duplication, one case showed combined deletions and duplications, one case showed monosomy and one case showed trisomy 14 combined with monosomy X. Compared with frozen ET, the most frequent trisomy in the miscarriage products from fresh ET was trisomy 16, followed by trisomy 22 and 15 (Fig. 4). Representative examples of the SNP results are shown in Fig. 5.

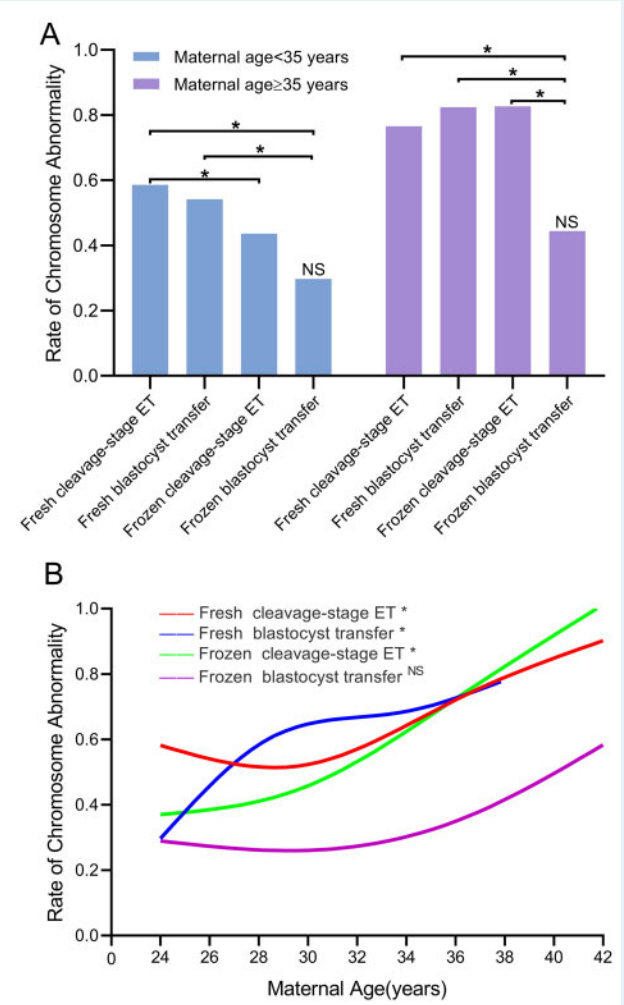

Figure 3. Comparisons of age-related changes in chromosome abnormalities among the four ET strategies. (**A**) NS indicates that no significant difference was detected between the two frozen blastocyst transfer groups, $P > 0.05$. * indicates that a significant difference was detected between the two groups, $P < 0.05$. (**B**) Associations between maternal age and chromosomal abnormalities in four embryo transfer strategies: NS indicates that no significant association was detected; $P > 0.05$; * indicates that a significant association was detected, $P < 0.05$. ET, embryo transfer.

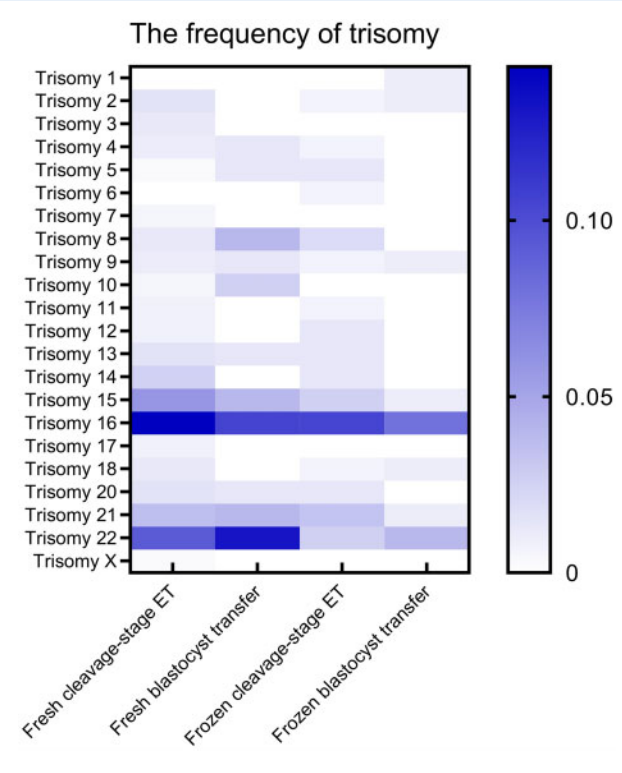

Figure 4. Distribution of trisomy occurrence with different chromosomes.

Discussion

Our study suggested that maternal age, transfer of blastocysts/cleavage-stage embryos and transfer of fresh/frozen embryos were significantly associated with the frequencies and profiles of cytogenetic abnormalities in products of early miscarriages. The transfer of frozen embryos or embryos at the blastocyst stage contributed to the decrease in chromosomally abnormal miscarriages, especially the decrease in trisomy. For patients at an advanced age, frozen blastocyst transfers were associated with a decreased frequency of abnormal karyotypes in miscarried conceptuses. This study assessed the crucial clinical factors that may be associated with the frequency of abnormal karyotypes in early miscarriages, and this information can be used to counsel patients regarding the causes of miscarriage and devise suitable treatment plans.

A previous multicenter randomized controlled trial found that frozen ET was associated with a higher rate of live births than fresh ET cycles(Chen *et al.*, 2016; Wei *et al.*, 2019), which may be related to the relatively better endometrial receptivity than that in fresh ET cycles (Shapiro *et al.*, 2011) since the hyper exposure of the endometrium to ovarian stimulation causes an alteration in gene expression and histological and structural abnormalities (Basir *et al.*, 2001; Bourgain and Devroey, 2003; Mirkin *et al.*, 2004; Horcajadas *et al.*, 2005). Interestingly, we observed a lower rate of chromosomally abnormal miscarriages in frozen ET than fresh ET, and unveiling the underlying causes may help clinicians to interpret ET strategies from a new perspective. Previous studies have indicated that a well-functioning endometrium can distinguish and exclude embryos with abnormal chromosomes better than a dysfunctional endometrium, which may lead to fewer implantations of aneuploid embryos (Teklenburg *et al.*, 2010; *et al.*, 2010; Weimar et al., 2012; Larsen *et al.*, 2013). Therefore, we speculate that an efficacious embryo selection by the endometrium in frozen ET cycles may reduce the likelihood of poor-quality embryo implantation, leading to a lower rate of chromosomally abnormal miscarriages. Further research is needed to unveil the underlying mechanisms involved in the embryo selection of the endometrium in different ET cycles. Although the impact of ART on the epigenome and adverse pregnancy outcomes is still disputed

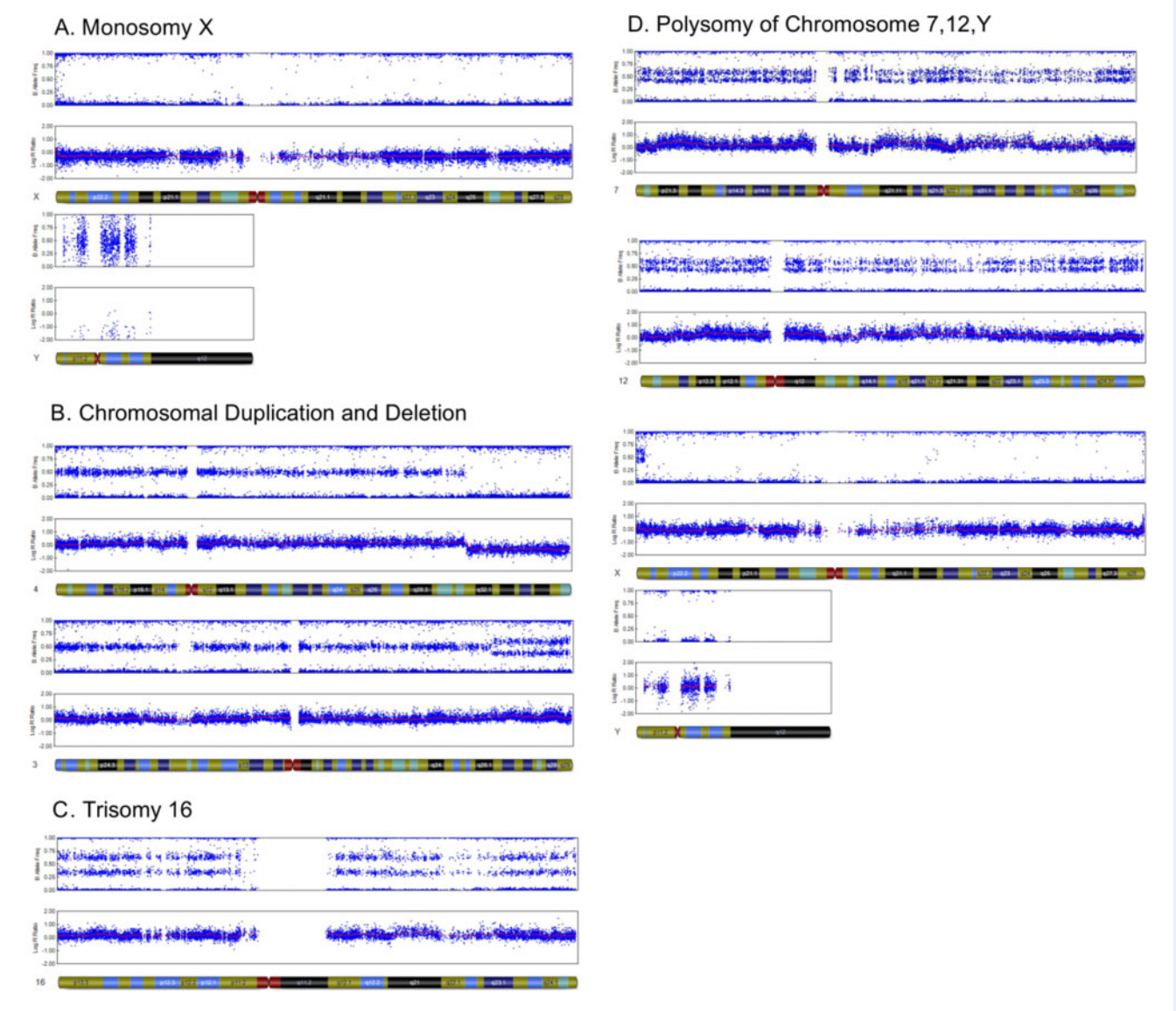

Figure 5. Representative examples of SNP results. (**A**) A monosomy of chromosome X. (**B**) Coexistence of structural duplication ranging from 3q26.1 to qter and deletion ranging from 4q31.3 to qter. (**C**) Trisomy of chromosome 16. (**D**) Polysomy involving chromosomes 7, 12 and Y simultaneously. SNP, single-nucleotide polymorphism.

(Lazaraviciute *et al.*, 2014), epigenetic mechanisms should also not be neglected in miscarried conceptuses of frozen ET. DNA methylation acting at the centromere may contribute to the maintenance of chromosomal stability (Scelfo and Fachinetti, 2019), which may be the epigenetic mechanism of chromosomally abnormal embryos *in vitro*. Human IVF/ICSI studies indicated a difference between fresh and frozen ET in placental DNA methylation levels, with DNA methylation levels of frozen ET consistent with *in vivo* conceptions (Estill *et al.*, 2016; Ghosh *et al.*, 2017), suggesting that embryo cryopreservation may temper the epigenetic alterations induced by IVF/ICSI. Unlike the miscarried conceptuses in our study, the study samples in previous studies were obtained at delivery, thus the epigenetic mechanisms that are suggested cannot be applied and deducted directly to explain our results. Further research into these associations is warranted.

In our study, blastocyst-stage ET was associated with fewer chromosomal abnormalities than cleavage-stage ET in the POC of first-trimester miscarriages; this finding may be explained by the following: compared with those in blastocyst-stage embryos, chromosomal aneuploidies and double-stranded DNA breaks are more frequent in cleavage-stage embryos because Day 2 to Day 3 embryos have less capacity to correct cell cycle errors, repair DNA damage and adjust misaligned chromosomes that occur as a consequence of challenging environments (Babariya *et al.*, 2017). Some cleavage-stage embryos with poor morphological quality or aneuploidy fail to reach the blastocyst stage during *in-vitro* culture; thus, the chromosomal instability phenotype is prevalent among cleavage-stage embryos during early embryogenesis after IVF (Vanneste *et al.*, 2009; Glujovsky *et al.*, 2016). Despite *in-vitro* culture selection, a large percentage of embryos with

chromosomal abnormalities can successfully develop into blastocysts and achieve temporarily successful implantation (Rubio et al., 2007).

Age is a generally acknowledged factor that affects aneuploidy in the embryo or miscarriage of the conceptus (Grande et al., 2012). A study carried out by Staessen demonstrated that among women younger than 36 years, the incidence of chromosomal abnormalities can be reduced from 39% in cleavage-stage embryos to 20% in blastocysts, and among women aged over 36 years, the chromosomal abnormality rates in cleavage-stage embryos and blastocyst-stage embryos are 59% and 35%, respectively (Staessen et al., 2004, 2008). In our study, age-related changes in the frequency of chromosomal abnormalities were discovered in fresh/frozen cleavage-stage ET and fresh blastocyst transfer cycles; however, in contrast to the other three ET groups and the previously acknowledged increase in the prevalence of chromosomal abnormalities with maternal age in miscarried conceptuses (Grande et al., 2012), no association between age and the chromosomal abnormality frequencies was detected in the miscarriage POC from the frozen blastocyst transfer cycles. Among the patients at an advanced age, frozen blastocyst transfer showed superiority, with the lowest frequency of chromosomally abnormal miscarriages over the other ET strategies. It seems that the transfer of frozen blastocysts can attenuate the effect of stress caused by advancing age on chromosomally abnormal miscarriages.

In our study, trisomy accounted for almost 30% of all chromosomal aberrations in POC, and chromosomal abnormality types did not appear to be random, as trisomy 16 tended to occur more frequently. Previous studies suggest that trisomy 16 is the most common trisomy leading to miscarriage, which may be related to fragile sites in the chromosome that are susceptible to breakage during embryogenesis (Benn, 1998; Babariya et al., 2017).

Underlying confounders that may be associated with chromosomally abnormal miscarriages were either excluded or adjusted by the regression model in our study. For example, 115 patients with PCOS were included in our study; studies investigating the associations between PCOS and embryonic chromosome aberrations are inconclusive, with a lower, higher and comparable aneuploidy rate reported in patients with PCOS compared to normal woman (Weghofer et al., 2007; Wang et al., 2016; Li et al., 2019). Although a lower rate of chromosomally abnormal miscarriages was observed in the patients with PCOS in our study, after multivariate logistic regression, PCOS was not a key factor influencing embryonic aneuploidy in POC.

Although the ICSI procedure has been reported to cause a higher incidence of aneuploidy than IVF (Lathi and Milki, 2004). Most studies examining the cytogenetic results of miscarriage POC following IVF and ICSI have shown that ICSI may not contribute to an increase in the aneuploidy rate in abortuses following ICSI (Ma et al., 2006; Kushnir and Frattarelli, 2009). In our study, the fertilization method (IVF and ICSI) was also found to be not significantly associated with embryonic aneuploidy in the POC, and in the subsequent data analysis, the fertilization method was not included in the regression model. The rate of chromosome aberrations in POC ranges from 50% to 85% among natural cycles and ranges from 44.8% to 61.3% in IVF/ICSI cycles (Zhang et al., 2018); no significant difference was observed in the frequency of chromosomal abnormalities in the POC from IVF/ICSI treatments and natural conceptions (Martínez et al., 2010). However, limited research has specifically compared fresh/frozen cleavage-stage embryos/blastocysts. Our study shows that the chromosomal abnormality rate in POC varied from 33.7% to 66.7% among the different ET strategies.

Strengths and limitations

Previous studies have evaluated ET strategies from the perspectives of improving endometrial receptivity and increasing the embryo implantation rate and live birth rate. We analyzed the underlying benefits of frozen blastocyst transfer from the perspective of reducing chromosomal abnormality miscarriage, which may promote comprehensive consultation and recommendations for patients. In addition, the SNP-based CMA we used for karyotype analysis is more accurate and precise than traditional G-band analysis, rendering our results more reliable. However, there are limitations in this study. First, this study was of retrospective design; thus, potential bias factors cannot be fully identified and addressed. Second, we cannot draw any definite conclusions from our results regarding the adequate safety of embryo cryopreservation in ongoing pregnancy, and the mechanism underlying the decreased frequencies of chromosomally abnormal miscarriages in frozen blastocyst transfer needs to be properly addressed. Third, a mosaicism below a certain level of 10–20% may be difficult to reveal (Cross et al., 2007).

Conclusion

The frequencies of chromosomal abnormalities in miscarried conceptuses are independently associated with fresh/frozen ET and embryo stage. The transfer of frozen blastocysts in patients undergoing IVF/ICSI treatment may attenuate the stress of advancing age on aneuploidy in embryos, thereby increasing the efficacy of ART treatment by reducing the probability of miscarriage associated with embryonic chromosomal abnormalities.

Data availability

The data underlying this article cannot be shared publicly due to the privacy of individuals that participated in the study. The data will be shared on reasonable request to the corresponding author.

Authors' roles

J.L.: study design, analysis and interpretation of data and drafting and revision of the manuscript. L.H., Q.Y., S.D., B.S. and F.Z.: data collection. H.S.: data analysis. J.X. and W.N.: chromosomal microarray analysis. Y.G.: study conception and design.

Funding

This work was supported by the Natural Science Foundation of China (Grant No.81571409), Science and Technology Research Project of Henan (Grant No. 172102310009) and Medical Science and Technology Research Project of Henan (Grant No. 201701005).

Conflict of interest

The authors declare no competing interests.

References

Babariya D, Fragouli E, Alfarawati S, Spath K, Wells D. The incidence and origin of segmental aneuploidy in human oocytes and preimplantation embryos. *Hum Reprod* 2017;**32**:2549–2560.

Basir GS, O WS, Ng EH, Ho PC. Morphometric analysis of peri-implantation endometrium in patients having excessively high oestradiol concentrations after ovarian stimulation. *Hum Reprod* 2001;**16**:435–440.

Benn P. Trisomy 16 and trisomy 16 Mosaicism: a review. *Am J Med Genet* 1998;**79**:121–133.

Bourgain C, Devroey P. The endometrium in stimulated cycles for IVF. *Hum Reprod Update* 2003;**9**:515–522.

Chen Z-J, Shi Y, Sun Y, Zhang B, Liang X, Cao Y, Yang J, Liu J, Wei D, Weng N *et al*. Fresh versus frozen embryos for infertility in the polycystic ovary syndrome. *N Engl J Med* 2016;**375**:523–533.

Conlin LK, Thiel BD, Bonnemann CG, Medne L, Ernst LM, Zackai EH, Deardorff MA, Krantz ID, Hakonarson H, Spinner NB. Mechanisms of mosaicism, chimerism and uniparental disomy identified by single nucleotide polymorphism array analysis. *Hum Mol Genet* 2010;**19**:1263–1275.

Cross J, Peters G, Wu Z, Brohede J, Hannan GN. Resolution of trisomic mosaicism in prenatal diagnosis: estimated performance of a 50K SNP microarray. *Prenat Diagn* 2007;**27**:1197–1204.

Daum H, Meiner V, Hacohen N, Zvi N, Eilat A, Drai-Hasid R, Yagel S, Zenvirt S, Frumkin A. Single-nucleotide polymorphism-based chromosomal microarray analysis provides clues and insights into disease mechanisms. *Ultrasound Obstet Gynecol* 2019;**54**:655–660.

Estill MS, Bolnick JM, Waterland RA, Bolnick AD, Diamond MP, Krawetz SA. Assisted reproductive technology alters deoxyribonucleic acid methylation profiles in bloodspots of newborn infants. *Fertil Steril* 2016;**106**:629–639.e10.

Ghosh J, Coutifaris C, Sapienza C, Mainigi M. Global DNA methylation levels are altered by modifiable clinical manipulations in assisted reproductive technologies. *Clin Epigenet* 2017;**9**:14.

Glujovsky D, Farquhar C, Quinteiro Retamar AM, Alvarez Sedo CR, Blake D. Cleavage stage versus blastocyst stage embryo transfer in assisted reproductive technology. *Cochrane Database Syst Rev* 2016;CD002118.

Grande M, Borrell A, Garcia-Posada R, Borobio V, Muñoz M, Creus M, Soler A, Sanchez A, Balasch J. The effect of maternal age on chromosomal anomaly rate and spectrum in recurrent miscarriage. *Hum Reprod* 2012;**27**:3109–3117.

Hipp H, Crawford S, Kawwass JF, Chang J, Kissin DM, Jamieson DJ. First trimester pregnancy loss after fresh and frozen in vitro fertilization cycles. *Fertil Steril* 2016;**105**:722–728.

Horcajadas JA, Riesewijk A, Polman J, van Os R, Pellicer A, Mosselman S, Simón C. Effect of controlled ovarian hyperstimulation in IVF on endometrial gene expression profiles. *Mol Hum Reprod* 2005;**11**:195–205.

Kushnir VA, Frattarelli JL. Aneuploidy in abortuses following IVF and ICSI. *J Assist Reprod Genet* 2009;**26**:93–97.

Larsen EC, Christiansen OB, Kolte AM, Macklon N. New insights into mechanisms behind miscarriage. *BMC Med* 2013;**11**:154.

Lathi RB, Milki AA. Rate of aneuploidy in miscarriages following in vitro fertilization and intracytoplasmic sperm injection. *Fertil Steril* 2004;**81**:1270–1272.

Lazaraviciute G, Kauser M, Bhattacharya S, Haggarty P, Bhattacharya S. A systematic review and meta-analysis of DNA methylation levels and imprinting disorders in children conceived by IVF/ICSI compared with children conceived spontaneously. *Hum Reprod Update* 2014;**20**:840–852.

Li Y, Wang L, Xu J, Niu W, Shi H, Hu L, Zhang Y, Zhang M, Bao X, Zhang N *et al*. Higher chromosomal aberration rate in miscarried conceptus from polycystic ovary syndrome women undergoing assisted reproductive treatment. *Fertil Steril* 2019;**111**:936–943.e2.

Ma S, Philipp T, Zhao Y, Stetten G, Robinson WP, Kalousek D. Frequency of chromosomal abnormalities in spontaneous abortions derived from intracytoplasmic sperm injection compared with those from in vitro fertilization. *Fertil Steril* 2006;**85**:236–239.

Martínez MC, Méndez C, Ferro J, Nicolás M, Serra V, Landeras J. Cytogenetic analysis of early nonviable pregnancies after assisted reproduction treatment. *Fertil Steril* 2010;**93**:289–292.

Meldrum DR, Casper RF, Diez-Juan A, Simon C, Domar AD, Frydman R. Aging and the environment affect gamete and embryo potential: can we intervene? *Fertil Steril* 2016;**105**:548–559.

Mirkin S, Nikas G, Hsiu J-G, Díaz J, Oehninger S. Gene expression profiles and structural/functional features of the peri-implantation endometrium in natural and gonadotropin-stimulated cycles. *J Clin Endocrinol Metab* 2004;**89**:5742–5752.

Ozawa N, Ogawa K, Sasaki A, Mitsui M, Wada S, Sago H. Maternal age, history of miscarriage, and embryonic/fetal size are associated with cytogenetic results of spontaneous early miscarriages. *J Assist Reprod Genet* 2019;**36**:749–757.

Pylyp LY, Spynenko LO, Verhoglyad NV, Mishenko AO, Mykytenko DO, Zukin VD. Chromosomal abnormalities in products of conception of first-trimester miscarriages detected by conventional cytogenetic analysis: a review of 1000 cases. *J Assist Reprod Genet* 2018;**35**:265–271.

Roque M, Haahr T, Geber S, Esteves SC, Humaidan P. Fresh versus elective frozen embryo transfer in IVF/ICSI cycles: a systematic review and meta-analysis of reproductive outcomes. *Hum Reprod Update* 2019;**25**:2–14.

Rubio C, Rodrigo L, Mercader A, Mateu E, Buendía P, Pehlivan T, Viloria T, De los Santos MJ, Simón C, Remohí J *et al*. Impact of chromosomal abnormalities on preimplantation embryo development. *Prenat Diagn* 2007;**27**:748–756.

Scelfo A, Fachinetti D. Keeping the centromere under control: a promising role for DNA methylation. *Cells* 2019;**8**:912.

Shah MS, Cinnioglu C, Maisenbacher M, Comstock I, Kort J, Lathi RB. Comparison of cytogenetics and molecular karyotyping for chromosome testing of miscarriage specimens. *Fertil Steril* 2017;**107**:1028–1033.

Shapiro BS, Daneshmand ST, Garner FC, Aguirre M, Hudson C, Thomas S. Evidence of impaired endometrial receptivity after ovarian stimulation for in vitro fertilization: a prospective randomized trial comparing fresh and frozen-thawed embryo transfer in normal responders. *Fertil Steril* 2011;**96**:344–348.

Shi Y, Sun Y, Hao C, Zhang H, Wei D, Zhang Y, Zhu Y, Deng X, Qi X, Li H *et al*. Transfer of fresh versus frozen embryos in ovulatory women. *N Engl J Med* 2018;**378**:126–136.

Staessen C, Platteau P, Van Assche E, Michiels A, Tournaye H, Camus M, Devroey P, Liebaers I, Van Steirteghem A. Comparison of blastocyst transfer with or without preimplantation genetic diagnosis for aneuploidy screening in couples with advanced maternal age: a prospective randomized controlled trial. *Hum Reprod* 2004;**19**:2849–2858.

Staessen C, Verpoest W, Donoso P, Haentjens P, Van der Elst J, Liebaers I, Devroey P. Preimplantation genetic screening does not improve delivery rate in women under the age of 36 following single-embryo transfer. *Hum Reprod* 2008;**23**:2818–2825.

Teklenburg G, Salker M, Heijnen C, Macklon NS, Brosens JJ. The molecular basis of recurrent pregnancy loss: impaired natural embryo selection. *Mol Hum Reprod* 2010;**16**:886–895.

Teklenburg G, Salker M, Molokhia M, Lavery S, Trew G, Aojanepong T, Mardon HJ, Lokugamage AU, Rai R, Landles C *et al*. Natural selection of human embryos: decidualizing endometrial stromal cells serve as sensors of embryo quality upon implantation. *PLoS One* 2010;**5**:e10258.

Vanneste E, Voet T, Le Caignec C, Ampe M, Konings P, Melotte C, Debrock S, Amyere M, Vikkula M, Schuit F *et al*. Chromosome instability is common in human cleavage-stage embryos. *Nat Med* 2009;**15**:577–583.

Wang Q, Luo L, Lei Q, Lin M-M, Huang X, Chen M-H, Zeng Y-H, Zhou C-Q. Low aneuploidy rate in early pregnancy loss abortuses from patients with polycystic ovary syndrome. *Reprod Biomed Online* 2016;**33**:85–92.

Weghofer A, Munne S, Chen S, Barad D, Gleicher N. Lack of association between polycystic ovary syndrome and embryonic aneuploidy. *Fertil Steril* 2007;**88**:900–905.

Wei D, Liu J-Y, Sun Y, Shi Y, Zhang B, Liu J-Q, Tan J, Liang X, Cao Y, Wang Z *et al*. Frozen versus fresh single blastocyst transfer in ovulatory women: a multicentre, randomised controlled trial. *Lancet* 2019;**393**:1310–1318.

Weimar CHE, Kavelaars A, Brosens JJ, Gellersen B, de Vreeden-Elbertse JMT, Heijnen CJ, Macklon NS. Endometrial stromal cells of women with recurrent miscarriage fail to discriminate between high- and low-quality human embryos. *PLoS One* 2012;**7**:e41424.

Zhang T, Sun Y, Chen Z, Li T. Traditional and molecular chromosomal abnormality analysis of products of conception in spontaneous and recurrent miscarriage. *BJOG: Int J Obstet Gy* 2018;**125**:414–420.