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Comparison of two protocols of blastocyst biopsy submitted to preimplantation genetic testing for aneuploidies: a randomized controlled trial

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Abstract

Purpose To compare the effectiveness of two protocols of blastocyst biopsy submitted to preimplantation genetic testing for aneuploidies (PGT-A).

Methods This is a randomized controlled trial of a cohort of 221 patients undergoing PGT-A. 106 female patients aged ≤ 40 years with no less than 8 mature oocytes retrieved and ≥ 3 good-quality embryos on day 3 were randomly assigned to the day-3 hatching-based TE biopsy. The remaining 115 females aged ≤ 40 years with ≥ 8 MII oocytes obtained and no less than 3 high-quality embryos on day 3 were assigned to the TE biopsy without hatching group (also called the new biopsy group). The primary outcome was measured by a live birth after the first embryo transfer.

Results The live birth rate did not differ significantly between the two groups (50.00% vs. 59.26%, $P > 0.05$, OR 1.46; 95% CI 0.78–2.70). There was no significant between-group difference in the rates of implantation, clinical pregnancy, and miscarriage. However, the frozen blastocyst rate was significantly lower in the day-3 hatching-based TE biopsy compared with the new biopsy group (47.54% vs. 53.96%, $P < 0.05$, OR 1.29; 95% CI 1.08–1.56).

Conclusions Our study provides strong evidence that the new blastocyst biopsy method exhibits advantages over day-3 hatching-based TE biopsy method. Using this method, we were able to obtain more blastocysts to perform trophectoderm biopsy in patients subjected to PGT-A.

Keywords Preimplantation genetic testing for aneuploidies (PGT-A) · Blastocyst biopsy · Embryonic aneuploidy · Next-generation sequencing · Frozen embryo transfer

Introduction

The ultimate goal of assisted reproductive technology (ART) is to select the embryo with the higher implantation potential to obtain a healthy newborn. Currently, embryo selection is based mainly on morphological criteria, including pronuclear morphology, cleavage rates, the evenness of blastomeres, and the degree of fragmentation [1, 2], although time-lapse technology has also been introduced as a new embryo-choosing strategy. However, it is a very subjective and low-efficiency assessment strategy. Centers for Disease Control and Prevention reported that only 19% of transferred embryos resulted in successful delivery [3]. What causes a morphologically normal embryo's failure to implant and prevents it from developing into a healthy live birth? The principal reason is aneuploidy. Embryonic aneuploidy is prevalent in IVF cycles, leading to low implantation rates, repeated IVF failures, and early pregnancy loss [4]. There is a weak correlation between the morphological criteria and the chromosomal status of the embryos; even the embryos with the highest morphological scores retain a significant risk of aneuploidy [5, 6]. Consequently, selecting euploid embryos to transfer without affecting their implantation potential is of crucial importance.

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The initial strategy for screening euploid embryos is fluorescence in situ hybridization (FISH). However, the clinical outcomes of this technique are disappointing. It has been demonstrated that one or two blastomeres from day-3 embryo biopsy resulted in a negative effect and lower delivery rates as compared to non-biopsied embryos by FISH [7–9]. In addition, only a partial karyotype can be evaluated with FISH technology.

Another euploid selection strategy is to perform a trophectoderm biopsy at the blastocyst stage, which is widely applied in PGT-A with indications of advanced maternal age, recurrent implantation failure, repeated spontaneous abortions, and severe male factor infertility [10–12]. This strategy has been shown to produce higher singleton live birth rates in a retrospective study and a prospective study in young, normal-prognosis patients [5, 13].

At present, there are two protocols of blastocyst biopsy that differ by the time of zona opening before biopsy. The first method requires a creation of a hole of about 5 μm by laser on day-3 cleavage embryos to make trophectoderm cells herniate through the hole for biopsy [14]. The second is to open the zona pellucida instantly before biopsy on day-5 or -6 blastocysts [15, 16]. To the author's knowledge, there is a lack of certainty about which of these is optimal. Hence, we designed a randomized, controlled trial to compare the two protocols of blastocyst biopsy submitted to PGT-A.

Materials and methods

Study design

The study was performed at the Center for Reproductive Medicine, Shandong University. From November 2015 to July 2016, 236 women who underwent PGT-A signed informed consent for possible randomization into this study. Inclusion criteria were: female age ≤ 40 years, ≥ 8 mature oocytes, and no less than 3 good-quality embryos on day 3 according to Puissant's criteria [17]. An embryologist, except for the operators, was specialized for generating random allocation sequence by Excel and assigning the participants on day 3 of embryo development. The participants were unknown to the group they were assigned. The subject selection process was detailed in Fig. 1. The primary outcome of the study was measured by the live birth rate, the secondary outcome measures referred to the implantation rate, clinical pregnancy rate, and miscarriage rate after the first embryo transfer, and the exploratory outcome was evaluated by the frozen blastocyst rate. This study was reviewed and approved by the Institutional Review Board of Reproductive Medicine, Shandong University.

Ovarian stimulation, ICSI, embryo culture, and grading

There were no restrictions concerning the ovarian stimulation protocols. The ovarian stimulation and oocyte collection were performed as previously described [18]. All the patients in the study underwent intracytoplasmic sperm injection (ICSI) due to the possibility of genetic testing. ICSI was carried out under an inverted microscope (Nikon, Japan, Eclipse Ti-U). Sequential culture media supplied by Vitrolife (G-IVF, G1 and G2) (Scandinavicn IVF Science, Sweden; 10135, 10127 and 10131) was used for all procedures. Sequentially cultured embryos on day 3 after oocyte retrieval included not only 2PN cleavage embryos but also 1PN and 0PN embryos on day 3 containing ≥ 4 cells with a grade 2 and over according to Puissant's criteria [17]. Blastocyst quality was evaluated on day 5 and 6 based on the criteria by Gardner and Lane [19]. Two equally skilled embryologists took turns at performing embryo grading and trophectoderm biopsy weekly to reduce the cases of discordance between them.

Blastocyst biopsy procedure

In this study, all the blastocysts assessed 4BC and better underwent trophectoderm biopsy. For the day-3 hatching-based TE biopsy group, a 5-μm opening was made by laser (RI, England, Saturn Active) before transferring the embryos into extended culture on the afternoon of day 3 [20]. This allowed some trophectoderm cells to herniate through the hole before biopsy on day 5 or 6. For the new biopsy group, the zona pellucida was opened immediately before biopsy on day 5 or 6 [21–23]. Approximately 3–5 trophoblast cells were sucked and removed from biopsied blastocysts by laser-mediated drilling, then the trophoblast cells were rinsed for three times in 1% PVP (Scandinavicn IVF Science, Sweden, 10111), after which the cells were enclosed into PCR tubes containing 2 μl phosphate-buffered saline (PBS) (Solarbio, America, P1020). Following whole-genome amplification (WGA) of the trophoblast cells, PGT was carried out in the DA8600 sequencing platform (DA AN GENE, China, DA8600) according to the manufacturer's protocol. A mosaic embryo was defined as a chromosomal ratio level exceeding 20% of the aneuploid cells in this study. In addition, the biopsied cells were tested in batches, typically a few weeks later. For consistency, all blastocyst biopsy procedures were carried out by the two equally skilled embryologists.

Blastocyst vitrification, warming, and embryo transfer

Vitrification was performed using the Mukaida protocol with cryoloop [24]. The base medium contained 5 mg/ml

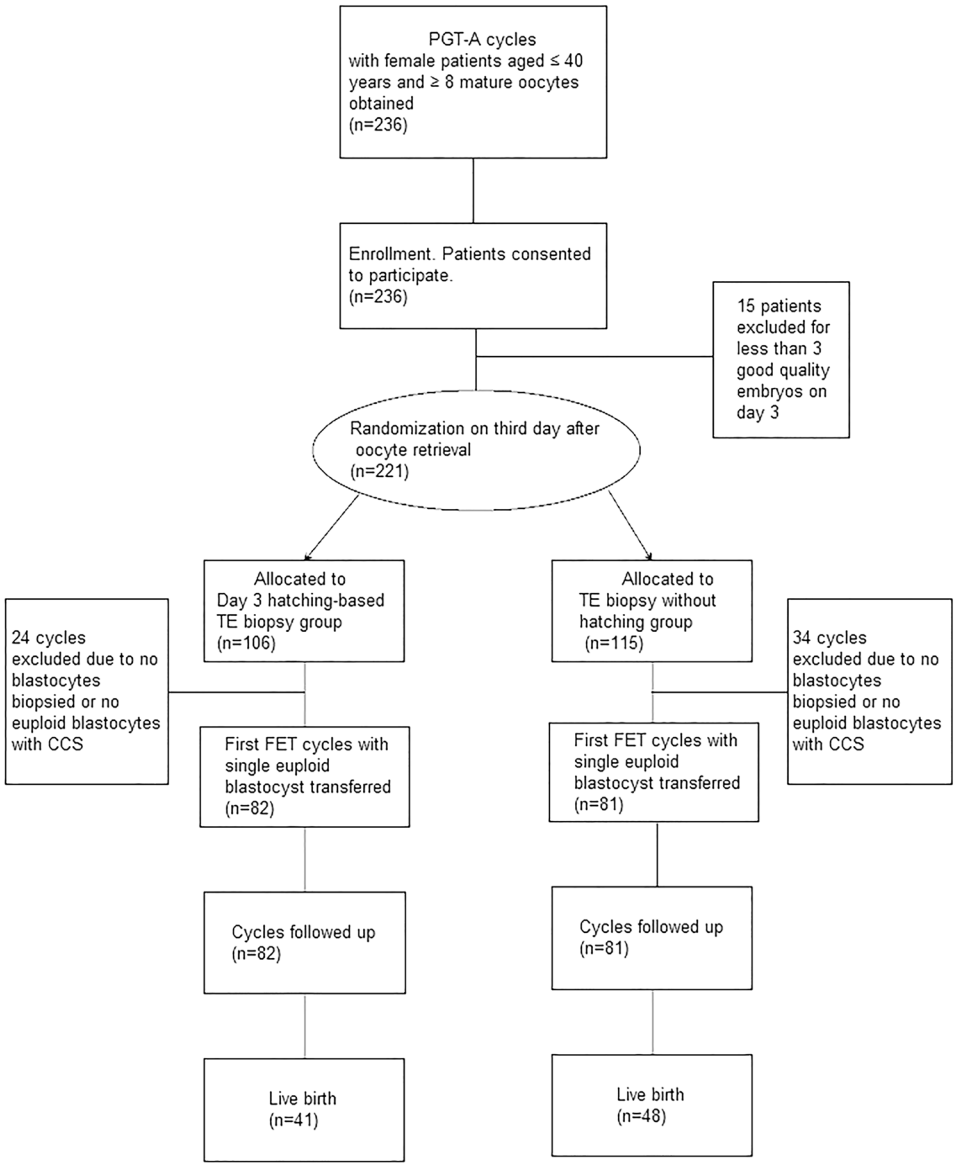

Figure 1 Flow of patients through the trial

HSA in HEPES-buffered modified hTF medium. Initially, blastocysts were put into the base medium, then the blastocysts were transferred into the base medium containing 7.5% (v/v) DMSO and 7.5% (v/v) EG (vitrification solution I), after 2 min the blastocysts were suspended in the base medium containing 15% (v/v) DMSO and 15% (v/v) EG, 10 mg/ml Ficoll 70 (Pharmacia Biotech, Sweden, CB9248463) and 0.65 mol/l sucrose (vitrification solution II) for 30 s before being quickly plunged into liquid nitrogen. Warming was carried out in a four-well multidish using the Mukaida protocol. Briefly, blastocysts were incubated in base medium containing 0.33 mol/l sucrose (thawing solution I), base medium containing 0.2 mol/l sucrose (thawing solution II), and base medium for 2 min, 3 min, and 5 min at 37℃, respectively.

All the patients recruited in the study underwent frozen embryo transfer. According to PGT results, only euploid blastocysts could be transferred. Vitrified euploid blastocysts were thawed on the morning of transfer day and hatching was assisted by laser with pulse length of 0.180 mS, applied for the thawed blastocysts 30 min after thawing. The blastocysts were incubated for 4–5 h before transfer, and only the expanded blastocysts were transferred. Twelve days of luteal support was routinely provided after FET irrespective of pregnancy. For patients with ongoing pregnancy, progesterone was continued for 10 weeks of gestation, and after confirming the presence of the fetal heart, the dosage was gradually reduced.

Diagnosis of pregnancy

The "implantation" was defined as the number of cardiac activities observed 7 weeks after transfer in this study. Observation of cardiac activity 7 weeks after transfer was regarded as a clinical pregnancy. Miscarriage was defined as a pregnancy loss at < 20–28 weeks of gestation.

Statistical analysis

The software used for statistical analysis was SPSS version 16.0. The Student's *t* distribution and χ^2 test, or Fisher's exact test, were used to analyze the mean and proportional values. A *P* value of < 0.05 was considered statistically significant.

Results

Baseline characteristics

The baseline characteristics were compared between the day-3 hatching-based TE biopsy group with 82 subjects and the new biopsy group involving 81 patients. As Table 1 indicates, the two groups were equivalent as evidenced by any of the examined baseline characteristics. Furthermore, the survival and expanding rates after vitrification-warming were 100% in both groups (Table 2).

Live birth rate and secondary outcomes

As shown in Table 3, the live birth rate in the day-3 hatching-based TE biopsy group was similar with that in the TE biopsy without hatching group (50.00% vs. 59.26%, $P > 0.05$, OR 1.46; 95% CI 0.78–2.70). In addition, there was no significant difference in the implantation, clinical pregnancy, and miscarriage rates between the two groups [(58.54% vs. 65.43%, $P > 0.05$) (57.32% vs. 64.20%, $P > 0.05$, OR 1.25; 95% CI 0.67–2.33) (12.77% vs. 7.69%, $P > 0.05$, OR 0.57; 95% CI 0.15–2.1), respectively].

The exploratory outcomes

As shown in Table 2, the two groups were similar in the mean number of PGT-A cycles, the oocytes obtained and

Table 1 The demographics of day-3 hatching-based TE biopsy and TE biopsy without hatching groups

	Hatching ($n = 82$)	Without hatching ($n = 81$)	*P* value
Female age (years) (± SD)	30.72 ± 3.89	30.73 ± 3.86	0.99[a]
BMI (kg/m^2)	24.07 ± 3.42	23.31 ± 3.63	0.17[a]
Years of infertility	2.53 ± 2.12	2.77 ± 2.46	0.51[a]
Primary infertility (%)	28.05% (23/82)	30.86% (25/81)	0.69[b]
Basal serum FSH (IU/l)	6.17 ± 1.25	6.18 ± 1.46	0.95[a]
AMH (ng/ml)	6.35 ± 4.38	6.07 ± 3.69	0.65[a]
E2 on hCG trigger day (pg/ml)	4460.53 ± 2107.02	4871.36 ± 2135.72	0.22[a]
Indications			
AMA (%)	13.41% (11/82)	12.35% (10/81)	0.84[b]
RIF (%)	31.71% (26/82)	20.99% (17/81)	0.12[b]
RPL (%)	26.83% (22/82)	34.57% (28/81)	0.28[b]
Others (%)	28.05% (23/82)	32.10% (26/81)	0.57[b]
Stimulation protocols			
Long protocol (%)	69.51% (57/82)	67.90% (55/81)	0.82[b]
Short protocol (%)	10.98% (9/82)	17.28% (14/81)	0.25[b]
GnRH-antagonist protocol (%)	17.07% (14/82)	14.81% (12/81)	0.69[b]
Sperm analysis parameters			
Volume	3.91 ± 1.53	3.55 ± 1.32	0.11[a]
Concentration (× 10^6/ml)	48.49 ± 35.72	48.48 ± 38.75	0.99[a]
Rapid forward motility	38.81% ± 17.30%	43.30% ± 16.79%	0.10[a]
Normal forms	4.66% ± 2.74%	4.54% ± 2.65%	0.78[a]
Endometrial thickness (cm)	0.90 ± 0.14	0.93 ± 0.16	0.26[a]

Values are presented as number (%) or mean ± SD

AMA advanced maternal age, *RIF* repeated IVF failures, *RPL* recurrent spontaneous pregnancy loss, *Others* chromosome abnormalities or combined the above

[a] *t* test

[b] χ^2 test or Fisher's exact test

Table 2 Embryology laboratory data of day-3 hatching-based TE biopsy and TE biopsy without hatching groups

	Hatching ($n=82$)	Without hatching (n=81)	*P* value
Mean number of PGS cycle	1.33 ± 0.57	1.41 ± 0.80	0.47[a]
Mean number of oocytes retrieved	16.38 ± 7.18	14.77 ± 5.47	0.11[a]
Mean number of MII oocytes	14.71 ± 7.06	13.16 ± 4.55	0.10[a]
2PN oocytes (%)	78.34% (951/1214)	78.85% (839/1064)	0.76[b]
Good-quality embryos on day 3 (%)	57.52% (547/951)	58.76% (493/839)	0.60[b]
Sequentially cultured embryos (%)	80.40% (976/1214)	79.42% (845/1064)	0.56[b]
Frozen blastocysts (%)	47.54% (464/976)	53.96% (456/845)	0.006[b]
Mean number of embryos transferred	1	1	1[a]
Day-5 blastocysts transferred (%)	81.71% (67/82)	79.01%(64/81)	0.66[b]
Morphologic score of embryos transferred			
≥ 4BA (%)	37.80% (31/82)	48.15% (39/81)	0.18[b]
≤ 4BB (%)	62.20% (51/82)	51.85% (42/81)	0.18[b]
Survival rate (%)	100.00% (82/82)	100.00% (81/81)	1[b]

Values are presented as number (%) or mean ± SD

[a] *t* test

[b] χ^2 test or Fisher's exact test

Table 3 Clinical outcomes of day-3 hatching-based TE biopsy and TE biopsy without hatching groups

	Hatching ($n=82$)	Without hatching ($n=81$)	*P* value	OR (95% CI)
Implantation (%)	58.54% (48/82)	65.43% (53/81)	0.36[a]	–
Clinical pregnancy (%)	57.32% (47/82)	64.20% (52/81)	0.37[a]	1.25 (0.67–1.33)
Miscarriage (%)	12.77% (6/47)	7.69% (4/52)	0.40[a]	0.57 (0.15–2.10)
Live birth (%)	50.00% (41/82)	59.26% (48/81)	0.24[a]	1.46 (0.78–2.70)

[a] χ^2 test or Fisher's exact test

Table 4 PGS results of day-3 hatching-based TE biopsy and TE biopsy without hatching groups

	Hatching ($n=82$)	Without hatching ($n=81$)	*P* value
Euploid blastocyst (%)	44.83% (208/464)	49.78% (227/456)	0.13[a]
Aneuploid blastocyst (%)	54.09% (251/464)	49.12% (224/456)	0.13[a]
Mosaic blastocyst (%)	0.86% (4/464)	1.10% (5/456)	0.72[a]
No-results blastocyst (%)	0.22% (1/464)	0.00% (0/456)	0.32[a]

[a] χ^2 test or Fisher's exact test

mature oocytes, the rates of 2PN oocytes, good-quality embryos on day 3, and sequentially cultured embryos. However, the rate of frozen blastocyst was markedly lower in the day-3 hatching-based TE biopsy (47.54% vs. 53.96%, $P<0.05$, OR 1.29; 95% CI 1.08–1.56) (Table 2). In addition, as Table 4 indicates, the rates of euploid, aneuploid, mosaic, and no-results blastocysts did not differ between the two groups.

To investigate the clinical outcomes of single euploid blastocyst transfers from different morphological grades, 48 cycles were employed from the two groups, including 40 cycles transferred with one-euploid blastocyst graded 4AA and 8 cycles transferred with 4BC. Table 5 illustrates that the implantation, clinical pregnancy, live birth, and miscarriage rates did not differ between the two groups (57.50% vs. 75.00%, $P>0.05$; 55.00% vs. 75.00%, $P>0.05$; 50.00% vs. 75.00%, $P>0.05$; 9.09% vs. 0.00%, $P>0.05$).

Discussion

This study provides evidence that the zona pellucida should be opened immediately before trophectoderm biopsy on day 5 or 6, rather than on day 3. Although the clinical pregnancy, live birth, and miscarriage rates were similar between the two protocols, the rate of frozen blastocysts was significantly lower in the day-3 hatching-based TE biopsy group. Our explanation for this difference is that opening the zona on day 3 may have a negative effect on embryo development from the cleavage stage to the blastocyst stage by extending

Table 5 Clinical outcomes of FETs with a single 4AA or 4BC euploid blastocyst transferred

	4AA	4BC	*P* value
Implantation (%)	57.50% (23/40)	75.00% (6/8)	0.36[a]
Clinical pregnancy (%)	55.00% (22/40)	75.00% (6/8)	0.29[a]
Miscarriage (%)	9.09% (2/22)	0.00% (0/6)	0.44[a]
Live birth (%)	50.00% (20/40)	75.00% (6/8)	0.20[a]

[a]χ^2 test or Fisher's exact test

the time of in vitro manipulation, which may lower the embryo potential compared with TE biopsy without hatching protocol. With the new approach, inner cell mass (ICM) can be precisely located to the opposite side of the zona hole rather than anywhere with the day-3 hatching-based TE biopsy protocol, thus avoiding potential injury to the ICM. This result is in agreement with a recent retrospective study by Capalbo et al., which revealed that the new method for trophectoderm biopsy was more convenient than the day-3 hatching-based TE biopsy method [21]. Furthermore, there were only a few trophoblast cells herniated after day-3 hatching, causing inconvenience to the biopsy operation. The presence of a hole in the zona and the subsequent precocious hatching of the trophoblast cells prior to their full expansion with the day-3 hatching-based method might have an negative effect on a proper grading of the ICM and TE quality, which was also a bias involved in this study. To the best of our knowledge, this study is the first randomized controlled trial to specifically compare the different trophectoderm biopsy protocols. The results of our trial add to the existing body of evidence and provide a scientific basis for the extensive application of the new blastocyst biopsy method.

We aimed to discern whether the lower rate of frozen blastocysts in the day-3 hatching-based TE biopsy group resulted in less and/or poorer quality embryos on day 3, compared to the TE biopsy without hatching group. We compared the rates of 2PN oocytes, good-quality embryos on day 3, and sequentially cultured embryos between the two groups and found that they were similar. For consistency, cleavage embryo and blastocyst grading in the two groups was carried out by the same two senior embryologists. Given all of this, we believe that the decreased frozen blastocyst rate in the day 3 group is attributable to the zona-opening procedure by laser on day 3.

In this study, we have recorded that the clinical outcome of transferring a high-quality euploid blastocyst was equivalent to that of transferring a lower quality euploid balstocyst. This implies that blastocyst morphology was not associated with its viability if the blastocyst was euploid. Low-quality embryos should therefore be included as performing trophectoderm biopsy. This would support Capalbo's study, which demonstrated that low-quality euploid blastocysts could obtain the same ongoing implantation rate (45.2%) as that of blastocysts of high morphological quality (51.4%) [21]. Additionally, our results also agree with several independent studies, which demonstrated that morphologically normal blastocysts retain a significant risk of aneuploidy and that some euploid embryos have poor morphology [5, 6, 25].

Routinely, the blastocysts assessed as 4CB on day 5 are incubated up to day 6 in our lab, if the ICM improves (with a grade ≥ B) on day 6, we will perform trophectoderm biopsy in PGT-A cycles, otherwise, the blastocyst will be given up. The exclusion of these blastocysts from trophectoderm biopsy would elicit a bias of the study, since the priority of ICM and/or TE remains controversial. Some scientific societies such as the Spanish ASEBIR reported that the ICM is less (or even not) significant compared to the TE in evaluating blastocyst's implantation potential. On the contrary, Irani et al. found ICM grade is a better predictor of implantation than other components of embryo grading [26].

The current study also has two limitations. The first one is the relatively small sample size of the 4BC grade blastocyst transfer group compared with that of the 4AA group. The second one is that the other two protocols of blastocyst biopsy, the day 5/6 hatching-based method and the morula stage hatching-based method, were not included in this study; a more comprehensive study will be required to confirm our results in the future.

In conclusion, the results of this study offer strong evidence that the new blastocyst biopsy protocol exhibits advantages over the day-3 hatching-based TE biopsy protocol. The new method allows for the acquisition of more blastocysts to perform trophectoderm biopsy on PGT-A patients. Further studies will be needed to confirm and elaborate on our conclusions.

Author contributions LH: data management/analysis, manuscript writing/editing, ZHB: data management/analysis, manuscript writing/editing, YGL: data collection, LM: manuscript editing, MSY: manuscript editing, ZHZ: data collection, WKL: study design, manuscript writing/editing.

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Compliance with ethical standards

Conflict of interest All the authors declare no conflict of interest with respect to the authorship and/or publication of this article.

Ethical approval All procedures performed in this study involving human participants were in accordance with the ethical standards of the Reproductive Medicine, Shandong University Research Ethics Com-

mittee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in this study.

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