Chapter

Next-Generation Sequencing Revealed that High Proportion of Human Embryos Resulted from Donor Eggs Are Segmental Chromosome Abnormal

Xiangli Niu, Yanping Lao, Yan Sun and Weihua Wang

Abstract

High proportion of human embryos produced by in vitro fertilization (IVF) are aneuploidy or have segmental chromosomal errors. Not only a whole chromosome aneuploidy, but also small errors in a chromosome, such as microdeletion can be detected by current next-generation sequencing (NGS) for preimplantation genetic testing (PGT). The prevalence of aneuploidy in donor egg IVF was significantly different between fertility clinics. In the present study, we examined whether different embryo biopsy procedures affect embryonic aneuploidies in donor egg IVF. We did not find significant differences in the samples with abnormal chromosomes between two biopsy methods. When we further analyzed the samples with abnormal chromosomes, we found that 64.0–80.7% of the abnormalities were whole chromosome aneuploidies while 19.3–36.0% were segmental chromosome abnormalities. High embryo implantation rates were obtained after transferring screened euploid blastocysts. These results indicate that blastocyst biopsy procedures may not significantly affect embryo's chromosomal status, but PGT by high-resolution NGS revealed that high proportions of human embryos derived from donor eggs are not only aneuploidy, but also segmental chromosome abnormal, and screening of small chromosomal errors by NGS is beneficial to patients who use donated eggs for infertility treatment.

Keywords: aneuploidy, blastocyst, donor eggs, human, in vitro fertilization, preimplantation genetic testing, segmental chromosome abnormalities

1. Introduction

Embryonic aneuploidy in human in vitro fertilization (IVF) is very common and is one of the factors reducing embryo implantation and causing birth defects. Although aneuploidy is mainly observed in the embryos from patients with advanced maternal ages [1–3], it is also very common in the embryos from young patients and oocyte donors [4–9]. The frequencies of aneuploid embryos produced by IVF have been widely studied [4–12] by examination of all chromosomes with microarray and next generation of sequencing (NGS) through preimplantation genetic testing for

aneuploidies (PGT-A) [13–17]. With PGT-A by NGS, not only a whole chromosome aneuploidy can be detected, but also segmental chromosome abnormalities (deletion and duplication) can be detected [18–21]. Segmental chromosome abnormalities typically represent regional losses or gains in one or more chromosomes. The size of a segmental abnormalities detectable by current NGS platforms is as small as 1 Mb, however, for PGT-A, usually 10 Mb and above are detected and reported.

Some segmental chromosome abnormalities may cause miscarriage and birth defect, while others may result in developmental delay and/or intellectual disability if the transfer of such embryos produce live birth. It has been found that the prevalence of embryonic aneuploidy in donor egg IVF was significantly different between fertility clinics indicating that clinical and laboratory procedures may be related to the occurrence of embryonic aneuploidies [12]. Embryo biopsy is a complicated and invasive laboratory procedure that involves several embryo manipulations during culture, so it may affect embryo's quality including aneuploidies. Therefore, in the present study, to examine whether embryo biopsy procedures affect embryonic aneuploidies in donor egg IVF, blastocysts were biopsied by two different biopsy methods and then the samples were examined by NGS. Collected data were analyzed in terms of the rates of embryos with whole chromosome aneuploidies and segmental chromosome abnormalities. Clinical outcomes, such as pregnant rate, live birth rate and embryo implantation rate were also analyzed.

2. Materials and methods

2.1 Ethical statement

The patients signed the consents for all laboratory and clinical procedures including embryo biopsy for PGT-A. The data was collected from medical records at the clinic and laboratory, and the study with PGT-A was approved by New England Institutional Review Board (NEIRB 14–504).

2.2 Donor stimulation

Donors for IVF treatment underwent controlled ovarian stimulation with a combination of daily injection of 75–300 IU recombinant follicle-stimulating hormone (Gonal-F, EMD Serono, MA, USA) and 75–300 IU of a combination of follicle stimulating hormone and luteinizing hormone (Menopur, Ferring Pharmaceuticals, NJ, USA). On day 5–7, 0.25 mg gonadotropin releasing hormone antagonist (Cetrotide, EMD Serono) was given daily until triggering for oocyte maturation by gonadotropin-releasing hormone agonist (Lupron) or human chorionic gonadotropin (hCG). Oocytes were retrieved at 35–36 hours after the trigger and then cultured in Global™Total medium (Origio Inc., CT, USA) at 37°C in an atmosphere of 5.5% CO_2, 6% O_2, and balanced N_2 under humidified or dry conditions.

2.3 Oocyte insemination and embryo culture

Oocytes were inseminated by intracytoplasmic sperm injection (ICSI) after cumulus cells were removed by using hyaluronidase (Fujifilm-Irvine Scientific) at 3–4 hours after oocyte retrieval and metaphase II oocytes were injected 4–5 hours after retrieval. After insemination, oocytes were cultured in Global™Total medium at 37°C in an atmosphere of 5.5% CO_2, 6% O_2, and balanced N_2 under humidified or dry conditions.

Fertilization was assessed 16–18 hours after insemination, and normal fertilization was characterized by two distinct pronuclei and two polar bodies. Fertilized

oocytes were further cultured in the Global™Total medium and embryo quality was evaluated by an inverted microscope on day 3, 5, or 6.

2.4 Blastocyst biopsy

Two biopsy methods were used in the present study. The first is a traditional two-step method in which a small hole in zona pellucida was opened by laser pulses on cleavage embryos at Day 3. As shown in **Figure 1**, when embryo developed to blastocyst at day 5 or later and some cells from blastocysts hatched from the hole, 5–10 cells were aspirated to a biopsy pipette and then cells were separated from blastocyst proper by mechanical pulling and laser pulses.

The second is a modified and simplified one-step method with less embryo manipulation and less laser application. Hole opening in the zona pellucida was not performed on day 3 embryos. Blastocysts were directly processed for biopsy. The details for this method are as the follows: As shown in **Figure 2**, blastocyst for biopsy was held to a proper position (**Figure 2A**) in which inner cell mass (ICM) was on the 6–9 O'clock position, a small hole in the zona pellucida was opened (**Figure 2B**) on the 3 O'clock position by one laser pulse with the ZILOS-tk™ laser system (Hamilton Thorn Bioscience Inc., MA, USA). A 20 μm polished biopsy pipette (Sunlight Medical, Jacksonville, FL, USA) was inserted to inside zona pellucida through the hole and a few trophectoderm cells on the 12–2 O'clock position were aspired into biopsy pipette (**Figure 2B**). After biopsy pipette was pulled out of the zona (**Figure 2C**), biopsy pipette together with blastocyst was moved to the top the holding pipette (**Figure 2D**), and the biopsy pipette was pull down against the holding pipette so that the cells inside the biopsy pipette were completely separated from blastocyst proper (**Figure 2E**).

After biopsy, biopsied cells were washed individually, transferred to PCR tubes, and stored at −20°C freezer until processing for PGT-A by commercial genetic testing company. Blastocysts were individually cryopreserved by vitrification for later frozen embryo transfer (FET). Blastocysts were classified as abnormal if they

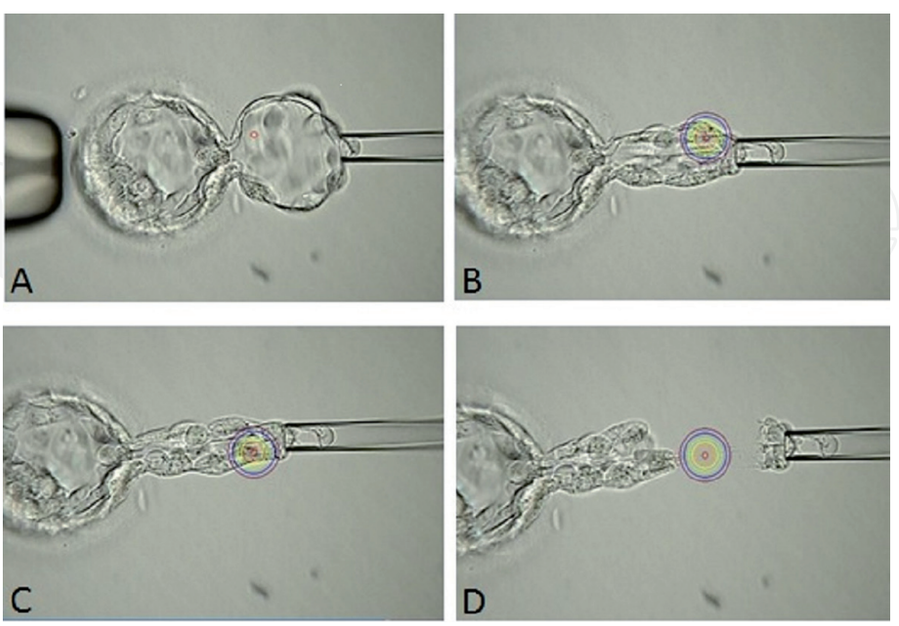

Figure 1.
Procedures for two-step blastocyst biopsy. A blastocyst with some trophectoderm cells being hatched from the hole in the zona pellucida opened on day 3 and the blastocyst is held to a proper position for biopsy (A). After a few trophectoderm cells are aspirated into biopsy pipette, one laser pulse is applied on upside of the cells (B) and another lase pulse is applied to the bottom side of cells (C) during mechanical pulling. Extra laser pulses may be necessary during pulling until the cells are completely isolated from blastocyst proper (D).

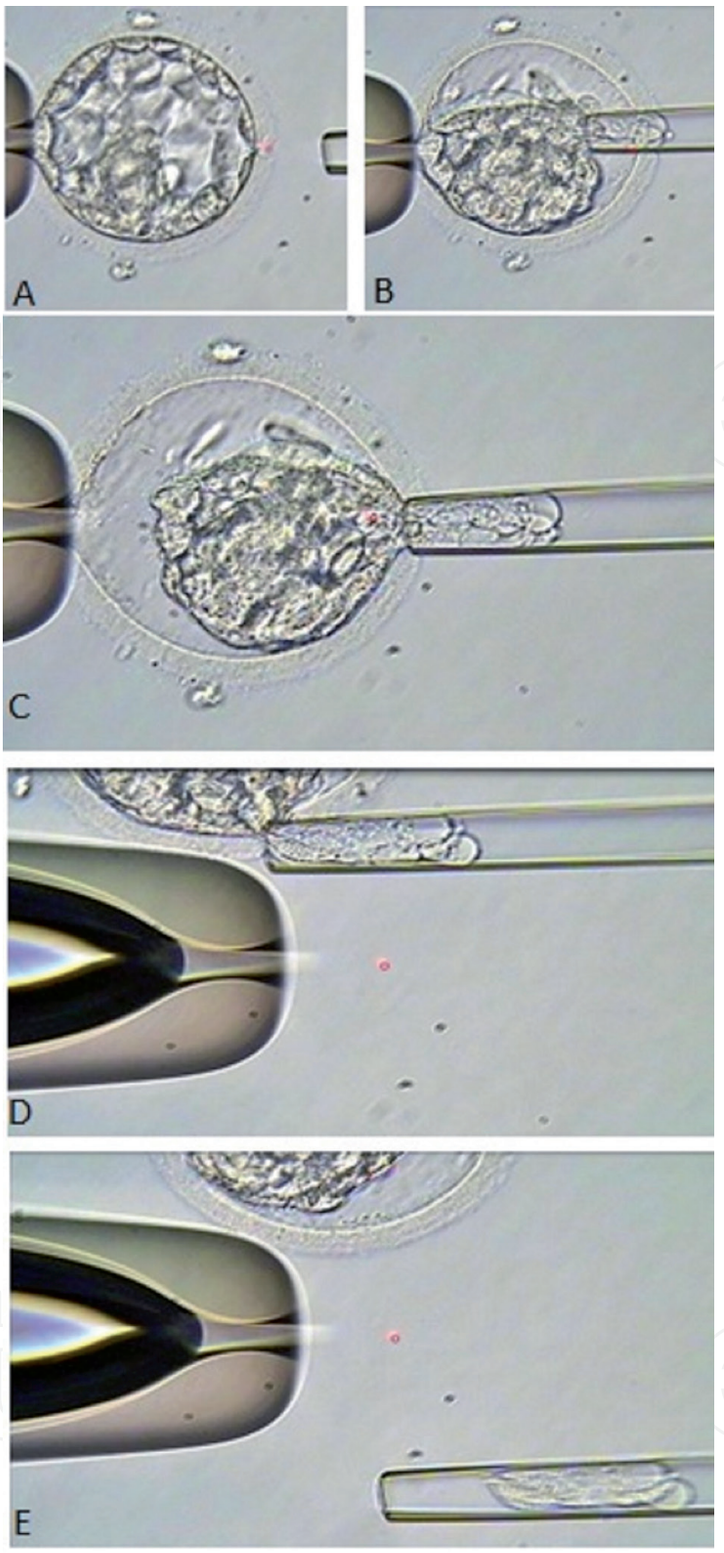

Figure 2.
Procedures for one-step blastocyst biopsy. A blastocyst is held to a proper position for biopsy and a small hole is opened in the zona by a laser pulse at 3 O'clock position (A). A biopsy pipette is inserted into the zona through the hole and a few trophectoderm cells are aspirated into biopsy pipette after blastocyst is collapsed or during collapsing from 12 to 2 O'clock position (B). Biopsy pipette is pull out of the zona (C) and the biopsy pipette together with blastocyst is moved to the top front of holding pipette (D). Biopsy pipette is pull down against the holding pipette to cut the cell connection between blastocyst proper and aspirated cells at the tip of biopsy pipette (E).

had any chromosomal error(s). The abnormal samples were further divided into aneuploidy if they had gain and/or missing of a chromosome(s) and segmental abnormal if they had only deletion and/or duplication in a chromosome(s).

2.5 Blastocyst vitrification, warming and transfer

Blastocysts were vitrified using a vitrification device (Cryotop, Vitristraw or Mini straw) and kit (Fujifilm-Irvine Scientific, Irvine, CA, USA). Both equilibration solution and vitrification solution were warmed in original vials at 37°C for at least 30 min before use. Briefly, collapsed blastocysts were equilibrated in 100 μl drop (without oil cover) of equilibration solution for 2 min, and then 45 seconds in 100 μl drop (without oil cover) of vitrification solution (both steps were performed on a 37°C warming stage) before loading to vitrification device. All blastocysts were vitrified individually and then stored in liquid nitrogen until warming for FET.

For warming, blastocysts were exposed to a thawing solution (Fujifilm-Irvine warming kit) at 37°C for 1 min, transferred to a dilution solution for 3 min and finally to a washing solution for 10 min with a solution change after 5 min at room temperature. After completion of the warming process, zona pellucida in the blastocysts were further cut by laser pulses to open 1/4–1/5 (2D image size) of zona pellucida and then cultured in Global™Total medium for 2–4 h before transfer.

For preparation of the transfer, patients received estradiol (Estrace, Warner Chilcott, NJ, USA) orally or vaginally, and estradiol patch (Estradiol Transdermal System, Noven Pharmaceuticals, NJ, USA) every three days, as well as progesterone that was administered on 15th day of estradiol treatment. Blastocysts were transferred on the sixth or seventh day of progesterone administered, and progesterone was continued daily until the first serum β-hCG test two weeks after transfer. Ongoing pregnancy was supported by continued estradiol and progesterone until 11 weeks of pregnancy. Pregnancy was initially confirmed 14 days after embryo transfer by a serum β-hCG assay. Four weeks after embryo transfer, when a gestational sac and a heartbeat appeared, the patient was diagnosed as having a clinical pregnancy. Live birth rates were calculated based on the number of live birth and number of transfers.

2.6 Statistical analysis

Interval data was analyzed by one-way analysis of ANOVA. The differences between groups were compared with chi square test. If the P value was less 0.05, the difference was considered to be statistically significant.

3. Results

To examine whether day 3 zona hole opening by laser pulse affected embryo development, blastocyst development between embryos with or without this procedure were compared. As shown in **Table 1**, similar blastocyst development rates (64.7 vs. 64.3%) were observed between two groups. Other parameters, such as egg donor's ages (26.5 ± 3.0 vs. 25.6 ± 2.6), and fertilization rates (86.4 vs. 88.8%) were also similar between two groups.

As shown in **Table 2**, after biopsy, the proportions of samples without tested results due to low quantity of DNA or no DNA in the samples were similar between two biopsy methods (3.6 vs. 4.4%), resulting in 96.4% of the samples biopsied with one-step method and 95.6% of the samples biopsied with two-step method were successfully amplified. It was found that euploid blastocyst rates were similar between two groups (63.4 vs. 64.0%).

Chromosome abnormalities include whole chromosome aneuploidies (extra and/or missing chromosomes), and segmental chromosome abnormalities, such as chromosome deletion and duplication. As shown in **Table 2**, no differences were

	Zona hole opening at Day 3 embryo		
	-	+	P Value
# of cases	61	45	NA
Donor age (Mean ± SD)	26.5 ± 3.0	25.6 ± 2.6	0.45
No. of eggs inseminated	1050	726	NA
No. of eggs fertilized (%)	907 (86.4)	645 (88.8)	0.12
No. of blastocysts (%)	587 (64.7)	415 (64.3)	0.88

NA: Not applicable.

Table 1.
Development of human embryos with or without laser zona hole opening that was performed on cleavage stage embryos at day 3.

	One-step method	Two-step method	P value
# of blastocysts biopsied	527	407	NA
# of samples without test results (%)	19 (3.6)	18 (4.4)	0.53
# of samples with test results (%)	508 (96.4)	389 (95.6)	0.53
# of euploid blastocysts (%)	322 (63.4)	249 (64.0)	0.85
# of embryos with abnormal chromosomes	186 (36.6)	140 (36.0)	0.85
# of aneuploid blastocysts (%)	119 (64.0)	113 (80.7)	0.06
# of segmental abnormalities (%)	67 (36.0)	27 (19.3)	0.06
# of samples with ≥2 abnormal chromosomes	45 (37.8)	50 (44.2)	0.32

NA: Not applicable.

Table 2.
Comparison of chromosomal abnormalities in the blastocysts after biopsy by one-step and two-step methods.

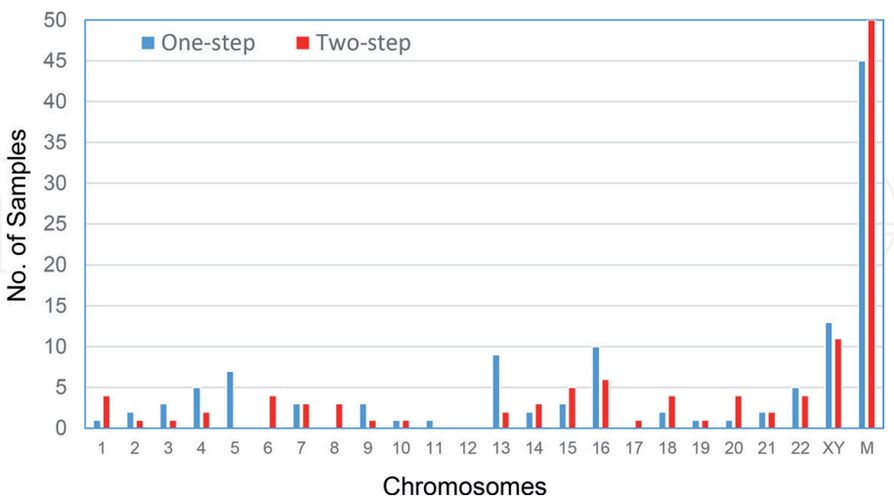

Figure 3.
Distribution of chromosomes in the aneuploid blastocysts biopsied with one-step and two-step method. Data represent the number of samples with a single abnormal chromosome and multiple (M: ≥2) abnormal chromosomes.

observed in the samples with whole chromosome aneuploid rates or segmental abnormalities between two biopsy methods. Samples with multiple chromosomal abnormalities were also similar between two biopsy methods.

	One-step method	Two-step method	P value
# of transfers	47	41	NA
Mean age of recipients	42.0 ± 6.8	42.9 ± 6.7	0.24
# of chemical pregnancy (%)*	37 (78.7)	26 (63.4)	0.11
# of clinical pregnancy (%)**	35 (74.5)	25 (61.0)	0.18
# of live birth (%)	33 (70.2)	24 (58.5)	0.25
# of blastocysts transferred	59	63	NA
# of blastocyst implanted	37(62.7)	31 (49.2)	0.11

**Positive beta hCG.*
***Fetus with heartbeat.*
NA: Not applicable.

Table 3.
Comparison of clinical outcomes after transfer of euploid blastocysts biopsied by one-step and two-step methods from donor egg cycles.

As shown in **Figure 3**, aneuploidies occurred in all chromosomes except chromosome 12 and the differences for each chromosome were not statistically significant between two methods.

As shown in **Table 3**, transfer of euploid blastocysts biopsied by one-step method had higher chemical pregnancy (78.7 vs. 63.4%), clinical pregnancy (74.5 vs. 61.0%), live birth (70.2 vs. 58.5%) and embryo implantation rates (62.7 vs. 49.2%) as compared with transfer of euploid blastocysts biopsied by two-step method. Although these rates were not statistically significant between two groups, improved clinical outcomes were observed when one-step biopsy method was used.

4. Discussion

Recently Munne *et al.* found that euploidy rate in human embryos produced by donor egg IVF differed significantly between infertility clinics [12], but they did not analyze the cause(s) related to these differences. Because they collected data from multiple IVF clinics and each clinic used different biopsy methods, which may make it difficult to analyze these factors. In the present study, to minimize the effects of maternal age-related aneuploidy formation in the embryos [6, 13–15, 17–20], we also used donor egg IVF cycles to examine whether biopsy methods affect embryo aneuploidies. Our data indicate that biopsy methods do not affect embryonic aneuploidies, however, simplified biopsy method may improve embryo implantation that may be benefited from reduced embryo manipulations and limited laser applications. We also found that high proportions of human embryos from donor egg IVF are not only whole chromosome aneuploidy, but also have segmental chromosome abnormalities.

Although most of embryonic aneuploidies have already occurred before oocyte and sperm are collected for IVF due to meiotic error(s) during oocyte and sperm development [1–3], some of aneuploidies may be caused by mitotic errors, or suboptimal in vitro conditions and/or in vitro manipulations [1]. Rigorous temperature control during oocyte manipulations can maintain meiotic spindle integrity that may prevent meiosis error during final oocyte maturation after egg retrieval [1]. While embryo biopsy for PGT-A is still an invasive laboratory procedure, thus different methods may affect the embryo quality including chromosome integrity. Since laser was used to zona hole opening and blastocyst biopsy in human IVF, it has made the biopsy procedure to be easy [21]. However, excess use of laser pulses

may be harmful to embryos and eventually would affect embryo development. For example, laser pulse(s) are applied on both cleavage embryos and blastocysts for the traditional two-step biopsy. The blastomeres next to the laser pulses may have different degrees of heat injuring, some injuries can be seen immediately or after further culture, while minor injuries may not be able to see under microscope during culture. Because the biopsied cells are mostly originated from these cells next to the position with laser pulses, chromosomes in some of these cells may be affected, which would eventually increase the rates of chromosomal abnormalities. However, based on our results observed in the present study, these manipulations of embryos do not affect chromosome integrity in the biopsied cells thus the aneuploidies after two-step biopsy were not increased as compared with one-step biopsy method. These results indicate that the chromosome abnormalities during embryo development, if occurs, are not from biopsy procedures.

However, biopsy procedures did affect embryo implantation. Previous studies with non-donor egg IVF found that blastocyst rates and live birth rates were reduced when day 3 zona opening was performed for two-step biopsy [22, 23]. Although we did not find the reduced blastocyst development after day 3 embryo manipulation in the present study, both embryo implantation and live birth rates were reduced when two-step biopsy procedure was used. These results indicate that laser pulses for zona hole opening at day 3 embryos may have detrimental effects on subsequent embryo development and embryo implantation ([22, 23], current study). We did not observe the differences in the blastocyst development after day 3 embryo manipulation in the present study as compared with no day 3 embryo manipulation, which may be due to good quality of oocytes from donors as compared oocytes from patients, thus some blastomeres might be affected, but overall blastocyst development rate was not reduced. Zona hole opening on day 3 embryos by laser pulses may affect embryo development especially if the perivitelline space is small or laser power is too large, thus the detrimental effects were caused by over-heating from laser pulses.

Although the statistical differences of embryo implantation rates between two biopsy methods were not significant due to small cycle numbers in the study by Zhao et al. [22] and in our current study, the differences were significant in the study by Rubino et al. in which more IVF cycles were examined [23]. When we reviewed the clinical outcomes by one-step and two-step biopsy methods in these studies, we found that the overall live birth rates could be increased by approximately 10% (9.26–12.7%) if one-step biopsy method was used, irrespective of small number of cycles or large number of cycles ([22, 23] and the current study) were analyzed.

Another reason for reduced embryo implantation after two-step biopsy may be resulted from blastocyst biopsy procedures. The traditional two-step blastocyst biopsy is performed by mechanical pulling and laser pulses. Heating from laser pulses would also cause injuries to the cells exposed to laser pulses, which would negatively affect embryo quality. Our one-step biopsy procedure is similar as that reported previously [22–24] but some modifications has been made. Cells were aspirated inside zona pellucida that is same as that used by Rubino et al. [23]. However, the cells aspirated into biopsy pipette were separated from blastocyst proper by mechanical blunt dissection, not by mechanical pulling and laser pulses, which is same as that reported by Zhao et al. [24].

The summarized benefits of our method are as the follows: First, one-step method does not need to have embryos to be exposed to laser pulse at day 3, which has been found to be detrimental to blastocyst development [22, 23]. Second, trophectoderm cells are aspirated inside zona pellucida, so that the fertilization of oocytes for PGT-A can be performed by either ICSI or regular IVF, and the contamination by cumulus cells or sperm can be minimized and avoided. Third, the separation of testing cells from blastocyst proper is made by mechanical blunt dissection,

not by mechanical suction/pulling and multiple laser pulses, thus the further injuries by laser pulses on isolated cells and blastocyst proper can be avoided. And the last, ICM may hatch from the hole in some blastocysts if zona opening is done at day 3 embryos, thus biopsy need to be done on a different position [23], which would further affect embryo's implantation competence.

A previous study reported monozygotic twins when two-step biopsy was used [23]. In the present study, we did not observe any monozygotic twins after transfer of blastocysts biopsied by either one-step or two-step method, and this may be attributed to the zona cutting (1/4–1/5) in all frozen blastocysts after warming. We performed blastocyst vitrification after blastocysts were completely collapsed, and the blastocysts were still at collapsed status after warming. The perivitelline space was still very wide after warming, thus laser cutting of a large portion of zona pellucida did not have any injury to the blastocysts. This procedure may avoid monozygotic twins after blastocyst biopsy.

Although biopsy procedures did not affect aneuploid formation in donor egg IVF, the proportions of embryos from donor egg IVF with chromosomal abnormalities are very high [8, 9, 12, 14, 15]. In the present study, we found that these chromosome abnormalities include the whole chromosome aneuploidies and segmental chromosome abnormalities. It has been estimated that ~32% of segmental abnormalities are originated from meiosis [25]. However, most segmental abnormalities originate from mitosis and are present in a mosaic pattern [25, 26]. It has been found that segmental abnormalities can occur in any chromosome, and the frequency of deletions and duplications is roughly equal [25].

It has been reported that approximately ~6–15% of blastocysts from human IVF have segmental abnormalities when evaluated by current PGT-A methods with different analysis platforms [25, 27, 28]. The incidence of blastocysts with only segmental abnormalities is about 2.4%–7.5% of all samples examined [25, 27–29]. However, in the present study, the segmental chromosome abnormalities accounts for approximately 20–40% of the abnormalities, or around 6–12% of all samples examined, which were higher than previous reports [25, 27, 28]. These differences may be attributed to different PGT analysis platforms because the resolutions and accuracies are different between platforms. The high-resolution PGS platforms, such as NGS, can detect more small chromosome errors than previous microarray and low-resolution platforms. This may also be explanation that PGT by NGS improves pregnancy outcomes compared with array comparative genomic hybridization in single thawed euploid embryo transfer cycles [16].

It has been reported that the incidence of segmental abnormalities in human embryos do not correlate with patient age [25, 27, 28]. This may be the reason that high rates were observed in the embryos derived from young and healthy egg donors. Transfer of these embryos would result in failed implantation, miscarriage, or possibly liveborn congenital syndromes if carried to term [29, 30]. Some syndromes and conditions may be related to development delay and intellectual disabilities such as 1q21.1 deletion syndrome, 16p11.2 deletion syndrome and 1p36 deletion syndrome [31], thus screening of these syndromes that have small segmental chromosome abnormalities may be necessary in human IVF.

5. Conclusions

In conclusion, the outcomes from two previous studies [22, 23] and the current study indicate that one-step blastocyst biopsy can improve blastocyst implantation rate and live birth rate by ~10% in non-donor IVF patients [22, 23] and donor egg IVF patients (current study), suggesting that one-step biopsy method is superior

to two-step method. Although blastocyst biopsy procedures may not affect the incidence of aneuploidies and/or segmental chromosome abnormalities, they affect embryo's implantation competences. Current PGT by high-resolution NGS reveals that high proportions of human embryos derived from donor eggs are not only whole chromosome aneuploidies, buy also segmental abnormal. Therefore, screening of these chromosome abnormalities may reduce embryo implantation failure, early miscarriage, birth defect, developmental delay and/or intellectual disability.

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Conflict of interest

The authors declare no conflict of interest.

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