INTRODUCTION

Preimplantation genetic testing for aneuploidy (PGT-A) was developed as a clinical tool for improving implantation rates and decreasing the risk of miscarriage. However, the efficacy of this technique is still controversial. Higher implantation rates per transfer are expected compared with the control group, but the live birth rate per oocyte retrieval may be lower than in the control group. Thus, misdiagnosis associated with lower diagnostic sensitivity and specificity, and the embryonic damage caused during embryo biopsy are the major disadvantages of PGT-A. Biopsy technique can be divided into two categories: targeting of the blastomere during the cleavage stage and targeting of the trophectoderm (TE) during the blastocyst stage. Fluorescent in situ hybridization (FISH) protocols require specimens with a wider nucleus diameter on glass slide; this approach is appropriate for blastomere biopsy, which can be useful for obtaining larger nuclei than observed in TE cells. In contrast, more advanced platforms, such as assisted hatching, may be more effective in improving implantation rates.
as next-generation sequencing (NGS) and microarray comparative genomic hybridization (aCGH), require optimum DNA from several cells for amplification are appropriate for blastocyst biopsy. TE biopsy for PGT was first reported 15 years ago,\(^4\) with the development of comprehensive chromosomal screening (CCS) technique, it is becoming a major technique for PGT-A. The Japan Society of Obstetrics and Gynecology (JSOG) has prohibited PGT-A screening since 1998; however, in 2016, JSOG initiated a clinical study of PGT-A for couples with recurrent in vitro fertilization failure and pregnancy loss (Clinical Trials.gov. as UMIN000026104).\(^5\) In the near future, there will be great need for a standardized embryo biopsy technique in Japan; however, the gold standard TE biopsy procedure has not been established yet. We outline the current status of blastocyst biopsy, and introduce technical tips for practitioners who are studying PGT.

**1.1 | Biopsy method; Hatching or non-assisted hatching?**

The first clinical report of preimplantation genetic diagnosis (PGD) using cleavage-stage embryo biopsy was published by Handyside in 1990.\(^6\) At the same time, researchers began studying trophoderm biopsy and demonstrated the feasibility of this method for PGD.\(^7,8\) Initially, cleavage-stage embryo biopsy predominated,\(^9-13\) particularly that using FISH protocols.\(^14-23\) The first live birth case following blastocyst biopsy for PGD was reported in 2002.\(^24\) McArthur et al\(^25\) described the details of trophoderm biopsy procedure and reproductive outcomes of PGD using this technique with FISH or real-time quantitative polymerase chain reaction (qPCR) in 2005. Notably, in their approach, zona breaching was performed on day 3 or 4, and 2-9 cells from the herniated trophoderm were teased free from the remaining trophoderm with laser pulse.\(^25,26\) This protocol was frequently applied for clinical trials; however, researchers also proposed another approach, that is, non-assisted hatching method. Capalbo et al\(^27\) reported a direct aspiration technique for biopsy of the trophoderm without assisted hatching in 2014, demonstrating the benefits of this protocol in daily laboratory work. Because the timing of biopsy for hatching blastocyst is critical, excess herniation of trophoderm may induce incarceration (Figure 1A). There is no evidence for the association between embryo incarceration and embryo development. However, practitioner should still avoid causing excess trophoderm herniation. Continuous observation using time-lapse monitoring technology and direct TE aspiration with non-assisted hatching are helpful for detecting and preventing such early incarceration. Non-assisted hatching has disadvantages. For example, if blastocyst collapse occurs during TE cell aspiration, the biopsy procedure may be difficult and be associated with a higher risk of losing the inner cell mass (ICM) area. Furthermore, blastocyst collapse may extend the biopsy time, despite recommendations that the biopsy procedure should be performed within 3 minutes.\(^28\) No published clinical data have shown which method is better for biopsy.

**1.2 | Optimal timing of zona drilling**

When establishing standard approaches for trophoderm biopsy,\(^25,26\) it is essential to consider the timing of zona breaching during the cleavage or blastocyst stage (Figure 1B). Zona breaching is associated with a risk of ICM incarceration, and practitioners must be careful to avoid physical damage to the ICM during the biopsy procedure. Zona breaching during the cleavage stage could increase ICM incarceration; however, few studies have reported this phenomenon. Rubino et al\(^29\) reported a double zona drilling method as a coping technique for ICM incarceration with an incidence of 3.0% (6/199) (Figure 2). Additionally, Gu et al\(^30\) reported an incidence of 5.5% (590/10 730) in human blastocysts. In contrast, animal data showed incidence rates of 44.2% (42/95)\(^31\) and 38.6% (49/127; N. Aoyama, Kato Ladies Clinic, unpublished data) for ICM incarceration with zona breaching during the cleavage stage, indicating large differences between human and mice. Importantly, animal experiments are performed using time-lapse monitoring, whereas clinical data do not always include such detailed information. Furthermore, 52.5% (5633/10 730) of cases were fully hatched blastocysts, which could be included many cases of ICM incarceration.\(^30\) Onodera et al\(^31\) suggested that the location of 8-shaped hatching influences ICM formation in mouse blastocysts, however, this concept is still controversial. Indeed, clinical data have shown that ICM incarceration does not increase monzygotic twinning delivery.\(^30\) If a part of ICM is aspirated together with TE cells during biopsy, although it depends on the number of TE cells aspirated, several cells reduction from the ICM may not have a huge effect on the embryo development after biopsy. This is because it has been hypothesized that ICM splitting may generate monzygotic twining during the repeat of collapse and expansion in the process of blastulation in a clinical study with time-lapse,\(^32\) or in an 8-shaped hatching animal experiment.\(^33\) However, not only the number of cell compensation, but also the chorionicity and amnioticity are also very important for normal fetal development after embryo splitting.\(^34\) Moreover, cell fate has already been determined at the blastocyst stage\(^35\) and ICM is formed by two cell lines, which are epiblasto and primitive endoderm in early blastocyst stage.\(^36\) Therefore, full attention should be paid to TE biopsy, and the double laser zona drilling may be effective to avoid impact on ICM.

Additionally, zona breaching during the cleavage stage may induce herniation in early-stage blastocyst, which are thought to have fewer cells. Although the total cell number in blastocysts have not been fully elucidated, in 1989, Hardy et al\(^37\) showed that mean numbers of TE cells were 37.9, 40.3, and 80.6 at 5, 6, and 7 days after insemination, respectively. In contrast, another study by Fong et al\(^38\) reported that the cell numbers ranged from 160.9 to 236.7 in morphologically high-quality blastocysts and from 43.7 to 84.0 in poor-grade blastocyst on day 6 morning. Additionally, a recent report showed that the cell number were 225.2 and 121.1 in hatching and non-hatching blastocyst.\(^39\) Thus, there may be greater differences among grades of blastocysts and
| (A) Biopsy method: hatching or non-assisted hatching? |
|-----------------------------------------------|
| **Hatching**                                 |
| • Trophoderm herniation is extensively used for TE biopsy. (2005, McArthur, Fertil Steril.). |
|                                           |
| **Non-hatching**                             |
| • Perivitelline space dilation by expelling medium using a fine pipet makes it easier to aspirate TE cells (2014, Capalbo, Hum Reprod.). |
|                                           |
| Excess hatching may induce incarceration.    |
|                                           |
| **Is Non-hatching biopsy safe?**             |
| • There is no evidence demonstrating whether incarceration influences embryo development after biopsy. However, it may be preferable to avoid cryopreservation of segmentalized embryos through small zona pellucida hole. Practitioners may pay attention to the progression of hatching after zona breaching. |
|                                           |
| • Conventional embryo culture system up to the expanded blastocyst stage is conducted and no laser assisted breach is performed for the duration of culture. Practitioners can coordinate the timing of biopsy. However, the incidence of collapse during TE suction may be higher than in the assisted hatching protocol. |

| (B) Timing of zona drilling: 8 cell or blastocyst stage? |
|----------------------------------------------------------|
| **8 cell stage**                                         |
| • Higher risk of ICM incarceration                        |
| • Avoid ICM biopsy; Double laser drilling is recommended (2014, Rubino, Eur J Obstet Gynecol Reprod Biol.). |
| • Wider perivitelline space                              |
| • Easily handled for zona drilling                       |
|                                           |
| **Blastocyst stage**                                    |
| • Lower risk of ICM incarceration                        |
| • Evaluation of morphological assessment of blastocyst   |
| • Narrow perivitelline space                            |
| Immerse blastocyst in hypertonic medium with 0.5 M sucrose solution to dilate perivitelline space. |

| (C) Size of hatching point (slit): small or large?      |
|----------------------------------------------------------|
| **Small hole hatching**                                  |
| • Zona hole size dictates whether 8-shaped-hatching or U-shaped-hatching occurs. Small holes minimize the area of the cut plane of TE, whereas large holes lead to larger cut planes. Higher thermal energy is needed for dissection of thick TE cells and may be more invasive. |
| • Suctioning pipettes (inner diameter 20-40µm) are commercially available, and smaller pipettes thin clumps of TE. |
|                                           |
| **Large hole hatching**                                 |
|                                           |

| (D) Trophoderm biopsy; Flicking? or pulling?             |
|----------------------------------------------------------|
| **Flicking**                                             |
| • Flicking may be recommended for TE dissection; however there is no evidence that flicking is better than pulling. Additionally, it requires time to learn this technique. |
|                                           |
| **Pulling**                                              |
| • Easy and simple technique                             |
| • It has been still unclear the toxicity of heat diffusion which must lie below 100°C, then a flick with quick movement within minimum range and number of laser pulse would be recommended. |

**FIGURE 1** Summary of the critical point of trophoderm biopsy
1.3.1 An appropriate number of biopsied TE cells is required to maintain implantation potential

The number of biopsied cells is one of the most critical factors affecting TE biopsy. The general consensus among researchers is that this number may be 5 cells. However, it is difficult to apply this in the clinical setting, and attempts have been shown to result in poor-grade TE and fragmentation. Fewer cell biopsies may be less invasive but result in a higher risk of amplification failure; whereas increased cell biopsies may lead to a lower risk of amplification failure but increase blastocyst disruption. Several clinical reports have described the incidence of DNA amplification and inconclusive results using a CCS device, at rates of 12.5% and 4.5-8.2% by polar body biopsy, 2.9%-12.3% and 0%-18.6% by single blastomere isolation, and 0%-4.0% and 0%-4.6% by TE, respectively (Table 1). Blastocyst biopsy results in lower amplification failure rates because of the superior cell numbers obtained from biopsy comparing with that of polar body or blastomere biopsy. Cimadomo et al showed that 8.0 trophectoderm cells were required for successful DNA amplification and conclusive diagnostic results; thus, the most suitable cell number for biopsy may be 5-10 cells rather than 1-5 cells. Overall, the use of these invasive techniques for TE biopsy may reduce the rate of live births by 5% live births.

1.3.2 When should we perform TE biopsy?

No conclusion has yet been reached regarding the best timing of TE biopsy, and we interpret that a point of contention may be the number of biopsied cell per the total cell number. The day 3 zona opening method may prompt hatching in early-stage blastocysts, which have 40-60 cells. On the other hand, zona opening in developing or developed blastocyst stage, which have 60-100 cell can avoid hatching in blastocyst with a fewer cell number. Goossens et al reported that the removal of one or two cells of blastomere in cleavage stage had a significant influence on embryo development on Day 5, that is; removing a smaller number of cells by embryo biopsy is less invasive for embryo development. Namely, removing 5 to 10 TE cells by biopsy may be less invasive for expanded blastocyst, which have a larger number of total cells than early-stage blastocyst, which have fewer cells. Actually, we performed TE biopsy when the blastocysts reached full size (>160 µm) with the hatching method in the pilot study of PGT-A, because there is no way to assess the total cell number.

On the other hand, from the results of our clinical study, blastocysts, which have earlier developmental speed, showed better reproductive outcomes such as clinical pregnancy and live birth rate; meanwhile, smaller size blastocysts (<160 µm) until day 7 had little chance to achieve clinical pregnancy. We hypothesize that biologically high-potential embryos reaching full-size blastocyst until 130 hours after insemination have a higher chance to be euploid, similar to the report that day 7 blastocysts showed a lower euploidy rate. Finally, the timing of biopsy may be better in the developed or developing blastocyst, which have larger cell numbers. Additionally, if an embryo has an earlier developmental speed, such as developed blastocyst in day 5, favorable result may be obtained.

1.3.3 Size of hatching point and time length to perform the biopsy

In general, single TE biopsy should be performed within 3 minutes. This can be achieved using a simple biopsy. However, thick TE samples may be difficult to disect from blastocysts. Thinning the TE area of the cut plane makes biopsy less difficult; thus, we suggest that smaller zona holes may produce less constriction of the hatching site for micro-cutting (Figure 1C). However, this process may be more complicated. Indeed, if a practicioner ignores the optimal timing of biopsy, a large 8-shaped hatching site may be generated, resulting in incarceration (Figure 1A). To prevent this, a time-lapse system may be useful. Furthermore, the size of the suction pipette for TE may be altered to minimize the area of the cut plane; several suction pipettes with inner diameters of 20-40 µm have been developed and are available from commercial sources.
| Outcomes (WGA and diagnostic) | Reproductive outcomes |
|-------------------------------|------------------------|
|                               | PGT-A group            | Control group | P-value | Reference                  |
|                               | Age | Result | Age | Result |                          |           |
| Biopsy sample: PB1/PB2        |     |        |     |        |                            |           |
| 1-2                           | 39.5| LB/transfer 26.4% (29/110) | 38.4| LB/transfer 14.9% (60/403) | .015 | 2015, Feichtinger, PLoS One. |
| 1-2                           | 38.6| Pregnancy/1st transfer 38% (57/149) | 38.6| Pregnancy/1st transfer 32% (54/171) | .71 | 2018, Verpoest, Hum Reprod. |
| Biopsy sample: Blastomere     |     |        |     |        |                            |           |
| 1                             |     |        |     |        |                            |           |
| DOP-PCR                       | 12.3% (9/73) | metaphase-CGH | 0.0% (0/64) | N/A, a validation report using surplus embryos | 2000, Wells, Mol Hum Reprod. |
| ① GenomePlex ② SurePlex      | 11.2% (18/161) | aCGH (BlueGnome) | 3.5% (5/143) | Pregnancy rate 57.9%/BT (44/76) 42.3%/OR (44/104) | N/A | N/A |
| MDA                           | 5.5% (5/91) | aCGH (8K) | 18.6% (16/86) | N/A, a validation report using surplus embryos | 2013, Mertzaniou, Hum Reprod. |
| PicoPLEX WGA                 | 8.3% (21/252) | Ion PGM Sequencing | 34.0 | Implantation rate 61.5% (40/65) | 0.001 | 2015, Lukaszuk, Fertil Steril. |
| qPCR                          | 1.4% (7/483) | N/A | 32.2 | Implantation rate 79.8% (107/134) | N/A | N/A |
| ① SurePlex ② SurePlex qPCR  | 1.5% (3/195) | aCGH (24sure V3) | 6.3%* (12/191) | Implantation rate 64.0% (32/50) | N/A | N/A |
| ① aCGH (24sure V3) ② NG5 {VeriSeq} | 1.4% (6/418) | aCGH (24sure) | 0.0% (0/418) | Implantation rate 66.2% (88/133) | P = .56 | N/A |
| ① aCGH (24sure) ② NG5 (HiSeq 2000) | 2.1% (9/427) | aCGH (24sure) | 0.0% (0/427) | Implantation rate 70.2% (92/131) | N/A | N/A |
| Biopsy sample: Trophectoderm  |     |        |     |        |                            |           |
| 5                             |     |        |     |        |                            |           |
| qPCR                          | 1.4% (7/483) | N/A | 32.2 | Implantation rate 79.8% (107/134) | N/A | N/A |
| ① aCGH (24sure V3) ② NG5 (VeriSeq) | 1.4% (6/418) | aCGH (24sure) | 0.0% (0/418) | Implantation rate 66.2% (88/133) | P = .56 | N/A |
| ① aCGH (24sure) ② NG5 (HiSeq 2000) | 2.1% (9/427) | aCGH (24sure) | 0.0% (0/427) | Implantation rate 70.2% (92/131) | N/A | N/A |

(Continues)
1.3.4 | Trophectoderm biopsy technique using flicking or pulling

The first solid-state laser to be used for ART, which was applied to trap spermatozoa, was neodymium:yttrium–aluminum–garnet (Nd:YAG) at a wavelength of 1064 nm\(^{61,62}\); however, this method can have the adverse effect of potential risk of DNA damage from ultraviolet wavelength. Thereafter, erbium:YAG laser, which operates at a wavelength of 2900 nm was introduced\(^{63,64}\); however, it required direct contact using laser fiber, raising concerns related to damage and contamination. Furthermore, holmium:yttrium–scandian–gallium–garnet (Ho:YSGG)\(^{65,66}\) laser using at 2100 nm wavelength exhibited different absorption behavior in water than earlier lasers; moreover, it required quartz slides. Several studies have applied ArF,\(^{67}\) KrF,\(^{68}\) XeCl\(^{69}\); however, they were impracticable. Finally, a non-contact 1480-nm diode laser-induced micro-drilling procedure has been introduced\(^{70}\); six types of lasers are now available for assisted reproductive technology based on infrared-emitting diodes currently in the market.\(^{71}\) The use of this approach is helpful for shortening the duration and reducing the complexity of zona breaching and TE biopsy. However, the potential disadvantages of this approach have not been clarified. For example, the toxicity of heat diffusion, which must remain below 100°C, has not been determined.

Does the laser-assisted biopsy introduce mosaic or chaotic changes to biopsied cells? This discussion has just started, and there is also a controversy. At American Society for Reproductive Medicine meeting in 2017, Kelka et al\(^ {72}\) concluded that laser-assisted trophectoderm biopsy (TEB) did not have an impact on DNA profiles, however, at the European Society of Human Reproduction and Embryology meeting in 2019, Herrero et al\(^ {73}\) concluded that laser assisted TEB may increase the risk of mosaicism. This mechanism may not be complicated; the number of laser shots and power of laser pulse may influence the induction of mosaicism. However, sample quality and biopsy technique may influence the results. Further investigation is needed to assess this mechanism, and it is necessary to conclude whether flicking method is superior to pulling method (Figure 1D).

1.3.5 | Tubing procedure

Blastocoel fluid and blastocyst culture medium contain cell-free DNA, and PGT-A protocols have already been established using liquid biopsy technique\(^ {74,75}\) and culture media.\(^ {76-79}\) The efficacy of these minimally invasive PGT approaches is controversial,\(^ {80-83}\) and regardless of diagnostic accuracy of PGT. These data showed biopsied TE samples are contaminated with higher concentrations of cell-free DNA. Therefore, practitioners should be careful to avoid this contamination and may apply a washing step to improve purity. Furthermore, retrieval of fragmented TE cells may increase inconclusive results. Accordingly, practitioners should avoid suction of fragmented TE during the biopsy procedure. Unamplified samples mainly result from the absence of TE cells, and recent data have shown that the rate is <1.5% when analyzing 5 TE cells using a whole-genome amplification kit. To decrease the
TE biopsy methods were used; however, the gold standard trophoderm (TE) biopsy procedure has not established yet. Based on our experience, zona breeching on days 5-6 after insemination has greater benefits than that on days 3-4. Moreover, biopsy of herniated TE samples simplifies and shortens the procedure, and a non-assisted hatching protocol may make the approach more complex if blastocysts collapse during TE suction. Recent studies have indicated that excess laser pulses may induce a higher frequency of mosaicism; thus, a rapid flick within the minimum range and fewer laser pulses are recommended. Smaller zona holes produce thinner TE cells at the hatching point, which may facilitate biopsy. Five to 10 TE cells are recommended for biopsy to decrease amplification failure. Achieving proficiency in these various techniques (Table 2) is expected to lead to optimal results. Since, biopsy techniques have not been discussed extensively in the literature, we hope that this review will facilitate further advancements in the study of this procedure.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

HUMAN AND ANIMAL RIGHTS

This article does not contain any studies with human and animal subjects performed by the any of the authors. This review focused on the literatures previously published and it did not contain ethical issues with human subjects; thus, there was no need for the study to be evaluated by an ethical committee.

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