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ARTICLE

Does slow embryo development predict a high aneuploidy rate on trophectoderm biopsy?

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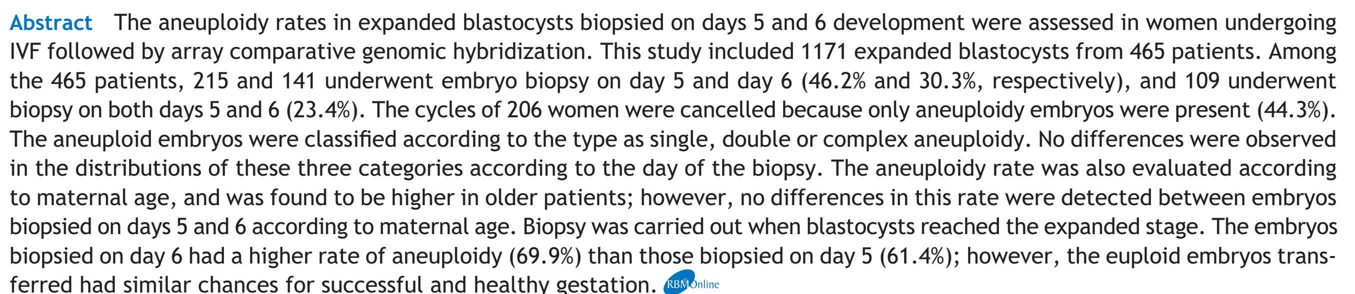

Abstract The aneuploidy rates in expanded blastocysts biopsied on days 5 and 6 development were assessed in women undergoing IVF followed by array comparative genomic hybridization. This study included 1171 expanded blastocysts from 465 patients. Among the 465 patients, 215 and 141 underwent embryo biopsy on day 5 and day 6 (46.2% and 30.3%, respectively), and 109 underwent biopsy on both days 5 and 6 (23.4%). The cycles of 206 women were cancelled because only aneuploidy embryos were present (44.3%). The aneuploid embryos were classified according to the type as single, double or complex aneuploidy. No differences were observed in the distributions of these three categories according to the day of the biopsy. The aneuploidy rate was also evaluated according to maternal age, and was found to be higher in older patients; however, no differences in this rate were detected between embryos biopsied on days 5 and 6 according to maternal age. Biopsy was carried out when blastocysts reached the expanded stage. The embryos biopsied on day 6 had a higher rate of aneuploidy (69.9%) than those biopsied on day 5 (61.4%); however, the euploid embryos transferred had similar chances for successful and healthy gestation. RBMOnline

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KEYWORDS: array-CGH, embryo aneuploidy, embryo culture, trophectoderm biopsy

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Introduction

Preimplantation genetic diagnosis (PGD) and preimplantation genetic screening (PGS) increase the chance of achieving a viable pregnancy without a single-gene defect or aneuploidy (Adler et al., 2014). Ten per cent of human pregnancies are affected by monosomy or trisomy; however, this rate can exceed 50% in women approaching the end of their reproductive lives (Nagaoka et al., 2012). Although embryos are typically selected according to morphology, PGS is the best method for selecting chromosomally healthy embryos for transfer because aneuploidy occurs at a higher rate in oocytes and spontaneous abortion (Sugiura-Ogasawara et al., 2012) or implantation failure (Margalioth et al., 2006).

Currently, extended culturing to the blastocyst stage enables biopsy samples to be obtained for PGS, which results in high diagnostic accuracy. The trophectoderm can therefore be more accurately identified, allowing biopsied cells to be obtained without causing damage to the inner cell mass. These improvements have resulted in significant increases in the implantation and pregnancy rates (Forman et al., 2013; Scott et al., 2013b, 2013c). Fewer embryos, however, reach the blastocyst stage in extended cultures, as demonstrated by a genetic analysis of 15,169 trophectoderm biopsies showing that 50% of patients had three or fewer blastocysts available for biopsy and that 20% had only a single blastocyst (Scott et al., 2013a). Therefore, an efficient laboratory with an extended culture system is required for blastocyst biopsy, and appropriate cryopreservation techniques are needed to allow for sufficient time to conduct genetic analyses.

Moreover, some embryos exhibit a slow rate of development during cleavage but may reach the early blastocyst stage on day 5, thus preventing safe biopsy at the appropriate stage. Those embryos are commonly kept in culture until day 6 to reach the appropriate developmental stage for biopsy. It is not clear, however, whether the slow rate of embryo development itself and the consequent extension of embryo culturing for an additional day of development (day 6) are independently related to any type of genetic disease. We hypothesized that a slow rate of development might be associated with a higher risk of embryo aneuploidy. Therefore, this study evaluated the aneuploidy rates in biopsied expanded blastocysts on days 5 and 6 of development in patients undergoing IVF with PGS by aCGH.

Materials and methods

Experimental design, patients and inclusion criteria

In this retrospective cohort study, 1171 expanded blastocysts were evalutated from 465 women who underwent fertility treatment with IVF and array comparative genomic hybridization (aCGH) between February 2014 and May 2015 at Huntington Reproductive Medicine, Sao Paulo, Brazil. Written informed consent was obtained from all patients before treatment, and the patients consented to the use of their retrospective data in scientific publications. According to the ethical guidelines, institutional review board approval was not required for this study because of its retrospective nature and because the data were anonymized.

Patients and ovarian stimulation

All couples included in this study had embryos that were biopsied for PGS by aCGH. All patients with an abnormal karyotype or family history of genetic disease were excluded. The patients received ovarian stimulation according to the routine protocols of the clinic. Briefly, pituitary blockade was achieved with a GnRH antagonist (0.25 mg Orgalutran, MSD, Kenilworth, NJ, USA) or agonist (Lupron, Abbott, North Chicago, IL, USA) according to standard protocols (Al-Inany et al., 2016; Pacchiarotti et al., 2016; Siristatidis et al., 2015). Ovarian stimulation was conducted using recombinant FSH (Gonal, Merck Serono, Germany or Puregon, MSD, USA), combined (or not) with human menopausal gonadotrophin (Menopur, Ferring, Switzerland), initiated on day 2 or 3 of the menstrual cycle. The initial dose was determined according to a previous antral follicle count, and the dose was adjusted according to the ovarian response. Follicle development was monitored every 2 days by ultrasonographic assessment of follicle growth. When at least two follicles measuring 18 mm or more in diameter were present, final oocyte maturation was triggered with 250 μg recombinant HCG (rhCG, Ovidrel®, Merck Serono, Switzerland). Oocyte aspiration was carried out under sedation at 35–36 h after recombinent HCG triggering.

Intracytoplasmic sperm injection and embryo culture

Follicular fluid was examined, and oocytes were identified and cultured for 3 h after retrieval to achieve final maturation, following denudation and assessment for the presence of the first polar body, indicating mature metaphase II. Then, metaphase II oocytes were fertilized by intracytoplasmic sperm injection (Palermo et al., 1992). Normally fertilized oocytes, defined by the presence of two pronuclei and two polar bodies, were cultured in groups between days 1 and 3 in 1 ml of cell culture medium (G-1 Plus, Vitrolife, Sweden) supplemented with 10% synthetic serum substitute (SSS, Irvine Scientific) under a layer of paraffin oil (OVOIL, Vitrolife). From day 3 until the blastocyst stage (D5 or D6), the embryos were cultured in 1 ml medium containing 10% human albumin (CSCM, Irvine Scientific, USA) under a layer of paraffin oil. The embryos were incubated in triple gas incubators (90% N_2, 5% O_2 and 5% CO_2).

Trophectoderm biopsy, genetic screening and embryo transfer

The blastocysts were morphologically classified according to Gardner et al. (2000), and all expanded blastocysts with a grade of 3 or higher were biopsied on day 5 ($n = 730$) or day 6 ($n = 441$) for genetic analysis. The time of biopsy was selected according to embryonic growth and blastocyst expansion, and only expanded blastocysts were biopsied. During the early blastocyst stage, the embryo has not expanded enough for herniation to occur, which prevents the removal of satisfactory amount of trophectoderm cells for analysis. At this stage, it is difficult to identify the inner cell mass, and cell removal can interfere with embryo quality and development (McArthur et al., 2005). The blastocysts were ob-

served by two embryologists on the morning and afternoon of day 5 and on the morning of day 6. As soon as both embryologists detected the presence of a grade 3 blastocyst (expanded blastocyst), biopsy was carried out for all embryos, independent of the inner cell mass and trophectoderm quality.

For blastocyst biopsy, a narrow channel was created in the zona pellucida using a laser (Hamilton Thorne®, USA) on day 3. On day 4 or day 6 of embryonic development, trophectoderm cells extruding from the expanded blastocyst were gently removed using suction, and laser pulses were focused on the cell junctions to safely remove a few cells without disrupting the inner cell mass. The cells were washed and placed into polymerase chain reaction tubes labelled with the corresponding embryo number and sent to the reference laboratory on dry ice for 24-chromosome analysis by aCGH. All analyses were carried at the same reference laboratory (Igenomix, Brazil) using standardized procedures.

The following two options were available for the couples with embryos biopsied on day 5 according to their schedule: embryos were kept in culture, and euploid blastocysts were transferred on day 6; or embryos were vitrified immediately after trophectoderm biopsy, and warmed embryos were transferred during a subsequent endometrial preparation cycle (Glujovsky et al., 2010). All embryos biopsied on day 6 were vitrified after trophectoderm biopsy, and euploid embryos were warmed and transferred during a subsequent cycle after endometrial preparation. All blastocysts were vitrified and warmed using vitrification kits (Vitringa, Brazil), according to the manufacturer's instructions.

Data analysis

The embryos were individually classified as euploid or aneuploid. The aneuploidy rate per patient was calculated by dividing the number of aneuploid embryos by the total number of embryos biopsied and genetically evaluated. The aneuploid embryos were further categorized according to the involvement of a single chromosome (single aneuploidy), two chromosomes (double aneuploidy), or three or more chromosomes (complex aneuploidy) (Fragouli et al., 2014).

The clinical pregnancy rate was calculated by dividing the number of positive pregnancies, confirmed by the presence of a gestational sac on ultrasound, by the number of patients who underwent embryo transfer. The patient demographic data were evaluated using descriptive statistics, including the means and frequencies. Continuous variables were analysed using Student's t-test to compare means and Pearson's X^2 test to compare frequencies. Regression analyses were conducted to evaluate the associations between variables. SPSS version 22 (IBM SPSS Software, USA) was used for data analyses, and $P \leq 0.05$ was considered statistically significant.

Results

A total of 730 out of 1171 embryos were biopsied on day 5 (62.3%), and 441 were biopsied on day 6 (37.7%). One day-6 blastocyst could not be genetically evaluated owing to amplification failure. The couples indicated for IVF plus aCGH had the following characteristics: advanced maternal age (≥38 years [61.9%]), a history of successive IVF failures (13.6%), recurrent spontaneous abortion (9.9%), severe male factor (5.4%), and a desire for screening (9.2%). Among the 465 patients who underwent ovarian stimulation, 215 and 141 underwent embryo biopsy on day 5 and day 6, respectively (46.2% and 30.3%, respectively), and 109 underwent biopsy on both days (day 5/6) during the same cycle (23.4%). The transfers of 206 patients were cancelled because all embryos were aneuploid (44.3%). The general demographic characteristics and laboratory data of the patients studied and the cycle outcomes are shown in **Table 1**.

The aneuploidy rate was also evlauated according to maternal age ranges (25–34, 35–37, 38–40, and 41–48 years of age). As expected, the aneuploidy rate was higher in the embryos from the older patients, but it did not significantly

Table 1 General characteristics of patients and intracytoplasmic sperm injection cycles.

Variables	*Total*	*Day 5*	*Day5/6*	*Day 6*	*P-value*
Number of patients	465	215	109	141	–
Age (years) (mean ± SD)	38.5 ± 3.9	38.3 ± 4.0	38.2 ± 3.9	39.0 ± 3.6	NS
Anti-Müllerian level (ng/ml) (mean ± SD)	1.9 ± 2.0	2.0 ± 2.0	1.8 ± 2.0	1.7 ± 2.0	NS
Number of oocytes retrieved (mean ± SD)	10.3 ± 7.1	10.2 ± 8.1[a,b]	13.3 ± 5.9[a,c]	8.3 ± 5.1[b,c]	<0.001
Number of MII oocytes (mean ± SD)	8.1 ± 6.0	8.3 ± 7.2[a,b]	10.5 ± 4.5[a,c]	6.1 ± 3.9[b,c]	<0.001
Fertilization rate (%)	76.4%	75.9%	75.8%	77.8%	NS
Number of embryos biopsied (mean ± SD)	2.5 ± 1.6	2.3 ± 1.4[a,b]	4.0 ± 1.7[a,c]	1.7 ± 1.0[b,c]	<0.001
Aneuploidy rate (%)	65.6%	61.4%[a]	68.6%	69.9%[a]	NS
Number (%) of cancelled cycles	206 (44.3%)	91 (42.3%)[a]	36 (33.0%)[b]	79 (56.0%)[a,b]	0.005
Number of cycles with embryos transferred	197	131	6	60	–
Number of embryos transferred (mean ± SD)	1.4 ± 0.5	1.4 ± 0.5[a]	2.0 ± 0.0[a,b]	1.3 ± 0.4[b]	0.002
Implantation rate (%)	41.5%	41.8%	33.3%	41.5%	NS
Clinical pregnancy rate (%)	44.6%	43.8%	50.0%	45.8%	NS
Live birth rate (%)	35.8%	34.4%	50.0%	37.3%	NS

The numbers sharing the same letter are significantly different: a, b, c: $P < 0.05$.
NS, not significant.

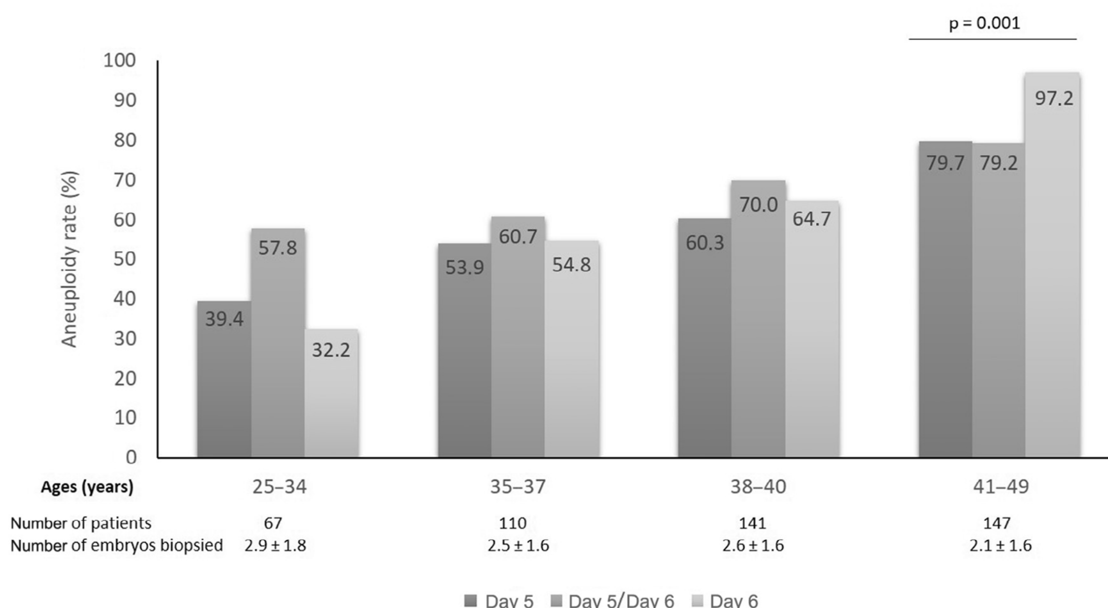

Figure 1 Embryo aneuploidy rates for patients who underwent embryo biopsy on either day 5 or day 6 and for those who underwent embryo biopsy on both days, stratified according to maternal age range. Number of embryos biopsied expressed as mean ± SD.

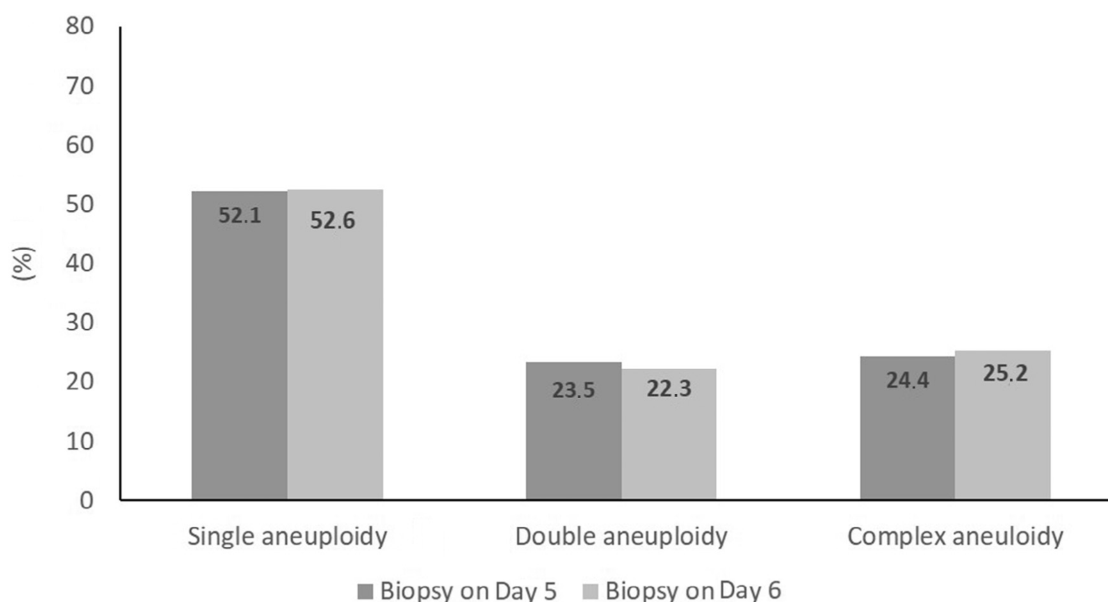

Figure 2 Percentages of aneuploid embryos biopsied on day 5 and day 6 according to the type of aneuploidy among the genetically abnormal embryos.

differ between the embryos biopsied exclusively on day 5 or day 6 and those biopsied on both day 5 and day 6 for the patients aged 25–40 years. Among the patients older than 40 years, the aneuploidy rate was significantly higher in embryos biopsied on day 6 only ($P = 0.001$) (**Figure 1**).

The aneuploidy rate was also examined according to the type of aneuploidy (single, double, or complex). No differences in the distributions of these three presentations were detected according to the day of biopsy (**Figure 2**).

Discussion

Since the first successful pregnancy achieved with preimplantation diagnosis in the 1990s (Handyside et al., 1992), the use of this technology has significantly expanded. Fluorescence in-situ hybridization has been used to examine biopsies from day 3 embryos for many years, and has been considered the best approach for evaluating chromosomal

integrity. Examination of only a few chromosomes, however, is possible using fluorescence in-situ hybridization, and this technique does not improve the pregnancy rate (Sermon et al., 2004). More recent advances in PGD include the use of modern incubators, as well as several improvements in culture media, permitting trophectoderm biopsy at the blastocyst stage, which yields a higher number of cells and allows for evaluation of all chromosomes by aCGH (Scott et al., 2013b). In addition, the biopsy protocol was revised to recommend biopsy on day 5 instead of day 3, minimizing the potential negative developmental effects on the blastocyst (Scott et al., 2013a). To carry out biopsy with minimal damage to the embryo, the inner cell mass must be accurately identified. Therefore, the change in the protocol allows for biopsy to be carried out at the expanded blastocyst stage, which is typically reached on day 5 or day 6 of development. In accordance with these criteria, 1171 embryos that reached the expanded blastocyst stage on day 5 or day 6 were evaluated. The patients underwent expanded blastocyst biopsy either on D5, on D6 or on both days (D5/D6). Analysis of the IVF cycles of these patients revealed some differences among the groups. Although the patient ages, anti-Müllerian hormone levels and fertilization rates were similar, a higher number of oocytes were collected from the patients who underwent embryo biopsy on day 5 and day 6. This increased availability of metaphase II oocytes allowed for biopsy of a larger number of embryos from these patients.

A study evaluating the implantation and pregnancy rates of embryos transferred on day 5 or day 6 in donation cycles revealed that the pregnancy rate was higher on day 5 with fresh transfer compared with that on day 6 (51.0% versus 33.3%); however, this difference was not observed for frozen-thawed transfers (63.6% versus 58.9%). The investigators suggested that the lower pregnancy rates detected in the fresh cycles were due to non-synchronization of the endometrium and not to an embryonic factor (Shapiro et al., 2008). Another study compared the pregnancy rates of expanded blastocysts transferred on day 5 or day 6 and a third group of blastocysts that expanded on day 5 but were transferred a day later (day 6). The authors found that the pregnancy rates were similar for the day 5-expanded blastocysts transferred on day 5 and day 6 (60.0% and 64%) but that the rate was lower for the day 6-expanded blastocysts (27.3%). The authors suggested, however, that endometrial factors do not influence the pregnancy rate, as the rates for transfers carried out on day 5 and day 6 were similar for embryos that reached the expanded blastocyst stage on day 5 (Elgindy and Elsedeek, 2012).

The clinical pregnancy rates did not significantly differ between the embryos transferred on only day 5 or day 6. This result was expected because the embryos were genetically evaluated in our study, and only euploid embryos were transferred. Therefore, although the aneuploidy rate in the blastocysts that expanded on day 6 was almost 10% higher than that in the blastocyst that expanded on day 5, blastocysts that were actually transferred had no structural or numerical chromosomal abnormalities. These findings suggest that once genetic screening is carried out and only euploid blastocysts are transferred, the development rate does not affect implantation success. Transfer of a mix of day 5 and day 6 embryos was carried out for only six patients, and because of this small patient number, this group could not be included in the comparisons.

We further analysed the type of aneuploidy according to the day of biopsy and found no significant differences in the distribution of aneuploidy types. Published research lacks comparisons of the complexity of aneuploidy between day 5 and day 6 blastocysts; however, it is clearly influenced by maternal age (Franasiak et al., 2014). The aneuploidy rates in day 5 and day 6 embryos according to age ranges were evlauated, and women aged over 40 years were found to have a higher aneuploidy rate in slow-growing embryos, i.e., day 6-expanded blastocysts), whereas the aneuploidy rates were similar between day 5 and day 6 embryos from the younger patients. These findings suggest that the aneuploidy rate is not associated with the rate of embryo development among younger patients; however, they indicate that the presence of aneuploidy is associated with slower embryo development among older patients. The results of this study are not sufficient to confirm a causal relationship between the rate of embryo development and aneuploidy in ageing patients. It is, however, known that oocytes of older patients exhibit an impaired development potential, which may be associated with both aneuploidy and slower development (Keefe et al., 2015).

The cycles of 206 patients were cancelled because only aneuploid embryos were present (44.3%). This high percentage of patients with no euploid embryos for transfer can be explained by the fact of most of patients in this study presented with an indication for embryo genetic screening, such as implantation failure, recurrent spontaneous abortion, advanced maternal age (>39 years), severe male factor, or both. Less than 10% of the patients underwent embryo genetic screening without a direct indication. Additionally, the mean patient age was 38.5 years, which is considered an advanced reproductive age for women. Furthermore, a higher percentage of cycles were cancelled owing to the availability of only aneuploid embryos for the patients with exclusively day-6 expanded blastocysts. This observation supports the notion that day 6-expanded blastocysts have a higher aneuploidy rate. Moreover, only six patients underwent mixed day 5/day 6 blastocyst transfers, and all patients had two embryos transferred, which resulted in an increased pregnancy rate; however, the mean number of embryos transferred for the patients with only day 5 or day 6 blastocysts was lower.

This study has some limitations, as the embryos transferred were selected based on aCGH analysis. Most of the patients who had a mixture of day 5 and day 6 blastocysts available only had day 5 blastocysts transferred. In addition, the effects of infertility factors other than age on the aneuploidy rate were not considered owing to the small number of patients with each factor. Reaching the proper blastocyst stage for biopsy is important, and continuous embryo observation or a time-lapse approach could increase the probability of carrying out biopsy at the correct time. The embryos were observed at three time points: day 5 morning, day 5 afternoon and day 6 morning. A time-lapse approach would allow for continuous observation of embryos, so that the exact point of entry into the expanded stage could be identified. Additionally, such an approach would avoid manipulation of embryos outside of the incubator.

Biopsy is carried out at the expanded blastocyst stage, which is when it is technically the most feasible, regardless of whether this stage is reached on day 5 or day 6. On the

basis of our findings, we suggest that slower embryo development is associated with aneuploidy in older women. When aCGH is applied and only euploid embryos are transferred, however, the success rate is not affected, and day 5- and day 6-expanded blastocysts have the same pregnancy potential.

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