Cleavage-stage biopsy significantly impairs human embryonic implantation potential while blastocyst biopsy does not: a randomized and paired clinical trial

Richard T. Scott Jr., M.D.,[a,b] Kathleen M. Upham, B.S.,[a] Eric J. Forman, M.D.,[b] Tian Zhao, M.S.,[a] and Nathan R. Treff, Ph.D.[a,b,c]

[a] Reproductive Medicine Associates of New Jersey, Morristown; [b] Division of Reproductive Endocrinology, Department of Obstetrics, Gynecology, and Reproductive Sciences, Robert Wood Johnson Medical School, Rutgers University, New Brunswick; and [c] Department of Genetics, Rutgers–State University of New Jersey, Piscataway, New Jersey

Objective: To determine if cleavage- or blastocyst-stage embryo biopsy affects reproductive competence.
Design: Paired randomized clinical trial.
Setting: Academic-assisted reproduction program.
Patient(s): Attempting conception through IVF.
Intervention(s): After selecting two embryos for transfer, one was randomized to biopsy and the other to control. Both were transferred within shortly thereafter. The biopsy was submitted for microarray analysis and single-nucleotide polymorphism (SNP) profiling. Buccal DNA obtained from the neonate after delivery had microarray analysis and SNP profile compared with that of the embryonic DNA. A match confirmed that the biopsied embryo implanted and developed to term, whereas a nonmatch indicated that the control embryo had led to the delivery.
Main Outcome Measure(s): Paired analysis of the delivery rates of the transferred embryos. Either twin delivery or failure to deliver represents equivalent outcomes for the biopsied and control embryos. In contrast, singletons were determined to be from the biopsied or the control embryo.
Result(s): Blastomere biopsy on day 3 of development resulted in a significant reduction in sustained implantation. Only 30% of biopsied embryos had sustained implantation and ultimately developed into live-born infants versus 50% of unbiopsied controls, a relative reduction of 39%. In contrast, sustained implantation rates were equivalent (51% vs. 54%) for biopsied and control blastocysts.
Conclusion(s): Cleavage-stage biopsy markedly reduced embryonic reproductive potential. In contrast, trophectoderm biopsy had no measurable impact and may be used safely when embryo biopsy is indicated.
Clinical Trial Registration Number: NCT01219504. (Fertil Steril® 2013;100:624–30. ©2013 by American Society for Reproductive Medicine.)
Key Words: Embryo biopsy, IVF, preimplantation genetic diagnosis, DNA fingerprinting
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Reprint requests: Richard T. Scott Jr., M.D., Reproductive Medicine Associates of New Jersey, 140 Allen Road, Basking Ridge, New Jersey 07920 (E-mail: rscott@rmanj.com).

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Genetic evaluation of human embryos during in vitro development provides patients with an opportunity to significantly reduce their risk of delivering a child with a genetic abnormality and to improve implantation rates in women attempting conception through in vitro fertilization (IVF). These technologies have been used in clinical practice

since the first reports more than 20 years ago (1–3). Although preimplantation genetic diagnosis (PGD) for single gene defects significantly reduced the risk of an affected child, initial efforts at aneuploidy screening to improve clinical outcomes produced discouraging results (4, 5). One putative explanation has been the potential for a negative impact of removing cells from the embryo. Perhaps the adverse effect of embryo biopsy abrogates the positive impact of selecting chromosomally normal embryos.

In considering this possibility, it is critical to understand that embryo biopsies may be done at different stages of development (i.e., cleavage or blastocyst) which may pose differing risks to subsequent development. Although the decision to biopsy at a specific stage of development is based on a number of clinical and technical considerations (6–11), the traditional approach has been to biopsy on culture day 3 at the cleavage stage. In fact, 88% of the almost 25,000 reported PGD cases had been performed at the cleavage stage of embryogenesis, whereas <1% had been performed at the blastocyst stage at the time of one report (7).

Despite extensive use of blastomere biopsy, studies evaluating the developmental impact compared with cleavage-stage embryos have been limited in scope (11–15). Although initial indirect evidence that a lack of significant impact on in vitro preimplantation development was encouraging, reports that have compared clinical outcomes after embryo transfer found a reduction in reproductive potential of 40% when two blastomeres instead of only one were removed (12–14). In another study, a 59% reduction in reproductive potential was observed for single-blastomere-biopsied embryos which failed to produce a PGD result (no genetic selection), compared with nonbiopsied control embryos (4). Although these studies have provided some insight regarding the impact of biopsy on embryonic reproductive potential, the results are not definitive.

A major limitation with these studies relates to selection of appropriate control subjects. Patients seeking PGD tend to have either a better prognosis for normal embryonic development (fertile couples who are carriers of genetic mutations) or a lesser prognosis (infertile couples with multiple failed treatment cycles) than their age-controlled peers. Given the number of factors that influence assisted reproduction outcomes, selecting an equivalent control group becomes a virtual impossibility.

Efforts to compare two treatment cycles in the same patient are even less useful. Patients completing the two cycles required in that design will have, by definition, failed their first treatment cycle, which creates an obvious bias. Moreover, even if the order of the treatment cycles were randomized with one-half undergoing PGD in the first cycle and one-half in a subsequent cycle, many important variables would remain inadequately controlled. Cycle-to-cycle variability in intrinsic oocyte quality, follicular stimulation dynamics, day-to-day drift in laboratory conditions and culture media, embryo transfer technique and efficiency, endometrial receptivity, and hormonal milieu in the luteal phase and during gestation would all remain inadequately controlled and would invalidate this from being a truly paired analysis with the only significant variable being embryo biopsy. To adequately control for these and numerous other known and unknown variables, impractically large sample sizes would be necessary (16).

A more powerful approach to define the impact of embryo biopsy on reproductive potential would be to take the two best embryos from the same treatment cycle and randomly assign one to undergo biopsy and the other to act as control. The two embryos would then be transferred simultaneously in the same cycle and the outcomes for each of the two embryos determined. The biopsied and control embryos would have equivalent outcomes if either a twin pregnancy or a failed cycle ensue. In those cases where a singleton pregnancy develops, DNA from the embryo biopsy may be compared with the DNA from the conceptus with the use of previously established single-nucleotide polymorphism (SNP) microarray–based DNA fingerprinting methods to determine which of the two embryos implanted (17–19). If biopsy has no impact, then the biopsied and nonbiopsied embryos would have an equivalent prevalence amongst singleton pregnancies. In contrast, if one group or the other was overrepresented among singletons, then an impact would be confirmed and an estimate of the magnitude of that impact attained. The paired design and analysis within each transfer would eliminate all known and unknown patient-specific variables, including those mentioned above.

This prospective paired randomized controlled trial design was used in the present study to provide the first rigorously controlled class I data on the safety of human embryo biopsy.

MATERIALS AND METHODS

Patients

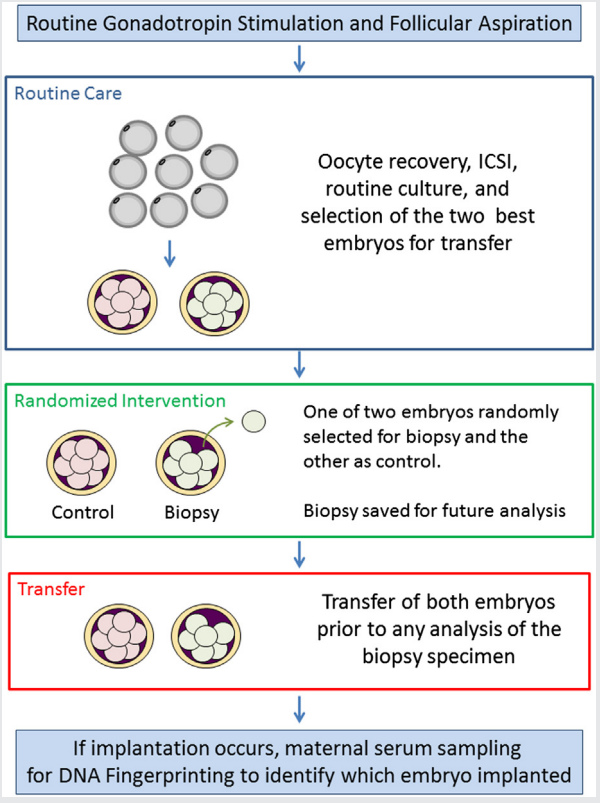
The study examined infertile couples attempting conception through IVF at Reproductive Medicine Associates of New Jersey from April 2008 to September 2012. Inclusion criteria were a female partner <35 years old with normal ovarian reserve screening (day 3 FSH levels <12 U/L and a basal antral follicle count ≥12) and a male partner with ejaculated sperm with >100,000 total motile spermatozoa and ≥1% normal forms. Exclusion criteria were a diagnosis of chronic anovulation, endometrial insufficiency, more than one earlier failed fresh IVF cycle, or a clinical indication for PGD.

Experimental Design

Patients received routine care inclusive of the day of development selected for embryo transfer (day 3 cleavage vs. day 5 blastocyst). The intent of the protocol was specifically not to alter any aspect of clinical or laboratory care provided to the patient. The study design is summarized in Figure 1.

The two best embryos were selected for transfer based on standard morphologic criteria used within the laboratory. All decisions were made completely without regard to participation in the study. The powerful paired design allows each patient to serve as her own control, which eliminated myriad variables, such as cycle-to-cycle variability in cohort quality, endometrial receptivity, the hormonal milieu, and transfer efficiency.

FIGURE 1



Study design to assess the impact of biopsy at the cleavage stage (day 3) on embryonic reproductive potential. The randomized paired experimental design allowed each patient to serve as her own control. An equivalent methodology was used to determine if trophectoderm biopsy at the blastocyst stage affected embryonic potential. ICSI = intracytoplasmic sperm injection.

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On the day of transfer, the embryos which had been selected for transfer were randomly designated as either A or B. A numbered sealed envelope was then opened which directed that either embryo A or B be biopsied and that the other would be the control. The order of the randomization which had been used to create the sheets which were sealed in the envelopes was created by a computer-based randomization program. Cleavage stage and blastocyst stage each had their own randomization schedule and group of envelopes to assure that the distribution of results were the same in both groups. Block randomization in groups of ten were used for both randomizations. The randomization table and sealed envelopes were created by the principal investigator (R.T.S.), and the envelopes were opened at the time of the randomization by the senior embryologist (K.M.F.).

The designated embryo underwent biopsy and then both were transferred together within 3 hours before any analysis of the biopsy. The biopsy specimen was placed in lysis buffer and held in reserve for future DNA fingerprinting. With resulting pregnancies, DNA fingerprinting of the conceptus was used to determine whether it was derived from the biopsied (self) or nonbiopsied (sibling) embryo.

Comparison of outcomes of biopsied and nonbiopsied embryos was performed with the use of a paired test of proportions (McNemar). All materials described in this study were collected and evaluated with Institutional Review Board approval (Western IRB, Olympia, Washington) and informed patient consent. This study was also registered with ClinicalTrials.gov under the identifier NCT01219504 (www.clinicaltrials.gov).

Embryo Biopsy

Individual cleavage-stage embryos randomized to the biopsy group were placed on a heated (37°C) micromanipulation stage (Olympus 1X70). With the use of a holding pipette (Origio), the embryo was rotated so that a blastomere with a clear and distinct nucleus was at the 3-o'clock position. Using an infrared 1.48-μm diode laser (Hamilton-Thorne Research) and 2–4 1-ms single pulses at 100% power, a 20–25 μm hole was made in the zona pellucida adjacent to the blastomere to be removed. The desired blastomere was gently aspirated with a biopsy pipette (Origio) while avoiding contact or disruption of the remaining cells of the embryo. The blastomere was then placed into polymerase chain reaction (PCR) tubes for future DNA analysis. The nonbiopsied embryo was handled identically in every way including being removed from the incubator and handled within the laboratory with the exception of the biopsy itself.

In those cases where the decision was made to perform the transfer at the blastocyst stage, assisted hatching was performed on all day 3 embryos at the cleavage stage in a manner identical to that described for cleavage-stage biopsy. This is the standard of care within our laboratory. On the day of blastocyst transfer, the blastocyst randomized to the biopsy was rotated with the use of gentle aspiration from a biopsy pipette until the herniating trophectoderm cells were located at the 3-o'clock position. With the use of gentle aspiration from a biopsy pipette (Origio) and 1–3 1-ms infrared 1.48-μm diode laser (Hamilton-Thorne Research) single pulses at 100% power on the herniating trophectoderm cells, approximately 4–5 cells were removed. The biopsied trophectoderm cells were placed intact into PCR tubes for DNA analysis. DNA from the female and male partners and the conceptus (villi or buccal swab) were analyzed as previously described (17–19). All microarray data described in this study were deposited at the Gene Expression Omnibus under the accession number GSE30374 (http://www.ncbi.nlm.nih.gov/geo/).

Data Analysis

It should be emphasized that overall delivery rates per transfer are of no analytic value with this experimental design and were not used in any matter during the formal data analysis. Delivery rates might be 100% and everything would seem optimal, but if the pregnancy always resulted from the biopsied embryo then there would be a dramatic difference in outcomes which is not reflected in delivery rates. The end point is sustained implantation: the probability that each individual embryo will implant and progress to delivery of a live-born infant.

There were three possible outcomes for each study patient. First, both embryos might implant and progress to delivery of twins. This represents equivalent outcomes for the biopsied and control embryos. If the patient does not conceive or miscarries, neither embryo was competent and equivalent outcomes are also attained. In contrast, when a singleton delivers, by definition the two embryos had different outcomes.

A McNemar chi-square for concurrence was used to determine if the proportion of singletons resulting from the biopsied and nonbiopsied embryo were equivalent or significantly different. If a difference was found, the overall magnitude of the effect was determined by looking at the overall implantation rates of the biopsied and nonbiopsied embryos. This included all transferred embryos, including those transfers that led to twins or failed to lead to pregnancy.

These data were used to estimate the magnitude of the effect, but given the paired nature of the experimental design they were not used to determine significance. An alpha error of 0.05 was considered to be significant. The study was powered to detect a 15% difference in implantation rates between sibling embryos (Dupont and Plummer; Vanderbilt University Department of Statistics; PS: Power and Sample Size Calculation).

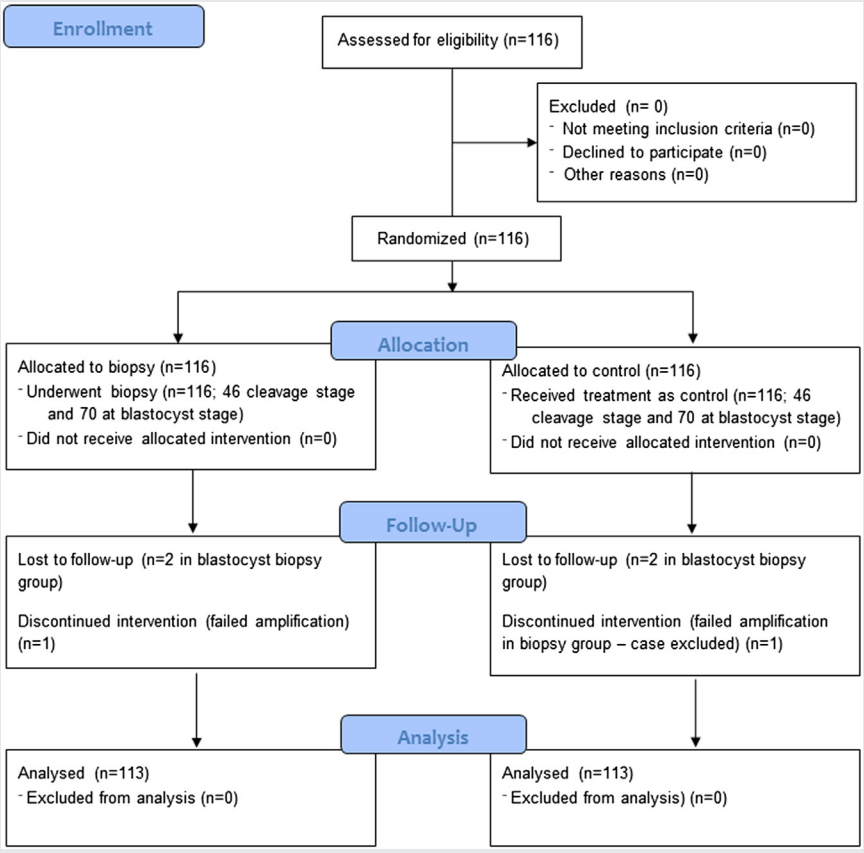
RESULTS

A total of 116 patients participated in the study. Progression of patients through the study is shown in Figure 2. Forty-six patients underwent transfer at the cleavage stage and 70 at the blastocyst stage. All 46 (100%) of the cleavage-stage embryos randomized to the biopsy group were successfully biopsied and evaluated with interpretable results. Sixty-nine (99%) of the 70 blastocysts randomized to the biopsy group were successfully biopsied and produced interpretable results (one failed to amplify). Thus, 46 cleavage stage pairs and 69 blastocyst stage pairs were available for analysis.

There were no significant differences in the morphologic characteristics of those embryos randomized to biopsy versus those randomized to control. Specifically, number of cells (7.7 $\pm$ 0.1 vs. 7.7 $\pm$ 0.1), percentage fragmentation (5.6 $\pm$ 0.8% vs. 5.9 $\pm$ 0.8%), and assigned grade (1 as best and 5 as worst; 2.1 $\pm$ 0.1 vs. 2.2 $\pm$ 0.1) were all equivalent.

Among the cleavage-stage transfers, 25 (54%) of the 46 patients delivered, with a sustained implantation rate of 40.2%. However, the analysis is paired so that the embryos within each transfer are compared to each other. When evaluating the embryo-specific outcomes from these cleavage-stage pairs, 12 had both embryos deliver (equivalent), 21

FIGURE 2



Consolidated Standards of Reporting Trials flow diagram detailing patient recruitment and progression through the study protocol. Of 116 enrolled patients, 113 completed the study and provided evaluable data.

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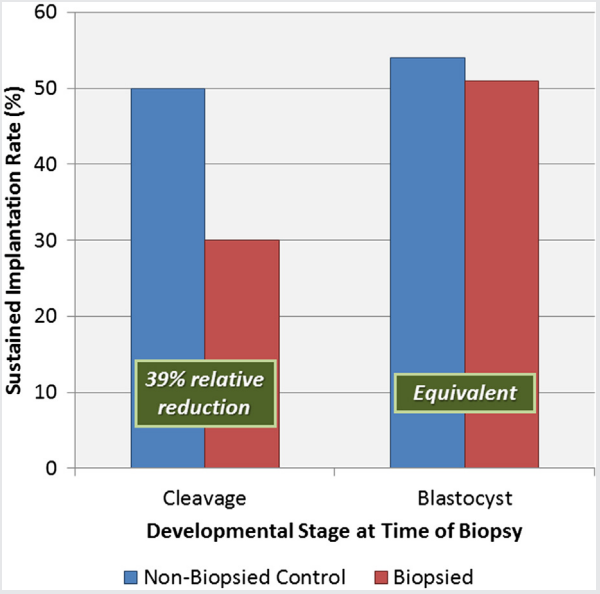
had both embryos fail to implant (equivalent), and 13 had a singleton delivery (differential outcome). Eleven of the 13 singletons were from the nonbiopsied embryo. Analysis of these outcomes shows a significant diminution in the probability of implantation of the biopsied embryos ($P=.02$). A total of 14 biopsied embryos (30.4%) and 23 nonbiopsied embryos (50%) implanted and displayed fetal cardiac activity (Fig. 3). The absolute reduction in implantation rates was 19.6%. The risk of the procedure may also be expressed by the relative reduction in the probability of an embryo having sustained implantation and progressing to delivery. The decline in implantation rates from 50% to 30.4% represents a relative reduction of 39.1% [1 − (30.4%/50%)]. This relative decrease may be the best overall estimate of the attributable risk of the procedure.

The blastocysts that underwent trophectoderm biopsy had equivalent morphologic grades compared with those randomized to the control group, specifically, expansion scores (4.0 vs. 4.0) and inner cell mass scores (10% graded as "A" and 90% "B" vs. 9% graded as "A" and 91% graded as "B")

Among the 70 blastocyst-stage transfers, one sample failed to amplify and two patients were lost to follow-up. Sixty-seven transfers were available for evaluation. Forty-three (64%) of these 67 delivered. Evaluation of the embryo-specific outcomes for the 67 blastocyst pairs demonstrated that 27 transfers had both embryos deliver (equivalent), 24 had neither progress to delivery (equivalent), and 16 had a singleton delivery (differential outcome). There was no difference in the probability of a singleton resulting from the biopsied embryo (n = 7) or nonbiopsied embryo (n = 9; $P=.804$). When considering all embryos transferred, 34 biopsied embryos (51%) and 36 nonbiopsied embryos (54%) had sustained implantation and progressed to delivery (Fig. 3). This nonsignificant difference represents an absolute reduction of 3%.

FIGURE 3



Implantation rates following a randomized paired analysis of the effects of cleavage- and blastocyst-stage biopsies on embryo reproductive potential. Sustained implantation and delivery of the biopsied embryo were significantly reduced compared with its control sibling when biopsy was performed on day 3 at the cleavage stage (McNemar chi-square: $P<.03$). A similar paired analysis demonstrated that the developmental potential of embryos undergoing trophectoderm biopsy at the blastocyst stage was equivalent to the nonbiopsied control siblings.

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Aneuploidy Rate Data

The same microarray analysis that allowed DNA fingerprinting essential to the paired design of this study provided aneuploidy results on the biopsied embryos. The aneuploidy rate was 41.3% (19/46) for day 3 embryos and 42.7% (30/79) for blastocysts. There are no data from the embryo in each pair which was not biopsied. As such, no comparison is possible between the biopsied and nonbiopsied embryos.

DISCUSSION

This paired study clearly demonstrates that the stage of embryonic development when biopsy is performed significantly affects the safety of the procedure. Trophectoderm biopsy at the blastocyst stage had no meaningful impact on the developmental competence of the embryo as measured by implantation and delivery rates, although blastomere biopsy at the cleavage stage produced a dramatic 39% relative reduction in the probability that an embryo would implant and progress to delivery.

It is important to realize that this study did not compare the impacts of blastomere biopsy on day 3 versus trophectoderm biopsy on day 5. The paired nature of the design meant that each transfer was compared only with itself: day 3 biopsied versus day 3 control and day 5 biopsy versus day 5 control. It is difficult to imagine a scenario where some embryos within a cohort would be biopsied on day 3 and others would be evaluated on day 5. As such, this study did not address that unlikely scenario.

Given the dramatic findings of this study, it is prudent to consider if the diminution might be attributable to factors other than the biopsy itself. One factor might be the experience or technique of the embryologists (20, 21). Explanations for the failure of cleavage-stage PGD in randomized controlled trials have included a purported lack of appropriate experience of the embryologists performing the biopsy, thus resulting in a detrimental impact of embryo biopsy that outweighs the putative benefits of PGD-based embryo selection (20–22). In the present study, all procedures were performed by two senior embryologists at a program with more than a decade of PGD experience including >10,000 blastomere biopsies. One of the embryologists actually trained with and worked for one of the major laboratories that has advocated the use of blastomere biopsy–based PGD and suggested that only well trained and highly experienced embryologists (such as their own) might produce optimal results (20, 21). Finally, cleavage-stage biopsies in the present study were performed according to the most highly advocated methodology (20).

The 29% sustained implantation rate of blastomere-biopsied embryos observed in this study was similar to the 27% implantation rates observed in an earlier report involving "experienced" centers where blastomere biopsy was performed in patients of similar mean maternal age (~32 years) (20). It seems to be exceedingly unlikely that the adverse impact of cleavage-stage biopsy might legitimately be attributed to operate inexperience or methodologic error.

It might be noted that the biopsied embryos were exposed to calcium-magnesium–free medium whereas the control embryos were not. This medium has previously been shown to facilitate cleavage-stage biopsy without affecting blastulation and is widely used (23). Although it is unlikely that such a large diminution in outcomes could be attributed to that brief exposure, it remains an almost essential part of the process. Any adverse effect, even if not from the removal of cellular material, should be considered that is intrinsic to the overall biopsy procedure.

All transferred embryos in the present study underwent laser-assisted hatching, so this intervention cannot account for a negative impact on one subgroup of embryos. The preponderance of human data indicates that this procedure does not have a negative effect on IVF outcomes (24). It is possible that having had a cell removed on day 3, displacement of other blastomeres, particularly at the time of transfer, might be more common. Therefore, although laser-assisted hatching cannot explain the difference in implantation rates, the combination of hatching and biopsy might produce a milieu where the risk of displacement is increased.

The aneuploidy rates were similar in those embryos that were biopsied on day 3 and those biopsied at the blastocyst stage. There were no data on the nonbiopsied embryos, and as such there was no possibility of comparing aneuploidy rates within each pair.

Although aneuploidy is very important clinically, it should have no impact on the results of this study because of the within-patient nature of the randomization. Every cohort of embryos by definition has its own aneuploidy rate. Given that the two embryos selected for transfer had equivalent morphology, each of the selected embryos had the same probability of being aneuploid. Given that the randomization occurred after the embryos were selected for transfer, the probability of an embryo being aneuploid was the same in both the biopsy and the control groups and thus should not have affected results. It should also be emphasized that this analysis was not done until weeks after the embryos were transferred and had no influence on any aspect of clinical care.

The observation that trophectoderm biopsy is safer than a blastomere biopsy implies that improved clinical outcomes following PGD may be best realized by performing biopsies at the blastocyst stage of embryo development. This is supported by the observation that the only studies to provide level I evidence for the benefit from aneuploidy screening used blastocyst-stage biopsy (25, 26). One potential explanation as to why blastocyst-stage biopsy is safer than cleavage-stage biopsy, is that the procedure involves removal of a smaller proportion of the embryo's total cellular content (27). For example, a single blastomere taken from an 8-cell embryo represents 13% of the total content, although an approximately 5-cell trophectoderm biopsy taken from an expanded blastocyst (200–220 cells in our program) represents only 2%–3% of the total content. Another contributing factor might be the certainty that only extra-embryonic (trophectoderm) cells are biopsied. In contrast, the lineage-specific developmental fate of an individual blastomere is unpredictable by morphology, despite molecular genetic evidence that commitment occurs at the cleavage stage (28). Finally, it is possible that blastocysts possess increased tolerance to manipulation compared with cleavage-stage embryos as a result of having already undergone embryonic genome activation (29).

In the end, a variety of factors may contribute to a significant diminution in outcomes following cleavage-stage biopsy on day 3 whereas none is observed following trophectoderm biopsy on day 5. It is sufficient to say that cleavage-stage biopsy is detrimental and that our data suggest that trophectoderm biopsy is safe, at least regarding the ability of the embryos to progress to delivery. The potential for impact on postnatal events remains to be studied.

Because PGD at the cleavage stage may significantly compromise clinical outcomes, biopsy at the cleavage stage of embryogenesis should be viewed skeptically. In addition, as new and more reliable genomic-based methodologies for embryo assessment become available to enhance selection, their application should include blastocyst rather than cleavage-stage biopsies. Stated briefly, cleavage-stage biopsy of human embryos may represent a violation of one of the principal precepts of medical ethics—*primum non nocere* or "first do no harm."

Most clinical laboratories do not have genetics laboratories capable of providing aneuploidy screening results quickly enough to allow trophectoderm biopsy followed by fresh transfer in the same cycle. Employing 24 chromosome aneuploidy screening would require those programs to vitrify those embryos after biopsy with subsequent warming and transfer in a future cycle when the endometrium is synchronous. This represents a difficult clinical scenario, and clinicians should consider the impact of cryopreservation when deciding how best to implement aneuploidy screening. This would include adverse effects, such as the risk of the vitrification and warming processes, as well as potential positive effects by avoiding transfer into a hyperstimulated endometrium. Further class I data will be needed to truly understand how best to use this technology in that setting.

Finally, not all laboratories perform blastomere and trophectoderm biopsy using the specific protocols employed in this study. Their individual results might be better or worse. In the event that the other laboratories might have a greater expertise in performing blastomere biopsy, the potential for better outcomes, i.e., a less severe or even negligible effect on embryonic potential, exists. However, at this time these data represent the most rigorous evaluation of this question. Until such time as other laboratories demonstrate safety by applying a similarly powerful study design, there remains insufficient evidence that blastomere biopsy can be safely performed without impacting the reproductive potential of human embryos.

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