ORIGINAL ARTICLE: ASSISTED REPRODUCTION

Impact of multiple blastocyst biopsy and vitrification-warming procedures on pregnancy outcomes

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Objective: To assess the impact of multiple blastocyst biopsy and vitrification-warming procedures on clinical outcomes.
Design: Retrospective study.
Setting: Private fertility clinic.
Patient(s): Preimplantation genetic diagnosis (PGD) patients undergoing comprehensive chromosome screening, including monogenic disorder and chromosome rearrangement cases.
Intervention(s): Warming and transfer of euploid blastocysts biopsied and vitrified-warmed once (group 1 [G1, control]; n = 2,130), biopsied once but vitrified-warmed twice (group 2 [G2]; n = 34), or biopsied and vitrified-warmed twice (group 3 [G3]; n = 29).
Main Outcome Measure(s): Thaw (for transfer) survival rate and clinical pregnancy rate (CPR).
Result(s): The thaw survival rates were 98.4% for G1, 97.3% for G2, and 93.3% for G3, with once biopsied and vitrified-warmed embryos being significantly higher than twice biopsied and vitrified-warmed embryos (G1 vs. G3; P=.032). There was a slight reduction in CPR with an additional vitrification-warming (G1 54.3% vs. G2 47.1%) and larger reduction with an additional embryo biopsy (G2 47.1% vs. G3 31.0%), but neither difference was statistically significant. However, the combined effect of both additional biopsy and vitrification-warming resulted in a significantly reduced CPR (G1 54.3% vs. G3 31.0%; P=.013).
Conclusion(s): This study indicates that blastocysts biopsied and vitrified-warmed twice have reduced clinical outcomes compared with blastocysts biopsied and vitrified-warmed once. PGD patients should be advised that performing a second biopsy and vitrification-warming in cases of failure to obtain a result from initial biopsy will reduce the chance of pregnancy. Patients with inherited disorders may elect to proceed with the second biopsy and vitrification to avoid transfer of embryos with the genetic condition. (Fertil Steril® 2017;■:■–■. ©2017 by American Society for Reproductive Medicine.)
Key Words: Blastocyst, preimplantation genetic screening, trophectoderm biopsy, vitrification

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The successful cryopreservation of excess embryos is an important component of assisted conception programs, with vitrification widely recognized as the criterion standard method (1, 2). There are many benefits of embryo cryopreservation, including the adoption of a single-embryo transfer policy to reduce the risk of multiple pregnancies and maximize the cumulative pregnancy rate (3, 4). Evidence also suggests that vitrified-warmed embryo transfers have equivalent or higher pregnancy rates and improved neonatal outcomes compared with fresh embryo transfers (5–8), which is hypothesized to be due to avoidance of deleterious effects from hormone stimulation on endometrial preparation and receptivity. In addition, embryo cryopreservation is critical to preimplantation genetic diagnosis (PGD) programs, allowing time for genetic testing of embryo biopsies for chromosomal content and inherited genetic disorders.

Embryo vitrification is performed with the use of high concentrations of cryoprotectants and ultrarapid cooling to avoid detrimental ice crystal formation (9). Although many studies have indicated vitrification to be a safe and efficient practice (5–8), it is unclear if multiple vitrification-warmings are detrimental to assisted conception outcomes. One reason this question has arisen is due to the request for chromosome screening on already cryopreserved embryos by patients hoping to improve their chance of pregnancy or reduce the risk of miscarriage from a given embryo transfer. Taylor et al. (10) attempted to address this question in their twice cryopreserved-warmed preimplantation genetic screening (PGS) population consisting of blastocysts both

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initially slow-frozen (n = 7) and vitrified (n = 12). They reported a lower embryo survival rate after warming compared with control samples, 87.5% vs. 98.3%, with the three twice cryopreserved-warmed embryos that failed to survive all being initially slow-frozen. However, pregnancy outcomes from their 14 euploid twice cryopreserved-warmed blastocyst transfers were similar to those of the control group.

Another reason for twice vitrifying-warming embryos is failure to achieve a result from the initial biopsy specimen, thus also requiring a second trophectoderm biopsy. This is particularly critical for inherited single-gene disorder and chromosomal rearrangement cases, and is increasingly being considered by PGS patients hesitant to transfer an embryo with unknown chromosomal status. However, there is concern that removal of too many trophectoderm cells could be detrimental to an embryo's pregnancy potential (11), and very little information is available on outcomes of twice-biopsied blastocysts. Zhang et al. (12) reported ten single euploid embryo transfers with twice biopsied and vitrified-warmed blastocysts, which resulted in five live births, although no control groups were presented. Likewise, Minasi and Greco (13) noted as unpublished data that they performed eight single-embryo transfers with the use of blastocysts biopsied twice but cryopreserved once, resulting in four live births. Further studies are required to determine if an additional blastocyst trophectoderm biopsy, as well as an additional vitrification-warming, is detrimental to pregnancy and neonatal outcomes.

In this retrospective analysis we analyzed the impact of multiple blastocyst biopsy and vitrification-warming procedures on clinical outcomes.

MATERIALS AND METHODS

Study Design

This retrospective study analyzed the impact of multiple blastocyst biopsy and vitrification-warming procedures on clinical outcomes at the Australian private fertility clinic Genea (previously Sydney IVF; Sydney, Liverpool, Northwest, and Canberra clinics). The first part of this study examined biopsy outcomes of vitrified embryos warmed with the intention of biopsy for PGD from January 28, 2013, to September 12, 2016. Data were separated into blastocysts that were vitrified without biopsy and blastocysts that were biopsied and vitrified but failed to produce a PGD result. All PGD cases were included regardless of reason for PGD or intended screening method. The second part of the study examined the outcomes of single or double trophectoderm biopsy and once or twice vitrified euploid blastocysts that were warmed with the intention of uterine transfer from May 29, 2014 (first transfer), to May 15, 2017. Data included PGD cycles undergoing PGS with the use of next-generation sequencing (NGS) or comparative genomic hybridization (CGH), with or without testing for single-gene disorders or chromosome rearrangements. The data were separated into three groups: blastocysts biopsied and vitrified-warmed once (group 1), blastocysts biopsied once but vitrified-warmed twice (group 2), and blastocysts both biopsied and vitrified-warmed twice (group 3). In both parts of the study, embryos were first vitrified as blastocysts (including very early blastocysts) on day 5 or 6, with or without trophectoderm biopsy, with a minority of the nonbiopsied blastocysts being imported from external clinics. Ethical approval for retrospective cohort studies with the use of deidentified patient clinical data was granted by Genea's Human Research Ethics Committee in December 2012.

General Assisted Reproduction Procedures

Ovarian stimulation, oocyte collection, and fertilization with sperm were performed as described previously (6, 14). Embryo culture to the blastocyst stage was performed in groups of up to five embryos as described previously (6) with either in-house manufactured sequential media identical in formulation to Sydney IVF embryo culture medium suite (Cook Medical) or Gems sequential embryo culture media (Genea Biomedx). A minority of embryos were cultured in the Geri time-lapse incubator with the use of Gems one-step media (Genea Biomedx) as described by the manufacturer. Note that these details may not be applicable to blastocysts imported from other clinics. Blastocysts were scored on the morning of day 5 of development onward and just before vitrification according to a simplified Gardner blastocyst grading system, whereby grade 1, grade 2, and grade 3 were considered to be an excellent, good, and poor-quality embryo, respectively (15). Blastocysts confirmed as euploid were warmed and transferred as described previously (6).

Embryo Hatching and Blastocyst Biopsy

Assisted hatching was performed on PGD-designated day-3 cleavage-stage embryos with the use of a Zilos TK Laser (Hamilton Thorne Biosciences) to create a 10-μm opening in the zona pellucida, with the exception of embryos cultured in the Geri time-lapse incubator which had assisted hatching on day 4. On day 5, blastocysts were assessed for the presence of trophectoderm herniating from the zona breach location. Blastocysts with suitable trophectoderm herniation underwent biopsy as described previously (16), whereas embryos not yet suitable for biopsy were reassessed after an additional 6–24 hours of culture. Embryo biopsies were immediately stored at −20°C for preservation of DNA for genetic analyses. After trophectoderm biopsy blastocysts were allowed to recover in culture for a minimum of 1 hour and then cryopreserved by means of vitrification.

For cryopreserved nonbiopsied blastocysts designated for PGD, embryos were mostly warmed in the late afternoon and hatched (if not already done previously), then cultured overnight and assessed for biopsy early the next morning. The exception to this was embryos warmed, biopsied, and revitrified on the same day. Similarly, cryopreserved biopsied blastocysts requiring a second biopsy because of unsuccessful genetic analysis were mostly warmed in the late afternoon, cultured overnight, and assessed for biopsy early the next morning. The exception to this was fully hatched blastocysts, which were warmed in the morning just before assessment for rebiopsy. Note that there was one warmed day-6 blastocyst that required culture for 2 nights before being suitable for biopsy.

Preimplantation Genetic Screening

Blastocyst biopsies were screened for chromosome content (for both the detection of gross aneuploidy in PGS patients as well as the detection of malsegregants for translocation PGD patients) as described previously (17). In brief, DNA was amplified with the use of the Sureplex whole genome amplification kit (Rubicon Genomics), then analyzed by means of CGH with the use of either Agilent 60K oligonucleotide microarrays (Agilent Technologies) or Bluegnome 24 Sure BAC arrays (Illumina), or alternatively by NGS performed with the use of the Veriseq PGS kit and Miseq sequencer (Illumina).

For embryos that underwent screening for a single-gene disorder, the patient-specific polymerase chain reaction (PCR) test that was designed specifically for their family before the commencement of the initial stimulation cycle was applied. The test consists of informative short tandem repeat (STR) linkage markers to track the inheritance of the affected chromosome(s) as well as a direct test to detect the familial mutation(s), typically using DNA resequencing or fragment size analysis with the use of capillary electrophoresis. Patients undergoing single-gene testing also underwent chromosome aneuploidy screening to help in the selection of the embryo with the best potential for a positive pregnancy outcome.

For embryos that underwent screening for translocations and where there was involvement of an imprinted chromosome (chromosomes 6, 7, 11, 14, and 15), an additional PCR test with the use of STR markers spanning the length of the specific chromosome was available to patients for embryos where no abnormality was detected in the initial aneuploidy PGS. The presence of both maternal and paternal DNA patterns in the biopsy sample eliminated the risk of uniparental disomy (UPD) for the chromosome. The inclusion of this UPD test was dependent on outcomes from patient counseling with a clinical geneticist and the patient's perspective on invasive prenatal testing further into any established pregnancy.

Vitrification and Warming

Blastocyst vitrification was performed with the use of solutions from the Sydney IVF Blastocyst Vitrification kit (Cook IVF) or Gems Vitrification Set (Genea Biomedx) and the open Cryotop device (Kitazato) or CVM Vitrification Kit (Cryologic) by means of a modified protocol as described in detail previously (6, 18). Alternatively, some embryos from the control group (group 1) were vitrified-warmed using the semiautomated Gavi system (Genea Biomedx) per the manufacturer's instructions. Note that details may not be applicable to imported vitrified blastocysts from other clinics. Embryos were warmed with the use of the Gems Warming Set (Genea Biomedx), then blastocysts were placed in blastocyst medium and allowed to recover for ~2 hours before either embryo transfer or, if required, blastocyst biopsy for PGD and revitrification.

Outcomes Measured

Embryo culture day was based on the number of nights that embryos were cultured, which was necessary owing to group 2 and group 3 embryos being cultured on two separate occasions. Blastocysts were considered to have survived vitrification-warming if $\geq$75% of cells were intact after warming (note that blastocysts not recovered from the vitrification device were considered not to have survived) (19). Maternal age (of patients undergoing a single-embryo transfer) was calculated in years at the time of oocyte retrieval. Biochemical pregnancies were those with $\geq$50 mIU/mL β-hCG in circulating blood at ~11 days after embryo transfer, with the biochemical pregnancy rate calculated as biochemical pregnancies per blastocyst transfers. Fetal heart pregnancies were those with fetal heart motion detected by ultrasound at ~4.5 weeks after embryo transfer, with the fetal heart pregnancy rate calculated as fetal heart pregnancies per blastocyst transfers. Live births were reported for embryo transfers up to September 12, 2016 (only embryo transfers with complete birth data), with the live birth rate calculated as live births per blastocyst transfers (up to September 12, 2016). Subclinical miscarriages were positive biochemical pregnancies that did not result in fetal heart pregnancies, with the subclinical miscarriage rate calculated as subclinical pregnancies per biochemical pregnancies. Clinical miscarriages were fetal heart pregnancies that did not result in live births (including stillbirths), restricted to embryo transfers up to September 12, 2016, with the clinical miscarriage rate calculated as clinical miscarriages per fetal heart pregnancies (embryo transfers up to September 12, 2016). Gestational age of live births was calculated in weeks by determining the number of days between embryo transfer and end of pregnancy plus 19 days, divided by 7. Preterm births were live births born before 37 weeks of gestation. Low birth weight babies were those live newborns born <2,500 g. Small-for-gestation-age newborns were those live births with birth weight below the 10th percentile for gestation age (weeks, rounded down), based on the reference range of Beeby et al. (20). Neonatal deaths were live-born babies that died during the first 28 days of life. Stillbirths were babies of $\geq$20 weeks gestation born with no signs of life.

Statistical Analysis

Statistical analysis of continuous data was performed by means of the Mann-Whitney *U* test owing to a significant skew in data distribution, as demonstrated by the Kolmogorov-Smirnov test (Minitab18). Statistical analysis of contingency tables was performed with the use of Pearson chi-square test or, if any expected frequency was $\leq$4, by a two-tailed Fisher exact test. In cases where the sample size was too large for the Fisher exact test, the *Z* test was used. A *P* value of <.05 was considered to be statistically significant.

RESULTS

The first part of this study examined biopsy outcomes of vitrified embryos warmed with the intention of biopsy for PGD over a 32-month period. There were 376 embryos warmed from a total of 173 patients, with embryos being intended for screening for PGS, inherited genetic disorders (single-gene disorder or chromosome rearrangement cases), or both. The embryos fell into two categories: those that were vitrified without biopsy, and those that were biopsied and vitrified but

TABLE 1

Biopsy outcomes of vitrified embryos warmed for genetic testing.

Outcome	Vitrified embryos	Biopsied vitrified embryos
Warming	234	142
Recovered	233 (99.6%)	142 (100%)
Survived	220 (94.0%)	136 (95.8%)
Fate		
Biopsied	165 (70.5%)[f]	116 (81.7%)[f]
Vitrified	161[c]	102[d]
Embryo transfer[a]	4	12
Discarded	0	2[e]
Nonbiopsied	69 (29.5%)[f]	26 (18.3%)[f]
Vitrified	4	1
Embryo transfer	6	0
Discarded	58	25
Not recovered[b]	1	0

Note: Embryos warmed for biopsy for preimplantation genetic diagnosis (PGD) and/or PGS were either vitrified (without biopsy) or previously biopsied and vitrified but failed to produce a result. Note that of the 234 vitrified nonbiopsied embryos, 52 were transferred from external clinics.

[a] Embryos had same-day PGD testing to provide a result before transfer. The majority of these embryos (13/16) were screened only for single-gene disorders, chromosome rearrangements, or sex screening (for medical purposes), sometimes in combination with chromosome 21 analysis.

[b] Not recovered from the vitrification device after warming.

[c] A subset of these embryos, with no genetic anomalies identified, were warmed for embryo transfer (group 2: once biopsied, twice vitrified embryos).

[d] A subset of these embryos, with no genetic anomalies identified, were warmed for embryo transfer (group 3: twice biopsied, twice vitrified embryos).

[e] These embryos had same-day PGD testing and were discarded because they carried a genetic disorder.

[f] Statistically significant difference between groups ($P<.05$). Note that statistical significant differences were assessed only for outcomes where rates (percentages) are shown.

Bradley. Repeated embryo biopsy and vitrification. Fertil Steril 2017.

failed to produce a PGD result. As presented in Table 1, 70.5% of the nonbiopsied vitrified embryos and 81.7% of the biopsied vitrified embryos that were warmed achieved a biopsy ($P=.016$). For nonbiopsied vitrified embryos, there was no significant difference in the biopsy rate for those embryos transferred from external clinics (n = 52) for PGD testing and those generated internally (n = 182): 63.5% and 72.5%, respectively ($P=.206$; no further data shown). The higher rate of biopsy for vitrified-warmed embryos that were previously biopsied likely reflects the fact that these embryo were earlier found to have been hatching suitable trophectoderm cells to be biopsied. All biopsied embryos were of suitable quality for revitrification or transfer; note that the two biopsied embryos that were discarded were of sufficient quality for use but were found to be abnormal by same-day PGD. Of the biopsied embryos that underwent genetic testing, 50.5% were suitable for clinical use, 43.1% were unsuitable owing to chromosome abnormalities and/or being affected by an inheritable genetic disorder, and 6.4% failed to achieve a test result (Supplemental Table 1; available online at www.fertstert.org).

The second part of this study examined the impact of multiple blastocyst biopsy and vitrification-warming procedures on embryo transfer outcomes over a ~3-year period. Data was sorted into three groups that varied according to the number of times embryos were biopsied and vitrified-warmed: blastocysts biopsied and vitrified-warmed once (group 1; control), blastocysts biopsied once but vitrified-warmed twice (group 2), and blastocysts biopsied twice and vitrified-warmed twice (group 3). The data were restricted to PGD cycles where PGS was performed with the use of comprehensive chromosome screening (CCS), regardless of whether additional screening for single-gene disorders or chromosome rearrangements was performed; the pooling of cases undergoing CCS with or without screening for inherited genetic disorders was justifiable based on similar pregnancy outcomes in the control group (group 1; data not shown). The characteristics at freeze of these embryos and their warming outcomes are presented in Table 2. Embryo quality at final

TABLE 2

Warming outcomes of embryos subjected to multiple blastocyst biopsy and vitrification-warming procedures.

Outcome	Group 1	Group 2	Group 3
Group characteristics			
Biopsy number	1	1	2
Vitrification-warm number	1	2	2
Warms	2183	37	30
Freeze characteristics			
Quality[a]			
Excellent or good	1993 (91.3%)[d]	31 (86.1%)	21 (72.4%)[d]
Poor	190 (8.7%)[d]	5 (13.9%)	8 (27.6%)[d]
Culture day[b,c]			
5	1252 (57.4%)[d,e]	0[d]	0[e]
6	931 (42.6%)[d,e]	28 (80.0%)[d]	18 (60.0%)[e]
7	0[d,e]	7 (20.0%)[d]	11 (36.7%)[e]
8	0[d]	0	1 (3.3%)[d]
Warming outcomes			
Recovered	2177 (99.7%)[d]	37 (100%)	29 (96.7%)[d]
Survived	2148 (98.4%)[d]	36 (97.3%)	28 (93.3%)[d]
Transferred	2149 (98.4%)	36 (97.3%)	29 (96.7%)

Note: Freeze characteristics and warming outcomes refer to the final vitrification-warm prior to embryo transfer.

[a] There was one embryo each for both group 2 and group 3 that could not be assessed for quality before vitrification owing to collapse during or after biopsy.

[b] Culture day for group 2 and group 3 is a best estimate based on the number of nights that an embryo spent in culture.

[c] Missing data for two embryos from group 2 (imported from external clinics).

[d,e] Statistically significant difference between the two marked groups in a row ($P<.05$).

Bradley. Repeated embryo biopsy and vitrification. Fertil Steril 2017.

TABLE 3

Pregnancy outcomes of embryos subjected to multiple blastocyst biopsy and vitrification-warming procedures.

Outcome	Group 1	Group 2	Group 3
Embryo transfers	2130	34	29
Biochemical pregnancies	1331 (62.5%)[b]	19 (55.9%)[c]	11 (37.9%)[b,c]
Fetal heart pregnancies	1156 (54.3%)[b]	16 (47.1%)	9 (31.0%)[b]
Subclinical miscarriages	175 (13.1%)	3 (15.8%)	2 (18.2%)
Live births[a]	734/1468 (50.0%)[b]	10/26 (38.5%)	6/22 (27.3%)[b]
Multiple births[a]	15/734 (2.0%)	0/10 (0%)	0/6 (0%)
Clinical miscarriages[a]	52/786 (6.6%)	0/10 (0%)	0/6 (0%)

Note: All data are single-embryo transfers; data are not presented for double-embryo transfers (nine from group 1 and one from group 2).
[a] Data restricted to transfers up to September 12, 2016. In this time frame there were 1,468, 26, and 22 embryo transfers for groups 1 to 3, respectively.
[b,c] Statistically significant difference between the two marked groups in a row ($P<.05$).

Bradley. Repeated embryo biopsy and vitrification. Fertil Steril 2017.

freeze was considerably lower for those embryos twice biopsied and vitrified-warmed; there were 8.7%, 13.9%, and 27.6% poor-quality embryos from groups 1 to 3, respectively, with the difference between groups 1 and 3 being statistically significant ($P<.001$). This translated into a lower survival rate after warming for transfer for embryos twice biopsied and vitrified-warmed: 98.4%, 97.3%, and 93.3% from groups 1 to 3, respectively, with the difference between groups 1 and 3 being statistically significant ($P=.032$).

Pregnancy outcomes from the transfer of PGD embryos with no detectable abnormalities subjected to multiple blastocyst biopsy and vitrification-warming procedures are presented in Table 3, with patient demographics presented in Supplemental Table 2 (available online at www.fertstert.org). The data demonstrated that pregnancy and birth outcomes were highest in the control group, with a single blastocyst biopsy and vitrification (group 1), followed by the single biopsy but twice vitrified-warmed group (group 2), and lowest in the twice biopsied and vitrified-warmed group (group 3). However, with the exception of the biochemical pregnancy rate, the differences between the groups in outcomes were statistically significant only between group 1 and group 3 transfers. The fetal heart pregnancy rates were 54.3%, 47.1%, and 31.0% for groups 1 to 3, respectively ($P=.013$, group 1 vs. group 3), and live birth rates 50.0%, 38.5%, and 27.3% for groups 1 to 3, respectively ($P=.034$, group 1 vs. group 3). Regarding group 2 embryos, there was no difference in outcomes between embryos transferred from external clinics as nonbiopsied vitrified embryos (n = 11) and those generated internally (n = 23); for example, the fetal heart pregnancy rates were 45.5% and 47.8%, respectively ($P=.888$; no further data shown).

The neonatal outcomes of babies born from embryos subjected to multiple blastocyst biopsy and vitrification-warming procedures are presented in Table 4. Although the low number of births from group 2 (n = 10) and group 3 (n = 6) need to be taken into consideration, the neonatal outcomes appeared similar between the groups. The preterm birth rates were 7.8%, 10.0%, and 0% for groups 1 to 3, respectively, and low birth weight rates were 5.1%, 10.0%, and 0% for groups 1 to 3, respectively.

DISCUSSION

Blastocyst biopsy and vitrification-warming are critical components of a successful PGD program, and both procedures are widely regarded to be safe and effective (5–8). However, in some cases it is necessary to repeat biopsy and/or vitrification-warming procedures on embryos. In our clinic the need for repeated blastocyst vitrification has primarily arisen from patients with already vitrified nonbiopsied blastocysts wishing their embryos to undergo PGS to ensure transfer of euploid embryos, as well as less commonly after the discovery of an inheritable genetic condition in patients with already vitrified embryos. The requirement for repeated blastocyst biopsy has arisen owing to failure of genetic testing of the initial biopsy from PGD cases as well as from PGS cases where patients decline to transfer embryos with unknown

TABLE 4

Neonatal outcomes of babies born from embryos subjected to multiple blastocyst biopsy and vitrification-warming procedures.

Outcome	Group 1	Group 2	Group 3
Singleton births	706	10	6
Live births	704	10	6
Gestational age (wk)[a]	39.1 (38.3–39.9)[b]	38.7 (38.1–38.9)	39.1 (38.5–40.1)
Live birth weight (kg)[a]	3.4 (3.0–3.7)[c]	3.2 (2.7–3.8)	3.4 (3.1–3.8)
Preterm births	55 (7.8%)[b]	1 (10.0%)	0
Low birth weight babies	36 (5.1%)[c]	1 (10.0%)	0
Small for gestational age	55 (7.9%)[c]	2 (20.0%)	1 (16.6%)
Neonatal deaths	1 (0.1%)	0	0
Still births	2	0	0

Note: All data are single-embryo autologous transfers with singleton births, restricted to transfers up to September 12, 2016. Because of the low number of births in group 2 and group 3, statistically comparisons were deemed to be inapplicable.
[a] Median (interquartile range).
[b] Missing data for one case.
[c] Missing data for four cases.

Bradley. Repeated embryo biopsy and vitrification. Fertil Steril 2017.

chromosome status (note that most cases also require a second vitrification-warming to allow sufficient time for genetic testing). These scenarios have resulted in blastocysts biopsied once but vitrified-warmed twice and blastocysts biopsied and vitrified-warmed twice, with little known about the clinical outcomes of these embryos. The present retrospective study analyzed the outcomes of these embryos to shed light on the impact of multiple blastocyst biopsy and vitrification-warming procedures.

When comparing embryo transfer outcomes of once biopsied but twice vitrified-warmed embryos with those of single biopsied and vitrified-warmed embryos, we found that an additional vitrification-warming resulted in lower, though not statistically different, pregnancy outcomes: The fetal heart pregnancy rate was 13% lower and the live birth rate 23% lower. Although this is the largest study to date examining blastocyst outcomes from repeated vitrification-warming, it should be noted that clinical outcomes were derived from only 34 blastocyst transfers (26 for live birth rate) and results might simply reflect low numbers, bias, and confounding. A few other studies have examined outcomes of repeated vitrification (with or without biopsy), but numbers were lower than reported here and data were pooled from both slow-freezing and vitrification cases and/or embryos cryopreserved at various developmental stages (10, 21–23). Our data therefore provide better evidence on the effect of twice vitrifying-warming blastocysts, suggesting that multiple procedures may result in some minor loss of pregnancy potential, although these findings need to be confirmed in larger studies.

When considering whether to perform PGS on nonbiopsied vitrified embryos, thus resulting in single biopsied but twice vitrified-warmed embryos, a number of other outcome measures need to be taken into consideration, including the biopsy rate and abnormality rate. Of the 234 vitrified embryos warmed for biopsy, 29.5% did not achieve a biopsy, the majority of which were discarded. However, of the 165 vitrified-warmed embryos that achieved a biopsy, all of which were of sufficient quality for either revitrification or transfer (same-day PCR testing), 41.2% were found to be unsuitable for clinical use owing to chromosome abnormalities or genetic disorders. Although this abnormality rate would vary depending on maternal age and testing performed (and genetic disorder if relevant), the opportunity to biopsy for PGD would mean that many patients avert a futile embryo transfer by avoiding the use of a clinically unsuitable embryo. In these cases patients would be able to move into a new stimulated IVF cycle faster and avoid the medical issues associated with some pregnancy failures, such as miscarriage, that could result from the presence of chromosome abnormalities typically detected by means of PGS. Whether warming of vitrified blastocysts for biopsy and revitrification to allow for PGS and thus transfer of a euploid embryo is justified compared with transferring a vitrified-warmed untested blastocyst remains to be determined.

In PGD programs it is not uncommon for embryos to fail genetic testing, and in our own clinic we have previously reported a failure rate of 5.0% from more than 5,000 PGS blastocysts with the use of CGH or NGS (17). This is similar to others, including Capalbo et al. who reported a failure rate of 2.7% from almost 1,000 PGS blastocysts with the use of CGH (24) and Minasi et al. who reported a failure rate of 4.9% from 1,122 blastocysts screened for inherited genetic disorders in combination with PGS using CGH (25). We therefore also examined the impact of performing a second blastocyst biopsy because of failed genetic testing. When comparing twice biopsied and vitrified-warmed embryos with single biopsied but twice vitrified-warmed embryos, we found that a second biopsy resulted in a 34% reduction of the fetal heart pregnancy rate and 29% reduction of the live birth rate, although neither difference was statistically significant owing to insufficient data. This reduction in pregnancy outcomes from repeated blastocyst biopsy is in line with Neal et al.'s recent finding that embryos with the highest (4th quartile) DNA biopsy content had significantly lower pregnancy outcomes compared with lower (1st–3rd quartile) DNA concentrations (26). Embryologists must therefore balance the need to obtain enough trophectoderm cells during blastocyst biopsy to achieve a result while minimizing the loss of trophectoderm cells to avoid possible negative effects on embryo potential.

In reality, the retesting of failed PGS embryos requires an additional biopsy and additional vitrification, so we compared the outcomes of twice biopsied and vitrified-warmed embryos with single biopsied and vitrified-warmed embryos. Our results show a large and statistically significant reduction in pregnancy and birth outcomes with a second biopsy and vitrification-warming: The fetal heart pregnancy rate was 43% lower and the live birth rate 45% lower. Again, the low number of embryo transfers, only 29 from twice biopsied and vitrified-warmed embryos (22 for live birth rate), needs to be kept in mind, and it is possible that results may vary between clinics, although we note that our clinic is very experienced with blastocyst biopsy and vitrification (6, 19, 27–29). Our finding that a second biopsy and vitrification-warming significantly reduces the chance of pregnancy should be discussed with patients considering retesting of PGD embryos that fail to produce a result. At the same time, the benefits of repeated testing to allow for transfer of embryos with no detectable abnormalities should also be considered. In some PGS cases it may be advisable to proceed with embryo transfer in the absence of known chromosome status with discussions on prenatal screening options if a pregnancy results. In PGD cases with inherited genetic conditions a second biopsy may be unavoidable, and here, if possible, we would recommend same-day PCR testing followed by transfer of unaffected embryos without revitrification.

This study also examined neonatal outcomes of babies born from embryos subjected to multiple blastocyst biopsy and vitrification-warming procedures. Although the number of births was low, just ten from single biopsied but twice vitrified-warmed embryos and six from twice biopsied and vitrified-warmed embryos, we thought it was prudent to look for "red flags." Although many studies have shown equivalent or better neonatal outcomes from vitrified-warmed embryos compared with fresh transfers (5–7, 30–32), a few studies have associated cryopreserved cycles with increased maternal complications such as placental

disorders and pregnancy-induced hypertension (32–35). Regarding embryo biopsy, a very recent study by Hasson et al. (36) found no association between blastocyst biopsy and adverse obstetrical or neonatal outcomes; that study performed a cohort analysis of blastocyst-biopsied fresh PGD cycles and fresh intracytoplasmic sperm injection cycles (89 and 166 births, respectively). However, it is not inconceivable that there is a threshold for the number of trophectoderm cells that can be biopsied before affecting these outcomes and that this "threshold" might be breached with repeated blastocyst biopsy for some embryos. Although our neonatal outcomes regarding gestational age and birth weight from multiple blastocyst biopsy and vitrification-warming groups are reassuring, unfortunately our procedures do not stringently capture data on pregnancy and neonatal complications (although we note that two cases of gestational diabetes from single biopsied but twice vitrified-warmed births were recorded). Further studies are required to investigate whether multiple blastocyst biopsy and vitrification-warming procedures affect obstetrical, perinatal, and neonatal outcomes.

In summary, our data suggest that performing an additional biopsy or an additional vitrification-warming on blastocysts results in lower but statistically similar outcomes. Whether performing an additional vitrification-warming to allow for PGS of nonbiopsied vitrified embryos is justified in the absence of risk factors considered to outweigh the possible negative consequences remains to be determined. Furthermore, our data suggest that twice biopsying and vitrifying-warming blastocysts to allow for retesting of PGD embryos that fail to produce a result significantly reduces an embryo's pregnancy potential. As such, the current evidence recommends caution when considering performing a second blastocyst biopsy for PGS patients. Patients with genetically inherited conditions who have embryos of unknown status in storage would likely consider the associated risk of not retesting to be greater than the risk of reduced pregnancy potential as a result of multiple biopsy and vitrification-warming procedures.

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