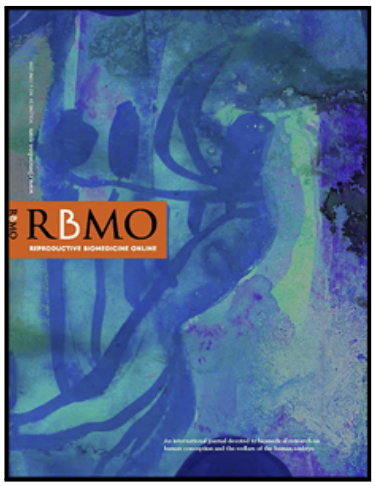
Journal Pre-proof



Multiple cryopreservation-warming cycles, coupled with blastocyst biopsy, negatively impact IVF outcomes

A. Aluko , D.A. Vaughan , A.M. Modest , A.S. Penzias , M.R. Hacker , K.L. Thornton , D. Sakkas

PII: S1472-6483(20)30635-0
DOI: https://doi.org/10.1016/j.rbmo.2020.11.019
Reference: RBMO 2588

To appear in: *Reproductive BioMedicine Online*

Received date: 22 April 2020
Revised date: 13 November 2020
Accepted date: 25 November 2020

Please cite this article as: A. Aluko , D.A. Vaughan , A.M. Modest , A.S. Penzias , M.R. Hacker , K.L. Thornton , D. Sakkas , Multiple cryopreservation-warming cycles, coupled with blastocyst biopsy, negatively impact IVF outcomes, *Reproductive BioMedicine Online* (2020), doi: https://doi.org/10.1016/j.rbmo.2020.11.019

Multiple cryopreservation-warming cycles, coupled with blastocyst biopsy, negatively impact IVF outcomes

AUTHORS

Aluko A[1,2], Vaughan DA[1,2,3], Modest AM[1,2], Penzias AS[1,2,3], Hacker MR[1,2,4], Thornton KL[1,2,3], Sakkas D[3]

1. Department of Obstetrics and Gynecology, Beth Israel Deaconess Medical Center, Boston, MA
2. Department of Obstetrics, Gynecology, and Reproductive Biology, Harvard Medical School, Boston, MA
3. Boston IVF, Waltham, MA
4. Department of Epidemiology, Harvard T. H. Chan School of Public Health, Boston, MA

KEY MESSAGE

In embryos undergoing preimplantation genetic testing for aneuploidy (PGT-A), live birth rates are significantly higher in those subjected to one, as opposed to two, cryopreservation-warming cycles.

ABSTRACT

Research Question: Do multiple cryopreservation-warming cycles, coupled with blastocyst biopsy, negatively impact IVF outcomes?

Design: Patients undergoing in vitro fertilization (IVF) with homologous single embryo transfer, and who underwent trophectoderm biopsy for PGT-A from 2013 through 2017, were divided into 3 groups based on degree of embryonic micromanipulation: once-biopsied, once-cryopreserved (Group BC, n=2603), once-biopsied, twice-cryopreserved (Group CBC, n=95) and twice-biopsied, twice-cryopreserved (Group BCBC, n=15). The primary outcome was live birth; secondary outcomes included positive serum pregnancy test, clinical pregnancy, and miscarriage.

Results: Group CBC had a significantly lower chance of live birth (aRR 0.57, 95% CI 0.41-0.79) and clinical pregnancy (aRR 0.67, 95% CI 0.53-0.85) compared to Group BC. Miscarriage rates were similar between Groups BC (6.6%) and CBC (8.4%).

Conclusions: Multiple cryopreservation-warming cycles, coupled with blastocyst biopsy, negatively impact IVF outcomes. While PGT-A is thought to improve reproductive outcomes on a per transfer basis, caution must be exercised in counseling patients on the possibility of diminishing returns due to further embryonic micromanipulation after an embryo has been cryo-preserved.

Key Words: cryopreservation, vitrification, biopsy, PGT-A, embryo

INTRODUCTION

An expanding body of literature suggests that outcomes in frozen embryo transfer (FET) cycles are at least equivalent, if not superior, to those of fresh embryo transfer cycles(Anon 2016). This has led many assisted reproductive technology (ART) centers to lower the threshold for converting a planned fresh embryo transfer cycle to "cryo-all" or favor planned "cryo-all" over fresh embryo transfer cycles from the outset. The trend in FET cycles has paralleled the increasing utilization of preimplantation genetic testing for aneuploidy (PGT-A). PGT-A as an adjunct to in vitro fertilization (IVF) has evolved from the removal and testing of a single cell after three days of embryo growth to the sampling of multiple nuclei from the syncytium of blastocyst trophectoderm on days five through seven of culture. Numerous laboratory advancements, such as extended embryo culture, the evolution of molecular genetic diagnostics, and near-universal adoption of vitrification in lieu of the slow cooling (commonly referred to as slow freeze) technique, have all influenced this shift globally. The advent of trophectoderm biopsy and vitrification of blastocysts further advanced diagnostic capabilities compared to single cell biopsy (Scott *et al.* 2013b).

The combination of trophectoderm biopsy and cryopreservation of a blastocyst with a FET at a future date involves a single cryopreservation-warming cycle. A second cryopreservation-warming cycle can be performed if results of the fresh biopsy are indeterminate or if a patient with untested cryopreserved blastocysts decides to perform PGT-A. Two independent studies have reported indeterminate result rates of approximately 2.5% (Cimadomo *et al.* 2018, Neal *et al.* 2019). In such cases embryos often are warmed for re-biopsy and analysis, thereby experiencing two biopsies and two cryopreservation-warming cycles prior to intrauterine transfer (Parriego *et al.* 2018). In the case of cryopreserved blastocysts that have not been previously biopsied, PGT-A sometimes is performed for sex selection, testing for newly discovered genetic disorders, or in an attempt to improve outcomes following an unsuccessful

IVF cycle or pregnancy loss. These embryos ultimately undergo one biopsy and two cryopreservation-warming cycles.

These micromanipulations and stressors on the embryo have the potential to influence embryo survival and, ultimately, cycle outcome; however, there is a paucity of high-quality data evaluating the effects of multiple cryopreservation-warming cycles and biopsies on IVF outcomes (Taylor *et al.* 2014, Bradley *et al.* 2017, Neal *et al.* 2017, Cimadomo *et al.* 2018, Parriego *et al.* 2018). Thus, we aimed to characterize reproductive outcomes associated with multiple cryopreservation-warming cycles with primary or repeat trophectoderm biopsy.

MATERIALS AND METHODS

We conducted a retrospective cohort study of patients undergoing IVF at a single university-affiliated infertility practice from July 1, 2013 to July 1, 2017. We included only homologous, single embryo transfer cycles, initially cultured to the blastocyst stage. All aneuploidy testing was performed by trophectoderm biopsy. Cycles were stratified into three groups according to the sequence and number of cryopreservation-warming cycles and/or trophectoderm biopsies: freshly biopsied once prior to cryopreservation (Group BC, where B denotes biopsy and C denotes cryopreservation); biopsied once following initial cryopreservation, then re-cryopreserved (Group CBC); freshly biopsied, cryopreserved, re-biopsied following indeterminate result of the initial biopsy, then re-cryopreserved (Group BCBC). All data was abstracted from electronic medical records. The primary outcome was live birth. Secondary outcomes included positive serum pregnancy test, clinical pregnancy (defined as a sonographic intrauterine pregnancy with a gestational sac) and miscarriage. We also calculated embryo survival rates. This retrospective cohort study was approved by the Institutional Review Board at Beth Israel Deaconess Medical Center (Protocol 2017P000056).

Clinical and Laboratory Procedures

Controlled ovarian stimulation was conducted using gonadotrophins and either a gonadotropin-releasing hormone agonist or antagonist as outlined previously (Vaughan *et al.* 2017). Each cycle was monitored through serum estradiol measurements and transvaginal ultrasound until at least three follicles measured 15 to 20 mm, at which time either 250 micrograms of recombinant human chorionic gonadotropin (hCG), 10,000 units of urinary hCG, or 80 units of leuprolide acetate was typically administered. Oocyte retrieval occurred 36-38 hours after ovulation trigger, and embryos were cultured to the blastocyst stage (day 5, 6 or 7 of culture), at which time they were cryopreserved with or without antecedent trophectoderm biopsy for PGT-A based on provider recommendation and patient preference. All embryos were cryopreserved through vitrification, using the Irvine Vitrification and Warming kits (Irvine Scientific, Santa Ana, CA), per manufacturer instructions. Over 98% of the blastocyst vitrification procedures were performed using Cryolock® vitrification devices. Any non-PGT-A blastocysts were artificially collapsed using a laser prior to cryopreservation. Blastocyst quality at first cryopreservation was classified according to the Gardner alpha-numeric criteria (Gardner *et al*. 2000) and defined as: good (>3 expansion stage with a BB or better), fair (blastocyst with any C grading) and poor (early blastocyst of grade 1 or 2). Upon warming, if a blastocyst exhibited <20% lysis, it was transferred. If the blastocyst exhibited >20% lysis it was rechecked after 30 minutes. If, after 30 minutes, it had deteriorated and exhibited >40% lysis it was not transferred. At our center, more than 90% of trophectoderm biopsies were analyzed by a single laboratory, which reported results as either euploid or aneuploid without characterizing levels of putative embryonic mosaicism.

Over the study period, 1.5% of all analyzed embryos had complex aneuploidies and 7.6% of embryos had whole deletions and duplications. For all cycles in which embryos were biopsied for PGT-A, only euploid embryos (as determined by the testing laboratory) were transferred. Embryo transfers were performed using a standard protocol; luteal support was provided by either

vaginal (Endometrin 100mg three times daily, [Ferring Pharmaceuticals], Crinone 90mg twice daily [Merck Serono]) or intramuscular progesterone (50mg progesterone in oil, daily).

Statistical analysis

Group BC (once-biopsied, once-cryopreserved embryos) served as the reference group. Data are presented as proportion or median and interquartile range (IQR). We calculated risk ratios (RR) and 95% confidence intervals (CI) using generalized estimating equations with an independent correlation matrix to account for repeated cycles from the same woman and cycles using oocytes from the same retrieval. For the primary outcome, we calculated the incidence of live birth, which is reported using the terminology "live birth rate" in order to remain consistent with existing literature. RRs were adjusted for age at retrieval, number of oocytes retrieved, prior miscarriage, day of embryo cryopreservation, number of prior cycles, infertility diagnosis (male factor, female factor, or both), and embryo quality. A p-value <0.05 was considered statistically significant.

RESULTS

Demographics

A total of 2,713 FET cycles met inclusion criteria. In 2,603 cycles, embryos underwent trophectoderm biopsy prior to cryopreservation and were determined to be euploid prior to warming and transfer (Group BC), whereas 95 cycles involved embryos that were biopsied once and cryopreserved twice prior to transfer (Group CBC). In 15 cycles, embryos were biopsied twice and cryopreserved and warmed twice prior to transfer (Group BCBC). From July 2016 to December 2019, 2.3% (386/16,789) of biopsied blastocysts returned an inconclusive result. Of the inconclusive result embryos that were re-biopsied, 135/235 (57.4%) were ultimately determined to be euploid; however, many were not prioritized for transfer. Of the additional re-biopsied embryos, 86 (33.4%) were abnormal and the remainder failed to provide a result.

The median age at retrieval and transfer was similar for all groups. Group BC had the highest proportion of nulligravid patients. The prevalence of secondary infertility and the number of prior cycles were higher in Groups CBC and BCBC compared to Group BC. The patient demographic and cycle characteristics of each group are outlined in Table I.

Embryo Survival

Embryo survival rates following the initial cryopreservation-warming cycle were 98.2%, 94.6% and 93.3% in Groups BC, CBC, and BCBC, respectively. Groups CBC and BCBC underwent a second cryopreservation-warming cycle yielding embryo survival rates of 98.9% and 100.0%, respectively.

Pregnancy Outcomes

The incidence of a positive pregnancy test was higher in Group BC (72.5%), our reference group, than in Group CBC (57.9%). A similar pattern was observed for clinical pregnancy and live birth. The live birth rate was higher in Group BC (55.1%) than in Group CBC (28.4%). A sub-analysis of patients without a previous miscarriage showed the same pattern for the live birth rate in Groups BC (55.1%), CBC (28.4%) and BCBC (53.8%). After controlling for age at retrieval, number of oocytes retrieved, prior miscarriage, day of embryo vitrification, number of prior cycles, reason for ART, and embryo quality, the adjusted RR (aRR) for clinical pregnancy was significantly lower in Group CBC, compared to Group BC (aRR: 0.67, 95% CI: 0.53-0.85). Compared to Group BC, the live birth rate was significantly lower in Group CBC (aRR: 0.57, 95% CI: 0.41-0.79). There was no significant difference in miscarriages between Group BC and CBC. No miscarriages were observed in Group BCBC. Table II shows the risk of clinical outcomes stratified by degree of embryo micromanipulation.

DISCUSSION

In the present study of embryos undergoing PGT-A, clinical outcomes were superior in those subjected to one, as opposed to two, cryopreservation-warming cycles. Embryos that underwent a single biopsy and two cryopreservation-warming cycles (Group CBC) had lower clinical pregnancy and live birth rates compared to embryos that underwent a single biopsy and a single cryopreservation-warming cycle (Group BC). This study provides sufficient numbers of this currently rare event to serve as preliminary insight into the effect that the growing utilization of double vitrification and/or double biopsy has on outcomes.

We postulate that the mechanical trauma of multiple cryopreservation-warming cycles in the setting of trophectoderm biopsy is detrimental to the embryo and impacts reproductive outcomes. Interestingly, the live birth rate in our twice-cryopreserved cohort (Group CBC, 28.4%) was lower than what has been reported in prior studies—38.5%[5], 50.0%[6], and 63.2%[7] (Table III). While institutional variability in laboratory technique may account for these discrepancies, inherent differences in patient characteristics also may play a role. Patients who are at high risk for poor outcomes or who have a history of failed IVF cycles may elect to biopsy previously cryopreserved embryos in an effort to optimize subsequent cycles. In fact, we observed a higher number of prior cycles in group CBC than the reference group. Although we attempted to adjust for potential confounders, certain negative prognostic factors, such as differences in clinical protocols and blastocyst culture conditions, may have remained unaccounted for.

Survival rates after the second warm were comparable (>98%) among the study groups. However, we were surprised to find a small decrease in survival rates after initial warm in group CBC, when compared to group BC. Because the vitrification process remained standardized over our study period, we postulate that differential survival may be due to differences in embryo quality between the groups. As such, we adjusted for embryo quality in our analysis of pregnancy outcomes.

In our study, the small sample size of embryos exposed to two trophectoderm biopsies and cryopreservation-warming cycles precluded our ability to draw meaningful conclusions regarding this particular cohort (Group BCBC). Patients and clinicians rarely prioritize the transfer of embryos that have undergone multiple biopsies. Consequently, few studies have characterized IVF outcomes in this group, with conflicting results (Cimadomo *et al.* 2018). The largest study to date on IVF cycles involving re-biopsied embryos (n=49) reported comparable live birth rates after trophectoderm re-biopsy and re-vitrification (38.8%) and standard frozen embryo transfer of an euploid embryo (42.9%) (Cimadomo *et al.* 2018). These results contrast a recent analysis of 36 re-biopsied embryos, which demonstrated a statistically lower ongoing pregnancy rate in re-biopsied embryos (50.0%), compared to those that had experienced a single biopsy and vitrification/warming cycle (66.8%) (Neal *et al.* 2019). Of note, Neal et al found no difference in outcomes when comparing re-biopsied embryos to embryos that had undergone one biopsy and two vitrification/warming cycles (Neal *et al.* 2019). The mechanism by which multiple biopsies impact reproductive outcomes remains speculative. An analysis of the relative content of DNA in single trophectoderm biopsies found that biopsy samples that contained high amounts of DNA were associated with lower live birth rates. This suggests that high cellular content of a biopsy sample may either diminish the accuracy of PGT-A diagnosis or mechanically damage the embryo itself (Neal *et al.* 2017). Further studies are necessary to characterize reproductive outcomes in embryos that have undergone multiple biopsies; however, it may be muddied by the observation that laboratories may differ in their ability to perform PGT-A and vitrification (Munné *et al.* 2017).

It is difficult to parse out reasons for the poorer outcomes noted in Group CBC in the present study. A number of studies looking at the individual manipulations have not shown detrimental effects (Kumasako *et al.* 2009, Koch *et al.* 2011, Stanger *et al.* 2012). For example, a single vitrification cycle alone has been shown to be highly efficient (Rienzi *et al.* 2017). In contrast, vitrification following slow freezing leads to a significant decrease in live birth rates

(Zheng *et al.* 2017). In mouse re-vitrification experiments, there were no ill effects on blastocyst development; however, there were fewer fetuses per embryo transferred in the re-vitrified group (Sheehan *et al.* 2006). This could indicate that some cell loss does occur, which may not impact blastocyst development but could have consequences for fetal development. We reviewed data on 10 twice-vitrified, un-biopsied blastocysts at our institution from July 1, 2013 to July 1, 2017. Eight out of the 10 embryos survived the second warm, 5 were transferred, and 2 resulted in a live birth (the remaining 3 transfers failed to result in a positive pregnancy test). A second vitrification in the absence of embryo biopsy was uncommonly performed at our institution and reserved for cases in which a uterine transfer was unexpectedly cancelled due to a patient "no-show," a last-minute decision to transfer one as opposed to two embryos, or technical inability to perform the procedure. In addition to the above, the practice of multiple freezing has been reported in a number of studies (Macnamee *et al.* 1990, Kumasako *et al.* 2009, Koch *et al.* 2011, Stanger *et al.* 2012) however a robust investigation into the impact is still lacking.

In relation to trophectoderm biopsy, Scott et al. performed double transfers of biopsied and non-biopsied blastocysts and tracked live births by DNA fingerprinting (Scott *et al.* 2013b). When considering all embryos transferred, 51% of biopsied embryos and 54% of un-biopsied embryos had sustained implantation and progressed to delivery. They concluded that trophectoderm biopsy did not have a significant impact on reproductive outcomes (Scott *et al.* 2013b). Contrary to this report, Neal et al found that trophectoderm biopsies with high relative DNA content were associated with a lower live birth rate, suggesting that biopsy size (and cell number reduction) may affect outcomes (Neal *et al.* 2017). Timing of the biopsy may have some unknown consequence in relation to our study. There may be a difference between a biopsy of a blastocyst prior to vitrification and after, leading to the speculation that differences may result due to the biopsy timing relative to vitrification, rather than the number of cryopreservation cycles. A final variable to consider is the time outside the incubator and manipulation of the embryos. Although these different steps have been shown to be sufficiently safe individually, it is

plausible that their cumulative effect could explain our findings. The American Society for Reproductive Medicine practice committee report on PGT-A concluded that PGT-A likely will be part of a future multidimensional approach to embryo screening and selection, although there is insufficient evidence to recommend the routine use of blastocyst biopsy with aneuploidy testing in all infertile patients (Penzias 2018). The report made no recommendation on thaw/warm, biopsy, and re-vitrification for PGT-A due to paucity of data.

The overall benefit of PGT-A remains controversial with conflicting data. The advantages and disadvantages vary, depending on whether data is examined on a per retrieval or a per transfer basis (Scott *et al.* 2013a, Forman *et al.* 2014, Penzias 2018, Munné *et al.* 2019, Murphy *et al.* 2019). The recently published Single Embryo Transfer of Euploid Embryo (STAR) trial represents the best insight into the current status of the practice of PGT-A, where randomization occurred with at least 2 high quality blastocysts available for transfer, and outcomes were reported on an intention to treat or on a per transfer basis (Munné *et al.* 2019). While the authors found a statistically significant increase in ongoing embryo implantation rates in patients over the age of 35, no increase in implantation rates was observed in the younger age group (Munné *et al.* 2019).

The examination of outcomes derived from embryos with varying degrees of micromanipulation is relevant as PGT-A becomes increasingly utilized and the present study adds substantially to the small body of literature on this topic. A key strength of this study is that all embryos were vitrified in the same fashion and all transfers were performed at a single university-affiliated center with limited change in clinical practice over the course of the study. Culture media, culture volumes, incubators, gas phase and air handling systems were also the same during the study period. The study is limited by its retrospective nature, which led to incomplete data for certain covariates and the need to pool different quality blastocysts in different study groups. An additional limitation was our inability to assess the effects of delayed vitrification (i.e. warming

blastocysts then culturing them overnight prior to re-vitrification), as this allows time for embryos to "heal," and may have positive effects (Vitale *et al.* 1997).

In conclusion, our study emphasizes the need to take pause and consider the degree to which we manipulate embryos in the quest to transfer a "euploid" embryo. These stressors and micromanipulation techniques may negatively impact clinical pregnancy rates and ultimately lower live birth rates. In addition, the long-term effects of these additional stressors on offspring are unknown. Further studies are necessary to characterize outcomes in embryos with varying degrees of embryonic micromanipulation and to determine which patients may benefit most from these additional techniques.

ACKNOWLEDGEMENTS

None

FUNDING

This work was conducted with support from Harvard Catalyst | The Harvard Clinical and Translational Science Center (National Center for Advancing Translational Sciences, National Institutes of Health Award UL 1TR002541) and financial contributions from Harvard University and its affiliated academic healthcare centers.

CONFLICT OF INTEREST

No conflicts of interest to declare

Table I: Baseline demographics and clinical characteristics for each cycle stratified by the degree of embryo micromanipulation

	Group BC n=2603	Group CBC n=95	Group BCBC n=15
Number of biopsies	1	1	2
Number of cryopreservation-warming cycles	1	2	2
Age at retrieval (years)	36.2 (33.5 – 38.9)	33.9 (31.7 – 37.7)	37.0 (34.9 – 38.8)
Age at transfer (years)	36.7 (33.8 – 39.3)	35.0 (32.0 – 39.1)	37.5 (35.1 – 39.1)
Body mass index (kg/m^2)	24.0 (21.6 – 27.5)	24.0 (21.6 – 27.5)	24.9 (23.1 – 27.5)
Current smoker	19 (0.7)	1 (1.1)	0 (0.0)
Type of infertility			
Primary	1865 (71.6)	40 (42.1)	8 (53.3)
Secondary	738 (28.4)	55 (57.9)	7 (46.7)
Reason for IVF			
Male infertility	517 (19.9)	24 (25.3)	4 (26.7)
Female infertility	1605 (61.7)	39 (41.1)	7 (46.7)
Other	423 (16.3)	8 (8.4)	3 (20.0)
Unexplained	58 (2.2)	24 (25.3)	1 (6.7)
Gravidity			
0	1865 (71.6)	40 (42.1)	8 (53.3)
1	263 (10.1)	21 (22.1)	2 (13.3)
2+	475 (18.2)	34 (35.8)	5 (33.3)
Prior miscarriage			
0	2223 (85.4)	88 (92.6)	13 (86.7)
1	222 (8.5)	6 (6.3)	1 (6.7)
2+	158 (6.1)	1 (1.1)	1 (6.7)
Parity			
0	2202 (84.6)	67 (70.5)	8 (53.3)
1	308 (11.8)	24 (25.3)	7 (46.7)
2+	93 (3.6)	4 (4.2)	0 (0.0)
Number of prior cycles	1 (0 – 2)	3 (2 – 4)	2 (1 – 3)
Oocytes retrieved in fresh cycle	15 (10 – 21)	20 (14 – 30)	11 (7 – 21)
ICSI			
Yes	1447 (55.6)	38 (40.0)	9 (60.0)
No	1156 (44.4)	57 (60.0)	6 (40.0)
Day of embryo cryopreservation			
5	1528 (58.7)	80 (84.2)	11 (73.3)
6	1058 (40.6)	15 (15.8)	4 (26.7)
7	17 (0.7)	0 (0.0)	0 (0.0)
Embryo quality at first cryopreservation			
Good	2446 (94.0)	92 (96.8)	14 (93.3)
Fair	21 (0.8)	3 (3.2)	1 (6.7)
Poor	126 (4.8)	0 (0.0)	0 (0.0)
Unknown	10 (0.4)	0 (0.0)	0 (0.0)

Endometrial thickness at embryo transfer (mm)	9.3 (8.1 – 10.8)	8.8 (7.9 – 10.2)	8.5 (7.7 – 12.1)
Natural cycle frozen embryo transfer	314 (12.1)	7 (7.4)	3 (20.0)

Data presented as median (interquartile range) or n (%)
IVF: *In vitro* fertilization; ICSI: intracytosplasmic sperm injection

Table II: Clinical outcomes per embryo transfer stratified by the degree of embryo micromanipulation

	Group BC n=2603	Group CBC n=95	Group BCBC n=15
Number of biopsies	1	1	2
Number of cryopreservation-warm cycles	1	2	2
Positive pregnancy test	1888 (72.5)	55 (57.9)	9 (60.0)
Crude RR (95% CI)	Reference	0.80 (0.67 – 0.95)	0.83 (0.55 – 1.2)
Adjusted RR (95% CI)*	Reference	0.82 (0.68 – 0.99)	0.84 (0.57 – 1.3)
Clinical pregnancy[§]	1624 (62.4)	37 (38.9)	7 (46.7)
Crude RR (95% CI)	Reference	0.62 (0.50 – 0.78)	0.75 (0.47 – 1.2)
Adjusted RR (95% CI)*	Reference	0.67 (0.53 – 0.85)	0.77 (0.48 – 1.2)
Miscarriage	171 (6.6)	8 (8.4)	0 (0.0)
Crude RR (95% CI)	Reference	1.2 (0.67 – 2.5)	-
Adjusted RR (95% CI)*	Reference	1.3 (0.64 – 2.7)	-
Live birth	1434 (55.1)	27 (28.4)	7 (46.7)
Crude RR (95% CI)	Reference	0.52 (0.38 – 0.71)	0.85 (0.53 – 1.3)
Adjusted RR (95% CI)*	Reference	0.57 (0.41 – 0.79)	0.87 (0.55 – 1.4)

Data are presented as n (%) or risk ratio (RR) and 95% confidence interval (CI).

* Adjusted for age at retrieval, number of oocytes retrieved, prior miscarriage, day of embryo vitrification, number of prior cycles, reason for *in vitro* fertilization, embryo quality

[§] Pregnancy outcomes were missing for 12 clinical pregnancies in Group BC, 1 in Group CBC. There were 7 stillbirths in Group BC and 1 stillbirth in Group CBC. Therefore, the number of live births and miscarriages do not sum to the number of clinical pregnancies

- Risk ratios could not be calculated due to zero outcomes.

Table III: Summary of literature on pregnancy outcomes following multiple biopsies and vitrification-warming cycles

	Group BC	Group CBC	Group BCBC
Number of biopsies	1	1	2
Number of vitrification-warming cycles	1	2	2
Live birth rate			
Bradley et al. 2017	50.0% (734/1468)	38.5% (10/26)	27.3% (6/22)
Ciamodo et al. 2018	42.9% (1211/2825)	Not reported	38.8% (19/49)
Aluko et al. 2020 [§]	52.5% (442/842)	28.9% (22/76)	41.7% (5/12)
Composite outcome of ongoing pregnancy and live birth rate			
Taylor et al. 2014	54.0% (61/113)	50.0% (6/12)	0.0% (0/2)
Ongoing pregnancy rate			
Neal et al. 2019	66.8% (2366/3542)	63.2% (98/155)	50.0% (18/36)

Percentages indicate live birth rate. Denominators represent the total number of embryos in each cohort. Numerators represent the total number of embryos that resulted in a live birth.

[§] Present study

References

Anon, 2016. Assisted reproductive technology national summary report. Centers for Disease Control and Prevention, American Society for Reproductive Medicine, Society for Assisted Reproductive Technology.

Bradley, C.K., Livingstone, M., Traversa, M.V., McArthur, S.J., 2017. Impact of multiple blastocyst biopsy and vitrification-warming procedures on pregnancy outcomes. Fertility and sterility. 108, 999-1006

Cimadomo, D., Rienzi, L., Romanelli, V., Alviggi, E., Levi-Setti, P.E., Albani, E., Dusi, L., Papini, L., Livi, C., Benini, F., Smeraldi, A., Patassini, C., Ubaldi, F.M., Capalbo, A., 2018. Inconclusive chromosomal assessment after blastocyst biopsy: Prevalence, causative factors and outcomes after re-biopsy and re-vitrification. A multicenter experience. Human reproduction (Oxford, England). 33, 1839-1846

Forman, E.J., Hong, K.H., Franasiak, J.M., Scott, R.T., 2014. Obstetrical and neonatal outcomes from the best trial: Single embryo transfer with aneuploidy screening improves outcomes after in vitro fertilization without compromising delivery rates. American journal of obstetrics and gynecology. 210, 157.e1-6

Gardner, D.K., Lane, M., Stevens, J., Schlenker, T., Schoolcraft, W.B., 2000. Blastocyst score affects implantation and pregnancy outcome: Towards a single blastocyst transfer. Fertility and sterility. 73,1155-8.

Koch, J., Costello, M.F., Chapman, M.G., Kilani, S., 2011. Twice-frozen embryos are no detriment to pregnancy success: A retrospective comparative study. Fertility and sterility. 96, 58-62

Kumasako, Y., Otsu, E., Utsunomiya, T., Araki, Y., 2009. The efficacy of the transfer of twice frozen-thawed embryos with the vitrification method. Fertility and sterility. 91, 383-6

Macnamee, M., Wick, K., Rainsbury, P., Sathanandan, M., Brinsden, P., Edwards, R.G., 1990. How robust are early human embryos? Lancet (London, England). 336, 636

Munné, S., Alikani, M., Ribustello, L., Colls, P., Martinez-Ortiz, P.A., McCulloh, D.H., Group, R.P., 2017. Euploidy rates in donor egg cycles significantly differ between fertility centers. Human reproduction (Oxford, England). 32, 743-749

Munné, S., Kaplan, B., Frattarelli, J.L., Child, T., Nakhuda, G., Shamma, F.N., Silverberg, K., Kalista, T., Handyside, A.H., Katz-Jaffe, M., 2019. Preimplantation genetic testing for aneuploidy versus morphology as selection criteria for single frozen-thawed embryo transfer in good-prognosis patients: A multicenter randomized clinical trial. Fertility and sterility. 112, 1071-1079.e7

Murphy, L.A., Seidler, E.A., Vaughan, D.A., Resetkova, N., Penzias, A., Toth, T.L., Thornton, K.L., Sakkas, D., 2019. To test or not to test? A framework for counselling patients on preimplantation genetic testing for aneuploidy (pgt-a). Human reproduction (Oxford, England). 34, 268-275

Neal, S.A., Franasiak, J.M., Forman, E.J., Werner, M.D., Morin, S.J., Tao, X., Treff, N.R., Scott, R.T., 2017. High relative deoxyribonucleic acid content of trophectoderm biopsy adversely affects pregnancy outcomes. Fertility and sterility. 107, 731-736.e1

Neal, S.A., Sun, L., Jalas, C., Morin, S.J., Molinaro, T.A., Scott, R.T., 2019. When next-generation sequencing-based preimplantation genetic testing for aneuploidy (pgt-

a) yields an inconclusive report: Diagnostic results and clinical outcomes after re biopsy. Journal of assisted reproduction and genetics. 36, 2103-2109

Parriego, M., Coll, L., Vidal, F., Boada, M., Devesa, M., Coroleu, B., Veiga, A., 2018. Inconclusive results in preimplantation genetic testing: Go for a second biopsy? Gynecological endocrinology : the official journal of the International Society of Gynecological Endocrinology. 35, 90-92

Penzia,s A, Bendikson, K., Butts, S., Coutifaris, C., Falcone, T., Fossum, G., Gitlin, S., Gracia, C., Hansen, K., La Barbera, A., 2018. The use of preimplantation genetic testing for aneuploidy (pgt-a): A committee opinion. Fertility and sterility. 109, 429-436

Rienzi, L., Garcia, C., Maggiulli, R., LaBarbera, A.R., Kaser, D.J., Ubaldi, F.M., Vanderpoel, S., Racowsky, C., 2017. Oocyte, embryo and blastocyst cryopreservation in art: Systematic review and meta-analysis comparing slow-freezing versus vitrification to produce evidence for the development of global guidance. Human reproduction update. 23, 139-155

Scott, R.T., Upham, K.M., Forman, E. J., Hong, K. H., Scott, K.L., Taylor, D., Tao, X., Treff, N.R., 2013a. Blastocyst biopsy with comprehensive chromosome screening and fresh embryo transfer significantly increases in vitro fertilization implantation and delivery rates: A randomized controlled trial. Fertility and sterility. 100, 697-703

Scott, R.T., Upham, K.M., Forman, E.J., Zhao, T., Treff, N.R.,, 2013b. Cleavage-stage biopsy significantly impairs human embryonic implantation potential while

blastocyst biopsy does not: A randomized and paired clinical trial. Fertility and sterility. 100, 624-30

Sheehan, C.B., Lane, M., Gardner, D.K., 2006. The cryoloop facilitates re-vitrification of embryos at four successive stages of development without impairing embryo growth. Human reproduction (Oxford, England). 21, 2978-84

Stanger, J., Wong, J., Conceicao, J., Yovich, J., 2012. Vitrification of human embryos previously cryostored by either slow freezing or vitrification results in high pregnancy rates. Reproductive biomedicine online. 24, 314-20

Taylor, T.H., Patrick, J.L., Gitlin, S.A., Michael, W.J., Crain, J.L., Griffin, D.K., 2014. Outcomes of blastocysts biopsied and vitrified once versus those cryopreserved twice for euploid blastocyst transfer. Reproductive biomedicine online. 29, 59-64

Vaughan, D.A., Leung, A., Resetkova, N., Ruthazer, R., Penzias, A.S., Sakkas, D., Alper, M., 2017. How many oocytes are optimal to achieve multiple live births with one stimulation cycle? The one-and-done approach. Fertility and sterility. 107, 397-404.e3

Vitale, N.J., Myers, M.W., Denniston, R.S., Leibo, S.P., Godke, R.A., 1997. In-vitro development of refrozen mouse embryos. Human reproduction (Oxford, England). 12, 310-6

Zheng, X., Chen, Y., Yan, J, Wu, Y., Zhuang, X., Lin, S., Zhu, J., Lian, Y., Qiao, J., Liu, P., 2017. Effect of repeated cryopreservation on human embryo developmental potential. Reproductive biomedicine online. 35, 627-632

AUTHOR BIOGRAPHY

Dr. Ashley Aluko is a chief resident in Obstetrics and Gynecology at Beth Israel Deaconess Medical Center. She received her medical degree at Harvard Medical School. Dr. Ashley Aluko will begin her fellowship in Reproductive Endocrinology and Infertility at the Ronald O. Perelman and Claudia Cohen Center for Reproductive Medicine (CRM) in July 2020.