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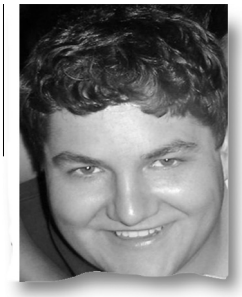
ARTICLE

Outcomes of blastocysts biopsied and vitrified once versus those cryopreserved twice for euploid blastocyst transfer

Tyl H Taylor [a,b], Jennifer L Patrick [a], Susan A Gitlin [c], J Michael Wilson [a], Jack L Crain [a], Darren K Griffin [b,*]

[a] *Reproductive Endocrinology Associates of Charlotte, 1524 E. Morehead St., Charlotte, NC 28207, USA;* [b] *University of Kent, Department of Biosciences, Canterbury CT2 7NJ, United Kingdom;* [c] *Eastern Virginia Medical School, Jones Institute for Reproductive Medicine, Department of Obstetrics and Gynecology, 601 Colley Avenue #316, Norfolk, VA 23507, USA*

[*] Corresponding author. *E-mail address:* d.k.griffin@kent.ac.uk (DK Griffin).



Tyl H Taylor, MS is currently pursuing his doctorate in genetics at the University of Kent in the UK. Tyl was born in Tennessee, grew up in Nevada and graduated from Randolph-Macon College in Virginia. He is currently an embryologist for Reproductive Endocrinology Associates of Charlotte.

Abstract Trophectoderm biopsy with comprehensive chromosome screening (CCS) has been shown to increase implantation and pregnancy rates. Some patients desire CCS on previously cryopreserved blastocysts, resulting in blastocysts that are thawed/warmed, biopsied, vitrified and then warmed again. The effect of two cryopreservation procedures and two thawing/warming procedures on outcomes has not been effectively studied. Cycles were divided into two groups: group 1 patients underwent a cryopreserved embryo transfer with euploid blastocysts that were vitrified and warmed once; group 2 patients had a cryopreserved embryo transfer of a euploid blastocyst that was cryopreserved, thawed/warmed, biopsied, vitrified and warmed. Groups 1 and 2 included 85 and 17 women aged 35.6 ± 3.9 and 35.3 ± 4.9 years, respectively (not significantly different). Blastocyst survival in group 1 (114/116, 98.3%) and survival of second warming in group 2 (21/24, 87.5%) was significantly different ($P = 0.0354$). There was no difference between biochemical (68.2% and 62.5%) and clinical (61.2% and 56.3%) pregnancy rates, implantation rate (58.4% and 52.4%) and live birth/ongoing pregnancy rate (54.0% and 47.6%) between groups 1 and 2, respectively. Although it is unconventional to thaw/warm, biopsy, revitrify and rewarm blastocysts for cryopreserved embryo transfer, the results indicate that outcomes are not compromised.

KEYWORDS: aneuploidy, blastocyst, biopsy, IVF, vitrification, warming

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Introduction

Typically, only good-quality blastocysts derived from a fresh cycle of IVF are utilized in trophectoderm biopsy with comprehensive chromosome screening (CCS). This approach has yielded pregnancy outcomes higher than standard morphological assessment alone (Scott et al., 2013a). Clinical pregnancy rates utilizing this technology are 60–75%, which is comparable to anonymous oocyte donation (Grifo et al., 2013). However promising, the utilization of this technology is typically limited to blastocysts derived from fresh IVF cycles.

In order to benefit from trophectoderm biopsy and CCS, a patient has to undergo a fresh IVF procedure or have zygote- or cleavage-stage embryos previously cryopreserved thawed and cultured to the blastocyst stage. There are a large number of patients who have had IVF previously and have good-quality, unbiopsied blastocysts cryopreserved (Zhu et al., 2013). These patients could simply want to utilize the current technology or they have previous outcomes that may warrant utilization of CCS with trophectoderm biopsy. For example, if a patient suffered a miscarriage or had failed attempts with fresh embryos, they may choose to utilize trophectoderm biopsy and CCS on previously cryopreserved blastocysts to allow for the transfer of a euploid embryo.

Blastocyst biopsy involves the removal of 3–10 cells from the trophectoderm of blastocysts on either day 5 or 6 of culture (Scott et al., 2013b). If a blastocyst is biopsied on day 5, it is possible to get results by day 6 for a fresh transfer. Most clinics do not conduct on-site CCS; therefore, a majority of the time, blastocysts are vitrified post biopsy. Current research indicates that the transfer of an embryo into an unstimulated uterus may yield higher pregnancy outcomes than a transfer during a fresh cycle (Shapiro et al., 2011).

After biopsy, the sample is sent to the genetics laboratory while the blastocysts remain cryopreserved awaiting results. Even in the hands of the most experienced embryologist and geneticists, readings are not possible 100% of the time (Harton et al., 2011). Therefore, it is possible that the CCS report would reveal a 'no result'. In this particular instance, the patients are left with a cryopreserved blastocyst that has no genetic result.

Although biopsy, obtaining CCS results and transfer without the need to vitrify can be achieved, particularly with methods such as 4-h quantitative real-time PCR, this approach cannot be utilized by every IVF clinic due to logistics (Treff and Scott, 2013). Because of this, blastocysts with a 'no result', as well as blastocysts that have been previously cryopreserved without undergoing trophectoderm biopsy during the fresh cycle, would need to be thawed/warmed for biopsy or rebiopsy and subsequently cryopreserved again while awaiting CCS results. Furthermore, if euploid, these blastocysts would undergo an additional warming procedure before being transferred into the uterus. Few studies have focused on patients that have previously cryopreserved blastocysts, which undergo thawing/warming, biopsy, vitrifying and a second warming prior to a cryopreserved embryo transfer. The purpose of this study was to test the hypothesis that blastocysts that were previously cryopreserved can be successfully utilized for subsequent trophectoderm biopsy and CCS and to determine the clinical efficiency of those blastocysts when used in a subsequent cryopreserved embryo transfer cycle.

Materials and methods

This retrospective chart review was deemed exempt by Sterling Institutional Review Board. Patients attending Reproductive Endocrinology Associates of Charlotte from 1 January 2009 to 31 April 2013 were included in this study. Cycles were subdivided into two groups (**Figure 1**). Group 1 ($n = 85$ cycles, 116 blastocysts) consisted of patients who underwent the traditional method of trophectoderm biopsy and CCS, by having their oocytes retrieved via IVF, embryos cultured to the blastocyst stage and all viable blastocysts biopsied and vitrified according to laboratory protocol. Group 2 consisted of cycles that had cryopreserved blastocysts and subsequently desired to have their blastocysts biopsied ($n = 19$ cycles, 70 blastocysts) or those who desired a rebiopsy due to a 'no result' ($n = 2$ cycles, three blastocysts). Outcomes consisted of biochemical pregnancy (positive β-human chorionic gonadotrophin test), clinical pregnancy (visualization of gestational sac on ultrasound), fetal cardiac activity and ongoing/live birth rate.

In-vitro fertilization and embryo culture

Oocytes were retrieved under ultrasound guidance and placed in HEPES-buffered solution (Cooper Surgical, Trumbull, CT, USA) and 10% serum protein substitute (Cooper Surgical) overlaid with oil (Irvine Scientific, Santa Ana, CA, USA). All oocytes were designated for intracytoplasmic sperm injection and trimmed and stripped of excess cumulus cells, as described by Taylor et al. (2006). Oocytes were separated based on maturity and placed back into the incubator. After 2 h, all mature oocytes underwent intracytoplasmic sperm injection (Nagy et al., 1995).

Because this study occurred over a long period of time, two different culture systems were utilized. From January 2009 to August 2012, sequential media (Cooper Surgical) and 10% serum protein substitute overlaid with oil was used. From September 2012 to April 2013, continuous sequential culture media (Irvine Scientific) and 10% serum substitute supplement (Irvine Scientific) overlaid with oil was utilized. Regardless of culture system, all oocytes and embryos were cultured in 95% N_2, 5% CO_2 and 98% humidity.

Trophectoderm biopsy and array comparative genomic hybridization

With the aid of a laser (Zilos-tk; Hamilton Thorne, Beverly, ME, USA), all embryos from group 1 underwent assisted hatching on day 3. Group 2 included blastocysts previously cryopreserved, these were thawed/warmed and assisted hatching was performed during the thawing/warming procedure while the blastocyst was compacted. Only blastocysts that presented with a good-quality inner cell mass and trophectoderm were biopsied. Blastocysts were placed in a drop of modified human tubal fluid (Irvine Scientific) and 10% serum substitute supplement. Gentle suction was

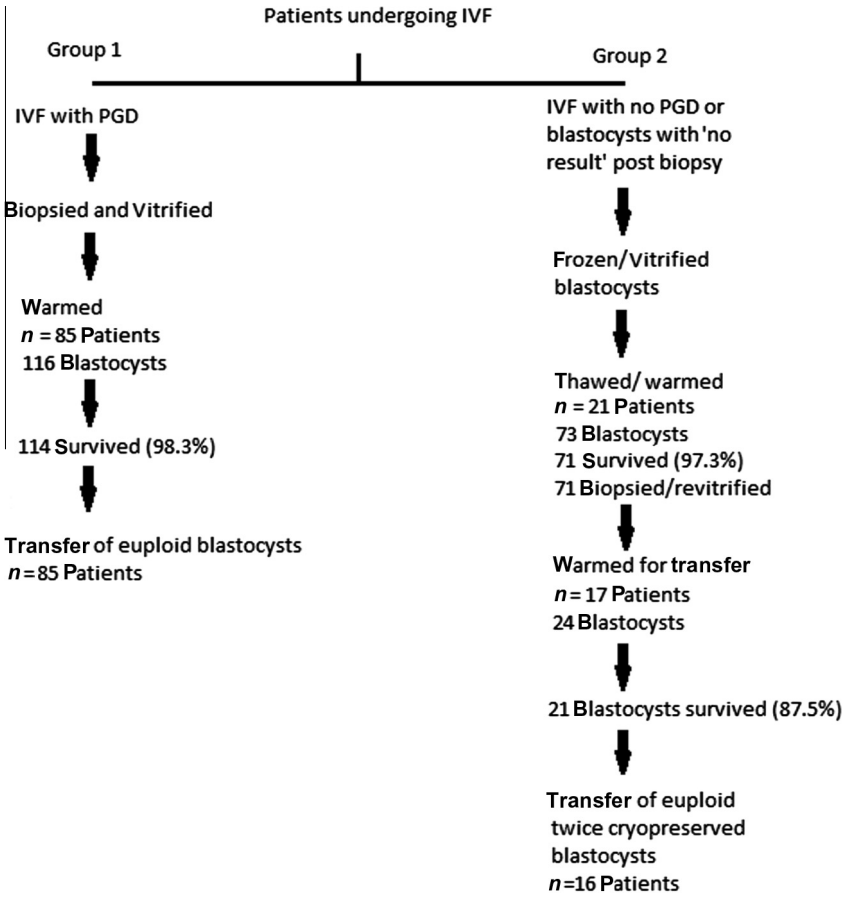

Figure 1 Flow chart of study design. PGD = preimplantation genetic diagnosis.

applied to the blastocyst via a holding pipette (Cook Medical, Bloomington, IL, USA). The hatching trophectoderm was located at the 3 o'clock position. A biopsy pipette (Cook Medical) was used to gently aspirate the trophectoderm into the bore of the needle. Laser pulses were used to 'cut' the trophectoderm away from the blastocyst. Care was taken to minimize the number of laser pulses needed to remove a piece of the trophectoderm. The piece of trophectoderm was prepared for array comparative genomic hybridization (Genesis Genetics, Detroit, MI, USA).

Briefly, the tube with the sample of the trophectoderm was amplified according to manufacturer's instructions (Sureplex; Rubicon Genomics/Bluegnome, Ann Arbor, MI, USA). Those samples that produced an amplification result were labelled with Cy3 dye for sample DNA and Cy5 dye for reference male DNA (Bluegnome), according to manufacturer's instructions. Separately, the sample DNA and reference DNA were denatured at 74°C. After hybridization, the sample and test DNA were mixed together and added to the microarray (Bluegnome). The microarrays were hybridized overnight at 47°C. After hybridization, microarrays were washed at room temperature for 10 min in 2 × saline sodium citrate (SSC) with 0.05% Tween 20, 10 min in 1 × SSC, 5 min in 0.1 × SSC at 60°C and finally 2 min in 0.1 × SSC. Microarray slides were scanned and analysed by Bluefuse (Bluegnome) (Harton et al., 2013).

Vitrification

Blastocysts were individually vitrified immediately following trophectoderm biopsy. Blastocysts were placed in equilibration solution (Irvine Scientific) for 15 min, rinsed in vitrification solution (Irvine Scientific) for $<$1 min, placed on a Cryolock (Biodiseno, Atlanta, Georgia, USA) and plunged into liquid nitrogen.

Warming

The Cryolock was uncapped under liquid nitrogen and plunged into 37°C warming solution (Irvine Scientific). The blastocyst was 'knocked' off the cryolock and left in the warming solution for 1 min. The blastocyst was transferred to dilute solution (Irvine Scientific) for 3 min and finally washing solution (Irvine Scientific) for 10 min. For group 2, where the blastocyst had not been subjected to assisted hatching in the fresh cycle, assisted hatching was performed in the washing solution. After warming, blastocysts were placed into continuous culture media and 20% serum substitute supplement overlaid with oil. The culture media was changed in October 2013, therefore prior to that date, blastocysts were placed in sequential blastocyst media and 20% serum protein supplement. Twenty blastocysts were initially cryopreserved utilizing a slow-freeze protocol prior to the introduction of vitrification. Therefore, those

blastocysts were thawed according to manufacturer's instructions (Cooper Surgical).

Regardless of how embryos were initially cryopreserved, vitrification was used for the second cryopreservation of all blastocysts after trophectoderm biopsy.

One-way ANOVA tests were utilized for the continuous variables and chi-squared test for independence was utilized for categorical variables. When comparing two categorical variables with $n \leq 5$, a Fisher's exact t-test was used. Regardless of test, significance was set at $P < 0.05$. Logistical regression was conducted to adjust for women's age and to assess the relationship between twice-cryopreserved blastocysts and implantation.

Results

Only patients who underwent a cryopreserved embryo transfer with euploid blastocysts were included in this study. Group 1 included 85 cycles, in women aged 35.6 ± 3.9 years, who underwent a cryopreserved embryo transfer with euploid blastocysts derived from a fresh cycle (once vitrified; Supplementary Table 1, available online). Group 2 included 17 cycles, in women aged 35.3 ± 4.9 years, who underwent a cryopreserved embryo transfer with euploid blastocysts derived from previously cryopreserved, thawed/warmed, biopsied (or rebiopsied), vitrified and rewarmed blastocysts (twice cryopreserved; Supplementary Table 2). There are multiple scenarios that may require this treatment approach: (i) vitrification, warming, biopsy, revitrification and rewarming; (ii) slow freezing, thawing, biopsy, vitrification and warming; and (iii) biopsy, vitrification, warming, rebiopsy, revitrification and rewarming. Therefore, group 2 was further subdivided into these three scenarios (Table 1).

Of the 73 total blastocysts from group 2 that were thawed/warmed for trophectoderm biopsy and revitrified, 20 of the blastocysts were initially frozen utilizing a slow-freeze protocol. Of those 20 blastocysts, 18 (90.0%) survived the initial thaw, were biopsied and vitrified. From those, only nine blastocysts were warmed for a cryopreserved embryo transfer (Table 1). A total of 53 vitrified blastocysts were warmed from group 2 and all 53 survived (100%). From those, 12 blastocysts were warmed for a cryopreserved embryo transfer (Table 1).

Blastocyst survival rate following warming of blastocysts from group 1 (114/116, 98.3%) compared with initial thawing/warming from group 2 (71/73, 97.3%) was not significantly different. However, the survival rate for blastocysts undergoing second warming in group 2 was significantly lower (21/24, 87.5%) compared with the survival rate for blastocysts warmed in group 1 (114/116 blastocysts, 98.3%; $P = 0.0354$; Table 1). From group 2, those blastocysts that underwent two vitrification and warming events showed the highest survival rate post second warming (12/12, 100%), compared with those that were initially slow frozen (7/9, 77.8%) and those that underwent two biopsies (2/3, 66.7%); however, the differences failed to reach statistical significance (Table 1).

From group 1, a total 113 euploid blastocysts were transferred in 85 cycles, with an average of 1.3 ± 0.6 blastocysts per transfer (Table 1). From group 2, a total of 21 euploid blastocysts were transferred in 16 cryopreserved embryo transfer cycles with an average of 1.3 ± 0.5 blastocysts per transfer (Table 1). For the cycle in which no blastocysts survived on the second warming, the two blastocysts were initially slow frozen. The blastocysts survived the initial thaw and were biopsied; however, they did not survive the second warming.

Biochemical (58/85, 68.2% versus 10/16, 62.5%) and clinical pregnancy (52/85, 61.2% versus 9/16, 56.3%) rates and implantation rate (66/113, 58.4% versus 11/21, 52.4%) were not significantly different between group 1 and group 2, respectively (Table 1).

Logistical regression analysis adjusted for maternal age revealed that when blastocysts are exposed to two cryopreservation–thawing/warming events and biopsy, implantation rate was not affected (OR 0.7013, 95% CI 0.2444–2.0123).

Discussion

These data demonstrate that previously cryopreserved blastocysts can be successfully thawed/warmed, biopsied, revitrified, rewarmed and utilized for CCS. Trophectoderm biopsy with CCS has been implicated in higher pregnancy rates and lower spontaneous abortions (Forman et al., 2012). However, research has focused on the biopsy, vitrification and subsequent cryopreserved embryo transfer of euploid blastocysts. A previous case report by Peng et al. (2011) demonstrated that a twice-vitrified and warmed blastocyst in conjunction with trophectoderm biopsy can achieve live birth. However, as far as is known, the present cohort is the first to demonstrate the effectiveness of utilizing previously cryopreserved blastocysts for use with trophectoderm biopsy and CCS.

In this study, thawing/warming, biopsy and revitrification were all performed on the same day, which may have caused the significantly lower warming survival rates in group 2. Therefore, an extra day may allow the blastocysts to recover from the warming and biopsy procedure. This treatment approach may prove advantageous with day-5 blastocysts as it has been shown that there is no difference in pregnancy rates if a blastocyst is transferred on day 5 or day 6 (Elgindy and Elsedeek, 2012). However, in the case of day-6 blastocysts, an extra day would make that embryo a day-7 blastocyst; and day-7 blastocysts have been shown to result in lower pregnancy rates compared with day-5/6 blastocysts (Kovalevsky et al., 2013). Interestingly, some reports indicate similar pregnancy and implantation rates between vitrified day-5, -6 and -7 blastocysts (Hiraoka et al., 2008, 2009a).

In the two cryopreserved embryo transfer cycles of twice-biopsied (due to a 'no result') and twice-cryopreserved blastocysts, neither resulted in a pregnancy (Table 1). Of the three blastocysts that were twice biopsied and twice cryopreserved, two (66.7%) survived the second warming (Table 1). The lower survival rate could be attributed to the low numbers or possible removal of too many cells from the trophectoderm. However, research has shown that the trophectoderm biopsy procedure is not detrimental to implantation and live birth rates (Scott et al., 2013b). Conversely, any additional intervention has the potential to

Table 1 Characteristics of euploid blastocyst transfer cycles with a vitrified and warmed blastocyst compared with those using twice-cryopreserved and -rewarmed blastocysts (group 2).

Characteristic	*Group 1*	*Group 2*			
Intervention	*Biopsy/vit/warm (n = 85)*	*Vit/warm/biopsy/revit/rewarm (n = 9)*	*Slow/thaw/biopsy/vit/warm (n = 6)*	*Biopsy/vit/warm/rebiopsy/revit/rewarm (n = 2)*	*Total (n = 17)*
Patient age at retrieval (years)	35.6 ± 3.9	35.3 ± 4.8	34.1 ± 5.5	39.0 ± 2.8	35.3 ± 4.9
Cryopreserved blastocysts thawed/warmed	116	12	9	3	24
Blastocysts survived	114 (98.3)[a,b]	12 (100.0)	7 (77.8)[b]	2 (66.7)	21 (87.5)[a]
Transfers					
Total transfers	85	9	5	2	16
Total embryos transferred	113	12	7	2	21
Embryos per transfer	1.3 ± 0.6	1.3 ± 0.5	1.2 ± 0.8	1.0 ± 0.0	1.3 ± 0.5
Biochemical pregnancies/transfer	58 (68.2)	6 (66.7)	4 (80.0)	0 (0)	10 (62.5)
Clinical pregnancies/transfer	52 (61.2)	5 (55.6)	4 (80.0)	0 (0)	9 (56.3)
Fetal cardiac activity/transfer	49 (57.6)	5 (55.6)	3 (60.0)	0 (0)	8 (50.0)
Implantation/per transfer	66 (58.4)	6 (50.0)	5 (71.4)	0 (0)	11 (52.4)
Live birth or ongoing pregnancy/transfer	61 (54.0)	6 (50.0)	4 (57.1)	0 (0)	10 (47.6)

Values are mean ± SD, *n*, or *n* (%).
Slow = slow freezing; vit = vitrification.
[a] $P = 0.0354$.
[b] $P = 0.0258$.

damage an embryo; therefore, it is plausible that twice-biopsied blastocysts are severely impacted by the biopsy procedure due to the removal of too many trophoblast cells. Furthermore, blastocysts that have been biopsied once prior to vitrification already have a hole in the zona pellucida. This hole allows for the direct exposure of cells to cryoprotectant, which may affect warming survival rates. The artificial collapse of blastocysts with the laser results in a similarly sized hole in the zona pellucida and improves vitrification outcomes (Iwayama et al., 2011; Mukaida et al., 2006). Likewise, the vitrification of blastocysts that are hatching or hatched from the zona pellucida is a common practice with IVF. Thus, the poor results in the twice-biopsy group seem to be due to the biopsy procedure itself rather than direct exposure of the cells to the cryoprotectant.

There are multiple reports showing that cryopreservation of embryos twice or cryopreservation of oocytes followed by cryopreservation of the resultant embryos does not affect outcomes. However, these reports focus on vitrification at two different stages of development. Cobo et al. (2013) showed no differences in live births between fresh embryos and vitrified oocytes that were later vitrified at either the cleavage or blastocyst stage. Likewise, pregnancies have been achieved from vitrified cleaving embryos that were warmed and revitrified at the blastocyst stage (Hiraoka et al., 2009b). Montag et al. (2006) demonstrated that previously frozen oocytes that were thawed and cryopreserved again at the pronuclear stage can result in a live birth.

Interestingly, there is little literature describing the outcomes of embryos twice cryopreserved on the same day utilizing a slow-freeze protocol. This may be due to the lack of sufficient data concerning the subject or the inability of the slow-freeze protocols to offer this type of procedure successfully. In this study, 20 blastocysts were initially cryopreserved using slow-freeze protocols. Of those, 18 survived the initial thaw (90.0%), while all of the previously vitrified blastocysts survived the initial warming. Furthermore, of the 24 twice-warmed blastocysts, three (12.5%) did not survive the second warming; of these three, two were previously cryopreserved with a slow protocol. This may indicate that blastocysts cryopreserved with slow-freeze protocols are not as able to survive two cryopreservation procedures compared with vitrified blastocysts, and larger studies are needed to confirm this conclusion.

Lastly, a majority of patients desiring CCS on previously cryopreserved blastocysts utilize this treatment approach for family balancing or because a miscarriage occurred during their initial fresh transfer. Patients that utilize CCS initially are typically those that present with advanced maternal age or diminished ovarian reserve; therefore, controlling for these two patient populations is difficult. Further studies, utilizing larger subsets of patients are needed to confirm this study's findings.

Although cryopreserved embryo transfer cycles utilizing euploid blastocysts that have been subjected to two cryopreservation and two thawing/warming events are slightly compromised, the overall viability of this procedure is encouraging. Even though survival appears to be lower, the overall data suggests similar outcomes. Current blastocyst vitrification protocols support the ability to vitrify, warm, biopsy, revitrify and rewarm blastocysts for subsequent cryopreserved embryo transfer.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.rbmo.2014.03.001.

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