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Double vitrification and warming of blastocysts does not affect IVF implantation rates, or birth outcomes

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Abstract

Research question: Does double blastocyst vitrification and warming affect pregnancy rates from embryos subjected to PGT-A testing?

Design: This is a retrospective observational analysis of embryo transfers performed at a single Centre between January 2017 and August 2022. The double vitrification (DV) group included frozen blastocysts that were vitrified after 5-7 days of culture, warmed, biopsied (either once or twice) and re-vitrified. The single vitrification (SV) group included fresh blastocysts that were biopsied at 5-7 days, and then vitrified.

Results: Comparison of the 84 DV blastocysts and 729 control SV blastocysts indicated that the DV embryos were frozen later in development and had expanded more than the SV embryos. Of the 813 embryo transfer procedures reported in this study, 452 resulted in the successful delivery of healthy infants (56%). There were however no significant differences between DV and SV embryos in the pregnancy rates achieved after single embryo transfer (55% vs 56%). Logistic regression indicated that while reduced pregnancy rates were associated with increasing maternal age at oocyte collection and at embryo transfer, and with longer culture prior to freezing, DV was not a significant predictor of outcome.

Conclusions: Blastocyst DV was not shown to impact pregnancy rates. While caution is necessary due to the study size, no effects of DV on miscarriage rates, birth weight or gestation period were noted. These data offer reassurance given the absence of influence of DV on pregnancy rates after PGT-A.

Keywords: blastocyst, double vitrification, single vitrification, embryo biopsy, preimplantation genetic testing for aneuploidy, PGT-A

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Introduction

The use of assisted reproductive technology (ART) has to date, led to the birth of more than ten million infants worldwide (Adamson et al., 2023), but despite impressive progress, many of the embryos created are not viable (Niederberger et al., 2018). The key reason for this is believed to be the relatively high rate of numerical chromosomal abnormalities seen in human embryos. Increased aneuploidy is associated with advancing maternal age but can arise paternally in meiosis or from post-zygotic events (Charalambous et al., 2023; Mikwar et al., 2020). Pre-implantation genetic testing for aneuploidy (PGT-A) commonly employs next generation sequencing (NGS) to screen all chromosomes to determine the ploidy status of embryos (Simopoulou et al., 2021). One of the challenges of using this technique, however, is that it requires the embryos that are being tested to be vitrified until the test results are available. This in turn brings challenges for testing previously frozen embryos, as this requires the embryos to be warmed, a biopsy to be performed with the embryos then needing to be refrozen (Wilding et al., 2019). This technique is referred to as double vitrification (DV).

Improvements in vitrification technologies (Mandawala et al., 2016), and a move away from the routine transfer of multiple embryos (Schoolcraft and Katz-Jaffe, 2013), has led the widespread generation and cryopreservation of surplus embryos. 'Freeze-all' cycles (in which all putatively viable embryos generated from a cycle are frozen, with no fresh embryo transfer taking place) are now commonplace, and may be used as mean of reducing the risk of complications such as ovarian hyperstimulation and by providing a more physiological uterine environment at subsequent transfer (Acet et al., 2022; Evans et al., 2014; Roque et al., 2013; Yang et al., 2022). While vitrification is now a routine element of practice, DV remains limited to specific clinical scenarios. Examples include implantation failure prior to genetic testing,

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embryos preserved from prior cycles, emergency FET cancellations and "no-result" embryos post-diagnosis. Its increasing use in these circumstances raises the question of the efficacy of DV. Studies in this area are limited, of small sample size, and have produced conflicting results; some studies suggest that DV may have an impact on clinical outcomes such as implantation rates (Aluko et al., 2021), and live birth rates (Zheng et al., 2017), whilst others show no difference in pregnancy outcomes when embryos that have undergone multiple rounds of vitrification are transferred (Theodorou et al., 2022). A recent systematic review and meta-analysis (Wang et al., 2023) of the results of 14 studies that have investigated the effects of embryo re-cryopreservation concluded that the approach can impact embryo viability and IVF success rates, but that neonatal outcomes are not affected. This would appear to be contradictory, and such a lack of consensus limits the guidance that clinicians can provide their patients with when they are interested in testing their previously cryopreserved embryos by PGT-A.

With the above in mind, the primary aim of this study was therefore to investigate the impact of DV versus single vitrification (SV) on pregnancy rate for patients undergoing PGT-A, focussing on the vitrification and transfer of blastocysts only. Secondary outcomes assessed were live birth and miscarriage rates, birth weight and gestation length.

Materials and methods

This is retrospective analysis of 813 embryo transfers performed following PGT-A testing at a single UK centre. The warmed and transferred embryos had been exposed to either SV or DV. SV embryos were subjected to trophectoderm biopsy, vitrified, warmed, and then transferred. DV embryos were either: 1. Vitrified without undergoing biopsy or PGT-A, and

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then warmed, biopsied, and re-vitrified / warmed for a second time prior to transfer, or, 2. When initial PGT-A had not been successful, warming, biopsy and vitrification took place twice before transfer. *i.e.,* all embryos were biopsied, with some DV embryos being biopsied twice. As a result, all embryos had PGT-A results available, and had either been vitrified and warmed once (the SV group) or twice (the DV group). The data presented were collected from patients that had made the informed decision to genetically test (PGT-A) their embryos following counselling in accordance with the guidelines from the Human Embryo and Fertilisation Authority (HFEA).

All patients underwent assisted reproduction treatment between January 2017 and August 2022. Exclusion criteria for analysis included one or both partners being known carriers of a chromosomal abnormality, known genetic carriers couples at risk of generating an embryo affected by an autosomal recessive single gene disorder and/or at least one partner being a carrier of a known autosomal dominant disorder. The use of patient data in this analysis was reviewed by The London Women's data protection team and approved by the University of Kent Research Ethics Advisory Group, with approval number (CREAG115-07-23). High quality embryos were defined as those graded AA, AB, BA, or BB, and lower quality as CB, BC, or CC, in accordance with the ACE/NEQAS embryo grading scheme.

Ovarian stimulation protocol and embryo culture conditions

All patients underwent ovarian stimulation of multiple follicle development using recombinant or urinary FSH, or a combination of both, alongside cotreatment with a GnRH antagonist to prevent premature luteinisation. Triggering of oocyte maturation was carried out when the leading follicle was >18mm using a subcutaneous injection of either hCG or a

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GNRH agonist. Oocyte retrieval was performed within 35 to 37 hours. For IVF cases, co-incubation with sperm occurred within four hours of oocyte retrieval. Similarly, for ICSI, oocytes were denuded using hyaluronidase media within four hours of retrieval, and a single sperm was then injected into each. After both IVF and ICSI, fertilisation was checked 16-18 hours later. All embryos were cultured in an Embryoscope (Vitrolife) device, an incubator with in-built time-lapse capabilities, to the blastocyst stage in continuous single culture medium (Vitrolife) pre-equilibrated the day before at 37°C in an atmosphere of 6% CO^2. Uninterrupted embryo culture continued for up to seven days post IVF/ICSI, or until blastocyst formation and expansion. Embryos were subsequently transferred in a non-stimulated cycle to control for uterine receptivity.

Embryo vitrification, warming and biopsy procedures

The vitrification and warming procedures used was consistent for all embryos. All blastocysts used in this analysis were vitrified using the Irvine Scientific® protocol, at room temperature. Post double witnessing, embryos were moved to a labelled freezing dish, and were equilibrated for 12 minutes in a M-199 HEPES buffered medium containing 7.5% dimethyl sulfoxide, 7.5% ethylene glycol, 20% dextran serum supplement (DSS) and gentamicin. Afterwards, blastocysts were moved to vitrification solution, M-199 HEPES buffered medium containing 15% DMSO, 15% ethylene glycol, 0.5 M sucrose, 20% DSS, and gentamicin. Embryos were loaded into Cryotop® devices (Kitazato BioPharma Co. Ltd. Fuji, Shizuoka, Japan) with minimal freezing media before plunging into liquid nitrogen.

In the SV group, embryos were biopsied upon reaching the blastocyst stage, after which they were vitrified. In contrast, in the DV group, the warming process occurred on the morning of

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the scheduled biopsy procedure. Post double witnessing, the Cryotop® device was instantly submerged in pre-equilibrated at 37°C warming solution (Irvine Scientific), M-199 HEPES Buffered Medium containing 1 M sucrose, 20% DSS, and gentamicin and for one minute, before being moved to a solution that contained a lower concentration of sucrose (0.5 M) for three minutes. Subsequently, embryos were moved to a sucrose-free washing solution for five minutes. After warming, embryos were moved to pre-equilibrated embryo culture media (Vitrolife) and cultured in Embryoscope incubators (Vitrolife). Blastocyst viability and re-expansion was checked two hours post warming. Embryos were deemed ready for biopsy once they had achieved an expansion score of 4 according to the ACE/NEQAS embryo grading scheme. The time this takes can vary from the minimum two hours to extending culture to day 6 or day7 if necessary. All biopsies were performed by one experienced senior embryologist. Trophectoderm (TE) biopsy was subsequently performed either on day 5, 6 or 7 of culture to determine the ploidy status of the embryos by the removal of five to seven TE cells as previously described (Gorodeckaja et al., 2020; Thornhill et al., 2012)

Embryo transfer and the establishment of pregnancy

Embryos reported to be euploid were warmed on request for transfer (mosaic embryos were excluded). Endometrial preparation was achieved using hormonal replacement therapy (HRT) in 96% of the frozen embryo transfer cycles. 6.5% of the patients that received SV embryos (n = 48) underwent endometrial receptivity (ER) testing prior to transfer, while no ER testing was performed on the patients that received DV embryos. If an early follicular baseline scan was normal, treatment was initiated by administering 6-10 mg/day of oestradiol valerate (Progynova, Bayer), to support endometrial development. When exposure to oestradiol and endometrial thickness was considered sufficient, luteal support was commenced using

vaginal progesterone pessaries (Cyclogest 400mg, L.D. Collins and Co. Ltd) administered thrice daily. After five days of progesterone treatment, a single euploid embryo was warmed and cultured for a minimum of two hours to confirm viability and allow for re-expansion, with embryo transfer occurring between two and five hours post-warming. Luteal support continued until a pregnancy was confirmed by measuring levels of hCG and then continued until at least 8 weeks gestation. An ultrasound scan was performed at approximately eight weeks of gestation to confirm the presence of an intra-uterine viable pregnancy.

Data analysis

All data were analysed in R version 4.2.2 (R Core Team, 2022), using RStudio (RStudio Team, 2020). Embryo storage time was calculated as the difference between the oocyte age at collection and maternal age at the time of transfer. Quantitative variables were analysed using a Mann-Whitney U test and qualitative variables were analysed by comparing DV and SV embryos by two-proportion Z tests, or, for the degree of expansion and the day of embryo freezing, by Pearson's Chi-squared test. Logistic regression, using the MASS (Venables and Ripley, 2002) package, was used to identify factors that affect pregnancy rates via stepwise regression with both forward and backward selection and AIC used for model selection. Odds ratios (OR) and confidence intervals were then calculated using the questioner package (Barnier et al., 2023). The relationships between measured factors and both gestation period and birthweight were investigated by stepwise linear regression.

Results

In total, 84 euploid DV embryos were thawed, 100% of these survived and were transferred. 736 euploid SV embryos were thawed, with seven not surviving the warming procedure (99%

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survival rate). A breakdown of the euploid embryos that were transferred is shown in Figure 1. Baseline parameters of the transferred DV and SV embryos are shown in Table 1. Analysis of these data indicated that the two groups did not differ in respect to either maternal or embryo age but identified that the DV embryos were stored for longer. Further to this, the women receiving SV embryos had slightly higher body mass index (BMI). More DV embryos were derived via IVF rather than via ICSI, and the DV embryos were frozen for the second time later and had expanded more (Table 1). Importantly, analysis indicated that vitrifying and warming embryos twice rather than once did not negatively impact the pregnancy, miscarriage, or live birth rate. Of the 813 transfers reported here, 452 achieved a successful live birth (55.6%). Analysis of live births also indicated that the SV and DV groups did not significantly differ in the method of delivery, sex, gestation period or birth weight (Table 1).

Logistic regression was then used to analyse the relationship between the number of times euploid embryos had been vitrified (and hence warmed), embryo and maternal age, embryo storage time, maternal BMI, degree of expansion, day of freezing and the success of the transfer as assessed by the live birth rate. Logistic regression of these data indicates that the live birth rate was greater with IVF (60%) than with ICSI (53%) (OR 1.35, 95% CI of 1.003 to 1.816), that transfer of embryos obtained from women of greater age oocyte collection was associated with lower success (OR 0.96, 95% CI 0.921 to 0.994), and that lower quality embryos (those graded as CB, BC or CC) performed worse than high quality (AA, AB, BA or BB) ones (OR 0.53. 95% CI 0.377 to 0.740). The effects of these measured variables on gestation period and birth weight were then investigated by stepwise linear regression. This indicated that none of the analysed factors significantly affected gestation period, whilst increasing BMI

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was associated with a minor increase in birth weight ($F_{1,450}$ = 5.88, p = 0.016, coefficient = 0.014, 95% CI 0.003 to 0.026).

Discussion

In this study, no differences in terms of pregnancy rates, or measures such as gestation period or birthweight were detected between euploid embryos that were vitrified and warmed once and those that were vitrified and warmed twice. As would be expected, embryos in the DV group were frozen significantly later and therefore showed greater blastocyst expansion when compared with SV blastocysts. Logistic regression indicated that greater embryo age was associated with lower success, and that success was greater with IVF than with ICSI. Given that there is no clear answer as to whether IVF or ICSI is more successful across the piece in the ART sector, this would be an interesting future avenue to explore. Unsurprisingly, this analysis also indicated that blastocysts scored as lower quality (CB, BC, or CC) performed worse than those classified as high quality (AA, AB, BA, or BB). As expected, given the conflicting results in the literature when considering single and double vitrification (Aluko et al., 2021; De Vos et al., 2020; Li et al., 2023; Peng et al., 2011; Taylor et al., 2014; Theodorou et al., 2022; Zheng et al., 2017), our conclusion concurs with the results of some previous work, but does not agree with the results of the others. Studies that concluded that DV did not affect outcomes include: Peng *et al*., (2011) who reported the first live birth following double vitrification and trophectoderm biopsy; Taylor *et al*., (2014), who compared the outcomes of blastocysts biopsied and vitrified a single time, with those that were cryopreserved twice; Theodorou and colleagues (2022), who studied the potential effects of double vitrification and warming on both cleavage stage and blastocyst stage and De Vos *et al*., (2020) that not only analysed clinical outcomes, but also reported positive findings when

performing neonatal follow ups of the resulting children. Conversely, Aluko *et al.*, 2021 indicated that blastocyst biopsy coupled with multiple rounds of freezing negatively affects IVF outcomes, whilst both Zheng and colleagues (2017) and Li *et al*., 2023, found that whilst neonatal outcomes were not affected, there was a correlation between repeated cryopreservation and pregnancy loss, and ultimately live birth rates. This study provides additional assurance regarding the use of the double vitrification approach and highlights that further comparisons of the potential effects of single versus double biopsy in conjunction with double vitrification could be of interest. Given however that all interventions can cause harm, particularly when procedures are performed by inexperienced hands, the skill of the embryologist is critical to produce favourable outcomes.

Our data also included some blastocysts that were transferred on day 7 and that resulted in live births (3 of 13 transferred day 7 blastocysts in our data). The transfer of day 7 blastocysts has previously been shown to be associated with lower pregnancy rates compared with day 5 or 6 blastocysts (Kovalevsky et al., 2013) and with an increased risk of the offspring being heavier, and very large for the gestational age (Huang et al., 2020). Interestingly, no significant difference in pregnancy rates is seen between transfer days. This result is consistent with Hiraoka *et al*. (2008, 2009a) who observed similar pregnancy and implantation rates between blastocysts vitrified on days 5, 6 or 7, and with Du *et al*., (2018), who found no statistical difference in the live birth weight, the incidence of stillbirths or congenital abnormalities for transferred day 7 embryos compared with their day 5 and day 6 counterparts (Du et al., 2018; Hiraoka et al., 2009, 2008). Work by Greco *et al*., (2015) corroborates with these studies, and takes things one step further with a healthy baby boy being the result of a day 7 embryo transfer following triple biopsy (polar body, day 3 and blastocyst) and double vitrification

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(Greco et al., 2015). Alongside these, and other studies of note (Cimadomo et al., 2022; Insogna et al., 2021), our findings suggest that extending the culture of embryos beyond day 6 could be valuable if clinically necessary, a practice that is not currently commonly observed in many IVF clinics.

In conclusion, in this study, embryos that were subjected to two rounds of vitrification were not compromised either in terms of their quality, or in their potential to establish, and maintain a pregnancy. This is reassuring for patients and clinicians alike, but more studies, of larger sample sizes are still required to confirm these results.

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Legend to Figure:

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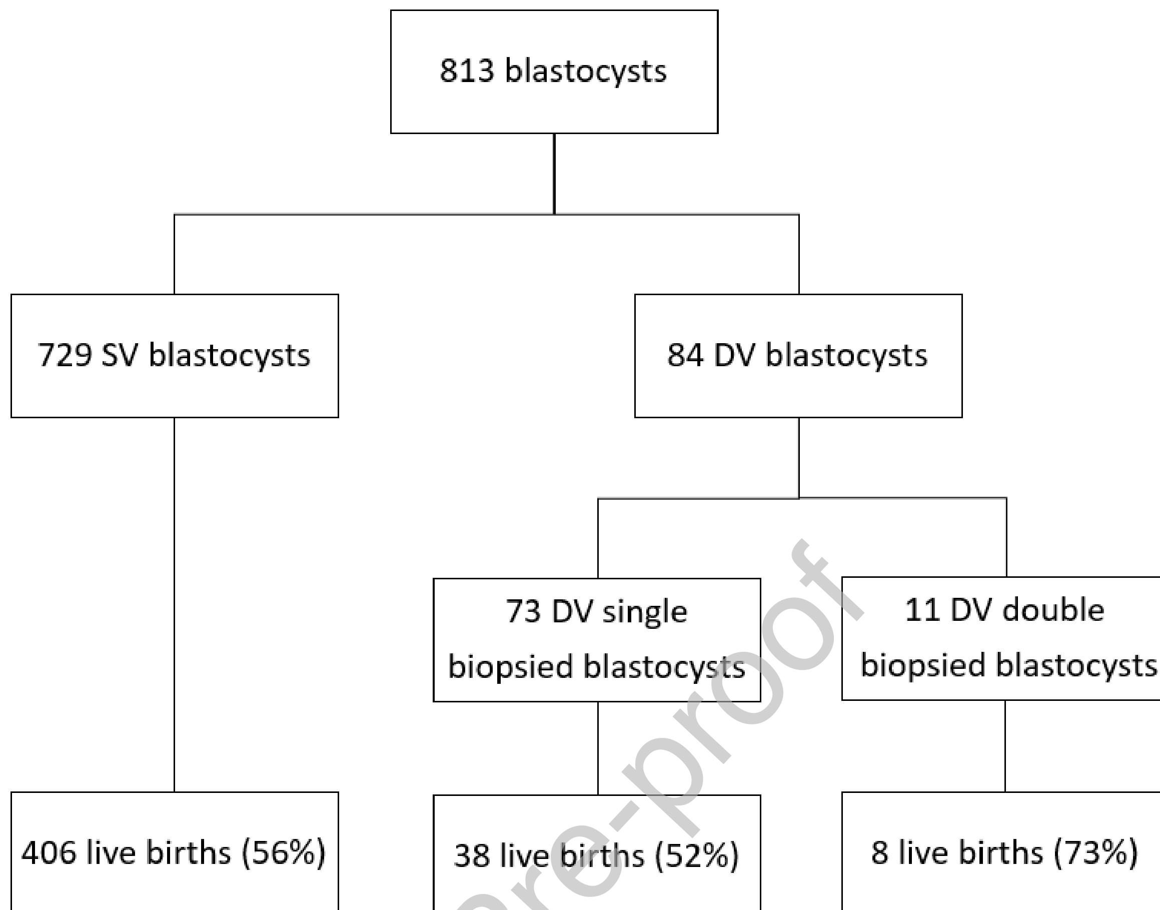

Figure 1: Flow chart showing the total number of euploid embryos (blastocysts) that were transferred in this retrospective analysis, and the proportions of live births. Abbreviations: DV, double vitrification; SV, single vitrification.

Table 1: Baseline characteristics and outcomes. Values are expressed as number (percentage) or mean ± standard deviation of the mean. Abbreviations: DV, double vitrification; IVF, *in vitro* fertilisation; ICSI, intracytoplasmic sperm injection; SV, single vitrification. High quality embryos were defined as those graded AA, AB, BA, or BB, and lower quality as CB, BC, or CC. Quantitative variables were analysed using a Mann-Whitney U test and qualitative variables were analysed by comparing DV and SV embryos by two-proportion Z tests or, for the degree of expansion and the day of embryo freezing, by Pearson's Chi-squared test. NS denotes a non-significant test result.

	Single vitrification	Double vitrification	Test
Maternal age at transfer (years)	37.9 ± 3.9	38.7 ± 3.5	NS
Oocyte age at collection (years)	36.9 ± 3.8	36.5 ± 3.5	NS

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Embryo storage time (years)	1.03 ± 1.6	2.20 ± 2.6	W = 42930, $p < 0.001$
BMI	24.9 ± 4.5	23.9 ± 3.9	W = 26590, $p = 0.048$
ICSI/IVF	65%/35%	54%/46%	$X^2 = 4.00$, $p = 0.045$
Day of embryo freezing (day 5, 6 or 7)	77.6%/20.7%/1.6%	61.9%/36.9%/1.2%	$X^2 = 11.4$, $p = 0.003$
Expansion (grade 3, 4, 5 or 6)	4.1%/85.0%/6.7%/4.1%	2.4%/61.9%/19.0%/16.7%	$X^2 = 41.7$, $p < 0.001$
Embryo quality (high, AA, AB, BA or BB / lower, CB, BC or CC)	78.2%/21.8%	71.4%/28.6%	NS
Positive pregnancy test	471/729 (65%)	56/84 (67%)	NS
Miscarriage rate	65/471 (14%)	10/56 (18%)	NS
Live birth rate	406/729 (56%)	46/84 (55%)	NS
Vaginal delivery / C-section	94/312 (23.2%/76.8%)	9/37 (19.6%/80.4%)	NS
Sex (male/female)	216/190 (53.2%/46.8%)	21/25 (45.7%/54.3%)	NS
Gestation period (weeks)	38.7 ± 1.7	38.9 ± 1.3	NS
Birth weight (kg)	3.4 ± 0.5	3.3 ± 0.5	NS

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Biography

Balsam Al Hashimi, an HCPC-registered clinical scientist, holds an MSc in clinical embryology from the University of Leeds. She serves as the Deputy Lab Manager and Lead Embryologist in Genetics at the London Women's Clinic (LWC) and is currently registered as a PhD student at the University of Kent.

Key Message

Double vitrification of blastocyst has not shown to have any impact on pregnancy rate while caution is warranted due to the study size. No notable influence on birth weight or gestation period was evident. These findings provide assurance regarding the efficacy and safety of double vitrification.

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