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ARTICLE

The effect of pipette- and laser-induced blastocyst collapse before vitrification on their re-expansion and clinical outcome after warming

BIOGRAPHY

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KEY MESSAGE

Expanded blastocysts that were artificially collapsed by laser before vitrification showed a significantly faster re-expansion than blastocysts that were collapsed by means of an intracytoplasmic sperm injection pipette and than the unmanipulated control group. No statistically significant differences were seen in clinical outcomes for any group.

ABSTRACT

Research question: What are the effects of pipette- versus laser-assisted artificial blastocyst collapse (ABC) on the morphokinetics of warmed blastocyst re-expansion, and what is the potential effect on treatment outcomes?

Design: Surplus blastocysts were extracted from 203 patients. These were divided into three groups: study group A, artificial collapsed by the aspiration of blastocoel fluid with a pipette; study group B, trophectoderm opened with a laser pulse; control group, no manipulation before vitrification was performed. During the 5-year study period, 257 associated single-warm blastocyst transfers were scheduled. The start and duration of the re-expansion process before transfer were annotated. Pregnancy and live birth data were also collected for the transfers.

Results: The overall blastocyst survival rate was 96.9%, with no effect observed as a result of the two ABC methods. The re-expansion of blastocysts in study group B was initiated significantly sooner after warming (0.50 ± 0.37 h) than in group A (0.79 ± 0.56 h) or the control group (1.22 ± 1.00 h). The duration of the re-expansion process was significantly reduced in study groups A ($P = 0.021$) and B ($P = 0.004$) compared with the control group. The embryos of participants who achieved a live birth had a significantly ($P < 0.001$) faster start of re-expansion (0.60 ± 0.42 h) than the embryos in those who did not produce an ongoing pregnancy (1.05 ± 0.92 h).

Conclusions: Laser-treated blastocysts exhibited substantially shorter re-expansion times. Because faster re-expansion of the blastocyst is associated with positive treatment outcomes, the laser technique should be prioritized over the pipetting technique if ABC is considered.

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*Corresponding author. E-mail address: Thomas.ebner@kepleruniklinikum.at (T. Ebner). https://doi.org/10.1016/j.rbmo.2024.104476 1472-6483/© 2024 Reproductive Healthcare Ltd. Published by Elsevier Ltd. All rights are reserved, including those for text and data mining, AI training, and similar technologies.
Declaration: The authors report no financial or commercial conflicts of interest.

KEY WORDS

Artificial collapse
Blastocoelic fluid
Implantation rate
Live birth rate
Time-lapse
Vitrification

INTRODUCTION

Compared with cleavage-stage embryo transfer, extended embryo culture and transfer at the blastocyst stage offers unique advantages, such as the selection of embryos with higher developmental potential, a decreased probability of transferring embryos with chromosome abnormalities, better endometrial synchronization and, ultimately, higher implantation rates that allow the maintenance of pregnancy chances while reducing multiple gestations (*Reh et al., 2010*; *Shapiro et al., 2002*; *Zhang et al., 2021*). However, the implementation of a single-blastocyst transfer policy relies on successful vitrification and adequate storage of supernumerary embryos (*Casper and Yanushpolsky, 2016*; *Kim et al., 2021*; *Nagy et al., 2020*; *Sundhararaj et al., 2017*). Simultaneously, clinical practices such as 'freeze-all blastocysts' for delayed frozen embryo transfer are becoming popular as they efficiently prevent the risk of ovarian hyperstimulation syndrome (*Mizrachi et al., 2020*; *Mourad et al., 2017*; *Vlaisavljević et al., 2017*).

The selection methods for fresh blastocyst transfer are based on relatively simple morphological criteria, namely the degree of expansion and the quality of the inner cell mass (ICM) and trophectoderm (*Chen et al., 2014*; *Ebner et al., 2009*; *Kovačič et al., 2004*; *Sivanantham et al., 2022*; *Wirleitner et al., 2016*), but applying similar grading criteria after warming is a less common strategy (*Ebner et al., 2017*). Morphokinetic evaluation of blastocysts after warming, particularly annotation of the re-expansion process, has recently been proposed as a predictor of implantation, pregnancy and live birth (*Ebner and Shebl, 2018*; *Mensing et al., 2023*; *Mirzazadeh et al., 2016*).

Although the efficiency of blastocyst vitrification is robust across medically assisted reproduction centres, there are some variations between protocols and laboratories, such as the different compositions of vitrification solutions, the use of open or closed vitrification systems and whether interventions such artificial blastocoel collapse (ABC) on blastocysts before vitrification or assisted hatching after warming are performed (*Boyard et al., 2022*; *Casciani et al., 2023*; *Kovačič et al., 2022*).

ABC aims to remove most of the fluid from the blastocoelic cavity to reduce the severity of osmotic changes when the blastocysts are exposed to cryoprotectants, and to decrease the chance of intracellular ice crystal formation (*Boyard et al., 2022*; *Mukaida et al., 2006*; *Vanderzwalmen et al., 2002*). Because there is a notable association between the volume of the blastocoelic cavity before vitrification and the survival rate after warming, some IVF laboratories prefer to remove the blastocoelic fluid by artificially collapsing blastocysts from the expanded stage onwards before vitrification. Different methods have been described as follows: a hyperosmotic solution to aspirate blastocysts into pipettes with a slightly smaller diameter than that of the blastocyst, aspirating blastocoelic fluid with an intracytoplasmic sperm injection (ICSI) pipette or creating a hole in the blastocoele with a laser pulse (*Mukaida et al., 2006*).

However, the routine use of this invasive procedure remains controversial (*Boyard et al., 2022*; *Kovačič et al., 2022*). Kovačič and colleagues found higher survival and a non-significant 5% increase in live birth rate in artificially collapsed blastocysts compared with intact ones (*Kovačič et al., 2022*). Moreover, a recent meta-analysis reported significantly higher blastocyst survival and clinical pregnancy rates in artificially collapsed blastocysts compared with non-manipulated ones (*Boyard et al., 2022*). Although these authors underlined the need for further clinical studies with larger populations, the meta-analysis by *Boyard and collaborators (2022)* stressed its limitations due to the variability of the collapsing techniques used in the pooled studies (*Boyard et al., 2022*). A uniform reaction of the blastocyst to external mechanical stresses should not be assumed as some authors have noted that differences in the timing of re-expansion could be associated with the collapse technique used (*Desai et al., 2008*; *Ebner and Shebl, 2018*).

Therefore, due to the lack of consensus regarding the efficacy of ABC in improving post-warming outcomes, this study assessed the effects of ICSI pipette- versus laser-assisted ABC before vitrification on the morphokinetics of blastocyst re-expansion after warming. Secondary outcomes were the biochemical pregnancy, pregnancy, miscarriage and live birth rates.

MATERIALS AND METHODS

Study design

This retrospective observational study analysed warming cycles that were conducted between January 2017 and December 2021. During this period, ABC was performed using either an ICSI pipette (study group A) or a laser pulse (study group B). All blastocysts to be vitrified from one ICSI cycle were included in the same manipulation group. The control group consisted of blastocysts that remained unmanipulated prior to vitrification. No randomization was performed to enrol the patients in the study or control groups. The study's design is illustrated in FIGURE 1.

All patients who had planned a single vitrified-warmed ICSI blastocyst transfer and had at least one expanded or hatching vitrified blastocyst were considered for inclusion in this study. This approach (FIGURE 1) led to the inclusion of 203 patients and 257 associated vitrification-warming cycles during the study period. Consequently, the last live births were expected in the fourth quarter of 2022.

Ethical statement

Before the submission of this dataset, ethical approval was sought from and granted by the Ethical Committee of the Medical Faculty of Kepler University (Linz, Austria; 1176/2022, approval date 12 July 2023). As all the techniques applied were standard laboratory practice, no informed consent was sought from the participants.

Patient cohort and fresh cycles

According to the present inclusion criteria (ICSI cycles only), the male partners did not reach the criteria of normozoospermia, including five cases of azoospermia (2.5%) and two cases of frozen-thawed spermatozoa (1.0%), except for three men (1.5%) who had a normal sperm sample but also a history of complete fertilization failure with conventional IVF. Regarding the female participants (34.1 ± 4.8 years old at the time of vitrification), the indication for medically assisted reproduction treatment was approximately 10% each due to endometriosis (n = 19) and polycystic ovary

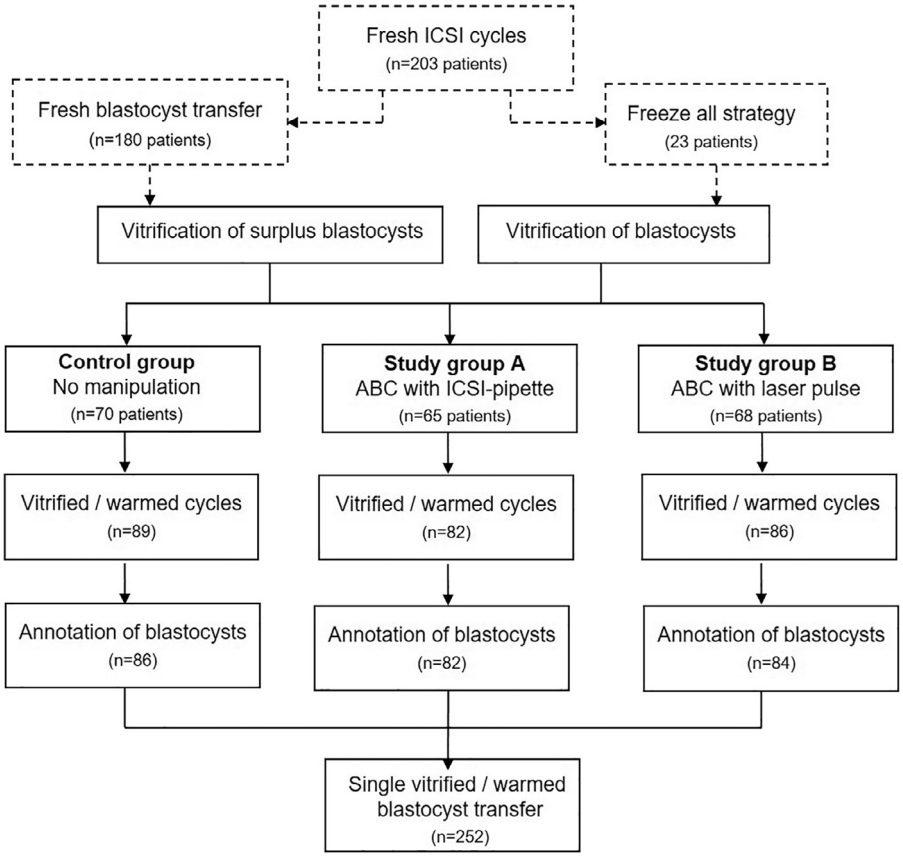

FIGURE 1 Schematic presentation of the study design. The dashed lines mark the preclinical phase. ABC, artificial blastocyst collapse; ICSI, intracytoplasmic sperm injection.

syndrome (n = 22), while 5.9% (n = 12) of the women showed bilateral blockage of the Fallopian tubes. Apart from the participants with polycystic ovary syndrome, all patients exhibited a normal-to-good ovarian response, which was reflected in adequate overall anti-Müllerian hormone concentrations (4.5 ± 3.7 ng/ml). Further details regarding the demographic data and potential confounders are provided in Supplementary Table 1.

After ovarian stimulation with either a long agonist (n = 32) or an antagonist (n = 171) protocol, a mean number of 10.1 ± 5.9 cumulus-oocyte complexes were collected. Of these, 8.5 ± 5.2 (84.2%) contained mature oocytes that were suitable for ICSI. The following day 7.3 ± 4.9 (80.4%) of the eggs were fertilized. Zygotes were cultured in 30 μl drops up to day 5 in either one-step medium (n = 80; Global Total LP, Cooper Surgical, USA) or sequential media (n = 100; Origio Sequential Blast, Cooper Surgical, USA). The embryos from the remaining participants (n = 23) were split between the two types of culture medium (Supplementary Table 1).

The blastocyst quality was annotated using the Gardner and Schoolcraft score (*Gardner and Schoolcraft, 1999*). As shown in FIGURE 1, all the participants underwent a fresh day 5 blastocyst transfer (n = 180), except for those considered for freeze-all cycles (n = 23).

Artificial collapse of blastocysts

Surplus blastocysts of a certain quality on day 5 were potential candidates for cryopreservation (no vitrification was performed on day 6). Blastocysts from the fully expanded stage onwards (Gardner and Schoolcraft score ≥3) had to of BB quality or better (ICM, trophectoderm) to be cryopreserved. In this study, this involved blastocysts showing an average diameter of 193 μm (grade 4) to 198 μm (grade 5) (*Ebner et al., 2016*).

Based on previous studies, only fully expanded and hatching blastocysts (Gardner and Schoolcraft scores 4 and 5) were subjected to ABC (*Hiraoka et al., 2004*; *Mukaida et al., 2006*; *Vanderzwalmen et al., 2002*). Two methods were used in the present study to artificially collapse the blastocysts. In group A, the blastocyst was fixed using a holding pipette (Microtech, Czech Republic), with the ICM being held at the 12 o'clock position, and an ICSI pipette (Microtech, Czech Republic) was inserted into the blastocoelic cavity at the 3 o'clock position. By gentle aspiration, blastocoelic fluid was removed until the ICM came into contact with the opposite trophectoderm layer. This active procedure ensured that almost all the fluid was aspirated from the blastocoel (FIGURE 2a, b). In study group B, one single laser pulse (1.5 ms; Octax NaviLase, Vitrolife, Sweden) was set at the interface of two trophectoderm cells (distant from the ICM), aiming to cause passive drainage of the blastocoelic fluid (FIGURE 2c, d). In contrast to the pipette-based approach, blastocyst shrinkage was not immediate but could take up to 5 min (*Van Landuyt et al., 2015*). The control group consisted of intact blastocysts without artificial blastocoel reduction.

Vitrification of blastocysts

A commercially available kit (GM501 VitriStore Freeze, Gynemed, Germany) was used to vitrify the blastocysts; the process is based on a two-step exposure to

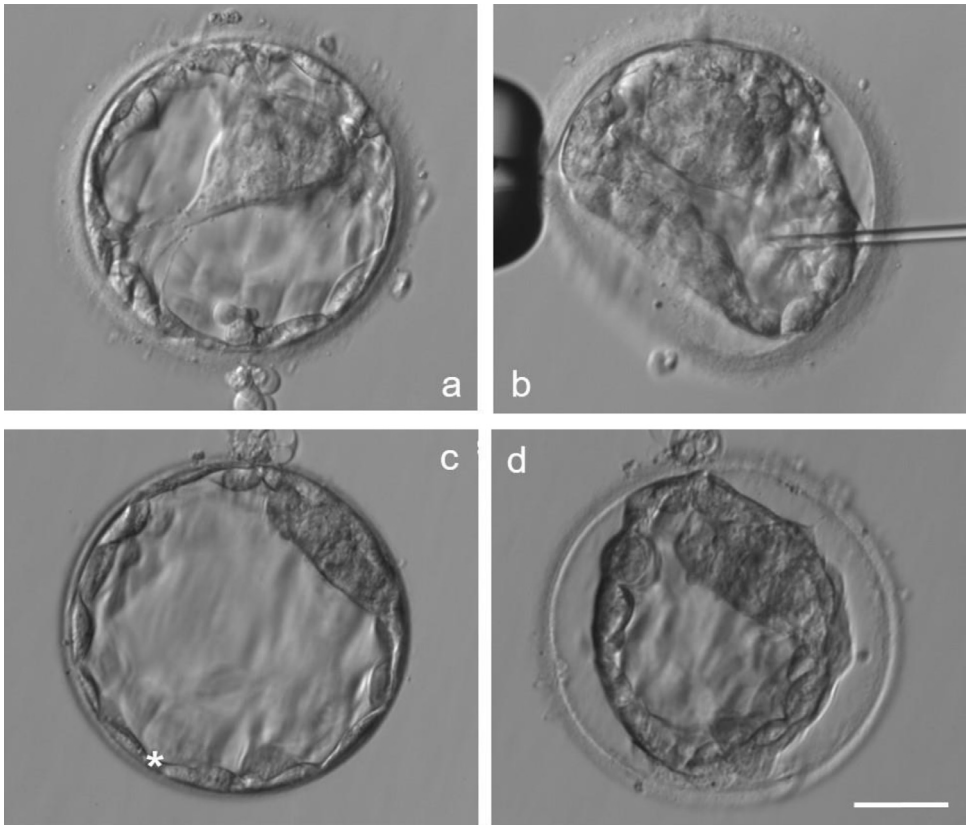

FIGURE 2 Pipette-assisted (a, b) and laser-assisted (c, d) blastocyst collapsing technique. (a) Blastocyst (5aa) status just before aspiration began. (b) Final stage of artificial collapse after the liquid was removed from the blastocoel. (c) A 5AA blastocyst at the time the laser pulse was set (* indicates the position). (d) The same blastocyst 2 min after laser manipulation. Scale bar = 50 μm.

increasing concentrations of the permeable cryoprotectants ethylene glycol and dimethyl sulfoxide (EG/DMSO). All the solutions were used at room temperature. All blastocysts were exposed to a 2 min pre-incubation step in a medium containing phosphate-buffered saline and human serum albumin (VitriStore Pre-vitrification Medium, Gynemed, Germany). After this, the embryos were incubated for 1.5 min in vitrification solution 1 (1.5 mol/l), while the higher molarity of EG/DMSO in vitrification solution 2 (5 mol/l) required a shorter incubation period of only 30 s. The blastocysts were then immediately and individually placed on the tip of a Cryotop device (Dibimed Biomedical Supply, Spain) using a relatively small volume of vitrification solution 2 (0.1 μl). The carrier was then directly plunged into liquid nitrogen. The open device was covered with an outer protective straw for further storage in liquid nitrogen in a designated container (Arpege 170, Air Liquide, Austria).

Vitrification-warming cycles

Blastocysts were warmed using a complementary warming kit (GM501 VitriStore Thaw, Gynemed) according to the manufacturer's instructions. It comprised four different warming media with decreasing concentrations of sucrose (0.5, 0.25, 0.125 and 0 mol/l). To ensure very high warming rates, special care was taken to detach the Cryotop from the outer straw and plunge it into the warming solution 1 (0.5 mol/l) in one rapid working step. To facilitate immediate plunging, 0.5 ml of warming medium 1 was not covered with mineral oil, while in the other three the warming medium (30 μl drops each) had an oil overlay (GM501 Mineral Oil, Gynemed, Germany). After incubation in the four warming solutions (3, 2, 2 and 1 min), blastocysts were transferred to a 4-well dish containing 1 ml of pre-equilibrated and prewarmed culture medium to be considered for later transfer; in all cases, this medium was identical to the last medium the fresh blastocyst had been in contact with prior to vitrification. Consequently, the medium used was either Origio Sequential Blast or Global Total LP.

Immediately after transfer to the 4-well dish, all post-warming surviving blastocysts (*Ebner et al., 2009*) had their zona pellucida routinely opened by a series of 3–4 laser pulses (1.8 ms each; Octax NaviLase) to avoid any potential negative impact of cryopreservation on zona pellucida hardening (*Liebermann and Tucker, 2006*; *Vanderzwalmen et al., 2003*). In all cases, the aim was a funnel-shaped complete opening of the zona pellucida involving a full-thickness defect with the outer diameter of the ablation being approximately 40 μm.

Immediately after assisted hatching, warmed blastocysts were directly transferred to culture dishes (CultureCoin, Esco Medical, Germany) specially designed for use in an associated time-lapse incubator (MIRI TL, Esco Medical, Germany). In detail, 30 μl drops of culture media covered with 7 ml of mineral oil were pre-equilibrated at an atmosphere of 6.2% CO_2 (corresponding to a pH of 7.30–7.32) and 5% O_2 in a humidified atmosphere at 37.0°C. The time between contact with warming medium 1 and loading of the warmed blastocysts into the wells of the CultureCoin was ≤10 min. This rapid proceeding and image frequency

(5 min) ensured that all re-expansion events were captured.

Blastocyst transfers were scheduled no earlier than 4 h after warming to increase the chances of annotating the whole re-expansion process in the MIRI TL incubators. As previously described (*Ebner et al., 2017*), the morphokinetic events of interest were the start of re-expansion (tRE), the completion of re-expansion (tCRE) and the start of hatching through the artificially created gap (tAH). The actual duration of the re-expansion process was calculated from some of these variables (tCRE − tRE). The presence and number of collapses and cytoplasmic strings were also recorded.

Approximately 12% (n = 30) of the vitrified-warmed transfers were performed in a natural cycle, whereas the rest were performed in artificial hormone replacement cycles using an endometrial preparation protocol with oestradiol valerate starting on day 2 or 3. Oestradiol was administered using a step-up approach with a maximum dose of 6 mg per day until the pregnancy test. Progesterone was administered for luteal phase support of all the cycles, and was either stopped in the case of a negative pregnancy test or continued until at least the seventh week of gestation.

Biochemical pregnancy was defined as an ascending human chorionic gonadotrophin value (the first human chorionic gonadotrophin determination was 14 days after blastocyst transfer) with no gestational sac being detected by ultrasonography. Clinical pregnancy, however, was determined by one or more gestational sacs with heart activity at 6–8 gestational weeks. The spontaneous loss of a clinical pregnancy before 22 weeks of gestational age was designated as a miscarriage. Live birth was defined as delivery of a live-born baby at 24 or more weeks of gestation.

Statistical analysis

The statistical package R (V 4.0.3, R Core Team, Austria) was used to perform the statistical analyses. First, a Kolmogorov–Smirnov test for normal distribution was applied. As neither the data nor the residuals from the analysis of variance (ANOVA) demonstrated a normal distribution, non-parametric statistics were used. Accordingly, independent samples were analysed using median tests. Post-hoc tests used Bonferroni correction to account for multiple testing. For nominal data, the selection between the chi-squared and Fisher's exact tests was driven by the sample size of the contingency table cells; Fisher's exact test was used when the chi-squared test was not applicable due to cells having counts of less than 5. ANOVA was used to compare the metric data within the three groups.

RESULTS

Cryo-survival

Out of the 257 vitrified/warmed blastocyst transfers that were originally scheduled, 252 (98.1%) were performed. Cryotransfer cycles were cancelled when the only available cryopreserved blastocysts did not survive the warming process (n = 5). On three occasions an additional blastocyst had to be warmed because the first choice did not survive, which led to an overall survival rate of 96.9% (252/260). The survival rates did not differ between study groups A (82/83, 98.8%) and B (84/87, 96.6%) and the control group (86/90, 95.6%) (P = 0.20 and 0.73, respectively).

Morphokinetic parameters

TABLE 1 shows the annotated re-expansion parameters of the 252 blastocysts that survived. Out of these, 212 blastocysts (84.1%) completed their re-expansion, and 124 (49.2%) had already started to hatch at the time of transfer. Significantly more ($P < 0.001$) blastocysts completed re-expansion in study groups A and B (92.7% and 94.1%, respectively) compared with the unmanipulated controls (66.3%). The same holds true for the percentage of blastocysts that started to hatch (57.3%, 58.3% and 32.6%; P = 0.0012 for study group A, $P < 0.001$ for study group B).

Group B started their re-expansion after warming (tRE) significantly earlier than did the blastocysts from group A (P = 0.005) and the control group ($P < 0.001$). Both ABC groups reached tCRE significantly earlier (P = 0.005 and $P < 0.001$, respectively) than the control group. The same trend was seen for tAH (P = 0.007 and $P < 0.001$, respectively). This phenomenon led to a significant difference in the duration of the re-expansion process (tCRE − tRE) in study group A (P = 0.021)

TABLE 1 MORPHOKINETIC PARAMETERS OF THE BLASTOCYST RE-EXPANSION PROCESS AFTER WARMING IN THE CONTROL AND STUDY GROUPS

Expansion timepoint (h)	Study group A	Study group B	Control group	Total	*P*-value[a]
	Pipette	Laser	Not collapsed		
tRE	0.79 ± 0.56[c]	0.50 ± 0.37[b]	1.22 ± 1.00	0.83 ± 0.08	<0.001
tCRE	2.15 ± 0.89[c]	1.78 ± 0.87[b]	3.04 ± 1.03	2.25 ± 1.05	<0.001
tAH	2.58 ± 0.85[d]	2.08 ± 0.77[b]	3.57 ± 0.92	2.61 ± 1.00	<0.001
tCRE − tRE	0.80 ± 0.63[e]	0.54 ± 0.39[c]	0.76 ± 0.72	0.69 ± 0.58	<0.001

Data are presented as mean ± standard deviation.

[a] Compared using analysis of variance; pairwise post-hoc tests used Bonferroni corrections for multiple testing. Further significant pairs are indicated using superscripts, and all the *P*-values can be found in Supplementary Table 2.

[b] $P < 0.001$ versus the control group

[c] $P \leq 0.005$ versus the control group

[d] $P = 0.007$ versus the control group

[e] $P = 0.021$ versus the control group.

tRE, time of start of re-expansion; tCRE, time of completion of re-expansion; tAH, time of start of hatching through the artificially created gap; tCRE − tRE, duration of the re-expansion process.

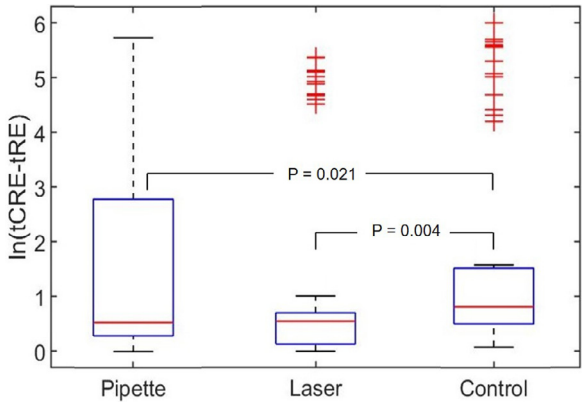

FIGURE 3 Box plot of the duration of re-expansion (hours post t0 which equals mid-time of ICSI) in both study groups and the control group. Each box represent the interquartile range, and the red line inside the box represents the median. Outliers are shown using a plus symbol. A post-hoc test with Bonferroni correction was used. tCRE – tRE, time of completion of re-expansion minus time of start of re-expansion (in minutes). Pipette and laser: artificial blastocyst collapse methods; control: not collapsed.

and study group B ($P = 0.004$) compared with the control group (FIGURE 3), with group B showing the shortest time.

Blastocoel re-expansion started and was completed significantly earlier in the group of patients with a live birth (tRE: 0.60 ± 0.42 h, $P < 0.001$; tCRE: 1.95 ± 0.94 h, $P = 0.012$) compared with those who did not conceive (1.05 ± 0.92 h and 2.46 ± 1.12 h). The cohort of patients who experienced pregnancy loss (including biochemical pregnancies and miscarriages) did not differ in timing from the live-birth group ($P = 0.45$ for tRE, $P = 0.88$ for tCRE). However, participants with pregnancy loss still showed a significantly earlier tRE (0.66 ± 0.45 h) than those who did not become pregnant ($P = 0.013$).

Of interest, spontaneous collapses (13.1–17.1%) and cytoplasmic strings (9.3–12.2%) were rarely seen after warming (TABLE 2). Due to the low number of transferred blastocysts being positive for these features, no analysis of could be undertaken of the potential correlation with treatment outcome.

Blastocyst characteristics

TABLE 2 shows the characteristics of the blastocysts included in this study. As mentioned above, 252 vitrified/warmed Gardner and Schoolcraft grade 4 or 5 blastocysts resulted in a single blastocyst transfer. A total of 132 blastocysts (52.4%) were graded as AA before vitrification and were homogeneously distributed between groups A and B and the control group (51.2%, 59.5%, and 46.5%, respectively).

The same holds true for BB grade blastocysts, which represented the lowest quality vitrified blastocysts and were also equally represented in the three groups (12.2%, 7.1% and 7.0%, respectively). TABLE 2 highlights that the quality of blastocysts for single-blastocyst transfer was comparable between the three groups.

Clinical outcome

Among the 252 single vitrified/warmed blastocyst transfers, biochemical pregnancy was achieved in 121 cases (48.0%). The associated clinical pregnancy rate was found to be 37.3% ($n = 94$). Two ectopic pregnancies, one medical abortion (due to a major cerebral malformation) and eight miscarriages reduced the proportion of live births to 32.9% ($n = 83$). There were 5.3% ($n = 5$) monozygotic twin pregnancies that were derived from either study group A ($n = 3$) or study group B ($n = 2$). Consequently, babies were born from 78 singleton and five twin pregnancies. All 43 male and 45 female babies were healthy; however, two children derived from a twin pregnancy and four singletons had to be referred to the neonatal intensive care unit.

As highlighted in TABLE 3, the method used for ABC was not related to the cryotransfer outcome because neither the biochemical ($P = 0.24$) nor the clinical ($P = 0.41$) pregnancy rate or the live birth rate ($P = 0.63$) differed among the three groups. This observation did not change when the pipette and laser data were pooled ($P = 0.094$ for biochemical pregnancy, $P = 0.36$ for clinical pregnancy

TABLE 2 QUALITIES AND MORPHOLOGICAL FEATURES OF THE BLASTOCYSTS THAT WERE TRANSFERRED

Features	Study group A	Study group B	Control group	*P*-value
	Pipette	Laser	Unmanipulated	
No. of cycles	82	86	89	
No. of frozen transfers	82	84	86	
Blastocyst expansion				
Grade 4	56 (68.3)	58 (69.0)	49 (57.0)	0.07
Grade 5	26 (31.7)	26 (31.0)	37 (43.0)	0.09
Inner cell mass				
Grade A	67 (81.7)	76 (90.5)	66 (76.7)	0.17
Grade B	15 (18.3)	8 (9.5)	20 (23.3)	0.08
Trophectoderm				
Grade A	53 (64.6)	59 (70.2)	60 (69.8)	0.38
Grade B	29 (35.4)	25 (29.8)	26 (30.2)	0.58
No. of embryos with				
Collapses	14 (17.1)	11 (13.1)	14 (16.3)	0.79
Strings	10 (12.2)	8 (9.5)	8 (9.3)	0.64

Data are presented as *n* or *n* (percentage).

A chi-squared test was used for the analysis.

Collapses and strings were identified after warming. No information is available on the presence of the two morphisms in the fresh blastocysts.

TABLE 3 CLINICAL OUTCOME OF VITRIFIED/WARMED SINGLE-BLASTOCYST TRANSFER OF CONTROL AND MANIPULATED EMBRYOS

Clinical outcomes	Study group A	Study group B	Control group	*P*-value
	Pipette	Laser	Not collapsed	
No. of transfers	82	84	86	
Biochemical pregnancy[a]	41 (50.0)	45 (53.6)	35 (40.7)	0.24
Clinical PR[b]	32 (39.0)	34 (40.5)	28 (32.6)	0.41
Live birth rate[c]	28 (34.1)	29 (34.5)	25 (29.1)	0.63
Multiple PR	3/32 (9.4)	2/34 (5.9)	0	0.13
Miscarriage rate[d]	4/32 (12.5)	5/34 (14.7)	3/28 (10.7)	0.70
Malformations	0	0	0	

Data are presented as *n* or *n* (percentage).

P-values were calculated using a Fisher's exact test for multiple pregnancy and miscarriage rates, and chi-squared tests for all other parameters.

[a] Defined as an ascending HCG value (the first HCG determination was 14 days after blastocyst transfer).

[b] Presence of one or more gestational sacs with heart activity at 6–8 weeks of gestation.

[c] Defined as the delivery of a live-born baby at ≥24 weeks of gestation.

[d] Spontaneous loss of a clinical pregnancy before 22 weeks of gestational age.

HCG, human chorionic gonadotrophin; PR, pregnancy rate.

rate and live birth rate). The number of miscarriages did not differ between the study and control groups.

DISCUSSION

While there is common position that blastocyst expansion is a positive predictor of fresh cycle treatment outcome, provided that the ICM and trophectoderm are of good quality (*Du et al., 2016*; *Huang et al., 2019*), the associated rise in blastocoelic fluid volume and its potential effect on survival and transfer outcomes in vitrified/warmed blastocysts is still a matter of concern (*Ebner et al., 2009*; *Morató et al., 2010*). The risk of less manageable osmotic changes during blastocyst exposure to cryoprotectants and ice crystal formation during vitrification has driven scientists to solve these problems by artificially reducing the large blastocoel fluid volume in expanded and hatching blastocysts before cryopreservation.

The first methods that emerged for blastocoelic fluid removal were based on osmotic shock (*Iwayama et al., 2011*) or mechanical removal of fluid, referred to as artificial blastocyst collapse (*Darwish and Magdi, 2016*; *Mukaida et al., 2006*; *Vanderzwalmen et al., 2003*). Indeed, such a drainage of the blastocoelic fluid was found to considerably increase the blastocyst survival rate after warming (*Boyard et al., 2022*; *Darwish and Magdi, 2016*; *Kovačič et al., 2022*; *Mitsuhata et al., 2019*; *Mukaida et al., 2006*; *Vanderzwalmen et al., 2003*) compared with untreated control blastocysts. However, this finding could not be confirmed in the present dataset, in which the survival, clinical pregnancy and live birth rates did not differ between the study and control groups.

Immediate survival upon warming is an important aspect that can help estimate the implantation potential of a warmed blastocyst (*Ebner et al., 2009*), but embryologists usually prefer to culture these blastocysts for a couple of hours to check for blastocoel re-expansion, which is thought to be a key predictor of viability (*Allen et al., 2022*; *Ebner et al., 2009*; *Shu et al., 2009*; *Wang et al., 2017*). In this context, the implementation of time-lapse imaging and continuous observation of blastocoel dynamics has broadened the spectrum of quality assessment approaches for post-warming blastocysts. While some authors have used this innovative technique to retrospectively screen videos for signs of re-expansion (*Maezawa et al., 2014*), or to document changes in key morphological structures of warmed blastocysts (*Coello et al., 2017*), others have precisely annotated the initiation and/or completion of the re-expansion process (*Coello et al., 2017*; *Ebner et al., 2017*).

Kovačič and colleagues reported that viable warmed blastocysts can follow one of the four types of dynamic re-expansion pattern (linear, non-linear, shrinkage/linear or linear growth with spontaneous collapse), which further complicates the comparison of the datasets available in the literature *Kovačič et al., (2018b)*. There is evidently still a certain lack of standardization in assessing blastocoel re-expansion dynamics (*Hershko-Klement et al., 2022*; *Kovačič et al., 2018a*). However, assessing blastocyst re-expansion ability or analysing growth dynamics post-warming seems reasonable from a physiological perspective. Re-expanding bovine blastocysts show greater glucose and pyruvate uptake (*Gardner et al., 1996*) and expanding human blastocysts have a higher oxygen consumption (*Maezawa et al., 2014*) than their non-expanding counterparts. More recently, new evidence has been published for fast blastocoel re-expansion after warming as a reliable indicator of treatment success (*Ebner et al., 2017*; *Lin et al., 2017*; *Mensing et al., 2023*; *Shu et al., 2009*).

The degree to which the manipulation during ABC before vitrification may affect blastocyst re-expansion dynamics after warming was unclear until recently, when two studies addressing this potential dependence were published (*Ebner et al., 2017*; *Kovačič et al., 2018b*). Surprisingly, the two groups showed different results. While Ebner and co-workers observed a notable delay in the re-expansion of collapsed blastocysts after warming, Kovačič and colleagues reported substantially faster re-expansion in blastocysts that were collapsed (*Ebner et al., 2017*; *Kovačič et al., 2018b*). The main difference between the two studies is the methods of ABC and blastocoel drainage before cryopreservation, which could influence blastocoel re-expansion kinetics. In the case of Ebner and co-workers blastocysts were manipulated with an ICSI pipette, whereas Kovačič and colleagues used a laser pulse for ABC. The present results corroborate this hypothesis: blastocysts that were collapsed with the ICSI pipette were slower to re-expand than those that did not collapse, and the laser-assisted collapse of blastocysts had the fastest re-expansion post-warming.

Rapid re-expansion is a feature of viable blastocysts (*Mensing et al., 2023*) and identifies a cohort of healthy blastocysts with a functional Na^+/K^+-ATPase pump. It has been reported that murine blastocysts that had been shrunk using laser showed improved re-expansion kinetics because of an effective stress response, which was

measured using endoplasmic reticulum stress markers such as activating transcription factor 4 and heat shock protein 90-alpha (*Frank et al., 2019*).

Using a pipette, embryologists can actively control the speed of drainage and decrease the blastocoel fluid volume. In the current experiment, as much fluid as possible was removed from the blastocoel in blastocysts in group A, causing the opposite trophectoderm cells to come into contact with the ICM. This strength of cohesion in blastocysts collapsed by the pipette might indeed have been the cause of the observed delay in tRE, because no such close contact between the two cell lineages was observed in most cases in laser-treated blastocysts. A similar effect was also noticed in the study of *Taborin and Kovačič (2019)*. Laser-induced shrinkage resulted in less standardized drainage patterns with interindividual variability in blastocyst response to a standardized laser pulse of 1.5 ms.

Many factors, such as pre-vitrification blastocoel size (*Park et al., 2022*), hatching status, cell number and/or internal pressure, may have contributed to the divergent responses. It took minutes for the laser-treated blastocysts to reach a state of equilibrium, at which point no further drainage was observed. Although most of the blastocoelic fluid leaked out of the blastocysts of group B, the ICM rarely came into contact with the opposite trophectoderm. The absence of direct contact might have promoted a faster initiation of re-expansion upon warming of the blastocysts.

If the survival rate and morphokinetics of artificially shrunken blastocysts are improved, higher pregnancy or cumulative pregnancy rates can be expected. Such relationships with clinical pregnancy rates have been reported over the years (*Boyard et al., 2022*; *Darwish and Magdi, 2016*; *Hur et al., 2011*; *Levi-Setti et al., 2016*). However, it should be clarified that such a benefit was not observed in the present clinical outcomes, as neither of the two evaluated ABC techniques increased clinical pregnancy or live birth rates compared with the unmanipulated control group. This is not in line with the literature because there is evidence of a positive effect of blastocoelic shrinkage, with both a pipette (*Hur et al., 2011*; *Mitsuhata et al., 2019*) and laser (*Wang et al., 2017*), on the live birth rate. By contrast, the only prospective evidence available (*Kovačič et al., 2022*; *Van Landuyt et al., 2015*) and one meta-analysis (*Boyard et al., 2022*) confirmed the current findings and did not support a potential association between ABC and live births.

The live birth rate may also have been affected by the relatively high miscarriage rate in the present dataset. Although this was within an acceptable range (*Devine et al., 2021*; *Honnma et al., 2012*; *Pakes et al., 2020*), the fact that most cycles were artificial could have contributed to the miscarriage rate because there is evidence that natural cycles perform better in this regard (*Vinsonneau et al., 2022*).

Another peculiarity related to the present clinical outcomes and worthy of discussion is the trend toward a higher monozygotic twinning (MZT) rate (5.9–9.4%) in ABC blastocysts. Although high rates of MZT for laser-induced ABC have also been reported elsewhere (*Kamel Mohamed et al., 2022*), unmanipulated blastocytes usually show a lower MZT rate (*Eliasen et al., 2021*; *Hazir et al., 2022*). It has previously been postulated that 'a series of (fresh) collapses that artificially generate cytoplasmic strings could eventually lead to MZT if the ICM is mechanically stressed by the contractions' (*Ebner et al., 2020*). Grøndahl and colleagues published a case report of conjoined twins after fresh single transfer of a blastocyst that showed both collapses and cytoplasmic strings (*Grøndahl et al., 2022*). Interestingly, another time-lapse video of a fresh blastocyst with ICM splitting, resulting in monochorionic triamniotic triplets, showed at least one cytoplasmic string and two distinct collapses (*Sutherland et al., 2019*). However, other authors have not found these two morphokinetic parameters to be relevant in predicting MZT (*Eliasen et al., 2021*).

There is a suggestion that loose ICM might further contribute to the observed twinning phenomenon (*Otsuki et al., 2016*), although this theory has not been unchallenged (*Eliasen et al., 2021*). Alternatively, multiple openings in the same blastocyst (ICSI and laser pulse) and assisted hatching might have influenced natural hatching process (*Schimmel et al., 2014*) and could have contributed to the phenomenon of MZT (*Skiadas et al., 2008*). However, it should be noted that, in the present study, only 1 in 5 (20%) MZT events were associated with a collapse after warming in a blastocyst with a loose ICM. This hypothesis of a theoretical association between collapses and MZT certainly deserves further investigation on a larger scale.

This current study demonstrated some limitations, including the retrospective nature of the analysis and the uncontrolled grouping of cases into three comparison groups, which opened up the possibility for certain confounders to influence the results. Therefore, most of these were compared in a descriptive analysis, and comparability between the groups was confirmed.

In summary, the descriptive analysis here suggests that artificially reducing blastocoelic fluid using either pipette or laser techniques may lead to high cryo-survival rates. In particular, laser-treated blastocysts exhibited a significantly shorter re-expansion process than those collapsed with a pipette or those left unmanipulated. As faster re-expansion dynamics are considered a positive predictor of treatment outcome, the laser technique could be prioritized over the pipetting technique when considering ABC. However, it is important to note that the retrospective nature of this study and the lack of adjustment for potential confounders, both known and unknown, limit the strength of these conclusions. Further research is necessary to explore the potential correlation between ABC techniques and an increased incidence of MZT, taking into account a more comprehensive analysis to adjust for confounding factors.

DATA AVAILABILITY

Data will be made available on request.

ACKNOWLEDGEMENTS

The authors would like to thank Gudrun Schappacher-Tilp for statistical expertise.

FUNDING

This work was partially supported by a travel grant from ASEBIR, the Spanish Society of Biology of Reproduction (01/2018). Iris Martínez-Rodero was awarded a scholarship from the Spanish Ministry of Science, Innovation and Universities (BES-2017-081962). This work was also partly funded by the Slovenian Research Agency (research programme P3-0327).

AUTHOR CONTRIBUTIONS

I.M.-R. and T.E. conceived and designed the experiments; I.M.-R. and T.E. performed the experiments; O.S, S.E., J.L. and T.E. provided the resources and analysed the data; I.M.-R., B.K. and T.E. wrote the manuscript.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.rbmo.2024.104476.

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Received 1 March 2024; received in revised form 26 July 2024; accepted 30 September 2024.