RESEARCH PAPER

Comparative Assessment of Early Cleavage-Stage Vitrification Followed by Culture Versus Direct Blastocyst Cryopreservation for Improved Embryo Selection and Pregnancy Outcomes In IVF

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Abstract
Background: The optimal stage for embryo vitrification remains a subject of investigation in assisted reproductive technology (ART). This study compared cleavage-stage vitrification followed by blastocyst culture with direct blastocyst vitrification to determine their relative effects on embryological and clinical outcomes.
Method: In a prospective randomized controlled study, 720 women undergoing *in vitro* fertilization (IVF) were allocated to cleavage-stage vitrification (Group A; n = 360) or direct blastocyst vitrification (Group B; n = 360). A total of 703 participants (97.6%) completed per-protocol analysis. Key embryological indicators—including embryo yield, post-thaw viability, morphology, and blastocyst development—were assessed, along with clinical outcomes such as implantation, pregnancy, and live birth rates.
Results: Group A showed higher embryo yield (5.6 ± 1.8 vs. 4.9 ± 1.6), better post-thaw survival (97.2% vs. 92.4%), and more intact embryos (94.1% vs. 88.6%). Blastocyst formation (85.6% vs. 78.1%) and good-quality blastocysts (72.3% vs. 63.5%) were also higher in Group A. Clinical outcomes favoured Group A, including implantation (54.1% vs. 45.6%), clinical pregnancy (61.8% vs. 50.9%), and live birth rates (52.6% vs. 42.4%). Cumulative pregnancy probability was higher in Group A (78.4% vs. 65.7%).
Conclusion: Cleavage-stage vitrification with post-thaw blastocyst culture enhances embryo quality and improves key IVF outcomes compared with direct blastocyst vitrification, supporting its preferential use in clinical practice.

Keywords: IVF; cleavage-stage vitrification; blastocyst culture; embryo survival; live birth rate; ART outcomes
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Introduction

Embryo cryopreservation and subsequent transfer are pivotal components of modern assisted reproductive technology (ART) programmes, enabling maximization of cumulative pregnancy rates. They also provide flexibility in cycle planning and help mitigate the risk of ovarian hyperstimulation syndrome (Liu et al. 2022). Within this context, vitrification has largely supplanted slow freezing as the preferred technique due to its superior post-thaw survival and clinical outcomes (Debrock et al. 2015). However, the optimal stage for embryo vitrification and subsequent culture—namely early cleavage stage versus blastocyst stage—remains a topic of ongoing investigation and clinical debate. Traditional approaches favouring cleavage-stage embryos (48 hours or 72 hours) vitrification have the advantage of a larger number of embryos available for banking, potentially increasing cumulative transfer opportunities. For example, earlier studies reported that embryos vitrified at the early cleavage stage showed high survival rates (≈ 93 % for intact blastomere survival), although these rates were slightly lower than those observed after blastocyst-stage vitrification (Cobo et al. 2012). On the other hand, blastocyst culture and vitrification inherently incorporated by a self-selection mechanism: only embryos robust enough to progress to the blastocyst stage are vitrified, thereby potentially enhancing implantation and live birth potential (Albahlol et al. 2022). Meta-analyses have demonstrated higher implantation rates with blastocyst transfer compared with cleavage-stage embryo transfer (Zeng et al. 2017).
Despite these advantages, extending embryo culture to the blastocyst stage also carries certain limitations:

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extended culture may lead to cycle cancellations when blastocyst formation fails and can reduce the number of vitrified embryos available for transfer (Cornelisse et al. 2024). Moreover, recent large cohort data indicate that although blastocyst transfer may reduce time to live birth, the cumulative live birth rate (CLBR) might not differ significantly between cleavage-stage and blastocyst-stage transfer in good-prognosis patients. The effect of vitrification stage on perinatal and obstetric outcomes is also a relevant concern; for example, a case study reported, obstetrical-perinatal complications after blastocyst transfer were increased (Ma et al. 2024). In the scenario of frozen-thawed embryo cycles, the strategy of vitrifying at early cleavage stage followed by subsequent culture to blastocyst (rather than direct blastocyst vitrification) has been proposed as a hybrid approach that might combine advantages of both early vitrification (preservation flexibility) and blastocyst culture (selection for developmental potential). Indeed, Albahlol et al. (2022) reported significantly improved clinical and ongoing pregnancy rates when post-thawed cleavage embryos were extended in culture to blastocyst stage versus non-extended culture (Clinical Pregnancy Rate(CPR) 59.7% vs 21.4%, $p < 0.0001$). These data suggest that post-thaw embryo culture may enhance embryo selection and synchrony with the endometrium, thereby optimizing outcomes. Nonetheless, the literature remains limited regarding rigorous comparative assessment of "early cleavage-stage vitrification → blastocyst culture" versus "direct blastocyst vitrification" in terms of embryo survival, pregnancy rate, implantation rate, cumulative live birth rate, and perinatal outcomes. Could the hybrid approach enable improved embryo selection, greater transfer flexibility, and higher pregnancy outcomes without reducing embryo yield or increasing risks? Investigation of this query is timely, especially given the increasing utilization of freeze-all cycles, vitrified embryo transfers, and extended culture systems in IVF laboratories (Mart Rodero et al. 2025).

Methodology

Study design

A prospective, randomized, and controlled clinical trial was conducted over a period of 24-months at a tertiary fertility centre to evaluate the efficacy of two embryo cryopreservation strategies in *in vitro* fertilization–embryo transfer (IVF-ET) cycles. Eligible participants were randomly split into two groups. Group A underwent embryo vitrification at the early cleavage stage (4-8 celled stage), with post-thaw culture continued to the blastocyst stage before uterine transfer. Group B participants had embryos cultured to the blastocyst stage in vitro, followed by direct vitrification and subsequent warming prior to transfer, without additional culture. Embryo quality was assessed using standard morphological criteria, and vitrification was performed using an open carrier system with validated cryoprotectants. Endometrial preparation was achieved using hormone replacement protocols, and embryo transfers were performed under transabdominal ultrasound guidance. Primary outcome measures included clinical pregnancy rate, while secondary outcomes encompassed embryo survival, implantation, and live birth rates. Ethical approval was obtained from the institutional review board, and informed consent was secured in accordance with the Declaration of Helsinki and Indian Council of Medical Research guidelines (Shenfielde et al., 2021; Zegers-Hochschild et al. 2017).

Participant selection frame work

Women aged 21 to 38 years undergoing IVF and intracytoplasmic sperm injection (ICSI) cycles were considered eligible for inclusion in this randomized clinical trial. Inclusion criteria included the presence of four or more high-quality embryos based on morphological grading, endometrial thickness ≥7 mm with a trilaminar pattern, and a clinical diagnosis of tubal factor infertility, mild male factor infertility, unexplained infertility, and related indications. Participants were excluded if they exhibited severe endometriosis (Stage III/IV), diminished ovarian reserve defined as Anti-Müllerian Hormone(AMH) <1 ng/mL with fewer than four oocytes retrieved, untreated hydrosalpinx, congenital uterine anomalies, acquired uterine anomalies, parental chromosomal abnormalities, and a documented history of recurrent implantation failure (defined as three or more unsuccessful IVF attempts). These criteria ensured a homogeneous study population suitable for assessing the effect of embryo cryopreservation strategy on clinical outcomes (Poli et al. 2022; Alpha Scientists in Reproductive Medicine & European Society of Human Reproduction and Embryology - Special Interest Group of Embryology, 2011).

Controlled Ovarian Stimulation (COS)

Controlled ovarian stimulation (COS) was initiated on Day2/Day3 of the menstrual cycle using a flexible GnRH antagonist approach. Participated clients received daily subcutaneous injections of recombinant follicle-stimulating hormone (rFSH) at doses ranging from 150 to 300 International Units (IU), with individual adjustments based on transvaginal ultrasonography findings and serum estradiol concentrations. Antagonist administration (antagonist dosage of 0.25 mg/day) commenced when the lead follicle reached ≥ 14 mm. Final oocyte maturation was triggered with a single subcutaneous dose of recombinant human chorionic gonadotropin (r-hCG, 250 µg) when atleast three or more follicles reached a diameter of ≥18 mm. Oocyte retrieval was performed 35–36 hours post-trigger under transvaginal ultrasound guidance and conscious sedation (Bosch et al. 2016; Griesinger 2012).

Process of fertilization and embryo culture

Intracytoplasmic sperm injection (ICSI) was performed on all mature oocytes to ensure consistent fertilization outcomes and minimize inter-cycle variability.
Following microinjection, zygotes were cultured in a

low-oxygen tri-gas incubator (5% CO_2, 5% O_2, 90% N_2) to mimic physiological in vivo conditions. Sequential culture media tailored for early cleavage and blastocyst development stages were utilized to support optimal embryo progression. In Group A, embryos exhibiting optimal morphology defined as 4 - 8 celled with ≤10% cytoplasmic fragmentation—were selected for vitrification. In Group B, embryos were cultured until Day 5, and only those that developed into good-quality blastocysts (≥3BB), as per the Gardner and Schoolcraft grading system, were vitrified for later transfer (Isa et al. 2023; Wirleitner et al. 2016).

Vitrification method

Embryo vitrification in both study groups was performed using Cryotec ready-to-use vitrification media, specifically the Cryotec Equilibration Solution (ES) and Cryotec Vitrification Solution (VS), following the standardized protocol. Embryos were first exposed to the equilibration solution for approximately 12–15 minutes, followed by rapid transfer to the vitrification solution for no more than 60 seconds. Subsequently, embryos were carefully loaded onto standard vitrification carriers (Cryolock) and immediately plunged into liquid nitrogen at −196°C to achieve ultra-rapid cooling. This method, designed to prevent intracellular ice crystal formation, enhances embryo viability and post-warming survival (Kuwayama et al. 2005; Rienzi et al. 2017). All vitrification procedures were conducted by trained embryologists using a sterile and aseptic technique under laminar airflow conditions to ensure optimal outcomes.

Warming and post-thaw culture

Embryo warming for both groups was performed using Cryotec commercially available warming media, following the manufacturer's standardized protocol to ensure consistency and viability. In Group A, vitrified cleavage-stage embryos were thawed and immediately cultured in sequential blastocyst media s to facilitate further development to blastocyst stage. Only blastocysts achieving a morphological grade of ≥3BB were deemed suitable and subsequently transferred into the uterine cavity under ultrasound guidance. In Group B, vitrified blastocysts were thawed and incubated for 2–4 hours to assess re-expansion, morphological integrity, and viability. Transfers were carried out on the same day following confirmation of post-warming survival and quality. This approach ensured optimal synchronization between embryo development and endometrial receptivity, improving implantation potential (Van Landuyt et al. 2013; Capalbo et al. 2020).

Endometrial preparation and embryo transfer

Endometrial preparation was conducted using a standardized artificial hormone replacement therapy (HRT) to ensure optimal synchronization between the endometrium and embryo development. Oral estradiol valerate (6 mg/day) was initiated on Day 2 of the menstrual cycle and continued until the endometrium achieved a thickness of ≥7 mm with a trilaminar pattern, as confirmed by transvaginal ultrasonography. Upon achieving the desired endometrial parameters, vaginal micronized progesterone (600 mg/day) was administered to support luteal phase development. Embryo transfer was scheduled on the sixth day of progesterone exposure (P+5), corresponding to the implantation window, and performed under transabdominal ultrasound guidance using a soft transfer catheter. Depending on embryo quality and patient-specific factors, either one or two blastocysts were transferred, in accordance with best practice guidelines to optimize pregnancy outcomes while minimizing the risk of multiple gestation (Wang et al. 2025; Bu et al. 2019).

Statistical analysis

Statistical analyses were performed using IBM SPSS software version 26.0 (IBM Corp. Armonk, NY, USA). Continuous variables were presented as mean ± standard deviation (SD) and assessed for normality using the Shapiro–Wilk test. Depending on data distribution, comparisons between groups were made using independent samples t-tests or one-way ANOVA. Categorical variables were analyzed using the Chi-square test and Fisher's exact test, when required due to small sample sizes. Time-to-pregnancy and cumulative pregnancy rates were evaluated using Kaplan–Meier survival analysis, with log-rank tests applied for group comparisons. A two-tailed p-value of less than 0.05 was considered statistically significant for all analyses (Altman, 1991; Motulsky, 2014).

Results

Participant enrolment, allocation, screening, allocation, eligibility and study completion

A total of 840 women were screened for eligibility, with 420 candidates assessed for each intervention group. Following screening, 120 participants (14.3%) were excluded before randomization. This included 60 participants from each group due to factors such as poor ovarian response, suboptimal endometrial parameters. The remaining 720 participants (85.7%) were randomized equally into Group A (cleavage-stage vitrification with post-thaw blastocyst culture) and Group B (direct blastocyst vitrification). The mean number of embryos vitrified per participant was slightly higher in Group A (5.6 ± 1.8) compared to Group B (4.9 ± 1.6). Post-thaw embryo survival was notably higher in Group A (97.2%) than in Group B (92.4%), and the rate of blastocyst formation after warming was also superior in Group A (85.6%) relative to Group B prior to vitrification (78.1%). The mean follow-up duration was 12.2 ± 1.3 months in Group A and 12.0 ± 1.4 months in Group B. A total of 17 participants (2.3%) were lost to dropouts 8, (2.2%) from Group A and 9 (2.5%) from Group B. Ultimately, 703 participants (97.6%) completed the study per protocol, 352 from Group A (97.8%), 351(97.5%) from Group B and 100% compliance with ethical approval and informed consent

was achieved across both groups. The entire study was completed over a 24-month period (Table. 1).

Table 1. Participant screening and eligibility distribution between group A and group B

Parameter	**Group A (Cleavage-Stage Vitrification + Blastocyst Culture)**	**Group B (Direct Blastocyst Vitrification)**	**Total (n = 840)**
Participants screened	420	420	840
No. of Participants excluded	60 (14.3 %)	60 (14.3 %)	120 (14.3 %)
Participants randomized (n, %)	360 (85.7 %)	360 (85.7 %)	720 (85.7 %)
Embryos vitrified per cycle	5.6 ± 1.8	4.9 ± 1.6	—
Post-thaw survival rate (%)	97.2	92.4	—
Blastocyst formation after warming (%)	85.6	78.1	—
Duration of follow-up (months)	12.2 ± 1.3	12.0 ± 1.4	—
Dropouts / Lost to follow-up (n, %)	8 (2.2 %)	9 (2.5 %)	17 (2.3 %)
Completed per-protocol Analysis (n, %)	352 (97.8 %)	351 (97.5 %)	703 (97.6 %)
Ethical clearance and informed consent	Obtained from all participants	Obtained from all participants	100% compliance
Duration of the study (months)	24	24	24

Controlled ovarian stimulation

A total of 720 participants underwent controlled ovarian stimulation (COS), with 360 in each group. The mean duration of stimulation was comparable between Group A (10.4 ± 1.6 days) and Group B (10.2 ± 1.5 days), with no significant difference (p = 0.284). The total recombinant follicle-stimulating hormone (FSH) dose administered was comparable between the groups 2225 ± 410 IU in Group A and 2280 ± 430 IU in Group B (p = 0.312).. Peak serum estradiol levels on the day of trigger were slightly higher in Group A (2375 ± 520 pg/ml) than in Group B (2298 ± 505 pg/ml), though not statistically significant (p = 0.198). The average number of follicles ≥17 mm was 12.3 ± 3.1 in Group A and 11.8 ± 2.9 in Group B (p = 0.127). Oocyte retrieval rates were high and comparable between groups, at 96.8% for Group A and 96.1% for Group B (p = 0.412). The mean number of oocytes retrieved was 13.4 ± 3.6 in Group A versus 12.9 ± 3.4 in Group B (p = 0.176), and the proportion of mature (MII) oocytes was 86.2% and 84.9%, respectively (p = 0.294). The incidence of ovarian hyperstimulation syndrome (OHSS) was low and similar between the groups, with 4 cases (1.1%) in Group A and 3 cases (0.8%) in Group B (p = 0.682), indicating that the stimulation protocol was safe across both strategies (Table 2).

Table 2. Controlled ovarian stimulation (COS) parameters in Group A and Group B

Stimulation Parameter	Group A (Cleavage-Stage Vitrification + Blastocyst Culture)	Group B (Direct Blastocyst Vitrification)	Total (n = 720)	p-Value
Participants undergoing COS	360	360	720	—
Duration of stimulation (days ± SD)	10.4 ± 1.6	10.2 ± 1.5	—	0.284
Total recombinant FSH dose (IU ± SD)	2225 ± 410	2280 ± 430	—	0.312
Peak serum estradiol (pg/ml ± SD)	2375 ± 520	2298 ± 505	—	0.198
Number of follicles ≥ 17 mm	12.3 ± 3.1	11.8 ± 2.9	—	0.127
Oocyte retrieval rate (%)	96.8	96.1	—	0.412
Number of oocytes retrieved (± SD)	13.4 ± 3.6	12.9 ± 3.4	—	0.176

Mature (MII) oocytes (%)	86.2	84.9	—	0.294
Incidence of OHSS (n, %)	4 (1.1 %)	3 (0.8 %)	7 (1.0 %)	0.682

Fertilization and embryo culture

Out of 720 randomized participants, 703 (352 in Group A and 351 in Group B) proceeded to ICSI. The mean number of oocytes injected per cycle was 12.1 ± 3.2 in Group A and 11.8 ± 3.4 in Group B, with no significant difference ($p = 0.368$). Normal fertilization rates, assessed by the presence of two pronuclei (2PN), were similar between the groups—81.7% in Group A and 80.6% in Group B ($p = 0.274$). The number of zygotes formed was also comparable: 9.9 ± 2.6 in Group A versus 9.6 ± 2.5 in Group B ($p = 0.317$). Cleavage rates were uniformly high in both groups, at 96.2% and 95.8%, respectively ($p = 0.458$). On Day 3, the number of embryos reaching cleavage stage was 9.1 ± 2.4 in Group A and 8.8 ± 2.3 in Group B ($p = 0.246$). However, statistically significant differences were observed in several key parameters. The number of embryos vitrified per cycle was significantly higher in Group A (5.6 ± 1.8) than in Group B (4.9 ± 1.6), with a p-value of 0.041, indicating a higher embryo yield with cleavage-stage vitrification followed by blastocyst culture. Additionally, the blastocyst formation rate post thaw was superior in Group A (85.6%) compared to Group B (78.1%), showing statistical significance ($p = 0.028$). The proportion of good-quality embryos available for transfer was also higher in Group A (72.3%) than in Group B (63.5%) ($p = 0.039$), and the post-thaw survival rate significantly favoured Group A (97.2%) over Group B (92.4%) **(**$p = 0.032$**)** **(**Table. 3**).**

Table 3. Fertilization and embryo culture outcomes in group A and group B

Parameter	Group A (Cleavage-Stage Vitrification + Blastocyst Culture)	Group B (Direct Blastocyst Vitrification)	Total (n = 720)	p-Value
Participants proceeding to ICSI	352	351	703	—
Oocytes injected per cycle	12.1 ± 3.2	11.8 ± 3.4	—	0.368
Normal fertilization rate (2PN %)	81.7	80.6	—	0.274
Number of zygotes formed	9.9 ± 2.6	9.6 ± 2.5	—	0.317
Cleavage rate (%)	96.2	95.8	—	0.458
Number of embryos reaching Day 3	9.1 ± 2.4	8.8 ± 2.3	—	0.246
Embryos selected for vitrification	≥ 4-8 celled, ≤ 10 % fragmentation)	Blastocysts ≥ 3BB (Isa et al. 2023)	—	—
Number of embryos vitrified per cycle	5.6 ± 1.8	4.9 ± 1.6	—	**0.041
Blastocyst formation rate (%)	85.6 (post-thaw extension)	78.1 (pre-vitrification)	—	**0.028
Good-quality embryos for transfer (%)	72.3	63.5	—	**0.039
Post-thaw survival rate (%)	97.2	92.4	—	**0.032

Significant difference ($p < 0.05$). (**) symbols indicates the high significant value.

Vitrification performance

A total of 703 participants underwent embryo cryopreservation, with 352 assigned to Group A and 351 to Group B. Embryo yield differed between groups, with Group A demonstrating a significantly higher number of embryos available for cryopreservation (5.6 ± 1.8) compared with Group B (4.9 ± 1.6; $p = 0.041$). Post-thaw performance consistently favored Group A. Cleavage-stage cryopreserved embryos exhibited a superior survival rate (97.2%) relative to blastocysts in Group B (92.4%; $p = 0.032$). The proportion of morphologically intact embryos after warming was also higher in Group A (94.1%) than in Group B (88.6%; $p = 0.028$). Viability following warming was enhanced in Group A, reflected by a markedly better blastocyst formation rate after culture (89.3%) in contrast to the re-expansion rate observed in Group B (80.7%; $p = 0.021$). Additionally, Group A recorded a significantly lower degeneration rate (2.8%) compared with Group B (7.6%; $p = 0.019$). Overall, these findings indicate that cleavage-stage cryopreservation followed by blastocyst development provides more robust structural preservation and improved post-warming viability than direct blastocyst-stage cryopreservation.

Table 4. Vitrification protocol performance in group A and group B

Parameter	Group A (Cleavage-Stage Vitrification + Blastocyst Culture)	Group B (Direct Blastocyst Vitrification)	Total (n = 720)	p-Value
Participants undergoing vitrification	352	351	703	—
Embryo developmental stage at vitrification	Day 2 – Day 3 (4–8 cells)	Day 5 – Day 6 (≥ 3BB blastocysts)	—	—
Equilibration time (min)	12	15	—	—
Vitrification exposure (s)	≤ 60 ± 5	≤ 60 ± 5	—	—
Storage temperature (°C)	−196	−196	—	—
Number of embryos vitrified	5.6 ± 1.8	4.9 ± 1.6	—	0.041 *
Vitrification time per cycle	9.2 ± 1.5	8.8 ± 1.4	—	0.237
Post-thaw survival rate (%)	97.2	92.4	—	0.032 *
Morphologically intact embryos (%)	94.1	88.6	—	0.028 *
Re-expansion rate after warming (%)	89.3	80.7	—	0.021 *
Degeneration rate (%)	2.8	7.6	—	0.019 *

* Significant at $p < 0.05$

Warming and post-thaw culture

A total of 703 participants underwent embryo warming, with 352 in Group A (cleavage-stage vitrification with blastocyst culture) and 351 in Group B (direct blastocyst vitrification). The survival rate after warming was significantly higher in Group A (97.2%) compared to Group B (92.4%) (p = 0.031), indicating better cryotolerance in the cleavage-stage vitrification method. Additionally, a significantly greater proportion of embryos in Group A showed full re-expansion post-warming (89.3%) versus Group B (80.7%) (p = 0.024), reflecting superior post-thaw viability. Partial survival with slight degeneration was observed in 5.6% of embryos in Group A and 8.9% in Group B (p = 0.047), while the rate of complete degeneration was significantly lower in Group A (2.8%) than in Group B (7.6%) (p = 0.019). The post-thaw culture duration differed substantially: Group A embryos were cultured for a mean of 60 ± 6 hours to allow progression to the blastocyst stage, whereas Group B embryos were transferred within 3 ± 1 hours after warming. Among warmed embryos, the blastocyst formation rate post-thaw in Group A was 85.6%, with 72.3% reaching good-quality blastocyst grades (≥3BB), significantly higher than the 63.5% observed in Group B (p = 0.038). The number of embryos transferred per cycle was similar between groups (1.7 ± 0.5 in Group A vs. 1.8 ± 0.4 in Group B; p = 0.278). However, a significantly lower proportion of embryos were discarded due to poor morphology in Group A (3.4%) compared to Group B (6.9%) (p = 0.041) (Table. 5).

Table 5. Post-warming survival and culture outcomes in group A and group B

Parameter	Group A (Cleavage-Stage Vitrification + Blastocyst Culture)	Group B (Direct Blastocyst Vitrification)	Total (n =720)	p-Value
Participants undergoing embryo warming	352	351	703	—
Survival rate after warming (%)	97.2	92.4	—	0.031 *

Embryos showing full re-expansion (%)	89.3	80.7	—	0.024 *
Partial survival / slight degeneration (%)	5.6	8.9	—	0.047 *
Completely degenerated embryos (%)	2.8	7.6	—	0.019 *
Post-thaw culture duration (hours)	60 ± 6	3 ± 1	—	—
Blastocyst formation rate post-thaw (%)	85.6	—	—	—
Good-quality blastocysts for transfer (≥ 3BB %)	72.3	63.5	—	0.038 *
Embryos selected for transfer	1.7 ± 0.5	1.8 ± 0.4	—	0.278
Time from warming to transfer (hours)	60 ± 5	4 ± 1	—	—
Embryos discarded due to poor morphology (%)	3.4	6.9	—	0.041 *

* Significant difference ($p < 0.05$)

Endometrial reparation and embryo transfer outcomes

Endometrial preparation was successfully completed in all 703 participants, with 352 in Group A and 351 in Group B. The duration of estradiol administration was similar between the groups, with 12.4 ± 2.1 days in Group A and 12.2 ± 2.3 days in Group B (p = 0.316). Prior to progesterone initiation, the mean endometrial thickness was 9.1 ± 0.8 mm in Group A and 9.0 ± 0.9 mm in Group B (p = 0.428), and the proportion of participants achieving a trilaminar endometrial pattern was high in both groups—98.6% in Group A and 97.9% in Group B (p = 0.512). All embryo transfers were conducted under ultrasound guidance, and the progesterone exposure prior to transfer was consistent across both groups at 5.0 ± 0.3 days. The number of blastocysts transferred per cycle was 1.7 ± 0.5 in Group A and 1.8 ± 0.4 in Group B (p = 0.284), with 100% of transfers performed using ultrasound-guided soft catheter techniques. The rate of atraumatic or easy transfers was high and comparable—97.4% in Group A and 96.8% in Group B (p = 0.612). The endometrial receptivity score (ERS) was 8.7 ± 0.6 in Group A and 8.6 ± 0.5 in Group B (p = 0.289), indicating favourable receptivity in both cohorts. Notably, Group A demonstrated a significantly higher implantation rate (54.1%) compared to Group B (45.6%) (p = 0.042), as well as a higher clinical pregnancy rate (61.8% vs. 50.9%, p = 0.036 (Table. 6).

Table 6. Endometrial preparation and embryo transfer outcomes in group A and group B

Parameter	Group A (Cleavage-Stage Vitrification + Blastocyst Culture)	Group B (Direct Blastocyst Vitrification)	Total (n = 703)	p-Value
Participants undergoing endometrial preparation	352	351	703	—
Duration of estradiol administration (days)	12.4 ± 2.1	12.2 ± 2.3	—	0.316
Endometrial thickness (mm) before progesterone	9.1 ± 0.8	9.0 ± 0.9	—	0.428
Percentage achieving trilaminar pattern (%)	98.6	97.9	—	0.512
Progesterone supplementation duration before transfer (days)	5.0 ± 0.3	5.0 ± 0.3	—	—
Type of embryo transfer	Blastocyst after post-thaw culture	Directly warmed blastocyst	—	—
Number of blastocysts transferred	1.7 ± 0.5	1.8 ± 0.4	—	0.284
Transfer method (% ultrasound-guided)	100	100	—	—
Easy/atraumatic transfer rate (%)	97.4	96.8	—	0.612

Endometrial receptivity score (ERS)	8.7 ± 0.6	8.6 ± 0.5	—	0.289
Implantation rate (%)	54.1	45.6	—	0.042 *
Clinical pregnancy rate (%)	61.8	50.9	—	0.036 *

* Significant difference ($p < 0.05$)

Comparative statistical analysis of clinical and embryological parameters

Comparison of outcomes between Group A and Group B demonstrated a clear advantage for the cleavage-stage vitrification strategy. Group A achieved a higher blastocyst formation rate (85.6%) compared with Group B (78.1%). Post-thaw performance also favored Group A, which showed a superior survival rate (97.2%) relative to Group B (92.4%), along with a greater proportion of good-quality blastocysts (72.3% vs. 63.5%). Clinically, Group A recorded higher implantation (54.1% vs. 45.6%), clinical pregnancy (61.8% vs. 50.9%), ongoing pregnancy (55.4% vs. 44.8%), and live birth rates (52.6% vs. 42.4%) compared with Group B. Although the miscarriage rate was lower in Group A (9.1%) than in Group B (12.7%), the difference was not substantial. Importantly, Group A required fewer embryo transfer attempts to achieve pregnancy, highlighting improved embryo competence and selection efficiency. Overall, the cumulative pregnancy probability was considerably higher in Group A (78.4%) than in Group B (65.7%), underscoring the clinical advantage of cleavage-stage cryopreservation over direct blastocyst vitrification (Table. 7).

Table 7. Statistical analysis of key clinical and embryological outcomes between group A and group B

Outcome Parameter (%)	Group A (Cleavage-Stage Vitrification + Blastocyst Culture)	Group B (Direct Blastocyst Vitrification)	p-Value	Inference
Number of oocytes retrieved	13.4 ± 3.6	12.9 ± 3.4	0.176	NS – no significant difference
Fertilization rate	81.7	80.6	0.274	NS
Blastocyst formation rate	85.6	78.1	0.028 *	Significant
Post-thaw survival rate (%)	97.2	92.4	0.032 *	Significant
Good-quality blastocysts (≥ 3BB %)	72.3	63.5	0.039 *	Significant
Implantation rate (%)	54.1	45.6	0.042 *	Significant
Clinical pregnancy rate	61.8	50.9	0.036 *	Significant
Ongoing pregnancy rate (%)	55.4	44.8	0.041 *	Significant
Miscarriage rate	9.1	12.7	0.322	NS
Live birth rate	52.6	42.4	0.039 *	Significant
Number of transfer attempts to achieve pregnancy	1.3 ± 0.4	1.8 ± 0.6	0.008 *	Significant
Cumulative pregnancy probability (Kaplan–Meier %)	78.4	65.7	0.017 *	Significant cumulative advantage in Group A

* $p < 0.05$ = statistically significant; NS = not significant

Discussion

This study provides a comprehensive comparison of two embryo cryopreservation strategies used in IVF-ET cycles: cleavage-stage vitrification followed by post-thaw blastocyst culture (Group A) and direct blastocyst vitrification (Group B). Both the studies demonstrated high procedural feasibility and patient compliance, with 720 women randomized and a per-protocol completion rate of 97.6% across both groups. While participant screening, allocation, and follow-up durations were comparable, Group A consistently outperformed Group B in several key embryological and clinical parameters. Group A showed a significantly higher mean number of embryos vitrified per cycle (5.6 ± 1.8 vs. 4.9 ± 1.6), better post-thaw survival (97.2% vs. 92.4%), and enhanced blastocyst formation after warming (85.6% vs. 78.1%). These findings align with prior studies suggesting that cleavage-stage vitrification followed by extended culture offers a more efficient embryo selection mechanism and improved cryotolerance (Cobo et al. 2012; Van Landuyt et al. 2013). The lower degeneration rates and higher re-expansion rates in Group A further confirm the biological robustness of this approach. Clinically, Group A achieved superior implantation (54.1% vs. 45.6%), clinical pregnancy (61.8% vs. 50.9%), and live birth rates (52.6% vs.

42.4%), consistent with earlier reports highlighting the enhanced synchrony between the embryo and endometrium when blastocyst culture follows cleavage-stage freezing (Albahlol et al. 2022; Capalbo et al. 2020). Moreover, Group A required fewer embryo transfer attempts to achieve pregnancy, and the cumulative pregnancy probability based on Kaplan–Meier analysis was significantly higher in this group (78.4% vs. 65.7%), underscoring its clear clinical advantage.

The present study demonstrates that while controlled ovarian stimulation (COS) parameters were largely comparable between the two groups, cleavage-stage vitrification followed by post-thaw blastocyst culture (Group A) yielded superior embryological outcomes compared to direct blastocyst vitrification (Group B). Both groups showed similar stimulation duration (10.4 ± 1.6 vs. 10.2 ± 1.5 days), total gonadotropin dose, estradiol levels, and oocyte retrieval rates, indicating homogeneity in baseline ovarian response. These findings are consistent with earlier evidence suggesting that COS outcomes remain independent of the selected cryopreservation strategy (Bosch et al., 2016; Griesinger, 2012). Post-fertilization outcomes significantly favoured Group A. While fertilization and cleavage rates were similar between groups, Group A exhibited a statistically higher number of embryos vitrified per cycle (5.6 ± 1.8 vs. 4.9 ± 1.6; $p = 0.041$), a better blastocyst formation rate (85.6% vs. 78.1%; $p = 0.028$), and a higher proportion of good-quality embryos suitable for transfer (72.3% vs. 63.5%; $p = 0.039$). These improvements may be attributed to the self-selection inherent process in blastocyst culture post-thaw, which allows only viable embryos to progress, thereby enhancing developmental competence (Isa et al. 2023; Wirleitner et al. 2016). Moreover, the post-thaw survival rate was significantly better in Group A (97.2%) than in Group B (92.4%) ($p = 0.032$). The early-stage embryos possess higher cryotolerance and structural integrity during vitrification and extended culture (Cobo et al. 2012). These findings are consistent with Van Landuyt et al. (2013), who reported improved embryo viability and transfer success when cleavage-stage vitrification was followed by blastocyst culture, as opposed to direct blastocyst freezing.

The findings from this study highlight a clear advantage of cleavage-stage vitrification followed by post-thaw blastocyst culture (Group A) over direct blastocyst vitrification (Group B) in terms of vitrification protocol performance. Group A demonstrated significantly higher embryo yield (5.6 ± 1.8 vs. 4.9 ± 1.6; $p = 0.041$), improved post-thaw survival rate (97.2% vs. 92.4%; $p = 0.032$), and greater morphological integrity post-warming (94.1% vs. 88.6%; $p = 0.028$). The re-expansion rate, a reliable predictor of implantation competence, was also markedly superior in Group A (89.3% vs. 80.7%; $p = 0.021$), while the degeneration rate was significantly lower (2.8% vs. 7.6%; $p = 0.019$). These results suggest that early-stage embryos are more resilient to cryo-injury and better suited for downstream culture and selection. These outcomes are consistent with prior studies reporting higher cryosurvival and developmental competence for cleavage-stage embryos. Kuwayama et al. (2005) emphasized the role of ultra-rapid vitrification in minimizing intracellular ice formation, a critical factor for successful thaw outcomes. Similarly, Cobo et al. (2012) found that vitrification at the cleavage stage resulted in superior survival and clinical performance in donor egg cycles. Rienzi et al. (2017) further confirmed the reliability of vitrification across stages but noted improved performance when using early-stage embryos under controlled laboratory conditions.

The outcomes of embryo warming and post-thaw culture further reinforced the biological advantages of cleavage-stage vitrification followed by extended blastocyst culture (Group A) compared to direct blastocyst vitrification (Group B). Group A exhibited a significantly higher post-thaw survival rate (97.2% vs. 92.4%; $p = 0.031$) and a greater proportion of embryos with full re-expansion (89.3% vs. 80.7%; $p = 0.024$), both of which are key indicators of embryo viability. The degeneration rate was markedly lower in Group A (2.8%) compared with Group B (7.6%; p = 0.019), resulting in substantially fewer embryos exhibiting partial survival or morphological compromise 3 in Group A and 11 in Group B. These results align with findings from Van Landuyt et al. (2013) and Capalbo et al. (2020), reporting that extended culture after early-stage vitrification enhances post-warming developmental potential and morphological integrity. Importantly, Group A achieved a higher blastocyst formation rate post-thaw (85.6%) and a significantly larger proportion of good-quality blastocysts for transfer (72.3% vs. 63.5%; $p = 0.038$), underscoring the effectiveness of post-thaw developmental screening. In terms of endometrial preparation and embryo transfer, both groups achieved comparable endometrial thickness, trilaminar pattern rates, and progesterone exposure durations, suggesting that hormonal priming was uniformly effective. However, Group A demonstrated a significantly higher implantation rate (54.1% vs. 45.6%; $p = 0.042$) and clinical pregnancy rate (61.8% vs. 50.8%; $p = 0.036$). These improved outcomes reflect to enhance synchrony between the embryo and endometrium, facilitated by extended post-thaw culture and morphological selection, as supported by Wang et al. (2025) and Bu et al. (2019).

The comparative statistical analysis between cleavage-stage vitrification with extended blastocyst culture (Group A) and direct blastocyst vitrification (Group B) revealed a consistent and significant clinical advantage for Group A across multiple embryological and pregnancy-related outcomes. While the number of oocytes retrieved and fertilization rates were statistically comparable between the groups, Group A demonstrated superior blastocyst formation rates (85.6% vs. 78.1%; $p = 0.028$) and post-thaw survival rates (97.2% vs. 92.4%; $p = 0.032$), suggesting improved developmental potential and cryotolerance of embryos vitrified at the cleavage stage and subsequently cultured. Furthermore, the proportion of good-quality blastocysts (≥3BB) was

significantly higher in Group A (72.3%) compared to Group B (63.5%) ($p = 0.039$), indicating better post-thaw embryo quality. These findings support previous studies by Cobo et al. (2012) and Rienzi et al. (2017), which emphasized that early-stage embryo vitrification coupled with blastocyst culture enables natural selection of competent embryos and preserves developmental integrity. Clinically, Group A achieved significantly higher implantation (54.1% vs. 45.6%; $p = 0.042$), clinical pregnancy (61.8% vs. 50.9%; $p = 0.036$), ongoing pregnancy (55.4% vs. 44.8%; $p = 0.041$), and live birth rates (52.6% vs. 42.4%; $p = 0.039$), demonstrating that improved embryological performance resulted in superior clinical outcomes. Although miscarriage rates did not differ significantly, the reduced incidence in Group A (9.1% vs. 12.7%; $p = 0.322$) suggests a possible trend toward better embryo-endometrial synchrony and viability. Additionally, Group A required significantly fewer transfer attempts to achieve pregnancy (1.3 ± 0.4 vs. 1.8 ± 0.6; $p = 0.008$), highlighting procedural efficiency and patient benefit. Finally, the cumulative pregnancy probability based on Kaplan–Meier analysis was significantly higher in Group A (78.4%) than Group B (65.7%) ($p = 0.017$), aligning with Van Landuyt et al. (2013), reported long-term benefits of early-stage vitrification with post-warming embryo development. These results advocate for the clinical adoption of cleavage-stage vitrification followed by blastocyst culture as a superior strategy in IVF methods.

Conclusion

This study provides compelling evidence that cleavage-stage embryo vitrification followed by extended in vitro culture to the blastocyst stage yields superior clinical and embryological outcomes compared to direct blastocyst vitrification. Participants in the cleavage-stage vitrification group consistently demonstrated higher embryo survival rates, better blastocyst formation, and a greater proportion of high-quality embryos. These improvements translated into significantly enhanced implantation rates, clinical and ongoing pregnancy rates, as well as live birth outcomes. Additionally, patients in this group required fewer embryo transfer attempts to achieve pregnancy and exhibited a higher cumulative pregnancy probability. Importantly, no significant differences were observed in ovarian stimulation and in fertilization metrics between the conducted groups, underscoring that the advantage lies specifically in the timing and strategy of cryopreservation. The findings support the notion that early-stage vitrification, followed by a post-thaw culture period, allows for better selection of viable embryos and improved synchrony with endometrial receptivity. This is considered as a more effective and clinically beneficial approach in optimizing assisted reproductive outcomes. Future studies are being done to analyse if this strategy will help in adopting SET (single embryo transfers) technique to improve perinatal outcomes and live birth rates

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