BMC Pregnancy and Childbirth

RESEARCH

Open Access

Embryonic and neonatal outcomes following double vitrification/thawing: a systematic review and meta-analysis

Arezoo Maleki-Hajiagha[1,2], Anahid Shafie[1], Saeede Rezayi[1], Mahnaz Marvi[3], Rana Karimi[3] and Fardin Amidi[1,4*]

Abstract

Objectives This systematic review and meta-analysis aimed to evaluate the impact of double vitrification/thawing (DVT) versus single vitrification/thawing (SVT) on key embryonic and neonatal outcomes.

Data extraction Information sources included systematic search in PubMed, Scopus, and Cochrane databases up to September 7, 2024. Data from each qualifying study were extracted by two reviewers using a standardized electronic data gathering form.

Data Analysis Mantel-Haenszel odds ratio (MHOR) and mean difference (MD) with 95% confidence intervals (CI) were calculated using both fixed and random-effects models. Subgroup analyses were based on biopsy status, number of biopsy rounds, extended culture between rounds of vitrification, and embryo transfer strategy.

Results A total of 35 studies involving 46,749 embryo transfer cycles were included. After excluding studies that used slow freezing, 28 studies were included in the meta-analyses. The findings indicated that DVT is associated with significant reductions in cryosurvival rates (MHOR: 0.4; CI: 0.3 to 0.8; $P<0.01$), biochemical pregnancy (MHOR: 0.7; CI: 0.6 to 0.8; $P<0.01$), clinical pregnancy (MHOR: 0.7; CI: 0.5 to 0.8; $P<0.01$), and live birth rates (MHOR: 0.6; CI: 0.5 to 0.7; $P<0.01$). Additionally, there was a significant increase in the miscarriage rate (MHOR: 1.4; CI: 1.2 to 1.7; $P<0.01$). No significant differences were found in neonatal outcomes.

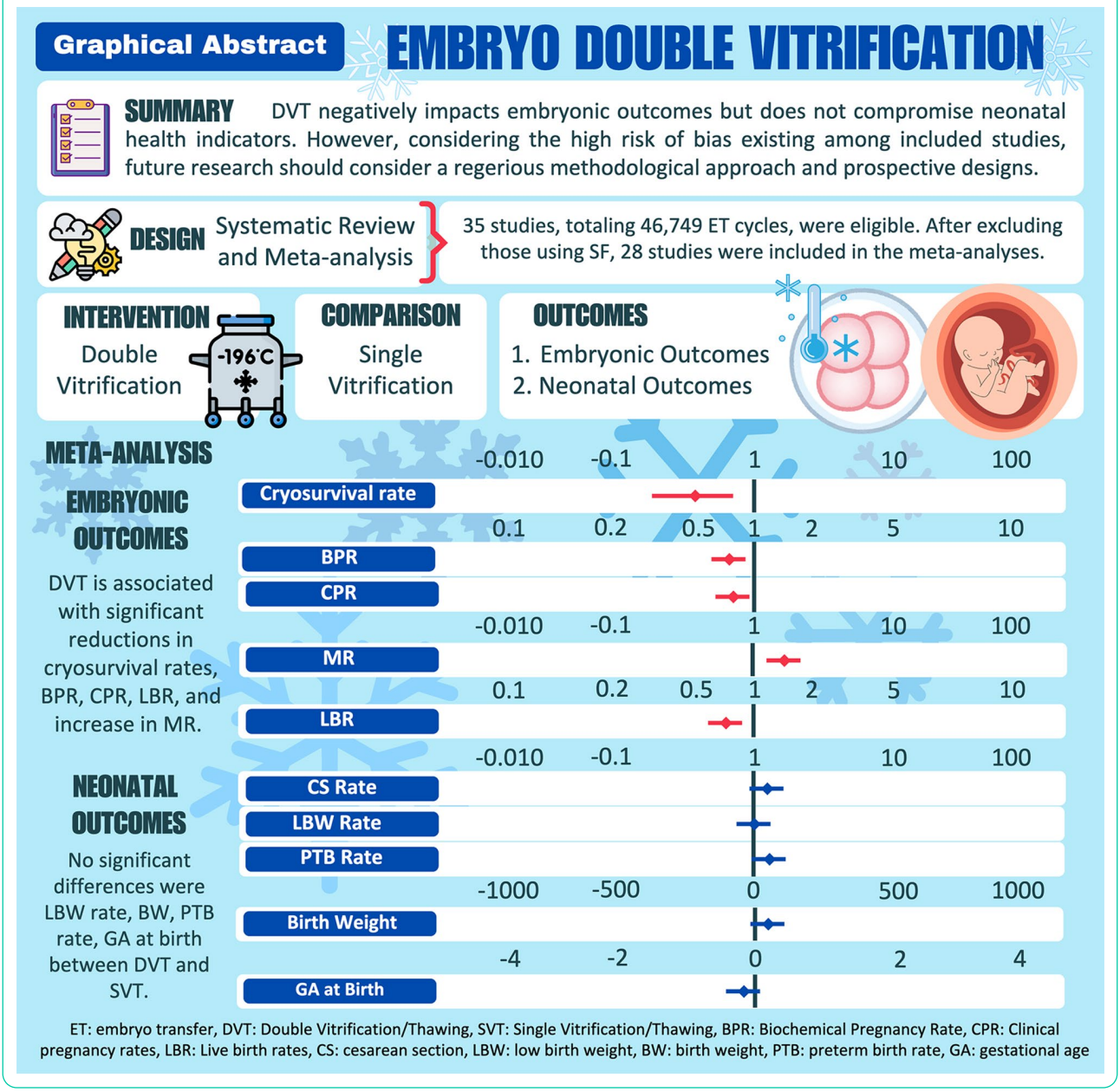
Conclusion Poor-quality evidence suggests that the transfer of double-vitrified embryos might be associated with significantly lower rates of cryosurvival, pregnancy, and live births; however, it does not appear to affect neonatal outcomes such as birth weight and gestational age at birth. Given the small sample size in some subgroups, the high risk of selection, confounding and missing data biases, and the high level of heterogeneity for some outcomes, these findings should be interpreted cautiously.

Keywords Double cryopreservation, Double vitrification, Repeated freezing, Embryo survival, Cryoinjury, Cryodamage

*Correspondence:
Fardin Amidi
Famidi@tums.ac.ir

Full list of author information is available at the end of the article

Graphical Abstract



Introduction

The practice of repeated cryopreservation in assisted reproductive technology (ART) has become increasingly common due to the widespread adoption of elective freeze-all and single embryo transfer (SET) strategies. These strategies, while effective in minimizing the risks of ART-related complications like ovarian hyperstimulation syndrome or multiple pregnancies [1–3], often resulting in surplus embryos. Surplus embryos may undergo multiple rounds of vitrification, although this is less common compared to single vitrification. Additionally, for preimplantation genetic testing (PGT), embryos may be subjected to one or more biopsies during the PGT process, further complicating their cryopreservation history. The need for repeated biopsy may be due to inconclusive diagnoses, test failures, or genetic testing on already cryopreserved, non-biopsied embryos [4].

Despite the prevalence of these practices, the impact of repeated rounds of cryopreservation and biopsy on embryonic and neonatal outcomes remains a topic of debate. Some studies have suggested that double cryopreservation can negatively affect reproductive outcomes,

including significantly lower survival rates [5] lower live birth rates (LBR), reduced implantation potential [6, 7], and lower clinical and ongoing pregnancy rates [8]. Conversely, other studies have found no significant differences in neonatal outcomes [9, 10]. Addressing these inconsistencies is crucial for improving clinical practices and patient outcomes in ART. This will guide patient expectations and enhance clinical decision-making.

Unlike the existing reviews, the present review applied a more comprehensive methodological approach in several aspects. Unlike Wang et al., [11] who included only non-biopsied embryos, our review encompassed both biopsied and non-biopsied embryos, providing a more comprehensive analysis. Additionally, Bickendorf et al. [12] and Piani et al. [13] focused solely on biopsied embryos and compared single and double biopsy, whereas our study reported results from both single and double biopsy procedures and also compared each approach together to provide a more comprehensive picture of these scenarios. We also took several other factors into account, such as embryo transfer (ET) strategy and culture status, to provide more homogeneous data. Furthermore, we performed several sensitivity analyses and a detailed quality assessment approach to provide more reliable and robust data. The current review aimed at providing a comprehensive understanding of the impact of embryo double vitrification/thawing (DVT) on a wide range of clinical and neonatal outcomes.

Methods

This systematic review and meta-analysis aimed to compare embryonic and neonatal outcomes between embryos subjected to double cryopreservation and single cryopreservation. Our methodology adhered to the Cochrane Handbook for Systematic Reviews of Interventions and the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Appendix S2).

Eligibility criteria

Inclusion criteria

We included all types of interventional studies, such as clinical trials and quasi-experimental studies, as well as all kinds of retrospective and prospective observational studies, including case-control and cohort studies. These studies needed to compare double cryopreservation with single cryopreservation concerning at least one of the specified outcomes. Full-text articles and conference abstracts were eligible for inclusion if they provided sufficient data for statistical analyses.

Exclusion criteria

We excluded studies that used cryopreserved oocytes, as well as any controls other than double cryopreservation. This also includes case reports, case series, reviews, editorials, animal studies, in-vitro studies, and studies that lacked sufficient methodological or result details. To maintain consistency, we excluded studies that employed slow freezing methods from our meta-analyses, ensuring that all included studies utilized the same cryopreservation technique. Vitrification, which is the most current and widely accepted cryopreservation method, enhances the relevance of this review to current clinical practices. By excluding slow freezing studies, we simplify interpretation and eliminate the variable of cryopreservation method, allowing us to more confidently attribute differences in outcomes to other factors, such as biopsy status. So, for the meta-analysis phase, study groups included DVT and single vitrification/thawing (SVT).

Search strategy

We conducted a systematic search in PubMed, Scopus, and Cochrane databases from inception to September 7, 2024. Additional studies were identified through hand-searching references and citation lists. No restrictions were applied on date, language, document type, geographical region, or publication status. Search terms included combinations of "Recryopreservation," "revitrification," "refrozen," "twice," "repeated," "double," "multiple," "Cryopreservation," "vitrification," "freezing," "Embryo," "blastocyst," and "zygote." Grey literature was explored through databases like Open Grey and clinical trial registries. (For detailed search strategy, see Appendix S1 file)

Study selection

Relevant studies were organized into a structured Excel database, with duplicates removed. Two independent reviewers (A.S. and S.R.) screened titles and abstracts against eligibility criteria. Full texts of potentially relevant studies were retrieved for comprehensive evaluation. Discrepancies were resolved through discussion with a third reviewer (A.M-H.). A full list of studies that were excluded by abstract and full-text screening with reasons for exclusion is provided in Supplementary File S2.

Data extraction

Data from each qualifying study were extracted and verified by two reviewers (R.K. and M.M.) using a standardized electronic data capture form. Extracted data included:

Baseline characteristics

Methodological aspects, technical and clinical considerations (devices, media, post-thaw culture duration, embryonic manipulations, controlled ovarian stimulation, developmental Stage at ET, and endometrial preparation protocol).

Outcome measures

Key embryonic and neonatal outcomes investigated in this review are defined as the following:

- *Embryo Cryosurvival Rate*: This is calculated as the number of surviving embryos divided by the total number of cryopreserved embryos. Several morphological criteria are used in studies to assess whether an embryo has sustained significant damage after thawing. These include cell membrane integrity, blastomere appearance, blastocoel cavity re-expansion, cellular granularity, and mitotic activity. We will consider any definitions of embryonic survival used in the included studies as eligible for our analysis.
- *Biochemical Pregnancy Rate (BPR)*: This refers to the number of chemical pregnancies diagnosed by the detection of beta human chorionic gonadotropin (BhCG) in blood or urine, relative to the total number of ET cycles.
- *Clinical Pregnancy Rate (CPR)*: This is the number of intrauterine clinical pregnancies diagnosed by ultrasonographic observation of fetal heartbeats, divided by the total number of ET cycles.
- *Miscarriage Rate (MR)*: This represents any type of pregnancy loss occurring before 20 weeks of gestation, relative to the total number of pregnancies. We consider all forms of miscarriage—biochemical, clinical, spontaneous, induced, early, or late—as part of this rate. Ideally, the total number of positive BhCG tests is used as the number of pregnancies; however, in some studies [4, 5, 7, 9, 14–20] that only reported clinical miscarriages or did not provide sufficient data for biochemical pregnancies, the number of clinical pregnancies was used for calculating the MR.
- *LBR*: This is the proportion of all ET cycles that resulted in the birth of at least one live baby, counted as the birth of one or more live babies from a single pregnancy, regardless of whether this involves singletons or multiples.
- *Cesarean Section (C/S) Rate*: This indicates the number of cesarean deliveries per total number of births.
- *Gestational Age (GA) at Birth*: This refers to the mean and standard deviation (SD) of GA (weeks) at the time of birth. If GA was reported in days in the included studies, we converted these values to weeks for consistency.
- *Rate of Preterm Birth (PTB)*: This measures the number of births occurring before 37 weeks of gestation relative to the total number of births.
- *Birth Weight (BW)*: This denotes the mean and SD of neonate's weight (grams) at birth.
- *Rate of Low Birth Weight (LBW)*: This is the number of neonates weighing less than 2500 g at birth relative to total number of neonates.

Quality appraisal

We employed the ROBINS-E (Risk of Bias in Non-randomized Studies - of Exposures) tool to assess the risk of bias in included studies.

Statistical analysis

Embryonic and neonatal outcomes were extracted, and the Mantel-Haenszel (MH) odds ratio (OR) with 95% confidence intervals (CI) was calculated for each categorical endpoint, and the mean difference (MD) with 95% CI was calculated for the numerical endpoint (BW and GA at birth). The pooled MHOR with 95% CI was obtained using a random-effects model. Heterogeneity was assessed using forest plots, chi-square-based Q statistic, and I^2 value, with significance P value or $I^2 > 40\%$. Statistical analyses were performed using Comprehensive Meta-Analysis (CMA) software (Version 3.0, Biostat Inc., USA).

Subgroup analyses

Biopsy status and number of Biopsy rounds

The primary intervention in this review was DVT. Previous reviews have typically included studies involving DVT solely in either biopsied or non-biopsied embryos. In contrast, our review includes studies using DVT in both biopsied and non-biopsied embryos, followed by subgroup analyses based on biopsy status.

Biopsy status involves additional embryonic manipulations that can affect embryo viability and implantation rates. Additionally, there is a hypothesis that opening the zona during biopsy may expose embryonic cells to direct contact with cryoprotectant agents (CPA), potentially increasing their toxic effects on blastomeres. On the other hand, transferring euploid embryos may lead to better ET outcomes compared to non-biopsied ETs. However, different indications for PGT (e.g., advanced maternal age, recurrent pregnancy loss, or known genetic conditions) may be associated with varying baseline risks and outcomes, adding another layer of complexity to the analysis.

In the first stage of our analyses, we combined the data from both single and double biopsy procedures into a single subgroup. The reason for merging these groups was to enhance the study's statistical power and to concentrate on the primary variable of interest: the DVT process. By combining this data, we aimed to improve our understanding of the effects of DVT.

Furthermore, merging the single and double biopsy groups allows for a more robust analysis by increasing the overall sample size and statistical power. Notably, out

of the 31,042 biopsied embryos, only 2,222 (less than 5%) underwent two rounds of biopsy. Given this relatively small number, we believed that it would not significantly influence our findings.

In the second stage, to ensure precise and comprehensive data, we also conducted subgroup analyses based on the number of biopsy rounds, comparing outcomes for no biopsy, one round of biopsy, and two rounds of biopsy. This review thus provides a thorough understanding of double vitrification across all biopsy types.

ET strategy

This variable compared different ET strategies, including single blastocyst transfer (SBT) or other strategies, including multiple blastocyst transfers, cleavage stage transfers, or a combination of both strategies. This analysis helps determine if using a uniform ET strategy can produce more homogeneous findings, reducing variability and heterogeneity in study results.

Extended embryo culture

We compared the extended culture (EC) periods between the first thawing (at 2PN or cleavage stage) and second vitrification (at blastocyst stage) versus no culturing (re-vitrification at same developmental stage/day of first thawing).

We also intended to perform subgroup analyses based on maternal age, type of insemination (IVF or ICSI), study design (prospective or retrospective), vitrification device (open or closed system), and the presence or absence of other embryonic manipulations (such as artificial/assisted hatching or zona opening for biopsy or artificial shrinkage). However, most studies did not report exact or enough data. Due to these inconsistencies, these variables could not be considered for subgroup analyses.

Sensitivity analyses

To ensure the robustness of our findings, we first conducted a sensitivity analysis by excluding studies with a very high risk of bias from the meta-analysis. This step aimed to verify the consistency and reliability of our results, ensuring that our conclusions were based on comprehensive and thoroughly reviewed studies. If moderate or high levels of heterogeneity (> 40%) persisted after this initial sensitivity analysis, we performed an additional step by also excluding studies with a high risk of bias.

Publication bias

Publication bias for each outcome was assessed using funnel plots. The trim-and-fill analysis was conducted to adjust for any 'missing' studies due to publication bias. Additionally, Egger's test was performed to statistically assess the presence of publication bias.

Results

Summary of the literature search

Our initial search across electronic databases retrieved a total of 2,956 records from databases, with an additional eight records found through manual and citation searching. This included 701 from PubMed and 2,255 from Scopus. After removing 349 duplicate records, 2,615 records were screened. Of these, 2,559 records were excluded based on the screening criteria.

Fifty-six reports were sought for retrieval, and all were successfully retrieved. These reports were assessed for eligibility, resulting in the exclusion of 21 reports for the following reasons: inclusion of both fresh and frozen cycles ($n = 1$), use of frozen gametes followed by FET ($n = 3$), repetitive congress abstract ($n = 1$), lack of a favorable control group ($n = 5$), case reports ($n = 3$), not reporting outcomes of interest ($n = 1$), and animal studies ($n = 6$).

Ultimately, 35 studies were included in this systematic review with a total of 46,749 ET cycles. This included both biopsied and non-biopsied embryos across two groups. In the control group, there were 28,332 biopsied embryos and 13,473 non-biopsied embryos, resulting in a total of 41,805 ET cycles. In the double vitrification group, there were 2,710 biopsied embryos (2,222 single-biopsied and 488 double-biopsied) and 2,234 non-biopsied embryos, resulting in a total of 4,944 ET cycles.

Finally, in the meta-analysis phase, 7 reports [8, 21–26] were excluded from the meta-analysis due to the use of a slow freezing strategy. Notably, two studies [27, 28] that applied both slow freezing and vitrification strategies reported their data separately, allowing us to include the vitrification data in the meta-analysis. This left 28 studies included in the meta-analyses.

The study flow of our literature search and study selection is depicted in the PRISMA 2020 flow diagram [29] (Fig. 1). For transparency, we have also provided a complete list of the screened studies, along with their details and reasons for exclusion at each screening phase, in Appendix S5 File.

Study characteristics

Table 1 summarizes the key methodological characteristics of all included studies. All records were designed as retrospective cohort studies spanning various countries and publication dates. The majority of the studies were published between 2018 and 2024, with a few earlier studies dating back to 2001. Most of the studies were full-text articles, with a few presented as congress abstracts.

Table 2 represents details of technical considerations in reviewed studies

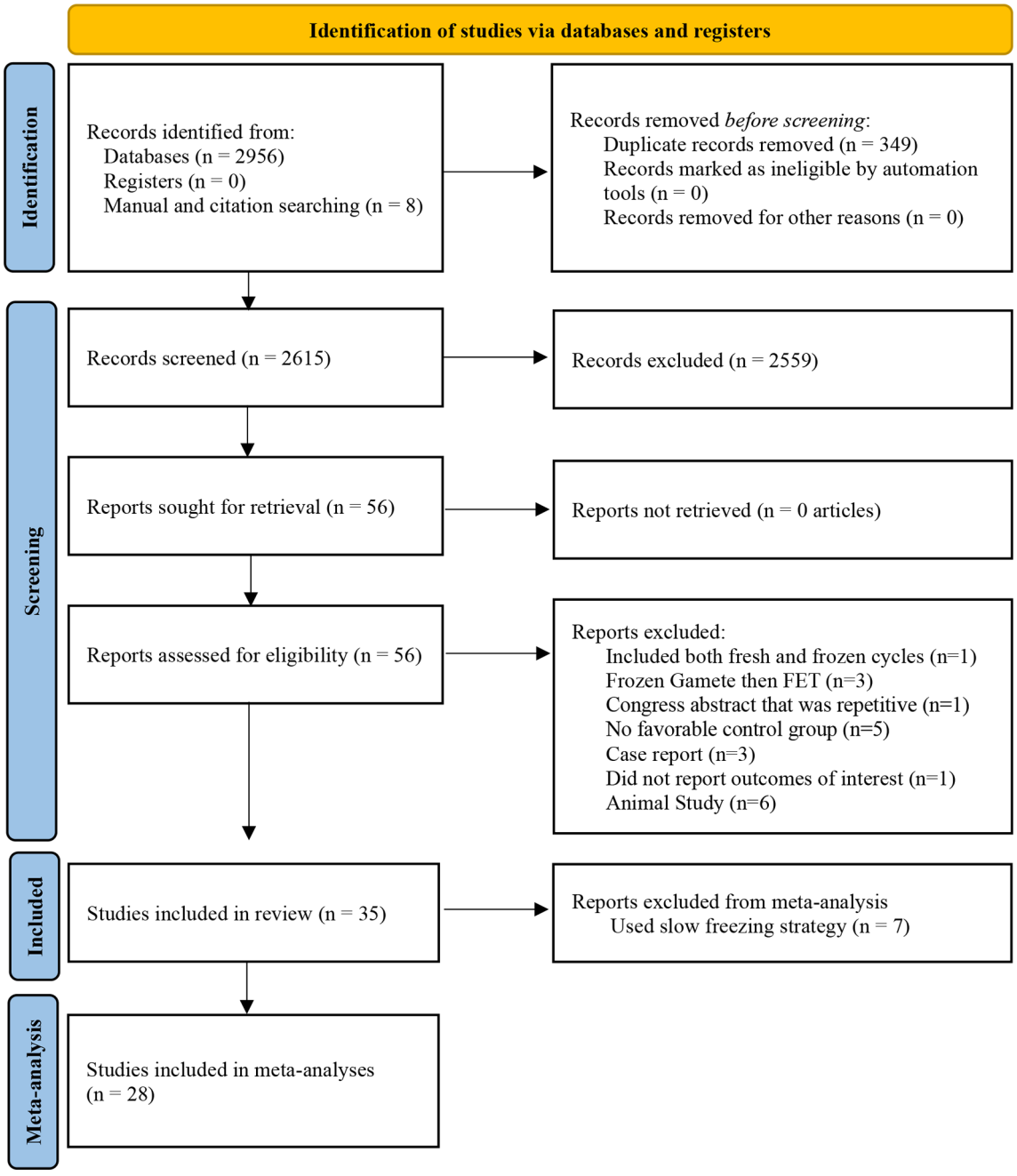


Fig. 1 PRISMA (preferred reporting items for systematic reviews and meta-analyses) 2020 flow diagram

Quality and risk of bias assessment

The summary of the risk of bias assessment is shown in Fig. 2. A total of 36 studies were evaluated for risk of bias using the ROBINS-E tool. The final scoring was as follows: 7 studies were rated as having a low risk of bias, seven studies had some concerns, 12 studies were rated as high risk, and 10 studies were rated as having a very high risk of bias.

- Confounding Bias: 17 studies were scored as high risk because they did not report on confounding factors or adjust their analyses accordingly.
- Measurement of Exposure: 6 studies were scored as high risk due to using slow freezing or a mix of slow freezing and vitrification methods, which have different effects on outcomes.
- Selection of Participants: 2 studies were judged to be at high risk of bias because they had groups with single vitrification and single biopsy compared

Table 1 Methodological characteristics of all included studies

Study ID	Location	Design	Article Type	Non-Biopsied		Biopsied			Mean or Median Age of Participants at ET day	Mean or Median Age of Participants at Egg Collection Day	Sample Size (Number of Patients)	Sample Size (Number of ET Cycles)	Included In Meta-analysis
				SCNB	DCNB	SCSB	DCSB	DCDB					
Theodorou et al., 2024	UK	Retrospective cohort	F			*	*	*	SCSB: 37 (34–40); DCSB: 38 (35–40); DCDB: 39 (35–41)	SCSB: 36 (33–39); DCSB: 36 (34–38); DCDB: 37 (34–39)	SCSB = 1684; DCSB = 312; DCDB = 50	SCSB = 1684; DCSB = 312; DCDB = 50	Yes
Vanderhoff et al., 2024	USA	Retrospective cohort	F			*	*	*	N/M	SCSB: 37.12 ± 4.00; DCSB: 35.64 ± 3.59; DCDB: 37.00 ± 4.02	SCSB = 1684; DCSB = 94; DCDB = 27	SCSB = 1602; DCSB = 68; DCDB = 19	Yes
Al Hashimi et al., 2024	UK	Retrospective cohort	F			*	*	*	SCSB: 37.9 ± 3.9; DCSB + DCDB: 38.7 ± 3.5	SCSB: 36.9 ± 3.8; DCSB + DCDB: 36.5 ± 3.5	N/M	SCSB = 729; DCSB = 73; DCDB = 11	Yes
Oraiopoulou et al., 2024	Greece	Retrospective cohort	F	*	*				SCNB: 33.3 ± 5.7; DCNB: 32.1 ± 6.7		SCNB = 450; DCNB = 252	SCNB = 450; DCNB = 252	Yes
Wang et al., 2024	China	Retrospective cohort	F	*	*				SCNB: 31 (28, 33); DCCNB: 31 (27, 35)	N/M	SCNB: 360; DCCNB: 360	SCNB: 360; DCNB: 360	Yes
Huang et al., 2024	China	Retrospective cohort	F	*	*				SCNB: 32.60 ± 4.07; DCNB: 31.77 ± 4.18	N/M	N/M	SCNB: 337; DCNB: 73	Yes
He et al., 2024	China	Retrospective cohort	F	*	*				SCNB: 29.32 ± 3.13; DCNB: 28.00 ± 2.85		SCNB: 8034; DCNB: 66	SCNB: 8034; DCNB: 66	Yes
Li et al., 2023	China	Retrospective cohort	F			*	*		SCSB: 33.38 ± 4.01; DCSB: 32.70 ± 3.93	SCSB: 33.38 ± 4.01; DCSB: 32.70 ± 3.93	SVSB: 177; DVSB: 30	SVSB: 177; DVSB: 30	Yes
Makieva et al., 2023	Switzerland	Retrospective cohort	F	*	*				SVNB: 36.61 ± 4.2; DVNB: 36.65 ± 4.1	SVNB: 35.7 ± 4.3; DVNB: 35.06 ± 4.2	N/M clearly	SVNB: 310; DVNB: 97	Yes
Zhang et al., 2023	China	Retrospective cohort	F			*	*		N/M	SCSB: 31.00; DCSB: 33.00	SCSB: 112; DCSB: 154	SCSB: 97; DCSB: 117	Yes
Nanassy et al., 2023	Germany	Retrospective cohort	F	*	*				SCNB: 33.6 ± 0.30; DCNB: 32.5 ± 0.39	N/M	DCNB: 122; SCNB: 189	DCNB: 122; SCNB: 189	Yes
Shen et al., 2023	China	Retrospective cohort	F	*	*				N/M	SCNB: 30.4 ± 4.4; DCNB: 29.3 ± 4.1	SCNB: 592; DCNB: 55	SCNB: 592; DCNB: 55	Yes
Brolinson et al., 2023	USA	Retrospective cohort	CA			*	*	*	N/M	N/M	N/M	SCSB: 7238; DCSB: 600; DCDB: 124	Yes
Nohales et al., 2023	Spain	Retrospective cohort	F			*		*	Mean or median of age is N/M (range 18–45 years old)	N/M	N/M	SCSB: 4562; DCDB: 71	Yes
Makieva et al., 2022	Switzerland	Retrospective cohort	CA	*	*				DCNB: 36.6 ± 4.4; SCNB: 36.5 ± 4.4	DCNB: 35.1 ± 4.4; SCNB: 35.9 ± 4.1	DCNB: 103; SCNB: 349	DCNB: 103; SCNB: 349	Yes
Theodorou et al., 2022	UK	Retrospective cohort	F			*	*		SCSB: 38 (36–40); DCSB: 39 (36–42)	SCSB: 38 (36–40); DCSB: 37.5 (34–41)	N/M clearly	SCSB: 97; DCSB: 117 (VT1 at day 5:146; VT1 at day 3: 97)	Yes
Oraiopoulou et al.,. 2022	Greece	Retrospective cohort	CA	*	*				SCNB: 33 ± 5.9; DCNB: 32.8 ± 5.9	N/M	SCNB: 172; DCNB: 233	SCNB: 172; DCNB: 233	Yes

Table 1 (continued)

Study ID	Location	Design	Article Type	Non-Biopsied		Biopsied			Mean or Median Age of Participants at ET day	Mean or Median Age of Participants at Egg Collection Day	Sample Size (Number of Patients)	Sample Size (Number of ET Cycles)	Included In Meta-analysis
				SCNB	DCNB	SCSB	DCSB	DCDB					
Wang et al., 2022	China	Retrospective cohort	F	*	*				SCNB: 36.26 ± 5.11; DCNB: 35.91 ± 4.56	N/M	SCNB: 294; DCNB: 98	SCNB: 294; DCNB: 98	Yes
Mizobe et al., 2021	Japan	Retrospective cohort	F	*	*				SCNB: 34.46 ± 4.32; DCNB: 34.91 ± 3.89	N/M	SCNB: 306; DCNB: 82	SCNB: 306; DCNB: 82	Yes
Aluko et al., 2021	USA	Retrospective cohort	F			*	*	*	SCSB: 36.7 (33.8–39.3); DCSB: 35.0 (32.0–39.1); DCDB: 37.5 (35.1–39.1)	SCSB: 36.2 (33.5–38.9); DCSB: 33.9 (31.7–37.7); DCDB: 37.0 (34.9–38.8)	SCSB: 2603; DCSB: 95; DCDB: 15	SCSB: 2603; DCSB: 95; DCDB: 15	Yes
Wang et al., 2021	China	Retrospective cohort	F	*	*				SCNB: 31.09 ± 4.86; DCNB: 32.07 ± 5.59	N/M	SCNB: 244; DCNB: 216	SCNB: 244; DCNB: 216	Yes
Hallamaa et al., 2021	Finland	Retrospective case–control	F	*	*				N/M	SCNB: 33 (4.1); DCNB: 32 (4.2)	SCNB: 304; DCNB: 89	SCNB: 304; DCNB: 89	Yes (with some adjustments)
Neal et al., 2019	USA	Retrospective cohort	F			*	*	*	SCSB: 36.0 ± 4.4; DCSB: 35.8 ± 5.6; DCDB: 36.2 ± 4.4	SCSB: 34.8 ± 4.4; DCSB: 32.0 ± 4.4; DCDB: 35.5 ± 4.4	N/M	SCSB: 3542; DCSB: 155; DCDB: 36	Yes
Schlenker et al., 2019	USA	Retrospective cohort	CA			*	*	*	SCSB: 37.3 ± 3.4; DCSB: 35.1 ± 3.7; DCDB: 37.8 ± 3.8	N/M	N/M	SCSB: 93; DCSB: 326; DCDB: 93	Yes
Farhi et al., 2019	Israel	Retrospective cohort	F	*	*				SCNB: 31.8 ± 3.2; DCNB: 30.8 ± 3.6	N/M	SCNB: 50; DCNB: 25	SCNB: 50; DCNB: 25	No
Wilding et al., 2019	UK	Retrospective cohort	F			*	*		SCSB: 40.1 ± 3.6; DCSB: 38.1 ± 4.1	N/M	SCSB: 220; DCSB: 54	SCSB: 26; DCSB: 19	No
Gunnala et al., 2018	USA	Retrospective cohort	CA			*	*		SCSB: 36.3 ± 4.1; DVSB: 34.4 ± 4.2	N/M	N/M	SCSB: 1028; DVSB: 125	Yes
Cimadomo et al., 2018	Italy	Retrospective cohort	F			*		*	Total population: 38.5 ± 3.9	N/M	N/M	SCSB:2825; DCDB: 49	Yes
Bradley et al., 2017	Australia	Retrospective cohort	F			*	*	*	N/M	N/M	N/M	SCSB: 2130; DCSB: 34, DCDB: 29	Yes
Zheng et al., 2017	China	Retrospective cohort	F	*	*				DCNB: 31.55 ± 4.16; SCNB: 31.45 ± 3.86	N/M	SCNB: 444; DCNB: 127	SCNB: 444; DCNB: 127	No
Taylor et al., 2014	USA	Retrospective cohort	F			*	*	*	N/M	SCSB: 35.6 ± 3.9; DCSB: 35.3 ± 4.8 (2 V) and 34.1 ± 5.5 (SF + V); DCDB: 39.0 ± 2.8	SCSB: 85; DCSB: 14; DCDB: 2	SCSB: 85; DCSB: 14; DCDB: 2	Yes (with some adjustments)
Stanger et al., 2012	Australia	Retrospective cohort	F	*	*				N/M	Nine couples < 35, nine couples: 35–39, seven > = 40 or more	N/M	SCNB: 506; DCNB: 30	No

Table 1 (continued)

Study ID	Location	Design	Article Type	Non-Biopsied		Biopsied			Mean or Median Age of Participants at ET day	Mean or Median Age of Participants at Egg Collection Day	Sample Size (Number of Patients)	Sample Size (Number of ET Cycles)	Included In Meta-analysis
				SCNB	DCNB	SCSB	DCSB	DCDB					
Murakami et al., 2011	Japan	Retrospective cohort	F	*	*				SCNB: 34.1 ± 4.1; DCNB: 33.8 ± 3.7	N/M	SCNB: 277; DCNB: 92	SCNB: 277; DCNB: 92	No
Koch et al., 2011	Australia	Retrospective cohort	F	*	*				Total population age: 32 ± 4.4	N/M	N/M clearly	SCNB: 40; DCNB: 52	No
Kumasako et al., 2009	Japan	Retrospective cohort	F	*	*				SCNB: 35.5 ± 4.3; DCNB: 34.8 ± 3.8	N/M	SCNB: 159; DCNB: 49	SCNB: 201; DCNB: 50	Yes
Check et al., 2001	USA	Retrospective cohort	F	*	*				DCNB: 36.2 ± 7.2; SCNB: 33.4 ± 4.4	N/M	SCNB: 14; DCNB: 14	SCNB: 12; DCNB: 12	No

CA: Congress Abstract; DCDB: Double cryopreservation and double biopsy; DCNB: Double cryopreservation and no biopsy; DCSB: Double cryopreservation and single biopsy; F: Full Text; SCNB: Single cryopreservation and no biopsy; SCSB: Single cryopreservation and single biopsy

to double vitrification and double biopsy, without evaluating double vitrification and single biopsy. One study selected live births first and then retrospectively evaluated neonatal outcomes. The remaining studies were rated as having some concerns because they were designed retrospectively, which could introduce selection bias.

- Post-Exposure Interventions: All studies that applied biopsy along with vitrification were scored as having some concerns, as this intervention could cause significant variation in findings.
- Missing Data: Many studies did not mention how they handled missing data and were rated as having some concerns. Abstracts with poorly described methodologies were rated as having a very high risk of bias.

Overall, the evaluation highlighted significant variability in study design and reporting, with a notable number of studies lacking adjustments for confounding factors and clear handling of missing data. These findings underscore the need for more rigorous methodological standards in future research to ensure the reliability and validity of results.

Results of meta-analysis

Considering the numerous statistical analyses and subgroups included in the present review, all results, including overall analyses (fixed and random effects models), subgroup analyses, sensitivity analyses, and publication bias, are summarized in Table 3 to prevent confusion. Detailed information for each outcome measure are depicted in the forest plots (see Appendix S3 file).

Cryosurvival rate

The meta-analysis included 10 studies assessing the cryosurvival rate following DVT compared to SVT. One study [7] did not provide explicit total embryo counts. Therefore, we assumed the total number of embryos in the control and intervention groups based on the provided high-grade and low-grade counts. One study [27] did not report the survival rate in the control group, so it was excluded from the meta-analysis due to the lack of necessary details for accurate comparison.

The random-effects model indicated a statistically significant decrease in cryosurvival rate following DVT (MHOR: 0.4; 95% CI: 0.3 to 0.8; I^2: 40%) (Table 3 and Supplementary Fig. 1).

Subgroup analyses

A significant reduction in cryosurvival rate was found for biopsied embryos after DVT. In contrast, the reduction of cryosurvival rate was not significant in non-biopsied embryos (Supplementary Fig. 2). For subgroup analysis

Table 2 Details of technical considerations in reviewed studies

Study ID	Biopsy		First cryopreservation			Second cryopreservation			Embryo storage time	Post thaw culture before ET	Other embryonic manipulations	ART and ET cycle characteristics				
	Developmental stage	Technique	Method	Device and media	Embryonic developmental stage	Method	Device and media	Embryonic developmental stage				COS protocol	Insemination protocol	ET strategy	Developmental stage in time of ET	Endometrial preparation protocol
Theodorou et al., 2024	Blastocyst	Laser assisted ZO	V	N/M	Blastocyst	V	N/M	Blastocyst	N/M	N/M	LAH	Mixed	Either IVF or ICSI	SET	Blastocyst	Mixed (HRT, Modified natural, and natural)
Vanderhoff et al., 2024	Blastocyst	Laser assisted ZO	V	Device: Cryo Locks (Irvine Scientific, USA); VTK: Irvine Scientific (Irvine Scientific, USA)	Blastocyst	V	Same as 1th CP	Blastocyst	N/M	N/M	LAH	Mixed	Either IVF or ICSI	SET	Blastocyst	Natural/ Modified natural/ Programmed
Al Hashimi et al., 2024	Blastocyst	N/M	V	Device: Cryotop (Kitazato, Japan), VTK: Irvine Scientific (Irvine Scientific, USA)	Blastocyst	V	Same as 1th CP	Blastocyst	SCSB: 1.03 ± 1.6 years; DCSB + DCDB: 2.20 ± 2.6 years	2–5 h hrs	N/M	GnRH antagonist	Either IVF or ICSI	SET	Blastocyst	HRT (96%), natural cycle (4%)
Oraiopoulou et al., 2024	N/A	N/A	V	Device: Cryotec (Reprolife, Japan); VTK: Vit Kit (Irvine Scientific, USA)	SCNB: Blastocyst; DCNB: Cleavage (= 208 ETs) or Blastocyst (44 ETs)	V	Same as 1th CP	Blastocyst	N/M	2–4 h	N/M	Mixed	N/M	Mixed (SET and MET)	Blastocyst	HRT
Wang et al., 2024	N/A	N/A	V	Device: Cryotop (Kitazato, Japan); VTK: Kitazato (Japan)	Blastocyst	V	Same as 1th CP	Blastocyst	N/M	2 h	LAS	GnRh Agonist or Antagonist	Either IVF or ICSI	SET	Blastocyst	Mixed (HRT and Natural)
Huang et al., 2024	N/A	N/A	V	Device: Cryotop (Kitazato, Japan); VTK: Kitazato (Japan)	SCNB: Blastocyst; DCNB: Cleavage (= 62 ETs) or Blastocyst (= 11 ETs)	V	Same as 1th CP	Blastocyst	N/M	N/M	LAS	Mixed	Either IVF or ICSI	Mixed (SET and MET)	Blastocyst	N/M
He et al., 2024	N/A	N/A	V	Device: Cryotop (Kitazato, Japan); VTK: Kitazato (Japan)	Blastocyst	V	Same as 1th CP	Blastocyst	N/M	2 h	N/M	Mixed	Either IVF or ICSI	SET	Blastocyst	Mixed (HRT, Natural, GnRH agonist-HRT)
Li et al,. 2023	Blastocyst	Laser assisted ZO	V	N/M	Blastocyst	V	N/M	Blastocyst	N/M	N/M	N/M	Mixed	Either IVF or ICSI	SET	Blastocyst	Mixed (HRT, Natural, GnRH agonist-HRT)
Makieva et al., 2023	2PN Zygots	N/A	V	Device: Cryotop (Kitazato, Japan), VTK: N/M	SCNB: Blactocyst; DCNB: 2PN Zygotes	V	Same as 1th CP	Blastocyst	3 Months to 2 years	at least 2 h	LAS (just for expansion grade >= 4)	Mixed	Either IVF or ICSI	SET	Blastocyst	Mixed (HRT and Natural)
Zhang et al,. 2023	Blastocyst	Laser assisted ZO	V	Device: Cryotop (Kitazato, Japan). VTK: EG, DMSO, and sucrose (Sigma, USA)	Blastocyst	V	Same as 1th CP	Blastocyst	N/M	4–5 h	LAH	Mixed	Either IVF or ICSI	SET	Blastocyst	Mixed (HRT and Natural)
Nanassy et al., 2023	N/A	N/A	V	Device: Cryotop (Kitazato, Japan); VTK: Kitazato (Japan)	SCNB: Blactocyst; DCNB: 2PN Zygotes	V	Same as 1th CP	Blastocysts	N/M	N/M	N/M	GnRH Antagonist	Either IVF or ICSI	Mixed (SET and MET)	Blastocyst	HRT
Shen et al., 2023	N/A	N/A	V	Device: Cryotop (Kitazato, Japan); VTK: Kitazato (Japan)	Cleavage or Blastocysts	V	Same as 1th CP	Blastocysts	N/M	N/M	LAS and LAH	GnRH Antagonist or GnRH Agonist	Either IVF or ICSI	SET	Blastocyst	Mixed (HRT, Modified natural, and natural)
Brolinson et al., 2023	N/M	N/M	V	N/M	Blastocyst	V	N/M	Blastocyst	N/M	N/M	N/M	N/M	N/M	N/M	Blastocyst	N/M

Table 2 (continued)

Study ID	Biopsy		First cryopreservation			Second cryopreservation			Embryo storage time	Post thaw culture before ET	Other embryonic manipulations	ART and ET cycle characteristics				
	Developmental stage	Technique	Method	Device and media	Embryonic developmental stage	Method	Device and media	Embryonic developmental stage				COS protocol	Insemination protocol	ET strategy	Developmental stage in time of ET	Endometrial preparation protocol
Nohales et al., 2023	Blastocyst	Laser assisted ZO	V	Device: Cryotop (Kitazato, Japan); VTK: Kitazato (Japan)	Blastocyst	V	Cryotop (Kitazato, Japan), Kitazato VK (Japan)	Blastocyst	N/M	2–4 h	N/M	GnRH agonist or GnRH antagonist	ICSI	SET	Blastocyst	HRT
Makieva et al., 2022	N/A	N/A	V	N/M	SCNB: Blactocyst; DCNB: 2PN Zygotes	V	N/M	Blastocyst	N/M	N/M	N/M	N/M	Either IVF or ICSI	SET	Blastocyst	N/M
Theodorou et al., 2022	Blastocyst	Laser assisted ZO	V	Device: N/M; VTK: Sydney IVF Blastocyst VK (Cook Medical)	SCNB: Blactocyst; DCNB: Cleavage (= 97 ETs) or Blastocyst (= 146 ETs)	V	Same as 1th CP	Blastocyst	Group 1: 0 (0–1) years; Group 2: 1 (1–3) years; Group 3: 0 (0–1) years	N/M	LAH (just for No biopsied embryos)	Mixed	Either IVF or ICSI	SET	Blastocyst	Mixed (HRT, Modified natural, and natural)
Oraiopoulou et al,. 2022	N/A	N/A	V	N/M	Cleavage or Blastocyst	V	N/M	Cleavage/ Blastocyst	N/M	N/M	N/M	N/M	N/M	N/M	Cleavage (day 2) or Blastocyst (day 5)	N/M
Wang et al., 2022	N/A	N/A	V	Device: N/M; VTK: Kitazato (Japan)	Cleavage	V	Same as 1th CP	Cleavage	N/M	2 h	N/M	long GnRH agonist or GnRH antagonist	Either IVF or ICSI	SET	Cleavage	Mixed (HRT and natural cycles)
Mizobe et al., 2021	N/A	N/A	V	Device: CryoTip (Kitazato, Japan)	Blastocysts	V	Same as 1th CP	Blastocysts	N/M	2–3 h	N/M	GnRH agonist or antagonist FSH protocol	Either IVF or ICSI	SET	Blastocyst	HRT
Aluko et al., 2021	Blastocyst	N/M	V	Device: Cryolock; VTK: Irvine kits (Irvine Scientific, CA)	Blastocyst	V	Same as 1th CP	Blastocyst	N/M	N/M	LAS (just for Nonbiopsied embryos)	Either GnRH Antagonist or GnRH Agonist	Either IVF or ICSI	SET	Blastocyst	HRT
Wang et al., 2021	N/A	N/A	V	Device: Cryotop (Kitazato, Japan); VTK: Kitazato (Japan)	Blastocyst	V	Same as 1th CP	Blastocyst	N/M	2–4 h	LAS (just for Fully expanded embryos)	GnRH antagonist	IVF	Mixed (SET and MET)	Blastocyst	N/M
Hallamaa et al., 2021	N/A	N/A	SCNB: SF = 122, V = 182; DCNB: SF = 52, V = 37	Various types of devices and medias	Cleavage or Blastocyst	V	Various types of devices and medias	Cleavage or Blastocyst	N/M	N/M	N/M	N/M	N/M	Mixed (SET and MET)	SCNB: Cleavage = 68, Blastocyst = 238; DCNB: Cleavage = 17, Blastocyst = 72	HRT, natural cycles
Neal et al., 2019	Blastocyst	N/M	V	N/M	Blastocyst	V	N/M	Blastocyst	N/M	N/M	N/M	N/M	N/M	SET	Blastocyst	N/M
Schlenker et al., 2019	N/M	N/M	V	N/M	N/M	V	N/M	N/M	N/M	N/M	N/M	N/M	N/M	SET	Blastocyst	HRT

Table 2 (continued)

Study ID	Biopsy		First cryopreservation			Second cryopreservation			Embryo storage time	Post thaw culture before ET	Other embryonic manipulations	ART and ET cycle characteristics				
	Developmental stage	Technique	Method	Device and media	Embryonic developmental stage	Method	Device and media	Embryonic developmental stage				COS protocol	Insemination protocol	ET strategy	Developmental stage in time of ET	Endometrial preparation protocol
Farhi et al., 2019	N/A	N/A	SF	Device: Cryogenic vials (Nalge Nunc International, Denmark), SFM: Quinn's kit	SCNB: seems to be at blastocyste. DCNB: Cleavage	SF	Same as 1th CP	Blastocyst	N/M	N/M	N/M	N/M	N/M	Mixed (SET and MET)	Blastocyst	N/M
Wilding et al., 2019	Blastocyst	N/M	SF	N/M	SCSB: Blastocyst. DCSB: Cleavage = 6 EE, Blastocyst = 25 EE	V	Cryotop devices and Kitazato VK (Kitazato, Japan)	Blastocyst	N/M	N/M	N/M	GnRH antagonist	ICSI	N/M	Blastocyst	HRT
Gunnala et al., 2018	N/M	N/M	V	N/M	Blastocyst	V	N/M	Blastocyst	N/M	N/M	N/M	N/M	N/M	N/M	Blastocyst	N/M
Cimadomo et al., 2018	Blastocyst	TE biopsy without ZO on Day 3	V	N/M	Blastocyst	V	N/M	Blastocyst	N/M	N/M	N/M	N/M	ICSI	SET	Blastocyst	N/M
Bradley et al., 2017	Blastocyst	Laser assisted ZO	V	Various types of devices and medias	Blastocyst	V	Same as 1th CP	Blastocyst	N/M	2 h	LAH	N/M	N/M	N/M	Mixed	N/M
Zheng et al., 2017	N/A	N/A	SF	Device: Cryostraws. SFM: 1,2-PROH (Sigma, USA)	SCNB: Blastocyst; DCNB: Cleavage	V	Device: Cryotop (Kitazato, Japan), VTK: EG, DMSO, and sucrose (Sigma, USA)	Blastocyst	N/M	2 h	LAS	either long or short protocols	Either IVF or ICSI	Mixed (SET and MET)	Blastocyst	HRT, natural cycles
Taylor et al., 2014	Blastocysts	Laser assisted ZO	SCSB: all were V; DCNB: SF = 5 ETs, V = 11 Ets	SF: (Cooper Surgical), V: Device: Cryolock (Biodiseno, USA); VTK: Irvine Scientific kit	Blastocysts	V	Same as 1th V	Blastocysts	N/M	N/M	N/M	N/M	ICSI	Mixed (SET and MET)	Blastocyst	N/M
Stanger et al., 2012	N/A	N/A	SF or V	SF: PROH and sucrose. V: Device: Cryotop (Kitazato, Japan); VTK: Vit Kit (Irvine Scientific)	Oocyte, 2PN, Cleavage, Blastocysts	V	Same as 1th V	Cleavage or Blastocyst	N/M	0.5–3 h	N/M	N/M	N/M	SET	Cleavage or Blastocyst	HRT
Murakami et al., 2011	N/A	N/A	SF	Device: Plastic straw; SFM: EG and sucrose	2PN, Cleavage	V	Device: Cryotop strip (Kitazato, Fuji, Japan); VTK: EG, DMSO, and sucrose	Blastocyst	N/M	At least 3 h	MAH	Either long or GnRH antagonist	Either IVF or ICSI	Mixed (SET and MET)	Cleavage (day 3), Blastocyst (day 5)	HRT
Koch et al., 2011	N/A	N/A	SF	Device: CBS straws (Cryo Bio System, I.M.V. Technologies); SFM: Quinn's Advantage Cleavage and Blastocyst (Cooper SAGE)	2PN, Cleavage, Blastocyst	SF	Device: CBS straws (Cryo Bio System, I.M.V. Technologies); SFM: Quinn's Advantage Cleavage and Blastocyst (Cooper SAGE)	Cleavage or Blastocyst	N/M	N/M	N/M	Long or short GnRH agonist	Either IVF or ICSI	Mixed (SET and MET)	Cleavage or Blastocyst	N/M

Table 2 (continued)

Study ID	Biopsy		First cryopreservation		Second cryopreservation			Embryo storage time	Post thaw culture before ET	Other embryonic manipulations	ART and ET cycle characteristics				
	Developmental stage	Technique	Method	Device and media	Embryonic developmental stage	Method	Device and media	Embryonic developmental stage					COS protocol	Insemination protocol	ET strategy	Developmental stage in time of ET	Endometrial preparation protocol
Kumasako et al., 2009	N/A	N/A	V	Device: Plastic straw; VTK: Kitazato, Japan)	2PN Zygots, Morula, blastocysts	V	Same as 1th CP	Morula, blastocysts	N/M	SCNB: 3 Days; DCNB: N/M	N/M		N/M	Either IVF or ICSI	Mixed (SET and MET)	Cleavage or Blastocyst	HRT
Check et al., 2001	N/A	N/A	SF	PROH	2PN, Cleavage	SF	PROH	Cleavage	N/M	2–3 cell embryos: 2 h; >=4 cell embryos: 24 h	CAH with ATS		N/M	N/M	Mixed (SET and MET)	Cleavage	N/M

ATS: acidic Tyrode's solution; CAH: Chemically assisted hatching; CP: Cryopreservation; DCDB: Double cryopreservation and double biopsy; DCNB: Double cryopreservation and no biopsy; DCSB: Double cryopreservation and single biopsy; DMSO: dimethyl sulfoxide; EE: Euploid Embryos; EG: ethylene glycol; ETs: Embryo Transfers; LAH: Laser assisted hatching; LAS: Laser Assisted Artificial shrinkage; MAH: Mechanically assisted hatching; MET: Multiple embryo transfer; PROH: propanediol; SCNB: Single cryopreservation and no biopsy; SCSB: Single cryopreservation and single biopsy; SET: Single embryo transfer; SF: Slow freezing; SFM: Slow freezing Media; TE: trophectoderm; V: Vitrification; VK: Vitrification Kits

based on rounds of biopsy (single or double), one study [10] just reported merged data for single and double-biopsied embryos. So, we were not able to include this study in either of the subgroup analyses. We found that neither single nor double biopsy rounds made a difference in findings (Supplementary Fig. 3). Studies that used a mixed ET strategy showed a significant reduction in cryosurvival rate, but results for the SBT strategy were not the same (Supplementary Fig. 4).

Publication bias and sensitivity analyses

After conducting a sensitivity analysis and excluding studies with a very high risk of bias, the reduction in cryosurvival rate was not statistically significant, and the heterogeneity level decreased significantly to 0% (Supplementary Fig. 5).

Egger's regression test revealed a substantial risk of publication bias (P-value 1-tailed: 0.043, P-value 2-tailed: 0.086), while the trim-and-fill method still indicated a significant decrease in cryosurvival rate (Supplementary Fig. 6).

BPR

The meta-analysis included 18 studies assessing the BPR following DVT compared to SVT. The random-effects model yielded a pooled MHOR of 0.7 (95% CI: 0.6 to 0.8), showing a significant decrease in BPR after DVT ($P < 0.001$; I^2: 25%) (Supplementary Fig. 7).

Subgroup analyses

Both biopsied and non-biopsied embryos showed a significant decrease in BPR after DVT with low heterogenity (Supplementary Fig. 8). The number of biopsy rounds made no difference in these findings (Supplementary Fig. 9). Both ET strategies also were associated with a significant decrease in BPR (Supplementary Fig. 10). However, subgroup analysis based on culture status made some differences. Transfer of double-vitrified embryos that underwent EC between did not show a significant change in BPR, while in double-vitrified embryos that immediately underwent the second round of vitrification, a significant decrease in BPR was observed (Supplementary Fig. 11).

Publication Bias and sensitivity analyses

Sensitivity analysis by excluding studies with a very high risk of bias confirmed our primary findings on a significant decrease in BPR following DVT (Supplementary Fig. 12).

The publication bias analysis using Egger's regression test and trim-and-fill method showed no publication bias (Supplementary Fig. 13).

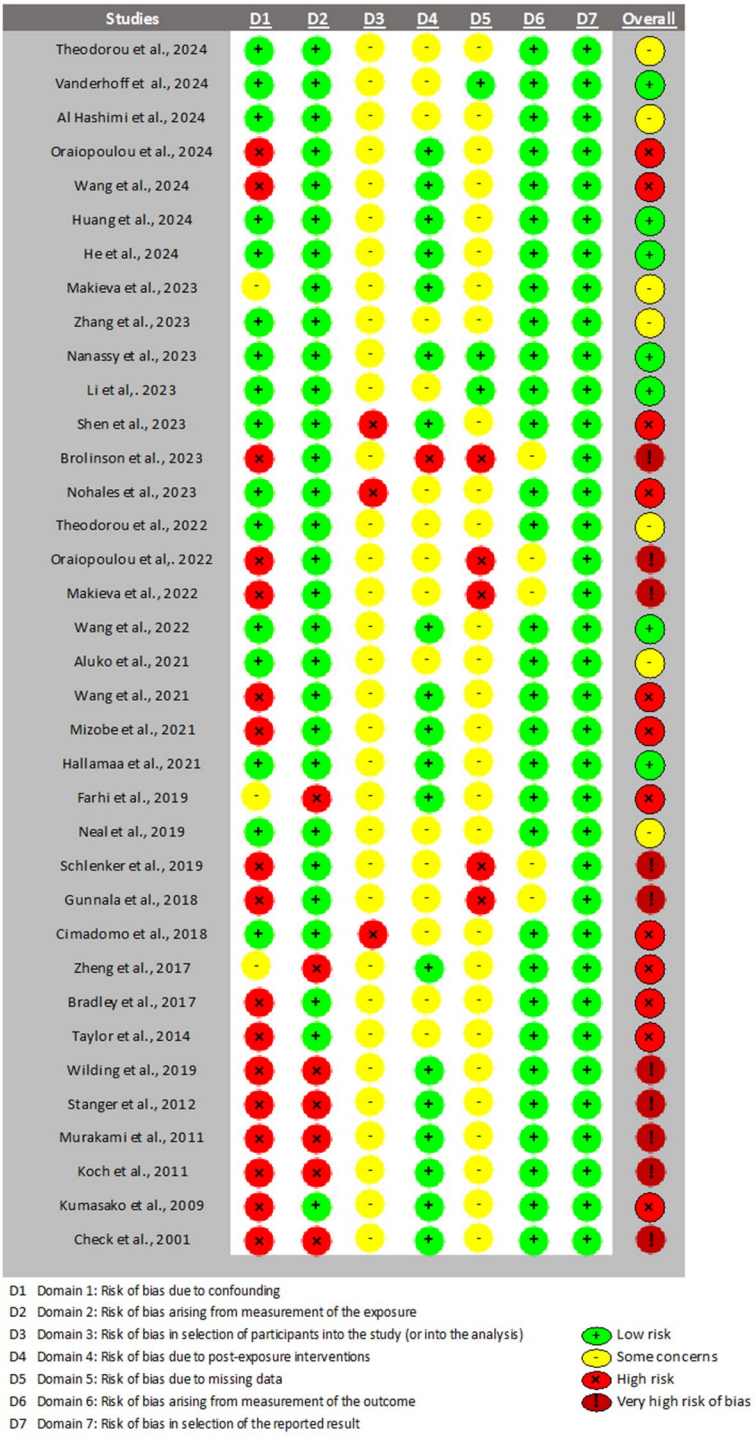


Fig. 2 Risk of Bias according to ROBINS-E Tool

CPR

The meta-analysis included 27 studies assessing the CPR following DVT compared to SVT. The random-effects model yielded a pooled MHOR of 0.7 (95% CI: 0.6 to 0.7), indicating a significant decrease in CPR following DVT ($P < 0.001$; $I^2 = 54\%$) (Supplementary Fig. 14).

Subgroup analyses

Subgroup analysis showed that DVT is associated with a significant reduction in CPR whether it was coupled with biopsy or not (Supplementary Fig. 15). The number of biopsy rounds showed similar findings (Supplementary Fig. 16). Findings for Non-biopsied embryos were significantly heterogeneous in both subgroup analyses

Table 3 Summary of study key findings

Outcome measure		Statistical model	Number of studies	Effect size and test of null				Heterogeneity	
				MHRR	95%CI	Z	*P*	I^2 (%)	*P*
Cryosurvival Rate									
Total		Fixed-effects	10	0.44 *	0.31 to 0.62	-4.69	**0.00**	41	0.08
		Random-effects		0.46 *	0.27 to 0.80	-2.74	**0.00**		
Subgroup analyses	Biopsy status	Biopsied	6	0.38 *	0.18 to 0.80	-5.16	**0.01**	46.5	0.09
		Non-biopsied	4	0.66 *	0.35 to 1.23	-1.28	0.19	0	0.81
	Number of biopsy rounds	Single Biopsied	5	0.36 *	0.14 to 0.93	-2.09	**0.03**	62	0.03
		Double Biopsied	5	0.30 *	0.10 to 0.93	-2.09	**0.03**	12	0.33
		Non-biopsied	4	0.66 *	0.35 to 1.23	-1.28	0.19	0	0.81
	ET strategy	SBT	6	0.61 *	0.26 to 1.45	-1.18	0.26	0	0.72
		Mixed	4	0.41 *	0.20 to 0.82	-4.11	**0.01**	46	0.08
Sensitivity analysis		Excluding VHR	8	0.62 *	0.37 to 1.03	-1.840	0.06	0	0.77
Publication bias			4 ‡	0.35 #	0.21 to 0.59	-	-	-	-
Biochemical pregnancy rate									
Total		Fixed-effects	18	0.71 *	0.66 to 0.78	-7.64	**0.00**	25	0.15
		Random-effects		0.71 *	0.64 to 0.79	-6.04	**0.00**		
Subgroup analyses	Biopsy status	Biopsied	10	0.75 *	0.64 to 0.86	-3.89	**0.00**	28.5	0.18
		Non-biopsied	8	0.66 *	0.56 to 0.78	-5.03	**0.00**	14.5	0.31
	Number of biopsy rounds	Non-Biopsied	8	0.66 *	0.56 to 0.78	-5.03	**0.00**	0	0.83
		Single Biopsied	9	0.82 *	0.73 to 0.92	-3.24	**0.00**	14	0.31
		Double Biopsied	6	0.42 *	0.32 to 0.54	-6.53	**0.00**	0	0.43
	ET strategy	SBT	7	0.69 *	0.59 to 0.82	-4.36	**0.00**	24	0.24
		Mixed	11	0.73 *	0.62 to 0.85	-3.92	**0.00**	33	0.13
	Culture status	EC	4	0.99 *	0.79 to 1.24	-0.05	0.95	0	0.78
		No EC	14	0.72 *	0.64 to 0.81	-5.59	**0.00**	13	0.30
Sensitivity analysis		Excluding VHR	16	0.72 *	0.63 to 0.82	-4.87	**0.00**	32	0.10
Publication bias			0 ‡	0.71 #	0.64 to 0.79	-	-	-	-
Clinical pregnancy rate									
Total		Fixed-effects	27	0.67 *	0.62 to 0.72	-10.66	**0.00**	54	0.00
		Random-effects		0.69 *	0.52 to 0.78	-6.00	**0.00**		
Subgroup analyses	Biopsy status	Biopsied	14	0.67 *	0.59 to 0.76	-6.20	**0.00**	31	0.12
		Non-biopsied	13	0.73 *	0.58 to 0.91	-2.74	**0.00**	68	0.00
	Number of biopsy rounds	Single Biopsied	12	0.71 *	0.61 to 0.83	-4.35	**0.00**	39	0.08
		Double Biopsied	9	0.52 *	0.42 to 0.63	-6.29	**0.00**	0	0.44
		Non-biopsied	13	0.73 *	0.58 to 0.91	-2.74	**0.00**	68	0.00
	ET strategy	SBT	18	0.73 *	0.64 to 0.84	-4.30	**0.00**	43	0.04
		Mixed	9	0.61 *	0.50 to 0.76	-4.45	**0.00**	42	0.07
	Culture status	EC	6	0.86 *	0.66 to 1.11	-2.00	0.25	48	0.09
		No EC	20	0.64 *	0.56 to 0.74	-10.54	**0.00**	56.5	0.00
Sensitivity analysis		Excluding VHR	16	0.72 *	0.63 to 0.82	-4.87	**0.00**	32	0.10
Publication bias			7 ‡	0.62 #	0.54 to 0.70	-	-	-	-
Miscarriage rate									
Total		Fixed-effects	23	1.37 *	1.20 to 1.56	4.79	**0.00**	42.5	0.01
		Random-effects		1.40 *	1.16 to 1.70	3.48	**0.00**		

Table 3 (continued)

Miscarriage rate									
Subgroup analyses	Biopsy status	Biopsied	11	1.35 *	1.03 to 1.77	2.22	**0.02**	42	0.07
		Non-biopsied	12	1.44 *	1.08 to 1.92	2.51	**0.01**	45	0.04
	Number of biopsy rounds	Single Biopsied	8	1.31 *	0.94 to 1.83	1.60	0.11	48.5	0.06
		Double Biopsied	7	1.52 *	0.95 to 2.42	1.75	0.07	18	0.29
		Non-biopsied	12	1.44 *	1.08 to 1.92	2.51	**0.01**	45	0.04
	ET strategy	SBT	15	1.34 *	1.00 to 1.78	2.00	**0.04**	52	0.01
		Mixed	8	1.44 *	1.13 to 1.84	2.98	**0.00**	25	0.23
	Culture status	EC	4	1.30 *	0.94 to 1.82	1.59	0.11	0	0.48
		No EC	17	1.49 *	1.16 to 1.92	3.15	**0.00**	54.5	0.00
Sensitivity analyses		Excluding VHR	21	1.40 *	1.11 to 1.71	2.93	**0.00**	41	0.00
		Excluding HR and VHR	13	1.33 *	0.99 to 1.79	1.91	0.055	49	0.02
Publication bias			0 ‡	1.40 #	1.16 to 1.70	-	-	-	-
Live birth rate									
Total		Fixed-effects	24	0.63 *	0.589 to 0.69	-11.24	**0.00**	63	0.00
		Random-effects		0.65 *	0.56 to 0.75	-5.76	**0.00**		
Subgroup analyses	Biopsy status	Biopsied	13	0.65 *	0.54 to 0.79	-4.39	**0.00**	62	0.00
		Non-biopsied	11	0.66 *	0.52 to 0.84	-3.37	**0.00**	66.5	0.00
	Number of biopsy rounds	Single Biopsied	12	0.69 *	0.56 to 0.84	-3.49	**0.00**	62	0.02
		Double Biopsied	9	0.54 *	0.38 to 0.76	-3.45	**0.00**	41	0.09
		Non-biopsied	11	0.66 *	0.52 to 0.84	-3.37	**0.00**	66.5	0.00
	ET strategy	SBT	15	0.72 *	0.59 to 0.88	-3.22	**0.00**	63.5	0.00
		Mixed	9	0.56 *	0.46 to 0.67	-6.27	**0.00**	45	0.06
	Culture status	EC	4	0.77 *	0.55 to 1.09	-1.43	0.15	58	0.07
		No EC	19	0.62 *	0.52 to 0.73	-5.39	**0.00**	66	0.00
Sensitivity analyses		Excluding VHR	21	0.67 *	0.56 to 0.80	-4.35	**0.00**	66	0.00
		Excluding HR and VHR	13	0.72 *	0.58 to 0.88	-3.11	**0.00**	61	0.00
Publication bias			1 ‡	0.63 #	0.55 to 0.73	-	-	-	-
Cesarean section rate									
Total		Fixed-effects	8	1.69 *	1.21 to 2.36	3.09	0.00	63	0.00
		Random-effects		1.53 *	0.84 to 2.79	1.42	0.15		
Subgroup analyses	Biopsy status	Biopsied	3	1.26 *	0.73 to 2.16	0.83	0.40	0	0.62
		Non-biopsied	5	1.80 *	0.67 to 4.85	1.17	0.24	77	0.00
Sensitivity analysis			7	1.22 *	0.80 to 1.86	0.95	0.34	19	0.28
Publication bias			0 ‡	1.69 #	1.21 to 2.36	-	-	-	-
Low birth weight rate									
Total		Fixed-effects	7	0.86 *	0.47 to 1.56	-0.50	0.62	0	0.75
		Random-effects		0.93 *	0.52 to 1.69	-0.21	0.75		
Subgroup analyses	Biopsy status	Biopsied	3	1.11 *	0.34 to 3.58	0.17	0.86	0	0.50
		Non-biopsied	4	0.88 *	0.44 to 1.75	-0.35	0.72	0	0.59
Publication bias			0 ‡	0.93 #	0.52 to 1.69	-	-	-	-
Birth Weight (gr)									
Total		Fixed-effects	10	40.25 †	-27.04 to 107.54	1.17	0.24	0	0.52
		Random-effects		40.25 †	-27.04 to 107.54	1.17	0.24		

Table 3 (continued)

Birth Weight (gr)									
Subgroup analyses	Biopsy status	Biopsied	4	6.87 †	-149.56 to 163.32	0.08	0.93	50	0.11
		Non-biopsied	6	70.33 †	18.51 to 159.18	1.55	0.12	0	0.96
	ET strategy	SBT	7	63.67 †	-65.24 to 192.59	0.97	0.33	0	0.30
		Mixed	3	35.15 †	-53.10 to 123.41	0.78	0.43	16	0.67
Publication bias			0 ‡	40.25 #	-27.04 to 107.54	-	-	-	-
Preterm birth rate									
Total		Fixed-effects	11	1.27 *	0.89 to 1.81	1.37	0.17	0	0.92
		Random-effects		1.33 *	0.93 to 1.90	1.61	0.10		
Subgroup analyses	Biopsy status	Biopsied	3	1.12 *	0.367 to 3.43	0.20	0.83	0	0.81
		Non-biopsied	8	1.36 *	0.93 to 1.98	1.62	0.10	0	0.77
	ET strategy	SBT	7	1.31 *	0.88 to 1.90	1.31	0.10	0	0.86
		Mixed	4	1.43 *	0.65 to 3.13	0.94	0.36	0	0.58
Publication bias			0 ‡	1.33 #	0.93 to 1.90	-	-	-	-
Gestational Age at Birth (weeks)									
Total		Fixed-effects	8	-0.12 †	-0.41 to 0.16	-0.85	0.39	34	0.15
		Random-effects		-0.17 †	-0.56 to 0.21	-0.86	0.39		
Subgroup analyses	Biopsy status	Biopsied	2	0.19 †	-0.27 to 0.66	0.81	0.41	0	0.95
		Non-biopsied	6	-0.33 †	-0.83 to 0.15	-1.33	0.18	35	0.17
	ET strategy	SBT	5	−0.026 †	-0.88 to 0.36	-0.82	0.40	54	0.06
		Mixed	3	-0.01 †	-0.48 to 0.45	-0.06	0.95	0	0.47
Publication Bias			1 ‡	-0.13 #	-0.42 to 0.15	-	-	-	-

*Mantel–Haenszel odds ratio (MHOR)

†Mean difference

#Adjusted effect size according to trim-and-fill method

‡Indicating number of potentially missing studies according to trim-and-fill method

(68%). Both mixed and SBT strategies showed a significant decrease in CPR (Supplementary Fig. 17). Similar to BPR, subgroup analysis based on culture status made some differences in findings. Reduction in CPR was non-significant for double vitrified embryos who underwent EC; However, in those that immediately underwent V2, reduction in CPR was statistically significant (Supplementary Fig. 18).

Publication Bias and sensitivity analyses

The sensitivity analysis, which excluded studies with a very high risk of bias, confirmed the primary finding of a significant reduction in CPR. This analysis also significantly reduced the heterogeneity level to 32%. (Supplementary Fig. 19).

The publication bias analysis using Egger's regression test showed no significant publication bias. Adjusted effect size, accounting for seven potentially missing studies according to the trim-and-fill method, still indicated a significant decrease in CPR (Supplementary Fig. 20).

MR

The meta-analysis included 23 studies assessing the MR following DVT compared to SVT. One study [30] was not included as data for MR was reported for clinical abortions, while we intended to consider all types of pregnancy loss (either biochemical or clinical) before 22 weeks as miscarriage. The random-effects model yielded a pooled MHOR of 1.4 (95% CI: 1.1 to 1.7), indicating a significant increase in MR ($P<0.001$; $I^2 = 42.5\%$) Supplementary Fig. 21).

Subgroup analyses

Both biopsied and non-biopsied embryos showed a significant increase in MR (Supplementary Fig. 22). However, subgroup analysis based on the number of biopsy rounds showed different results. Despite non-biopsied embryos, neither single nor double biopsy rounds showed a significant increase in MR (Supplementary Fig. 23). Both mixed ET and SBT strategies showed a significant increase in MR (Supplementary Fig. 24).

EC between W1 and V2 showed a non-significant increase in MR, while immediate re-vitrification showed a significant increase in MR (Supplementary Fig. 25).

Publication bias and sensitivity analyses

The sensitivity analysis, which excluded studies with a very high risk of bias, still showed a significant reduction in CPR with moderate heterogeneity (41%) (Supplementary Fig. 26). To ensure the robustness of these findings, an additional sensitivity analysis was performed. Further analysis caused the increased MR to reach a non-significant level and increased the heterogeneity level to 49% (Supplementary Fig. 27).

Egger's regression test and trim-and-fill method revealed no publication bias (Supplementary Fig. 28).

LBR

The meta-analysis included 24 studies assessing the LBR following DVT compared to SVT. The random-effects model yielded a pooled MHOR of 0.6 (95% CI: 0.5 to 0.7), showing a significant decrease in LBR ($P < 0.001$; I^2 = 63%) (Supplementary Fig. 29).

Subgroup analyses

Subgroup analysis showed that neither biopsy status (Supplementary Fig. 30) nor the number of biopsy rounds (Supplementary Fig. 31) would change primary findings or heterogeneity level. The same findings were observed for ET strategy subgroups (Supplementary Fig. 32), while subgroup analysis by culture status made substantial changes in findings. Despite immediate vitrification, EC between W1 and V2 showed a non-significant reduction in LBR (Supplementary Fig. 33).

Publication bias and sensitivity analyses

The sensitivity analysis, which excluded studies with a very high risk of bias, still showed a significant reduction in CPR with significant heterogeneity (66%) (Supplementary Fig. 34). An additional sensitivity analysis also showed a significant reduction in LBR but did not significantly reduce the heterogeneity level (61%) (Supplementary Fig. 35).

The publication bias analysis using Egger's regression test showed no publication bias. The trim-and-fill method identified just one potentially missed study, but the overall conclusion remained consistent (Supplementary Fig. 36).

C/S rate

The meta-analysis included 8 studies assessing the C/S rate following DVT compared to SVT. The fixed-effects model yielded a pooled MHOR of 1.7 (95% CI: 1.2 to 2.4), indicating a statistically significant increase in C/S rate (P: 0.002), while the random-effects model produced a pooled MHOR of 1.5 (95% CI: 0.8 to 2.8), showing no significant increase (P: 0.156). The overall heterogeneity was high (I^2: 63%) (Supplementary Fig. 37).

Subgroup analyses

Subgroup analysis showed that both biopsied and non-biopsied embryos had no significant increase in C/S rate (Supplementary Fig. 38). Subgroup analysis based on other variables was not possible due to small number of studies.

Publicaton bias and sensitivity analyses

The sensitivity analysis, which excluded one study with a high risk of bias, revealed no significant risk of CS with DVT. This analysis also significantly reduced the heterogeneity level to 19% (Supplementary Fig. 39).

Egger's regression test and the trim-and-fill method showed no publication bias (Supplementary Fig. 40).

LBW rate

The meta-analysis included 7 studies assessing the LBW rate following DVT compared to SVT. The random-effects model indicated no statistically significant difference in LBW rate (pooled MHOR: 0.9; 95% CI: 0.5 to 1.7; P: 0.830; I^2 = 0%) (Supplementary Fig. 41).

Subgroup analyses

Biopsy status showed no significant difference in LBW rate either in biopsied or non-biopsied embryos (Supplementary Fig. 42).

Publicaton bias and sensitivity analyses

Sensitivity analysis was not performed as none of the included studies were rated as very high risk of bias. Egger's regression test and the trim-and-fill method showed no publication bias (Supplementary Fig. 43).

BW

The meta-analysis included 10 studies assessing the BW following DVT compared to SVT. Six studies were excluded as one [6] did not report the standard deviation (SD), four reported BW as median and interquartile range (IQR) [12, 27, 28, 31], and one [9] reported BW as Estimated Marginal Means (EMM) without providing the unadjusted means. One study [32] reported the standard error of the mean (S.E.M) that were converted to SD. The random-effects model showed no significant difference in BW following DVT (pooled MD: 40.2 g; 95% CI: -27 to 107.5; P: 0.241; I^2: 0%) (Supplementary Fig. 44).

Subgroup analyses

Both biopsied and non-biopsied embryos had no significant difference in BW (Supplementary Fig. 45). Subgroup analysis by ET type showed that there is no significant

difference in BW in mixed or SBT strategies (Supplementary Fig. 46).

Publicaton bias and sensitivity analyses

Sensitivity analysis was not performed as none of the included studies were rated as very high risk of bias. Egger's regression test and the trim-and-fill method showed no publication bias (Supplementary Fig. 47).

PTB rate

The meta-analysis included 11 studies assessing the PTB rate following DVT compared to SVT. Random-effects model indicated no statistically significant increase in PTB rate (pooled MHOR: 1.3; 95% CI: 0.9 to 1.9; P: 0.107; I^2: 0%) (Supplementary Fig. 48).

Subgroup analyses

PTB rate was not affected by DVT in biopsied or non-biopsied embryos (Supplementary Fig. 49). Same findings were found regarding ET sterategies (Supplementary Fig. 50).

Publicaton bias and sensitivity analyses

Sensitivity analysis was not performed as none of the included studies were rated as very high risk of bias. Egger's regression test and the trim-and-fill method showed no publication bias (Supplementary Fig. 51).

GA at birth

The meta-analysis included 8 studies assessing GA at birth following DVT compared to SVT. Five studies were excluded as four [27, 31, 33, 34] reported GA at birth as median and IQR and one [9] reported GA as EMM without reporting unadjusted means. One study [32] reported the standard S.E.M. For the purposes of this meta-analysis, we converted the S.E.M to SD.

The random-effects model indicated no statistically significant difference in GA at birth (pooled MD: -0.17 weeks; 95% CI: -0.5 to 0.2; P: 0.390; I^2 = 33.86%) (Supplementary Fig. 52).

Subgroup analyses

Biopsy status (Supplementary Fig. 53) and ET srategies (Supplementary Fig. 54) posed no difference in primary findings.

Publicaton bias and sensitivity analyses

Sensitivity analysis was not performed as none of the included studies were rated as very high risk of bias. Egger's regression test showed no publication bias. The trim-and-fill method indicated one study was trimmed, but the adjusted point estimate was not changed (Supplementary Fig. 55).

Discussion

Potential biological mechanisms behind detrimental effects of DVT

Existing literature primarily focuses on the disruption of molecular pathways following vitrification, with only a few studies examining the effects of repeated vitrification on the embryo genome. Most of the available research is based on animal models, with Wang et al. [35] being one of the few studies focusing on human blastocysts.

Numerous studies have shown that vitrification alters the transcriptional profiles of preimplantation embryos, affecting genes involved in apoptosis, stress responses, pluripotency, zygotic genome activation, cell differentiation, and implantation [14, 36–39]. An experimental study on mice exploring the effects of re-cryopreservation found that re-cryopreserved mouse blastocysts exhibited higher expression levels of apoptotic genes (BAX and CASP3) [36]. Wang et al.'s [35] study on human blastocysts confirmed these findings, showing that recryopreservation altered genes involved in apoptosis.

ER stress, which plays a crucial role in cell proliferation and survival [40], was shown to be involved in the re-cryopreservation process, with elevated expression of GRP78, XBP1s, and CHOP. Recryopreservation may impair cell adhesion, activate ER stress, lead to apoptosis, and ultimately result in implantation failure [35]. Cryopreservation induces the expression of ER stress markers such as ATF4, ATF6, GRP78, and CHOP [40]. Inhibiting ER stress during in-vitro culture improves embryo competency and cryo-tolerance by reducing expression level of oxidative stress, apoptosis, and ER stress-induced genes [41].

Additionally to apoptosis and ER stress, animal studies have demonstrated that vitrification can affect DNA integrity in blastocysts [42]. The same findings were found for the DNA integrity of human blastocyst after repeated vitrification according to TUNEL and the protein expression of Caspase-3 and cleaved Caspase-3 [35].

Wang et al. [35] revealed 291 differentially expressed genes in double-vitrified embryos. These DEGs were mainly related to cell-cell communication, extracellular matrix degradation, and integrin cell surface interactions.

The other potential mechanism involved in the reduced implantation potential of doble-vitrified embryos is related to the impaired function of trophectoderm (TE) cells. Maintaining normal TE cell function is crucial for successful implantation and fetal development [43]. DVT leads to lower β-hCG levels and impaired TE function and implantation potential of re-cryopreserved blastocysts [35].

Altogether, these findings highlight that preimplantation embryo development is affected by DVT through perturbations in apoptosis, ER stress response, cell-cell communication, and impaired TE cell function. Despite

these findings, our understanding of the mechanisms underlying the effects of DVT is still limited, and more studies are needed in this area.

Summary of study findings

Effect of DVT on embryonic outcomes

The findings of this review are consistent across different outcomes, indicating that DVT generally has a detrimental impact on embryonic outcomes and reproductive success. Our results revealed a significant reduction in cryosurvival rates for embryos undergoing DVT, with an approximate decrease of 56%. This reduction was more pronounced in biopsied embryos, suggesting that the additional stress from biopsy procedures may exacerbate the negative impact of DVT. Although moderate heterogeneity was observed in the studies, indicating some variability in outcomes, the overall effect remained consistent. An important consideration regarding the cryosurvival rate is that, after performing a sensitivity analysis by excluding studies with a very high risk of bias, the heterogeneity level has been reduced to zero. Consequently, the statistical significance approached a non-significant level ($p = 0.06$), although there was still a trend toward decreased cryosurvival.

DVT was associated with a significant decrease in BPR (29%). Subgroup analyses showed that both biopsied and non-biopsied embryos experienced reduced BPR, with moderate heterogeneity. This suggests that the negative impact of DVT on BPR is consistent across different embryo types and transfer strategies.

The CPR also showed a significant decrease in DVT (33%). Both biopsied and non-biopsied embryos exhibited reduced CPR, with biopsied embryos showing slightly lower heterogeneity. This indicates that while DVT negatively impacts CPR, the effect is somewhat consistent across different embryo conditions.

MR also showed a significant increase (37%) with DVT. Biopsy staus or ET strategy did not make a difference in findings. Interestingly, the risk of MR was different across embryos with different numbers of biopsies. DVT was associated with a significant increase in MR (1.41 times higher) in non-biopsied embryos. The MR was highest (1.52 times higher) in double-biopsied embryos, although this increase was not statistically significant. However, in single-biopsied embryos, MR was the lowest (1.31 times higher) and was not statistically significant. This finding suggests that while DVT increases the risk of miscarriage, the number of biopsy rounds and the euploid status of embryos also play crucial roles. Single biopsied embryos, often euploid, show a non-significant increase in MR, suggesting that their chromosomal normality may reduce miscarriage risk.

The LBR was significantly lower (37%) in embryos undergoing DVT. Both biopsied and non-biopsied embryos showed reduced LBR, with moderate to high heterogeneity. This indicates that DVT negatively impacts the likelihood of live births, regardless of biopsy status or ET strategy.

One interesting finding in subgroup analyses was that the extended culture appears to mitigate some of these negative effects. We found that when embryos undergo an extended culture period between the first and second vitrification, their BPR, CPR, LBR, and MR were comparable to those undergoing SVT. This could be because the extended culture period allows embryos to recover from the initial vitrification stress. Additionally, this interval provides an opportunity to identify and discard damaged embryos, ensuring only the more viable ones proceed to the second vitrification. In contrast, embryos that immediately undergo the second vitrification show a significant decrease in all embryonic outcomes, likely due to the lack of recovery and selection time. The findings of the subgroup analyses will be discussed in detail in the following subsections.

Effect of DVT on neonatal outcomes

The analysis revealed a significant increase in the C/S rate following DVT in the fixed-effects model, although this was not confirmed in the random-effects model. The lack of confirmation in this model indicates variability among the studies. This model accounts for differences between studies, suggesting that the increase in the C/S rate might not be a universal finding and could vary depending on specific study conditions or populations. Subgroup analysis based on biopsy status made no changes in results. The moderate heterogeneity suggests variability among the included studies, but the overall effect remains robust. The meta-analysis found no significant difference in LBW rate between DVT and SVT. This was consistent across both fixed-effects and random-effects models, with low heterogeneity indicating minimal variability among the included studies. Subgroup analysis by biopsy status also showed no significant differences, suggesting that DVT does not adversely affect the likelihood of LBW. Similarly, there was no significant difference in BW between DVT and SVT. Both fixed-effects and random-effects models showed consistent results, with low heterogeneity. Subgroup analyses by biopsy status and ET strategy confirmed these findings, indicating that DVT does not significantly impact BW.

The analysis showed no significant increase in the PTB rate following DVT. This was consistent across both fixed-effects and random-effects models, with low heterogeneity. Subgroup analyses by biopsy status and ET type also showed no significant differences, suggesting that DVT does not increase the risk of PTB. The meta-analysis found no significant difference in GA at birth between DVT and SVT. The random-effects model

showed consistent results, with moderate heterogeneity indicating some variability among the included studies. Subgroup analysis by biopsy status also showed no significant differences, suggesting that DVT does not significantly affect GA at birth. The findings of the subgroup analyses will be discussed in detail in the following subsections.

Comparison of study findings with existing literature

Our findings are consistent with two recent systematic reviews by Bickendorf et al. [12] and Piani et al. [13], who reported that both "double biopsy + double vitrification" and "single biopsy + double vitrification" significantly reduced live birth and CPR and elevated MR, which aligns with our observations. Similarly, another systematic review revealed the systematic review and meta-analysis by Wang et al. on the effect of recryopreservation on embryo viability and IVF outcomes and found that recryopreservation led to decreased embryo survival and CPR. Their findings also highlighted a lower LBR and increased MR associated with re-cryopreservation, which parallels our observations regarding the negative impact of double vitrification on clinical outcomes.

Robustness of study findings

The robustness of our study findings was evaluated through various methods, including subgroup analyses, sensitivity analyses, strict quality assessments, and investigations of publication bias using several techniques.

Subgroup analyses and heterogenity assessment

The subgroup analysis was performed to address potential sources of heterogeneity. The biopsy status and number of biopsy rounds were considered the most essential subgroup analyses. Similar to previous systematic reviews [12, 13], we found no differences between single and double biopsies in most of the embryonic outcomes, and the heterogeneity levels remained similar to the combined single and double biopsy data, ensuring that our combined results are reliable. The only exceptional finding was related to MR. In combined analyses, we found a significantly increased MR in both biopsied and non-biopsied embryos. In the separated subgroup analyses, the same trends of increased MR were found in both single and double-biopsied groups, although it was not statistically significant. This finding may indicate that multiple biopsies could add stress, but their euploid status might still offer some protection.

An important issue that should be noted is that there is a complicated interplay between biopsy and DVT. When an embryo undergoes double rounds of biopsy, it is mandatory to perform DVT. The same rule applies to single biopsied embryos, which must undergo at least one round of vitrification after biopsy (either at the cleavage or blastocyst stage) to be preserved. This coupling presents a unique challenge in distinguishing the effects of DVT from those of the biopsy procedures. This means that any observed outcomes are caused by both interventions, making it difficult to attribute specific effects to the biopsy or the vitrification process alone in a clinical context.

The transfer of biopsied euploid embryos may result in better outcomes for ET. However, the biopsy procedure, whether conducted as a single or double procedure, involves removing cells from the embryo for genetic testing. This extra manipulation can introduce stress and potential damage to the embryo. When these damages occur alongside the DVT process, the effects of the biopsy can be magnified, making it challenging to determine the impact of vitrification on its own.

Also, the baseline risks associated with different indications for PGT indeed play a significant role in influencing the outcomes of double-vitrified biopsied embryos. For instance, recurrent implantation failure (RIF) is a common indication for PGT, and patients with RIF often have unexplained underlying issues that can affect implantation success, such as genetic and immunological factors [44, 45]. Women of advanced maternal age are at higher risk for chromosomal abnormalities in embryos [46]. Severe male factor infertility can result in higher rates of aneuploidy in embryos [47]. While PGT can help identify embryos with normal chromosomes, the baseline risk associated with sperm quality can still influence overall outcomes. This is because some genetic issues in sperm, such as DNA fragmentation and epigenetic changes, are often not detected through current routine genetic tests. These abnormalities are closely associated with poor pregnancy outcomes [48].

On the other hand, non-biopsied embryos may be derived from parents with higher incidence and better prognosis, as they might not be complicated by the baseline risk factors that biopsied embryos face. However, the varying local regulations and indications for PGT make it challenging to draw definitive conclusions. In some countries, PGT is used for sex selection in fertile parents without any reproductive issues, while in some other countries, the high cost of biopsy and PGT can limit their use to cases with very high-risk factors. These disparities highlight the need for careful consideration of baseline risks and local practices when interpreting the outcomes of DVT cycles coupled with biopsy.

The final critical point concerning biopsy is that various factors influence the outcomes. It's not just the biopsy status or the number of biopsies performed; the methods used, the equipment and media employed, the embryonic developmental stage, and even the experience and skill level of the operator all play significant roles.

The other two subgroups we included were culture status and ET strategy. EC could decrease heterogeneity for BPR and MR, and the ET strategy could reduce heterogeneity for cryosurvival rates with SBT and MR with mixed strategies. Nonetheless, these strategies, similar to the biopsy status, did not significantly influence overall heterogeneity for other outcomes. Several additional factors, like study methodology, vitrification media/device, maternal age, cause of infertility, type of insemination, embryo quality, and additional embryonic manipulations, might still be responsible for the high heterogeneity observed in some outcomes. Addressing these variables in future research could help further reduce heterogeneity and improve the precision of the findings.

Also, the vitrification process is performed manually in almost all settings around the world, making it highly dependent on the skill and experience of the operator. Additionally, a broad range of protocols, media, and equipment are used in different settings, which means the vitrification process can be influenced by many technical and operator-specific factors. This can introduce significant uncontrollable heterogeneity in findings.

Sensitivity analyses

Sensitivity analyses were performed for all embryonic outcomes, while it was not possible for neonatal outcomes except for the CS rate. The primary sensitivity analyses confirmed the robustness of our findings for most of the outcomes by revealing the same primary results and producing an acceptable heterogeneity level, including cryosurvival, BPR, CPR, and CS. The results for CS after the sensitivity analysis are more reliable as the heterogeneity was significantly reduced, and the discrepancy between the random effects and fixed effects models in the primary analysis was eliminated after sensitivity analysis, with both models showing no significant difference between DVT and SVT.

MR and LBR underwent an additional sensitivity analysis due to persistent heterogeneity. However, this extra step did not reduce the heterogeneity levels for either outcome. For MR, the significance level even reached a non-significant level. In meta-analyses, including more studies generally increases statistical power, allowing for more precise estimates of effect sizes. However, this inclusion can also introduce greater heterogeneity due to variations in study designs, populations, and methodologies. While excluding studies with a high risk of bias or other issues can reduce heterogeneity, it may also decrease the overall power of the analysis. Therefore, changes in results after such exclusions do not necessarily indicate that the primary analyses are incorrect. Instead, they highlight the influence of specific studies on the overall findings and the importance of considering potential sources of bias and heterogeneity. Reduced MR compared to primary analyses should be interpreted with caution, as it may simply be a result of reduced power, given that the level of heterogeneity did not decrease with additional sensitivity analysis.

Risk of bias and quality assessments

A comprehensive quality assessment was performed using Cochrane's quality assessment tool (ROBINS-E). Unfortunately, most studies were rated as having a high risk or a very high risk of bias. These findings highlight several key areas of concern. The most prevalent causes of low quality were the risk of bias due to not handling confounding variables, not reporting missing data, and selection bias.

Many studies failed to adjust for confounding factors, and in some cases, they did not even report these variables. Such biases can greatly affect the interpretation of the results, potentially leading to an overestimation or underestimation of the true effects of DVT. The initial significant confounding factors in these studies include patient characteristics (such as variations in age, BMI, and underlying fertility issues), which can influence outcomes but were not consistently controlled for across studies. The second confounder was the differences in characteristics of double-vitrified embryos, including the initial embryo quality and developmental stage at the time of vitrification, which can affect survival and implantation rates. The third group of confounders is related to technical considerations, like variability in laboratory protocols (such as culture media and vitrification techniques), operator's skill and experience level, and biopsy techniques, which can also pose confounding effects. Considering the confounding effect of the cryopreservation method, we excluded studies that used the slow-freezing method from meta-analyses and included only studies that used the vitrification method for both rounds of cryopreservation. One of the other essential technical variables reported in a few studies was the cryo-storage duration. According to a recently published systematic review, it seems that prolonged embryo storage over 12 months is associated with poor fertility outcomes [49]. Considering the detrimental effects we found in DCP embryos, it appears to be mandatory for future studies to assess different cryo-storage durations on the reproductive potential of DCP embryos. Finally, differences in clinical practices, including the insemination method, post-thaw embryo culture, the timing of ET, and luteal phase support, are the other confounders that were not properly reported or handled in most of the studies.

Unfortunately, except for three studies, none of the other included studies reported missing data. Proper handling of missing data, such as using multiple imputation or sensitivity analyses, and clear reporting of these methods are necessary to ensure validity.

Another major concern is that all the included studies were designed as retrospective studies, which introduces potential selection bias. In retrospective designs assessing DVT, embryologists might unintentionally select the top-ranking thawed embryos on a straw for ET and preserve the remaining lower-quality embryos for the second vitrification. Conversely, they may choose embryos that appear more resilient for a second round of freezing to increase their chances of survival. These practices can under- or overestimate the detrimental effects of DVT on embryonic outcomes, as embryos with specific qualities, based on the embryologist's opinion, are selected to undergo DVT.

Post-exposure interventions involving extra manipulations, such as zona opening for biopsy, artificial shrinkage, or assisted hatching, posed another challenge. These interventions could cause significant variation in findings, making it difficult to isolate the effect of DVT from other procedures.

In this review, we rated all abstracts with poorly described methodologies, lack of rigorous review, and quality appraisal as having a very high risk of bias, as they are prone to various methodological flaws. Also, we excluded such studies in sensitivity analyses.

Limitations and strengths

One of the primary limitations identified was the potential bias and low quality of included studies. These limitations pose significant challenges to our findings, as they can lead to over- or underestimation of the effects of DVT on embryonic and neonatal outcomes. The high heterogeneity in some outcomes suggests that other unmeasured confounding factors might influence the results, further complicating the interpretation. Therefore, the findings of this review should be interpreted with caution, and there is a need for more rigorous research methodologies to ensure reliable and valid results.

The other limitation of our study is the small sample sizes in some subgroup analyses. This limitation can affect the power of the subgroup analyses and the generalizability of findings. Although post hoc power analysis is generally not recommended and can be misleading, we performed power calculations for all subgroups (See Appendix S5). Considering the lower power in some subgroups, we observed a decrease in heterogeneity levels compared to the main analyses, suggesting more precise estimates within these subgroups. Also, we included all eligible studies in our meta-analysis, ensuring a comprehensive assessment of the available evidence.

For instance, regarding culture status, the number of eligible included studies ranged only form 4 to 6 for the EC subgroup, potentially impacting the corresponding findings. However, the significant decrease in heterogeneity levels across all evaluated outcomes suggests that EC may still have a potential protective effect against the detrimental effects of DVT. Nonetheless, the results of these subgroup analyses should be interpreted with caution. This is particularly true for less common outcomes like miscarriage, LBW, and PTB, where the low number of included studies and the low rate of recorded events mean the sample size might not accurately reflect the actual risk. Additionally, findings on neonatal outcomes should be interpreted with caution since, considering the low number of included studies, most subgroup analyses were not feasible or contained very few studies. Furthermore, sensitivity analyses were not possible for most of these outcomes. Meanwhile, as the heterogeneity level for most of these outcomes was zero or low and most included studies were rated to have a low or moderate risk of bias, the results can be considered relatively reliable.

Despite these limitations, the review has strengths. Using the ROBINS-E tool for evaluating the risk of bias provides a systematic assessment of the included studies, identifying key areas of concern and overall quality. Detailed subgroup analyses based on biopsy status, ET type, and other factors allow for a nuanced understanding of how different variables influence outcomes. Many analyses showed low heterogeneity, indicating consistent results across studies and enhancing the reliability of the findings.

Implications of findings in clinical practice

The lack of significant differences in LBW rate, BW, PTB rate, and GA at birth between DVT and SVT suggests that while DVT may increase the C/S rate, it does not adversely affect other key neonatal outcomes. The inconsistency between the fixed effects and random effects models for S/C in the primary analyses indicated that while there is some indication that DVT could lead to a higher C/S rate, this is not a definitive conclusion. However, the sensitivity analysis, which excluded one high-risk study, eliminated this discrepancy. Clinicians should consider that this probable risk may not apply uniformly to all patients, and risk evaluation needs to be approached on a case-by-case basis. Patients should be informed about the possibility of an increased C/S rate with DVT but also reassured that the evidence is not conclusive. This balanced information can help them make more informed decisions about their treatment options.

Given the variability in outcomes based on biopsy status and ET strategy, personalized treatment plans that consider these factors may optimize results. Clinicians should tailor their approaches based on individual patient characteristics and the specifics of their ART procedures. Our finding on the effect of biopsy rounds on MR underscores the importance of considering the number of

biopsies and the chromosomal status of embryos when using DVT. Minimizing invasive procedures and ensuring euploid embryos can help improve overall outcomes in ART procedures involving DVT.

One of the concerns during routine cryopreservation programs is the exposure of blastomeres to CPA, which may have toxic effects on these cells. With additional embryonic manipulations involving zona opening, such as artificial shrinkage or biopsy, the likelihood of this exposure increases. Furthermore, repeated cryopreservation can result in more prolonged contact with these toxic agents. Our data also showed that the detrimental effect of DVT on cryosurvival was more pronounced in biopsied embryos. Therefore, in DVT programs, clinical embryologists may benefit from newly emerging strategies like rapid vitrification-thawing. These approaches, which have been tested in routine cryopreservation programs, may minimize the contact of embryos with CPA, potentially reducing the underlying cellular damage [50]. However, their effectiveness in the context of DVT specifically has not yet been evaluated and requires further investigation.

Considering the great variability among vitrification techniques and the operator-dependent nature of this procedure, clinical embryologists may benefit from using recently introduced semi-automated or automated vitrification devices for DVT [51, 52]. The clinical validation and efficiency of these techniques are not yet proven, but they might change the outcomes of vitrification, whether SVT or DVT and provide more reproducible findings.

The other important implication of our findings was the effect of EC between rounds of vitrification. The timing and conditions between vitrification rounds are crucial. Allowing an EC period not only aids in recovery but also may help in selecting healthier embryos, thereby improving overall outcomes.

Patients should be fully informed about the potential risks and benefits of DVT versus SVT. This includes discussing the increased likelihood of cryodamage, and decreased chance of pregnancy and live birth on one hand and the lack of significant impact on other neonatal outcomes on the other hand, enabling patients to make informed decisions about their treatment options.

Finally, clinicians are encouraged to be cautious about the potential detrimental effects of DVT. However, considering the low quality of existing primary studies, the high levels of heterogeneity and risk of bias, and the retrospective nature of these studies, there is still no consensus.

Recommendations for future research

We recommend future studies to assess some of the ambiguous findings of this review. For example, more studies are needed to clarify the relationship between DVT and neonatal outcomes. Additionally, our observations regarding the mitigating effects of EC on double-vitrified embryos should be addressed in future studies.

To mitigate various methodological concerns in previous studies, future research should minimize different sources of bias by adhering to rigorous methodological standards, including consistent measurement methods, proper handling of missing data, and thorough reporting of confounding factors and interventions. Future studies should provide detailed reporting of patient characteristics, embryo quality, and clinical practices to identify and adjust for potential confounders.

As mentioned earlier, all included studies were designed as retrospective studies. Given the ethical and practical challenges, a randomized clinical trial or even an observational prospective study on DVT versus SVT may not be feasible or ethical. In clinical practice, the first choice is typically the transfer of single vitrified high-quality embryos, making selection bias due to the preference for transferring high-quality embryos inevitable. Additionally, couples with double-vitrified embryos might not return for the transfer of their remaining double-vitrified embryos later for various reasons, such as achieving pregnancy. This makes the follow-up process very challenging in prospective settings.

Despite the technical and ethical limitations present, several considerations can enhance the quality of future studies. One approach is to establish specific inclusion and exclusion criteria, focusing only on eligible DVT cycles instead of including all embryos. In a prospective study design, researchers can pre-plan the selection of embryos undergoing DVT using predefined criteria. Additionally, conducting all DVT procedures with a single operator can help minimize variations due to differing skill levels.

Standardizing post-exposure interventions, such as the culture time between the first and second vitrification, the vitrification protocol, and biopsy techniques, can also contribute to improved quality. Even if not all of these measures can be implemented, researchers should measure these variables and adjust their findings accordingly to better control for confounding factors. This approach can help reduce biases and enhance the reliability of the results.

For future studies, it is crucial to consider the effect of the baseline cause of biopsy. Researchers should predefine the indications for biopsy and avoid including all biopsy causes in DVT cycles. This approach will help better control the level of heterogeneity and confounding factors, leading to more accurate and reliable results.

Additionally, designing more basic science studies on embryos that are candidates for being discarded or those extra embryos that couples do not intend to preserve for any reason can be valuable. With informed consent

from the parents and proper ethical considerations, these embryos can be used in advanced biological studies to evaluate the true effect of DVT in arandomized and well-controlled setting. Also, this approach can help better understand the molecular and cellular mechanisms behind the effects of DVT on embryos.

We also recommend prospective long-term follow-up studies to assess the lasting effects of DVT on offspring health and developmental outcomes.

Conclusion

This systematic review and meta-analysis demonstrated that DVT negatively impacts various embryonic outcomes. This includes reduced rates of cryosurvival, biochemical pregnancy, clinical pregnancy, live birth, alongside an increase in MR. These effects appear consistent regardless of biopsy status or ET strategy. EC between the first and second rounds of vitrification may help mitigate some of the negative impacts of DVT. However, because the subgroup sizes were small, this finding should be interpreted with caution.

Furthermore, the review indicates that DVT does not significantly influence neonatal outcomes such as CS rate, LBW, BW, PTB, or GA at birth. Nevertheless, the limited number of studies presenting these outcomes and the low prevalence of LBW and PTB in the overall included ET cycles, these findings are not conclusive. Additionally, none of the studies evaluated the long-term health effects on offspring born from double-vitrified embryos.

It is important to note that most of the studies included in this review were assessed as having a high or very high risk of bias due to factors such as confounding variables, participant selection, post-exposure interventions, handling of missing data, and retrospective design. Given these limitations, the results should be approached with caution. There is a pressing need for more rigorous research methodologies to yield reliable and valid outcomes.

Abbreviations

ART	Assisted reproductive technology
BPR	Biochemical Pregnancy Rate
BW	Birth Weight
C/S	Cesarean Section
COS	Controlled Ovarian Stimulation
CPR	Clinical Pregnancy Rate
DCDB	Double cryopreservation double biopsy
DCNB	Double cryopreservation no biopsy
DCP	Double cryopreservation
DCSB	Double cryopreservation single biopsy
DVT	Double vitrification/thawing
SVT	Single vitrification/thawing
EC	Extended culture
ET	Embryo transfer
GA	Gestational Age
HRT	Hormone replacement therapy
LBR	Live birth rates
LBW	Low Birth Weight
MET	Multiple embryo transfer
MR	Miscarriage Rate
PGT	Preimplantation genetic testing
PTB	Preterm Birth
SBT	Single blastocyst transfer
SCNB	Single cryopreservation no biopsy
SCP	Single cryopreserved
SCSB	Single cryopreservation single biopsy
SET	Single embryo transfer

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12884-025-07311-x.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Author contributions

All authors contributed to the study conception and design and commented on previous versions of the manuscript. All authors read and approved the final manuscript. A detailed contribution statement is provided below: AM-H: Data collection (systematic search, and risk of bias assessment), manuscript original draft preparation, statical analyses, and study graphs and graphical abstract designing; AS: Data collection (study selection, data extraction, and risk of bias assessment) and manuscript editing; SR: Data collection (study selection and data extraction) and manuscript editing; RK: Data collection (study selection and data extraction); MM: Data collection (study selection and data extraction); FA: Study conceptualization; supervision, interpretation of results; manuscript critical review and editing.

Funding

The research team received no specific grant from public, commercial, or not-for-profit funding agencies for performing or reporting this study.

Data availability

The full list of screened studies with the reason of exclusion is available in an Excel file (Appendix S4). Other study data including the data collection forms and data extracted from included studies, data used for meta-analyses, and results of subgroups power analyses are available in another Excel file (Appendix S5).

Declarations

Ethics approval and consent to participate

NA.

Consent for publication

NA.

Competing interests

The authors declare no competing interests.

Registration of systematic review

Our study has been duly registered in the PROSPERO International prospective register of systematic reviews. The approval code for our registration is CRD42024597287, and the approval date is 23/10/2024.

Author details

[1]Department of Anatomy, School of Medicine, Tehran University of Medical Sciences, Pour Sina St., Ghods St., Keshavarz Blvd, Tehran, Iran
[2]Students' Scientific Research Center (SSRC), Tehran University of Medical Sciences, Tehran, Iran
[3]Department of Obstetrics and Gynecology, Arash Women's Hospital, Tehran University of Medical Sciences, Tehran, Iran

[4]Department of Infertility, Moheb Yas Hospital, Tehran University of Medical Sciences, Tehran, Iran

Received: 7 December 2024 / Accepted: 10 February 2025
Published online: 26 February 2025

References

1. Maheshwari A, Bell JL, Bhide P, Brison D, Child T, Chong HY, et al. Elective freezing of embryos versus fresh embryo transfer in IVF: a multicentre randomized controlled trial in the UK (E-Freeze). Hum Reprod. 2022;37. https://doi.org/10.1093/humrep/deab279.
2. Boynukalin FK, Turgut NE, Gultomruk M, Ecemis S, Yarkiner Z, Findikli N et al. Impact of elective frozen vs. fresh embryo transfer strategies on cumulative live birth: do deleterious effects still exist in normal & hyper responders? PLoS One. 2020;15. https://doi.org/10.1371/journal.pone.0234481
3. Wei D, Legro RS, Chen ZJ. The cumulative live birth rate after a freeze-only strategy versus a conventional fresh embryo transfer strategy: a call for more level 1 evidence. BMC Med. 2020;18. https://doi.org/10.1186/s12916-019-1479-2
4. Cimadomo D, Rienzi L, Romanelli V, Alviggi E, Levi-Setti PE, Albani E, et al. Inconclusive chromosomal assessment after blastocyst biopsy: prevalence, causative factors and outcomes after re-biopsy and re-vitrification amulticenter experience. Hum Reprod. 2018;33. https://doi.org/10.1093/humrep/dey282.
5. Wang M, Jiang J, Xi Q, Li D, Ren X, Li Z, et al. Repeated cryopreservation process impairs embryo implantation potential but does not affect neonatal outcomes. Reprod Biomed Online. 2021;42. https://doi.org/10.1016/j.rbmo.2020.11.007.
6. Oraiopoulou C, Karagianni M, Papatheodorou A, Toumpa O, Papadopoulou M, Christophoridis N, et al. Double vitrification of embryos adversely affects clinical outcomes. JBRA Assist Reprod. 2024;28:399. https://doi.org/10.5935/1518-0557.20240014.
7. Huang Y, Cheng Y, Zhang M, Chen Y, Zhou R, Lin D, et al. Effect of repeated vitrification of human embryos on pregnancy and neonatal outcomes. J Ovarian Res. 2024;17. https://doi.org/10.1186/s13048-024-01370-y.
8. Koch J, Costello MF, Chapman MG, Kilani S. Twice-frozen embryos are no detriment to pregnancy success: a retrospective comparative study. Fertil Steril. 2011;96. https://doi.org/10.1016/j.fertnstert.2011.04.034.
9. Theodorou E, Jones BP, Cardenas Armas DF, Heath C, Serhal P, Ben-Nagi J. Live birth rate following a euploid blastocyst transfer is not affected by double vitrification and warming at cleavage or blastocyst stage. J Assist Reprod Genet. 2022;39. https://doi.org/10.1007/s10815-022-02440-0.
10. Al Hashimi B, Linara-Demakakou E, Harvey SC, Harvey KE, Griffin DK, Ahuja K, et al. Double vitrification and warming of blastocysts does not affect pregnancy, miscarriage or live birth rates. Reprod Biomed Online. 2024;49:104103. https://doi.org/10.1016/J.RBMO.2024.104103.
11. Wang X, Mao R, Wang M, Long R, Jin L, Zhu L. The effect of recryopreservation on embryo viability and outcomes of in vitro fertilization: a systematic review and meta-analysis. Fertil Steril. 2023;120. https://doi.org/10.1016/j.fertnstert.2023.03.001.
12. Bickendorf K, Qi F, Peirce K, Wang R, Natalwala J, Chapple V, et al. Impacts of double biopsy and double vitrification on the clinical outcomes following euploid blastocyst transfer: a systematic review and meta-analysis. Hum Reprod. 2024. https://doi.org/10.1093/HUMREP/DEAE235.
13. Li Piani L, Petrone P, Brutto M, De Vos A, Van Der Kelen A, Vaiarelli A, et al. A systematic review and meta-analysis of double trophectoderm biopsy and/or cryopreservation in PGT: balancing the need for a diagnosis against the risk of harm. Hum Reprod Update. 2024. https://doi.org/10.1093/HUMUPD/DMAE031.
14. Theodorou E, Chronopoulou E, Ozturk O, Brunetti X, Serhal P, Ben-Nagi J. Impact of double trophectoderm biopsy on reproductive outcomes following single euploid blastocyst transfer. Eur J Obstet Gynecol Reproductive Biology. 2024;298:35–40. https://doi.org/10.1016/J.EJOGRB.2024.04.033.
15. Nohales M, Coello A, Martin A, Insua F, Meseguer M, de los Santos MJ. Should embryo rebiopsy be considered a regular strategy to increase the number of embryos available for transfer? J Assist Reprod Genet. 2023;40. https://doi.org/10.1007/s10815-023-02875-z.
16. Makieva S, Sachs MK, Xie M, Velasco A, El-Hadad S, Kalaitzopoulos DR, et al. Double vitrification and warming does not compromise the chance of live birth after single unbiopsied blastocyst transfer. Hum Reprod Open. 2023;2023. https://doi.org/10.1093/hropen/hoad037.
17. Oraiopoulou C, Karagianni M, Toumpa O, Ioannidou D, Papadopoulou M, Christophoridis N, et al. O-065 the effect of double vitrification on embryo reproductive potential and clinical outcome. Hum Reprod. 2022;37. https://doi.org/10.1093/humrep/deac104.079.
18. Wang Y, Chen D, Cai B, Huang D, Xu Y, Ding C. Effects of repeated cryopreservation of cleavage-stage embryos on pregnancy and perinatal outcomes of single embryo transfer. Clin Exp Obstet Gynecol. 2022;49. https://doi.org/10.31083/j.ceog4911244.
19. Mizobe Y, Kuwatsuru Y, Kuroki Y, Fukumoto Y, Tokudome M, Moewaki H, et al. The effect of repeated cryopreservation and thawing using cryotip on the clinical outcomes of embryos. Reprod Med Biol. 2021;20. https://doi.org/10.1002/rmb2.12365.
20. Kumasako Y, Otsu E, Utsunomiya T, Araki Y. The efficacy of the transfer of twice frozen-thawed embryos with the vitrification method. Fertil Steril. 2009;91. https://doi.org/10.1016/j.fertnstert.2007.11.079.
21. Farhi J, Elizur S, Yonish M, Seidman DS, Shulman A, Schiff E, et al. Assessment of a double freezing approach in the management of surplus embryos in IVF. Reprod Biomed Online. 2019;38. https://doi.org/10.1016/j.rbmo.2018.11.010.
22. Wilding M, Terribile M, Parisi I, Nargund G. Thaw, biopsy and refreeze strategy for PGT-A on previously cryopreserved embryos. Facts Views Vis Obgyn. 2019;11.
23. Zheng X, Chen Y, Yan J, Wu Y, Zhuang X, Lin S, et al. Effect of repeated cryopreservation on human embryo developmental potential. Reprod Biomed Online. 2017;35. https://doi.org/10.1016/j.rbmo.2017.08.016.
24. Stanger J, Wong J, Conceicao J, Yovich J. Vitrification of human embryos previously cryostored by either slow freezing or vitrification results in high pregnancy rates. Reprod Biomed Online. 2012;24. https://doi.org/10.1016/j.rbmo.2011.11.013.
25. Murakami M, Egashira A, Murakami K, Araki Y, Kuramoto T. Perinatal outcome of twice-frozen-thawed embryo transfers: a clinical follow-up study. Fertil Steril. 2011;95. https://doi.org/10.1016/j.fertnstert.2010.12.038.
26. Check JH, Brittingham D, Swenson K, Wilson C, Lurie D. Transfer of refrozen twice-thawed embryos do not decrease the implantation rate. Clin Exp Obstet Gynecol. 2001;28.
27. Hallamaa M, Seikkula J, Willman S, Ollila H, Jokimaa V. Pregnancy potential and perinatal outcomes of embryos cryopreserved twice: a case–control study. Reprod Biomed Online. 2021;43. https://doi.org/10.1016/j.rbmo.2021.06.028.
28. Taylor TH, Patrick JL, Gitlin SA, Michael Wilson J, Crain JL, Griffin DK. Outcomes of blastocysts biopsied and vitrified once versus those cryopreserved twice for euploid blastocyst transfer. Reprod Biomed Online. 2014;29. https://doi.org/10.1016/j.rbmo.2014.03.001.
29. Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. BMJ. 2021;372. https://doi.org/10.1136/bmj.n71.
30. Neal SA, Sun L, Jalas C, Morin SJ, Molinaro TA, Scott RT. When next-generation sequencing-based preimplantation genetic testing for aneuploidy (PGT-A) yields an inconclusive report: diagnostic results and clinical outcomes after re biopsy. J Assist Reprod Genet. 2019;36. https://doi.org/10.1007/s10815-019-01550-6.
31. Bradley CK, Livingstone M, Traversa MV, McArthur SJ. Impact of multiple blastocyst biopsy and vitrification-warming procedures on pregnancy outcomes. Fertil Steril. 2017;108. https://doi.org/10.1016/j.fertnstert.2017.09.013.
32. Nanassy L, Schoepper B, Schultze-Mosgau A, Depenbusch M, Eggersmann TK, Hiller RAF, et al. Evaluation of live birth rates and perinatal outcomes following two sequential vitrification/warming events at the zygote and blastocyst stages. J Assist Reprod Genet. 2023;40. https://doi.org/10.1007/s10815-023-02909-6.
33. Shen X, Ding M, Yan Y, Huang C, Wang S, Zhou J, et al. Perinatal outcomes of singletons following double vitrification-warming procedures: a retrospective study using propensity score analysis. BMC Pregnancy Childbirth. 2023;23. https://doi.org/10.1186/s12884-023-05369-z.
34. Zhang Q, Yu W, Jin C, Ni T, Zhou T, Zhao Q, et al. Impact of multiple vitrification-warming procedures and insemination methods on pregnancy and neonatal outcomes in preimplantation genetic testing for aneuploidy. Reproductive Sci. 2023;30. https://doi.org/10.1007/s43032-023-01177-0.
35. Wang M, Zhou J, Long R, Li Y, Gao L, Mao R, et al. Recryopreservation impairs blastocyst implantation potential via activated endoplasmic reticulum stress pathway and induced apoptosis. MedComm (Beijing). 2024;5:e689. https://doi.org/10.1002/MCO2.689.

36. Li J, Xiong S, Zhao Y, Li C, Han W, Huang G. Effect of the re-vitrification of embryos at different stages on embryonic developmental potential. Front Endocrinol (Lausanne). 2021;12. https://doi.org/10.3389/fendo.2021.653310.
37. Kopeika J, Thornhill A, Khalaf Y. The effect of cryopreservation on the genome of gametes and embryos: principles of cryobiology and critical appraisal of the evidence. Hum Reprod Update. 2015;21. https://doi.org/10.1093/humupd/dmu063.
38. Mamo S, Bodo S, Kobolak J, Polgar Z, Tolgyesi G, Dinnyes A. Gene expression profiles of vitrified in vivo derived 8-cell stage mouse embryos detected by high density oligonucleotide microarrays. Mol Reprod Dev. 2006;73. https://doi.org/10.1002/mrd.20588.
39. Zhou G, Zeng Y, Guo J, Meng Q, Meng Q, Jia G, et al., et al. Vitrification transiently alters Oct-4, Bcl2 and P53 expression in mouse morulae but does not affect embryo development in vitro. Cryobiology. 2016;73. https://doi.org/10.1016/j.cryobiol.2016.08.011.
40. Lin T, Lee JE, Kang JW, Shin HY, Lee J, Bin, Jin D, Il. Endoplasmic reticulum (ER) stress and unfolded protein response (UPR) in mammalian oocyte maturation and preimplantation embryo development. Int J Mol Sci. 2019;20. https://doi.org/10.3390/ijms20020409.
41. Khatun H, Ihara Y, Takakura K, Egashira J, Wada Y, Konno T et al. Role of endoplasmic reticulum stress on developmental competency and cryo-tolerance in bovine embryos. Theriogenology. 2020;142. https://doi.org/10.1016/j.theriogenology.2019.09.042
42. Kader A, Agarwal A, Abdelrazik H, Sharma RK, Ahmady A, Falcone T. Evaluation of post-thaw DNA integrity of mouse blastocysts after ultrarapid and slow freezing. Fertil Steril. 2009;91. https://doi.org/10.1016/j.fertnstert.2008.04.049.
43. Hernandez-Nieto C, Lee J, Alkon-Meadows T, Briton-Jones C, Sandler B, Copperman A, et al. Biological relevance of trophectoderm morphology: initial β-hCG measurements correlate with trophectoderm grading on euploid frozen embryo transfers. J Assist Reprod Genet. 2022;39. https://doi.org/10.1007/s10815-022-02553-6.
44. Indications. Management of preimplantation genetic testing for monogenic conditions: a committee opinion. Fertil Steril. 2023;120. https://doi.org/10.1016/j.fertnstert.2023.03.003.
45. Carvalho F, Coonen E, Goossens V, Kokkali G, Rubio C, Meijer-Hoogeveen M, et al. ESHRE PGT Consortium good practice recommendations for the organisation of PGT. Hum Reprod Open. 2020;2020. https://doi.org/10.1093/hropen/hoaa021.
46. Laguna Rodriguez T, Serrano Molina M, Romero Olmedo MDLS, De Andrés Cara M, Rico Nieto E, Fernández González MJ, et al. P-253 clinical efficacy of PGT-A according to maternal age and embryo quality in blastocyst stage. Hum Reprod. 2023;38. https://doi.org/10.1093/HUMREP/DEAD093.611.
47. Cozzolino M, Pellegrini L, Fasulo DD, Pellicer A, Galliano D. P-510 the impact of the severe male factor in the egg donation cycles using preimplantation genetic test for aneuploidy. Hum Reprod. 2024;39. https://doi.org/10.1093/HUMREP/DEAE108.850.
48. Fan PL. Sperm genetic abnormality testing in recurrent pregnancy loss cases: a narrative review. Middle East Fertil Soc J. 2023;28:1–6. https://doi.org/10.1186/S43043-023-00149-3/METRICS
49. Zolfaroli I, Romeu Villarroya M, Serralta García LB, Rubio Rubio JM, Monzó Miralles A. Impact of prolonged embryo storage on reproductive and neonatal outcomes: a systematic review and meta-analysis. J Assist Reprod Genet. 2024;41:2691–700. https://doi.org/10.1007/S10815-024-03283-7/METRICS.
50. Schiewe MC, Reichelderfer R, Wozniak K, De Romana C, Nordbak M, Baek K, et al. Ultra-fast vitrification and rapid elution of human oocytes: part I. Germinal vesicle model validation. Reprod Biomed Online. 2024;49. https://doi.org/10.1016/j.rbmo.2024.104691.
51. Barberet J, Ducreux B, Bruno C, Guilleman M, Simonot R, Lieury N, et al. Comparison of oocyte vitrification using a semi-automated or a manual closed system in human siblings: survival and transcriptomic analyses. J Ovarian Res. 2022;15. https://doi.org/10.1186/s13048-022-01064-3.
52. Miwa A, Noguchi Y, Hosoya K, Mori Y, Sato T, Kasahara Y, et al. Equivalent clinical outcome after vitrified-thawed blastocyst transfer using semi-automated embryo vitrification system compared with manual vitrification method. Reprod Med Biol. 2020;19. https://doi.org/10.1002/rmb2.12320.

Publisher's note