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Research Article

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The Developmental Stage at Cryopreservation in Assisted Reproduction: A Systematic Review and Meta-Analysis of Pregnancy Outcomes

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Abstract

To date, there is no consensus in embryo developmental stages for cryopreservation. The present study aimed to investigate the impact of embryo developmental stages at cryopreservation on pregnancy outcomes of frozen embryo transfer. Systematic review and meta-analysis of relevant studies identified through MEDLINE literature search was performed. The primary outcome was live birth/delivery rate, and the secondary outcomes included implantation rate, ongoing pregnancy rate, clinical pregnancy rate, miscarriage rate, and multiple pregnancy rate. The protocol of this systematic review has been registered on PROSPERO 2017 (registration number: CRD42017072828). Five studies met the eligibility criteria were included in the present review. The outcomes of embryos frozen at different stages but transferred at the same stage were analyzed and compared. Embryos frozen at non-blastocyst showed a significant higher delivery/live birth rate than those cryopreserved at blastocyst (odds ratio=1.37; 95% confidence interval, 1.13-1.66) in the setting of frozen embryo transfer with blastocysts. There was only a limited number of studies with analyzable data for comparisons. The literature varied substantially in study design and methodology applied. Although a significant difference was observed toward an improved delivery/live birth rate for blastocyst transfer with embryos frozen at non-blastocyst stage, future studies are required to further corroborate this finding.

Keywords: Frozen Embryo Transfer; Embryo Stage, Cryopreservation, In Vitro Fertilization, Assisted Reproductive Technology

Introduction

Since the first pregnancy with frozen-thawed human embryo succeeded in 1983, assisted reproductive technologies (ARTs) have benefited infertile couples who could not conceive without treatment [1]. The progress in embryo cryopreservation technologies further advanced the success rate of ART. In addition to patients' characteristics, the efficacy of embryo transfer is influenced by multiple variables, including hormone supplementation protocols, embryo quality, developmental stages of embryo transferred, cryopreservation methods, culture media, and synchronization between the embryo and endometrial development.

Among all variables mentioned previously, embryo cryopreservation has become a critical and indispensable part of ART. The methods of cryopreservation can be categorized into conventional slow-freezing method and vitrification, the former is known for its lower survival rates for blastocysts, which makes vitrification gradually replaces the dominance of the conventional slow-freezing method [2]. Vitrified-thawed embryos have equivalent or even better clinical outcomes as compared with the slow-freezing method in both the cleavage and blastocyst stage embryos [3-8].

In frozen embryo transfer, factors such as extended culture after thawing/warming may further impact pregnancy outcomes. It is suggested that extended culture to the blastocyst stage enables self-selection of viable cells and thus leading to a higher implantation rate [9]. Some studies also showed that, although cumulative pregnancy rates were similar between cleavage-stage and blastocyst embryos, the transfer frequency before pregnancy was significantly lower in the blastocyst group, indicating shorter time

to pregnancy and reduced medical cost [10]. Moreover, for ART complications such as multiple pregnancy, ectopic pregnancy, and miscarriage, it was reported that blastocysts advantage over cleavage-stage embryos. With a lower number of transferred embryos, blastocyst transfer was logically expected to have a lower multiple pregnancy rate and therefore could be a better strategy to prevent the incidence of multiple pregnancy [10].

Some studies suggested other advantages of replacing frozen-thawed blastocysts. Fang et al. have shown that patients received frozen-thawed Day-5 blastocysts had a lower ectopic pregnancy rate as compared with frozen-thawed Day-3 embryos and fresh embryos [11]. Nevertheless, prolonged culture results in fewer embryos available for transfer and thus increases the cancellation rate of the transfer cycle. On the other hand, blastocyst culture before cryopreservation rather than after thawing may be preferred by clinics for cost-effectiveness issue.

The outcomes of embryo transferred at different developmental stages (the cleavage stage [day 2 or day 3] and the blastocyst stage [day 5 or day 6]) following fresh or fresh and subsequent frozen embryo transfer (cumulative outcomes) have been broadly investigated and well-reviewed [9]. Nevertheless, only limited studies have specifically investigated and compared the outcomes of frozen embryo transfer with different developmental stage cryopreservation. Although the technology of cryopreservation has greatly advanced in the past decade, there is still no clear consensus on which developmental stage an embryo should be cryopreserved. Additionally, no meta-analyses have specifically investigated whether the developmental stages at which an embryo was frozen would influence the outcomes of embryo transfer.

For the reasons mentioned above, a question was raised: for patients who need frozen-thawed embryo transfer, do embryos frozen at different developmental stages affect the outcomes of assisted reproduction? To answer this question, we systematically examined literature regarding comparisons of embryo cryopreserved at different developmental stages (2PN [2 pronuclear], cleavage, or blastocyst stages) but transferred at the same stage, and subsequently analyzed the pregnancy outcomes following the frozen-embryo transfer.

Methods

Registration number

The protocol for this systematic review has been registered in PROSPERO (available on https://www.crd.york.ac.uk/prospero/display_record.asp?ID=CRD42017072828).

Study selection and search strategy

Both prospective and retrospective studies that met eligibility criteria and investigated the outcomes of frozen embryo transfer for ART were to be included in this review. The inclusion criteria were as follows: (1) women/couples undergoing in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) and subsequently receiving frozen embryo transfer (FET); (2) women/couples transferred with embryos cryopreserved using either slow-freezing or vitrification method; (3) studies showed the outcomes of embryos frozen at different stages but transferred at the same stage.
The primary outcome was live birth/delivery rate. The secondary outcomes included clinical pregnancy rate, implantation rate, ongoing pregnancy rate, miscarriage rate, and multiple pregnancy rate.

In July 2019, the literature search was conducted in PubMed electronic database covering articles published between 2002 and 2017, with keywords including ((vitrify*) OR (cooling) OR (frozen) OR (freez*) OR (cryopreserve*) OR (cryotransfer)) AND (transfer) AND (embryo) AND ((stage) OR (day) OR (development*) OR (pronuclear) OR (cleavage) OR (blastocy*)). All searches were limited to human studies published in the language of English and excluded reviews, editorials, and case reports. The papers without presenting comparisons between different developmental stages at the time of freezing were excluded. Studies with mixed and inseparable results of embryos frozen using vitrification and slow-freezing were excluded as well.

This study was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) statement (Table S1 PRISMA Checklist).

Supporting Information

Table: S1 PRISMA checklist

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	3-5
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	5
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	5
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	5-6
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	5-6
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	5-6
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	6-7
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	6-7
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	6-7
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	6-7
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	6-7
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I2) for each meta-analysis.	6-7
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	N/A
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	N/A
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	8

Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	8
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	8
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	8-9
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	8-9
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	N/A
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	N/A
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	10-13
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	14
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	14-15
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	15

From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(7): e1000097. doi:10.1371/journal.pmed1000097

Data extraction and quality assessment

The abstracts retrieved from the search were screened by two independent reviewers (JYPH and ACHL), after which the full articles meeting the inclusion criteria were retrieved. The references cited in retrieved articles were reviewed. Subsequently, the eligibility was assessed, and the data were extracted independently by the two authors (JYPH and ACHL). The risk of bias for eligible cohort studies was evaluated using the Newcastle-Ottawa Scale. Any discrepancies were resolved by consensus, and all authors critically analyzed the results.

Data Synthesis and Analysis

In the meta-analysis, the outcomes of blastocyst transfer of embryos frozen at the non-blastocyst stage were compared with those frozen at the blastocyst stage. In the case of cleavage stage transfer, the outcomes of embryos cryopreserved at the 2PN stage versus the cleavage stage were analyzed. For insufficient or missing data within the literature, attempts were made to contact the investigators of individual studies via e-mail. Subsequently, dichotomous outcomes of eligible studies for meta-analysis were calculated and expressed in terms of odds ratios (ORs) with 95% confidence intervals (CIs) and combined with Stata (version 13; StataCorp LLC, College Station, TX) using Mantel-Haenszel model (fixed effect) and the DerSimonian and Laird method (random effect). Heterogeneity among studies was assessed by I^2 and chi-squared tests. Funnel plots were not generated when there were less than 10 studies, as the plots could be misleading [12].

Results

Study selection and quality assessment

A total of 885 potentially relevant studies was identified via the electronic search on the PubMed. Additional nine articles were identified through reviewing the retrieved articles. Following the screening of abstracts, 856 studies were excluded, and 38 studies were reviewed for eligibility. After reading the manuscripts and assessing the inclusion criteria and methodology, five remaining articles fulfilling the inclusion criteria were included in this review (Figure 1).

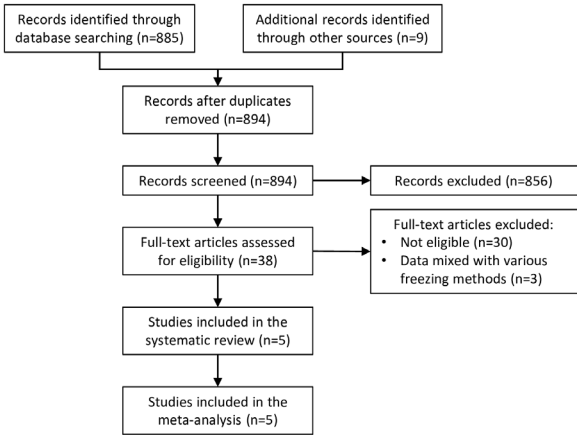

Figure 1: PRISMA flow diagram

All five studies were retrospectively conducted as single-center studies. It was found that the methodology varied substantially between studies. While one study vitrified embryos, four studies utilized slow-freezing protocols. Three papers investigated the impact of developmental stages on the outcomes of embryo transfer with surplus embryos following fresh cycles, and the rest two studies involved whole embryo freezing (freeze-all). The characteristics of these studies are summarized in Table 1, and the risks of bias were assessed and summarized in Table 2.

Table 1: Characteristics of All Studies Included

Study	Ref	Study type	Freezing method	Freeze-all	Duration of study	Duration of study
Veeck (2003)	15	Retrospective	Slow freezing	No	Jan 1995-Jun 2002	794 cycles
Noyes et al. (2009)	16	Retrospective	Slow freezing	No	2000-2006	706 cycles
Moragianni et al. (2010)	17	Retrospective	Slow freezing	No	Mar 2000-Mar 2008	546 patients
Mesut et al. (2011)	18	Retrospective	Slow freezing	Partial	2004-2009	2,531 cycles
Cobo et al. (2012)	19	Retrospective	Vitrification	Partial (for OHSS-risk patients, impaired endometrium pattern, or high progesterone levels)	Jan 2007- Dec 2010	3,057 cycles

OHSS, ovarian hyperstimulation syndrome.

Table 2: Newcastle-Ottawa Scale Summary Assessment of Risk of Bias

Study	Selection				Comparability	Outcome			Total number of stars
	Representativeness of the exposed cohort	Selection of the nonexposed cohort	Ascertainment of exposure	Outcome not present at start of study		Assessment of outcome	Length of follow-up	Adequacy of follow up	
Cobo et al. (2012)			*		*	*	*	*	*****
Mesut et al. (2011)			*		*	*	*	*	*****
Moragianni et al. (2010)			*		*	*	*	*	*****
Noyes et al. (2009)			*		*	*	*	*	*****
Veeck (2003)			*		*	*	*	*	*****

Each study is assessed with eight domains categorized into three groups, Selection, Comparability, and Outcome. A study can be awarded a maximum of one star for each domain except the Comparability, which can be awarded for a maximum of two stars for important or additional factor [13-17].

Synthesis of Results

To explore the impact of the frozen stage to the clinical outcomes of embryo transfer, the results of studies comparing embryos frozen at different stages but transferred at the same stage were synthesized for further analysis. Two scenarios were investigated, embryos transferred at the cleavage or blastocyst stages.

For blastocyst embryo transfer, the outcomes of blastocyst versus non-blastocyst cryopreservation were analyzed. The forest plots of primary and secondary outcomes are displayed in Figs. 2 and 3, respectively. The results showed that frozen at the non-blastocyst stage was associated with a significant improvement in live birth/delivery rate as compared with embryos cryopreserved at the blastocyst stage (2652 thawing cycles; 3 studies; OR=1.37; 95%CI, 1.13-1.66). Nevertheless, no significant difference was observed in clinical pregnancy rate (2652 thawing cycles; 3 studies; OR=0.92; 95%CI, 0.76-1.12), implantation rate (3821 embryos; 2 studies; OR=0.89; 95%CI, 0.76-1.05), and miscarriage rate (581 clinical pregnancies; 3 studies; OR=0.79; 95%CI, 0.49-1.25). With respect to ongoing pregnancy rate, there was only one study identified for this outcome measure and no statistical significance was observed.

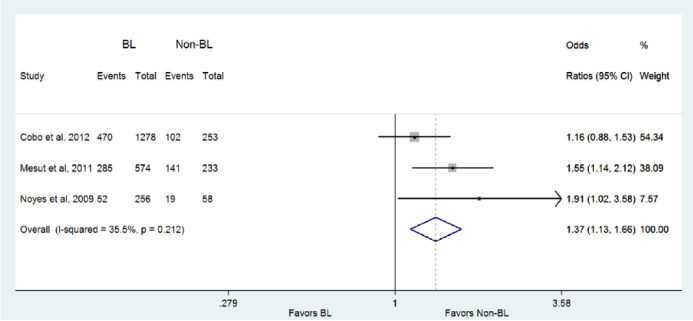

Figure 2: Forest plot of primary outcome for embryos transferred at blastocyst. Meta-analyses of live birth/delivery rate of embryos frozen at blastocyst and non-blastocyst were displayed

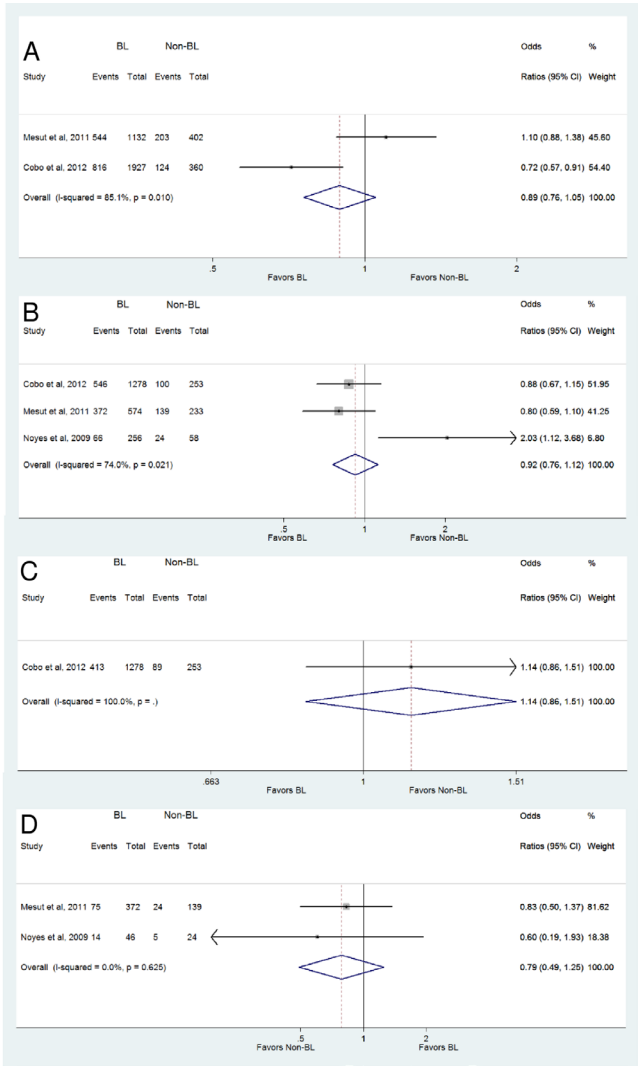

Figure 3: Forest plots of secondary outcomes for embryos transferred at blastocyst. Meta-analyses of (A) implantation rate, (B) clinical pregnancy rate, (C) ongoing pregnancy rate, and (D) miscarriage rate of embryos frozen at blastocyst and non-blastocyst were displayed

For cleavage stage transfer, the outcomes of embryos cryopreserved at the 2PN stage were compared with those frozen at the cleavage stage. As shown in Figure 4, the forest plots of live birth/delivery rate, implantation rate, clinical pregnancy rate, and miscarriage rate are displayed (no data were available for ongoing pregnancy rate and multiple pregnancy rate). Only one study meeting the eligibility criteria was identified for the outcomes of live birth/delivery rate, implantation rate, and miscarriage rate. For clinical pregnancy rate, no difference was found between 2PN- and cleavage-stage transfer (805 transfers; 2 studies; OR=1.24; 95% CI, 0.

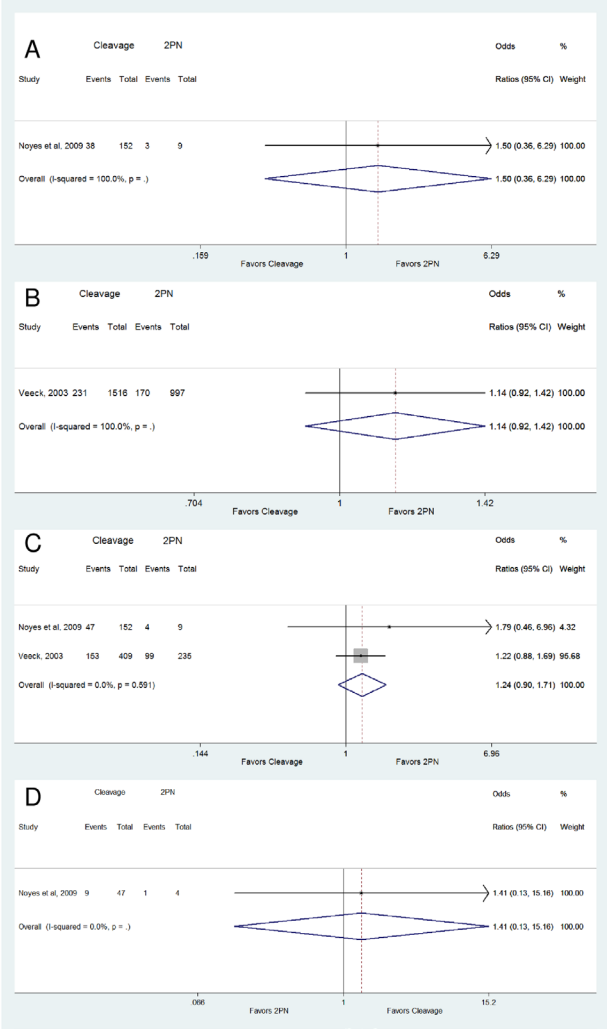

Figure 4: Forest plots of embryos transferred at cleavage stage. Meta-analyses of (A) live birth/delivery rate, (B) implantation rate, (C) clinical pregnancy rate, and (D) miscarriage rate of embryos frozen at cleavage and 2PN stages were shown

Discussions

The success with frozen embryo transfer has dramatically improved in recent years. Embryo freezing allows the surplus good-quality embryos in the fresh cycle can be preserved. In addition, freeze-all of embryos serves as a favorable option for patients at high risk of OHSS during the fresh cycle. Nevertheless, with the increasing need for frozen embryo transfer, there is still no consensus on which developmental stage embryos should be cryopreserved. For this reason, a systematic review and meta-analysis was conducted to provide insight into the influence of developmental stages for embryo freezing. To our knowledge, this is the first systematic review and meta-analysis that specifically investigated whether the developmental stages at which an embryo was frozen would impact the outcomes of frozen embryo transfer. The current review analyzed the available results regarding the impact of developmental stage at frozen on the outcomes of ART following frozen-thawed embryo transfer. Five articles were identified through systematic search. As all studies were carried out and retrospectively reviewed at a single center, different institutional policies thereby directly resulted in different protocols in culture media, freezing protocol, the number of embryos replaced, as well as stimulation protocols. Hence, the design and methodology applied to these studies varied substantially. In addition to the high level of heterogeneity, the definitions and outcomes evaluated also

differed between the studies.

For most clinics or hospitals, the number of cleavage embryos replaced is often greater than that of blastocyst embryos due to different implantation potential between cleavage embryos and blastocysts. Of the five studies included, significant differences in the number of embryos replaced between the cleavage and blastocyst stages were found in three studies, whereas the rest two had a comparable number of embryos replaced (Table S2). In the present review, we compared outcomes of embryos cryopreserved at different stages but transferred at the same stage in order to eliminate the impact of implantation potential from different stage of embryos.

Supporting Information

Table: S2 Summary of the number of embryo replaced

REFERENCE	NUMBER OF EMBRYO REPLACED		P VALUE	FROZEN METHOD
	CLEAVAGE BLASTOCYST P VALUE DAY 2/DAY 3	BLASTOCYST DAY 5/DAY 6		
(MESUT ET AL. 2011)	3.0±0.7	Day 5: 2.2±0.6 Day 6: 1.9±0.6 Day 3→Day 5: 2.2±0.7 Day 3→Day 6: 2.0±0.7	NS	Slow freezing
MORAGIANNI ET AL. 2010)	Day 1→2: 2.90±1.39 Day 3: 2.55±1.35	Day 5: 1.79±0.86	0.0001	Slow freezing
(NOYES ET AL. 2009)	Day 2-3→CSE: 2.6±0.1	2PN→BL: 2.7±0.2 Day 2-3→BL: 2.2±0.1 Day 5→BL: 2.3±0.1 Day 6→BL: 2.3±0.1 Days 5&6→BL: 2.0±0.1	NA	Slow freezing
(VEECK 2003)	NA	NA	NA	Slow freezing
(COBO ET AL. 2012)	Day 2: 1.9±0.8 Day 3: 1.8±0.6	Day 5: 1.5±0.6 Day 6: 1.5±0.6	<0.05	Vitrification

NS, not significant; NA, not available: FET, frozen embryo transfer; CSE, transferred at cleavage stage; BL, transferred at blastocyst stage.

In consideration of the fact that freezing methods significantly influenced the survival and pregnancy outcomes of frozen embryo transfer, the present review has prospectively designed to exclude studies with mixed results of the different freezing methods [3,4,8,18]. For this reason, three studies have been excluded from the search results, including one randomized controlled trial (RCT) and two population-based studies based on registry databases. Although registry-based studies have a large study population, limitations such as inconsistent data collection, information unavailable, and potential variability in reporting outcomes are hardly avoided. Therefore, these studies are not suitable to be combined and analyzed in the systematic review and meta-analysis.

This study aimed to elucidate whether embryos frozen at different developmental stages would affect the pregnancy outcomes. About a dozen studies were excluded because they focused on the outcomes of the cleavage versus blastocyst stage transfer. Post-thaw culture was only applied in five studies, which were therefore included in this review. Through combining results from different studies, embryos frozen at different stages but transferred at the same stage were allowed to be compared. In our results, no difference was observed in primary and secondary outcomes of cleavage embryo transfer group. Nevertheless, for blastocyst embryo transfer group, there was a significantly higher delivery/live birth rate with embryos frozen at the non-blastocyst stage as compared with those cryopreserved at the blastocyst stage. In comparison with embryos transferred soon after thawing, extended culture of non-blastocyst embryos after thawing provides a longer recovery time and opportunity to select better blastocyst, and therefore they were relatively competent while being replaced.

Most studies identified in the present review performed frozen embryo transfer using supernumerary embryos of the retrieval cycle. For women with a high risk of ovarian hyper stimulation syndrome (OHSS) or adverse endocrinological profile, freezing all embryos provides a valuable alternative when a fresh transfer is not advantageous. In addition, because top-quality embryos are generally transferred in fresh cycles, cryopreservation of supernumerary embryos may result in lower rates in pregnancy outcomes. Ideally, freeze-all strategies are better to be evaluated as primary therapies. In the current review, only one RCT focused on exploring the outcomes of cryopreserved-embryo transfer exclusively in patients

undergoing freeze-all [19]. In the randomized trial conducted by Shapiro et al., embryos frozen at the 2PN or blastocyst stages were both transferred at the blastocyst stage [19]. However, this study was not included in the present review because the results of embryo cryopreservation using vitrification or slow-freezing were mixed and inseparable, which makes the study ineligible for inclusion. In contrast to Shapiro's study, the three studies included for meta-analysis in this review were all retrospective, with heterogeneity in design. Thus, further randomized controlled research is suggested before drawing definitive conclusions.

Blastocyst transfer is suggested to improve embryo-endometrial synchronicity and enable self-selection of viable embryos through the period of extended culture. Thus, culturing of embryos to the blastocyst stage may serve as a suitable strategy without compromising the transfer outcomes. However, for blastocyst transfer, freezing at an earlier stage other than blastocyst markedly increases the workload imposed on embryologists for the embryo freezing processes. Moreover, if preimplantation genetic test for aneuploidy or embryo biopsies are scheduled, blastocyst extended culture before cryopreservation may be mandatory. However, in the current study, significant higher live birth/delivery rates were noted in a setting of non-blastocyst embryo cryopreservation and post-thawed extended culture to blastocyst and transfer. Thus, whether an extended culture should be performed before or after cryopreservation needs multi-facet evaluations.

There are some limitations in the studies included in the present review. No RCTs were included in the final search results so that no results from RCTs were available for the meta-analyses. Studies with extended culture after thawing were limited. Therefore, the analyzable data for data synthesis were restricted. Moreover, some studies only investigated the pregnancy outcome by the number of transfer or thawing cycle without providing information of subject number, which may significantly affect the outcome variable. In addition to the pregnancy outcomes analyzed in the present review, other clinical considerations such as ectopic pregnancy rate, time to live birth, cost-effectiveness, and neonatal outcomes are also of great value. Nevertheless, these outcomes are less frequently investigated and therefore were not included in the present review.

Conclusions

The present review summarizes pregnancy outcomes from five articles evaluating the developmental stages of frozen embryo transfer following IVF or ICSI. Meta-analyses were conducted for the outcomes of embryos frozen at different stages but transferred at the same stage. Although there were no significant differences in various outcomes for frozen-thawed embryo transferred at cleavage stage group, we found that there was a significant difference in blastocyst embryo transfer group. Embryos frozen at non-blastocyst showed a statistically higher live birth/delivery rate as compared with those cryopreserved at blastocyst in the setting of blastocyst frozen embryo transfer cycles. There is currently no sufficient evidence to recommend a specific embryonic stage for cryopreservation in couples undergoing ART. Nevertheless, the favorable live birth/delivery rate of extended culture to blastocyst after non-blastocyst thawing provides a new insight with regards to future clinical practices. Randomized controlled trials are warrant to explore the impact of embryo stage at freezing on pregnancy outcomes.

Data Availability

All data generated or analyzed during this study are included in this published article (and its Supplementary Information files).

Author Contributions

J.Y.P.H. contributed to the study design, acquisition of data, analysis, and interpretation of data, as well as article drafting and revising. A.C.H.L. contributed to the acquisition of data, interpretation of data, and revision of the article. All authors read and approved the final manuscript and participated in the decision to submit the manuscript for publication.

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Disclosure of Potential Conflicts of Interest

ACHL and JYPH claim no conflicts of interest. There are no additional declarations from the authors relevant to this research relating to employment, consultancy, products in development, patents, or revenues from marketed products to declare.

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