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REVIEW

The effect of extended cryo-storage following vitrification on embryo competence: a systematic review and meta-analysis

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Abstract

Purpose Few studies explored whether prolonged cryo-storage after vitrification affects embryo competence and perinatal outcomes. This systematic review and meta-analysis aims at highlighting any putative impact of cryo-storage duration on cryo-survival, miscarriage, live birth and major malformations.

Methods A systematic review was performed using MEDLINE (PubMed), ISI Web of Knowledge, Scopus and Embase databases up to June 2021. Data were combined to obtain a pooled OR, and meta-analysis was conducted using a random effects model. Out of 1,389 screened abstracts, 22 papers were assessed for eligibility, and 5 studies were included ($N = 18{,}047$ embryos). Prolonged cryo-storage was defined as > 12 months ($N = 3389$ embryos). Subgroup analysis was performed for untested vitrified cleavage stage embryos ($N = 1739$ embryos) and for untested and euploid vitrified blastocysts ($N = 13{,}596$ and 2712 embryos, respectively).

Results Survival rate, miscarriage, live birth and major malformation rates were all similar in the two groups.

Conclusion These data further support the safety of long-term cryo-storage of human embryos beyond 12 months. This is reassuring for good prognosis patients with surplus embryos, couples seeking a second child from supernumerary embryos and women postponing the transfer for clinical or personal reasons.

Keywords Cryo-storage · Vitrification · Cryo-survival · Live birth rate · Embryo

Introduction

The ability to cryopreserve reproductive specimens has dramatically changed the daily practice in IVF clinics [1]. Cryopreservation techniques are routinely performed nowadays to store oocytes and embryos even for long periods until future use [2, 3]. Prolonged storage of oocytes is currently adopted for many medical or social reasons, including fertility preservation in patients at risk of premature ovarian insufficiency, like patients facing gonadotoxic treatments, women delaying childbearing for personal, professional, financial or psychological reasons and egg donation programs [2, 4, 5]. In addition, the use of this technology improved the cumulative live birth rate by allowing repeated embryo transfers with surplus frozen embryos from a single ovarian stimulation cycle in patients with impaired endometrial receptivity or undergoing preimplantation genetic testing (PGT). In general, the introduction of cycle segmentation and delayed embryo transfer also decreased the risk of severe ovarian hyperstimulation syndrome (sOHSS) [6, 7]. Indeed, as demonstrated by the last release of the European IVF registries, cryopreservation has dramatically spread in the last years resulting in an overall reduction of the maternal, gestational and perinatal risks associated with multiple gestations, mainly thanks to the systematic application

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of an elective single embryo transfer (SET) policy [8, 9]. Initially, the slow-freezing method provided reasonably good outcomes [10], but IVF laboratories rapidly switched their practice towards vitrification once this protocol was proven simpler, cheaper, faster and safer than slow-freezing [11] and as efficient as fresh transfers in terms of pregnancy, miscarriage and live birth rates [12–15]. Vitrification, indeed, represents the gold standard worldwide for either oocyte or embryo cryopreservation [16, 17]. Of note, current evidence suggests that the pregnancies achieved through warmed embryos are at lower risk of premature delivery and low birth weight compared to those obtained from fresh transfers [9]. On the other hand, an increased frequency of hypertensive disorders, large for gestational age and high birth weight has been observed [9]. However, no difference has been reported in terms of congenital anomalies and perinatal mortality [18].

Cryopreservation is therefore an established technique and an integral part of the routine practice of IVF laboratories. Nonetheless, although many oocytes and embryos are cryo-stored in liquid nitrogen for long periods, the evidence is still limited as for the putative impact of prolonged cryo-storage on oocyte and embryo viability, developmental and reproductive competence, as well as on neonatal outcomes. So far, a single meta-analysis included data from seven studies and reported no impact on pregnancy outcome [19]. The present systematic review of the most recent literature aims at comprehensively highlighting the putative impact of long-term cryo-storage on embryo cryo-survival and clinical outcomes, as miscarriage, live birth and major neonatal malformation rates. We focused only on papers adopting vitrification, as this cryopreservation protocol is safer and more frequently used nowadays, we set a clear time cut-off (12 months) to outline short- and long-term cryo-storage and we further stratified the results according to stage of transfer (cleavage or blastocyst) and chromosomal competence (euploid or untested embryos), because of the intrinsic association of these features with the clinical outcomes downstream.

Material and methods

Protocol

This study was exempt from institutional review board approval. We adhered to PRISMA guidelines [20].

Eligibility criteria

We used the patients, intervention, comparison and outcomes (PICO) model to select our study population (see Supplementary Table 1). We included studies in which the survival rate of the embryos (blastocyst/cleavage-stage) cryopreserved for more than 12 months was compared with the one of those cryopreserved for less or equal to 12 months in infertile women candidates for IVF.

Information sources and search

We searched the MEDLINE (PubMed), ISI Web of Knowledge, Scopus and Embase databases up to June 2021. We also hand-searched the reference lists of relevant studies and reviews. Combinations of the following keywords and search terms were used: (Oocyte OR Metaphase II OR cleavage-stage embryo OR day3 embryo OR blastocyst OR day5 embryos) AND (vitrification OR slow freezing OR warming OR thawing) AND (cryo-storage OR duration OR length) AND (survival OR pregnancy OR implantation OR live birth OR gestational outcomes OR perinatal outcomes OR post-natal outcomes). No time or language restriction was adopted, and queries were limited to human studies.

Study selection, data collection and data items

Four authors (S.C., A.G., A.T., F.G) evaluated titles and abstracts. Duplications were removed using Endnote online software and manually. Disagreements were resolved by discussion with two additional authors (D.C., R.M.). Case series, case reports, books, congress abstracts and grey literature were not included in the analysis.

Risk of bias, summary measures and synthesis of the results

The risk of bias and quality assessment of the included studies were performed adopting the Newcastle–Ottawa Scale (NOS) [21]. Three authors (S.C., D.C., A.C.) independently assessed the risk bias for each included study. The most experienced author (R.M.) resolved conflicts. The NOS score was used to evaluate the included studies, and each study was judged based on three issues: selection of the study group; comparability between groups; and ascertainment of exposed and not exposed cohorts. The primary outcome was the live birth rate per transferred embryo. Secondary outcomes were cleavage-stage embryo or blastocyst cryo-survival rate after warming, miscarriage rate per clinical pregnancy (i.e. the visualization of at least one gestational sac with foetal heartbeat) and rate of major malformations among newborns (defined as abnormalities that have medical, surgical or cosmetic significance according to [22]). Data were extracted independently by two reviewers (S.C., D.C.), and discrepancies were resolved by discussion with the most experienced authors (A.C., R.M.). Publication bias of the primary outcome was evaluated analysing the funnel plots both visually and formally with the trim and fill

method [23] and the Egger test [24]. These evaluations were performed using ProMeta 3.0 software.

Data extraction

Data were extracted independently by two reviewers (S.C., D.C,) using predefined data fields and study quality indicators. In detail, we developed a data extraction sheet based on the Cochrane data extraction template for non-RCTs (https://dplp.cochrane.org/data-extraction-forms). Disagreements and discrepancies were resolved by discussion with the senior authors (A.C., R.M.). In case of missing data, the authors were contacted by email address.

Subgroup analysis

Subgroup analysis was carried out evaluating vitrified-warmed embryos at the blastocyst stage (euploid or untested) and at the cleavage stage (untested).

Statistical analysis

Statistical analysis was carried out using the RevMan software (The Nordic Cochrane Centre, The Cochrane Collaboration. Review Manager version 5.4). Data from prolonged cryopreserved embryos (> 12 months) versus cryopreserved embryos for less than or equal to 12 months were combined to obtain a pooled odds ratio. A more conservative approach using random effects model was adopted. Between-study heterogeneity was addressed using I^2 which represents the percentage of total variation in the estimated effect across studies. An I^2 value over 50% indicates substantial heterogeneity. p-values < 0.05 were considered statistically significant.

Results

Study selection and characteristics

A total of 2,123 articles were identified using databases (Fig. 1). Duplications were removed by Endnote Online and manually ($n = 734$). Abstracts and titles ($N = 1389$) were reviewed by four authors (S.C., A.G., A.T., F.G.). Disagreements were resolved by discussion with two additional authors (D.C., R.M.) Twenty-two full-text articles were assessed for eligibility, but 17 of them were excluded because they did not fulfil the inclusion criteria (Fig. 1). Overall, five studies were finally included ($N = 18,047$ embryos). The characteristics of the studies are shown in Table 1. The risk of bias within studies is reported in Table 1. The group of prolonged cryo-storage (> 12 months) included 3389 embryos. The subgroup of untested vitrified cleavage stage embryos included 1739 embryos, while the subgroups of untested and euploid vitrified blastocysts included 13,596 and 2712 embryos, respectively.

Survival rate per warmed embryo

Survival rate was reported in all included studies ($N = 18,047$ embryos) [25–29]. The overall odds ratio did not show any significant differences between study groups (Fig. 2). Significant difference was observed only in the subgroup analysis that included untested blastocysts (OR 0.67, 95% CI 0.47–0.97, $p = 0.03$, $I^2 = 78\%$; Fig. 2).

Live birth rate per transferred vitrified-warmed embryo

Live birth rate was assessed in all studies ($N = 16,658$ transferred embryos) [25–29]. The overall odds ratio did not reveal any differences between study groups (Fig. 3). No differences were observed in the subgroup analysis including blastocyst and cleavage stages embryos (Fig. 3).

Miscarriage rate per clinical pregnancy

Miscarriage rate was assessed in all studies ($N = 7062$ clinical pregnancies) [25–29]. The overall odds ratio did not reveal any differences between study groups (Fig. 4). No differences were observed in the subgroup analysis including blastocyst and cleavage stages embryos (Fig. 4).

Major malformation rate per newborn

Major malformations were assessed in all studies ($N = 5574$ newborns) [25–29]. The overall odds ratio did not reveal any differences between study groups (Fig. 5). No differences were observed in the subgroup analysis including blastocyst and cleavage stages embryos (Fig. 5).

Risk of bias across studies

The risk of a significant bias across studies regarding the primary outcome was excluded by Egger's test ($p = 0.912$) and confirmed by the trim and fill method (Supplementary Fig. 1).

Discussion

Cryopreservation techniques are routinely adopted in IVF clinics nowadays to store reproductive specimens for long time periods until future use. Although long-term cryopreservation is thought to pause cell metabolism and ageing, conflicting results are now available as some authors

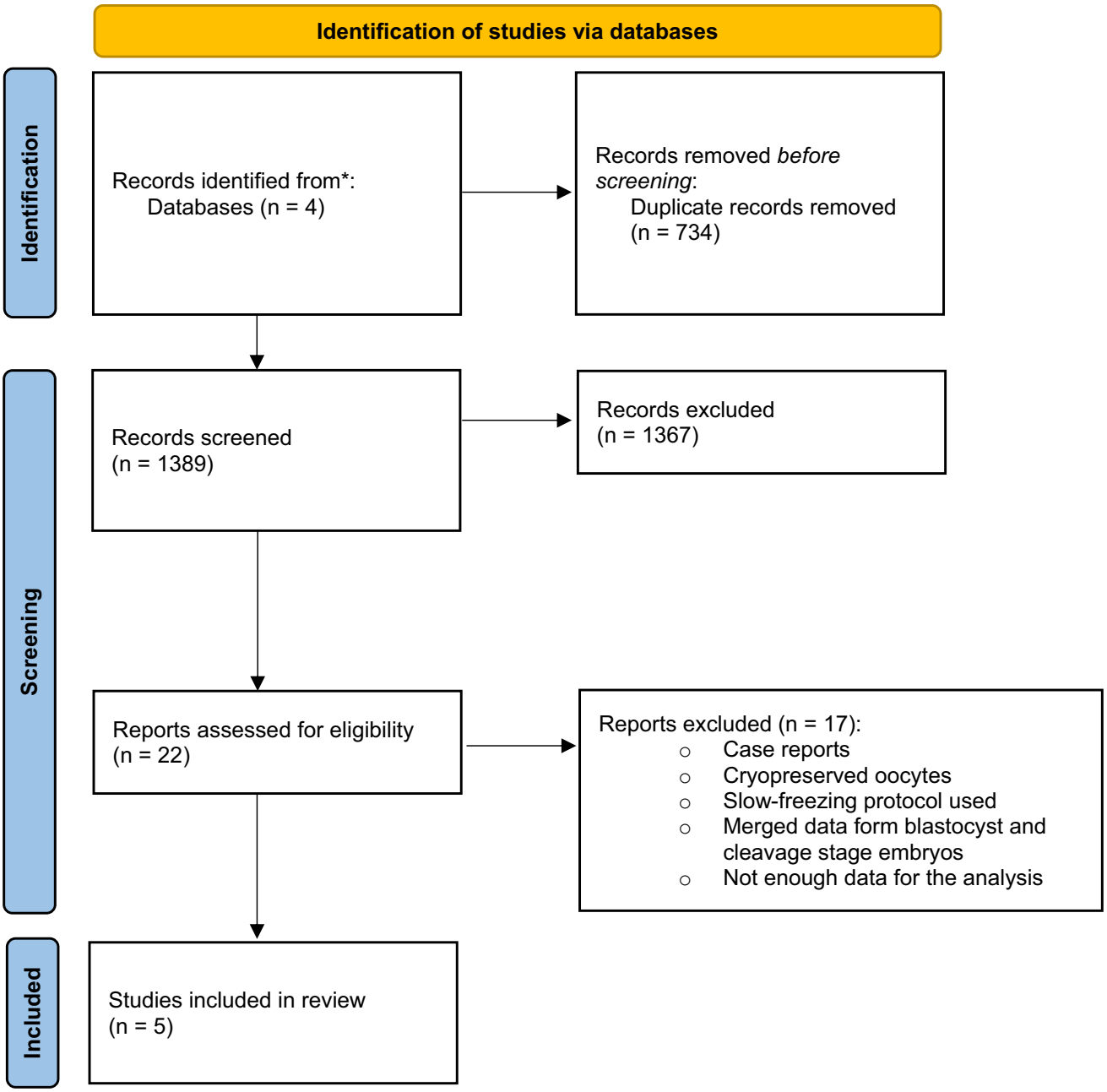

Fig. 1 Flow chart. Adapted from Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. BMJ 2021;372:n71. https://doi.org/10.1136/bmj.n71

questioned a putative harmful effect deriving from prolonged cryo-storage on oocyte and embryo competence [30]. In addition, the toxic effects of a prolonged exposure to cryoprotectant agents or a potential contamination of liquid nitrogen may have a detrimental impact in the long run [31, 32]. We can also speculate that other factors such as temperature fluctuations due to frequently opening the cryotank may have an impact. Nevertheless, many case reports have been published across the years showing successful pregnancies and healthy live births from either oocytes or embryos stored 3–14 years before [33–37]. Indeed, human embryos cryopreserved for 18 years were shown to maintain the same pluripotency as fresh embryos [38].

Recently, Ma et al. conducted a dose–response meta-analysis investigating the putative association between the duration of cryo-storage and pregnancy outcome [19]. They reported no impact of long-term storage lasting up to 8 years, comparing the lowest versus the highest category of storage time per each outcome in each study. To complement their investigation, reinforce the current level of

Table 1 List and characteristics of the 5 papers included in the meta-analysis. *NOS* Newscastle-Ottawa Score

Reference	Design	Clinical policy	Warmed embryos	Mean maternal age (yr)	Data adjusted for confounders	Clusters of cryo-storage duration (months)	NOS
Cimadomo (2021)	Retrospective	Euploid vitrified blastocysts	2712	38	Yes	≤2, 2–3, 4–6, 7–12, 13–24, 25–36, > 36	7
Ueno (2018)	Retrospective	Untested vitrified blastocysts	8736	38.2	No	0–2, 2–12, 13–97	7
Lee (2021)	Retrospective	Untested vitrified blastocysts	2868	35.8	Yes	0–6, 7–12, 13–24, ≥25	6
Wirleitner (2013)	Retrospective	Untested vitrified blastocysts	1992	36	No	0–3, 4–6, 7–12, 13–24, 25–36, 37–48, 49–72	6
Li (2017)	Retrospective	Untested vitrified cleavage-stage embryos	1739	31.2	No	1–3, 4–6, 7–12, 13–24, 25–60	5

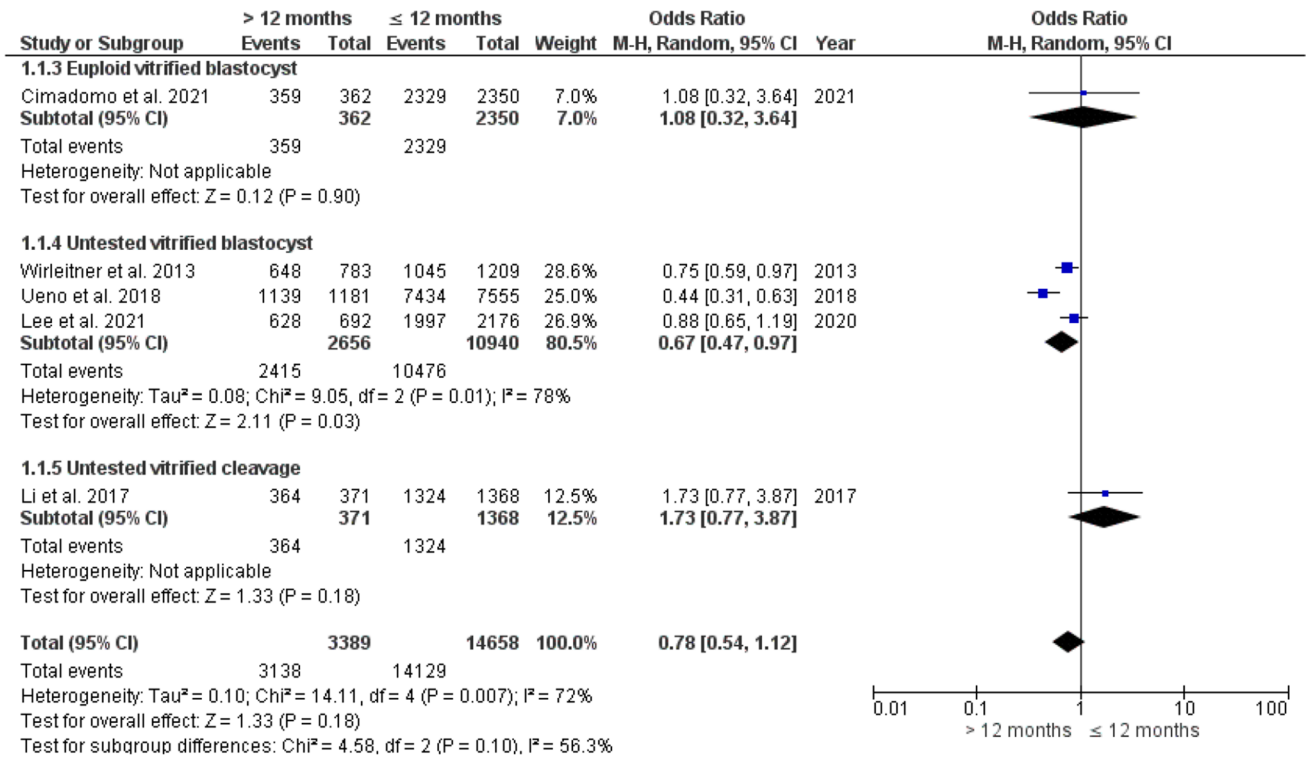

Fig. 2 Forest plot showing the effect of prolonged cryopreservation on cryo-survival rate per warmed embryo

evidence and improve the clinical utility of this information, we have conducted this systematic review and meta-analysis by identifying a clear and clinically reasonable cut-off (12 months) to define long-term cryo-storage, including only studies that adopted vitrification, and further clustering the results according to stage of embryo transfer (cleavage or blastocyst) and chromosomal competence of the transferred embryos (whether aneuploidy testing was performed or not). A comprehensive overview of the outcomes was conducted, which included cryo-survival, miscarriage, live birth and major malformation rates. After revision by four independent authors, 17 studies were excluded [33–37, 39–49] because (i) they were just case reports (some discussed above), (ii) they were focused only on oocytes, (iii) slow-freezing protocol was adopted, (iv) they reported merged data including both cleavage and blastocyst stage embryos and (v) it was not possible to obtain the full-text manuscript or to extract the data required for the analysis. Finally, five studies overall were selected in this meta-analysis [25–29]. We clustered the data from each study in two groups, namely prolonged cryo-storage (> 12 months) versus ≤ 12 months. Previously, Ma et al. [19] reported that cryo-storage duration up to 8 years did not influence pregnancy outcomes after frozen embryo transfer. In our view, this interesting information is poorly usable clinically though, since the embryos transferred beyond this time limit (i) are few in Ma's meta-data

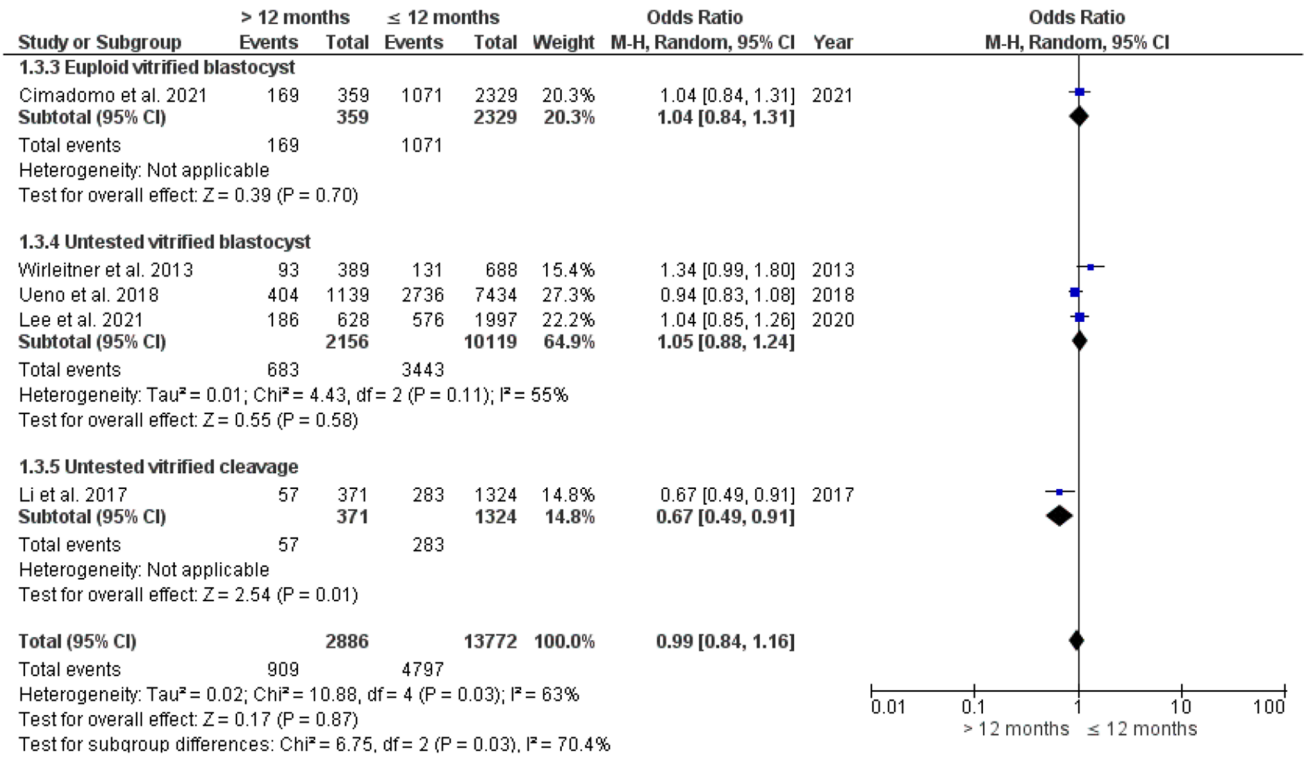

Fig. 3 Forest plot showing the effect of prolonged cryopreservation on live birth rate per transferred embryo

and, therefore, their claim might be under-powered, (ii) are most probably of a poor morphological quality and therefore intrinsically less competent and (iii) are hardly transferred during typical IVF cycles. On the contrary, our stricter 12 months cut-off is deemed more reasonable from a clinical perspective, since it applies to several instances like (i) supernumerary embryos left for transfer after several failed attempts in patients producing a large number of blastocysts, (ii) freeze-all cycles for patients requiring time-consuming clinical management before transfer (e.g. reproductive surgery, nutritional counselling and corrective actions, previous miscarriage or gestational/perinatal complications, other detailed gynaecological investigations), (iii) postponement of the pregnancy due to personal reasons (e.g. social, economic, psychological or familiar issues) and (iv) second attempts of conception after a delivery from a single cohort of embryos. At last, this design ensures a large sample size in both arms of the meta-analysis, thereby improving the statistical robustness of our conclusions.

We observed a significantly lower survival rate in untested vitrified-warmed blastocysts for more than 1 year (90.9% vs. 95.8%, OR 0.67, 95%CI 0.47–0.97, $p = 0.03$). While this outcome might be easily imputed to the fact that worse quality and slower blastocysts within each cohort are transferred later, some concerns about the consistency in defining "degeneration after warming" across different clinics can be raised. In fact, cryo-survival rates were largely variable between the 5 included studies, ranging 82.8–99.2%. With this regard, some guidance has been provided by the Alpha Consensus, where the experts state that blastocysts may undergo multiple morphological changes after warming, including collapse of the blastocoele, cellular lysis and partial degeneration, still being viable [50]. A skilled embryologist, via visual examination of the extent and localization of cellular degeneration, shall allow the transfer of blastocysts with areas of degeneration variable between 0 and 20% [50] and should target 75% and 90% as competence and benchmark values of cryo-survival, respectively. Similarly, for cleavage stage embryos, ≥ 50% of the blastomeres should be intact, and the competence and benchmark values for cryo-survival are 55% and 70%, respectively. Of note, the evaluation should be conducted after 2 h from warming, but the studies included here showed a large heterogeneity also in the timing of observation. Perhaps, a more standardized protocol for embryo morphological assessment after warming would be useful in order to limit the subjective definition of this critical outcome across embryologists from different clinics. Moreover, a further intrinsic bias in the evaluation of this outcome is the following: different regulations exist across countries about the definition of an embryo that can be discarded based on its morphological assessment. Specifically, in some countries like Italy, any "viable" embryo shall be either transferred or cryopreserved (Law 40/2004), while in other countries the law is less restrictive, and it leaves

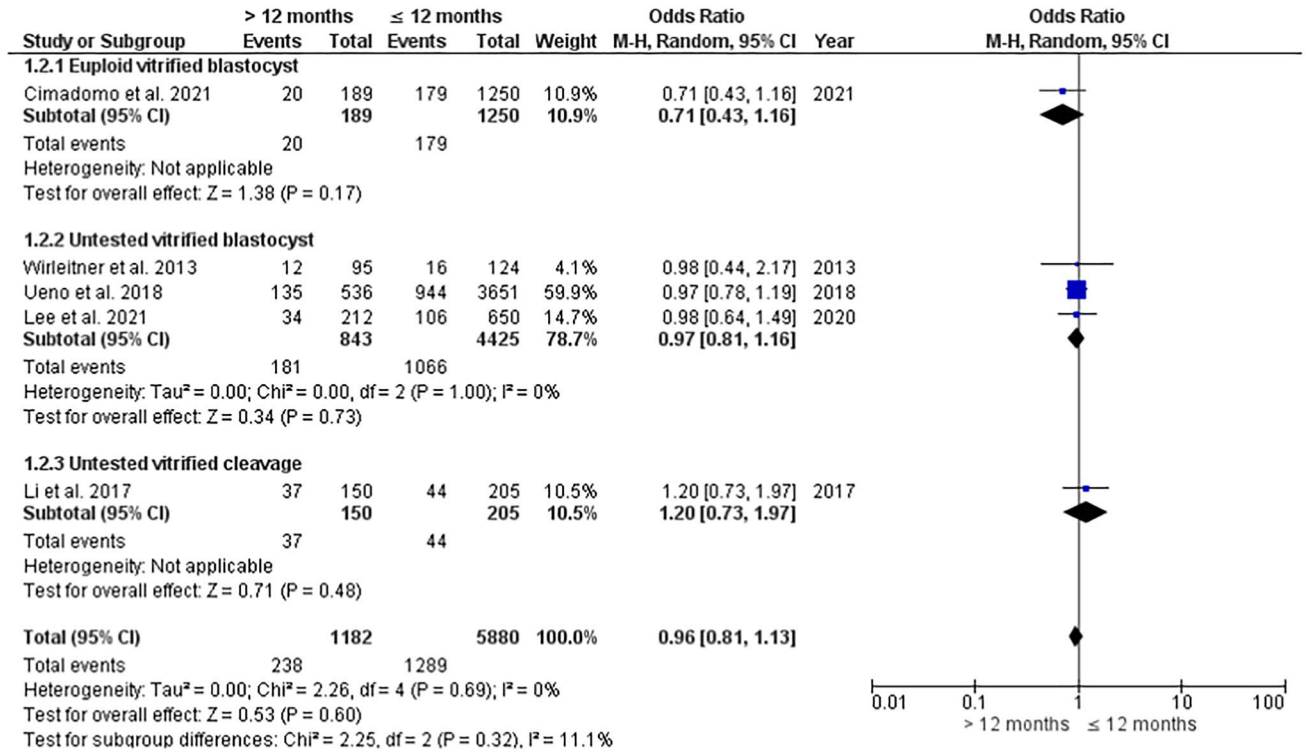

Fig. 4 Forest plot showing the effect of prolonged cryopreservation on miscarriage rate per clinical pregnancy

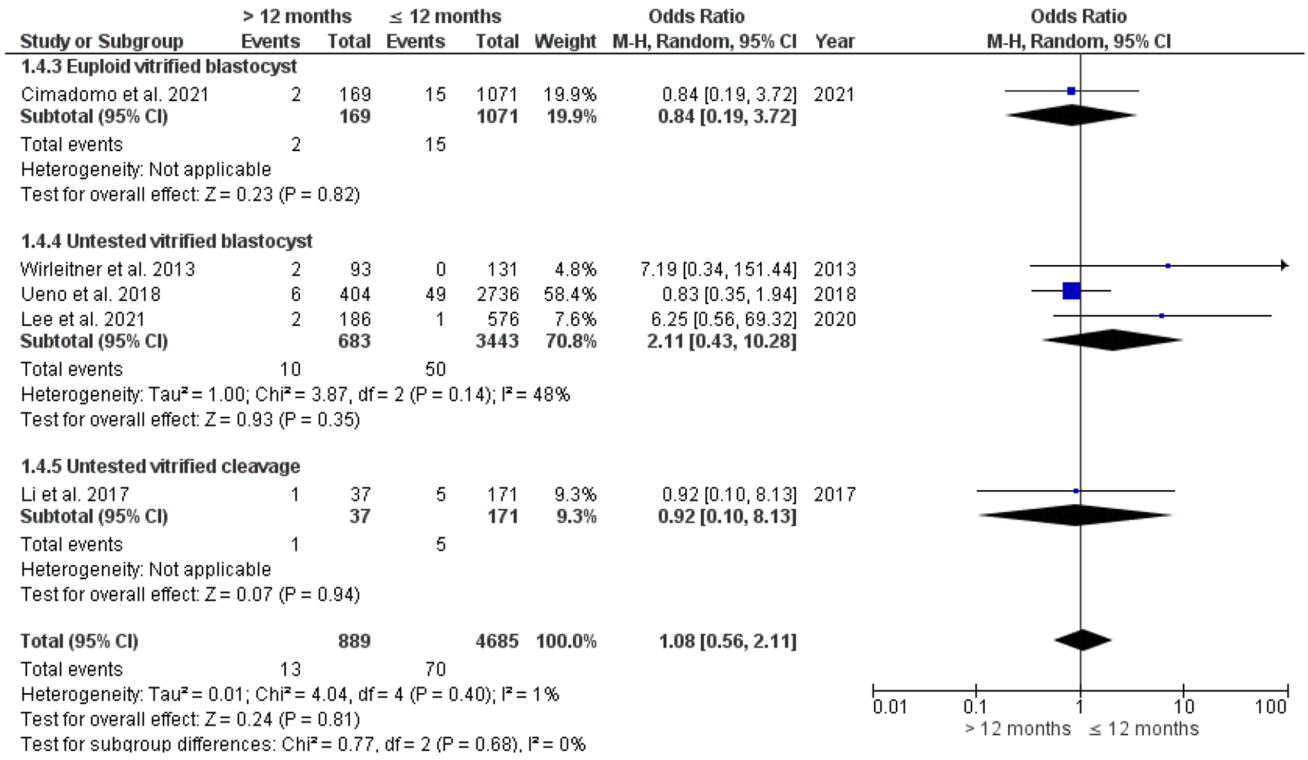

Fig. 5 Forest plot showing the effect of prolonged cryopreservation on major malformation rates per newborn

more room for embryologists' subjective evaluation of outcomes such as cryo-survival. In other terms, the definition of poor-quality embryos not eligible for transfer is extremely variable and subject to low inter-operator concordance [51], especially when these embryos show areas of degeneration or do not re-expand after warming [52].

Despite the slightly lower cryo-survival among untested vitrified-warmed blastocysts, all other outcomes (i.e. miscarriage, live birth and major malformations) were reassuringly similar in the two groups and in all sub-groups under analysis. Of note, two of the studies adjusted the results also for patients' and/or embryological confounders (e.g. female age at the time of oocyte retrieval and embryo transfer, embryo morphological quality, day of embryo development), thereby further strengthening the absence of consequences deriving from long-term storage on the main IVF outcomes. The prevalence of major malformations at birth reported across all studies was also comparable among the two study groups. Nevertheless, a long-term follow-up of the babies born would still be desirable.

The main limitation of the present review is that it is based on retrospective studies, which so far represent the only source of evidence published. In addition, we observed heterogeneity in terms of chromosomal status of the embryos (euploid and untested embryos) and of stage of preimplantation development (cleavage stage and blastocyst stage). To compensate for these limitations, we adopted a conservative approach using random effects model and carried out subgroup analyses considering the aforementioned features.

In our opinion, any information highlighting the safety of a clinical procedure so extensively and widely used worldwide such as cryopreservation, for which the level of evidence is still rather limited (especially with respect to "long-term" cryo-storage), is worth being published. Our meta-analyses, as well as Ma's, are complementary in strengthening the concept that embryo long-term cryopreservation is safe, from a multicenter perspective, at different stages and with different protocols. This information is certainly key for the scientific community and IVF professionals, but most importantly for IVF couples, especially (i) good prognosis patients with surplus embryos, (ii) women seeking a second child from supernumerary embryos and (iii) women postponing their transfer for clinical or personal reasons.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10815-022-02405-3.

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Author contribution S.C., D.C., R.M. and G.F. conceived the study, drafted and edited the manuscript; A.C. performed the statistical analysis and contributed to the drafting and editing the manuscript; S.C., D.C., R.M., A.T., F.G. and A.G. conducted the study selection; G.G., A.V, A.R., C.A., F.M.U and L.R. contributed to the final interpretation of data and editing of the manuscript. All authors gave their final approval.

Declarations

Competing interests The authors declare no competing interests.

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