Storage Time of Cryopreserved Embryos and Pregnancy Outcomes: A Dose-Response Meta-Analysis

Lagerdauer kryokonservierter Embryos und Schwangerschaftsausgang: eine Metaanalyse der Dosis-Wirkungs-Beziehung

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ABSTRACT

Purpose Cryopreservation techniques have become an essential part of assisted reproduction technology. Embryos may be cryopreserved for several years before transfer, and the safety of long-term cryopreservation needs to be considered. This dose-response meta-analysis was conducted to evaluate whether there were dose-response relationships between the storage time of cryopreserved embryos and pregnancy outcomes such as survival rate, implantation rate, miscarriage rate, clinical pregnancy rate, and congenital malformation rate.

Methods After searching the databases PubMed, Embase, MEDLINE, CCRT and related reviews up until June 4, 2020, seven studies were included for analysis. Two reviewers extracted the relevant information and independently assessed the study quality using the Newcastle-Ottawa scale. Potential linear or non-linear dose-response relationships were assessed with a random-effect dose-response meta-analysis.

Results No dose-response association was found between duration of embryo cryostorage and survival rate, implantation rate, miscarriage rate, clinical pregnancy rate or congenital malformation rate.

Conclusion The interval between the start of embryo cryopreservation and frozen/thawed embryo transfer does not influence pregnancy outcomes.

ZUSAMMENFASSUNG

Zielsetzung Kryokonservierungstechniken sind inzwischen zu einem wesentlichen Bestandteil der assistierten Reproduktionstechnologie geworden. Embryos können mehrere Jahre lang kryokonserviert werden, bevor sie transferiert werden. Das bedeutet, dass man sich auch wegen der Sicherheit der langfristigen Kryokonservierung Gedanken machen muss. Diese Metaanalyse wurde durchgeführt, um herauszufinden, ob es eine Dosis-Wirkungs-Beziehung zwischen der Lagerdauer von kryokonservierten Embryos und dem Schwangerschaftsausgang gibt, z. B. ob die Kryokonservierung sich auf die Überlebensrate, Implantationsrate, Fehlgeburtsrate, klinische Schwangerschaftsrate und angeborene Fehlbildungsrate auswirkt.

Methoden Es wurde eine Datenbanksuche von PubMed, Embase, MEDLINE und CCRT bis zum 4. Juni 2020 durchgeführt, und verschiedene Überblicksdarstellungen wurden zusätzlich überprüft. Insgesamt wurden 7 Studien in die Analyse aufgenommen. Die Daten wurden von 2 Wissenschaftlern extrahiert, die die Daten unabhängig voneinander unter Zuhilfenahme der Newcastle-Ottawa-Skala auswerteten. Potenzielle lineare bzw. nicht lineare Dosis-Wirkungs-Beziehungen wurden mithilfe einer Zufallseffekt-Metaanalyse überprüft.

Ergebnisse Es gab keine Dosis-Wirkungs-Beziehung zwischen der Lagerdauer kryokonservierter Embryos und der Überlebensrate, Implantationsrate, Fehlgeburtsrate, klinischen Schwangerschaftsrate oder angeborenen Fehlbildungsrate.

Schlussfolgerung Die Zeitspanne zwischen der Kryokonservierung von Embryos und dem Transfer von eingefrorenen/aufgetauten Embryonen hat keine Auswirkung auf den Schwangerschaftsausgang.

Introduction

Since the first successful pregnancy following transfer of a cryopreserved embryo was reported in 1983 [1], cryopreservation techniques have become an essential part of assisted reproduction technology (ART). According to statistics from the European Society for Reproductive Medicine and Embryology (ESHRE), the number of frozen/thawed embryo transfer (FET) cycles carried out in Europe were 129 693 cycles in 2011, and the percentage of FET cycles out of the total number of cycles rose from 28 % in 2010 to 32 % in 2011. In many countries such as Switzerland, Finland, Netherland, Sweden, and Iceland, the percentages of FET cycles exceed 50 % [2].

Embryos are cryopreserved for many medical or social reasons, including to preserve supernumerary embryos, decrease the risk of ovarian hyperstimulation syndrome (OHSS), preserve the fertility of women who need ovarian resection, radiotherapy or chemotherapy, and for embryo donation programs. Cryopreservation has resulted in a global reduction in the number of multiple pregnancies as it reduces the number of embryos transferred in a single transfer. Societal pressures have also played a significant role in delaying childbearing, with cryopreservation now an available choice.

In recent years, with the sustainable increase in the number of IVF/ICSI cycles, the improvements in ovulation induction and laboratory techniques, as well as the emphasis on fertility preservation, the number of frozen embryos has increased, and many of them have been frozen for several years. Although embryos kept in long-term cryostorage can result in live births, the safety of long-term cryopreservation needs to be considered, as many manipulations could change cryostorage conditions such as liquid nitrogen levels or tank temperatures, thus damaging the viability of the embryos. In a mouse model, it was found that survival rate, fertilization rate and embryonic development of mouse oocytes were significantly affected by cryopreservation storage times [3]. However, several cohort studies [4 – 10] showed that long-term storage (< 8 years) of embryos had no negative effect on pregnancy outcomes. Live births after transferring embryos cryopreserved for more than 10 years were reported [11, 12]. Overall, how long frozen embryos can remain in storage and whether the storage time influences pregnancy outcomes remains controversial, and the dose-response relationship between the duration of time embryos remain in cryostorage and pregnancy outcomes has not been investigated.

A dose-response meta-analysis offers a potential solution to the aforementioned questions. It enables both linear and non-linear dose-response relationships to be evaluated by pooling multiple studies to create greater statistical power [11, 12]. Therefore, we carried out a dose-response meta-analysis to examine the relationship between duration of storage time and pregnancy outcomes.

Material and Methods

Inclusion and exclusion criteria

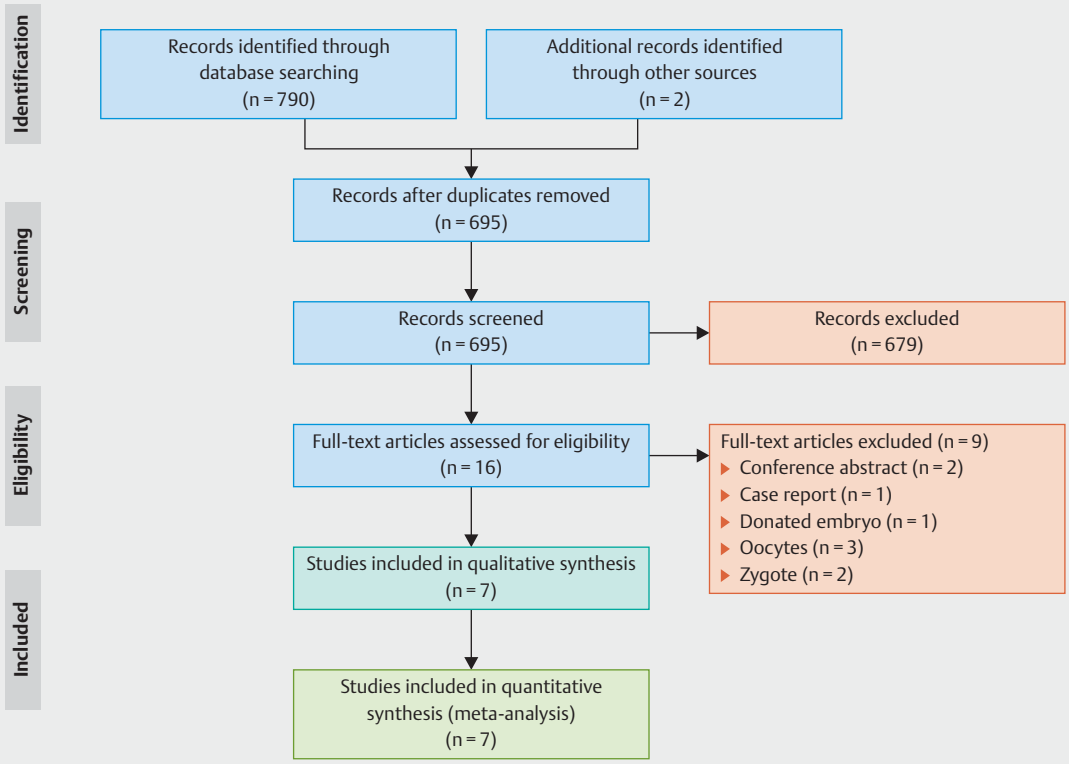
To be included for analysis, a study had to meet all of the following criteria:

1. It was a cohort study;
2. The population was human;
3. The study must look at the duration of storage time of cryopreserved embryos;
4. The embryo was not donated;
5. Investigated subjects were blastocysts or cleavage stage embryos.

Case-series, case-control studies, and abstracts were ruled out. Studies that presented insufficient data with regard to our prespecified outcome parameters were also excluded.

Search strategy and study selection

Electronic searches of PubMed, Embase, MEDLINE and the Cochrane Controlled Trials Register (CCTR) were carried out, covering the period from the start of the respective database up until June 4, 2020. Subjects had to be human, but there was no restriction with regard to region, publication type, or language (see Supplemental Table **S1** for the detailed search strategy). Reference lists of identified articles were also searched. The titles and abstracts of selected articles were independently analyzed by two of the authors to evaluate whether they complied with the inclusion criteria. If necessary, the full text of an article was carefully scanned. Any disagreements between reviewers were resolved by consensus. Studies that evaluated the association between storage time and pregnancy outcomes were included for meta-analysis.



▶ **Fig. 1** Study selection process for the meta-analysis.

Data extraction and quality assessment

Two reviewers independently extracted data from the full-text copies of all of the included studies, using a standardized form. Information obtained from the studies, including author, publication year, country where the study was conducted, study design, number of patients, duration of follow-up, developmental stage of embryo, cryopreservation technique, methods of fertilization, number of cases, details of storage time, etc., were analyzed. The same reviewers independently evaluated the quality of the studies using the Newcastle-Ottawa scale (NOS) [15]. Disagreements between reviewers were resolved by consensus.

Data synthesis and analysis

All statistical analyses were conducted using Review Manager (version 5.3, Cochrane Collaboration, Oxford, UK) and STATA (version 14.0, STATA Corp, College Station, TX, USA). The meta-analysis was performed in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-analysis (PRISMA) guidelines [16]. Endpoints of the analysis were pregnancy outcomes, including survival rate, implantation rate, clinical pregnancy rate, miscarriage rate, live birth rate and congenital malformation rate.

Dichotomous outcome data from individual trials were analyzed using odds ratio (OR) or risk difference (RD). The 95% confidence intervals (CI) were also computed for individual trials [17]. A random-effects model was utilized. We used "month" as the unit of storage time since it was reported by most of the included studies. When a study only reported the range of storage times, we used the average values of the lower and upper limits of the category. When the highest category was open ended, its value was calculated as 1.2 times the lower limit. When the lowest category was open ended, its value was calculated as the average of the upper limit and 0.

We considered the lowest category of storage time as the reference category and calculated the OR or RD and the respective 95% CI using a random-effects model weighted with the Mantel-Haenszel method [18]. We also examined cryopreservation technique-specific effects by conducting separate meta-analyses for both vitrification and the slow-freezing rapid-thawing method. We only carried out a dose-response meta-analysis when it had statistical significance. For a dose-response meta-analysis, we first explored linear trends between storage time and outcome using the method described by Greenland and Longnecker [13, 19]. Next, we estimated potential non-linear trends using restricted cubic splines with three knots in the dose-response regression model [13, 14].

Heterogeneity was analyzed by I^2 test. A value for I^2 of 0% indicated no observed heterogeneity, and higher values showed increased heterogeneity. $P < 0.05$ was considered statistically significant. A sensitivity analysis was done to test which study was the reason for the heterogeneity.

Results

Trial flow and study characteristics

After searching databases and reference lists, we initially identified 792 articles (▶ **Fig. 1**). After screening the titles and abstracts, 16 potentially eligible articles were retrieved for full-text screening. However, a further 9 articles were excluded (2 conference abstracts, 1 case report, 1 study which investigated donated embryos, and 5 other studies which looked at oocytes or zygotes). Therefore, only 7 articles were included in this analysis. Details on the articles included in our analysis are summarized in ▶ **Table 1**. Supplemental Table **S2** shows the calculated OR or RD for different storage times. All included studies have been published as full manuscripts, and most have been evaluated as moderate-quality studies, except one which was considered a high-quality study (see Supplemental Table **S2**).

One article [4] included all cycles with a single embryo transfer. Only two studies [4, 7] reported maternal age at the time of oocyte retrieval. One study [5] did not describe the methods of fertilization used, and the other two studies [4, 7] only focused on blastocysts. For the analyses relating to specific cryopreservation techniques, four studies reported using vitrification and three studies reported using slow-freezing. All results are shown in ▶ **Fig. 2**.

Meta-analysis

Survival rate

Four studies assessed the survival rate. The pooled OR of the survival rate for the highest versus the lowest category of storage time was 0.74 (95% CI [0.44, 1.23]) (▶ **Fig. 2a**) with significant heterogeneity between studies ($I^2 = 76\%$, $p = 0.006$), suggesting no association between storage time and survival rate. The results of the cryopreservation technique-specific analysis showed a pooled OR for vitrification and slow-freezing of 0.67 (95% CI [0.33, 1.37]) and 0.92 (95% CI [0.72, 1.18]), respectively, which indicated no association between storage time and survival rate for either vitrification or slow-freezing.

Implantation rate

The implantation rate was reported in five studies. Pooling the results showed no significant differences in implantation rates (OR 1.05, 95% CI [0.78, 1.42]) (▶ **Fig. 2b**) and no significant heterogeneity ($I^2 = 36\%$, $p = 0.18$). Cryopreservation technique-specific analysis showed a pooled OR of 0.84 (95% CI [0.57, 1.24]) and 1.24 (95% CI [0.90, 1.72]) for vitrification and slow-freezing, re-

▶ **Table 1** Summary of studies included in the dose-response meta-analysis of storage time and pregnancy outcomes.

Study (year)	Region	Design	Number of patients/ thaw cycles	Duration	Developmental stage of embryo	Cryopreservation technique	Methods of fertilization	Pregnancy outcomes	Storage time frame	Average storage time	NOS scores
Ueno 2018	Japan	retrospective	7409/8736	Jan 2007 to Dec 2015	blastocyst	vitrification	IVF	SR, cPR, MR, LBR, CMR	0–2 months; 2–13 months; 13–97 months	NA	7
Li 2017	China	retrospective	735/786	Jan 2013 to Oct 2013	cleavage-stage embryos	vitrification	NA	SR, IR, cPR, MR, LBR, CMR	1–3 months; 4–6 months; 7–12 months; 13–24 months; 25–60 months	NA	5
Liu 2014	China	retrospective	NA/867	Jan 2005 to March 2012	cleavage-stage embryos	slow freezing	IVF	SR, IR, cPR, MR, LBR	12–23 months; 24–35 months; 36–47 months; > 48 months	NA	5
Wirleitner 2013	Austria	retrospective	NA/603	Jan 2009 to Apr 2012	blastocysts	vitrification	IVF, ISCI IMSI	SR, IR, cPR, MR, LBR, CMR	0–3 months; 3–6 months; 6–12 months; 12–24 months; 24–36 months; 36–48 months; 47–72 months	446 days	6
Aflatoonian 2013	Iran	retrospective	651/651	Jan 2009 to Jan 2012	cleavage-stage embryos	vitrification	IVF, ISCI	IR, cPR	< 90 days; 90–365 days; 365–730 days; 730–1095 days; > 1095 days	296.72 ± 301.82 days	5
Ashrafi 2011	Iran	retrospective	222/247	March 2006 to March 2008	cleavage-stage embryos	slow freezing	IVF, ISCI	IR, cPR	≤ 180 days; > 180 days	170 (53–1671) days	5
Riggs 2010	USA	retrospective	NA/537	Nov 1986 to Feb 2007	cleavage-stage embryos	slow freezing	IVF	LBR	30–100 days; 101–365 days; 366–730 days; 731–1095 days; > 1095 days	346 ± 492 days	5

IVF = in vitro fertilization, ICSI = intracytoplasmic sperm injection, IMSI = intracytoplasmic morphologically selected injection, SR = survival rate, IR = implantation rate, cPR = clinical pregnancy rate, MR = miscarriage rate, LBR = live birth rate, CMR = congenital malformation rate

spectively. There was no association between storage time and implantation rate.

Clinical pregnancy rate

Six studies reported the clinical pregnancy rate. No significant difference in the clinical pregnancy rate was found (OR 0.94, 95% CI [0.83, 1.07]) (▶ **Fig. 2c**) and there was no significant heterogeneity between studies ($I^2 = 1\%$, $p = 0.41$). Cryopreservation technique-specific analysis showed a pooled OR of 0.91 (95% CI [0.80, 1.03]) for vitrification and 1.22 (95% CI [0.85, 1.75]) for slow-freezing, respectively. No association was found between storage time and clinical pregnancy rate.

Miscarriage rate

The miscarriage rate was calculated for four studies. The pooled results revealed no significant differences in miscarriage rates for the highest compared to the lowest category of storage time (OR 1.05, 95% CI [0.85, 1.29]) (▶ **Fig. 2d**) and no significant heterogeneity ($I^2 = 0.0\%$, $p = 0.46$). After carrying out cryopreservation technique-specific analysis, we got a pooled OR of 1.20 (95% CI [0.74, 1.96]) for vitrification and 1.11 (95% CI [0.38, 3.26]) for slow-freezing, respectively, showing no association between storage time and miscarriage rate.

Live birth rate

We performed a meta-analysis of the live birth rates of five trials. The pooled OR of the live birth rate of the highest compared to the lowest category of storage time was 0.99 (95% CI [0.78, 1.25]) (▶ **Fig. 2e**) with no significant heterogeneity ($I^2 = 29\%$, $p = 0.23$). After carrying out cryopreservation technique-specific analysis, the pooled OR was 0.90 (95% CI [0.79, 1.03]) for vitrification and 1.37 (95% CI [0.76, 2.46]) for slow-freezing, respectively. The results showed no association between storage time and live birth rate.

Congenital malformation rate

Congenital malformation rates were reported in three studies using vitrification. No significant differences were found (RD −0.00, 95% CI [−0.02, 0.01]) (▶ **Fig. 2f**) and there was no significant heterogeneity between studies ($I^2 = 0\%$, $p = 0.70$). The results showed no association between storage time and congenital malformation rate.

Dose-response analysis

The results of the highest versus the lowest category of storage time showed no significant differences for all pregnancy outcomes. It suggests that there is no linear or non-linear association between the duration of embryo cryostorage and pregnancy outcomes.

Sensitivity analysis and publication bias

Six of the seven cohorts were of moderate quality, and one was considered high quality according to the criteria of the NOS. All were therefore included in the sensitivity analysis which showed no significant changes in any of the outcomes, except for the survival rate. After eliminating one study, the survival rate was found to be significantly lower for the highest category of storage time following vitrification [10].

We did not construct funnel plots for publication bias or perform meta-regression analyses because of the limited number of studies included in this meta-analysis.

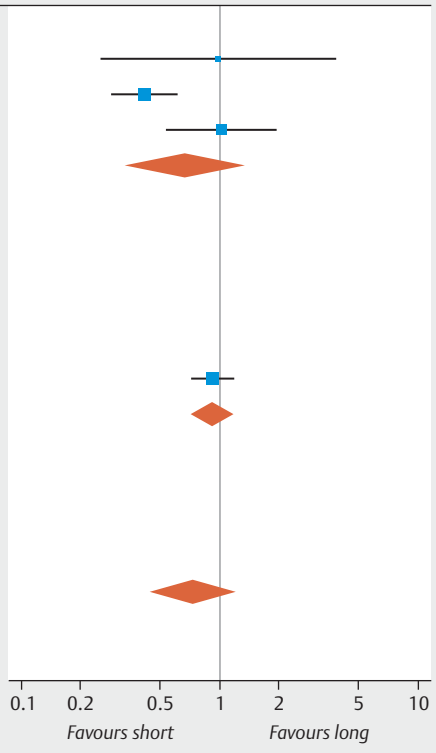
Discussion

Embryos which have been cryopreserved for several years may still result in live births after transfer. However, there is an issue with the safety of long-term storage. To the best of our knowledge, this is the first dose-response meta-analysis investigating an association between the duration of cryostorage and pregnancy outcomes. We found that a long storage time (less than 8 years) did not influence pregnancy outcomes of FET cycles.

Our group has previously reported on live births achieved with a cohort of human embryos which were cryopreserved for more than 12 years [11]. Another study showed that human embryos cryopreserved for 18 years maintained their pluripotency similar to fresh embryos and were not adversely affected by the long duration of cryopreservation [20].

During long-term storage, the viability of embryos may be influenced, mainly by temperature fluctuations and radiation. Pogozhykh et al. [21] investigated the impact of temperature fluctuations on frozen stored placental multipotent stromal cells to simulate repeated temperature fluctuations in biobanking or interruptions to the cold chain due to transportation and stocking events. They found that the quantity, viability, and metabolic parameters of these cells were influenced by both the number of cycles with temperature fluctuations and the fluctuation range. The increasing number of apoptotic changes was only related to the number of cycles of temperature fluctuations. However, the differentiation potential of these cells was not significantly compromised. Gamma radiation is a common research tool used to explore the impact of cryostorage times. There was no detrimental effect on the morphological appearance, development into morulae and blastocysts, implantation rate, or live birth rates when mouse embryos were exposed up to 200 cGy radiation, which is equal to 2000 years of background radiation [22]. However, problems related to development, i.e., increased DNA damage, decreased body length, increased mortality rates, and increased number of morphological deformities, were observed in zebrafish embryo exposed to 1 Gy gamma radiation [23].

Most individual studies included in this study showed no association between storage time and pregnancy outcomes, which is in accordance with our results. Only one study showed a significant decrease in survival rates with increasing duration of cryostorage. However, using multivariable logistic regression analysis, Ueno et al. found that only the time from insemination to vitrification correlated with survival rates but not the duration of cryostorage [4]. In contrast, a recently published study, which investigated the effect of the storage time of embryos on pregnancy outcomes in 24 698 patients following the first embryo transfer cycles, found the rates of implantation, clinical pregnancy, multiple pregnancy, and live births decreased with prolonged storage time, although no significant difference in survival rates was found between the different groups [24]. Although the sample



Study or subgroup	Long Events	Long Total	Short Events	Short Total	Weight	Odds ratio M-H, random, 95% CI
2.1.1 Vitrification						
Li 2017	119	122	283	290	10.2%	0.98 (0.25, 3.86)
Ueno 2018	1139	1181	4631	4702	31.2%	0.42 (0.28, 0.61)
Wirleitner 2013	69	83	283	341	23.8%	1.01 (0.53, 1.92)
Subtotal (95% CI)		**1386**		**5333**	**65.2%**	**0.67 (0.33, 1.37)**
Total events	1327		5197			
Heterogeneity: $\tau^2 = 0.25$; $\chi^2 = 6.21$, df = 2 (p = 0.04); $I^2 = 68\%$						
Test for overall effect: Z = 1.10 (p = 0.27)						
2.1.2 Slow-freezing						
Liu 2014	308	421	1042	1393	34.8%	0.92 (0.72, 1.18)
Subtotal (95% CI)		**421**		**1393**	**34.8%**	**0.92 (0.72, 1.18)**
Total events	308		1042			
Heterogeneity: not applicable						
Test for overall effect: Z = 0.68 (p = 0.50)						
Total (95% CI)		**1807**		**6726**	**100.0%**	**0.74 (0.44, 1.23)**
Total events	1635		6239			
Heterogeneity: $\tau^2 = 0.18$; $\chi^2 = 12.53$, df = 3 (p = 0.006); $I^2 = 76\%$						
Test for overall effect: Z = 1.16 (p = 0.25)						
Test for subgroup differences: $\chi^2 = 0.67$, df = 1 (p = 0.41); $I^2 = 0\%$						

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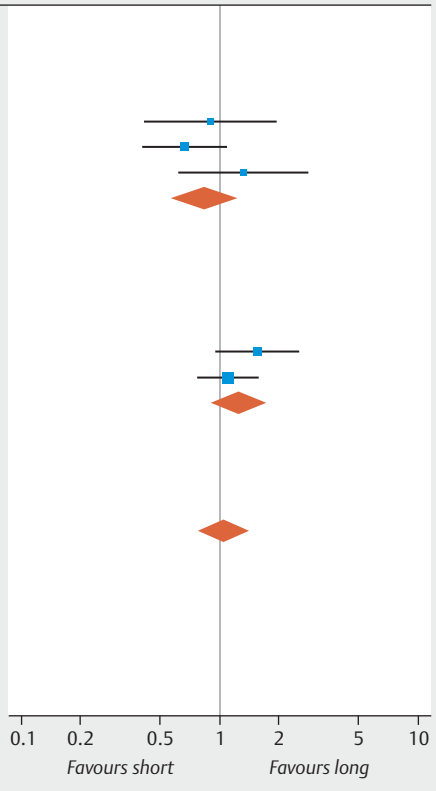

Study or subgroup	Long Events	Long Total	Short Events	Short Total	Weight	Odds ratio M-H, random, 95% CI
2.2.1 Vitrification						
Aflatoonian 2013	9	48	73	355	11.9%	0.89 (0.41, 1.92)
Li 2017	28	117	91	282	22.2%	0.66 (0.40, 1.08)
Wirleitner 2013	12	40	46	186	12.3%	1.30 (0.61, 2.77)
Subtotal (95% CI)		**205**		**823**	**46.4%**	**0.84 (0.57, 1.24)**
Total events	49		210			
Heterogeneity: $\tau^2 = 0.01$; $\chi^2 = 2.24$, df = 2 (p = 0.33); $I^2 = 11\%$						
Test for overall effect: Z = 0.88 (p = 0.38)						
2.2.2 Slow-freezing						
Ashrafi 2011	38	393	33	506	22.6%	1.53 (0.94, 2.50)
Liu 2014	54	230	137	624	31.0%	1.09 (0.76, 1.56)
Subtotal (95% CI)		**623**		**1130**	**53.6%**	**1.24 (0.90, 1.72)**
Total events	92		170			
Heterogeneity: $\tau^2 = 0.01$; $\chi^2 = 1.22$, df = 1 (p = 0.27); $I^2 = 18\%$						
Test for overall effect: Z = 1.31 (p = 0.19)						
Total (95% CI)		**828**		**1953**	**100.0%**	**1.05 (0.78, 1.42)**
Total events	141		380			
Heterogeneity: $\tau^2 = 0.04$; $\chi^2 = 6.28$, df = 4 (p = 0.18); $I^2 = 36\%$						
Test for overall effect: Z = 0.33 (p = 0.74)						
Test for subgroup differences: $\chi^2 = 2.29$, df = 1 (p = 0.13); $I^2 = 56.4\%$						

b

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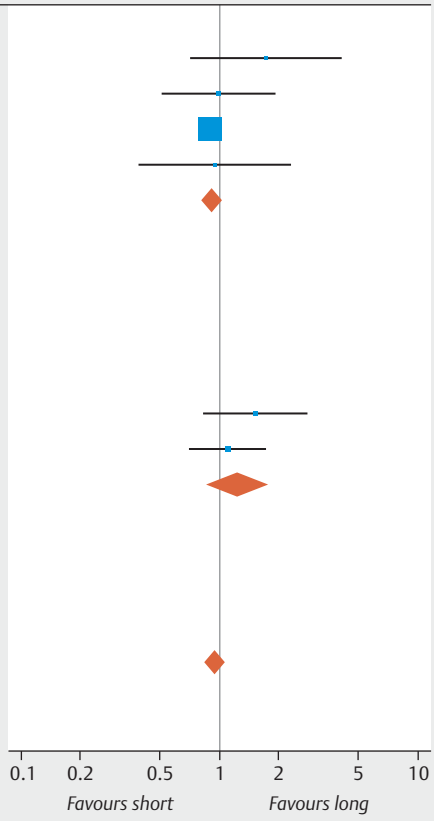
Study or subgroup	Long Events	Long Total	Short Events	Short Total	Weight	Odds ratio M-H, random, 95% CI
2.3.1 Vitrification						
Aflatoonian 2013	9	25	47	189	2.0%	1.70 (0.70, 4.10)
Li 2017	22	46	71	147	3.5%	0.98 (0.51, 1.90)
Ueno 2018	536	1139	2309	4630	80.8%	0.89 (0.78, 1.02)
Wirleitner 2013	10	26	40	100	2.0%	0.94 (0.39, 2.27)
Subtotal (95% CI)		**1236**		**5066**	**88.2%**	**0.91 (0.80, 1.03)**
Total events	577		2467			
Heterogeneity: $\tau^2 = 0.00$; $\chi^2 = 2.06$, df = 3 (p = 0.56); $I^2 = 0\%$						
Test for overall effect: Z = 1.50 (p = 0.13)						
2.3.2 Slow-freezing						
Ashrafi 2011	30	98	27	119	4.1%	1.50 (0.82, 2.76)
Liu 2014	43	102	142	354	7.6%	1.09 (0.70, 1.70)
Subtotal (95% CI)		**200**		**473**	**11.8%**	**1.22 (0.85, 1.75)**
Total events	73		169			
Heterogeneity: $\tau^2 = 0.00$; $\chi^2 = 0.71$, df = 1 (p = 0.40); $I^2 = 0\%$						
Test for overall effect: Z = 1.08 (p = 0.28)						
Total (95% CI)		**1436**		**5539**	**100.0%**	**0.94 (0.83, 1.07)**
Total events	650		2636			
Heterogeneity: $\tau^2 = 0.00$; $\chi^2 = 5.05$, df = 5 (p = 0.41); $I^2 = 1\%$						
Test for overall effect: Z = 0.94 (p = 0.35)						
Test for subgroup differences: $\chi^2 = 2.28$, df = 1 (p = 0.13); $I^2 = 56.1\%$						



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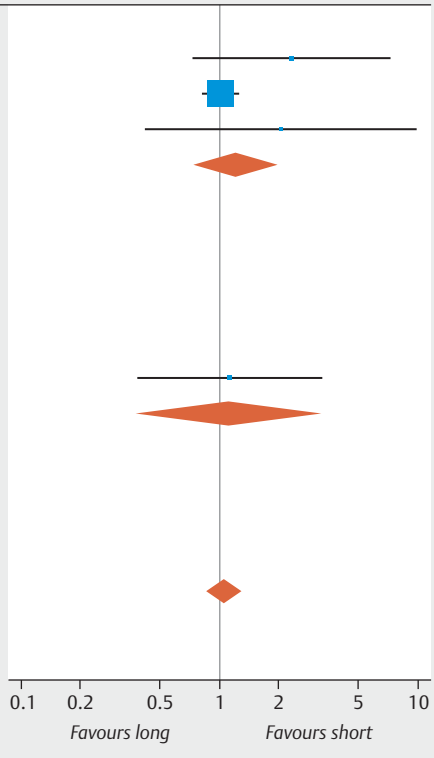
Study or subgroup	Long Events	Long Total	Short Events	Short Total	Weight	Odds ratio M-H, random, 95% CI
2.4.1 Vitrification						
Li 2017	6	22	10	71	3.2%	2.29 (0.72, 7.24)
Ueno 2018	135	536	580	2309	91.4%	1.00 (0.81, 1.25)
Wirleitner 2013	3	10	7	40	1.7%	2.02 (0.42, 9.80)
Subtotal (95% CI)		**568**		**2420**	**96.3%**	**1.20 (0.74, 1.96)**
Total events	144		597			
Heterogeneity: $\tau^2 = 0.07$; $\chi^2 = 2.58$, df = 2 (p = 0.28); $I^2 = 22\%$						
Test for overall effect: Z = 0.75 (p = 0.45)						
2.4.2 Slow-freezing						
Liu 2014	5	43	15	142	3.7%	1.11 (0.38, 3.26)
Subtotal (95% CI)		**43**		**142**	**3.7%**	**1.11 (0.38, 3.26)**
Total events	5		15			
Heterogeneity: not applicable						
Test for overall effect: Z = 0.20 (p = 0.84)						
Total (95% CI)		**611**		**2562**	**100.0%**	**1.05 (0.85, 1.29)**
Total events	149		612			
Heterogeneity: $\tau^2 = 0.00$; $\chi^2 = 2.59$, df = 3 (p = 0.46); $I^2 = 0\%$						
Test for overall effect: Z = 0.44 (p = 0.66)						
Test for subgroup differences: $\chi^2 = 0.02$, df = 1 (p = 0.90); $I^2 = 0\%$						



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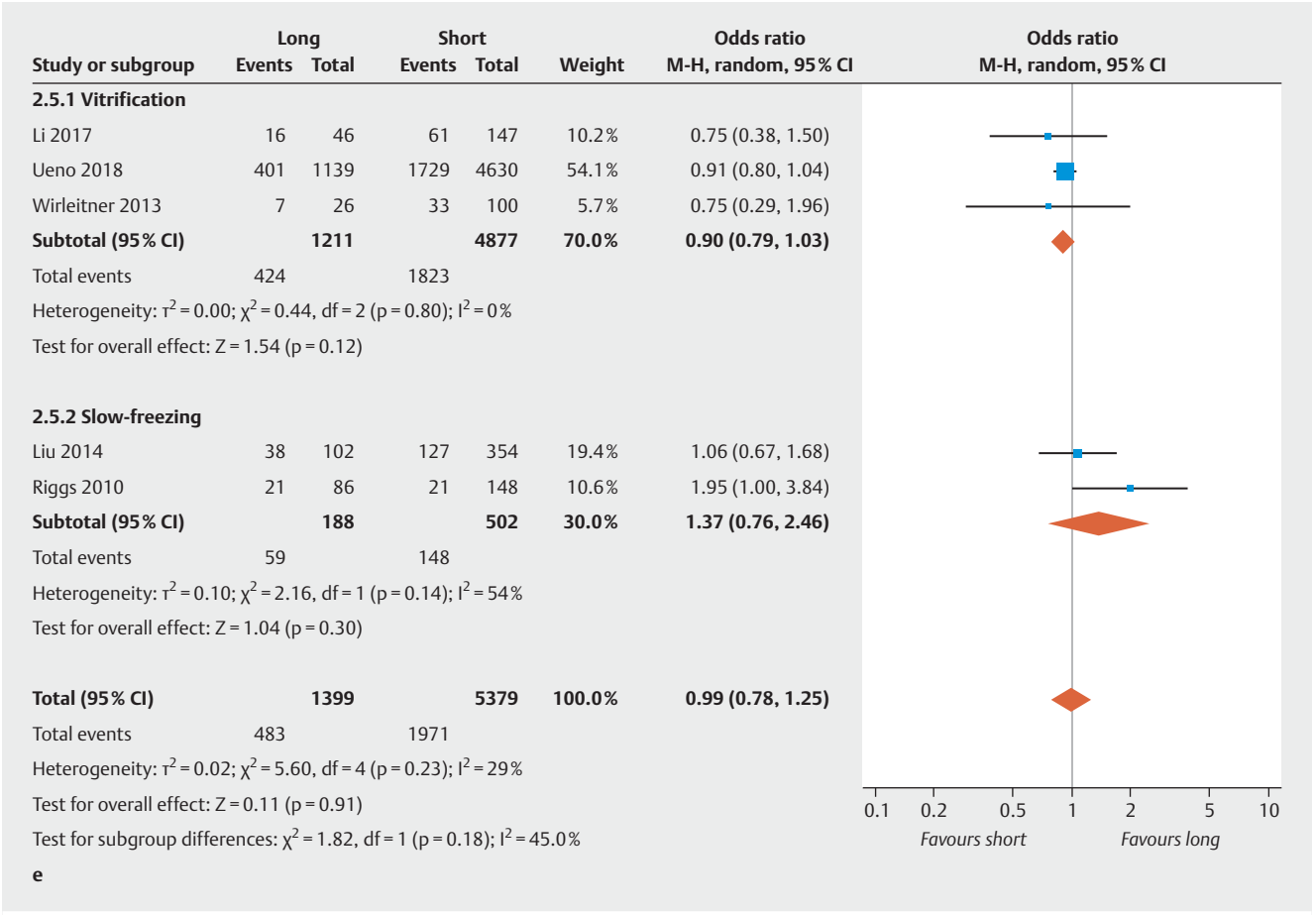

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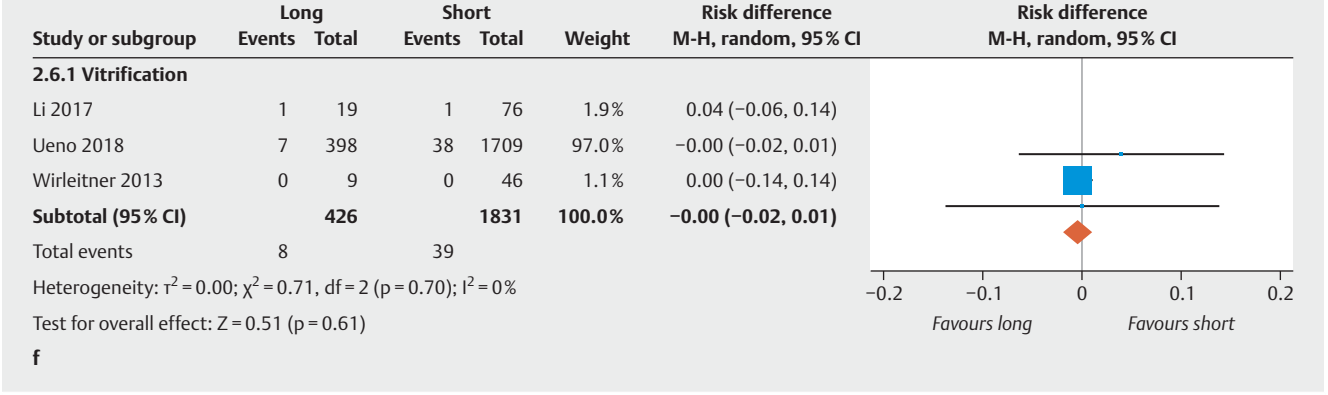

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▶ **Fig. 2** Summary of OR or RD of pregnancy outcomes, the highest vs. the lowest storage time category. **a** Survival rate. **b** Implantation rate. **c** Clinical pregnancy rate. **d** Miscarriage rate. **e** Live birth rate. **f** Congenital malformation rate.

size of this study is the largest to date, the storage time-frame was one of the shortest compared to the studies we included in our meta-analysis. All embryos were cryopreserved by vitrification, and most embryos (92.8%) were in the cleavage stage when they were transferred. Surprisingly, the clinical pregnancy rate and live birth rate were significant lower in the group with times of more than 3 months compared with the group with times of less than 3 months. This result challenges the principles of cryobiology and should be treated with caution. We speculate that other factors such as temperature fluctuations due to frequently opening the liquid tank may have had an impact in addition to the 3-month storage time.

There are many potential confounders, such as female age at the time of oocyte retrieval, female BMI, infertility type, infertile years, causes of infertility, parity, embryo quality, stage of embryo development, and number of transferred embryos, which can influence the results. Although there is no question that female age at the time of oocyte retrieval is one of the major confounders

[25], it is still not known whether female age at the time of embryo transfer matters. However, we could not draw any conclusions since only two studies provided these data [4, 7]. This should be investigated in future studies.

In vitrification, embryos are cooled much more rapidly to prevent the formation of ice crystals and this is therefore presumed to cause less damage to embryos. But the high concentration of cryoprotectants used in vitrification might be cytotoxic and lead to osmotic shock [26]. The main problem with slow-freezing is the formation of intracellular ice, resulting in cell damage and developmental arrest [27]. Compared to vitrification, slow-freezing may cause additional damage to embryos which is not visible on microscopic examination but may adversely affect embryo viability [28]. We found survival rates, implantation rates, clinical pregnancy rates and live birth rates were higher for vitrification than for slow-freezing, which was also in accordance with the results of previous papers [26, 29]. As this technique significantly increases embryo survival rates compared to slow-freezing, it has led to an improvement in clinical outcomes of cryopreserved cycles and made fertility preservation an available option for patients. Vitrification is now considered to be the best choice to cryopreserve both human oocytes and embryos, due to its high survival rate [30]. It is worth mentioning that the implantation rate (OR 0.84, 95% CI [0.57, 1.24]), clinical pregnancy rate (OR 0.91, 95% CI [0.80, 1.03]) and live birth rate (OR 0.90, 95% CI [0.79, 1.03]) for long storage time groups all showed a decreasing trend for vitrification. Conversely, implantation rates (OR 1.24, 95% CI [0.90, 1.72]), clinical pregnancy rates (OR 1.22, 95% CI [0.85, 1.75]) and live birth rates (OR 1.37, 95% CI [0.76, 2.46]) in long storage time groups had a tendency to increase following slow-freezing. It is not clear why all three outcomes tended in opposite directions for vitrification and slow-freezing, although the differences between groups did not meet conventional levels of statistical significance. There are several points we need to consider. First, only limited data on patients' characteristics were available. For example, female age at the time of oocyte retrieval and embryo transfer are important confounders. However, few studies reported female age. Another important issue was the reason why patients delayed the timing of FET. A reasonable explanation was that female patients became pregnant in a previous embryo transfer cycle. Therefore, they delayed the timing of the next FET. They might have been expecting a better prognosis when they underwent FET in the next pregnancy. In addition, our analysis only included seven studies. If the number of studies had been higher, the results might be different.

Although storage time did not influence pregnancy outcomes in our study, in clinical practice, manipulations, such as repeated opening of the cryo-tank, transportation of specimens or laboratory procedures over time, may influence pregnancy outcomes. It was thought that annual cleaning and registering might decrease the frozen-thawed survival rate of sperm specimens with longer storage times (5–15 years) [31]. The composition of the preimplantation human embryo culture media and its stability during storage varied [32]. It is possible that the embryos stored for a long time were not the best, as embryologists would thaw the best embryos first. These outcomes should therefore be continuously monitored. Embryo cryopreservation is also a social problem, and issues such as the duration of cryopreservation, the number of cryopreserved embryos, the consent rules for storing embryos, etc., all need legislation [33, 34].

To the best of our knowledge, this is the first meta-analysis investigating the relationship between the duration of embryo cryostorage and pregnancy outcomes. We tried to include all suitable studies without restriction with regard to region, publication type, and language, and investigated the quantitative relation between embryo cryostorage duration and pregnancy outcomes using a dose-response meta-analysis. The current evidence supports the idea that cryopreservation does not affect the differentiation potential of embryos and offers women the opportunity to have their own healthy children in the future. Our meta-analysis had several limitations. Firstly, the meta-analysis was based on a small number of studies and should therefore be treated with caution. Secondly, the OR or RD and 95% CI were estimated using a random-effects model which led to information bias. Thirdly, the maximum storage time we evaluated was 96 months. Moreover, the developmental stage of embryos differed. Lastly but not least, our meta-analysis was conducted using summarized statistics rather than individual data. Acquiring and examining individual data would give a more accurate picture of the dose-response relationship and offer better control of potential residual confounders.

In conclusion, our dose-response meta-analysis showed that long-term storage of human cryopreserved embryos did not impact pregnancy outcomes.

Conflict of Interest

The authors declare that they have no conflict of interest.

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