The safety of long-term cryopreservation on slow-frozen early cleavage human embryos

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Abstract

Objective To evaluate the impact of cryopreservation storage time on cleavage-stage embryo survival rate, pregnancy rate, implantation rate, singleton birth weight, and live birth rate.

Methods This study was a retrospective analysis, including 867 thaw cycles and 3,367 embryos. Women who underwent IVF-FET cycles between 2005 and 2012 were analyzed. The patients were divided into four groups, as follows: group 1 (12–23 months); group 2 (24–35 months); group 3 (36–48 months); and group 4 (≥ 48 months).

Results The storage time did not have a significant effect on survival, damage rate of the blastomeres, implantation rate, pregnancy rate, singleton birth weight, and live birth rate for embryos frozen at cleavage stages.

Conclusion Storage time did not influence the survival and pregnancy outcomes of slow-frozen early cleavage human embryos. The developmental potential of cryopreserved human embryos with different storage times does not appear to have a negative influence on further development.

Capsule Presence of storage time did not influence the survival and pregnancy outcome of slowfrozen early cleavage human embryo.

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Survival · Singleton birth weight · Live birth rate

Introduction

Cryopreservation of surplus embryos is widely used in human-assisted reproduction because the embryos can be thawed in subsequent treatment cycles. Cryopreservation of surplus embryos is also a strategy currently established for preservation of female fertility in which a woman can undergo a cycle of IVF and create embryos for later use. The number and time cryopreserved embryos are in storage has increased. Nevertheless, we do not know the length of time cryopreserved embryos remain viable after long-term cryopreservation. Although theoretical models have speculated that a mammalian embryo can be stored for several thousand years [1–3], direct experimental confirmation of this estimate is still lacking. Testart et al. [4] reported that the survival rate of embryos and the pregnancy rate were reduced after several months of cryopreserving human embryos. Mozdarani & Moradi [5] showed that the viability of murine embryos is reduced after long-term cryopreservation. In contrast, some studies have reported no effects of storage time on embryonic survival or pregnancy outcomes [6–8]. Moreover, studies on the safety of cryopreservation on human embryos have been limited. Pal et al. [9] demonstrated a significant impact of post-thaw blastomere survival on the outcome of FET cycles. We know that embryos with ≥ 50 % surviving blastomeres (with clear cellular boundaries and no fragmentation) have a successful thaw in cleavage-stage frozen embryos [10]. Thus, a portion of surviving blastomeres is damaged. Some studies have shown that the duration of cryostorage does or does not affect post-thaw survival [4, 6–8]; studies on the blastomere damage rate are also limited.

Our primary aim was to evaluate the impact of cryopreservation storage on embryo survival, the damage rate of

surviving embryos, clinical pregnancy rate, birth weight, and live birth rate.

Materials and methods

Patients

The retrospective study was approved by the Ethics Committee of Peking University Third Hospital. Patients who underwent thaw cycles of cryopreserved embryos between January 2005 and March 2012 at the Reproductive Medical Centre of Peking University Third Hospital were analyzed.

Freezing protocol

A slow freezing protocol with 1,2-propanediol (PROH; Sigma Chemical Co., Sigma-Aldrich, St. Louis, MO, USA) as a cryoprotectant was used [11]. Freezing and thawing solutions consisted of cryoprotectant in phosphate-buffered saline supplemented with 20 % v/v human serum albumin (HSA; LifeGlobal, USA). The freezing procedure was performed at room temperature. Briefly, the embryos were first incubated in 1.5 mol/l PROH freezing solution for 15 min, and then moved to a final solution of 1.5 mol/l PROH+0.1 mol/l sucrose for 15 min and aspirated into cryostraws. Cooling was carried out in a programmable planar freezer (Kryo Ten Series; Planer Products, Sunbury-on Thames, UK) at a rate of $-3^{\circ}\text{C}/\text{min}$ to -7°C , at which point seeding was induced manually. Cooling was then

continued at rates of $-0.3^{\circ}\text{C}/\text{min}$ to -30°C and $-50^{\circ}\text{C}/\text{min}$ to -150°C before storage in liquid nitrogen until thawing.

Embryo thawing and transfer

A total of 3,367 embryos were thawed. Embryo storage time ranged from 12 to 119 months for all day 3 cleavage embryos. The embryos were divided into the following four groups based on the storage time (1–4): group 1, between 12 and 23 months ($n=360$); group 2, between 24 and 35 months ($n=291$); group 3, between 36 and 47 months ($n=112$); and group 4, > 48 months ($n=104$). Maternal age was not significantly different between the groups.

On the day of FET, the embryos were thawed rapidly by removing straws from storage, exposed to air for 40 s, and immersed in a water bath at 30°C for 1 min. Embryos were sequentially placed in 5-min baths at room temperature with decreasing PROH concentrations (1.0 mol/l, then 0.5 mol/l, and finally 0.0 mol/l), while the sucrose concentration was kept constant (0.2 mol/l) to remove the cryoprotectant. Thawed embryos were transferred to culture medium at 37°C . Day-3 (D3) cleavage embryos of only intermediate or high quality were cryopreserved (embryos that developed to the 4–8 cell stage or higher with a morphology score ≤ 3). Embryo grading followed the criteria introduced by Veeck [12]. Intact blastomere survival $\geq 50\%$ (with clear cellular boundaries and no fragmentation) identified a successful thaw in cleavage-stage frozen embryos. Embryos were transferred in natural cycles or estrogen-progesterone hormonally-supplemented cycles.

Table 1 Patients and cycles characteristics

Characteristic	Group1 ($n=360$)	Group2 ($n=291$)	Group3 ($n=112$)	Group4 ($n=104$)	P value
Age (years)	33.22 $\pm$ 3.75	33.27 $\pm$ 3.54	33.18 $\pm$ 3.34	34.01 $\pm$ 3.55	0.451
Natural cycles	250	188	82	87	
Artificial cycles	110	103	30	17	
Thaw cycles (n)	360	291	112	104	
Transfer cycles (n)	354 (98.33 %)	284 (97.59 %)	110 (98.21 %)	102 (98.07 %)	
Embryos thawed	1,393	1,132	421	421	
Survival embryos	1,042 (74.80 %)	875 (77.30 %)	330 (78.38 %)	308 (73.16 %)	0.407
Embryos transferred	624	610	237	230	
Body mass index	22.44 $\pm$ 2.92	23.07 $\pm$ 13.39	21.92 $\pm$ 2.09	22.46 $\pm$ 2.32	0.826
The cycle of three damaged types					
Type 0	159	122	42	38	
Type 1	110	89	34	31	
Type 2	91	80	36	35	

Data are presented as numbers (%) or mean $\pm$ SD

Type 0, no visualization of damaged blastomeres in embryos of one transfer cycle; Type 1, 1 damaged blastomere in embryos of one transfer cycle; Type 2, ≥ 2 damaged blastomeres in embryos of one transfer cycle

Table 2 The storage time and pregnancy outcomes

Storage time (month)	Group1	Group2	Group3	Group4	P value
Embryos transferred	2.63±0.56	2.60±0.59	2.56±0.60	2.58±0.60	0.513±0.56
Clinical pregnancy rate (%)	40.12	39.08	45.45	42.16	0.687
Live birth rate (%)	35.88	32.04	36.36	37.25	0.221
Implantation rate (%)	22.01	22.32	25.27	23.57	0.689
High-quality embryo rate (%)	70.74	72.04	68.38	65.20	0.405
Singletons birthweight (g)	3,261.84±633.29	3,386.92±502.59	3,239.66±526.17	3,458.62±335.16	0.664
Low birthweight (<2,500 g)	6 (7.69 %)	3 (4.61 %)	4 (13.33 %)	1 (3.44 %)	0.522
Gestational weeks	37.72±2.09	37.79±1.53	37.79±1.77	38.21±1.39	0.729

Data are presented as numbers (%) or mean ± SD

The effects of the time of cryopreservation on the birth rate of transferred embryos, and the birth weight were evaluated. Two or three embryos were transferred to patients.

The number of lysed blastomeres

Intact blastomere survival ≥ 50 % (with clear cellular boundaries and no fragmentation) identified a successful thaw in cleavage-stage frozen embryos. Blastomeres were considered to be damaged when they were lysed, degenerated or dark. Transferred embryos according to the number of damaged blastomeres after cryopreservation were divided into the following types (Table 1):

Type 0, no visualization of damaged blastomeres in embryos of one transfer cycle;

Type 1, 1 damaged blastomere in embryos of one transfer cycle;

Type 2, ≥ 2 damaged blastomeres in embryos of one transfer cycle.

Data collection

In the present study patients were excluded who received pre-implantation genetic diagnosis (PGD). Furthermore, only data

of live births after 20 weeks gestation were included in the data analysis.

Statistical analysis

Descriptive data are presented as the mean with 1 SD. The basic characteristics of patients were compared using Student's *t*-tests (continuous variables) and categorical variables were evaluated with chi-square tests. P values <0.05 was considered significant. Statistical analyses were performed using SPSS software (version 17.0; SPSS, Chicago, IL, USA).

Results

In order to make a valid comparison among the four groups, we reviewed the outcomes of 867 thaw cycles which met the inclusion criteria, including 354 transfer cycles (group 1), 384 transfer cycles (group 2), 110 transfer cycles (group 3), and 102 transfer cycles (group 4). There were 867 thaw cycles. A total of 3,367 embryos were thawed. Basic patient characteristics, including mean female age, viable embryos, and body mass index, did not differ among the four groups (Table 1). According to the grade of blastomere lysis among the four groups, approximately 70 % of the post-thaw embryos had ≤ 1

Table 3 Results of multiple regression analysis among live born

	Single birthweight (grams)				Gestational age at birth (weeks)			
	β	<i>t</i>	<i>P</i> -value	95%CI	β	<i>t</i>	<i>P</i> -value	95%CI
Maternal age	0.143	0.014	0.989	(−19.757–20.043)	−0.016	−0.505	0.614	(−0.079–0.047)
Maternal BMI	11.846	0.930	0.354	(−13.280–36.972)	−0.005	−0.097	0.179	(−0.013–0.0252)
storage time	36.283	1.089	0.278	(−29.463–102.029)	0.006	0.004	0.954	(−0.203–0.216)
type of cycle	8.779	0.014	0.839	(−76.284–93.842)	−0.195	−0.103	0.156	(−0.464–0.075)
No. of transfer embryos	105.837	1.513	0.132	(−32.152–243.826)	0.304	1.370	0.172	(−0.134–0.742)

β is the regression coefficient; 95 % CI is confidence interval

blastomere damaged, and approximately 30 % of post-thaw embryos had ≥ 2 blastomeres damaged.

The survival rates and the outcomes of embryo transfer are shown in Table 2. There was no difference between the four groups in terms of survival rates (79.58 %, 79.80 %, 82.40 %, and 77.38 %), implantation rates (22.01 %, 22.32 %, 25.27 %, and 23.57 %), clinical pregnancy rates (40.12 %, 39.08 %, 45.45 %, and 42.16 %), and live birth rates (35.88 %, 32.04 %, 36.36 %, and 37.25 %). Moreover, the singleton birth weights were $3,261.84 \pm 633.29$ g, $3,386.92 \pm 502.59$ g, $3,239.66 \pm 526.17$ g, and $3,458.62 \pm 335.16$ g in the four groups, respectively. No significant differences were observed.

Finally, multiple linear regression was used to determine the relationship between storage time and birth weight and gestational age in neonates with maternal age, BMI, number of transferred embryos, and type of cycle. We found the birth weight and gestational age were negative associated with storage time, maternal age, maternal BMI, type of cycle and number of transfer embryo in Table 3.

Discussion

In this analysis of 3,367 thawed embryos in 867 thaw cycles, storage time had no significant effect on thaw survival or pregnancy outcome. We also show that singleton birth weight is not affected by different storage times.

Earlier studies yielded contradictory results. Testart et al. [4] found a decrease in survival rate on embryos with longer storage time, but did not control for pre-frozen morphology. In agreement with our study, previous studies [6, 8] have also shown that the length of cryopreservation time had no significant effects on the survival or pregnancy outcomes of human embryos. Cohen et al. [6] reported that storage time did not have a significant effect on survival or clinical pregnancy rate, but did not provide live birth data. Riggs et al. [8] also found that storage time did not have a significant effect on blastomere survival, clinical pregnancy rate, and live birth rate, but birth weights were not provided.

Abbeel et al. [13] reported that fully intact embryos had a higher birth rate than damaged embryos [13]. In our study, storage time had no significant effect on thaw survival or pregnancy outcomes in the four groups. We found that approximately 70 % of post-thaw embryos had one or less than one damaged blastomeres in the four groups. This may account for the lack of significant differences in the four groups.

Human cryopreserved embryos appear to be relatively stable with no obvious deleterious time effects on pregnancy outcomes. Limited studies exist with respect to obstetric outcomes. It is well-known that birth weight is a measure commonly used for the assessment of perinatal outcome, which is related to morbidity and mortality [14]. Therefore, more and more IVF centers have focused on the effect of in vitro

embryo culture on neonatal birth weight. Singleton birth weights did not differ significantly based on storage time in our study. In the present study, we studied singleton birth weights. Moreover, longitudinal child health should be the focus of a follow-up study to confirm that cryopreservation of embryos is a safe procedure. Currently an increasing number of IVF centers use the vitrification technique, so the survival and pregnancy outcomes of long-term cryopreservation on vitrified embryos will be an important issue.

Multiple linear regression analysis was performed in order to find the relationship between confounding factors and birthweight. The maternal height was found to have the correlation with the birthweight from the previously published data in our center [15]. Surprisingly, in our study maternal BMI was found to have no correlation with the birthweight. It is possible that the number of the live birth was small and the basic condition (i.e., age, BMI...) of the patients had little difference with each other.

The theoretical models have speculated that mammalian embryos can be stored for several thousand years [1]. However, these models are limited by the significant differences between animal and human physiology. Additionally, the animal models assume optimized storage conditions, but in the process of practical application, the storage conditions may change temporarily because we often open the tank to remove or store embryos.

In China, a large number of infertility patients had their first baby after undergoing IVF treatment and supernumerary embryos were frozen. After several years, the infertility patients want to have a second baby. In addition, there are patients who have repeated implantation failure and patients who desire preservation of ovarian function. Thus, the safety of cryopreservation for human fertility preservation is an important issue.

Although retrospective, our analysis benefits from the large sample size. We conclude that these findings provide reassurance about cleavage-stage embryos under strictly controlled laboratory conditions.

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Conflict of interest None declared.

Author's Roles Conceived and designed the study: P.L., J.Q.; Collected data and wrote the paper: L.Q.L., Y.L.; Assisted to collected data: M.L., S.L.L.; Assisted to write the paper: J.H., X.L.R.

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