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FERTILITY PRESERVATION

Effects of duration of cryo-storage of mouse oocytes on cryo-survival, fertilization and embryonic development following vitrification

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Abstract
Purpose To investigate the effects of cryo-storage duration in liquid nitrogen on oocyte cryo-survival, fertilization and embryonic development following vitrification and warming.
Methods Mature mouse oocytes were vitrified with McGill Cryoleaf and stored in liquid nitrogen for a period of 8–10 days, 90–92 days and 180–182 days, respectively. After warming, the survived oocytes were inseminated by intracytoplasmic sperm injection (ICSI) and cultured for 120 h. The rates of oocyte cryo-survival, cleavage and embryonic development were compared.
Result(s) The oocyte cryo-survival rate declined following cryo-storage duration for 180–182 days (90.4±7.9%) compared to that of the other two groups (97.4±3.0% and 98.0±3.3%). The fertilization rate in the group of 180–182 days (66.6±22.0%) was also significantly reduced ($P<0.05$) compared with the groups of 8–10 days (92.2±10.8%) and 90–92 days (94.7±9.1%). In addition, the number of embryos developed to the blastocyst stage declined significantly ($P<0.05$) following long cryo-storage duration (72.1±8.2%, 25.2±3.8% and 5.5±13.6%, respectively).
Conclusion(s) The cryo-survival, fertilization rate and embryonic development of mouse oocytes are affected significantly, in an adverse manner, by the cryo-storage duration in liquid nitrogen.

Keywords Oocyte · Cryo-storage · Vitrification · Liquid nitrogen · Cryo-survival

Capsule The cryo-survival, fertilization rate and embryonic development of mouse oocytes are affected significantly by the duration of cryo-storage in liquid nitrogen.

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Introduction

Since Chen reported the first pregnancy after cryopreservation of human oocytes, recent oocyte freezing methods have been improved dramatically, especially with the development of vitrification method [1]. To date, more than 1,000 successful live births resulting from cryo-preserved human oocytes have been reported [2–12]. Vitrification seems to be a successful and promising technique for oocyte cryopreservation, resulting in several hundred healthy live births recently [10, 11, 13–15]. With the improved efficiency of oocyte vitrification, it has been suggested that establishing oocyte cryo-banking with the oocyte vitrification method would be beneficial to many women [16].

Most of the successful cases of oocyte cryopreservation have been reported to involve cryo-storage of oocytes for

a relatively short duration in liquid nitrogen before warming. However, evidence provided by these reports do not lend support for the practice of oocyte cryopreservation in long-term cryo-banking that may require long duration of cryo-storage [17]. Theoretically, oocytes stored in liquid nitrogen should be relatively safe without any obvious undesirable biological and metabolic changes in the ooplasm. Parmegiani et al. reported that human oocytes can be safely cryo-preserved by slow-freezing method with plastic straw and stored for several years based on the results of oocyte survival after thawing, fertilization, cleavage, embryo quality and development, implantation, and birth from oocytes stored in liquid nitrogen [18].

Regarding cryo-storage systems, especially those related to vitrification methods, there are 'closed' and 'open' systems to store oocytes or embryos in the liquid nitrogen following cryopreservation. 'Closed' system refers to the oocytes or embryos that do not make direct contact with liquid nitrogen during storage, and the 'open' system refers to the oocytes or embryos that are in direct contact with liquid nitrogen during storage. Although a previous study showed that oocyte vitrification with 'open' system does not have an adverse effect on the subsequent fertilization and embryo development as well as clinical pregnancy rates [19], there is a paucity of information about the effects of duration of oocyte cryo-storage on the subsequent cryo-survival, fertilization and embryonic development following 'open' vitrification system. Interestingly, Cobo et al. reported that storage of vitrified oocytes in a vapour-phase nitrogen storage freezer compared to a traditional liquid nitrogen tank storage for up to 10 months does not adversely affect oocyte cryo-survival, fertilization and embryonic development as well as clinical pregnancy rates when the 'open' vitrification system was applied [20].

Therefore, for application of human oocyte vitrification in the clinic, it is of great urgency to systematically investigate the influence of cryopreservation duration in liquid nitrogen on the outcomes of the vitrified human oocytes. The objective of this study was to investigate, in the mouse model, the effects of cryo-storage duration in liquid nitrogen on oocyte cryo-survival and fertilization as well as embryonic development using the 'open' vitrification system.

Materials and methods

Chemicals

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO), unless otherwise stated.

Oocyte collection and sperm preparation

Outbred CD1 mice (8–10 weeks of age) were used for oocyte collection, and male CD1 mice (12–14 weeks of age) were used for sperm collection. All of the mice were kept under pathogen-free condition with a 30–60% humidity, a temperature ranging from 21 to 24°C, a light cycle of 12 h light:12 h darkness, and were given free access to sterile food and water. The Animal Care Committee of McGill University approved all the experimental procedures carried out in the study. Female mice were super-ovulated by an intraperitoneal injection of 10 IU of pregnant mare's serum gonadotropin (PMSG) and 48 h later followed by an intraperitoneal injection of 10 IU of human chorionic gonadotropin (HCG). Cumulus-oocyte-complexes (COCs) were collected from the ampullar region of the oviduct 14 h after HCG injection. Cumulus cells were dispersed by incubating the COCs for 1 min in a solution containing 80 units/mL of hyaluronidase, and the cells were then removed by pipetting gently. The denuded metaphase-II (M-II) oocytes were used for the experiments.

For sperm collection, the male mice were sacrificed by cervical dislocation. Both epididymides were excised and sperm were collected from the cauda region of each epididymis. The contents of each cauda epididymis were squeezed out by a pair of forceps and then transferred immediately into a drop (0.4 mL) of modified human tubal fluid (mHTF) containing 0.9% bovine serum albumin (BSA). The sperm were incubated in the mHTF for 90 min at 37°C in 5% CO_2 in humidified air to induce capacitation.

Oocyte vitrification and warming procedures

The vitrification and warming procedures were similar to those reported by Huang et al. [21]. Briefly, the oocytes were suspended in an equilibrated solution containing 7.5% ethylene glycol (EG), 7.5% 1.2-propanediol (PROH) and 10% fetal bovine serum (FBS) in Dulbecco's phosphate buffered saline (DPBS) for 3 min. The oocytes were then transferred to vitrification solution (containing 15% EG, 15% PROH, 0.5M sucrose and 10% FBS in DPBS) for 45–60 s at room temperature. 4–5 oocytes were loaded onto a McGill Cryoleaf (MediCult Company, Denmark) which was then immediately plunged into liquid nitrogen for storage. For warming, the McGill Cryoleaf was immersed directly in a 37°C-thawing solution (containing 1.0M sucrose in 10% FBS-supplemented DPBS) for 1 min. The thawed oocytes were transferred to 0.5M and 0.25M sucrose in 10% FBS-supplemented DPBS for 3 min, respectively, and then washed twice with washing medium (10% FBS in DPBS) before they were transferred to culture medium at 37°C in 5% CO_2 in humidified air. Cryo-survival rate of the oocytes was assessed 2 h after

incubation. Cryo-survived oocytes were identified by the morphological appearance of membrane integrity and discoloration of the ooplasm. The surviving oocytes were used further for the subsequent experiments.

Insemination with intracytoplasmic sperm injection (ICSI) and embryo culture in vitro

ICSI was performed by using an Olympus light microscope equipped with Narishigue micromanipulators and a Piezo system (Prime Tech, Japan). After sperm capacitation, 5 μL of sperm suspension was mixed in a droplet consisted of 5 μL of 12% PVP solution under paraffin oil which was previously prepared in a 60-mm petri dish (FALCON, USA). Prior to ICSI, oocytes were transferred from mHTF medium into a droplet of Hepes buffered mHTF medium under paraffin oil. With the sperm/PVP suspension in the same dish, only highly motile sperm with a morphologically normal head were selected, and the head was separated from the tail by applying a few Piezo pulses. The sperm head was injected into the oocyte by using a Piezo drive unit [22]. After injection, the oocytes were washed thoroughly with mHTF medium and then transferred into a droplet containing 50 μL of embryo maintenance medium (SAGE Media, USA) under paraffin oil for culture at 37°C in 5% CO_2 in humidified air. Following ICSI, fertilization rate was assessed by cleavage of the survived oocytes 16–18 h after ICSI, and embryonic development to 8-cell stage was observed 70–72 h after ICSI. Blastocyst development was assayed 120–122 h after ICSI. The rates of cleavage, embryonic development to 8-cell stage and blastocyst development were based on the number of oocytes survived after ICSI.

Assessment of quality of blastocysts with differential staining

The number of inner cell mass (ICM) and trophectoderm (TE) cells in the blastocysts was determined with the method developed by Handyside and Hunter [23] with modifications. Briefly, the zona pellucida was removed from the blastocysts by culturing in mHTF containing 0.5% protease for 15 min at 37°C in 5% CO_2 in humidified air. After rinsing with 0.5% PVP/PBS for 5 min, the naked blastocysts were transferred to Hepes-mHTF (1:3) containing rabbit anti-mouse splenocyte antiserum and were incubated in the solution for 20 min at 37°C in 5% CO_2 in humidified air. After being washed 3 times with 0.5% PVP/PBS for 15 min, the blastocysts were immersed in Hepes-mHTF (1:5) containing guinea pig complement for another 20 min at 37°C in 5% CO_2 in humidified air. After rinsing with 0.5% PVP/PBS, the blastocysts were stained with a staining solution (that contained 5 μg/mL of Hoechst 33342 and 5 μg/mL of Propidium iodide (PI) in 0.5% PVP/PBS) for 15 min at room temperature. The stained blastocysts were mounted on light microscope slides with droplets of mounting medium. Following mounting, the number of TE and ICM cells was counted under the UV light with a blue filter. The nuclei in the TE cells were indicated by the presence of red-pink color while the nuclei in the ICM cells were indicated by the presence of blue color.

Experimental design

Following vitrification, the oocytes were stored in liquid nitrogen for 8–10 days, 90–92 days and 180–182 days, respectively. After thawing, the oocytes in each group were assessed respectively for the cryo-survival, fertilization (2-cell cleavage) and embryonic development as well as the quality of blastocysts as previously described [24]. The control group consisted of freshly collected oocytes without vitrification. 4–5 females with 15–20M-II oocytes each super-ovulation were used for each replicate, and pooled oocytes distributed randomly to three groups. Oocytes in all three groups were frozen within a short intensive period. The frozen oocytes were warmed, ICSI and subsequent in vitro culturing at three different interval.

Statistical analysis

All the data were reviewed by the statistician for the department of Clinical epidemiological study in Peking University Third Hospital. The differences of the number of oocytes collected, the total cell number of blastocysts and the ratio of ICM/TE in each group were analyzed by ANOVA and a Fisher protected least significant difference test. The difference in oocyte cryo-survival, fertilization and embryonic development rates were analyzed by ANOVA test, and quality control between each replicate were analyzed by ANOVA just shown as *Mean* ± *SD* for different rates. The SPSS 17.0 statistical software package was employed in this analysis. Differences with a P value of <0.05 (two-tailed) were considered as statistically significant.

Results

As shown in Table 1, the cryo-survival rate in the group of oocytes with a cryo-storage duration of 180–182 days (90.4± 7.9%) was significantly reduced ($P<0.05$) compared to the other two groups (97.4±3.0% and 98.0±3.3%, respectively) following warming.

Following insemination by ICSI, although the cleavage rates were not different between the experimental groups with a shorter cryo-storage duration of 8–10 days (92.2±10.8%) and 90–92 days (94.7±9.1%) and the control group (92.0± 4.9%), the cleavage rate was significantly reduced in the

Table 1 Effect of duration of cryo-storage of mouse oocytes in liquid nitrogen on cryo-survival rates following vitrification and warming (six replicates)

Cryo-storage duration (days)	No. of oocytes vitrified	No. of oocytes survived	Survival rates (mean±D%)	95% Confidence interval (CI)
8–10	118	115	97.4±3.0[a]	92.6~102.2
90–92	119	117	98.0±3.3[a]	95.8~100.3
180–182	126	117	90.4±7.9[b]	83.1~97.8

[a] Indicates that there was no difference in the cryo-survival rates between the group of oocytes with a cryo-storage duration of 8–10 and 90–92 days ($P>0.05$)

[b] Indicates the cryo-survival rate in the group of oocytes with a cryo-storage duration of 180–182 days was significantly reduced compared to the other two groups ($P<0.05$)

group with a longer cryo-storage duration of 180–182 days (66.6±22.0%) compared with the other two experimental groups and the control group (Table 2). The embryos developed to 8-cell stage in the group of cryo-storage for 90–92 days was significantly reduced (60.8±1.5%) compared with the group of cryo-storage for 8–10 days (91.2±7.1%) and the control group (90.0±3.9%). The number of embryos developed to 8-cell stage was further reduced after a longer duration of cryo-storage for 180–182 days (15.2±22.0%, $P<0.05$) compared with the other groups. Furthermore, the percentage of embryos developed to the blastocyst stage in the groups of cryo-storage duration for 90–92 days and 180–182 days were 25.2±3.8% and 5.5±13.6% respectively, which were significantly lower ($P<0.05$) than the group of cryo-storage duration for 8–10 days (72.1±8.2%) and the control (81.4±12.8%).

The total cell number of individual blastocyst was also found to be significantly reduced ($P<0.05$) in the groups of cryo-storage duration for 90–92 days (79.0±44.2) and 180–182 days (73.0±35.5) compared with the group of cryo-storage for 8–10 days (101.3±39.4) and the control (99.1±47.1). In addition, the ratio of ICM/TE was significantly lower ($P<0.05$) in the group of cryo-storage duration for 180–182 days (9.8±17.0) compared with the other groups (21.5±18.6, 16.7±15.6 and 18.4±18.6 respectively, Table 3).

Discussion

The results of the present study demonstrated that the cryo-storage duration of mouse oocytes in liquid nitrogen detrimentally affect the subsequent cryo-survival, fertilization and embryonic development following vitrification and warming.

It is generally believed that the biological activities of cells, even oocyte and embryo would be stopped completely when they are stored at the temperature of liquid nitrogen (−196°C). So far most studies focussed on the quality of embryos cryo-stored at −196°C and reported that embryo quality is not expected to be influenced by the duration of cryo-storage [25–29]. Studies carried out in mice showed that a cumulative dose of background radiation applied to frozen mouse embryos during a simulated 2,000-year storage period had no detectable effect on their viability [30]. Experiments carried out in sheep indicated that embryos could safely survive for up to 13 years [27]. Therefore, it appears that the duration of cryo-storage will not affect the outcome of frozen embryos. Several studies carried out in humans indicated that long-term cryo-storage did not affect embryo cryo-survival and pregnancy outcome in the IVF or embryo donation program [31, 32]. However, a previous study reported that increased rates in embryonic cell death were found associated with cryo-storage of

Table 2 Effect of duration of cryo-storage of mouse oocytes in liquid nitrogen on fertilization and embryonic development following vitrification and warming (six replicates)

Cryo-storage duration (days)	No. of oocytes inseminated	No. of oocytes cleaved (mean±SD%)	No. of embryos developed to 8-cell stage (mean±SD%)	No. of embryos developed to blastocyst stage (mean±SD%)
Control	100	94 (92.0±4.9)[a]	83 (90.0±3.9)[a]	72 (81.4±12.8)[a]
8–10	60	55 (92.2±10.8)[a]	49 (91.2±7.1)[a]	40 (72.1±8.2)[a]
90–92	90	67 (94.7±9.1)[a]	42 (60.8±1.5)[b]	15 (25.2±3.8)[b]
180–182	82	50 (66.6±22.0)[b]	9 (15.2±22.0)[c]	3 (5.5±13.6)[c]

In the column of *cleavage rate*, [b] indicates that the cleavage rate was significantly reduced in the group with a longer cryo-storage duration of 180–182 days compared with the other two experimental groups and the control group, which were similar indicated by [a]. In the column of *8-cell stage*, [b] indicates that the embryos developed to 8-cell stage in the group of cryo-storage for 90–92 days was significantly reduced compared with the group of cryo-storage for 8–10 days and the control group, which were similar indicated by [a]. The number of embryos developed to 8-cell stage was further reduced after a longer duration of cryo-storage for 180–182 days compared with the other groups, indicated by [c]. Furthermore, the percentage of embryos developed to the blastocyst stage in the groups of cryo-storage duration for 90–92 days and 180–182 days were 25.2±3.8% and 5.5±13.6% respectively, which were significantly lower than the group of cryo-storage duration for 8–10 days and the control, indicated by [a, b, c]

Table 3 Effect of duration of cryo-storage of mouse oocytes in liquid nitrogen on the quality of blastocysts following vitrification and warming

Cryo-storage period (days)	No. of blastocysts examined	No. of TE in each blastocyst (mean±SD)	No. of ICM in each blastocyst (mean±SD)	Total cell numbers in each blastocyst (mean±SD)	Ratio of ICM/TE (mean±SD%)
Control	21	85.1±40.3[a]	15.8±13.7[a]	99.1±47.1[a]	21.5±18.6[a]
8–10	25	87.6±34.9[a]	13.7±10.1[a]	101.3±39.4[a]	16.7±15.6[a]
90–92	15	66.8±37.1[b]	12.1±11.5[a]	79.0±44.2[b]	18.4±18.6[a]
180–182	3	64.3±20.5[b]	8.6±15.0[b]	73.0±35.5[b]	9.8±17.0[b]

In terms of the number of TE, [a] indicates that there was no difference between the group with cryo-storage for 8–10 days and the control group, and [b] indicates that there was no difference between the group with cryo-storage for 90–92 days and 180–182 days, while significantly reduced compared with the other two groups. In terms of the number of ICM, [a] indicates that there was no difference between the groups with cryo-storage for 8–10 days, 90–92 days and the control group, and [b] indicates that ICM was significantly reduced in the group with cryo-storage for 180–182 days compared with the three groups. In terms of total cell number, [a] indicates that there was no difference between the group with cryo-storage for 8–10 days and the control group, and [b] indicates that there was no difference between the groups with cryo-storage for 90–92 days and 180–182 days, while significantly reduced compared with the other two groups. In terms of the ratio of ICM/TE, [a] indicates that there was no difference between the groups with cryo-storage for 8–10 days, 90–92 days and the control group, and [b] indicates that ICM was significantly reduced in the group with cryo-storage for 180–182 days compared with the three groups

embryos for a period of only several months, and embryos with the cryo-duration less than 1–2 months produced higher pregnancy rate than embryos cryo-preserved for more than 2 months [33]. To date, only several studies of live births have been reported resulting from long-term cryo-preserved human embryos at zygote stage or cleavage stage with a duration of 7.5 years, 8 years, 8.9 years and 12 years, respectively [34–37]. Based on an analysis of 11,768 cryo-preserved human embryos, recently Riggs et al. [31] have reported that the "oldest" zygotes resulting in live births were stored for 9.2 years, and the "oldest" cleavage embryos resulting in live births was stored for 5 years, but no pregnancies resulted from embryos that had been cryo-preserved for more than 10 years. The results indicated that there are some negative effects of long-term cryo-storage on human embryo viability [31]. All those studies suggest that cryo-duration in liquid nitrogen may affect the outcome of embryo cryopreservation, and long cryo-duration probably damages to the viability of embryos. This tendency was also noticed in our study that cryo-duration affects oocyte cryo-survival and subsequent fertilization and early embryonic development.

In terms of oocyte cryopreservation, in animal models, information about the effect of oocyte cryo-storage duration on cryo-survival and subsequent fertilization and embryonic development is limited. Regarding human oocyte cryopreservation, there have been reported of live births resulting from slow-freezing with plastic straws of oocytes with subsequent cryo-storage for up to 4, 5 and 6 years respectively [38, 39]. Only one clinical trial study involving a sample size of 228 slow-freezing oocytes indicated that, with the increase of cryo-storage duration from 1 to 48 months, some decline in oocyte survival, fertilization, cleavage rate and embryo development was noted, although the decline was not significant [18]. Due to the fact that many factors could affect the outcome of human oocyte cryopreservation, such as the difference in age of women, duration of infertility and lack of proper controls, it would be premature to draw a solid conclusion. Therefore, further studies are required to confirm whether the cryo-storage duration affects the subsequent cryo-survival and fertilization as well as embryonic development following cryopreservation.

It has been reported that no thermally driven reactions occur in cells in aqueous systems at −196°C. On the contrary, many important photo-physical events or chemical syntheses have been reported to produce some form of hazards in liquid nitrogen [40]. As a result of photo-physical events, direct ionizations from background irradiation or cosmic rays are known as the source of damage at such low temperature, such as the formation of free radicals and the production of breaks in macromolecules as a direct result of "hits" by background ionizing radiation or cosmic rays. For example, ionizing radiation on oxygen dissolved in liquid nitrogen causes the formation of free radicals due to the rapid decomposition of ozone (O_3) [41]. There is also evidence that oxides of nitrogen are formed in liquid nitrogen enhancing the yield of ozone by a catalytic effect [41–43]. These can cause DNA breaks or other deleterious damage to DNA since enzymatic repair cannot occur at such low temperature [44]. In that study, 8–10 weeks old female mice were used [24], suggesting that there might be a tendency of declining embryonic development following the increasing oocyte cryo-duration in liquid nitrogen. Although it is not clear why duration of oocyte cryo-storage affects the subsequent embryonic development, it may be due to the results of DNA breakages of the oocytes during cryo-storage or other deleterious damage to oocyte DNA during cryo-storage. It seems that the longer cryo-duration will cause the worse damage of oocytes, which could cause disorder of gene expression in subsequent embryonic development.

Further study is required to verify the relationship between duration of cryo-storage and DNA damage of oocytes in the liquid nitrogen.

In conclusion, cryo-storage duration of vitrified mouse oocytes evidently affects the oocyte viability post-thawing, resulting in poor fertilization and embryonic development. Our results suggest that the effects of duration of cryo-storage in liquid nitrogen on oocyte cryo-survival, subsequent fertilization and embryonic development as well as pregnancy following vitrification should be further investigated and confirmed, especially when the procedure is used for the fertility cryopreservation of human oocytes.

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