J. Reprod. Engineer. 2011; 14: 19–28.
http://sre.ac.affrc.go.jp/Contents.htm

Journal of REPRODUCTION ENGINEERING JRE

= Original Article =

Development of a low toxicity and completely serum-free vitrification system combining a Cryo-Nano-Hole vitrification container with antifreeze polyamino-acid (carboxylated poly-L-lysine) for assisted reproductive technology (ART)

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ABSTRACT

In protocols for conventional vitrification, cells can be injured easily because of toxicity to the cells and dehydration due to high concentration of cryoprotectant agents (CPA). In this study, we describe a novel vitrification system, achieving ultra rapid cooling rates with minimal concentration of CPA, in which a Cryo-Nano-Hole vitrification container and carboxylated poly-L-lysine (PLL: Antifreeze Polyamino-acid) are used. The Cryo-Nano-Hole vitrification container (10 nl/hole) enabled vitrification of mouse and human blastocysts using a completely serum-free medium and a low concentration of CPA (15 or 20% ethylene glycol (EG) + 10% PLL) in the range used for slow freezing. The survival rates of mouse and human blastocysts after vitrification with this system are similar to those in conventional vitrification protocols with 47% CPA (15% EG + 15% dimethyl sulfoxide (DMSO) + 17% (0.5M) Sucrose with 20% serum). These results indicate that a Cryo-Nano-Hole vitrification system with carboxylated PLL is useful tool for the safe and reliable cryopreservation of human embryos. Our experiments demonstrate the importance of accurate minimum volume control and use of low concentration of CPA in the vitrification. In conclusion, mouse and human blastocysts can be cryopreserved successfully in a Cryo-Nano-Hole vitrification container with a low concentration of CPA solution without serum. It is expected that this system will be efficiently used for human ART.

Key words: vitrification, low toxicity, serum-free, Cryo-Nano-Hole vitrification container, antifreeze polyamino-asid (carboxylated poly-L-lysine)

INTRODUCTION

In assisted reproductive technology (ART), the cryopreservation of oocytes/embryos has made it possible to implant them into the best uterine environment and is one of the therapeutic technologies required to obtain a high pregnancy rate. The vitrification method was reported by Luyet in 1937 [14], but it took a long time until the method was practically used on

Submitted: November 18, 2011
Accepted: December 29, 2011
Advance Publication in Website: December 30, 2011

embryos [24]. There is close causality between the CPA concentrations needed for vitrification and the volume and the cooling rate during vitrification. If the volume during vitrification is reduced, the cooling rate increases, making it possible to reduce the concentration of CPA required for vitrification [4,10,25]. In order to reduce the concentration of CPA, plans to reduce the amount of solution have been devised during the last decade, and various kinds of vitrification containers have been invented and used for clinical purposes [11,12,15,22,26, 27].

The Cryotop method, which is currently considered to be one of the global standard methods, has made it possible to reduce the concentration of cell membrane-permeating CPA added to the vitrification solution by half to 30% (15% ethylene glycol (EG) + 15% dimethyl sulfoxide (DMSO)) [10,11]. However, a concentration of 17% (0.5 M) of disaccharide (either sucrose or trehalose) is required for the vitrification solution, as a cell membrane-nonpermeating CPA. The total concentration of CPA is 47.1%, and the total osmotic pressure is 4000 mOsmol/kg, which is much higher than that of the culture medium (260−300 mOsmol/kg), causing concern about the toxicity of the CPA to the ovum/embryo, and the possibility of osmotic shock.

Recently, Matsumura *et al.* developed an antifreeze polyamino acid (carboxylated poly-L-lysine: PLL) [16,17], an amphoteric macromolecular compound containing both cationic and anionic substituents (polyampholyte), by chemically modifying polylysine, a CPA which is highly safe and frequently used as a food additive in substitution for DMSO, which had raised concerns of toxicity. In other words, polylysine was made to react with succinic anhydride to block more than approximately 50% of its amino groups. Since PLL has an affinity for cells because of its amino groups, it has a protective effect on the cell membrane; and in addition, because it also contains a large number of carboxyl groups in its molecule, it has a high affinity for water and helps excrete the intracellular water outside immediately when the cells are being frozen. They reported that since a low toxic CPA is used as a substitute for DMSO, PLL is effective in the cryopreservation of many cultured cells such as L929, MG63, HT1680, B16F1, Caco2, MC3T3, and KB cells. In addition, it has been reported that PLL can also be applied to the vitrification of human induced pluripotent stem cells (iPS cells) [18].

In this study, we developed a vitrification system in which toxicity and osmotic shock could be suppressed as much as possible by combining the use of Cryo-Nano-Hole vitrification containers and carboxylated PLL, and we examined the system's efficacy and safety by using mouse and human blastocysts.

MATERIALS AND METHODS

Preparation of mouse blastocysts

The sampling of mouse expanded blastocysts was conducted in accordance with the operating manual for the handling of mouse embryos [1].

Preparation of human blastocysts

The experiments were performed after approval to conduct research was obtained from the hospital's ethics committee and to the Japan Society of Obstetrics and Gynecology. After informed consent was obtained from patients, day 5 or day 6 (day 0 means a fertilization day) blastocysts (fresh or preserved by vitrification), which would be waste, were used for vitrification experiments.

Cryo-Nano-Hole vitrification containers

Figure 1A and 1B shows a picture of a Cryo-Nano-Hole vitrification container [19, 20], and Figure 2 shows the operation method. The container was manufactured by injection molding a highly safe resin (patent application was completed in 2009). The size of the distal portion designed to carry the ovum was as follows: length: 6 mm, width: 0.5 mm, thickness: 150 μm or 200 μm. It was perforated with 250 μm-diameter holes at intervals of 0.5 mm from its tip. Each perforating hole had a cylindrical shape (diameter at the bottom × height = 250 μm × 150 μm), and a volume of approximately 10 nl.

Vitrification method

The mouse and human blastocysts were allowed to cool to room temperature for 5 min in a basic culture medium (HBM). HBM was added 12 mM HEPES (Dojindo, 346-00015) and 8 mM Na-HEPES (Wako, 105593) into a complete serum-free basic culture medium (BM) [7]. We used a vitrification solution (VS) made of a mixture of HBM (0.1% PVA (Sigma, P8136), 1% Ficoll 70 (GE health care Bio sciences AB, 17-0310-10), and 0.05% Hyaluronan (Nakalai tesque, 18237-41) were added), 15% EG (Sigma, 324558) as a

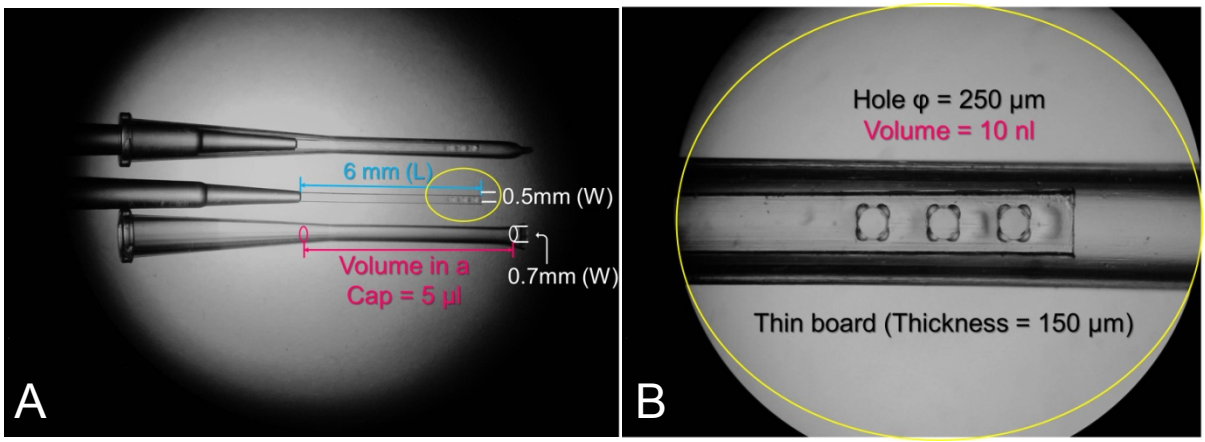

Figure1. The appearance (A) and the magnification (B) of Cryo-Nano-Hole vitrification container
The size of the distal portion designed to carry the ovum was as follows: length: 6 mm, width: 0.5 mm, thickness: 150 µm or 200 µm. It was perforated with 250 µm-diameter holes at intervals of 0.5 mm from its tip. Each perforating hole had a cylindrical shape (diameter at the bottom × height = 250 µm × 150 µm), and a volume of approximately 10 nl.

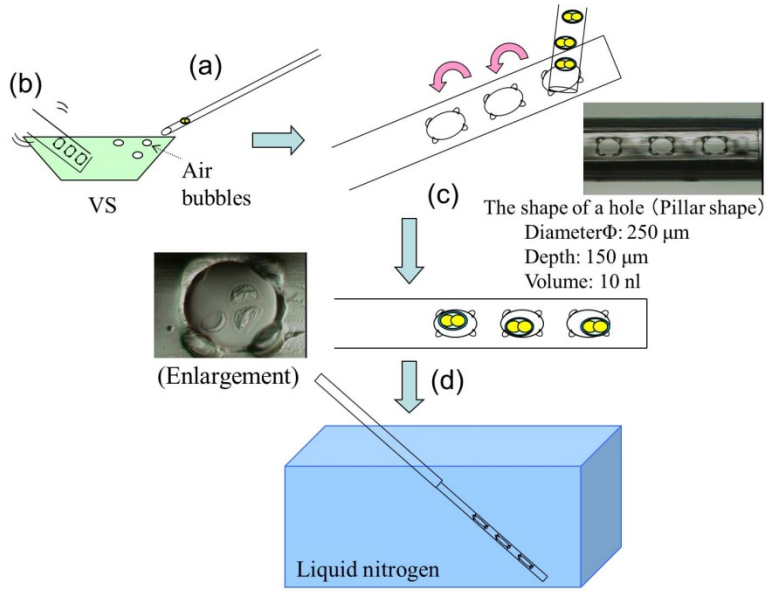

Figure 2. Protocols for Cryo-Nano-Hole vitrification container
(a) Ovum/embryo were held in the pipette,
(b) The container was swung, and air in a hole is removed, and filled with vitrification solution (VS) solution,
(c) Put ovum/embryo in the hole with very small amount of VS solution,
(d) It's put in liquid nitrogen promptly after held ovum/embryo in the hole.

CPA, and 10% carboxylated PLL. The oocyte and embryo manipulations were all performed at room temperature. First, mouse expanded blastocysts were immersed in Equilibration solution (ES: 15% EG solution) for 5–10 min for equilibration, equilibrated for 3 min in a VS (total CPA is 25% consisting of 15% EG + 10% PLL), transferred into the hole of a Cryo-Nano-Hole vitrification container that had been filled with VS solution beforehand, and immediately put into liquid nitrogen for storage directly. Two controls were used: VS (total CPA is 32% consisting of 7.5% EG + 7.5% DMSO + 0.5 M Sucrose [17%]) previously described by Mizuno *et al.* [19] was used as control number 1, and VS (total CPA is 47% consisting of 15% EG + 15% DMSO + 0.5 M Sucrose [17%] (Vitrification Kit: Kitazato Corporation, Fuji, Japan) previously described by Kuwayama *et al.* [11] was used as control number 2.

Human blastocysts were equilibrated by immersion in ES (15% EG solution) for 8–10 min, subjected to equilibration treatment for 3 min in a VS solution (20% or 15% EG + 10% PLL + 0.2 M (7%) Sucrose), transferred into the hole of a Cryo-Nano-Hole vitrification container that had been filled with VS

Table. The results of vitrified mouse blastocysts using various vitrification and warming solutions with a Cryo-Nano-Hole container and carboxylated poly-L-lysine.

Groups	Sucrose concentration (M)	Dilution steps	Total number of blastocysts examined	% (No.) of Survived blastocysts	% (No.) of developed blastocysts	Average total cell number*	% of offspring production** (No. offspring /total transferred embryos)
Experiment group[a]	1	3	50	98 (49)	94 (47)	86.2	48.6 (18/37)
	0.5	2	50	100 (50)	96 (48)	89.8	55.2 (21/38)
	0.25	1	50	96 (48)	92 (46)	82.6	48.5 (18/36)
	0	0	50	90 (45)	86 (43)	88.2	48.5 (16/33)
Control group-1[b]	1	3	50	100 (50)	98 (49)	84.2	48.7 (19/39)
	0.5	2	50	96 (48)	94 (47)	91.6	56.8 (21/37)
	0.25	1	50	98 (49)	94 (47)	85.3	48.6 (18/37)
Control group-2[c]	1	2	50	98 (49)	96 (48)	84.0	47.4 (18/38)

*Ten embryos were evaluated. **All offspring produced normally in all 8 groups.
[a]15% ethylene glycol (EG) + 10% carboxylated poly-L-lysine, complete serum free (total CPA: 25%). [b]7.5% EG + 7 .5% dimethyl sulfoxide (DMSO) + sucrose 0.5 M (17%), complete serum free (total CPA: 32%) (Mizuno et al. [19]). [c]15% EG + 15% DMSO + 0.5 M (17%) sucrose + 20% serum (total CPA: 47%) (Vitrification Kit: Kitazato Corporation, Fuji, Japan).
No difference was found among 8 groups in each column.

solution (10 nl or less) beforehand, and immediately and directly put into liquid nitrogen for storage.

Warming

Mouse and human blastocysts that had been vitrified and stored in Cryo-Nano-Hole vitrification containers were warmed at room temperature and were equilibrated for 1 min by direct immersion into a warming solution (WS: HBM containing disaccharides (sucrose or trehalose) at 4 different concentrations, namely 1 M, 0.5 M, 0.25 M, and 0 M), which had been warmed at 37°C. After warming was performed, when the disaccharides (sucrose or trehalose) were at a concentration of 1 M in the WS, the concentrations were decreased stepwise in 3 steps to 0.5 M and 0.25 M. At a concentration of 0.5 M, equilibration was performed in 2 steps (at 0.5 M and at 0.25 M), for 3 min each. Finally, washing was performed 3 times in a 20-µl microdrop prepared with BM [7], and cell culture was performed continuously from the stage of expanding blastocysts up the stage of hatching blastocysts.

Evaluation of the viability of mouse and human blastocysts preserved by vitrification

The vitrified embryos were photographed before vitrification, immediately after warming, and after 12–24 hours of incubation (Figures 3 and 4). Viability was evaluated on the basis of the morphological observation from the pictures, and the survival rate and the growth rate were calculated. Blastocyst survival was defined by its expansion, intact cellular membrane, and normal zona pellucida. Its growth was defined by its development, expansion and hatching.

Quality Evaluation of mouse and human blastocysts preserved by vitrification

For quality evaluation, some of the embryos that survived and developed were fixed on glass slides, and the total cell number (TCN) of blastocysts was counted by nuclear fluorescence staining with 4', 6-diamidino-2-phenylindole (DAPI; Sigma, D9542).

The outcomes of the transplantation of vitrified mouse embryos in recipients were as follows: The blastocysts that survived and developed after vitrification and warming were transplanted into the uterus of the recipient female ICR mice (CLEA Japan Inc., Tokyo, Japan) under general anesthesia (third day of pseudopregnancy after mating with vasectomized ICR,males (CLEA Japan) = 2 days after confirmation of mating). Next, in the afternoon of the 16th day after transplantation, in order to prevent the newborn mice from being killed and eaten by the mothers, the embryos were collected by cesarean section, and their normality was confirmed. The extracted fetuses were nurtured and fed by foster parent mice that had been prepared in

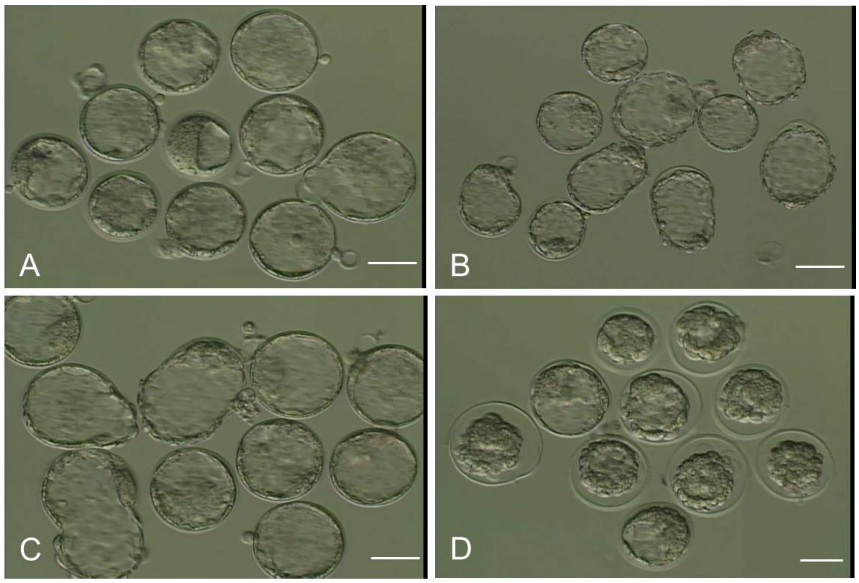

Figure 3. The survivability after vitrification of the mouse blastocyst which carried out vitrification preservation by the Cryo-Nano-Hole vitrification container and carboxylated PLL (15% EG + 10% PLL and no Sucrose (Total CPA 25%))
A) Before vitrification, B) After vitrification, C) 24 h after vitrification and D) 48 h after vitrification. Scale bars represent 100 μm.

advance. The young mice that were obtained by implantation of vitrified embryos were observed and examined for the presence or absence of external malformation or behavioral disorders during their developmental process and were confirmed to be normal.

Statistical analysis

A chi-square (χ^2) test with Yates's correction was used, and a value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Viability of mouse blastocysts after vitrification

The survivability and growth of mouse blastocysts after vitrification were calculated from the results obtained from 10 replications (using a total of 400 samples). Table shows the survival rate, the growth rate, and the mean total cell number of mouse blastocysts after vitrification, the live offspring production rate, and the rate of production of normal offspring. Regarding the survival rate, the growth rate, the mean total cell number, the live offspring production rate, and the rate of production of normal offspring in each experimental lot (total CPA 32%), similar results were obtained with the VS used in control 1 (total CPA 32%: 7.5% EG + 7.5% DMSO + 0.5 M (17%)sucrose) [19] and the VS used in control 2 (total CPA 47%: 15% EG + 15% DMSO + 0.5M (17%) sucrose, Kitazato Vitrification Kit), and no difference was found. The differences in the types of disaccharide (sucrose or trehalose) used in the WS and the differences in the concentrations (4 concentrations, including 1.0 M, 0.5 M, 0.25 M, and 0 M) had no influence on the survival rate, growth rate, mean total cell count, live offspring production rate, and the rate of production of normal offspring. In addition, the offspring born in all the experimental lots were all normal, and the observation of the course of their growth showed no external malformation or behavioral disorder, and it was confirmed that they had reproductive ability. The normal offspring rate was 100% in all experimental groups.

Viability of day 5 and day 6 human blastocysts after vitrification

Eight fresh human blastocysts were vitrified in a Cryo-Nano-Hole vitrification system, and as a result, all 8 were viable, including 5 from the lot using a VS containing 20% EG + 10% PLL + 0.2M (7%) sucrose, and 3 from the lot using a VS containing 15% EG + 10% PLL + 0.2 M (7%) sucrose. The cultures showed good growth and morphology after 12-24 h of continuous culture, and there was no morphological change that would indicate damage caused by vitrification (Figure 3). In addition, 10 human blastocysts that had been stored by vitrification were re-vitrified in a Cryo-Nano-Hole vitrification system, and as a result, all 10 lots using a VS

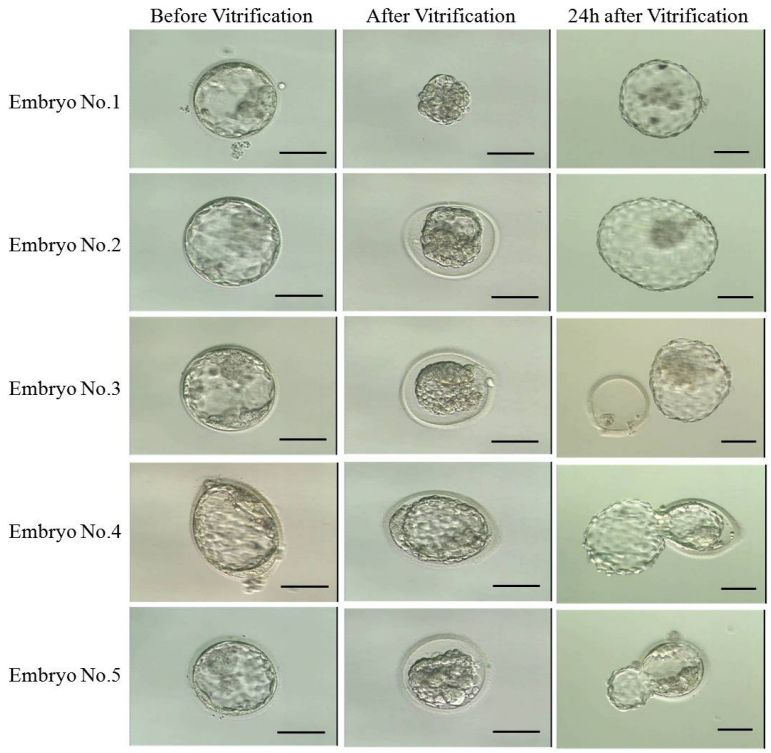

Figure 4. The survivability after vitrification of the human blastocyst which carried out vitrification preservation by the Cryo-Nano-Hole vitrification container and carboxylated PLL (20% EG + 10% PLL +0.2M (7%) Sucrose (total CPA: 37%))
The cultures showed good growth and morphology after 12–24 h of continuous culture, and there was no morphological change that would indicate damage caused by vitrification. Scale bars represent 100 μm.

containing 20% EG + 10% PLL + 0.2 M (7%) sucrose were viable and showed good growth and morphology after 16-20 h of continuous culture. There was no morphological change indicating damage caused by storage by vitrification.

DISCUSSION

In the 1990s, cryopreservation techniques for assisted reproductive technology (ART) started to switch over from slow freezing methods to vitrification. As for vitrification containers, containers of various shapes have been developed from straws in order to reduce the working volume during vitrification [10–12,15,22,25–27]. The disadvantage of vitrification is that it causes cytotoxicity and osmotic shock due to high concentrations of CPA. To solve this problem, the amount of cooling fluid was reduced and the cooling rate was accelerated in order to make it possible to reduce the concentrations of CPA, and various reports have been made regarding methods for preserving oocytes/embryos in very small amounts of vitrification solution [11,12,15, 22,24,26,27].

The relation between the container and the cooling rate during vitrification has been reported by many researchers [11,12,15,22,26,27]. Rall and Fahy [24] reported for the first time that they successfully achieved the vitrification of mouse embryos by using 0.25-ml straws and a vitrification solution containing a 6.5 M CPA cocktail in a container, and that the cooling rate was then 2,500°C/min. Under the same conditions, when slush nitrogen was used as a refrigerant, the cooling rate was 4,000°C/min; with the open pulled straw (OPS) method, [27] the cooling rate was 5,300°C/min; and when the OPS method and slush nitrogen were combined, the cooling rate was 10,000–20,000°C/min [21]. With the grid method for electron microscopes [4], the cooling rate was 11,000-14,000°C/min, and in a combination of slush nitrogen and the grid method for electron microscopes, the cooling rate was 24,000–30,000°C/min [2].

Kuwayama *et al.* [10] used the Cryotop method to reduce the volume during vitrification from 1.5 μl as in the OPS method [27] to less than 0.1 μl (=100 nl), to reduce the cooling rate from 16,000°C/min to

23,000°C/min, and to accelerate the speed of the warming process from 14,000°C/min to 42,000°C/min. They reported that as a result, the viability rate of human oocytes at the metaphase-II (MII) stage after vitrification improved [3]. However, in both reports, the total concentration of CPA added was higher than 47%.

Meanwhile, Risco *et al.* [25] reported that they achieved a cooling rate of 250,000°C/min by using a highly heat-conductive quartz capillary (diameter, 0.18 mm; thickness, 0.01mm) as a vitrification container and slush nitrogen (–205 to –210°C) as a refrigerant; that they were able to reduce to 2 M the concentration of CPA (propanediol) required for vitrification, and that this fact was consistent with Bartron's theory [3] on the cooling rate-dependence of the molar concentration of CPA, which is essential for vitrification.

In addition, Demirci *et al.* [5] have previously reported that by taking a cell storage solution containing lymphocytic cells, mouse embryonic stem (ES) cells, cell lines derived from fetal skin, liver cell lines, and atrial cell lines of monkeys, and by dropping droplets of the storage solution directly into liquid nitrogen and by filtering it through the desired filter size, it is possible to store the cells in very small droplets measuring 100-200 μm in diameter, and to vitrify the cells by using low concentrations of CPA (1.5 M propanediol). However, because this method consists of dropping very small droplets directly into liquid nitrogen, there were problems such as the need for special equipment, the difficulty to ensure that all droplets are the same volume, the low utilization rate of the cells due to the fact that droplets larger than the prescribed size are discarded, and the fact that the process of collection of droplets after their filtration to the prescribed size are time-consuming. However, in the vitrification of oocytes/embryos, the problem is that even if their viability rate after storage is good, the subsequent embryonic development rate and the offspring yield after implantation are known to be low; and Demirci *et al.* [5] did not mention anything about the application of the process to germinal cells and fertilized egg cells. These reports described attempts to reduce CPA, but because some kind of human-derived serum components were used in order to protect the cells during vitrification, the possibility of infection by unknown viruses cannot be denied.

In order to overcome the problems faced by existing vitrification systems, Mizuno *et al.* tried to develop a completely serum-free vitrification system with low-toxicity, and in order to minimize the volume used during conventional vitrification from the order of microliters to the order of nanoliters, they developed Cryo-Nano-Hole vitrification containers [19,20]. A plated-through hole was opened at the tip of the container, the volume of the vitrification solution was minimized, and the container was made so that it could easily hold a volume of vitrification solution of 10 nl or less. The base solution of the complete serum-free vitrification solution was a complete serum-free culture medium [7] into which we added PVA, hyaluronan, and Ficoll, which are substitutes for serum components such as albumin, and which have cytoprotective properties during vitrification. In vitrification systems that have been developed and used in clinical settings so far, 30% or higher concentrations of CPA were required in order to prevent ice crystal formation during vitrification [8,10,11]. It has been revealed that through the development of Cryo-Nano-Hole vitrification containers, it could be reduced to less than half, namely 15% or 12% (approximately the same as in the slow freezing method [19,20]), both in mouse oocytes/embryos and human oocytes/embryos. Reports from studies of mouse embryo transfers have also confirmed the safety of the containers. The factor that contributed to the high viability rates of mouse and human oocytes/embryos was suggested to be the fact that Cryo-Nano-containers minimized the vitrification volume to less than 10 nl in a stable manner. As a result, the conditions allowed much faster cooling and warming than in the Cryotop method [8,10,11]; thus, there was less exposure to temperature ranges that have harmful effects on the viability of oocytes/embryos [3,24].

It is generally recognized that when a solution changes and becomes solid during vitrification, its volume does not change; however, because research papers pertaining to areas of low-temperature physics and cryobiology have reported that microscopic deformations and cracks also occur during vitrification [23], additional innovations were necessary in order to ensure that oocytes/embryos are not damaged by such deformations and cracks occurring during vitrification or warming. Through the use of Cryo-Nano-Hole vitrification containers, oocytes/embryos are vitrified while being stored in a cylindrical hole of about 10 nl of volume, and this might have reduced the deformations.

Recently, Lee *et al.* [13] reported that they successfully achieved the vitrification of metaphase II (MII) mouse oocytes by using highly heat-conductive quartz (crystal) capillaries in vitrification containers, slush nitrogen (prepared by vacuum treatment at –210°C) as a refrigerant, and 1, 2-propanediol + 0.5M (17.1%) trehalose containing a concentration of CPA similar to that in storage by slow freezing (total concentration of CPA: 28.5%). However, this method requires the use of specific quartz capillaries that are not commercially available and of slush nitrogen that needs to be prepared with equipment designed specifically for that purpose.

There have been numerous reports on the efforts to develop vitrification containers [10–12,15,22,25–27], but there are no reports of the development of CPA for vitrification solutions with low toxicity and that cause less osmotic shock. In the future, there is an urgent need to search for and adopt CPA other than the existing DMSO, EG, propanediol, sucrose, and trehalose, which would have low or no toxicity, and which would cause less or no osmotic shock. Matsumura *et al.* have developed carboxylated PLL, a unique amphoteric polyelectrolyte [16,17]. Carboxylated PLL has a protective effect on the cell membrane because of its affinity for cells due to amino (cation) radicals. In addition, because of the large number of carboxyl (anion) radicals inside its molecule, it has a high affinity for water, and because it helps excrete the intracellular water outside immediately when the cells are being frozen, it has a protective effect on cells during cryopreservation [16,17]. Previous reports have shown that since carboxylated PLL is a low-toxicity CPA used as an alternative to DMSO under completely serum-free conditions, it is effective in the cryopreservation of human iPS cells and a large number of cultured cells such as L929, MG63, HT1680, B16F1, Caco2, MC3T3, and KB cells [16–18].

In this study, mouse blastocysts were vitrified by combining the use of Cryo-Nano-Hole vitrification containers and carboxylated PLL, and it was found that by using a VS into which only 15% EG + 10% PLL were added, the resulting viability rate after vitrification, implantation rate, mean total cell count, rate of production of live offspring after transplantation were similar to those obtained with Kitasato Vitri kit which is considered to be in accordance with global standards (using Cryotop and in which 15% EG + 15% DMSO + 0.5 M sucrose were added to the VS) (Table). In addition, the offsprings that were born from the transplantation of mouse blastocysts were all normal, and the observation of the course of their growth showed that there were no individuals with external malformations or behavioral disorders, and that by mating, they had normal reproductive ability, confirming the safety of the vitrification system described in this study. Furthermore, to determine whether this vitrification system can be applied to human assisted reproductive technology, human blastocysts that were handled as waste were vitrified as a preliminary experiment, and the results showed a viability rate of 100% (n=18) after vitrification. The vitrified human blastocysts showed good growth and morphology after 12 h of incubation at warm temperature; there were no morphological changes that indicated damage caused by vitrification, indicating that the method was effective on human blastocysts as it was on mouse embryos (Figure 3).

Interestingly, the conventional vitrification solution procedures requires the use of about 30% intracellular penetrating CPA, and in addition, as extracellular CPA, about 0.5 M (17.1%) of disaccharides such as sucrose or trehalose are needed. These cause an intracellular dehydration and a severe shrinkage of the cells. Then, the osmotic pressure reaches 4000 mOsmol/kg, which is much higher than that of the culture medium (260–300 mOsmol/kg), and which, along with toxicity, causes concern about a possible osmotic shock in the ovum/embryo. On the other hand, because the osmotic pressure reaches lower than 1000 mOsmol/kg by addition of 10% carboxylated PLL, dehydration by removal of free water (which is necessary for vitrification) is performed, but this does not cause a severe shrinkage like during the use of disaccharides such as 0.5 M (17.1%) sucrose or trehalose [18]. Because carboxylated PLL are polyelectrolytes with both cationic and anionic substituents (polyampholytes), they exert a cryoprotective effect due to the carboxylation activity of these 2 substituents; in other words, their protective effect on the cell membrane is due to the cellular affinity of the amino (cation) radicals, the high affinity of carboxyl (anion) radicals for water, and the dehydrating effect on intracellular free water [16,17].

The findings in this study showed the possibility of the clinical use of a complete serum-free vitrification system with low toxicity and low risk of osmotic shock. However, proficient skills and experience are needed for

the preparation and the determination of a stable volume in terms of nanoliters, and the manipulation of embryos under such conditions. In addition, preliminary experiments showed a 100% viability rate of human blastocysts after vitrification, but since the experiments were conducted on as few as 8 samples, supplementary tests on a large number of human blastocysts will be expected to be conducted in the future; and optimization and additional improvement regarding the usage of containers and carboxylated PLL will be expected to accomplish a system, which will allow a safer and highly effective vitrification.

The history of the application of the vitrification technology to human ART is new, and 10 years have passed since the first child-bearing through the vitrification method was reported in 1999 [9]. According to the statistics of the Japan Society of Obstetrics and Gynecology in 2008 [6], the number of treatments using frozen embryos has shown a tendency to increase year after year, and the treatment outcome was better than that using fresh embryos.

In this study, we achieved production of offsprings by combining the use of Cryo-Nano-Hole vitrification containers and PLL in a transplantation of mouse blastocysts, which had been vitrified using a vitrification solution without DMSO or sucrose as additives; and reproduction tests on the offsprings confirmed the safety of the vitrification system described in this study.

This study will potentially open the door to the development of low-toxicity, highly safe, and completely serum-free methods for the vitrification of human ovum, spermatozoids, and embryos. Clinical applications are expected in the future.

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