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Research Article

A Comparison of Open Cryotop Device and Hemi-Straw in Sealed Straw for Mouse Oocytes Vitrification

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Abstract

Mouse oocytes were exposed to an equilibration solution, consisting of Dulbecco's Phosphate Buffered Saline (D-PBS), 7.5% Ethylene Glycol (EG) and 7.5% Dimethyl Sulfoxide (DMSO) for 5 minutes, followed by a vitrification solution consisting of D-PBS, 15% EG, 15% DMSO and 0.6 M sucrose for 30 seconds. They were placed on a Cryotop device and vitrified by direct exposure to liquid nitrogen or on a hemi-straw and vitrified by an exposure to cooled air inside a 0.5-mL straw. After one week of storage in liquid nitrogen, they were warmed and fertilized by Intracytoplasmic Sperm Injection (ICSI), using a Piezo micromanipulator. There was no significant difference in oocytes survival, fertilization rates, and the developmental rate of expanded blastocyst from fertilized oocytes in both groups. The numbers of inner cell mass and trophectoderm cells in both groups were also similar. The fertilization rate of vitrified oocytes and first cleavage rate were comparable to those of non-vitrified controls. However, development from the 4-cell stage up to the expanding blastocyst stage in the vitrified group was significantly lower than those in the control. In conclusion, a hemi-straw closed vitrification device was as effective as the open Cryotop device. Survived oocytes from vitrification could suffer subtle injury that resulted in lower developmental potential than fresh oocytes.

Keywords: Vitrification; Cryotop; Hemi straw closed system; Intracytoplasmic sperm injection

Introduction

Chilling injury, solution effects, and formation of intracellular ice crystal are major causes of cell damage during a slow programmable freezing. Vitrification, which is free from these deleterious effects, is emerging as an attractive alternative to the traditional slow freezing method [1,2]. The first baby from vitrified human oocytes was reported in 1999, using an open-pulled straw method [3]. Several devices have now been employed for oocytes and embryos vitrification in an open system, such as an electron-microscope grid [4], Cryoleaf [5], Cryoloop [6] and Cryotop [7]. Cryotop vitrification has been successfully utilized to cryopreserve human oocytes in many clinics around the world with excellent results, and is now becoming a "benchmark method" for oocyte cryopreservation [8,9].

One drawback of the open system is that microorganisms, such as fungal spores, yeasts, bacteria and viruses, could potentially be transmitted through the liquid nitrogen (LN_2) during freezing or storage [10,11]. To overcome this problem, a closed system of vitrification and storage is recommended. However, there is still controversy whether the survival rate in a closed system is comparable to that of an open vitrification system [12]. In our unit, we used a 0.25 mm hemi-straw as a container, and inserted it into a 0.5 mm straw inside a pre-cooled aluminum cylinder, which was placed in liquid nitrogen before the procedure [13]. With this hemi-straw closed vitrification system (HS-CS), we achieved a significantly higher embryo survival and blastocyst formation rates than those cryopreserved by slow programmable freezing.

The objective of this study was to compare our system with the open Cryotop device, using mouse oocytes as a model. The survival rates of vitrified/warmed oocytes, their fertilization rates by intracytoplasmic sperm injection using Piezo micromanipulation, and subsequent embryo development were investigated.

Materials and Methods

The Animal Ethics Committees of Suranaree University of Technology approved this study. An international and national guideline for ethical conduct in the use of animals for research was followed.

Chemicals and media

All reagents were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA) unless otherwise stated. CZB-HEPES (CZB-H) medium [14] was used as a holding medium, and KSOM medium [15] was used for embryo culture.

Collection and preparation of matured (MII) oocytes

Outbred Institute of Cancer Research (ICR) mice were purchased from the National Animal Institute, Mahidol University, Bangkok, Thailand. They were kept in a temperature- and humidity-controlled room, under a 12-hour light and 12-hour dark cycles. Six- to eight-week old ICR female mice were superovulated by an intraperitoneal injection (IP) of 7.5 IU pregnant mare serum gonadotropin (PMSG; Folligon®, Intervet, UK), followed 48 hours later by an IP injection of 7.5 IU human chorionic gonadotropin (hCG; Chorulon®, Intervet, UK). They were sacrificed by cervical dislocation 14 to 16 hours post hCG injection. A 27G needle was used to dissect and release Cumulus Oocytes-Complexes (COCs) from the ampulla of oviducts. COCs

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Table 1: Survival rates of oocytes vitrified on the Cryotop and HS-CS system and embryo development after piezo-ICSI of vitrified/warmed and fresh oocytes.

Group	Oocytes	Survival rate	Fertilization by ICSI	2-Cell 26 h	4-Cell 38 h	8-Cell 50 h	Morula 62 h	EarBL 80	ExBL 100
Cryotop	160	155/160	110/155	102/110	90/110*	62/110*	43/110*	37/110*	30/110*
		96.86%	70.97%	92.73%	81.82%	56.36%	39.08%	33.64%	27.27%
HS-CS	160	158/160	110/158	104/110	88/110*	62/110*	43/110*	38/110*	32/110*
		98.75%	69.62%	94.55%	80.00%	56.36%	39.08%	34.55%	29.09%
Fresh (control)	160	_	114/160	110/114	100/114*	80/114*	73/114*	68/114*	63/114*
			71.25%	96.49%	87.72%	70.18%	64.05%	59.65%	55.26%

EarBL: Early Blastocyst stage; ExBl: Expanding Blastocysts and Beyond; *indicates *P*<0.05, (Chi-square tests).

Table 2: Cells numbers in vitrification and control groups after differential staining.

Group	Blastocyts (N)	Trophectoderm (TE) Mean ± SD	Inner Cell Mass (ICM) Mean ± SD	Total Cell Mean ± SD	ICM to TE ratio Mean ± SD
Cryotop	10	45.5 ± 11.6	18.0 ± 3.8	63.5 ± 12.3	0.41 ± 0.11
Hemistraw	10	45.4 ± 16.7	18.0 ± 3.9	63.4 ± 19.9	0.42 ± 0.10
Fresh oocytes	10	57.8 ± 14.8	25.2 ± 5.3	83.0 ± 18.0	0.45 ± 0.11

were denuded in CZB-H medium containing 0.2% hyaluronidase. Denuded oocytes were washed five times with CZB-H medium, and pooled in a drop of 75 µL KSOM medium under oil at 37°C in a humidified atmosphere of 6% CO_2 in air. Oocytes were allocated to a non-vitrification group (control) and two vitrification groups. In the control group, oocytes were left undisturbed for 2 h before insemination by Intracytoplasmic Sperm Injection (ICSI). In the vitrification groups, oocytes were immediately vitrified either by the open Cryotop device or hemi-straw in outer straw closed system.

Vitrification and warming

Cryotop vitrification method: Five oocytes were transferred at a time into equilibration solution, consisting of Dulbecco's Phosphate Buffered Saline (D-PBS), 7.5% Ethylene Glycol (EG) and 7.5% Dimethyl Sulfoxide (DMSO) for 5 minutes, and then into vitrification solution consisting of D-PBS, 15% EG, 15% DMSO and 0.6 M sucrose for 30 seconds. The oocytes, with <1µL of vitrification solution, were loaded on the surface of a Cryotop device (Kitazato Supply Co., Fujunomiya, Japan), and immediately immersed into liquid nitrogen (LN_2) and stored for at least 1 week. Vitrified oocytes were warmed by directly immersing the tip of the Cryotop into 3 mL of warm (37 °C) 1.0 M sucrose in D-PBS supplemented with 20% Fetal Bovine Serum (FBS) for 5 minutes, and then into 0.5 M, 0.25 M and 0 M sucrose in D-PBS supplemented with 20% FBS for 5 minutes interval. Oocytes with intact cytoplasm and zona pellucida were regarded as survived. They were washed four times and left in KSOM medium at 37°C under a humidified atmosphere of 6% CO_2 in air for 2 hours before (ICSI).

Vitrification on a hemi-straw closed system (HS-CS)

Our home-made system of vitrification was previously described [13]. In brief, it consisted of two aluminum cylinders. The inner one had eight grooves in a vertical positon that could accommodate eight 0.5-mL straws. It fitted into a cavity in the outer cycliner. The cylinders were immersed in LN_2 for 15 to 20 minutes before use. The 0.5-mL straws were heat sealed at the plug-end and inserted into the holes of the pre-cooled aluminum cylinder. Five oocytes were transferred at a time into the equilibration and vitrification solution, as described in the previous section. Oocytes with <1µL of vitrification solution were loaded onto the tip of a 0.25 mL hemi-straw, which was made by cutting open the lower one-third of the straw and shortened the opposite end so that it could be inserted completely into the outer 0.5-mL straw. In this system, oocytes were vitrified by cooled air inside the outer straw, with no contact with liquid nitrogen. After vitrification, The top end of the outer straw was heat sealed to form a completely closed system. The straws were directly immersed into the LN_2 tank for storage for at least 1 week before warming.

For warming, The outer straw was placed in liquid nitrogen, and cut open at its top end. The hemi-straw, with vitrified oocytes, was removed and immediately immersed into warming solutions at 37 °C, consisting of 1.0 M, 0.5 M, 0.25 M and 0 M sucrose and 20% FBS, at 5 minutes interval, as previously described. Survived oocytes were washed and incubated in KSOM medium at 37 °C in a humidified atmosphere of 6% CO_2 in air for 2 hours before ICSI.

Sperm preparation

Epididymal spermatozoa were collected from 8- to 10-week old ICR male mice. The spermatozoa were placed at the bottom of a 0.50 mL Eppendorf tube, containing 200 µl of CZB-H medium, and left for swim up at 37 °C in a humidified atmosphere of 6% CO_2 in air for 1 hour. Approximately 50 µL of supernatant were collected and suspended in 50 µL of 7% Polyvinylpyrrolidone (PVP) medium in a 90 mm ICSI dish covered with mineral oil.

Piezo- ICSI procedure

A PMAS-CT150 Piezo unit (Prime Tech, Ibaraki, Japan) attached to an IX71 inverted microscope (Olympus, Tokyo, Japan) was used for ICSI. A flat-tip pipette, with an inner diameter of 5.5-7.5 µm and an outer diameter of 6.5-8.5 µm, was used to decapitate the spermatozoon by drawing its head into the injection pipette up to the head-midpiece boundary. Several pulses were applied with a foot switch, using a setting intensity of three, and frequency of six.

A holding pipette (inner diameter 20 µm; outer diameter 90-100 µm) connected to a MO188NE micromanipulator (Narishige, Tokyo, Japan) was used to hold the oocyte gently, such that its metaphase plate is located at the 6 or 12 o'clock position. Five oocytes were injected per dish by drawing five sperm heads into the ICSI pipette at an interval of approximately 100 µm. Under a magnification of 200 x, the oocyte plasma membrane and the tip of the injection pipette were

brought into sharp focus. The flat-tip of the pipette was advanced to contact the surface of the zona and the Piezo driver was activated with the foot switch (intensity = 3, frequency = 3) as the pipette was pushed through the zona until the zona was almost penetrated. The Piezo impact force was then stopped, and the pipette was advanced to complete the opening in the zona. Ejected the zona plug and placed the ICSI pipette in contact with the plasma membrane, and stretched it towards the holding pipette. Activated the footswitch once for a quick pulse with an intensity of one and frequency of one. The plasma membrane visibly relaxed along the shaft of the injection needle, indicating that it had been ruptured. One sperm head was deposited in the oocyte cytoplasm and the ICSI pipette was gently withdrawn. The procedure was repeated until all oocytes were injected. Allowed oocytes to remain in the ICSI dish for 10 minutes to recover, and then washed them four times in KSOM culture medium.

In vitro embryo culture

ICSI-derived embryos were cultured in KSOM medium, at 37 °C under an atmosphere of 6% CO_2 in air. Embryo development was recorded at 26 hours (2-cell), 38 hours (4-cell), 50 hours (8-cell), 62 hours (morula), 80 hours (early blastocyst) and 100 hours (expanded blastocyst) post-ICSI.

Differential staining of blastocysts

Differential staining of the expanded blastocysts was performed as previously described [16], with slight modifications. Briefly, expanded blastocysts were treated with 0.1 mg/mL Propidium Iodide (PI) and 0.2% Triton X-100 dissolved in D-PBS for 35 seconds. They were then exposed to 25 µg/mL Hoechst 33342 in 99.5% ethanol for 4 minutes, and mounted on a glass slide in a glycerol droplet. Expanded blastocysts were flattened by a glass cover slip, and examined under an excitation wavelength of 330-385 nm with an IX71 epifluorescence microscope (Olympus, Tokyo, Japan). The nuclei of the Trophectoderm (TE) cells were stained by both PI and Hoechst solutions, and appeared as red or pink. The nuclei of the Inner Cell Mass (ICM) cells were stained blue only by the Hoechst dye. A digital image of each expanded blastocyst was taken, and the counts of both cell types were recorded. The numbers of ICM and TE were counted separately in expand blastocysts that had clearly distinguishable populations of blue and red nuclei.

Statistical analysis

Chi-square tests were used to compare the proportion of survived oocytes, fertilization rates by Piezo ICSI, and embryo developmental rate up to the expanding blastocysts stage. The average number of nuclei in the blastocysts in each group was compared by Analysis Of Variance (ANOVA), using Statistical Analysis System (SAS; SAS Institute Inc., Carry, NC). Differences between groups were considered to be statistically significant at a probability (P) value of <0.05.

Results

Four hundred and eighty metaphase II oocytes of good quality were collected and divided equally into one control and two vitrification groups (Table 1). There was no significant difference in oocytes survival rates between Cyrotop and HS-CS vitrification groups (P=0.2517). There was also no significant difference in the fertilization rates by Piezo ICSI of oocytes in the two vitrified groups and control (P=0.9436). Neither was there a significant difference in the cleavage rate of fertilized oocytes at 26 hours post-ICSI in the three groups (P=0.4591). Although there was no significant difference in the proportion of embryos that became 4-cell, 8-cell, morula, early blastocysts, and expanding/hatching blastocysts in the two vitrification groups, there was a signficant difference from the control at 38 hours post-ICSI (4-cell stage) onward (P=0.0043 at 38 hours, and P <0.0001 at 50, 62, 80, and 100 hours post-ICSI). There was no significant difference in the mean number of TE (p=0.1087; ANOVA test) cells of expanded blastocysts among the three groups ($P>0.05$, Table 2). The mean number of ICM cells in the two vitrification groups was not statistically different, but it was significantly fewer than that in the control (p=0.0027; Tukey HSD post-hoc tests). Similarly, there was no significant difference in the number of total cells in the expanding blastocysts in the two vitrification groups, but the mean number of cells was significantly fewer than that in the non-vitrified control (P=0.0421; Tukey HSD post-hoc tests). There was no significant difference in the ICM/TE ratio (p=0.6874, ANOVA tests)

Discussion

Current vitrification systems have variations in the composition of equilibration, vitrification, and warming media, equilibration time, carrier devices, cooling and warming parameters, and storing methods. However, they could be broadly classified into two categories: the "open" and the "closed" system [12]. An open system allows a direct contact between the sample and liquid nitrogen, while the closed system separates the sample from liquid nitrogen during the entire cooling, storage and warming procedures [17]. To date, most IVF centers worldwide vitrify embryos and oocytes in an open system [12]. There is still a debate whether one system is better than the others. Advocates of the open system claim that it has a better efficiency and consistency than the closed system, and the risk of disease transmission is nil or very minimal [12,18].

Kuwayama et al. [18] reported that both the open- (Cryotop) and closed- (Cryotip) vitrification system for human blastocysts resulted in similar survival and pregnancy rates (97% vs. 93% and 59% vs. 51%, respectively). In their study, they were comparing the whole system of open or closed vitrification, using different vitrification/warming media, cooling and warming parameters, and different carrier devices. In this study, we compared an open device (Cryotop) with our in-house closed system of vitrification on a hemi-straw, using the same equilibration, vitrification, and warming media, equilibration time, and warming parameters. Similar to their study, we found no difference in mouse oocytes survival rate and the ability of ICSI-derived embryos to develop into blastocysts after vitrification with an open or closed device.

The idea behind the invention of an open container is that a direct contact of the specimen with liquid nitrogen will greatly increase the cooling rate [12]. With a high cooling rate, the concentration of cryoprotectants in the vitrification solutions can be dramatically decreased to a level that is less toxic to oocytes and embryos. It is a popular belief that vitrification requires an extremely high cooling rate of 10,000 °C or more [1]. Since such a high cooling rate cannot be achieved with a closed vitrification system, an open system has been favored despite the concern of potential infectious contamination [12]. Although the speed of cooling during vitrification is important,

there is a limit beyond which an increase in the cooling rate will not result in a higher survival rate at a given concentration of cryoprotectants. A vitrification medium containing 15%-20% EG and 15%-20% DMSO, as used in this and most other vitrification systems, might not require a very high cooling rate to achieve optimum results. Indeed many previous studies had shown that a much slower cooling rate in a hermetically isolated cooling device was enough to obtain appropriate vitrification. Nowshari and Brem [19] showed that increasing the cooling rate from 1,200 °C/minute to 10,300 °C/minute was not beneficial to their mouse embryo vitrification system. A cooling rate of only 400 °C/minute was shown to be effective in the vitrification of mouse embryos in a double straw [20]. For vitrification of human oocytes and embryos, a moderately low cooling rate of 120 °C/minute [21] to 400 °C/minute [22] was shown to be appropriate. In our closed vitrification system, the cooling rate was approximately 900 °C/minute [13].

When compared with non-vitrified control, there was no difference in fertilization rate, cleavage rate of fertilized oocytes, or developmental rate up to 2-cell stage. However, from the 4-cell stage onward there was a significant reduction in embryo developmental potential, which became more obvious at the expanding blastocyst stage or beyond. This suggested that survived oocytes could sustain a subtle cryo-injury to ultra structure, such as the mitochondria or other important organelles that could exert a late negative effect on future embryonic development [23].

In conclusion, our closed vitrification system is as effective as the Cryotop open device for mouse oocytes vitrification. It is simple and easy to operate, and also eliminates or minimizes the risk of contamination. Moreover, it is much cheaper than the commercial devices and may be suitable for use in developing countries where resource is limited. Further study should be done on discarded human oocytes, before the technique can be adapted for human use in the IVF laboratory.

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