The effect of minimal concentration of ethylene glycol (EG) combined with polyvinylpyrrolidone (PVP) on mouse oocyte survival and subsequent embryonic development following vitrification

Yao Wang · Osamu Okitsu · Xiao-Ming Zhao · Yun Sun ·
Wen Di · Ri-Cheng Chian

Received: 27 August 2013 / Accepted: 29 October 2013 / Published online: 21 November 2013
© Springer Science+Business Media New York 2013

Abstract

Purpose Vitrification techniques employ a relatively high concentration of cryoprotectant in vitrification solutions. Exposure of oocytes to high concentrations of cryoprotectant is known to damage the oocytes via both cytotoxic and osmotic effects. Therefore, the key to successful vitrification of oocytes is to strike a balance between the usage of minimal concentration of cryoprotectant without compromising their cryoprotective actions.

Methods The minimal concentration of ethylene glycol (EG) on mouse oocyte survival and subsequent embryonic development was evaluated following vitrification-warming and parthenogenetic activation. Polyvinylpyrrolidone (PVP) combined with EG on mouse oocyte survival and subsequent embryonic development as well as morphology of the spindle

and chromosome alignment were also evaluated. Vitrification system was adapted with JY Straw and the cooling rate was approximately 442–500 °C/min. In contrast, the warming rate was approximately 2,210–2,652 °C/min.

Results Survival rate of oocytes increased significantly when 15 % EG was combined with 2 % PVP in vitrification solution (VS). The effect of combination of EG and PVP was not significant when the concentration of EG was 20 % and higher. Although there were no significant differences in embryonic development, the percentage of abnormal spindle and chromosome alignment was significantly higher in the oocytes without 2 % PVP in VS.

Conclusions Our data provide a proof of principle for oocyte vitrification that may not require a high concentration of cryoprotectant. There are synergic effects of EG combined with PVP for oocyte vitrification, which may provide important information to the field in developing less cytotoxic VS.

Capsule Oocyte vitrification may not require a high concentration of cryoprotectant. There are synergic effects of EG combined with PVP for oocyte vitrification.

Y. Wang · X.-M. Zhao · Y. Sun · W. Di (✉)
Center for Reproductive Medicine, Key Laboratory for Assisted
Reproduction and Genetics, Department of Obstetrics and
Gynecology, Ren Ji Hospital, School of Medicine, Shanghai Jiao
Tong University, Shanghai, China
e-mail: diwen163@163.com

Y. Wang · R.-C. Chian (✉)
Division of Reproductive Biology, Department of Obstetrics and
Gynecology, McGill University, Montreal, Canada
e-mail: ri-cheng.chian@mcgill.ca

O. Okitsu
Division of Reproductive Medicine, Miyage Clinic, Okayama, Japan

R.-C. Chian
State Key Laboratory of Reproductive Medicine, Center for Clinical
Reproductive Medicine, The First Affiliated Hospital of Nanjing
Medical University, Nanjing, China

Keywords Vitrification · Cryoprotectant · Concentration ·
Oocytes · Embryonic development

Introduction

Cryopreservation of oocytes, embryos and ovarian tissues are the three main options in female fertility preservation. Each option has its own advantages and disadvantages. Oocyte cryopreservation can be applied to women who have no male partner, object to the use of donor sperm, or do not agree with embryo cryopreservation for various personal, financial, religious or moral reasons. In addition, to be a fertility preservation option for young women undergoing potentially sterilizing medical and surgical treatments, cryobanking of oocytes can also benefit a large population of single women who wish to delay motherhood. However, oocyte cryopreservation is not

a viable option for pre-pubescent girls. In their cases, ovarian tissue cryopreservation is the only remaining option [16, 17].

Slow-freezing methods that were considered the gold standard for embryo cryopreservation were applied in initial attempts in freezing oocytes. However, the slow-freezing methods for oocyte cryopreservation were met with low survival rates [31, 40, 41]. Fortunately, with recent advance of vitrification techniques, there has been an important improvement in the efficacy of oocyte cryopreservation in terms of survival and pregnancy rates [1, 4–7, 9, 10, 20, 27, 33, 41]. It now appears that oocyte vitrification can clinically be applied as a standard procedure for female fertility cryopreservation [12, 38].

Although oocyte vitrification leads to pregnancy and live birth rates comparable to those achieved in fresh oocytes for in vitro fertilization (IVF) [38], there are concerns about the technology used for oocyte vitrification in the relatively high concentration of cryoprotectant, such as ethylene glycol (EG) and dimethyl sulphoxide (DMSO) or propylene glycol (1, 2-propandiol, PROH). Exposure of oocytes to high concentration of cryoprotectants is known to damage the oocytes via both cytotoxic and osmotic effects [18]. Currently there is no standard protocol applied to oocyte vitrification. It seems that all present oocyte vitrification protocols use a relatively high concentration of cryoprotectant compared to slow-freezing protocols. Therefore, the key to successful vitrification of oocytes is to strike a balance between the use of minimal concentration of cryoprotectant without compromising their cryoprotective actions.

Polyvinylpyrrolidone (PVP) has been used as an additive in the freezing solution and successfully produced the first live birth following a cryopreservation of 8-cell stage mouse embryos [45]. However, PVP is not widely used for cryopreservation, because it has been reported that the effect of PVP on its cryoprotective function does not seem significant [11, 37]. In addition, there has been an attempt to replace human serum albumin (HSA) with PVP for a vitrification of mouse 8-cell stage embryos with unclear results [39]. PVP tends to increase viscosity of the freezing solution and also form interactions through hydrogen bonding water, thereby decreasing the propensity for ice crystal formation [14]. Recently, PVP has been used in conjunction with cryoprotectant to freeze bovine oocytes in order to improve and increase survival rates [2]. However, the exact functional role of PVP in freezing solutions during vitrification is still unclear.

The objectives of the present study are to determine the effect of minimal concentration of EG on oocyte survival and subsequent embryonic development following vitrification-warming. In addition, the effect of PVP combined with EG on oocyte survival and subsequent embryonic development following vitrification-thawing were also evaluated.

Materials and methods

Chemicals

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

Animals

Mice (CD1, females 8–10 weeks old) were used in this study. The mice were housed in a temperature- and light-controlled room with free access to food and water under a photoperiod of 12 h in the light and 12 h in the dark. The experimental protocols and animal handling procedures were reviewed and approved by Animal Ethics Committee of McGill University.

Source of oocytes

Female CD1-strain mice were subjected to superovulation protocol involving intraperitoneal (IP) injection of 5 IU pregnant mare's serum gonadotropin (PMSG) followed 48 h later by IP injection of 5 IU human chorionic gonadotropin (HCG). The mice were killed 14 h after HCG injection, and the oviducts were dissected and placed into a Petri dish containing modified human tubal fluid (m-HTF-HEPES) [32] supplemented with 1.0 mg/mL bovine serum albumin (BSA). The cumulus-oocyte complexes (COCs) were released by tearing the ampullae of the oviducts. The cumulus cells were removed enzymatically using 75 U/mL hyaluronidase and by mechanical dissociation using a glass pipette. The denuded oocytes were then washed with m-HTF-HEPES at room temperature before being subjected to cryopreservation experiments. The denuded oocytes were then assessed for their meiotic maturation status. Only morphologically normal metaphase II (MII) stage oocytes as judged by the presence of a first polar body (PB1) were selected in the study.

Cooling and warming procedures

After 1 h of oocyte denuding, the oocytes were suspended in Dulbecco's phosphate buffered saline (DPBS) based equilibration solution (ES) for 3 min at room temperature (RT) and then transferred to a DPBS based vitrification solution (VS) for 1 min at room temperature. The oocytes were immediately loaded onto JY Straw (JieYing Laboratory Inc, Longueuil, Quebec, Canada) with a straw cover (Fig. 1), and then gently plunged into liquid nitrogen (LN₂) for vitrification. The loading section of this device does not contact LN₂ directly. Therefore, the cooling speed (Fig. 2a) inside of JY Straw has been measured using a special Thermometer (www.anritsu-meter.co.jp) with a probe (BS-11 K-015-TS1-ANP) and the cooling rate was calculated as approximately 442–500 °C/min. In contrast, the cooling speed (Fig. 2b) was also

Fig. 1 Structure of JY Straw being used in the experiments. **a** JY Straw has two parts: an oocyte loading part and a straw cover part; **(b)** After putting the straw cover tightly, JY Straw is gently plunged in LN₂. This is not a device where the loading part contacts LN₂ directly. There is a space between the loading part and the straw cover. Therefore, the cooling rate may not be as high as when a direct contact to LN₂ is performed

measured when the probe directly plunged into LN₂, and the cooling rate was calculated as approximately 1,326–1,500 °C/min.

For the thawing procedure, the loading part of JY Straw was directly inserted into a DPBS based thawing solution (TS) containing 1.0 M sucrose at room temperature for 3 min. The warming rate (Fig. 3) has also been measured, and calculated as approximately 2,210–2,652 °C/min. Then the oocytes were transferred to 0.5 M and 0.25 M sucrose solution for 3 min respectively. Finally the oocytes were washed twice in washing medium for 3 min before the transfer to 5 % CO₂ incubator at 37 °C for culture.

Survival assessment of oocytes after vitrification-warming

Survival of the oocytes was assessed morphologically based on plasma membrane integrity and discoloration of the ooplasm after the oocytes were recovered from vitrification-warming procedures.

Embryonic development of oocytes following parthenogenetic activation

The oocytes that survived were activated parthenogenetically by exposure to 8.5 mM strontium chloride in calcium-free KSOM medium for 2 h based on the methodology described by Huang et al. [19] and then the oocytes were transferred to 1.5 mM Cytochalasin B (CB) in mHTF medium containing 3.0 mg/mL BSA for 2 h of culture. The oocytes were washed twice and then placed into 50 µL microdrops under mineral oil in modified KSOM containing 1.0 mg/mL BSA (mKSOM) [23] for the embryonic developmental culture. After 24 h of parthenogenetic activation, whether the oocytes activated or not were confirmed by 2-cell cleavage. The cleaved embryos were cultured until day 5 (120 h after activation) without

Fig. 2 Cooling curve of JY Straw for oocyte vitrification. **a** The cooling rate of the inside of JY Straw after plunging it into LN₂. The drop of temperature from 25 °C to −196 °C required approximately 26–30 s. Therefore, the cooling rate was approximately 442–500 °C/min; **(b)** In contrast, the cooling rate was approximately 1,326–1,500 °C/min when the loading part was inserted into LN₂ in direct contact without the straw cover

changing the medium. At the end of the culture, the percentage of blastocyst formation was assessed.

Observation of meiotic spindle and chromosome alignment of oocytes

After vitrification-warming, the oocytes that survived were fixed for immune-fluorescent staining of tubulin and chromatin for ultrastructure analysis as described by Liu and Keefe [25]. Briefly, after thawing, the oocytes were incubated in 5.0 % CO₂ incubator at 37°C for 2 h, and then the oocytes were fixed in 4 % paraformaldehyde for 5 min. Following the fixation, the oocytes were washed in Dulbecco's phosphate-buffered saline (DPBS) and transferred to 0.01 % Triton X-

Fig. 3 Warming curve of JY Straw for oocyte thawing. The loading part of JY Straw was inserted into 25 °C Thawing Solution (TS) directly. An increase of temperature from −196 °C to 25 °C required approximately 4–5 s. Therefore, the warming rate was approximately 2,210–2,652 °C/min

100 in DPBS for 40 min at room temperature. The oocytes were then washed extensively and blocked at 4 °C in a wash medium (DPBS supplemented with 0.02 % NaN_3 , 0.2 % nonfat dry milk, 2 % goat serum, 2 % BSA, and 0.1 mol/L glycine) for 45 min at room temperature. After rinsing with DPBS, the oocytes were incubated with β -tubulin monoclonal antibody diluted 1:500 in DPBS at 4 °C overnight. Then,

washing twice in DPBS, the microtubulins were stained with fluorescein isothiocyanate (FITC)–conjugated antimouse IgG (Molecular Probes, Eugene, OR, USA) diluted 1:320 in DPBS for 60 min in the dark at room temperature. Subsequently, washing three times in DPBS, the oocytes were mounted onto a slide under a coverslip in the Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA), containing 4,6-diamidino-2-phenylindole (DAPI).

Oocytes images were acquired using a Zeiss LSM780 confocal microscope with a Dioid (405 nm) laser for a nuclear labeling (DAPI) and an Argon multi-line (488 nm) laser for a spindle (FITC). The imaging used was 63 $\times$ /1.40 oil DIC Plan-Apochromat objective and analyzed with LSM 5 Image Browser or Image J. The localization of tubulin and chromatin was revealed by the green signal of FITC and the blue signal of DAPI, respectively. Morphologically normal meiotic spindles and chromosome alignments were defined as bipolar spindle/aligned chromosomes: bipolar organization, with pointed or flattened poles, and microtubules converging at both poles and all chromosomes present and evenly aligned at the equatorial plate (Fig. 4a). Otherwise, the meiotic spindles and chromosome configuration were considered to be abnormal. It usually appeared as a disarranged or multipolar spindle/nonaligned chromosomes (Fig. 4b); bipolar spindles, clusters of disorganized microtubules, and chromosomes with varying degree of misalignment (Fig. 4c); severely

Fig. 4 Normal and abnormal meiotic spindles and chromosome alignments of the oocytes. **a** Oocyte with normal microtubules converging at both poles and all chromosomes presented and evenly aligned at the equatorial plate; **(b)** Oocyte with abnormal multipolar spindles and multiple chromosomes appeared; **(c)** Oocyte with abnormal bipolar spindles, clusters of disorganized microtubules, and chromosomes with varying degree of misalignment; **(d)** Oocyte with abnormal severely disarranged or absent spindle/dispersed or absent chromosomes

disarranged or absent spindle/dispersed or absent chromosomes (Fig. 4d).

Experimental design

All solutions were prepared using base medium, DPBS supplemented with 20 % fetal bovine serum (FBS, heat inactivated). Following vitrification, the oocytes were stored in LN₂ at least for 1 week before thawing for the experiments.

Effect of EG concentrations on oocyte survival following vitrification-warming The oocytes were equilibrated in the following ES containing 0.00 %, 1.88 %, 3.75 %, 7.50 % and 15.00 % EG, respectively. The oocytes were then transferred to the following VS containing 0.50 M sucrose with 3.75 %, 7.50 %, and 15.00 % and 30.00 % EG for vitrification, respectively. After warming, the oocyte survival rates were compared in each group.

Effect of PVP concentrations combined with 15.00 % EG on oocyte survival following vitrification-warming The oocytes were equilibrated in ES containing 7.50 % EG, and vitrified in VS contained 0.5 M sucrose and 15.00 % EG with 0.0 %, 0.5 %, 1.0 % and 2.0 % PVP, respectively. After warming, the oocyte survival rates were compared in each group.

Effect of EG concentrations combined with 2.00 % PVP on survival of oocytes following vitrification-warming The oocytes were equilibrated in ES containing 7.50 %, 10.00 %, 12.50 % and 15.00 % EG, respectively. Then, accordingly, the oocytes were transferred in VS containing 0.50 M sucrose with 15.00 %, 20.00 %, 25.00 % and 30.00 % EG, respectively. Each concentration was divided into with and without 2.00 % PVP. After warming, the oocyte survival rates were compared in each group.

Effect of 2.00 % PVP on embryonic development following vitrification-warming and parthenogenetic activation The oocytes were equilibrated in ES containing 7.5 % EG, and then the oocytes were vitrified in VS containing 0.5 M sucrose and 15.0 % EG with and without 2.0 % PVP. After warming, the oocyte survival rates were compared, and then the oocytes were artificially activated to compare the embryonic development.

Effect of 2.00 % PVP on spindle and chromosome alignment of oocytes following vitrification-warming The oocytes were equilibrated in ES containing 7.5 % EG, and then vitrified in VS containing 0.5 M sucrose and 15.0 % EG with and without 2.0 % PVP. After warming, the oocytes were fixed and stained for spindle and chromosome alignment observation.

Statistical analysis

Statistical analyses were performed with SPSS 16.0 software. Student *t*-test was used to analyze the differences in the percentages of oocyte survival, embryonic development and abnormality. *P* value of <0.05 was considered to be statistically significant differences.

Results

Effect of EG concentrations on oocyte survival following vitrification-warming

As shown in Table 1, the minimal functional concentration of EG was 7.5 % in VS for oocytes following vitrification-warming. The survival rates of the oocytes in final concentration of 7.5 %, 15.0 % and 30.0 % EG were 49.3 % (68/138), 75.0 % (105/140) and 93.6 % (132/141), respectively. There were significant differences among the groups (*P* < 0.01).

Effect of PVP concentrations combined with 15.00 % EG on oocyte survival following vitrification-warming

The survival rates of oocytes were 73.3 % (85/116), 77.5 % (86/111), 80.4 % (86/107) and 92.5 % (112/121) respectively when the final concentration of 15 % EG in VS combined with 0.0 %, 0.5 %, 1.0 % and 2.0 % PVP, respectively (Table 2). Although there were no differences in the survival rates of oocytes after vitrification-warming among 0.0 %, 0.5 % and 1.0 % PVP groups, those were significantly different (*P* < 0.05) compared to 2.0 % PVP group.

Effect of EG concentrations combined with 2.00 % PVP on survival of oocytes following vitrification-warming

As shown in Table 3, there were significant differences (*P* < 0.05) in the oocyte survival rates in the final concentration of 15 % EG in VS with (91.4 % = 74/81) and without (80.5 % =

Table 1 Effect of EG minimal concentration on survival of mouse oocytes following vitrification-warming (6 replicates)

Concentrations of EG (%)	No. of oocytes vitrified	No. of oocytes survived (%)
0.00	60	0 (0.0)
3.75	60	0 (0.0)
7.50	138	68 (49.3) ^a
15.00	140	105 (75.0) ^b
30.00	141	132 (93.6) ^c

^{abc} Significantly different

Table 2 Effect of PVP minimal concentration combined with 15 % EG on survival of mouse oocytes following vitrification-warming (10 replicates)

Concentrations of PVP (%)	No. of oocytes vitrified	No. of oocytes survived (%)
0.0	116	85 (73.3) ^a
0.5	111	86 (77.5) ^a
1.0	107	86 (80.4) ^a
2.0	121	112 (92.5) ^b

Different superscripts in the column indicate significant differences ($P < 0.05$)

62/77) 2.0 % PVP. However, there were no differences in the survival rates of oocytes after vitrification-warming when the final concentration of 20.0 %, 25.0 % and 30.0 % EG, regardless of with and without 2.0 % PVP for oocyte cryopreservation.

Effect of 2.00 % PVP on embryonic development following vitrification-warming and parthenogenetic activation

The survival rates of oocytes following vitrification-warming were significantly different ($P < 0.05$) when the final concentration of 15.0 % EG in VS with (91.4 % = 203/222) and without (75.3 % = 192/255) 2.0 % PVP (Table 4). There were no differences in the activation rates of oocytes with (98.5 % = 130/132) and without (96.9 % = 126/130) PVP. Also, there were no differences for embryonic development to 8-cell stage (40.0 % = 52/130 vs. 32.5 % = 41/126) and blastocyst stage (30.0 % = 39/130 vs. 21.4 % = 27/41) in the two groups. However, the percentages of embryonic development to 8-cell stage and blastocyst stage were significantly reduced ($P < 0.05$) compared to fresh oocytes (87.6 % = 99/113 and 81.4 % = 92/113, respectively).

Table 3 Effect of EG concentrations combined with 2.0 % PVP on survival of mouse oocytes following vitrification-warming (4 replicates)

Concentrations of EG (%)	With (+) or without (–) PVP	No. of oocytes vitrified	No. of oocytes survived (%)
15.0	+	81	74 (91.4) ^a
	–	77	62 (80.5) ^b
20.0	+	78	72 (92.3) ^a
	–	80	68 (85.0) ^a
25.0	+	75	69 (92.0) ^a
	–	76	68 (89.5) ^a
30.0	+	78	75 (96.2) ^a
	–	79	72 (91.1) ^a

Different superscripts in each group indicate significant differences ($P < 0.05$)

Effect of 2.00 % PVP on spindle and chromosome alignment of oocytes following vitrification-warming

As shown in Table 5, the percentages of normal spindle and chromosome alignment of oocytes were 70.3 % (26/37) and 45.5 % (15/33) respectively when the oocytes vitrified with and without 2.00 % PVP in 15.0 % EG of VS plus 0.50 M sucrose. There were significant differences ($P < 0.05$) between oocytes vitrified with and without 2.0 % PVP. Accordingly, the abnormal morphology of oocytes following vitrification-warming with (29.7 % = 11/37) and without (54.5 % = 18/33) were also significant differences ($P < 0.05$). In addition, both oocytes vitrified with and without 2.0 % PVP, the percentages of abnormal spindle and chromosome alignment were significantly increased compared to the fresh oocytes without vitrification-warming (8.6 % = 3/35).

Discussion

The present study demonstrates that the minimal concentration of 15.0 % EG as a cryoprotectant combined with 2.0 % PVP can vitrify mouse oocytes successfully using JY Straw system in terms of survival, embryonic developmental and morphological observation.

Almost all in the field have believed that the cooling rate is the most critical factor for oocyte vitrification. In order to accomplish this, a variety of devices that can obtain relatively high cooling rates have been used. Initially, replacement devices, such as electron microscope (EM) grid [28, 46], open pull straw (OPS) [42, 43], nylon mesh [29], cryoloop [22], or plastic sticker [3] were employed to plunge the devices into LN₂ directly. Later on, commercial devices, such as the cryotop [3, 21] and cryoleaf [4, 6] were developed, in which the vitrified samples were able to make direct contact with LN₂ in order to obtain a high speed of cooling rate, such as >20,000 °C/min, for vitrification [21].

Until recently, it was unknown what the optimal cooling and warming rates with a very small amount (<1.0 µl) of solution and samples should be [24]. Recent studies with mouse models indicate that the lethality of slow warming is a consequence of the growth of small intracellular ice crystals by re-crystallization [34, 35]. This means that the cooling rate is of less consequence than the warming rate during vitrification and warming procedures [30]. Interestingly, these authors also demonstrated that a very high warming rate relative to a high cooling rate is the essential element to survival in a vitrification procedure [36]. The results from the present study supports their results that oocyte vitrification may not require such a high cooling speed.

Table 4 Effect of 2 % PVP combined with 15.0 % EG on mouse oocyte survival and embryonic development following vitrification-warming and parthenogenetic activation (10 replicates)

With (+) or without (–) PVP	No. of oocytes vitrified	No. of oocytes survived (%)	No. of oocytes used for activation	No. of oocytes activated (%)	No. of 8-cell stage embryos	No. of blastocysts developed (%)
+	222	203 (91.4) ^a	132	130 (98.5) ^a	52 (40.0) ^a	39 (30.0) ^a
–	255	192 (75.3) ^b	130	126 (96.9) ^a	41 (32.5) ^a	27 (21.4) ^a
Fresh	—	—	114	113 (99.1) ^a	99 (87.6) ^b	92 (81.4) ^b

Different superscripts in each column indicate significant differences ($P < 0.05$)

The toxicity of cryoprotectant has been recognized as a critical barrier to further advancement of the field. In fact, the toxicity of cryoprotectant has been considered as the most limiting factor to development of successful cryopreservation protocols for challenging cells and tissues [13]. The toxicity of cryoprotectant can be considered by its chemical self and the change of osmolarity of solution after adding the cryoprotectant into the vitrification solution, which affect directly microtubule assembly/disassembly, inducing a disorganization of the spindle microtubules and chromosomal scattering as well as microfilament network [15].

The strategy to deal with reducing the toxicity of cryoprotectant is to employ less concentration of cryoprotectant in the vitrification solution but not to reduce their efficiency. Therefore, the lower the concentration of cryoprotectant will be, the lower the toxicity of the cryoprotectant, which may decrease the detrimental effect to cells or tissues without reducing the functional role of cryoprotectant. Interestingly, a recent report indicated that the high survival of mouse oocytes obtained after vitrification with a high warming rate in lower concentration of a standard slow-freezing solution suggested that the cooling rate is relatively of less consequence compared to the warming rate [36]. The results from our study also indicates that the high survival rate of oocytes can be achieved after vitrification and warming with relatively lower concentration of cryoprotectant (ie, EG) combined with 2.0 % PVP.

PVP is the soluble homopolymer of N-vinyl-2-pyrrolidone, and is sometimes called povidone. PVP is one of the most commonly used polymers in medicine because of its solubility in water and its low cytotoxicity [26]. It has been used widely in pharmaceutical and food industries because it possesses excellent characteristic properties, acting as a vehicle for drugs and as a plasma expander as well as a food additive. It has been known that cells of the reticuloendothelial (RES) could retain PVP, resulting in incorporation into macrophages by pinocytosis [44]. PVP is not only frequently added to medium as a substitute for macromolecules in serum, but are also useful for preventing loss of oocytes and embryos due to sticking to the glass or plastic surface of culture dishes or handling tools [8].

Although PVP was shown to have a low cytotoxicity, there were few papers about its function on vitrification [2, 11, 14, 37, 39, 45]. The results of the present study demonstrate that addition of 2.0 % PVP into the vitrification solution is not only increasing the survival rate of oocytes after vitrification-warming, but also is beneficial to the micro-spindle and chromosome alignment of oocytes, resulting in higher embryonic development. It is not clear about the mechanism of PVP in preventing the damage from vitrification and warming procedures. This may need further investigation in the future.

In conclusion, the mouse oocytes can be vitrified successfully by relatively slow cooling rate with high survival rate using a minimal concentration of 15 % EG combined with

Table 5 Effect of 2 % PVP combined with 15.0 % EG on spindle and chromosome alignment of mouse oocyte following vitrification-warming

Frozen with (+) or without (–) PVP	No. of oocytes examined	No. of oocytes with (%):				
		Normal morphology	Abnormal morphology			
			A	B	C	Total
+	37	26 (70.3) ^a	1 (2.7) ^a	3 (8.1) ^a	7 (18.9) ^a	11 (29.7) ^a
–	33	15 (45.5) ^b	5 (15.2) ^b	3 (9.1) ^a	10 (30.2) ^b	18 (54.5) ^b
Fresh	35	32 (91.4) ^c	0 (0.0) ^a	1 (2.9) ^a	2 (5.7) ^c	3 (8.6) ^c

Different superscripts indicate the significant differences in each column ($P < 0.05$)

2.0 % PVP. Further understanding of the principle of vitrification is required in the field. Therefore, there is still a room for improvement of oocyte vitrification technology.

Acknowledgements We thank Dr. Bao-Zeng Xu for technical assistance for immune-fluorescent staining of tubulin and chromosomes. YW was supported by the Key Program for Basic Research of the Science and Technology Commission of Shanghai Municipality, China (Grant No. 12JC1405800).

Authors' roles YW performed the experiments and analyzed data. OO performed the experiment of measuring cooling and warming rates. XMZ, YS assisted the experiments and helped to analyze the data. WD designed study, analyzed the experiments, RCC designed study, analyzed the experiments, and wrote the manuscript.

Funding This study was supported by McGill Reproductive Center, McGill University Health Center (MUHC) and a grant from China Natural Science Foundation (No. 81270746) to RCC.

Conflict of interest YW, OO, XMZ, YS, WD have nothing declared. RCC designed and invented JY Straw.

References

- Cai LB, Qian XQ, Wang W, Mao YD, Yan ZJ, Liu CZ, et al. Oocyte vitrification technology has made egg-sharing donation easier in China. *Reprod Biomed Online*. 2012;24:186–90.
- Checura CM, Seidel Jr GE. Effect of macromolecules in solutions for vitrification of mature bovine oocytes. *Theriogenology*. 2007;67: 919–30.
- Chian RC, Kuwayama M, Tan L, Tan J, Kato O, Nagai T. High survival rate of bovine oocytes matured in vitro following vitrification. *J Reprod Dev*. 2004;50:685–96.
- Chian RC, Son WY, Huang J, Cui S, Buckett WM, Tan SL. High survival rates and pregnancies of human oocytes following vitrification: preliminary report. *Fertil Steril*. 2005;84 Suppl 1:S36.
- Chian RC, Huang JYJ, Tan SL, Lucena E, Saa A, Rojas A, et al. Obstetric and perinatal outcome in 200 infants conceived from vitrified oocytes. *Reprod Biomed Online*. 2008;16:608–10.
- Chian RC, Gilbert L, Huang JYJ, Demirtas E, Holzer H, Benjamin A, et al. Live birth after vitrification of in vitro matured human oocytes. *Fertil Steril*. 2009;91:372–6.
- Chian RC, Gilbert L, Huang JYJ, Son WY, Holzer H, Cui SJ, et al. Obstetrical outcomes following vitrification of oocytes matured in vivo or in vitro. *Fertil Steril*. 2009;91:2391–8.
- Chung JT, Tosca L, Huang TH, Niwa K, Chian RC. The effect of polyvinylpyrrolidone on bovine oocyte maturation in vitro and subsequent fertilization and embryonic development. *Reprod Biomed Online*. 2007;15:198–207.
- Cobo A, Meseguer M, Remohí J, Pellicer A. Use of cryo-banked oocytes in an ovum donation programme: a prospective, randomized, controlled, clinical trial. *Hum Reprod*. 2010;25: 2239–46.
- Cobo A, Remohí J, Chang CC, Nagy ZP. Oocyte cryopreservation for donor egg banking. *Reprod Biomed Online*. 2010;23:341–6.
- Dumoulin JC, Bergers-Janssen JM, Pieters MH, Engelsu ME, Geraedts JP, Evers JL. The protective effects of polymers in the cryopreservation of human and mouse zona pellucida and embryos. *Fertil Steril*. 1994;62:793–8.
- Edgar DH, Gook DA. A critical appraisal of cryopreservation (slow cooling versus vitrification) of human oocytes and embryos. *Hum Reprod Update*. 2012;18:536–54.
- Fahy GM, Levy DI, Ali SE. Some emerging principles underlying the physical properties, biological actions, and utility of vitrification solutions. *Cryobiology*. 1987;24:196–213.
- Fuller BJ. Cryoprotectants: the essential antifreezes to protect life in the frozen state. *Cryo Letters*. 2004;25:375–88.
- Galeati G, Spinaci M, Vallorani C, Buccì D, Porcu E, Tamanini C. Pig oocyte vitrification by cryotop method: effects on viability, spindle and chromosome configuration and in vitro fertilization. *Anim Reprod Sci*. 2011;127:43–9.
- Gook DA. History of oocyte cryopreservation. *Reprod Biomed Online*. 2011;23:281–9.
- Gook DA, Edgar DH. Human oocyte cryopreservation. *Hum Reprod Update*. 2007;13:591–605.
- Huang J, Tan SL, Chian RC. Fertility preservation for female. *J Reprod Contracep*. 2006;17:109–28.
- Huang JY, Chen HY, Tan SL, Chian RC. Effect of choline-supplemented sodium-depleted slow freezing versus vitrification on mouse oocyte meiotic spindles and chromosome abnormalities. *Fertil Steril*. 2007;88:1093–100.
- Katayama KP, Stehlik J, Kuwayama M, Kato O, Stehlik E. High survival rate of vitrified human oocytes results in clinical pregnancy. *Fertil Steril*. 2003;80:223–4.
- Kuwayama M, Vajta G, Kato O, Leibo SP. Highly efficient vitrification method for cryopreservation of human oocytes. *Reprod Biomed Online*. 2005;11:300–8.
- Lane M, Schoolcraft WB, Gardner DK. Vitrification of mouse and human blastocysts using a novel cryoloop container-less technique. *Fertil Steril*. 1999;72:1073–8.
- Lawitts JA, Biggers JD. Culture of preimplantation embryos. *Methods Enzymol*. 1993;225:153–64.
- Leibo SP, Pool TB. The principal variables of cryopreservation: solutions, temperatures, and rate changes. *Fertil Steril*. 2011;96: 269–76.
- Liu L, Keefe DL. Ageing-associated aberration in meiosis of oocytes from senescence-accelerated mice. *Hum Reprod*. 2002;17:2678–85.
- Lopes CM, Felisberti MI. Mechanical behaviour and biocompatibility of poly(1-vinyl-2-pyrrolidinone)-gelatin IPN hydrogels. *Biomaterials*. 2003;24:1279–84.
- Lucena E, Bernal DP, Lucena C, Rojas A, Moran A, Lucena A. Successful ongoing pregnancies after vitrification of oocytes. *Fertil Steril*. 2006;85:108–11.
- Martino A, Songsasen N, Leibo SP. Development into blastocysts of bovine oocytes cryopreserved by ultra-rapid cooling. *Biol Reprod*. 1996;54:1059–69.
- Matsumoto H, Jiang JY, Tanaka T, Sasada H, Sato E. Vitrification of large quantities of immature bovine oocytes using nylon mesh. *Cryobiology*. 2001;42:139–44.
- Mazur P, Seki S. Survival of mouse oocytes after being cooled in a vitrification solution to -196°C at 95° to $70,000^{\circ}\text{C/min}$ and warmed at 610° to $118,000^{\circ}\text{C/min}$: A new paradigm for cryopreservation by vitrification. *Cryobiology*. 2011;62:1–7.
- Porcu E, Fabbri R, Seracchioli R, Ciotti PM, Magrini O, Flamigni C. Birth of a healthy female after intracytoplasmic sperm injection of cryopreserved human oocytes. *Fertil Steril*. 1997;68:724–6.
- Quinn P, Moinipanah R, Steinberg JM, Weathersbee P. Successful human in vitro fertilization using a modified human tubal fluid medium lacking glucose and phosphate ions. *Fertil Steril*. 1995;63: 922–4.
- Rienzi L, Cobo A, Paffoni A, Scarduelli C, Capalbo A, Vajta G, et al. Consistent and predictable delivery rates after oocyte vitrification: an observational longitudinal cohort multicentric study. *Hum Reprod*. 2012;27:1606–12.

34. Seki S, Mazur P. Effect of warming rate on the survival of vitrified mouse oocytes and on the recrystallization of intracellular ice. *Biol Reprod.* 2008;79:727–37.
35. Seki S, Mazur P. The dominance of warming rate over cooling rate in the survival of mouse oocytes subjected to a vitrification procedure. *Cryobiology.* 2009;59:75–82.
36. Seki S, Mazur P. Ultra-rapid warming yields high survival of mouse oocytes cooled to -196°C in dilutions of a standard vitrification solution. *PLoS One.* 2012;7:6058.
37. Shaw JM, Kuleshova LL, MacFarlane DR, Trouson AO. Vitrification properties of solutions of ethylene glycol in saline containing PVP, ficoll, or dextran. *Cryobiology.* 1997;35: 219–29.
38. The Practice Committees of the American Society for Reproductive Medicine and the Society for Assisted Reproductive Technology. Mature oocyte cryopreservation: a guideline. *Fertil Steril.* 2013;99: 37–43.
39. Titterton JL, Robinson J, Killick SR, Hay DM. Synthetic and biological macromolecules: protection of mouse embryos during cryopreservation by vitrification. *Hum Reprod.* 1995;10:649–53.
40. Tucker M, Wright G, Morton P, Shanguo L, Massey J, Kort H. Preliminary experience with human oocyte cryopreservation using 1,2-propanediol and sucrose. *Hum Reprod.* 1996;11:1513–5.
41. Tucker MJ, Morton PC, Wright G, Sweitzer CL, Massey JB. Clinical application of human egg cryopreservation. *Hum Reprod.* 1998;13: 3156–9.
42. Vajta G, Holm P, Greve T, Callesen H. Vitrification of porcine embryos using the Open Pulled Straw (OPS) method. *Acta Vet Scand.* 1997;38:349–52.
43. Vajta G, Holm P, Kuwayama M, Booth PJ, Jacobsen H, Greve T, et al. Open Pulled Straw (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos. *Mol Reprod Dev.* 1998;51:53–8.
44. Wang YB, Lou Y, Luo ZF, Zhang DF, Wang YZ. Induction of apoptosis and cell cycle arrest by polyvinylpyrrolidone K-30 and protective effect of alpha-tocopherol. *Biochem Biophys Res Commun.* 2003;308:878–84.
45. Whittingham D. Survival of mouse embryos after freezing and thawing. *Nature.* 1971;233:125–6.
46. Yoon TK, Kim TJ, Park SE, Hong SW, Ko JJ, Chung HM, et al. Live births after vitrification of oocytes in a stimulated in vitro fertilization-embryo transfer program. *Fertil Steril.* 2003;79:1323–6.