Asia-Pacific Journal of Science and Technology: Volume: 22. Issue: 04. Article ID.: APST-22-04-07. Research Article

APST

Asia-Pacific Journal of Science and Technology

https://www.tci-thaijo.org/index.php/APST/index

Published by the Faculty of Engineering, Khon Kaen University, Thailand

Effects of polyvinyl alcohol on vitrification of mature and immature bovine oocytes

Chatree Chumnandee[1,3], Pinich Wangsomnuk[2], Saksiri Sirisathien[1,*]

[1] Department of Surgery and Theriogenology, Faculty of Veterinary Medicine, Khon Kaen University, Khon Kaen, 40002, Thailand.
[2] Department of Biology, Faculty of Science, Khon Kaen University, Khon Kaen, 40002, Thailand.
[3] Division of Animal Science, Faculty of Agriculture and Technology, Nakhon Phanom University, Nakhon Phanom, 48000, Thailand.
*Corresponding author: saksiri@kku.ac.th

Abstract

The objective of this study was to evaluate the putative effect of low molecular weight (9000-10000 Da) polyvinyl alcohol (PVA) as an ice blocking agent on vitrification of mature (metaphase II stage; MII) and immature (germinal vesicle stage; GV) bovine oocytes. In experiment 1, effect of two concentrations of PVA (0.1% and 1%) on the viability of vitrified-warmed MII oocytes was determined using fluorescein diacetate staining. The results showed that the viability of vitrified-warmed MII oocytes was not significantly different among groups (vitrified control; 65.6%, +0.1 %PVA; 83.3%, and +1.0 %PVA; 73.7%). In experiment 2, effect of 0.1% PVA on fertilization and developmental competence of vitrified-warmed MII oocytes was determined. The blastocyst development of oocytes in 0.1%PVA group was significantly higher ($p \leq 0.05$) than that of vitrified control group (10.1% and 3%, respectively) but significantly lower ($p \leq 0.05$) than that of fresh control group (59.1%). In experiment 3, the maturation rate of vitrified-warmed GV oocytes was determined. The percentages of vitrified-warmed GV oocytes reaching MII stage were not different among three maturation periods (22h IVM; 33.3%, 24 h IVM; 54.5%, and 26 h IVM; 31.3%). In experiment 4, effect of 0.1% PVA on fertilization and developmental competence of vitrified-warmed GV oocytes was determined. The proportions of blastocysts were not different between vitrified control and 0.1% PVA group (1.5% and 0.8%, respectively). In conclusion, the results showed that a low molecular weight PVA at a concentration of 0.1% can improve vitrification of mature bovine oocytes but lacking positive effect with immature oocytes.

Keywords: Bovine oocyte, Cryopreservation, Ice blocking agent, Polyvinyl alcohol, Vitrification

1. Introduction

Progress in cryopreservation of oocytes plays an important role in the widely implementation of reproductive biotechnology to both human and animal [1]. In animal, for example, cryopreserved oocytes could be a steady source for a large amount of oocytes required in the somatic cell nuclear transfer [2 & 3]. Cryopreserved oocytes could be stored and later used with the appropriate male when the genetic merit of female is known. For human, cryopreserved oocytes allow long term storage of female gametes through the cryobanking [4 & 5]. Oocytes cryopreservation also provides a fertility option for women undergoing malignant cancer therapy [6]. Additionally, cryopreservation of oocytes has become useful as a model in the area cryobiology [7]

Oocytes from a variety of species have been successfully cryopreserved [8]. Live offspring from cryopreserved oocytes have been reported in several species such as cattle [9], pig [10], mouse [11], and human [12]. Although both immature (at germinal vesicle stage; GV) and mature (at metaphase II stage; MII) oocytes are cryopreservable, the MII stage oocytes have been more traditionally cryopreserved and the progress in the cryopreservation of GV stage oocytes has been limited [13]. The MII stage oocytes are known to be vulnerable to cryoinjury at their meiotic spindles [14]. On the other hand, GV stage oocytes have not yet organized the spindles

at the time of cryopreservation, however, the gap junction mediated the communication between oocytes and cumulus cells are highly susceptible to the freeze-thaw process [7 & 15].

Currently, there are two types of cryopreservation methods known as slow freezing and vitrification. Vitrification is an ultra-rapid freezing rate method in which the aqueous solution can be transformed into the solid form (glass-like) without ice crystal formation [16]. Studies comparing slow freezing and vitrification showed higher survival rates of oocytes after vitrification [17 & 18]. In general, results from vitrification of oocytes are promising, but not satisfying. High survival rates have been achieved in several studies, however, the proportions of blastocyst development from vitrified oocytes remain to be improved [19 & 20]. Several studies have shown the blastocyst rate at below 5% [21 & 22].

Theoretically, ice crystal formation should not occur in vitrification under the typical condition. However, formation of ice crystals might not be completely prevented during the whole process [23]. Ice crystals have damaging effects to the oocytes. Ice crystals not only directly injure the cells but also increase the salt concentrations of water resulting in dehydration the cells. Substances such as antifreeze proteins [24] and ice blocking agent [23] have been included into vitrification solution to control the formation of ice crystals. The antifreeze proteins can be obtained naturally in small amount from their sources making them less attractive for routine applications.

Polyvinyl alcohol (PVA) is a synthetic agent whose various molecular weights ranging from 9,000-150,000 Da [23]. PVA of molecular weights 30,000-70,000 Da has been used previously as macromolecules substitute for serum in cryopreservation of ruminants oocytes [25-27]. However, the effective PVA as an ice blocking agent has been reported at lower molecular weights [23]. Vieira et al. [9] reported the vitrification of bovine oocytes using a relatively high molecular weight PVA (30,000-70,000) as an ice blocking agent with limited success. The lowest molecular weight of commercially available PVA at the time of conducting this experiment was 9,000-10,000 Da. Therefore, the present study was aimed to examine the putative effects of PVA of molecular weight 9,000-10,000 Da on the viability and subsequent development to blastocyst stage of bovine oocytes after vitrification at mature (MII) and immature stages (GV).

2. Materials and methods

2.1 Collection of oocytes and in vitro maturation (IVM)

Bovine ovaries were transported from a local slaughterhouse in Khon Kaen province to laboratory and used within 3 h. Cumulus oocyte complexes (COCs) were collected from follicles of diameter 2-6 mm. by aspiration technique using 18-gauge needle and 5 ml syringe. Only COCs with homogeneous cytoplasm and at least 2 layers of cumulus cell were selected. A group of 20 COCs was matured in 100 µl of maturation medium comprised of TCM-199 with Earle's salt (M0650, Sigma, USA) supplemented with 5% fetal bovine serum (FBS), 10 ng/ml epidermal growth factor (EGF, E9644, Sigma, USA) and 0.025 Armour Unit/ml follicle stimulating hormone (FSH, F-2283, Sigma, USA). The maturation drops were covered with mineral oil (M8410, Sigma, USA) and cultured at 38.6 ℃ in 5% CO_2 humidified air atmosphere.

2.2 Vitrification and warming of oocytes

TCM-199 with 20% FBS was used as the basal medium for vitrification. The vitrification solution (VS) was ethylene glycol (EG, 03750, Fluka, USA) combined with dimethyl sulfoxide (DMSO, 23500.260, VWR BDH Prolabo, UK) in a basal medium. The vitrification processes were carried out on a 38.5 ℃ warm plate.

Experiment 1. Vitrification was performed according to glass micropipettes (GMPs) technique described by Kong et al. [28] with minor modifications. Briefly, glass pasteur pipettes were pulled with a pipette puller (model PB-7, Narishige, Japan) until the inner diameter was decreased from 1.2 mm. to approximately 0.2 mm. The GMPs were broke at the narrowest point and sterilized by flushing with 70% ethanol and heat at 60 ℃. A group of 4 mature oocytes were equilibrated in vitrification solution 1 (VS1; 10%EG + 10%DMSO) for 2 min. Subsequently, the oocytes were placed into vitrification solution 2 (VS2; 20% EG + 20% DMSO and 0.5 M sucrose) with two different concentrations of PVA (360627, Sigma-Aldrich, USA) (0.1%PVA or 1.0%PVA). Oocytes were loaded (1-2 oocytes per GMP) into the tip of GMP by capillary effect and immediately immersed into liquid nitrogen. The processes from exposure to VS2 to immersing into liquid nitrogen were completed within 30 s. GMP was inserted into 0.5 ml semen straw as a cab cover and stored in liquid nitrogen.

Experiment 2. Mature oocytes were partially denuded from cumulus cells by exposing to 0.025 % hyaluronidase (H3506, Sigma, USA) solution for 5 min [29]. Vitrification was carried out using to the microdroplet technique described by Papis et al. [29] with some modifications. A group of 3-4 oocytes were equilibrated in VS1 for 2 min followed by placing into VS2. Immediately, the group of oocytes was directly dropped from the glass pasteur pipette into liquid nitrogen which contained in a styrofoam box. The time from exposure of oocytes in VS2 to dropped into liquid nitrogen was within 30 s. The volume of microdroplet was

approximately 2±0.56 µl. For storage, the microdroplets were kept into 1.5 ml cryotube (20-25 oocytes per cryotube) and stored in liquid nitrogen.

Experiment 3 and 4. The COCs (GV stage oocytes) were partially denuded from cumulus cells by exposing to 0.025 % hyaluronidase solution for 5 min before vitrification. The vitrification process was carried out as described in experiment 2.

Warming was performed on a 38.5 ℃ warm plate in four wells plate. For GMP method (experiment 1), placing the tip of GMP directly into thawing solution 1 (TS1) (basal medium and 0.26 M sucrose) for 20 s and then gently expelled into medium to complete 1 min. For microdroplet method (experiment 2, 3 and 4), pouring the microdroplets to TS1 for 1 min. After that, the oocytes were placed into thawing solution 2 (TS2) (basal medium and 0.13 M sucrose) for 2 min and then washed two times in basal medium for 5 min each time. Finally, the oocytes were placed in an IVM medium for 1-2 h.

2.3 Evaluation of viability of oocytes

The vitrified-warmed oocytes were assessed for viability using fluorescein diacetate (FDA, F7378, Sigma, USA) staining, as described by Shi et al. [30] with modifications. Briefly, oocytes were incubated with 2 mg/ml pronase (P8340, Sigma, USA) for 4 min to remove zona pellucida. Subsequently, the oocytes were incubated with 2.5 µg/ml FDA for 5 min. Thereafter, the oocytes were placed on slides. The cover glass with 4 points of vaseline was softly pressed down until it touched oocytes. The oocytes were observed under a fluorescent microscope (250-CF Series, Zeiss, Jena, Germany). Oocytes with regular, spherical shape and without lysis at all level of fluorescent staining were classified as viable oocytes. Oocytes with colorless were classified non-viable.

2.4 Evaluation of nuclear maturation

Nuclear maturation status was determined by using a fluorescent dye staining technique. Oocytes were incubated with 2 mg/ml pronase for 4 min to remove zona pellucida. Oocytes were fixed in 2.5% glutaraldehyde for 2 min and washed in PBS. Oocytes were stained with 10 µg/ml Bis-benzemide (H3569, Hoechst 33342, Molecular Probes, USA) for 5 min and then washed thoroughly. After that, the stained oocytes were placed in 0.22 M anti-fading (DABCO, 10981, Sigma-Aldrich, USA), covered with glass slide and mounted with nail varnish. The stained oocytes were examined under magnification 400× using a fluorescent microscope.

2.5 In vitro fertilization (IVF)

The spermatozoa were prepared by using swim up technique. Briefly, two straws of frozen semen from a single bull were thawed in a water bath at 37 ℃ and 110 µl of semen were dropped under 1.5 ml of the medium TALP-HEPES in eight of 12×75 mm. polystyrene tubes. After incubation for 45 min at 38.5 ℃, the top layer 1 ml of medium from each tube was pooled in a 15 ml tube. The spermatozoa were centrifuged at 200×g for 10 min and then the supernatant was discarded to leave minimal volume of medium. The pellet was aspirated in 80 µl and determined for sperm concentration. The IVF drops were prepared with TALP-IVF medium supplemented with 1 mg/ml BSA (A9418, Sigma, USA) and 10 µg/ml heparin (H4784, Sigma, USA). The IVF was obtained by adding an appropriate volume of the spermatozoa to achieve the final concentration of 2×10^6 spermatozoa/ml in 100 µl drops. The matured oocytes and spermatozoa were co-incubated at 38.6 ℃ in 5% CO_2 humidified air atmosphere for 16-18 h.

2.6 In vitro culture (IVC)

The presumptive zygotes were cultured at 38.6 ℃, 5%CO_2 and 5%O_2 in SOF medium with 0.05 mM glutamine (G8540, Sigma, USA) without glucose until 72 h post insemination (hpi). The embryos with at least 4 cells were cultured in SOF medium with 0.5 mM citrate (C7129, Sigma, USA) until 144 hpi and then placed into TCM-199 medium with 2% FBS until day 8. Cleavage was evaluated on day 3, the embryos at least 8 cells on day 6, and the blastocyst on day 8 of culture.

2.7 Experimental design

2.7.1 Experiment 1: Viability of vitrified-warmed MII bovine oocytes

The oocytes for vitrification were cultured for 22 h while the oocytes for control group (non-vitrification) were cultured for 24 h. This experiment was to evaluate effect of PVA on viability of vitrified-warmed MII (n = 106) bovine oocytes. The bovine oocytes matured in vitro for 22 h were randomly assigned to 3 groups: 1) vitrified

control; vitrified without supplementation of PVA, 2) +0.1%PVA; vitrified with supplementation of 0.1%PVA, and 3) +1.0%PVA; vitrified with 1.0%PVA.

2.7.2 Experiment 2: Developmental competence of vitrified-warmed MII bovine oocytes

The oocytes for vitrification were incubated into 1 µM Taxol (T7402, Paclitaxel, Sigma, USA) for 15 min prior to vitrification [31]. This experiment was to evaluate effect of PVA on developmental competence of MII (n = 366) bovine oocytes after vitrification. MII bovine oocytes were randomly divided into 3 groups: 1) fresh control; fresh oocytes, matured in vitro for 24 h, 2) vitrified control; vitrified without supplementation of PVA, and 3) +0.1%PVA; vitrified with supplementation of 0.1%PVA.

2.7.3 Experiment 3: Effect of vitrification on maturation of GV bovine oocytes

This experiment was to determine the effect of vitrificaton on maturation rate of GV bovine oocytes (n = 115). The vitrified-warmed GV oocytes were cultured for three different periods (22, 24, and 26 h). GV bovine oocytes were randomly divided into 4 groups: 1) fresh control; fresh oocytes, matured in vitro for 24 h, 2) 22 h IVM; vitrified and matured in vitro for 22 h, 3) 24 h IVM; vitrified and matured in vitro for 24 h, and 4) 26 h IVM; vitrified and matured in vitro for 26 h.

2.7.4 Experiment 4: Developmental competence of vitrified-warmed GV bovine oocytes

The vitrified-warmed GV stage oocytes were cultured for 24 h and incubated into 1 µM Taxol for 15 min before vitrification. This experiment was to evaluate developmental competence of GV (n = 319) bovine oocytes after vitrification. GV bovine oocytes were randomly divided into 3 groups: 1) fresh control; fresh oocytes, matured in vitro for 24 h, 2) vitrified control; vitrified without supplementation of PVA, and 3) +0.1%PVA; vitrified with supplementation of 0.1%PVA.

2.8 Statistical analysis

Data were analyzed by one-way ANOVA by LSD's test. All data were conducted by SPSS program (IBM SPSS Statistics for Windows, Version 19.0, Armonk, NY: IBM Crop) and presented as the mean±SD. The p-value of statistical significance was set at $p \leq 0.05$.

3. Results

3.1 Viability of vitrified-warmed MII bovine oocytes

The viability of vitrified-warmed MII bovine oocytes with different concentrations of PVA is presented in Table 1. The results showed that PVA had no effect on viability of vitrified-warmed MII oocytes. The proportions of viable oocytes and non-viable oocytes including degenerated cytoplasm and rupture of zona pellucida were not different among groups. The representative pictures of viable and non-viable oocytes are showed in Figure 1.

3.2 Developmental competence of vitrified-warmed MII bovine oocytes

The percentages of cleavage, embryos with at least 8 cells, and blastocyst (Figure 2) of vitrified-warmed bovine oocytes were significantly lower than that of the fresh control group ($p \leq 0.05$) (Table 2). However, the proportion of blastocyst in +0.1%PVA group was significantly higher than that of vitrified control groups ($p \leq 0.05$).

3.3 Effect of vitrification on maturation of GV bovine oocytes

The process of vitrification and warming had no effect on speed of progress through maturation of oocytes (Table 3). The percentage of oocytes reaching MII stage (Figure 3) at 24 h IVM was comparable to those at 22 h IVM and 26 h IVM groups. However, the nuclear maturation of all vitrified-warmed GV groups were significantly lower than that of fresh control group ($p \leq 0.05$).

3.4 Developmental competence of vitrified-warmed GV bovine oocytes

Supplementation of PVA in vitrification solution had no effect on developmental competence of vitrified-warmed GV bovine oocytes. The proportion of blastocyst in the +0.1%PVA group was not different from that of vitrified control group (Table 4).

Table 1 Viability of vitrified-warmed MII bovine oocytes with different concentrations of PVA

Group	No. of oocytes	Non-viable (%)	Viable (%)
Vitrified control	32	11 (32.9±26.0)	21 (67.1±26.0)
+0.1 %PVA	36	6 (15.3±20.0)	30 (84.7±20.0)
+1.0 %PVA	38	10 (26.4±14.6)	28 (73.6±14.6)

Note: Data in each group were pooled from six replications.

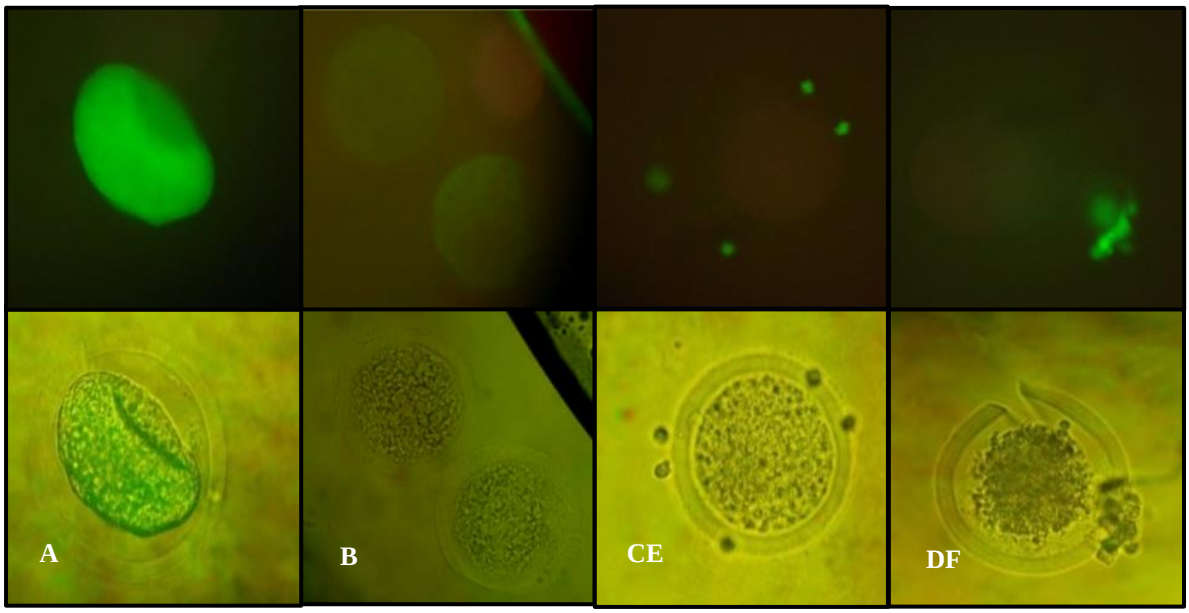

Figure 1 Viability of vitrified-warmed MII bovine oocytes based on FDA staining. Above row: fluorescence microscope images; below row: phase-contrast microscope images. A); viable oocyte with high fluorescent staining, B); viable oocyte with low fluorescent staining, C); degenerated cytoplasm oocyte, and D); ruptured zona pellucida oocyte. Magnification 400×.

Table 2 Developmental competence of vitrified-warmed MII bovine oocytes with different concentrations of PVA

Group	No. of oocytes	Cleavage (%)	≥ 8 cells embryo (%)	Blastocyst (%)	Blastocyst per cleavage (%)
Fresh control	66	61 (91.8±6.3)^{a}	55 (82.8±10.2)^{c}	39 (58.7±10.4)^{e}	39/61 (63.9±10.3)^{h}
Vitrified control	101	56 (58.3±15.8)^{b}	29 (31.5±17.1)^{d}	3 (3..2±3.7)^{f}	3/56 (5.0±6.2)^{i}
+0.1% PVA	99	44 (44.0±8.6)^{b}	28 (27.9±6.6)^{d}	10 (9.8±6.5)^{g}	10/44 (21.4±12.4)^{j}

Note: Data in each group were pooled from four replications. Values with different letters within a column denote significant difference ($p \leq 0.05$)

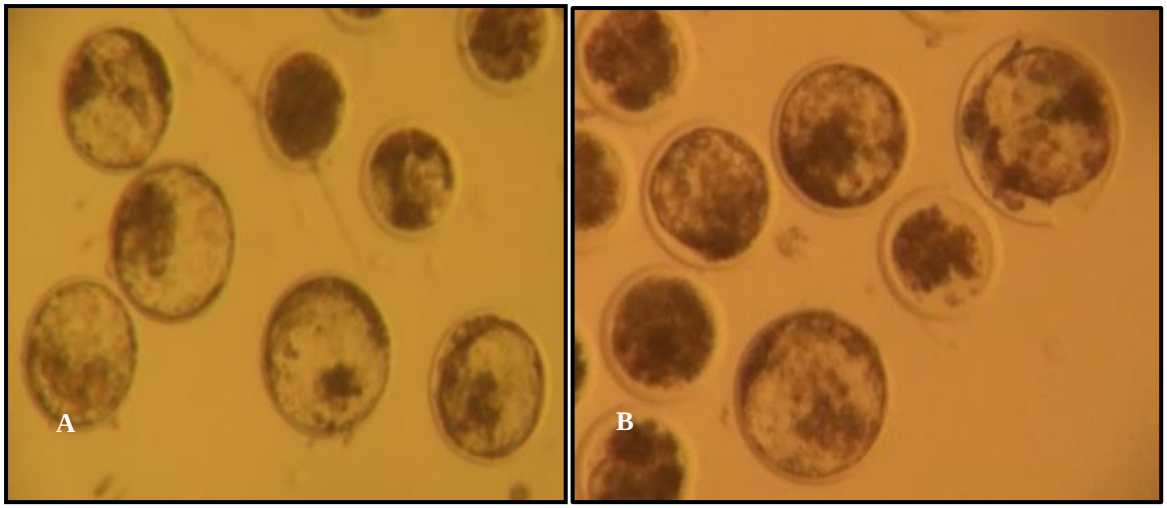

Figure 2 Expanded blastocysts developed from A); fresh control group and B); vitrified-warmed MII with 0.1%PVA. Magnification 40×.

Table 3 Nuclear maturation status of vitrified-warmed GV bovine oocytes after different periods of maturation

Group	No. of oocytes	Nuclear maturation stages				
		GV (%)	GVBD (%)	MI (%)	AI-TI (%)	MII (%)
Fresh control	29	0 (0.0±0.0)	0 (0.0±0.0)^{a}	0 (0.0±0.0)^{c}	0 (0.0±0.0)^{f}	29 (100.0±0.0)^{h}
22 h IVM	21	0 (0. ±0.00)	4 (20.0±26.5)^{b}	3 (12.2±10.7)^{d,e}	7 (32.2±13.5)^{g}	7 (35.6±22.2)^{i}
24 h IVM	33	2 (6.7±11.5)	3 (5.6±9.6)^{a,b}	1 (1.9±3.2)^{c,d}	9 (25.7±3.9)^{g}	18 (54.6±18.5)^{i}
26 h IVM	32	0 (0.0±0.0)	5 (15.2±26.2)^{b}	6 (18.5±8.6)^{e}	11 (34.8±22.4)^{g}	10 (31.5±11.7)^{i}

Note: Data in each group were pooled from five replications. Values with different letters within a column denote significant difference ($p \leq 0.05$). germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), C); anaphase I (AI), telophase I (TI), and metaphase II (MII)

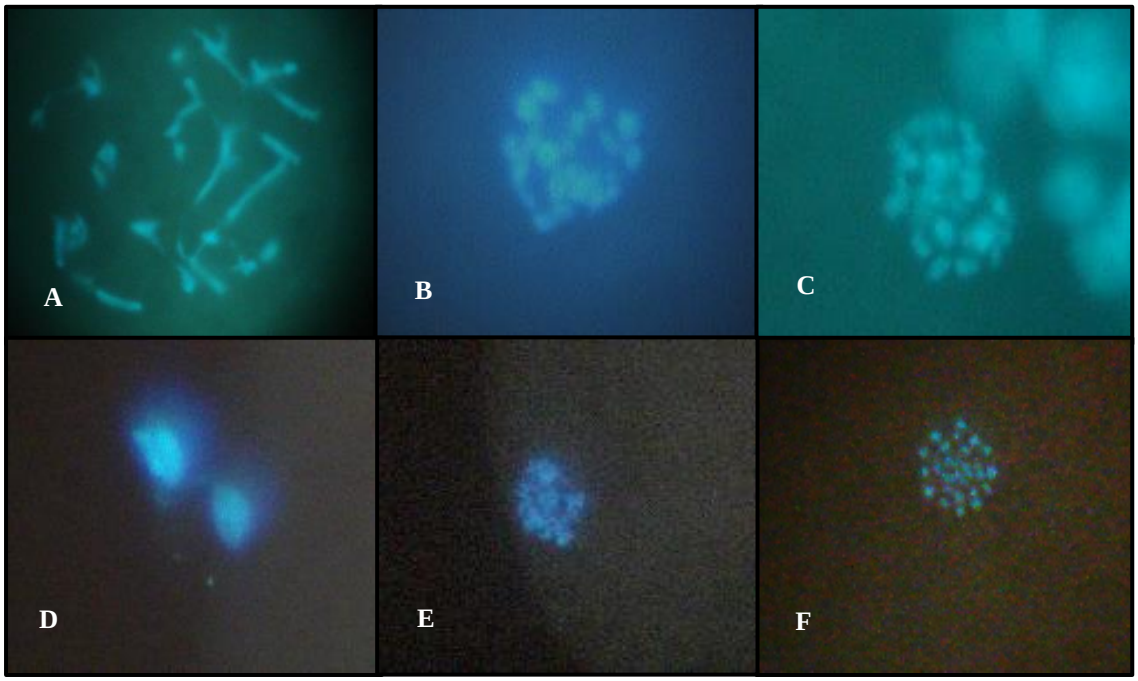

Figure 3 Meiotic status of vitrified-warmed GV bovine oocytes at difference stages. A); germinal vesicle breakdown (GVBD), B); metaphase I (MI), C); anaphase I (AI), D); telophase I (TI), E); MII , and F); MII of fresh control group. Magnification 400×.

Table 4 Fertilization and developmental competence of vitrified-warmed GV bovine oocytes with or without PVA

Group	No. of oocytes	Cleavage (%)	≥ 8 cells embryo (%)	Blastocyst (%)	Blastocyst per cleavage (%)
Fresh control	58	50 (85±18.2)[a]	46 (75.9±17.5)[c]	34 (58.3±20.4)[e]	34/50 (66.5±11.0)[h]
Vitrified control	138	53 (39.2±8.7)[b]	13 (9.7±4.3)[d]	2 (1.4±1.3)[f]	2/53 (4.1±3.5)[i]
+0.1%PVA	123	44 (35.8±1.5)[b]	13 (10.6±3.1)[d]	1 (0.8±1.3)[f]	1/44 (2.2±3.8)[i]

Note: Data in each group were pooled from three replications. Values with different letters within a column denote significant difference ($p \leq 0.05$)

4. Discussion

A high cooling rate is vital for the successful vitrification of oocytes. The microdroplet technique was chosen for vitrification of oocytes in this study. The microdroplet technique is a container-less vitrification allowing direct exposure of the solution into liquid nitrogen thus eliminating the insulation effect of the container wall [32]. In addition, this technique permits the microdroplets containing vitrified oocytes to be dissolved in a large volume of thawing solution. Within each microdroplet, several oocytes (3-4 oocytes per droplet) were enclosed in a minimum volume of vitrification solution, average 2±0.56 µl in this study, and numerous vitrified oocytes can be warmed at the same time (20-25 vitrified oocytes in each time). The size of droplet employed in this study was slightly smaller than a previous study in which a group of 5-8 oocytes were contained in a 6±2 µl of vitrification solution [29].

In our study, we found no positive effect of PVA on viability of vitrified-warmed MII bovine oocytes determined by the FDA staining. The FDA dye is a non-polar compound making it membrane permeable. Once inside the cells, FDA is hydrolyzed by esterases and fluorescein is produced. Because fluorescein is a polar compound, it is impermeable to intact cell membrane, and therefore it accumulates in the cytoplasm of the cells to allow observation [33]. The overall viability of vitrified-warmed oocytes in our study was approximately 74% which was comparable to other studies [31 & 34]. However, the fertilization rates of vitrified-warmed oocytes in this study were lower than the viability rates determined by FDA staining. This is agreement with previous studies which suggest that the FDA test is an assessment for immediate survival of oocytes but not for further developmental competence of oocytes [35]. Sripunya et al. [36] reported a 24% reduction in fertilization rate compared to the viability test from FDA staining. Similarly, Shi et al. [30] obtained only 5% fertilization rate of vitrified-warmed porcine oocytes despite achieving the viability rate at 53%. Various ultrastrustural changes have been identified with cryopreserved oocytes such as dilation of smooth endoplasmic reticulum (SER) [37], vacuolization in mitochondria [7 & 38], meiotic spindle disassembly [30], and reduction in cortical granules [14] including DNA fragmentation of metaphase II chromosomes [39]. This probably explained the discrepancy between the high viability and low developmental competence of cryopreserved oocytes.

Supplementation of 0.1% PVA as an ice nucleation blocking agent proved to be useful. The percentage of blastocyst from vitrified MII oocytes in the +0.1% PVA group was significantly higher than that of the vitrified control group. A relatively high molecular weight PVA (30,000-70,000 Da) has been previous used as a serum substitute in vitrification of oocytes [26 & 27]. The results of our study supported the notion that low molecular weight PVA at low concentration was an effective ice blocking agent as reported by Wowk et al. [40]. Deller et al. [41] studied the effect of 9 kDa PVA on cryopreservation of sheep red blood cells and founded that PVA at concentrations as low as 0.1% had a potent ice crystal inhibition activity. The action of PVA as an ice blocking agent is to bind on the surface of newly formed ice crystals and hence blocking any additional supplementary water molecules in the area of growth [40]. Since PVA cannot penetrate the cell membrane therefore it can only suppress extracellular ice crystals. Although the formation of extracellular ice crystal was not investigated in this study, it was likely that extracellular ice growth partly contribute to cryodamages.

The average of maturation rate of vitrified-warmed GV stage oocytes in this study was approximately 40%. From several studies, maturation rates of vitrified-warmed GV stage bovine oocytes were found to be in the range of 45-85% [42 19 & 20]. Our results showed that a large portion (approximately 30%) of vitrified-warmed GV stage oocytes were arrested at the anaphase I-telophase I (AI-TI) stage. However, the time required for progression through meiotic division was not affected by vitrification. Clearly, the reduction in maturation rate of vitrified-warmed GV stage oocytes could not simply overcome with longer incubation time.

The overall fertilization rates (average 37%) of vitrified-warmed GV stage oocytes in our study were comparable with other studies [34, 42]. The fertilization rates were corresponding with the percentages of oocytes reaching the MII stage. Supplementation of PVA had no improving effect on developmental competence of

vitrified-warmed GV stage oocytes. Our finding was in agreement with other authors who found no positive effect of PVA on developmental competence of vitrified-warmed GV stage bovine oocytes [9 & 34].

We observed that 75% of cleavages from vitrified at GV stage were arrested at 8-16 cells compared to only 35% of cleavages arresting at 8-16 cells when oocytes were vitrified at MII stage. This might imply that the GV stage oocytes are more susceptible to cryopreservation than the MII stage oocytes. Previous studies have reported that GV stage oocytes are extremely sensitive to cryopreservation due to the disruption between cumulus cells and oocytes by destruction of gap junction [7 & 15]. The communication between oocytes and cumulus cells was found to be crucial for coordination of nuclear and cytoplasmic maturation [43].

5. Conclusions

In conclusion, the results of the present study indicated that vitrification process induced impairment on developmental competence of both GV and MII stage bovine oocytes. A low molecular weight (9,000-10,000 Da) PVA can improve developmental competence of MII stage oocytes. The vitrification and warming processes are necessary to be further optimized to achieve an acceptable developmental competence.

6. Acknowledgements

This work was supported by the Office of the Higher Education Commission and by research publication fund from a post graduate student of the Faculty of Veterinary Medicine, Khon Kaen University, Thailand

7. References

[1] Palasz, A.T., Mapletoft, R.J., 1996. Cryopreservation of mammalian embryos and oocytes: recent advances. Biotechnology Advances 14, 122-149.

[2] Dinnyes, A., Dai, Y., Jiang, S., Yang, X., 2000. High developmental rates of vitrified bovine oocytes following parthenogenetic activation, in vitro fertilization, and somatic cell nuclear transfer. Biology of Reproduction 63, 513-518.

[3] Atabay, E.C., Takahashi, Y., Katagiri, S., Nagano, M., Koga, A., Kanai, Y., 2004. Vitrification of bovine oocytes and its application to intergeneric somatic cell nucleus transfer. Theriogenology 61, 15-23.

[4] Gosden, R.G., 2005. Prospects for oocyte banking and in vitro maturation. Journal of the National Cancer Institute. Monographs 34, 60-3.

[5] Mara, L., Casu, S., Carta, A., Dettena, M., 2013. Cryobanking of farm animal gametes and embryos as a means of conserving livestock genetics. Animal Reproduction Science 138, 25-38.

[6] Grifo, J.A., Noyes, N., 2009. Delivery rate using cryopreserved oocytes is comparable to conventional in vitro fertilization using fresh oocytes: potential fertility preservation for female cancer patients. Fertility and Sterility 93, 391-396.

[7] Fuku, E., Xia, L., Downey, B.R., 1995. Ultrastructure changes in bovine oocytes cryopreserved by vitrification. Cryobiology 32, 139-56.

[8] Saragusty, J., Arav, A., 2011. Current progress in oocyte and embryo cryopreservation by slow freezing and vitrification. Reproduction 141, 1-19.

[9] Vieira, A.D., Forell, F., Feltrin, C., Rodrigues, J.L., 2008. Calves born after direct transfer of vitrified bovine in vitro-produced blastocysts derived from vitrified immature oocytes. Reproduction in Domestic Animals 43, 314–318.

[10] Somfai, T., Yoshioka, K., Tanihara, F., Kaneko, H., Noguchi, J., Naomi, K., Nagai, T., Kikuchi, K., 2014. Generation of Live Piglets from Cryopreserved Oocytes for the First Time Using a Defined System for In Vitro Embryo Production. PLoS ONE 9, e97731.

[11] Kohaya, N., Fujiwara, K., Ito, J., Kashiwazaki, N., 2013. Generation of Live Offspring from Vitrified Mouse Oocytes of C57BL/6J Strain. PLoS ONE 8, e58063.

[12] Kim, T.J., Hong, S.W., 2011. Successful live birth from vitrified oocytes after 5 years of cryopreservation. Journal of Assisted Reproduction and Genetics 28, 73-6.

[13] Massip, A., 2003. Cryopreservation of bovine oocytes: current status and recent developments. Reproduction Nutrition Development 43, 325-330.

[14] Morato, R., Mogas, T., Hyttel, P.M., 2008a. Ultrastructure of bovine oocytes exposed to Taxol prior to OPS vitrification. Molecular Reproduction and Development 75, 1318-26.

[15] Diez, C., Duque, P., Gomez, E., Hidalgo, C.O., Tamargo, C., Rodriguez, A., 2005. Bovine oocyte vitrification before or after meiotic arrest: effects on ultrastructure and developmental ability. Theriogenology 64, 317-333.

[16] Rall, W.F., Fahy, G.M., 1985. Ice-free cryopreservation of mouse embryos at -196 degrees C by vitrification. Nature 313, 573-557.

[17] Cao, Y.-X., Xing, Q., Li, L., Cong, L., Zhang, Z.-G., Wei, Z.-L., Zhou, P., 2009. Comparison of survival and embryonic development in human oocytes cryopreserved by slow-freezing and vitrification. Fertility and Sterility 92, 1306–1311.

[18] Fasano, G., Vannin, A.S., Biramane, J., Delbaere, A., Englert, Y., 2010. Cryopreservation of human failed maturation oocytes shows that vitrification gives superior outcomes to slow cooling. Cryobiology 61, 243-247.

[19] Sprícigo, J.F.W., Morais, K., Ferreira, A.R., Machado, G.M., Gomes, A.C.M., Rumpf, R., Franco, M.M., Dode, M. a. N., 2014. Vitrification of bovine oocytes at different meiotic stages using the Cryotop method: assessment of morphological, molecular and functional patterns. Cryobiology 69, 256–265.

[20] Ezoe, K., Yabuuchi, A., Tani, T., Mori, C., Miki, T., Takayama, Y., Beyhan, Z., Kato, Y., Okuno, T., Kubayashi, T., Kato, K., 2015. Developmental Competence of Vitrified-Warmed Bovine Oocytes at the Germinal-Vesicle Stage is Improved by Cyclic Adenosine Monophosphate Modulators during In Vitro Maturation. PLoS ONE 10, e0126801.

[21] Gupta, M.K., Uhm, S.J., Lee, H.T., 2007. Cryopreservation of immature and in vitro matured porcine oocytes by solid surface vitrification. Theriogenology 67, 238-248.

[22] Aono, A., Nagatomo, H., Takuma, T., Nonaka, R., Ono, Y., Wada, Y., Abe, Y., Takahashi, M., Watanabe, T., Kawahara, M., 2013. Dynamics of intracellular phospholipid membrane organization during oocyte maturation and successful vitrification of immature oocytes retrieved by ovum pick-up in cattle. Theriogenology 79, 1146–1152.e1.

[23] Wowk, B., Leitl, E., Rasch, C.M., Mesbah-Karimi, N., Harris, S.B., Fahy, G.M., 2000. Vitrification enhancement by synthetic ice blocking agents. Cryobiology 40, 228-236.

[24] O'Neil, L., Paynter, S.J., Fuller, B.J., Shaw, R.W., DeVries, A.L., 1998. Vitrification of mature mouse oocytes in a 6 M Me_2SO solution supplemented with antifreeze glycoproteins: the effect of temperature. Cryobiology 37, 59-66.

[25] Naitana, S., Ledda, S., Loi, P., Leoni, G., Bogliolo, L., Dattena, M., Cappai, P., 1997. Polyvinyl alcohol as a defined substitute for serum in vitrification and warming solutions to cryopreserve ovine embryos at different stages of development. Animal Reproduction Science 48, 247–256.

[26] Asada, M., Ishibashi, S., Ikumi, S., Fukui, Y., 2002. Effect of polyvinyl alcohol (PVA) concentration during vitrification of in vitro mature bovine oocytes. Theriogenology 58, 1199-1208.

[27] Checura, C.M., Seidel, G.E., Jr., 2007. Effect of macromolecules in solutions for vitrification of mature bovine oocytes. Theriogenology 67, 919-930.

[28] Kong, I.K., Lee, S.I., Cho, S.G., Cho, S.K., Park, C.S., 2000. Comparison of open pulled straw (OPS) VS glass micropipette (GMP) vitrification in mouse blastocyst. Theriogenology 53, 1817-1826.

[29] Papis, K., Shimizu, M., Izaike, Y., 2000. Factors affecting the survivability of bovine oocytes vitrified in droplets. Theriogenology 54, 651-58.

[30] Shi, W.Q., Zhu, S.E., Zhang, D., Wang, W.H., Tang, G.L., Hou, Y.P., Tian, S.J., 2006. Improved development by Taxol pretreatment after vitrification of in vitro matured porcine oocytes. Reproduction 131, 795-804.

[31] Morató Roser, Izquierdo Dolors, Albarracín José Luis, Anguita Begoña, Palomo María Jesús, Jiménez-Macedo Ana Raquel, Paramio María Teresa, Mogas Teresa, 2007. Effects of pre-treating in vitro-matured bovine oocytes with the cytoskeleton stabilizing agent taxol prior to vitrification. Molecular Reproduction and Development 75, 191–201.

[32] Kim, D.-H., Park, H.-S., Kim, S.-W., Hwang, I.-S., Yang, B.-C., Im, G.-S., Chung, H.-J., Seong, H.-W., Moon, S.-J., Yang, B.-S., 2007. Vitrification of Immature Bovine Oocytes by the Microdrop Method. Journal of Reproduction and Development 53, 843–851.

[33] Green, V.S., Stott, D.E., Diack, M., 2006. Assay for fluorescein diacetate hydrolytic activity: optimization for soil samples. Soil Biology and Biochemistry 38, 693-701.

[34] Zhou, X.L., Naib, A.A., Sun, D.W., Lonergan, P., 2010. Bovine oocyte vitrification using the cryotop method: effect of cumulus cells and vitrification protocol on survival and subsequent development. Cryobiology 61, 66-72.

[35] Yang, B.-C., Im, G.-S., Chang, W.-K., Lee, Y.-K., Oh, S.-J., Jin, D.-I., Im, K.-S., Lee, C.-K., 2003. Survival and In Vitro Development of Immature Bovine Oocytes Cryopreserved by Vitrification, Survival and In Vitro Development of Immature Bovine Oocytes Cryopreserved by Vitrification. Asian-Australasian Journal of Animal Sciences, Asian-Australasian Journal of Animal Sciences 16, 23–28.

[36] Sripunya, N., Liang, Y., Panyawai, K., Srirattana, K., Ngernsoungnern, A., Ngernsoungnern, P., Ketudat-Cairns, M., Parnpai, R., 2014. Cytochalasin B efficiency in the cryopreservation of immature bovine oocytes by Cryotop and solid surface vitrification methods. Cryobiology 69, 496–499.

[37] Schmidt, M., Hyttel, P., Avery, B., Greve, T., 1995. Ultrastructure of in vitro matured bovine oocytes after controlled freezing in 10% glycerol. Animal Reproduction Science 37, 281-90.

[38] Dai, J., Wu, C., Muneri, C.W., Niu, Y., Zhang, S., Rui, R., Zhang, D., 2015. Changes in mitochondrial function in porcine vitrified MII-stage oocytes and their impacts on apoptosis and developmental ability. Cryobiology 71, 291–298.
[39] Stachowiak, E.M., Papis, K., Kruszewski, M., Iwanenko, T., Bartomiejezyk, T., Modlinski, J.A., 2009. Comparison of the levels of DNA damage using Comet assay in bovine oocytes subjected to selected vitrification methods. Reproduction in Domestic Animals 44, 653-8.
[40] Wowk, B., 2002. Polyvinyl alcohol compounds for inhibition of ice growth. US6391224B1.
[41] Deller, R.C., Vatish, M., Mitchell, D.A., Gibson, M.I., 2014. Synthetic polymers enable non-vitreous cellular cryopreservation by reducing ice crystal growth during thawing. Nature Communications 5, 3244.
[42] Faheem, M.S., Baron, E., Carvalhais, I., Chaveiro, A., Pavani, K., da Silva, F.M., 2014. The effect of vitrification of immature bovine oocytes to the subsequent in vitro development and gene expression. Zygote 26, 1-10.
[43] Gilchrist, R.B., Lane, M., Thompson, J.G., 2008. Oocyte-secreted factors: regulators of cumulus cell function and oocyte quality. Human Reproduction Update 14, 159–177.