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In Vitro Growth and Maturation of Vitrified-Warmed Bovine Oocytes Collected from Early Antral Follicles

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Abstract. Cryopreservation of growing oocytes enriches the choice of timing and location of artificial embryo production. However, completion of oocyte growth after warming is crucial when using such cryopreserved oocytes. Our research objective was to develop a sequential system that incorporates cryopreservation of growing bovine oocytes and their subsequent *in vitro* growth. Oocyte-granulosa cell complexes with a mean oocyte diameter of approximately 100 μm were vitrified-warmed and then cultured for 14 days. The percentage of surviving oocytes following cryopreservation and 14-day culture was approximately 80%. More than half of the surviving oocytes were capable of maturing to metaphase II after *in vitro* maturation; the rate was comparable to that of control oocytes grown *in vitro* without cryopreservation. Taken together, the combined protocols for vitrification-warming of growing oocytes and subsequent *in vitro* growth can produce oocytes capable of undergoing meiotic maturation.

Key words: Bovine oocyte, *In vitro* growth, *In vitro* maturation, Vitrification-warming

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Cryopreservation of immature oocytes is expected to allow greater flexibility in the timing and location of artificial embryo production. Cryopreservation of fully grown but immature oocytes using vitrification rather than conventional freezing has been studied in mice [1], cattle [2–4], sheep [5, 6], pigs [7, 8] and humans [9], and offspring have been obtained from mice and cattle [1, 2]. The cohort of growing oocytes in the ovary is a potentially larger source of oocytes compared with those that are fully grown. However, growing oocytes that are incapable of maturing must achieve their full growth to be utilized as functional gametes. Thus, the efficacy of cryopreservation of growing oocytes is dependent on the quality of the vitrification-warming technology and culture of oocytes after warming.

A technique involving vitrification of growing oocytes and subsequent *in vitro* growth has been used in mice [10–12]. Oocytes have been vitrified in the form of isolated pre-antral follicles [11, 13], ovarian cortical strips [13] or intact ovaries [10]. After warming, they have been cultured as oocyte-granulosa cell complexes [10, 11] or isolated follicles [13]. Some oocytes have been shown to have acquired competence to become fertile during *in vitro* growth [10, 11]. Similar studies have been conducted in larger animals such as cattle [14] and goats [15]. However, the capabilities of the oocytes of these animals remain to be investigated. Because viable oocytes were obtained after cryopreservation in these previous studies, the present study addressed issues related to *in vitro* oocyte growth with respect to the development of maturational competence. We previously reported a 14-day culture system for bovine oocytes in which full size was achieved from approximately the late mid-growth phase [16]. The present study aimed to establish a combined technology to produce mature bovine oocytes: vitrification-warming of growing oocytes and subsequent culture of the recovered oocytes.

Growing oocytes were isolated from early antral follicles and subjected to stepwise pre-equilibration as described in the Methods section and Table 1. The morphologies of the oocyte-granulosa cell complexes at the end of pre-equilibration are shown in Fig. 1A. Prior to transfer to liquid nitrogen (LN_2), the complexes were treated with a medium containing 0.5 M sucrose, in which the oocytes shrank quickly. After cryopreservation, the complexes were warmed in another medium containing 0.5 M sucrose. The oocytes remained shrunken while being washed in media supplemented with decreasing concentrations of sucrose. Figure 1B shows the complexes in the medium containing 0.2 M sucrose. The oocytes partially regained their spherical shape after transfer to a medium without cryoprotectants (Fig. 1C). Only oocytes that did not exhibit any sign of degradation, such as insubstantial cytoplasm or membrane disruption, were used for culture (Day 0). On Day 1, the complexes were attached to tissue culture membrane inserts (Fig. 1D). After vitrification-warming, morphologies of the oocytes were re-examined to determine the percentage of surviving oocytes. The results of four replicate experiments indicated that vitrification-warming resulted in the death of 7.8 ± 6.6% (SD) of oocytes.

The oocyte-granulosa cell complexes grew and formed dome-like structures (Fig. 1E–G), except when oocyte degeneration occurred (Fig. 1G). Figure 2A shows the percentage of surviving oocytes on Day 14. Of the oocytes present on Day 1, 84.9 ± 7.2% were recovered alive as oocyte-cumulus cell complexes. These oocytes are hereafter referred to as vitrified-warmed *in vitro*-grown (VW-IVG) oocytes. The percentage of VW-IVG oocytes was significantly lower

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Table 1. Stepwise pre-equilibration of oocyte-granulosa cell complexes using progressively increasing concentrations of ethylene glycol (EG) and dimethyl sulfoxide (DMSO)

Pre-equilibration step[a]	Concentration in the base medium (20 µl) at each step		Cumulative concentration		Cumulative volume (µl)
	EG	DMSO	EG	DMSO	
0[b]	0.00%	0.00%	0.00%	0.00%	20
1	0.60%	0.60%	0.30%	0.30%	40
2	1.20%	1.20%	0.60%	0.60%	60
3	1.80%	1.80%	0.90%	0.90%	80
4	2.40%	2.40%	1.20%	1.20%	100
5	3.00%	3.00%	1.50%	1.50%	120
6	3.60%	3.60%	1.80%	1.80%	140
7	4.20%	4.20%	2.10%	2.10%	160
8	4.80%	4.80%	2.40%	2.40%	180
9	5.40%	5.40%	2.70%	2.70%	200
10[c]	6.00%	6.00%	3.00%	3.00%	220

[a] Each step, except steps 0 and 10, was followed by a 1-min incubation period. [b] At step 0, the complexes were only transferred to culture plates to initiate pre-equilibration. Step 0 was immediately followed by step 1. [c] The last step of pre-equilibration was followed by a 5-min incubation period.

than that of control IVG oocytes (97.1 ± 5.1%, $P<0.05$). Figure 2B depicts the distribution of the oocyte volume before and after *in vitro* growth. The increases in oocyte volume from Day 1 to Day 14 were 67.9% and 58.9% for control IVG oocytes and VW-IVG oocytes, respectively. The mean diameter of control IVG oocytes increased from 100.9 ± 3.3 µm to 119.8 ± 5.2 µm ($P<0.01$), whereas that of VW-IVG oocytes increased from 99.7 ± 3.6 µm to 116.3 ± 5.5 µm ($P<0.01$). On an average, VW-IVG oocytes were smaller than control IVG oocytes ($P<0.01$).

The cumulus cell mass, which was compact when collected on Day 14 (Fig. 3A), underwent expansion following hormone-induced oocyte maturation (Fig. 3B). Expanded cumulus cells were removed with the help of hyaluronidase to examine the presence of the first polar body. The polar body was extruded in more than half of the oocytes, irrespective of whether they had been vitrified or not (Fig. 3C). In three replicate experiments, the oocytes were fixed to examine the meiotic stage by nuclear staining (Table 2). The percentage of oocytes at metaphase II was similar for both VW-IVG and control IVG oocytes (Table 2). In a preliminary experiment, one of the embryos derived from a VW-IVG oocyte developed to the blastocyst stage following somatic nuclear cell transfer using cumulus cells as nuclei donors. Taken together, the combined protocols that were modified in the present study for vitrification-warming of growing bovine oocytes and subsequent *in vitro* growth can produce oocytes capable of undergoing meiotic maturation.

Methods

Chemicals

Unless otherwise specified, chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). The concentration of supplemental fluid in the culture medium is expressed as a volume/volume percentage.

Collection and vitrification-warming of oocyte-granulosa cell complexes

Early antral follicles that were 0.5–0.8 mm in diameter were dissected from the ovaries of Japanese Black or F_1 (Japanese Black × Holstein) cows collected from an abattoir. The complexes consisting of oocytes and cumulus/granulosa cells were withdrawn from the follicles using previously described methods [16]. The collection medium comprised a modified minimum essential medium (MEM, pH 7.2; Nissui Pharmaceutical, Tokyo, Japan) that was buffered with 20 mM HEPES and 5 mM sodium bicarbonate. The medium was supplemented with 1 mM sodium pyruvate, 3 mM glutathione, 5 mM taurine, 1 mM ascorbic acid 2-glucoside (AA2G; Hayashibara, Okayama, Japan) and 20 mg/ml polyvinylpyrrolidone [PVP; w/v, molecular weight (MW) 360,000]. Only healthy-looking oocytes were used.

Vitrification of the complexes was characterized by a serial 10-step pre-equilibration procedure adapted from the method developed by Aono *et al.* for immature mouse oocytes [1]. In the present study, we aimed to maintain the complexes in a small volume of the pre-equilibration medium and at the same time to increase the concentration of cryoprotectants gradually. Accordingly, a series of 20-µl aliquots of media containing progressively increasing concentrations of ethylene glycol (EG) and dimethyl sulfoxide (DMSO) was added to the complexes. The base medium (BM) used for cryopreservation was TCM 199 (Life Technologies, Carlsbad, CA, USA) containing 20 mM HEPES, 5 mM sodium bicarbonate, 0.14 mM kanamycin sulfate, 1 mM sodium pyruvate, 3 mM glutathione, 5 mM taurine, 2 mM dimethylthiourea, 1 mM AA2G and 5% fetal bovine serum (FBS; HyClone, Logan, UT, USA). The oocyte-granulosa cell complexes (approximately 20 complexes at a time) were placed in a well of a K-1 Repro Plate (Kitazato Corporation, Shizuoka, Japan) with 20 µl of BM (step 0 in Table 1). Then, from step 1 to step 10, 20 µl of BM containing progressively increasing concentrations of EG and DMSO was added every 1 min. The concentrations of EG

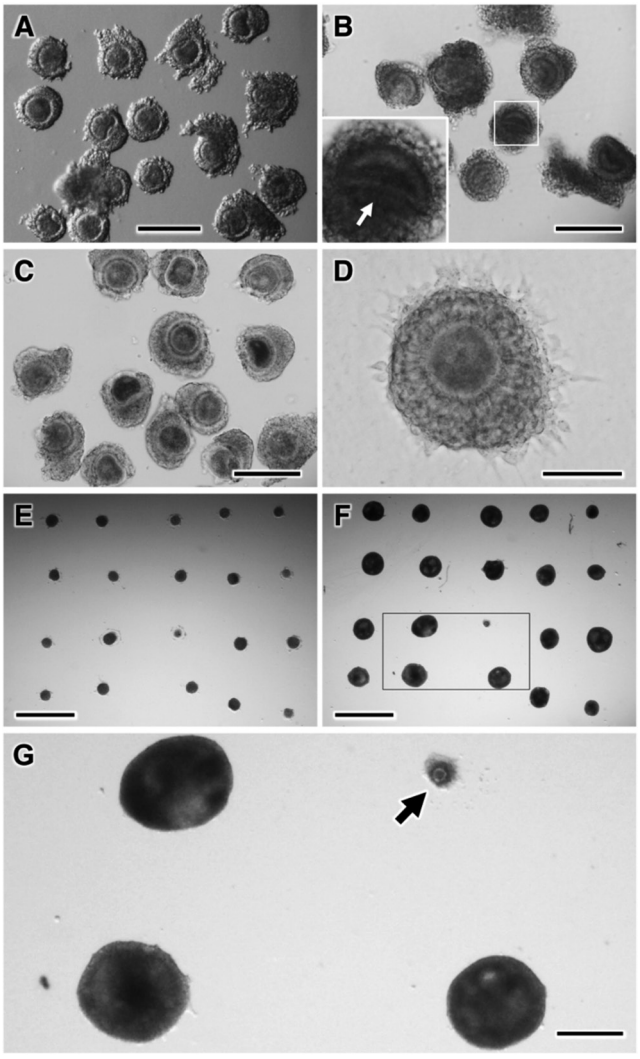

Fig. 1. Morphology of bovine oocyte-granulosa cell complexes at various stages of vitrification-warming and *in vitro* growth. (A) Complexes in the pre-equilibration medium at step 10, prior to vitrification. (B) Complexes in a medium containing 0.2 M sucrose after recovery from storage in LN_2. A representative oocyte is shown enlarged in the inset (arrow). Note the shrinkage of oocytes. (C) Complexes that regained their spherical shape before transfer to the culture medium. (D) Complexes attached to the culture membrane insert on Day 1. (E) Complexes on Day 6 and (F) on Day 14. (G) Enlarged image of the complexes enclosed by a rectangle in F. The oocyte without a dome-like structure (arrow) shows degeneration. Bars: 250 µm (A, B, and C), 100 µm (D), 2 mm (E and F) and 500 µm (G).

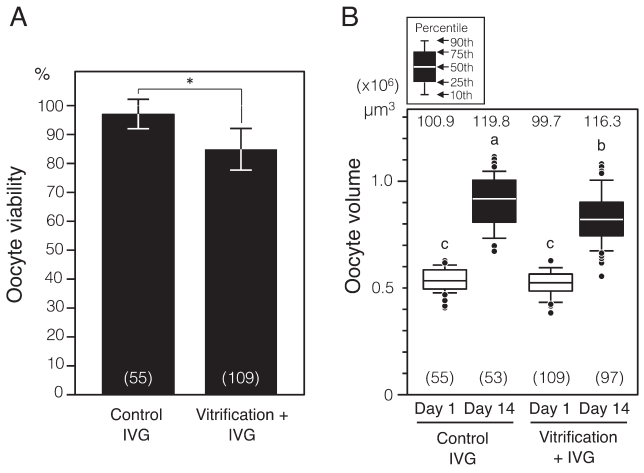

Fig. 2. Viability and growth of vitrified-warmed oocytes after the 14-day culture period. (A) Percentage of oocytes that survived for 14 days. The data represent mean ± standard deviation values from four replicate experiments. The asterisk indicates a significant difference ($P<0.05$; Student's *t*-test). (B) Distributions of oocyte volume on Days 1 and 14. The numbers above the box plots indicate the mean oocyte diameter (µm). $^{a–c}$ Different letters indicate significant differences ($P<0.05$; Tukey's test). The total number of oocytes is shown in parentheses.

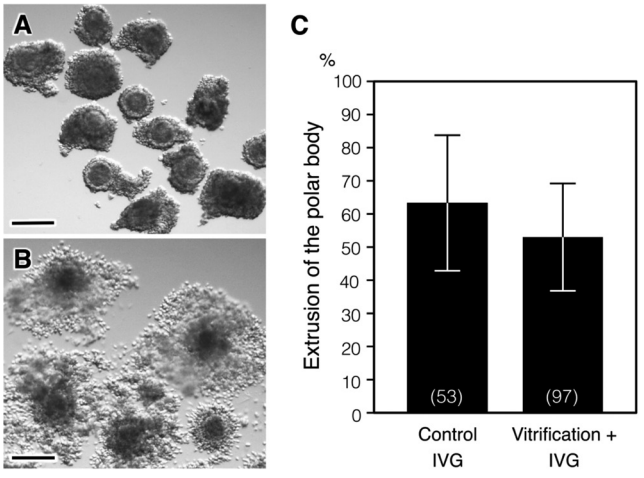

Fig. 3. *In vitro* maturation of oocytes that were vitrified-warmed and cultured *in vitro* for 14 days. (A) Oocytes covered with compact cumulus cell mass after collection on Day 14. (B) Expanded cumulus cells after hormone-induced oocyte maturation. Bar: 200 µm. (C) Percentage of *in vitro*-grown (IVG) oocytes that extruded the first polar body. The data represent mean ± standard deviation values from four replicate experiments. The total number of oocytes is indicated in parentheses.

and DMSO and the total volume of the medium after each step are summarized in Table 1. At step 10, the concentrations of EG and DMSO in BM (total 220 µl) were both 3% (Table 1), and this final step was followed by incubation for 5 min (Fig. 1A). The complexes were then transferred to the vitrification medium, i.e., BM containing 15% EG, 15% DMSO and 0.5 M sucrose [1]. After three transfers in the vitrification medium (lasting approximately 2 min in total), the complexes were placed on a Millicell 12 mm PCF membrane (EMD Millipore, Billerica, MA, USA), and the excess medium was immediately removed by placing sterilized paper underneath the membrane (17.7 ± 3.2 complexes per membrane; mean ± SD). The inserts were then plunged into LN_2 and held in 15-ml centrifuge tubes with holes at the top and bottom. The complexes were maintained in

Table 2. *In vitro* maturation of bovine oocytes that were grown *in vitro* either after vitrification-warming (VW) or immediately after isolation from early antral follicles

VW	No. of oocytes[a]	No. [mean ± SD (%)] of oocytes				
		GV	MI	A/T	MII	Others[b]
−	36	0 (0 ± 0)	8 (24.2 ± 11.6)	2 (6.7 ± 4.7)	19 (52.1 ± 8.7)	7 (17.1 ± 10.0)
+	64	8 (8.9 ± 9.8)	13 (24.0 ± 10.5)	2 (2.4 ± 1.7)	33 (55.3 ± 14.2)	8 (9.4 ± 6.8)

GV, germinal vesicle; MI, metaphase I; A/T, anaphase I or telophase I; MII, metaphase II. [a] Data from three replicate experiments. [b] Oocytes with spindle abnormality, cytoplasmic degeneration or pronucleus-like structure are included.

LN_2 for at least 2 days. When the complexes were recovered from storage in LN_2, they were transferred to BM supplemented with 0.5 M sucrose at 37 C, followed by a 3-step washing process in BM containing decreasing concentrations of sucrose: 0.2 M (3 min), 0.1 M (3 min) and 0.05 M (1 min). The complexes were then washed in the dissection medium described above, and only healthy-looking oocytes were cultured.

In vitro growth of vitrified-warmed oocytes

Modified TCM 199 (M3769) medium [17] was used to culture growing oocytes. TCM 199 was supplemented with 1 mM sodium pyruvate, 40 mg/ml PVP (MW 360,000), 0.14 mM kanamycin sulfate, 20 ng/ml androstenedione, 2 mM hypoxanthine, 0.3 mM cysteine, 150 µM AA2G, 5% FBS, 5 ng/ml bone morphogenetic protein-7 (R&D Systems, Minneapolis, MN, USA), 0.685 mM GlutaMAX (Life Technologies), 1% MEM vitamin mixture (Life Technologies) and 0.5% StemPro nutrient supplements (Life Technologies). The oocyte-granulosa cell complexes, either vitrified-warmed or freshly isolated from early antral follicles, were placed in groups on membrane inserts (Millicell, PICM ORG 50; EMD Millipore) that were set in Falcon 351007 dishes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) with 1 ml and 5 ml of the culture medium on the membrane insert (top) and the Falcon dish (bottom), respectively. The oocytes were incubated at 38.5 C in atmospheres of 5% O_2, 5% CO_2 and 90% N_2 from Day 0 to Day 3 and in 5% CO_2 in air from Day 3 to Day 14. On Day 1, the oocyte diameter excluding the zona pellucida was measured using an ocular micrometer at a magnification of 150×. Half of the medium was replaced with fresh medium on Days 3, 6, 9 and 12. After the 14-day culture period, the oocyte-cumulus cell complexes were extracted from the domes using a fine pipette. The oocytes were considered alive when they appeared normal (with a round shape and smooth surface) and had at least half of their surfaces covered with cumulus cells.

In vitro maturation of oocytes

Maturation of oocytes was conducted in microdrops of the culture medium (5–10 µl/complex) that were covered with paraffin oil in Falcon 351008 dishes (Becton, Dickinson and Company). The culture medium consisted of modified TCM 199 [17] supplemented with 100 ng/ml follicle-stimulating hormone, 500 ng/ml luteinizing hormone (National Institute of Diabetes and Digestive and Kidney Diseases, Baltimore, MD, USA) and 10 ng/ml epidermal growth factor (Upstate Biotechnology, Lake Placid, NY, USA). The oocytes were incubated in an atmosphere of 5% CO_2 in air at 38.5 C with high humidity. Cumulus cells that were mucified during oocyte maturation were dispersed with 0.1% hyaluronidase and removed from the oocytes using a narrow-bore pipette. The oocytes were then mounted on slides, fixed with acetic alcohol and stained using acetic orcein.

Data presentation and statistical analyses

Unless specified, percentage data are presented as mean percentages of at least three independent experimental replicates; the variations between experiments are described by standard deviations of the means. For statistical analyses of percentages, the data were transformed using an arcsine transformation. Comparisons between two groups were conducted using the Student's *t*-test. For multiple comparisons, Tukey's tests were used. P values <0.05 were considered significant.

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