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—Original Article—

Prematurational Culture with 3-Isobutyl-1-methylxanthine Synchronizes Meiotic Progression of the Germinal Vesicle Stage and Improves Nuclear Maturation and Embryonic Development in *In Vitro*-grown Bovine Oocytes

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Abstract. The objective of this study was to clarify the effects of prematurational culture (pre-IVM) supplemented with 3-isobutyl-1-methylxanthine (IBMX) on nuclear and cytoplasmic maturation of *in vitro*-grown bovine oocytes. In experiment 1, oocytes (95 μm in diameter) derived from early antral follicles (0.5–1 mm in diameter) were cultured for 12 days for *in vitro* growth (IVG). IVG oocytes with a normal appearance were subjected to examinations of diameter and chromatin structure in the germinal vesicle (GV) before IVM. In addition, percentages of metaphase II (M II) were examined after IVM. Regardless of pre-IVM, the mean diameters of IVG oocytes were about 115 μm. The proportions of GV3 (50.0%) and M II stages (80.1%) of IVG oocytes with pre-IVM were higher than those without pre-IVM (28.0 and 49.4%, respectively). In experiment 2, the fertilizability and developmental competence of IVG oocytes were examined. Regardless of pre-IVM, the normal fertilization rates of IVG oocytes were similar (around 70%) but were lower than that of *in vivo*-grown oocytes (88.0%). Cleavage and blastocyst rates of IVG oocytes with pre-IVM (63.0 and 26.1%, respectively) were higher than those without pre-IVM (45.8 and 12.7%, respectively). The blastocyst rate based on cleaved IVG oocytes with pre-IVM (41.7%) was similar to that of *in vivo*-grown oocytes (48.7%), although the cleavage rate of IVG oocytes with pre-IVM was lower than that of *in vivo*-grown oocytes. In conclusion, pre-IVM with IBMX improved the maturational and developmental competences of IVG oocytes, probably due to promotion of their chromatin transition and synchronization of meiotic progression.

Key words: Early antral follicle**,** Fertilization, Germinal vesicle, IBMX, Pre-IVM culture

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In bovine ovaries, there are large numbers of growing follicles, offering a large pool of oocytes for *in vitro* production to obtain the embryos of genetically valuable animals. To utilize these oocytes efficiently, studies to improve *in vitro* growth (IVG) culture systems that enable oocytes to acquire the competences for maturation, fertilization and development to blastocysts *in vitro* have been performed recently [1–3]. The birth of calves by transferring embryos derived from IVG oocytes cultured for 14 days was reported [1, 2]. However, the blastocyst rate was 3–18% [1–3], which is lower than with the conventional method (30–40%) for *in vivo*-grown oocytes derived from antral follicles (≥ 2 mm in diameter) [4]. In our previous study, we clarified that the optimal duration of IVG is 12 days, since a longer duration (14 days) of IVG culture resulted in reduced cumulus cell viability, which was related to low developmental competence of oocytes, and shorter duration (10 days) resulted in lower nuclear maturation. Furthermore, prematurational culture (pre-IVM) for 20 h in medium supplemented with phosphodiesterase (PDE) inhibitor (3-isobutyl-1-methylxanthine, IBMX) improved nuclear maturation, resulting in a high blastocyst rate (43% based on cleaved oocytes) in bovine IVG oocytes derived from early antral follicles (0.5–1 mm in diameter) [5]. However, why and how pre-IVM contributes to the improvement of developmental competence are still unclear.

It is well known that the chromatin structure in the germinal vesicle (GV) is subjected to dynamic modifications during oocyte growth [6], and transitions in large-scale chromatin structure have been shown to be essential for growing oocytes to acquire maturational and developmental competences in bovine [7] and mice [8]. Recently, it was reported that bovine oocytes at the GV stage could be divided into 4 stages by the degree of chromatin condensation (GV 0–3) [7, 9] and that oocytes at GV stages 2 and 3 had higher developmental competence than others [9]. In a previous study, we confirmed that most IVG oocytes were at the GV stage before and after pre-IVM; however, the details of the GV structure were not investigated [5]. Therefore, determination of the chromatin structure in the GV of IVG oocytes is necessary to investigate the effect of pre-IVM on the development of IVG oocytes.

Full oocyte maturation involves both nuclear and cytoplasmic events that support normal fertilization and early embryonic development [10]. In a previous study, we showed that the nuclear maturation rate of IVG oocytes was more than 70% by subjecting them to pre-IVM, and was close to that of *in vivo*-grown oocytes (about 90%). However, the cleavage rate (55%) of IVG oocytes was significantly lower than that of *in vivo*-grown oocytes (86%) [5]. One of the causes of low cleavage in IVG oocytes may be inadequate cytoplasmic maturation. Bovine oocytes that had not completed their growth phase at the

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time of submission to an *in vitro* embryo production system failed to cleave, even if the nuclear maturation rate was almost the same [11]. Therefore, cytoplasmic maturation must be evaluated to deal with the effect of pre-IVM, which can be done by assessing pronuclear formation in fertilized oocytes [12–14]. Oocytes with inadequate cytoplasmic maturation have been reported to be incapable of male pronuclear formation in bovine [12] and porcine oocytes [14, 15], and improved cytoplasmic maturation produces high fertilization and development rates in *in vitro*-matured bovine oocytes [16]. To clarify the effect of pre-IVM on the cytoplasmic maturation of IVG oocytes, we should investigate the fertilization and developmental ability of IVG oocytes with/without pre-IVM.

In the present study, we aimed to clarify the effect of pre-IVM supplemented with PDE inhibitor (IBMX) on the nuclear and cytoplasmic maturation of IVG oocytes, chromatin structure of the GV and competences of nuclear maturation, fertilization and early embryonic development of IVG oocytes with/without pre-IVM. In the present study, we used 12-day IVG oocytes for all experiments because the maturation rates of 12- and 14-day IVG oocytes with/without pre-IVM were similar but blastocyst development of 14-day IVG oocytes was impaired by extending the IVG duration in our previous study [5].

Materials and Methods

Chemicals

All the chemicals used for this study were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated.

Collection of early antral follicles and in vitro growth culture of oocyte-granulosa cell complexes

Bovine ovaries obtained at a local abattoir were kept in plastic bags at 20 C and were transported to the laboratory within 6–10 h of collection. After washing 3 times in physiological saline, sliced ovarian cortex tissues (< 1 mm thick) were prepared using a surgical blade (No. 11, Feather Safety Razor, Osaka, Japan) and stored in TCM199 (Cat #31100-035, Invitrogen, Grand Island, NY, USA) supplemented with 0.1% polyvinyl alcohol, 25 mM HEPES, 10 mM sodium bicarbonate (Kanto Chemical, Tokyo, Japan) and 50 μg/ml gentamicin sulfate (isolation medium, pH 7.4 at 37 C) as described elsewhere [17]. Under a stereomicroscope, early antral follicles (0.5 to 1 mm in diameter) were dissected from the sliced ovarian tissues using a surgical blade (No. 20, Feather) and fine forceps. Oocyte-cumulus-granulosa complexes (OCGCs) were isolated from early antral follicles using a pair of fine forceps. The OCGCs with a normal appearance were cultured individually in a 96-well culture plate (Falcon 353872, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) with 200 μl of growth medium for 12 days at 39 C in humidified air with 5% CO_2. The growth medium was HEPES-buffered TCM199 (Cat #12340-030, Invitrogen) supplemented with 0.91 mM sodium pyruvate, 1 μg/ml estradiol-17β, 5% fetal calf serum (FCS; Invitrogen), 4 mM hypoxanthine, 4% polyvinylpyrrolidone (MW 360,000) and 50 μg/ml gentamicin sulfate. At the onset of IVG culture, OCGCs were photographed under an inverted microscope (CK 40, Olympus, Tokyo, Japan) with an attached CCD camera (Moticam 2000, Shimadzu Rika, Tokyo, Japan), and the diameters of oocytes (excluding the zona pellucida) were determined using software (Motic Images Plus 2.2s, Shimadzu). Every 4 days of IVG culture, half (100 μl) of the growth medium was replaced with the same amount of fresh medium.

Pre-IVM and IVM of IVG oocytes

After 12 days of IVG culture, oocytes surrounded by several layers of cumulus cells were collected from morphologically normal OCGCs and submitted to IVM with or without pre-IVM. Pre-IVM of IVG oocytes was performed as they were cultured individually in each well of micro-well plates (Mini Trays 163118, NUNC, Roskilde, Denmark) filled with 6 ml of HEPES-buffered TCM-199 (Cat #12340-030) supplemented with 0.2 mM sodium pyruvate, 2×10^{-6} units/ml follicle-stimulating hormone (FSH, from porcine pituitary), 0.5 mM IBMX, 1 μg/ml estradiol-17β, 10% FCS and 50 μg/ml gentamicin sulfate at 39 C under 5% CO_2 in air for 20 h [5]. For IVM, oocytes were cultured individually in each well of micro-well plates filled with 6 ml of IVM medium, which was HEPES-buffered TCM199 supplemented with 0.2 mM sodium pyruvate, 0.02 units/ml FSH, 1 μg/ml estradiol-17β, 10% FCS and 50 μg/ml gentamicin sulfate, at 39 C under 5% CO_2 in air for 22 h [18].

Oocytes collected from antral follicles of 2 to 8 mm in diameter (*in vivo*-grown oocytes) served as controls. IVM of *in vivo*-grown oocytes was conducted as described previously [19]. In brief, cumulus-oocyte complexes (COCs) were incubated in droplets of IVM medium, the same as used for IVG oocytes (about 10 COCs/50 μl), covered with paraffin oil at 39 C under 5% CO_2 in air for 22 h.

IVF and IVC of inseminated oocytes

IVF was performed using frozen semen according to a procedure described previously [20] with slight modification. In brief, motile sperm (5×10^6 sperm/ml) separated from thawed semen by a Percoll gradient (45 and 90%) were co-incubated with COCs in a 100-μl droplet (about 10 COCs per droplet) of modified Brackett and Oliphant isotonic medium [21] containing 3 mg/ml fatty acid-free BSA and 2.5 mM theophylline [22] for 18 h at 39 C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2.

IVC of inseminated oocytes (presumptive zygotes) was performed as described previously [19, 23]. Briefly, after co-incubation with sperm, presumptive zygotes were freed from cumulus cells by vortexing and washing three times in the culture medium. Cumulus-free zygotes were cultured for 6 days in a 30-μl droplet of the culture medium at 39 C under 5% CO_2, 5% O_2 and 90% N_2. The culture medium was a modified synthetic oviduct fluid containing 1 mM glutamine, 12 essential amino acids for basal medium Eagle, 7 nonessential amino acids for minimum essential medium and 10 μg/ml insulin [19] and was further supplemented with 5 mM glycine, 5 mM taurine, 1 mM glucose and 3 mg/ml fatty acid-free BSA [23]. After 2 days (about 30 h) and 6 days (about 150 h) of IVC, cleavage and development of presumptive zygotes to the blastocyst stage were assessed, respectively. Total cell numbers of blastocysts obtained after 6 days of IVC were counted using an air-drying method [22].

Evaluation of oocyte chromatin configuration, nuclear status and fertilization

Oocytes were denuded from cumulus cells by vortexing and

photographed under an inverted microscope with an attached CCD camera, and the diameters of oocytes (excluding the zona pellucida) were determined using software. Then oocytes were fixed in 60% methanol in Dulbecco's phosphate buffer solution for 30 min at 4 C and stained with 5 μg/ml Hoechst 33342 for 15 min. The chromatin configuration of the GV in denuded oocytes was evaluated under an inverted microscope (TE300, Nikon, Tokyo, Japan) equipped with a fluorescence system using an appropriate filter (UV-2A, Nikon). Oocytes at the GV stage were classified according to the degree of chromatin condensation, as described previously [9]: GV0, with a diffuse filamentous pattern of chromatin in the whole nuclear area; GV1, with a few foci of chromatin condensation; GV2, with chromatin further condensed into distinct clumps or strands; and GV3, with chromatin condensed in a single clump.

After IVM, denuded oocytes were stained with 1% aceto-orcein and examined for their nuclear status as described elsewhere [24]. Nuclear statuses were divided into GV, germinal vesicle breakdown (GVBD), metaphase I (M I), anaphase I/telophase I (A I/T I) and metaphase II (M II) by observation under a phase contrast microscope.

After IVF, the cumulus-free oocytes were stained with 1% aceto-orcein and examined for their fertilization as described elsewhere [20]. The oocytes were considered fertilized when they had an enlarged sperm head or male pronucleus with a corresponding sperm tail. Normal fertilization was defined as oocytes having a single male pronucleus or a single enlarged sperm head.

Experimental design

Experiment 1: GV status and diameters of IVG oocytes were examined before and after pre-IVM (about 10–15 oocytes per replicate). A total of 102 IVG oocytes derived from 147 OCGCs were used (69.4%, 102/147) (Table 1). After IVM culture, nuclear statuses of IVG oocytes were investigated (about 15–20 oocytes per replicate). A total of 184 IVG oocytes derived from 243 OCGCs were subjected to IVM with or without pre-IVM (75.7%, 184/243) (Table 2).

Experiment 2: IVG oocytes after IVM with or without pre-IVM (about 30–40 oocytes per replicate) were subjected to IVF and IVC. Their fertilization was examined by using a total of 331 IVG oocytes derived from 528 OCGCs (62.7%, 331/528) (Table 3). Cleavage and development to the blastocyst stage were also examined by using a total of 355 IVG oocytes derived from 497 OCGCs (71.4%, 355/497) (Table 4).

Statistical analysis

Maturation, fertilization, cleavage and blastocyst rates and cell numbers in blastocysts were analyzed by one-way ANOVA followed by Tukey-Kramer's HSD test. Percentages of oocytes with each chromatin configuration were compared by chi-square test. All statistical analyses were performed using software (JMP version 8.02, SAS Institute, Cary, NC, USA).

Results

Experiment 1

As shown in Table 1, the mean diameter of isolated oocytes before IVG culture was around 95 μm. The mean diameters of IVG oocytes with or without pre-IVM were similar and were smaller than that of *in vivo*-grown oocytes ($P<0.05$). After pre-IVM, the proportion of IVG oocytes at GV1 was lower and that at the GV3 stage was higher than that before pre-IVM ($P<0.05$). These values after pre-IVM were similar to those of *in vivo*-grown oocytes.

As shown in Table 2, the M II rate of IVG oocytes with pre-IVM was higher than that without pre-IVM ($P<0.05$) and similar to that of *in vivo*-grown oocytes.

Experiment 2

As shown in Table 3, total and normal fertilization rates of IVG oocytes with pre-IVM were similar to those without pre-IVM. However, the total fertilization rate of IVG oocytes with pre-IVM tended to be lower ($P=0.05$) and the normal fertilization rate was lower than those of *in vivo*-grown oocytes ($P<0.05$). Polyspermy rates were similar and <10% in all experimental groups.

As shown in Table 4, the cleavage rate of IVG oocytes with pre-IVM was higher than that without pre-IVM ($P<0.05$) but lower than that of *in vivo*-grown oocytes ($P<0.05$). The blastocyst rate of IVG oocytes with pre-IVM based on cleaved oocytes was similar to that of *in vivo*-grown oocytes ($P=0.61$), although that based on inseminated oocytes tended to be lower than that of *in vivo*-grown oocytes ($P=0.05$). The blastocyst rate based on inseminated and cleaved IVG oocytes with pre-IVM tended to be higher than those of IVG oocytes without pre-IVM ($P=0.08$ and $P=0.17$, respectively). Cell numbers in blastocysts in IVG oocytes with and without pre-IVM tended to be lower than that of the control ($P=0.25$ and $P=0.18$, respectively).

Discussion

When IVG oocytes were subjected to pre-IVM with IBMX, the percentages of IVG oocytes at GV2 and GV3 were 29 and 50%, respectively. These results are similar to those of *in vivo*-grown oocytes (35 and 49%, respectively) and consistent with those previously reported (31 and 45%, respectively) [9]. The higher M II rate at 22 h IVM culture and higher blastocyst rate of IVG oocytes with pre-IVM may be caused by meiotic progression and synchronization to GV2-3 of IVG oocytes during pre-IVM. By promoting and synchronizing to GV2-3, fertilization may occur at appropriate timing for embryonic development in IVG oocytes with pre-IVM.

In the present study, the M II rate of IVG oocytes without pre-IVM was lower than that with pre-IVM, and the percentages of M I and A I/T I stages were higher in IVG oocytes without pre-IVM. Despite the low M II rate of IVG oocytes without pre-IVM, the fertilization rate was similar to that with pre-IVM. These results indicate that IVG oocytes without pre-IVM might reach the M II stage during IVF culture and suggest that extension of the IVM duration to more than 22 h may increase the percentage of M II oocytes but also increase the variation in the timing of M II arrival. Previous reports indicated that the oocyte's ability to develop into a blastocyst was not acquired immediately after M II arrival and that it might develop gradually after the completion of nuclear maturation [25, 26]. If oocytes were inseminated immediately after M II arrival, the cleavage and blastocyst rates were shown to be significantly reduced [26]. In the present study, slower meiotic progression of IVG oocytes without pre-IVM may have resulted in sperm penetration immediately after the completion of nuclear maturation and led to lower cleavage and

Table 1. Chromatin structure and diameter of IVG oocytes before and after pre-IVM

Pre-IVM	No. of oocytes (replicates)	Diameter of oocytes (μm*)		% of oocytes at each GV stage		
		Before IVG	After IVG	GV1	GV2	GV3
Before	50 (4)	95.0 ± 4.4	115.5 ± 4.8[a]	40.0[a]	32.0	28.0[a]
After	52 (3)	94.3 ± 4.0	116.2 ± 5.1[a]	21.2[b]	28.8	50.0[b]
Control**	65 (3)	-	125.3 ± 4.0[b]	15.4[b]	35.4	49.2[b]

[a, b] Values with different superscripts within columns are significantly different (P<0.05). * Mean ± SD. ** The oocytes immediately after collection from antral follicles (2 to 8 mm in diameter) served as *in vivo*-grown controls. GV1, with a few foci of chromatin condensation; GV2, with chromatin further condensed into distinct clumps or strands; GV3, with chromatin condensed in a single clump.

Table 2. Effect of pre-IVM on nuclear maturation of IVG oocytes

Pre-IVM	No. of oocytes (replicates)	% of oocytes at each stage				
		GV	GVBD	M I	A I/T I	M II
–	85 (5)	5.0 ± 7.2	2.1 ± 2.9	26.6 ± 9.4[a]	15.8 ± 10.7[a]	49.4 ± 10.4[a]
+	99 (5)	1.3 ± 2.8	0	15.9 ± 4.3[b]	2.7 ± 6.1[b]	80.1 ± 9.6[b]
Control*	120 (5)	0	0	9.6 ± 2.9[b]	1.7 ± 2.4[b]	88.7 ± 1.9[b]

[a, b] Values (mean ± SD) with different superscripts within columns are significantly different (P<0.05). * The oocytes collected from antral follicles (2 to 8 mm in diameter) served as *in vivo*-grown controls. GV, germinal vesicle; GVBD, germinal vesicle breakdown; M I, metaphase I; A I/T I, anaphase I/telophase I; M II, metaphase II.

Table 3. Effect of pre-IVM on fertilization of IVG oocytes

Pre-IVM	No. of oocytes (replicates)	% normal fertilization			% of polyspermy	% total fertilization
		% of ESH	% of 2PN	% of subtotal		
–	124 (5)	8.7 ± 9.3	56.8 ± 12.4	65.6 ± 7.9[a]	7.5 ± 5.1	73.1 ± 4.4[a]
+	207 (7)	14.0 ± 12.3	58.9 ± 16.3	73.0 ± 12.6[a]	6.3 ± 3.0	79.7 ± 13.6[ab]
Control*	148 (6)	20.6 ± 18.5	66.7 ± 17.3	87.3 ± 6.2[b]	5.1 ± 4.6	93.1 ± 4.9[b]

[a, b] Values (mean ± SD) with different superscripts within columns are significantly different (P<0.05). * The oocytes collected from antral follicles (2 to 8 mm in diameter) served as *in vivo*-grown controls. ESH, enlarged sperm head; 2PN, two pronuclei.

Table 4. Effect of pre-IVM on embryonic development of IVG oocytes

Pre-IVM	No. of oocytes (replicates)	% of cleaved oocytes	% of blastocysts based on		Cell no. in blastocysts (n)
			Inseminated oocytes	Cleaved oocytes	
–	148 (5)	45.8 ± 9.0[a]	12.7 ± 7.1[a]	26.9 ± 10.4[a]	125 ± 60 (18)
+	207 (5)	63.0 ± 5.5[b]	26.1 ± 6.9[ab]	41.7 ± 11.4[ab]	137 ± 57 (54)
Control*	170 (6)	83.0 ± 5.8[c]	40.3 ± 11.4[b]	48.7 ± 13.8[b]	156 ± 72 (68)

[a–c] Values (mean ± SD) with different superscripts within columns are significantly different (P<0.05). * The oocytes collected from antral follicles (2 to 8 mm in diameter) served as *in vivo*-grown controls.

embryonic development. A previous study examined the effects of meiotic arrest during pre-IVM on maturational and developmental competence of *in vivo*-grown oocytes [27], and it showed that the time required for nuclear maturation of oocytes with pre-IVM was shorter than the time required for oocytes without pre-IVM. Therefore, we should examine the details of meiotic progression of IVG oocytes with or without pre-IVM in a future study.

The cleavage rate (83.0%) and normal fertilization rate (87.3%) in *in vivo*-grown oocytes were similar and higher than those of IVG oocytes in the present study. On the other hand, cleavage rates of IVG oocytes with pre-IVM and without pre-IVM were only 63.0 and 45.8%, despite their normal fertilization rates being 73.0 and 65.6%, respectively. The reason for this discrepancy between normal fertilization and cleavage rates in IVG oocytes is not clear. It suggests that cytoplasmic maturation in IVG oocytes might have been inadequate and could not support oocyte cleavage, although we did not identify a difference in pronucleus formation in all experimental groups. Early embryonic development is dependent on stored maternal RNAs and proteins synthesized during oogenesis and oocyte maturation [28]. It was reported that there was a concomitant decrease in total RNA

synthesis once the oocyte diameter increased beyond 110 μm and that RNA synthesis ceased in oocytes ≥ 120 μm in diameter [29]. These results may indicate that lack of some types of mRNA in IVG oocytes results in low cleavage rates because the diameters of the IVG oocytes were between 110 and 120 μm. However, the cleavage rate in IVG oocytes with pre-IVM was improved compared with that without pre-IVM. This may indicate that IVG oocytes store some RNAs/proteins during pre-IVM to support subsequent cleavage and development. Under the present IVG system, cleaved IVG oocytes with pre-IVM showed high developmental competence similar to that of *in vivo*-grown oocytes, although oocytes derived from early antral follicles could not grow to ≥ 120 μm in diameter. A previous study reported that bovine *in vivo*-grown oocytes with a diameter < 115 μm could not acquire nuclear and/or cytoplasmic maturation [30]. In contrast, it was reported that IVG oocytes in mice exhibited similar developmental competence to *in vivo*-grown oocytes, even if the mean diameter of the IVG oocytes (around 70 μm) was smaller than that of the *in vivo*-grown oocytes (around 80 μm) [31]. Our results suggest that cleaved IVG oocytes with pre-IVM acquire similar developmental competence to *in vivo*-grown oocytes, even at around 115 μm in diameter.

In conclusion, pre-IVM improved the nuclear maturational and developmental competences of IVG oocytes, probably due to promotion of their chromatin transition and synchronization of meiotic progression. The present culture system achieved a higher nuclear maturation rate of IVG oocytes but low fertilization and cleavage rates. This may be due to sub-optimal conditions, leading to insufficient cytoplasmic maturation of IVG oocytes. In a future study, we should investigate the cytoplasmic status of IVG oocytes during pre-IVM.

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