Effects of pre-maturational culture duration on developmental competence of bovine small-sized oocytes

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Abstract

We investigated the effects of pre-maturational (pre-IVM) culture on the developmental competence of small-sized bovine oocytes (110 and <115 μm). Oocytes were cultured with 3-isobutyl-1-methylxanthine (IBMX) for 0, 5, or 10 h and subjected to in vitro maturation, fertilization, and culture. The cleavage rate (73%) of small-sized oocytes with 5 h pre-IVM was higher than those with 0 and 10 h pre-IVM (61 and 62%, respectively). The blastocyst rate (16%) of embryos derived from small-sized oocytes with 5 h pre-IVM was higher than those with 0 and 10 h pre-IVM (9 and 8%, respectively). In addition, small-sized oocytes with 5 h pre-IVM had a higher mean cell number in blastocysts (134.1±34.8) than those with 0 and 10 h pre-IVM (100.2±17.2 and 107.8±23.7, respectively). In conclusion, the pre-IVM of small-sized oocytes with IBMX for 5 h improved the developmental competence of bovine oocytes, as well as the quality of blastocysts.

Keywords*:* Oocytes, IBMX, Pre-IVM duration

Bovine oocytes obtained from slaughterhouse-derived ovaries and from ovum pick-up (OPU) from live cows are widely used for *in vitro* embryo production (IVEP). Previous studies have focused on establishing a suitable system to efficiently employ these oocytes [1-4]. However, the oocytes aspirated from these ovaries vary in size, and development to the blastocyst stage is known to increase with larger follicular and oocyte diameters [5]. It was reported that the percentage of oocytes of 115 to < 120 μm in size that achieve metaphase II (MII) and develop to blastocyst stage was higher than that of oocytes sized 110 to < 115 μm [6]. Furthermore, there was a positive correlation between oocyte diameter and follicular size [7]. When OPU is performed, follicles more than 2 mm in diameter are generally aspirated, but the number of harvested oocytes is limited [8]. It was also reported that the mean diameter of oocytes collected from follicles of 2-3 mm in diameter was 112.9 μm [7], thus, the improvement of small-sized oocytes (110 to <115 μm) should be attempted for the effective IVEP.

Previous studies have demonstrated improvement of the developmental competence of oocytes by inhibiting germinal vesicle breakdown (GVBD) and holding oocytes at the germinal vesicle (GV) stage before *in vitro* maturation (IVM), because oocytes require time to acquire full developmental competence during meiotic arrest [9]. We speculated that oocytes collected by OPU, especially small-sized oocytes, need time prior to IVM to acquire developmental competence, and this time is different than that for large-sized oocytes (≥115 μm). In our previous study [10], we showed that culture with 3-isobutyl-1-methylxanthine (IBMX) for 20 h before IVM culture (pre-IVM) had a positive effect on the developmental competence of oocytes derived from 12 days of *in vitro* growth (IVG) culture; however, pre-IVM treatment for 20 h did not improve the rate of development to the blastocyst stage in oocytes derived from 14 days of IVG culture. In addition, we found that the low developmental rate of 14-day IVG oocytes was associated with reduced integrity of the cell membranes of cumulus cells [10]. We also showed that several small-sized oocytes (<115 μm) derived from antral follicles (2-8 mm) started to degenerate and that some of the cumulus cell process endings lost gap junctions with the oocytes [11], because the cow is a mono-ovulator and most of the oocytes used for IVEP are destined to degenerate. Therefore, the effect of pre-IVM treatment with IBMX on the developmental competence of small-sized

oocytes derived from antral follicles (2-8 mm) is unclear. In the present study, we collected bovine oocytes from slaughterhouse-derived ovaries and divided them into small-sized (110 to <115 μm) and large-sized (≥115 μm) oocytes and cultured them for 0, 5, or 10 h with IBMX before IVM culture. The oocytes were then submitted to IVM, *in vitro* fertilization (IVF), and *in vitro* culture (IVC) and their development to the blastocyst stage was examined.

Immediately before pre-IVM culture, all small- and large-sized oocytes were at metaphase II (MII; 19/19 and 22/22, respectively). Among the small-sized oocytes, the proportions of oocytes that reached metaphase I (MI) and overall meiotic resumption when treated with IBMX for 5 h were lower than those treated for 10 h pre-IVM ($P < 0.05$) (Table 1). However, the proportion of oocytes at GV stage was higher ($P < 0.05$) when the oocytes were treated for 5 h than when they were treated for 10 h pre-IVM. Similarly, in large-sized oocytes, the percentage of MI oocytes and the overall meiotic resumption were higher ($P < 0.05$) in oocytes treated for 10 h than in those treated for 5 h pre-IVM, whereas lower percentages at the GV stage were observed in oocytes treated for 10 h than in those treated for 5 h pre-IVM ($P < 0.05$). After IVM culture, the overall meiotic resumption was similar between the small and the large-sized oocytes, regardless of the duration of pre-IVM treatment (Table 2). In addition, the percentage of MII oocytes was similar among oocytes of the same size category that were treated for 0, 5, and 10 h pre-IVM. However, the percentage of MII oocytes among those treated for 0 and 10 h pre-IVM was higher among large-sized than among small-sized oocytes ($P < 0.05$), while the percentage of MII oocytes among those treated for 5 h pre-IVM tended to be higher among large-sized than among small-sized oocytes ($P = 0.06$). On the other hand, the rate of MI oocytes was higher among small-sized oocytes than among large-sized oocytes, regardless of the duration of pre-IVM treatment ($P < 0.05$).

As shown in Fig. 1, the major effects of the size of oocytes and the duration of pre-IVM treatment were evident through cleavage and blastocyst rates ($P < 0.05$), but not through the cell numbers of blastocysts ($P > 0.05$). Namely, the cleavage rate (73%) of embryos derived from the small-sized oocytes treated for 5 h pre-IVM was higher ($P < 0.05$) than that of those treated for 0 and 10 h pre-IVM (61 and 62%, respectively) (Fig. 1). However, the cleavage rates were lower in small-sized oocytes than in large-

sized oocytes, regardless of the duration of pre-IVM treatment ($P < 0.05$). This difference may be caused by the lower maturation rate in small-sized oocytes than in large-sized oocytes. Based on inseminated oocytes, the blastocyst rate (16%) of embryos derived from small-sized oocytes subjected to 5 h pre-IVM treatment was higher ($P < 0.05$) than that of those subjected to 0 and 10 h pre-IVM treatment (9 and 8%, respectively), but was lower ($P < 0.05$) than that of large-sized oocytes (31%). In addition, blastocysts derived from small-sized oocytes treated for 5 h pre-IVM had a higher mean cell number (134.1 ± 34.8) than those derived from oocytes treated for 0 and 10 h pre-IVM (100.2 ± 17.2 and 107.8 ± 23.7, respectively). A previous mouse and bovine study using cumulus-oocyte complex (COC) [12] demonstrated that IBMX treatment for 1-2 h pre-IVM increased COC cAMP levels 100-fold and improved embryo cleavage, blastocyst rates, and embryo quality. Additionally, the pre-IVM treatment had a positive influence on the developmental competence of oocytes in pigs by improving cytoplasmic maturation [13]. In the present study, we also added low concentration of FSH during pre-IVM treatment, expecting to increase cAMP levels in oocytes. Sugimura *et al*. [14] reported that bovine oocytes treated with IBMX for 2 h followed by 22 h IVM with FSH significantly enhanced the ability of oocytes to develop to blastocysts. They suggested that pre-treatment with IBMX enhanced the effectiveness of FSH at improving oocyte developmental competence [14]. In the present study, we cultured bovine oocytes with simultaneous addition of IBMX and FSH for 5 h, and we did not observe a significant increase in blastocyst rate among large-sized oocytes. However, the synergistic effect of IBMX and FSH on developmental competence was observable in small-sized oocytes after 5 h of pre-IVM treatment. These results may suggest that the proper duration of pre-IVM treatment is different for large- and small-sized oocytes. We should thus determine the optimal pre-IVM treatment duration for oocytes of different sizes or oocytes derived from follicles of different sizes (estrous cycle), as well as the dynamics of cAMP concentrations in oocytes during pre-IVM treatment with IBMX and FSH, in a future study.

Large-sized oocytes were previously reported to show higher developmental competence than small-sized oocytes, and bovine oocytes derived from larger follicles exhibited stronger mitochondrial activity and a higher proportion of blastocyst development than those from smaller follicles [15]. Our results

112 demonstrated that the pre-IVM treatment of small-sized oocytes improved their developmental
113 competence. The intracellular secondary messenger cAMP plays an important role in the regulation of
114 mitochondrial activity in mammalian cells [16-18]. IBMX prevents the deprivation of cAMP in COCs,
115 which significantly increases cAMP levels and further enhances meiosis progression in oocytes, similar to
116 what occurs during *in vivo* oocyte maturation [19]. Furthermore, mitochondrial activity of bovine oocytes
117 increases during follicular development, and stronger mitochondrial activity is accompanied by greater
118 developmental competence of immature oocytes [15]. We showed that the mitochondrial activity of *in*
119 *vitro* grown oocytes of 105.9-122.7 mm in diameter increased during pre-IVM treatment and was
120 accompanied by the acquisition of developmental competence [20]. In the present study, cAMP
121 concentrations in oocytes may have been increased by the addition of IBMX and a low concentration of
122 FSH to the pre-IVM medium, and mitochondrial activity before the IVM culture may also have increased;
123 it is possible that these phenomena improved the developmental competence of small-sized oocytes
124 treated for 5 h pre-IVM. The extension of the pre-IVM treatment to 10 h may have resulted in the aging
125 of oocytes, subsequently inducing oocyte or cumulus cell degradation and reducing not only
126 mitochondrial activity before IVM culture, but also the developmental competence of oocytes.
127 In the case of oocytes with a diameter of ≥ 115 µm, 5- and 10-h pre-IVM treatment did not have any
128 detrimental effects (aging) on the cleavage and blastocyst rates; however, the cell number in blastocysts
129 significantly increased after the 5- and 10-h pre-IVM treatment ($P < 0.05$) compared to untreated oocytes.
130 These results indicate that embryo quality may be improved by pre-IVM treatment due to improved
131 cytoplasmic maturation.
132 In conclusion, the results of our study show that pre-IVM treatment with FSH and IBMX for 5 h
133 improved the developmental competence of bovine oocytes of a diameter between 110 and < 115 µm as
134 well as of those of a diameter of ≥ 115 µm. These results suggest that the approach to enhance the
135 developmental competence of oocytes using the pre-IVM treatment for 5 h, regardless of oocyte diameter,
136 will ultimately be useful in the management of IVEP in bovines. In the present study, we showed the

benefits of pre-IVM treatment for only 5 and 10 h pre-IVM; therefore, the appropriate duration of pre-IVM treatment should be determined in a future study.

Methods

Chemicals

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

Collection of cumulus oocyte complexes (COCs)

Bovine ovaries were obtained from a local abattoir. They were transported to the laboratory within 6 h of collection in plastic bags at 20°C. After three washes in sterile physiological saline, follicles (2-8 mm in diameter) were aspirated using an 18-gauge needle attached to a 10-ml syringe containing TALP working medium, supplemented with 10% fetal bovine serum (FBS, Invitrogen, Grand Island, NY, USA). Aspirated follicular fluid was pooled in 50-ml conical tubes and allowed to settle. COCs were searched for under a stereomicroscope and washed three times in TALP working medium; those with more than three layers of cumulus cells and a uniform cytoplasm were selected for further processing. The diameters of oocytes (excluding the zona pellucida) were measured using an ocular scale attached to the stereomicroscope and then divided into COCs having oocytes with diameters between 110 and < 115 μm (small-sized) and those having oocytes with a diameter of ≥ 115 μm (large-sized).

Pre-IVM treatment and IVM of COCs

COCs were submitted to IVM with or without pre-IVM treatment, as described previously [21]. Briefly, COCs were incubated in droplets of pre-IVM medium (approximately 10 COCs/50 μl), which was modified from IVM medium containing 0.5 mM IBMX, and a lower FSH concentration (2×10^{-6} units/ml, from the porcine pituitary), and covered with paraffin for 0, 5, or 10 h. The IBMX stock solution was diluted to 500 mM in dimethyl sulfoxide, and 10 μl of the stock solution was mixed with 10 ml pre-

IVM medium, yielding a final concentration of IBMX of 0.5 mM. The maturation medium consisted of HEPES-buffered TCM199 supplemented with 0.2 mM sodium pyruvate, 0.02 units/ml FSH, 1 μg/m1 estradiol-17β, 10% FBS, and 50 μg/ml gentamicin sulfate, covered with paraffin at 39°C for 22 h in a 5% CO_2 atmosphere. The total culture periods were 22, 27, or 32 h in the 0, 5, and 10 h pre-IVM groups, respectively.

Evaluation of the oocyte nuclear status after pre-IVM treatment

After pre-IVM and/or IVM, oocytes were denuded from cumulus cells by vortexing and stained with 1% aceto-orcein. The nuclear status was classified as germinal vesicles (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), or metaphase II (MII) by observation under a phase-contrast microscope [3].

In vitro fertilization (IVF) and subsequent culture (IVC)

IVF using frozen semen was performed according to a previously described procedure [22] with slight modifications. Briefly, motile sperm (5×10^6 sperm/ml) separated in a Percoll gradient (45% and 90%) were incubated with COCs in a 100-μl droplet (approximately 10 COCs per droplet) of modified Brackett and Oliphant isotonic medium [23] containing 3 mg/mL fatty acid-free BSA and 2.5 mM theophylline [24] at 39°C for 18 h in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2. IVC of inseminated oocytes (presumptive zygotes) was performed as previously described [25]. Briefly, after an incubation with sperm, presumptive zygotes were freed from cumulus cells by vortexing and washing three times in culture medium. Cumulus-free zygotes were cultured at 39°C for 6 days in 30-μl droplets of culture medium in a 5% CO_2, 5% O_2, and 90% N_2 atmosphere. The culture medium consisted of modified synthetic oviduct fluid containing 1 mM glutamine, 12 essential amino acids for basal medium Eagle, seven non-essential amino acids for minimum essential medium, 10 μg/ml insulin, 5 mM glycine, 5 mM taurine, 1 mM glucose, and 3 mg/ml fatty acid-free BSA. Cleavage and blastocyst rates were assessed after 2 days (approximately 30 h) and 6 days (approximately 150 h) of IVC, respectively. The total cell number in blastocysts obtained after 6 days of IVC was counted using an air-drying method [24].

Statistical analysis

All statistical analyses were performed using JMP software version 11.0.0 (SAS Institute, Cary, NC, USA). The oocyte nuclear status was analyzed by the chi-square test. Interactions among the size of collected COCs, duration of the pre-IVM culture, and developmental competence were compared by two-way ANOVA followed by Turkey-Kramer's HSD as a *post-hoc* test. Differences of $P < 0.05$ were regarded as significant.

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Competing interests

Authors declare that they have no competing interests.

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Figure Legend

Figure 1 Cleavage rates, blastocyst rates, and blastocyst cell numbers for small-sized (110 to < 115 μm in diameter) and large-sized oocytes (≥ 115 μm in diameter) after 0, 5, and 10 h of pre-IVM treatment with IBMX. The number in the bar is the number of oocytes, and the number of replicates is in parentheses.

* Asterisk indicates a significant difference between experimental groups ($P < 0.05$).

[a,b] Different letters indicate a significant difference among small-sized oocytes ($P < 0.05$).

[x,y] Different letters indicate a significant difference among large-sized oocytes ($P < 0.05$).

Figure 1

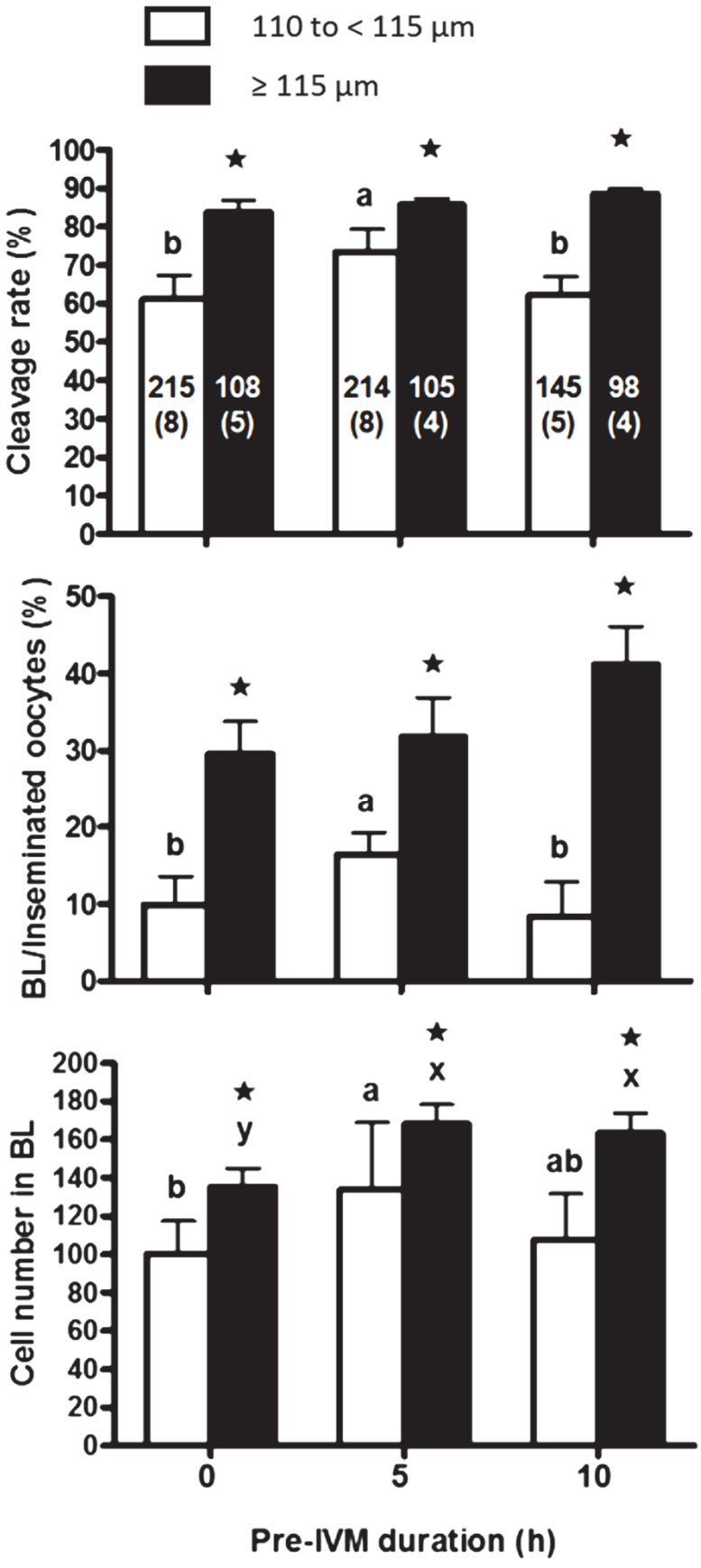
110 to < 115 µm
≥ 115 µm
Cleavage rate (%)
BL/Inseminated oocytes (%)
Cell number in BL
215 (8)
108 (5)
214 (8)
105 (4)
145 (5)
98 (4)
b
a
b
b
a
b
b
y
a
x
ab
x
0
5
10
Pre-IVM duration (h)

Table 1 Effects of pre-IVM durations on the nuclear status of oocytes before IVM

Oocyte diameter (μm)	Pre-IVM (h)	No. of oocytes (replicates)	No. of oocytes in each meiotic stage (%)					GVBD-MII
			GV	GVBD	MI	MII	Deg.	
110 to < 115	5	101(5)	93 (92.1)[a)]	0 (0)	6 (5.9)[a)]	0 (0)	2 (0.2)[a)]	6 (5.9)[a)]
	10	117 (5)	49 (41.9)[b)]	1(0.9)	63 (53.8)[b)]	0 (0)	4 (3.4)[b)]	64 (54.7)[b)]
≥ 115	5	54 (4)	47 (87.0)[a)]	1(1.9)	5 (9.5)[a)]	0 (0)	1(1.8)	6 (11.1)[a)]
	10	64 (3)	35 (54.7)[b)]	0 (0%)	28 (43.8)[b)]	0 (0)	1(1.6)	28 (43.8)[b)]

GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, metaphase I; MII, metaphase II.

[a,b)] Within a column, values without a common superscript differed significantly ($P < 0.05$).

Table 2 Effects of pre-IVM durations on the nuclear status of oocytes after IVM

Oocyte diameter (μm)	Pre-IVM (h)	No. of oocytes (replicates)	No. of oocytes in each meiotic stage (%)					GVBD-MII
			GV	GVBD	MI	MII	Deg.	
110 to < 115	0	49 (3)	5 (10.2)	6 (12.2)	9 (18.4)*	26 (53.1)	2 (4.1)	40 (81.6)
	5	40 (3)	1 (2.5)	0 (0)	11 (27.5)*	27 (67.5)	1 (2.5)	38 (95)
	10	43 (3)	0 (0)	0 (0)	13 (30.2)*	28 (65.1)	2 (4.6)	41 (95.3)
≥ 115	0	44 (3)	1 (2.3)	3 (6.8)	2 (4.5)	37 (84.1)*	1 (2.3)	42 (95.5)
	5	36 (3)	1 (2.8)	1 (2.8)	3 (8.4)	31 (86.1)	0 (0)	35 (97.2)
	10	34 (3)	0 (0)	0 (0)	2 (5.9)	32 (94.1)*	0 (0)	34 (100)

GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, metaphase I; MII, metaphase II.

*Asterisks indicate the difference among different oocyte groups of the same sizes and with the same duration of pre-IVM treatment ($P < 0.05$).