Title: Extension of the culture period for the *in vitro* growth of bovine oocytes in the presence of bone morphogenetic protein-4 increases oocyte diameter, but impairs subsequent developmental competence

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Running Head: PROLONGED OOCYTE CULTURE WITH BMP-4

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ABSTRACT

Bone morphogenetic protein-4 (BMP-4) inhibits luteinization of granulosa cells during *in vitro* growth (IVG) culture of bovine oocytes; however, oocytes derived from a 12-day IVG were less competent for development than *in vivo*-grown oocytes. We herein investigated whether an extended IVG culture with BMP-4 improves oocyte growth and development to blastocysts after *in vitro* fertilization. Oocyte-granulosa cell complexes (OGCs) were cultured for 14 or 16 days with BMP-4 (10 ng/mL), while a 12-day culture with BMP-4 served as the *in vitro* control. OGC viability was maintained for the 16-day culture with BMP-4 (83.2%), but was significantly lower without BMP-4 (58.9%) than the control (83.0%). Prolong-cultured oocytes at 16 days had statistically greater diameter (114.6 μm) than the control (111.7 μm). IVG oocytes with BMP-4 for the 16-day culture had a similar nuclear maturation rate to the control (approximately 67%); however, blastocyst rates in BMP-4 treated oocytes of 14- (1.8%) and 16-day (0%) IVG were statistically lower than that of 12-day IVG (9.0%). In conclusion, BMP-4 maintained OGC viability and promoted oocyte growth in a prolonged culture, but impaired the developmental competence of oocytes. Prolonged culture may not be an appropriate strategy for enhancing the developmental competence of IVG oocytes.

Key words: *BMP-4, bovine oocyte, developmental competence,* in vitro *growth*

INTRODUCTION

In vitro-grown oocytes are a potential oocyte source for the *in vitro* production of embryos in the cattle industry. Although live calves derived from *in vitro*-grown oocytes have been reported by several research groups, the low viability of oocytes during *in vitro* growth (IVG) culture (Yamamoto *et al.* 1999; Senbon & Miyano 2002; Huang *et al.* 2016) and the developmental competence of IVG oocytes (Yamamoto *et al.* 1999; Senbon & Miyano 2002; Huang *et al.* 2016) remain problematic. The adequate IVG culture of oocytes is a prerequisite for better subsequent development acquisition. In the currently developed IVG culture system, theca cell-free oocyte-granulosa cell complexes (OGCs) are used. Theca cells support the follicular structure and provide androgens to granulosa cells for estrogen synthesis. In addition, theca cells produce some growth factors; however, their effects on the *in vitro* growth of oocytes remain unclear.

One type of growth factor produced by theca cells is bone morphogenetic proteins (BMPs), which regulate oocyte growth (Young & McNeilly 2010). BMP-4, one of the products exclusively supplied by theca cells, has been shown to regulate the growth and functions of granulosa cells by suppressing apoptosis and enhancing the secretion of estradiol, while delaying luteinization and/or atresia by reducing progesterone secretion *in vitro* (Knight & Glister 2003; Kayamori *et al.* 2009; Yamashita *et al.* 2011). A previous *in vivo* study also showed that BMP-4 mRNA was expressed at high levels in dominant follicles,

while it was very low or undetectable in atretic follicles from rat ovaries (Erickson & Shimasaki 2003). These findings demonstrated the anti-luteinization and/or anti-atresia roles played by BMP-4 in order to maintain the “health” of follicles. We previously confirmed the anti-luteinizing effects of BMP-4 on granulosa cells co-cultured with bovine oocytes, and oocytes showed a healthy appearance after a 12-day IVG culture with BMP-4 (Yang *et al.* 2016). However, oocytes did not grow to a similar size to *in vivo*-grown oocytes, and their developmental competence was low. Cho *et al.* (2008) reported that oocytes grew to a larger size after a 16-day IVG culture in the presence of fibroblast growth factor 7, which is another theca-derived growth factor. Development to blastocyst from oocytes cultured for 14 days followed *in vitro* fertilization (IVF) or parthenogenetic activation have also been reported in other groups (Hirao *et al.* 2012; Oi *et al.* 2015). Therefore, we speculate that maintaining the “health” of granulosa cells using BMP-4 may allow oocytes to grow for a longer period (for 16 days) and reach 120 μm, a diameter indicating full developmental competence (Otoi *et al.* 1997). The present study was undertaken to investigate the effects of an extension in the IVG culture period and treatment with BMP-4 on oocyte growth and their subsequent developmental competence.

MATERIALS AND METHODS

This study was conducted entirely *in vitro* using abattoir-derived materials. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless indicated otherwise.

Collection of OGCs and IVG culture

Bovine ovaries obtained from a slaughterhouse were transported to the laboratory within 6 to 10 h after collection. After three washes with saline, sliced ovarian cortex strips (<1 mm thick) were prepared using a surgical blade (No. 11). Early antral follicles (0.5-1 mm in diameter) were dissected from cortex strips using a surgical blade (No. 20) under a stereomicroscope in TCM199 (Invitrogen; Grand Island, NY, USA) supplemented with 0.1% polyvinyl alcohol, 25 mM HEPES, 10 mM sodium bicarbonate, and 50 μg/mL gentamicin sulfate (isolation medium, pH 7.4), as described elsewhere (Huang *et al.* 2013). Follicles were punctured to release OGCs using a pair of fine forceps, and OGCs with a normal appearance were individually cultured in a well of 96-well culture plates (Primaria™ 353872, Corning, NY, USA) with 200 μL of growth medium at 39°C in humidified air with 5% CO_2. Growth medium consisted of HEPES-buffered TCM199 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. In order to evaluate the effects of the IVG culture duration and BMP-4 treatment on OGC viability, OGCs were cultured for 12 or 16 days with or without 10 ng/mL BMP-4 (HumanZyme, Inc., Chicago, IL, USA). Half of the medium was replaced at 4, 8, and 12 days of culture.

OGCs cultured for 12 days with BMP-4 served as an *in vitro*-grown control. OGC viability was assessed at the end of the IVG culture. Morphologically normal OGCs enclosed by several compact layers of granulosa cells were considered viable (Fig. 1). In addition, the diameters of IVG oocytes were measured before and after the IVG culture using an inverted microscope connected to a CCD camera (Moticam 2000; Shimadzu Rika Corporation, Tokyo, Japan) and image processing software (Motic Images Plus 2.2s, Shimadzu), as previously described (Huang *et al.* 2013). The diameter of artificially denuded oocytes following an *in vitro* maturation (IVM) culture was considered to be the diameter after the IVG culture.

IVM of IVG oocytes

Cumulus-oocyte complexes (COCs) isolated from viable OGCs were individually cultured in micro-well plates (MiniTrays 163118; NUNC, Roskilde, Denmark) containing 6 mL of IVM medium for 22 h at 39°C, in a humidified atmosphere with 5% CO_2 as previously described (Nagano *et al.* 2013). The IVM medium consisted of HEPES-buffered TCM199 supplemented with 0.2 mM sodium pyruvate, 0.02 IU/mL FSH (from the porcine pituitary), 1 μg/mL estradiol-17β, 10% FCS, and 50 μg/mL gentamicin sulfate. After the IVM culture, denuded oocytes were stained with 1% (W/V) aceto-orcein, and oocytes classified as metaphase II (MII) by observations under a phase-contrast microscope were considered to be matured (Nagano *et al.* 2006). For evaluating the competence of nuclear maturation of oocytes immediately after IVG culture, we did not carry out a 10-h pre-IVM culture (Huang *et al*. 2016), because

106 the pre-IVM culture significantly improved nuclear maturation rate (approximately 90%) and the
107 difference between BMP-4 treated groups became unclear (Yang *et al*. 2016).
108
109 ***In vitro* fertilization (IVF) and subsequent culture**
110 Groups cultured for 12, 14 or 16 days with BMP-4 were used to evaluate *in vitro* developmental
111 competence because 16-day IVG oocytes without the BMP-4 treatment showed low OGC viability. After
112 the IVG culture, a 10-h pre-IVM culture and 22-h IVM culture were performed as previously described
113 (Huang *et al*. 2016). Pre-IVM medium, which was modified from IVM medium, additionally contained
114 0.5 mM 3-isobutyl-1-methylxanthine, a phosphodiesterase inhibitor, and lower FSH concentration
115 (2×10^{-6} IU/mL). IVF was performed according to a previously described procedure (Takahashi &
116 Kanagawa 1998). Briefly, approximately 10 COCs were incubated in a 100-μL droplet of IVF medium
117 with frozen-thawed motile sperm (5×10^{6} sperm/mL) separated by a Percoll gradient (45 and 90%) at
118 39°C for 18 h in a humidified atmosphere with 5% CO_2, 5% O_2, and 90% N_2. IVF medium was modified
119 Brackett and Oliphant isotonic medium (Brackett & Oliphant 1975) containing 3 mg/mL fatty acid–free
120 BSA and 2.5 mM theophylline. After IVF, approximately 20-28 cumulus-free presumptive zygotes were
121 cultured in a 30-μl droplet of culture medium at 39°C for 6 days in a humidified atmosphere with 5%
122 CO_2, 5% O_2, and 90% N_2 (Takahashi *et al.* 1996). Culture medium consisted of modified synthetic
123 oviduct fluid containing 1 mM glutamine, twelve essential amino acids for basal medium Eagle, seven

non-essential amino acids for minimum essential medium, 10 μg/mL insulin, and 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 and 6 days of culture, respectively. Cell numbers in blastocysts, as a parameter of embryo quality, were counted using an air-drying method (Takahashi & First 1992).

In vivo-grown oocytes collected from antral follicles (2-8 mm in diameter) were submitted to IVM, IVF, and subsequent culture as an *in vivo* control. In IVM, they were cultured in droplets (approximately 10 COCs/50 μL) covered with paraffin oil (Nagano *et al.* 2013).

Statistical analysis

Multiple sets of data were compared by one-way analysis of variance with Tukey's multiple comparison test. Two sets of data were compared with the Student's *t*-test. Statistical analyses were performed using JMP Pro software (version 12.0.1, SAS Institute, Cary, NC, USA). All data are expressed as the mean ± SD.

RESULTS

As shown in Figure 2, OGC viabilities were around 85% in 12 days of IVG culture in the presence or

absence of BMP-4. When the duration was extended from 12 to 16 days, OGCs showed similar viability in the presence of BMP-4 (83.2%, $P > 0.05$) to that of oocytes cultured for 12 days with BMP-4 (83.0%). However, in the absence of BMP-4 (58.9%, $P < 0.05$) in the 16-day culture, viability was lower than those in the absence of BMP-4 for 12 days and in the presence of BMP-4 for 12 and 16 days.

[Figure 2]

After the 16-day IVG culture, oocytes derived from the BMP-4 treatment had a larger diameter than that cultured for 12 days with BMP-4 ($P < 0.05$), although the diameters of oocytes cultured for 12 and 16 days without the BMP-4 treatment were similar (Table 1). No significant differences were observed in oocyte diameter or nuclear maturation between 16-day IVG oocytes with and without the BMP-4 treatment ($P > 0.05$).

As shown in Table 2, 16-day IVG oocytes with the BMP-4 treatment had significantly lower cleavage rate (13.8%) than those of 12- and 14-day IVG oocytes (50.3 and 36.0%, respectively; $P < 0.01$). Blastocyst rates of 14- and 16-day IVG oocytes (1.8 and 0%, respectively) were also lower than that of 12-day IVG oocytes with the BMP-4 treatment (9.0%; $P < 0.05$). All IVG oocytes had lower cleavage rates, blastocyst rates, and cell numbers in blastocysts than those of the *in vivo* control ($P < 0.01$).

[Table 1]

[Table 2]

DISCUSSION

In the present study, an extension of the culture period from 12 to 16 days for the IVG of OGCs in medium including 10 ng/mL BMP-4 maintained their viability and enhanced oocyte growth. One possible reason for the positive effects of BMP-4 on oocyte growth *in vitro* may be the anti-luteinization function of BMP-4, which has been confirmed in dispersed granulosa cells (Glister *et al.* 2004; Yamashita *et al.* 2011) and granulosa cells enclosed in OGCs (Yang *et al.* 2016). Since granulosa cells from atretic bovine follicles were reportedly more luteinized than cells from "healthy" follicles in culture (Henderson *et al.* 1987), the BMP-4 treatment may contribute to maintaining OGC with "healthy" granulosa cells, resulting in high OGC survival. Another possible reason for improved OGC survival may be the anti-apoptotic effects of BMP-4. BMP-4 has been shown to reduce granulosa cell apoptosis by suppressing the release of caspase-activated DNase via Survivin, a member of the inhibitor of apoptosis family (Kayamori *et al.* 2009).

Bovine early antral follicles (0.5-1 mm) take 8-10 days to grow to antral follicles (8 mm) (Adams *et al.* 2008); however, bovine oocytes appear to grow slower *in vitro*. Previous studies comparing 10, 12, and 14-day IVG cultures found that 10 days was inadequate for oocyte growth and nuclear maturation, while 14 days impaired oocyte viability and subsequent developmental competence (Huang *et al.* 2013, 2014).

178 Therefore, under culture conditions without BMP-4, we concluded that the optimal IVG duration for
179 bovine oocytes derived from early antral follicles was 12 days based on previous findings (Huang *et al.*
180 2013, 2014). In mice, oocyte diameter was found to increase linearly with extensions in the length of the
181 culture duration for oocytes (Hirao & Miyano 2008). Furthermore, in cattle, a 16-day IVG culture
182 achieved a diameter of approximately 120 μm (Cho *et al*. 2008), which indicated that oocytes had full
183 developmental competence (Otoi *et al.* 1997). Thus, by maintaining high OGC viability, a longer culture
184 duration may result in better oocyte growth and the subsequent acquisition of competence. As expected,
185 the 16-day IVG culture with BMP-4 maintained OGC survival and resulted in further oocyte growth;
186 however, the size of oocytes did not achieve that of *in vivo*-grown oocytes, which averaged 120 μm in
187 diameter (Huang *et al.* 2013; Yang *et al.* 2016). In addition, oocyte nuclear maturation was not improved
188 and developmental competence was impaired as culture duration has been extended to 14 or 16 days.
189 Oocytes have been suggested to suffer aging problems if they are cultured for longer than necessary
190 (Hirao & Miyano 2008). Although 16-day IVG oocytes under the present IVG system showed similar
191 nuclear maturation competence, the developmental competence of embryos derived from 16-day IVG
192 were inferior to those from 12-day IVG, suggesting that oocyte aging is closely associated with impaired
193 cytoplasmic maturation. During 12 to 16 days of IVG culture, oocytes grew at a much slower rate (0.2-0.7
194 μm/day) than that (approximately 1.2 μm/day) in the initial 12 days. The retarded oocyte growth may be a
195 sign of aging problems. Cho *et al.* (2008) showed an increase in oocyte diameters after a 16-day IVG

196 culture; however, they did not report the viability of OGCs or their developmental competence. These
197 results suggest that extending the culture period to more than 12 days is not an appropriate strategy to
198 promote oocyte growth in cattle.

199 In the present study, the developmental rate to blastocysts (9.0%) of oocytes derived from 12-day IVG
200 culture without the BMP-4 treatment group was higher than that in our previous study (1.3%) (Yang *et al.*
201 2016). In the present study, we utilized different bull semen from that used in the previous study (Yang *et*
202 *al.* 2016). We speculate that the difference stems from the difference of intrinsic developmental
203 competence of sperm as described previously (Palma & Sinowatz 2004).

204 In conclusion, the present study showed that a 16-day IVG culture in the presence of BMP-4
205 maintained oocyte survival and promoted oocyte growth. However, it did not improve the competence of
206 the nuclear maturation of oocytes, and impaired their developmental competence; also 14-day IVG with
207 BMP-4 impaired the developmental competence. An extension in the culture duration is not a good
208 strategy to promote oocyte growth, presumably due to cytoplasm-associated aging problems in oocytes.
209 In further studies, we need to develop an IVG culture system that enhances oocyte growth without
210 extending the culture period.

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213 **ACKNOWLEDGMENTS**

This work was supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science, awarded to M. Nagano (Nos. 25450441 and 16K08043). Yinghua Yang was sponsored by the China Scholarship Council. We thank Genetic Hokkaido Association for the donation of frozen bull spermatozoa.

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320

Figure legend

Figure 1 Representative morphology of OGCs after 16 days of IVG culture.

OGCs assessed as viable and degenerated were shown in A and B, respectively.

Scale bar = 100 μm.

Figure 2 Effects of the IVG culture duration and BMP-4 treatment on OGC viability.

OGC collected from early antral follicles (0.5-1 mm in diameter) were cultured for a normal culture duration (12 days) with BMP-4 or for a prolonged duration (16 days) with or without BMP-4 (10 ng/mL). OGC viability was assessed at the end of the IVG culture (see Figure 1). Data are expressed as the mean values (n = 4) and error bars mean SD. Different letters (a, b) indicate significant differences ($P < 0.05$).

Fig. 1

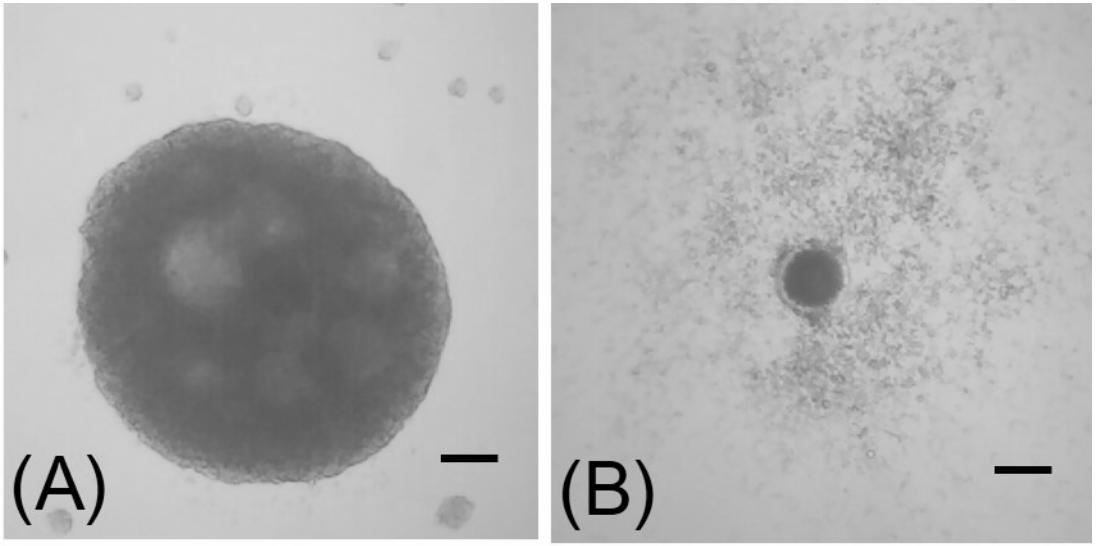

Fig. 2

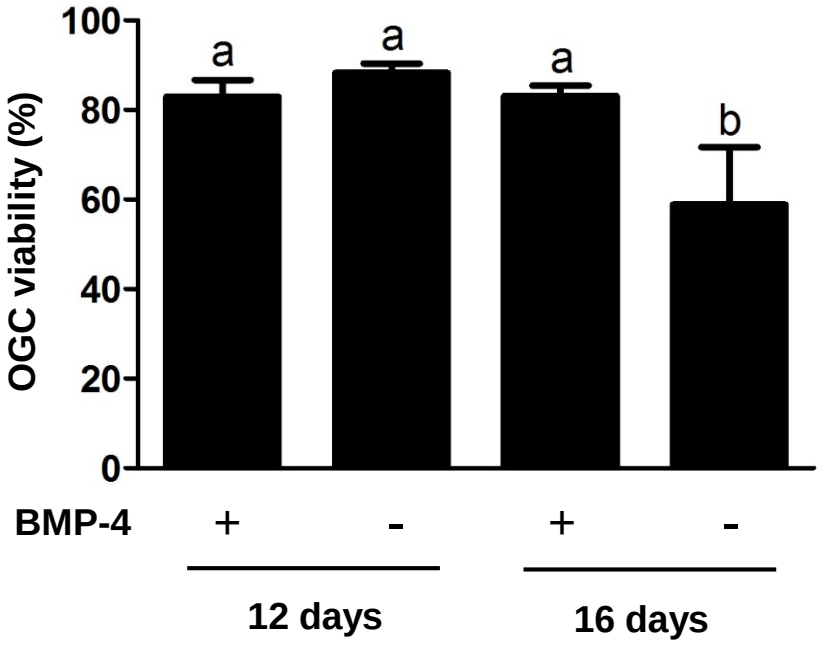

Table 1. Effects of the IVG culture duration and BMP-4 treatment on oocyte diameter and nuclear maturation

IVG culture duration (days)	BMP-4 treatment	No. of oocytes (replicates)	Oocyte diameter (μm)		% nuclear maturation†
			before IVG	after IVM	
12	+	76 (4)	97.5 ± 5.5	111.7 ± 5.7[b]	67.1 ± 11.7
	-	73 (3)	97.7 ± 3.1	113.2 ± 4.1[ab]	47.7 ± 13.5
16	+	69 (4)	98.6 ± 3.9	114.6 ± 3.5[a]	67.7 ± 15.3
	-	48 (4)	97.8 ± 3.9	113.9 ± 3.4[a]	50.7 ± 11.2

[a,b] Values with different superscripts differ significantly ($P < 0.05$).

† Oocytes that develop to metaphase II after IVM culture.

Data are expressed as mean ± SD.

Table 2. Effects of the IVG culture duration on the developmental competence of oocytes treated with BMP-4

Oocyte	IVG culture duration (days)	No. of oocytes (replicates)	% of cleaved oocytes	% of blastocysts	Cell no. in blastocysts (n)
In vitro-grown	12	82 (3)	50.3 ± 13.2[b]	9.0 ± 3.7[b]	78.3 ± 38.7[b] (7)
	14	97 (4)	36.0 ± 12.5[b]	1.8 ± 3.5[c]	70.0 ± 29.7[b] (2)
	16	80 (4)	13.8 ± 7.5[c]	0[c]	-
In vivo-grown†	-	82 (4)	87.5 ± 5.4[a]	36.4 ± 2.2[a]	145.3 ± 61.5[a] (30)

† Oocytes collected from antral follicles (2-8 mm in diameter) served as the *in vivo*-grown control.

[a,b,c] Values with different superscripts differ significantly ($P < 0.05$).

Data are expressed as mean ± SD.