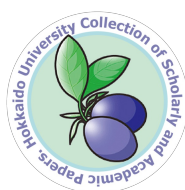




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Title: Extension of the culture period for the *in vitro* growth of bovine oocytes in the presence of bone morphogenetic protein-4 increases oocyte diameter, but impairs subsequent developmental competence

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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 μ m) than the control (111.7 μ m). 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% CO₂. 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% CO₂ 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

the pre-IVM culture significantly improved nuclear maturation rate (approximately 90%) and the difference between BMP-4 treated groups became unclear (Yang *et al.* 2016).

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

Groups cultured for 12, 14 or 16 days with BMP-4 were used to evaluate *in vitro* developmental competence because 16-day IVG oocytes without the BMP-4 treatment showed low OGC viability. After the IVG culture, a 10-h pre-IVM culture and 22-h IVM culture were performed as previously described (Huang *et al.* 2016). Pre-IVM medium, which was modified from IVM medium, additionally contained 0.5 mM 3-isobutyl-1-methylxanthine, a phosphodiesterase inhibitor, and lower FSH concentration (2×10^{-6} IU/mL). IVF was performed according to a previously described procedure (Takahashi & Kanagawa 1998). Briefly, approximately 10 COCs were incubated in a 100- μ L droplet of IVF medium with frozen-thawed motile sperm (5×10^6 sperm/mL) separated by a Percoll gradient (45 and 90%) at 39°C for 18 h in a humidified atmosphere with 5% CO₂, 5% O₂, and 90% N₂. IVF medium was modified Brackett and Oliphant isotonic medium (Brackett & Oliphant 1975) containing 3 mg/mL fatty acid-free BSA and 2.5 mM theophylline. After IVF, approximately 20-28 cumulus-free presumptive zygotes were cultured in a 30- μ L droplet of culture medium at 39°C for 6 days in a humidified atmosphere with 5% CO₂, 5% O₂, and 90% N₂ (Takahashi *et al.* 1996). Culture medium consisted of modified synthetic 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).

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

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

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

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

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221 REFERENCES

222

223 Adams GP, Jaiswal R, Singh J, Malhi P. 2008. Progress in understanding ovarian follicular dynamics in
 224 cattle. *Theriogenology* **69**, 72-80.

225

226 Brackett BG, Oliphant G. 1975. Capacitation of rabbit spermatozoa *in vitro*. *Biology of Reproduction* **12**,
 227 260-274.

228

229 Cho JH, Itoh T, Sendai Y, Hoshi H. 2008. Fibroblast growth factor 7 stimulates *in vitro* growth of oocytes
 230 originating from bovine early antral follicles. *Molecular Reproduction and Development* **75**,
 231 1736-1743.

232

233 Erickson GF, Shimasaki S. 2003. The spatiotemporal expression pattern of the bone morphogenetic
 234 protein family in rat ovary cell types during the estrous cycle. *Reproductive Biology and*
 235 *Endocrinology* **1**, 9.

236

237 Glister C, Kemp CF, Knight PG. 2004. Bone morphogenetic protein (BMP) ligands and receptors in
 238 bovine ovarian follicle cells: actions of BMP-4, -6 and -7 on granulosa cells and differential
 239 modulation of Smad-1 phosphorylation by follistatin. *Reproduction* **127**, 239-254.

240

- Henderson KM, McNatty KP, Smith P, Gibb M, O'Keeffe LE, Lun S, *et al.* 1987. Influence of follicular health on the steroidogenic and morphological characteristics of bovine granulosa cells *in vitro*. *Journal of Reproduction and Fertility* **79**, 185-193.
- Hirao Y, Miyano T. 2008. *In vitro* growth of mouse oocytes: oocyte size at the beginning of culture influences the appropriate length of culture period. *Journal of Mammalian Ova Research* **25**, 56-62.
- Hirao Y, Shimizu M, Iga K, Takenouchi N. 2012. Optimization of oxygen concentration for growing bovine oocytes *in vitro*: constant low and high oxygen concentrations compromise the yield of fully grown oocytes. *Journal of Reproduction and Development* **58**, 204-211.
- Huang W, Kang SS, Nagai K, Yanagawa Y, Takahashi Y, Nagano M. 2016. Mitochondrial activity during pre-maturational culture in *in vitro*-grown bovine oocytes is related to maturational and developmental competences. *Reproduction, Fertility and Development* **28**, 349-356.
- Huang W, Kang SS, Yanagawa Y, Yang Y, Takahashi Y, Nagano M. 2014. Effects of *in vitro*-growth culture duration on fertilizability of bovine growing oocytes and proliferation of cells surrounding oocytes. *Japanese Journal of Veterinary Research* **62**, 135-141.
- Huang W, Nagano M, Kang SS, Yanagawa Y, Takahashi Y. 2013. Effects of *in vitro* growth culture duration and prematuration culture on maturational and developmental competences of bovine oocytes derived from early antral follicles. *Theriogenology* **80**, 793-799.
- Kayamori T, Kosaka N, Miyamoto A, Shimizu T. 2009. The differential pathways of bone morphogenetic protein (BMP)-4 and -7 in the suppression of the bovine granulosa cell apoptosis. *Molecular and Cellular Biochemistry* **323**, 161-168.
- Knight PG, Glister C. 2003. Local roles of TGF- β superfamily members in the control of ovarian follicle development. *Animal Reproduction Science* **78**, 165-183.
- Nagano M, Kang SS, Koyama K, Huang WP, Yanagawa Y, Takahashi Y. 2013. *In vitro* maturation system for individual culture of bovine oocytes using micro-volume multi-well plate. *Japanese Journal of Veterinary Research* **61**, 149-154.
- Nagano M, Katagiri S, Takahashi Y. 2006. ATP content and maturational/developmental ability of bovine

- 277 oocytes with various cytoplasmic morphologies. *Zygote* **14**, 299-304.
- 278
- 279 Oi A, Tasaki H, Munakata Y, Shirasuna K, Kuwayama T, Iwata H. 2015. Effects of reaggregated
280 granulosa cells and oocytes derived from early antral follicles on the properties of oocytes grown
281 in vitro. *Journal of Reproduction and Development* **61**, 191-197.
- 282
- 283 Otoi T, Yamamoto K, Koyama N, Tachikawa S, Suzuki T. 1997. Bovine oocyte diameter in relation to
284 developmental competence. *Theriogenology* **48**, 769-774.
- 285
- 286 Palma GA, Sinowatz F. 2004. Male and female effects on the *in vitro* production of bovine embryos.
287 *Anatomia, Histologia, Embryologia* **33**, 257-262.
- 288
- 289 Senbon S, Miyano T. 2002. Bovine oocytes in early antral follicles grow in serum-free media: effect of
290 hypoxanthine on follicular morphology and oocyte growth. *Zygote* **10**, 301-309.
- 291
- 292 Takahashi Y, First NL. 1992. *In vitro* development of bovine one-cell embryos: Influence of glucose,
293 lactate, pyruvate, amino acids and vitamins. *Theriogenology* **37**, 963-978.
- 294
- 295 Takahashi Y, Hishinuma M, Matsui M, Tanaka H, Kanagawa H. 1996. Development of *in vitro*
296 matured/fertilized bovine embryos in a chemically defined medium: influence of oxygen
297 concentration in the gas atmosphere. *Journal of Veterinary Medical Science* **58**, 897-902.
- 298
- 299 Takahashi Y, Kanagawa H. 1998. Effect of oxygen concentration in the gas atmosphere during *in vitro*
300 insemination of bovine oocytes on the subsequent embryonic development *in vitro*. *Journal of*
301 *Veterinary Medical Science* **60**, 365-367.
- 302
- 303 Yamamoto K, Otoi T, Koyama N, Horikita N, Tachikawa S, Miyano T. 1999. Development to live young
304 from bovine small oocytes after growth, maturation and fertilization *in vitro*. *Theriogenology* **52**,
305 81-89.
- 306
- 307 Yamashita H, Murayama C, Takasugi R, Miyamoto A, Shimizu T. 2011. BMP-4 suppresses progesterone
308 production by inhibiting histone H3 acetylation of StAR in bovine granulosa cells *in vitro*.
309 *Molecular and Cellular Biochemistry* **348**, 183-190.
- 310
- 311 Yang Y, Kanno C, Huang W, Kang SS, Yanagawa Y, Nagano M. 2016. Effect of bone morphogenetic
312 protein-4 on *in vitro* growth, steroidogenesis and subsequent developmental competence of the

313 oocyte-granulosa cell complex derived from bovine early antral follicles. *Reproductive Biology*
314 *and Endocrinology* **14**, 3.

315

316 Young JM, McNeilly AS. 2010. Theca: the forgotten cell of the ovarian follicle. *Reproduction* **140**,
317 489-504.

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321 **Figure legend**

322

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

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

325 Scale bar = 100 μ m.

326

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

328 OGC collected from early antral follicles (0.5-1 mm in diameter) were cultured for a normal culture

329 duration (12 days) with BMP-4 or for a prolonged duration (16 days) with or without BMP-4 (10 ng/mL).

330 OGC viability was assessed at the end of the IVG culture (see Figure 1). Data are expressed as the mean

331 values ($n = 4$) and error bars mean SD. Different letters (a, b) indicate significant differences ($P < 0.05$).

Fig. 1

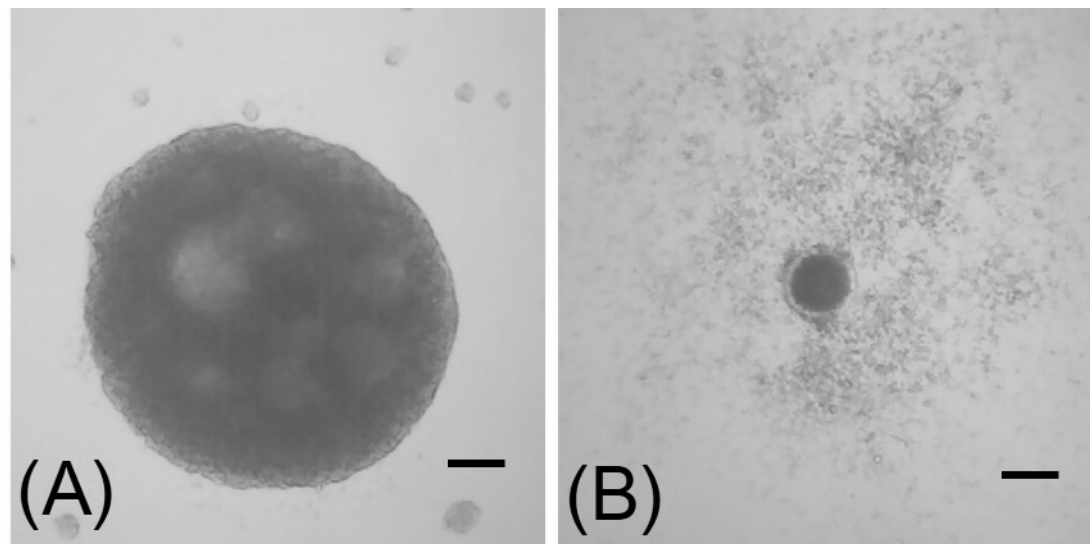


Fig. 2

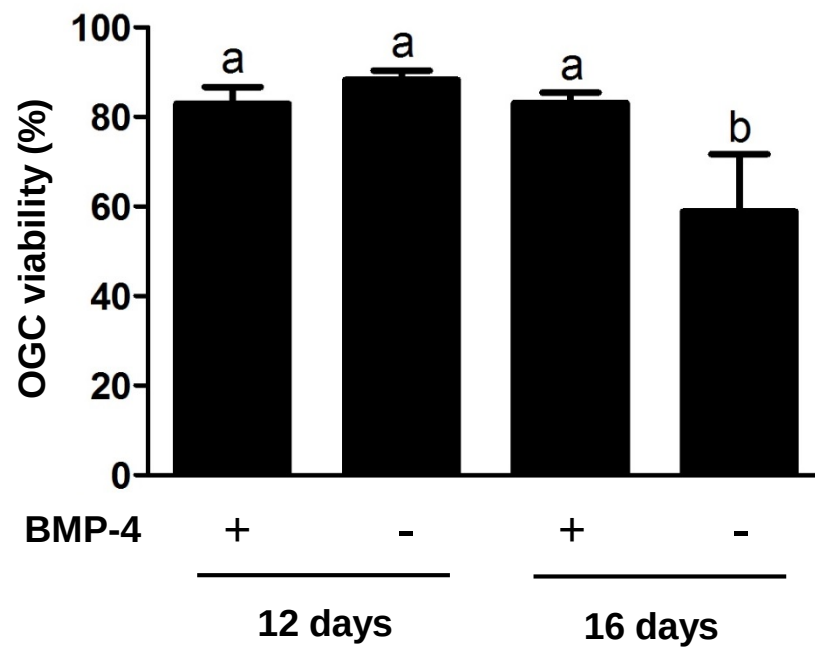


Table 1. Effects of the IVG culture duration and BMP-4 treatment on oocyte diameter and nuclear maturation

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 $\pm$ 5.5	111.7 $\pm$ 5.7 ^b	67.1 $\pm$ 11.7
	-	73 (3)	97.7 $\pm$ 3.1	113.2 $\pm$ 4.1 ^{ab}	47.7 $\pm$ 13.5
16	+	69 (4)	98.6 $\pm$ 3.9	114.6 $\pm$ 3.5 ^a	67.7 $\pm$ 15.3
	-	48 (4)	97.8 $\pm$ 3.9	113.9 $\pm$ 3.4 ^a	50.7 $\pm$ 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 $\pm$ 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)
<i>In vitro</i> -grown	12	82 (3)	50.3 $\pm$ 13.2 ^b	9.0 $\pm$ 3.7 ^b	78.3 $\pm$ 38.7 ^b (7)
	14	97 (4)	36.0 $\pm$ 12.5 ^b	1.8 $\pm$ 3.5 ^c	70.0 $\pm$ 29.7 ^b (2)
	16	80 (4)	13.8 $\pm$ 7.5 ^c	0 ^c	-
<i>In vivo</i> -grown†	-	82 (4)	87.5 $\pm$ 5.4 ^a	36.4 $\pm$ 2.2 ^a	145.3 $\pm$ 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 $\pm$ SD.