



Title	Effects of in vitro growth culture duration and prematuration culture on maturational and developmental competences of bovine oocytes derived from early antral follicles
Author(s)	Huang, Weiping; Nagano, Masashi; Kang, Sung-Sik; Yanagawa, Yojiro; Takahashi, Yoshiyuki
Citation	Theriogenology, 80(7), 793-799 https://doi.org/10.1016/j.theriogenology.2013.07.004
Issue Date	2013-10-15
Doc URL	http://hdl.handle.net/2115/53559
Type	article (author version)
File Information	THERIO-D-13-00173.pdf



[Instructions for use](#)

1 Effects of in vitro growth culture duration and pre-maturation culture on maturational
2 and developmental competences of bovine oocytes derived from early antral follicles

3

4

5 Weiping Huang, Masashi Nagano*, Sung-Sik Kang, Yojiro Yanagawa, Yoshiyuki

6 Takahashi

7

8

9 Laboratory of Theriogenology, Department of Veterinary Clinical Sciences, Graduate

10 School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

11

12

13 *Corresponding author (M. Nagano): Tel.: +81-11-706-5232, Fax: +81-11-706-5232

14 e-mail: mnaga@vetmed.hokudai.ac.jp

1 **Abstract**

2

3 Bovine ovaries offer a large pool of oocytes that could be used for in vitro production
4 of embryos of genetically valuable animals. The effects of IVG culture duration (10,
5 12 and 14 days) on the viability and growth of bovine oocytes derived from early
6 antral follicles (0.5 to 1 mm in diameter) in this study. In addition, the effect of
7 pre-IVM culture with PDE inhibitor (IBMX) on nuclear maturation of IVG oocytes
8 was also evaluated. In Experiment 1, oocyte viability observed after 10 or 12 days of
9 IVG culture was greater ($P < 0.05$) than that observed after 14 days of culture. Oocyte
10 diameters and proportions of oocytes at M II stage were greater ($P < 0.05$) when 12 or
11 14 days of IVG culture were used when compared to 10 days. In addition, the
12 proportion of oocytes at M II stage were greater ($P < 0.05$) when pre-IVM culture was
13 performed for oocytes derived from 12 and 14 days of IVG culture. When 12 and 14
14 days of IVG culture followed by pre-IVM culture were compared in Experiment 2,
15 cumulus cell membrane integrity was greater ($P < 0.05$) after 12 days. Blastocyst
16 production rate for oocytes obtained after 12 days of IVG culture (24.5%) were
17 greater ($P < 0.05$) than for oocytes obtained after 14 days (9.9%). In conclusion, 12
18 days IVG followed by pre-IVM culture was considered the optimal processing system
19 for bovine oocytes derived from early antral follicles when oocyte viability, diameter,
20 maturation, and development competences were considered.

21 *Keywords:* early antral follicle, IBMX, IVG duration, oocyte growth, pre-IVM

1 **1. Introduction**

2

3 Large numbers of growing follicles in bovine ovaries offer a large pool of oocytes for
4 in vitro maturation, fertilization, and culture (IVM/IVF/IVC) to produce embryos of
5 genetically valuable animals. Although the production of live offspring from oocytes
6 obtained from early antral follicles has been reported, the developmental competence
7 to blastocysts of these oocytes is low (4 to 18% based on IVM oocytes) [1-3]. To
8 utilize these oocytes efficiently, it is important to develop culture systems for in vitro
9 growth (IVG) that permit oocytes to acquire the competency for undergoing
10 maturation, fertilization, and development to blastocysts in vitro.

11

12 In previous studies, growing oocytes (approximately 95 μm in diameter) derived from
13 early antral follicles were cultured in vitro for 7 to 16 days, grew to 107 to 117 μm in
14 diameter, and some of them acquired maturational and developmental competency [1,
15 4-6]. However, the optimal duration of IVG culture for growing bovine oocytes is not
16 known. In mice, the appropriate length of the IVG culture period was related to the
17 oocyte size at the start of culture [7]. Previous studies reported that extending the IVG
18 period from 10 to 12 days reduced blastocyst production rates in mice, although
19 nuclear maturation rates were similar [8,9]. These studies suggest that longer culture
20 duration has protracted detrimental effects on oocyte developmental competence.
21 Determining the optimal IVG culture duration for growing bovine oocytes will

1 contribute to improve their maturational and developmental competences.

2

3 Nuclear maturation rates obtained after IVM of in vivo-derived bovine oocytes larger
4 than 115 μm in diameter are generally high (around 85%) [10]. However, maturation
5 rates of IVG oocytes grown to more than 115 μm in diameter are considerably lower
6 (30 to 65%) [5, 11]. Since growing oocytes accumulate compounds essential for
7 maturation, fertilization, and embryogenesis during folliculogenesis [12,13], low
8 maturational competence of IVG oocytes might be caused by the absence of crucial
9 oocyte cytoplasmic components (inadequate cytoplasmic maturation). This was
10 suggested by the observation that oocyte cytoplasmic maturation was improved by the
11 temporary maintenance of oocytes in meiotic arrest immediately before (pre-IVM)
12 IVM culture [14]. Previous studies demonstrated that phosphodiesterase (PDE)
13 inhibitors prevented the meiotic resumption of bovine oocytes [15,16] and improved
14 nuclear maturation and blastocyst production rates when used in pre-IVM culture
15 [17-19]. However, there have been no reports investigating the effects of pre-IVM
16 culture with PDE inhibitor on the maturational and developmental competences of
17 IVG oocytes.

18

19 The objectives of the present study were to evaluate the effects of IVG culture
20 duration (10, 12 and 14 days) on the viability and growth of bovine oocytes derived
21 from early antral follicles. In addition, the effect of pre-IVM culture with PDE

1 inhibitor (3-isobutyl-1-methylxanthine, IBMX) on nuclear maturation of IVG oocytes
2 was also evaluated.

3

4 **2. Materials and Methods**

5

6 *2.1. Chemicals*

7

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

10

11 *2.2. Collection of early antral follicles and IVG oocytes*

12

13 Bovine ovaries obtained at a local abattoir were kept in plastic bags at 20 °C and were
14 transported to the laboratory within 6 to 10 h of collection. After 3 washes in
15 physiological saline, sliced ovarian cortex tissues (< 1 mm thick) were prepared
16 using a surgical blade (No. 11) and stored in TCM199 (Invitrogen; Grand Island, NY,
17 USA) supplemented with 0.1% polyvinyl alcohol, 25 mM HEPES, 10 mM sodium
18 bicarbonate and 50 µg/mL gentamicin sulfate (isolation medium, pH 7.4 at 37°C) as
19 described elsewhere [4]. Under a stereo microscope, early antral follicles (0.5 to 1 mm
20 in diameter) were dissected from the sliced ovarian tissues using a surgical blade (No.
21 20). Oocyte-cumulus-granulosa complexes (OCGCs) were isolated from follicles

1 using a pair of fine forceps and those with normal appearance were individually
2 cultured in 96-well culture plates (Falcon 353872' Becton Dickinson, Franklin Lakes,
3 NJ, USA) with 200 μ L of the growth medium for 10 to 14 days at 39 °C in humidified
4 air with 5% CO₂. Growth medium consistent of HEPES-buffered TCM199
5 (Invitrogen) supplemented with 0.91 mM sodium pyruvate, 1 μ g/mL estradiol-17 β ,
6 5% fetal calf serum (FCS; Invitrogen), 4 mM hypoxanthine, 4% polyvinylpyrrolidone
7 (MW 360,000), and 50 μ g/mL gentamicin sulfate. At the onset of IVG culture,
8 OCGCs were photographed (Fig. 1A) under an inverted microscope (CK 40, Olympus,
9 Tokyo, Japan) attached with a CCD camera (Moticam 2000, Shimadzu Rika
10 Corporation, Tokyo, Japan), and the diameters of oocytes (excluding the zona
11 pellucida) were determined using software (Motic Images Plus 2.2s, Shimadzu).
12 During IVG culture, half (100 μ L) of the growth medium was replaced by the same
13 amount of fresh medium every 4 days.

14

15 *2.3. IVM of in vivo-derived and IVG oocytes*

16

17 In vivo-derived oocytes collected from antral follicles (2 to 8 mm in diameter) were
18 submitted to IVM as described previously [20]. Briefly, cumulus-oocyte complexes
19 (COCs) were incubated in droplets of IVM medium (about 10 COCs/50 μ L) covered
20 with paraffin oil for 22 h at 39 °C under 5% CO₂ in air. Maturation medium consisted
21 of HEPES-buffered TCM199 supplemented with 0.2 mM sodium pyruvate, 0.02

1 units/mL FSH (from porcine pituitary), 1 µg/mL estradiol-17β, 10% FCS, and 50
2 µg/mL gentamicin sulfate. Oocytes grown in vitro surrounded by several layers of
3 cumulus cells recovered from morphologically normal OCGCs were selected for IVM.
4 The IVM procedure for IVG oocytes without pre-IVM culture was the same as for in
5 vivo-derived oocytes. Oocytes subjected to pre-IVM culture were cultured
6 individually in micro-well plates (Mini Trays 163118; NUNC, Roskilde, Denmark)
7 filled with 6 mL of HEPES-buffered TCM-199 supplemented with 0.2 mM sodium
8 pyruvate, 2×10^{-6} units/mL FSH, 0.5 mM IBMX, 1 µg/mL estradiol-17β, 10% FCS,
9 and 50 µg/mL gentamicin sulfate at 39 °C under 5% CO₂ in air. After 20 h of pre-IVM
10 culture, IVG oocytes were transferred to the micro-well plates filled with 6 mL of the
11 same IVM medium used for in vivo-derived oocytes, and cultured for another 22 h at
12 39 °C under 5% CO₂ in air.

13

14 *2.4. Evaluation of oocyte nuclear status*

15

16 Oocytes were denuded from cumulus cells by vortexing and stained with 1%
17 aceto-orcein as described elsewhere [21]. Nuclear statuses were classified as germinal
18 vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (M I), anaphase
19 I/telophase I (A I/T I) and metaphase II (M II) by observation under a phase contrast
20 microscope.

21

1 2.5. *In vitro fertilization and culture*

2

3 In vitro fertilization was performed using frozen semen according to a procedure
4 described previously [22] with slight modifications. Briefly, motile sperm (5×10^6
5 sperm/mL) separated by Percoll gradient (45 and 90%) were co-incubated with COCs
6 in a 100- μ L droplet (about 10 COCs per droplet) of modified Brackett & Oliphant
7 isotonic medium [23] containing 3 mg/mL fatty-acid-free BSA and 2.5 mM
8 theophylline [24] for 18 h at 39 °C in a humidified atmosphere of 5% CO₂, 5% O₂ and
9 90% N₂. In vitro culture of inseminated oocytes (presumptive zygotes) was performed
10 as previously described [20,25]. Briefly, after co-incubation with sperm, presumptive
11 zygotes were freed from cumulus cells by vortexing and washing three times in
12 culture medium. Cumulus-free zygotes were cultured for 6 days in 30- μ L droplets
13 of culture medium at 39 °C under 5% CO₂, 5% O₂ and 90% N₂. The culture medium
14 consisted of modified synthetic oviduct fluid containing 1 mM glutamine, 12 essential
15 amino acids for basal medium Eagle, 7 non-essential amino acids for minimum
16 essential medium, 10 μ g/mL insulin, and 5 mM glycine, 5 mM taurine, 1 mM glucose,
17 and 3 mg/mL fatty-acid-free BSA. Cleavage and blastocyst production rates were
18 determined after 2 d (about 30 h) and 6 d (about 150 h) of IVC, respectively. Total
19 live cell numbers of blastocysts obtained after 6 d of IVC were counted using an
20 air-drying method [24].

21

1 2.6. *Evaluation of cumulus cell membrane integrity*

2

3 Evaluation of membrane integrity of cumulus cells was performed in accordance with
4 a previous report [26] with slight modifications. Briefly, IVG oocytes with cumulus
5 cells were incubated for 10 min in the dark at 39 °C in 3 mL of isolation medium
6 supplemented with 5 µg/mL propidium iodide (PI). Oocytes were then washed twice
7 in isolation medium and transferred to a glass-bottomed dish containing 3 mL of
8 isolation medium. Stained IVG oocytes were observed under an inverted microscope
9 equipped with a fluorescence system using an appropriate filter (G-2A, Nikon).
10 Cumulus cells with a damaged cell membrane fluoresced red. Each IVG oocyte was
11 scored for the membrane integrity of cumulus cells as follows: Grade 1: less than
12 one-quarter of cumulus cells were stained by PI; Grade 2: one-quarter to one-half of
13 cumulus cells were stained; or Grade 3: more than one-half of cumulus cells were
14 stained (Fig. 2).

15

16 2.7. *Experimental design*

17

18 2.7.1. *Experiment 1*

19

20 A total of 699 OCGCs (14 to 66/replicate) were used to evaluate the effects of 10, 12
21 or 14 days IVG culture on oocyte viability, growth, and nuclear maturation. Oocyte

1 viability was evaluated based on morphological appearance; oocytes were considered
2 to be viable when completely enclosed by a healthy granulosa cell layer at the end of
3 IVG (Fig. 1B) and isolated oocytes had cytoplasm with normal appearance and
4 several layers of cumulus cells (Fig. 2 A1, B1, and C1). Oocyte diameters were
5 measured before and after IVG culture; measurements of cumulus-denuded oocytes
6 after IVM were considered to be the diameters after IVG. Viable IVG oocytes (10 to
7 20/replicates) were used to examine maturation competence after IVM with or
8 without pre-IVM culture. In vivo-derived oocytes collected from antral follicles were
9 also subjected to IVM and served as control.

10

11 2.7.2. *Experiment 2*

12

13 This experiment was conducted to further evaluate the processes that in Experiment I
14 resulted in high oocyte viability and nuclear maturation rates, i.e. IVG for 12 or 14
15 days and pre-IVM culture. A total of 118 oocytes (approximately 20/replicate) derived
16 from 171 OCGCs were used for evaluation of cumulus cell integrity immediately after
17 IVG culture. Oocyte nuclear statuses immediately after IVG culture and after
18 subsequent pre-IVM culture were examined in a total of 213 IVG oocytes (10 to 20
19 oocytes/replicate). To examine oocyte developmental competence, IVF was
20 performed using semen from a bull using a total of 338 IVG oocytes derived from 541
21 OCGCs (approximately 30/replicate). In vivo-derived oocytes collected from antral

1 follicles were also subjected to IVM/IVF/IVC and served as control.

2

3 *2.8. Statistical analysis*

4

5 All statistical analyses were performed using JMP software version 8.02 (SAS
6 Institute, Cary, NC, USA). The effects of culture conditions on oocyte diameter and
7 viability, cleavage and blastocyst production rates, and blastocyst cell number were
8 analyzed by one-way ANOVA and Tukey-Kramer's HSD test. The effects of culture
9 conditions on oocyte nuclear status and on cumulus cells membrane integrity were
10 analyzed by chi-square test.

11

12 **3. Results**

13

14 In Experiment 1, oocyte viability observed after 10 or 12 days of IVG culture was
15 greater ($P < 0.05$) than that observed after 14 days of culture (Table 1). Oocyte
16 diameters and proportions of oocytes at M II stage were greater ($P < 0.05$) when 12 or
17 14 days of IVG culture were used when compared to 10 days. In addition, the
18 proportion of oocytes at M II stage were greater ($P < 0.05$) when pre-IVM culture was
19 performed for oocytes derived from 12 and 14 days of IVG culture (Table 2). Oocyte
20 diameters and proportions of oocytes at M II stage were greater ($P < 0.05$) for in
21 vivo-derived oocytes than for IVG oocytes cultured for 10, 12 or 14 days.

1

2 In Experiment 2, cumulus cell membrane integrity was greater ($P < 0.05$) after 12 than
3 14 days of IVG culture (Table 3). Regardless of IVG duration, all oocytes were
4 arrested at the GV stage before pre-IVM culture. After pre-IVM culture, the
5 proportion of oocytes obtained after 12 days IVG in GV stage was greater ($P < 0.05$)
6 than that of oocytes obtained after 14 days (Table 4). Blastocyst production rate for
7 oocytes obtained after 12 days IVG culture was greater ($P < 0.05$) than for oocytes
8 obtained after 14 days; cleavage rates and live blastocyst cell numbers were not
9 affected by IVG duration. There was no difference in blastocyst production rates
10 based on the number of cleaved oocytes between oocytes obtained after 12 days IVG
11 and in vivo-derived oocytes, although the cleavage rate of 12-day IVG oocytes was
12 lower ($P < 0.05$) than that of in vivo-derived oocytes. Blastocysts originating from in
13 vivo-derived oocytes had a larger ($P < 0.05$) number of live cells than those from IVG
14 oocytes (Table 5).

15

16 **4. Discussion**

17

18 In the present study, oocyte viability after 14 days IVG culture was significantly lower
19 (55%) than after 10 or 12 days (approximately 70%), although the viability after 14
20 days was similar to that observed in a previous study using a similar culture system
21 [2]. Recently, several studies have reported higher survival rates (approximately 80%)

1 after IVG culture for 14 days [3,27]. This discrepancy in viability rates may be
2 explained by the use of different culture media and/or different culture methods, since
3 in these studies OCGCs were cultured in medium containing ascorbic acid
4 2-glucoside, with half of the medium replaced every other day. In the present study,
5 we replaced half of the medium at 4-day intervals. During 4-day culture without
6 medium replacement, some detrimental products may have accumulated.

7

8 After 12 or 14 days IVG culture, oocyte diameters were similar and larger than that of
9 10-day IVG oocytes. These results indicated that oocytes were growing until 12 days
10 of culture, but growth was not supported beyond this time under our present IVG
11 system. The lower nuclear maturation rate of 10-day IVG oocytes might have been
12 caused by insufficient growth (approximately 110 μm in diameter). Meiotic
13 competence of bovine oocytes is obtained gradually during folliculogenesis [14,28].
14 Meiotic competence for bovine in vivo-derived oocytes increases as the diameter
15 increases from 110 μm (42% M II rate; [29]) to 115 μm (85% M II rate; [10]). In the
16 present study oocytes reached approximately 115 μm after 12 or 14 days IVG and the
17 diameter was similar to that observed after 14 days in previous reports [2,3,27].
18 However, similarly to previous studies [5,11], maturation rates for 12- and 14-day
19 IVG oocytes without pre-IVM culture were low (approximately 54%). When IVG
20 oocytes were subjected to 20 h of pre-IVM culture with IBMX (PDE inhibitor),
21 nuclear maturation rates of 12- and 14-day IVG oocytes after IVM were significantly

1 improved to more than 70%. It was been reported that gap junctions are essential for
2 oocyte meiotic regulation by allowing the passage of small regulatory molecules [30].
3 Previous studies that used a pre-IVM culture system with PDE inhibitor for in
4 vivo-derived oocytes showed that the intra-oocyte level of cAMP was maintained and
5 loss of gap junction was inhibited in cattle [18] and pig [17]. These observations
6 suggest that intracellular events that are essential for oocyte nuclear maturation
7 occurred during pre-IVM culture.

8

9 In the present study, 12 days of IVG culture better supported the development of
10 oocytes to blastocyst when compared to 14 days IVG. Although only IVG oocytes
11 exhibiting normal morphology were used for IVM and IVF, 14-day IVG oocytes
12 showed lower membrane integrity of cumulus cells and higher GVBD rate after
13 pre-IVM culture compared with 12-day IVG oocytes. It has been reported that
14 acquisition of oocyte developmental competence is dependent on nutrients and
15 soluble factors supplied by cumulus cells [30,31]. Our results suggest that the longer
16 duration of IVG culture leads to the decline in cumulus cells membrane integrity
17 resulting in lower developmental competence of IVG oocytes. Although blastocyst
18 production rate of 12-day IVG oocytes as a function of cleaved embryos was similar
19 to that of in vivo-derived oocytes in the present study, cleavage rate and blastocyst
20 cell numbers were still lower than those of in vivo-derived oocytes. These differences
21 may be due to insufficient growth of oocytes in vitro. Under the present IVG system,

1 oocytes did not grow to more than 120 μm in diameter, which was reported to be the
2 diameter at which oocytes acquired full developmental competence [10]. Since in the
3 present study we only evaluated the effects of 20 h pre-IVM culture, further studies
4 are required to determine the optimal duration of pre-IVM culture. Moreover, research
5 and development of use of oocytes from early antral follicles would greatly benefit
6 from the development of novel methods to recover OCGCs, since morphologically
7 normal OCGCs could be collected from only approximately 60% of isolated early
8 antral follicles (data not shown).

9

10 In conclusion, 12 days was considered the optimal duration for IVG culture of bovine
11 oocytes derived from early antral follicles when oocyte viability, diameter, maturation,
12 and development competences were considered. Shorter IVG culture duration (10
13 days) was associated with reduced oocyte diameter and maturation competence,
14 whereas longer duration (14 days) was associated with reduced cumulus cell
15 membrane integrity and development competence. In addition, pre-IVM culture for 20
16 h improved nuclear maturation of IVG oocytes.

17

18 **Acknowledgments**

19

20 Part of this study was supported by Grants-in-Aid for Scientific Research (Nos.
21 21780286 and 21580339) from the Japan Society for the Promotion of Science to M.

1 Nagano and Y. Takahashi. We thank Genetics Hokkaido Association for the donation
2 of frozen bull semen.

3

4 **References**

5

6 [1] Yamamoto K, Otoi T, Koyama N, Horikita N, Tachikawa S, Miyano T.

7 Development to live young from bovine small oocytes after growth, maturation and
8 fertilization in vitro. *Theriogenology* 1999;52:81-9.

9 [2] Hirao Y, Itoh T, Shimizu M, Iga K, Aoyagi K, Kobayashi M, et al. In vitro growth

10 and development of bovine oocyte-granulosa cell complexes on the flat substratum:

11 effects of high polyvinylpyrrolidone concentration in culture medium. *Biol Reprod*

12 2004;70:83-91.

13 [3] Hirao Y, Shimizu M, Iga K, Takenouchi N. Optimization of oxygen concentration

14 for growing bovine oocytes in vitro: constant low and high oxygen concentrations

15 compromise the yield of fully grown oocytes. *J Reprod Dev* 2012;58:204-11.

16 [4] Harada M, Miyano T, Matsumura K, Osaki S, Miyake M, Kato S. Bovine oocytes

17 from early antral follicles grow to meiotic competence in vitro: effect of FSH and

18 hypoxanthine. *Theriogenology* 1997;48:743-55.

19 [5] Senbon S, Miyano T. Bovine oocytes in early antral follicles grow in serum-free

20 media: effect of hypoxanthine on follicular morphology and oocyte growth. *Zygote*

21 2002;10:301-9.

- 1 [6] Hirao Y, Shimizu M, Iga K, Takenouchi N. Growth of bovine oocyte-granulosa
2 cell complexes cultured individually in microdrops of various sizes. *J Reprod Dev*
3 2009;55:88-93.
- 4 [7] Hirao Y, Miyano T. In vitro growth of mouse oocyte: oocyte size at the beginning
5 of culture influences the appropriate length of culture period. *J Mamm Ova Res*
6 2008;25:56-62.
- 7 [8] Liu J, Rybouchkin A, Van der Elst J, Dhont M. Fertilization of mouse oocytes
8 from in vitro-matured preantral follicles using classical in vitro fertilization or
9 intracytoplasmic sperm injection. *Biol Reprod* 2002;67:575-9.
- 10 [9] Nonowaki S, Takahashi K, Horiuchi T. Effect of preantral mouse follicle culture
11 period on meiotic maturation and developmental competence of oocytes. *Reprod Med*
12 *Biol* 2010;9:83-9.
- 13 [10] Otoi T, Yamamoto K, Koyama N, Tachikawa S, Suzuki T. Bovine oocyte
14 diameter in relation to developmental competence. *Theriogenology* 1997;48:769-74.
- 15 [11] Endo M, Kawahara RM, Cao F, Kimura K, Kuwayama T, Monji Y, et al.
16 Estradiol supports in vitro development of bovine early antral follicles. *Reproduction*
17 2012.
- 18 [12] Eppig JJ, O'Brien M, Wigglesworth K. Mammalian oocyte growth and
19 development in vitro. *Mol Reprod Dev* 1996;44:260-73.
- 20 [13] Marteil G, Richard-Parpaillon L, Kubiak JZ. Role of oocyte quality in meiotic
21 maturation and embryonic development. *Reprod Biol* 2009;9:203-24.

- 1 [14] Gilchrist RB, Thompson JG. Oocyte maturation: emerging concepts and
2 technologies to improve developmental potential in vitro. *Theriogenology*
3 2007;67:6-15.
- 4 [15] Bilodeau S, Fortier MA, Sirard MA. Effect of adenylate cyclase stimulation on
5 meiotic resumption and cyclic AMP content of zona-free and cumulus-enclosed
6 bovine oocytes in vitro. *J Reprod Fertil* 1993;97:5-11.
- 7 [16] Thomas RE, Armstrong DT, Gilchrist RB. Differential effects of specific
8 phosphodiesterase isoenzyme inhibitors on bovine oocyte meiotic maturation. *Dev*
9 *Biol* 2002;244:215-25.
- 10 [17] Ozawa M, Nagai T, Somfai T, Nakai M, Maedomari N, Fahrudin M, et al.
11 Comparison between effects of 3-isobutyl-1-methylxanthine and FSH on gap
12 junctional communication, LH-receptor expression, and meiotic maturation of
13 cumulus-oocyte complexes in pigs. *Mol Reprod Dev* 2008;75:857-66.
- 14 [18] Luciano AM, Franciosi F, Modina SC, Lodde V. Gap junction-mediated
15 communications regulate chromatin remodeling during bovine oocyte growth and
16 differentiation through cAMP-dependent mechanism(s). *Biol Reprod* 2011;85:1252-9.
- 17 [19] Nogueira D, Cortvrindt R, De Matos DG, Vanhoutte L, Smitz J. Effect of
18 phosphodiesterase type 3 inhibitor on developmental competence of immature mouse
19 oocytes in vitro. *Biol Reprod* 2003;69:2045-52.
- 20 [20] Takahashi Y, Hishinuma M, Matsui M, Tanaka H, Kanagawa H. Development of
21 in vitro matured/fertilized bovine embryos in a chemically defined medium: influence

1 of oxygen concentration in the gas atmosphere. J Vet Med Sci 1996;58:897-902.

2 [21] Nagano M, Katagiri S, Takahashi Y. ATP content and
3 maturational/developmental ability of bovine oocytes with various cytoplasmic
4 morphologies. Zygote 2006;14:299-304.

5 [22] Takahashi Y, Kanagawa H. Effect of oxygen concentration in the gas atmosphere
6 during in vitro insemination of bovine oocytes on the subsequent embryonic
7 development in vitro. J Vet Med Sci 1998;60:365-7.

8 [23] Brackett BG, Oliphant G. Capacitation of rabbit spermatozoa in vitro. Biol
9 Reprod 1975;12:260-74.

10 [24] Takahashi Y, First NL. In vitro development of bovine one-cell embryos:
11 Influence of glucose, lactate, pyruvate, amino acids and vitamins. Theriogenology
12 1992;37:963-78.

13 [25] Takahashi Y, Kanagawa H. Effects of glutamine, glycine and taurine on the
14 development of in vitro fertilized bovine zygotes in a chemically defined medium. J
15 Vet Med Sci 1998;60:433-7.

16 [26] Uchikura K, Nagano M, Hishinuma M. Prediction of maturational competence of
17 feline oocytes using supravital staining of cumulus cells by propidium iodide. Zygote
18 2012;20:333-7.

19 [27] Taketsuru H, Hirao Y, Takenouchi N, Iga K, Miyano T. Effect of androstenedione
20 on the growth and meiotic competence of bovine oocytes from early antral follicles.
21 Zygote 2012;20:407-15.

- 1 [28] Sanchez F, Smitz J. Molecular control of oogenesis. *Biochim Biophys Acta*
2 2012;1822:1896-912.
- 3 [29] Fair T, Hyttel P, Greve T. Bovine oocyte diameter in relation to maturational
4 competence and transcriptional activity. *Mol Reprod Dev* 1995;42:437-42.
- 5 [30] Sutton ML, Gilchrist RB, Thompson JG. Effects of in-vivo and in-vitro
6 environments on the metabolism of the cumulus-oocyte complex and its influence on
7 oocyte developmental capacity. *Hum Reprod Update* 2003;9:35-48.
- 8 [31] Tanghe S, Van Soom A, Nauwynck H, Coryn M, de Kruif A. Minireview:
9 Functions of the cumulus oophorus during oocyte maturation, ovulation, and
10 fertilization. *Mol Reprod Dev* 2002;61:414-24.

11

1 Figure captions

2

3 Fig. 1. Morphology of oocyte-cumulus-granulosa cell complexes (OCGCs) cultured

4 individually in 96-well plates. (A) Isolated OCGCs before IVG culture; (B) viable

5 OCGCs after IVG culture, arrowhead indicates oocyte surrounded by healthy

6 granulosa cell layers; and (C) degenerated OCGCs after 14 days of IVG culture. Scale:

7 100 μm .

8

9 Fig. 2. Bright field and fluorescent images after propidium iodide staining of IVG

10 oocytes. Fluorescent images were used to evaluate cumulus cells membrane integrity:

11 Grade 1 (A1, A2) - less than one-quarter of cumulus cells were stained; Grade 2 (B1,

12 B2) - one-quarter to one-half of cumulus cells were stained; and Grade 3 (C1, C2) -

13 more than one-half of cumulus cells were stained. Scale: 100 μm .

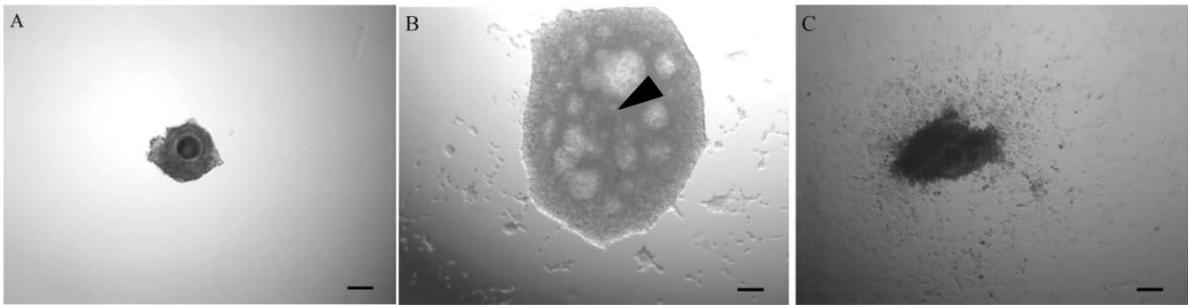


Fig. 1

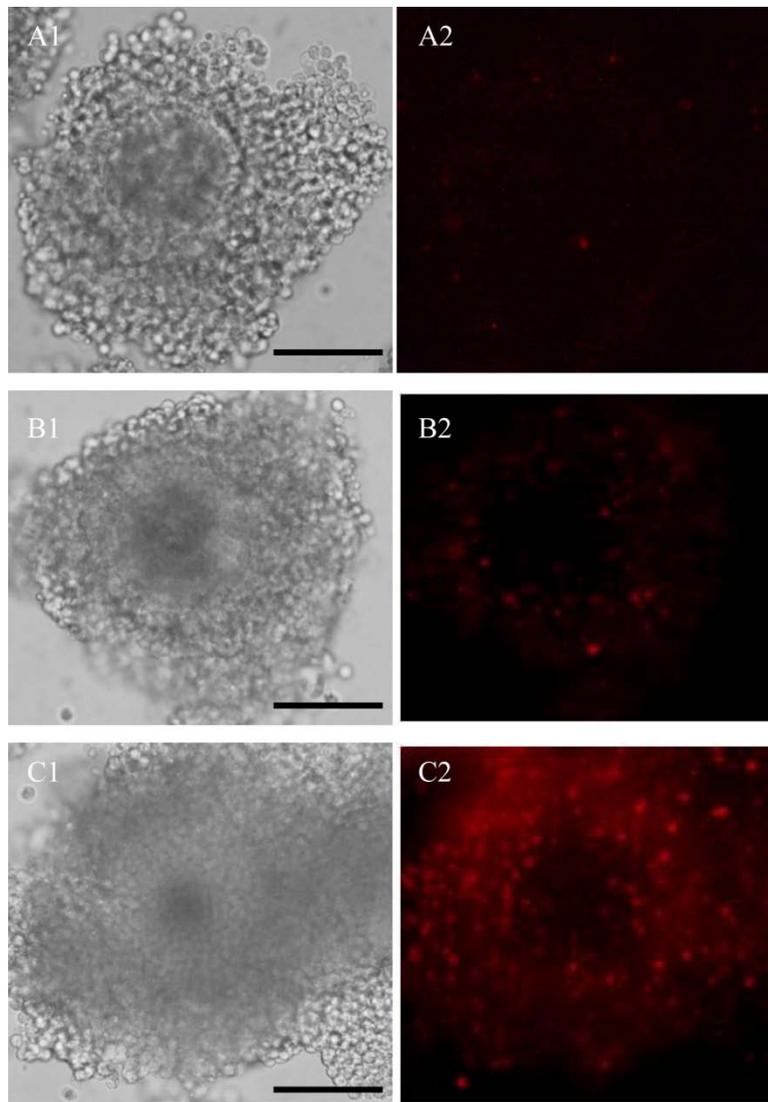


Fig. 2

Table 1. Oocyte viability (mean \pm SD) according to the duration of in vitro growth (IVG) culture of bovine oocyte-cumulus-granulosa cell complexes (OCGCs) from early antral follicles.

IVG duration (d)	No. of OCGCs (replicates*)	Viability (%)
10	171 (3)	69.1 \pm 7.2 ^a
12	237 (6)	70.4 \pm 4.6 ^a
14	291 (9)	55.4 \pm 9.1 ^b

*14 to 66 OCGCs/replicate. ^{a,b}Rows with different superscripts differ (P < 0.05).

Table 2. Effects of in vitro growth (IVG) duration and pre-in vitro maturation (IVM) culture on the diameter (mean \pm SD) and nuclear maturation of bovine oocytes from early antral follicles. GV: germinal vesicle, GVBD: germinal vesicle breakdown, M I: metaphase I, A I/T I: anaphase I/telophase I, M II: metaphase II, Deg: degeneration.

IVG duration (d)	Pre-IVM	No. of oocytes (replicates*)	Oocytes diameter (μm)		% of oocytes at each stage					
			Before IVG	After IVG**	GV	GVBD	MI	AI/TI	MII	Deg
10	-	61 (3)	95.4 \pm 3.8	109.8 \pm 4.6 ^{a†}	1.6	0	45.9 ^{a†}	16.4	36.1 ^{a†}	0
10	+	56 (3)	95.3 \pm 5.9	110.4 \pm 4.6 ^{x†}	3.6	0	42.9 ^{x†}	5.4	48.2 ^{x†}	0
12	-	69 (3)	96.0 \pm 5.4	115.5 \pm 4.4 ^{b†}	0	0	20.3 ^{b†}	20.3	53.6 ^{bA†}	5.8
12	+	97 (4)	95.5 \pm 5.1	115.9 \pm 5.4 ^{y†}	0	0	18.6 ^{y†}	10.3	70.1 ^{yB†}	1.0
14	-	91 (5)	95.2 \pm 6.7	114.6 \pm 5.7 ^{b†}	3.3	6.6	31.9 ^{abA†}	4.4	53.8 ^{bA†}	0
14	+	67 (5)	95.5 \pm 4.1	115.8 \pm 4.9 ^{y†}	0	0	14.9 ^{yB}	6.0	76.1 ^{yB†}	3.0
Control***	-	50 (2)	-	120.4 \pm 3.2	0	0	6.0	0	94.0	0

*10 to 20 oocytes/replicate. **Oocyte diameter was measured after IVM with or without pre-IVM. ***Oocytes collected from antral follicles (2 to 8 mm in diameter) served as in vivo-derived controls. ^{a,b}Rows with different superscripts differ within pre-IVM (-) groups ($P < 0.05$). ^{x,y}Rows with different superscripts differ within pre-IVM (+) group ($P < 0.05$). ^{A,B}Rows with different superscripts differ within the same duration of IVG ($P < 0.05$). [†]Rows with superscript differ from controls ($P < 0.05$).

Table 3. Cumulus cells membrane integrity according to the duration of in vitro growth (IVG) culture of bovine oocytes from early antral follicles. Membrane integrity was evaluated with propidium iodide staining: Grade 1 - less than one-quarter of cumulus cells were stained; Grade 2 - one-quarter to one-half of cumulus cells were stained; and Grade 3 - more than one-half of cumulus cells were stained.

Duration of IVG (d)	No. of oocytes (replicates*)	Grade 1 (%)	Grade 2 (%)	Grade 3 (%)
12	58 (3)	75.9 ^a	15.5 ^a	8.6 ^a
14	60 (3)	26.7 ^b	36.7 ^b	36.7 ^b

*Approximately 20 oocytes/replicate. ^{a,b}Rows with different superscripts differ (P < 0.05).

Table 4. Effects of in vitro growth (IVG) duration and pre-in vitro maturation (IVM) culture on and nuclear maturation of bovine oocytes from early antral follicles. GV: germinal vesicle, GVBD: germinal vesicle breakdown, M I: metaphase I, Deg: degeneration.

IVG duration (d)	Pre-IVM	No. of oocytes (replicates*)	% of oocytes at each stage			
			GV	GVBD	MI	Deg.
12	before	31 (3)	100	0	0	0
14	before	49 (3)	98.0	2.0	0	0
12	after	60 (3)	95.0 ^a	5.0	0	0
14	after	73 (3)	82.2 ^b	5.5	8.2	4.1

*10 to 20 oocytes/replicate. ^{a,b}Rows with different superscripts differ (P < 0.05).

Table 5. Effects of in vitro growth (IVG) duration on cleavage and blastocyst production rates and blastocysts cell number (mean \pm SD) obtained with bovine oocytes from early antral follicles.

IVG duration (d)	No. of oocytes (replicates*)	Cleavage (%)	Blastocyst production (%) based on		Blastocyst cell number (n)
			Inseminated oocytes	Cleaved oocytes	
12	164 (5)	55.7 \pm 11.0 ^a	24.5 \pm 9.7 ^a	43.2 \pm 10.3 ^a	127.0 \pm 47.2 ^a (39)
14	174 (5)	49.7 \pm 8.5 ^a	9.9 \pm 3.4 ^b	19.7 \pm 4.1 ^b	110.6 \pm 49.7 ^a (18)
Control**	142 (5)	86.6 \pm 4.4 ^b	42.4 \pm 9.3 ^c	48.9 \pm 10.2 ^a	167.7 \pm 64.6 ^b (61)

*Approximately 30 oocytes/replicate. **Oocytes collected from antral follicles (2 to 8 mm in diameter) served as in vivo-derived controls.

^{a,b,c}Rows with different superscripts differ ($P < 0.05$).