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

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Abstract

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

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

1. Introduction

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

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

contribute to improve their maturational and developmental competences.

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

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

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

2. Materials and Methods

2.1. Chemicals

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

2.2. Collection of early antral follicles and IVG oocytes

Bovine ovaries obtained at a local abattoir were kept in plastic bags at 20 °C and were transported to the laboratory within 6 to 10 h of collection. After 3 washes in physiological saline, sliced ovarian cortex tissues (< 1 mm thicks) were prepared using a surgical blade (No. 11) and stored 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 at 37°C) as described elsewhere [4]. Under a stereo microscope, early antral follicles (0.5 to 1 mm in diameter) were dissected from the sliced ovarian tissues using a surgical blade (No. 20). Oocyte-cumulus-granulosa complexes (OCGCs) were isolated from follicles

using a pair of fine forceps and those with normal appearance were individually cultured in 96-well culture plates (Falcon 353872' Becton Dickinson, Franklin Lakes, NJ, USA) with 200 μL of the growth medium for 10 to 14 days at 39 °C in humidified air with 5% CO_2. Growth medium consistent of HEPES-buffered TCM199 (Invitrogen) supplemented with 0.91 mM sodium pyruvate, 1 μg/mL estradiol-17β, 5% fetal calf serum (FCS; Invitrogen), 4 mM hypoxanthine, 4% polyvinylpyrrolidone (MW 360,000), and 50 μg/mL gentamicin sulfate. At the onset of IVG culture, OCGCs were photographed (Fig. 1A) under an inverted microscope (CK 40, Olympus, Tokyo, Japan) attached with a CCD camera (Moticam 2000, Shimadzu Rika Corporation, Tokyo, Japan), and the diameters of oocytes (excluding the zona pellucida) were determined using software (Motic Images Plus 2.2s, Shimadzu). During IVG culture, half (100 μL) of the growth medium was replaced by the same amount of fresh medium every 4 days.

2.3. IVM of in vivo-derived and IVG oocytes

In vivo-derived oocytes collected from antral follicles (2 to 8 mm in diameter) were submitted to IVM as described previously [20]. Briefly, cumulus-oocyte complexes (COCs) were incubated in droplets of IVM medium (about 10 COCs/50 μL) covered with paraffin oil for 22 h at 39 °C under 5% CO_2 in air. Maturation medium consisted of HEPES-buffered TCM199 supplemented with 0.2 mM sodium pyruvate, 0.02

units/mL FSH (from porcine pituitary), 1 μg/mL estradiol-17β, 10% FCS, and 50 μg/mL gentamicin sulfate. Oocytes grown in vitro surrounded by several layers of cumulus cells recovered from morphologically normal OCGCs were selected for IVM. The IVM procedure for IVG oocytes without pre-IVM culture was the same as for in vivo-derived oocytes. Oocytes subjected to pre-IVM culture were cultured individually in micro-well plates (Mini Trays 163118; NUNC, Roskilde, Denmark) filled with 6 mL of HEPES-buffered TCM-199 supplemented with 0.2 mM sodium pyruvate, 2×10^{-6} units/mL FSH, 0.5 mM IBMX, 1 μg/mL estradiol-17β, 10% FCS, and 50 μg/mL gentamicin sulfate at 39 °C under 5% CO_2 in air. After 20 h of pre-IVM culture, IVG oocytes were transferred to the micro-well plates filled with 6 mL of the same IVM medium used for in vivo-derived oocytes, and cultured for another 22 h at 39 °C under 5% CO_2 in air.

2.4. Evaluation of oocyte nuclear status

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

2.5. In vitro fertilization and culture

In vitro fertilization was performed using frozen semen according to a procedure described previously [22] with slight modifications. Briefly, motile sperm (5×10^6 sperm/mL) separated by Percoll gradient (45 and 90%) were co-incubated with COCs in a 100-μL droplet (about 10 COCs per droplet) of modified Brackett & Oliphant isotonic medium [23] containing 3 mg/mL fatty-acid-free BSA and 2.5 mM theophylline [24] for 18 h at 39 °C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2. In vitro culture of inseminated oocytes (presumptive zygotes) was performed as previously described [20,25]. Briefly, after co-incubation with sperm, presumptive zygotes were freed from cumulus cells by vortexing and washing three times in culture medium. Cumulus-free zygotes were cultured for 6 days in 30-μL droplets of culture medium at 39 °C under 5% CO_2, 5% O_2 and 90% N_2. The culture medium consisted of modified synthetic oviduct fluid containing 1 mM glutamine, 12 essential amino acids for basal medium Eagle, 7 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 production rates were determined after 2 d (about 30 h) and 6 d (about 150 h) of IVC, respectively. Total live cell numbers of blastocysts obtained after 6 d of IVC were counted using an air-drying method [24].

2.6. Evaluation of cumulus cell membrane integrity

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

2.7. Experimental design

2.7.1. Experiment 1

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

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

2.7.2. Experiment 2

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

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

2.8. Statistical analysis

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

3. Results

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

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

4. Discussion

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

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

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

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

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

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

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

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Figure captions

Fig. 1. Morphology of oocyte-cumulus-granulosa cell complexes (OCGCs) cultured individually in 96-well plates. (A) Isolated OCGCs before IVG culture; (B) viable OCGCs after IVG culture, arrowhead indicates oocyte surrounded by healthy granulosa cell layers; and (C) degenerated OCGCs after 14 days of IVG culture. Scale: 100 μm.

Fig. 2. Bright field and fluorescent images after propidium iodide staining of IVG oocytes. Fluorescent images were used to evaluate cumulus cells membrane integrity: Grade 1 (A1, A2) - less than one-quarter of cumulus cells were stained; Grade 2 (B1, B2) - one-quarter to one-half of cumulus cells were stained; and Grade 3 (C1, C2) - more than one-half of cumulus cells were stained. Scale: 100 μm.

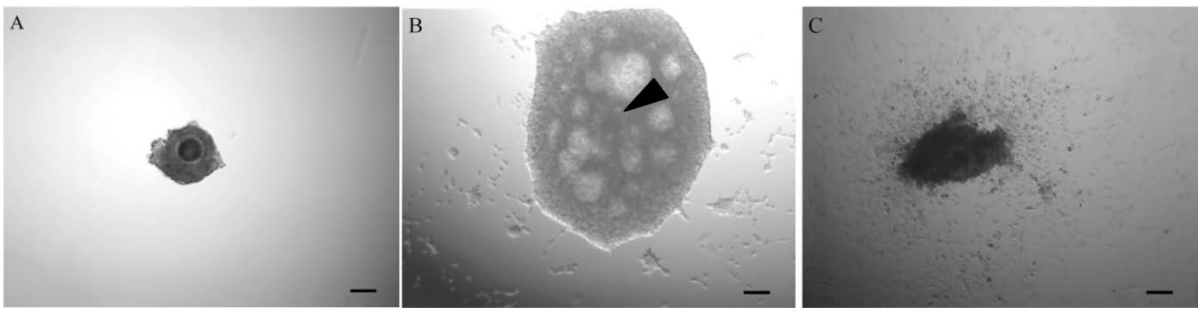

Fig. 1

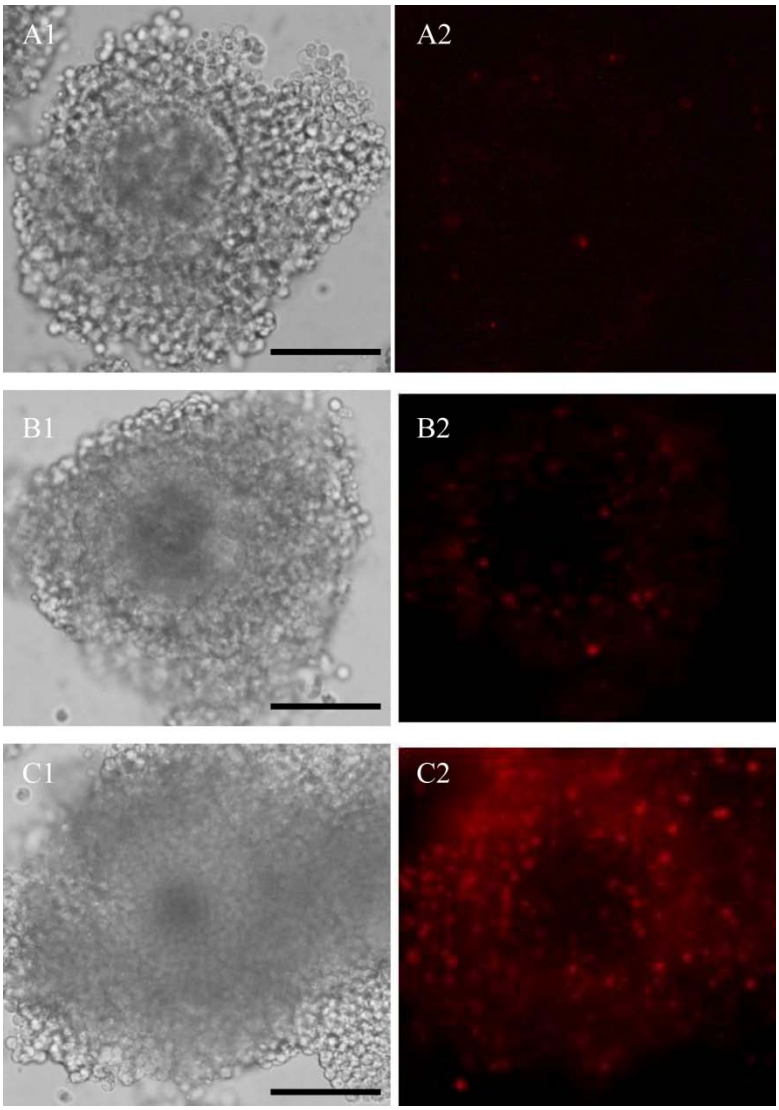

Fig. 2

Table 1. Oocyte viability (mean ± 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 ± 7.2[a]
12	237 (6)	70.4 ± 4.6[a]
14	291 (9)	55.4 ± 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 ± 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 ± 3.8	109.8 ± 4.6^{a†}	1.6	0	45.9^{a†}	16.4	36.1^{a†}	0
10	+	56 (3)	95.3 ± 5.9	110.4 ± 4.6^{x†}	3.6	0	42.9^{x†}	5.4	48.2^{x†}	0
12	-	69 (3)	96.0 ± 5.4	115.5 ± 4.4^{b†}	0	0	20.3^{b†}	20.3	53.6^{bA†}	5.8
12	+	97 (4)	95.5 ± 5.1	115.9 ± 5.4^{y†}	0	0	18.6^{y†}	10.3	70.1^{yB†}	1.0
14	-	91 (5)	95.2 ± 6.7	114.6 ± 5.7^{b†}	3.3	6.6	31.9^{abA†}	4.4	53.8^{bA†}	0
14	+	67 (5)	95.5 ± 4.1	115.8 ± 4.9^{y†}	0	0	14.9^{yB}	6.0	76.1^{yB†}	3.0
Control***	-	50 (2)	-	120.4 ± 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 whithin 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 ± 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 ± 11.0[a]	24.5 ± 9.7[a]	43.2 ± 10.3[a]	127.0 ± 47.2[a] (39)
14	174 (5)	49.7 ± 8.5[a]	9.9 ± 3.4[b]	19.7 ± 4.1[b]	110.6 ± 49.7[a] (18)
Control**	142 (5)	86.6 ± 4.4[b]	42.4 ± 9.3[c]	48.9 ± 10.2[a]	167.7 ± 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 ($P < 0.05$).