Studies on *in vitro* maturation/fertilization/development and mitochondrial activity of *in vitro*-grown bovine oocytes derived from early antral follicles

（初期胞状卵胞由来体外発育牛卵子の体外成熟/受精/発生およびミトコンドリア活性に関する研究）

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Contents

Abbreviations

2PN	two pronuclei
A I	anaphase I
ANOVA	analysis of variance
ATP	adenosine triphosphate
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CC	chromosomal condensation
COC	cumulus oocyte complex
dbcAMP	dibutyryl cyclic AMP
DCHFDA	2', 7'-dichlorodihydrofuorescein diacetate
DPBS	Dulbecco's phosphate buffered saline
ESH	enlarged sperm head
FCS	fetal calf serum
FSH	follicle stimulating hormone
GV	germinal vesicle
GVBD	germinal vesicle breakdown
h	hour
HEPES	4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid
HSD	honestly significant difference

IBMX	3-isobutyl-1-methylxanthine
IU	international unit
IVC	*in vitro* culture
IVF	*in vitro* fertilization
IVG	*in vitro* growth
IVM	*in vitro* maturation
IVP	*in vitro* embryo production
JC-1	5,5’,6,6’-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine iodide
M I	metaphase I
M II	metaphase II
min	minute
mRNA	messenger ribonucleic acid
OCGC	oocyte cumulus granulosa complex
PDE	phosphodiesterase
PI	propidium iodide
RNA	ribonucleic acid
ROS	reactive oxygen species
T I	telophase I
TCM-199	tissue culture medium 199

Preface

In 1989, it was reported that mouse growing oocytes derived from preantral follicles can be develop to live young after growth, maturation and fertilization *in vitro* (Eppig and Schroeder 1989). After this report, many basic researches for *in vitro*-growth (IVG) culture of oocytes derived from preantral or early antral follicles were performed in mouse (Eppig *et al.* 2000; O'Brien *et al.* 2003), cattle (Yamamoto *et al.* 1999; Hirao *et al.* 2004), pig (Hirao *et al.* 1994; Hashimoto *et al.* 2007) and sheep (Newton *et al.* 1999; Cecconi *et al.* 2004). In murine IVG, the efficiency of embryo production is very high (dela Pena *et al.* 2002; Demeestere *et al.* 2005; Miyano 2005) and it is speculated that the number of offspring available from one female using IVG technique can overcome that by natural mating and parturition (Eppig and O'Brien 1998). Therefore, the IVG technique has become very important to produce genetically valuable animals efficiently. In cattle, IVG of oocytes derived from preantral follicles has not succeeded yet, but two offspring were produced by IVG of oocytes derived from early antral follicles (<1 mm in diameter) (Yamamoto *et al*. 1999; Hirao *et al*. 2004). If effective IVG system for bovine growing oocytes is developed, it can be used not only for valuable animal production but also for clarifying the mechanisms of oocyte acquisition of maturational/developmental competences. Therefore, it is necessary to develop the IVG system which allows bovine growing oocytes to acquire the competences for undergoing nuclear maturation, fertilization and development to blastocysts *in vitro*.

In mouse, the many basic researches for IVG system were performed (Eppig *et al*. 1996; Hartshorne 1997; Eppig *et al*. 2000; Thomas *et al*. 2003; Demeestere *et al*. 2005; Miyano 2005; Liu *et al*. 2006; Thomas and Vanderhyden 2006; Picton *et al*. 2008; Desai *et al*. 2010), and the optimal culture duration (Liu *et al.* 2002; Nonowaki *et al.* 2010) was also clarified. In bovine IVG system, the optimal duration of IVG culture is not known, although growing oocytes derived from early antral follicles were subjected to IVG for 7 to 16 days (Harada *et al.* 1997; Yamamoto *et al.* 1999; Senbon and Miyano 2002; Hirao *et al.* 2009). In addition, after 14 (Endo *et al.* 2013) or 16 days (Senbon and Miyano 2002) of IVG culture, the diameter of bovine growing oocytes became around 115 μm, and their nuclear maturation rates (30 to 65%) were lower than those (around 85%) of *in vivo*-grown oocytes which reached 115 μm in diameter (Otoi *et al.* 1997). It is speculated that the low maturational ability of IVG oocytes may be caused by the absence of certain crucial oocyte cytoplasmic components (inadequate cytoplasmic maturation), because growing oocytes accumulate compounds essential for maturation, fertilization, and embryogenesis during folliculogenesis (Eppig *et al.* 1996; Marteil *et al.* 2009).

Previous studies suggested that cytoplasmic maturation of mammalian oocytes was improved by temporal meiotic arrest using phosphodiesterase (PDE) inhibitor immediately before *in vitro* maturation (IVM) (Hashimoto *et al*. 2002; Gilchrist and Thompson 2007; Albuz *et al*. 2010). The chromatin structure in germinal vesicle (GV) of bovine oocytes was also modulated during the culture immediately before IVM (pre-IVM) in the medium supplemented by PDE inhibitor and the development to blastocysts was enhanced (Luciano *et al.* 2011).

These results suggest that some cytoplasmic changes which support early embryonic development and the modulation of GV structure may occur simultaneously during pre-IVM culture.

During follicular development, the increase of mitochondrial activity has been described in rabbit (Kanaya *et al.* 2007) and bovine oocytes (Machatkova *et al.* 2012). In mammalian oocytes, it is reported that the higher mitochondrial activity is associated with greater meiotic competence (Egerszegi *et al.* 2010; Machatkova *et al.* 2012) and higher developmental ability to blastocysts (Kanaya *et al.* 2007). For this reason, mitochondrial activity can be used as a criterion for the evaluation of cytoplasmic maturation and developmental competence of mammalian oocytes.

In chapter I, the author examined the effect of IVG culture duration (10, 12 and 14 days) on the viability, growth of bovine oocytes derived from early antral follicles (0.5 to 1 mm in diameter) and the effect of pre-IVM using PDE inhibitor, 3-isobutyl-1-methylxanthine (IBMX), on the nuclear maturation and development to blastocysts of IVG oocytes.

In chapter II, the GV structure of IVG oocytes was examined before and after pre-IVM culture. Then the relationship between GV structure and nuclear maturation, fertilization and early embryonic development of IVG oocytes were examined.

In chapter III, for clarifying the status of cytoplasmic maturation of IVG oocytes, the mitochondrial activity in IVG oocytes during pre-IVM (0, 10 or 20 h) was evaluated. Also for investigating the relationship between mitochondrial activity during pre-IVM culture and subsequent maturational/developmental competences of IVG oocytes, the meiotic progression

and development to blastocysts in IVG oocytes with different pre-IVM duration were examined. Moreover, to confirm the developmental ability of IVG oocytes beyond blastocyst stage, the author also attempted the embryos transfer using IVG oocytes with pre-IVM.

Chapter I

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

Introduction

Large numbers of growing follicles in bovine ovaries offer a large pool of oocytes for *in vitro* maturation, fertilization (IVF), and culture (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) (Yamamoto *et al*. 1999; Hirao *et al*. 2004; Hirao *et al*. 2012). To utilize these oocytes efficiently, it is important to develop culture systems for 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 be the size of 107 to 117 μm in diameter, and some of them acquired maturational and developmental competency (Harada *et al.* 1997; Yamamoto *et al.* 1999; Senbon and Miyano 2002; Hirao *et al.* 2009). However, the optimal duration of IVG culture for growing bovine oocytes is not known. In mouse, the

appropriate length of the IVG culture period was related to the oocyte size at the start of culture (Hirao and Miyano 2008). Previous studies reported that extending the IVG period from 10 to 12 days reduced blastocyst rates in mouse, although nuclear maturation rates were similar (Liu *et al.* 2002; Nonowaki *et al.* 2010). 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*-grown bovine oocytes larger than 115 μm in diameter are generally high (around 85%) (Otoi *et al*. 1997). However, maturation rates of IVG oocytes grown to more than 115 μm in diameter are considerably lower (30 to 65%) (Senbon and Miyano 2002; Endo *et al*. 2013). Since growing oocytes accumulate compounds essential for maturation, fertilization, and embryogenesis during folliculogenesis (Eppig *et al.* 1996; Marteil *et al.* 2009), low maturational competence of IVG oocytes might be caused by the inadequate cytoplasmic maturation. This was suggested by the observation that oocyte cytoplasmic maturation was improved by the temporal maintenance of oocytes in meiotic arrest immediately before IVM culture (Gilchrist and Thompson 2007). Previous studies demonstrated that PDE inhibitors prevented the meiotic resumption of bovine oocytes (Bilodeau *et al.* 1993; Thomas *et al.* 2002) and improved nuclear maturation and blastocyst rates when used in pre-IVM culture (Nogueira *et al.* 2003; Luciano *et al.* 2011). 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 this chapter 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 (IBMX) on nuclear maturation of IVG oocytes was also evaluated.

Materials and Methods

Chemicals

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

Collection of early antral follicles and IVG culture of oocyte-granulosa cell complexes

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 TCM-199 (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 (Harada *et al.* 1997). 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 and Company, 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 consisted of HEPES-buffered TCM-199 (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. 1-1A) under an inverted microscope (CK 40, Olympus, Tokyo, Japan) with an attached 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 Rika). During IVG culture, half (100 µl) of the growth medium was replaced by the same amount of fresh medium every 4 days.

Pre-IVM and IVM of IVG oocytes

In vivo-grown oocytes collected from antral follicles (2 to 8 mm in diameter) were submitted to IVM as described previously (Takahashi *et al.* 1996). 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 TCM-199 supplemented with 0.2 mM sodium pyruvate, 0.02 units/ml follicle stimulating hormone (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*-grown oocytes. Oocytes subjected to pre-IVM 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*-grown oocytes, and cultured for another 22 h at 39°C under 5% CO_2 in air (Nagano *et al.* 2013).

Evaluation of oocyte nuclear status

Oocytes were denuded from cumulus cells by vortexing and stained with 1% aceto-orcein as described elsewhere (Nagano *et al.* 2006). Nuclear statuses were classified as 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.

IVF and IVC of inseminated oocytes

IVF was performed using frozen semen according to a procedure described previously (Takahashi and Kanagawa 1998) 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/droplet) of modified Brackett & Oliphant isotonic medium

(Brackett and Oliphant 1975) containing 3 mg/ml fatty-acid-free BSA and 2.5 mM theophylline (Takahashi and First 1992) for 18 h at 39°C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2.

IVC of inseminated oocytes (presumptive zygotes) was performed as previously described (Takahashi *et al.* 1996; Takahashi and Kanagawa 1998). 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 about 150 h in a 30-µl droplet 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 rates were determined after 30 h and about 150 h of IVC, respectively. Total cell numbers of blastocysts obtained after about 150 h of IVC were counted using an air-drying method (Takahashi and First 1992).

Evaluation of cumulus cell membrane integrity

Evaluation of membrane integrity of cumulus cells was performed in accordance with a previous report (Uchikura *et al.* 2012) 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. 1-2).

Experimental design

Experiment 1-1

A total of 699 OCGCs (14 to 66 OCGCs/replicate) were used to evaluate the effects of 10, 12 or 14 days of 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. 1-1B) and isolated oocytes had cytoplasm with normal appearance and several layers of cumulus cells (Fig. 1-2 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 oocytes/replicate) were used to examine maturational competence after IVM with or without pre-IVM culture. *In vivo*-grown oocytes collected from antral follicles were also subjected to IVM and served as control.

Experiment 1-2

To examine the effect of IVG duration on developmental competence, oocytes after 12 or 14 days of IVG culture, which showed higher nuclear maturation rates in Experiment 1-1, were subjected to IVF and IVC after IVM with pre-IVM culture. A total of 118 oocytes (20 oocytes/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 (about 30 oocytes/replicate). *In vivo*-grown oocytes collected from antral follicles were also subjected to IVM/IVF/IVC and served as control.

Statistical analysis

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

Results

Experiment 1-1

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 1-1). Oocyte diameters and proportions of oocytes at M II stage were greater ($P < 0.05$) when 12 or 14 days of IVG culture were 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 1-2). Oocyte diameters and proportions of oocytes at M II stage were greater ($P < 0.05$) for *in vivo*-grown oocytes than for IVG oocytes cultured for 10, 12 or 14 days.

Experiment 1-2

Cumulus cell membrane integrity was greater ($P < 0.05$) after 12 than 14 days of IVG culture (Table 1-3). 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 of IVG in GV stage was greater ($P < 0.05$) than that of oocytes obtained after 14 days (Table 1-4). Blastocyst rate for oocytes obtained after 12 days of IVG culture was greater ($P < 0.05$) than for oocytes obtained after 14 days; cleavage rates and blastocyst cell numbers were not affected by IVG duration (Table 1-5). There was no difference in blastocyst rates based on the number of cleaved oocytes between oocytes obtained after 12 days of IVG and

in vivo-grown oocytes, although the cleavage rate of 12-day IVG oocytes was lower ($P < 0.05$) than that of *in vivo*-grown oocytes. Blastocysts originated from *in vivo*-grown oocytes had a larger ($P < 0.05$) number of blastocyst cells than those from IVG oocytes.

Table 1-1. Oocyte viability according to the duration of IVG culture of bovine OCGCs from early antral follicles

IVG duration (days)	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]

[a,b] Values (mean ± SD) with different superscripts are significantly different ($P < 0.05$).

Table 1-2. Effects of IVG duration and pre-IVM culture on the diameter and nuclear maturation of bovine oocytes from early antral follicles

IVG duration (days)	Pre-IVM	No. of oocytes (replicates)	Oocytes diameter* (μm)		% of oocytes at each status					
			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

*Oocyte diameter (mean ± SD) was measured after IVM with or without pre-IVM.

** The oocytes collected from antral follicles (2 to 8 mm in diameter) served as *in vivo*-grown controls.

^{a,b} Values with different superscripts differ within pre-IVM (-) groups ($P < 0.05$).

^{x,y} Values with different superscripts differ within pre-IVM (+) group ($P < 0.05$).

^{A,B} Values with different superscripts differ within the same duration of IVG ($P < 0.05$).

† Values with superscript differ from controls ($P < 0.05$).

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.

Table 1-3. Cumulus cell membrane integrity according to the duration of IVG of bovine oocytes from early antral follicles

Duration of IVG (days)	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]

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

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; or Grade 3: more than one-half of cumulus cells were stained.

Table 1-4. Effects of IVG duration and pre-IVM culture on and nuclear maturation of bovine oocytes from early antral follicles

IVG duration (days)	Pre-IVM	No. of oocytes (replicates)	% of oocytes at each status			
			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

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

GV, germinal vesicle; GVBD, germinal vesicle breakdown; M I, metaphase I; Deg, degeneration.

Table 1-5. Effects of IVG duration on cleavage and blastocyst rates and blastocysts cell number obtained with bovine oocytes from early antral follicles

IVG duration (days)	No. of oocytes (replicates)	% of cleaved oocytes	% of blastocysts based on		Cell no. in blastocysts (n)
			Inseminated oocytes	Cleaved oocytes	
12	164 (5)	55.7 ± 11.0[a]	24.5 ± 9.7[a]	43.2 ± 10.3[a]	127 ± 47[a] (39)
14	174 (5)	49.7 ± 8.5[a]	9.9 ± 3.4[b]	19.7 ± 4.1[b]	111 ± 50[a] (18)
Control*	142 (5)	86.6 ± 4.4[b]	42.4 ± 9.3[c]	48.9 ± 10.2[a]	168 ± 65[b] (61)

[a-c] Values (mean ± SD) with different superscripts within columns are significantly different ($P < 0.05$).

* The oocytes collected from antral follicles (2 to 8 mm in diameter) served as *in vivo*-grown controls.

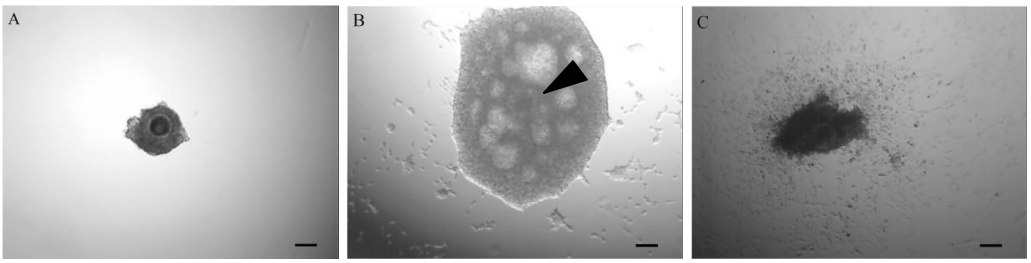


Fig. 1-1. Morphology of oocyte-cumulus granulosa cell complexes (OCGCs) cultured individually in 96-well plates. (A) Isolated OCGCs before IVG culture; (B) viable OCGCs after IVG culture, arrowhead indicates oocyte surrounded by healthy granulosa cell layers; and (C) degenerated OCGCs after 14 days of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

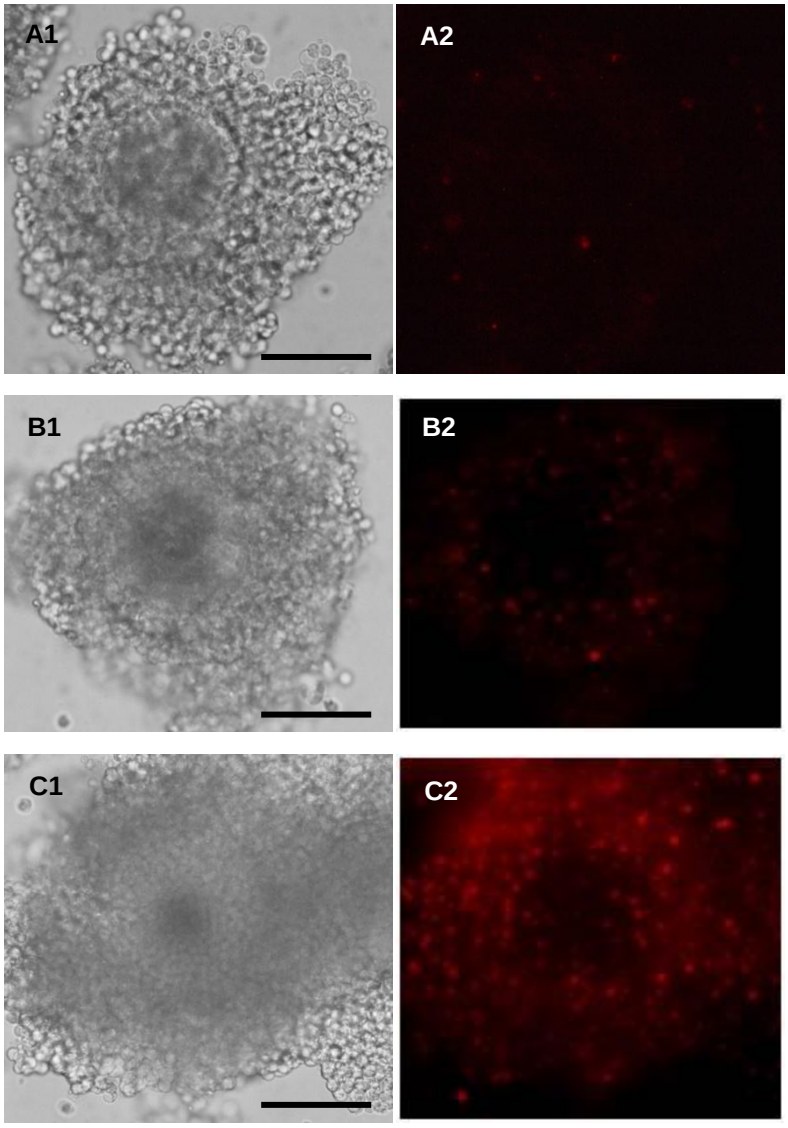


Fig. 1-2. Bright field and fluorescent images after propidium iodide staining of IVG oocytes. Fluorescent images were used to evaluate cumulus cell membrane integrity: Grade 1 (A1, A2): less than one-quarter of cumulus cells were stained; Grade 2 (B1, B2): one-quarter to one-half of cumulus cells were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

Discussion

In the present study, oocyte viability after 14 days of 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 (Hirao *et al.* 2004). Recently, several studies have reported higher survival rates (approximately 80%) after IVG culture for 14 days (Hirao *et al.* 2012; Taketsuru *et al.* 2012). 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, the author 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 of 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 period under the 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 (Gilchrist and Thompson 2007; Sanchez and Smitz 2012). Meiotic competence for bovine *in vivo*-grown oocytes increases as the diameter increases from 110 μm (42% M II rate) (Fair *et al*. 1995) to 115 μm (85% M II rate) (Otoi *et al*. 1997). In the present study, oocytes reached approximately 115 μm after 12 or 14 days of IVG and the diameter was similar to that observed after 14 days in previous reports (Hirao *et al.*

2004; Hirao *et al.* 2012; Taketsuru *et al.* 2012). However, similarly to previous studies (Senbon and Miyano 2002; Endo *et al.* 2013), 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 has been reported that gap junctions are essential for oocyte meiotic regulation by allowing the passage of small regulatory molecules (Sutton *et al.* 2003). Previous studies that used a pre-IVM culture system with PDE inhibitor for *in vivo*-grown oocytes showed that the intra-oocyte level of cAMP was maintained and loss of gap junction was inhibited in cattle (Luciano *et al.* 2011) and pig (Ozawa *et al.* 2008). 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 blastocysts when compared to 14 days of 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 developmental competence in oocytes is dependent on nutrients and soluble factors supplied by cumulus cells (Tanghe *et al.* 2002; Sutton *et al.* 2003). These results suggest that the longer duration of IVG culture leads to the decline in cumulus cell membrane integrity, resulting in lower developmental competence of IVG oocytes. Although blastocyst rate of 12-day IVG oocytes as a function of cleaved embryos was similar to that of *in vivo*-grown oocytes in this chapter, cleavage rate and blastocyst cell numbers were still lower than those of *in vivo*-grown 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, the size reported to acquire full developmental competence (Otoi *et al.* 1997). Since the author evaluated only the effects of 20 h pre-IVM culture in the present study, further studies are required to determine the optimal duration of pre-IVM culture.

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

Summary

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) was evaluated. In addition, the effect of pre-IVM culture with IBMX on nuclear maturation of IVG oocytes was also evaluated. In Experiment 1-1, 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 1-2, cumulus cell membrane integrity was greater ($P < 0.05$) after 12 days. Blastocyst 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 of IVG followed by pre-IVM culture was considered to be the optimal processing system for bovine oocytes derived from early antral follicles when oocyte viability, diameter, maturational and developmental competences were considered.

Chapter II

Pre-maturational culture with IBMX synchronizes meiotic progression of the germinal vesicle stage and improves nuclear maturation and embryonic development in IVG bovine oocytes

Introduction

In chapter I, the author clarified that the optimal duration of IVG is 12 days since a longer duration (14 days) of IVG culture resulted in reduced cumulus cell viability, which was related to low developmental competence of oocytes, and shorter duration (10 days) resulted in lower nuclear maturation. Furthermore, pre-IVM for 20 h in medium supplemented with IBMX improved nuclear maturation, resulting in a high blastocyst rate (43.2% based on cleaved oocytes) in bovine IVG oocytes derived from early antral follicles. However, why and how pre-IVM contributes to the improvement of developmental competence are still unclear.

It is well known that the chromatin structure in GV is subjected to dynamic modifications during oocyte growth (Tan *et al.* 2009), and transitions in large-scale chromatin structure have been shown to be essential for growing oocytes to acquire maturational and developmental competences in cattle (Luciano *et al.* 2011) and mouse (Zuccotti *et al.* 1998). Recently, it was reported that bovine oocytes at the GV stage could be divided into 4 stages by the degree of chromatin condensation (GV 0 to GV 3) (Lodde *et al*. 2007; Luciano *et al*. 2011), and that oocytes at GV 2 and 3 had higher developmental competence than others (Lodde *et al.* 2007).

In chapter I, the author confirmed that most IVG oocytes were at the GV stage before and after pre-IVM; however, the details of the GV structure were not investigated. Therefore, determination of GV structure of IVG oocytes is necessary to investigate the effect of pre-IVM on the development of IVG oocytes.

Full oocyte maturation involves both nuclear and cytoplasmic events that support normal fertilization and early embryonic development (Crozet *et al.* 1995). In the previous chapter, the author showed that the nuclear maturation rate of IVG oocytes was more than 70% by subjecting them to pre-IVM culture, and was close to that of *in vivo*-grown oocytes (about 90%). However, the cleavage rate (55.7%) of IVG oocytes was significantly lower than that of *in vivo*-grown oocytes (86.6%). One of the causes of low cleavage in IVG oocytes may be inadequate cytoplasmic maturation. Bovine oocytes that had not completed their growth phase at the time of submission to IVF failed to cleave, even if the nuclear maturation rate was almost the same (Fair and Hyttel 1997). Therefore, cytoplasmic maturation must be evaluated to deal with the effect of pre-IVM culture, which can be done by assessing pronuclear formation in fertilized oocytes (Mattioli *et al.* 1991; Chian *et al.* 1994; Swain and Pool 2008). Oocytes with inadequate cytoplasmic maturation have been reported to be incapable of male pronuclear formation in bovine (Chian *et al.* 1994) and porcine oocytes (Iritani *et al.* 1978; Mattioli *et al.* 1991), whereas improved cytoplasmic maturation results in high fertilization and development rates in *in vitro*-matured bovine oocytes (Izadyar *et al.* 1998). To clarify the effect of pre-IVM culture on the cytoplasmic maturation of IVG oocytes, the fertilization and developmental ability of IVG oocytes with/without pre-IVM should be investigated.

In this chapter, the author aimed to clarify the effect of pre-IVM supplemented with IBMX on the nuclear and cytoplasmic maturation of IVG oocytes, GV structure and competences of nuclear maturation, fertilization and early embryonic development of IVG oocytes with/without pre-IVM.

Materials and Methods

Collection of early antral follicles and IVG culture of oocyte-granulosa cell complexes

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 their collection as described in chapter I. The OCGCs with normal appearance were cultured individually in a 96-well culture plate (Falcon 353872, Becton Dickinson and Company, Franklin Lakes, NJ, USA) with 200 μl of growth medium for 12 days at 39°C in humidified air with 5% CO_2. The growth medium was HEPES-buffered TCM-199 (Cat #12340-030, Invitrogen) supplemented with 0.91 mM sodium pyruvate, 1 μg/ml estradiol-17β, 5% 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 under an inverted microscope (CK 40, Olympus, Tokyo, Japan) with an attached 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 Rika). Every 4 days of IVG culture, half (100 μl) of the growth medium was replaced with the same amount of fresh medium.

Pre-IVM and IVM of IVG oocytes

After 12 days of IVG culture, oocytes surrounded by several layers of cumulus cells were collected from morphologically normal OCGCs and submitted to IVM with or without pre-IVM as described in chapter I. Briefly, they were cultured individually in each well of 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 (from porcine pituitary), 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 for 20 h and then cultured in the same manner as pre-IVM using IVM medium, which was HEPES-buffered TCM-199 supplemented with 0.2 mM sodium pyruvate, 0.02 units/ml FSH, 1 μg/ml estradiol-17β, 10% FCS, and 50 μg/ml gentamicin sulfate (Nagano *et al.* 2013).

Oocytes collected from antral follicles of 2 to 8 mm in diameter (*in vivo*-grown oocytes) served as controls. IVM of *in vivo*-grown oocytes was conducted as described previously (Takahashi *et al.* 1996). In brief, COCs were incubated in droplets of IVM medium, the same as used for IVG oocytes (about 10 COCs/50 μl) covered with paraffin oil at 39°C under 5% CO_2 in air for 22 h.

IVF and IVC of inseminated oocytes

IVF was performed using frozen semen according to a procedure described previously (Takahashi and Kanagawa 1998) with slight modification. In brief, motile sperm (5×10^6 sperm/ml) separated from thawed semen by a Percoll gradient (45 and 90%) were co-incubated with COCs in a 100-μl droplet (about 10 COCs/droplet) of modified Brackett &

Oliphant isotonic medium (Brackett and Oliphant 1975) containing 3 mg/ml fatty-acid-free BSA and 2.5 mM theophylline (Takahashi and First 1992) for 18 h at 39°C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2.

IVC of inseminated oocytes (presumptive zygotes) was performed as described previously (Takahashi *et al.* 1996; Takahashi and Kanagawa 1998). Briefly, after co-incubation with sperm, presumptive zygotes were freed from cumulus cells by vortexing and washing three times in the culture medium. Cumulus-free zygotes were cultured for about 150 h in a 30-µl droplet of the culture medium at 39°C under 5% CO_2, 5% O_2 and 90% N_2. The culture medium was a modified synthetic oviduct fluid containing 1 mM glutamine, 12 essential amino acids for basal medium Eagle, 7 nonessential amino acids for minimum essential medium and 10 µg/ml insulin (Takahashi *et al.* 1996) and was further supplemented with 5 mM glycine, 5 mM taurine, 1 mM glucose and 3 mg/ml fatty-acid-free BSA (Takahashi and Kanagawa 1998). After 30 h and about 150 h of IVC, cleavage and development of presumptive zygotes to the blastocyst stage were assessed, respectively. Total cell numbers of blastocysts obtained after about 150 h of IVC were counted using an air-drying method (Takahashi and First 1992).

Evaluation of oocyte chromatin configuration, nuclear status and fertilization

Oocytes were denuded from cumulus cells by vortexing, and photographed under an inverted microscope with an attached CCD camera, and the diameters of oocytes (excluding the zona pellucida) were determined using software. Then oocytes were fixed in 60% methanol in Dulbecco's phosphate buffered saline (DPBS) for 30 min at 4°C and stained with 5

μg/ml Hoechst 33342 for 15 min. The chromatin configuration of the GV in denuded oocytes was evaluated under an inverted microscope (TE300, Nikon, Tokyo, Japan) equipped with a fluorescence system using an appropriate filter (UV-2A, Nikon). Oocytes at the GV stage were classified according to the degree of chromatin condensation, as described previously (Lodde *et al.* 2007): GV 0, with a diffuse filamentous pattern of chromatin in the whole nuclear area; GV 1, with a few foci of chromatin condensation; GV 2, with chromatin further condensed into distinct clumps or strands; and GV 3, with chromatin condensed in a single clump.

After IVM, denuded oocytes were stained with 1% aceto-orcein and examined for their nuclear status as described elsewhere (Nagano *et al.* 2006). Nuclear statuses were divided into GV, GVBD, M I, A I/T I and M II by observation under a phase contrast microscope.

After IVF, the cumulus-free oocytes were stained with 1% aceto-orcein and examined for their fertilization as described elsewhere (Takahashi and Kanagawa 1998). The oocytes were considered fertilized when they had an enlarged sperm head or male pronucleus with corresponding sperm tail. Normal fertilization was defined as oocytes having a single male pronucleus or a single enlarged sperm head.

Experimental design

Experiment 2-1

GV stage and diameters of IVG oocytes were examined before and after pre-IVM (10 to 15 oocytes/replicate). A total of 102 IVG oocytes derived from 147 OCGCs were used. After IVM culture, nuclear statuses of IVG oocytes were investigated (15 to 20 oocytes/replicate). A total of 184 IVG oocytes derived from 243 OCGCs were subjected to

IVM with or without pre-IVM.

Experiment 2-2

IVG oocytes after IVM with or without pre-IVM (30 to 40 oocytes/replicate) were subjected to IVF and IVC. Their fertilization was examined by using a total of 331 IVG oocytes derived from 528 OCGCs. Cleavage and development to the blastocyst stage were also examined by using a total of 355 IVG oocytes derived from 497 OCGCs.

Statistical analysis

Maturation, fertilization, cleavage and blastocyst rates, and cell numbers in blastocysts were analyzed by one-way ANOVA followed by Tukey-Kramer's HSD test. Percentages of oocytes with each chromatin configuration were compared by chi-square test. All statistical analyses were performed using software (JMP version 10, SAS Institute, Cary, NC, USA).

Results

Experiment 2-1

As shown in Table 2-1, the mean diameter of isolated oocytes before IVG culture was around 95 μm. The mean diameters of IVG oocytes with or without pre-IVM were similar, and were smaller than that of *in vivo*-grown oocytes ($P < 0.05$). After pre-IVM, the proportion of IVG oocytes at GV 1 was lower and that at GV 3 stage was higher than that before pre-IVM ($P < 0.05$). These values after pre-IVM were similar to those of *in vivo*-grown oocytes.

As shown in Table 2-2, M II rate of IVG oocytes with pre-IVM was higher than that without pre-IVM ($P < 0.05$) and similar to that of *in vivo*-grown oocytes.

Experiment 2-2

As shown in Table 2-3, total and normal fertilization rates of IVG oocytes with pre-IVM were similar to those without pre-IVM. However, the total fertilization rate of IVG oocytes with pre-IVM tended to be lower ($P = 0.05$) and the normal fertilization rate was lower than those of *in vivo*-grown oocytes ($P < 0.05$). Polyspermy rates were similar and <10% in all experimental groups.

As shown in Table 2-4, the cleavage rate of IVG oocytes with pre-IVM was higher than that without pre-IVM ($P < 0.05$), but lower than that of *in vivo*-grown oocytes ($P < 0.05$). The blastocyst rate of IVG oocytes with pre-IVM based on cleaved oocytes was similar to that of *in vivo*-grown oocytes ($P = 0.61$), although that based on inseminated oocytes tended to be lower than that of *in vivo*-grown oocytes ($P = 0.05$). The blastocyst rate based on inseminated and cleaved IVG oocytes with pre-IVM tended to be higher than those of IVG oocytes without pre-IVM ($P = 0.08$ and $P = 0.17$, respectively). Cell numbers in blastocysts in IVG oocytes with and without pre-IVM tended to be lower than that of the control ($P = 0.25$ and $P = 0.18$, respectively).

Table 2-1. Chromatin structure and diameter of IVG oocytes before and after pre-IVM

Pre-IVM	No. of oocytes (replicates)	Diameter of oocytes (μm*)		% of oocytes at each GV stage		
		Before IVG	After IVG	GV 1	GV 2	GV 3
Before	50 (4)	95.0 ± 4.4	115.5 ± 4.8[a]	40.0[a]	32.0	28.0[a]
After	52 (3)	94.3 ± 4.0	116.2 ± 5.1[a]	21.2[b]	28.8	50.0[b]
Control**	65 (3)	-	125.3 ± 4.0[b]	15.4[b]	35.4	49.2[b]

[a, b] Values with different superscripts within columns are significantly different (P < 0.05).

*Mean ± SD.

**The oocytes immediately after collection from antral follicles (2 to 8 mm in diameter) served as *in vivo*-grown controls.

GV 1, with a few foci of chromatin condensation; GV 2, with chromatin further condensed into distinct clumps or strands; GV 3, with chromatin condensed in a single clump.

Table 2-2. Effect of pre-IVM on nuclear maturation of IVG oocytes

Pre-IVM	No. of oocytes (replicates)	% of oocytes at each status				
		GV	GVBD	M I	A I/T I	M II
-	85 (5)	5.0 ± 7.2	2.1 ± 2.9	26.6 ± 9.4[a]	15.8 ± 10.7[a]	49.4 ± 10.4[a]
+	99 (5)	1.3 ± 2.8	0	15.9 ± 4.3[b]	2.7 ± 6.1[b]	80.1 ± 9.6[b]
Control*	120 (5)	0	0	9.6 ± 2.9[b]	1.7 ± 2.4[b]	88.7 ± 1.9[b]

[a, b] Values (mean ± SD) with different superscripts within columns are significantly different (P < 0.05).

*The oocytes collected from antral follicles (2 to 8 mm in diameter) served as *in vivo*-grown controls.

GV, germinal vesicle; GVBD, germinal vesicle breakdown; M I, metaphase I; A I/T I, anaphase I/telophase I; M II, metaphase II.

Table 2-3. Effect of pre-IVM on fertilization of IVG oocytes

Pre-IVM	No. of oocytes (replicates)	% normal fertilization			% of polyspermy	% total fertilization
		% of ESH	% of 2PN	% of subtotal		
-	124 (5)	8.7 ± 9.3	56.8 ± 12.4	65.6 ± 7.9[a]	7.5 ± 5.1	73.1 ± 4.4[a]
+	207 (7)	14.0 ± 12.3	58.9 ±16.3	73.0 ± 12.6[a]	6.3 ± 3.0	79.7 ± 13.6[ab]
Control*	148 (6)	20.6 ± 18.5	66.7 ± 17.3	87.3 ± 6.2[b]	5.1 ± 4.6	93.1 ± 4.9[b]

[a, b] Values (mean ± SD) with different superscripts within columns are significantly different ($P < 0.05$).

*The oocytes collected from antral follicles (2 to 8 mm in diameter) served as *in vivo*-grown controls.

ESH, enlarged sperm head; 2PN, two pronuclei.

Table 2-4. Effect of pre-IVM on embryonic development of IVG oocytes

Pre-IVM	No. of oocytes (replicates)	% of cleaved oocytes	% of blastocysts based on		Cell no. in blastocysts (n)
			Inseminated oocytes	Cleaved oocytes	
-	148 (5)	45.8 ± 9.0[a]	12.7 ± 7.1[a]	26.9 ± 10.4[a]	125 ± 60 (18)
+	207 (5)	63.0 ± 5.5[b]	26.1 ± 6.9[ab]	41.7 ± 11.4[ab]	137 ± 57 (54)
Control*	170 (6)	83.0 ± 5.8[c]	40.3 ± 11.4[b]	48.7 ± 13.8[b]	156 ± 72 (68)

[a-c] Values (mean ± SD) with different superscripts within columns are significantly different ($P < 0.05$).

*The oocytes collected from antral follicles (2 to 8 mm in diameter) served as *in vivo*-grown controls.

Discussion

When IVG oocytes were subjected to pre-IVM with IBMX, the percentages of IVG oocytes at GV 2 and 3 were 28.8 and 50.0%, respectively. These results are similar to those of *in vivo*-grown oocytes (35.4 and 49.2%, respectively) and consistent with those previously reported (31.1 and 44.6%, respectively) (Lodde *et al*. 2007). The higher M II rate at 22 h IVM culture and higher blastocyst rate of IVG oocytes with pre-IVM may be caused by meiotic progression and synchronization to GV 2 and 3 of IVG oocytes during pre-IVM culture. By promoting and synchronizing to GV 2 and 3, fertilization may occur at appropriate timing for embryonic development in IVG oocytes with pre-IVM.

In the present study, M II rate of IVG oocytes without pre-IVM was lower than that with pre-IVM, and the percentages of M I and A I/T I stages were higher in IVG oocytes without pre-IVM. Despite the low M II rate of IVG oocytes without pre-IVM, the fertilization rate was similar to that with pre-IVM. These results indicate that IVG oocytes without pre-IVM might reach M II stage during IVF culture and suggest that extension of IVM duration to more than 22 h may increase the percentage of M II oocytes but also increase the variation in the timing of M II arrival. Previous reports indicated that the oocyte's ability into develop to a blastocyst was not acquired immediately after M II arrival and that it might develop gradually after the completion of nuclear maturation (Kubiak 1989; Dominko and First 1997). Also, if oocytes were inseminated immediately after M II arrival, the cleavage and blastocyst rates were shown to be significantly reduced (Dominko and First 1997). In the present study, slower meiotic

progression of IVG oocytes without pre-IVM may have resulted in sperm penetration immediately after the completion of nuclear maturation and led to lower cleavage and embryonic development. A previous study examined the effects of meiotic arrest during pre-IVM culture on maturational and developmental competences of *in vivo*-grown oocytes (Hashimoto *et al.* 2002), and it showed that the time required for nuclear maturation of oocytes with pre-IVM was shorter than the time required for oocytes without pre-IVM. Therefore, the details of meiotic progression of IVG oocytes with or without pre-IVM should be examined in a future study.

The cleavage rate (83.0%) and normal fertilization rate (87.3%) in *in vivo*-grown oocytes were similar in the present study. On the other hand, cleavage rates of IVG oocytes with pre-IVM and without pre-IVM were only 63.0 and 45.8%, despite their normal fertilization rates being 73.0 and 65.6%, respectively. The reason for this discrepancy between normal fertilization and cleavage rates in IVG oocytes is not clear. It suggests that cytoplasmic maturation in IVG oocytes might have been inadequate and could not support oocyte cleavage, although the author did not identify a difference in pronucleus formation in all experimental groups. Early embryonic development is dependent on stored maternal RNAs and proteins synthesized during oogenesis and oocyte maturation (Memili and First 2000). It was reported that there was a concomitant decrease in total RNA synthesis once the oocyte diameter increased beyond 110 μm and that RNA synthesis ceased in oocytes ≥120 μm in diameter (Fair *et al.* 1996). These reports may indicate that lack of some types of RNA in IVG oocytes results in low cleavage rates because the diameters of IVG oocytes were between 110 and

120 µm. However, the cleavage rate in IVG oocytes with pre-IVM was improved compared with that without pre-IVM. This may indicate that IVG oocytes store some RNAs/proteins during pre-IVM to support subsequent cleavage and development. Under the present IVG system, cleaved IVG oocytes with pre-IVM showed high developmental competence similar to that of *in vivo*-grown oocytes, although oocytes derived from early antral follicles could not grow to more than 120 µm in diameter. A previous study reported that bovine *in vivo*-grown oocytes with a diameter around 115 µm could not acquire full nuclear and/or cytoplasmic maturation (Otoi *et al.* 1997). In contrast, it was reported that IVG oocytes in mouse exhibited similar developmental competence to *in vivo*-grown oocytes, even if the mean diameter of the IVG oocytes (around 70 µm) was smaller than that of the *in vivo*-grown oocytes (around 80 µm) (Eppig and O'Brien 1998). The present results suggest that cleaved IVG oocytes with pre-IVM acquire similar developmental competence to *in vivo*-grown oocytes, even at around 115 µm in diameter.

In conclusion, pre-IVM improved the nuclear maturational and developmental competences of IVG oocytes, probably due to promotion of their chromatin transition and synchronization of meiotic progression. The present culture system achieved a higher nuclear maturation rate of IVG oocytes, but low fertilization and cleavage rates. This may be due to sub-optimal conditions, leading to insufficient cytoplasmic maturation of IVG oocytes. In further study, the cytoplasmic status of IVG oocytes during pre-IVM culture should be investigated.

Summary

The objective of this chapter was to clarify the effects of pre-IVM supplemented with IBMX on nuclear and cytoplasmic maturation of bovine IVG oocytes. In Experiment 2-1, 12-day IVG oocytes with a normal appearance were subjected to examinations of diameter and chromatin structure in the GV before IVM. In addition, percentages of M II were examined after IVM. Regardless of pre-IVM, the mean diameters of IVG oocytes were about 115 μm. The proportions of GV 3 and M II stages of IVG oocytes with pre-IVM were higher than those without pre-IVM ($P < 0.05$). In Experiment 2-2, the fertilizability and developmental competence of IVG oocytes were examined. Regardless of pre-IVM, the normal fertilization rates of IVG oocytes were similar (around 70 %) but were lower than that of *in vivo*-grown oocytes ($P < 0.05$). Cleavage and blastocyst rates of IVG oocytes with pre-IVM were higher than those without pre-IVM ($P < 0.05$). The blastocyst rate based on cleaved IVG oocytes with pre-IVM was similar to that of *in vivo*-grown oocytes, although the cleavage rate of IVG oocytes with pre-IVM was lower than that of *in vivo*-grown oocytes ($P < 0.05$). In conclusion, pre-IVM with IBMX improved the maturational and developmental competences of IVG oocytes, probably due to promotion of their chromatin transition and synchronization of meiotic progression.

Chapter III

Mitochondrial activity during pre-maturational culture in bovine IVG oocytes related to maturational and developmental competences

Introduction

In the previous chapters, the maturational and the developmental competences of IVG oocytes were improved by pre-IVM for 20 h in medium supplemented with IBMX compared to those without pre-IVM, and the stage of GV in most IVG oocytes were synchronized from GV 1 to GV 2 or 3 during pre-IVM culture. From the results, the author speculates that the improvement of maturational and developmental competences of IVG oocytes is probably caused by synchronization of meiotic progression. It was also reported that *in vivo*-grown bovine oocytes with 18 to 21 h pre-IVM showed higher developmental competence to blastocysts than that without pre-IVM (Hashimoto *et al.* 2002). However, their results showed higher developmental competence of oocytes with pre-IVM compared to that without pre-IVM, regardless of the synchronization of meiotic progression. Hashimoto *et al.* (2002) concluded that immature oocytes required time to acquire developmental competence during meiotic arrest, and speculated that translation or posttranslational modification of proteins or transcription of mRNAs itself might increase in oocytes during the period. Therefore, it is necessary to investigate the cytoplasmic changes in IVG oocytes during pre-IVM culture.

Mitochondria play important roles in oocyte maturation and subsequent embryonic development by providing a steady source of adenosine trisphosphate (ATP) through oxidative phosphorylation (Nagano *et al.* 2006; Tarazona *et al.* 2006; Van Blerkom 2011). In mammalian oocytes, the mitochondrial activity increased with follicular development (Kanaya *et al.* 2007; Machatkova *et al.* 2012), and the higher mitochondrial activity before the resumption of meiosis was associated with greater meiotic competence (Egerszegi *et al.* 2010; Machatkova *et al.* 2012) and higher developmental competence to blastocysts (Kanaya *et al.* 2007). Therefore, it is speculated that the mitochondrial activity in IVG oocytes increases during pre-IVM culture. On the other hand, it was reported that reactive oxygen species (ROS) such as hydroxyl radical, hydrogen peroxide was generated by the mitochondrial respiratory chain (Fleury *et al.* 2002) and ROS suppressed the developmental competence of oocytes (Hashimoto *et al.* 2000).

In this chapter, for clarifying the relationship between maturational/developmental competences of IVG oocytes and mitochondrial activity, the mitochondrial activity and ROS generation in IVG oocytes during pre-IVM culture were evaluated. Then the competences of nuclear maturation and development to blastocysts in IVG oocytes with different duration of pre-IVM were examined. Moreover, to confirm the developmental ability of IVG oocytes beyond blastocyst stage, the author also attempted the embryos transfer using IVG oocytes with 10 h pre-IVM.

Materials and Methods

Collection of early antral follicles and IVG culture of oocyte-granulosa cell complexes

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 their collection as described in chapters I and II. The OCGCs with normal appearance were cultured individually in a 96-well culture plate (Falcon 353872, Becton, Dickinson and company, Franklin Lakes, NJ, USA) with 200 µl of the growth medium for 12 days at 39°C in humidified air with 5% CO_2 as described in chapters I and II. The growth medium was HEPES-buffered TCM-199 (Cat #12340-030, Invitrogen) supplemented with 0.91 mM sodium pyruvate, 1 µg/ml estradiol-17β, 5% FCS (Invitrogen), 4 mM hypoxanthine, 4% polyvinylpyrrolidone (MW 360,000), and 50 µg/ml gentamicin sulfate. Every 4 days of IVG culture, half (100 µl) of the growth medium was replaced with the same amount of fresh medium.

Pre-IVM and IVM of IVG oocytes

After 12-day IVG culture, oocytes surrounded by several layers of cumulus cells were collected from morphologically normal OCGCs and submitted to IVM with or without pre-IVM as described in chapter II. Briefly, pre-IVM culture of IVG oocytes was performed as they were cultured individually in each well of micro-well plates (Mini Trays 163118, NUNC, Roskilde, Denmark) filled with 6 ml of HEPES-buffered TCM-199 (Cat #12340-030) supplemented with 0.2 mM sodium pyruvate, 2×10^{-6} units/ml FSH (from porcine pituitary), 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 for 0, 10 or 20 h. For IVM, oocytes were cultured individually in each well of micro-well plates filled with 6 ml of IVM medium, which was HEPES-buffered TCM-199 supplemented with 0.2 mM sodium pyruvate, 0.02 units/ml FSH, 1 μg/ml estradiol-17β, 10% FCS and 50 μg/ml gentamicin sulfate, at 39°C under 5% CO_2 in air for 22 h (Nagano *et al.* 2013).

Oocytes collected from antral follicles of 2 to 8 mm in diameter (*in vivo*-grown oocytes) served as controls. IVM of *in vivo*-grown oocytes was conducted as described previously (Takahashi *et al.* 1996). In brief, COCs were incubated in droplets of IVM medium that was the same as used for IVG oocytes (about 10 COCs/50 μl) covered with paraffin oil at 39°C under 5% CO_2 in air for 22 h.

IVF and IVC of inseminated oocytes

IVF (day 0) was performed using frozen semen according to a procedure described previously (Takahashi and Kanagawa 1998) with slight modification. In brief, motile sperm (5 × 10^6 sperm/ml) separated from thawed semen by a Percoll gradient (45 and 90%) were co-incubated with COCs in a 100-μl droplet (about 10 COCs/droplet) of modified Brackett & Oliphant isotonic medium (Brackett and Oliphant 1975) containing 3 mg/ml fatty-acid-free BSA and 2.5 mM theophylline (Takahashi and First 1992) for 18 h at 39°C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2.

IVC of inseminated oocytes (presumptive zygotes) was performed as described

previously (Takahashi and Kanagawa 1998). Briefly, after co-incubation with sperm, presumptive zygotes were freed from cumulus cells by vortexing and washing three times in the culture medium. Cumulus-free zygotes were cultured for about 150 h in a 30-μl droplet of the culture medium at 39°C under 5% CO_2, 5% O_2 and 90% N_2. The culture medium was a 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, 5 mM glycine, 5 mM taurine, 1 mM glucose and 3 mg/ml fatty-acid-free BSA instead of polyvinyl alcohol (Takahashi and Kanagawa 1998). After 30 h (day 2) and about 150 h (day 7) of IVC, cleavage and development of presumptive zygotes to the blastocyst stage were assessed, respectively. Total cell numbers of blastocysts obtained after about 150 h of IVC were counted using an air-drying method (Takahashi and First 1992).

Evaluation of oocyte nuclear status

After IVM, oocytes were denuded from cumulus cells by vortexing. Denuded oocytes were stained with 1% aceto-orcein and examined for their nuclear status as described elsewhere (Nagano *et al.* 2006). Nuclear statuses were divided into GV, GVBD, chromosomal condensation (CC), M I, A I/T I and M II by observation under a phase contrast microscope.

Evaluation of mitochondrial activity in IVG oocytes during pre-IVM culture

IVG oocytes after pre-IVM were treated with 500 IU/ml hyaluronidase in DPBS for 10 min

and then the oocytes were denuded from cumulus cells by repeat pipetting with a fine pipette. Denuded oocytes were incubated for 15 min in the dark at 37°C in DPBS supplemented with 1 μM 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine iodide (JC-1) and 10% FCS as described previously (Ge *et al.* 2012) with slight modification. Images of oocytes were acquired using digital fluorescence microscope (BZ-9000, Keyence, Osaka, Japan) and the mean fluorescence intensity of the images was calculated by analysis software (BZ-H2A, Keyence). Membrane potential of mitochondria was calculated as the ratio of florescence intensity of activated mitochondria, expressed as red fluorescence of JC-1 staining, to less-activated mitochondria, expressed as green fluorescence of JC-1 staining (Δψm, red/green fluorescent intensity).

Assessment of ROS generation in IVG oocytes during pre-IVM culture

IVG oocytes after pre-IVM were denuded from cumulus cells by vortexing. Denuded oocytes were incubated for 15 min in the dark at 37°C in DPBS supplemented with 10 μM 2', 7'-dichlorodihydrofuorescein diacetate (DCHFDA) and 10% FCS as described previously (Hashimoto *et al.* 2000) with slight modification. Images of oocytes were acquired using digital fluorescence microscope and the mean fluorescence intensity of the images was calculated by analysis software. ROS in oocytes was defined as mean fluorescence intensity of oocytes.

Experimental design

Experiment 3-1

After 0, 10 and 20 h pre-IVM, a total of 83 IVG oocytes derived from 129 OCGCs were used for the evaluation of mitochondrial activity in oocytes (6 to 7 oocytes/replicate). Also the ROS generation in IVG oocytes was assessed during pre-IVM culture using a total of 61 IVG oocytes derived from 94 OCGCs (6 to 7 oocytes/replicate).

Experiment 3-2

To examine relationship between the mitochondrial activity and the maturational and developmental competences of IVG oocytes with different pre-IVM duration, a total of 419 IVG oocytes derived from 635 OCGCs (10 to 30 oocytes/replicate) were subjected to IVM with 0, 10 or 20 h pre-IVM culture. After 10 and 22 h IVM culture, their nuclear statuses were investigated. Before the examination of nuclear status, some of IVG oocytes after 22 h IVM (with 0, 10 and 20 h per-IVM: n=53, 71 and 37, respectively) were provided to the measurement of their diameter as described in chapters I and II. IVG oocytes with 10 or 20 h pre-IVM were subjected to IVM/IVF/IVC and examined their cleavage, development to the blastocyst stage and cell numbers in blastocysts using a total of 357 IVG oocytes derived from 503 OCGCs (25 to 40 oocytes/replicate). *In vivo*-grown oocytes after IVM served as controls.

Experiment 3-3

For evaluating the embryonic development beyond the blastocyst stage, eleven fresh blastocysts (day 7) obtained after IVC were transferred by transcervically to a recipient heifer

synchronized at day 8 of estrus by CIDR synch (Katagiri and Takahashi 2006). Transcervical uterine flushing using two-way foley catheter (Fujihira Industry, Tokyo, Japan) was performed at 16 days of estrus for embryo collection, and collected embryos were evaluated their developmental stage. In addition, two blastocysts were also transferred to another heifer synchronized by same protocol, and her pregnancy was diagnosed at 29 and 62 days of estrus by ultrasonography (5 MHz, HS101V, Honda electronics, Tokyo, Japan).

Statistical analysis

All data were analyzed by one-way ANOVA followed by Tukey-Kramer's HSD test. All statistical analyses were performed using software (JMP version 10, SAS Institute, Cary, NC, USA).

Results

Experiment 3-1

Mitochondrial activity in IVG oocytes at 10 h pre-IVM was higher than those at 0 and 20 h pre-IVM ($P < 0.05$) (Fig. 3-1). ROS in IVG oocytes with 0 h pre-IVM was higher than those at 10 and 20 h pre-IVM ($P < 0.05$) (Fig. 3-2).

Experiment 3-2

After 10 h IVM, M I rate (82.7%) of IVG oocytes with 10 h pre-IVM tended to be higher

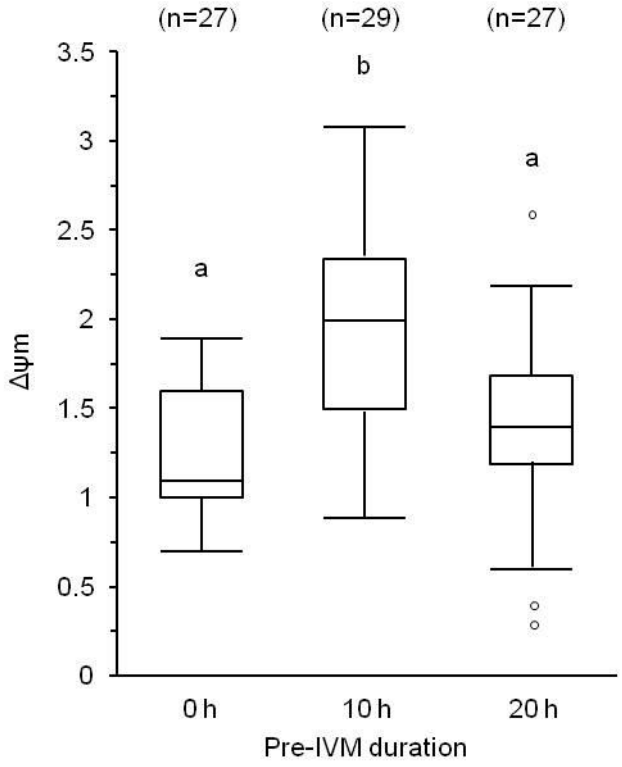
than that with 0 h pre-IVM (48.3%, P = 0.08) and their GVBD/CC rate (1.5%) tended to be lower than those with 0 h (37.3%, P = 0.09) and 20 h pre-IVM (34.2%, P = 0.12) (Table 3-1). After 22 h IVM, M II rates of IVG oocytes with 10 h (91.5%) and 20 h pre-IVM (79.0%) were higher than that with 0 h pre-IVM (50.5%, P < 0.05), and similar to that of *in vivo*-grown oocytes (88.6%) (Table 3-2). However, the percentage of M I stage were significantly higher in IVG oocytes with 20 h pre-IVM (19.8%) than 10 h pre-IVM (5.5%, P < 0.05). The mean diameter of IVG oocytes after 22 h IVM was 115.2 ± 3.4 μm (range: 105.9 to 122.7 μm, n = 161).

As shown in Table 3-3, cleavage (79.4%) and blastocyst rates (38.9%) of IVG oocytes with 10 h pre-IVM were higher than those with 20 h pre-IVM (63.0 and 25.8%, respectively; P < 0.05), and similar to those of *in vivo*-grown oocytes (82.7 and 36.7%, respectively). However, blastocyst rates based on cleaved oocytes were similar regardless of pre-IVM duration. Also total cell numbers in blastocysts in IVG oocytes with 10 h pre-IVM was similar to that of the control (P = 0.38).

Experiment 3-3

The seven embryos (63.6%, 7/11) were recovered from recipient heifer at 16 days of estrus. The six embryos were elongated ranging from 40 to 100 mm (70.0 ± 21.9 mm) (Fig. 3-3), and the one showed retardation in the length (30 mm). Another heifer served embryo transfer was confirmed as pregnant by detection of an embryo (50%, 1/2) with heartbeat on 29 and 62 days of estrus.

A



B

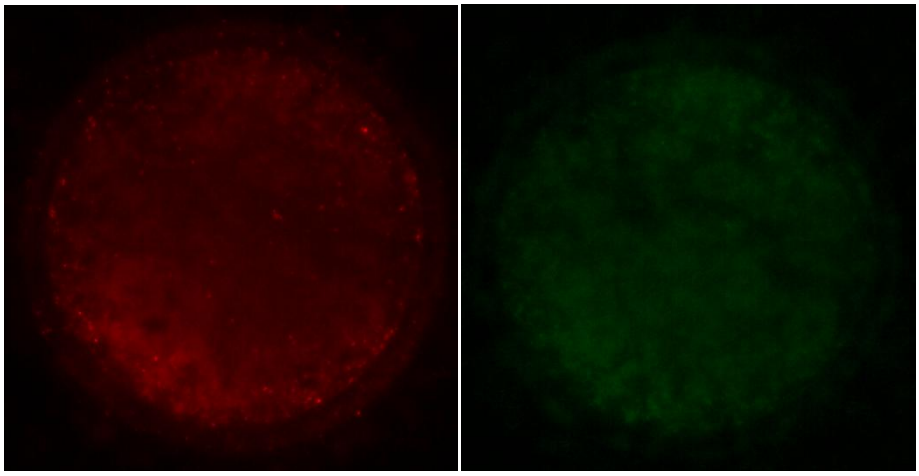


Fig. 3-1. Mitochondrial activity (Δψm) of IVG oocytes at different pre-IVM duration (A).
The ratio of red to green fluorescence was quantified as an indicator of mitochondrial activity (B).
Lines of the boxes delineate the 25th, 50th, and 75th percentiles and the whiskers depict the 10th and 90th percentiles of a population. The circles represent the outliers.
[a, b] Different characters means significant difference ($P < 0.05$).
Numbers in parentheses are the number of IVG oocytes used (5 replicates).

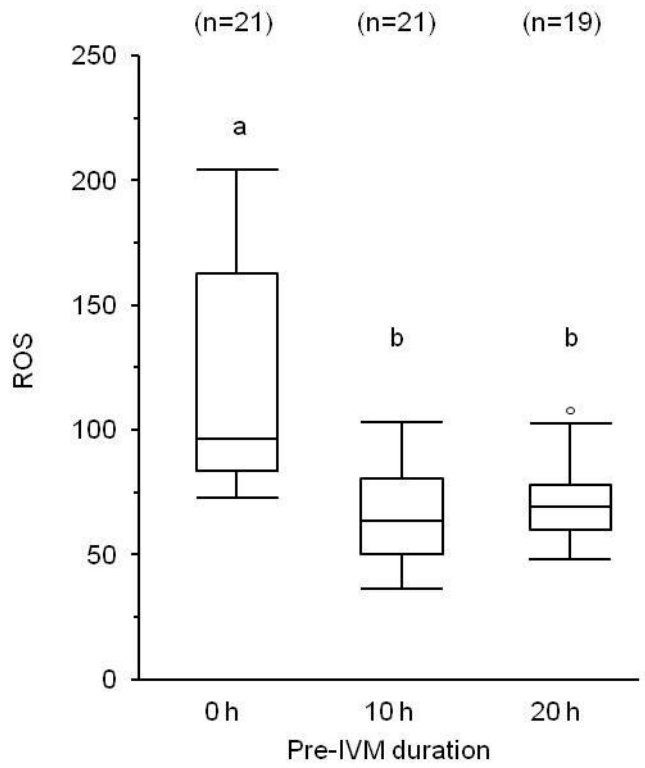


Fig. 3-2. Reactive oxygen species (ROS) in IVG oocytes after different pre-IVM duration. Lines of the boxes delineate the 25th, 50th, and 75th percentiles and the whiskers depict the 10th and 90th percentiles of a population. The circles represent the outliers.

[a,b] Different characters means significant difference ($P < 0.05$).

Numbers in parentheses are the number of IVG oocytes used (3 replicates).

Fig. 3-3. The embryo (100 mm) recovered from cattle at 16 days of estrus was elongated. Scale bars represent 5 mm.

Table 3-1. Effect of pre-IVM duration on meiotic resumption of IVG oocytes after 10 h IVM

Pre-IVM duration (h)	No. of IVM (replicates)	% of oocytes at each status				
		GV	GVBD/CC	M I	M II	Deg
0	33 (3)	14.4 ± 15.5	37.3 ± 27.7	48.3 ± 20.2	0	0
10	49 (3)	13.5 ± 13.7	1.5 ± 2.6	82.7 ± 16.1	0	2.2 ± 3.9
20	30 (3)	7.5 ± 6.6	34.2 ± 8.0	60.0 ± 8.7	2.7 ± 4.8	0
Control*	40 (3)	0	14.0 ± 13.4	86.0 ± 13.4	0	0

*The oocytes collected from antral follicles (2 to 8 mm in diameter) served as *in vivo*-grown controls.

GV, germinal vesicle; GVBD/CC, germinal vesicle breakdown/chromosomal condensation; M I, metaphase I; M II, metaphase II; Deg, degenerate.

Table 3-2. Effect of pre-IVM duration on nuclear maturation of IVG oocytes after 22 h IVM

Pre-IVM duration (h)	No. of IVM (replicates)	% of oocytes at each status				
		GV	GVBD	M I	A I/TI	M II
0	94 (5)	5.0 ± 7.2	3.9 ± 3.9	25.4 ± 7.2^{a}	14.2 ± 10.7	50.5 ± 10.1^{a}
10	117 (5)	0	0	5.5 ± 5.2^{c}	3.8 ± 4.8	91.5 ± 2.3^{b}
20	96 (5)	1.3 ± 2.8	0	19.8 ± 12.6^{ab}	0	79.0 ± 12.0^{b}
Control*	103 (5)	0	0	9.6 ± 2.7^{bc}	1.8 ± 2.5	88.6 ± 2.6^{b}

$^{a-c}$ Values (mean ± SD) with different superscripts within columns are significantly different ($P < 0.05$).

*The oocytes collected from antral follicles (2 to 8 mm in diameter) served as *in vivo*-grown controls.

GV, germinal vesicle; GVBD, germinal vesicle breakdown; M I, metaphase I; A I/T I, anaphase I/telophase I; M II, metaphase II.

Table 3-3. Effect of pre-IVM duration on embryonic development of IVG oocytes

Pre-IVM duration (h)	No. of oocytes (replicates)	% of cleaved oocytes	% of blastocysts based on		Cell no. in blastocysts (n)
			Inseminated oocytes	Cleaved oocytes	
10	164 (5)	79.4 ± 2.4[a]	38.9 ± 4.2[a]	49.0 ± 5.0	141 ± 62 (49**)
20	193 (5)	63.0 ± 5.6[b]	25.8 ± 7.3[b]	41.2 ± 12.0	135 ± 59 (51)
Control*	150 (5)	82.7 ± 7.6[a]	36.7 ± 3.4[a]	44.7 ± 6.4	156 ± 55 (55)

[a,b] Values (mean ± SD) with different superscripts within columns are significantly different (P < 0.05).

*The oocytes collected from antral follicles (2 to 8 mm in diameter) served as *in vivo*-grown controls.

**Total cell numbers in blastocysts were evaluated in four replicates, because, some of blastocysts after IVC were transferred.

Discussion

In the present study, mitochondrial activity peaked at 10 h pre-IVM in IVG oocytes, which showed higher maturational and developmental competences to blastocysts. Furthermore, most (more than 80%) of IVG oocytes with 10 h pre-IVM and *in vivo*-grown oocytes reached M I stage after 10 h IVM culture, and around 90% of them reached M II stage after 22 h IVM culture, indicating that nuclear maturation of IVG oocytes were enhanced and synchronized by 10 h pre-IVM. It was reported that reducing nuclear asynchrony during IVM by temporal meiotic arrest using dibutyryl cyclic AMP (dbcAMP) appeared to enhance nuclear maturation of porcine oocytes, and it was speculated that the improvement was caused by enhancement of cytoplasmic maturation during temporal meiotic arrest (Somfai *et al.* 2003). In the present study, the author used IBMX instead of dbcAMP for arresting meiotic resumption, and IBMX prevented the decrease of cAMP in bovine oocytes (Bilodeau *et al.* 1993). The cAMP level in IVG oocytes might be elevated by 10 h pre-IVM, which increased the mitochondrial activity and resulted in the nuclear maturation improvement compared to those by 0 or 20 h pre-IVM.

It was reported that bovine oocytes derived from larger follicles (> 6 mm in diameter) showed a higher proportion of blastocyst development (Lonergan *et al.* 1994) and higher mitochondrial activity (Machatkova *et al.* 2012) than those from small follicles (< 6 mm in diameter). These reports suggest that mitochondrial activity in bovine oocytes increases during follicular development and that higher mitochondrial activity is associated with greater developmental competence. The present pre-IVM culture system may have mimicked

follicular development in the aspect of the dynamics of mitochondrial activity. In the present study, the author added FSH and IBMX to pre-IVM medium. FSH can increases cAMP concentration in cumulus-enclosed mouse oocytes (Webb *et al.* 2002) and IBMX maintains intra-oocyte cAMP levels (Bilodeau *et al.* 1993) in bovine oocytes. Also, cAMP can regulate mitochondrial activity (Papa 2006; Carlucci *et al.* 2008). It was reported that cAMP concentration in bovine *in vivo*-grown oocytes increased and blastocyst development became high by addition of IBMX and forskolin in pre-IVM culture medium (Albuz *et al.* 2010). Forskolin, adenylate cyclase activator, potently increases intra-oocyte cAMP level like as FSH (Thomas *et al.* 2002). The present results suggest that mitochondrial activity was improved by the increase of cAMP concentration in IVG oocytes until 10 h pre-IVM, and the developmental competence of IVG oocytes increased. However, the blastocyst development of IVG oocytes was decreased by extending pre-IVM duration to 20 h. In further study, it is necessary to investigate cAMP production during pre-IVM culture.

It was reported that mitochondrial activity and ATP content in mouse oocytes decreased by ROS and subsequent disruption of the spindle in M II oocytes occurred (Zhang *et al.* 2006). In addition, it was speculated that the developmental competence of rabbit IVG oocytes decreased when mitochondrial activity was impaired by ROS (Kanaya *et al.* 2007). In the present study, there was no significant difference between ROS in IVG oocytes with 10 and 20 h pre-IVM although the mitochondrial activity in IVG oocytes with 20 h pre-IVM was decreased. The result indicates that low mitochondrial activity in IVG oocytes with 20 h pre-IVM is not probably due to ROS production. The mitochondrial function related to not only high

production of ROS, but also some other factors such as Ca^{2+} overload or over expression of the pro-apoptotic Bcl-2 proteins, which can result in release of cytochrome-c and activate the caspase pathway leading to apoptosis and cell death (Belizario *et al.* 2007). Therefore, the study about apoptotic program induction during pre-IVM culture should be performed in further study.

Following embryo transfer of blastocysts derived from IVG oocytes with 10 h pre-IVM, 6 out of 7 elongated embryos showed normal length (40 to 100 mm) similar to *in vivo*-derived bovine embryos (Betteridge *et al.* 1980; Gustafsson and Ploen 1986) and 1 out of 2 embryos maintained pregnancy. These results suggest that IVG oocytes with 10 h pre-IVM acquired full developmental competence as same as *in vivo*-grown/IVM oocytes and probably *in vivo*-grown/*in vivo*-matured oocytes. On the other hand, mean diameter of IVG oocytes was 115 μm consistent with previous chapters and smaller than *in vivo*-grown oocytes (more than 120 μm). A previous study reported that bovine *in vivo*-grown oocytes around 115 μm in diameter could not acquire full nuclear and/or cytoplasmic maturation (Otoi *et al.* 1997). However, it is reported that IVG oocytes in mouse exhibited similar developmental competence to *in vivo*-grown oocytes, even if the mean diameter of IVG oocytes (around 70 μm) was smaller than that of *in vivo*-grown oocytes (around 80 μm) (Eppig and O'Brien 1998). These results suggest that the developmental competence of IVG oocytes cannot be evaluated only by oocyte diameter.

In conclusion, the author clarified that mitochondrial activity in bovine IVG oocytes was peaked at 10 h pre-IVM culture with IBMX, and closely correlated with the nuclear maturation

and developmental competence of IVG oocytes. The IVG oocytes with 10 h pre-IVM can acquire high maturational and developmental competences as same as *in vivo*-grown oocytes. However, the extension of pre-IVM duration to 20 h reduced the mitochondrial activity and the developmental competence of IVG oocytes.

Summary

The objective of this chapter was to investigate the dynamics of mitochondrial activity in bovine IVG oocytes during pre-IVM culture and the developmental competence of those subjected to different pre-IVM duration. After 12-day IVG culture, oocytes were cultured for 0, 10 or 20 h with IBMX as pre-IVM. Mitochondrial activity in IVG oocytes after 10 h pre-IVM was highest in all pre-IVM duration ($P < 0.05$). In addition, cleavage and blastocyst rates of embryos derived from IVG oocytes with 10 h pre-IVM were higher than those with 20 h pre-IVM ($P < 0.05$), and similar to those of *in vivo*-grown oocytes. These results demonstrate that IVG oocytes with 10 h pre-IVM have higher developmental competence to blastocysts associated with the increase of mitochondrial activity in IVG oocytes. To confirm the developmental ability of IVG oocytes with 10 h pre-IVM to grow beyond blastocyst stage *in vivo*, embryo transfer was attempted. Transferred embryos developed to elongated embryonic stage (63.6%, 7/11) in the recipient uterus at day 16 of estrus. One pregnancy was confirmed at day 62 (50%, 1/2). In conclusion, IVG oocytes with 10 h pre-IVM can acquire high developmental competence of IVG oocytes as same as *in vivo*-grown oocytes.

Summary and Conclusions

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. However, the developmental competence to transferable embryos of these oocytes is low. 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*. For improving the bovine IVG system, the present studies were conducted to use culture system supplemented with phosphodiesterase inhibitor (3-isobutyl-1-methylxanthine, IBMX) immediately before IVM (pre-IVM) and to clarify the optimal duration of IVG culture and the effects of pre-IVM with IBMX on nuclear and cytoplasmic maturation of IVG oocytes.

In chapter I, the viability and growth of oocytes after different IVG durations (10, 12 and 14 days) were examined. Nuclear maturation (metaphase II, M II) of oocytes with normal appearance obtained at 10-, 12- and 14-day IVG was also examined after IVM with or without pre-IVM culture. The viability of oocytes after 14-day IVG was lower than those after 10- and 12-day IVG ($P < 0.05$). The mean diameters of 12- and 14-day IVG oocytes were larger than that of 10-day IVG oocytes ($P < 0.05$). M II rate of 10-day IVG oocytes was lower than those of 12- and 14-day IVG oocytes, regardless of pre-IVM culture ($P < 0.05$). M II rates of 12- and 14-day IVG oocytes with pre-IVM were higher than those without pre-IVM ($P < 0.05$). After 12- and 14-day IVG, the developmental competence and nuclear status of IVG oocytes as well

as the membrane integrity of cumulus cells were examined. After being subjected to pre-IVM and IVM, the blastocyst rate of 12-day IVG oocytes was higher than that of 14-day IVG oocytes ($P < 0.05$), but was lower than that of *in vivo*-grown oocytes ($P < 0.05$). The percentage of oocytes arrested at the germinal vesicle (GV) stage derived from 12-day IVG was higher than that from 14-day IVG immediately after pre-IVM culture ($P < 0.05$). The proportion of 12-day IVG oocytes having cumulus cells with intact membrane was also higher than that of 14-day IVG oocytes ($P < 0.05$). These results demonstrate that 12-day IVG culture is suitable for oocyte growth, and prolonging IVG culture reduces the integrity of cumulus cells, and the viability and developmental ability of IVG oocytes.

In chapter II, 12-day IVG oocytes with normal appearance were subjected to examinations of diameter and GV structure before IVM. In addition, percentages of M II were examined after IVM. Regardless of pre-IVM, the mean diameters of IVG oocytes were about 115 μm. The proportions of GV 3 and M II stages of IVG oocytes with pre-IVM were higher than those without pre-IVM ($P < 0.05$). The fertilizability and developmental competence of IVG oocytes were examined. Regardless of pre-IVM, normal fertilization rates of IVG oocytes were similar, but were lower than that of *in vivo*-grown oocytes ($P < 0.05$). Cleavage and blastocyst rates of IVG oocytes with pre-IVM were higher than those without pre-IVM ($P < 0.05$). The blastocyst rate based on cleaved IVG oocytes with pre-IVM was similar to that of *in vivo*-grown oocytes, although the cleavage rate of IVG oocytes with pre-IVM was lower than that of *in vivo*-grown oocytes. These results demonstrate that pre-IVM with IBMX improved the maturational and developmental competences of IVG oocytes, probably due to promotion of

their chromatin transition and synchronization of meiotic progression.

In chapter III, mitochondrial activity in IVG oocytes were investigated at different pre-IVM duration. Moreover, meiotic progression and development to blastocysts of IVG oocytes were examined. Mitochondrial activity in IVG oocytes at 10 h pre-IVM was higher than those at 0 and 20 h pre-IVM ($P < 0.05$). After 10 h IVM, M I rate of IVG oocytes with 10 h pre-IVM tended to be higher than that with 0 h ($P = 0.08$), and similar to that of *in vivo*-grown oocytes. After 22 h IVM, M II rate of IVG oocytes with 10 h pre-IVM was higher than those with 0 h and 20 h pre-IVM ($P < 0.05$), and similar to that of *in vivo*-grown oocytes. Cleavage and blastocyst rates of IVG oocytes with 10 h pre-IVM were higher than those with 20 h pre-IVM ($P < 0.05$), and similar to those of *in vivo*-grown oocytes. These results demonstrate that IVG oocytes with 10 h pre-IVM showed higher development to blastocysts associated with the increase of mitochondrial activity in IVG oocytes. To confirm the developmental ability of IVG oocytes with 10 h pre-IVM beyond blastocyst stage, embryo transfer was attempted. Transferred embryos developed to elongated embryonic stage (63.6%, 7/11) in the recipient uterus at day 16 of estrus. One pregnancy was confirmed at day 62 (50%, 1/2).

In conclusion, the present studies demonstrate that 12 days of IVG followed by 10 h pre-IVM culture with IBMX is considered as the optimal IVG system for bovine growing oocytes derived from early antral follicles, and that this IVG system can produce the oocytes having the high maturational and developmental competences as same as *in vivo*-grown oocytes.

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