



HOKKAIDO UNIVERSITY

Title	Studies on the aging-related changes in bovine oocytes during in vitro maturation
Author(s)	古山, 敬祐
Degree Grantor	北海道大学
Degree Name	博士(獣医学)
Dissertation Number	甲第11277号
Issue Date	2014-03-25
DOI	https://doi.org/10.14943/doctoral.k11277
Doc URL	https://hdl.handle.net/2115/56177
Type	doctoral thesis
File Information	Keisuke_Koyama.pdf



**Studies on the aging-related changes in bovine oocytes
during *in vitro* maturation**

(体外成熟培養における牛卵子の老化に関する研究)

Keisuke KOYAMA

Abbreviations

AI: artificial insemination

ART: assisted reproductive technology

ATP: adenosine triphosphate

A-I: anaphase I

BSA: bovine serum albumin

COCs: cumulus oocyte complexes

DCF: 2',7'-dichlorofluorescein diacetate

DCHFDA: 2',7'-dichlorodihydrofluorescein diacetate

DPBS: Dulbecco's phosphate-buffered saline

EGA: embryonic genome activation

ET: embryo transfer

FCS: fetal calf serum

FSH: follicle stimulating hormone

GAGs: glycosaminoglycans

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

hpi: hour post insemination

IVC: *in vitro* culture

IVF: *in vitro* fertilization

IVM: *in vitro* maturation

IVP: *in vitro* production

MPF: maturation-promoting factor

M-I: metaphase I

M-II: metaphase II

OPU: ovum pick-up

PN: pronucleus

ROS: reactive oxygen species

SD: standard deviation

TCM: tissue culture medium

T-I: telophase I

Contents

Preface	1
Chapter 1	
Estimation of the optimal timing of fertilization for embryonic development of <i>in vitro</i>-matured bovine oocytes based on the times of nuclear maturation and sperm penetration	
Introduction	3
Materials and Methods	5
Results	9
Discussion	11
Summary	18
Chapter 2	
Aging-related changes in <i>in vitro</i>-matured bovine oocytes: oxidative stress, mitochondrial activity and ATP content after nuclear maturation	
Introduction	19
Materials and Methods	21
Results	27
Discussion	29
Summary	38
Summary and Conclusion	39
Acknowledgements	41
References	42
Summary in Japanese	51

Preface

During the past three decades, marked advances have been made in the field of assisted reproductive technology (ART). In livestock production, ART has already become imperative technology: *in vitro* production (IVP), embryo transfer (ET), ovum pick-up (OPU) and artificial insemination (AI). *In vitro* maturation (IVM), one of the ARTs, is the technology that produces the fertile oocytes from immature oocytes. In many species, the offspring derived from *in vitro*-matured oocytes have been produced (mouse: Schroeder and Eppig 1984, cattle: Critser *et al.* 1986; Hanada *et al.* 1986, sheep: Crozet *et al.* 1987, pig: Mattioli *et al.* 1989, human: Cha *et al.* 1991). In cattle, the productive efficiency of blastocysts from oocytes subjected to IVM has been improved throughout the last decades, and IVM results in about 40% of bovine oocytes subjected to IVM developing to the blastocyst stage (Lonergan and Fair 2014; Wrenzycki and Stinshoff 2013). However, the developmental competence of *in vitro*-matured bovine oocytes is still lower than that of *in vivo*-matured bovine oocytes (Rizos *et al.* 2002). Therefore, further researches for IVM of bovine oocytes are required for additional improvement of IVM systems.

Nuclear and cytoplasmic maturation is essential for oocytes to acquire developmental competence during IVM (Eppig 1996; Ferreira *et al.* 2009). Nuclear maturation is defined as a meiotic progression from germinal vesicle to metaphase II (M-II). On the other hand, cytoplasmic maturation including a variety of processes occurs within the cytoplasm of the oocytes: the redistribution of cytoplasmic organelles, and the storage of mRNA, proteins and transcription factors (Ferreira *et al.* 2009). After the completion of nuclear maturation, oocytes gradually accomplish cytoplasmic maturation and acquire developmental competence. Then, they maintain that competences of fertilization and embryonic development for a certain period, but eventually undergo deterioration in their quality and lose developmental competence. This phenomenon is called

“oocyte aging” (Miao *et al.* 2009). It is thought that the oocyte aging is one of the factors causing the low efficiency of blastocyst yield *in vitro* (Agung *et al.* 2006; Long *et al.* 1994; Rispoli *et al.* 2011; Ward *et al.* 2002). In humans and rodents, oocyte aging is defined as numerous morphological and cellular alterations (reviewed in Lord and Aitken 2013; Miao *et al.* 2009). Moreover, recent studies on murine oocytes have indicated the possibility that oxidative stress acts as a trigger for a cascade of several events associated with oocyte aging and one of the aging-related changes caused by oxidative stress is mitochondrial dysfunction (Lord and Aitken 2013). However, there are few data on aging-related changes in bovine oocytes and even the timing of aging of bovine oocytes during IVM culture is unclear. Therefore, research on aging in bovine oocytes will contribute to the development of methods for preventing aging in *in vitro*-matured bovine oocytes, and eventually to the improvement of ART efficiency. Moreover, it has been proposed that bovine oocytes may be a better model than murine for assessing the risks of human ART (Leidenfrost *et al.* 2011; Malhi *et al.* 2005). This is because important similarities to humans include the emergence of follicular waves, the number of waves during the menstrual/estrous cycle, selection of a dominant follicle and ovulation of a single follicle (Malhi *et al.* 2005).

Therefore, the author focused on clarifying the aging-related changes in bovine oocytes during IVM in this study. In chapter 1, to estimate the timings when bovine oocytes acquire and lose their developmental competence during IVM culture, the embryonic development of *in vitro*-matured bovine oocytes based on the times of nuclear maturation and sperm penetration was investigated. In chapter 2, aging-related changes in *in vitro*-matured bovine oocytes were investigated, especially focusing on the functions of mitochondria during IVM culture and early embryonic development.

Chapter 1

Estimation of the optimal timing of fertilization for embryonic development of *in vitro*-matured bovine oocytes based on the times of nuclear maturation and sperm penetration

Introduction

Nuclear and cytoplasmic maturation is essential for oocytes to acquire developmental competence (Eppig 1996; Ferreira *et al.* 2009). After the completion of nuclear maturation, oocytes gradually accomplish cytoplasmic maturation and acquire developmental competence. This competence is maintained for a certain period, but oocytes eventually undergoes deterioration in quality and lose developmental competence, which is called “oocyte aging” (Miao *et al.* 2009). However, the optimal timing for fertilization to achieve proper embryonic development of *in vitro*-matured bovine oocytes remains unclear, and the developmental competence of *in vitro*-matured bovine oocytes is lower than that of *in vivo*-matured oocytes (Rizos *et al.* 2002). One of the reasons for the low developmental competence of *in vitro*-matured bovine oocytes may be that fertilization occurred at a suboptimal timing for oocytes. Therefore, clarification of the optimal timing for fertilization of *in vitro*-matured bovine oocytes will contribute to improve IVP efficiency and basic research on oocyte aging.

Many researchers have examined the effect of the duration of IVM on the embryonic development of bovine oocytes (Agung *et al.* 2006; Long *et al.* 1994; Merton *et al.* 2003; Park *et al.* 2005; Ward *et al.* 2002), but their optimal maturation culture periods for yielding a high blastocyst rate were not consistent (18 to 24 h). Although it was reported that the timing of fertilization after achieving nuclear maturation affected the blastocyst development of IVM oocytes (Dominko and First 1997), no studies determined the relationship between the timing of sperm penetration of nuclear-matured oocytes and subsequent embryonic development. The kinetics of meiotic progress during IVM has

been shown to be affected by the addition of substances to IVM medium (Izadyar *et al.* 1996; Suss *et al.* 1988), temperature during IVM culture (Edwards *et al.* 2005), periods of ovary conservation (Iwata *et al.* 2003), or the size of follicles from which oocytes are retrieved (Iwata *et al.* 2004). It was reported that the timing of sperm penetration was influenced by bull variation and conditions of *in vitro* fertilization (IVF) (Saeki *et al.* 1995; Takahashi and First 1993). There is a possibility that this inconsistency of optimal maturation culture periods is caused by the different timing of nuclear maturation and sperm penetration.

In previous studies, bovine oocytes at about 30 h after the initiation of IVM were considered as aged or slightly aged oocytes and used to investigate aging-related changes (Rispoli *et al.* 2011; Somfai *et al.* 2011; Sugimura *et al.* 2012). This was because the oocytes matured for 30 h before IVF showed a low developmental rate to blastocysts (Rispoli *et al.* 2011). However, since it takes several hours for bovine oocytes to be penetrated by sperm after starting IVF (Rispoli *et al.* 2011; Takahashi and First 1993), studies on the characteristics of aged oocytes should not be based on developmental competence corresponding to the duration of IVM, but instead on the timing of sperm penetration. Exact identification of the changes in the developmental competence based on the timing of sperm penetration may contribute to the establishment of an *in vitro* model for basic research on oocyte acquisition of developmental competence and oocyte aging.

In the present study, for estimation of the optimal timing of fertilization for achieving proper embryonic development of *in vitro*-matured bovine oocytes correctly and for clarifying the characteristics of oocytes possessing high developmental competence, the author investigated the time of nuclear maturation and sperm penetration during IVM and IVF, and also examined the effect of the time of sperm penetration after nuclear maturation on the embryonic development of bovine oocytes.

Materials and Methods

Chemicals

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

***In vitro* maturation and fertilization of bovine oocytes**

IVM was performed as previously described (Takahashi *et al.* 1996). Briefly, bovine cumulus oocyte complexes (COCs) aspirated from follicles (2 to 8 mm in diameter) of slaughterhouse-derived ovaries were cultured for various periods (12 to 30 h) under a humidified atmosphere of 5% CO₂ in air at 39°C using droplets of IVM medium (about 10 COCs/50 µl). IVM medium was composed of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered tissue culture medium (TCM)-199 (Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS, Invitrogen), 0.02 units/ml follicle stimulating hormone (FSH, from porcine pituitary), 1 µg/ml estradiol-17β, 0.2 mM sodium pyruvate and 50 µg/ml gentamicin sulfate.

IVF was conducted using frozen-thawed semen from one Holstein bull according to a procedure described previously (Takahashi and Kanagawa 1998a) with slight modifications. In brief, motile sperm (2×10^6 sperm/ml) separated from thawed semen using a Percoll (GE Healthcare, Buckinghamshire, UK) gradient (45 and 90%) were co-incubated with COCs in droplets of IVF medium (about 10 COCs/100 µl). IVF medium was composed of modified Brackett and Oliphant isotonic medium (Takahashi and First 1993) containing 3 mg/ml fatty acid-free bovine serum albumin (BSA), 2.5 mM theophylline, 20 µM penicillamine, 10 µM hypotaurine and 1 µM epinephrine. Co-incubation of COCs and sperm was performed for 4 to 18 h under 5% CO₂, 5% O₂ and 90% N₂ at 39°C.

Evaluation of the nuclear status of matured and fertilized oocytes

After various periods of IVM or IVF, oocytes were freed from the cumulus cells by vortexing. Denuded oocytes were fixed with ethanol:acetic acid (3:1) and stained with a 1% aceto-orcein solution. Their meiotic stages and fertilization statuses (sperm penetration and pronucleus (PN) formation) were examined under a phase-contrast microscope (Nagano *et al.* 2006a; Nagano *et al.* 2006b). Oocytes that reached the M-II stage were defined as nuclear-matured, and oocytes having enlarged sperm head(s) were defined as penetrated by sperm.

***In vitro* culture and evaluation of subsequent embryonic development**

To determine the developmental competences of oocytes, inseminated oocytes were assigned to *in vitro* culture (IVC) according to a procedure described previously (Takahashi *et al.* 1996; Takahashi and Kanagawa 1998b). In brief, inseminated oocytes were freed from the cumulus cells by vortexing at 18 h post insemination (hpi). Cumulus-free oocytes were washed three times and cultured for 6 days under 5% CO₂, 5% O₂ and 90% N₂ at 39°C in droplets of IVC medium (about 30 oocytes/30 µl). IVC 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 and 10 µg/ml insulin, and further supplemented with 5 mM glycine, 5 mM taurine, 1 mM glucose and 3 mg/ml fatty acid-free BSA. After 44 to 48 and 166 to 169 hpi (2 and 7 days after IVF, respectively), cleavage (developmental stage of cleaved embryos) and development to the blastocyst stage were assessed, respectively. All blastocysts were subjected to counting of the total number of cells by an air-drying method (Takahashi and First 1992).

Experimental design

In Experiment 1-1, to estimate the timing when 50% of oocytes reached the M-II stage, oocytes were subjected to IVM for 14, 16, 17, 18, 19, 20 and 22 h and their nuclear statuses were determined. A total of 645 COCs were subjected to IVM culture. After that, the time when 50% of oocytes accomplished nuclear maturation was computed using the percentage values of M-II oocytes at each IVM time point. In brief, the percentage values were modeled using the Gompertz equation, and the time to reach the point of 50% in the modeled Gompertz curve was defined as the time when 50% of oocytes reached the M-II stage. The Gompertz equation can be specified as follows: $y = a \times \exp(-e^{-b(x-c)})$ (y = M-II rate, x = duration of IVM, a , b and c = parameters of the equation).

In Experiment 1-2, to investigate the relationship between IVM duration and the timing of fertilization, oocytes after 12, 14, 18, 22, 26 and 30 h of IVM were assigned for 4, 8, 12 and 18 h of IVF and their fertilization statuses were determined. A total of 696 COCs were subjected to IVM and IVF. Then, the timings when 50% of oocytes were penetrated by sperm and formed two pronuclei were estimated using the Gompertz equation, the same as in Experiment 1-1. The relationships between the duration of IVM and the time from IVF start to sperm penetration and PN formation were also analyzed.

In Experiment 1-3, to investigate the timing when M-II oocytes have the highest developmental competence, oocytes subjected to 12, 14, 18, 22, 26 and 30 h of IVM and 18 h of IVF were cultured and examined for cleavage and development to the blastocyst stage. A total of 663 oocytes were subjected to IVC. In addition, the author estimated the optimal timing for embryonic development of *in vitro*-matured bovine oocytes by analyzing the results of nuclear maturation, sperm penetration and subsequent embryonic development.

Statistical analysis

Data of nuclear maturation and fertilization, subjected to arcsine transformation (Experiments 1-1 and 1-2), and of embryonic development (Experiment 1-3) were analyzed using one-way analysis of variance followed by Tukey-Kramer's honestly significant different test as a post hoc test. Nonlinear regression analyses for the kinetics of nuclear maturation, sperm penetration and PN formation after the initiation of IVM or IVF (Experiments 1-1 and 1-2) were performed by the Gompertz equation. Linear regression analyses were performed for expressing the relationships between the duration of IVM and the time from IVF start to sperm penetration and PN formation (Experiment 1-2). Quadratic regression analysis to express the relationship between the period from nuclear maturation to sperm penetration and blastocyst rate was performed (Experiment 1-3). The level of statistical significance was set at $P < 0.05$. Statistical analyses were performed using the software JMP version 10.0.2 (SAS Institute, Cary, NC, USA) and StatView version 5.0 (Abacus Concepts, Berkeley, CA, USA).

Results

Experiment 1-1

The data of the nuclear status at each time point of IVM and the Gompertz curve fitted to the M-II rate are shown in Fig. 1-1. At 14 h after IVM start, more than 80% of oocytes were at the metaphase I (M-I) stage. Although no oocyte reached the M-II stage, some oocytes (16.6%) were at the anaphase or telophase I (A-I/T-I). At 16 h after IVM start, 14.3% of oocytes reached M-II. At 18 h after IVM start, more than half of oocytes reached M-II (64.5%). After 19 h of IVM start, the M-II rate reached a plateau. By the Gompertz equation ($r^2 = 0.906$), the time when 50% of oocytes reached the M-II stage was estimated to be 17.5 h after the initiation of IVM.

Experiment 1-2

The data of sperm penetration and PN formation at each time point of IVF in oocytes subjected to different IVM culture periods are shown in Fig. 1-2. The Gompertz curves fitted to these values are also shown in Fig. 1-2. At 4 hpi, no oocyte was penetrated by sperm in the groups with 12, 14 and 18 h of IVM, and some oocytes were penetrated by sperm in the groups with 22, 26 and 30 h of IVM. At 8 hpi, in the groups with 14 and 18 h of IVM, some oocytes (27.9 and 27.4%) were penetrated by sperm and the penetration rate was significantly higher in the groups with 26 and 30 h of IVM than in the others ($P < 0.05$). Few oocytes showed PN formation in all groups at 8 hpi. At 12 hpi, more than 60% of oocytes showed PN formation in the group with 30 h of IVM, and the percentages of PN formation in the groups with 26 and 30 h of IVM were higher than in the others ($P < 0.05$). At 18 hpi, the percentages of sperm penetration and PN formation in the group with 12 h of IVM were lowest ($P < 0.05$), and those in the groups with 18, 22, 26 and 30 h of IVM were similar. The proportions of polyspermic oocytes were lower than 10%, regardless of the durations of IVM and IVF (data not shown). The regression equation of the estimated times when 50% of oocytes were penetrated by

sperm after IVM culture for 12 to 30 h was “ $y = -0.328x + 15.7$ ” ($r^2 = 0.905$, $P < 0.01$), which indicated that a longer duration of IVM resulted in faster sperm penetration. The regression equation of the estimated times when 50% of oocytes showed PN formation after IVM culture for 14 to 30 h was “ $y = -0.280x + 19.6$ ” ($r^2 = 0.979$, $P < 0.01$). These two regression lines were almost parallel, and the time period from sperm penetration to PN formation was similar regardless of the IVM duration.

Experiment 1-3

The data of embryonic development in oocytes subjected to different IVM durations are shown in Table 1-1. At 2 days after IVF, the cleavage rate (≥ 2 -cell) in the group with 12 h of IVM was lower than that in the group with 22 h of IVM ($P < 0.05$). The proportion of cleaved oocytes that developed to ≥ 5 cells (beyond the third cell cycle) was significantly higher in the groups with 22 and 26 h of IVM than in that with 14 h ($P < 0.05$) and tended to be higher in the group with 22 h of IVM than in that with 12 h ($P = 0.09$). At 7 days after IVF, the percentage of blastocysts based on the number of inseminated oocytes in the group with 22 h of IVM was higher than those in the groups with 12 and 30 h ($P < 0.05$). The total number of cells in blastocysts was higher in the group with 22 h of IVM than in the groups with 12 and 18 h of IVM ($P < 0.05$).

The correlation between the period from nuclear maturation to sperm penetration and blastocyst rate based on the number of inseminated oocytes is shown in Fig. 1-3. The developmental rate to the blastocyst stage (based on the number of inseminated oocytes: 44%) peaked when sperm penetration occurred at 12.2 h after achieving nuclear maturation (i.e., 29.7 h after the initiation of IVM), and the optimal IVM duration was estimated to be about 21 h.

Discussion

From the estimation in the present study, bovine oocytes matured for about 21 h before IVF have the highest rate of development to the blastocyst stage. This is in agreement with the results of previous reports (Agung *et al.* 2006; Long *et al.* 1994). In the present study, 50% of oocytes reached the M-II stage after 17.5 h of IVM culture. This meiotic progression of oocytes is in agreement with the finding of a previous report in which a similar IVM system was employed (Sirard *et al.* 1989).

The present study demonstrated that the time of sperm penetration was affected by IVM duration: a longer duration of IVM resulted in faster sperm penetration (interval from IVF start to sperm penetration: $y = -0.328x + 15.7$). In the present IVF system (Takahashi and First 1992; Takahashi and First 1993), sperm capacitation mainly depended on the glycosaminoglycans (GAGs) produced by cumulus cells (Chen *et al.* 1990; Salustri *et al.* 1992), and the production of GAGs from cumulus cells increased during IVM culture (Chen *et al.* 1990). Therefore, in the present IVF conditions, sperm may have difficulty penetrating oocytes after short-term IVM culture, but easily penetrate them after long-term IVM culture. The results also showed that the time period from sperm penetration to PN formation was similar regardless of the IVM duration. On the other hand, a previous report indicated that M-II oocytes recovered after 16 h of IVM culture and immediately subjected to IVF required a longer period for PN formation than those cultured for another 8 h before being subjected to IVF (Dominko and First 1997). This inconsistency may be due to the delay of sperm penetration after short-term IVM culture in the present study.

The results of the time from nuclear maturation (50% M-II at 17.5 h) to sperm penetration and blastocyst development indicated that oocytes acquired the highest developmental competence at around 12 h after achieving nuclear maturation (30 h after the initiation of IVM culture, Fig. 1-3). This estimated time may be comparable to a previous report by Dominko and First (1997). They indicated that oocytes extruded a first polar body after 16 h of IVM required another 8 h of culture to

acquire high developmental capacity. In their IVF system, the presence of a sperm head in the cytoplasm was first observed at 5 hpi, regardless of the time of M-II or the time of insemination. Therefore, the time of sperm penetration is estimated to be about 13 h after achieving nuclear maturation (29 h after the initiation of IVM culture), and our findings support their results.

In the present study, the cleavage rate, the blastocyst rate based on the number of inseminated oocytes and the total cell number in blastocysts were lower in the group with 12 h of IVM than in the group with 22 h. This result may reflect lower sperm penetration rate at 18 hpi in the group with 12 h of IVM than that in the group with 22 h. Another possible explanation for this result is inadequate cytoplasmic maturation of oocytes. As described above, under the present IVF system, the timing of sperm penetration depended on the function of cumulus cells. To clarify the time of oocyte acquisition of developmental competence, an IVF system that can allow sperm to penetrate oocytes whenever we want should be developed.

Although the percentages of sperm penetration at 18 hpi and cleavage in groups with 22 and 30 h of IVM were similar, the developmental competence to the blastocyst stage of the group with 30 h of IVM was significantly lower than that of the group with 22 h. It has been reported that mitochondria can play important roles in the production of adenosine triphosphate (ATP) for fertilization and pre-implantation embryo development, and act as stores of intracellular calcium and proapoptotic factors (Ferreira *et al.* 2009; Wang *et al.* 2009). Tarazona *et al.* (2006) suggested the important role of ATP produced by mitochondria for the embryonic genome activation (EGA) process. In bovine oocytes, minor and major EGAs were observed at the 1- to 4-cell and 8- to 16-cell stages and were found to be responsible for successful subsequent embryonic development (Badr *et al.* 2007). Therefore, it is speculated that the low percentages of cleavage and blastocyst development are caused by the failure of the essential EGA process in aged oocytes. In future study, EGAs, the

mitochondrial activity and ATP content of oocytes derived from different culture durations of IVM should be examined.

The present study showed that oocytes penetrated by sperm at around 30 h after the initiation of IVM culture had the highest developmental competence and that they began to lose their developmental competence gradually after this timing. In previous studies, bovine oocytes at about 30 h after IVM were considered as aged or slightly aged oocytes and used to reveal aging-related changes (Rispoli *et al.* 2011; Somfai *et al.* 2011). However, after 30 h of IVM, degradation of the microfilament-rich domain overlying the spindle in bovine oocytes was not observed, which had been observed in porcine aged oocytes (Somfai *et al.* 2011). Moreover, maturation-promoting factor (MPF) activity was similar to that in bovine oocytes matured for 24 and 32 h (Rispoli *et al.* 2011), although a decrease in activity of MPF in bovine oocytes matured for 40 h was observed (Tian *et al.* 2002). Because aged oocytes were defined as those deteriorated in quality and lost developmental competence (Rispoli *et al.* 2011), from the present result and previous results, the author recommends that bovine oocytes at 30 h after the initiation of IVM should not be considered as aged oocytes, but considered as fully matured ones.

In conclusion, in the present culture system, bovine oocytes acquire their highest developmental competence at around 12 h after achieving nuclear maturation (i.e., around 30 h after the initiation of IVM culture). To achieve stable IVP of bovine embryos, it is recommended to use the IVF system that permits oocytes to be penetrated by sperm around this timing. In addition, in the case of conducting basic studies on oocyte acquisition and loss of developmental competence by using the present culture system, bovine oocytes at 30 h after the initiation of IVM should be considered as fully matured oocytes, not as aged ones.

Table 1-1. The effects of the timing of IVF start after IVM (duration of IVM) on embryo development.

Timing of IVF start after IVM (duration of IVM: h)	No. of oocytes (replicates)	Cleavage and developmental stage at 2 days after IVF start			Blastocyst development at 7 days after IVF start		
		% ≥ 2 cells /oocytes	% ≥ 3 cells /cleaved	% ≥ 5 cells /cleaved	% blastocysts / oocytes	% blastocysts /cleaved	Total cell no. in blastocyst (n)
12	104 (4)	67.2 $\pm$ 4.8 ^{a)}	73.2 $\pm$ 6.7	9.4 $\pm$ 12.5 ^{a,b)}	32.8 $\pm$ 7.8 ^{a)}	49.2 $\pm$ 13.1	131.5 $\pm$ 54.9 ^{a)} (34)
14	106 (4)	77.4 $\pm$ 2.5 ^{a,b)}	69.5 $\pm$ 8.2	6.0 $\pm$ 7.4 ^{a)}	35.6 $\pm$ 7.3 ^{a,b)}	46.1 $\pm$ 10.2	153.3 $\pm$ 62.6 ^{a,b)} (33)
18	109 (4)	78.9 $\pm$ 3.0 ^{a,b)}	70.2 $\pm$ 20.3	17.2 $\pm$ 11.2 ^{a,b)}	38.4 $\pm$ 5.9 ^{a,b)}	48.6 $\pm$ 7.2	133.5 $\pm$ 61.2 ^{a)} (42)
22	136 (5)	80.5 $\pm$ 6.0 ^{b)}	78.5 $\pm$ 9.9	28.2 $\pm$ 7.6 ^{b)}	50.0 $\pm$ 9.5 ^{b)}	62.6 $\pm$ 14.0	173.3 $\pm$ 75.6 ^{b)} (68)
26	108 (4)	78.7 $\pm$ 5.5 ^{a,b)}	73.1 $\pm$ 6.9	27.9 $\pm$ 5.2 ^{b)}	42.7 $\pm$ 9.1 ^{a,b)}	54.7 $\pm$ 12.7	147.8 $\pm$ 72.4 ^{a,b)} (46)
30	100 (4)	71.1 $\pm$ 9.9 ^{a,b)}	69.8 $\pm$ 6.1	13.8 $\pm$ 9.4 ^{a,b)}	29.0 $\pm$ 4.4 ^{a)}	40.8 $\pm$ 2.6	156.1 $\pm$ 58.3 ^{a,b)} (29)

^{a, b)} Different superscripts within the same column differ significantly ($P < 0.05$). Data are presented as means $\pm$ standard deviation (SD).

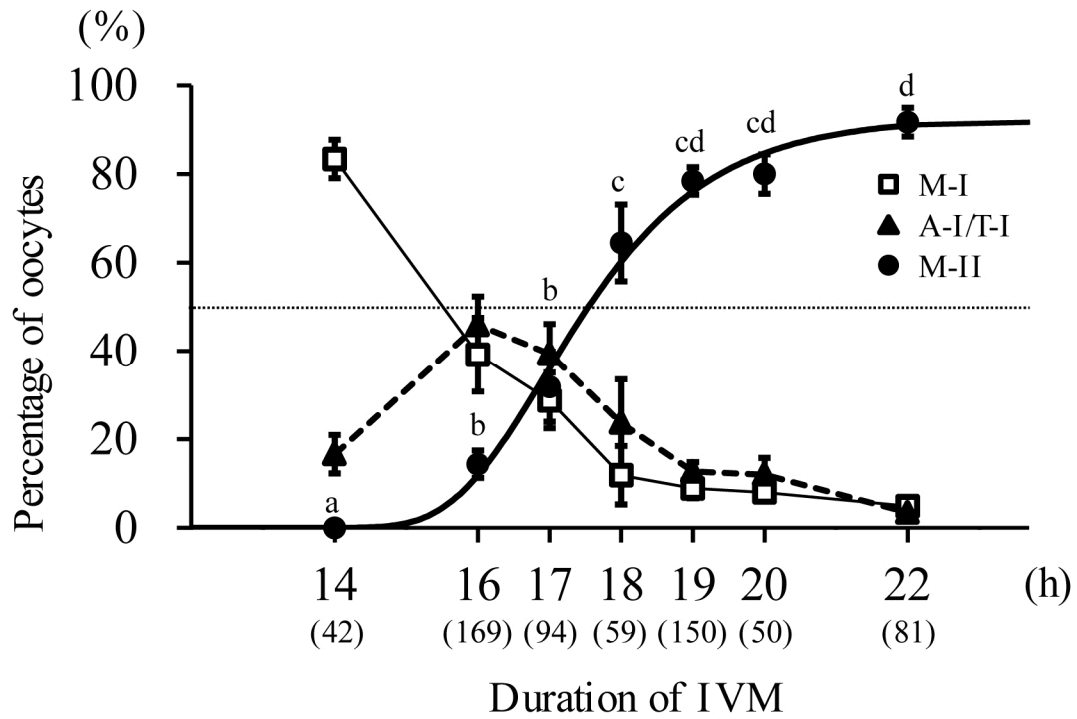


Fig. 1-1. Meiotic progression of bovine oocytes during IVM.

Gompertz curve (bold solid line) is fitted to M-II rate.

The equation of this curve is $y = 92.7 \times \exp(-e^{-0.8(x-16.9)})$.

Dotted line shows 50%. ^{a,b,c,d} Values with different characters differ significantly in the percentage of M-II oocytes ($P < 0.05$). Three to 8 replicates were performed and 42 to 169 oocytes were used in each group. Numbers in parentheses show the number of oocytes used.

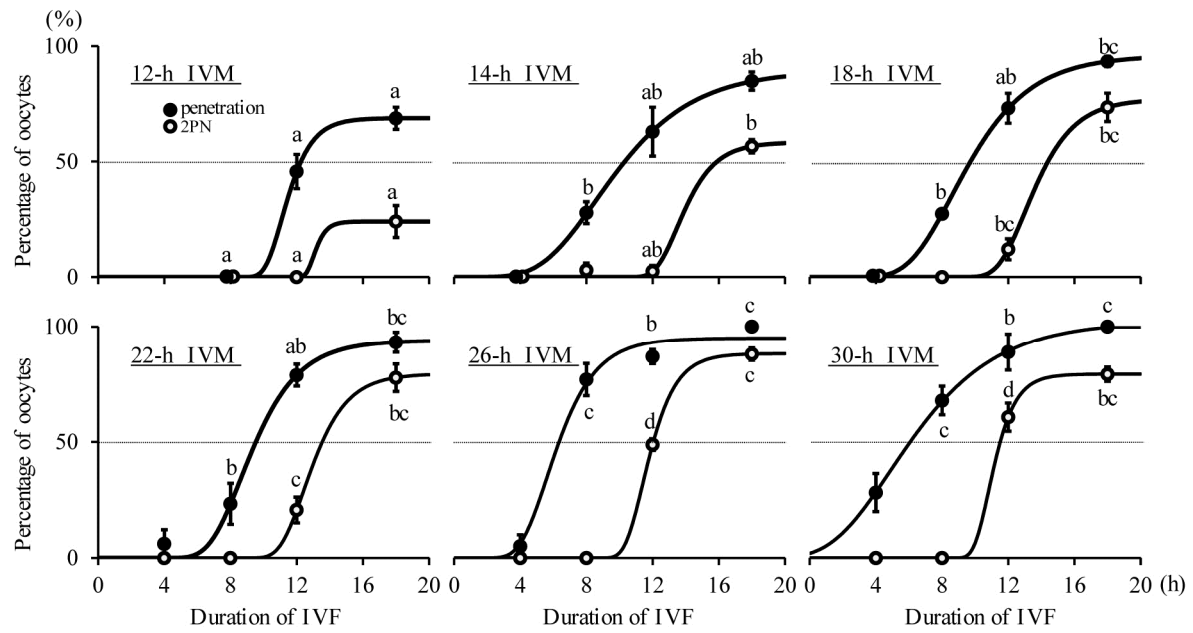


Fig. 1-2. The effects of duration of IVM on sperm penetration and PN formation after IVF.

Gompertz curves (bold solid line) were fitted to the data of sperm penetration and PN formation.

The equations of the curves for the percentage of oocytes penetrated were $y = 68.8 \times \exp(-e^{-0.990(x-11.1)})$ ($r^2 = 0.871$), $89.6 \times \exp(-e^{-0.306(x-8.55)})$ ($r^2 = 0.877$), $96.0 \times \exp(-e^{-0.382(x-8.59)})$ ($r^2 = 0.964$), $93.9 \times \exp(-e^{-0.519(x-8.64)})$ ($r^2 = 0.934$), $94.9 \times \exp(-e^{-0.615(x-5.62)})$ ($r^2 = 0.946$), $1.02 \times 10^2 \times \exp(-e^{-0.281(x-4.88)})$ ($r^2 = 0.874$) for 12, 14, 18, 22, 26 and 30 h of IVM, respectively.

The equations of the curves for the percentage of oocytes with 2PN were $y = 24.1 \times \exp(-e^{-2.24(x-13.0)})$ ($r^2 = 0.706$), $58.4 \times \exp(-e^{-0.775(x-13.5)})$ ($r^2 = 0.970$), $77.1 \times \exp(-e^{-0.608(x-13.0)})$ ($r^2 = 0.950$), $79.4 \times \exp(-e^{-0.693(x-12.4)})$ ($r^2 = 0.949$), $88.2 \times \exp(-e^{-0.912(x-11.4)})$ ($r^2 = 0.991$), $79.3 \times \exp(-e^{-1.12(x-10.8)})$ ($r^2 = 0.966$) for 12, 14, 18, 22, 26 and 30 h of IVM, respectively.

Dotted line shows 50%. ^{a,b,c,d} Values with different characters differ significantly in the percentage of sperm penetration and PN formation at each time post-insemination ($P < 0.05$). Each group had 2 to 4 replicates and 21 to 46 oocytes.

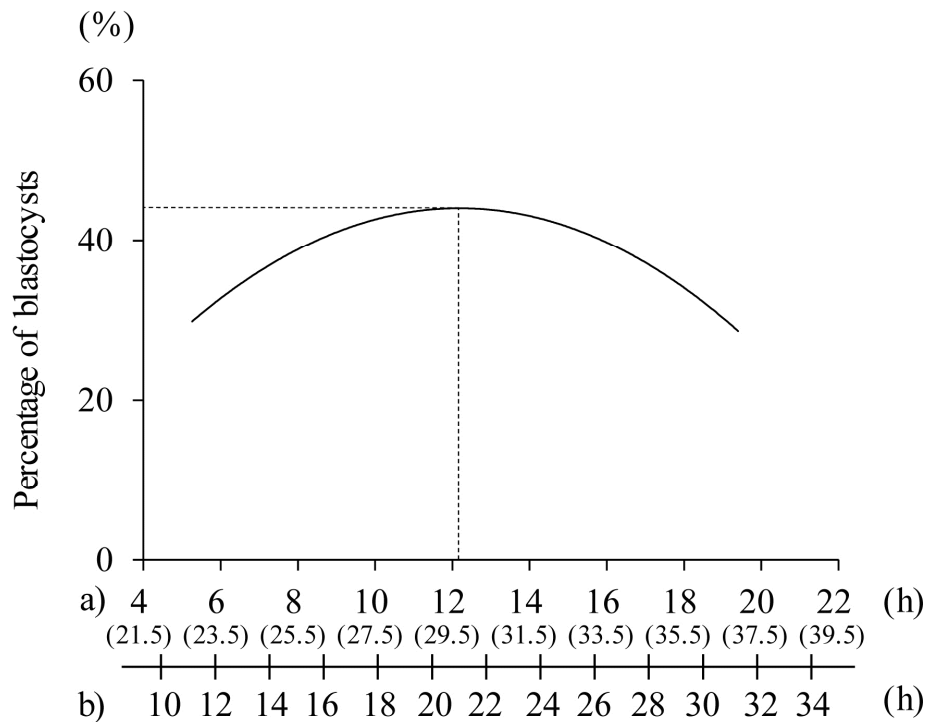


Fig. 1-3. The regression curves describing the relationship between the time from nuclear maturation to sperm penetration and the percentage of blastocysts.

Time from initiation of IVM to sperm penetration and duration of IVM corresponding to time from nuclear maturation to sperm penetration are described on the x-axis. a) Time from nuclear maturation to sperm penetration is shown, and the time from initiation of IVM to sperm penetration is also shown in parentheses. b) Duration of IVM culture.

Regression equation was $y = 7.23x - 0.297x^2$ ($r^2 = 0.963$, $P < 0.01$).

Dotted lines show the time from nuclear maturation to sperm penetration and blastocyst rate when the developmental rate to the blastocyst stage reached its maximum value.

Summary

The objective of this research was to estimate the optimal timing for fertilization to achieve proper embryonic development of *in vitro*-matured bovine oocytes. Firstly, COCs were subjected to IVM for 14 to 22 h. The timing when 50% of oocytes reached the M-II stage was estimated to be 17.5 h after IVM start. Secondly, using oocytes subjected to IVM for 12 to 30 h, sperm penetration was examined after 4 to 18 h of IVF. A significant negative correlation between IVM duration and the timing when 50% of oocytes were penetrated by sperm after IVF start was observed ($P < 0.01$). Finally, oocytes subjected to 12 to 30 h of IVM were inseminated and cultured for 6 days to examine embryonic development. In the group with 22 h of IVM, the percentages of cleaved embryos and blastocysts showed highest values in all groups. According to the regression equation describing the time from nuclear maturation to sperm penetration (x) and the percentage of blastocysts (y) ($y = 7.23x - 0.297x^2$, $P < 0.01$), the blastocyst rate peaked when sperm penetration occurred at 12.2 h after achieving nuclear maturation. In conclusion, under the present IVM/IVF conditions, it was estimated that oocytes acquired their highest developmental competence at about 30 h after IVM start, and thus the optimal IVM duration was calculated to be about 21 h.

Chapter 2

Aging-related changes in *in vitro*-matured bovine oocytes: oxidative stress, mitochondrial activity and ATP content after nuclear maturation

Introduction

In humans and domestic animals, it is well known that post-ovulatory aging of oocytes at the M-II stage adversely affects the outcome of ART, such as AI (Saacke *et al.* 2000), IVF (Agung *et al.* 2006; Harrison *et al.* 1988), and intracytoplasmic sperm injection (Emuta and Horiuchi 2001; Yanagida *et al.* 1998). In humans and rodents, post-ovulatory aging of oocytes is defined as numerous morphological and cellular alterations, and causes a decrease in fertilization and embryonic development (reviewed in Lord and Aitken 2013; Miao *et al.* 2009). In bovines, both *in vivo* and *in vitro* aging of oocytes cause a decrease in fertilization and embryonic development (Agung *et al.* 2006; Long *et al.* 1994; Rispoli *et al.* 2011; Roelofs *et al.* 2006; Saacke *et al.* 2000; Ward *et al.* 2002). However, there are few data on aging-related changes in bovine oocytes. Research on aging in bovine oocytes will contribute to the development of a method for preventing aging in *in vitro*-matured bovine oocytes, and eventually to improvement of ART efficiency.

Recently, studies on murine oocytes have indicated the possibility that oxidative stress acts as a trigger for a cascade of several events associated with oocyte aging and one of the aging-related changes caused by oxidative stress is mitochondrial dysfunction (Lord and Aitken 2013). It has been reported that the aging of murine oocytes causes increased oxidative stress (Takahashi *et al.* 2009), mitochondrial dysfunction (Wilding *et al.* 2001; Zhang *et al.* 2011) and decreased intracytoplasmic levels of ATP (Chi *et al.* 1988). In *in vitro*-matured bovine oocytes, the changes in level of oxidative stress (Morado *et al.* 2009), mitochondrial activity (Nabenishi *et al.* 2012; Tarazona *et al.* 2006) and

ATP content (Merton *et al.* 2012; Nagano *et al.* 2006b; Stojkovic *et al.* 2001; Tamassia *et al.* 2004) during IVM culture for less than 24 h were determined, but there are few data on the changes in these parameters associated with bovine oocyte aging. Therefore, it is still unclear whether the extension of IVM duration in bovine oocytes causes the aging-related changes in oxidative stress, mitochondrial activity and ATP content.

The present study was conducted to clarify the aging-related changes related to oxidative stress, mitochondrial activity and ATP content in *in vitro*-matured bovine oocytes, and to mitochondrial activity in embryos at around the 8-cell embryo stage derived from *in vitro*-fertilized bovine oocytes. Firstly, the author confirmed the competence of fertilization and embryonic development in oocytes subjected to 22 and 30 to 34 h of IVM. Then, the author examined reactive oxygen species (ROS) production, mitochondrial activity, and ATP content in oocytes after 20, 30, and 40 h of IVM culture, and also examined the mitochondrial activity in embryos at 72 h after IVF.

Materials and Methods

***In vitro* maturation and fertilization of bovine oocytes**

IVM was performed as previously described (Takahashi *et al.* 1996). Briefly, bovine ovaries (mostly Holstein breed) 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. Bovine COCs were aspirated from small antral follicles (2 to 8 mm in diameter). The COCs with brown-colored ooplasm surrounded by intact cumulus investments (Nagano *et al.* 2006a) were washed twice in HEPES-buffered Tyrode's medium (Bavister *et al.* 1983) supplemented with 3 mg/ml BSA (fraction V), 0.2 mM sodium pyruvate and 50 µg/ml gentamicin sulfate. The COCs were then cultured for 20 to 40 h under a humidified atmosphere of 5% CO₂ in air at 39°C in droplets of IVM medium (about 10 COCs/50 µl). IVM medium was composed of HEPES-buffered TCM-199 (Invitrogen) supplemented with 10% FCS (Invitrogen), 0.02 units/ml FSH (from porcine pituitary), 1 µg/ml estradiol-17β, 0.2 mM sodium pyruvate and 50 µg/ml gentamicin sulfate.

IVF was conducted using frozen-thawed semen from one Holstein bull according to a procedure described previously (Takahashi and Kanagawa 1998a) with slight modifications. In brief, motile sperm (2×10^6 sperm/ml) separated from thawed semen using a Percoll (GE Healthcare) gradient (45 and 90%) were co-incubated with COCs in droplets of IVF medium (about 10 COCs/100 µl). IVF medium was composed of modified Brackett and Oliphant isotonic medium (Takahashi and First 1993) containing 3 mg/ml fatty acid-free BSA, 2.5 mM theophylline, 20 µM penicillamine, 10 µM hypotaurine and 1 µM epinephrine. Co-incubation of COCs and sperm was performed for 18 h under 5% CO₂, 5% O₂ and 90% N₂ at 39°C.

Evaluation of the fertilized oocytes

After 18 h of IVF, oocytes were freed from the cumulus cells by vortexing. Denuded oocytes were fixed with ethanol: acetic acid (3:1) and stained with a 1% aceto-orcein solution. Their fertilization statuses (sperm penetration and PN formation) were examined under a phase-contrast microscope (Nagano *et al.* 2006a; Nagano *et al.* 2006b). Oocytes having enlarged sperm head(s) or male PN were defined as penetrated by sperm, and the following categories of oocytes penetrated by sperm were recorded: 1) oocytes with male and female pronuclei or with an enlarged sperm head and anaphase II/telophase II chromosome (normal fertilization), 2) oocytes with more than two enlarged sperm heads or male pronuclei (polyspermy), and 3) oocytes with an enlarged sperm head and female PN or with male PN and telophase II chromosome (asynchronous fertilization).

***In vitro* culture and evaluation of subsequent embryonic development**

To determine the developmental competences of oocytes, inseminated oocytes were assigned to IVC according to a procedure described previously (Takahashi *et al.* 1996; Takahashi and Kanagawa 1998b). In brief, inseminated oocytes were freed from the cumulus cells by vortexing at 18 hpi. Denuded oocytes were washed three times and cultured for 6 days under 5% CO₂, 5% O₂ and 90% N₂ at 39°C in droplets of IVC medium (25 to 30 oocytes/30 µl). IVC 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 and 10 µg/ml insulin, and further supplemented with 5 mM glycine, 5 mM taurine, 1 mM glucose and 3 mg/ml fatty acid-free BSA. After 2 and 7 days of IVF (44 to 48 and 166 to 170 hpi, respectively), cleavage and development to the blastocyst stage were assessed, respectively. All blastocysts were subjected to counting of the total number of cells by an air-drying method (Takahashi and First 1992).

Measurement of ROS in individual oocytes

The quantity of H_2O_2 produced by individual oocytes was measured as ROS according to a previous report (Marei *et al.* 2012). ROS in oocytes can be quantified by measuring 2',7'-dichlorofluorescein diacetate (DCF) (Nasr-Esfahani *et al.* 1990). DCF fluorescence is generated by H_2O_2 from 2',7'-dichlorodihydrofluorescein which is formed by intracellular esterase from 2',7'-dichlorodihydrofluorescein diacetate (DCHFDA).

After IVM culture, oocytes were freed from the cumulus cells by vortexing. Denuded oocytes with a polar body were incubated with 10 μ M DCHFDA in Dulbecco's phosphate-buffered saline (DPBS) supplemented with 10% FCS for 15 min at 39°C and washed in DPBS. Stained oocytes were transferred to a slide glass with a small amount of DPBS and pressed gently with a cover slide. Their fluorescence emissions were then observed under a fluorescence microscope using an appropriate filter (BZ-9000; Keyence, Osaka, Japan), and the sectioned fluorescent images of each oocyte were acquired at 2 μ m interval. The mean green fluorescent intensity of each oocyte, which represents the H_2O_2 level, was calculated by analysis software (BZ-H2A, Keyence).

Evaluation of mitochondrial activity in individual oocytes and embryos

Denuded oocytes or embryos at 72 hpi were stained with JC-1 (Cell Technology Inc., Mountain View, CA, USA) as described previously (Tarazona *et al.* 2006) with slight modifications. JC-1 is a fluorescent dye that accumulates in mitochondria and shows the membrane potential across the matrix membrane (Reers *et al.* 1991). JC-1 fluorescence has two emission peaks, with red fluorescence (JC-1 dimers) indicating high-polarized mitochondria (high membrane potential) and green fluorescence (JC-1 monomers) indicating low-polarized mitochondria (low membrane potential) (Reers *et al.* 1991). Mitochondrial activity can be evaluated by the intensity of the red/green fluorescence (Nabenishi *et al.* 2012; Tarazona *et al.* 2006; Wilding *et al.* 2001).

Briefly, they were incubated with 1 μ M JC-1 and 1 μ g/ml Hoechst 33342 in DPBS supplemented with 10% FCS for 15 min at 37°C and washed twice in DPBS. Stained oocytes or embryos were transferred to a slide glass with a small amount of DPBS and pressed gently with a cover slide. They were then observed under a fluorescence microscope (BZ-9000). The distributions of JC-1 dimers with red fluorescence and monomers with green fluorescence were detected using the red filter and green filter of the microscope, respectively. Mitochondrial activity of the oocytes at the M-II stage or cleaved oocytes (evaluated by Hoechst staining) was determined by the intensity of the red/green fluorescence using software (BZ-H2A).

Measurement of ATP content in individual oocytes

After IVM culture, oocytes were freed from the cumulus cells by vortexing. The ATP content of individual oocytes with a polar body was measured according to a previous report (Nagano *et al.* 2006b; Rieger 1997). Briefly, a denuded oocyte was washed four times in the sample buffer and transferred to 25 μ l of the sample buffer in a 1.5 ml tube. The sample buffer consisted of 99.0 mM NaCl, 3.1 mM KCl, 0.35 mM NaH₂PO₄, 21.6 mM sodium lactate, 10.0 mM HEPES, 2.0 mM CaCl₂, 1.1 mM MgCl₂, 25.0 mM NaHCO₃, 1.0 mM sodium pyruvate, 0.1 mg/ml of gentamicin sulfate and 6.3 mg/ml of BSA (Rieger 1997). These tubes were placed in boiling water for 3 min to inactivate the endogenous phosphatases and then frozen at -80°C until assay. All assay reagents were purchased as a kit (ATP bioluminescent somatic cell assay kit, FL-ASC) and prepared according to the manufacturer's instructions. The ATP stock solution was diluted to concentrations of 0.16 to 10 pmol/25 μ l in sample buffer for the ATP standards. The ATP standards and samples in 1.5 ml tubes were kept on ice, and 50 μ l of ice-cold somatic cell-releasing agent was added to all tubes. After the tubes were kept on ice for 5 min, the contents of the tubes were transferred to a white 96-well plate (Labsystems, Tokyo, Japan). Thereafter, 100 μ l of assay mix was added to each well at 5 sec

intervals and held at room temperature for 5 min to pass through the initial chemiluminescence flash period. ATP content in an oocyte was quantified by measuring the luminescence (Luminesensor JNR AB-2100, Atto, Tokyo, Japan).

Experimental design

In Experiment 2-1, bovine oocytes after 22 and 30 to 34 h of IVM were assigned for 18 h of IVF and their fertilization statuses were determined. In addition, the percentages of cleavage and development to the blastocyst stage were evaluated. Out of 473 oocytes subjected to IVM and IVF, 351 oocytes were subjected to IVC (20 to 30 oocytes/replicate), and the remaining 122 oocytes were used for the fertilization evaluation (10 to 13 oocytes/replicate).

In Experiment 2-2, oocytes subjected to IVM for 20, 30 and 40 h served in the determination of ROS, mitochondrial activity and ATP content. The result in chapter 1 indicated the time when 50% of oocytes reached the M-II stage was 17.5 h after the initiation of IVM, and the times when 50% of oocytes were penetrated by sperm after IVM culture for 22 and 30 to 34 h were around 30 and 40 h after the initiation of IVM culture, respectively. Namely, oocytes subjected to IVM for 20, 30 and 40 h were considered as “oocytes immediately after M-II arrival”, “oocytes with high developmental competence”, and “aged oocytes”, respectively. For ROS measurement, a total of 78 oocytes were subjected to IVM, and oocytes with polar body clearly observed under stereomicroscope were used for the experiment (10 to 13 oocytes/replicate). Also for evaluating mitochondrial activity and ATP content, a total of 216 oocytes were subjected to IVM, and oocytes with a polar body clearly observed under stereomicroscope were used for the experiment (9 to 14 oocytes/replicate). In addition, oocytes were then subjected to 22 h (penetrated by sperm at around 30 h after starting IVM) and 34 h (penetrated by sperm at around 40 h after starting IVM) of IVM, IVF and IVC, and the cleavage and developmental stage of embryos were confirmed by the number of nuclei stained with Hoechst 33342

at 72 hpi. Mitochondrial activities of cleaved embryos were then examined. Distribution of high-polarized mitochondria in embryos was also examined, and the percentage of embryos with high-polarized mitochondria at the periphery of blastomeres was recorded. In the experiment, 20 and 21 embryos derived from the oocytes subjected to 22 and 34 h of IVM were used (1 replicate).

Statistical analysis

Data of fertilization and embryonic development (Experiment 2-1) and mitochondrial activity of embryos at 3 days after IVF (Experiment 2-2) were analyzed by Student's *t*-test. ROS, mitochondrial activity, and ATP content of oocytes after IVM culture (Experiment 2-2) were analyzed using one-way analysis of variance followed by Tukey-Kramer's honestly significant different test as a post-hoc test. Data of embryonic development and distribution of high-polarized mitochondria in embryos was analyzed by Fisher's exact test (Experiment 2-2). The level of statistical significance was set at $P < 0.05$. Statistical analyses were performed using JMP software version 10.0.2 (SAS Institute).

Results

Experiment 2-1

The data of fertilization is shown in Table 2-1. The percentages of normal fertilization, polyspermy and sperm penetration were similar regardless of IVM duration. However, the percentage of oocytes that formed PN asynchronously in the group subjected to 30 to 34 h of IVM was higher than in the group subjected to 22 h ($P < 0.01$). In the group subjected to 30 to 34 h of IVM, the percentages of delay in male and female PN formation were 10.4 and 1.3%, respectively. As shown in Table 2-2, the cleavage rate tended to be lower in the group subjected to 30 to 34 h of IVM than in the group subjected to 22 h ($P = 0.06$). The percentages of blastocysts based on the number of inseminated and cleaved oocytes in the group subjected to 30 to 34 h of IVM were also lower than in the group subjected to 22 h ($P < 0.01$). Total cell numbers in blastocysts were similar regardless of IVM duration.

ROS production in oocytes after different IVM culture periods is shown in Fig. 2-1. Mean intensity of DCF fluorescence in the group subjected to 40 h of IVM was similar to that of the groups subjected to 20 and 30 h, and that in the group subjected to 30 h of IVM was lower than that in the group subjected to 20 h of IVM ($P < 0.01$). The mitochondrial activity and the ATP content in oocytes of different IVM culture periods are shown in Fig. 2-2. The mitochondrial activity in the group subjected to 40 h of IVM was higher than in the group subjected to 20 h ($P < 0.01$). The mitochondrial activity in the group subjected to 30 h of IVM showed an intermediate value between the groups subjected to 20 and 40 h of IVM. The ATP content in the group subjected to 40 h of IVM was higher than in the other groups ($P < 0.01$).

At 72 hpi, the percentage of ≥ 8 -cell stage embryos in the group subjected to 22 h of IVM (60.0%) was higher than in the group subjected to 34 h of IVM (23.8%) ($P < 0.05$). As shown in Fig. 2-3, the mitochondrial activity of embryos at 72 hpi was similar regardless of IVM duration; however, the

percentage of embryos having high-polarized mitochondria at the periphery of blastomeres (Fig. 2-4) was higher in the group subjected to 34 h of IVM (81.0%) than in the group subjected to 22 h (30.0%) ($P < 0.01$).

Discussion

In the present study, normal fertilization rate was similar regardless of IVM duration, but developmental competence to blastocyst stage was low in the bovine oocytes subjected to 30 to 34 h of IVM. These results were consistent with the results of previous studies (Agung *et al.* 2006; Long *et al.* 1994; Rispoli *et al.* 2011; Ward *et al.* 2002). Moreover, asynchronous fertilization has been frequently observed in bovine oocyte subjected to 30 to 34 h of IVM. The delay in male PN formation might be due to the deficiency of male PN growth factor in the group subjected to 30 to 34 h of IVM (Saeki *et al.* 1991; Thibault *et al.* 1975). The reason for the delay in female PN formation is unclear, but a similar delay in female PN formation was observed in fertilized oocytes from aged hamsters (Suzuki *et al.* 1996).

In contrast to the past report that cytoplasmic ROS in oocytes increased with oocytes aging in murine and swine (Lord *et al.* 2013; Takahashi *et al.* 2009; Tang *et al.* 2013), the extension of IVM culture up to 40 h did not cause the increased cytoplasmic ROS in the present study. Moreover, although it has been reported that mitochondrial activity and ATP content in human, murine and porcine oocytes decrease with aging (Chi *et al.* 1988; Hao *et al.* 2009; Igarashi *et al.* 2005; Wilding *et al.* 2001; Zhang *et al.* 2011), the mitochondrial activity and ATP content in bovine oocytes subjected to 40 h of IVM were highest among all IVM durations in the present study. Therefore, it is thought that the depression of developmental competence in aged oocytes was not due to the mitochondrial dysfunction and the decrease of intracytoplasmic levels of ATP caused by oxidative stress. On the other hand, ROS in the oocytes after 30 h of IVM was lower than in the oocytes after 20 h of IVM, and this result showed that the oocytes after 30 h of IVM had high competence to protect against oxidative stress. Therefore, it is thought that one of the reasons of this high developmental competence oocytes penetrated by sperm at around 30 h after the initiation of IVM was due to the low oxidative stress in oocytes by high competence to protect against oxidative stress. In a previous

study (Takahashi *et al.* 2009), it was reported that cumulus cells prevented the increase of ROS during *in vitro* aging. Although the author used only oocytes having complete cumulus investments, the relationship between the function of cumulus cells and ROS generation during IVM culture should be examined in further study.

Van Blerkom and Davis (2006) indicated that the subplasmalemmal domains, including high-polarized mitochondria, were extruded as fragments from human embryo blastomeres and this phenomenon caused failure to cleave during early embryonic development. In the present study, high-polarized mitochondria were frequently observed at the periphery of blastomeres in embryos derived from aged oocytes. These high-polarized mitochondria at the periphery of blastomeres might have already been extruded from blastomeres like the previous report (Van Blerkom and Davis 2006), and it is possible that the extrusion of high-polarized mitochondria from blastomeres causes lower early embryonic development of aged oocytes. However, in the present study, it was unable to confirm the clear extrusion of high-polarized mitochondria from blastomeres because bovine embryo contained a large number of lipid droplets in their ooplasm unlike human embryos (Kruip *et al.* 1983) and it was necessary to press the embryos with a cover slide for observing the mitochondria stained by JC-1. In future study, the localization of high-polarized mitochondria in embryos derived from the aged bovine oocytes should be examined in detail by electron microscope analysis.

Although the present study does not clarify the direct relationship between the enhanced mitochondrial activity and low developmental competence of bovine oocytes, the present results suggest that enhanced mitochondrial activity may be one of the reasons for low developmental competence of *in vitro*-aged bovine oocytes. High-polarized mitochondria in oocytes are associated with elevated levels of ATP generation (Van Blerkom 2008). Tamassia *et al.* (2004) indicated that ATP content in OPU-derived oocytes did not increase during IVM culture, but ATP content in abattoir-derived oocytes did increase. Also it has been reported that OPU-derived bovine oocytes

kept high developmental competence during longer periods of IVM culture than abattoir-derived oocytes (Merton *et al.* 2012). These previous reports indicate that OPU-derived oocytes can control mitochondrial activity and this ability contributes to maintaining high developmental competence during long periods. In the present study, blastocysts derived from oocytes subjected to 30 to 34 h of IVM culture (penetrated by sperm at around 40 h after the initiation of IVM culture) had a similar number of cells to those derived from oocytes subjected to 22 h of IVM culture. This suggests that some oocytes at around 40 h after IVM start maintain their developmental competence. In future study, it is necessary to examine the mechanisms of maintenance of mitochondrial activity in *in vitro*-matured bovine oocytes and the relationship between the mitochondrial activity and developmental competence in detail.

In conclusion, the present study suggests that low developmental competence of aged bovine oocytes is probably not due to low mitochondrial activity or low ATP content, but high mitochondrial activity at fertilization and localization of high-polarized mitochondria at the periphery of blastomeres during early embryonic development. Enhanced mitochondrial activity seems to have detrimental effects on the developmental competence of bovine oocytes, and it is possibly related with oocyte aging *in vitro*. Future detailed studies on the relationship between enhanced high mitochondrial activity at fertilization and subsequent embryo development should be conducted.

Table 2-1. The effect of IVM duration of bovine oocytes on fertilization at 18 h after IVF

Duration of IVM (h)	No. of oocytes (replicates)	% of oocytes with			Total penetration (%)
		normal fertilization*	polyspermy	asynchronous fertilization**	
22	45 (4)	84.6±8.4	8.6±9.2	0.0±0.0 ^{a)}	93.2±8.7
30 to 34	77 (7)	79.1±13.7	9.2±10.5	11.7±6.9 ^{b)}	100±0.0

^{a,b)} Values (mean ± SD) with different superscripts within columns are significantly different ($P < 0.01$).

* normal fertilization: male and female pronuclei or an enlarged sperm head and anaphase II/telophase II chromosome

** asynchronous fertilization: an enlarged sperm head and female PN or male PN and telophase II chromosome

Table 2-2. The effect of IVM duration of bovine oocytes on embryonic development at 2 and 7 days after IVF

Duration of IVM (h)	No. of embryos (replicates)	% $\geq$ 2-cell /oocytes	% blastocyst /oocytes	% blastocyst /cleaved	Total cell no. in blastocysts (n)
22	161 (6)	81.1 $\pm$ 5.6 ^{A)}	51.6 $\pm$ 9.4 ^{a)}	64.1 $\pm$ 13.1 ^{a)}	172.5 $\pm$ 77.5 (83)
30 to 34	190 (7)	71.1 $\pm$ 10.6 ^{B)}	24.2 $\pm$ 8.8 ^{b)}	33.8 $\pm$ 11.4 ^{b)}	157.6 $\pm$ 56.5 (45)

^{a,b)} Values (mean $\pm$ SD) with different superscripts within the same column differ significantly ($P < 0.01$).

^{A,B)} Values (mean $\pm$ SD) with different superscripts within the same column tended to be different ($P = 0.06$).

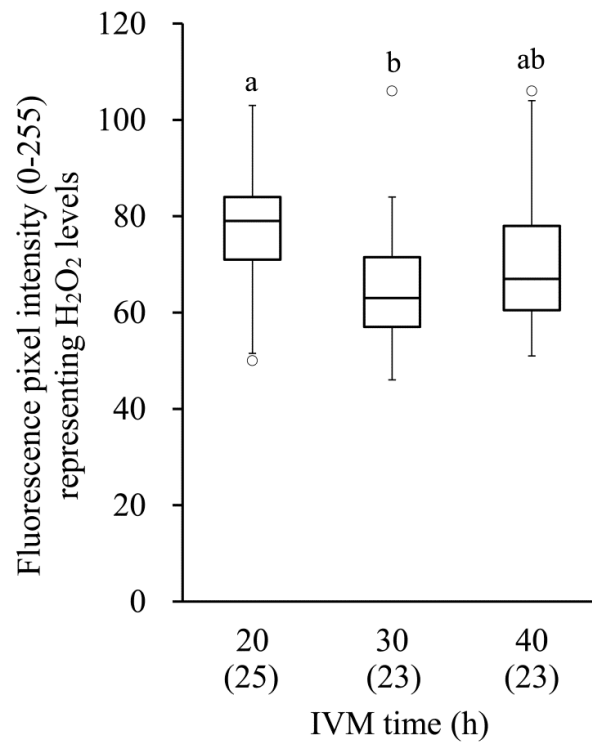


Fig. 2-1. Boxplots of the mean green intensity of DCF fluorescence in oocytes after IVM culture, which represents the H_2O_2 level.

The lower and upper ± 1.5 quartile are indicated by whiskers, the lower and upper ends of the boxes indicate the 25th and 75th quartiles, and the line across the middle of the box identifies the median sample value. The circles represent the outliers. ^{a,b} Values with different characters differ significantly among the three IVM groups ($P < 0.01$). Numbers of oocytes used are indicated in parentheses (2 replicates).

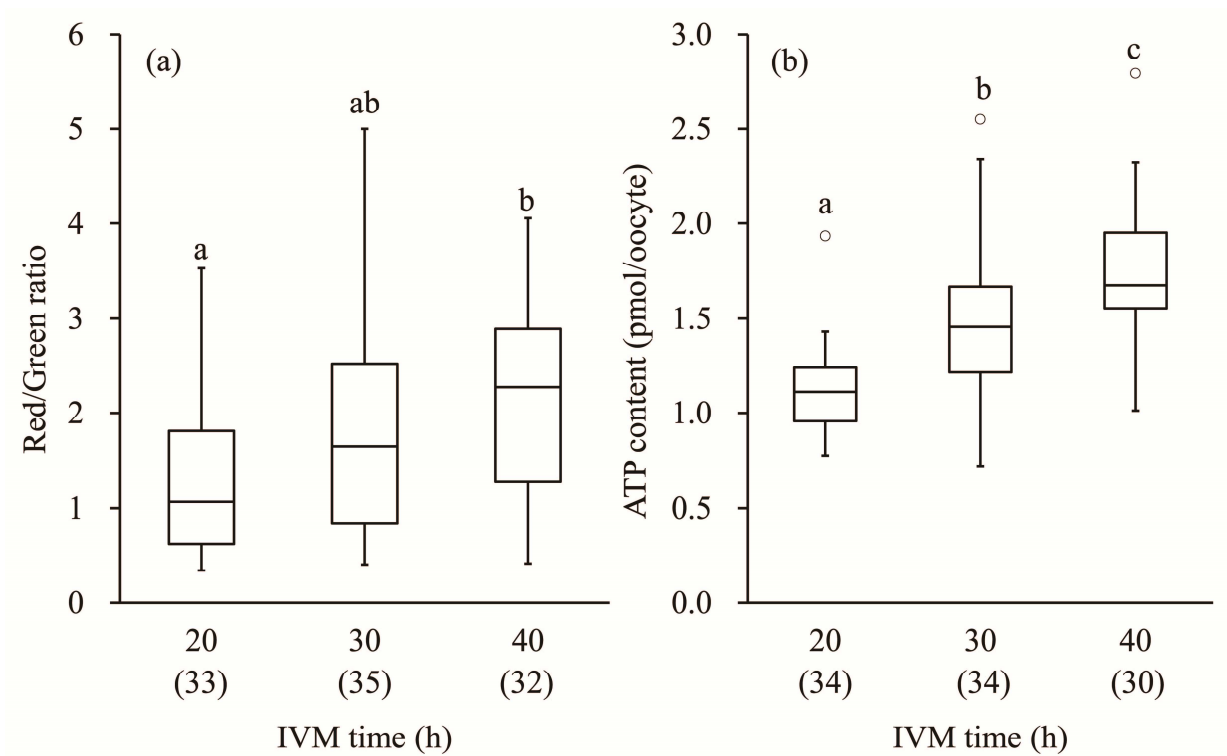


Fig. 2-2. Boxplots of mitochondrial activity (a) and ATP content (b) in oocytes after IVM culture.

The lower and upper ± 1.5 quartile are indicated by whiskers, the lower and upper ends of the boxes indicate the 25th and 75th quartiles, and the line across the middle of the box identifies the median sample value. The circles represent the outliers. ^{a,b,c} Values with different characters differ significantly among the three IVM groups ($P < 0.01$). Numbers of oocytes used are indicated in parentheses (3 replicates).

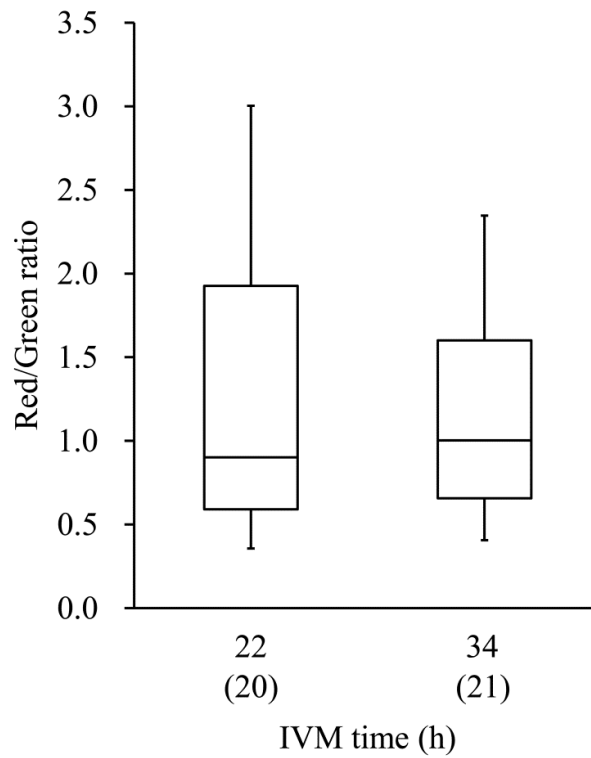


Fig. 2-3. Mitochondrial activity in ≥ 2 -cell embryos derived from the oocytes subjected to 22 and 34 h of IVM.

The lower and upper ± 1.5 quartile are indicated by whiskers, the lower and upper ends of the boxes indicate the 25th and 75th quartiles, and the line across the middle of the box identifies the median sample value. Numbers of oocytes used are indicated in parentheses (1 replicate).

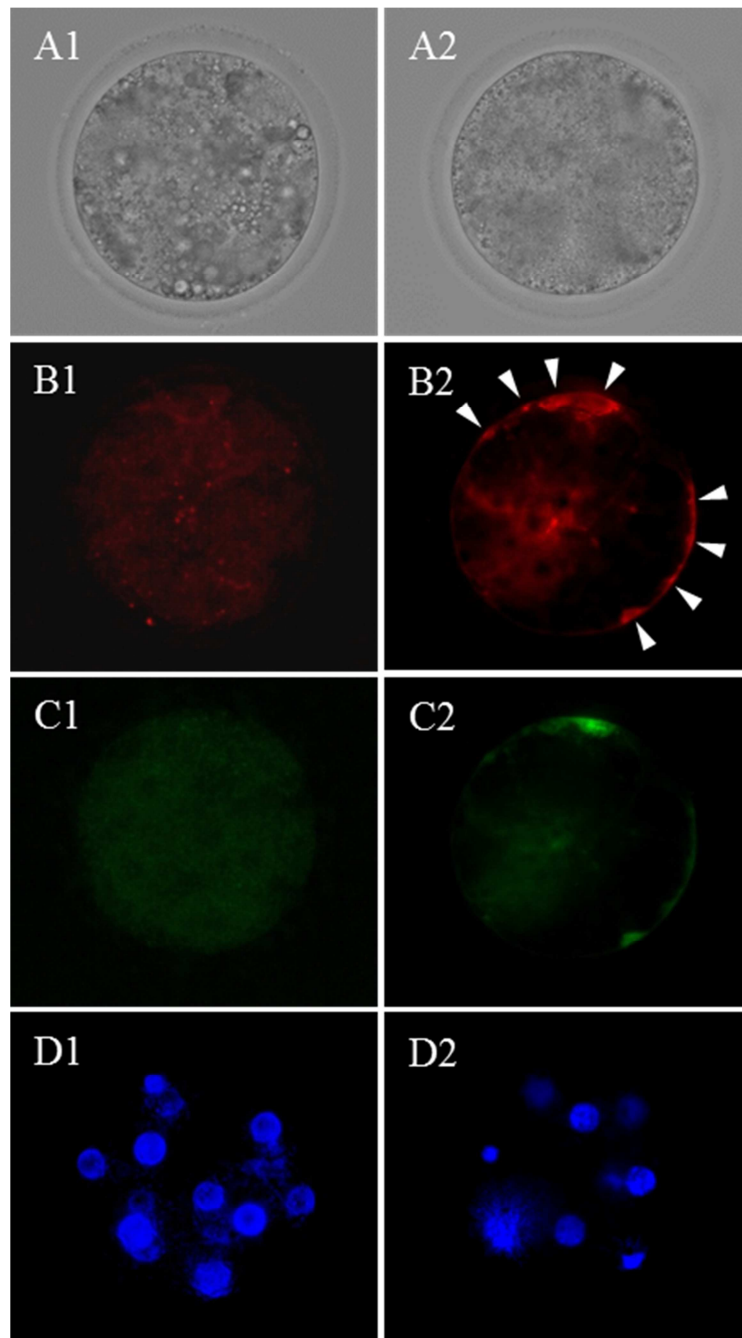


Fig. 2-4. Bright field (A) and fluorescent micrographs (B, C and D) of embryos (72 hpi) derived from the oocytes subjected to 22 (1) and 34 h (2) of IVM culture.

B, high-polarized mitochondria stained by JC-1: C, low-polarized mitochondria stained by JC-1: D, nuclei stained by Hoechst 33342. Twelve and 8 cells are observed in D1 and 2, respectively.

The embryo derived from the oocyte subjected to 22 h of IVM shows high-polarized mitochondria at the periphery of blastomeres (white arrows: B2).

Summary

The objective of this research was to clarify the aging-related changes in *in vitro*-matured bovine oocytes. Firstly, the author examined the fertilization and embryonic development of bovine oocytes after 22 and 30 to 34 h of IVM. The oocytes after 30 to 34 h of IVM (penetrated by sperm at around 40 h after starting IVM) showed a lower developmental rate to blastocysts ($P < 0.01$), although normal fertilization rates were similar regardless of IVM duration. In the next experiment, ROS (evaluated by H_2O_2), mitochondrial activity and ATP content in oocytes after 20, 30 and 40 h of IVM were examined. ROS in the group subjected to 30 h of IVM showed lowest value in all groups. The mitochondrial activity and the ATP content in the group subjected to 40 h of IVM were higher than in the group subjected to 20 h ($P < 0.01$), and those in the group subjected to 30 h of IVM showed intermediate values in all groups. Thereafter, mitochondrial activities at 3 days after IVF in embryos derived from the oocytes subjected to 22 and 34 h of IVM were evaluated. In the group subjected to 34 h of IVM, high-polarized mitochondria were frequently observed at the periphery of blastomeres. The results suggest that high mitochondrial activity observed in oocytes at prolonged duration after IVM start and localization of high-polarized mitochondria at the periphery of blastomeres during early embryonic development may be associated with the low developmental competence in aged bovine oocytes.

Summary and Conclusion

Nowadays IVP of bovine embryos is general technique and commercially used; however, the developmental competence of *in vitro*-matured bovine oocytes is still lower than that of *in vivo*-matured bovine oocytes. Nuclear and cytoplasmic maturation are essential for oocytes to acquire developmental competence during IVM. After the completion of nuclear maturation, oocytes gradually accomplish cytoplasmic maturation and acquire developmental competence. Then, they maintain that competences of fertilization and embryonic development for a certain period, but eventually undergo deterioration in their quality and lose developmental competence. This phenomenon is called “oocyte aging”. In this thesis, the author conducted the study to estimate the timing when bovine oocytes acquire and lose their developmental competence during IVM culture and investigate aging-related changes in *in vitro*-matured bovine oocytes, especially focusing on the functions of mitochondria during IVM culture and early embryonic development.

In chapter 1, the objective was to estimate the timing when bovine oocytes acquire and lose their developmental competence during IVM culture. The embryonic development of *in vitro*-matured bovine oocytes based on the times of nuclear maturation and sperm penetration was investigated. Firstly, COCs were subjected to IVM for 14 to 22 h. The timing when 50% of oocytes reached the M-II stage was estimated to be 17.5 h after IVM start. Secondly, using oocytes subjected to IVM for 12 to 30 h, sperm penetration was examined after 4 to 18 h of IVF. A significant negative correlation between IVM duration and the timing when 50% of oocytes were penetrated by sperm after IVF start was observed. Finally, oocytes subjected to 12 to 30 h of IVM were inseminated and cultured for 6 days to examine embryonic development. It was showed that bovine oocytes had their highest developmental competence at around 12 h after achieving nuclear maturation (i.e., around 30 h after the initiation of IVM culture), and began to lose gradually their developmental competence after this

timing. Therefore bovine oocytes at 30 h after the initiation of IVM should be considered as fully matured oocytes, not as aged ones.

In chapter 2, the objective was to clarify the aging-related changes in *in vitro*-matured bovine oocytes. The oocytes subjected to IVM for 20 h (oocytes immediately after M-II arrival), 30 h (fully matured oocytes with high developmental competence) and 40 h (aged oocytes) served in the determination of ROS production (evaluated by H_2O_2), mitochondrial activity and ATP content. ROS in the oocytes after 30 h of IVM was lower than in the oocytes after 20 h of IVM, and this result showed that the oocytes after 30 h of IVM had high competence to protect against oxidative stress. The mitochondrial activity and ATP content in bovine oocytes subjected to 40 h of IVM were highest among all IVM durations. In addition, mitochondrial activities at 3 days after IVF in embryos derived from the oocytes subjected to 22 h (penetrated by sperm at around 30 h after starting IVM) and 34 h (penetrated by sperm at around 40 h after starting IVM) of IVM were evaluated. In the group subjected to 34 h of IVM, high-polarized mitochondria were frequently observed at the periphery of blastomeres. These high-polarized mitochondria at the periphery of blastomeres might have already been extruded from blastomeres. It is suggested that high mitochondrial activity observed in oocytes at prolonged duration after IVM start and localization of high-polarized mitochondria at the periphery of blastomeres during early embryonic development may be associated with the low developmental competence in aged bovine oocytes.

The present study demonstrates that bovine oocytes acquire their highest developmental competence at around 30 h after the initiation of IVM culture and then begin to lose their developmental competence gradually. Moreover, it is showed that, in contrast to the past reports, the mitochondria activity increases with aging of *in vitro*-matured bovine oocytes. Therefore, this depression of developmental competence in *in vitro*-matured bovine oocytes is possibly related with the enhanced mitochondrial activity.

Acknowledges

The author would like to acknowledge Emeritus Professor Yoshiyuki Takahashi, Hokkaido University, and Associate Professor Masashi Nagano, Laboratory of Theriogenology, Graduate School of Veterinary Medicine, Hokkaido University, for valuable advice to this manuscript and review of the present manuscript.

The author is deeply grateful to Professor Mitsuyoshi Takiguchi, Laboratory of Veterinary Internal Medicine, Graduate School of Veterinary Medicine, Hokkaido University, and Professor Toshio Tsubota, Laboratory of Wildlife Biology and Medicine, Graduate School of Veterinary Medicine, Hokkaido University, for reviewing manuscript, valuable advices and suggestions.

The author especially thanks to Assistant Professor Yojiro Yanagawa, Laboratory of Theriogenology, Graduate School of Veterinary Medicine, Hokkaido University, for providing encouragement and valuable advice. The author is indebted to Mr. Sung-Sik Kang, and Mr. Weiping Huang, Laboratory of Theriogenology, Graduate School of Veterinary Medicine, Hokkaido University, for their great supports. Then, the author thanks to all my colleagues in the Laboratory of Theriogenology, Graduate School of Veterinary Medicine, Hokkaido University.

Finally, the author would like to express my gratitude to my parents and wife for supporting me all through the time.

Reference

- Agung B, Otoi T, Wongsrikeao P, Taniguchi M, Shimizu R, Watari H, Nagai T, 2006: Effect of maturation culture period of oocytes on the sex ratio of *in vitro* fertilized bovine embryos. *J Reprod Dev* **52**, 123-127.
- Badr H, Bongioni G, Abdoon AS, Kandil O, Puglisi R, 2007: Gene expression in the *in vitro*-produced preimplantation bovine embryos. *Zygote* **15**, 355-367.
- Bavister BD, Leibfried ML, Lieberman G, 1983: Development of preimplantation embryos of the golden hamster in a defined culture medium. *Biol Reprod* **28**, 235-247.
- Cha KY, Koo JJ, Ko JJ, Choi DH, Han SY, Yoon TK, 1991: Pregnancy after *in vitro* fertilization of human follicular oocytes collected from nonstimulated cycles, their culture *in vitro* and their transfer in a donor oocyte program. *Fertil Steril* **55**, 109-113.
- Chen L, Wert SE, Hendrix EM, Russell PT, Cannon M, Larsen WJ, 1990: Hyaluronic acid synthesis and gap junction endocytosis are necessary for normal expansion of the cumulus mass. *Mol Reprod Dev* **26**, 236-247.
- Chi MM, Manchester JK, Yang VC, Curato AD, Strickler RC, Lowry OH, 1988: Contrast in levels of metabolic enzymes in human and mouse ova. *Biol Reprod* **39**, 295-307.
- Critser ES, Leibfried-Rutledge ML, Eyestone WH, Northey DL, First NL, 1986: Acquisition of developmental competence during maturation *in vitro*. *Theriogenology* **25**, 150.
- Crozet N, Huneau D, Desmedt V, Theron MC, Szollosi D, Torres S, Sevellec C, 1987: *In vitro* fertilization with normal development in the sheep. *Gamete Res* **16**, 159-170.
- Dominko T, First NL, 1997: Timing of meiotic progression in bovine oocytes and its effect on early embryo development. *Mol Reprod Dev* **47**, 456-467.
- Edwards JL, Saxton AM, Lawrence JL, Payton RR, Dunlap JR, 2005: Exposure to a Physiologically

- Relevant Elevated Temperature Hastens *In vitro* Maturation in Bovine Oocytes. J Dairy Sci **88**, 4326-4333.
- Emuta C, Horiuchi T, 2001: Effects of timing of activation and aging of bovine oocytes fertilized by intracytoplasmic sperm injection (ICSI) on cleavage and subsequent embryonic development *in vitro*. J Reprod Dev **47**, 399-405.
- Eppig JJ, 1996: Coordination of nuclear and cytoplasmic oocyte maturation in eutherian mammals. Reprod Fertil Dev **8**, 485-489.
- Ferreira EM, Vireque AA, Adona PR, Meirelles FV, Ferriani RA, Navarro PA, 2009: Cytoplasmic maturation of bovine oocytes: structural and biochemical modifications and acquisition of developmental competence. Theriogenology **71**, 836-848.
- Hanada A, Shioya Y, Suzuki T, 1986: Birth of calves from non-surgical transfer of blastocysts originated from *in vitro* fertilized oocytes matured *in vitro*. Proc 78th Ann Meet Jpn Soc Zootech Sci, 18.
- Hao Z, Liu S, Wu Y, Wan P, Cui M, Chen H, Zeng S, 2009: Abnormal changes in mitochondria, lipid droplets, ATP and glutathione content, and Ca^{2+} release after electro-activation contribute to poor developmental competence of porcine oocyte during *in vitro* ageing. Reprod Fertil Dev **21**, 323-332.
- Harrison KL, Wilson LM, Breen TM, Pope AK, Cummins JM, Hennessey JF, 1988: Fertilization of human oocytes in relation to varying delay before insemination. Fertil Steril **50**, 294-297.
- Igarashi H, Takahashi T, Takahashi E, Tezuka N, Nakahara K, Takahashi K, Kurachi H, 2005: Aged mouse oocytes fail to readjust intracellular adenosine triphosphates at fertilization. Biol Reprod **72**, 1256-1261.
- Iwata H, Hashimoto S, Ohota M, Kimura K, Shibano K, Miyake M, 2004: Effects of follicle size and electrolytes and glucose in maturation medium on nuclear maturation and developmental

- competence of bovine oocytes. *Reproduction* **127**, 159-164.
- Iwata H, Ohota M, Hashimoto S, Nagai Y, 2003: Free oxygen radicals are generated at the time of aspiration of oocytes from ovaries that have been stored for a long time. *Zygote* **11**, 1-5.
- Izadyar F, Colenbrander B, Bevers MM, 1996: *In vitro* maturation of bovine oocytes in the presence of growth hormone accelerates nuclear maturation and promotes subsequent embryonic development. *Mol Reprod Dev* **45**, 372-377.
- Kruip TAM, Cran DG, Beneden THv, Dieleman SJ, 1983: Structural changes in bovine oocytes during final maturation *in vivo*. *Gamete Res* **8**, 29-47.
- Leidenfrost S, Boelhauve M, Reichenbach M, Gungor T, Reichenbach HD, Sinowatz F, Wolf E, Habermann FA, 2011: Cell arrest and cell death in mammalian preimplantation development: lessons from the bovine model. *PLoS One* **6**, e22121.
- Lonergan P, Fair T, 2014: The ART of studying early embryo development: Progress and challenges in ruminant embryo culture. *Theriogenology* **81**, 49-55.
- Long CR, Damiani P, Pinto-Correia C, MacLean RA, Duby RT, Robl JM, 1994: Morphology and subsequent development in culture of bovine oocytes matured *in vitro* under various conditions of fertilization. *J Reprod Fertil* **102**, 361-369.
- Lord T, Aitken RJ, 2013: Oxidative stress and ageing of the post-ovulatory oocyte. *Reproduction* **146**, R217-227.
- Lord T, Nixon B, Jones KT, Aitken RJ, 2013: Melatonin prevents postovulatory oocyte aging in the mouse and extends the window for optimal fertilization *in vitro*. *Biol Reprod* **88**, 67.
- Malhi PS, Adams GP, Singh J, 2005: Bovine model for the study of reproductive aging in women: follicular, luteal, and endocrine characteristics. *Biol Reprod* **73**, 45-53.
- Marei WF, Wathes DC, Fouladi-Nashta AA, 2012: Differential effects of linoleic and alpha-linolenic fatty acids on spatial and temporal mitochondrial distribution and activity in bovine oocytes.

- Reprod Fertil Dev **24**, 679-690.
- Mattioli M, Bacci ML, Galeati G, Seren E, 1989: Developmental competence of pig oocytes matured and fertilized *in vitro*. Theriogenology **31**, 1201-1207.
- Merton JS, de Roos AP, Mullaart E, de Ruigh L, Kaal L, Vos PL, Dieleman SJ, 2003: Factors affecting oocyte quality and quantity in commercial application of embryo technologies in the cattle breeding industry. Theriogenology **59**, 651-674.
- Merton JS, de Roos APW, Koenen EPC, Roelen BAJ, Vos P, Mullaart E, Knijn HM, 2012: Bovine OPU-derived oocytes can be matured *in vitro* for 16-28 h with similar developmental capacity. Reprod Domest Anim **47**, 1037-1042.
- Miao Y-L, Kikuchi K, Sun Q-Y, Schatten H, 2009: Oocyte aging: cellular and molecular changes, developmental potential and reversal possibility. Hum Reprod Update **15**, 573-585.
- Morado SA, Cetica PD, Beconi MT, Dalvit GC, 2009: Reactive oxygen species in bovine oocyte maturation *in vitro*. Reprod Fertil Dev **21**, 608-614.
- Nabenishi H, Takagi S, Kamata H, Nishimoto T, Morita T, Ashizawa K, Tsuzuki Y, 2012: The role of mitochondrial transition pores on bovine oocyte competence after heat stress, as determined by effects of cyclosporin A. Mol Reprod Dev **79**, 31-40.
- Nagano M, Katagiri S, Takahashi Y, 2006a: Relationship between bovine oocyte morphology and *in vitro* developmental potential. Zygote **14**, 53-61.
- Nagano M, Katagiri S, Takahashi Y, 2006b: ATP content and maturational/developmental ability of bovine oocytes with various cytoplasmic morphologies. Zygote **14**, 299-304.
- Nasr-Esfahani MH, Aitken JR, Johnson MH, 1990: Hydrogen peroxide levels in mouse oocytes and early cleavage stage embryos developed *in vitro* or *in vivo*. Development **109**, 501-507.
- Park YS, Kim SS, Kim JM, Park HD, Byun MD, 2005: The effects of duration of *in vitro* maturation of bovine oocytes on subsequent development, quality and transfer of embryos.

- Theriogenology **64**, 123-134.
- Reers M, Smith TW, Chen LB, 1991: J-aggregate formation of a carbocyanine as a quantitative fluorescent indicator of membrane potential. *Biochemistry* **30**, 4480-4486.
- Rieger D, 1997: Batch analysis of the ATP content of bovine sperm, oocytes, and early embryos using a scintillation counter to measure the chemiluminescence produced by the luciferin-luciferase reaction. *Anal Biochem* **246**, 67-70.
- Rispoli LA, Lawrence JL, Payton RR, Saxton AM, Schrock GE, Schrick FN, Middlebrooks BW, Dunlap JR, Parrish JJ, Edwards JL, 2011: Disparate consequences of heat stress exposure during meiotic maturation: embryo development after chemical activation vs fertilization of bovine oocytes. *Reproduction* **142**, 831-843.
- Rizos D, Ward F, Duffy P, Boland MP, Lonergan P, 2002: Consequences of bovine oocyte maturation, fertilization or early embryo development *in vitro* versus *in vivo*: implications for blastocyst yield and blastocyst quality. *Mol Reprod Dev* **61**, 234-248.
- Roelofs JB, Graat EA, Mullaart E, Soede NM, Voskamp-Harkema W, Kemp B, 2006: Effects of insemination-ovulation interval on fertilization rates and embryo characteristics in dairy cattle. *Theriogenology* **66**, 2173-2181.
- Saacke RG, Dalton JC, Nadir S, Nebel RL, Bame JH, 2000: Relationship of seminal traits and insemination time to fertilization rate and embryo quality. *Anim Reprod Sci.* **60-61**, 663-677.
- Saeki K, Kato H, Hosoi Y, Miyake M, Utsumi K, Iritani A, 1991: Early morphological events of *in vitro* fertilized bovine oocytes with frozen-thawed spermatozoa. *Theriogenology* **35**, 1051-1058.
- Saeki K, Nagao Y, Hoshi M, Nagai M, 1995: Effects of heparin, sperm concentration and bull variation on *in vitro* fertilization of bovine oocytes in a protein-free medium. *Theriogenology* **43**, 751-759.

- Salustri A, Yanagishita M, Underhill CB, Laurent TC, Hascall VC, 1992: Localization and synthesis of hyaluronic acid in the cumulus cells and mural granulosa cells of the preovulatory follicle. *Dev Biol* **151**, 541-551.
- Schroeder AC, Eppig JJ, 1984: The developmental capacity of mouse oocytes that matured spontaneously *in vitro* is normal. *Dev Biol* **102**, 493-497.
- Sirard MA, Florman HM, Leibfried-Rutledge ML, Barnes FL, Sims ML, First NL, 1989: Timing of nuclear progression and protein synthesis necessary for meiotic maturation of bovine oocytes. *Biol Reprod* **40**, 1257-1263.
- Somfai T, Kikuchi K, Kaneda M, Akagi S, Watanabe S, Mizutani E, Haraguchi S, Dang-Nguyen TQ, Inaba Y, Geshi M, Nagai T, 2011: Cytoskeletal abnormalities in relation with meiotic competence and ageing in porcine and bovine oocytes during *in vitro* maturation. *Anat Histol Embryol* **40**, 335-344.
- Stojkovic M, Machado SA, Stojkovic P, Zakhartchenko V, Hutzler P, Goncalves PB, Wolf E, 2001: Mitochondrial distribution and adenosine triphosphate content of bovine oocytes before and after *in vitro* maturation: correlation with morphological criteria and developmental capacity after *in vitro* fertilization and culture. *Biol Reprod* **64**, 904-909.
- Sugimura S, Matoba S, Hashiyada Y, Aikawa Y, Ohtake M, Matsuda H, Kobayashi S, Konishi K, Imai K, 2012: Oxidative phosphorylation-linked respiration in individual bovine oocyte. *J Reprod Dev* **58**, 636-641.
- Suss U, Wuthrich K, Stranzinger G, 1988: Chromosome configurations and time sequence of the first meiotic division in bovine oocytes matured *in vitro*. *Biol Reprod* **38**, 871-880.
- Suzuki H, Moriguchi M, Kida R, Moro Y, 1996: Delay in ovulation and fertilization and asynchronous pronuclear development in aged hamsters. *J Reprod Dev* **42**, 15-22.
- Takahashi T, Igarashi H, Kawagoe J, Amita M, Hara S, Kurachi H, 2009: Poor embryo development

- in mouse oocytes aged *in vitro* is associated with impaired calcium homeostasis. *Biol Reprod* **80**, 493-502.
- Takahashi Y, First NL, 1992: *In vitro* development of bovine one-cell embryos: Influence of glucose, lactate, pyruvate, amino acids and vitamins. *Theriogenology* **37**, 963-978.
- Takahashi Y, First NL, 1993: *In vitro* fertilization of bovine oocytes in the presence of theophylline. *Anim Reprod Sci* **34**, 1-18.
- Takahashi Y, Kanagawa H, 1998a: Effect of oxygen concentration in the gas atmosphere during *in vitro* insemination of bovine oocytes on the subsequent embryonic development *in vitro*. *J Vet Med Sci* **60**, 365-367.
- Takahashi Y, Kanagawa H, 1998b: Effects of glutamine, glycine and taurine on the development of *in vitro* fertilized bovine zygotes in a chemically defined medium. *J Vet Med Sci* **60**, 433-437.
- Takahashi Y, Hishinuma M, Matsui M, Tanaka H, Kanagawa H, 1996: Development of *in vitro* matured/fertilized bovine embryos in a chemically defined medium: influence of oxygen concentration in the gas atmosphere. *J Vet Med Sci* **58**, 897-902.
- Tamassia M, Nuttinck F, May-Panloup P, Reynier P, Heyman Y, Charpigny G, Stojkovic M, Hiendleder S, Renard JP, Chastant-Maillard S, 2004: *In vitro* embryo production efficiency in cattle and its association with oocyte adenosine triphosphate content, quantity of mitochondrial DNA, and mitochondrial DNA haplogroup. *Biol Reprod* **71**, 697-704.
- Tang DW, Fang Y, Liu ZX, Wu Y, Wang XL, Zhao S, Han GC, Zeng SM, 2013: The disturbances of endoplasmic reticulum calcium homeostasis caused by increased intracellular reactive oxygen species contributes to fragmentation in aged porcine oocytes. *Biol Reprod* **89**, 124.
- Tarazona AM, Rodriguez JI, Restrepo LF, Olivera-Angel M, 2006: Mitochondrial activity, distribution and segregation in bovine oocytes and in embryos produced *in vitro*. *Reprod Domest Anim* **41**, 5-11.

- Thibault C, Gerard M, Menezo Y, 1975: Preovulatory and ovulatory mechanisms in oocyte maturation. *J Reprod Fertil* **45**, 605-610.
- Tian XC, Lonergan P, Jeong BS, Evans AC, Yang X, 2002: Association of MPF, MAPK, and nuclear progression dynamics during activation of young and aged bovine oocytes. *Mol Reprod Dev* **62**, 132-138.
- Van Blerkom J, 2008: Mitochondria as regulatory forces in oocytes, preimplantation embryos and stem cells. *Reprod Biomed Online* **16**, 553-569.
- Van Blerkom J, Davis P, 2006: High-polarized ($\Delta\psi_m$ (HIGH)) mitochondria are spatially polarized in human oocytes and early embryos in stable subplasmalemmal domains: developmental significance and the concept of vanguard mitochondria. *Reprod Biomed Online* **13**, 246-254.
- Wang LY, Wang DH, Zou XY, Xu CM, 2009: Mitochondrial functions on oocytes and preimplantation embryos. *J Zhejiang Univ Sci B* **10**, 483-492.
- Ward F, Enright B, Rizos D, Boland M, Lonergan P, 2002: Optimization of *in vitro* bovine embryo production: effect of duration of maturation, length of gamete co-incubation, sperm concentration and sire. *Theriogenology* **57**, 2105-2117.
- Wilding M, Dale B, Marino M, di Matteo L, Alviggi C, Pisaturo ML, Lombardi L, De Placido G, 2001: Mitochondrial aggregation patterns and activity in human oocytes and preimplantation embryos. *Hum Reprod* **16**, 909-917.
- Wrenzycki C, Stinshoff H, 2013: Maturation environment and impact on subsequent developmental competence of bovine oocytes. *Reprod Domest Anim* **48 Suppl 1**, 38-43.
- Yanagida K, Yazawa H, Katayose H, Suzuki K, Hoshi K, Sato A, 1998: Influence of oocyte preincubation time on fertilization after intracytoplasmic sperm injection. *Hum Reprod* **13**, 2223-2226.

Zhang N, Wakai T, Fissore RA, 2011: Caffeine alleviates the deterioration of Ca^{2+} release mechanisms and fragmentation of *in vitro*-aged mouse eggs. Mol Reprod Dev **78**, 684-701.

Summary in Japanese

牛の体外受精・胚移植技術、特に体外成熟卵子を用いた体外受精技術は目覚ましく発展し、牛の増殖・改良に貢献している。現在では、牛体外成熟卵子から胚移植後に着床可能な胚盤胞が発生する割合は約 40%にまで向上したが、体内成熟卵子に比べて体外成熟卵子の発生能は低く、牛卵子の体外成熟培養系の改善が期待されている。

卵胞内の未成熟卵子は第一減数分裂前期で分裂が停止している状態から黄体形成ホルモンの刺激等を受け、減数分裂を再開する。そして、第二減数分裂中期 (M-II 期) に達した後に、再び減数分裂が停止する。この M-II 期に達するまでの過程を核成熟と言う。卵子は核成熟後に正常な受精および発生に必要な細胞質の機能的変化である細胞質成熟を完了し、発生能を獲得すると考えられている。発生能を獲得した卵子はしばらくの間、発生能を維持した後、これを徐々に失う。この発生能の低下が卵子の老化と考えられている。ヒトやマウスの卵子では、老化に伴う形態学的あるいは細胞学的な変化が報告されているが、牛卵子においては老化に関する研究がほとんど行われておらず、牛卵子が核成熟に達した後の発生能獲得および低下時期は不明である。体外成熟により高い胚盤胞への発生率を得るためには、老化により発生能が低下する前に卵子は受精する必要がある。そこで本研究では、牛卵子を成熟培養して核成熟に達した後の発生能獲得および低下時期を推定するとともに、老化過程にある卵子内のミトコンドリア活性に関連した項目の変化について検討した。

第1章は、牛卵子の体外成熟時における発生能獲得および低下時期を推定することを目的とした。まず本研究で用いた培養系における核成熟完了時期および媒精後の精子侵入時期を明らかにするために、食肉検査場由来の牛卵巣から回収した卵子を用いて成熟培養および体外受精を行った。その結果、核成熟完了時期は成熟培養開始後 17.5 時間であり、成熟培養時間と媒精から精子侵入までの時間の間には負の相関が見られた。次に、成熟培養、体外受精および発育培養を行い、核成熟完了から精子侵入までの時間と胚盤胞への発生率との関連性を調べた。その結果、核成熟完

了後約 12 時間 (成熟培養開始後約 30 時間) に精子が侵入した卵子の胚盤胞への発生率が最も高くなると推定された。また、精子侵入時期が成熟培養開始 30 時間以降になることによって、卵子の胚盤胞への発生率は低下することが推定された。以上の結果から、本研究の条件で成熟培養した牛の未成熟卵子は、成熟培養開始後約 30 時間で発生能が最も高くなり、その後、発生能が低下することが明らかとなった。

第 2 章は、体外成熟時における老化に伴う牛卵子内での変化を明らかにすることを目的とした。成熟培養開始後 20 時間 (核成熟完了直後の卵子)、30 時間 (高い発生能を有する卵子) および 40 時間 (老化卵子) 目の牛卵子内における活性酸素種 (H_2O_2) 産生量、ミトコンドリア活性および ATP 含有量を調べた。その結果、成熟培養開始後 30 時間目の卵子内の H_2O_2 量は最も低く、この時期の卵子の発生能が高くなるのは、抗酸化能が高いためと推察された。また、成熟培養開始後 40 時間経過した老化卵子では、ミトコンドリア活性が高まり、ATP 含有量も多くなった。次に、成熟培養 22 あるいは 34 時間後に媒精して、その 3 日後に得られた胚の割球中のミトコンドリア活性を調べた。その結果、成熟培養 34 時間後に媒精して得られた胚 (精子侵入は成熟培養開始後 40 時間目と推定) においては、高活性のミトコンドリアが割球辺縁に多く見られた。また、割球辺縁に存在するミトコンドリアは、割球外に放出されていると推定された。以上の結果から、成熟培養開始から長時間経過した老化卵子で見られる胚発生能の低下は、活性化したミトコンドリアが初期胚発生時に割球辺縁へ局在することに関連していることが示唆された。

本研究の結果、牛の体外成熟卵子の発生能は、核成熟後約 12 時間、すなわち成熟培養開始後約 30 時間目に最も高くなり、その後は徐々に低下することが明らかとなった。また、成熟培養開始後 40 時間経過した老化卵子ではミトコンドリア活性が高まり、ATP 含有量が増加していることが明らかになった。さらに、体外成熟後に老化した牛卵子はミトコンドリアを制御する能力を失い、ミトコンドリアの活性化により体外受精後の発生能が低下することが示唆された。