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Application of *In Vitro* Maturation to Assisted Reproductive Technology

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Abstract. *In vitro* maturation (IVM) of immature oocytes is an important reproductive technology that generates mature oocytes that are capable of supporting preimplantation embryo development and full development to turm. The developmental competence of oocytes after *in vitro* fertilization (IVF) was supported in cumulus-cell density dependent manner. The developmental competence of oocytes matured under low oxygen (5% O_2) tension was higher than that of oocytes matured under high oxygen tensinon (20% O_2) due to the decrease of reactive oxygen content in the oocyte. To expand the availability of female gamete, an attempt to culture the growing oocytes have been made. We have focused attention on the mitochondrial activity of *in vitro* grown (IVG) oocytes and shown that L-Carnitine, which acts as a carrier of fatty acids across the inner mitochondrial membrane for subsequent β-oxidation, supported the viability of growing oocytes. Nowadays, IVM is gaining acceptance in assisted reproductive technology in cattle and human.

Key words: Cumulus cell density, Meiotic arrest, Mitochondrial activity of growing oocytes, Oocyte transportation, Transvaginal oocyte pick up

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Animal breeders, especially cattle breeders have been trying to get as many offspring from their genetically superior or important animals as possible. Thus, hormonal induction of multiple ovulations, followed by non-surgical collection of the embryos and embryo transfer to recipients is widely used. This multiple ovulation and embryo transfer procedure can be repeated in the same animal about 4 times per year in cattle. However, the yield of transferable embryos is highly variable. No more than an average of 20 embryos per year per animal can be obtained in cattle [1]. Feedback on the endocrinological regulation of follicle growth and maturation also limits the number of oocytes that can mature and develop *in vivo* [2]. Thus embryo production *in vivo* by means of super-ovulation may lead to less than 100 embryos over the lifespan of an individual cow, whereas approximately 100,000 gametes are present in the ovaries. If more gametes could be matured, fertilized and developed *in vitro*, production would increase correspondingly. Oocytes from slaughterhouse ovaries [3–5] have been used for the production of bovine embryos *in vitro*. Moreover, *in vitro* embryo production from live cows has been established [6]. However, the ability of bovine oocytes matured *in vitro* to develop to the morula/blastocyst stage is less than that of *in vivo*-matured oocytes [7, 8]. The decrease in the developmental competence of *in vitro*-matured oocytes may be due to insufficient cytoplasmic maturity.

In this paper, 1) bovine oocyte-pick-up (OPU) from live cows, 2) transportation of bovine immature oocytes, 3) improvement of *in vitro* maturation (IVM) of bovine immature oocytes, 4) application of IVM culture to human assisted reproductive technology (ART), 5) IVM following meiotic inhibition with butyrolactone I under low oxygen tension, and 6) *in vitro* growth (IVG) of pig and rabbit growing oocytes were described.

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Oocyte Collection

Ultrasound-guided transvaginal aspiration of bovine immature oocytes has been proven to be a successful and repeatable technique for the production of embryos and calves from donors with normal estrous cycles, problem donors and pregnant donors. Threfore, transvaginal oocyte aspiration followed by IVM, *in vitro* fertilization (IVF) and *in vitro* culture (IVC) has been thought to be a potential alternative to superovulation. A number of vacuum pressures have been used in various trials with the OPU technique in humans, ranging from 40 to 300 mmHg, with most of the trials using a vacuum pressure of about 100 mmHg. The combination of aspiration vacuum with the inner diameter of aspiration needle was crucial for the quality of cumulus-oocyte complexes (COCs) [9–11]. For example, optimal aspiration pressure were 40 mmHg when a 18-gauge needle was used and 120 mmHg when a 21-gauge needle was used [11]. However, recovery rate of oocytes by transvaginal aspiration from live cows was lower than that of syringe aspiration of ovaries from slaughtered cows [11]. The difficulty in grasping the ovaries correctly in live cows may be the cause of the low recovery rates. We assessed effect of the frequency of the ultrasonic transvaginal probe on the visualization of bovine follicles using 5.0 and 7.5 MHz probes. The number of follicles that had a clear outline with the 7.5 MHz probe was greater than that obtained with 5.0 MHz probe (Table 1) [12]. Furthermore, the recovery rates of oocytes and COCs based on the number of aspirated follicles obtained with the 7.5 MHz probe were greater than those obtained with the 5.0 MHz probe (Table 2) [12]. Today, the recovery rate of oocytes by transvaginal aspiration from live cows is almost the same as that of syringe aspiration of ovaries from

Table 1. The number of bovine small follicles (3 to 5 mm in diameter) of different clarity

Probe frequency	Number of small follicles visualized per ovary ± SEM		
	Follicles	With clear outline	With obscure outline
7.5 MHz	9.9 ± 1.1	9.0 ± 1.1[a]	0.9 ± 0.4[a]
5.0 MHz	7.5 ± 0.6	3.2 ± 0.8[b]	4.3 ± 0.8[b]

All data were obtained from 10 bovine ovaries which were obtained from a local slaughter abattoir and were selected randomly. [a,b]Values with different superscripts within the same column differ significantly (P<0.01).

Table 2. Effect of the frequency of the ultrasonic transvaginal probe on bovine oocyte collection from 3 cows

Probe frequncy	No. of follicles aspirated	Recovery rates[a] (%)			
		Total	COCs[b]	Denuded oocytes[c]	All others[d]
7.5 MHz	120	81.7[e]	65.8[e]	7.5	8.3
5.0 MHz	120	44.2[f]	35.8[f]	3.3	5.0

Oocyte aspiration was carried out 6 times on 20 follicles per treatment using 3- to 10-mm follicles of 6 Japanese-black-beef cows (6 to 7 yr old). [a] Number of collected oocytes per numbers of aspirated follicles. [b] Oocytes surrounded with more than 1 layer of cumulus cells. [c] Oocytes partially or completely denuded of their somatic cells. [d] Oocytes with expanded cumulus cells and degenerated oocytes. [e,f] Values with different superscripts within the same column differ significantly (P<0.01).

slaughtered cows when using a combination of 18 gauge aspiration needle with aspiration pressure of 40 mmHg with the 7.5 MHz probe [12].

Oocyte Transportation

In vitro maturation of bovine oocytes recovered from ovaries of slaughtered or live cows has become a prevalent source for such procedures as IVF, cloning, and other related reproductive technologies. Because of the limited time during which an oocyte remains viable and can be fertilized or activated, and the limited number of oocytes that can be collected on any given day, to develop the techniques to preserve bovine oocytes at germinal-vesicle stage will give great impact on the research and commercial applications. In the process of the spread of the technique of transvaginal oocyte retrieval, the case, to collect oocytes in the places that are vast distance from the *in vitro* embryo production laboratory, is increasing. Thus, the successful preservation of oocytes at germinal-vesicle stage using a simple and easy way will make it possible to transport oocytes collected from cows by ultrasound-guided transvaginal aspiration and will increase the availability of the collection of bovine oocytes. We attempted the oocytes transportation method using a protein synthesis inhibitor, cycloheximide (CHX). Oocytes were maintained in meiotic arrest by 10 μg/ml CHX in 25-mM HEPES-buffered TCM 199 (H199) at 39 C or synthetic oviduct fluid (HSOF) at 20 or 39 C in air for 24 h. The developmental rate of oocytes arrested in HSOF at 20 C to the blastocyst stage after IVF was similar to that of non-arrested oocytes, but was significantly higher (P<0.05) than that of oocytes arrested at 39 C in H199 or in HSOF (Fig. 1) [13]. In consideration of oocyte transportation conditions, we also investigated the meiotic arrest of oocytes maintained in a 0.25-ml straw by CHX individually with 10 μl HSOF at 20 C in air for 24 h. The developmental rate of oocytes treated with CHX individually was similar to that of those treated with CHX in 50-μl droplet of HSOF at 20 C (Fig. 2) [13]. Furthermore, two calves were delivered after embryo transfer which were developed from oocytes maintained in meiotic arrest in a plastic straw. The data showed bovine oocytes could be stored and transported under air with CHX treatment. This will make it possible to transport oocytes collected from cows by ultrasound-guided transvaginal aspiration and will increase the availability of donor cows.

In Vitro Maturation

Cumulus-cell density

It has been suggested that both the presence of cumulus cells and cell-oocyte contact are crucial for oocyte maturation to attain developmental competence [14, 15]. In addition, the developmental competence of bovine oocytes surrounded with corona cells was induced in a cell density-dependent manner in the maturation medium (Table 3) [16]. This supporting mechanism has been shown to be partialy due to a soluble factors secreted from cumulus cells [16–18]. Moreover, the developmental competence of oocytes retrieved from live cows and matured in proper cumulus cell density was similar to that of oocytes retrieved from slaughterhouse (Table 4).

Nuclear maturation under low oxygen tension

Several studies have indicated that mammalian embryos develop better at reduced oxygen tensions: (a) oxygen at the atmospheric level was found to be toxic to mammalian embryos, probably due to the formation of free oxygen radicals [19]; (b) The oxygen tension in the female reproductive tract is very low compared with that of atmospheric oxygen [20–28]; (c) *In vitro* embryo development

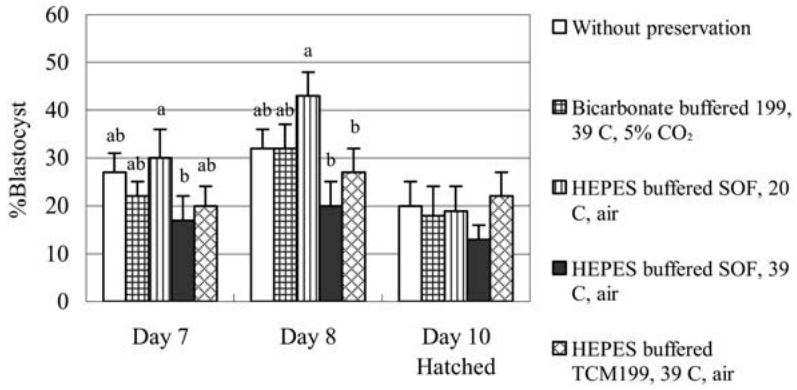

Fig. 1. Developmental competence of bovine oocytes meiotically arrested by treatment with cycloheximide (CHX) in HEPES-buffered synthetic oviduct fluid (HSOF) at 20 or 39 C under air following release from CHX, *in vitro* maturation and fertilization. All data were obtained from 5 replications with 20 embryos. [a,b] Values with different superscripts within each column differ ($P<0.05$).

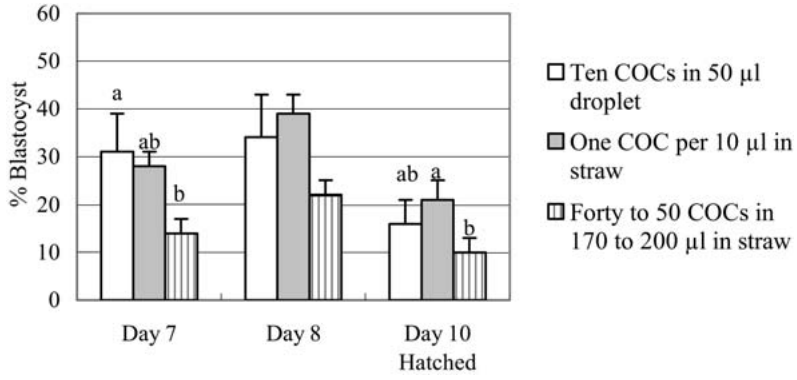

Fig. 2. Developmental competence of bovine oocytes meiotically arrested by treatment with CHX in 50-μl droplets, in a plastic straw individually, or in a group following release from CHX, *in vitro* maturation and fertilization. All data were obtained from 5 replications with 20 embryos. [a,b] Values with different superscripts within each column differ ($P<0.05$).

was improved by using an oxygen concentration that is lower than that in the atmosphere [29, 30]. It has been shown that the partial pressure of oxygen in follicular fluid is 22–97 mmHg (3–13%) [31]. Five percent oxygen has been suggested to be an optimal concentration for mouse and hamster oocytes in order to complete nuclear maturation and produce a polar body [32, 33]. In cattle, however, the proportion of oocytes that matured to the metaphase II stage was much lower under 5 or 10% O_2 than that of oocytes matured under 20% O_2 [34]. In addition, Biggers *et al*. [35] demonstrated that pyruvate, as a sole energy substrate, directly supports meiotic maturation of mouse oocytes under 20% O_2 (high oxygen tension). However, our data demonstrated that under 5% O_2 (low oxygen tension), glucose, not pyruvate, is critical for meiotic maturation and ATP production of bovine COCs [36]. Thus, it was conceivable that the mechanisms that control the completion of meiotic maturation and the production of ATP are different between COCs cultured under low oxygen tension and those cultured under high oxygen tension. It has been reported that a factor termed hypoxia-inducible factor I (HIF I) [37] induces the expression of erythropoietin under low oxygen tension in human hepatoblastoma cells. Subsequently, transient transfection studies have revealed that HIF I binding sites are involved in the regulation of a number of hypoxically inducible genes including genes encoding glycolytic enzymes [38–40] and glucose transporters [41, 42]. Furthermore, aerobic metabolism such as mitochondrial respiration is inhibited by low oxygen tension. Accordingly, ATP production shifts from the TCA cycle and the mitochondrial respiratory chain to anaerobic glycolysis [43, 44]. We showed that glucose supported meiotic maturation of bovine COCs in a dose-dependent manner (up to 20 mM) under 5% O_2. Thus, glucose is thought to be a major energy substrate that supports the meiotic maturation of bovine oocytes under low oxygen tension, and the lower involve-

Table 3. Effect of cumulus cell density *in vitro* maturation of bovine corona-enclosed oocytes on the developmental competence after *in vitro* fertilization and development

Droplet size	Density of cumulus cells* (cells/ml)	Maturation (Mean % ± SEM)		Fertilization (Mean % ± SEM)			Development (Mean % ± SEM)	
		Ex[a]	MII[b]	Ex[a]	Penetrated	With FMP[c]	Ex[a]	Blastocyst[d]
Control	4.2×10^6	53	44 (83 ± 3)[e]	49	45 (93 ± 7)[e]	32 (66 ± 12)[e]	109	33 (32 ± 3)[e]
1 to 2 μl	8.0×10^6	51	0 (0 ± 0)[f]	48	27 (58 ± 12)[f]	8 (16 ± 6)[f]	113	0 (0 ± 0)[f]
5 μl	3.2×10^6	53	39 (73 ± 5)[e]	44	35 (80 ± 9)[e]	28 (64 ± 4)[e]	113	36 (31 ± 3)[e]
10 μl	1.6×10^6	50	40 (81 ± 3)[e]	53	44 (84 ± 10)[f]	33 (63 ± 8)[e]	115	40 (33 ± 4)[e]
50 μl	0.32×10^6	50	34 (77 ± 6)[e]	47	40 (85 ± 9)[e]	28 (59 ± 6)[e]	115	19 (17 ± 4)[g]
250 μl	0.06×10^6	51	41 (81 ± 8)[e]	48	40 (84 ± 8)[e]	27 (56 ± 4)[e]	113	14 (12 ± 3)[g]

All data were obtained from 5 replications. *Total cell number of cumulus cells in culture. [a] Number of oocytes or embryos examined. [b] Arrested at metaphase II. [c] With female and male pronuclei. [d] Number of embryos developed to blastocysts. Control: Ten cumulus-oocyte complexes were cultured in 50 μl droplet. [e-g] Values with different superscripts within each column are significantly different (P<0.05).

Table 4. Developmental competence of bovine oocytes collected by ultrasound-guided transvaginal follicular puncture after *in vitro* fertilization

Source	Number of embryos (Mean % ± SEM)		
	cultured	cleaved	developed to the blastocyst stage
Live cow	45	31 (69 ± 5)	11 (37 ± 5)
Slaughterhouse ovary	35	25 (72 ± 8)	10 (42 ± 11)

All data were obtained from 4 replicates. *Corona-enclosed oocytes retrieved from live cows were matured in 10-μl droplet to adjust proper cumulus-cell density.

ment of pyruvate in meiotic maturation may be due to the decrease in the total amount of available oxygen.

Cytoplasmic maturation under low oxygen tension

Oxygen at the atmospheric level has been shown to be detrimental to mammalian embryos [45–51] and to bovine oocytes [36], due to the generation of reactive oxygen species (ROS) [19, 36]. We also showed that oocytes matured under high oxygen tension contained greater ROS than oocytes matured under low oxygen tension (Fig. 3) [36]. Moreover, the developmental competence of oocytes matured under high oxygen tension was lower than that of oocytes matured under low oxygen tension (Fig. 4). On the other hand, higher glucose concentration in the medium during *in vitro* maturation decreased the developmental competence of bovine oocytes under high oxygen tension (5% CO_2 in air) due to the generation of ROS and the decrease of the intracellular glutathione content [52]. Thus, it is believed that low oxygen tension during *in vitro* maturation supports the developmental competence of bovine oocytes by means of decreasing intracellular ROS contents of oocytes.

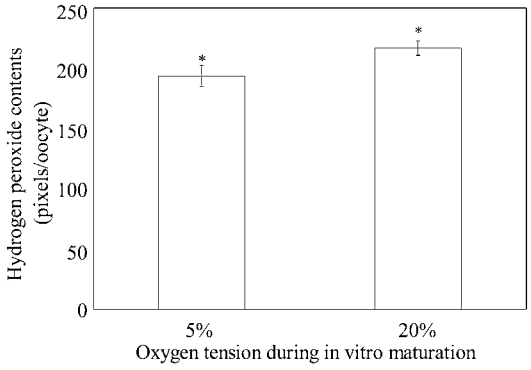

Fig. 3. Intracellular reactive oxygen contents of bovine oocytes matured *in vitro* under different oxygen tension. *P<0.05 by unpaired *t* test.

Application to Human ART

IVM of human immature oocytes following retrieval of those oocytes has been proposed as a potential alternative to conventional *in vitro* fertilization (IVF) treatment following ovarian stimulation. The procedure is becoming more accepted and more and more babies have been born worldwide following IVM since the first report of successful pregnancy following this procedure in 1994 [53]. Moreover, several improvements to the IVM protocol (cryopreservation; hCG priming before oocyte retrieval) have expanded the availability of IVM. However, this procedure is performed in

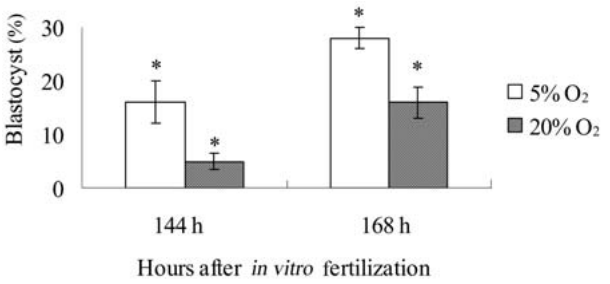

Fig. 4. Developmental competence to the blastocyst stage of bovine oocytes matured under different oxygen tension. Data were obtained from 5 replicates with 20 embryos each. *P<0.05 by unpaired *t* test within each column.

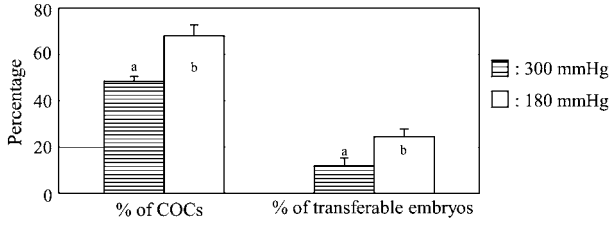

Fig. 5. Percentage of oocytes categorized into cumulus-oocytes complexes (COCs) and developmental rates to transferable embryos of oocytes retrieved with 300 or 180 mmHg following IVM and IVF. The retrieved oocytes were divided into 2 categories based on the presence and number of cumulus cell layers surrounding oocytes and the quality of the oocytes according to Hashimoto *et al*. [11] with some modifications. Oocytes in COCs had 4 or more cumulus cell layers and those with less than 4 cumulus cell layers and all others were categorized into "others". Transferable embryos were estimated on Day 2 or Day 3 after insemination according to the grading system depending on the numbers of blastomeres (Day 2: above 2 blastomeres; Day 3: above 6 blastomeres) except for embryos containing a multinuclear blastomere on Day 2 [54]. [a, b] P<0.05 by unpaired *t* test.

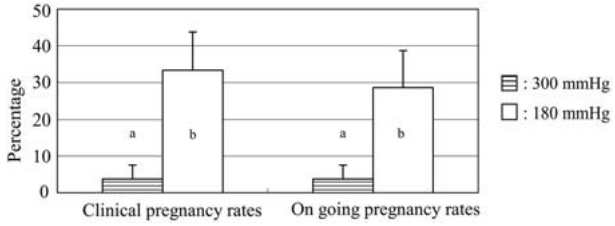

Fig. 6. Clinical pregnancy rates and on-going pregnancy rates per retrieval cycle of oocytes retrieved with 300 or 180 mmHg following IVM, IVF and ET. The clinical pregnancy and the on-going pregnancy rates were determined by identification of a gestational sac (GS) ultrasonographically and development beyond 10 weeks of gestation, respectively. [a, b] P<0.05 by unpaired *t* test.

only a limited number of institutes worldwide because the effects of human immature oocyte retrieval conditions on the subsequent development are not well understood. Thus, we assessed the effects of different aspiration vacuums during human oocyte retrieval with a 20 gauge single lumen needle on the developmental competence of immature oocytes following IVM, IVF and embryo transfer (ET) based on the data of cattle [11]. The percentage of COCs of retrieved oocytes in 180 mmHg (70%) was significantly higher (P<0.05) than that of oocytes in 300 mmHg (46%, Fig. 5) [54]. Moreover, the developmental rate of oocytes obtained by lower aspiration vacuum to transferable embryos (24%, Fig. 5) was higher (P<0.05) than that obtained by higher aspiration vacuum (13%). As a result, the on-going pregnancy rates per retrieval cycle in lower aspiration vacuum were improved compared with those in higher aspiration vacuum (Fig. 6). Nowadays, more than nine hundred live babies were delivered from IVM oocytes in the world [our unpublished data] including twice-vitrified case [55] and monozygotic twin [56].

Meiotic Arrest

Fully grown, prophase I-arrested oocytes, which are competent to resume meiotic maturation, are maintained in meiotic arrest within ovarian follicles by inhibitory factors produced by the follicle. When oocytes are removed from Graafian follicles and cultured *in vitro* under suitable conditions, they resume meiosis spontaneously and progress to the metaphase II stage. The ability of bovine oocytes matured *in vitro* to develop to the morula/blastocyst stage is less than that of *in vivo*-matured oocytes [7. 8]. The decrease in the developmental competence of *in vitro*-matured oocytes may be due to insufficient cytoplasmic maturity [7, 8, 57]. To improve the developmental competence of bovine immature oocytes, various approaches have been attempted. Several studies have examined the effects of holding oocytes at the germinal vesicle (GV) stage before *in vitro* maturation, because oocytes might require time to acquire developmental competence during meiotic arrest [58–60]. This idea is based on the fact that mammalian oocytes are arrested at the diplotene stage until the GnRH surge occurs and meiotic maturation resumes. On the other hand, oocytes aspirated from follicles resume meiotic maturation spontaneously. Furthermore, the developmental competence of oocytes collected from large follicles has been shown to be higher than that of oocytes collected from small follicles [61–63].

Various agents have been shown to inhibit GV breakdown (GVBD) of bovine oocytes. Such agents include drugs to elevate intracellular cAMP [64, 65], inhibitors of protein synthesis or phosphorylation [58, 66–68], follicular fluid [64, 69], granulosa or theca cells [70–72], follicle hemisections [73, 74], and follicle wall [75]. Oocytes cultured in intact follicles for 48 h appeared to acquire a greater developmental competence than those that matured directly following aspiration [76]. The effects of various kinase inhibitors on embryo development following IVM and IVF are summerized in Table 5. These studies show that controlling spontaneous meiotic progression by manupilating MPF activity, either adversely affects [58, 68] or has no positive effect [59, 60, 77, 78] on subsequent oocyte developmental competence after IVF except for our data (Fig. 7) [79]. There were several differences between our data

Table 5. Influence of kinase inhibitors during pre-IVM culture on the developmental competence of embryos after IVF

Kinase inhibitors	Species	Developmental competence	Reference number
Butyrolactone I	Cattle	Improved	79
	Cattle	Unchanged	59
	Pig	Unchanged	77, 78
Roscovitine	Cattle	Unchanged	60
6-DMAP	Cattle	Decreased	58, 68

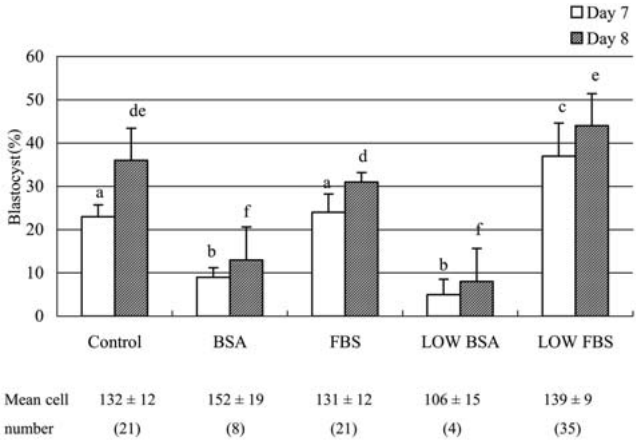

Fig. 7. The developmental competence of bovine oocytes after *in vitro* maturation and *in vitro* fertilization following the release from meiotic arrest in protein-free, BSA- or FCS-supplemented medium. Control: oocytes were matured directly following follicle aspiration. BSA: oocytes were cultured in arrest medium supplemented with BSA under 5% CO_2 in air. FBS: oocytes were cultured in arrest medium supplemented with FBS under 5% CO_2 in air. LOW BSA: oocytes were cultured in arrest medium supplemented with BSA under 5% CO_2, 5%O_2 and 90% N_2. LOW FBS: oocytes were cultured in arrest medium supplemented with FBS under 5% CO_2, 5%O_2 and 90% N_2. Data were obtained from 6 replications with 20 embryos. The numbers of blastocysts used for the estimation of cell number are shown in parentheses. $^{a\text{-}c,\ d\text{-}f}$ Different superscripts differ significantly ($P<0.01$ by Fisher's PLSD following ANOVA) in each developmental stage.

and others in methods for culturing immature oocytes during meiotic arrest. The primary difference is to culture under low oxygen tension (5% O_2) during meiotic arrest [79]. As descrived in IVM section, oxygen at the atmospheric level has been shown to be detrimental to mammalian embryos [29, 30] and to bovine oocytes [36], due to the generation of ROS [19, 36]. HIF I has been shown to induce the expression of erythropoietin under low oxygen tension in human hepatoblastoma cells [37]. Subsequently, transient transfection studies have revealed that HIF I-binding sites are involved in the regulation of a number of hypoxically inducible genes, such as vascular endothelial growth factor (VEGF) [80–83] and platelet-derived endothelial cell growth factor [84]. In addition, under low oxygen tension, the hypoxiainducible VEGF gene is not only transcribed, its mRNA stability increases [85]. VEGF is a major angiogenic factor; that is, it stimulates endothelial cell mitogenesis and capillary proliferation [80]. Human oocytes collected from follicles that contain a higher concentration of VEGF have a higher viability [86, 87]. These results imply that transcripts of genes that are expressed during meiotic arrest under low oxygen tension play beneficial roles in the subsequent development of oocytes. In fact, we observed different expression patterns of mRNA between meiotic arrested oocytes and non-treated oocytes (unpublished data). In addition, Marchal *et al*. [88] have also shown that immature porcine oocytes synthesize some proteins during meiotic arrest by roscovitine, a potent inhibitor of cyclin-dependent kinase.

An addition of sera to the media during meiotic arrest resulted in a higher developmental competence of oocytes than did addition of BSA [79]. Sera contain many components, including hormones, trace elements, and growth factors. Addition of hormones or growth factors to the maturation medium has been shown to enhance the developmental competence of oocytes after IVF (LH [90], GH [91], and epidermal growth factor [92, 93]). Our results suggest that components contained in sera also improve the environments of oocytes during meiotic arrest.

A combination of FBS supplementation into the medium and a decrease of oxygen tension during meiotic arrest provided the favorable conditions to enable developmentally incompetent oocytes to acquire developmental competence.

Mitochondrial Activity of *In Vitro* Grown Oocytes

The mammalian ovary contains a large number of oocytes in various growth stages. Nevertheless, most of these oocytes undergo atresia, and only a few oocytes obtain meiotic competence. It is important to develop an *in vitro* culture system that provides oocytes with a suitable environment to attain meiotic and developmental competence.

IVG of mammalian oocytes is described in several outstanding articles [94–98] In this section, mitochondrial activity of *in vitro* grown oocytes was discussed. The developmental competence of IVG oocytes after IVF is low compared with that of fully grown oocytes in mice [99], cattle [100], and rabbits (our unpublished data). The mitochondrial activity of rabbit oocytes increased as the follicles grew (Fig. 8) [101]. However, the mitochondrial activity of the IVG oocytes was significantly lower than that of ovulated oocytes and oocytes recovered from follicles with diameters of more than 700 μm. Furthermore, the expression levels of mitochondrial transcriptional factor A (TFAM) in the oocytes increased in a similar manner (Fig. 9). However, the expression levels of TFAM in the IVG oocytes was significantly lower than that of ovu-

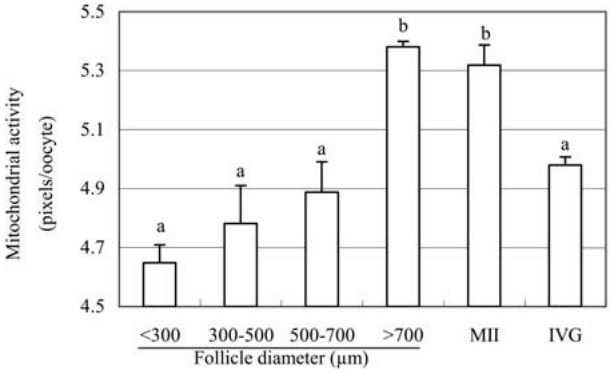

Fig. 8. Mitochondrial activities in rabbit oocytes from growing follicles, ovulated oocytes (MII), and *in vitro* grown oocytes (IVG). <300: Oocytes recovered from follicles with diameter of less than 300 μm. 300–500: Oocytes recovered from follicles with diameter of 300–500 μm. 500–700: Oocytes recovered from follicles with diameter of 500-700 μm. >700: Oocytes recovered from follicles with diameter of more than 700 μm. IVG: *In vitro* grown oocytes. MII: Ovulated oocytes. [ab] P<0.05 by Fisher's PLSD following ANOVA.

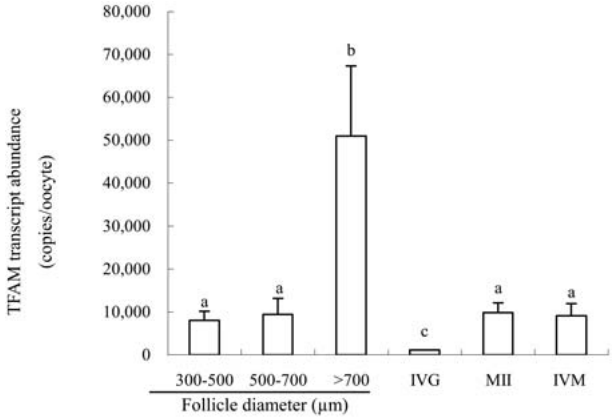

Fig. 9. Expression levels of TFAM in rabbit oocytes from growing follicles, *in vitro*-grown (IVG) oocytes, ovulated oocytes (MII), and *in vitro* matured (IVM) oocytes. Expression levels of TFAM were determined by quantitative PCR [a-c] P<0.05 by Fisher's PLSD following ANOVA.

lated oocytes and oocytes recovered from follicles with diameters of more than 300 μm. Moreover, the nuclei, but not the cytoplasm, of the IVG oocytes were able to support subsequent cleavage after artificial activation after nuclear-substitution [101]. These data indicated that the developmental competence of the IVG oocytes was impaired by mitochondrial dysfunction due to damage to the mitochondria during *in vitro* culture.

L-Carnitine (g-trimethylamino-b-hydroxybutyric acid) is a small water-soluble molecule which is a natural substance that acts as a carrier of fatty acids across the inner mitochondrial membrane for subsequent β-oxidation. It is essential for the normal mitochondrial oxidation of fatty acids and excretion of acyl-coenzyme A (acyl-CoA) esters, which are produced in some organic acidemias and affects adenosine triphosphate (ATP) levels [102]. Free carnitine was first isolated from bovine muscle in 1905, and only the L-isomer was found to be bioactive. L-Carnitine protects cell membrane and DNA against damage induced by free oxygen radicals and has a pivotal role in mitochondrial oxidation of long-chain fatty acids which increase energy supply to the cell. Mitochondrial dysfunction may lead to incomplete detoxification of the free radicals, which may lead to oxidative damage to macromolecules such as lipids, proteins, and DNA. The L-Carnitine levels may also affect ATP levels. L-Carnitine has a role in intramitochondrial fatty acid oxidation. Acyl-CoA esters cannot directly cross the mitochondrial inner membrane, and their entry into the mitochondrion is a major point for control and regulation of the β-oxidation of fatty acids [103]. Expression of fatty acid oxidation enzymes such as carnitine palmitoyl transferase 1 and medium-chain acyl-CoA dehydrogenase has been demonstrated in embryonic and fetal tissues and human placenta [104]. In addition, fatty acid oxidation may be important for generation of ATP necessary to meet the energy requirement of developing embryos [105, 106]. L-Carnitine decreased the apoptosis of granulose cells surrounding growing oocytes and improved the meiotic competence and the mitochondrial activity of porcine growing oocytes during *in vitro* culture (Fig. 10) [107]. Moreover, activity of mitochondria in porcine oocytes cultured in medium containing L-Carnitine was higher than that in oocytes cultured without L-Carnitine (Fig. 11). From these data, it is suggested that L-Carnitine support the meiotic competence of IVG oocytes by the decrease of apoptosis of granulosa cells and improvement of mitochondrial activity.

Conclusion

IVM of immature oocytes following their retrieval has been proposed as a potential alternative to conventional IVF treatment following ovarian stimulation. The procedure is becoming more accepted, and more calves and babies have been born worldwide. IVM following IVG of growing oocytes will provide us with more available mature oocytes from one individual. However, the production efficiency of fully grown oocytes is quite low. Our data showed that the low developmental competence of IVG oocytes is caused by a cytoplasm deficiency due to low mitochondrial activity and L-Carnitine, which acts as a carrier of fatty acids across the inner mitochondrial membrane for subsequent β-oxidation, supports the viability of growing oocytes. Improvement of cytoplasmic deficiency may lead to the expansion of available oocytes.

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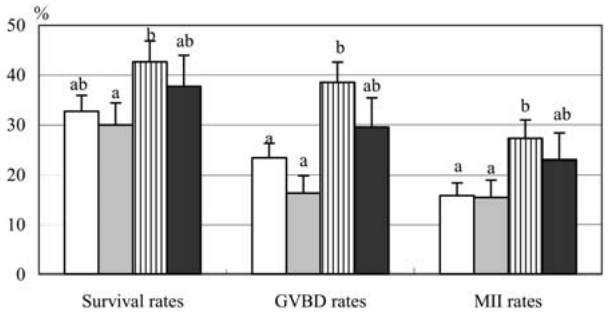

Fig. 10. Meiotic competence of porcine *in vitro* grown oocytes after *in vitro* maturation. □: control (0% L-carnitine); ▒: 0.002% L-carnitine; ▥: 0.02% L-carnitine; ■: 0.2% L-carnitine. OGCs were recovered from follicles with 300–700 μm in diameter. The proportions of survived oocytes after 14 days culture, of germinal-vesicle break down and of metaphase II oocytes after IVM were calculated based on the number of growing oocytes cultured. [ab] P<0.05 by ANOVA followed by Fisher's PLSD test.

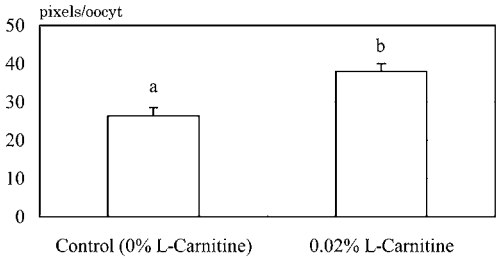

Fig. 11. Mitochondrial activity of porcine *in vitro* grown oocytes cultured in 0.02% L-Carnitine.[ab] P<0.05 by unpaired *t* test.

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