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—Original Article—

Aging-related Changes in *In Vitro*-matured Bovine Oocytes: Oxidative Stress, Mitochondrial Activity and ATP Content After Nuclear Maturation

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Abstract. The objective of this research was to clarify the aging-related changes in *in vitro*-matured bovine oocytes. Firstly, we examined the fertilization and embryonic development of bovine oocytes after 22 and 30–34 h of *in vitro* maturation (IVM). The oocytes after 30–34 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, reactive oxygen species (ROS), mitochondrial activity and ATP content in oocytes after 20, 30 and 40 h of IVM were examined. The lowest level of ROS was found in the group subjected to 30 h of IVM. The mitochondrial activity and ATP content in the group subjected to 40 h of IVM were higher than in the group subjected to 20 h of IVM ($P<0.01$), and those in the group subjected to 30 h of IVM showed intermediate values. Thereafter, the mitochondrial activities at 3 days after *in vitro* fertilization 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 present results suggest that high mitochondrial activity observed in oocytes after prolonged IVM culture 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.

Key words: Aging, ATP, Bovine oocytes, Mitochondria, ROS

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In humans and domestic animals, it is well known that postovulatory aging of oocytes at the metaphase II (M-II) stage adversely affects the outcome of assisted reproductive technologies (ART), such as artificial insemination [1], *in vitro* fertilization (IVF) [2, 3] and intracytoplasmic sperm injection [4, 5]. In humans and rodents, postovulatory aging of oocytes is defined as numerous morphological and cellular alterations and causes a decrease in fertilization and embryonic development, which has been previously reviewed [6, 7]. In bovines, both *in vivo* and *in vitro* aging of oocytes cause a decrease in fertilization and embryonic development [1, 2, 8–11]. 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 that one of the aging-related changes caused by oxidative stress is mitochondrial dysfunction [6]. It has been reported that the aging of murine oocytes causes increased oxidative stress [12], mitochondrial dysfunction [13, 14] and decreased intracytoplasmic levels of ATP [15]. In *in vitro*-matured bovine oocytes, the changes in level of oxidative stress [16], mitochondrial activity [17, 18] and ATP content [19–22] during *in vitro* maturation (IVM) culture for less than 24 h have been determined, but there is little 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.

In previous studies, bovine oocytes at about 30 h after IVM were treated as aged or slightly aged oocytes and used to investigate aging-related changes [9, 23, 24]. This was because the oocytes after 30 h of IVM showed a low developmental rate to blastocysts [9]. 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 [24]. Moreover, maturation-promoting factor (MPF) activity was similar to that in bovine oocytes matured for 24 and 32 h [9], although a decrease in activity of MPF in bovine oocytes matured for 40 h was observed [25]. Since it takes several hours for bovine oocytes to be penetrated by sperm after starting IVF [9, 26], studies on the characteristics

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of aged oocytes should not be based on developmental competence corresponding to the duration of IVM, but instead should be based on the timing of sperm penetration.

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, we confirmed the competence of fertilization and embryonic development in oocytes subjected to 22 and 30–34 h of IVM. We then examined reactive oxygen species (ROS), mitochondrial activity and ATP content after 20, 30 and 40 h of IVM culture and examined the mitochondrial activity in embryos at 72 h after IVF.

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 [27]. 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 6–10 h of collection. Bovine cumulus-oocyte complexes (COCs) were aspirated from small antral follicles (2 to 8 mm in diameter). The COCs with brown-colored ooplasm surrounded by intact cumulus investments [28] were washed twice in HEPES-buffered Tyrode's medium [29] supplemented with 3 mg/ml bovine serum albumin (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% CO_2 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, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS, Invitrogen), 0.02 units/ml follicle stimulating hormone (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 [30] 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 [26] 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% CO_2, 5% O_2 and 90% N_2 at 39 C.

Evaluation of the fertilized oocytes

After 18 h of IVF, oocytes were freed from 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 pronuclear formation) were examined under a phase-contrast microscope [19, 28]. Oocytes having an enlarged sperm head(s) or male pronucleus(ei) 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 pronucleus or with male pronucleus and telophase II chromosome (asynchronous fertilization).

In vitro culture and evaluation of subsequent embryonic development

To determine the developmental competence of oocytes, inseminated oocytes were assigned to *in vitro* culture (IVC) according to a procedure described previously [27, 31]. In brief, inseminated oocytes were freed from cumulus cells by vortexing at 18 h post insemination (hpi). Denuded oocytes were washed three times and cultured for 6 days under 5% CO_2, 5% O_2 and 90% N_2 at 39 C in droplets of IVC medium (25–30 oocytes/30 μl). IVC medium was a modified synthetic oviduct fluid containing 1 mM glutamine, 12 essential amino acids for Basal Medium Eagle, 7 nonessential 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 [32].

Measurement of ROS in individual oocytes

The quantity of H_2O_2 produced by individual oocytes was measured as the level of ROS according to a previous report [33]. ROS in oocytes can be quantified by measuring 2',7'-dichlorofluorescein diacetate (DCF) [34]. 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 μM 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 μm intervals. 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 [18] with slight modifications. JC-1 is a fluorescent dye that accumulates in mitochondria and shows the membrane potential across the matrix membrane [35]. 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) [35]. Mitochondrial activity can be evaluated by the intensity of the red/green fluorescence [13, 17, 18].

Briefly, denuded oocytes or embryos were incubated with 1 μM JC-1 and 1 μg/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 [19, 36]. Briefly, a denuded oocyte was washed four times in the sample buffer and transferred to 25 μl 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 NaH_2PO_4, 21.6 mM sodium lactate, 10.0 mM HEPES, 2.0 mM $CaCl_2$, 1.1 mM $MgCl_2$, 25.0 mM $NaHCO_3$, 1.0 mM sodium pyruvate, 0.1 mg/ml of gentamicin sulfate and 6.3 mg/ml of BSA [36]. 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 μl in sample buffer for the ATP standards. The ATP standards and samples in 1.5 ml tubes were kept on ice, and 50 μl 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 μl 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 (Luminescensor JNR AB-2100, Atto, Tokyo, Japan).

Experimental design

In Experiment 1, bovine oocytes after 22 and 30–34 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 (20–30 oocytes/replicate), and the remaining 122 oocytes were used for the fertilization evaluation (10–13 oocytes/replicate).

In the preliminary study, we examined the percentages of oocytes penetrated by sperm at 4, 8 and 12 h after starting IVF in bovine oocytes subjected to IVM for 22 or 30 h, and the times when more than 50% of oocytes were penetrated by sperm were estimated at 6 to 8 h after starting IVF (data not shown). Therefore, in Experiment 2, oocytes subjected to IVM for 20 h (immediately after M-II arrival [19]), 30 and 40 h were used for determination of ROS, mitochondrial activity and ATP content. The 30 and 40 h time points for IVM were considered the times when sperm penetration occurred in oocytes subjected to 22 and 30–34 h of IVM, respectively. For ROS measurement, a total of 78 oocytes were subjected to IVM, and oocytes with a polar body clearly observed under a stereomicroscope were used for the experiment (10–13 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 a stereomicroscope were used for the experiment (9–14 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. The mitochondrial activities of cleaved embryos were then examined. The 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 for fertilization and embryonic development (Experiment 1) and mitochondrial activity of embryos at 3 days after IVF (Experiment 2) were analyzed by Student's *t*-test. ROS, mitochondrial activity and ATP content of oocytes after IVM culture (Experiment 2) were analyzed using one-way analysis of variance followed by Tukey-Kramer's honestly significant different test as a post hoc test. Data for embryonic development and distribution of high-polarized mitochondria in embryos were analyzed by Fisher's exact test (Experiment 2). The level of statistical significance was set at $P<0.05$. Statistical analyses were performed using JMP version 10.0.2 (SAS Institute, Cary, NC, USA).

Results

Experiment 1

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

Experiment 2

The levels of ROS production in oocytes after different IVM culture

Table 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
30–34	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 pronucleus or male pronucleus and telophase II chromosome.

Table 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)	% ≥2 cells/ inseminated	% blastocysts/ inseminated	% blastocysts/ cleaved	Total cell no.in blastocysts (n)
22	161 (6)	81.1 ± 5.6[A]	51.6 ± 9.4[a]	64.1 ± 13.1[a]	172.5 ± 77.5 (83)
30–34	190 (7)	71.1 ± 10.6[B]	24.2 ± 8.8[b]	33.8 ± 11.4[b]	157.6 ± 56.5 (45)

[a,b] Values (mean ± SD) with different superscripts within the same column differ significantly (P<0.01). [A,B] Values (mean ± SD) with different superscripts within the same column tended to be different (P = 0.06).

periods are shown in Fig. 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 of IVM, 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. The mitochondrial activity in the group subjected to 40 h of IVM was higher than in the group subjected to 20 h of IVM (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. 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. 4) was higher in the group subjected to 34 h of IVM (81.0%) than in the group subjected to 22 h of IVM (30.0%) (P<0.01).

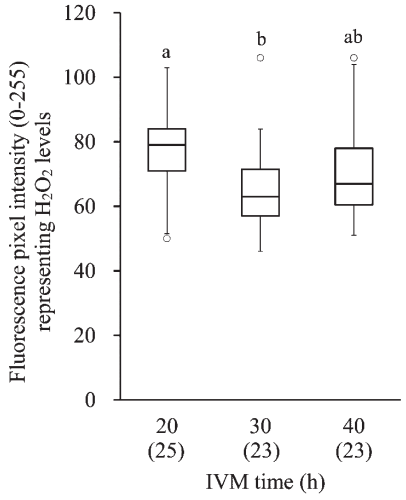


Fig. 1. Box plots of the mean green intensity of DCF fluorescence in oocytes after IVM culture, which represents the H_2O_2 level. The lower and upper ± 1.5 quartiles are indicated by whiskers, the lower and upper ends of the boxes indicate the 25th and 75th quartiles, and the line across the middle 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).

Discussion

In the present study, normal fertilization was similar regardless of IVM duration, but developmental competence to the blastocyst stage was low in the bovine oocytes subjected to 30–34 h of IVM. These results were consistent with the results of previous studies [2, 9–11]. Moreover, asynchronous fertilization has been frequently observed in bovine oocyte subjected to 30–34 h of IVM. The delay in male pronucleus formation might be due to the deficiency of male pronucleus growth factor in the group subjected to 30–34 h of IVM [37, 38]. The reason for the delay in female pronucleus formation is unclear, but a similar delay in female pronucleus formation was observed in fertilized oocytes from aged hamsters [39].

In contrast to the past report that the level of cytoplasmic ROS in oocytes increased with oocytes aging in murine and swine [12, 40, 41], extension of IVM culture up to 40 h did not cause the increased level of cytoplasmic ROS in the present study. Moreover, although it has been reported that mitochondrial activity and ATP contents in human, murine and porcine oocytes decrease with aging [13–15, 42, 43], the mitochondrial activity and ATP content in bovine oocytes subjected to 40 h of IVM was highest among all IVM durations in the present study. Therefore, it is thought that the decrease in developmental

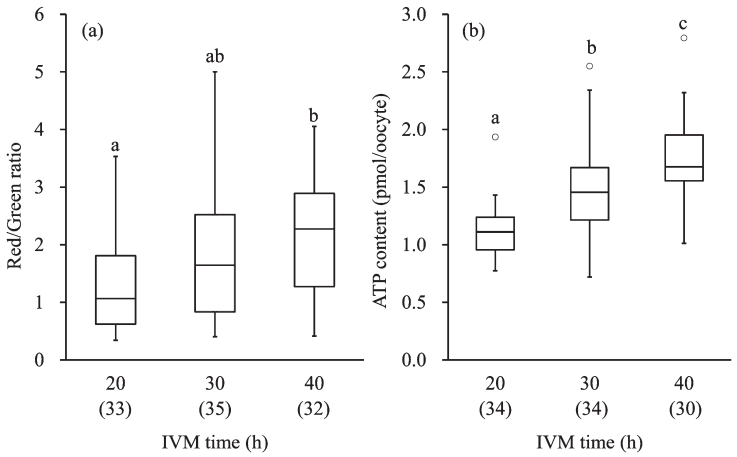


Fig. 2. Box plots of mitochondrial activity (a) and ATP content (b) in oocytes after IVM culture. The lower and upper ± 1.5 quartiles are indicated by whiskers, the lower and upper ends of the boxes indicate the 25th and 75th quartiles, and the line across the middle of the box identifies the median 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).

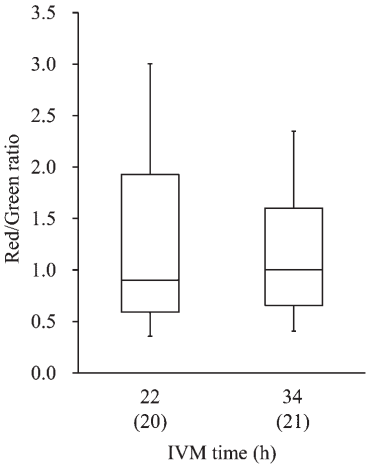


Fig. 3. Mitochondrial activity in ≥2-cell embryos derived from the oocytes subjected to 22 and 34 h of IVM. The lower and upper ± 1.5 quartiles are indicated by whiskers, the lower and upper ends of the boxes indicate the 25th and 75th quartiles, and the line across the middle of the box identifies the median sample value. Numbers of oocytes used are indicated in parentheses (1 replicate).

competence in oocytes after prolonged IVM culture was not due to the mitochondrial dysfunction and decrease in 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 themselves from oxidative stress. Therefore, it is thought that one of the reasons for this high developmental competence of oocytes subjected to 22 h of IVM (penetrated by sperm at around 30 h after the initiation of IVM) was due to the low oxidative stress in oocytes resulting from their high competence to protect themselves from oxidative stress. In a previous study [12], it was reported that cumulus cells prevented the increase of ROS during *in vitro* aging. Although we 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 [44] indicated that the subplasmalemmal domains, including high-polarized mitochondria, were extruded as fragments from human embryo blastomeres and that 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 oocytes with extended IVM culture. These high-polarized mitochondria at the periphery of blastomeres might have already been extruded from blastomeres like in the previous report [44], and it is possible that the extrusion of high-polarized mitochondria from blastomeres causes lower early embryonic development of oocytes with extended IVM culture. However, in the present study, we were unable to confirm the clear extrusion of high-polarized mitochondria from blastomeres because the bovine embryos contained a large number of lipid droplets in their ooplasm, unlike human embryos [45], and it was necessary to press the embryos with a cover slide to observe the mitochondria stained by JC-1. In future study, we should examine the localization of high-polarized mitochondria in embryos derived from oocytes with extended IVM culture in detail by electron microscope analysis.

Although the present study does not clarify the direct causal 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 bovine oocytes after an extended duration of IVM culture. High-polarized mitochondria in oocytes are associated with elevated levels of ATP generation [46]. Tamassia *et al*. [21] indicated that the ATP content in ovum pick up (OPU)-derived oocytes did not increase during IVM culture but that the ATP content in abattoir-derived oocytes did increase. Also, it has been reported that OPU-derived bovine oocytes retained high developmental competence during longer periods of IVM culture than abattoir-derived oocytes [22]. These previous reports indicate that OPU-derived oocytes can control mitochondrial activity and that this ability contributes to maintaining high developmental competence during long periods. In the present study, blastocysts derived from oocytes subjected to 30–34 h 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 subjected to more than 30 h of IVM maintain their developmental competence. In future studies, we should examine the mechanisms of maintenance of mitochondrial activity in *in vitro*-matured bovine oocytes and the relationship between mitochondrial activity and developmental competence in detail.

In conclusion, the present study suggests that low developmental competence of bovine oocytes with extended IVM culture is probably not due to low mitochondrial activity or low ATP content but is prob-

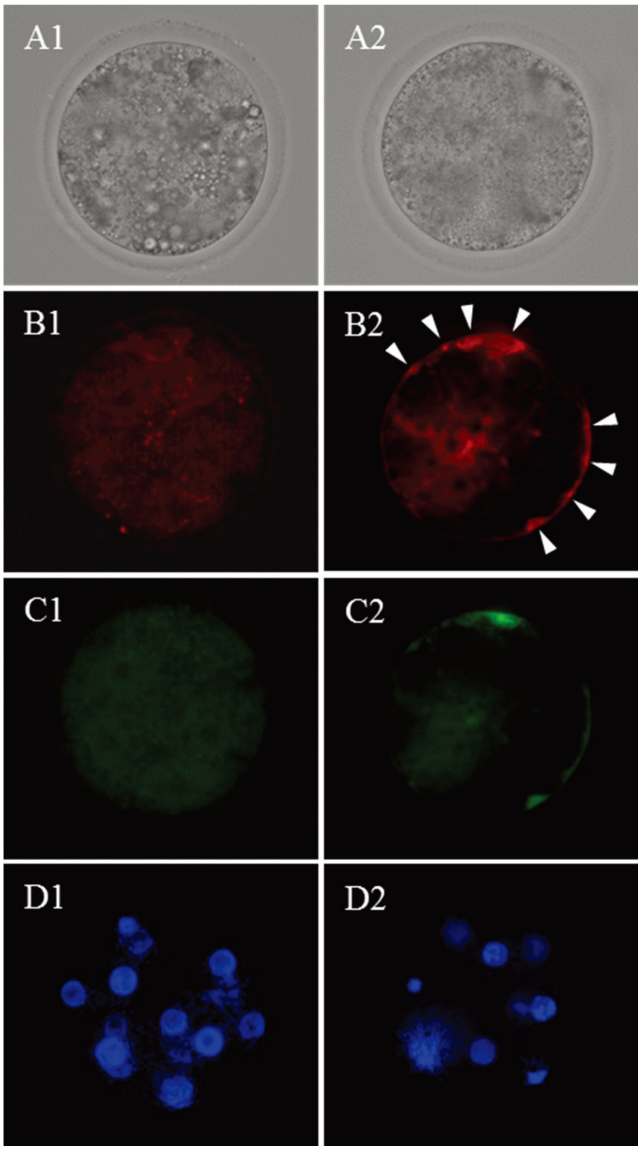


Fig. 4. Bright field (A) and fluorescent micrographs (B, C and D) of embryos (72 hpi) derived from the oocytes subjected to 22 (1) and 34 h (2) of IVM culture. B, high-polarized mitochondria stained by JC-1; C, low-polarized mitochondria stained by JC-1; D, nuclei stained by Hoechst 33342. Twelve and 8 nuclei 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).

able due to 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 this is possibly related to oocyte aging *in vitro*. Future detailed studies on the relationship between enhanced high mitochondrial activity at fertilization and subsequent embryo development should be conducted.

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