Full paper

Title: Mitochondrial activity during pre-maturational culture in *in vitro*-grown bovine oocytes related to maturational and developmental competences

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Running head: Mitochondrial activity and IVG oocyte development

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Abstract

The objective of this study was to investigate the dynamics of mitochondrial activity in *in vitro*-grown (IVG) bovine oocytes during pre-maturational culture (pre-IVM) and its relationship to their developmental competence upon being subjected to different pre-IVM durations. After 12-day IVG culture, oocytes were cultured for 0, 10 or 20 h with 3-isobutyl-1-methylxanthine (IBMX) as pre-IVM. Mitochondrial activity in IVG oocytes after 10 h pre-IVM was the highest among all the pre-IVM durations ($P < 0.05$). In addition, cleavage (79.4%) and blastocyst rates (38.9%) of embryos derived from IVG oocytes with 10 h pre-IVM were higher than those with 20 h pre-IVM (63.0 and 25.8%, respectively; $P < 0.05$) and similar to those of *in vivo*-grown oocytes (82.7 and 36.7%, respectively). To confirm the developmental ability of IVG oocytes with 10 h pre-IVM beyond the blastocyst stage *in vivo*, embryo transfer was attempted. Transferred embryos developed to the elongated embryonic stage (63.6%, 7/11) in the recipient uterus at Day 16 of oestrus, and a male calf was delivered (50%, 1/2). In conclusion, it was indicated that the mitochondrial activity of bovine IVG oocytes peaked at 10 h pre-IVM and was closely correlated with the nuclear maturation and developmental competences of IVG oocytes

Additional Keywords: early antral follicle, embryo transfer, IVG, mitochondria, ROS

Introduction

The development to blastocysts of bovine oocytes derived from in vitro-growth (IVG) culture was found to be markedly lower than that of in vivo-grown oocytes (Hirao et al. 2004). In our previous studies, we showed that the maturational and developmental competences of IVG oocytes were improved by shortening the IVG duration from 14 to 12 days (Huang et al. 2013, 2014) and by performing pre-maturational culture (pre-IVM) for 20 h in medium supplemented with phosphodiesterase (PDE) inhibitor (3-isobutyl-1-methylxanthine (IBMX)) between IVG and in vitro maturational (IVM) cultures (Huang et al. 2013, 2014). In one previous study (Huang et al. 2014), the normal fertilisation rates of IVG oocytes were similar (around 70%) regardless of pre-IVM, despite the low nuclear maturation rate of IVG oocytes without pre-IVM. Therefore, the authors speculated that IVG oocytes without pre-IVM achieved nuclear maturation during the culture for fertilisation, and that the improvement of maturational and developmental competences of IVG oocytes with pre-IVM is probably caused by synchronisation of meiotic progression because most IVG oocytes may be fertilised at an appropriate time for embryonic development. On the other hand, it was reported that in vivo-grown bovine oocytes with 18 to 21 h pre-IVM showed higher developmental competence to blastocysts than those without pre-IVM, regardless of the timing of fertilisation (Hashimoto et al. 2002). Hashimoto et al. (2002) concluded that immature bovine oocytes required a certain time to acquire developmental competence during meiotic arrest. For this reason, we speculate that cytoplasmic changes of oocytes occur during pre-IVM and this contributes to the acquisition of developmental competence. Therefore, it is necessary to investigate the cytoplasmic changes in IVG oocytes during pre-IVM culture.

Mitochondria play important roles in oocyte maturation and subsequent embryonic

development by being a steady source of adenosine triphosphate (ATP) through oxidative phosphorylation (Nagano et al. 2006; Tarazona et al. 2006; Van Blerkom 2011). In mammalian oocytes, mitochondrial activity increased with follicular development (Kanaya et al. 2007; Machatkova et al. 2012) and the higher mitochondrial activity before the resumption of meiosis was associated with higher competences in nuclear maturation (Egerszegi et al. 2010; Machatkova et al. 2012) and development to blastocysts (Kanaya et al. 2007). On the other hand, it was reported that reactive oxygen species (ROS) such as hydroxyl radical and hydrogen peroxide were generated by the mitochondrial respiratory chain (Fleury et al. 2002) and ROS suppressed the developmental competence of oocytes (Hashimoto et al. 2000). For this reason, there is a possibility that mitochondrial activity and ROS production change during pre-IVM culture and affect the nuclear maturation and embryonic development of IVG oocytes.

In the present study, in order to clarify the cause of the improvement of maturational and developmental competences of IVG oocytes by pre-IVM, we examined mitochondrial activity and ROS production during pre-IVM culture and the competences in nuclear maturation and development to blastocysts of IVG oocytes with different pre-IVM durations. Moreover, to confirm the developmental ability of IVG oocytes beyond the blastocyst stage, we also attempted to transfer the embryos derived from IVG oocytes into recipient cows.

Materials and Methods

Chemicals

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

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

Bovine ovaries obtained at a local abattoir were kept in plastic bags at 20°C and were transported to the laboratory within 6 to 10 h of their collection, as described previously (Huang et al. 2013, 2014). The oocyte–cumulus–granulosa complexes (OCGCs) with normal appearance were cultured individually in a 96-well culture plate (Becton, Dickinson and Co., Franklin Lakes, NJ, USA) with 200 μL of the growth medium for 12 days at 39°C in humidified air with 5% CO2, as described previously (Huang et al. 2013, 2014). The growth medium was HEPES-buffered TCM-199 (Invitrogen, Grand Island, NY, USA) supplemented with 0.91 mM sodium pyruvate, 1 μg mL–1 oestradiol-17β, 5% fetal calf serum (FCS; Invitrogen), 4 mM hypoxanthine, 4% polyvinylpyrrolidone (MW 360000) and 50 μg mL –1 gentamicin sulfate. Hypoxanthine was added to prevent premature meiotic resumption as described previously (Eppig and Downs 1987; Harada et al. 1997; Hirao et al. 2004). Every 4 days of IVG culture, half (100 μL) of the growth medium was replaced with the same amount of fresh medium.

Pre-IVM and IVM of IVG oocytes

After 12-day IVG culture, oocytes surrounded by several layers of cumulus cells were collected from morphologically normal OCGCs and submitted to IVM with or without pre-IVM, as described previously (Huang et al. 2013, 2014). Briefly, pre-IVM culture of IVG oocytes was performed as they were cultured individually in each well of micro-well plates (NUNC, Roskilde, Denmark) filled with 6 mL of HEPES-buffered TCM-199 supplemented with 0.2 mM sodium pyruvate, 2 × 10□6 units mL–1 follicle-stimulating hormone (FSH, from porcine pituitary), 0.5 mM IBMX, 1 μg mL–1 oestradiol-17β, 10% FCS and 50 μg mL–1 gentamicin sulfate at 39°C

under 5% CO_2 in air for 0, 10 or 20 h. For IVM, oocytes were cultured individually in each well of micro-well plates filled with 6 mL of IVM medium, which was HEPES-buffered TCM-199 supplemented with 0.2 mM sodium pyruvate, 0.02 units mL–1 FSH, 1 μg mL–1 oestradiol-17β, 10% FCS and 50 μg mL–1 gentamicin sulfate, at 39°C under 5% CO_2 in air for 22 h (Nagano et al. 2013).

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

IVF and IVC of inseminated oocytes

IVF (Day 0) was performed using frozen semen from a Holstein bull according to a procedure described previously (Takahashi and Kanagawa 1998) with slight modification. In brief, motile spermatozoa (5 × 106 spermatozoa mL–1) separated from thawed semen using a Percoll gradient (45 and 90%) were co-incubated with COCs in a 100-μL droplet (~10 COCs per droplet) of modified Brackett and Oliphant isotonic medium (Brackett and Oliphant 1975) containing 3 mg mL–1 fatty-acid-free bovine serum albumin (BSA) and 2.5 mM theophylline (Takahashi and First 1992) for 18 h at 39°C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2.

IVC of inseminated oocytes (presumptive zygotes) was performed as described previously (Takahashi and Kanagawa 1998). Briefly, after co-incubation with spermatozoa,

presumptive zygotes were freed from cumulus cells by vortexing and washing three times in the culture medium. Cumulus-free zygotes were cultured for ~150 h in a 30-μL droplet of the culture medium at 39°C under 5% CO2, 5% O2 and 90% N2. The culture medium was a modified synthetic oviduct fluid containing 12 essential amino acids for basal medium Eagle, 7 non-essential amino acids for minimum essential medium, and further supplementation with 1 mM glutamine, 5 mM glycine, 5 mM taurine, 1 mM glucose, 10 μg mL–1 insulin and 3 mg mL–1 fatty-acid-free BSA instead of polyvinyl alcohol (Takahashi and Kanagawa 1998). After 30 h (Day 2) and ~150 h (Day 7) of IVC, cleavage and development of presumptive zygotes to the blastocyst stage were assessed, respectively. Total cell numbers of blastocysts obtained after ~150 h of IVC were counted using an air-drying method (Takahashi and First 1992).

Evaluation of oocyte nuclear status

After pre-IVM and IVM, oocytes were denuded from cumulus cells by vortexing. Denuded oocytes were stained with 1% aceto-orcein and examined for their nuclear status as described elsewhere (Nagano et al. 2006). Nuclear statuses were divided into germinal vesicle (GV), germinal vesicle breakdown (GVBD), chromosomal condensation (CC), metaphase I (MI), anaphase I–telophase I (AI–TI) and metaphase II (MII) by observation under a phase-contrast microscope.

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

IVG oocytes after pre-IVM and IVM were treated with 500 IU mL–1 hyaluronidase in Dulbecco’s phosphate-buffered saline (DPBS) for 10 min and then the oocytes were denuded

153 from cumulus cells by repeat pipetting with a fine pipette. Denuded oocytes were incubated for
154 15 min in the dark at 37°C in DPBS supplemented with 1 μM
155 5,5□,6,6□-tetrachloro-1,1',3,3□-tetraethylbenzimidazolyl carbocyanine iodide (JC-1), 1 μg
156 mL–1 Hoechst 33342 and 10% FCS, as described previously (Ge et al. 2012) with slight
157 modification. Images of oocytes were acquired using a digital fluorescence microscope
158 (BZ-9000; Keyence, Osaka, Japan) and the mean fluorescence intensity of the images was
159 calculated using analysis software (BZ-H2A; Keyence). Nuclear status of IVG oocytes was
160 evaluated by blue fluorescence of Hoechst 33342 and oocytes having GV before IVM and
161 metaphase plate with polar body after IVM were subjected to the evaluation of mitochondrial
162 activity. Membrane potentials of mitochondria in GV and MII oocytes were calculated as the
163 ratio of florescence intensity of activated mitochondria, expressed as red fluorescence of JC-1
164 staining, to less-activated mitochondria, expressed as green fluorescence of JC-1 staining
165 (Δψm, red/green fluorescence intensity).
166
167 **Assessment of ROS generation in IVG oocytes during pre-IVM culture**
168 IVG oocytes after pre-IVM were denuded from cumulus cells by vortexing. Denuded
169 oocytes were incubated for 15 min in the dark at 37°C in DPBS supplemented with 10 μM
170 2',7'-dichlorodihydrofluorescein diacetate (DCHFDA) and 10% FCS, as described previously
171 (Hashimoto et al. 2000) with slight modification. Images of oocytes were acquired using a
172 digital fluorescence microscope and the mean fluorescence intensity of the images was
173 calculated using analysis software. ROS in oocytes were defined as the mean fluorescence
174 intensity of oocytes.

175

176 **Experimental design**

177 **Experiment 1**

178 After 0, 10 and 20 h pre-IVM, a total of 83 IVG oocytes derived from 129 OCGCs were
179 used for the evaluation of mitochondrial activity in oocytes (six to seven oocytes per replicate).
180 After IVM with 0, 10 and 20 h pre-IVM durations, a total 90 IVG oocytes at MII stage from 263
181 OCGCs were also used for the evaluation of mitochondrial activity (five to twelve oocytes per
182 replicate). In addition, the ROS generation in IVG oocytes was assessed during pre-IVM
183 culture using a total of 61 IVG oocytes derived from 94 OCGCs (six to seven oocytes per
184 replicate).

185

186 **Experiment 2**

187 To examine the relationship between mitochondrial activity and the maturational and
188 developmental competences of IVG oocytes with different pre-IVM durations, a total of 419
189 IVG oocytes derived from 635 OCGCs (10 to 30 oocytes per replicate) were subjected to IVM
190 with 0, 10 or 20 h pre-IVM culture. After 10 and 22 h IVM culture, their nuclear statuses were
191 investigated. Before the examination of nuclear status, some of the IVG oocytes after 22 h IVM
192 (with 0, 10 and 20 h pre-IVM: n = 53, 71 and 37, respectively) were provided for the
193 measurement of their diameter, as described previously (Huang et al. 2013, 2014). To confirm
194 meiotic arrest immediately before IVM culture, nuclear statuses of IVG oocytes after 0, 10 or
195 20 h pre-IVM were evaluated by using a total of 157 IVG oocytes derived from 254 OCGCs (11
196 to 27 oocytes per replicate). Moreover, IVG oocytes with 10 or 20 h pre-IVM were subjected to

IVM–IVF–IVC and examined for their cleavage, development to blastocyst stage and cell numbers in blastocysts using a total of 357 IVG oocytes derived from 503 OCGCs (25 to 40 oocytes per replicate). In vivo-grown oocytes after IVM served as controls.

Experiment 3

For evaluating embryonic development beyond the blastocyst stage, eleven fresh blastocysts (Day 7) obtained after IVC were transferred transcervically into a recipient heifer synchronised at Day 8 of oestrus by EAZI-BREED CIDR (Inter Ag, Te Rapa Road, Hamilton, NZ) synchronisation (Katagiri and Takahashi 2006). Transcervical uterine flushing using a two-way Foley catheter (Fujihira Industry, Tokyo, Japan) was performed at Day 16 of oestrus for embryo collection, and collected embryos were evaluated for their developmental stage. In addition, two blastocysts were also transferred into another heifer synchronised by the same protocol, and her pregnancy was diagnosed at Days 29 and 62 of oestrus by ultrasonography (5 MHz, HS101V; Honda Electronics, Tokyo, Japan).

Statistical analysis

All percentage data were subjected to arcsine transformation before statistical analysis. All data were analysed by one-way ANOVA or two-way ANOVA followed by Tukey-Kramer's honestly significant difference test. All statistical analyses were performed using software (JMP Version 10; SAS Institute, Cary, NC, USA).

Results

Experiment 1

There was no significant interaction by two-way ANONA between pre-IVM duration and with or without IVM culture in terms of mitochondrial activity. Mitochondrial activity in IVG oocytes at 10 h pre-IVM was higher than at 0 and 20 h pre-IVM ($P < 0.05$; Fig. 1). After IVM, the mitochondrial activities of IVG oocytes at MII stage were similar regardless of pre-IVM durations (Fig. 1). Also, great variability of mitochondrial activity among each oocyte type was observed compared with before IVM culture. Mitochondrial activity of IVG oocytes with 0 and 20 h pre-IVM was higher after IVM than before IVM ($P < 0.05$), although it was similar in IVG oocytes with 10 h pre-IVM both before and after IVM. ROS generation in IVG oocytes with 0 h pre-IVM was higher than in those with 10 and 20 h pre-IVM ($P < 0.05$; Fig. 2).

Experiment 2

After 10 h IVM, the MI rate (82.7%) of IVG oocytes with 10 h pre-IVM tended to be higher than in those with 0 h pre-IVM (48.3%, $P = 0.17$) and their GVBD/CC rate (1.5%) was lower than that of the 20 h pre-IVM group (34.2%, $P < 0.05$) and tended to be lower than the 0 h pre-IVM group (37.3%, $P = 0.08$; Table 1). After 22 h IVM, the MII rate of IVG oocytes with 10 h (91.5%) was higher than in those with 0 h (50.5%, $P < 0.01$) and 20 h pre-IVM (79.0%, $P < 0.05$) and similar to that of in vivo-grown oocytes (88.6%; Table 2). The percentage of MI-stage oocytes was significantly lower in IVG oocytes with 10 h pre-IVM (5.5%) than in the 0 h (25.4%, $P < 0.01$) and 20 h pre-IVM groups (19.8%, $P < 0.05$). The mean diameter of IVG oocytes after 22 h IVM was 115.2 ± 3.4 μm (range: 105.9 to 122.7 μm, n = 161). Before IVM culture, all IVG oocytes with 0 and 10 h pre-IVM were at GV stage (Table 3). The percentages (100%) of

GV-stage oocytes after 0 and 10 h pre-IVM were higher than after 20 h pre-IVM (90.0%, $P < 0.01$).

As shown in Table 4, cleavage (79.4%) and blastocyst rates (38.9%) of IVG oocytes with 10 h pre-IVM were higher than those with 20 h pre-IVM (63.0 and 25.8%, respectively; $P < 0.05$) and similar to those of in vivo-grown oocytes (82.7 and 36.7%, respectively). However, blastocyst rates based on cleaved oocytes were similar regardless of pre-IVM duration. In addition, total cell numbers in blastocysts of IVG oocytes with 10 h pre-IVM were similar to those of the control ($P = 0.38$).

Experiment 3

Seven embryos (63.6%, 7/11) were recovered from recipient heifers at Day 16 of oestrus. Six embryos were elongated, ranging from 40 to 100 mm (70.0 ± 21.9 mm; Fig. 3), and the other one showed retardation in terms of length (30 mm). Another heifer subjected to embryo transfer was confirmed as pregnant by detection of an embryo (50%, 1/2) with a heartbeat on Days 29 and 62 of oestrus. After 276 days of oestrus, the heifer delivered a healthy male offspring with 46 kg bodyweight.

Discussion

In the present study, mitochondrial activity in IVG oocytes was high after 10 h pre-IVM, and the oocytes showed higher maturational and developmental competence to blastocyst stage. Furthermore, most of the IVG oocytes (more than 80%) with 10 h pre-IVM and in vivo-grown oocytes reached the MI stage after 10 h IVM culture, and around 90% of them

reached the MII stage after 22 h IVM culture, indicating that the nuclear maturation of IVG oocytes was enhanced and synchronised by 10 h pre-IVM. On the other hand, when the pre-IVM duration was extended to 20 h, IVG oocytes at MII stage were decreased to around 80%, and also 10% of IVG oocytes started meiotic resumption spontaneously and some of the IVG oocytes were already degenerated. These results indicate that the extension of pre-IVM to 20 h induced the degradation of oocytes or cumulus cells, resulting in the lost of the gap junction between the IVG oocyte and cumulus cells. It was reported that reducing nuclear asynchrony during IVM by temporary meiotic arrest using dibutyryl cyclic AMP (dbcAMP) appeared to enhance the nuclear maturation and developmental competence of porcine oocytes (Somfai et al. 2003). It was also reported that increasing mitochondrial activity and ATP content during pre-IVM by using IBMX and forskolin improved the nuclear maturation and developmental competence of mouse oocytes (Zeng et al. 2013). It is well known that cAMP can be increased in oocytes by cAMP analogues (e.g. dbcAMP), activators of adenylate cyclase (e.g. FSH, forskolin) and PDE inhibitors (e.g. IBMX; Gilchrist and Thompson 2007). The intracellular second messenger, cAMP, plays a significant role in the regulation of mitochondrial activity in mammalian cells (Papa 2006; Carlucci et al. 2008) and further meiosis progression in oocytes (Thomas et al. 2002). It was reported that bovine oocytes derived from larger follicles (>6 mm in diameter) showed higher mitochondrial activity (Machatkova et al. 2012) and a higher proportion of blastocyst development (Lonergan et al. 1994) than those from small follicles (<6 mm in diameter). These reports suggest that mitochondrial activity in bovine oocytes increases during follicular development and that higher mitochondrial activity is associated with greater developmental competence of immature oocytes. The present pre-IVM

285 culture system may have mimicked follicular development in terms of the dynamics of
286 mitochondrial activity. By adding IBMX and FSH to pre-IVM medium, the cAMP concentration
287 in IVG oocytes may increase, resulting in the improvement of mitochondrial activity before IVM
288 culture, and may have led to the improved maturational and developmental competences of
289 IVG oocytes with 10 h pre-IVM in the present study.

290 After IVM culture, the mitochondrial activity in IVG oocytes at MII stage became similar
291 regardless of pre-IVM duration, because mitochondrial activities in IVG oocytes with 0 and 20
292 h were enhanced. It was reported that bovine IVM oocytes exhibited higher mitochondrial
293 activity than immature oocytes (Tarazona et al. 2006). This corresponded to our results of IVG
294 oocytes with 0 and 20 h pre-IVM. However, in vivo-matured porcine oocytes showed similar
295 mitochondrial activity to immature oocytes (Romek et al. 2011). This corresponded to our
296 results of IVG oocytes with 10 h pre-IVM. These results may indicate that IVG oocytes with 10
297 h pre-IVM have similar characteristics to in vivo-matured oocytes. In our previous study
298 (Koyama et al. 2014), we showed that the mitochondrial activity and ATP content were
299 increasing in parallel as the duration of IVM culture was extended, and that the developmental
300 competence of bovine oocytes did not correlate with them directly. It was also reported that
301 ATP content in ovum pick up-derived oocytes did not increase during IVM culture, but ATP
302 content in abattoir-derived oocytes did increase (Tamassia et al. 2004). From these results, we
303 speculate that IVG oocytes with 10 h pre-IVM may have similar competence of nuclear
304 maturation and development to blastocysts from in vivo-grown oocytes immediately collected
305 from live cows. On the other hand, IVG oocytes with 0 and 20 h pre-IVM may have not
306 acquired these competences yet and may have already lost them, respectively. In further

307 studies we should confirm the mitochondrial activity of in vivo-matured bovine oocytes.

308 ROS in IVG oocytes with 0 h pre-IVM were higher than in those with 10 and 20 h pre-IVM.
309 A previous study indicated that, when goat granulosa cells were treated with FSH in vitro,
310 catalase activity was stimulated significantly (Behl and Pandey 2002). In cumulus
311 cell-enclosed bovine oocytes, enzymatic antioxidant (superoxide dismutase, glutathione
312 peroxidase and catalase) systems can attenuate the effect of oxidative stress by scavenging
313 ROS (Cetica et al. 2001). In the present study, pre-IVM culture medium supplemented with
314 FSH may directly or indirectly neutralise ROS generation in oocytes. Our previous results
315 showed that the developmental rate to blastocysts (12.7%) without pre-IVM was significantly
316 lower than with 20 h pre-IVM (26.1%), despite the similar normal fertilisation rate (Huang et al.
317 2014). It is speculated that high ROS content in IVG oocytes without pre-IVM may result in a
318 lower developmental rate to blastocysts than with pre-IVM.

319 The mitochondrial activity and blastocyst development of IVG oocytes with 10 h pre-IVM
320 were decreased by extending the pre-IVM duration to 20 h in the present study, although there
321 was no significant difference in ROS content between IVG oocytes with 10 and 20 h pre-IVM.
322 This result indicates that the decreases of mitochondrial activity and developmental
323 competence in IVG oocytes with 20 h pre-IVM were probably not due to ROS production.
324 During apoptosis, mitochondria rapidly lose their transmembrane potential (Ly et al. 2003). It
325 was reported that the pro-apoptotic pathway can be promoted by the cAMP signalling pathway
326 in most cells (Insel et al. 2012) and that granulosa cells stimulated with forskolin and IBMX
327 exhibited a longer-lasting rise in intracellular cAMP, resulting in cell apoptosis (Aharoni et al.
328 1995). It was indicated that the duration of the intracellular rise in cAMP plays an important role

329 in controlling the extent of apoptosis in granulosa cells (Aharoni et al. 1995). Therefore,
330 investigation of cAMP concentration and apoptotic program induction during pre-IVM culture
331 should be conducted in future studies.

332 Following the embryo transfer of blastocysts derived from IVG oocytes with 10 h pre-IVM,
333 six out of seven elongated embryos showed a normal length (40 to 100 mm), similar to the rate
334 of in vivo-derived bovine embryos (Betteridge et al. 1980; Gustafsson and Ploen 1986), and
335 one out of two embryos was maintained in the pregnancy. After 276 days of oestrus, the heifer
336 delivered a male calf with normal bodyweight (46 kg). Large calf syndrome was not observed
337 in the present study although the oocytes were cultured for 3 weeks in vitro. These results
338 suggest that IVG oocytes with 10 h pre-IVM acquired full developmental competence the same
339 as in vivo-grown IVM oocytes and probably in vivo-grown in vivo-matured oocytes.

340 In conclusion, we clarified that the mitochondrial activity of bovine IVG oocytes peaked at
341 10 h pre-IVM culture with IBMX and was closely correlated with the nuclear maturation and
342 developmental competences of IVG oocytes. The IVG oocytes with 10 h pre-IVM can acquire
343 high maturational and developmental competences, the same as in vivo-grown oocytes.
344 However, the extension of pre-IVM duration to 20 h induced oocyte or cumulus cell
345 degradation and reduced the mitochondrial activity before IVM culture and the developmental
346 competence of IVG oocytes.

347

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Figure Legends

Fig. 1. Mitochondrial activity (Δψm) of IVG oocytes with different pre-IVM durations before and after 22 h IVM culture.

The ratio of red to green fluorescence was quantified as an indicator of mitochondrial activity. Lines of the boxes delineate the 25th, 50th and 75th percentiles and the whiskers depict the 10th and 90th percentiles of a population. The circles represent the outliers.

[a, b] Different characters indicate a significant difference between different pre-IVM duration ($P < 0.05$).

* Asterisk indicates a significant difference between same pre-IVM duration before IVM and after IVM ($P < 0.05$).

Numbers in parentheses are the numbers of IVG oocytes used (5 replicates before IVM and 3 replicates after IVM).

Fig. 2. Reactive oxygen species (ROS) in IVG oocytes at different pre-IVM durations.

Lines of the boxes delineate the 25th, 50th and 75th percentiles and the whiskers depict the 10th and 90th percentiles of a population. The circles represent the outliers.

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

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

Fig. 3. The embryo (length 100 mm) recovered from cattle at 16 days of estrus was elongated.

Bar represents 5 mm.

Fig. 1.

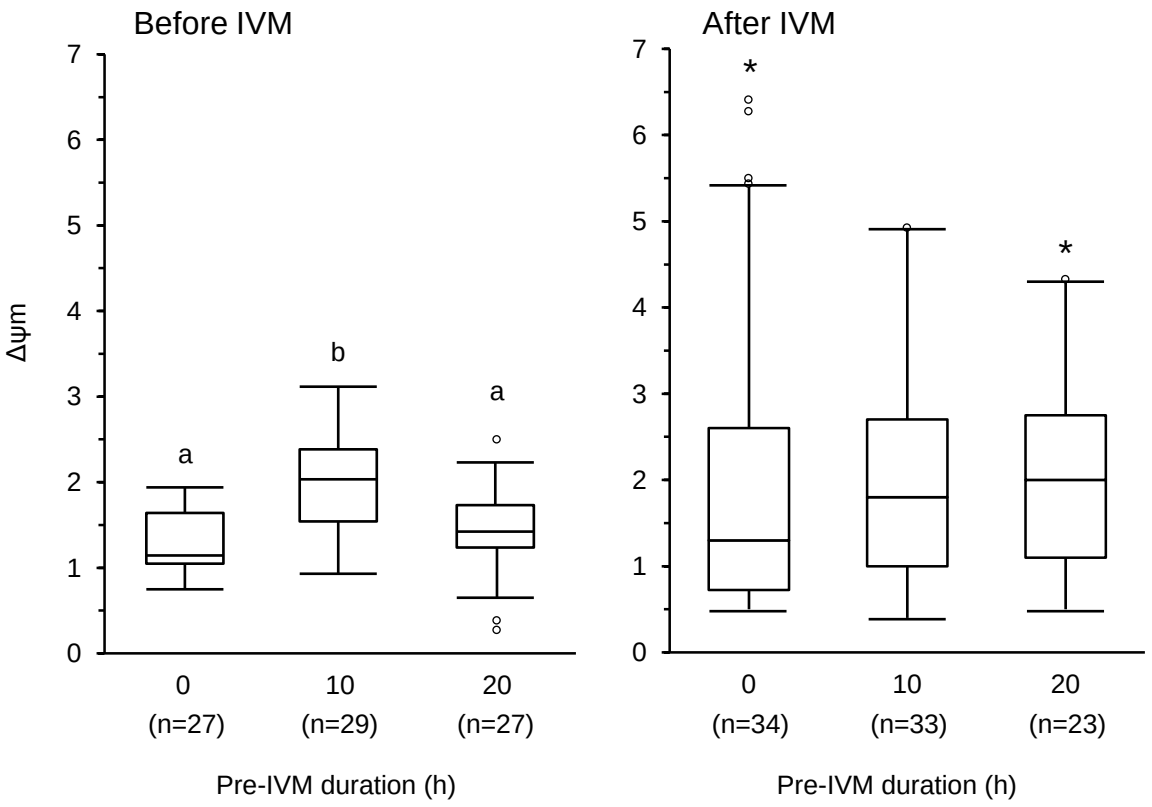
Before IVM
After IVM
Δψm
0
1
2
3
4
5
6
7
a
b
a
*
*
0
(n=27)
10
(n=29)
20
(n=27)
0
(n=34)
10
(n=33)
20
(n=23)
Pre-IVM duration (h)
Pre-IVM duration (h)

Fig. 2.

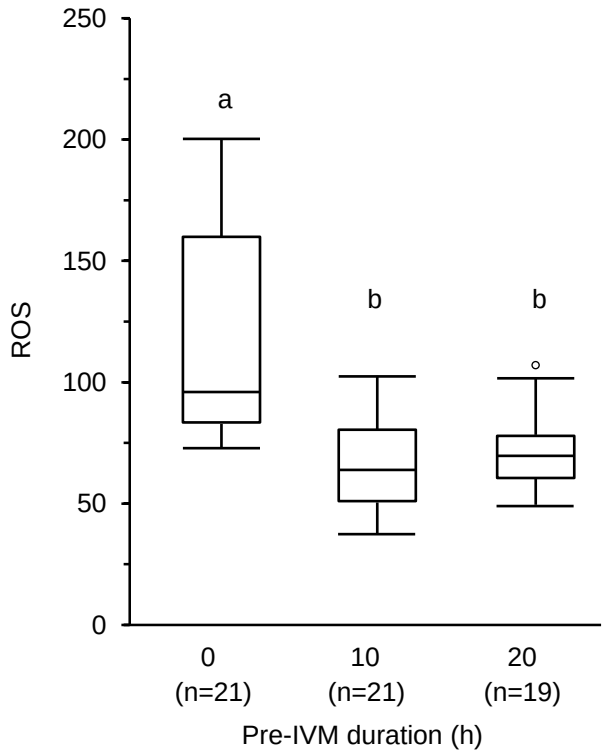
250
200
150
100
50
0
ROS
a
b
b
0
(n=21)
10
(n=21)
20
(n=19)
Pre-IVM duration (h)

Fig. 3.

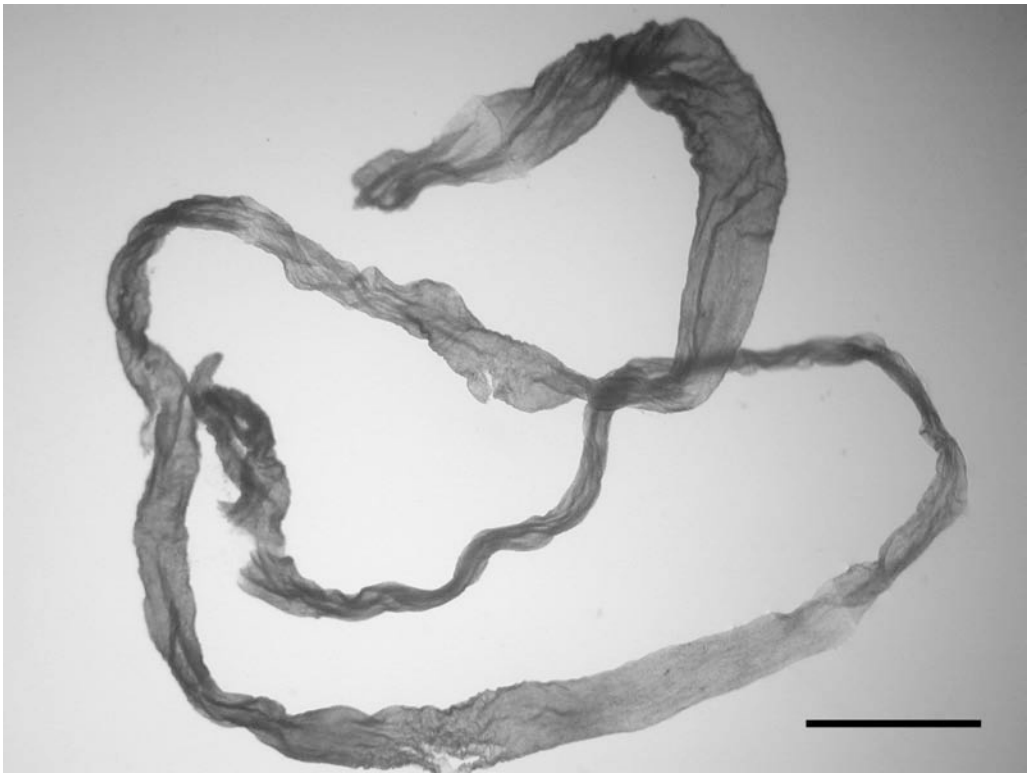

Table 1. Effect of pre-IVM duration on meiotic resumption of IVG oocytes after 10 h IVM

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

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

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

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

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

Values are mean ± SD.

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

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

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

Table 3. Effect of pre-IVM duration on meiotic arrest of IVG oocytes

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

GV, germinal vesicle; GVBD, germinal vesicle breakdown; M I, metaphase I

Pre-IVM duration (h)	No. of IVM (replicates)	% of oocytes at each stage			
		GV	GVBD	M I	Degeneration
0	42 (3)	100.0 ± 0.0[a]	0	0	0
10	60 (3)	100.0 ± 0.0[a]	0	0	0
20	55 (3)	90.0 ± 5.5[b]	5.7 ± 1.7	1.7 ± 2.9	2.6 ± 4.4

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

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

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

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

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