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## Effects of *in vitro*-growth culture duration on fertilizability of bovine growing oocytes and proliferation of cells surrounding oocytes

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### Abstract

The effects of *in vitro*-growth (IVG) culture duration (12 and 14 days) on the fertilizability and the developmental ability of IVG oocytes matured with pre-maturational culture, and on the proliferation of cells surrounding oocytes during IVG culture were examined. The fertilization and cleavage rates of 12- and 14-day IVG oocytes were similar; however, 14-day IVG oocytes showed lower blastocyst development rate than 12-day IVG oocytes. In addition, the number of cells surrounding oocytes increased until 12 days of IVG, but decreased at 14 days. The results indicated that the extension of IVG period beyond 12 days impaired the proliferation of cells surrounding oocytes, leading to the decrease of developmental ability but not fertilizability of 14-day IVG oocytes.

Key Words: early antral follicles, fertilization, IVG duration

The nuclear maturation rate and developmental rate 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 derived from antral follicles (2 to 8 mm in diameter)<sup>7,8,22</sup>. In our previous study<sup>9</sup>, the nuclear maturation rate of bovine IVG oocytes, which were derived from early antral follicles, were improved from 50% to more than 70% by subjecting them to 12- or 14-day IVG followed by pre-maturational (pre-IVM) culture for 20 hr in medium supplemented with

phosphodiesterase (PDE) inhibitor (3-isobutyl-1-methylxanthine, IBMX). We also showed that the developmental rate to blastocysts in 12-day IVG oocytes (25%) was higher than that in 14-day IVG oocytes (10%), although the cleavage rates of 12- and 14-day IVG oocytes were similar (around 50%)<sup>9</sup>. The nuclear maturation rate (70%) in IVG oocytes with pre-IVM was quite different from the cleavage rate (50%)<sup>9</sup>, although they were almost the same (around 90%) in *in vivo*-grown oocytes. The cause of this discrepancy is still unclear. One of the causes is speculated to be

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that fertilization does not occur normally in IVG oocytes, especially oocytes with 14-day IVG. Therefore we should determine the fertilizability of IVG oocytes with pre-IVM.

Another reason of low developmental competences may be due to the small diameter (around 115  $\mu\text{m}$ ) of IVG oocytes<sup>7,9,22</sup>, because it has been reported that bovine oocytes acquire the developmental competences completely after growing more than 120  $\mu\text{m}$  in diameter<sup>4,15</sup>. To support the oocyte growth more than 120  $\mu\text{m}$ , IVG culture duration should be extended; however, we also indicated that the extension of IVG culture duration beyond 12 days could not support the growth of IVG oocytes, and that cumulus cells surrounding oocytes with 14-day IVG were damaged compared to that with 12-day IVG<sup>9</sup>. In the previous study<sup>9</sup>, we did not investigate the cell proliferation during IVG culture. Also there are no reports investigated about granulosa cell proliferation during IVG culture of bovine oocytes. Therefore, for improving IVG culture systems, it is necessary to investigate the dynamics of the proliferation of cells supporting oocyte growth.

In the present study, we examined the fertilizability of 12- and 14-day IVG oocytes matured with pre-IVM, and confirmed their embryonic development using a bull sperm which were different from sperm used in the previous study<sup>9</sup>. In addition, the proliferation of cells surrounding oocytes was examined during IVG culture.

IVG were performed as described previously<sup>9</sup>. Briefly, bovine ovaries obtained at a local abattoir were transported to the laboratory, and oocyte-cumulus-granulosa complexes (OCGCs) were isolated from early antral follicles (0.5 to 1 mm in diameter). The OCGCs with a normal appearance were cultured individually in a 96-well culture plate (Falcon 353872, Becton Dickinson, Franklin Lakes, NJ, USA) with 200  $\mu\text{l}$  of the growth medium for 12 or 14 days at 39°C in humidified air with 5%  $\text{CO}_2$ . The growth medium was HEPES-buffered TCM199 (Cat #12340-030,

Invitrogen) supplemented with 0.91 mM sodium pyruvate (Sigma-Aldrich, St. Louis, MO, USA), 1  $\mu\text{g}/\text{ml}$  estradiol-17 $\beta$  (Sigma-Aldrich), 5% fetal calf serum (FCS; Invitrogen), 4 mM hypoxanthine (Sigma-Aldrich), 4% polyvinylpyrrolidone (MW 360,000, Sigma-Aldrich), and 50  $\mu\text{g}/\text{ml}$  gentamicin sulfate (Sigma-Aldrich). During IVG culture, half (100  $\mu\text{l}$ ) of the growth medium was replaced by the same amount of fresh medium every 4 days. At the onset of IVG culture, OCGCs were photographed under an inverted microscope (CK 40, Olympus, Tokyo, Japan) attached with a CCD camera (Moticam 2000, Shimadzu Rika Corporation, Tokyo, Japan), and the diameters of oocytes (excluding the zona pellucida) were determined using software (Motic Images Plus 2.2s, Shimadzu). Mean diameter of oocytes used in the study was  $96.3 \pm 4.9 \mu\text{m}$  (range: 81.7 to 106  $\mu\text{m}$ ). At 8, 12 and 14 days of IVG culture, the survivability of OCGCs was evaluated by their morphological appearance; oocytes completely enclosed by a healthy granulosa cell layer were considered to be viable<sup>9</sup>.

After 12 or 14 days of IVG culture, oocytes surrounded by several layers of cumulus cells were collected from morphologically normal OCGCs and submitted to IVM with pre-IVM<sup>9</sup>. Briefly, they were cultured individually in each well of micro-well plates (Mini Trays 163118, NUNC, Roskilde, Denmark) filled with 6 ml of HEPES-buffered TCM-199<sup>12</sup> supplemented with 0.2 mM sodium pyruvate,  $2 \times 10^{-6}$  units/ml follicle-stimulating hormone (FSH, from porcine pituitary, Sigma-Aldrich), 0.5 mM IBMX (Sigma-Aldrich), 1  $\mu\text{g}/\text{ml}$  estradiol-17 $\beta$ , 10% FCS and 50  $\mu\text{g}/\text{ml}$  gentamicin sulfate at 39°C under 5%  $\text{CO}_2$  in air for 20 hr and then cultured in the same manner as pre-IVM using IVM medium, which was HEPES-buffered TCM199 supplemented with 0.2 mM sodium pyruvate, 0.02 units/ml FSH, 1  $\mu\text{g}/\text{ml}$  estradiol-17 $\beta$ , 10% FCS, and 50  $\mu\text{g}/\text{ml}$  gentamicin sulfate.

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<sup>18</sup>). In brief, cumulus-oocyte complexes (COCs) were incubated in droplets of IVM medium that was the same as used for IVG oocytes (about 10 COCs/50  $\mu$ l) covered with paraffin oil at 39°C under 5% CO<sub>2</sub> in air for 22 hr.

*In vitro* fertilization (IVF) was performed using frozen semen from a bull according to a procedure described previously<sup>19</sup>) with slight modification. In brief, motile sperm ( $5 \times 10^6$  sperm/ml) separated from thawed semen by a Percoll gradient (45 and 90%, Pharmacia BioProcess, Uppsala, Sweden) were co-incubated with COCs in a 100- $\mu$ l droplet (about 10 COCs/droplet) of modified Brackett and Oliphant isotonic medium<sup>1</sup>) containing 3 mg/ml fatty-acid free bovine serum albumin (BSA, Sigma-Aldrich) and 2.5 mM theophylline (Sigma-Aldrich)<sup>17</sup>) for 18 hr at 39°C in a humidified atmosphere of 5% CO<sub>2</sub>, 5% O<sub>2</sub> and 90% N<sub>2</sub>. To evaluate the fertilization, cumulus-free oocytes were stained with 1% aceto-orcein and examined for their nuclear statuses as described elsewhere<sup>19</sup>). The oocytes were considered as fertilized when they had an enlarged sperm head (s) or male pronucleus (s) with corresponding sperm tail (s). Monospermic penetration was defined as oocytes having a single male pronucleus and a female pronucleus or a single enlarged sperm head with the chromosomes at anaphase/telophase II. Before the examination of the fertilization, the diameters of several IVG oocytes were measured.

To confirm the developmental competence to blastocysts, cumulus-free presumptive zygotes were cultured for 6 days in a 30- $\mu$ l droplet (about 30 embryos/droplet) of the culture medium covered with paraffin oil at 39°C under 5% CO<sub>2</sub>, 5% O<sub>2</sub> and 90% N<sub>2</sub>, as described previously<sup>18</sup>). The culture medium was a modified synthetic oviduct fluid<sup>18</sup>) with 3 mg/ml BSA instead of polyvinyl alcohol. After 2 days (about 30 hr) and 6 days (about 150 hr) of *in vitro* culture (IVC), cleavage and development of presumptive zygotes to the blastocyst stage were assessed, respectively. Total cell numbers of blastocysts obtained after 6

days of IVC were counted using an air-drying method<sup>17</sup>).

Some of the OCGCs judged as surviving at 0, 8, 12 and 14 days of IVG culture were used for counting the numbers of cells surrounding oocytes, that is, both cumulus and granulosa cells. For cell counting, the IVG medium was replaced by the same volume (200  $\mu$ l) of Dulbecco's phosphate-buffered saline (DPBS) containing 0.05% trypsin and 0.53 mM EDTA (Cat. #15400, Invitrogen). The OCGCs were incubated in DPBS for 10 min at 37°C and dispersed by vigorous pipetting at the end of the trypsin-EDTA treatment. The concentration of cells in the suspension was determined using a hemocytometer and the number of cells in a well was calculated.

Survival, fertilization, cleavage and blastocyst rates were compared by Chi-square test; however, analysis was performed by Fisher's exact test when the sample number was low. Numbers of cells surrounding oocytes in a well and blastocyst cell numbers were compared by one-way analysis of variance followed by Tukey-Kramer's honestly significant difference test. All statistical analyses were performed using software (JMP Pro version 10.0.2, SAS Institute, Cary, NC, USA).

Total fertilization and monospermic penetration rates of 12-day IVG oocytes were similar to those of 14-day IVG oocytes, but lower than those of *in vivo*-grown oocytes ( $P < 0.05$ , Table 1). Although the monospermic penetration rate of 14-day IVG oocytes was lower ( $P < 0.05$ ) than that of *in vivo*-grown oocytes, the total fertilization rates were similar. The polyspermy rate of 14-day IVG oocytes tended to be higher than those of *in vivo*-grown oocytes and 12-day IVG oocytes ( $P = 0.18$  and  $0.17$ , respectively). The mean diameters of 12- and 14-day IVG oocytes after IVF were similar ( $116.2 \pm 4.6$  and  $115.6 \pm 4.0$   $\mu$ m, respectively). These results suggest that the ability of polyspermy block in 14-day IVG oocytes may be impaired. It is well known that cortical granules play important

**Table 1. Effect of duration on fertilization of *in vitro*-grown oocytes**

Duration of IVG (days)	No. of oocytes <sup>x</sup> (replicates)	% of monospermic penetration			% of polyspermy	% of total fertilization
		% of ESH	% of 2PN	% of subtotal		
12	87 (3)	25.3 <sup>ab</sup>	50.6	75.9 <sup>a</sup>	3.4	79.3 <sup>a</sup>
14	84 (3)	16.7 <sup>a</sup>	58.3	75.0 <sup>a</sup>	8.3	83.3 <sup>ab</sup>
Control <sup>y</sup>	65 (3)	35.4 <sup>b</sup>	53.9	89.2 <sup>b</sup>	3.1	92.3 <sup>b</sup>

<sup>a, b</sup>Values with different superscripts within columns differ significantly ( $P < 0.05$ ).

<sup>x</sup>A total of 171 IVG oocytes derived from 258 OGCs were used (20–30 oocytes/replicate).

<sup>y</sup>The oocytes collected from antral follicles (2 to 8 mm in diameter) served as *in vivo*-grown control.

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

**Table 2. Effect of duration on development of *in vitro*-grown oocytes after IVF**

Duration of IVG (days)	No. of oocytes <sup>x</sup> (replicates)	% of cleaved oocytes	% of blastocysts based on		Cell no. in blastocysts (n)
			Inseminated oocytes	Cleaved oocytes	
12	110 (3)	60.0 <sup>a</sup>	25.5 <sup>a</sup>	42.4 <sup>a</sup>	129.0 ± 65.2 <sup>ab</sup> (28)
14	113 (3)	61.9 <sup>a</sup>	14.2 <sup>b</sup>	22.9 <sup>b</sup>	96.9 ± 36.4 <sup>b</sup> (16)
Control <sup>y</sup>	80 (3)	85.0 <sup>b</sup>	33.0 <sup>c</sup>	39.1 <sup>a</sup>	148.8 ± 66.3 <sup>a</sup> (32)

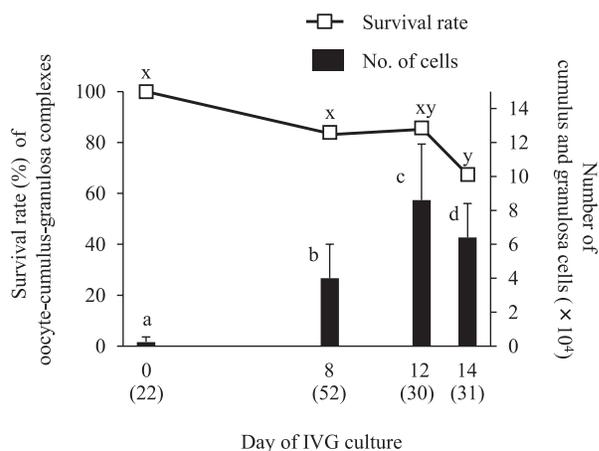
<sup>a-c</sup>Values with different superscripts within columns differ significantly ( $P < 0.05$ ).

<sup>x</sup>A total of 223 IVG oocytes derived from 332 OGCs were used (35–40 oocytes/replicate).

<sup>y</sup>The oocytes collected from antral follicles (2 to 8 mm in diameter) served as *in vivo*-grown control.

roles in the establishment of the block to polyspermy in bovine oocytes<sup>21</sup>, and bovine oocytes derived from follicles at different growth stages showed different statuses of cortical granules in ooplasm and their levels of developmental competence were different<sup>11,13</sup>. During oocyte maturation, clustered cortical granules were distributed and lined up next to the plasma membrane individually for preventing polyspermy, and the phenomenon was thought to be an indicator of cytoplasmic maturation of oocytes<sup>14</sup>. Therefore, it speculated that the ability of IVG oocytes distributing cortical granules (*i.e.*, the status of cytoplasmic maturation) may be different between oocytes after 12- and 14-day IVG. Organelle redistribution is essential for cytoplasmic maturation of oocytes and mitochondria also plays crucial roles during cytoplasmic maturation<sup>6</sup>. In further study, we should investigate the status of ooplasm including cortical granule distribution and mitochondrial activity relating to the cytoplasmic maturation of IVG oocytes.

The fertilization rate (around 75%) of IVG oocytes (Table 1) was almost the same as the expected nuclear maturation rate (70 to 75%)<sup>9</sup>, but the cleavage rate (around 60%, Table 2) was low compared to fertilization rate. These results indicate that most of the matured oocytes derived from IVG were fertilized normally. The reason for the discrepancy between normal fertilization and cleavage rates in IVG oocytes is not clear. It is suggested that cytoplasmic maturation in IVG oocytes might be inadequate and could not support oocyte cleavage, even if fertilization was accomplished. Early embryonic development is dependent on stored maternal RNAs and proteins synthesized during oogenesis and oocyte maturation<sup>10</sup>. It was reported that there was a concomitant decrease in total RNA synthesis once oocyte diameter increased beyond 110 μm and that RNA synthesis completely ceased in oocytes ≥120 μm in diameter<sup>5</sup>. These results may indicate that the lack of some types of mRNA in IVG oocytes results in low cleavage rates because the diameter of IVG oocytes was



**Fig. 1. Dynamics of survivability of oocyte-cumulus-granulosa complexes and mean numbers of cells surrounding oocyte during IVG culture.** Numbers in parentheses are the number of OCGCs used for counting the cell number in a well. <sup>a-d</sup>Values with different superscripts differ significantly ( $P < 0.05$ ). <sup>x, y</sup>Values with different superscripts differ significantly ( $P < 0.05$ ). Error bar means standard deviation.

between 110 and 120  $\mu\text{m}$ .

As shown in Table 2, 12-day IVG oocytes showed a higher blastocyst rate than 14-day IVG oocytes ( $P < 0.05$ ), although the cleavage rates were similar between IVG groups. There was no difference in the percentages of blastocysts based on cleaved oocytes of 12-day IVG oocytes and *in vivo*-grown oocytes, although the cleavage and blastocyst rates of *in vivo*-grown oocytes were higher than those of 12-day IVG oocytes ( $P < 0.05$ ). The blastocysts that originated from *in vivo*-grown oocytes had a larger number of cells than those from 14-day IVG oocytes ( $P < 0.05$ ). These results suggest that the intrinsic developmental abilities (probably the statuses of cytoplasmic maturation) of 12- and 14-day IVG oocytes and *in vivo*-grown oocytes are different. In further study, we should investigate about the relationship between cytoplasmic status of these oocytes and the developmental competence.

As shown in Fig. 1, the survival rate of OCGCs at 14 days of IVG was lower than those at 0 and 8 days ( $P < 0.05$ ) and tended to be lower than that at 12 days ( $P = 0.06$ ). The number of cells surrounding oocytes increased

until 12 days of IVG with increased culture duration, but decreased significantly at 14 days ( $P < 0.05$ ). In mice, early follicular development involves oocyte growth with a concomitant increase in the number of granulosa cells<sup>16</sup>, and these cells surrounding oocytes supply them with essential nutrients<sup>2,20</sup>. Therefore, it is important that granulosa and cumulus cells are healthy in order to support growth and the acquisition of maturational and developmental abilities in oocytes. At 14 days of IVG culture, a decrease of the number of cells surrounding oocytes was observed, and our previous study also showed low membrane integrity of cumulus cells<sup>9</sup>. These results indicate that the degradation of these cells start during the period from 12 to 14 days of IVG culture, leading to the decrease of developmental ability of 14-day IVG oocytes. It was reported that the addition of the fibroblast growth factor (FGF) 7 to IVG medium increased the diameter of 16-day IVG oocytes (about 117  $\mu\text{m}$ ) and improved the expression of several mRNAs in cultured OCGCs as similar to that of *in vivo*-grown OCGCs<sup>3</sup>. However the authors did not investigate the survival rate of OCGCs and the developmental competence of oocytes after 16-day IVG. They also reported no stimulatory effect of FGF7 on the proliferation of granulosa cells by the examination only at 16 days of IVG culture<sup>3</sup>. In further study, we should investigate about effects of the addition of several growth factors including FGF7 to IVG medium on the growth and developmental competence of IVG oocytes and on the proliferation and granulosa cells in detail.

In conclusion, although the fertilizability and cleavage were similar between 12- and 14-day IVG oocytes, 14-day IVG oocytes showed lower blastocyst development than 12-day IVG oocytes, which may have been due to impaired cytoplasmic status. The extension of IVG period impaired the proliferation and viability of cells surrounding oocytes, and also the developmental competence of IVG oocytes. Further study developing the culture system after 12 day of

IVG is required.

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