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—Original Article—

Efficient *in vitro* embryo production using *in vivo*-matured oocytes from superstimulated Japanese Black cows

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Abstract. We examined whether the use of *in vivo*-matured oocytes, collected by ovum pick-up (OPU) from superstimulated Japanese Black cows, can improve the productivity and quality of *in vitro* produced embryos. The cows were superstimulated by treatment with progesterone, GnRH, FSH and prostaglandin F2α according to a standardized protocol. The resulting *in vivo*-matured oocytes were collected by OPU and used subsequently for the other experiments. The immature oocytes from cows in the non-stimulated group were collected by OPU and then subjected to maturation *in vitro*. We found that the rate of normally distributed cortical granules of the matured oocyte cytoplasm in the superstimulated group was significantly higher than that in the non-stimulated group. The normal cleavage rate (i.e., production of embryos with two equal blastomeres without fragmentation) and freezable blastocyst rate were significantly higher in the superstimulated group than in the non-stimulated group. Among the transferable blastocysts, the ratio of embryos from normal cleavage was also significantly higher in the superstimulated group than in the non-stimulated group. For *in vivo*-matured oocytes, it was observed that the pregnancy rates were significantly higher when normally cleaved embryos were used for transfer. Taken together, these results suggest that high-quality embryos with respect to developmental kinetics can be efficiently produced with the use of *in vivo*-matured oocytes collected by OPU from superstimulated Japanese Black cows.

Key words: Bovine, Embryonic development, Embryo transfer, Ovum pick-up, Superstimulation

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Over the past several decades, multiple ovulation and embryo transfer (MOET) has been developed and widely used to accelerate genetic improvement in herds of cattle [1]. On the other hand, along with the development of the *in vitro* embryo production (IVP) system, including *in vitro* maturation (IVM), fertilization (IVF), and culture (IVC), embryos produced *in vitro* have also been increasingly utilized for calf production. Nowadays, the number of *in vitro*-produced embryos that are transferred into recipients is comparable to that of *in vivo*-produced embryos (International Embryo Transfer Society http://www.iets.org/comm_data.asp). Ovum pick-up (OPU) followed by IVP (OPU-IVP) is being largely utilized by commercial cattle breeders as the practicality of IVP dictates that oocytes be collected from living cows with superior genetic value [2]. In OPU, oocytes can be collected in any of the stages of estrus, including early pregnancy, and even from cows with reproductive disorders, juvenile calves, and prepubertal heifers [3–6]. In addition, use of OPU along with IVF allows for the flexibility in which the collected oocytes can be inseminated individually with semen from different bulls, increasing the chance of generating genetic variability in the resultant embryos [7]. These distinct advantages of OPU-IVP, in increasing the selection intensity and shortening the generation interval, suggest that OPU-IVP is a reliable alternative to MOET in the efficient genetic-improvement of cattle [7, 8]. Thus, the demand for OPU-IVP embryos in calf production is expected to rise.

However, despite the substantial improvements made in the efficiency of IVP protocols in livestock, the rate of embryos developing normally remains lower than that seen in *in vivo*-produced embryos [9]. In fact, pregnancy rates obtained in OPU-IVP procedures (transferring *in vitro*-produced blastocysts) are lower than that obtained in MOET procedures (transferring *in vivo*-produced blastocysts) [1, 10]. This suggests that the development of IVP systems enabling a more efficient production of embryos with higher viability is required. Oocyte quality at the start of IVP is considered the most critical factor that affects the outcome of the *in vitro*-produced embryos [7, 11]. Several studies in cattle have also shown that *in vivo*-matured oocytes have higher developmental competence than *in vitro*-matured oocytes as evidenced by their higher rates of development to the blastocyst stage [11–15]. A possible reason for this difference in developmental competence is that cytoplasmic maturation of *in vitro*-matured oocytes, including the distribution of intracellular organelles and molecules and the

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storage of maternal mRNAs and proteins, is compromised, whereas *in vivo*-matured oocytes developing under the proper conditions in follicles exhibit fully achieved cytoplasmic maturation [7]. Based on these observations, the use of *in vivo*-matured oocytes is considered reasonable for efficient embryo production by OPU-IVP.

Several studies have shown that the developmental kinetics of embryos in IVP are related to their developmental competence [16–19]. Notably, previous studies on bovine embryos, which analyzed images from time-lapse cinematography (TLC), revealed that the cleavage pattern at the first cell division after IVF is significantly correlated to *in vitro* developmental competence [20] as well as the pregnancy rates after embryo transfer [21, 22]. Higher pregnancy rates were observed in cows that received blastocysts that had undergone normal cleavage, i.e., in which embryos were unfragmented and had two equal-sized blastomeres at the first cleavage [21, 22]. Although further studies are needed to elucidate the factors that cause abnormal cleavage at the first cell division and the subsequent lowering of pregnancy rates, these findings indicate that pregnancy rates can be increased through the efficient production of blastocysts, which originate from embryos with a normal first cleavage pattern in OPU-IVP. However, to our knowledge, studies investigating the first cleavage pattern in embryos produced from *in vivo*-matured oocytes are lacking.

The present study aimed to investigate if the use of *in vivo*-matured oocytes collected from superstimulated Japanese Black cows increased the production of blastocysts that originated from normally cleaved embryos at first cell division, and if it contributed to enhancing pregnancy rates in OPU-IVF. In order to ascertain the above set goals we conducted four experiments, as follows: (1) in order to estimate the effect of superstimulation on the efficiency of oocyte collection by OPU in Japanese Black cows, we compared the ovarian status and number of oocytes recovered; (2) we compared the cytoplasmic maturation status between *in vivo*- and *in vitro*-matured oocytes by investigating the distribution of cortical granules (CGs), which is a known cytoplasmic maturation indicator due to its important role in preventing polyspermy during fertilization [23–26]; (3) in order to investigate if superstimulating cows prior to oocyte collection by OPU enhances the production of blastocysts with higher viability, we studied the *in vitro* development of blastocysts in embryos that originated from *in vivo*- and *in vitro*-matured oocytes while comparing their normal cleavage rates and morphological quality; and (4) to estimate the post-transfer viability of the resultant blastocyst we analyzed the pregnancy rates in cows that received blastocyst from either normally or abnormally cleaved embryos.

Materials and Methods

Animal care and use

Animals were treated according to preapproved animal care and use guidelines established by the Institutional Animal Care and Use Committee of Saga Prefectural Livestock Experiment Station, Japan.

Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

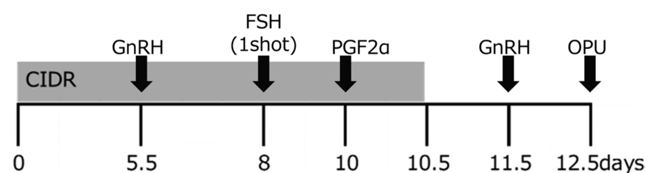

Fig. 1. Schematic diagram of the protocol for the collection of *in vivo*-matured oocytes. Superstimulation was performed according to Matoba *et al*. [27] with minor modifications; Cows received an intravaginal progesterone-releasing device (CIDR: controlled internal drug release) (Day 0), administration of 100 µg gonadotropin-releasing hormone (GnRH) on Day 5.5, a single administration of 30 Armour units of follicle stimulating hormone (FSH) on Day 8, and prostaglandin F2α (PGF2α) on Day 10; The progesterone-releasing device was removed on Day 10.5, and then 100 µg GnRH was administered a second time on Day 11.5 (0 h); Ovum pick-up (OPU) was conducted 25–26 h and 30 h after the second GnRH administration [GnRH: fertirelin acetate, FSH: in aluminum hydroxide gel, PGF2α: cloprostenol].

Donor cows and their management

The non-pregnant Japanese Black cows (n = 7, 6.4 ± 1.4 years old, 5.0 ± 1.3 parity) used in this study were raised in Saga Prefectural Livestock Experiment Station. The cows were housed in a stanchion barn with sufficient space and were fed according to Japanese feeding standards for beef cattle. At the start of the experiment, the cows had a mean body condition score of approximately 3.5 (BCS, scale 1 to 5 according to the Wagyu Registry Association's Guide).

Superstimulated and non-stimulated groups

For collection of *in vivo*-matured oocytes, superstimulation was performed as previously described by Matoba *et al*. [27] with minor modification (Fig. 1). Briefly, cows in the superstimulated group received an intravaginal progesterone-releasing device (CIDR; Pfizer, Tokyo, Japan) at a random stage of the estrus division on Day 0 (start of the superstimulation program). On Day 5.5, in order to induce dominant follicle ovulation, these cows received intramuscular administration of 100 µg fertirelin acetate–an analogue of gonadotropin-releasing hormone (GnRH) (Supolnen; Kyoritsu Seiyaku, Tokyo, Japan). This was followed by a single subcutaneous administration of follicle stimulating hormone (FSH) in aluminum hydroxide gel (30 Armour units, Antrin Al; Kyoritsu Seiyaku) on Day 8, and intramuscular administration of 500 µg prostaglandin F2α (PGF2α) analogue (Estrumate; Intervet, Tokyo, Japan) on Day 10. The progesterone-releasing device was removed on Day 10.5, and subsequently on Day 11.5 (0 h), 100 µg of GnRH was administered intramuscularly to induce a surge luteinizing hormone (LH) levels. The OPU and IVF procedures were conducted 25–26 h and 30 h after this second GnRH administration, respectively. Cows in the non-stimulated (control) group received no treatment before OPU, and OPU was conducted at a random stage of their estrus cycle.

Oocyte collection by OPU

The OPU procedure was performed according to the method previously described by Matoba *et al*. [27]. The oocyte collection procedure was as follows: 10 mg of xylazine hydrochloride (Celactal;

Bayer, Tokyo, Japan) was administered intramuscularly to sedate the study animals and epidural anesthesia was administered using 100 mg of lidocaine hydrochloride (LIDOCAINE Hydrochloride Injection; Pfizer, Tokyo, Japan) to facilitate the handling of the ovaries through the rectum. After the rectum was emptied and the perineal area was cleaned, all visible follicles of diameter ≥ 2 mm were aspirated using a real-time B-mode ultrasound scanner (ECHOPAL II, Hitachi Medical, Tokyo, Japan) equipped with a 7.5 MHz microconvex transducer and a 17 ga × 540 mm disposable needle (COVA Needle; Misawa Medical Industry, Tokyo, Japan). Follicular aspiration was conducted using an electric suction pump (FV4; Fujihira Industry, Tokyo, Japan) at a constant negative pressure of 130 mmHg (superstimulated group) and 120 mmHg (control group). Follicle contents were aspirated into 50 ml conical tubes that contained approximately 2 ml of Lactated Ringer's solution (Nippon Zenyaku Kogyo, Fukushima, Japan) supplemented with 10 IU/ml heparin (Heparin Sodium 5,000 units/5 ml for Injection; Mochida Pharmaceutical, Tokyo, Japan) and 0.5% (v/v) fetal bovine serum (FBS; ThermoFisher Scientific, Waltham, MA, USA) at 30ºC. The number and size of follicles aspirated from each cow were recorded. All oocyte recovery procedures were performed by the same veterinarian. Conical tubes that contained the follicular aspirates were transported to a laboratory and the cumulus-oocyte complexes (COCs) were collected using a Petri dish with a mesh filter (Cell Collector; Nipro Medical Industries, Gunma, Japan).

In vitro embryo production

In the non-stimulated group, the collected oocytes were subjected to IVM followed by IVF. The TCM-199 (Thermo Fisher Scientific), supplemented with 5% FBS, FSH (0.02 IU/ml; Kyoritsu Seiyaku, Tokyo, Japan), and gentamicin (50 µg/ml; Nacalai Tesque, Kyoto, Japan), was used as the IVM medium. Grade 1 to 3 immature oocytes were induced to mature in 100 µl droplets of IVM medium covered with liquid paraffin (Nacalai Tesque) and incubated at 38.5°C for 22 h in a humidified atmosphere of 5% CO_2 in air. In the superstimulated group, *in vivo*-matured oocytes that had expanded cumulus cells were washed three times with TCM-199 supplemented with 5% FBS and 50 µg/ml gentamicin (preservation medium). They were incubated in 100 µl droplets of preservation medium per approximately 20 oocytes at 38.5°C for approximately 3 h in a humidified atmosphere of 5% CO_2 in air (until the 30-h time point from the 2nd GnRH administration was reached).

For thawing, the straw containing frozen semen was immersed in warm water (37°C) for 20 sec. Spermatozoa were washed by centrifugation (800 × *g* for 10 min) in 90% (v/v) Percoll solution (GE Healthcare Bio-Sciences AB, Stockholm, Sweden). After removing the supernatant, the pellet was diluted with IVF100 solution (Research Institute for the Functional Peptides, Yamagata, Japan) and centrifuged at 600 × *g* for 5 min. The spermatozoa pellet was then diluted with IVF100 to prepare a final sperm-cell concentration of 3.0×10^6 sperm/ml. The IVF procedure was performed at 38.5°C under humidified air with 5% CO_2 for 6 h; with 20 to 25 oocytes per 100 µl sperm droplet covered with liquid paraffin.

After fertilization, cumulus cells were removed mechanically by pipetting in CR1aa medium that contained 5% FBS [28]. Microwell dishes (LinKID micro25; Dai Nippon Printing, Tokyo, Japan) were used for culturing in order to identify each individual zygote. Putative zygotes with one or two polar bodies were cultured in 100 µl droplets of CR1aa medium supplemented with 5% FBS in a microwell dish. The droplets were covered with liquid paraffin. Zygotes (n = 20 to 25) were placed into the microwells (one zygote per microwell) and cultured for 8 days at 38.5°C in a humidified atmosphere of 5% O_2, 5% CO_2, and balanced with N_2.

Staining of cortical granules (CG)

The distribution of CGs in oocytes matured *in vivo* and *in vitro* was evaluated as described previously [29]. Briefly, the expanded cumulus cells and zona pellucida were removed by pipetting the COCs in a solution of TCM-199 that contained 1 mg/ml actinase E (Nacalai Tesque, Kyoto, Japan). Zona-free oocytes were fixed in 4% (w/v) paraformaldehyde solution (pH 7.4) for 60 min, rinsed three times in Dulbecco's phosphate buffered saline (PBS) that contained 0.05% (w/v) polyvinylpyrrolidone (PVP; Nacalai Tesque), then permeabilized in 0.05% (w/v) PVP-PBS that contained 0.05% (v/v) TritonX-100 for 5 min, and washed three times using 0.05% (w/v) PVP-PBS. After permeabilization, zona-free oocytes were rinsed in blocking solution [PBS that contained 1% (w/v) bovine serum albumin] for 15 min and incubated in 10 µg/ml fluorescein isothiocyanate (FITC)-labeled Lens culinaris agglutinin (LCA) (LCA-FITC, FL-1041, Vector Laboratories, Burlingame, CA, USA) for another 30 min in the dark. Oocytes were washed three times in 0.05% (w/v) PVP-PBS and mounted on glass slides using mounting solution (Vectashield; Vector Laboratories). The CG distribution was observed using confocal laser microscopy (C1, Nikon, Tokyo, Japan, Excitation: 488 nm, Emission: 515 nm). Images of equatorial sections were captured by repeated laser scanning (five times in 5 sec) to improve the signal-to-noise ratio.

Embryo transfer and pregnancy diagnosis

Blastocysts in culture for 7 or 8 days were evaluated according to the criteria defined in the International Embryo Transfer Society manual [30], and only blastocysts graded as code 1 were used for embryo transfer. Fresh single blastocysts were transferred into the ipsilateral uterine horn of Holstein recipient cows at 7 or 8 days after estrus. Pregnancy was diagnosed using ultrasonography (iMAGO; ECM, France) at 35 to 40 days after estrus. Recipient cows were bred in five commercial dairy farms and were synchronized with modified CIDR-based ovulation-synchronization protocols as described previously [31].

Experimental design

We conducted the afore-mentioned four experiments, as follows-

Experiment 1: effect of superstimulation on efficiency of oocyte collection by OPU

To assess the effects of superstimulation on efficiency of oocyte collection, the follicular size and number, and number of recovered oocytes were recorded and compared between non-stimulated and superstimulated groups. In order to be of use in further experiments, the COCs to be collected were selected according to different classifications from the non-stimulated and superstimulated groups, respectively, as described by Matoba *et al*. [27]. In the non-stimulated group, the immature COCs were stored in TCM-199 that was supplemented with 5% FBS at 37°C and their quality was evaluated using

a previously described procedure [32]. The immature COCs were graded as follows: grade 1 had several layers of cumulus cells and homogeneous oocyte cytoplasm; grade 2 had one to three layers of cumulus cells; grade 3 had completely denuded oocytes; grade 4 had expanded cumulus cells, and grade 5 had degenerated oocytes. Only immature COCs graded 1 to 3 underwent IVM and were used in the subsequent experiments. In contrast, in the superstimulated group, morphological assessment of COCs was conducted to estimate the maturation status of recovered oocytes. Only oocytes that exhibited expanded cumulus cells were identified as matured oocytes and used in the subsequent experiments.

Experiment 2: the distribution of cortical granules (CGs) in ooplasm of *in vitro*- and *in vivo*-matured oocytes

The distribution pattern of CGs in the ooplasm is used as an indicator of cytoplasmic maturation [23–26]. In this study, we evaluated the maturation status of oocytes matured *in vitro* and *in vivo* in the non-stimulated and superstimulated groups, respectively, by observing the CG distribution pattern. LCA-FITC was used to stain oocytes in the non-stimulated and superstimulated groups after IVM (Fig. 2C) and collection by OPU (Fig. 2A), respectively. The CG distribution patterns in bovine ooplasm were classified according to the four patterns described by Hosoe and Shioya [26]. Pattern I had CGs distributed in clusters or large aggregates (Fig. 3A); Pattern II had CGs individually dispersed beneath the plasma membrane and partially clustered or aggregated (Fig. 3B); Pattern III had CGs completely dispersed beneath the plasma membrane (Fig. 3C); and Pattern IV had no CGs (Fig. 3D). Oocytes with CGs distributed in the cortical cytoplasm beneath the plasma membrane (patterns II and III) were classified as having normal distributions.

Experiment 3: *in vitro* development of embryos produced using *in vitro*- and *in vivo*-matured oocytes

To investigate whether oocyte collection from superstimulated cows enhances the production of blastocysts with higher viability, we compared the *in vitro* development of embryos obtained from *in vitro*- (non-stimulated group) and *in vivo*- (superstimulated group) matured oocytes. The first cleavage pattern in embryos was observed using an inverted microscope at 28 h post-insemination (hpi). This observation timing was chosen to evaluate blastocyst development because a previous study reported that almost all embryos, which developed to blastocyst stage, had completed their first cell division by this time point at the latest [21]. The first cleavage patterns of embryos were classified as follows: embryos with blastomeres of the same size without fragmentation (Fig. 4A) were classified as having undergone normal cleavage, and embryos with two blastomeres of different sizes (Fig. 4B), two blastomeres with a protrusion (Fig. 3C) or several small fragments (Fig. 4D), direct cleavage from the one-cell stage to three or four blastomeres (Fig. 4E) were classified as having undergone abnormal cleavage. The rates of cleavage and blastocyst development were measured at 50 hpi and on 8 days after IVC, respectively. The morphological quality of blastocysts was evaluated according to guidelines in the International Embryo Transfer Society manual [30]. Blastocysts graded as Code 1 and code 1 plus 2 were identified as freezable and transferable blastocysts, respectively.

Experiment 4: *in vivo* development of blastocysts developed from normally cleaved embryos after transfer

To compare the *in vivo* developmental competence between normally

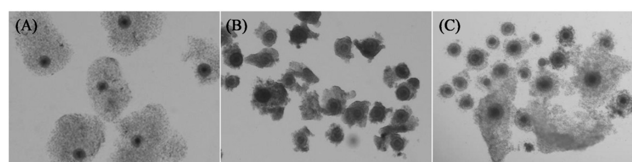

Fig. 2. Representative images of oocytes collected from non-stimulated and superstimulated cows. Oocytes collected by OPU from superstimulated and non-stimulated cows (A and B, respectively); Only oocytes surrounded by expanded cumulus cells were used as *in vivo*-matured oocytes (A); Oocytes collected from non-stimulated cows were subjected to *in vitro* maturation and then used as *in vitro*-matured oocytes (C).

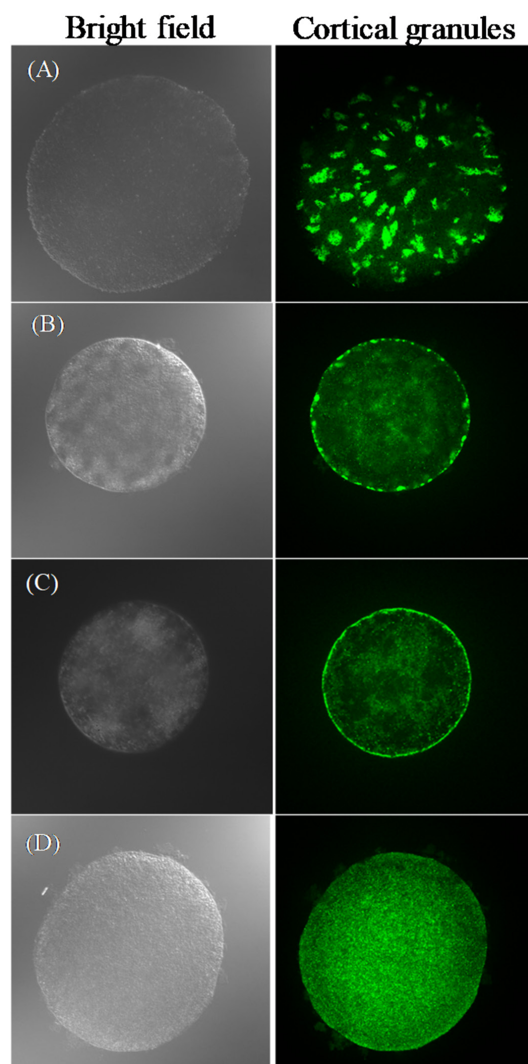

Fig. 3. Representative images of the distribution of cortical granules (CGs) in bovine matured oocytes. The distribution pattern of CGs in bovine ooplasm were classified according to the four patterns described by Hosoe and Shioya [26]: Pattern I – CGs distributed in clusters or large aggregates (A); Pattern II – CGs individually dispersed beneath the plasma membrane and partially clustered or aggregated (B); Pattern III – CGs completely dispersed beneath the plasma membrane (C); and Pattern IV - no CGs (D).

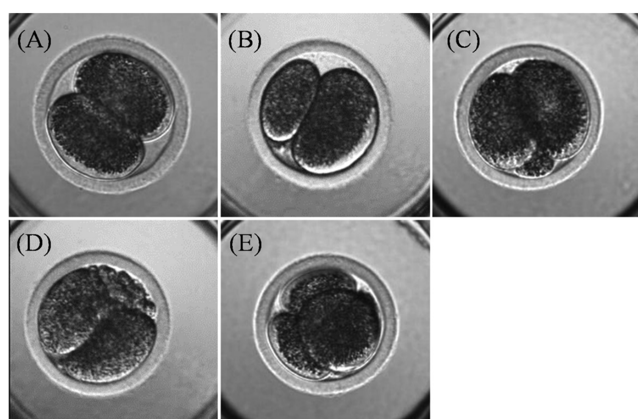

Fig. 4. Morphological classification of cleavage pattern of bovine embryos at the first cell cleavage. The cleavage patterns of embryos at the first cell division were classified as described by Somfai *et al*. [20]; Embryos with blastomeres of the same size without fragmentation (A) were classified as having undergone normal cleavage, and embryos with two blastomeres of different sizes (B), two blastomeres with a protrusion (C) or several small fragments (D), direct cleavage from the one-cell stage to three or four blastomeres (E) were classified as having undergone abnormal cleavage.

and abnormally cleaved embryos at the first cell division, blastocysts from *in vivo*-matured (superstimulated group) oocytes were transferred to Holstein recipient cows. Moreover, for comparison with *in vivo* developmental competence of embryos produced from *in vivo* oocytes, blastocysts from normally cleaved embryos in the non-stimulated group were also transferred to Holstein recipient cows. Blastocysts in culture for 7 or 8 days were used in all transfers. Pregnancy rates in recipient cows were measured at 35 to 40 days after estrus.

Statistical analyses

The distribution of CGs and *in vitro* development results were analyzed using a chi-squared test. The pregnancy rates were analyzed by Fisher's exact test. Other data were analyzed using a Student's *t*-test. All percentage data were arcsine-transformed prior to statistical analysis. P-values < 0.05 were considered statistically significant.

Results

Experiment 1: effect of superstimulation on efficiency of oocyte collection by OPU

The number of follicles and recovered oocytes in non-stimulated and superstimulated Japanese Black cows are shown in Table 1. The total number of follicles aspirated during each OPU session was similar between the non-stimulated and superstimulated groups. Numbers of large (> 8 mm) and medium (≥ 5 to ≤ 8 mm) follicles were higher in the superstimulated group than in the non-stimulated group (P < 0.01 and P < 0.05, respectively); whereas the number of small (< 5 mm) follicles was lower in the superstimulated group than in the non-stimulated group (P < 0.01). The representative images of oocytes collected from the superstimulated and non-stimulated cows are shown in Fig. 2A and 2B, respectively. No significant difference in the total number of recovered oocytes and the recovery rate (total number of oocytes / total number of follicles) was observed between the superstimulated and non-stimulated groups.

Experiment 2: the distribution of cortical granules (CGs) in ooplasm of in vitro- and in vivo-matured oocytes

Representative images of each of the observed distribution patterns of CGs are shown in Fig. 3. The oocytes with CGs distributed throughout the cortical cytoplasm were classified as having a normal distribution. The rate of oocytes showing normal CG distribution was higher in the superstimulated group than in the non-stimulated

Table 1. Number of follicles and recovered oocytes in non-stimulated (control) and superstimulated Japanese Black cows

	Non-stimulated	Superstimulated
No. of OPU sessions	7	6
No. of follicles at OPU		
Large follicles (> 8 mm)	1.1 ± 0.3 **	40.2 ± 4.2 **
Medium follicles (≥ 5 to ≤ 8 mm)	3.3 ± 0.7 *	15.5 ± 6.4 *
Small follicles (< 5 mm)	47.9 ± 8.9 **	7.5 ± 2.8 **
Total follicles	52.3 ± 8.9	63.2 ± 12.5
No. of oocytes		
Normal oocytes (grade 1 to 3)	29.0 ± 4.1	
Abnormal oocytes (grade 4 and 5)	5.4 ± 1.4	
Oocytes with expanded cumulus cells		36.0 ± 7.0
Oocytes with non-expanded cumulus cells		8.3 ± 1.7
Degenerated oocytes		2.0 ± 1.4
Total oocytes recovered	34.4 ± 3.7	46.3 ± 9.2
Recovery rate of oocytes (%) [a]	69.8 ± 5.6	73.8 ± 2.9

Oocytes were collected from non-stimulated and superstimulated cows prior to ovum pick-up (OPU). Data are expressed as means ± SEM per head per OPU session. [a] The percentages of total number of oocytes recovered to total number of follicles was denoted. Asterisks indicate that values within the same row are significantly different (*, P < 0.05; **, P < 0.01).

Table 2. Distribution patterns of cortical granules (CGs) in *in vitro*- (non-stimulated, control) and *in vivo*- (superstimulated) matured oocytes in Japanese Black cows

Group	No. of oocytes examined	No. of oocytes showing each distribution pattern of CGs				No. of oocytes showing normal CG distribution (%)
		I	II	III	IV	
Non-stimulated	26	11	10	4	1	14 (53.8) *
Superstimulated	31	5	11	14	1	25 (80.6) *

The distribution patterns of CGs in ooplasm were detected after *in vitro* maturation (non-stimulated group) or OPU (superstimulated group), and classified according to the four patterns shown in Fig. 3. Oocytes with CGs distributing cortical cytoplasm (patterns II and III) were classified as normal distribution. Asterisks indicate that values within the same column are significantly different (P < 0.05).

Table 3. *In vitro* development of embryos produced using *in vitro*- (non-stimulated, control) and *in vivo*- (superstimulated) matured oocytes

	Non-stimulated	Superstimulated
No. of putative zygotes cultured	200	204
No. of embryos cleaved (%) [a]	166 (83.0)	160 (78.4)
No. of normally cleaved embryos (%) [b]	86 (51.8) *	105 (65.6) *
No. of embryos developed to blastocyst stage on Day 8 (%) [b]	100 (60.2)	96 (60.0)
No. of IETS code 1 and 2 blastocysts (%) [b]	81 (48.8)	92 (57.5)
No. of IETS code 1 blastocysts (%) [b]	42 (25.3) *	59 (36.9) *
No. of IETS code 1 and 2 blastocysts from normal cleaved embryos (%) [b]	49 (29.5) **	73 (45.6) **

Oocytes collected from non-stimulated and superstimulated Japanese Black cows were subjected to *in vitro* and *in vivo* maturation in the non-stimulated and superstimulated groups, respectively, and used for *in vitro* fertilization. Data for the non-stimulated and superstimulated groups were obtained from 7 and 6 replicates, respectively. [a] The percentages of cleaved embryos at 50 hpi to cultured zygotes was denoted. [b] The percentages of embryos corresponding to each criterion in cleaved embryos was denoted. Asterisks indicate that values within the same row are significantly different (*, P < 0.05; **, P < 0.01).

group (P < 0.05; Table 2).

Experiment 3: in vitro development of embryos produced using in vitro- and in vivo-matured oocytes

In vitro developmental characteristics of the embryos obtained from *in vitro*- (non-stimulated group) and *in vivo*- (superstimulated group) matured oocytes are shown in Table 3. Although no differences in the cleavage rates and developmental rates (i.e., to blastocyst stage) were observed between the groups, the rates of normally cleaved embryos and code 1 (freezable) blastocysts were higher in the superstimulated group than in the non-stimulated group (P < 0.05). In addition, the rate of code 1 and 2 (transferable) blastocysts obtained from embryos with normal cleavage at first cell division was also higher in the superstimulated group than in the non-stimulated group (P < 0.01).

Experiment 4: in vivo development of blastocysts developed from normally cleaved embryos after transfer

In the superstimulated group, the pregnancy rate in transfer of blastocysts developed from normally cleaved embryos was 61.9% (13/21), whereas that in transfers of blastocysts developed from abnormally cleaved embryos was 12.5% (1/ 8). Thus, it can be seen that within the superstimulated group, pregnancy rates differed between normally and abnormally cleaved embryos (P < 0.05). Moreover, in the non-stimulated group, the pregnancy rate was 50.0% (5/ 10) when blastocysts from normally cleaved embryos were used for embryo transfer. However, the difference in pregnancy rates achieved by using normally cleaved embryos between the non-stimulated (50.0%) and superstimulated groups (61.9%) was not significant.

Discussion

The advantages offered by OPU-IVP technique over the MOET technique in enhancing cattle breeding, is offset by the low developmental competence of *in vitro*-produced embryos and this has been the most serious hindrance in the use of this technique for efficient calf production. Even with numerous efforts made over the last few decades, the developmental competence of *in vitro*-produced embryos has not yet become comparable to that of *in vivo*-produced embryos [33]. However, recent studies have demonstrated that developmental kinetics, especially the first cell division timing and pattern, can be used to select high viability embryos for transfer that would in turn result in higher pregnancy rates in recipient cows [20–22]. Thus, according to the current-state of knowledge, to improve pregnancy success, blastocysts should be selected prior to transfer not only by conventional morphological evaluation but also by developmental kinetics. Therefore, in the present study, we verified the hypothesis that the use of *in vivo*-matured oocytes could contribute to improving pregnancy rates by yielding larger number of embryos with higher developmental competence (in developmental kinetics terms).

We found that, in the superstimulated group, the numbers of large

(> 8 mm) and medium (≥ 5 to ≤ 8 mm) follicles were higher, whereas the number of small (< 5 mm) follicles was lower than that in the non-stimulated group. In contrast, no differences were observed in the total number of recovered oocytes and the recovery rate between the superstimulated and non-stimulated groups. These results were consistent with those of a previous study [27]. On the other hand, studies on the effects of superstimulation on the quality of oocytes collected by OPU, have shown that in bovines, quality of *in vitro* development of *in vivo*-matured oocytes is higher than that of *in vitro*-matured oocytes [11–14]. However, few studies have compared the cytoplasmic maturation status between *in vitro*- and *in vivo*-matured oocytes. Therefore, we investigated the cytoplasmic maturation status of *in vitro*- and *in vivo*-matured oocytes that were collected from non-stimulated and superstimulated cows, respectively. We found that rate of normal CG distribution was higher in the superstimulated group than in the non-stimulated group. The CGs are distributed near the plasma membrane of the oocytes during final maturation [25]. Only CGs that exist very close to the plasma membrane can fuse and release their contents into the perivitelline space [26, 34]. Exocytosis of CGs is very important in the prevention of polyspermy [26, 34]. A recent study showed that oocytes collected from cows superstimulated prior to OPU had a lower rate of polyspermy than oocytes collected from non-stimulated cows [35]. Thus, this finding in combination with our findings suggests that normal CGs distribution during cytoplasmic maturation of *in vivo*-matured oocytes could efficiently prevent polyspermy occurrence, whereas polyspermy would frequently occur *in vitro*-matured oocytes due to the abnormal distribution of their CGs.

In the present study, the rate of development to the blastocyst stage was similar between *in vitro*- and *in vivo*-matured oocytes that were collected from non-stimulated and superstimulated cows, respectively. However, based on morphological evaluation, it was observed that the quality of blastocysts was improved when *in vivo*-matured oocytes were used. This finding is consistent with that of previous studies, which have reported that *in vivo*-matured oocytes produced blastocysts with higher quality although *in vitro* development was not improved [27, 36]. In contrast, another study showed that the blastocyst developmental rate was higher with oocytes collected from superstimulated cows than with those from non-stimulated cows [15]. Furthermore, another study demonstrated that the use of *in vivo*-matured oocytes yielded more blastocysts than oocytes that were recovered just before the LH surge and were matured *in vitro* [13]. One possible reason for these discrepancies may be the differences in the superstimulation protocols used. Previous studies have reported that suitable superstimulation protocols including the dose of hormone differ depending on the breeds, and unsuitable superstimulation protocols may have negative effects on follicular development, ovulation rates and embryo viability in MOET [37, 38]. As the protocol used in the present study was designed with reference to the protocol designed for Holstein cows [27], further studies are required to specify the optimal protocols for the Japanese Black breed and other breeds of cow.

Quality evaluation of blastocysts prior to embryo transfer, in general, has been conducted by morphological observation. However, this method is a subjective evaluation, and results may vary among evaluators consequently having an adverse effect on the post embryo transfer pregnancy rates. Recent studies have shown that it is possible to track the developmental kinetics of individual embryo using TLC observation of the developing embryo in a microwell in the culture dish based on the well-of-the-well system [21, 22]. In addition, new criteria based on the blastomere number at the end of the first cleavage, and the presence or absence of multiple fragments at the end of the first cleavage were established for selecting bovine embryos with higher developmental competence [21]. However, because the TLC system is very expensive and skilled users are limited, this technique is not widely used in the field of calf production. In the present study, we estimated the first cleavage patterns without using the TLC system with reference to a previous study showing that embryos with high developmental competence could be selected by estimating the first cleavage patterns [21]. The TLC system used in the study revealed that the blastocysts from embryos with equal blastomeres after the first cleavage had higher pregnancy rates than the blastocysts formed from embryo that underwent abnormal cleavage (i.e., direct cleavage from one cell to three or four blastomeres, unequal blastomeres, multiple fragments, and protrusion formation), thus, indicating that the analysis of the first cleavage patterns may reduce the risk of pregnancy loss that occur following embryo transfers [22]. Our results are in concurrence with this study, we found that the pregnancy rate was significantly higher for normally cleaved embryos than for abnormally cleaved embryos even when embryos with same morphological quality at the blastocyst stage were transferred. Thus, our classification method seems to be reasonable in selecting blastocysts with higher post-transfer viability. However, because we observed the first cleavage pattern at one-time point (28 hpi), any embryo with three or four blastomeres at that time was identified as a directly (abnormally) cleaved embryo even if it had undergone the two-cell stage prior to observation. In our preliminary experiment using TLC system, about 10% of embryos identified as directly cleaved embryos had undergone the two-cell stage before 28 hpi (unpublished data). Therefore, further modifications including observation at multiple time points will be needed to identify their cleavage pattern more precisely. In addition, we showed that the pregnancy rate of normally cleaved embryos was similar between the superstimulated and non-stimulated groups. These results indicate that the post-transfer viability of normally cleaved embryos was high irrespective of whether *in vivo*- or *in vitro*-matured oocytes were used, suggesting that efficient production of normally cleaved embryos is important for improving the pregnancy rate. As the rate of obtaining transferable blastocysts from normally cleaved embryos in the superstimulated group was higher than that in the non-stimulated group, the use of *in vivo*-matured oocytes for OPU-IVP would contribute to its increased efficiency.

In conclusion, the present study showed that the use of *in vivo*-matured oocytes for OPU-IVP improved the production of embryos that underwent normal cleavage at the first cell division and freezable blastocysts. This beneficial effect of *in vivo*-matured oocytes on the developmental competence of embryos may be a result of proper maturation as evidenced by the normal distribution of CGs. In addition, higher pregnancy rates were observed in the transfers of blastocysts obtained from embryos that underwent normal cleavage. These findings suggested that the use of *in vivo*-matured oocytes collected by OPU from superstimulated Japanese Black cows contributed to efficient production of embryos with high developmental competence

in terms of morphology and developmental kinetics.

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