Optimizing production of in vivo-matured oocytes from superstimulated Holstein cows for in vitro production of embryos using X-sorted sperm

S. Matoba,^{*1,2} H. Yoshioka,^{*3} H. Matsuda,^{*} S. Sugimura,^{*4} Y. Aikawa,^{*} M. Ohtake,^{*} Y. Hashiyada,^{*} T. Seta,^{†5} K. Nakagawa,^{‡6} P. Lonergan,[‡] and K. Imai^{*7}

^{*}Reproductive Biology and Technology Division, National Livestock Breeding Center, 1 Odakurahara, Nishigo, Fukushima 961-8511, Japan

[†]Niigata Agricultural Research Institute Livestock Research Center, 178 Tanahire, Sanjo, Niigata 955-0143, Japan

[‡]School of Agriculture and Food Science, University College Dublin, Belfield, Dublin 4, Ireland

ABSTRACT

The present study aimed to establish an efficient system for the production of female embryos from dairy cows by in vitro fertilization (IVF) using X-sorted sperm and in vivo-matured oocytes collected by ovum pick up (OPU). Nonlactating Holstein cows ($n = 36$) were administered a controlled intravaginal progesterone-releasing (controlled internal drug release) device (d 0), underwent dominant follicle ablation (DFA) or ovulation by administration of 100 μg of GnRH on d 5, and were superstimulated with FSH and PGF_{2 α} , following standard procedures. Controlled internal drug release devices were removed on the evening of d 8 or on the morning of d 9, depending on the experiment. For LH surge induction, 200 μg of GnRH was administered on the morning of d 10 (0 h). In experiment 1, the peak (48.1%) of ovulating follicles was detected at 29 to 32 h after GnRH injection (0 h), and the range in the timing of the initiation of ovulation was less by timing from GnRH administration (30.0 ± 2.8 h) rather than by timing the onset of estrus (32.7 ± 4.7 h). Only 0.9% of total ovulated follicles were recorded before 26 h after GnRH injection. Therefore, OPU was carried out at 26 h and IVF occurred at 30 h after GnRH in experiments 2 and 3. In experiment 2, $83.3 \pm 10.8\%$

of oocytes with expanded cumulus cells had extruded the first polar body at 30 h after GnRH injection. The aim of experiment 3 was to compare the effect of either DFA or GnRH-induced LH surge before superstimulation on the efficiency of embryo production by IVF following superstimulation. Progesterone concentrations from d 10 to 12 in the DFA group were lower than those in the GnRH group. A greater proportion of recovered oocytes with expanded cumulus cells from ≥ 8 -mm follicles was observed in the DFA group than in the GnRH group (95.9 and 77.4%, respectively). Blastocyst rates in the DFA and GnRH groups (58.0 and 52.8%, respectively) did not differ from those of oocytes collected from nonstimulated OPU and matured in vitro (49.9%). However, the proportion of high-quality blastocysts was higher in the DFA group compared with the GnRH group (54.9 vs. 21.5%). Our results demonstrate that high rates of good-quality blastocysts can be produced by IVF with X-sorted frozen sperm using in vivo-matured oocytes collected by OPU from cows after DFA and superstimulation combined with ovulation induction.

Key words: in vivo-matured oocyte, sex-sorted sperm, in vitro fertilization, superstimulation

INTRODUCTION

The ability to produce calves of the desired sex is an attractive reproductive technology for dairy farmers wishing to breed replacement heifers. The potential benefits of using sexed semen have recently been outlined (Hutchinson et al., 2013). Two broad approaches to sex selection in cattle have been used: sexing embryos before transfer or the use of sex-sorted sperm to bias the sex ratio at fertilization. A variety of PCR-based techniques are available for sexing biopsies taken from embryos (Bondioli et al., 1989; Hasler et al., 2002). However, in most cases, this requires specialized equipment such as a micromanipulator and related PCR machines (Hasler et al., 2002). Moreover, embryo biopsy requires skill, increases the time required for

Received March 21, 2013.

Accepted October 12, 2013.

¹Corresponding author: matoba@affrc.go.jp

²Present address: NARO Institute of Livestock Grassland Science (NILGS), Tsukuba, Ibaraki 305-0901, Japan.

³Present address: National Livestock Breeding Center, Miyazaki Station, 5157-29 Hosono, Kobayashi, Miyazaki 886-0004, Japan.

⁴Present address: Department of Biological Production, Tokyo University of Agriculture and Technology, 3-5-8 Saiwaicho, Fuchu, Tokyo 183-8509, Japan.

⁵Present address: Niigata Central Livestock Hygiene Service Center, 686 Hataya, Nisikanku, Niigata 950-0423, Japan.

⁶Present address: Niigata Central Livestock Hygiene Service Center, Sado Branch, 264 Chigusa, Sado, Niigata 952-1209, Japan.

⁷Present address: Department of Sustainable Agriculture, College of Agriculture, Food and Environment Sciences, Rakuno Gakuen University, 582 Midorimachi, Bunkyo-dai, Ebetsu, Hokkaido 069-8501, Japan.

processing, and potentially reduces the viability of the embryo (Thibier and Nibart, 1995; Hasler et al., 2002; Hirayama et al., 2004).

Currently, sexed sperm is commercially available and represents an attractive alternative to embryo sexing (Seidel, 2003; Garner and Seidel, 2008; DeJarnette et al., 2009). The accuracy of the sexing procedure has been reported to be close to 90% (Seidel, 1999; Garner and Seidel, 2008; Schenk et al., 2009). Despite the potential use of this technology, fertility rates after AI with sexed sperm are lower than those achieved with conventional unsorted semen, possibly due to DNA damage caused to sperm by the sorting process, reduced sperm viability, and low sperm numbers per insemination (DeJarnette et al., 2008; Schenk et al., 2009; DeJarnette et al., 2011).

To increase the production of calves of the desired sex above that achievable with routine AI, multiple ovulation embryo transfer can be combined with AI with sex-sorted semen (Hayakawa et al., 2009; Soares et al., 2011). However, whether this potential is achievable in commercial practice has been questioned, particularly in cows, because of reduced fertilization rates when using low doses of X-sorted semen (Hayakawa et al., 2009; Peippo et al., 2009; Larson et al., 2010).

Ovum pick up (OPU) coupled with in vitro fertilization (IVF) has been reported to be an efficient method of embryo production compared with multiple ovulation embryo transfer, and requires far fewer sperm than required for AI (Merton et al., 2003; Imai et al., 2006). However, blastocyst developmental rates using oocytes recovered from small follicles and matured in vitro are typically low (<30 to 40%; Lonergan et al., 2003a,b). In contrast, blastocyst development from in vivo-matured oocytes is consistently higher than that from in vitro-matured oocytes (Greve et al., 1987; Marquant-Le Guienne et al., 1989; Dieleman et al., 2002; Rizos et al., 2002). Thus, IVF of in vivo-matured oocytes with sex-sorted sperm may be one way of maximizing the number of transferable female embryos in applied reproductive programs in dairy cattle.

The objective of this study was to establish an efficient program for the production of female embryos in dairy cattle by IVF using in vivo-matured oocytes collected by OPU and sex-sorted sperm. To achieve this, the specific aims were to (1) determine the optimum timing of aspiration of in vivo-matured oocytes from cows after superstimulation combined with LH surge induction using GnRH, (2) assess the morphology of aspirated oocytes, and (3) examine embryo production following IVF of in vivo-matured oocytes with X-sorted sperm following dominant follicle removal before superstimulation.

MATERIALS AND METHODS

The overall scheme for the collection of in vivo-matured oocytes using superstimulation and OPU and subsequent embryo production by IVF is illustrated in Figure 1.

Animal Management

The use of animals was subject to the regulations set out by the Law for the Humane Treatment and Management of Animals (Law No. 105, 1973) and notification no. 6 and no. 22 of the Japanese Guidelines for Animal Care and Use. All experimental procedures involving animals were approved by the Ethics Committee for Care and Use of Experimental Animals [National Livestock Breeding Center (NLBC), Fukushima, Japan].

Nonlactating Holstein cows were used ($n = 39$; parity = 2.5 ± 0.9 ; BCS at start of experiment = 3.39 ± 0.52). All cows were housed in a loose barn and fed based on the Japanese Feeding Standard for Dairy Cattle (National Agricultural Research Organization, 2006). The BCS was assessed on a 5-point scale (with 1 being extremely thin and 5 being extremely fat) with 0.25 increments (Ferguson et al., 1994).

Superstimulation

Donor cows received an intravaginal progesterone-releasing device [controlled internal drug release, (CIDR); Pfizer, Tokyo, Japan; CIDR insertion = d 0]. Before FSH administration in experiments 1 and 2, all follicles ≥ 8 mm in diameter were aspirated (dominant follicle ablation, DFA) on d 5. In experiment 3, a cross-over design was used; half of the animals underwent DFA and half received 100 μg of a GnRH analog (fetrelin acetate, Supolnen; Kyoritsu Seiyaku Co., Tokyo, Japan) to induce dominant follicle ovulation on d 5, and cows were subsequently switched to the opposite treatment. A total of 30 armour units (AU) of FSH (Antrin R10; Kyoritsu Seiyaku Co.) was administered twice daily for 4 d in decreasing doses (6, 6, 4, 4, 3, 3, 2, and 2 AU, respectively) from the evening of d 6 to the morning of d 10 to stimulate follicular growth. All cows received 0.225 mg of PGF_{2 α} (D-cloprostenol, Dalmazin; Kyoritsu Seiyaku Co.) at CIDR removal on the evening of d 8 (in experiment 3, CIDR removal was delayed until the morning of d 9 to avoid ovulation of FSH-stimulated follicles before OPU). Gonadotropin-releasing hormone (200 μg ; 0 h) was administered to induce the LH surge of growing follicles on the morning of d 10. All drugs were administered i.m. into the ischiorectal fossa.

Figure 1. Determination of the optimum timing of aspiration of in vivo-matured oocytes from cows after superstimulation combined with a GnRH-induced LH surge and protocol for the collection of in vivo-matured oocytes and subsequent embryo production by in vitro fertilization (IVF) using X-sorted sperm. All events in the boxes occurred in all experiments unless otherwise indicated. Key events are indicated by arrows and boxes along the timeline plot. CIDR = controlled internal drug release (intravaginal progesterone-releasing device); DFA = dominant follicle ablation; PGF_{2α} = D-cloprostenol (Dalmazin; Kyoritsu Seiyaku Co., Tokyo, Japan); OPU = ovum pick up; morning = 0900 h; afternoon = 1330 h; evening = 1650 h; OPU = 1000 to 1100 h and oocyte assessment or IVF = 1500 h.

Monitoring of Follicle Numbers

The number of antral follicles >2 mm was monitored ultrasonically (SSD-900; Hitachi Aloka Medical Ltd., Tokyo, Japan) on d 5, 10, and 11, corresponding to day of DFA, expected estrus (all experiments), and follicle ovulation (experiment 1) or OPU (experiments 2 and 3), respectively. Follicles were categorized as small (2 to <5 mm), medium (5 to <8 mm), or large (≥8 mm) in diameter.

Estrus Detection

Estrus was detected by the pedometric system (Gyuhō; Comtec Co. Ltd., Miyazaki, Japan) as previ-

ously described (Yoshioka et al., 2010). Briefly, activity was automatically recorded every hour. The onset of estrus was defined as increased activity per hour above the default threshold value for 3 consecutive hours. The end of estrus was deemed to have occurred when no increase in activity above the default threshold value was observed for 3 consecutive hours.

Blood Sampling and Hormone Assay

Blood samples were collected for analysis of LH, estradiol, and progesterone concentration as described below. Following collection, samples were kept on crushed ice, transported to the laboratory and centrifuged at approximately $2,000 \times g$ for 25 min at 4°C.

Then, plasma was collected and stored at -40°C until assayed. Concentration of plasma LH was measured by a time-resolved fluoroimmunoassay using Eu-labeled LH as described previously (Kaneko et al., 2002). All standards and plasma samples were assayed in duplicate. The sensitivity of the assay was 0.195 ng/mL. The intraassay coefficient of variation was 8.3%. Plasma estradiol and progesterone were measured by an enzyme immunoassay as described previously (Takenouchi et al., 1993, 2004). All standards and plasma samples were assayed in duplicate. The sensitivity of the estradiol and progesterone assays was 0.1 pg/mL and 1.25 ng/mL, respectively. Intraassay coefficient of variation of estradiol and progesterone were 0.25 and 3.73%, respectively. Results of this enzyme immunoassay were highly correlated with RIA results (Takenouchi et al., 1993, 2004).

Oocyte Recovery

A total of 785 oocytes were collected from superstimulated (672 oocytes and 21 cows) and nonstimulated (113 oocytes and 6 cows at random stages of the estrous cycle without any hormonal treatment) cows. Transvaginal recovery of in vivo-matured oocytes was carried out as previously described by Imai et al. (2006) at 25 to 26 h after GnRH administration on d 11. All visible follicles ≥ 5 mm in diameter were aspirated from cows in each group with a 17 gauge $\times$ 500 mm disposable needle attached to a 1,500-mm polyvinyl chloride tube (COVA Needle; Misawa Medical Industry Co. Ltd., Tokyo, Japan). A relatively high vacuum pressure (130 mm of Hg; aspiration rate approximately 24 mL/min) was used due to the presence the expanded sticky cumulus cells associated with in vivo maturation. The follicular contents were aspirated into a 50-mL conical tube containing approximately 5 mL of lactate Ringer's solution supplemented with 1% bovine serum (16170-078; Gibco Invitrogen, Life Technologies, Auckland, New Zealand) and 10 IU/mL heparin (Novo-Heparin Injection 1000; Aventis Pharma Ltd., Tokyo, Japan; aspiration medium). The conical tube was changed after every 5 to 6 follicle aspirations. Sedimented materials in the conical tubes were transferred to 90-mm Petri dishes and the remaining solution was filtered through an EmCon filter (Immuno System Inc., Spring Valley, WI). Cumulus oocyte complexes were collected under a stereomicroscope and stored in tissue culture medium 199 (TCM 199; 31100-035, Medium 199 powder; Gibco Invitrogen) buffered with 11 mmol of HEPES, 9 mmol of Na-HEPES, and supplemented with 5 mmol of sodium bicarbonate and 5% newborn calf serum (NBS; S0750-500; Biowest SAS, Nuaille, France; pH 7.3) until the end of the entire collection procedure. The in vitro

maturation (IVM) medium was TCM 199 (12340-030, Medium 199; Gibco Invitrogen) supplemented with 5% NBS. Oocytes were washed twice with IVM medium and were incubated in 50- μL droplets (approximately 15 oocytes in each droplet) of IVM medium covered with paraffin oil (Paraffin Liquid; Nacalai Tesque Inc., Kyoto, Japan) in 35-mm Petri dishes (153066 TC Dish 35 $\times$ 10 Vents Nunclon D Si; Nalge Nunc International, Rochester, NY) at 38.5°C in an atmosphere of 5% CO_2 in humidified air for approximately 3 h (i.e., until 30 h after GnRH administration).

Oocyte Fertilization and Embryo Culture In Vitro

Sperm preparation for IVF was as previously described (Abe et al., 2002; Blondin et al., 2009). Frozen X-sorted sperm (minimum 90% purity) from a single proven Holstein bull (Genetics Hokkaido, Hokkaido, Japan) (Hayakawa et al., 2009) were thawed in a 37°C water bath for 30 s, layered on a Percoll (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) density gradient (45 and 60%) and centrifuged at $740 \times g$ for 10 min at 37°C . After centrifugation, the pellet of sperm was resuspended in 5.5 mL of IVF-100 medium (Research Institute for Functional Peptides Co. Ltd., Yamagata, Japan) and centrifuged again at $540 \times g$ for 5 min at 37°C . The resulting supernatant was removed and the final concentration of sperm was adjusted to 5×10^6 sperm/mL in IVF-100. Only oocytes with expanded cumulus cells were used for fertilization (Figure 2). Oocytes were washed in IVF-100 and transferred to an 80- μL droplet of the sperm suspension covered with paraffin oil. Gametes were co-incubated for 6 h at 38.5°C in an atmosphere of 5% CO_2 in humidified air. Presumptive zygotes were denuded by gentle pipetting with a fine glass pipette in preincubated Charles Rosenkrans 1 medium (CR1; Rosenkrans et al., 1993) with amino acids (CR1aa; Imai et al., 2002) supplemented with 5% NBS and 0.25 mg/mL of linoleic acid albumin (CR1aa-LAA; L-8384; Sigma-Aldrich Co., St Louis, MO; Imai et al., 1997). Then, zygotes were cultured in 35-mm polystyrene-based microwells (Dai Nippon Printing Co. Ltd., Tokyo, Japan; Sugimura et al., 2010) in 125- μL droplets of CR1aa-LAA covered with paraffin oil at 38.5°C in a humidified atmosphere of 5% CO_2 , 5% O_2 , and 90% N_2 for 9 d. Cleavage, blastocyst formation, and quality of blastocysts were assessed on denudation, d 2 (i.e., 48 h postinsemination; day of IVF = d 0), and d 7 to 9, respectively. Evaluation of embryo quality was according to the criteria of the International Embryo Transfer Society (Stringfellow and Givens, 2010). Briefly, good-quality and transferable blastocysts were included Code 1 and Code 1 and 2, respectively (Stringfellow and Givens, 2010).

Figure 2. Oocytes surrounded with expanded (A) or non-expanded cumulus cells (B) at 30 h after GnRH administration. Color version available in the online PDF.

Oocyte Staining for the Determination of Maturation Status

To assess maturation status, after incubation for approximately 3 h (i.e., until 30 h after GnRH treatment), all oocytes were placed in 0.1% hyaluronidase (H-3506; Sigma-Aldrich Co.) in Ca- and Mg-free Dulbecco's PBS (Gibco Invitrogen) for approximately 3 min and denuded by pipetting. Extrusion of the first polar body (PB) was assessed and oocytes were stained with aceto-orcein to determine the meiotic status (Prentice et al., 2011). To achieve this, oocytes were mounted to a glass slide, fixed in ethanol:acetic acid (3:1) overnight and stained in 1% orcein in 45% acetic acid for 1 h and washed in 20% acetic acid and glycerol. Meiotic status was categorized as germinal vesicle, germinal vesicle breakdown, metaphase I, anaphase I, telophase I, or metaphase II (MII; Süß et al., 1988) under a phase-contrast microscope at 1,000 $\times$ magnification.

Embryo Sexing

After removal of the zona pellucida by pipetting with a fine Pasteur pipette, embryo sexing was carried out on a representative number of blastocysts ($n = 38$) using the loop-mediated isothermal amplification (LAMP) method as reported previously (Hirayama et al., 2004).

Experiment 1: Optimum Timing of Aspiration of In Vivo-Matured Oocytes

Cows ($n = 12$) were superstimulated and follicle numbers and timing of ovulation were recorded. The duration of the entire ovulatory process (i.e., the time taken for all superstimulated follicles to ovulate) was compared relative to the time from GnRH administra-

tion versus the time from onset of estrus to select the optimum time at which to carry out OPU in subsequent experiments. Blood plasma samples were collected from 0 h (just before GnRH treatment) to 10 h at hourly intervals for measurement of LH concentrations after GnRH administration on d 10. To confirm the change in concentration of estradiol and progesterone in cows during superstimulation, blood plasma samples were collected on d 6 to 11. Ovulation was assessed ultrasonographically every 3 h from 20 to 41 h inclusive and again at 53 h, and corpora lutea were counted at 125 h after GnRH administration on d 10.

Experiment 2: Assessment of Oocyte Morphology

To confirm the maturation status of oocytes recovered by OPU, morphological assessment of oocytes was carried out. All cows ($n = 9$) were superstimulated and the numbers of follicles ≥ 5 mm aspirated and oocytes recovered by OPU at 25 to 26 h after GnRH administration on d 10 were recorded. Recovered oocytes were classified as having expanded or non-expanded cumulus cells (Figure 2) 30 h after GnRH administration on d 10 (just before the expected peak of ovulation, based on the results of experiment 1). The presence of the first PB was assessed in oocytes with expanded cumulus (defined as expanded oocytes) immediately after collection and after 4-h culture. Oocytes ($n = 122$) from 6 cows were stained to determine meiotic status. In the IVM oocyte group, expanded oocytes after 22 to 24 h maturation in vitro were used for the same assessment.

Experiment 3: Development of an Efficient Sexed-Embryo Production System

Cows ($n = 12$) were superstimulated and the numbers of follicles aspirated and oocytes recovered by

Table 1. Ovarian response to superstimulation (experiment 1)

Item	No. of follicles ¹		Ovulation of ≥ 8 -mm follicles ²		No. of corpora lutea ³	
	≥ 8 mm	5 to < 8 mm	No.	% ⁴	No.	% ⁵
Mean $\pm$ SEM/cow	23.3 $\pm$ 5.1	12.4 $\pm$ 1.2	17.7 $\pm$ 3.2	86.3 $\pm$ 5.1	15.3 $\pm$ 2.8	88.4 $\pm$ 4.2

¹Estimated by ultrasonography at 11 h after GnRH injection (on day of estrus).

²Estimated by ultrasonography at 53 h after GnRH injection (on third day after onset of estrus).

³Estimated by ultrasonography at 125 h after GnRH injection (on fifth day after onset of estrus).

⁴Number of ovulated follicles as a percentage of the total number of follicles ≥ 8 mm.

⁵Number of corpora lutea as a percentage of the total number of ovulated follicles ≥ 8 mm.

OPU at 25 to 26 h after GnRH administration after superstimulation were recorded. Two methods of dominant follicle removal (DFA or ovulation induction by administration of 100 μ g of GnRH) on d 5 were compared. The CIDR removal occurred on the morning of d 9 to avoid ovulation based on the results of experiment 2 (1 cow had ovulated more than 10 follicles of ≥ 8 mm at OPU). Blood samples were collected for analysis of progesterone on the mornings of d 9 to 12 and the afternoon of d 10 (Figure 1). Assessment of follicle number occurred on d 5, 10, and 11. Aspirated oocytes were classified as described above. Then, expanded oocytes underwent IVF at 30 h after GnRH administration after culture in maturation media for 3 h on d 10. For comparison of IVF embryo production, immature oocytes aspirated by OPU from ≥ 2 -mm follicles of nonstimulated cows ($n = 6$) and matured (22–23 h) in vitro were considered as controls. A random selection of d-7 to -8 blastocysts ($n = 38$) derived from in vivo-matured oocytes were sex determined as described above.

Statistical Analysis

Data (means $\pm$ SEM) on the timing of ovulation after GnRH administration or estrus in experiment 1 were compared by the Tukey test by 2-way ANOVA using SigmaStat software (version 3.1.1; Systat Software Inc., San Jose, CA). Data (means $\pm$ SEM) on the repeated measures of the concentration of progesterone and the numbers and rates of follicles, oocytes, and

in vitro fertility were compared by PROC MIXED of SAS (version 9.3; SAS Institute Inc., Cary, NC). The PDIF command, incorporating the Tukey-Kramer test was applied to evaluate pairwise comparisons between treatment means. Rates of PB extrusion and the proportion of oocytes at the MII stage were analyzed by Student's *t*-test.

RESULTS

Experiment 1: Optimum Timing of Aspiration of In Vivo-Matured Oocytes

The number of ovulatory follicles ≥ 8 mm at estrus and subsequent corpora lutea are shown in Table 1. The variation around the start of ovulation was narrower when timed from GnRH administration (30.0 $\pm$ 2.8 h) than from the onset of estrus as defined by pedometry (32.7 $\pm$ 4.7 h; $P < 0.05$; Table 2). The LH concentration (3.6 $\pm$ 1.9 ng/mL) rapidly increased following GnRH administration (0 h), reached a peak (20.1 $\pm$ 3.2 ng/mL) at 2 h, and decreased from 2 h to basal concentrations (3.8 $\pm$ 0.5 ng/mL) at 6 h. The peak of ovulation occurred from 29 to 32 h after GnRH administration (Figure 3). Only 2 follicles (0.9% of ovulatory follicles in total) ovulated before 26 h after GnRH administration. Based on these observations, 25 to 26 h after GnRH administration was selected for the timing of OPU for experiments 2 and 3, and 30 h after GnRH administration was selected for the morphological assessment of in vivo-matured oocytes and

Table 2. Comparison of timing of ovulation from GnRH administration or onset of estrus (experiment 1)

Item	Ovulation time (h)				Duration of ovulation (h)
	From GnRH administration		From onset of estrus		
	Start	End	Start	End	
Mean $\pm$ SEM	30.0 $\pm$ 0.8*	34.8 $\pm$ 0.7†	32.7 $\pm$ 1.3*	37.4 $\pm$ 1.5†	4.8 $\pm$ 0.9
Range	27.5–36.5	30.5–39.5	26.5–42.5	32.5–48.5	0–12

† $P < 0.1$; * $P < 0.05$ (significant differences or tendency between GnRH and estrus groups).

fertilization. Plasma estradiol concentration (15.1 ± 2.6 pg/mL) peaked at estrus on d 10 and progesterone concentration rapidly declined from d 8 (8.6 ± 1.1 ng/mL), reached basal concentrations (0.5 ± 0.1 ng/mL) at d 9, and remained basal from d 9 to 11.

Experiment 2: Assessment of Oocyte Morphology

Data were presented as means $\pm$ standard error of the mean per head per OPU session. The numbers of follicles <8 mm at DFA and ≥ 8 mm at OPU were 42.2 ± 3.4 and 15.3 ± 4.2 , respectively. A total of 9.1 ± 2.4 oocytes ($61.7 \pm 5.6\%$) were recovered; 6.0 ± 1.6 ($70.8 \pm 9.4\%$) had expanded cumulus, 5.0 ± 1.5 ($82.2 \pm 10.6\%$) had extruded the first PB before culture, and 5.1 ± 1.6 ($83.3 \pm 10.8\%$) had extruded the first PB after 4 h of culture. Based on the results of nuclear staining, no differences were found in the proportion of oocytes with an extruded first PB or at MII between groups. One cow had already ovulated 13 follicles of ≥ 8 mm at the time of OPU. However, OPU was carried out on the 5 remaining follicles ≥ 8 mm. Based on this, to avoid ovulation before OPU, CIDR removal was delayed until the morning of d 9 in experiment 3 (Figure 1).

Experiment 3: Development of an Efficient Sexed-Embryo Production System

Plasma progesterone concentration in the DFA group rapidly decreased from d 9 to a nadir on the morning of d 10, and remained at this concentration until d 12 compared with the GnRH group ($P < 0.01$ or $P < 0.05$, respectively; Figure 4). No difference was detected in the number of follicles <8 mm at dominant follicle

removal, 5- to <8 -mm follicles at OPU, or recovered oocytes between the DFA and GnRH groups (Table 3). A higher number of ≥ 8 -mm follicles at OPU was observed in the GnRH group than the DFA group ($P < 0.05$). The greatest recovery of oocytes (recovered oocytes/observed follicles) was recorded for the DFA group compared with GnRH and nonstimulated OPU groups ($P < 0.05$). Recovery rates of oocytes with expanded cumulus from ≥ 8 -mm follicles were higher in the DFA group than the GnRH group ($P < 0.01$). Overall recovery rates of expanded oocytes from follicles 5 to <8 mm were not different between groups. The highest number and rates of good-quality blastocysts were achieved in the DFA group compared with the GnRH group ($P < 0.01$ or $P < 0.05$; Table 4). Following sex determination, 92.1% of embryos were female.

DISCUSSION

Here, we report the establishment of an efficient method of producing female embryos from Holstein dairy cows by IVF with X-sorted sperm of in vivo-matured oocytes collected by OPU after superstimulation. Maturation of the oocyte in the ovulatory follicle is initiated by the preovulatory surge of LH (Sunderland et al., 1994). In the present study, GnRH was administered on the day of expected estrus for induction of an LH surge and subsequent ovulation. Time of initiation of ovulation was in a narrower range when aligned with the time of GnRH administration rather than the time from onset of estrus as defined by pedometer measurements (Table 2). For determining the optimum timing of follicle aspiration, almost all (98.6%) follicles ovulated between 26 and 38 h after GnRH treatment

Figure 3. Ovulatory follicle emergence after GnRH administration (time = 0 h; experiment 1).

Figure 4. Plasma progesterone concentration following dominant follicle ablation (DFA; $\diamond$) or GnRH-induced ovulation ($\blacksquare$; mean $\pm$ SEM; experiment 3). Asterisks and dagger represent differences between DFA and GnRH groups at the same time point ($\dagger P < 0.1$; $* P < 0.05$; $** P < 0.01$). OPU = ovum pick up.

Table 3. Number of follicles and recovered oocytes in superstimulated cows following dominant follicle ablation (DFA) or GnRH-induced ovulation (experiment 3)¹

Item	Superstimulated		Nonstimulated
	DFA ² (n = 12)	GnRH ³ (n = 12)	OPU ⁴ (n = 6)
Follicles at DFA/GnRH administration (no.)			
<8-mm follicles	31.2 ± 3.2	33.1 ± 4.7	
Follicles at OPU (no.)			
≥8-mm follicles	16.4 ± 2.7*	26.1 ± 3.9*	
5- to <8-mm follicles	7.2 ± 1.3	7.8 ± 1.2	
Total of ≥5-mm follicles	23.6 ± 3.3†	33.8 ± 4.8†	
Oocytes			
Oocytes from ≥8-mm follicles (no.)	14.3 ± 2.6	20.6 ± 3.1	
Oocytes from 5- to <8-mm follicles (no.)	5.4 ± 1.1	4.1 ± 0.8	
Total recovery of oocytes [no. (%)]	19.8 ± 2.8 (83.5 ± 4.0*†)	24.6 ± 3.9 (71.3 ± 3.3*)	18.8 ± 3.7 (65.8 ± 6.8†)
Oocytes surrounded by expanded cumulus [no. (%)]			
From ≥8-mm follicles	13.6 ± 2.4 (95.9 ± 1.9**)	16.6 ± 3.0 (77.4 ± 5.4**)	
From 5- to <8-mm follicles	1.0 ± 0.2 (27.5 ± 8.3)	1.9 ± 0.6 (41.8 ± 11.6)	
Total	14.6 ± 2.5 (74.4 ± 4.4)	18.5 ± 3.4 (72.3 ± 5.1)	

¹Data are mean ± SEM per head per ovum pick-up (OPU) session in each group.²DFA: ≥8-mm dominant follicle ablation.³Administration of GnRH i.m.⁴Oocytes were aspirated ≥2-mm follicles.† $P < 0.1$; * $P < 0.05$; ** $P < 0.01$ (significant differences within a row).

(Figure 3) with the biggest proportion (48.1%) ovulating from 29 to 32 h after GnRH treatment; 86.3% of ovulatory follicles were ≥8 mm in diameter (Table 1).

The superior developmental competence of in vivo-matured oocytes compared with those matured in vitro is well known (Hendriksen et al., 2000; Rizos et al., 2002; Humblot et al., 2005). Humblot et al. (2005) reported higher blastocyst development following IVF of in vivo-matured oocytes collected 20 h after GnRH administration and immediately inseminated compared with oocytes matured in vitro. Oocytes for in vitro maturation are generally aspirated from follicles 2 to 6

mm in diameter at unknown stages of the cycle or the follicular wave (Imai et al., 2002, 2006). In contrast, during superstimulation, in vivo-matured oocytes are collected from healthy growing follicles under the influence of FSH. Indeed, in vivo-matured oocytes recovered after a GnRH-induced LH surge (49%) had a higher developmental competence than in vitro-matured oocytes (26%) obtained from 2- to 8-mm follicles from abattoir-derived ovaries (Hendriksen et al., 2000). Furthermore, in vivo-matured oocytes collected 20 h later after a GnRH-induced LH surge yielded more blastocysts than those recovered at the time of the preovulatory surge

Table 4. Embryo production in each group (experiment 3)¹

Item	Superstimulated		Nonstimulated ²
	DFA ³ (n = 9)	GnRH ⁴ (n = 9)	OPU (n = 6)
Oocytes			
Live oocyte ⁵ (no.)	12.4 ± 2.3	14.7 ± 3.6	13.8 ± 2.7
Cleaved [no. (%)]	10.9 ± 2.5 (83.8 ± 3.6)	12.1 ± 3.0 (84.0 ± 4.4)	10.8 ± 2.4 (75.4 ± 5.2)
Blastocysts [no. (%)]			
d-7 blastocyst	6.6 ± 2.0 (45.7 ± 9.3)	3.4 ± 1.3 (22.0 ± 5.2)	4.0 ± 1.1 (31.8 ± 8.2)
d-7 to -9 blastocyst (blastocysts/cleaved)	7.6 ± 2.5 (58.0 ± 10.9)	6.9 ± 2.3 (52.8 ± 8.6)	4.8 ± 1.1 (49.9 ± 11.0)
Transferable (transferable blastocysts until d 9)	7.1 ± 2.1 (85.1 ± 6.0)	5.5 ± 1.9 (62.3 ± 11.9)	3.7 ± 1.1 (67.4 ± 15.5)
Good-quality blastocyst (good blastocysts until d 9)	4.1 ± 1.0** (54.9 ± 7.5*)	0.9 ± 0.2** (21.5 ± 11.8*)	1.5 ± 0.4 (36.1 ± 14.9)

¹Data are mean ± SEM per head per ovum pick-up (OPU) session in each group.²Immature oocytes were inseminated after 22 to 24 h of maturation in vitro.³DFA: ≥8-mm dominant follicle ablation.⁴Administration of GnRH i.m.⁵Non-degenerated oocytes with expanded cumulus cells used for in vitro fertilization (IVF) of 9 of 12 animals in the DFA and the GnRH groups.* $P < 0.05$; ** $P < 0.01$ (significant differences within a row).

and matured in vitro (58.2 vs. 39.2%, respectively; Rizos et al., 2002).

To maximize the number of in vivo-matured oocytes recovered, management of the timing of follicular wave emergence can be achieved by dominant follicle removal before superstimulation with FSH (Bergfelt et al., 1997; Baracaldo et al., 2000; Bó et al., 2006). We compared dominant follicle removal by physical (i.e., DFA) and hormonal (i.e., GnRH-induced ovulation) means to control follicular wave emergence at 1.5 d before the initiation of superstimulation. The proportion of expanded oocytes from follicles ≥ 8 mm was higher in the DFA group than the GnRH group ($P < 0.01$; Table 3). Follicular wave emergence occurred 1 to 2 d after DFA by transvaginal ultrasound-guided aspiration of the 2 largest follicles or all follicles ≥ 5 mm in the ovaries at any time of the estrous cycle (Bergfelt et al., 1997; Baracaldo et al., 2000). Other approaches to the synchronization of follicular wave emergence include the administration of GnRH or LH from 3 d before to 5 d after superstimulation (Martinez et al., 1999; Bó et al., 2006). In the current study, GnRH administration 1.5 d before superstimulation was considered appropriate, based on the higher concentration of plasma progesterone from estrus to OPU compared with DFA ($P < 0.001$ and $P < 0.01$, respectively; Figure 4). High progesterone concentrations during final follicle growth have been shown to improve embryo quality following superstimulation (Nasser et al., 2011; Pursley and Martins, 2011; Rivera et al., 2011).

Worldwide, approximately 50% of in vivo-derived embryos transferred are frozen-thawed (Stroud, 2011). In contrast, 90% of IVP embryos are transferred fresh, reflecting their lower cryotolerance (Stroud, 2011). To be of practical use, it is important that blastocysts produced in vitro are of sufficiently high quality to survive cryopreservation. Moreover, for the commercial export/import of embryos, it is necessary to transport them frozen. In this study, the highest proportion of good quality blastocysts was obtained in the DFA group compared with both the GnRH and nonstimulated OPU groups ($P < 0.05$). Surprisingly, the yield of high-quality presumptive female blastocysts by d 9 from the DFA group was 3-fold higher than that from the nonstimulated OPU group (4.1 and 1.5, respectively).

CONCLUSIONS

The results of this study demonstrate that high rates of good-quality blastocysts can be obtained by IVF with X-sorted sperm using in vivo-matured oocytes collected by OPU from dairy cows.

ACKNOWLEDGMENTS

This study was supported by the “Research and development projects for application in promoting new policy of agriculture, forestry and fisheries” grant number 22016 to S. Matoba, H. Yoshioka, H. Matsuda, Y. Hashiyada, T. Seta, K. Nakagawa, and K. Imai. The authors acknowledge F. Aono and O. Kato at the Kato Ladies Clinic (Tokyo, Japan) and T. Seki, M. Maeda, and N. Sugawara at Iwaki Obstetrics and Gynecology (Iwaki, Japan) for advice on the collection of in vivo-matured oocytes; M. Nagai at Ishikawa Prefectural Livestock Research Center (Ishikawa, Japan) for making culture media; and N. Takenouchi at the National Agricultural Research Organization (NARO) Kyushu Okinawa Agricultural Research Center (Fukuoka, Japan) for the provision of antibodies for the estradiol and progesterone assays. We also thank the staff of the Reproductive Biology and Technology Division in the National Livestock Breeding Center (NLBC, Nishigo, Japan) for help in oocyte collection and animal management.

REFERENCES

- Abe, H., S. Yamashita, T. Satoh, and H. Hoshi. 2002. Accumulation of cytoplasmic lipid droplets in bovine embryos and cryotolerance of embryos developed in different culture systems using serum-free or serum-containing media. *Mol. Reprod. Dev.* 61:57–66.
- Baracaldo, M. I., M. F. Martinez, G. P. Adams, and R. J. Mapletoft. 2000. Superovulatory response following transvaginal follicle ablation in cattle. *Theriogenology* 53:1239–1250.
- Bergfelt, D. R., G. A. Bo, R. J. Mapletoft, and G. P. Adams. 1997. Superovulatory response following ablation-induced follicular wave emergence at random stages of the oestrous cycle in cattle. *Anim. Reprod. Sci.* 49:1–12.
- Blondin, P., M. Beaulieu, V. Fournier, N. Morin, L. Crawford, P. Madan, and W. A. King. 2009. Analysis of bovine sexed sperm for IVF from sorting to the embryo. *Theriogenology* 71:30–38.
- Bó, G. A., P. S. Baruselli, P. M. Chesta, and C. M. Martins. 2006. The timing of ovulation and insemination schedules in superstimulated cattle. *Theriogenology* 65:89–101.
- Bondiol, K. R., S. B. Ellis, J. H. Pryor, M. W. Williams, and M. M. Harpold. 1989. The use of male-specific chromosomal DNA fragments to determine the sex of bovine preimplantation embryos. *Theriogenology* 31:95–104.
- DeJarnette, J. M., M. A. Leach, R. L. Nebel, C. E. Marshall, C. R. McCleary, and J. F. Moreno. 2011. Effects of sex-sorting and sperm dosage on conception rates of Holstein heifers: Is comparable fertility of sex-sorted and conventional semen plausible? *J. Dairy Sci.* 94:3477–3483.
- DeJarnette, J. M., R. L. Nebel, and C. E. Marshall. 2009. Evaluating the success of sex-sorted semen in US dairy herds from on farm records. *Theriogenology* 71:49–58.
- DeJarnette, J. M., R. L. Nebel, C. E. Marshall, J. F. Moreno, C. R. McCleary, and R. W. Lenz. 2008. Effect of sex-sorted sperm dosage on conception rates in Holstein heifers and lactating cows. *J. Dairy Sci.* 91:1778–1785.
- Dieleman, S. J., P. J. M. Hendriksen, D. Viuff, P. D. Thomsen, P. Hyttel, H. M. Knijn, C. Wrenzycki, T. A. M. Kruip, H. Niemann, B. M. Gadella, M. M. Bevers, and P. L. A. M. Vos. 2002. Effects of in vivo prematuration and in vivo final maturation on developmental

- capacity and quality of pre-implantation embryos. *Theriogenology* 57:5–20.
- Ferguson, J. D., D. T. Galligan, and N. Thomsen. 1994. Principal descriptors of body condition score in Holstein cows. *J. Dairy Sci.* 77:2695–2703.
- Garner, D. L., and G. E. Seidel Jr. 2008. History of commercializing sexed semen for cattle. *Theriogenology* 69:886–895.
- Greve, T., K. P. Xu, H. Callesen, and P. Hyttel. 1987. In vivo development of in vitro fertilized bovine oocytes matured in vivo versus in vitro. *J. In Vitro Fert. Embryo Transf.* 4:281–285.
- Hasler, J. F., E. Cardey, J. E. Stokes, and P. Bredbacka. 2002. Non-electrophoretic PCR-sexing of bovine embryos in a commercial environment. *Theriogenology* 58:1457–1469.
- Hayakawa, H., T. Hirai, A. Takimoto, A. Ideta, and Y. Aoyagi. 2009. Superovulation and embryo transfer in Holstein cattle using sexed sperm. *Theriogenology* 71:68–73.
- Hendriksen, P. J. M., P. L. A. M. Vos, W. N. M. Steenweg, M. M. Bevers, and S. J. Dieleman. 2000. Bovine follicular development and its effect on the in vitro competence of oocytes. *Theriogenology* 53:11–20.
- Hirayama, H., S. Kageyama, S. Moriyasu, K. Sawai, S. Onoe, Y. Takahashi, S. Katagiri, K. Toen, K. Watanabe, T. Notomi, H. Yamashina, S. Matsuzaki, and A. Minamihashi. 2004. Rapid sexing of bovine preimplantation embryos using loop-mediated isothermal amplification. *Theriogenology* 62:887–896.
- Humblot, P., P. Holm, P. Lonergan, C. Wrenzycki, A.-S. Lequarré, C. G. Joly, D. Herrmann, A. Lopes, D. Rizos, H. Niemann, and H. Callesen. 2005. Effect of stage of follicular growth during superovulation on developmental competence of bovine oocytes. *Theriogenology* 63:1149–1166.
- Hutchinson, I. A., L. Shalloo, and S. T. Butler. 2013. Expanding the dairy herd in pasture-based systems: The role for sexed semen use on replacement heifers. *J. Dairy Sci.* 96:1312–1322.
- Imai, K., S. Kobayashi, Y. Goto, O. Dochi, and I. Shimohira. 1997. Cryopreservation of bovine embryos obtained by in-vitro culture of IVM-IVF oocytes in the presence of linoleic acid albumin. *Theriogenology* 47:347. (Abstr.)
- Imai, K., S. Matoba, O. Dochi, and I. Shimohira. 2002. Different factors affect developmental competence and cryotolerance in in vitro produced bovine embryo. *J. Vet. Med. Sci.* 64:887–891.
- Imai, K., M. Tagawa, H. Yoshioka, S. Matoba, M. Narita, Y. Inaba, Y. Aikawa, M. Ohtake, and S. Kobayashi. 2006. The efficiency of embryo production by ovum pick-up and *in vitro* fertilization in cattle. *J. Reprod. Dev.* 52(Suppl.):S19–S29.
- Kaneko, H., J. Todoroki, J. Noguchi, K. Kikuchi, K. Mizoshita, C. Kubota, and H. Yamakuchi. 2002. Perturbation of estradiol-feedback control of luteinizing hormone secretion by immunoneutralization induces development of follicular cysts in cattle. *Biol. Reprod.* 67:1840–1845.
- Larson, J. E., G. C. Lamb, B. J. Funnell, S. Bird, A. Martins, and J. C. Rodgers. 2010. Embryo production in superovulated Angus cows inseminated four times with sexed-sorted or conventional, frozen-thawed semen. *Theriogenology* 73:698–703.
- Lonergan, P., D. Rizos, A. Gutiérrez-Adán, T. Fair, and M. P. Boland. 2003a. Effect of culture environment on embryo quality and gene expression—Experience from animal studies. *Reprod. Biomed. Online* 7:657–663.
- Lonergan, P., D. Rizos, A. Gutierrez-Adan, T. Fair, and M. P. Boland. 2003b. Oocyte and embryo quality: Effect of origin, culture conditions and gene expression patterns. *Reprod. Domest. Anim.* 38:259–267.
- Marquant-Le Guenne, B., M. Gérard, A. Solari, and C. Thibault. 1989. In vitro culture of bovine egg fertilized either in vivo or in vitro. *Reprod. Nutr. Dev.* 29:559–568.
- Martinez, M. F., G. P. Adams, D. R. Bergfelt, J. P. Kastelic, and R. J. Mapletoft. 1999. Effect of LH or GnRH on the dominant follicle of the first follicular wave in beef heifers. *Anim. Reprod. Sci.* 57:23–33.
- Merton, J. S., A. P. W. de Roos, E. Mullaart, L. de Ruigh, L. Kaal, P. L. A. M. Vos, and S. J. Dieleman. 2003. Factors affecting oocyte quality and quantity in commercial application of embryo technologies in the cattle breeding industry. *Theriogenology* 59:651–674.
- Nasser, L. F., M. F. Sa Filho, E. L. Reis, C. R. Rezende, R. J. Mapletoft, G. A. Bo, and P. S. Baruselli. 2011. Exogenous progesterone enhances ova and embryo quality following superstimulation of the first follicular wave in Nelore (*Bos indicus*) donors. *Theriogenology* 76:320–327.
- National Agricultural Research Organization. 2006. Japanese Feeding Standard for Dairy Cattle. Japan Livestock Industry Association, Tokyo, Japan. (In Japanese).
- Peippo, J., K. Vartia, K. Kananen-Anttila, M. Rätty, K. Korhonen, T. Hurme, H. Myllymäki, A. Sairanen, and A. Mäki-Tanila. 2009. Embryo production from superovulated Holstein-Friesian dairy heifers and cows after insemination with frozen-thawed sex-sorted X spermatozoa or unsorted semen. *Anim. Reprod. Sci.* 111:80–92.
- Prentice, J. R., J. Singh, O. Dochi, and M. Anzar. 2011. Factors affecting nuclear maturation, cleavage and embryo development of vitrified bovine cumulus-oocyte complexes. *Theriogenology* 75:602–609.
- Pursley, J. R., and J. P. Martins. 2011. Impact of circulating concentrations of progesterone and antral age of the ovulatory follicle on fertility of high-producing lactating dairy cows. *Reprod. Fert. Dev.* 24:267–271.
- Rivera, F. A., L. G. D. Mendonça, G. Lopes Jr., J. E. P. Santos, R. V. Perez, M. Amstalden, A. Correa-Calderón, and R. C. Chebel. 2011. Reduced progesterone concentration during growth of the first follicular wave affects embryo quality but has no effect on embryo survival post transfer in lactating dairy cows. *Reproduction* 141:333–342.
- Rizos, D., F. Ward, P. Duffy, M. P. Boland, and P. Lonergan. 2002. Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: Implications for blastocyst yield and blastocyst quality. *Mol. Reprod. Dev.* 61:234–248.
- Rosenkrans, C. F., Jr., G. Q. Zeng, G. T. McNamara, P. K. Schoff, and N. L. First. 1993. Development of bovine embryos in vitro as affected by energy substrates. *Biol. Reprod.* 49:459–462.
- Schenk, J. L., D. G. Cran, R. W. Everett, and G. E. Seidel Jr. 2009. Pregnancy rates in heifers and cows with cryopreserved sexed sperm: Effects of sperm numbers per inseminate, sorting pressure and sperm storage before sorting. *Theriogenology* 71:717–728.
- Seidel, G. E., Jr. 1999. Sexing mammalian spermatozoa and embryos—State of the art. *J. Reprod. Fert. Suppl.* 54:477–487.
- Seidel, G. E., Jr. 2003. Sexing mammalian sperm—Intertwining of commerce, technology, and biology. *Anim. Reprod. Sci.* 79:145–156.
- Soares, J. G., C. M. Martins, N. A. T. Carvalho, A. C. Nicacio, A. L. Abreu-Silva, E. P. Campos Filho, J. R. S. Torres Júnior, M. F. Sá Filho, and P. S. Baruselli. 2011. Timing of insemination using sex-sorted sperm in embryo production with *Bos indicus* and *Bos taurus* superovulated donors. *Anim. Reprod. Sci.* 127:148–153.
- Stringfellow, D. A., and M. D. Givens. 2010. Manual of the International Embryo Transfer Society: A Procedural Guide and General Information for the Use of Embryo Transfer Technology Emphasizing Sanitary Procedures. 4th ed. International Embryo Transfer Society. International Embryo Transfer Society Inc., Champaign, IL.
- Stroud, B. 2011. IETS 2011 Statistics and data retrieval committee report. The year 2010 worldwide statistics of embryo transfer in domestic farm animals. *Embryo Transfer Newsletter* 29:14–23.
- Sugimura, S., T. Akai, T. Somfai, M. Hirayama, Y. Aikawa, M. Ohtake, H. Hattori, S. Kobayashi, Y. Hashiyada, K. Konishi, and K. Imai. 2010. Time-lapse cinematography-compatible polystyrene-based microwell culture system: A novel tool for tracking the development of individual bovine embryos. *Biol. Reprod.* 83:970–978.
- Sunderland, S. J., M. A. Crowe, M. P. Boland, J. F. Roche, and J. J. Ireland. 1994. Selection, dominance and atresia of follicles during the oestrous cycle of heifers. *J. Reprod. Fert.* 101:547–555.
- Süss, U., K. Wüthrich, and G. Stranzinger. 1988. Chromosome configurations and time sequence of the first meiotic division in bovine oocytes matured in vitro. *Biol. Reprod.* 38:871–880.

- Takenouchi, N., Y. Izaike, K. Ohshima, K. Shimada, and M. Takahashi. 1993. Enzyme immunoassay for determination of progesterone in bovine plasma. *Bull. Chugoku Natl. Agric. Exp. Stn.* 12:125–132. (In Japanese).
- Takenouchi, N., K. Oshima, K. Shimada, and M. Takahashi. 2004. The development of a sensitive enzyme immunoassay for the determination of estrone and estradiol-17 β in bovine blood plasma based on the same homologous combination with antiserum and steroid-enzyme conjugate. *J. Vet. Med. Sci.* 66:1315–1321.
- Thibier, M., and M. Nibart. 1995. The sexing of bovine embryos in the field. *Theriogenology* 43:71–80.
- Yoshioka, H., M. Ito, and Y. Tanimoto. 2010. Effectiveness of a real-time radiotelemetric pedometer for estrus detection and insemination in Japanese Black cows. *J. Reprod. Dev.* 56:351–355.