Ultrasound-Guided Follicle Aspiration and IVF in Dairy Cows Treated with FSH after Removal of the Dominant Follicle at Different Stages of the Estrous Cycle

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ABSTRACT. Recently, transvaginal ultrasound-guided follicle aspiration technology has been found to be of great value for *in vitro* fertilization (IVF) programs, even though the oocyte recovery rate and cleavage rate of transferrable embryos were low. In this study, we investigated the effect of the removal of the dominant follicle at different stages of the estrous cycle on the ovarian response of donor cows. Four experiments (EXPs) were devised. In EXP 1, 3 cows received 20 mg FSH on Day 1, ovulation occurred on Day 0, and on Day 3 follicles were aspirated. In EXP 2, the dominant follicle of the first wave was removed on Day 6 from 3 cows which received 20 mg FSH on Day 7 and on Day 9 follicles were aspirated. In EXP 3, 2 pregnant cows received 20 mg FSH on 70 d of pregnancy and 48 hr later follicles were aspirated a total of 5 times at 5-day intervals. In EXP 4, after ovulation on Day 0, 9 cows received 20 mg FSH on Days 8 to 14 of the estrous cycle and 48 hr after the last injection, follicles were aspirated once. The respective mean ± SD numbers of aspirated follicles and recovered oocytes were higher ($p<0.01$) in EXP 1 (13.4 ± 1.7 and 8.7 ± 2.3), EXP 2 (12.1 ± 1.4 and 7.7 ± 1.7) and EXP 3 (10.7 ± 2.1 and 7.0 ± 2.2) than in EXP 4 (5.8 ± 2.3 and 3.1 ± 1.6). The oocyte recovery rates were higher ($p<0.05$) in EXP 1, EXP 2 and EXP 3 than in EXP 4. Similarly, the respective numbers of viable oocytes and cleavage rates were higher in EXP 1, EXP 2 and EXP 3 (6.0 ± 1.3, 5.0 ± 1.1 and 4.6 ± 1.5 viable oocytes ($p<0.01$); 66, 73 and 65% cleavage rates ($p<0.05$)) than in EXP 4 (2.4 ± 1.1; 46%). The numbers of morulae and blastocysts were higher ($p<0.05$) in EXP 1, EXP 2 and EXP 3 than in EXP 4. In conclusion 1) removal of the dominant follicles from lactating and pregnant cows enabled viable oocytes to be recovered constantly and repeatedly by aspiration at different reproductive stages, and that viable blastocysts can be produced after IVF. 2) The presence or absence of a dominant follicle significantly affects the ovarian responses to FSH treatment. 3) This ultrasound-guided procedure proved to be an effective, repeatable and safe method for viable oocyte recovery from valuable pregnant donors. — KEY WORDS: cattle, dominant follicle removal, follicle aspiration, oocyte, ultrasound.

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The study of *in vitro* development of bovine embryos derived from oocytes matured, fertilized and cultured *in vitro* has been limited to oocytes recovered from ovaries of slaughtered cattle. Recently, several new techniques have been introduced for animal husbandry. In particular, a transvaginal ultrasound-guided aspiration procedure that enables viable oocytes to be collected from living valuable donor cows, followed by *in vitro* fertilization (IVF) programs has been described [20, 25, 26, 32, 33, 40, 41]. Trans-vaginal ultrasound-guided follicular aspiration techniques were initially used in humans [17]. Then, they were employed for cattle and horses. Several researchers have used ultra-sound-guided technology with the aim of increasing the number of oocytes obtained, and there are several reports on cattle concerning hormone-treated, non-treated and repeated aspiration from pregnant and non-pregnant donors, and donors at different reproductive stages [6, 21–23, 25–28, 36, 41]. Recently, the presence or absence of a dominant follicle was reported to affect superovulatory responses of dairy cattle significantly [5, 7, 14, 15, 18, 28, 30, 31]. The purpose of our experiments was (A) to examine whether the number of ovarian follicles, yield of viable oocytes, and number of embryos produced after *in vitro* fertilization (IVF) are affected by the absence or presence of a dominant follicle; and (B) to investigate the ovarian responses to FSH treatment at different stages of the estrous cycle in Holstein cows.

MATERIALS AND METHODS

Animals: Seventeen cyclic lactating Holstein cows (3–6 years old, 60–100 days post partum) were selected at random from June 1994 to August 1994 from the dairy herd at the Yamaguchi Prefectural Dairy Cooperative in Japan. The cows were housed in a confinement facility, which was exposed to the natural environment and consisted of a concrete walkway and dairy-type bedding with chips. The cows received a mixed ration of equal proportions by weight, of alfalfa cubes, good quality alfalfa hay and a barley and corn concentrate, which provided a diet containing approximately 17% crude protein (on a dry weight basis), and the total daily ration was adjusted according to each cow's milk production. The cows were milked twice a day and were checked for signs of estrous during the evening session.

FSH treatment procedure: The cows selected for EXP 1 to EXP 4, each received a total dose of 20 mg FSH (FSH-R: ANTLIN: Denka Pharmaceutical Co., Kawasaki, Japan). The cows in EXP 4 served as controls. FSH was administered as a single injection to stimulate follicular development. FSH-R (20 mg) was dissolved in 1.0–1.5 m*l* physiological saline and mixed well with 10 m*l* 30% w/v PVP (polyvinylpyrrolidone). Ovarian follicular development was monitored ultrasonographically daily from day 2 after estrous until the follicles were aspirated.

Experiment 1: After ovulation (Day=0), 3 cows received 20 mg FSH (dissolved in PVP plus saline) i.m. on Day 1 at 08:00 hr. On Day 3, 48 hr after the injection, ultrasound-guided transvaginal oocyte aspiration was performed. This experiment was carried out three times (total=9 times) on each cow.

Experiment 2: After ovulation (Day=0), the dominant follicle of the first wave was removed by surgical ablation on Day (n=3). These cows received 20 mg FSH (dissolved in PVP plus saline) i.m. on Day 7 at 08:00 hr. On Day 9, ultrasound-guided transvaginal oocyte aspiration was performed 48 hr after the injection. This experiment was carried out three times (total=9 times) on each cow.

Experiment 3: Two pregnant cows from 70 d to 100 d of pregnancy were used. On 70 d of pregnancy, each cow received 20 mg FSH (dissolved in PVP plus saline) and 48 hr later ultrasound-guided transvaginal oocyte aspiration was carried out at 5-day intervals (total=10 times) up to 100 d of pregnancy (D 0=day of breeding).

Experiment 4 (control): After ovulation (Day=0), 9 cows Dreceived 20 mg FSH (dissolved in PVP plus saline) i.m. on Days 8 to 14, inclusive of the estrous cycle. Ultrasound-guided transvaginal oocyte aspiration was performed once, 48 hr after the injection.

Transvaginal aspiration technique: Prior to follicular aspiration, epidural anaesthetic block (5–8 m*l* 2% w/v procaine) was induced to prevent abdominal straining and to relax the rectum, which was necessary for easy manipulation of the ovaries over a long period. The animals were restrained to minimize movement during follicular aspiration. The vulva and the perineal area of each cow were cleaned thoroughly and disinfected. For ultrasound guidance of the aspiration needle, a 5.5-MHz sector tip probe (Aloka) equipped with a 58-cm-long holder with a needle guide was used. This long probe carrier was inserted into the vagina while the ovaries were located and held against the sector tip by rectal manipulation, scanned in several planes and displayed on the monitor (Aloka ss500). A one-way special puncture needle (68 cm long; 18G) was pushed through the vaginal wall and inserted into the ovarian surface. The one-way needle was connected to a permanent rinsing tubing system (modified phosphate-buffered saline containing 0.5% w/v heparin). The diameters of the follicles aspirated in this study ranged from 3 to 13 mm. The aspirates were collected into 50-m*l* sterile plastic tubes. During aspiration, the collection tubes were stored in a container at 32°C. Immediately after aspiration, cumulus-oocyte complexes were separated from the flushing medium and the quality of the oocytes was evaluated.

Oocyte maturation and in vitro fertilization: The oocytes were washed 3 times with maturation medium (TCM-199, Earle's salt; Gibco, Grand Island, NY, U.S.A.) supplemented with 5% v/v superovulated cow serum (SCS) collected on Day 7, 0.01 mg/m*l* follicle stimulating hormone (FSH; Denka Pharmaceutical Co., Kawasaki, Japan) and 50 μg/m*l* gentamicin (Sigma Chemical Co., St Louis, MO, U.S.A.). Oocytes (5 to 10) with cumulus cells covering over one-third of their surfaces were introduced into the maturation medium and cultured for 21 hr at 38.5°C under 5% CO_2 in air. Then, the maturation medium (2.5 m*l*) in polystyrene culture dishes (35-mm diameter, Falcon 1008; Becton Dickinson Co. Ltd., Oxnard CA, U.S.A) was covered with mineral oil (E.R. Squibb & Son Inc., Princeton, NJ, U.S.A.). Thawed frozen semen from USA Holstein bulls was used for IVF. Frozen spermatozoa were thawed in a water bath (37°C) and washed twice with Brackett and Oliphant's [4] medium containing 2.5 mM caffeine (Caff=BO) by centrifugation at 500 g for 5 min for each wash. Then, the spermatozoa were resuspended in Caff-BO supplemented with 1% v/v bovine serum albumin (BSA, Sigma, U.S.A.) and 20 μg/m*l* heparin (Shimizu Pharmaceutical Co., Ltd. Shimizu, Japan) to produce a sperm concentration of 5×10^6/m*l*. A 100-m*l* aliquot of the sperm suspension was preincubated for 1 hr. *In vitro* matured oocytes were transferred into sperm microdrops (5 to 10 oocytes/microdrop) for insemination. After 5 hr, oocytes with adherent cumulus cells were washed by repeated pipetting in the culture medium and transferred for further development into polystyrene dishes (4-well multidish; Nunclon, Roskilde, Denmark) containing: medium-199 supplemented with 5% v/v SCS, 5 μg/m*l* insulin (Wako Pure Chemical Industries Ltd., Osaka, Japan) and 50 μg/m*l* gentamicin sulfate. The culture medium (0.5 m*l*) was then overlaid with mineral oil (0.5 m*l*). Adherent cumulus cells surrounding the embryos were removed by repeated pipetting 48 hr after insemination. The monolayer of cumulus cells adherent to the surface of the culture dish was not disrupted and the embryos were cultured on this layer. The culture medium was replaced with fresh medium 4 days after insemination. The cleavage rate and development of morulae and blastocysts were observed on Day 3 and Day 8. The embryos that developed into blastocysts were frozen using 1.8 M ethylene glycol according to our previous report [38]. Single embryos derived from EXP 3 were transferred nonsurgically directly to Holstein recipient cows. The recipients were checked for pregnancy at Day 60 by an ultrasonic scanning instrument (Aloka, Japan).

Statistical analysis: Data were analyzed for statistical significance using the Student's *t*-test. Data are presented as mean ± SD.

RESULTS

Ultrasonography: The ovarian response (the numbers and sizes of the follicles) in EXP 1, EXP 2, EXP 3 and EXP 4 are shown in Figs. 1, 2, 3 and 4. The average respective numbers of aspirated follicles and recovered oocytes were higher ($p<0.01$) in EXP 1 (13.4 ± 1.7 and 8.7 ± 2.3), EXP 2 (12.1 ± 1.4 and 7.7 ± 1.7) and EXP 3 (10.7 ± 2.1 and 7.0 ± 2.2) than in EXP 4 (5.8 ± 2.3 and 3.1 ± 1.6) (Table 1). The follicle sizes did not differ significantly and ranged from 3 to 13 mm (diameter) (Figs. 1 to 4). The recovery rates were higher ($p<0.05$) in EXP 1, EXP 2 and EXP 3 than in EXP 4

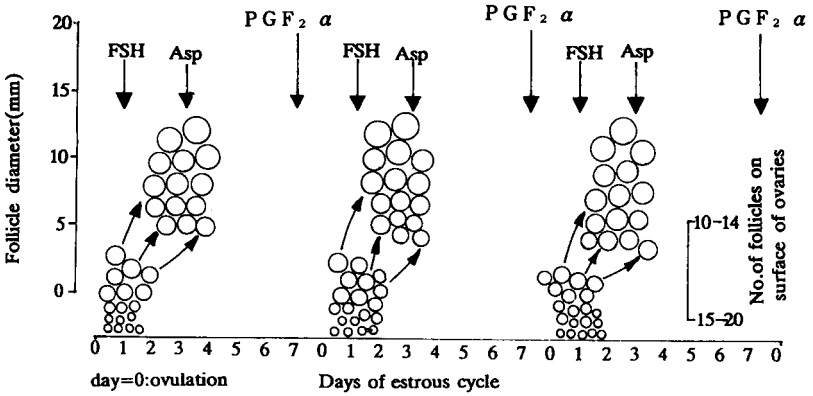

Fig. 1. The numbers and sizes of follicles in EXP 1. FSH ↓: 20 mg i.m. on day 1. Asp ↓: follicles were aspirated on day 3. $PGF_2\alpha$ ↓: Estrous (Day=0: ovulation) was synchronized using one injection of 20 mg Dinoprost (prostaglandin $F_2\alpha$, Pronalgon F).

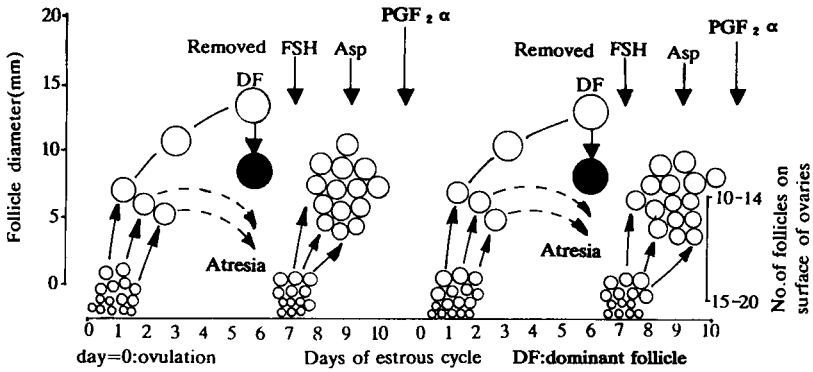

Fig. 2. The numbers and sizes of follicles in EXP 2. DF (dominant follicle) ○→●: the dominant follicle was removed by surgical ablation on day 6. FSH ↓: 20 mg i.m. on day 7. Asp ↓: follicles were aspirated on day 9. $PGF_2\alpha$ ↓: Estrous (Day=0: ovulation) was synchronized using one injection of 20 mg Dinoprost (prostaglandin $F_2\alpha$, Pronalgon F).

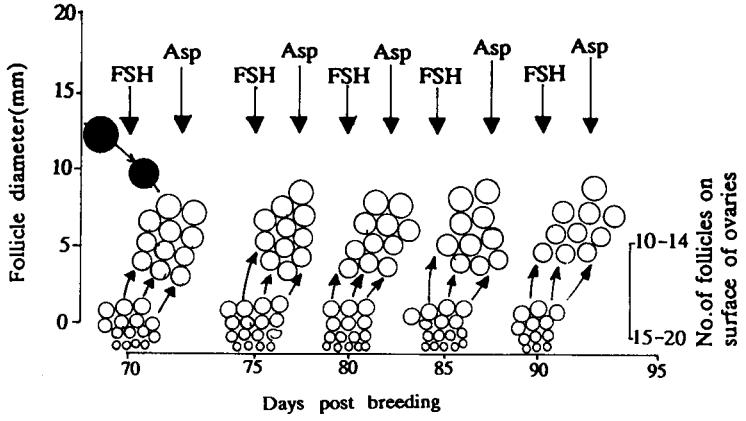

Fig. 3. The numbers and sizes of follicles in EXP 3. ●→●: Atresia phase of the dominant follicle. FSH ↓: 20 mg i.m. on 70 d of pregnancy. Asp ↓: 48 hr after the FSH injection, follicles were aspirated a total of 5 times at 5-day intervals up to 100 d of pregnancy (D 0=day of breeding).

(Table 1). Similarly, the respective numbers of viable oocytes and cleavage rates were higher ($p<0.01$ and $p<0.05$) in EXP 1 (6.0 ± 1.3 and 66%), EXP 2 (5.0 ± 1.1 and 73%) and EXP 3 (4.6 ± 1.5 and 65%) than in EXP 4 (2.4 ± 1.1

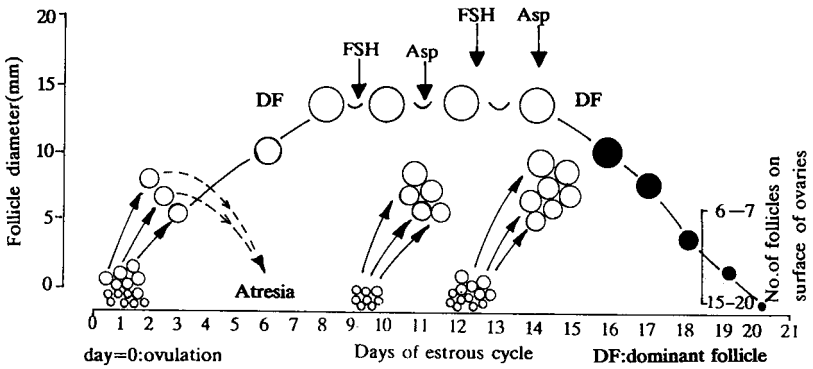

Fig. 4. The numbers and sizes of follicles in EXP 4. Day=0: ovulation of a normal estrous cycle. DF (dominant follicle) ○～○～○～○～●DF: Dominant phase of DF. FSH ↓: 20 mg i.m. on day 8 to 14. ASP ↓: follicles were aspirated once, 48 hr after the FSH injection.

Table 1. Mean number of aspirated follicles, oocytes, cleavage and blastocyst development *in vitro* following IVF of Cumulus Oocyte Complexes (COC) collected via ultrasound-guided follicular aspiration from cows at different reproductive stages

Experimental groups (Number of cows)	EXP 1 lactating cows (n=3)	EXP 2 lactating cows (n=3)	EXP 3 pregnant cows (n=2)	EXP 4 lactating cows (n=9)
Aspiration times	9	9	10	9
Number of aspirated follicles	13.4[a] ± 1.7	12.1[a] ± 1.4	10.7[a] ± 2.1	5.8[b] ± 2.3
recovered COC	8.7[a] ± 2.3	7.7[a] ± 1.7	7.0[a] ± 2.2	3.1[b] ± 1.6
viable COC	6.0[a] ± 1.3	5.0[a] ± 1.1	4.6[a] ± 1.5	2.4[b] ± 1.1
Recovery rate (%)	66.6[c] ± 20.2	64.5[c] ± 12.5	64.9[c] ± 15.0	49.5[d] ± 16.1
Cleaved COC	3.8[a] ± 1.6	3.6[a] ± 1.1	3.1[a] ± 1.5	1.2[b] ± 1.0
Cleavage rate (%)	65.5[c] ± 27.2	73.2[c] ± 11.3	65.0[c] ± 25.4	46.3[d] ± 40.6
Blastocysts	1.5[c] ± 1.0	1.4[c] ± 0.8	1.5[c] ± 0.8	0.7[d] ± 0.6

(Mean ± SD) Within columns, means with different superscripts are significant different (a), b) $p<0.01$; c), d), $p<0.05$.

and 46%). The numbers of morulae and blastocysts were also higher ($p<0.05$) in EXP 1, EXP 2 and EXP 3 than in EXP 4. Five expanded blastocysts were transferred to 5 recipients and resulted in 2 pregnancies (40%) which delivered two male calves.

DISCUSSION

The results of this investigation showed that at different phases of the estrous cycle of lactating dairy cattle, and stages of gestation in pregnant cows, ultrasound-guided transvaginal oocyte aspiration can be performed repeatedly and safely, and enabled the production of transferable embryos from IVF. The ovarian responses of dairy and pregnant cows to FSH were significantly greater ($p<0.01$) in the absence of a dominant follicle than in the presence of a dominant follicle. Similarly, other researchers reported that the presence or absence of a dominant follicle significantly affected the superovulatory responses of dairy cattle [3, 5, 7, 14, 15, 18, 28]. Recently, the follicular dynamics of ovarian function were investigated. A wave of follicular development in cattle is characterized by the synchronous growth of a number of small follicles followed by selection of a dominant follicle and consequent regression of the subordinates. Most estrous cycles consist of 2 or 3 follicular waves [2, 10–12, 16, 19, 24, 34, 35]. Wave emergence was detected on Day 0 (the day of ovulation) and Day 10 of two-wave estrous cycles and on Days 0, 9 and 16 of three-wave cycles [10, 12, 19]. The dominant follicle of a wave induces regression of the subordinates

and during its growth phase, the dominant follicle suppresses the emergence of the next wave [1, 2, 16, 18]. The apparent time of selection of the dominant follicle was coincident with the first significant drop in the serum FSH concentration, which occurred 1 to 3 days after wave emergence [1, 2]. In EXP 1, FSH (20 mg) was given to lactating cows on Day 1. In this ovarian state, exogenous FSH induced synchronized growth of subordinate smaller follicles and resulted in higher yields of oocytes recovered using ultrasound-guided aspiration on Day 3. In EXP 2, the dominant follicle was removed by surgical ablation with an ultrasound-guided aspiration needle on Day 6. FSH on Day 7 led to a greater ovarian response and resulted in higher yields of oocytes recovered on Day 9. In EXP 4, cows received FSH during the mid-phase of their normal estrous cycles. The ovarian responses and the numbers of aspirated follicles and recovered oocytes were significantly lower than in EXP 1, 2 and 3. During the mid-phase of the estrous cycle, the dominant follicle still exerts functional dominance over the first follicular wave and suppresses the subordinate follicles and the emergence of the next wave. Consequently, FSH treatment in the presence of a functional dominant follicle results in a reduced ovarian response and does not lead to the synchronized formation of a group of subordinate small follicles, nor does it elicit the emergence of the next wave, due to the inhibitory effect of the dominant follicle. In our study, surgical removal of the dominant follicle at an appropriate stage of the estrous cycle was most beneficial for the ovarian response. However, other investigators reported that the removal of the dominant follicle by hormonal methods induced no consistent positive effect [7, 29–31]. In the case of a three-wave cycle, we suggest that the removal of the dominant follicle in the second wave led to synchronized growth of a group of subordinate follicles of the third wave and resulted in a greater ovarian response to FSH. In pregnant cows, the wave-like pattern of follicular growth continues throughout the first 60 days of pregnancy and each wave results in a large dominant follicle corresponding to the mid-cycle and ovulatory follicles of the normal estrous cycle [39]. Meintjes *et al*. punctured follicles twice during the periods of 60–75 and 80–95 days of pregnancy and found that the results with FSH-stimulated pregnant cows and non-stimulated non-pregnant cows were similar, but the IVF cleavage rates were considerably higher in the former [22]. Similarly, other investigators found that repeated aspiration proved effective and safe for viable oocyte recovery during the first trimester of pregnancy cows [6, 22]. In EXP 3, FSH treatment on 70 d of pregnancy, a phase during which the dominant follicle exerts regressive effects, led to synchronized growth of a group of subordinate smaller follicles and the 5-day-interval repeated FSH treatment and aspiration schedule was beneficial for suppressing the emergence of dominant follicles and resulted in higher yields of viable oocytes. Otherwise, the aspiration interval seemed to be more important than FSH treatment for the recovery of viable oocytes from donors [6, 9, 13, 36, 37, 40, 42]. Furthermore, Fry *et al*. [8] reported that vaccination against inhibin on follicular development increased the numbers of aspirated follicles, but did not improve aspiration results. Consequently, the absence of the inhibitory effects of the dominant follicle leads to follicular wave synchronization and the emergence of the next wave that release viable oocytes resulting in higher yields of viable oocytes. Using ultrasound-guided technology, this procedure proved to be an effective, repeatable and safe method for viable oocyte recovery from valuable pregnant donors. However, Gibbons *et al*. [9] reported that aspiration frequency may have elevated endogenous FSH levels. An increase of endogenous FSH due to aspiration frequency probably overshadowed any effects from exogenous FSH. Further study is required to establish whether exogenous FSH treatment is needed every time when repeated aspiration is performed at short intervals.

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