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—Original—

Changes in Follicular Vascularity during the First Follicular Wave in Lactating Cows

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Abstract. Increase in the blood supply to individual follicles appears to be associated with follicular growth rates and the ability to become the dominant follicle, while reduced thecal vascularity appears to be closely associated with follicular atresia. Therefore, this study aimed to determine the real-time changes in the vascularity of the follicle wall during the first follicular wave in cycling Holstein cows. Normally cycling and lactating cows (n=5) were examined by transrectal color Doppler ultrasonography (the sensitivity for velocity: > 2 mm/sec) to determine the changes in the vasculature of the follicle wall (presence or absence of blood flow) and the diameter of follicles. A new follicular wave and ovulation were induced by GnRH injection at 48 h after an injection of $PGF_{2\alpha}$ analogue. The ovaries were scanned daily for 7 days after GnRH injection. Follicles >2.5 mm were classified into 3 groups by the changes in diameter as follows: 1) largest follicle, 2) second largest follicle, and 3) small follicles, which included all other follicles >2.5 mm. Before the follicle selection, there was no significant difference in the percentage of follicles with detectable blood flow between the subsequently determined largest and second largest follicles. After the follicle selection, the percentage of follicles with detectable blood flow significantly decreased among the second largest follicles. In addition, small follicles with detectable blood flow kept larger diameters than those without detectable blood flow from one day before the occurrence of follicle selection. It is likely that maintenance of follicle vasculature and appropriate blood supply to the larger follicles is essential for follicle dominance. In small follicles, the presence of blood flow within the wall also appears to be required for recruitment. Consequently, the data suggest that the change of the blood supply to an individual follicle closely relates to the dynamics of follicular growth in the first follicular wave in the cow.

Key words: Blood flow, Follicular wave, Selection, Deviation, Cow

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Selection of the dominant follicle in cattle occurs from a cohort of growing antral follicles, termed a follicular wave [1–3]. Two or three successive waves occur during the estrous cycle in cattle. The first wave (wave 1) appears around the time of ovulation and the second during mid-diestrus (wave 2). Following emergence of a group of follicles 4 to 5 mm diameter, the follicles develop in a common-growth phase for about 3 days. When the largest follicle reaches a mean diameter of about 8.5 mm, the common-growth phase ends and

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deviation in diameter begins, characterized by dissociation in growth rates and the formation of dominant and subordinate follicles [3]. After the end of the common growth phase, only the selected dominant follicle continues growing, while subordinate follicles undergo atresia [4]. The dominant follicle in the cow is normally identified when it reaches a diameter of 10 mm and is larger than the other follicles of the same wave. At this size, the dominant follicle acquires LH receptors on its granulosa cells and acquires ovulatory capacity in response to the LH surge [5].

During follicle growth, an extensive vascular plexus develops in the theca layer surrounding the avascular basement membrane and granulosa layer [6]. Morphological studies of bovine follicles have revealed that the major histological characteristic of the selected (dominant) follicle is its increased vascularity in the theca layer, when compared with unselected (atretic) follicles [7]. It has been suggested that insulin-like growth factor and estradiol (E_2) play key roles in the selection of a dominant follicle during the first wave in heifers and that both are reliable markers to predict the future dominant follicle [8]. Other studies have demonstrated a close correlation between intrafollicular concentration of E_2 and follicular vascularization in porcine and bovine ovaries [7, 9]. However, studies on the differences among follicles in terms of vascularity and growth rates around the time of follicle selection (deviation) have not been carried out in cattle.

Color Doppler ultrasonography is a useful, non-invasive tool for evaluating the vascular function of follicles, allowing visual observation of the blood flow in a delimited area in the wall of preovulatory follicles in humans [10] and cows [11]. In human, blood flow determinations of individual follicles by Doppler ultrasound provide an index of the intrafollicular environment and may be used to predict the developmental competence of the oocyte [12]. In cows, transrectal color Doppler ultrasonography demonstrated a clear difference in the vascularity of the wall of preovulatory follicles compared with anovulatory follicles [11]. The results of the above reports suggest that transrectal Doppler ultrasonography has potential for investigating follicle vasculature in cattle to identify the future dominant follicle at an early development stage or to predict follicle viability after selection. A recent study in mares quantified the daily changes in blood flow within the follicle wall around the time of diameter deviation and demonstrated considerable differences in blood flow area between the two largest follicles one day before diameter deviation [13]. The mean diameter of the largest follicle at deviation is about 22.5 mm in mares and about 8.5 mm in cattle [3]. The smaller follicular size at deviation in cattle represents a technical limitation for determination of blood flow velocities, although blood flow velocity has been accurately determined in healthy growing follicles of more than 10 mm of diameter [11].

The physiological mechanisms involved in the selection of the dominant follicle from identical follicular cohorts exposed to the same levels of circulating gonadotropins remain unclear. It has been suggested that the preferential delivery of gonadotropins and nutrients via a more highly developed vascular system in individual follicles plays a role in the selection and growth of the dominant follicle [14–17]. The present study was carried out to determine if color Doppler ultrasonography could distinguish future dominant follicles from subordinate follicles around the time of the establishment of follicle selection (deviation), and whether or not there is a temporal association between changes in follicular vascularity and follicular growth capacity during the first follicular wave in lactating cows.

Materials and Methods

Animals and ultrasound scanning

The animal experiment was carried out at the Field Center of Animal Science and Agriculture, Obihiro University, Japan. Experimental procedures complied with the Guide for Care and Use of Agriculture Animals of Obihiro University. Lactating Holstein cows were kept under the normal management program of the Field Center and fed daily with corn silage, hay and concentrate with free access to water. At the middle stage of the estrous cycle, cows received 500 μg of a prostaglandin (PG) $F_{2\alpha}$ analogue (cloprostenol [estrumate]; Sumitomo Pharm. Co., Osaka, Japan) i.m. to induce luteolysis. A new follicular wave and ovulation of the preovulatory follicle was induced by a GnRH analogue (Fertirelin acetate 100 μg; [Conceral]; Nagase Pharm. Co., Osaka, Japan)

injected 48 h after $PGF_{2\alpha}$. The day of GnRH injection was designated as Day 0.

Ultrasound scanning and blood collection were performed daily beginning on the day of $PGF_{2\alpha}$ treatment and continued until Day 7. To determine changes in the follicular diameter and follicle vascularity, color Doppler ultrasonography was used. The follicles were examined by transrectal ultrasonography using an ultrasound scanner (SSD-5500, Aloka Co., Tokyo, Japan) equipped with a 7.5-MHz convex transducer (UST-995–7.5, Aloka Co.). All follicles >2.5 mm present in both ovaries were tracked daily during the examination period. The two largest follicles were defined retrospectively according to the maximum attained diameter and were then used to define the day of deviation. During each ultrasonographic examination, the diameter of each follicle was determined by averaging the maximum and the transverse diameters as previously reported [13]. After morphological evaluation, the flow mode was activated for blood flow mapping. Color signals were used to generate images in which blood flow with a velocity higher than 2 mm/sec could be located as areas of color within the follicle wall. Forward flow is usually presented in red and reverse flow in blue. The degree of turbulence is indicated as color-coded signal. The brightness of the color is proportional to the velocity of flow within the vessel. Owing to technical limitations for measurement of blood velocities in small individual follicles, only the presence or absence of blood flow was assessed for each follicle. When a clearly visible red or blue spot (blood flow) was detected in the follicle wall, it was considered as a follicle with detectable blood flow.

Scan records (images) were stored on a Magneto Optical (MO) disk drive for a personal computer (Macintosh; Apple Corp., San Jose, CA) and then viewed on a monitor. The same image was used to calculate the diameter and whether or not the blood flow was detectable. After the last ultrasound scanning on Day 7, the dominant follicle content was evacuated by transvaginal ultrasound-guided follicular aspiration using a convex probe (UST-M15-21079 Aloka Co.) with an 18G needle connected to a 5-ml disposable syringe. Follicular fluid (FF) was collected to determine the concentrations of progesterone (P_4) and E_2.

Progesterone and estradiol determinations

Blood samples were obtained by caudal venipuncture just before each scanning using sterile 10 ml tubes containing 200 μl of stabilizer solution (0.3 M EDTA, 1% acetyl salicylic acid, pH 7.4). All tubes were immediately chilled in ice water for 10 min. and then, centrifuged at 3000 × g for 20 min at 4 C. Aspirated FF was centrifuged at 1000 × g for 5 min to remove granulosa cells. The obtained plasma and FF were stored at –30 C until determination of the concentrations of P_4 and E_2. At the end of the experiment, samples from each cow were analyzed in duplicate using a second antibody enzyme immunoassay (EIA). Steroids assays were performed after diethyl ether extraction. For E_2 determination, FF was diluted 50- or 500-fold so that concentrations in the EIA would be within the optimal range of the standard curve as reported previously for our laboratory [18]. The standard curve for P_4 ranged from 0.05 to 50 ng/ml, and the ED_{50} of the assay was 1.1 ng/ml [19]. The intra- and inter-assay coefficients of variation (CVs) were 4.7 and 6.5%, respectively. The recovery rate of P_4 (1 ng) added to 1 ml plasma samples was 92% (n=10). Likewise 40 μl of samples were analyzed for E_2. The standard curve ranged from 2 to 2000 pg/ml, and the ED_{50} of the assay was 72 pg/ml. The intra- and interassay CVs were 6.8 and 8.6%, respectively.

Statistical analysis

The diameters of follicles were expressed as means ± SEM, and compared with values at the same time point by repeated measures ANOVA followed by Student's t test. The percentages of the follicles with detectable blood flow in largest, second largest and small follicles were compared before and after follicle deviation using two-way ANOVA followed by Fisher's PLSD test. Differences were considered significant at $p<0.05$.

Results

The GnRH injection induced ovulation of the preovulatory follicle and the emergence of a new follicular wave in all the cows used in the present study. Plasma P_4 concentrations progressively increased throughout the experiment with CL development (Fig. 1). The mean number of follicles tracked during the examination was 1 dominant

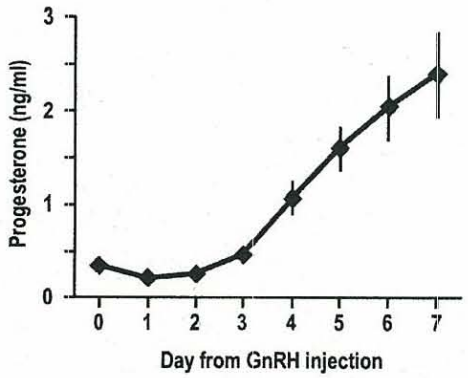

Fig. 1. Daily changes in the plasma concentration of progesterone after GnRH injection. Data are mean ± SEM concentrations of circulating progesterone (n=5 cows).

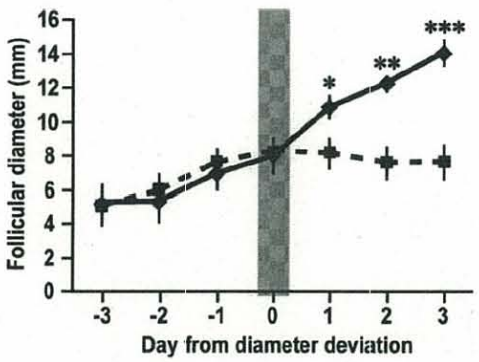

Fig. 2. Changes in the diameter of the largest follicle (◆) and second largest follicle (■). Data were normalized to the day of observed deviation (Day 0). Data are mean ± SEM for each time point (n=3–5). * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ versus values of second largest follicles.

follicle and 7 subordinate follicles per cow. A clear deviation in the growth rates between the two largest follicles occurred between 3 and 4 days after GnRH injection. Because the length of time from GnRH injection to follicle deviation varied among the cows, the data were normalized to the day of deviation based on the criteria of a previous study [5]. Briefly, the beginning of diameter deviation was defined as the beginning of the greatest difference in growth rates between the largest follicle and second largest follicle (observed deviation). Follicles were classified retrospectively into 3 groups based on the changes in diameter as follows: 1) the largest follicle, which grew continuously and attained the maximum diameter during the examination period; 2) the second largest follicle, which grew for 3 to 4 days up to the day of deviation; and 3) small follicles, which other follicles. The mean diameters of the two largest follicles from Day –3 to Day 3 normalized to the day of observed deviation are shown in Fig. 2. The largest follicles continuously grew up to 14.1 ± 0.6 mm on Day 3. The diameter of the second largest follicle increased from 5.1 ± 1.1 mm on Day –3 to 8.3 ± 0.6 mm on Day 0 and then decreased to 7.7 ± 0.9 mm on Day 3. Concentrations of E_2 and P_4 in FF aspirated from dominant follicles were 63.0 ± 21.4 ng/ml and 26.0 ± 6.9 ng/ml, respectively. Accordingly, the ratio of E_2 to P_4 concentration in FF was 2.4 ± 0.5 (n=5) confirming that the largest follicles classified by change in diameter were healthy and dominant on Day 7.

Variation of the blood flow within the follicle wall

Time-average maximum velocity could be measured in some large follicles on each day, but the follicles in which velocity could be measured varied from day to day with values ranging from 4.1 cm/sec to 10.4 cm/sec during the growth phase. Therefore, only the presence of blood flow was used to evaluate follicular vascularity in the present study. The color Doppler image detected blood flow in the follicle wall of most of the largest and second largest follicles and some small follicles during the growth phase (Fig. 3). After deviation had occurred, the blood flow was mainly detectable in the largest follicle.

Changes in the percentages of follicles with detectable blood flow during follicle deviation

Percentages of follicles with detectable blood flow during follicle deviation are shown in Fig. 4. Before the occurrence of diameter deviation (pre-deviation), there was no significant difference in the percentages with detectable blood flow between the two largest follicles. In small follicles, the percentage of follicles with detectable blood flow was lower than that for the future largest follicle and future second largest follicle. After the follicle deviation (post-deviation), the percentage of follicles with detectable blood flow significantly decreased in second largest follicle compared with

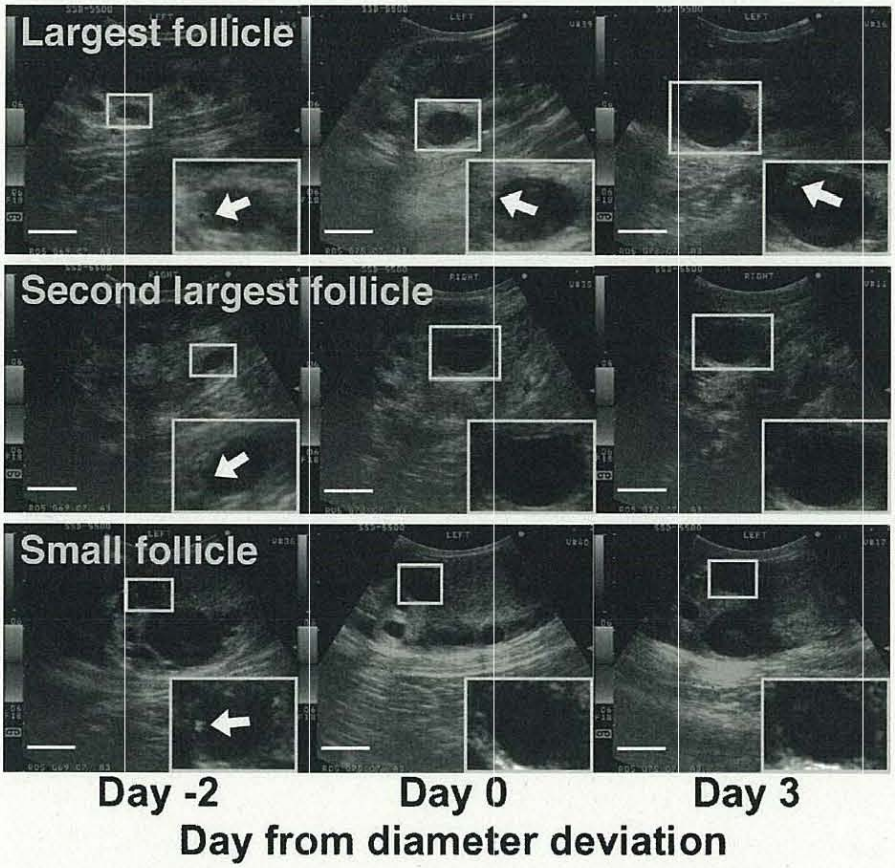

Fig. 3. Representative color Doppler images of the ovary around the day of follicle deviation. Encircled images showing largest (upper panel), second largest (middle panel) and small follicle (lower panel) at Days –2, 0 and 3 from follicle deviation. Enlarged follicle images of the encircled part showing typical changes in detectable blood flow. Arrow indicates areas with detectable blood flow within the follicle wall. Scale bar represents 1 cm.

values before deviation. During the post-deviation period, the percentage of follicles with detectable blood flow in the largest follicles was significantly higher than in the second largest follicles and small follicles.

Changes in the diameter of small follicles with detectable blood flow and undetectable blood flow

To clarify further the effect of blood flow on the development of small follicles, small follicles were further classified as small follicles (+), which showed detectable blood flow at least once during the examination period, and small follicle (–), which did not show any blood flow during the examination period. The mean diameter of small follicle (+) was significantly larger than that of small follicle (–) from Day -1 to Day 3 (Fig. 5).

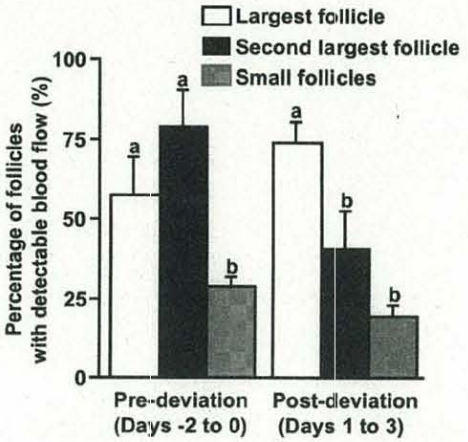

Fig. 4. Change in the percentage of follicles with detectable or undetectable blood flow around the day of follicle deviation. Data are mean ± SEM of each group of follicles. Different letter indicate differences ($p<0.05$) between groups.

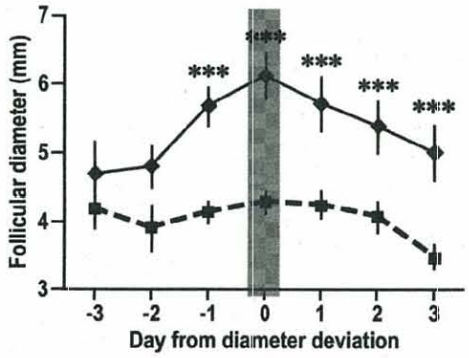

Fig. 5. Changes in the follicle diameter of small follicles with detectable blood flow (◆) and with undetectable blood flow (■) around the day of follicle diameter deviation. Data are mean ± SEM for each time period (n=6–20 follicles). *** $p<0.001$ versus values of small follicles with detectable blood flow.

Discussion

To the best of our knowledge, this is the first report on real-time changes in follicle vascularity associated with the selection of the dominant follicle during the first follicular wave in lactating cows. We used highly sensitive (2 mm/sec in velocity) color Doppler ultrasonography to identify blood flow area in the follicle wall. The ultrasound-derived images of blood flow revealed the time-related changes in appearance of detectable local blood flow in the largest, the second largest and small follicles during the first wave.

In the present study, the percentage of follicles with detectable blood flow before diameter deviation was not different between the largest and second largest follicles. Morphological study has shown that bovine healthy antral follicles with diameters of 3 to 7 mm develop dense capillary blood vessel networks in the theca layer [7]. These results suggest that there is no difference in vasculature and blood supply between the future dominant and subordinate follicles before follicle deviation. Furthermore, there is also no difference between each follicle in levels of mRNA for FSH receptor in the granulosa cells or mRNA for LH receptor in theca cells up to follicle deviation [20], suggesting that there is no difference in the responsiveness to gonadotropins between the two largest follicles at this stage. Equivalent supply of nutrients and gonadotropins provided by a developing vascular system equally contribute to a similar growth rate in both future dominant and subordinate follicles before follicle deviation.

After follicle deviation, our data show that the percentage of largest follicles with detectable blood flow was significantly higher than that of the second largest follicles and small follicles. It has been demonstrated that the capillary blood flow velocity of the dominant follicle is greater than that of the atretic follicles in ewes [21]. These results strongly suggest that blood supply is preferentially delivered to the dominant follicle rather than subordinate follicles after diameter deviation. It is evident that the dominant follicle maintains blood vessel and active angiogenesis [7, 22]. In bovine follicles, the expression of mRNA for angiogenic factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor, and concentration of VEGF protein in FF significantly increased accompanied with increasing follicular size and E_2 levels in FF [14]. In addition, a recent report suggested that the angiopoietin-Tie system that acts on angiogenesis in concert with VEGF controls the blood vessels to maintain active angiogenesis in developing bovine follicles [23]. In contrast, degenerative thecal capillary blood vessels are more abundant and the number of blood vessels is markedly reduced in atretic follicles [6, 7]. These findings indicate that active angiogenesis ensuring blood vessel network and blood supply to the follicle may play a crucial role in the determination of the follicle dominance and atresia.

Quite recently, the daily observation of equine ovaries using color Doppler ultrasonography demonstrated that velocities and the area of blood flow within the follicle wall began to decrease in the future subordinate follicle prior to deviation in diameter, whereas the blood flow continued to increase in the future dominant follicle during follicle selection [13]. In addition, mares with double dominant follicles showed no differences in diameter and blood flow in the follicle wall between the two largest follicles [13]. These data from mares and the present results from cows support the concept that different blood flow changes within the follicle wall influence the fate of follicles and that detectable blood flow and vasculature are associated with follicle viability.

It is interesting to note that the diameter of small

follicles with detectable blood flow was larger than those without detectable blood flow before follicle deviation. The present result agrees with a recent observation of non-dominant follicles during the luteal phase in which healthy follicles with 6.7 mm of mean diameter showed active angiogenesis, and that atretic small follicles with a mean diameter of 4.8 mm showed poorly developed capillaries in the follicle wall [7]. These results indicate that small, but healthy growing follicles maintain angiogenesis and blood flow during early follicular development. Recent studies have demonstrated that modification of follicular angiogenesis by inhibition of VEGF action or stimulation of VEGF production caused a delay or induction of follicular development, respectively [17, 24]. Taken together, we propose that angiogenesis and blood supply in follicles may be involved in not only selection of the dominant follicle but also early follicular development including follicular recruitment.

In conclusion, our results demonstrate that change in the number of follicles with detectable blood flow was associated with follicle selection. It is likely that maintenance of follicle vasculature and appropriate blood supply to the follicle is essential for follicle dominance. In small follicles, the presence of blood flow within the wall appears to be required for recruitment. Consequently, the data suggest that a change of the blood supply to individual follicles closely relates to the dynamics of follicular growth in the first follicular wave in the cow.

Acknowledgments

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