Haemodynamic Changes of the Superovulated Follicle as a Cause of Superovulation Variability in Cattle

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Abstract: The aim of this study was to explore the real time changes in the vascularity of growing superovulated follicles and to establish whether the blood flow of growing follicles can affect superovulation variability. Eight Holstein-Freisian cows received PGF2α 10 days after spontaneous ovulation. After 36 hours, all follicles larger than 5 mm were aspirated at day 0 (D0). Animals were given 28 Armour units (A.U) FSH 24h after aspiration and for 4 days (twice daily, 12h interval). On day 5, animals received a GnRH analogue. Blood samples were then collected daily and were used to detect estradiol (E2), progesterone (P4) and insulin like growth factor-1 (IGF-1) using an enzyme immunoassay (EIA). Results showed that neither the follicular diameter nor the follicle sectional areas (SA) are reliable parameters to predict the superovulation response. On the other hand, the blood area (BA) and blood area percentage were significantly (P<0.05) higher for ovulated (OF) than non-ovulated follicles (NOF). The (NOF) became atretic or continued to form luteal cysts. The P4 level was below 1ng/ml while E2 increased on day 3 to reach the peak. IGF-1 decreased on day 3 and then started to increase until ovulation. In conclusion, the haemodynamics of superovulated follicle could be considered a major source of superovulated variability and play a crucial role in controlling the superovulation result. [Journal of American Science 2010;6(10):744-751]. (ISSN: 1545-1003).

Key words: Cattle; superovulation; blood flow; color Doppler imaging, cyst

1. Introduction:

Superovulation variability constitutes one of the greatest problems associated with embryo transfer in cattle [1]. This variability in ovarian response has been related to many factors including 1) Gonadotrophin preparation, total dose and method of administration [1], 2) Ovarian status at the time of gonadotrophin treatment [2], 3) Environmental influence, age, breed, species and nutrition [1].

There have been many attempts to improve ovarian response using different gonadotrophin preparation such as follicle stimulating hormone (FSH) [3-5], equine chorionic gonadotrophin (eCG) [6, 7] and human menopausal gonadotrophin (hMG) [8, 9]. Some studies improved the number of the recruited follicles by administering Growth hormone (GH) as a pretreatment of FSH which increased the ovulation rate and number of transferable embryo [10]. Similarly, controlling the ovarian status at the time of gonadotrophin treatment by removing the dominant follicles mechanically [11-13], physiologically [14] and or removing the CL [15] improved the response but did not control the variability. Failure of many superovulated follicles, within the same animals, to ovulate has also been considered as crucial source of variability [16].

The importance of the follicle capillary angiogenesis and degeneration was confirmed as a determining factor of the fate of the follicles (continued growth versus atresia) [17]. It has been reported that the haemodynamic changes are involved in the cyclic remodeling of ovarian tissue during follicular growth, ovulation and new corpus luteum development [18]. It has also been reported that following selection of the dominant follicle, the blood flow area of the subordinate follicle disappeared; this was following by an increase of the blood flow area in the future dominant follicle [19]. In a superovulation regime, the FSH overcomes the selection stage because it is equally distributed over the entire growing follicles. However, many superovulated follicles still cannot ovulate.

The aim of this work is to study the haemodynamic changes of the superstimulated follicles and to establish whether the blood flow of growing follicles can affect superovulation variability.

2. Materials and Methods

Eight non-pregnant (Holstein-Fresian), four to five year old cows and of a body weight ranging from 400 to 500 kilograms were kept under normal management program at the Animal Science and Agriculture Farm, Obihiro University, Japan. From the gynaecological point of view, all animals were of
normal health, reproductive soundness, and were exhibiting normal estrous cycles.

Experimental design:
Eight animals were examined daily until spontaneous ovulation. On day 10 all animals received prostaglandin F2α (PGF2α; Estrumate, 5ml, Sumitomo pharm. Co., Osaka, Japan). After 36 hour, all follicles (≥ 5mm) were aspirated at day 0 (D0) by transvaginal ultrasound-guided follicle aspiration. For the ultrasound guidance of the aspiration needle, ultrasound scanner (SSD-5500, ALOKA CO., Ltd., Tokyo, Japan) was used and equipped with a 7.5 MHz transvaginal convex transducer (UST-M15-21079, ALOKA CO., Ltd.) with an 18-gauge single-lumen attached stainless steel needle guide.

All animals were given 5, 4, 3, 2 Armour units (A.U.) of porcine FSH (ANTRIN R 10, Kawasaki Pharm. Co., Kawasaki Japan) twice daily at 12 hour intervals for four consecutive days, respectively. On Day 5, ovulation was induced by GnRH analogue (Fertirelin acetate 100µg; Conceral; Nagase Pharm. Co., Osaka, Japan) which was injected 12h after the last dose of FSH.

Monitoring of the follicular development:
The growing follicles (≥ 2mm) were examined by transrectal ultrasonography. All scans were performed by the same investigator. Each follicle diameter was measured at its maximum diameter. After morphological evaluation, the power flow mode of the ultrasound scanner was activated for blood flow mapping. Colour signals were used to evaluate the blood flow around the entire perimeter of the follicle. The sectional area (SA) of the follicle was estimated by the following equation SA= π/4x (SD)², where SD is the sectional diameter [18]. The coloured area in the image that was obtained at the maximum diameter of the follicle was used as quantitative index to express the blood flow within the follicular wall. Areas of colour represent regions with a flow velocity higher than 2 cm⁻¹. Scan recorded images were stored on a Magneto optical (MO) disk drive (Maxoplix Corporation). The sectional area was calculated and the blood flow area (BA) was quantified using Image J program (version 1.62) developed at the USA National Instituted of Health (http://rsb.info.nih.gov/ij). The daily changes in the superovulated follicles were profiled using retrospective evaluation of ovarian sketches that provided topographical, dimensional and colored area. To overcome the problem of tracking large number of superovulated follicles, a sectional method of sketching follicles was used in which a multiple ovarian maps was made for each ovary, while moving the transducer from medial to lateral aspect of the ovary [20]. After ovulation, non-ovulated follicles (NOF) were examined. Follicles larger than 20 mm and sustained for 9 days with thick wall were considered as luteal cysts.

Blood collection and hormonal determination:
The blood samples were collected on D1 and every 24-h until D8 by caudal venipuncture using 10 ml heparinized tube. The concentration of P₄ was determined by double- antibody enzyme immunoassays (EIA) [21]. The recovery rate was 87%. The standard curve ranged from 0.05 to 50ng/ml, and the ED₅₀ (effective dose 50) of the assay was 7.3ng/ml. Intra- and interassay coefficients of variations (CVs) were 2.9 and 9.3%, respectively. EIA for estradiol-17β (E₂) was conducted as described previously[22]. The recovery rate was 85%. The standard curve ranged from 2 to 2,000pg/ml and ED₅₀ of the assay was 126.2pg/ml. Intra- and interassay (CVs) were 10.4 and 15.5%, respectively. The determination of insulin like growth factor (IGF-1) in plasma was performed by EIA as previously described [23]. The standard curve ranged from 0.39 to 50 ng/ml and ED₅₀ of assay was 4.0 ng/ml. Intra- and interassay (CVs) were 3.1 and 5.6 %, respectively.

Statistical analysis:
The day of follicular aspiration was considered as (=D0). The data of hormonal concentration, follicular sectional diameter (SD), follicular sectional area (SA), blood area (BA) and blood area percent were expressed as mean ± SEM. All data of ovulated (OF) and non-ovulated follicles (NOF) were analysed by repeated measures analysis of variance (ANOVA) to determine main effects of group and interaction of group by day. When main effect of group or group by day was observed, the difference of group means at specific time point were analyzed by the Student’s t-test using JMP statistical software (version 5.1; SAS Institute, Cary, NC, USA). The different means were significant at P<0.05.

3. Results:
Ovarian response to FSH treatment
The emergence of follicular growth started 24 h after aspiration. The recruited superovulated follicles (3-5mm) had nearly the same diameter and growth rate until the day of ovulation under complete absence of CL. The ovulation occurred 24-36h after GnRH treatment. The number of growing follicles, CL and cyst for each trial is presented in table 1.
Table 1: The result of superovulation treatment.

<table>
<thead>
<tr>
<th>Animal number</th>
<th>No. of growing follicles</th>
<th>No. of CL</th>
<th>No of Cyst</th>
<th>% of ovulated follicles</th>
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Follicular growth and hemodynamic changes from D1 to D5:

Images of (OF) and (NOF) during the course of treatment are presented in Fig 1. The follicular diameter and sectional area (SA) of the (OF) and (NOF) showed no significant difference (Fig 2, a, b). However, the percent of the blood area of (OF) was significantly (P<0.05) larger than that of (NOF) (12.3±1.01 Vs 2.9±0.47%; Fig 2.c). The blood sectional area (BA) of (OF) and (NOF) was similar on day 2 then significantly (P<0.05) diverted at day 3 and continued to increase concomitantly with the growth of the ovulated follicles (OF) (Fig 2.d).

The ovulatory follicles on day 5 were well vascularised with large area (0.14±0.02 cm²) of detected blood flow that surrounds their bases (Fig 1).

Hormonal profile during the Days of treatment:

Until D5, P₄ concentration was lower than 1ng/ml. The P₄ level started to increase on day 6 (following ovulation), while E₂ level increased on day 3. IGF-1 showed a transient decrease on day 3 (Fig 3).

Non-ovulated follicle (NOF) from day 7 to day 16:

The NOF get regressed or continued to grow and were then converted to luteal cyst with a diameter above the 20 mm on day 9 of the treatment. Although the blood sectional area (BA) of luteal cyst started to increase after D6 through D14, the percent of the blood area did not change (Fig 4).

Fig. 1 Representative images of the superovulated follicles of cows showing a gradual increase in the blood flow area as ovulation approaches (D5). Ovulatory follicle (*) and non-ovulatory follicle (Close arrow) show a clear difference in the vascular blood flow area (BA). Red colour indicates blood flow toward the transducer, and blue indicate blood flow away from the transducer. The colour gain of the flow mode was set to detect movement of at least 2cm s⁻¹.
Fig. 2. Changes in (a,b) the diameter and follicular sectional area and (c,d) percent of detected blood flow area and blood flow area (BA) in the follicular wall. Data points show mean ± SEM for each time period (n=8 cows). a,b Values with different letters are significantly different (P<0.05).

Fig. 3. Changes in the plasma concentration of progesterone (P₄), estradiol (E₂) and Insulin like growth factors-1 (IGF-1). Data points show mean ± SEM for each time period (n=8 cows).
Fig. 4. Changes (a) in the non-ovulatory follicular area and blood flow in the follicular wall and (b) diameter of the cystic non ovulatory follicles and percent of detected blood flow area. Data points show mean ± SEM for each time period (n=8 cows).

Fig. 5. Schematic diagram explain the mechanism by which the blood vasculature of superovulated follicles causing a variability in the superovulation response. At the beginning of the treatment, the blood vascular area did not differ significantly among recruited follicles. At day 3, all follicles were subjected to a transient decrease of IGF-1 with a transient and significant increase of the E2 level. Thus, E2 dominated the intra-follicular fluid of healthy follicle stimulating the production of VEGF which consequently increased the blood vascular area. At day 5, after the injection of the GnRH, the response of follicles to the LH surge was not influenced by LH pattern or frequency but by the amount of LH received by each follicle through its blood vascular network. So the follicles which had a large blood area ovulated while the follicle with small area converted to luteal cyst.

4. Discussion:
To our knowledge, this report is the first to document the follicular hemodynamic changes during superovulation in cattle.

In this superovulation protocol, the ovarian status was controlled in order to minimize the variability. Aspiration of all follicles larger than 5mm before the LH surge eliminated the negative effect of the dominant follicles and synchronized the follicular wave emergence [11-13], it suppressed the CL formation and maintained a nadir of P4 concentration [24] which improved the number of growing follicles [15]. However, not all superovulated follicles responded to GnRH treatment.

Results showed that the diameter and sectional area (SA) of the growing follicles were unreliable parameters to predict the superovulation response. On the contrary, the blood area percent was significantly different between small follicles from the commencement of the treatment. It was reported that the microvasculature of the growing follicles play an important role in folliculogenesis, ovulation and ovarian hormone production [25]. Although, the small follicles were nearly equal in size there was a certain degree of heterogeneity in follicular
vasculature which determined the fate of developing follicles. Scanning electron microscopy showed that the small atretic follicles had capillary vascular degeneration in comparison to the small healthy follicles [17].

The blood area (BA) of the (OF) and (NOF) in the current study differed significantly on D3. Such different was important in order to switch off the angiogenesis in the atretic follicles (NOF) and divert the ovarian blood flow to the (OF). Similarly, the blood flow area disappeared from the wall of subordinate follicle after the selection stage in order to divert the ovarian blood flow to the dominant follicle [19]. In gilt, the diversion between the same size follicles was controlled by vascular endothelial growth factors (VEGF) [26] which was not equally distributed on the same sized superovulated follicles. It was reported that injection of the VEGF directly in the ovary decrease the atretic follicles and increased the development of the antral follicles by increasing the thecal vasculature [27].

The E2 level was increased at D3. It was reported that the follicular fluid of healthy growing follicles was always E2-dominant while the fluid of the regressed follicles was P4-dominant [28]. This E2 could stimulate the production of VEGF [29] which increased the blood flow area to give the sufficient nutrient for the growing follicles. Moreover it increased the vessel permeability to deliver large size precursor like lipids or lipoproteins that is used by follicle cells to build up steroids [26].

The transient decrease of IGF-1 on day 3 may be the factor that switched off the angiogenesis in the degenerative follicles (NOF) to the healthy growing follicles (OF) by decreasing the level VEGF of (NOF). It has been reported that IGF-1 increased the production of VEGF in cattle granulosa cells culture [30] while injection of IGF-1 binding protein intra-follicular decreased the VEGF in mare [31].

The ovulatory follicles (OF) on day 5 were vascularised with large area of detected blood flow which surrounded their bases. Such changes in the region of blood flow were recorded in cattle[18, 19] and human ovulatory follicles [32]. Thus, the decrease in blood flow at the apex of ovulatory follicles could cause a localized necrosis in this area and facilitate the breakdown of the follicular wall [32]. On the other hand, the increase of the vascular blood flow at the base of the follicles raised the follicular permeability and consequently the intra-follicular pressure.

In this study, the (NOF) continued to grow and converted to luteal cyst which was confirmed by the ultrasonography changes in the wall [33]. It was previously hypothesized that the primary cause of the cystic formation is the deficiency in the pre-ovulatory LH surge or an aberrant release pattern of this hormone [34]. In the current study, giving external GnRH on day 5 controlled the LH surge and pattern [18]. So it seems that the capability of the blood vasculature network surrounding the follicle played a significant role in the distribution and the response to the LH surge. Follicles which had a large blood area (BA) and blood area percent received a sufficient LH amount for ovulation while the non-ovulatory follicles (small blood area and percent) converted to cyst.

Taken together, a mechanism whereby the superovulation response maybe affected by the blood vasculature of the follicles is hypothesized (Fig 5).

On commencing the treatment, the blood vascular area did not differ significantly among recruited follicles. On day 3, all follicles were subjected to a transient decrease of IGF-1 with a transient and significant increase of the E2 level. Thus, E2 dominated the intra-follicular fluid of healthy follicle stimulating the production of VEGF which consequently increased the blood vascular area. On day 5, following the injection of the GnRH, the response of follicles to the LH surge was not influenced by LH pattern or frequency but by the amount of LH received by each follicle through its blood vascular network. So the follicles which had a large blood area ovulated while the follicle with small area converted to a luteal cyst.

In conclusion, the haemodynamics of superovulated follicle is considered as a major source of superovulation variability and can play a crucial role in controlling the superovulation outcome.

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