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Vascularization to preovulatory follicle and corpus luteum-a valuable predictor of fertility in dairy cows

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ABSTRACT

The aim of the present study was to predict pregnancy rate based on vascularization to follicle and corpus luteum (CL). 26 Holstein Friesian cows were synchronized using Ovsynch protocol. On day 10 of the protocol, vascularization and morphological characteristics [sectional area (SA), volume (V) and mean diameter] of follicle was assessed and animals underwent artificial insemination (AI). Morphological evaluation and vascularization to CL was assessed on day 12 and 21 following AI and blood samples were obtained for estimation of plasma progesterone (P_4). Pregnancy diagnosis was performed on day 60 of AI and was classified as normal, complicated and non-pregnant. The overall conception rate was 76.92% (20/26); normal pregnancy was 53.85% (14/26). Complications observed in pregnancy were intrauterine growth retardation, late embryonic death and infection. Cows with a highly vascularized follicle (>550 pixel^2) underwent a normal pregnancy, whereas those that had moderately (250–550 pixel^2) and poorly (<250 pixel^2) vascularized follicle experienced complicated pregnancy or remained non-pregnant, respectively. On day 12, there was no significant variation ($P > 0.05$) between mean diameter, SA, V, luteal blood flow (LBF) or plasma P_4 concentration among CL of cows that remained pregnant (PCL), non-pregnant (NPCL) or that had a complicated pregnancy (CPCL). LBF alone was not beneficial in differentiating among the three groups ($P > 0.05$), but assessment of LBF along with turbulence to blood flow in day 21 CL proved highly valuable due to an increased turbulence in CPCL (66.67%) compared to PCL (16.67%). Assessment of turbulence and LBF on day 12 and 21 can also be used to predict luteolysis with accuracy.

1. Introduction

A calf per animal per year is the most favorable realization of every farmer/dairy enterprise. A successful pregnancy involves several important stages. Firstly, an ovarian follicle must develop and ovulate an oocyte capable of being fertilized and undergo embryonic development. Secondly, the oviductal and uterine environments must be suitable for gamete transport, fertilization, and subsequent embryonic development. Finally, the corpus luteum (CL) must function for a sufficient period of time for maternal recognition of pregnancy and maintain gestation [1]. Hemodynamic changes are involved in the cyclic remodeling of ovarian tissue that occurs during final follicular growth, ovulation and new CL development [2] during which angiogenesis plays a crucial role in the maintenance of ovarian structures.

Transrectal Doppler ultrasonography has been utilized increasingly for research and clinical studies of ovarian hemodynamics involving follicle and CL in large farm animals [3]. Blood flow determinations of individual preovulatory follicles provide an important index on the intrafollicular environment and may predict the developmental competence of the corresponding oocyte [4,5]. A study on 39 Holstein heifers proved that blood flow at the time of artificial insemination (AI) was greater in the preovulatory follicle wall of heifers that became pregnant than in heifers that did not [6]. Relationships between the echogenicity of CL and progesterone (P_4) concentrations have already been made with conventional gray-scale ultrasound in heifers [7] and in mares [8] and an increased blood flow has been proposed to augment the transfer of the embryonic signal for CL maintenance and for maternal recognition through the utero-ovarian pathway [9].

Therefore, assessment of blood flow to ovarian structures (follicle and CL) has been shown to give preliminary information regarding pregnancy rate [6]. However continuous assessment of

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follicle, CL and sustenance of pregnancy has not been studied. Early prediction of nature of pregnancy based on blood flow to follicle and CL will prove to be a boon for the dairy industry. Infertility and complications in pregnancy remains to contribute significantly to reduced conception rates in dairy farms. Since follicle and CL are ovarian structures easily visualized by ultrasonography and indispensable for a successful pregnancy, our study aimed at assessing blood flow to follicle and CL to predict fertility in dairy cows.

2. Materials and methods

2.1. Animals

The study was conducted on twenty-six healthy, reproductively normal and regular cycling postpartum Holstein Friesian cows (body weight: 300–350 kg, body condition score (BCS): 3.5 to 4, parity: 2 to 3). The cows were housed in an organized dairy farm in semi-loose housing system and were reared on green fodder (30–40 kg), wheat straw (2 kg), concentrate feed, mineral mixture and *ad libitum* drinking water.

2.2. Ovulation synchronization (Ovsynch)

Irrespective of the stage of estrus cycle, the cows were subjected to an ovulation synchronization protocol. Cows were treated with a GnRH analogue (Buserelin acetate, Receptal™, Intervet, India, im) on day 0 (20 μg) and day 9 (10 μg) along with a synthetic prostaglandin $F_{2\alpha}$ analogue (Cloprostenol Sodium, Vetmate™, Vetcare, Bangluru, India, 500 μg im) on day 7.

2.3. Study design

On the day of estrus (Day 10, of Ovsynch protocol), transrectal examination and ultrasonography were used to locate and measure the size of follicle respectively. Color flow mode (CFM) was used to assess the blood supply to the follicle, and timed AI was performed. Blood flow of the follicle was reassessed on day 11 (24hrs following AI), and AI was repeated if the follicle had not ovulated. Size and blood flow of CL was assessed on day 12 and 21 following AI (represented as day 12 and 21 respectively). Blood samples were collected on day 12 and 21 for estimation of P_4. Pregnancy diagnosis was performed in all the animals on day 60 of AI using ultrasonography.

2.4. Brightness mode (B-mode) and doppler ultrasonography

Transrectal ultrasonography was carried out using a battery operated B-mode ultrasound scanner equipped with a 7.5 MHz, linear-array transducer (Exago, ECM - Noveko International Inc., Angoulème, France) for assessing the size of follicle/CL. The frozen optimal scan images were used to determine the diameter (average of maximum length and transverse diameter) with the help of built-in, on-screen callipers. The volume (V) of the follicle and CL were estimated using the following equation for a modified prolate ellipsoid: $V = 0.523 \times A \times B^2$, in which A represents the maximum length and B represents the transverse diameter [10]. CL were described as CL with a single (central or eccentric)/multiple cavities or CL with incomplete luteinization of the follicular wall [11]. If a CL contained a cavity, volume of the cavity was determined and subtracted from the calculated CL volume except in CL with incomplete luteinization. In addition, the image obtained in a vertical plane from the apex to the base of the follicle/CL, designated as the overall image was used to determine the sectional area (SA) of the follicle/CL: $SA = \pi/4 \times (SD)^2$, where SD is the sectional diameter [10].

Thereafter, color flow mode (4000 Hz pulse repetition frequency and 27.5 dB gain) of the scanner was used for blood flow mapping of follicle/CL. The red color indicated blood-flow toward the transducer's face, and blue color indicated blood-flow away from the transducer's face [12]. When the transducer was placed close to the follicle/CL, the blood flow captured by the transducer appeared as different color intensities on the monitor. As the velocity of the flow increased, the color intensity increased. Forward flow with turbulence resulted in yellow and backward flow with turbulence resulted in cyan (blue-green) [13]. Transducer was positioned at the maximal diameter of the follicle/CL to achieve the maximal number of color pixels in the recorded image. The blood vessels supplying the structures were evaluated based on their diameter into small (<3 mm), medium (3–6 mm) and large (>6 mm).

2.5. Quantification of blood flow

Colored spots or pixel aggregates were selected from the images, extracted, and saved using Adobe Photoshop software (Adobe® Photoshop® CS3 Extended, Version 10.0, USA). Image J (Image J 1.45s, USA) was used to calculate total number of colored pixels, expressed as $pixel^2$.

2.6. Pregnancy diagnosis

Pregnancy was confirmed at Day 60. Fetus appeared as an echogenic structure inside a non-echogenic structure [14] using B-mode ultrasonography. Once the fetus was detected, image was frozen and crown-rump length (CRL, a straight line between the fetal crown and the origin of the tail), trunk diameter (TD, at level of umbilical cord attachment) and skull diameter (SkD, the widest diameter) were measured and the image was recorded in a few normal and other cases.

Blood flow velocity wave forms of the middle uterine artery were recorded in those cases which were suspected for fetal growth retardation/abnormalities and in normal cases for comparison. The middle uterine artery was located using CFM and switched to Pulsed-wave mode (4000 Hz pulse repetition frequency, 12 dB gain, 50% power and 20–60° Doppler angle) for determination of waveform. The waveform was evaluated for Resistance Index (RI) and Pulsatility Index (PI). A higher resistance or RI indicated lower perfusion to the particular organ and a high PI indicated decreased perfusion to distal tissues [12].

Based on the blood supply of follicle/CL, fetal characteristics and blood flow velocity waveforms, pregnancy was classified as normal, complicated and non-pregnant.

2.7. Blood sampling

Jugular vein blood samples (10 ml) were collected in heparinized vaccutainer vials prior to ultrasonography on day 12 and 21. Plasma was separated immediately after blood collection by centrifugation at $3000 \times g$ for 15 min. The plasma aliquots were stored at −20 °C for estimation of P_4.

2.8. Estimation of P_4

Plasma P_4 was assayed with a solid-phase radioimmunoassay using antisera raised in our laboratory [15]. Sensitivity of the assay was 0.1 ng/ml; intra- and inter-assay variation coefficients were 6.2% and 9.5%, respectively.

2.9. Statistical analysis

Numerical data is expressed as Mean ± SEM. Statistical analysis was performed using SPSS 16 [16]. Pearson's correlation coefficient