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SHORT COMMUNICATION

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Use of B–mode and Power Doppler ultrasonography of the uterus and preovulatory follicle to predict ovulation time in Holstein cows after heat synchronization

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Abstract

Aim of study: To evaluate the utility of B-mode and Power Doppler ultrasonography to predict ovulation time in Holstein cows by assessment of uterine and follicle measurements.

Area of study: Galicia, NW Spain

Material and methods: 33 Holstein cows were examined every 12 h until ovulation. Measurements for the ratio endometrium/myometrium (END/MYO), uterine lumen (UL), diameter of the dominant follicle (DF), and Power Doppler of the dominant follicle and corpus luteum were recorded. The times of onset of heat, maximum heat (MHA) and heat finalization were obtained from the database of monitoring devices. Blood samples were taken at each examination for progesterone (P4) determination. Data were analyzed using one-way ANOVA and Pearson's χ^2 tests.

Main results: For UL, time -6 (1.53 mm) with respect to ovulation (time 0) significantly differed from time -42 (5.70 mm). Concerning DF, significant differences were observed between time -6 (20.48 mm) and time -54 (16.60 mm). As for P4, significant differences were found between time -6 (0.34 ng/mL) and time -54 (1.03 ng/mL). Considering MHA, significant differences were observed for the UL between after and before/during groups; for DF, significant differences were found before and after MHA. As for heat, the UL significantly differed between after and before/during groups. Significant differences were found for the percentage of cows with Doppler signal in the ovulatory follicle and corpus luteum concerning MHA and heat factors.

Research highlights: The use of Power Doppler to predict ovulation time needs to be refined. The END/MYO and UL measurements could be useful to identify cows in heat, but inaccurate to determine ovulation.

Additional key words: ultrasound; blood flow; ovulation; reproductive tract; dairy cattle

Abbreviations used: AFT (after group); AI (artificial insemination); BEF (before group); DF (diameter of the dominant follicle); DUR (during group); END (endometrium thickness); END/MYO (ratio endometrium/myometrium); LH (luteinizing hormone); MHA (maximum heat activity); MYO (myometrium and perimetrium thickness); P4 (progesterone); PGF2α (prostaglandin F2α); UL (uterine lumen).

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Introduction

In order to achieve maximum success at artificial insemination (AI), capacitated sperm should be present in the female reproductive tract at ovulation time. Then, AI should be carried out close to the ovulation time.

The hormonal fluctuations during the oestrous cycle induce changes on the female reproductive tract at both uterine and ovary level (Carrière et al., 2010; DesCôteaux et al., 2010). The uterine wall presents oedema, and a variable quantity of mucus in the uterine lumen can be observed, which may be greater during oestrus. Besides, uterine blood flow velocity increases during proestrus and oestrus and returns to its lowest values after ovulation (Bollwein et al., 2000). Regarding the ovary, not only an increase in size of the preovulatory follicle can be observed, but also an increase in blood flow around the initiation of the luteinizing hormone (LH) surge that reaches its maximum just before ovulation (Carrière et al., 2010).

In addition, hormonal environment is responsible for the oestrus behaviour, which is characterized by standing immobile while being mounted, restlessness, sniffing the vulva of another cow, resting chin, and mounting (Roelofs et al., 2010). Following these signs, the well-known rule "AM-PM" has been established as a recommendation for AI after oestrus detection (Trimberger, 1948). Overall, it has been stated that the optimal time of insemination is 24 to 12 h before ovulation (Pursley et al., 1998; Roelofs et al., 2006a).

Nowadays, automatic milking machines and monitoring devices are the new tools to perform oestrus detection, and each day more farms are switching to this approach (Roelofs & Van Erp-Van Der Kooij, 2015; Saint-Dizier & Chastant-Maillard, 2018). These technologies can discern between the onset, middle and end of oestrus, and thus determine its duration and even the maximum heat activity.

Therefore, whether using observational oestrus detection or electronic devices, it is necessary to determine which oestrus characteristic features are better indicators of ovulation, aiming to perform AI at the appropriate time. Some studies have been conducted using behavioural signs (Layek et al., 2011), ultrasonographic features of the preovulatory follicle (Siddiqui et al., 2010), and progesterone (P4) concentrations (Roelofs et al., 2006b). Additionally, investigations on the relation between uterine wall changes and hormone concentration and fertility have been reported (Souza et al., 2011; Sugiura et al., 2018). To our knowledge, research on the combined evaluation of uterine features and size and vascularization of the preovulatory follicle to predict ovulation time, with the aid of monitoring devices to perform oestrus detection, has not been published. In addition, another value of this study is that it aims to simulate as closely as possible the conditions veterinarians have to face on farm work in a daily basis, so the results can have a direct application.

Consequently, the objective of this study was to assess the utility of B-mode ultrasonography to predict ovulation time by assessment of uterine and follicular measurements; additionally, we aimed to determine the usefulness of Power Doppler ultrasonography compared to B-mode to predict ovulation, by the assessment of blood flow to the preovulatory follicle.

Material and methods

Animals

A total of 33 Holstein cows were enrolled (parity 1-4). They were housed in a free-stall facility at "Granxa Campus Terra" (Castro de Rei, Spain). This farm has an automatic milking machine, and cows are milked ~3.2 times/day, with a mean milk production of 42.49 kg/cow-day. Cows had a body condition score of 2.75-3.5 (1-5), were fed a total mixed ration, and had *ad libitum* access to water. These cows had monitoring devices (Innogando, Lugo, Spain) that recorded real time information about their activity: number of steps, resting time, food intake, and rumination. The experiment was conducted in accordance with the European and Spanish Regulations for the protection of animals used for scientific purposes (Directive 2010/63/EU; RD 53/2013). The animal study was reviewed and approved by Ethics Committee of the University of Santiago de Compostela.

Study design

Only cows > 60 days in milk that had already cycled at least one time after the last calving were included. Routine reproductive examinations were carried out twice a month by a veterinarian, and all data were recorded on farm software (Gando Nuevas Tecnologías, Spain). Cows were enrolled in a modified synchronization protocol, G6G (Fig. S1 [suppl]), with 2 or 3 prostaglandin F2α (PGF2α) administrations (150 µg of PGF2α analogue Dinoprost, Enzaprost® T, Ceva Salud Animal S.A., Barcelona, Spain). Ovulation time was considered as time 0. Examinations were carried out every 12 h since 52 h after the administration of the second PGF2α until ovulation. The decision was based on the results obtained in a previous experiment (data not published), in which 86.7% of cows ovulated between 54 h and 90 h after PGF2α administration. Additionally, the times of onset of heat, maximum heat and heat finalization were obtained from the database of the monitoring devices.

Ultrasonography examination and image analysis

Ultrasonography examinations were performed using a ProVetScan SR-2C (New Veterinary Technologies, León, Spain), equipped with a multifrequency (6.5-8 MHz), linear-array transducer (Frequency 8.0 MHz, Gain 56%, PRF 2.0 K). Measurements for endometrium thickness (END), myometrium and perimetrium thickness (MYO), uterine lumen (UL), and diameter of the dominant follicle (DF)

were collected. Uterine wall measurements were taken at the uterine horns, just before the curvature, and UL was measured where the maximum content was found. Additionally, videos of the Power Doppler examination of the dominant follicle (PDF) and corpus luteum (PDCL) were recorded. A subjective evaluation was performed, classifying blood flow as absent or present (Fig. S2 [suppl]).

Blood sample collection and progesterone analysis

Blood samples were collected from the coccygeal vein immediately prior to each ultrasonography examination, centrifugated at 1500 g for 15 minutes, and serum was separated into 0.5 mL aliquots and frozen at -20 ºC until analysis.

Serum P4 concentrations were determined using a commercial progesterone ELISA kit (DRG-Progesterone-ELISA-EIA-1561, DRG-International, Inc., USA), following the manufacturer's instructions. The detection limit for P4 was 0-40 ng/mL, with an analytical sensitivity of 0.045 ng/mL. Optical densities were measured in a microplate reader (Multiskan-EX, Thermo Fisher Scientific Inc., Waltham, USA).

Statistical analysis

As throughout oestrus measurements for END and MYO respectively increase and decrease, the ratio END/MYO was calculated as an index to contemplate both events. Time of maximum heat activity (MHA) and heat, obtained from the monitoring devices database, were classified into three categories: before (BEF), during (DUR) or after (AFT), according to examination time with respect to MHA or heat.

A one-way ANOVA test was performed including the UL, DF, END/MYO, and P4 as dependent variables, and the time of examination (-54, -42, -30, -18 and -6 h with respect to ovulation time) as factor. This test was performed again with the same dependent variables and MHA and heat as factors. The Bonferroni test was used to perform post-hoc comparisons and homogeneity of variances was checked using Levene's test. Additionally, a Pearson's χ^2 test, including PDF and PDCL as dependent variables, and time of examination, MHA, and heat as the independent factors, was performed.

All analyses were conducted in SPSS version 28.0 for Windows (SPSS Inc, Chicago, IL, USA). Differences were considered significant at $p \leq 0.05$.

Results and discussion

Of the 33 cows, 8 (24%) did not respond to the treatment. In addition, 2 cows (6%) ovulated before 52 h and were excluded from de analysis. The distribution of ovulation times of the remaining cows (n = 23) is displayed in Fig. 1. Additionally, descriptive statistics are shown in Table 1. No statistically significant differences were observed in this experiment regarding 2 or 3 PGF2α administrations for the variables of interest.

Results for the one-way ANOVA test showed that, for the variable UL, time -6 (1.53 mm) significantly differed from time -42 (5.69 mm, p = 0.016). Concerning DF, significant differences were observed between time -6 (20.48 mm) and time -54 (16.60 mm, p = 0.038). As for P4, significant differences were found between time -6 (0.34 ng/mL) and time -54 (1.03 ng/mL, p = 0.013). No significant differences were found between examination times for END/MYO (p = 0.707). Considering MHA, statistically significant differences were observed for the UL between group AFT (1.60 mm) and groups BEF and DUR (4.90 mm (p = 0.002) and 4.84 mm (p = 0.009), respectively); similar results were obtained for DF, with significant differences between group BEF (18.63 mm) and group AFT (20.90 mm, p = 0.043). As for heat, the UL significantly differed between group AFT (1.57 mm) and groups BEF and DUR (5.09 and 4.25 mm, respectively, p = 0.002).

Results for the Pearson's χ^2 test showed that the percentage of cows with Doppler signal in the preovulatory

Table 1. Descriptive statistics (mean±SD) for the ratio endometrium/myometrium (END/MYO), uterine lumen (UL), diameter of the ovulatory follicle (DF), and serum progesterone concentration (P4) of 23 cows after PGF2α administration at different examination times before ovulation (0 h).

Time (h)[1]	END/MYO	UL (mm)	DF (mm)	P4 (ng/mL)
-6 (n=23)	2.36±0.90	1.53±2.63^{a}	20.48±3.92^{a}	0.34±0.48^{a}
-18 (n=23)	2.61±0.93	4.44±4.05^{a}	20.20±3.13^{a}	0.44±0.41^{a}
-30 (n=21)	2.84±1.19	4.14±3.74^{a}	19.98±3.47^{a}	0.37±0.26^{a}
-42 (n=13)	2.56±1.64	5.70±3.83^{b}	18.84±2.94^{a}	0.43±0.51^{a}
-54 (n=10)	2.52±0.80	4.71±4.48^{a}	16.60±3.47^{b}	1.03±1.15^{b}

[1] Due to different ovulation times, the number of animals (n) differ between examination times. ^{ab}: Means within a column lacking a common superscript differ ($p < 0.05$).

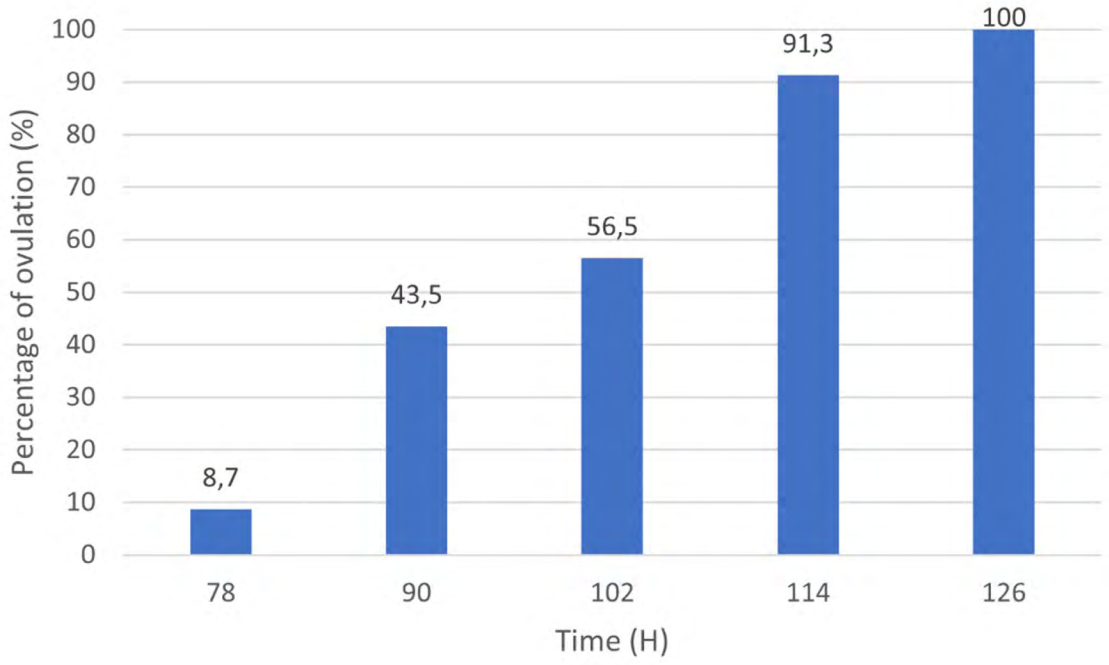

Figure 1. Cumulative distribution of ovulation times (hours) of 23 Holstein cows with respect to the last PGF2α administration (time 0 h) after a modified G6G synchronization protocol (the last GnRH was suppressed).

follicle at examination times -54, -42, -30, -18, and -6 h was 0%, 23.1%, 14.3%, 36.4%, and 54.2%, respectively ($p = 0.011$). For the variable PDCL, the percentage of cows with Doppler signal was 60.0%, 23.1%, 19.0%, 8.7%, and 0% ($p < 0.001$). Concerning MHA and heat, statistically significant differences were found for both variables (Table 2).

One important finding was the presence of Doppler signal in most preovulatory follicles few hours before ovulation, and the increase in the percentage of follicles with blood flow during and after MHA and heat. Blood flow to the preovulatory follicle is correlated to the increase concentration of oestradiol and the LH surge (Acosta et al., 2003; Pancarci et al., 2012). Moreover, Siddiqui et al. (2010) reported a biphasic increase and decrease in blood flow to the preovulatory follicle, with the first peak occurring 3 h after GnRH treatment and the second peak occurring 8-6 h before ovulation. Probably due to our study design (examinations carried out every 12 h), we were not able to identify the two peaks, but a progressive increase in follicles with detectable peripheral vascularization instead. Our results suggest that the identification of Doppler signal on the follicular wall might be of use as an indicator of ovulation within the following 6 to 18 h. Additionally, this identification took place mostly after MHA and heat, determined by electronic monitoring devices, which offers the possibility of a complementary use of these two approaches.

Regarding the UL, differences were observed among times of examination. During oestrus, the UL may show an accumulation of anechogenic content inside the uterine horns, that will normally disappear during the dioestrus phase (DesCôteaux et al., 2010). However, no significant differences were observed for UL at the examination times closer to ovulation (-18 and -6), in contrast with the PDF. Consequently, UL may be useful as a positive sign of heat, but it may not be an adequate parameter to estimate ovulation time. Special attention should be paid to the characteristics of the uterine content to discern between heat and pathological situations like endometritis (Sheldon et al., 2006).

As for END/MYO, no significant differences were found among examination times. The high level of circulating oestrogens during the perioestrus period is responsible

Table 2. Results for the Pearson's χ^2 test, including 23 Holstein cows, for the Doppler of the preovulatory follicle (PDF) and Doppler of the corpus luteum (PDCL) considering maximum heat activity (MHA) and heat, divided into 3 groups with respect to the time of examination: before (BEF), during (DUR), and after (AFT) MHA or heat according to the monitoring devices.

Variable	Factor	Factor group			SIG
		BEF	DUR	AFT	
PDF	MHA	16.2	28.6	50.0	0.015
	HEAT	14.3	38.5	43.5	0.030
PDCL	MHA	35.1	4.5	3.8	0.001
	HEAT	37.1	3.7	4.3	< 0.001

for the greater thickness and oedema of the uterine wall, that will normally decrease between 4 and 5 days after oestrus (DesCôteaux et al., 2010). A possible explanation for the lack of significance could be the fact that the first examination was delayed 52 h after PGF2α administration. Therefore, cows were already at the onset of heat. Nevertheless, no differences were found at the times closer to ovulation, which suggest that END/MYO could be a good parameter to identify cows in heat, but not to predict the time of ovulation. Additionally, it should be noted that endometrial thickness also increases during endometritis due to inflammation. Consequently, a differential diagnosis should be made taking into account other aspects such as the uterine fluid, time of the cycle and the diagnostic tools available like endometrial cytology (Dubuc et al., 2010).

Finally, we used Doppler ultrasonography and P4 serum levels to assess luteal function, since its accuracy has been previously described by numerous researchers (Siqueira et al., 2013; Rocha et al., 2019; Dubuc et al., 2020). In this case, similar results were obtained, as PDCL progressively decreased until heat, and was absent near ovulation.

In conclusion, the use of Power Doppler to predict the time of ovulation needs further research. Additionally, END/MYO and UL could be of use to identify cows in heat; however, their application to determine ovulation time seems to be inaccurate. Therefore, the difficulties to predict the exact moment of ovulation in field conditions have to be considered, and additional studies, including a bigger sample size, are needed.

Supplementary material (Figures S1 and S2) accompanies the paper on SJAR's website.

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