The vaginal and uterine blood flow changes during the ovsynch program and its impact on the pregnancy rates in Holstein dairy cows

Heba A. Sharawy1, AbdelRaouf O. Hegab1, Engy F. Risha2, Mohamed El-Adl3, Walid T. Soliman4, Mohamed A. Gohar4, Reham A. Fahmy5, Virginia M. Farag2, Kazuhiko Imakawa6, Fuller W. Bazer7, Daniela James8, Adel Zaghloul9, Abdelnasser A. Abdalla10, Mariam M. Rabie1 and Mohammed A. Elmetwally1*

Abstract

Aim: OvSynch is a hormonal protocol for synchronization of estrus and use of artificial insemination (AI) at an optimal time without adverse effects on the ovaries or uterus. This study investigated the use of noninvasive color Doppler ultrasound to assess changes in uterine and vaginal blood flow during the Ovsynch program for synchronization of estrus and its relation to the pregnancy rates in Holstein cows.

Materials and methods: The experimental cows received an intramuscular dose of 10 μg of a GnRH analogue (G1), followed 7 days later with an intramuscular injection of synthetic prostaglandin F2α (P: PGF2α) analogue (500 μg cloprostenol sodium), and given a 10 μg, injection of the GnRH analogue (G2) i.m. 48 h after the PGF2α treatment, and the cows were bred 14-16 h after. Uterine and vaginal perfusion were investigated by performing transrectal Doppler ultrasonography of both the uterine and vaginal arteries in Holstein cows at different time points during the Ovsynch program to determine: peak systolic velocity (PSV), time-averaged maximum velocity (TAMV), the volume of blood flow (BFV), pulsatility index (PI), resistance index (RI), resistance impedance (S/D) and diameters of uterine (UA) and vaginal (VA) arteries. Steroid hormones were also assayed. Transrectal ultrasonography (TUS) was performed at 32 and 60 days to confirm the pregnancy per artificial insemination (P/AI).

Results: The uterine PSV, TAMV, and PV were greater at the time of the cloprostenol sodium and second GnRH injections (p<0.05) than at the time of the first GnRH injection. The vaginal PSV, PV were greater at the time of the cloprostenol sodium than at the time of the first and second GnRH injections (p<0.05). The receiver operating characteristic curve (ROC curve) indicated a high correlation between the uterine and vaginal blood flow and the rate of the pregnancy (p<0.05). The area under the ROC curve was 0.920 and 0.87 (p<0.05) for vaginal and uterine arteries respectively at time of G2. The serum levels of progesterone, estrogen and cortisol were correlated with the P/AI (p<0.05). The P/AI significantly decreased from 43.9 % at 32 d to 35.37 % at 60 d.

Conclusion: These results indicate that noninvasive Doppler ultrasonography is a valid method to evaluate changes in the characteristics of uterine and vaginal blood flow in cows during the Ovsynch protocol. Furthermore, vaginal and uterine blood flow are two determinant factors for the higher conception rates in Holstein dairy cows.

*Correspondence: mmetwally@mans.edu.eg
1 Department of Theriogenology, Center for Reproductive Biotechnology, Faculty of Veterinary Medicine, Mansoura University, Mansoura 35516, Egypt
Full list of author information is available at the end of the article

© The Author(s) 2022. Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated in a credit line to the data.
Keywords: Ovsynch, Vaginal blood flow, Uterine blood flow, pregnancy rates; Cows

Background
The profitability of dairy cow operations is dependent mainly on reproductive performance, and reproductive management is one of the most important factors for increasing the profitability of dairy enterprises. Calving interval, milk production efficiency, herd replacement dynamics, and timing of the establishment of pregnancy during lactation impact the profitability of dairy herds [1]. Successful artificial insemination (AI) and conception rates after a voluntary waiting period (VWP) are two main determinants of time to the establishment of pregnancy postpartum. The VWP has traditionally been 60 days in dairy farms [2]. Dairy farm revenue is affected positively by maximizing the number of female calves born for replacements in the herd, minimizing replacement of cows due to reproductive disorders, and sustaining the length of the lactation curve when milk production is greatest [3].

Although there are numerous reproductive management strategies available for dairy farms, implementation of the best management program remains a major challenge for dairy producers owing to the complicated interactions of multiple biological and management factors affecting dairy herd dynamics and economics [4, 5]. The dairy industry has approved hormonal protocols for synchronizing estrus and ovulation as reproductive management systems to increase the overall reproductive performance of lactating cows [6]. Ovsynch protocols are important for increasing the use of AI with semen from bulls with desired genetic with respect to milk production daughters, but AI does not improve fertility in dairy cattle as conception rates (CR) for AI services after Ovsynch are similar to those for cows inseminated after observed natural estrus [4, 7]. New protocols, such as Presynch-Ovsynch and Double-Ovsynch, have been developed to improve the timing of insemination and fertility following AI at a timed AI (TAI) [8–10].

Doppler ultrasonography has broadened the utility of imaging from an anatomical to a physiological base [11]. This novel method has been used to assess healthy and pathological variations in uterine blood flow [12]. Earlier research, utilizing invasive techniques, evaluated blood flow in the reproductive system of cows to find rhythmic alterations related to changing concentrations of progesterone (P4) and estradiol (E2) in serum during the estrous cycle [13–15]. Color Doppler ultrasonography, a non-invasive technology developed two decades ago, is able to detect variations in blood flow throughout the estrous cycle of mares [16], sheep [17], and cows [18]. It has also been used to assess changes in blood flow during pregnancy [19, 20], uterine torsion [21]; and the puerperium [22, 23]. This imaging approach can be used to assess the vasculature of ovarian follicles [24], corpora lutea (CL) [25], ovulatory follicles [26], and the uterus [27].

To the best of our knowledge, uterine and vaginal blood flow in cows has not yet been studied during the estrus synchronization regime, and no attempts have been made to correlate the changes in the blood flow with subsequent pregnancy yield. The purpose of this study was to evaluate uterine and vaginal blood flow in cows undergoing an Ovsynch program for estrus synchronization and to investigate the relationship between the changes in uterine and vaginal blood flow and steroid hormone levels and pregnancy rates in Holstein dairy cows.

Results
Ovulation and pregnancy per insemination parameters
Pregnancy per AI at 32 and 60 d after AI and pregnancy loss are presented in Table 1. The overall P/AI was 43.9% (36/82) and 35.37 (29/82) for the first and second pregnancy checks, respectively (P = 0.001). All experimental cows were ovulated within 33±4.2 h after the second injections of GnRH.

The effects of GPG Ovsynch on uterine blood flow
Imaging of the uterine blood vessel was successful in all of our examinations. Blood flow parameters PSV, TAMV, BFV, PV (Fig. 1 A, B, C, D respectively), RI, PI, S/D, and uterine artery diameter (Fig. 2 A, B, C, D respectively) during the GPG management program for estrus synchronization in Holstein dairy cows underwent interesting changes. Uterine blood flow PSV was increased (P < 0.0001) at the time during which cows were injected with PGF2α and then the second injection of buserelin (94.01 ± 2.02 and 92.03 ± 1.15 cm/s, respectively) when compared with values at the time of the first injection of buserelin (67.26 ± 3.09cm/s). Similarly, the TAMV of the uterine blood flow increased (P <

Table 1 Pregnancy rate per artificial insemination (P/AI) for Holstein dairy cows exposed to GPG ovsynch program

| Pregnancy check | parity               | Prim-parous | Pleuri-parous | overall |
|-----------------|---------------------|-------------|--------------|---------|
| 32±3            | 41.9 (13/31)        | 45.09 (23/51)| 43.9 (36/82) |
| 60±3            | 32.25 (10/31)       | 37.2 (19/51) | 35.37 (29/82) |
| P value         | 0.002               | 0.01         | 0.001        |
Fig. 1  Peak systolic velocity (PSV) in the uterine artery, time-averaged maximum velocity (TAMV), blood flow volume (BFV), peak velocity (PV) changes in response to the GnRH-PGF2α management program. Values are means ± standard error of mean (SEM). Means with different superscripts (a, b, c) are significantly different (P < .05)

Fig. 2  Changes in pulsatility index (PI), resistance index (RI), systolic/diastolic(S/D), and diameter (D) changes in the uterine artery in response to the GnRH-PGF2α management program. Values are means ± standard error of mean (SEM). Means with different superscripts (a, b, c) are significantly different (P < .05)
The maximum increase occurred with the PGF2α and the second injection of buserelin acetate (39.09 ± 1.86 vs. 38.35 ± 2.14 cm/s) as compared to the time of the first injection of buserelin (23.22 ± 1.52 cm/s). In the same way, the peak velocity (PV) was increased (P < 0.0001) at the time during which cows were injected with PGF2α, and then the second injection of buserelin (87.12 ± 2.93 and 101.66 ± 1.93 cm/s, respectively) when compared with values at the time of the first injection of buserelin (59.72 ± 4.61 cm/s). The uterine artery blood flow volume (U_BFV) increased (P < 0.05) during the time when PGF2α was administered (12.58 ± 0.54 ml/min) compared to the times for the first and the second injections of buserelin (9.25 ± 0.47 and 5.83 ± 0.53 ml/min, respectively). The resistance impedance Doppler indices (RI, PI, S/D) for uterine blood flow during the GPG protocol for synchronization of estrus changed significantly. The resistance index (RI) and pulsatility index (PI) for uterine blood flow increased (P < 0.05) during the time of the first injection of buserelin (1.95 ± 0.02 vs. 0.91 ± 0.2, respectively) compared to the time of the PGFα injection (1.48 ± 0.02 vs. 0.77 ± 0.13, respectively) and the second injection of buserelin acetate (1.61 ± 0.01 vs. 0.83 ± 0.13, respectively). On the other hand, the S/D ratio of uterine blood flow increased (P < 0.05) at the time of the second injection of buserelin acetate (3.48 ± 0.07) compared to the time of the first injections of buserelin acetate and PGF2α (1.99 ± 0.08 and 2.54 ± 0.05, respectively). The diameter of the uterine artery (D) increased (P < 0.05) in response to PGFα and the second injection of buserelin acetate (0.65 ± 0.01 and 0.64 ± 0.01 mm, respectively) compared to the time of the first injections of buserelin acetate (0.56 ± 0.01 mm).

As illustrated in Table 2, concentrations of progesterone in serum were positively correlated with volume of blood flow through the uterine artery and TAMV (BFV: \( r^2 = 0.66, P < 0.01 \); TAMV: \( r^2 = 0.63, P < 0.05 \), respectively) and negatively correlated with the RI (RI: \( r^2 = -0.50, P < 0.05 \)). As for concentrations of cortisol in serum, they were positively correlated with U_BFV and TAMV (BFV: \( r^2 = 0.77, P < 0.01 \); TAMV: \( r^2 = 0.50, P < 0.05 \), respectively), but negatively correlated with RI (RI: \( r^2 = -0.51, P < 0.05 \)) and not correlated with PI (P > 0.05). There was a negative correlation between concentrations of E2 in serum and U_BFV and TAMV (BFV: \( r^2 = -0.38, P < 0.05 \); TAMV: \( r^2 = -0.30, P > 0.05 \), respectively).

Table 2 Pearson’s rank correlation coefficients for the relationships between concentrations of estrogen, progesterone and cortisol in serum and peak systolic velocity (PSV), blood flow volume (BFV), time-averaged maximum velocity (TAMV), pulsatility index (PI), resistance index (RI), systolic/diastolic velocity (S/D), and peak velocity (PV) with respect to blood flow through uterine artery (U) based on parameters measured using Doppler ultrasonography (D Parameters) in Holstein cows during the GnRH- PGF2α management program. Bold parameters are \( R \) value and reverse bolding indicates \( P \) value

|        | P4  | E2  | Cortisol | U-PSV | U-TAMV | U-BFV | U-PI | U-RI | U-S/D | U-D |
|--------|-----|-----|----------|-------|--------|-------|------|------|-------|-----|
| P4     | 1   | -0.18 | 0.94** | -0.17 | 0.63* | 0.66** | 0.04 | -0.5 | -0.49 | 0.39 |
| E2     | -0.18 | 1   | -0.26 | 0.02  | -0.7  | -0.38 | 0.08 | 0.27 | 0.05  | -0.20 |
| Cortisol | 0.94** | -0.26 | 1     | -0.23 | 0.5   | 0.77** | 0.19 | -0.51* | -0.12 | 0.38 |
| cortisol | 0.000 | 0.34 | 0.4   | 0.05  | 0.001 | 0.47  | 0.05 | 0.65 | 0.15  |
| U-PSV  | -0.17 | 0.02 | -0.23 | 1     | 0.14  | -0.52* | -0.21 | -0.23 | 0.43  | -0.07 |
| U-TAMV | 0.63* | -0.7 | 0.5*  | 0.14  | 1     | 0.37  | 0.66** | 0.05 | 0.1   | 0.7   |
| U-BFV  | 0.66** | -0.38 | 0.77** | -0.5* | 0.9   | 1     | -0.49 | -0.34 | -0.5*  | 0.22  |
| U-PI   | 0.007 | 0.15 | 0.001 | 0.04  | 0.01  | 0.05  | 0.2  | 0.61 | 0.41  |
| U-RI   | 0.04 | 0.08 | 0.19  | -0.2  | -0.5  | -0.49 | 1    | -0.21 | -0.85** | 0.2   |
| U-S/D  | 0.8  | 0.75 | 0.4    | 0.43  | 0.05  | 0.05  | 0.05 | 0.66 | 0.00  | 0.46  |
| U-D    | 0.05 | 0.31 | 0.05  | 0.4   | 0.16  | 0.2   | 0.66 | 0.78 | 0.48  |
| U-BFV  | -0.49 | 0.05 | -0.12 | 0.43  | 0.66** | -0.56* | -0.85** | -0.07 | 1     | 0.05  |
| U-PI   | 0.05 | 0.85 | 0.65  | 0.1   | 0.006 | 0.02  | 0.00 | 0.7  | 0.85  |
| U-RI   | 0.39 | -0.20 | 0.38  | -0.07 | 0.19  | 0.22  | 0.2  | -0.16 | -0.05  | 1     |
| U-D    | 0.14 | 0.47 | 0.15  | 0.7   | 0.4   | 0.46  | 0.4  | 0.8  |

*P<0.05; **P<0.01
Fig. 3 Changes in peak systolic velocity (PSV), time-averaged maximum velocity (TAMV), blood flow volume (BFV), and peak velocity (PV) in the vaginal artery in response to the GnRH:PGF2α management program. Values are means ± standard error of mean (SEM). Means with different superscripts (a, b, c) are significantly different (P < .05).

Fig. 4 The pulsatility index (PI), resistance index (RI), systolic/diastolic (S/D), and changes in diameter (D) of the vaginal artery in response to the GnRH:PGF2α management program. Values are means ± standard error of mean (SEM). Means with different superscripts (a, b) are significantly different (P < .05).

P < 0.15; TAMV: r² = -0.7, P < 0.01, respectively), but no correlation with uterine artery PI (P > 0.05).

The effects of GPG Ovsynch on vaginal blood flow
The Doppler parameters and the diameter of the vaginal artery changed during GPG ovsynch protocol (Figs. 3 and 4). Vaginal blood flow peak systolic velocity (PSV) and peak velocity (PV) increased (P < 0.05) at the time of injection of PGF2α (73.51 ± 0.93 and 146.5 ± 3.41 cm/s) compared to times of the first and the second injections of buserelin acetate (44.63 ± 1.05 vs 81.22 ± 3.42 and 54.28 ± 3.38 vs 111.45 ± 12.9 cm/s, respectively), as was TAMV of vaginal blood flow (P < 0.05). The maximum increase occurred at the second injection of buserelin (30.39 ± 0.42 cm/s) when compared to times of the first injection of buserelin and the injection of PGF2α (20.45 ± 1.59 and 23.22 ± 0.62 cm/s, respectively). Vaginal blood flow during the GPG estrus synchronization protocol changed significantly. The resistance index (RI) and the pulsatility index (PI) of the vaginal blood flow increased (P < 0.05) during the time of the first injection of buserelin (0.8 ± 0.01 vs. 1.82 ± 0.13, respectively) and the time when PGF2α was injected (0.81 ± 0.02 vs. 1.74 ± 0.02, respectively) compared to time of the second injection of buserelin acetate (0.67 ± 0.009 vs. 1.2 ± 0.03, respectively). Moreover, the S/D ratio of vaginal blood flow increased (P < 0.05) in response to PGF2α and a second injection of buserelin acetate (2.27 ± 0.003 and 2.27 ± 0.004 mm, respectively) compared to the first injection of buserelin acetate (0.24 ± 0.004 mm). As showed in Table 3, There was a positive correlation between the vaginal blood flow peak systolic velocity (PSV) and concentrations of both progesterone (PSV: r² = 0.75, P < 0.01) and cortisol in serum (PSV: r² = 0.76, P < 0.01). In contrast the concentrations of both progesterone and cortisol in serum were negatively correlated with vaginal blood flow resistance index (RI) (RI: r² = -0.44, P > 0.05 and RI: r² = -0.37, P < 0.1, respectively) and also with PI (PI: r² = -0.48, P > 0.05 and PI: r² = -0.54, P < 0.1, respectively). Concentrations for E2 in serum were negatively

Table 3 Pearson’s rank correlation coefficients for the relationships between concentrations of estrogen, progesterone and cortisol in serum and peak systolic velocity (PSV), blood flow volume (BFV), time-averaged maximum velocity (TAMV), pulsatility index (PI), resistance index (RI) systolic/diastolic velocity (S/D), and peak velocity (PV) of blood flow through vaginal artery (V) using Doppler ultrasonography parameters (D) in Holstein cows during the GnRH PGF2α management program. Bold parameters are r value and reverse bolding indicates P value

| P4  | E2  | Cortisol | V-PSV | V-TAMV | V-BFV | V-PI | V-RI | V-S/D | V-D |
|-----|-----|----------|-------|--------|-------|------|------|-------|-----|
| P4  | 1   | -0.18    | 0.94**| 0.75** | 0.98**| 0.33 | -0.48| -0.44| 0.69**| 0.49|
| P4  | 0.5 | 0.000    | 0.001 | 0.01   | 0.22  | 0.06 | 0.09 | 0.01  | 0.06 |
| E2  | -0.18| 1       | -0.26 | -0.31  | -0.09 | -0.17| -0.1  | 0.18  | -0.04| -0.27|
| E2  | 0.5 | 0.34     | 0.25  | 0.7    | 0.047 | 0.7  | 0.5   | 0.86  | 0.32 |
| Cortisol | 0.94** | -0.26 | 1    | 0.76**| 0.05  | 0.26 | -0.54**| -0.37 | 0.12  | 0.37 |
| cortisol | 0.000 | 0.34    | 0.001 | 0.83   | 0.34  | 0.03 | 0.16  | 0.64  | 0.17 |
| V-PSV | 0.75** | -0.31 | 0.76**| 1     | -0.13 | 0.1  | 0.34  | -0.31 | 0.4   | 0.41 |
| V-PSV | 0.001 | 0.25    | 0.001 | 0.62   | 0.7   | 0.2  | 0.24  | 0.1   | 0.12 |
| V-TAMV | 0.98  | -0.17  | -0.05 | -0.13  | 1     | 0.74**| -0.81*| -0.34 | -0.74*| 0.5   |
| V-TAMV | 0.01  | 0.7     | 0.83  | 0.62   | 0.001 | 0.01 | 0.21  | 0.01  | 0.05 |
| V-BFV | 0.33  | -0.17   | 0.26  | 0.1    | 0.74**| 1    | 0.02  | -0.3  | 0.25  | 0.82**|
| V-BFV | 0.22  | 0.047   | 0.34  | 0.7    | 0.001 | 0.9  | 0.26  | 0.35  | 0.000|
| V-PI  | -0.48 | -0.1    | -0.54*| 0.34   | -0.81*| 0.02 | 1     | -0.21 | 0.4   | -0.04|
| V-PI  | 0.06  | 0.72    | 0.03  | 0.2    | 0.01  | 0.9  | 0.43  | 0.13  | 0.88 |
| V-RI  | -0.44 | 0.18    | -0.37 | -0.31  | -0.34 | -0.3  | -0.21 | 1     | 0.18  | -0.46|
| V-RI  | 0.09  | 0.5     | 0.16  | 0.24   | 0.74* | 0.26 | 0.43  | 0.05  | 0.08 |
| V-S/D | 0.69**| -0.04  | 0.12  | 0.4    | 0.01  | 0.25 | 0.18  | 1     | 0.26  |
| V-S/D | 0.01  | 0.8     | 0.64  | 0.1    | 0.52  | 0.35 | 0.13  | 0.5   | 0.33  |
| V-D   | 0.49  | -0.27   | 0.37  | 0.41   | 0.5   | 0.82**| -0.04 | -0.46 | 0.26  | 1     |
| V-D   | 0.06  | 0.32    | 0.17  | 0.12   | 0.05  | 0.000| 0.88  | 0.08  | 0.33  |

*p<0.05; **p<0.01
correlated with the vaginal artery TAMV and BFV (TAMV: \(r^2 = -0.09\); BFV: \(r^2 = -0.17\), \(P < 0.5\), respectively).

The effects of the GPG Ovsynch protocol on concentrations of cortisol, progesterone, and estrogen in serum

As summarized in Table 4, concentrations of P4 in serum increased during the luteal growth phase of the Ovsynch protocol at the time when cloprostenol sodium was injected (10.43 ± 0.17 ng/dl, \(P < 0.05\)) as compared to times of the first and the second injections of buserelin acetate (0.54 ± 0.17 and 2.07 ± 1.12 ng/dl, respectively). In contrast, concentrations of E2 in serum increased (\(P < 0.05\)) during the time of the first injection of buserelin acetate (21.41 ± 0.99 pg/dl) and increased further during the time of the second injection of buserelin acetate (25 ± 1.83 pg/dl) compared to the time of the PGF2α injection (16.56 ± 0.78 pg/dl). Furthermore, concentrations of cortisol in serum increased (\(P < 0.05\)) at the time of the PGF injection (1.08 ± 0.02 ng/dl) when compared to the times of the first and second injections of buserelin acetate (0.49 ± 0.04 and 0.5 ± 0.02 ng/dl, respectively).

Prediction of pregnancy

ROC curves were constructed and the area under the curves could be seen in (Figs. 5 and 6). It was possible to set a cutoff value for every parameter taken into account (Table 5). The area under the curve examining the P4 level at the time of G2 shot as a predictor of pregnancy was found to be 0.839 and the best P4 cutoff value was 0.71 ng/ml with (sensitivity of 90% and a specificity of 37.5%; \(P < 0.003\)). The volume of vaginal artery blood flow volume (V_BFV) as a good predictor of pregnancy. At the time of the G2 shot, the V_BFV cutoff value was set at 0.90 ml/min with an increase in sensitivity (90.8%) and specificity (30.8%; \(P < 0.001\)). Regarding the other parameters, it was possible to set a cutoff value for U_BFV, cortisol, at the time of G2 shot that was (0.722 ml/min and 0.60 ng/ml, respectively), with a (sensitivity of 90% and 80% and a specificity of (33.33% and 40% ; respectively)

| Time points | Hormonal levels | Estrogen (pg/dl) | Cortisol (ng/dl) |
|-------------|-----------------|------------------|-----------------|
| First GnRH injection | 0.54 ±0.17\(^a\) | 21.41 ±0.99\(^a\) | 0.49 ±0.04\(^b\) |
| PGF2α injection | 10.43 ±0.17\(^b\) | 16.56 ±0.78\(^b\) | 1.08 ±0.02\(^a\) |
| Second GnRH injection | 2.07 ±1.12\(^b\) | 25 ±1.83\(^a\) | 0.5 ±0.02\(^b\) |

Concentrations (means ± standard error of mean (SEM)) of estrogen, progesterone and cortisol in serum from Holstein cows at the time of the initial treatments with GnRH and PGF2α in the GPG program. Means with different superscripts (a,b) are significantly different between time points (\(P < 0.05\)).

![Fig. 5](image_url)

**Fig. 5** ROC curve for predicting viable pregnancy by (A) uterine artery blood flow volume (U_BFV), (B) vaginal artery blood flow volume (V_BFV) at the time of g2 shots. The ROC curve was constructed by plotting the true positive rate (sensitivity) on the y-axis and the false positive rate (1-specificity) on the x-axis.
The area under the curve (AUC) examining the E2 level at the time of G2 shot as a predictor of pregnancy was found to be 0.875 and the best E2 cutoff value was 0.607 Pg/ml with (sensitivity of 87.5% and specificity of 42.86%; \( P < 0.001 \)). Also, AUC for uterine and vaginal blood flow were 0.870 and 0.804 respectively at the time of injection of G2.

Discussion

Transrectal color Doppler ultrasonography is a noninvasive method used to investigate the effects of a gonadotropin treatment during superovulation on uterine blood flow, as well as its relationship with steroid hormone levels, ovarian response, and the yield of embryos in dairy cows [28]. The present study characterized, for the first time, changes in uterine and vaginal blood flow during the period of application of the Ovsynch protocol in Holstein dairy cows. The P/AI was correlated significantly with the vaginal blood flow at time of insemination than the uterine blood flow according to the ROC curves analyses. The present study has high economic value because it guides the veterinarian to either give the injections of GnRH and/or PGF2α during the Ovsynch protocol on dairy farms. The results of the current study revealed significant variations in uterine and vaginal blood flow indices in Holstein cows undergoing the GPG estrus synchronization regime as well as P/AI. The overall P/AI in the current study was 43.09% and 35.37% at the first and second pregnancy checks. The decreased pregnancy percentage may be attributed to the decreased uterine and vaginal blood flow at the time of artificial insemination. This hypothesis was proved by the results of ROC curve analyses that indicated a positive correlation between the blood flow of both uterine and vaginal arteries and the success of the pregnancy at day 32 after TAI.

### Table 5

Receiver operating characteristic (ROC) curve of uterine blood flow, vaginal blood flow, serum progesterone, serum estrogen and serum cortisol at the G2 shots

| Items      | Cutoff value | AUC value | Sensitivity (%) | Specificity (%) | \( P \)-value |
|------------|--------------|-----------|-----------------|-----------------|--------------|
| U_BFV      | 0.722 ml/min | 0.870 ± 0.115 | 90%             | 33.33%          | \( P < 0.001 \) |
| V_BFV      | 0.90 ml/min  | 0.804 ± 0.07  | 90.8%           | 30.8%           | \( P < 0.03 \)  |
| Cortisol   | 0.60 ng/ml   | 0.820 ± 0.119 | 80%             | 40%             | \( P < 0.007 \) |
| Estrogen   | 0.607 pg/ml  | 0.875 ± 0.09  | 87.5%           | 42.86%          | \( P < 0.001 \) |
| Progesterone| 0.7143 ng/ml | 0.839 ± 0.116 | 90%             | 37.5%           | \( P < 0.003 \) |

\( P < 0.01, 0.007 \) respectively. The area under the curve (AUC) examining the E2 level at the time of G2 shot as a predictor of pregnancy was found to be 0.875 and the best E2 cutoff value was 0.607 Pg/ml with (sensitivity of 87.5% and specificity of 42.86%; \( P < 0.001 \)). Also, AUC for uterine and vaginal blood flow were 0.870 and 0.804 respectively at the time of injection of G2.
The effects of the first and second injections of buserelin acetate, as well as PGF2α significantly affected uterine blood flow. The peak systolic velocity in uterine blood flow and the TAMV of uterine blood flow were measured during the time of the GPG treatments. When comparing the first and second injections of buserelin, uterine artery BFV increased during the time of the PGF2α injection, indicating that blood flow increased at the time of AI and during the luteal phases of the estrous cycle [29]. Also, uterine blood flow increases during the proestrus and estrus phases of the estrous cycle of dairy cows [18]. Blood flow velocity is relatively constant during diestrus in response to high concentrations of progesterone [11]. Similarly, uterine blood flow in the present study was significantly positively correlated with concentrations of E2 and P4 in serum.

In the present study, concentrations of cortisol in serum were greater at the time of PGF2α injections than during the first and second injections of buserelin. The pattern of changes in the serum cortisol was similar to those reported previously for cows and mares. Prolactin and cortisol were present in greater concentrations in the blood during estrus in cows, while concentrations of cortisol were greater during the luteal phase of the estrous cycle in mares [30, 31].

In the current study, the resistance impedance indices (RI, PI, S/D) for uterine blood flow changed significantly in response to the G:PG program. When compared to the effects of injection of PGF2α and the second injection of buserelin acetate, the RI and PI of uterine blood flow increased significantly in response to the first injection of buserelin. The S/D ratio of uterine blood flow increased at the time of the second injection of buserelin. The variation in resistance impedance indices in the uterine arteries may be attributed to changes in concentrations of P4, E2 and cortisol in serum as well as changes in the diameter of the uterine arteries [18, 28]. Previous research established that increases in estradiol in plasma act as the primary vasodilator of the uterine artery, resulting in an increase in uterine blood flow [28, 32]. Similarly, changes in indices of uterine artery blood flow (PSV, PV, TAMV) were positively correlated with BFV ([11, 20].

It is worth noting that previous research has shown that RI increases during proestrus [18, 33]. During early estrus, the resistance index was strongly correlated with both the average maximum velocity and volume of blood flow in the uterine artery to perfuse the uterus. The highest correlation was found on days 0 and 1 of the estrous cycle, which corresponded to the first and second injection of buserelin in the current study [34]. On the other hand, they found a negative relationship between RI of uterine blood flow and concentrations of estradiol in serum, but the correlation between RI and concentrations of progesterone in serum was not significant. In the current study, there was a negative correlation between RI in the uterine artery and serum progesterone levels, but there was not a significant correlation with serum estradiol levels. Furthermore, concentrations of cortisol in serum were correlated positively with volume and TAMV for uterine arterial blood flow, negatively correlated with RI and S/D, and not significantly correlated with PI. These findings suggest that uterine blood flow changes at different times during the GPG program in dairy cows when there are changes in concentrations of steroid hormones in serum.

In this study, changes in vaginal artery blood flow in dairy cows were investigated for the first time during the G:PG Ovsynch program. A previous study of pregnant buffalo characterized changes in vaginal blood flow during pregnancy [35].

In the current study, changes in vaginal blood flow during the G:PG Ovsynch estrus synchronization program were significant. The PSV and PV for vaginal blood flow increased more at the time of PGF2α injections than at the time of the first and second injections of buserelin acetate. The increase in blood perfusion via the vaginal artery was characterized by decreasing PI and RI values in response to effects of gonadotropins on the ovaries (follicle development) and/or the increase in concentrations of estradiol in serum [28]. During estrus, estradiol acts as a major vasodilator, and there is an increase in blood flow as its concentrations increase in serum [28, 32].

When compared to the second injection of the buserelin acetate, the RI and PI for vaginal blood flow were significantly greater than during the first injection of buserelin and the injection of PGF2α. Furthermore, the S/D ratio of vaginal blood flow was greater in response to PGF2α and the second injection of buserelin acetate as compared to the response to the first injection of buserelin acetate. It is likely that the increase in vaginal blood flow in cows following gonadotropin treatment is due to the vasodilatory effect of increasing concentrations of estradiol, despite the fact that the increase in estradiol in serum was greater than the increase in BFV and decrease in PI, respectively [28, 36].

This is the first study in dairy cows to demonstrate the relationship between vaginal blood flow and concentrations of steroid hormones in serum during treatment with gonadotropins and PGF2α during the Ovsynch protocol. The concentrations of progesterone and cortisol in serum were correlated positively with PSV and BFV, but negatively correlated with RI and PI. These consistent changes in concentrations of steroids in serum likely account for the fluctuations in vaginal blood flow in Holstein dairy cows during the G:PG program. These
valuable results may be of economic importance at different times of the Ovsynch protocol with respect to either deciding to continue with the cows until they are inseminated or reinitiating the protocol without inseminating the cows. Accordingly, this may improve the reproductive performance of Holstein dairy cows.

Regarding the relationship between the uterine and vaginal arteries blood flow changes, the current study is considered as the first one indicating that the vaginal blood flow is correlated with the uterine blood flow changes and this may explain the difference in the conception and pregnancy rates at time of insemination. These changes are controlled in general by the concentrations of steroid hormones during different times of gonadotropin and/or prostaglandin injections during the ovsynch program in dairy cows.

**Conclusion**

The results of the current study revealed that noninvasive color Doppler ultrasound is a cost-effective tool for monitoring responses of Holstein dairy cows to hormones used in the Ovsynch protocol that may influence fertility.

**Materials and methods**

**Cows and farm management**

The Animal Care and Use Committee of the Faculty of Veterinary Medicine, Mansoura University, approved all procedures performed on the cows (M/158).

Lactating Holstein cows (n = 82) from the Dairy Unit of the Nobaria (Beheira Governorate) were enrolled between November 2021 and February 2022. Cows were housed in naturally ventilated barns with 10 rows of free stalls. Deep-bedded sand stalls, cooling fans over the feeding lane and, and sprinklers above the feed bunk for heat reduction during summer. The meal was designed to fulfill or surpass the nutritional needs of nursing Holstein cows that produce 45 kg of milk per day. All of the cows were provided with water ad libitum. All cows were managed strictly to minimize and control internal and external parasites, and they were dewormed on a regular basis. The cows were subjected to reproductive tests on a regular basis. The local veterinary authorities provided an annual vaccination system for the animals against endemic diseases such as foot and mouth disease, rift valley fever, and Pasteurella and clostridia. Cows were milked three times a day, at eight-hour intervals. The cows enrolled in the experimental procedures were about 60 ± 3 DIM.

**Study design**

The Ovsynch procedure was used to synchronize lactating nonpregnant primiparous (n = 31) and multiparous (n = 51) Holstein cows at various DIMs. Estrus was synchronized using ovsynch protocol [4]. The experimental cows received 10 μg of a GnRH analogue (Buserelin acetate: Receptal®, MSD animal health, Egypt) intramuscularly followed 7 days later with an intramuscular injection of (500 μg cloprostenol sodium; Estrumate intramuscularly; Essex, Munich, Germany), and given 10 μg, injection of the GnRH analogue i.m. (2.5ml/animal) 48h after the PGF2α treatment and the cows were bred 14-16 h then after. The ovaries were examined ultrasonographically 12, 24, and 36 h later to confirm that ovulation has occurred. Day 1 of the estrous cycle was defined as the day when the dominant follicle was no longer detectable due to it having ovulated and the pregnancy check was done by ultrasound imaging at day 33 post inseminations.

**Blood samples and hormonal assays**

After each ultrasonographic examination, blood samples from the coccygeal vein were taken. Within 1 h, serum (vacutainer tubes Plain®, Lab Supply, Egypt) was separated and frozen at -20 °C until analyzed. An established enzyme immunoassay was used to measure concentrations of P4 in serum progesterone [37]. Concentrations of P4 in serum were measured in duplicate using a commercial solid-phase, no-extraction RIA (Coat-a-count, Diagnostic Products Corp., Los Angeles, CA; ImmuChem Coated Tube, MP Biomedicals, Costa Mesa, CA). To assess the assay’s precision, control samples with high (6.0 ng/mL for) and low (0.3 ng/mL for) concentrations of P4 were analyzed. The sensitivity of the P4 assay was 0.03 ng/mL on average. The intra-assay coefficient of variation for samples with a high concentration of P4 was 10.6%, and 7.9% for low-concentration serum samples. The coefficient of variation for the samples was 4.9 percent.

**Assay for estradiol (E2) in serum**

Benzenetolune was used to extract E2 from serum and determine circulating concentrations of E2 concentrations. Duplicate samples were analyzed using a double antibody RIA. The analysis was carried out using a commercially available kit (MaiaZen Estradiol R-FA-120, Zen Tech SA, Liege, Belgium), as described previously [38]. The sensitivity of the assay was 0.3 pg/mL. A quality control sample (6.5 pg/mL E2) was included in quadruplicate. The intra-assay coefficient of variation was 16%.

**Concentrations of Cortisol in serum**

Concentrations of cortisol in serum were determined using an enzyme immunoassay described previously [39]. The intra-assay and inter-assay coefficients of variation in high (n = 6) and low cortisol pooled serum samples (n = 5) were 6.0 percent and 11.4 percent, and 4.2 percent and 8.4 percent, respectively.
Reproductive management and examinations of the reproductive tract using color Doppler ultrasonography

Cows were enrolled at the time of the first GnRH injection in the Ovsynch protocol and were healthy during clinical and gynecological examinations. All management procedures were carried out while the cows were restrained in the feed bunk by self-locking head gates. The uterine arteries of Holstein dairy cows were located and examined in accordance with a previously established research protocol [11]. The rudimentary umbilical artery, located cranial to the external iliac artery, was used to examine the uterine artery, a branch originating from the internal iliac artery (Fig. 7). The Doppler waveforms were obtained at this location by activating the pulsed Doppler function and modifying the Doppler gate over the uterine artery to fit the vessel’s diameter.

Except for the examination at the injection of second GnRH, performed between 18:00 and 20:00 p.m., all transrectal B-mode and color Doppler ultrasound examinations were performed by the same person (Heba Sharawy) between 7:00 and 11:00 a.m. All examinations were performed using the same ultrasound machine (Esaote MyLab 30X Vision, Esaote, Genova, Italy) with high-frequency linear transducers: 6–12 MHz with a filter of 100 Hz, power of 50%, pulse repetition frequency (PRF) of 4,500 Hz, and Doppler angle ranging from 0 to 40. To avoid continuous straining by the cows, an epidural anesthesia of 4 ml procaine hydrochloride (2% Procasel<sup>®</sup>; Selectavet, Weyarn-Holzolling, Germany) was administered immediately before measuring blood flow.

The time-averaged maximum velocity (TAMV), resistance index (RI), pulsatility index (PI), resistance impedance (S/D), peak velocity (PV) and blood flow volume (BFV), as well as the diameters of the uterine and vaginal arteries, were the Doppler indices that the device displayed for each waveform when using the automatic mode as previously reported [35]. B-mode images were used to measure the diameters of the uterine and vaginal arteries.

Pregnancy diagnosis

Transrectal ultrasound (TUS) was used to check for pregnancy in experimental cows 32±3 days after the first service if the cow hadn't been re-inseminated at detected estrus before. A positive pregnancy outcome was based on the amount of fluid in the uterus that was not echogenic and the size of the embryo compared to the expected stage of pregnancy. The fact that the embryo had a heartbeat was also used as proof that it was alive. The evidence of positive pregnancy was done by TUS 60±3 d after AI in all cows that were already known to be pregnant at the first examination unless the cow was left the herd.

Fig. 7 The uterine artery and vaginal artery are depicted diagrammatically to demonstrate the position for transrectal placement of an ultrasonography probe
Statistical analyses

The data were presented as means ± SEM for statistical analysis using SAS® (version 9.2, SAS Institute). The Shapiro–Wilk test was used to determine the normality of all variables’ distributions. To determine the effect of injection time on Doppler indices in uterine and vaginal arteries, as well as steroid hormone concentrations, a mixed model one-way analysis of variance was used, with time points as repeated measurements. Multiple pairwise comparisons were performed post hoc using Duncan’s error rate adjustment.

An ROC-curve is constructed using the results was used to determine the cutoff point to determine the relationship between the uterine and vaginal blood flow and the pregnancy per artificial insemination (P/AI). The experimental cows were assigned as pregnant (positive outcome) or not pregnant (negative outcome) at day 60 post insemination for the purpose of running the ROC curve analyses. The ROC analysis option of MedCalc (version 12.5.0.0; MedCalc Software BVBA) was used to create the ROC curves. Differences were considered significant at \( p \leq 0.05 \).

Abbreviations

AI: Artificial insemination; BVF: Blood flow volume; CI: Corpus luteum; CR: Conception rate; D: Diameter of the artery; DIM: Days in milk; G1: Time of first gonadotropins injection; G2: Time of second gonadotropins injection; GnRH: Gonadotropin releasing hormone; E2: Estradiol; P4: Progesterone; (P/AI): Pregnancy per artificial insemination; PGF2α: Prostaglandin F2 alpha; PI: Pulsatility index; PSV: Peak systolic velocity; PV: Peak velocity; RI: Resistance index; ROC: Receiver operating curve; S/D: Systolic / Diastolic velocity; SEM: Standard error of mean; TAI: Time-artificial insemination; TAMV: Time-averaged maximum velocity; TUS: Transrectal ultrasonography; UA: Uterine artery; VA: Vaginal artery; VWP: Voluntary waiting period.

Acknowledgements

The authors are grateful to the workers at the Dairy Unit of the Nobaria (Beheira Governorate) for their help during ultrasound imaging and blood sampling. The authors would like to thank Nagham Elsayed Elsheshtawy for help during ELISA assays.

Authors’ contributions

Heba Sharawy: Conceptualization, Methodology, Investigation, Data curation, Writing – original draft. AbdelRaouf O. Hegab: Data curation, Writing – original draft. Reviewing. Enpy F. Risha; Mohamed El-Adl; Vilia Carolina; Farag: conceptualization, Hormonal assay, writing of manuscript. Walid T. Soliman; Mohamed A. Gohar, Abdelnaser Abdallah, Mariam Rabie: Animal management and writing of the manuscript. Reham A. Fahmy; Adel Zaghoul: interpretation of Doppler data and writing of manuscript. Kazuhiro Imakawa; Fuller W. Bazer: Writing and final revision of the manuscript. Daniela James: manuscript writing and drawing the demograph for uterine and vaginal arteries in dairy cows. Mohammed A. Elmetwally: Conceptualization, Methodology, Investigation, Data curation, Statistic analyses, Writing – original draft. All authors reviewed the manuscript. The author(s) read and approved the final manuscript.

Funding

Open access funding provided by The Science, Technology & Innovation Funding Authority (STDF) in cooperation with The Egyptian Knowledge Bank (EKB).

Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

The Committee on the Ethics of Animal Experiments of the Faculty of Veterinary Medicine, Mansoura University Code No, approved the protocol M/158. All methods were carried out in accordance with relevant guidelines and regulations.

Consent for publication

Not applicable

Competing interests

The authors have declared that they have no competing interests.

Author details

1Department of Theiogenology, Center for Reproductive Biotechnology, Faculty of Veterinary Medicine, Mansoura University, Mansoura 35516, Egypt. 2Department of Clinical Pathology, Faculty of Veterinary Medicine, Mansoura University, Mansoura 35516, Egypt. 3Department of Biochemistry and Chemistry of Nutrition, Faculty of Veterinary Medicine, Mansoura University, Mansoura 35516, Egypt. 4Veterinary Department, Egyptian Armed Forces, Nasr City, Cairo, Egypt. 5Oncology center, Faculty of Medicine, Mansoura University, Mansoura 35516, Egypt. 6Laboratory of Molecular Reproduction, Research Institute of Agriculture, Tokai University, Kumamoto 862-8652, Japan. 7Department of Animal Science, Texas A&M University, College Station, Texas, USA. 8Faculty of Agricultural Sciences, University of Applied and Environmental Sciences U.D.C.A., Bogota, Colombia. 9Department of Surgery, Anesthesiology, and Radiology, Faculty of Veterinary Medicine, Mansoura University, Mansoura 35516, Egypt. 10Department of Internal Medicine, Infectious and Fish Diseases, Mansoura Veterinary Teaching Hospital, Mansoura University, Mansoura 35516, Egypt.

Received: 15 June 2022 Accepted: 9 September 2022

Published online: 17 September 2022

References

1. Giordano JO, Kalantari AS, Fricke PM, Wiltbank MC, Cabrera VE. A daily herd Markov-chain model to study the reproductive and economic impact of reproductive programs combining timed artificial insemination and estrus detection. J Dairy Sci. 2012;95(9):5442–60.
2. Miller RH, Norman HD, Kuhn MT, Clay JS, Hutchison JL. Voluntary waiting period and adoption of synchronized breeding in dairy herd improvement herds. J Dairy Sci. 2007;90(3):1594–606.
3. Giordano JO, Fricke PM, Wiltbank MC, Cabrera VE. An economic decision-making support system for selection of reproductive management programs on dairy farms. J Dairy Sci. 2011;94(12):6216–32.
4. Pursley JR, Mee MO, Wiltbank MC. Synchronization of ovulation in dairy cows using PGF2α and GnRH. Theriogenology. 1995;44(7):915–23.
5. Souza AH, Ayres H, Ferreira RM, Wiltbank MC. A new presynchronization system (Double-Ovsynch) increases fertility at first postpartum timed AI in lactating dairy cows. Theriogenology. 2008;70(2):208–15.
6. Olynik N, Wolf CA. Economic analysis of reproductive management strategies on US commercial dairy farms. J Dairy Sci. 2008;91(10):4082–91.
7. Fricke PM, Carvalho PD, Giordano JO, Valenza A, Lopes G, Amundson MC. Expression and detection of estrus in dairy cows: the role of new technologies. Animal. 2014;8(Suppl 1):134–43.
8. Fricke PM, Giordano JO, Valenza A, Lopes G, Amundson MC, Carvalho PD. Reproductive performance of lactating dairy cows managed for first service using timed artificial insemination with or without detection of estrus using an activity-monitoring system. J Dairy Sci. 2014;97(5):2771–81.
9. Stangalferro ML, Wijma RW, Giordano JO. Profitability of dairy cows submitted to the first service with the Presynch-Ovsynch or Double-Ovsynch system (Double-Ovsynch).
10. Voelbe BE, Rocha L, Scortegagna F, Stevenson JS, Mendonça LGD. Response of lactating dairy cows with or without purulent vaginal discharge to gonadotropin-releasing hormone and prostaglandin F2α. J Anim Sci. 2018;96(1):56–65.

11. Herzog K, Bollwein H. Application of Doppler ultrasonography in cattle reproduction. Reprod Domest Anim. 2007;42(Suppl 2):51–8.

12. Steer CV, Campbell S, Pampiglione JS, Kingstad CR, Mason BA, Collins WP. Transvaginal colour flow imaging of the uterine arteries during the ovarian and menstrual cycles. Hum Reprod. 1990;5(4):391–5.

13. Ford SP, Christenson RK, Chenault JR. Patterns of blood flow to the uterus and ovaries of ewes during the period of luteal regression. J Anim Sci. 1979;49(6):1510–6.

14. Ford SP, Christenson RK. Blood flow to uteri of sows during the estrous cycle and early pregnancy: local effect of the conceptus on the uterine blood supply. Biol Reprod. 1979;21(3):617–24.

15. Ford SP, Reynolds LP, Magness RR. Blood flow to the uterine and ovarian vascular beds of animals during the estrous cycle or early pregnancy. Biol Reprod. 1982;27(4):878–85.

16. Bollwein H, Maierl J, Mayer R, Stolla R. Transrectal color Doppler sonography of the A. uterina in cyclic mares. Theriogenology. 1998;49(8):1483–8.

17. Roman-Ponce H, Caton D, Thatcher WW, Lehrer R. Uterine blood flow in relation to endogenous hormones during estrous cycle and early pregnancy. Am J Physiol. 1983;245(6):R843–9.

18. Bollwein H, Meyer HH, Maierl J, Weber F, Baumgartner U, Stolla R. Transrectal Doppler sonography of uterine blood flow. Theriogenology. 2000;53(8):1541–52.

19. Honnens A, Voss C, Herzog K, Niemann H, Rath D, Bollwein H. Uterine blood flow measured by Doppler ultrasonography during the first 3 weeks of pregnancy in dairy cows. Theriogenology. 2000;53(8):1070–91.

20. Heppelmann M, Krüger L, Leidl S, Bollwein H. Transrectal Doppler sonography of uterine blood flow during the first two weeks after parturition in Simmental heifers. J Vet Sci. 2013;14(3):323–7.

21. Krueger L, Koerte J, Tsousis G, Herzog K, Flachowsky G, Bollwein H. Transrectal Doppler sonography of uterine blood flow during the first 12 weeks after parturition in healthy dairy cows. Anim Reprod Sci. 2009;114(1–3):23–31.

22. Acosta TJ, Hayashi KG, Ohtani M, Miyamoto A. Local changes in blood flow within the prevoluntary follicle wall and early corpus luteum in cows. Reproduction. 2003;125(5):759–67.

23. Acosta TJ, Yoshizawa N, Ohtani M, Miyamoto A. Local changes in blood flow within the early and midcycle corpus luteum after prostaglandin F2alpha injection in the cow. Biol Reprod. 2002;66(3):651–8.

24. Aislan S, Arslanbas D, Beindorff N, Bollwein H. Effects of induction of ovulation with GnRH or HCG on follicular and luteal blood flow in Holstein-Friesian heifers. Reprod Domest Anim. 2011;46(5):781–6.

25. Scully S, Evans ACO, Carter F, Duff P, Lonergan P, Crowe MA. Ultrasound monitoring of blood flow and echotexture of the corpus luteum and uterus during early pregnancy of beef heifers. Theriogenology. 2015;83(3):449–58.

26. Honnens A, Niemann H, Paul V, Meyer HHD, Bollwein H. Doppler sonography of the uterine arteries during a superovulatory regime in cattle. Uterine blood flow in superovulated cattle. Theriogenology. 2008;70(5):859–67.

27. Hassan M, Arshad U, Bilal M, Sattar A, Avais M, Bollwein H, et al. Luteal blood flow measured by Doppler ultrasonography during the first three weeks after artificial insemination in pregnant and non-pregnant Bos indicus dairy cows. J Reprod Dev. 2019;65(1):29–36.

28. Swanson LV, Haft HD, Morrow DA. Ovarian characteristics and serum LH, prolactin, progesterone and glucocorticoid from first estrus to breeding size in Holstein heifers. J Anim Sci. 1972;34(2):284–93.

29. Asa CS, Robinson JA, Gnthar OJ. Changes in plasma cortisol concentrations during the ovulatory cycle of the mare. J Endocrinol. 1983;99(2):329–34.

30. Greiss FC, Anderson SG. Effect of ovarian hormones on the uterine vascular bed. Am J Obstet Gynecol. 1970;107(6):829–36.

31. Bollwein H, Heppelmann M, Lütjenhaus J. Ultrasonographic doppler use for female reproduction management. Vet Clin North Am Food Anim Pract. 2016;32(1):149–64.

32. Bollwein H, Baumgartner U, Stolla R. Transrectal Doppler sonography of uterine blood flow in cows during pregnancy. Theriogenology. 2002;57(8):2053–61.

33. Elmetwally MA, Elshopeakey EG, Eldomany W, Eldesouky A, Samy A, Lenis YY, et al. Uterine, vaginal and placental blood flows increase with dynamic changes in serum metabolic parameters and oxidative stress across gestation in buffaloes. Reprod Domest Anim. 2021;56(1):142–52.

34. Tekay A, Martikainen H, Jouppila P. Blood flow changes in uterine and ovarian vasculature, and predictive value of transvaginal pulsed colour Doppler ultrasonography in an in-vitro fertilization programme. Hum Reprod. 1995;10(3):688–93.

35. Herzog K, Brockhan-Ludemann M, Kaske M, Beindorff N, Paul V, Niemann H, et al. Uterine blood flow is a more appropriate indicator for luteal function during the bovine estrous cycle than luteal size. Theriogenology. 2010;73(5):691–7.

36. Beam SW, Butler WR. Energy balance and ovarian follicle development prior to the first ovulation postpartum in dairy cows receiving three levels of dietary fat. J Biol Reprod. 1997;56(1):133–42.

37. Yoshida C, Nakao T. Response of plasma cortisol and progesterone after ACTH challenge in ovarioctomized lactating dairy cows. J Reprod Dev. 2005;51(1):99–107.