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Evaluation of endometrial vascular flow index and echogenicity following experimental induction of subclinical endometritis in dairy cows

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This study was conducted aiming to investigate impacts of experimentally induced endometritis on the vascular perfusion and echogenicity of the endometrium in dairy cows. Following estrus synchronization and applying cytological and bacteriological examinations, Holstein cows ($n=9$) were enrolled in the experiment. The endometrial blood flow and echogenicity in the middle of each uterine horn were evaluated two times a week until the subsequent estrus by power Doppler and two dimensional ultrasonography. On days 3 and 4 of the subsequent estrous cycle, 15 milliliters of a suspension containing pure culture of *Truperella pyogenes* ($8–15\times10^8$ cfu/mL) were inoculated into the body of uterus, and then endometrial blood flow and echogenicity were evaluated according to the applied procedure of the previous cycle. The provided results indicated different vascular perfusion and echogenicity among various days of normal estrous cycle, while after induction of endometritis, no significant difference observed. Comparing vascular perfusion between the normal and infectious estrous cycles also revealed significant difference in days 3, 10 and day 14 of two cycles. The differences of endometrial echogenicity between days 3 and 10 of normal and infectious estrous cycle were also significant. In conclusion, inducing endometritis significantly altered pattern of vascular perfusion and echogenicity of uterine horn during certain days of the estrous cycle.

Keywords Ultrasound, Uterine blood flow, Echogenicity, Power doppler, Endometritis

Considering, inevitable bacterial contamination of the uterine environment during the puerperal period and the prevalence of predisposing risk factors including metabolic disturbations of fresh cows during this period, the endometritis is a prevalent reproductive disorder in high-producing dairy cows. Many studies have investigated impacts of subclinical endometritis on reproductive performance of dairy cows. Based on their findings, the elevated number of polymorphonuclear neutrophils (PMNs) in endometrial samples above the normal threshold level, results in decreased pregnancy per AI, even in cows with no abnormal clinical signs. Considering this, extended calving to conception interval in the affected cows, and hence reduction of dairy economic enterprise is presumed[1–4].

Evaluation of endometrial cytology through biopsy is considered as gold standard method for diagnosis of the subclinical endometritis[5]. However, it's not a cow side and convenient test as it needs sample collection and preparation, followed by microscopy and enumeration of PMNs, which is time consuming. Lacking of precise, easy and practical cow side test can be considered as one of the major reason of unsuccessful management of subclinical endometritis in commercial dairy herds. The recent alternative methods for convenient diagnosis of subclinical endometritis including optical density assessment of uterine lavage sample (ULSOD), urinary strip test containing leukocyte esterase (LE), protein, and pH test, weredescribed[6]. Besides these new methods, plenty of researches were carried out to evaluate feasibility of diagnosing subclinical endometritis by transrectal ultrasonography. The presence of intrauterine fluids in ultrasound examination has been reported to be associated with decreased reproductive performance and increasing odds of diagnosis endometritis by cytology.

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Regardless of this initial reports, further researches revealed that, despite high specificity, the sensitivity of ultrasonography by considering intrauterine flow or assessment of endometrial thickness is low, compared to cytology or histopathology[7,8].

Considering local establishment of inflammatory condition during endometritis and subsequent hyperemia, edema and PMNs infiltration, the possibility of diagnosing subclinical endometritis by evaluating endometrial vascular perfusion and echogenicity for the purpose of increasing sensitivity of ultrasonography has rationale to be examined. Considering this, the aim of this study was to evaluate altrations of endometrial vascular perfusion and echogenicity, following induction of subclinical endometritis, through Power Doppler and real time B-mode ultrasonography. The power Doppler ultrasound, expresses the number of red blood cells flowing through the vessels per time unit. In this mode, the color maps display the integrated power of the Doppler signal rather than the mean Doppler frequency shift shown in conventional color Doppler mode. Hence, this method is based on the analysis of frequency shift followed by amplitude coding rather than the coding of the frequency shift of the reflected ultrasound[9]. Power Doppler has wider dynamic range and relative angle independence and it provides better delineation of tortuous vessels and it is more sensitive than conventional color Doppler for detecting low-flow vessels, vessels which run at unfavorable angles to the ultrasound beam, and vessel branching[10,11].

Materials and methods

This experiment was conducted at the farm animals veterinary teaching hospital of the university of Tehran, located in Mardabad, Alborz, Iran. The cyclic and clinically healthy Holstein cows ($n=13$) were primarily, enrolled in the study. The average age of studied cows was 43.6 ± 0.91 months, with parity number of 2.2 ± 0.21, and BCS of 3.55 ± 0.35 (1–5 scale). They had regular estrous cycles and average days in milk of 115 ± 21, and daily produced, 36.66 ± 1.92 L of milk. The cows were fed a mixed total daily ration, mainly silage, which is formulated by professional nutritionists according to their protein, vitamins, and energy requirements. The cows were fed three times a day with TMR, which is composed of corn silage, alfalfa, soybean meal, extruded corn, etc. to meet the minimum nutritional needs of the animals (NRC, 2001). The selected cows were synchronized by applying two consecutive injections of 500 µg of prostaglandin F2α (Cloprostenol; Vetaprost®, Aburaihan, Iran) 14 days apart. The endometrial cytology and bacteriological culture of external os of the cervix was done a day before the first PG injection, and the taken samples transported to the laboratory within 1 h. The selection criteria for ruling out of clinical or subclinical endometritis at the beginning the experiment were: the absence of purulent vaginal discharge during transrectal palpation and/or echogenic fluid in ultrasonography of uterus, negative bacteriological culture of the cervical discharge, and the endometrial cytology results ≤ 1% PMN. After ultrasonography of reproductive system and according to the provided results of cytology and bacteriological culture of the uterine lumen, eligible cows ($N=9$) were identified.

A pathogenic strain of *Trueperella pyogenes* was isolated from the uterus of a postpartum cow with clinical endometritis cultured on sheep blood agar plates (Oxoid; Hants, UK) at 37 °C for 48 h in an aerobic environment and preserved in semi-skimmed milk with 10% glycerol at − 80 °C. *T. pyogenes* was cultured from this bacterial stock on sheep blood agar plates and incubated at 37 °C for 48 h. Colonies were sub-cultured in 600 mL brain heart infusion broth (BHI; Oxoid) supplemented with 5% heat-inactivated fetal bovine serum at 37 °C for 48 h. The purity of the *T. pyogenes* broth and number of CFU/mL was determined by streaking 50 µl onto sheep blood agar plates that were incubated for 48 h. Bacterial concentration was determined by a viable plate count. For each cows, 15 mL of suspension, containing $8–15 \times 10^8$ cfu/mL *T. pyogenes* into the lumen of the uterus on days 3 and 4 of the estrous cycle using double guarded uterine catheter. Bacteriological culture of cervical discharge was done using sterile guarded swap, two days after the injection of bacterial suspension into the lumen of uterus and the swap samples transported to the laboratory within 1 h to be cultured.

Ultrasonographic endometrial blood flow and echogenicity in the middle of each horn were evaluated on days 0, 3, 7, 10, 14, 17 of estrous cycle (0 = standing heat). After induction of endometritis, endometrial blood flow and echogenicity in the middle of each horn were evaluated as previous cycle. Uterine lavage was done at the end of the experiment (on the day 20 of second estrous cycle) in order to evaluate the cytology and count the PMNs in the lumen of the uterus. Uterine lavage was conducted for endometrial cytology sampling as previously described[1,7]. Sterile saline solution (35 mL) was infused into the uterus by a 60 mL syringe attached to a 50 cm disposable plastic infusion rod. The pipette was inserted into the vagina within a protective plastic sheath, which was penetrated once the pipette was in the cranial vagina adjacent to the cervix. The pipette was then manipulated through the cervix and saline solution was infused into the uterus. The uterus was gently massaged for about 10 s, and some of the infused fluid was aspirated into the syringe via the same infusion pipette by negative pressure aspiration. Recovered fluid was transferred to a sterile plastic tube and placed on ice in a small container. Samples brought to the laboratory within 3 h and centrifuged at 700 × g for 6 min. A drop of sediment was streaked onto a clean microscopic slide. The slide was then air-dried and stained with modified Wright-Giemsa stain. Each slide was examined once by the clinician who collected the samples and once by a technician who was blinded regarding the sampling. Each examiner counted a minimum of 100 cells at 400 × magnification and a differential count (endometrial cells, PMNs, and squamous cells) was obtained to provide a quantitative assessment of endometrial inflammation. Samples must contain epithelial cells to confirm the correct site of collection. Cut off level of 5% PMNs count considered positive for diagnosis of subclinical endometritis.

The endometrial echogenicity and blood flow ultrasonography of uterine horns were performed by utilizing a real time B mode and Color power Doppler scanning, respectively (Sonosite, TITAN, USA, with 720 mW/cm2 ISPATA) equipped with a multifrequency (10 − 5 MHz) linear-array transrectal transducer (L52, TITAN 180 PLUS). Frequency of 7.5 MHz, gain mode of overall image, tissue scanning depth of 5 cm, mechanical index (MI) and soft tissue thermal index (TIS) of 0.3, was set as a default in ultrasound examinations. Constant setting was applied for B-Mode and power Doppler ultrasonography, in order to minimize variation in obtained data. All ultrasound examinations were done by the same investigator using the same parameters so as to eliminate

any inter-observer variations. Every ultrasound examination lasted for about 20 min and during examinations, while the animals were restrained in a stanchion. After locating the uterus, the transducer was placed over a cross section of each uterine horn in the vicinity of the junction of segments 2 and 3[12]. The junction is where the descending segment reaches the lower horizontal segment of a horn at larger curvature of uterus.

The images of each uterine horn were analyzed in the region of interest (ROI). The pixels were selected from images, extracted, and saved (GIF format) using Adobe Photoshop software (Adobe Systems). Image J 1.31v software (National Institutes of Health, Bethesda, MD) was used for calculation of the total number of interested pixels in each GIF-format image for evaluating echogenicity and vascular perfusion respectively. Based on brightness intensity, pixels of ultrasonographic images can be represented numerically (0-255) and its gray scale histogram can be plotted[13]. The Mean gray level (MGL), which is arithmetical average gray level of all pixels in the picture for evaluating echogenicity; and vascular flow index (VFI) for endometrial perfusion, were investigated. Vascular flow index (1-100) was assessed by combining results of vascularization and flow indices evaluation, which were defined as a ratio of color pixels and average of their color intensity. The provided data were analyzed by SPSS software version 16. According to the Kolmogorov-Smirnov normality test, and lack of normaldistribution, the Freidman test, which is non-parametric equivalent of repeated measures ANOVA, was applied. Differences with P-value < 0.05 considered significant.

Results

None of the studied cows has developed signs of clinical endometritis following treatment. The success of inducing subclinical endometritis following inoculation of *T. pyogenes*, was confirmed by the results of endometrial cytology, and increased number of PMNs from less than 1% at the beginning of the experiment to the range of 5–7% in all cases (Fig. 1). Bacteriological culture of external os was negative 3 days following inoculation of the bacteria, while the results were positive 10 days later in all cows. The mean vascular flow index (VFI) of both uterine horns was calculated by evaluating vascular index and flow index (Fig. 2). Comparing various days of normal estrus cycle showed that mean VFI was different (P value = 0.02) and VFI was significantly higher on days 7, 14 and 17 compared with days 3 and 10. (Table 1). Changes of VFI were not significant in the different days of the infected cycles (P value = 0.43).

Comparing, the mean endometrial VFIs between normal and infected cycles, showed that endometrial blood flow on days 3, 10 and 14 of the normal cycle were significantly lower compared to similar days of infected cycle. No significant changes existed among other days (Fig. 3). Evaluation of the mean uterine gray level of both uterine horns after B-Mode ultrasonography and plotting gray scale histogram (Fig. 4) showed significant difference in various days of normal estrous cycle (P value = 0.01). According to the provided results, it was significantly higher on days 7, 14 and 17 compared to day10. Conversely, changes of uterine MGL were not significant in the infected cycle (P value = 0.49) (Table 2). Comparing different days of two cycles revealed significantly higher uterine horns echogenicity on days 3 and 10 of infected cycle (Fig. 5).

Discussion

According to the previous reports, the most prevalent pathogens involved in the etiology of bovine endometritis are *T. pyogenes*, *Escherichia coli*, *Fusobacterium necrophorum*, and *Prevotella sp.*[14,15]. Moreover, the association between the presence of pathogenic bacteria like *T. pyogenes* and incidence of subclinical endometritis was reported before[1]. Presence of bacterial infection elicits detrimental effects on the endometrium, which results in subfertility or infertility of affected dairy cows[16,17]. Based on the provided results of previous studies, intrauterine infusions of *T. pyogenes* and *Escherichia coli* into postpartum beef cows under experimental conditions did not establish infection, unless peripheral plasma progesterone concentrations had started to increase[18]. Since, a mild clinical or subclinical endometritis was intended to induce in this study, *T. pyogenes* was selected for intrauterine inoculation. Moreover, to assure the success of inducing endometritis, theinoculation of bacteria was done on

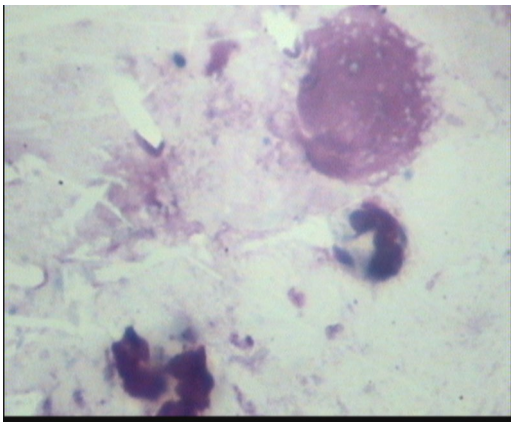

Fig. 1. Evaluating the uterine cytology by differential counting of PMNs after smear preparation and Giemsa staining (×400).

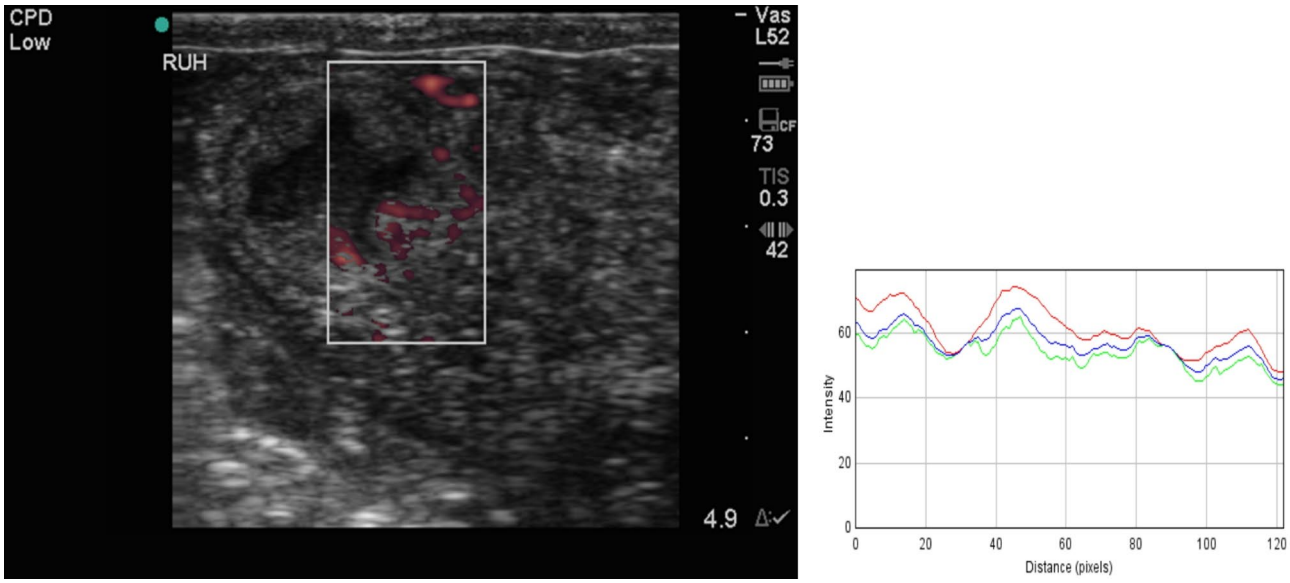

Fig. 2. Evaluation of vascular (ratio of color pixels) and flow (average color intensity of pixels) index in defined ROI by using Image j (National Institute of Health, USA) after color power Doppler ultrasonography.

	Min		Max		Mean		STD	
Day	Normal	infectious	Normal	infectious	Normal	infectious	Normal	infectious
0 (20)*	23.6	42.7	46.7	55.8	$36.44^{a, b,1}$	$49.44^{a,1}$	8.8	6.2
3	15.6	49.5	30.1	99.3	$24.52^{a,1}$	$66.34^{a,2}$	5.6	20.6
7	26.8	47.1	95.7	71.6	$49.82^{b,1}$	$59.06^{a,1}$	26.7	9.2
10	10.2	40.8	32.5	74.4	$22.12^{a,1}$	$53.6^{a,2}$	8.5	13.7
14	37.4	41.6	44	83.6	$40.36^{b,1}$	$67.94^{a,2}$	2.7	17.2
17	37.8	34.2	64.4	96.6	$46.02^{b,1}$	$60.04^{a,1}$	11.3	27.8

Table 1. VFI values during various days of normal and infectious estrous cycle. *Values of day zero of normal estrous cycle and day 20 of infectious cycle. Different letters in each columns and different numbers in each rows indicate significant difference.

day 3 and 4 of estrous cycle when the progesterone starts to increase, and according the provided results, all 9 cows developed subclinical endometritis.

The Doppler ultrasound was utilized in gynecologic studies in order to assess uterine involution by evaluating uterine blood flow, monitor fetal growth, and evaluate risk of pregnancy loss[19-21]. Inspirited from these medical researches, the application of color Doppler ultrasonography in cattle reproduction for clinical examination of ovaries and uterus during different phases of estrous cycle[22–24], pregnancy diagnosis[25–27], and puerperal period[28] is emerging[29–31]. The efficacy of power Doppler ultrasonography in diagnosing pelvic inflammatory disease and increasing success rate of IVF following follicular assessment was reported previous gynecologic studies[32,33]. Regardless of these medical reports, according to the best of our knowledge, the application of power Doppler ultrasound in studying cattle reproduction has not been reported yet, and for the first time it was used in this study for evaluation of vascular perfusion of endometrium following induction of endometritis.

According to the provided results, endometrial VFI significantly changed during estrous cycle and it was lower on days 3, and 10. The color Doppler ultrasonography of uterine arteries in previous researches, revealed significant changes of blood flow indices (Resistance and pulssatility indices) during estrous cycle, and according to reported results, lowest blood flow volume occurs after estrus and on day 10 of cycle[34]. Alteration in estradiol: progesterone ratio due to ovarian dynamics was considered the possible cause[35,36]. The estradiol, elicits vasodilatory effects in the reproductive system and increase blood flow due to induce of nitric oxide synthesis and decrease calcium uptake of potential sensory channels, through binding to its receptors in tunica media, while progesterone suppresses theses vasodilatory effect[37,38]. Evaluating VFI, through power Doppler ultrasonography in this study, demonstrated that local vascular perfusion of endometrium followed exactly the same pattern, and lowest values of VFI occurred on days 3 and 10 of estrous cycle, following decrease of estradiol level as a consequence of ovulation and follicular atresia, respectively.

It was reported that endometrial expression of pro-inflammatory transcriptomes including IL-1, IL-6, IL-8, IL-17 A, TNFα[39,40] and different prostaglandin synthase enzymes like L-PGDS, PGES, PGHS2 [41,42], together with inducible nitric oxide synthetase (iNOS), significantly upregulated in cows with subclinical endometritis[43].

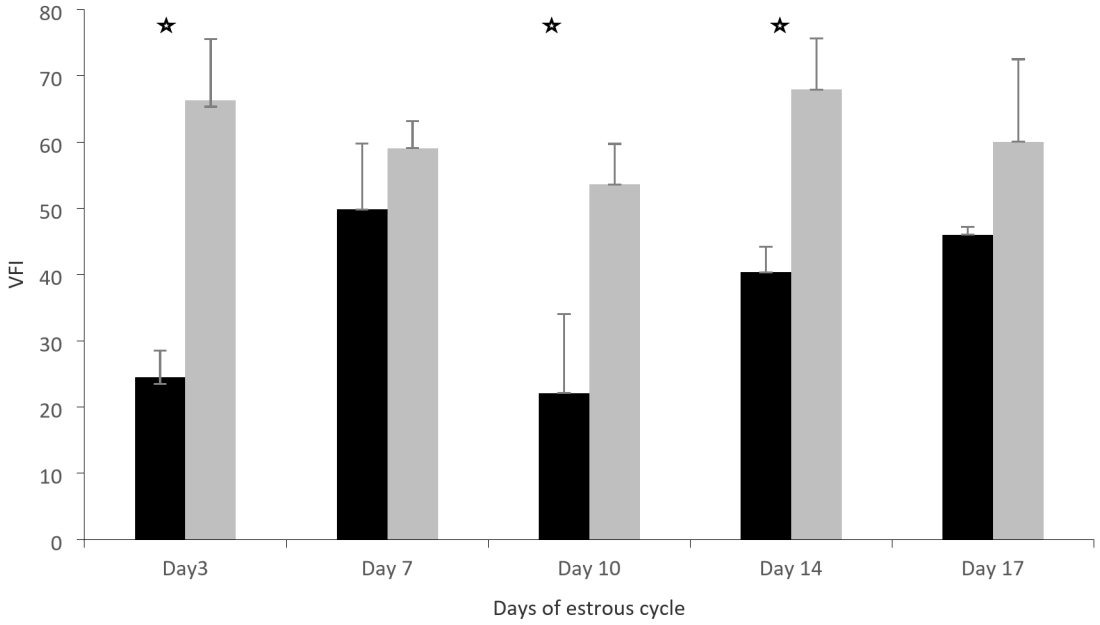

Fig. 3. Meanvascular flow index on different days of normal (black bars; $n = 9$) and infectious (gray bars; $n = 9$) cycle. VFI was significantly lower on days 3, 10 and 14 of normal estrous cycle compared to infectious cycle.

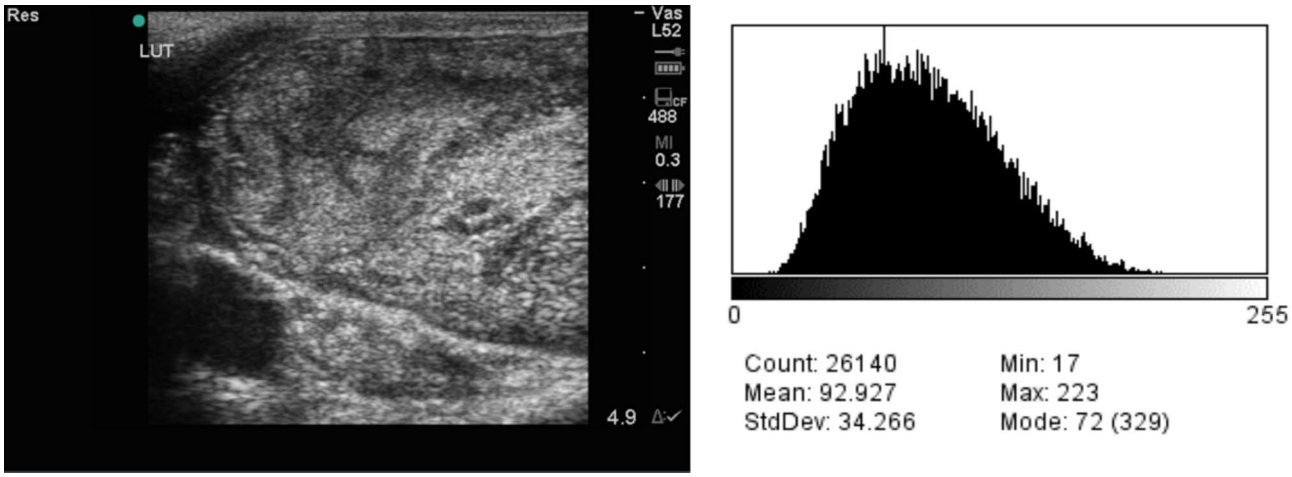

Fig. 4. B-Mode ultrasonography of uterine horn and evaluating mean gray level (MGL) by using gray scale histogram on day 14 of infectious cycle.

	Min		Max		Mean		STD	
Day	Normal	infectious	Normal	infectious	Normal	infectious	Normal	infectious
0 (20)*	37.7	46.5	61.3	66.5	$42.9^{a,1}$	$56.1^{a,1}$	11.1	8.05
3	32.3	56.5	53.5	91.6	$41.2^{a,1}$	$74.1^{a,2}$	9.4	15.6
7	42.5	48.1	84.4	62.7	$59.9^{b,1}$	$55.02^{a,1}$	16.2	5.9
10	25.7	39	45.7	69.1	32.8^{a}	$53.5^{a,2}$	7.8	14.1
14	47.2	55.1	79.8	94.7	$59.9^{b,1}$	$70.1^{a,1}$	15.8	14.6
17	52.9	50.7	81.7	69	$61.7^{b,1}$	$60.8^{a,1}$	12.1	6.9

Table 2. Uterine Horn MGL values during various days of normal and infectious estrous cycle. *Values of day zero of normal estrous cycle and day 20 of infectious cycle. Different letters in each columns and different numbers inn each rows indicate significant difference.

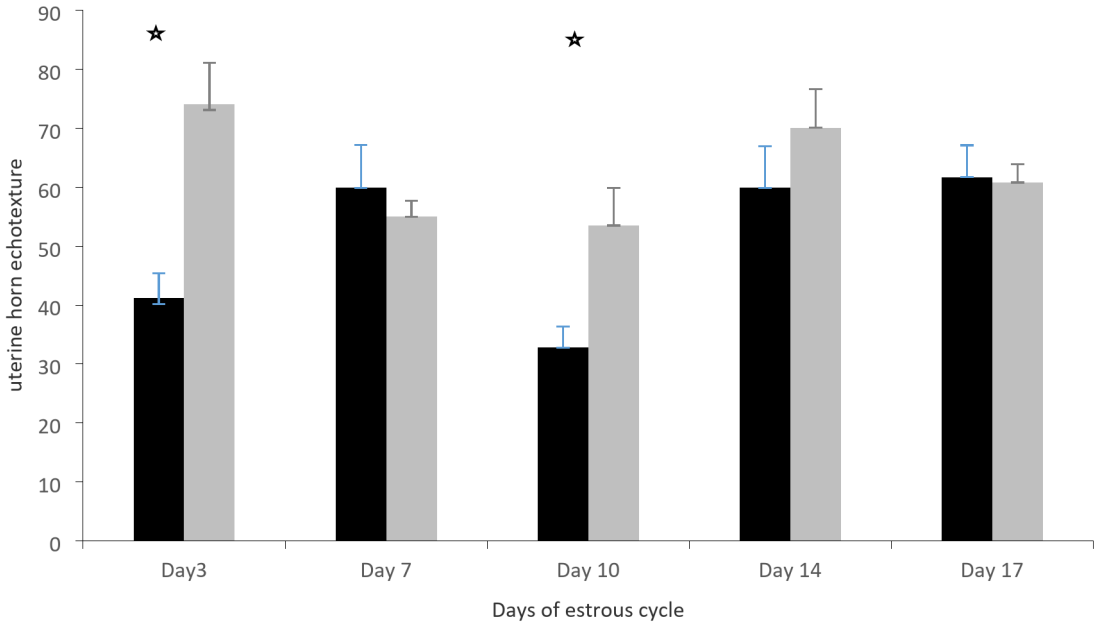

Fig. 5. Mean uterine horn echogenicity on different days of normal (black bars; $n = 9$) and infectious (gray bars; $n = 9$) cycle. Echogenicity was significantly higher on days 3 and 10 of infectious estrous cycle compare to normal cycle.

The effects of nitric oxide and upregulated prostaglandins result in vasodilation. Considering this, increasing vascular perfusion following subclinical endometritis is expected[44]. The extent of local vascular perfusion within tissues can be estimated in real time by color Doppler ultrasonography in the color-flow mode and can be quantified by the percentage of a given tissue with colored pixels and their intensity.

The effects of uterine diseases on uterine blood flow volume were evaluating previously and based on provided results, the blood flow volume and pulsatility index (PI) are influenced by puerperal uterine disease, and affected cows had significantly greater blood flow volume and lower PI on days 8 after calving[45,46]. Evaluating different blood flow characteristics including time average maximum velocity (TAMV), blood flow volume (BFV) and PI by color Doppler ultrasonography also showed significant changes of these parameters, from 1 h after treatment to up to 10 days later after induction of endometritis by intrauterine infusion of 720 mg polycresulen[47]. According to the result of this study, induction of subclinical endometritis significantly raised local vascular perfusion of the endometrium on days 3, 10 and 14 of estrous cycle. Presence of significant differences in certain days of two cycles, might be due to low vascular perfusion of endometrium on these days in normal condition due to low estradiol level. After induction of subclinical endometritis, significant difference of endometrial VFI in various days of normal estrous cycle vanished, and this can be explained by compensating effect of inflammatory mediators which prevented decrease of vascular perfusion after progression of luteal phase, due to presumed falling of estradiol level.

Considering, the uterine echotextutre and MGL analysis, changes of this parameter during estrous cycle has been reported, and based on provided results of previous studies, the endometrial echogenicity decreases until day 4 and then increases again after day 16 [48]. The results of this study showed that endometrial echogenicity was significantly higher on days 7, 14 and 17 compared to day10, which is similar to previous described pattern. Changes of endometrial echogenicity possibly occur due to fluctuations of estradiol level and its effect on endometrial edema and inflammation which can leads to increased MGL. No significant changes in endometrial echogenicity was seen in infected cycle, which might have occurred as a result of lacking reduction of uterine echogenicity due to endometritis. Different parameters including contrast, homogeneity, and MGL to evaluate changes of the endometrial echogenicity in cows with endometritis, can be investigated. It was reported that gray scale analysis (GSA) can be useful for monitoring uterine physiological changes[49].

According to results from evaluating endometrial echogenicity variables in postpartum cows with subclinical endometritis, significant linear decrease in homogeneity and linear increase in contrast occurs by increasing uterine inflammatory cells density, and these parameters have fair sensitivity and specificity for diagnosing SCE respectively. Despite of these two items, MGL remains relatively constant and does not alter following cellular infiltration density[50]. In contrast with this report findings, the results of present study showed that MGL significantly alter at least in particular days of estrous cycle following induction of subclinical endometritis. Inconsistency between these findings may arise from different design of two studies. Despite of different subjects, current result is in accordance with findings of another conducted research which reported significant changing of echogenecity and increasing MGL due to endometritis by comparing of uterine echogenicity parameters in different level of clinical endometritis and control group[51]. Significant increase of MGL occurred on days 3 and 10, which might reflect interfering effect of estradiol on this item.

In conclusion, the changing pattern of endometrial VFI and MGL is similar during estrous cycle and were significantly lower during early and mid-luteal phase. Moreover, both items significantly altered after induction of subclinical endometritis in mentioned time period. It seems that endometrial blood flow was apparently more diagnostic than endometrial echogenicity in subclinical endometritis, because the endometrial blood flow showed significant changes on days 3, 10 and 14 but the endometrial echogenicity showed significant changes only, on day 3 and 10 of the estrous cycles. Why, the alterations of endometrial vascular perfusion and echogenicity in certain days of the mid-luteal phase, were significantly different between the two cycles? Although endocrinologic assays were not included in this study, based on ultrasonographic observations of ovarian structures and their presumed hormonal secretions, it can be hypothesized that, subsiding estradiol level in the early and mid-luteal phase, and rising of progesterone as the dominant secreted hormone of the observed corpus luteum in the luteal phase, removed the interfering effect of estradiol, and hence difference between normal and infected cycles became detectable. In other words, in the late luteal phase, when the estradiol level starts to increase, the vascularity of endometrium in the normal and endometritis induced cows were less differed. More studies in larger scale, and with explained details about possible hormonal profile impacts, are needed to evaluate sensitivity and specificity of power Doppler ultrasonography in detectingthe subclinical endometritis, during various days of estrous cycle.

Data availability

Data is provided within the manuscript.

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Author contributions
Hamid Ghasemzadeh-Nava designed the study, clinical examinations was done by Majid Masoudifard, conducting of the study and data providing was done by Marzieh-Shafiee-Tabar, writing of the paper and statistical analysis was done by Maziar Kaveh Baghbadorani.

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Declarations

Competing interests
The authors declare no competing interests.

Ethical approval
Animal welfare conditions and all methods in this study were conducted in accordance with relevant guidelines and regulations and ARRIVE guidelines, based on the code of ethics of the faculty of veterinary medicine of the University of Tehran (IR.UT.VETMED.REC).

Consent for publication
This study was conducted after receiving the informed consent document for involving their owned animals in the study; approval for this study was obtained from the ethics committee of the Mardabad veterinary teaching hospital.

Additional information

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