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Endometrial echotexture variables in postpartum cows with subclinical endometritis

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ABSTRACT

The aim of this study was to evaluate endometrial echotexture changes on ultrasonographic digital images during subclinical endometritis using a computer-assisted image analysis program. Endometrial samples were collected from 140 Brown Swiss cows (days in milk = 35 ± 3) using a cytobrush method and classified as having a non-inflamed uterus ($n = 66$) and uterus with acute ($n = 42$), subacute ($n = 21$), and chronic ($n = 11$) inflammations. The mean cellular infiltration density was 0%, 31 ± 5%, 37 ± 6%, and 16 ± 8% for cows with non-inflamed uterus and cows with acute, subacute, and chronic uterine inflammations ($P < 0.0001$). As the cell infiltration density increased, both cervical diameter and mean gray level did not change. There were a liner decrease in homogeneity and a linear increase in contrast in response to increased cellular infiltration density. The sensitivity and specificity were 79.73% and 46.97% for the homogeneity value and 59.46% and 69.70% for the contrast value, respectively. In conclusion, monitoring endometrial echotexture alterations, especially homogeneity and contrast, changed depending on the cellular density and inflammation status and may be potential diagnostic markers for subclinical endometritis in cows.

1. Introduction

Real time B-mode ultrasonography has been one of the most commonly used diagnostic tools in farm animal reproduction since it was first used in 1980 in mares (Palmer and Driancourt, 1980), which later became available for cows (Pierson and Ginther, 1984; Reeves et al., 1984). Ultrasonography is an important routine tool for use in veterinary medicine to assess physiological and pathological changes in the reproductive system, which mainly covers detection of pregnancy at early stages, follicular dynamics in ovaries, and prediction of ovulation time as well as diagnosis of ovarian cysts, infections and tumors (Ginther, 2014). Contrast-enhanced and color Doppler ultrasonography, multi-dimensional ultrasonography, and computer-assisted image analysis (CAIA) are some of the advancements in ultrasonic imaging available to veterinary medicine (Nakamura et al., 2009; Morel et al., 2010; Cengiz et al., 2014).

Computer-assisted image analysis, a computer algorithm, is termed "echotexture analysis" and used in human (Chen et al., 2008) and veterinary (Arashiro et al., 2010) medicine. A two-dimensional ultrasonographic image is a matrix of square picture elements (pixels), which vary in grayscale values ranging from 0 (absolute black) to 255 (absolute White; Singh et al., 1997). This

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methodology permits a quantitative assessment of the density of each pixel present on an image. Thus, minor alterations on digital images of the tissues that cannot be distinguished by the human eye can be detected in numerical values (Siqueira et al., 2009), eliminating subjectivity in visual evaluation. The mean grey level (MGL), homogeneity (HOM), and contrast (CON) are major variables of the digital images to make inferences about tissue echotexture (Kucukaslan et al., 2014).

Endometritis is an inflammation, which is limited to endometrium and occurs at least 21 days after calving without exhibiting systemic signs of the condition (Gilbert et al., 2005). Clinical endometritis can be diagnosed according to symptoms such as abnormal vaginal discharge (a fetid watery red-brown or mucoprulent), uterine and cervical enlargement, whereas subclinical cases need specific examinations employing ultrasonography, cytology, or biopsy (Sheldon et al., 2006; Barlund et al., 2008). Although real time ultrasonography has been extensively used in animal reproduction (Ginther, 2014), it is inadequate to differentiate healthy and subclinical uterine inflammations (Barlund et al., 2008). In ultrasonography, increases in fluid accumulation and endometrial thickness are typical findings (Pierson and Ginther, 1987; Kähn, 2004). However, sensitivity and specificity of diagnosing subclinical endometritis by using ultrasonography is lower than cytology examinations. Endometrial cytology is thus preferred for the diagnosis, although it is an invasive procedure (Barlund et al., 2008). This experiment was conducted to evaluate variations in echotexture variables during subclinical endometritis using the CAIA method, a quantitative and objective assessment.

2. Materials and methods

2.1. Cows

The study was conducted on 140 (parity 1, $n = 22$; parity 2, $n = 37$; parity 3, $n = 29$; and parity ≥ 4, $n = 52$) Brown Swiss cows housed in Atatürk University Research Farm (39°54'E; 41°13'N; altitude of 1980 m). The cows that had no clinical and systemic signs of metritis (e.g., abnormal vaginal discharge, apathy, anorexia, and fever) in early postpartum (d < 21) and that had no clinical endometritis diagnosis (e.g., abnormal vaginal discharge in vaginoscopy and enlarged uterine horn in rectal palpation) on d 35 ± 3 postpartum were used in the study (Sheldon et al., 2006).

2.2. Endometrial sampling

The endometrial samples were collected using endocervical brushes (Plasti-med®, Istanbul, Turkey) as described by Kasimanickam et al. (2004). The brush was joined to another brush for passage through the cervix. A stainless steel catheter, which was placed into a plastic artificial insemination cover was used as the sampling instrument. The instrument was placed in a clean rectal palpation sleeve for protection from the vaginal and perianal contamination. Briefly, after cleaning the external vulvar area, the instrument was placed into the vagina. When the instrument reached to the external orificium of the cervix, the sleeve was punctured and the instrument was advanced through the cervix. The steel catheter was then retracted, while advancing the cytobrush through the plastic cover until it reached the endometrium. The uterus was slightly massaged while the cytobrush was twisting two or three times in a clockwise direction. Retraction of the cytobrush into the plastic cover was subsequently performed and the instrument was removed from the uterus. The smear was expelled onto a glass slide and fixed with methanol about 5 min before staining. The same researcher performed all endometrial samplings.

2.3. Cytological examination and classification of endometritis

The fixed samples were stained using Giemsa and examined under light microscopy. Postpartum subclinical endometritis was diagnosed based on the density of inflammatory cells (>5%) with the cytological examination (Oral et al., 2009). The samples were also subjected to the cytopathological classification based on presence of polymorpho-nuclear cells (PMN) and lymphocytes (LYM) to define inflammatory status (Schlafer and Miller, 2007). The samples with ≥5% PMN, 5% PMN + LYM, and 5% LYM were considered acute, sub-acute, and chronic, respectively.

2.4. Handling of digital images and measuring of cervical diameter

Digital images of the endometrium were collected by transrectal ultrasonography using a 7.5 MHz linear-array transducer connected to a portable B-mode ultrasonic scanner (Agroscan AL®, Noveko International Inc., Angoulême, France) as described previously (Kähn, 2004). The same machine setting standardized [i.e., Mode (B), depth (8 cm), MHz (7.5), focus zone, B-gain, B-brightness] was used throughout the study. To improve accuracy and prevision, at least three circular cross-sections of the uterus of each cow were recorded. The images from the point of larger curvature of uterus were recorded using a digital recorder (PMP-100®, Sigmatek, France). In addition to endometrial images, cervical diameters were also measured by the transrectal ultrasonography. The same researcher performed ultrasonography throughout the study to eliminate person-to-person variation.

2.5. Echotexture analysis

The outline of the echotexture analyses of the endometrium was previously described Cengiz et al. (2014). Briefly, the images saved in the non-compressed bitmap file format were analyzed using a custom-developed software (BS200 Pro® Image Processing and Analysis Software, BAB, Ankara, Turkey). Four regions of interests (ROI, 20×20 pixels) were identified on each image for MGL, HOM, and CON variables. The ROI areas were selected only for endometrial layer to avoid results being confounded by unintended evaluation of the myometrium and luminal fluid (Fig. 1).

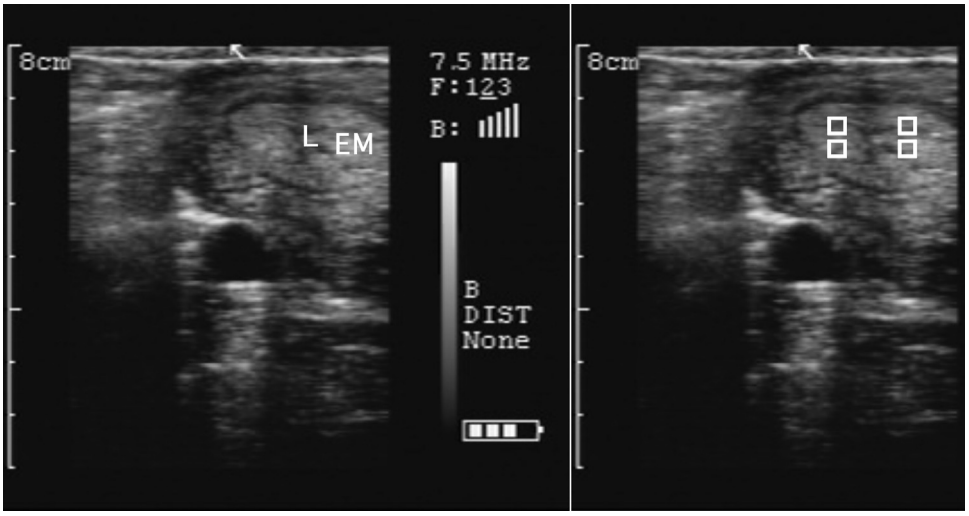

Fig. 1. Circular cross-section image of the uterus. L: Uterine lumen. EM: Endometrium. Squares represent the Region of Interests.

2.6. Statistical analysis

Analysis of variance was computed to determine if cellular infiltration varied with inflammatory category. The group mean differences were attained using the LSD lines option. The cellular infiltration (%) was regressed on cervical diameter and echotexture variables to elucidate associations using the REG Procedure. The receiver operating characteristics (ROC) curves for echotexture variables and inflammatory categories were established to compare the sensitivity (ability to detect uterine inflammation), specificity (ability to avoid misclassifying non-inflamed uterus as uterus with inflammation), positive likelihood ratio (low specificity or how much the odds of the uterine inflammation increase when a test is positive), negative likelihood ratio (low sensitivity or how much the odds of the uterine inflammation decreases when a test is negative), positive predictive value (the probability that a uterus with inflammation with a positive test result actually has the condition for which the test was conducted), and negative predictive value (the probability that a uterus with inflammation with a negative test result is actually free of the condition for which the test was conducted). Statistical significance was declared at $P < 0.05$ for all analyses (IBM SPSS Statistics version 20, IBM Corp., Released 2011, Armonk, NY).

3. Results

The mean cellular infiltration density was 0, 31 ± 5, 37 ± 6, and 16 ± 8% for cows with non-inflamed uterus ($n = 66$) and cows with acute ($n = 42$), subacute ($n = 21$), and chronic ($n = 11$) uterine inflammations (mean $\pm$ SE), which was in parabolic pattern ($P < 0.0001$; data not shown). Considering cytopathological classifications, cervical diameter and MGL remained unchanged. There were downward and upward parabolic patterns in HOM ($P < 0.0006$) and CON ($P < 0.0001$) in uterine tissues classified as non-inflamed and those with acute, subacute, and chronic inflammations, respectively (Fig. 2).

Fig. 3 depicts association of the cell infiltration density for cervical diameter and echotexture variables. There were no alterations in cervical diameter and MGL as the cellular infiltration density increased (Fig. 3). Despite low R^2, decreases in HOM ($P < 0.01$; $R^2 = 0.05$) and increases in CON ($P < 0.007$ occurred; $R^2 = 0.05$) as the cellular infiltration density increased.

The ROC curve analysis was done only for HOM and CON because of the significant association with the cellular infiltration density (Fig. 4). The HOM value indicated a greater sensitivity (79.73% compared with 59.46%), whereas the CON value indicated greater specificity (69.70% compared with 46.97%) for assessing uterine inflammation. Specificity and sensitivity evaluations resulted in similar true/false likelihood ratios (Table 1).

4. Discussion

Endometrial biopsy and histopathology were found to be the most reliable methods for diagnosis of subclinical endometritis and assessing postpartum uterine function and health (Bonnett et al., 1993; Chapwanya et al., 2010). However, endometrial biopsy was avoided due to being an invasive approach and possible negative effects on future fertility in cows where the procedure was performed (Etherington et al., 1988). Thus, endometrial cytology (cytobrush or endometrial lavage) is a preferable method (Barlund et al., 2008) in the diagnosis despite its application difficulties in field conditions. In previous studies, cut-off values based on percentage of PMN cells were used to define subclinical endometritis according to days in milk (DIM). Kasimanickam et al. (2004) suggested >18% and >10% of PMN as a diagnostic criteria for 20 to 33 and 34 to 47 DIM cows, respectively. However, some other studies suggested >6% to >8% PMN and >4% to >5% PMN as threshold values for 28–41 d and 40–60 d after parturition in the cows without clinical signs of endometritis (Gilbert

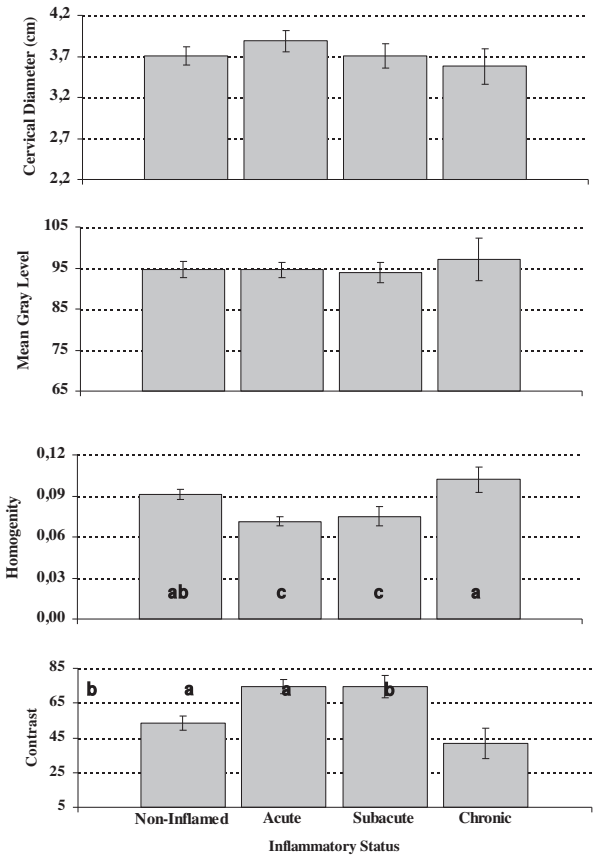

Fig. 2. Cervical diameter and echotexture variables of the uterus based on the cytopathological classification. The mean cell density was 0%, 31 ± 5%, 37 ± 6%, and 16 ± 8% for cows with a non-inflamed uterus ($n = 66$) and cows with acute ($n = 42$), subacute ($n = 21$), and chronic ($n = 11$) uterine inflammations (mean ± SE). Probability of significance was 0.58, 0.94, 0.0006, and 0.0001 for cervical diameter, mean gray level, homogeneity, and contrast, respectively. Bars with different superscripts differ ($P < 0.05$).

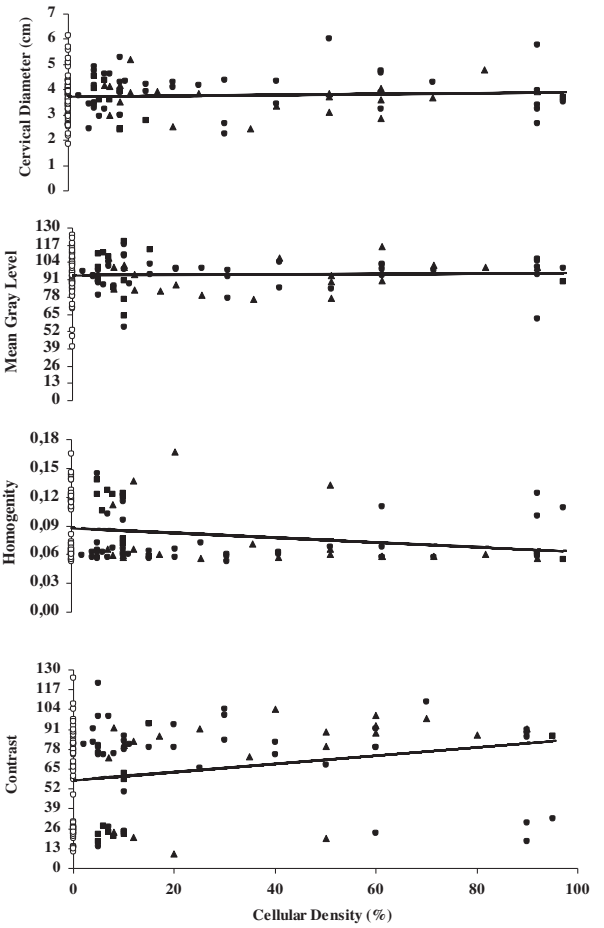

Fig. 3. Alterations in cervical diameter (cm) and endometrial echotexture variables based on the inflammatory cell density (%) by the cytopathological classification (○, non-inflamed, $n = 66$; ●, acute inflammation, $n = 42$; ▲, subacute inflammation, $n = 21$; ■, chronic inflammation, $n = 11$). The best-fit linear regression equations were Cervical Diameter = 3.72 + 0.002*Cellular Density ($P < 0.49$; $R^2 = 0.03$), Mean Gray Level = 94.54 + 0.011 × Cellular Density ($P < 0.80$; $R^2 = 0.0004$), Homogenity = 0.09 − 0.0003 × Cellular Density ($P < 0.01$; $R^2 = 0.05$), and Contrast = 57.75 + 0.27*Cellular Density ($P < 0.007$; $R^2 = 0.05$).

et al., 2005; Barlund et al., 2008; Galvao et al., 2009). In the presented study, both PMN and LYM were taken into the consideration when the samples were classified by cytological categorization because the predominant cell type was variable on the slides.

Ultrasonography findings such as increased amount of fluid accumulation in the uterine lumen (Kähn, 2004), increase in uterine horn and cervical diameter, and echotextural changes (Griffin and Ginther, 1992; Mateus et al., 2002) are associated with bacterial growth and delayed uterine involution after calving (Mateus et al., 2002; Drillich et al., 2005). Mateus et al. (2002) also reported that an increase in uterine diameter was obvious only with severe endometritis rather than mild endometritis. In the same study, similar uterine diameters were observed in the cows with a healthy uterus and mild endometritis cases 5 weeks after calving. Consistent with findings of Mateus et al. (2002), the mean cervical diameter in cows with an inflamed (both in cellular density and inflammatory status) and healthy uterus was similar to those found in the present

Table 1
Receiver operating curve parameters for endometrial echotexture in assessing uterine inflammation diagnosed by the cytopathological examination.[a]

Parameter	Criterion[c]	Sensitivity		Specificity		True/false[b]		Area under curve				
		Value	95% CI	Value	95% CI	+LR	–LR	Mean	SE	95% CI	*z*	*P*<
Homogenity	≤0.1061	79.73	68.8–88.2	46.97	34.6–59.7	1.50	0.43	0.65	0.05	0.56–0,72	3.10	0.002
Contrast	>77.8987	59.46	47.4–70.7	69.70	57.1–80.4	1.96	0.58	0.65	0.05	0.536–0.72	3.09	0.002

[a] Non-inflamed, $n = 66$ (47.1%); inflamed, $n = 74$, (82.9).
[b] LR = likelihood ratio.
[c] Corresponding with greatest Youden index (0.267 for homogeneity and 0.292 for contrast).

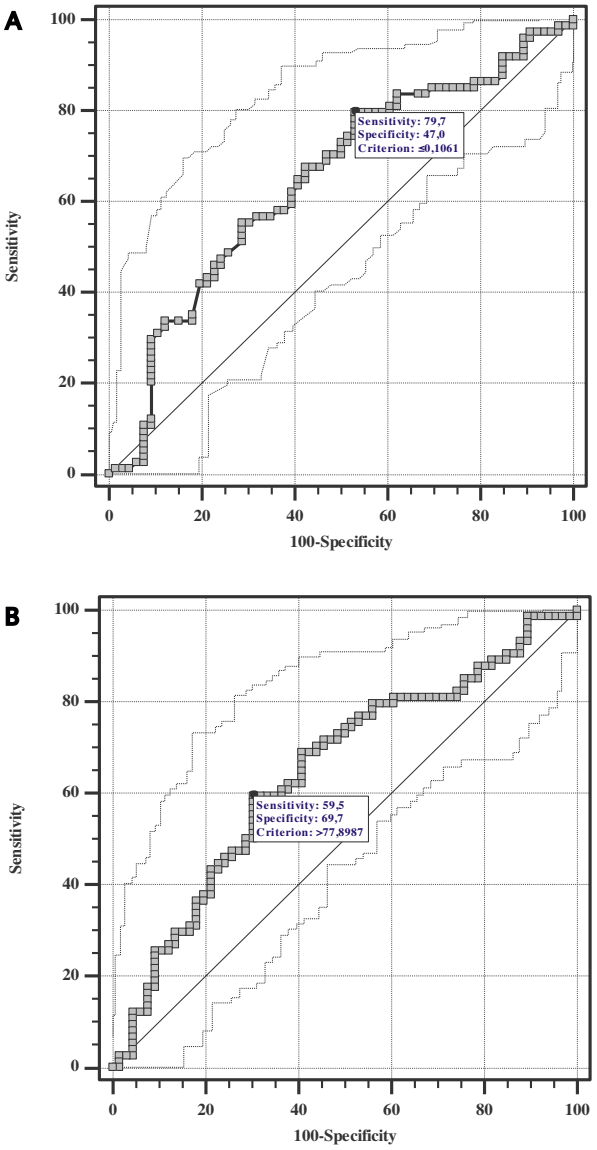

Fig. 4. Specificity and sensitivity of homogeneity (Panel A) and contrast (Panel B) in determining uterine inflammatory status (non-inflamed compared with inflamed).

study (Fig. 2). Cows with mild puerperal endometritis can recover spontaneously (Hoedemaker, 1998) without significant morphological changes (Mateus et al., 2002). Fluid accumulation and endometrial edema can also be observed when cows are in estrus (Barlund et al., 2008). Moreover, involution of uterus and cervix is not complete until 40–50 days postpartum (Sheldon et al., 2006); therefore, this criterion may not be reliable in diagnosis of subclinical endometritis in postpartum cows. Breed, age, parity and other factors could affect uterine and cervical involution (Sheldon et al., 2006) that did not influence findings in previous studies and thus were not evaluated in the present study. Alterations in endometrial thickness have also been considered for detection of subclinical endometritis by Barlund et al. (2008). However, assessments using this criterion could easily be influenced by location and positioning of probe.

While the HOM and CON values were influenced by acute and sub-acute inflammations, the values were similar to those for cows with a non-inflamed endometrium and chronic endometritis (Fig. 2). These findings might have been influenced by endometrial edema in these cows, which usually is present in cows with acute and subacute endometritis. As a result, differentiation of cows with a chronically inflamed and healthy uterus may not be possible by using the CAIA methodology.

The knowledge about ultrasonography in cows with subclinical endometritis is still inconsistent. Distinguishing pathological changes in the endometrium by real time ultrasonography is difficult in cows with subclinical inflammations. This shortfall in use of ultrasonography may be due to the inability to distinguish differences with the human eye. In recent years, some studies focused on quantitative echotextural changes in the endometrium during physiological states such as the sexual cycle, ovulation, luteolysis, and embryonic implantation in various animal species (Schmauder et al., 2008; Kauffold et al., 2010; Cengiz et al., 2014). In these studies, the changes were defined by quantitative values using the CAIA methodology. Kucukaslan et al. (2014) adapted this methodology in cattle to study endometritis and responsiveness to treatment. In the same study, the HOM value, representing the uniformity of pixel pair distributions in the tissue image decreased when severity of endometritis increased. Normally, an increase in HOM value indicates that there are few grey value combinations in the image, but all the value combinations are equally distributed (Raeth et al., 1985). In the present study, similar results were achieved for HOM and CON values, which decreased and increased with cellular infiltration density (Fig. 3). This could be related to disruption of the endometrial epithelium, infiltration and accumulations of inflammatory cells, vascular congestion and stromal edema (Bonnett et al., 1991; BonDurant, 1999; LeBlanc, 2008), suggesting that cytological changes might influence the distribution of gray tones rather than density in a ROI. However, low R^2 values for regression equations fitting the relationships of HOM and CON with the cellular infiltration density suggest consideration of other factors not taken into account in the present study. Although the MGL value was reported to be an indicator defining physiological changes (Kauffold et al., 2010; Cengiz et al., 2014), this value remained relatively constant depending upon the inflammatory status (Fig. 3) and with an increase in the cellular infiltration density (Fig. 3).

Ultrasonography is not a reliable method for detection of subclinical endometritis alone (Sheldon et al., 2006). Additionally, this technology has not been established in veterinary practice for routine diagnosis of chronic endometritis (Drillich et al., 2005). Evaluation of echotexture variables in veterinary theriogenology is a developing practice. To our knowledge there have been no studies to assess reliability of echotexture variables in assessment of uterine inflammation status. The present study revealed HOM had a greater sensitivity than CON for these assessments, whereas use of CON resulted in a greater

specificity than HOM in determination of uterine inflammation (Table 1; Fig. 4).

5. Conclusion

Ultrasonography is the most preferred diagnosis method in animal reproduction due to its feasible and non-invasive nature. In the present study, a novel assisted methodology was applied to ultrasonography and evaluated together with cytological results. The CAIA methodology is a potential diagnostic method in detection of subclinical endometritis as reflected by the negative association of HOM and positive association of CON with cellular infiltration density, while both having moderate sensitivity and specificity to confirm the uterine subclinical inflammation status. Future studies considering more fertility variables along with echotexture variables to diagnose endometritis are needed.

Conflict of interest

The authors declare that there are no conflicts of interest.

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