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RESEARCH ARTICLE

Subjective Scoring Method for Evaluation of Uterine Echotexture to Detect Early Pregnancy in Holstein Cows and Heifers

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ABSTRACT

In the study, it was aimed to develop a scoring scale, graded from 1 to 5, to identify different gray tones of the ultrasonographic images of uterus, and the pregnancy determination in cows was tried to make by the scoring technique, in the first 20 days after artificial insemination. Sixty Holstein cows (n:30) and heifers (n:30) were enrolled in the study. For each animal, two ultrasonographic images were taken (totally 1320 images) starting from the day of the artificial insemination (D0) until the 20th day (D20) on every other day. At the same time that images were taken, subjective scoring were made. Then, the images were evaluated to determine mean gray value (MGV) by ImageJ (ImageJ 1.53k; NIH, ABD) software. The obtained data at the end of the evaluations were analyzed in terms of age of animals (cow or heifer), the days of examinations (alternate days between D0 and D20) and the 28th day pregnancy status (pregnant or non-pregnant). Also score rates were given by one experienced and one inexperienced independent operators to determine scoring agreement level in terms of experience on the ultrasonographic examinations. The mean MGV values indicated statistically significant differences only between days of sampling ($p<0.05$). On the other hand, according to other main effects and interactions were not concluded with statistically significant differences ($p>0.05$). It has been determined that experienced (Rater 1) and inexperienced (Rater 2) raters have agreement ($p<0.001$) in poor level ($\kappa=0.15$). Moreover, scores of the Rater 1 (0.720) were observed that had higher level correlations than Rater 2 (0,541). The gray tone scoring analysis were concluded that the mean of gray tone scoring were not differed significantly in terms of means and two-way interactions between sampling time and pregnancy status ($p>0.05$). In conclusion, it has been observed that the 256 different shades of gray which is analyzed by computer assisted image analysis could be identified by human eye in a 5 graded scale. However, the endometrium of the ovulation side uterine horn reflects the same differentiation in the presence or absence of pregnancy, in the first 20 days.

Keywords: Cow, Echotexture, Heifer, Pregnancy, Scoring, Ultrasound image

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Holstein Irkı İnek ve Düvelerde Erken Gebeliğin Belirlenmesinde Uterus Ekotekstürünün Değerlendirilmesi için Subjektif Skorlama Yöntemi

ÖZ

Çalışmada uterusun ultrasonografik görüntülerinin farklı gri tonlarını belirlemek için 1 ile 5 arasında derecelendirilen bir skorlama skalası geliştirilmesi amaçlanmış ve çalışmada ineklerde tohumlama sonrası ilk 20 gün içinde bu skorlama yöntemi ile gebelik tespitinin gerçekleştirilmesi araştırılmıştır. Çalışmada 60 adet Holstein inek (n:30) ve düve (n:30) kullanıldı. Her bir hayvandan suni tohumlamanın yapıldığı günden başlayarak 20. güne kadar gün aşırı olmak üzere en az iki adet (toplam 1320 adet) ultrasonografik uterus görüntüsü alındı. Subjektif skorlamalar bir deneyimli ve bir deneyimsiz olmak üzere iki bağımsız uygulayıcı tarafından gerçekleştirildi. Aynı görüntüler, ImageJ (ImageJ 1.53k; NIH, ABD) yazılımı ile Ortalama Grilik Değeri (MGV) değerlerinin belirlenmesi açısından analiz edildi. Veriler, hayvanların yaşı (inek veya düve), muayene günleri (D0 - D20) ve 28. gün gebelik durumu (gebe veya gebe değil) açısından değerlendirildi. Ortalama MGV değerleri sadece örnekleme günleri arasında istatistiksel olarak anlamlı farklılıklar gösterdi ($p<0,05$). Diğer ana etkilere ve etkileşimlere göre elde edilen farklılıklar istatistiksel olarak anlamsız bulundu ($p>0,05$). Deneyimli (1. skorlayıcı) ve deneyimsiz (2. skorlayıcı) skorlayıcıların uyumlarının zayıf düzeyde ($\kappa=0,15$) olduğu belirlendi ($p<0,001$). Ayrıca, 1. skorlayıcının (0,720) 2. skorlayıcıya göre (0,541) MGV değerleri ile daha yüksek korelasyona sahip olduğu belirlendi. Gri tonu skorlama ile gebelik belirlenmesi analizinde anlamlı bir farklılık bulunmadı ($p>0,05$). Sonuç olarak, bilgisayar destekli görüntü analizi ile değerlendirilen grinin 256 farklı tonunun (MGV) insan gözü tarafından 5 dereceli bir ölçekte tanımlanabildiği sonucu elde edilmiştir. Ancak gebe olan ve olmayan inek ve düvelerde uterus endometriyumunun ekotekstür yapısının suni tohumlama sonrası ilk 20 gün içinde benzer olduğu gözlemlenmiştir.

Anahtar kelimeler: Düve, Ekotekstür, Gebelik, İnek, Skorlama, Ultrason görüntüsü

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INTRODUCTION

B-Mode ultrasound is a unique device that is used for monitoring the tissues and organs by collecting the reflected ultrasound waves and converting them to visual images in a wide gray scale between 0 (absolute black) and 255 (absolute white) (Pierson and Adams; 1995; Zagzebski, 1996). The gray scale appearance of the tissues and organs is called echotexture. Recently, the echotexture evaluation of different structures such as muscle, tendon, bone, nerve, liver and mammary gland has been used to detect physiological and pathological changes (Sarwall et al., 2015; Martinoli et al., 1993; Themistokleous et al., 2022; Dietrich et al., 1999; Lee et al., 2011). Moreover, the differentiation in echogenic conditions of the genital tract in farm animals have been tried to determine according to reproductive situations such as ovarian and testicular soundness, oestrus, insemination time, and pregnancy (Camela et al., 2019; Cengiz et al; 2014; Kauffold et al., 2010; McKinnon and Carnevale, 1993).

In cattle breeding, ultrasonography is used widely. Moreover, it is an indispensable part of the reproductive examinations (Cengiz; 2017; Scully et al., 2014). The examinations mostly depend on direct observation of the interested event, which takes a long time to become visible. For example, pregnancy determination in cows could be made by observation of embryo and embryonic heartbeats. However, pregnancy detection with 95% accuracy can be done as early as on 35th day after fertilization by this method (Ribadu and Nakao, 1999). It has been reported that different ultrasonographic evaluating techniques such as uterine echotexture, dominant follicle diameter, endometrial thickness, uterine volume, amount of fluid collected in the uterus, and evaluation of uterine vascularization can be used in pregnancy prediction by analyzing ultrasonographic images (Abdalla et al., 2021; Holm et al., 2015; Mee et al., 2009; Souza et al., 2011; Stevenson et al., 2008; Vasconcelos et al., 2001). However, all of the studies conducted on echogenicity have been based on computer assisted image analysis. Although the computer assisted image analysis has the advantage of achieving objective results, the inability to use it in real-time in clinical applications is observed as the most important restraint to the widespread use of this method. Therefore, it is needed to develop a real-time subjective scoring technique to evaluate the echotexture properties of ultrasonographic images.

Echotexture consists of three different measurements known as mean gray value (MGV), contrast and homogeneity. Among these measurements, MGV is the most likely to identify by the human eye. Because the eye is known that can identify color tones (almost 20 different gray color tones) better than the contrast changes of an image or the distribution homogeneity of colors (Pierson and Adams, 1995). In the study, it has been aimed to develop a scoring scale, graded from 1 to 5, to identify different gray tones of the ultrasonographic images of the ovulation side uterine horn. Moreover, the pregnancy determination in cows and heifers has been tried to make by this scoring technique, in the first 20 days after artificial insemination.

MATERIALS and METHODS

All procedures involving study animals in the experiment were approved by Ankara University Animal Experiments Local Ethics Committee (No: 2021–20–180). A total of sixty Holstein cows (n:30) and heifers (n:30) which were determined as healthy according to general examination, body condition scoring, and reproductive examination were enrolled in the study. Animals were conducted in a presynch-ovsynch synchronization program and artificially inseminated (Giordano et al., 2016; Gordon et al., 2010). For each animal, two ultrasonographic images were taken (totally 1320 images) starting from the day of the artificial insemination (D0) until the 20th day (D20) on every other day. At the same time that images were taken, subjective scoring was made for all of the images. Then, the images were transferred and stored in a hard drive until the image analysis were done. The MGV values determined by ImageJ (ImageJ 1.53k; NIH, ABD) software. The obtained data at the end of the evaluations were analyzed in terms of Groups (cow or heifer), Sampling Time (alternate days between D0 and D20) and Pregnancy Status (pregnant or non-pregnant). Also score rates were given by one experienced (experienced on the reproductive ultrasonography more than 10 years) and one inexperienced (no experience on the reproductive ultrasonography) independent operators to determine scoring agreement level in terms of experience on the ultrasonographic examinations.

Ultrasound images were collected from the ovulation side uterine horn on the mentioned days by 6,5-9,5 mHz linear rectal probe attached to a portable B-mode ultrasonography device (CTS 800, SIUI, China). It was noted that all images were taken from the same area, with the same probe position, and maximum contrast provided as much as possible. Standard settings of the ultrasound device were used throughout the study. For each of the imaging procedure, probe was located on the dorsal cavity of the related region of the uterus on the dorsoventral position, and longitudinal images were taken. Images were determined as the regions including all of the endometrium and stratum vascularis layers in the hyperechoic serosa lines were in the image.

The gray tone of the uterus tissue in the images were scored between the grades of 1 and 5 at the same time the images were taken according to white point accumulation in the interested area (Figure 1).

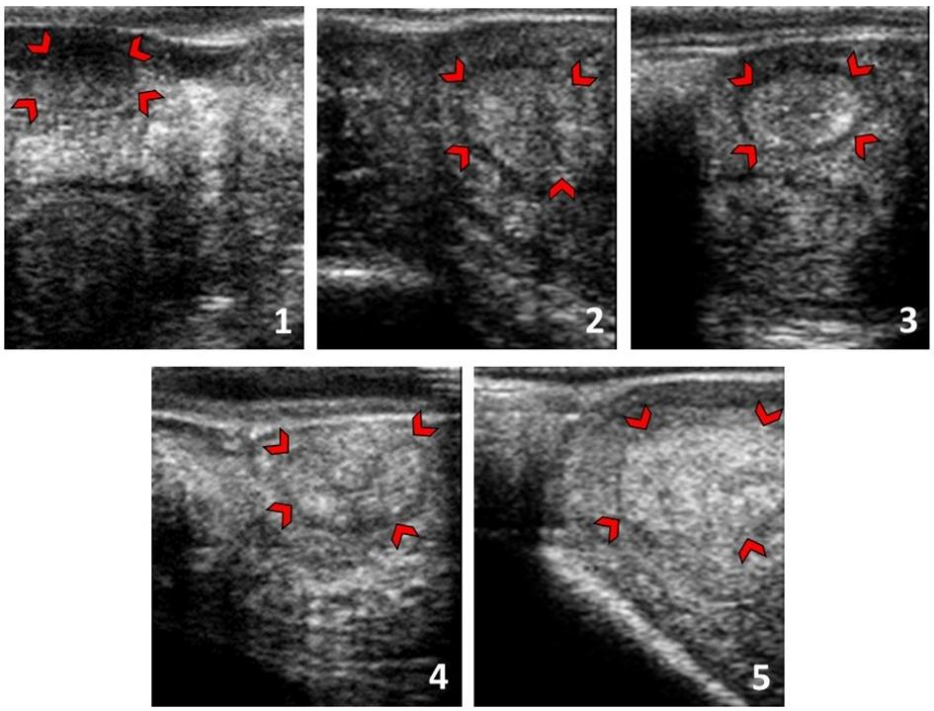

Figure 1: Ultrasonographic appearance of ovulation side uterine horn of heifers and cows. The red arrows heads in each image indicate the border of the uterus tissue that interested and scored by raters. The numbers in the lower corner of the right side of images indicate the Gray Tone Scores given for each sample. The gray tone of the uterus tissue in the images were scored according to white point accumulation in the interested area. Interested area was identified as including the entire endometrial tissue in the serosa layer for an image (same area with the ROI that was used in the MGV analysis). Score 1 represented almost black (0-20 % white point in interested area), Score 2 represented dark gray (20-40 % white point), Score 3 represented moderate gray (40-60 % white point), Score 4 represented light gray (60-80 % white point) and Score 5 represented almost white (80-100 % white point).

Interested area was the same area with the ROI that was used in the MGV analysis identified as including the entire endometrial tissue in the serosa layer for an image (Figure 2). Score 1 represented almost black (0-20 % white point in interested area), Score 2 represented dark gray (20-40 % white point), Score 3 represented moderate gray (40-60 % white point), Score 4 represented light gray (60-80 % white point) and Score 5 represented almost white (80-100 % white point). Scoring of the uterus was made by two independent raters (Rater 1 and Rater 2). Rater 1 was determined as an experienced rater when Rater 2 was enrolled as an inexperienced rater.

For MGV analysis ImageJ (ImageJ 1.53k; NIH, ABD) software was used. In the analysis, the whole region in each longitudinal view was evaluated. A single region of interest (ROI) was identified as including the entire endometrial tissue in the serosa layer for an image (Figure 2). The determined ROI's were analyzed objectively according to the MGV parameter (Scully vd., 2014).

Descriptive statistics for each variable were calculated and presented as "mean ± standard error of mean (SEM)". The variables were examined as parametric test assumptions. To test the effects of groups, pregnancy status, and sampling times, a linear mixed model for repeated measures was performed. Animals were included in the model as random effects, while group, pregnancy status, and sampling times were included as fixed effects. Besides, two-way and three-way interactions were also evaluated in the model. Variance components were used as the covariance structure in the established model since it resulted in the lowest Akaike information criterion (AIC). When a significant difference was obtained, all significant terms were compared using a simple effect analysis with Bonferroni correction. The model shown below was used for statistical analysis.

$$Y_{ijklmnoprs} = \mu + G_i + P_j + T_k + (G*P)_m + (G*T)_n + (P*T)_s + e_{ijklmnoprs}$$

Where, $Y_{ijklmnoprs}$, is the dependent variable; μ, is the overall mean; G_i, is the effect of the group (i= 2 classes; heifer and cow); P_j, is the effect of pregnancy status (j= 2 classes; pregnant and non-pregnant); T_k, is the effect of sampling time (k= 11 classes; Day 0 to 20); $(G*P)_m$, is the interaction of group and pregnancy status; $(G*T)_n$, is the interaction of group and sampling time; $(P*T)_s$, is the interaction of pregnancy status and sampling time; and $e_{ijklmnoprs}$, is the residual error.

For analysis of the gray tone scoring method descriptive statistics were calculated. The agreement level of the results obtained with the different raters was determined by calculating the inter-rater agreement (Cohen's kappa coefficient, $\varkappa$). Measure of agreement levels of $\varkappa < 0.20$, $0.20 < \varkappa \leq 0.40$, $0.40 <$

$\varkappa \leq 0.60$, $0.60 < \varkappa \leq 0.80$, and $\varkappa > 0.80$ indicated poor, fair, moderate, substantial and almost perfect agreement, respectively. Pearson correlation coefficient was performed to assess the correlation between MGV, Rater 1 scores, Rater 2 scores and median scores of raters. Differences with $p<0.05$ were considered statistically significant.

To test the differences of gray tone scores according to pregnancy status and sampling times, a linear mixed model for repeated measures was performed. Animals were included in the model as random effects, while pregnancy status, and sampling times were included as fixed effects. When a significant difference was obtained, all significant terms were compared using a simple effect analysis with Bonferroni correction. The model shown below was used for statistical analysis.

$$Y_{ijk} = \mu + P_i + S_j + (S*P)_k + e_{ijk}$$

Where; Y_{ijk}, is the dependent variable; μ, is the overall mean; P_i, is the effect of pregnancy status (j= 2 classes; pregnant and non-pregnant); S_j, is the effect of sampling time (k= 11 classes; Day 0 to 20); $(S*P)_k$, is the interaction of pregnancy status and sampling time ve e_{ijk}, is the residual error.

Statistical analyses were performed using IBM SPSS Statistics software Version 26.0.

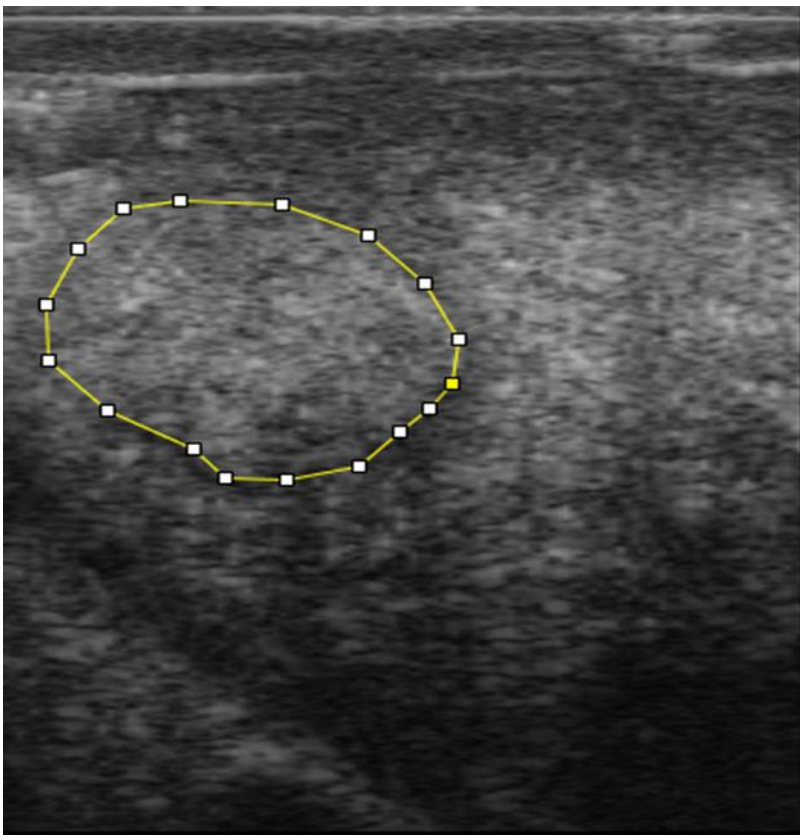

Figure 2: An example for determination of region of interest. A single ROI was identified as including the entire uterine tissue in the serosa layer for an image. The determined ROIs were analyzed according to the MGV.

RESULTS

Mean MGV values indicated statistically significant differences only between days of sampling ($p<0.05$). On the other hand, according to other main effects as Group and Pregnancy Status, statistical significance was not observed ($p>0.05$). Similarly, in the two- and three-way interactions were not concluded with statistically significant differences ($p>0.05$; Table 1).

In the analysis on scoring of the ultrasonographic images of uterus according to MGV values (Table 2), it has been determined that experienced (Rater 1) and inexperienced (Rater 2) raters have agreement ($p<0.001$) in poor level ($\varkappa=0.15$). Moreover, scores of the Rater 1 (0.720) were observed that had higher level correlations than Rater 2 (0.541; Table 3).

Pregnancy determination of cows and heifers by gray tone scoring was investigated by only according to the scores of Rater 1, because of poor agreement level ($\varkappa=0.15$; $p<0.001$) and high correlation coefficient (0.720; $p<0.001$). On the other hand, gray tone scoring analyses were conducted on according to Sampling Time and Pregnancy Status, independent from cow and heifer groups, in case there was no significant differences between cows and heifers according to MGV ($p>0.05$). It has been concluded that the mean of gray tone scoring were not differed significantly in terms of means and two-way interactions between sampling time and pregnancy status ($p>0.05$; Table 4).

Table 1. Means and interactions of MGV in pregnant (P) and non-pregnant (NP) cows and heifers evaluated from D0 to D20 (Mean ± SEM).

Sampling Time	Groups	
	Cow	Heifer
D0	94.25 ± 4.82	110.08 ± 6.13
D2	107.63 ± 4.64	100.72 ± 6.69
D4	96.01 ± 3.61	105.66 ± 4.5
D6	101.36 ± 3.22	95.32 ± 9.02
D8	103.45 ± 4.66	118.86 ± 5.23
D10	104.76 ± 4.57	118.11 ± 6.47
D12	107.43 ± 4.42	122.16 ± 10.47
D14	106.62 ± 3.47	111.51 ± 4.31
D16	106.64 ± 3.95	117.63 ± 4.81
D18	94.86 ± 3.62	96.02 ± 3.96
D20	98.43 ± 4.08	102.73 ± 6.5
Pregnancy Status		
Non-Pregnant	100.82 ± 1.41	107.72 ± 2.55
Pregnant	104.82 ± 2.63	111.74 ± 2.48
p		
Group (G)	0.138	
Sampling Time (S)	0.006	
Pregnancy Status (P)	0.342	
G*S	0.324	
G*P	0.982	
S*P	0.191	
G*S*P	0.941	

G: Group; **S:** Sampling Time; **P:** Pregnancy Status **G*S:** Interaction between G and S; **G*P:** Interaction between G and P; **S*P:** Interaction between S and P **G*S*P:** Interactions between G, S, and P

Table 2. Measurements of agreement level of two different and independent raters (Rater 1 and Rater 2) on gray tone scoring of ultrasonographic images of the ovulation side uterine horn.

			Rater 2 (Inexperienced)					Total
			Score 1	Score 2	Score 3	Score 4	Score 5	
Rater 1 (Experienced)	Score 1	N	29	24	5	0	0	58
		% Rater 1	50.0%	41.4%	8.6%	0.0%	0.0%	100.0%
		% Rater 2	19.6%	5.3%	0.9%	0.0%	0.0%	3.6%
	Score 2	N	77	214	178	69	8	546
		% Rater 1	14.1%	39.2%	32.6%	12.6%	1.5%	100.0%
		% Rater 2	52.0%	47.5%	33.8%	16.8%	10.3%	33.8%
	Score 3	N	35	175	297	239	32	778
		% Rater 1	4.5%	22.5%	38.2%	30.7%	4.1%	100.0%
		% Rater 2	23.6%	38.8%	56.4%	58.2%	41.0%	48.2%
	Score 4	N	7	38	47	102	38	232
		% Rater 1	3.0%	16.4%	20.3%	44.0%	16.4%	100.0%
		% Rater 2	4.7%	8.4%	8.9%	24.8%	48.7%	14.4%
	Score 5	N	0	0	0	1	0	1
		% Rater 1	0.0%	0.0%	0.0%	100.0%	0.0%	100.0%
		% Rater 2	0.0%	0.0%	0.0%	0.2%	0.0%	0.1%
Total		N	148	451	527	411	78	1615
		% Rater 1	9.2%	27.9%	32.6%	25.4%	4.8%	100.0%
		% Rater 2	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%

$\varkappa$ = 0.15 ; p<0.001

Table 3. Correlation analysis of two different and independent raters (Rater 1 and Rater 2) on gray tone scoring of ultrasonographic images of the uterus according to MGV.

	MGV	Rater 1 (Experienced)	Rater 2 (Inexperienced)
MGV	1	0.720***	0.541***

***. Correlation is significant at the 0.001 level (2-tailed).

Table 4. Means and interactions of Gray Tone Scoring in pregnant (P) and non-pregnant (NP) cows and heifers evaluated from D0 to D20 (Mean ± SEM).

Sampling Time	Pregnancy Status	
	NP	P
D0	2.28 ± 0.12	2.48 ± 0.16
D2	2.25 ± 0.17	2.29 ± 0.19
D4	2.62 ± 0.17	2.19 ± 0.18
D6	2.18 ± 0.14	1.80 ± 0.20
D8	2.41 ± 0.16	2.44 ± 0.14
D10	2.38 ± 0.17	2.12 ± 0.18
D12	2.44 ± 0.17	2.15 ± 0.30
D14	2.31 ± 0.14	2.43 ± 0.18
D16	2.16 ± 0.14	2.17 ± 0.15
D18	1.89 ± 0.14	1.84 ± 0.22
D20	1.85 ± 0.15	2.00 ± 0.24
p		
Sampling Time (S)	0.065	
Pregnancy Status (P)	0.311	
S*P	0.623	

S: Sampling Time; **P:** Pregnancy Status; **S*P:** Interaction between S and P

DISCUSSION

Detailed observation of the reproductive organs and evaluation of the structural differences in cows have been possible since the ultrasonographic imaging was integrated into veterinary medicine (Pierson and Ginther, 1984). The variations in the structure of the sonographic images occur as a result of different reproductive events such as estrous cycle, pregnancy, etc. Therefore, these events are observed as different echogenicity because of different level of steroid hormones to which the endometrium is exposed (Schmauder et al., 2008; Vollmerhaus, 1958). Different echogenicities of a B-Mode ultrasonograpcih image could be defined as different gray tones by the human eye that could identify almost 20 different gray tones (Pierson and Adams, 1995). It has been hypothesized that variations in the MGV of the B-Mode ultrasonographic images of the ovulation side uterine horn can be detected by human eye in a 5 graded (1-5) scale and pregnancy can be diagnosed with this scoring scale in the first 20 days after artificial insemination. According to the hypotheses, the aim of the study conducted was to discriminate the gray tones of the B-Mode ultrasonographic images of the endometrial tissue of the Holstein cows and heifers in a 5 graded scoring scale, and to identify pregnancies in terms of the subjective scoring method in the first 20 days after artificial insemination.

Scully et al. (2014) examined the sonographic images of uterus in cows between the 18th and 21st days following artificial insemination, and they reported that the only uterine echogenicity could not be a pregnancy determining parameter. In a similar study performed in heifers (Scully et al., 2015), uterine echogenicity of pregnant, non-pregnant (inseminated) and cyclic (non-inseminated) heifers was evaluated on the 7th, 11th, 14th, 16th, 18th days following ovulation. Although there were significant differences between cyclic and inseminated heifers, it was concluded that there was no difference in uterine echogenicity between heifers that were pregnant and non-pregnant after insemination. Similarly, in a study in which the ultrasonographic image of the uterus was evaluated by the Optic Density Image (ODI) analysis method, which is based on the determination of the white spot density in the image (Cengiz et al., 2017),

any significant difference was not found between the ODI values in pregnant and non-pregnant animals. In the present study, the MGV analysis has been concluded with similar results to the previous studies. According to these results, MGV of the ultrasonographic appearance of the ovulation side uterine horn of cows and heifers did not differ in terms of parturition history or present pregnancy status, but only the day of the images were taken. It could be though that the exposure of steroid hormones caused the similar differences in endometrial echotexture in cows and heifers in the first 20 days after artificial insemination.

To the best of knowledge, in reproductive medicine for cows any subjective scoring method has not been developed for using in pregnancy determination, yet. On the contrary, in mares, ultrasonographic examinations of the changes in the uterus can be detected subjectively by unique cart wheel structure. Thanks to the structural properties, endometrial folds change under effects of steriod hormones, which vary in the estrus cycle. Also, there are 4 (0-3) graded (McCue et al., 2011) and 6 (0-5) graded (Samper, 2010) subjective scoring methods for mare uterus, based on various echotexture and appearance of the endometrial folds. Moreover, these scoring methods is used to predict pregnancy in mares. In the present study, it has been tried to reveal a novel subjective scoring approach to evaluate the ultrasonographic examination of the endometrial tissue of cows and heifers. Totally 1320 B-Mode ultrasonographic images have been evaluated in a 5 graded scoring scale that has been conducted on the different tones of gray, and compared to the MGV values. It has been concluded that the MGV values could be detected by this novel scoring method. Also, scores that have been rated by experienced practitioner have been found more consequent than the inexperienced practitioner. However, the gray tone scoring method has been resulted as insufficient on prediction or definition of the pregnancy in cows and heifers in the first 20 days after artificial insemination. The fact that the computer assisted MGV has not been succeed on the identifying the pregnancy could indicated the insufficient results obtained from 5 graded gray tone scoring method.

CONCLUSION

In conclusion, it has been observed that the 256 different shades of gray (MGV) which is analyzed by computer assisted image analysis could be identifed by human eye in a 5 graded scale. On the other hand, experienced practitioners might detected the different tones of gray of the endometrial tissue in cows and heifers more coefficiently than inexperienced ones. However, the pregnancy in cows and heifers in the first 20 days could not be predicted or diagnosed by neighter computer assissted nor subjective methods in terms of the echotexture of endometrial tissue. According to the results, the endometrium of the ovulation side uterine horn reflects the same differentiation in the presence or absence of pregnancy, in the first 20 days. Otherwise, the success of the gray tone scoring method in identifying MGV changes could be used to evaluate different physiological and pathological conditions such as estrus cycle, ovulations time, uterine soundness, subclinical endometritis, ovarian cysts, etc. in further studies.

Conflict of interest: The author have no conflicts of interest to report.

Authors' Contributions: KTO contributed to the project idea, design and execution of the study, acquisition and analysed the data, drafted, wrote, edited and finalized the manuscript.

Ethical approval: All procedures applied on animals in the study were allowed by Ankara University Animal Experiments Local Ethics Committee (No: 2021-20-180).

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