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Polish Journal of Veterinary Sciences Vol. 25, No. 4 (2022), 525–533

DOI 10.24425/pjvs.2022.142040

Original article

Computer-assisted assessment of ovarian echotexture parameters in mares following changes after ovulation determined by ultrasonography

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Abstract

This study was carried out to determine the time-dependent changes in the ultrasonographic image of the ovary with computer-assisted analysis programs at certain intervals after ovulation and to determine whether computer-assisted analysis programs and ovulation programs can be managed in cases where the ovulation time is unknown. The study included 40 purebred Arab mares. The study was subdivided into 4 different time periods of 6 (Group 1), 12 (Group 2), 18 (Group 3) and 24 (Group 4) hours following ovulation. In addition, after ovulation and ultrasonographic examination, natural insemination was performed at 6, 12, 18 and 24 hours, and pregnancy examination and follow-up were performed at 15-30-45 days. In the echotexture analysis, mean grayness value (MGV) and contrast (CON) measurements were at different levels according to the time groups ($p<0.001$). Homogeneity (HOM) measurements were at different levels according to the time groups ($p<0.001$). A very strong, significant negative correlation was determined between MGV and pregnancy rates ($r=-0.91$, $p=0.01$, $p<0.05$). No significant relationship was observed between HOM values and pregnancy rates ($r=0.19$, $p=0.23$, $p>0.05$). A very strong, significant negative correlation was determined between CON and pregnancy rates ($r=-0.92$, $p=0.01$, $p<0.05$). It was concluded that the use of ultrasonographic echotexture in mares after ovulation provided very important information. In cases where the time of ovulation was not known, by looking at the values of echotexture parameters, it was seen that the highest pregnancy rates were at the 6th hour and the lowest pregnancy rates were at the 24th hour. As the echotexture parameters MGV and CON increased, it was determined that pregnancy rates decreased, but there was no relationship between them and the HOM value.

Key words: computer-aided analysis program, embryonic death, mare, ovarian echotexture, ovulation, pregnancy

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Introduction

Pre-ovulatory follicle development and ovulation in mares are different from other farm animal species. Compared to other species, the pre-ovulatory follicle diameter is larger in mares, and ovulation occurs internally through the ovulation fossa in the mare as compared to the ovum being released from the external surface of the ovary (Ginther et al. 2008, Aurich 2011). The developing follicle is firm on transrectal palpation but softens just before ovulation. The lumen of the follicle begins to fill with blood 6-12 hours after ovulation and results in the formation of corpora hemorrhagica (CH). A CH can usually be detected 2-3 days after ovulation. Clinically, the detection of a CH indicates that ovulation occurred at least 6-12 hours ago and it may be too late for insemination (Bowman 2011).

The ultrasonographic appearance of a tissue varies according to the histological structure and this is known as echotexture. Visual analysis of ultrasonographic images is made with the subjective evaluation of the examiner. However, the density and characteristics of tissue cannot be quantified by the human eye, which can only distinguish 18-20 different shades of gray. Computer-aided analysis (computer algorithm) allows for the objective evaluation of the image and determination of the visual analysis subjectivities by quantitative evaluation of the density of each pixel in an image (Singh et al. 2003). During ultrasonographic examinations, frozen images are recorded digitally and transferred to the computer environment for evaluation of echotexture parameters. Some parameters are used in echotexture analysis for selected examination areas (Region of Interest, ROI). The most commonly used parameters include mean grayness value (MGV), homogeneity (HOM) and contrast (CON) (Küçükaslan et al. 2014). In recent years, computer-assisted ultrasonographic image analysis has been used in the evaluation of reproductive tract structures including testicles (Gabor et al. 1998), ovarian follicles (Vassena et al. 2003), corpora lutea (CL) (Vassena et al. 2003, Davies et al. 2006, Liu et al. 2007) and the uterus (Ginther 2014, Korkmaz et al. 2021).

Embryonic and fetal losses are among the most important problems in equine breeding (Pycock 2001). Absence of the embryonic vesicle in mares can be detected with transrectal ultrasonographic examinations after the 10th day of fertilization. The causes leading to embryonic death can be classified as internal, external and embryonic factors. Internal factors include inflammatory or non-inflammatory diseases of the endometrium, progesterone deficiency, maternal age/reproductive status, lactation, insemination time in foal heat and insemination time according to ovulation (insemination of the mare after ovulation increases the rate of early embryonic loss) (Vanderwall 2008).

The aim of this study was to describe the changes in the ovarian echotexture in the first 24 hours after ovulation using a computer analysis program to monitor the time-related changes of transrectal ultrasound derived images. A further aim was to determine the relationship between mating and pregnancy status at this time.

Materials and Methods

Permission for this study was granted by the Animal Experiments Local Ethics Committee of Harran University (dated 2016/20).

Selection of animals and test protocol

In this study, subjects included 40 purebred Arab mares aged 4-8 years. The average age of the 40 mares in the study groups was 5.73±1.88 years. All the mares were kept for breeding without taking familial performance into consideration and without having been in horse races. No mare had any medical history of general or genital organ-related disease. The breeding documents of the mares were made available by the Ministry of Food, Agriculture and Livestock at the Şanlıurfa insemination station and animals were certified free of dourine, glanders, equine viral arthritis, infectious equine anemia, salmonella abortus-equine diseases and had no known fertility problems. To attain uniformity of the mares included in this study, the mares that had given birth recently (mare with foal) and those with no birth in the previous season (barren) were not included in the study, although those with a first mating (maiden) were included. The feed for the mares was prepared with oats, barley, corn, soybean meal, salt and feed additives. The mares were tied side by side with 3 m width for each mare in each station, in routine housing. The body condition score of the mares was between 5 and 6.

Examination of the ovaries was performed using rectal palpation and transrectal ultrasonographic examination techniques. Ovary inspections started on February 15, when the breeding season started. For mares in which follicular growth was detected in the ovaries, these evaluations were repeated at intervals of 1-3 days depending on the size of the follicle diameter. When a follicle size of 35 mm was detected, 1500 IU hCG was administered intravenously and ovarian ultrasound was performed at every half hour until ovulation was detected. In the ultrasonographic examination of the ovaries, it was assumed that ovulation had occurred from the lost of the pre-ovulatory follicle detected in the previous examination, imaging of the corpora lutea, and regression (absence) of endometrial oedema. When ovulation was first detected, Group 1 (n=10) consisted of naturally mated mares with ultrasound imaging at the 6th hour after ovulation detection. Group 2 (n=10) consisted of naturally mated mares with ultrasound imaging at the 12th hour after ovulation detection. Group 3 (n=10) consisted of naturally mated mares with ultrasound imaging at the 18th hour after ovulation detection. Group 4 (n=10) consisted of naturally mated mares with ultrasound imaging at the 24th hour after ovulation detection.

Insemination of the mares was performed in the following manner. The stallions (n=10) used in the study were aged 9-15 years, reproductively active for 5 years leading up to the study, and their body condition score ranged from 5-7. These stallions were fed under the same conditions as the mares with a mixture containing barley, oat, dry grass, dry clover, probiotics, and multivitamins. Natural insemination was performed on the mares at the 6th, 12th, 18th and 24th hours from the first detection of ovulation. All the mares that were inseminated were then examined three times on day 15, day 30, and day 45 day using transrectal ultrasonography for pregnancy examination.

Ultrasonography

Transrectal ultrasound examination of the ovaries and uterus was performed with a 5 MHz linear probe attached to a portable ultrasound device with B-mode capability (SonoSite Edge II Vet®, Providian Medical Equipment LLC, Highland Heights, Ohio, United States). For each time period following ovulation, three images of the site of ovulation were recorded using uniform depth, gain, focus, and brightness. All ultrasound examinations were performed by the same person.

Computer-aided image analysis

Digital echotexture analysis of the ovarian ultrasound images was performed on a personal computer (the ovary was not evaluated as a whole, only the luteal tissue). The images were saved in file type PNG and at 640x480 pixel resolution. Computer analysis was performed using special software to enhance grayscale comparison (ImageJ 1.42q; NIH, USA-Image Processing and Analysis Java). In the measurement analysis, polygon-style boundaries were created from each region of the structure formed after ovulation as the "region of interest", and the mean grayness value (MGV), homogeneity (HOM) and contrast (CON) measurements were made from the echotexture parameters.

Homogeneity (HOM): This is the gray value combinations of neighboring pixels in the micro or macro texture in the specified matrix. HOM values range from 0–1 (Raeth et al. 1985).

$$\text{HOM}= \sum_{(i,j)} p(i,j)\,2$$

HOM=homogeneity, *i,j*=the row *(i)* and column *(j)* index; *p(i, j)*=value in the section of the co-occurrence matrix

Contrast (**CON**): This is the total measurement of large gray value differences in the selected examination areas. It provides information about the macrotexture of the image. It is defined by Lefebvre et al. (2000) as

$$\text{CON}= \sum_{(i,j)\in ROI}(i,j)2 * p(i,j)$$

CON=contrast, *i,j*=the row *(i)* and column *(j)* index; *p(i, j)*=value in the section of the co-occurrence matrix

Mean Gray Value (**MGV**): This is the arithmetic mean grayness of all pixels in the image that defines its brightness. MGV ranges from 0 to 255. It is defined by Raeth et al. (1985) as

$$\mu_g= \frac{1}{N}\sum_{(x,y)} g(xy)$$

μ_g=mean gray level (values: 0-255); N=size of ROI in pixels; x,y=row (x) and column (y) index; g(xy)=gray level in pixels

Statistical analysis

Statistical analysis of the data was performed with SPSS for Windows vn.22.0 software (*Statistical Package for the Social Sciences*). The conformity of variables to normal distribution was examined using visual (histogram and probability graphs) and analytical methods (Shapiro-Wilk tests). Descriptive analyses were presented as mean ± standard deviation values for normally distributed variables. Evaluation of relevant data with normal distribution was made using the One-way ANOVA test. The homogeneity of the variances was determined using the Levene test. A value of $p<0.05$ was accepted as statistically significant. When there was a significant difference between the groups, it was compared with the post-hoc Tukey test. Correlation coefficients and statistical significance between pregnancy and echotexture were calculated using the Spearman test. Correlations were also calculated separately between the echotexture parameters.

Results

Findings on Follicle Development and First Ovulation of the Year

In the transrectal ultrasonographic examination performed on February 15, which is the beginning of the breeding season, the average diameter of active, pre-ovulatory follicles of all the mares was measured

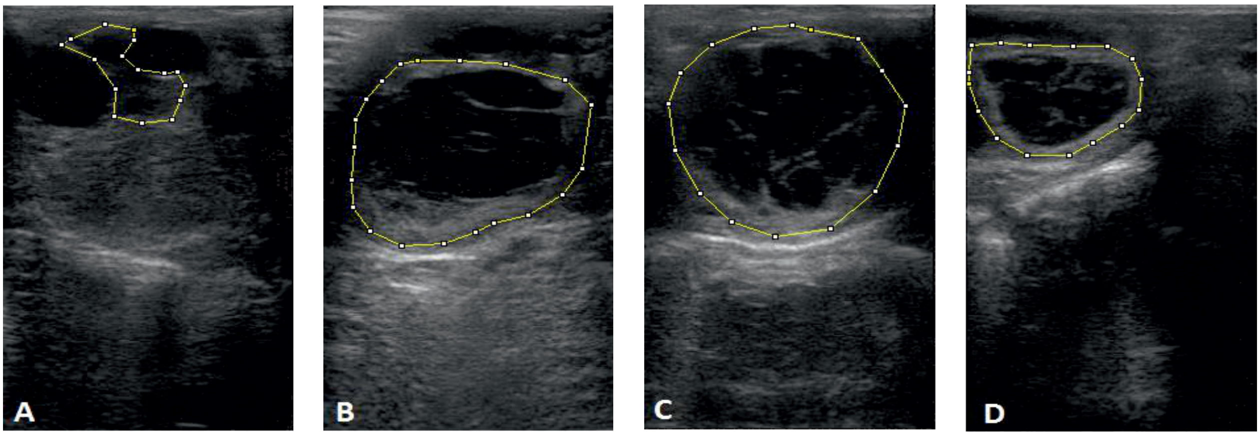

Fig. 1. Measurement of ovarian tissue with image analysis program. 6th hour (A) after ovulation, 12th hour (B) after ovulation, 18th hour (C) after ovulation, 24th hour (D) after ovulation.

Table 1. Mean echotexture values of the study mare groups.

Parameters	Groups	N		P (oneway ANOVA)	Difference (Groups)
MGV (mean grayness value)	Group 1 (6th hour)	10	27.47±1.04[a]	**0.000**	1.2<3.4 (p<0.001)
	Group 2 (12th hour)	10	37.82±0.54[b]		
	Group 3 (18th hour)	10	44.71±0.61[c]		
	Group 4 (24th hour)	10	55.30±0.53[d]		
HOM (homogeneity)	Group 1 (6th hour)	10	0.075±0.001[a]	**0.000**	1>2.3.4 (p<0.001)
	Group 2 (12th hour)	10	0.035±0.001[d]		
	Group 3 (18th hour)	10	0.045±0.001[c]		
	Group 4 (24th hour)	10	0.056±0.001[b]		
CON (contrast)	Group 1 (6th hour)	10	109.93±1.30[a]	**0.000**	1.2<3.4 (p<0.001)
	Group 2 (12th hour)	10	132.77±1.40[b]		
	Group 3 (18th hour)	10	150.65±1.68[c]		
	Group 4 (24th hour)	10	181.21±3.35[d]		

[a,b,c,d] Different letters in the same column represent a statistically significant difference. ANOVA: Analysis of variance

as 47.81 ± 3.76 mm. The first ovulation was recorded in February for 32 mares, in April for 5, and in May for 3.

Evaluations Regarding Echotexture

The average values of the study groups are presented in Table 1, and measurements are shown in Fig. 1. The mean grayness value (MGV) was measured in Group 1 (27.47 ± 1.04), Group 2 (37.82 ± 0.54), Group 3 (44.71 ± 0.61) and Group 4 (55.30 ± 0.53). It was lowest in Group 1 (the group in which an ultrasound image of the ovary was taken at the 6th hour after ovulation) and the highest in Group 4 (the group in which an ultrasound image of the ovary was taken at the 24th hour after ovulation). The MGV measurements showed a statistically significant difference between the groups (p<0.001). As a result of the paired comparison test conducted to determine the origin of the difference, Group 1 and Group 2 MGV measurements were found to be lower than those of Group 3 and Group 4 (p<0.001). The HOM measurements were statistically significantly different according to the time groups (p<0.001). As a result of the paired comparison test conducted to determine the origin of the difference, the Group 1 HOM measurements were found to be higher than those of Group 2, Group 3 and Group 4 (p<0.001). A statistically significant difference was determined between the groups in respect of the CON measurements (p<0.001). As a result of the paired comparison test, it was found that the Group 1 and Group 2 CON measurements were lower than those of Group 3 and Group 4 (p<0.001).

Table 2. Relationship between pregnancy rates and mean grayness value (MGV), homogeneity (HOM) and contrast (CON).

Parameters		MGV (mean grayness value)	HOM (homogeneity)	CON (contrast)
Pregnancy rate	r	**-0.91****	0.19	**-.92****
	p	0.01	0.23	0.01

** significant relationship

Table 3. Relationships Between MGV, HOM and CON.

Parameters		MGV (mean grayness value)	HOM (homogeneity)	CON (contrast)
MGV (mean grayness value)	r	1	**-.367***	**.935***
	p		0.02	0.01
HOM (homogeneity)	r	**-.367***	1	**-.317***
	p	0.02		0.04
CON (contrast)	r	**.935***	**-.317***	1
	p	0.01	.046	

* significant relationship

Pregnancy Rates and Findings Related to Embryonic Death

Ultrasound examinations performed on the 15th day after insemination and at regular intervals thereafter revealed pregnancy in 10/10 (100%) of the mares in Group 1, in 9/10 (90%) of the mares in Group 2, in 3/10 (30%) of the mares in Group 3 and in 1/10 (10%) of the mares in Group 4. In the examinations made on the 30th and 45th days, no embryonic death was observed and all pregnancies were maintained.

Correlation between echotexture parameters and pregnancy

The correlations between the echotexture parameters and the correlations between the echotexture parameters and pregnancy are presented in Table 2 and Table 3. A very strong, significant negative correlation was determined between MGV and pregnancy rates. As MGV increased, pregnancy rates decreased ($r=-0.91$, $p=0.01$, $p<0.05$). No significant relationship was observed between HOM values and pregnancy rates ($r=0.19$, $p=0.23$, $p>0.05$). A very strong, significant negative correlation was determined between CON and pregnancy rates. As CON values increased, pregnancy rates decreased ($r=-0.92$, $p=0.01$, $p<0.05$). A weak, significant negative relationship was determined between MGV and HOM values ($r=-0.367$, $p=0.02$, $p<0.05$). A very strong, significant positive relationship was determined between MGV and CON values ($r=0.935$, $p=0.01$, $p<0.05$). A weak, significant negative correlation was determined between CON and HOM values ($r=-0.317$, $p=0.04$, $p<0.05$).

Discussion

Ovulation time and exact detection of its occurrence in mares is critical for a number of reasons: to ensure that ovulation takes place within the time deemed appropriate after mating that occurs prior to ovulation, to decide the optimum timing so that fertilization of short-lived semen for artificial/natural insemination after ovulation is achieved, to accurately determine the embryo age required for embryo recovery, to determine the number of ovulations associated with the number of pre-existing follicles in order to manage twin pregnancies appropriately, and to exclude the pathological anovulation phenomenon by ensuring follicle rupture and collapse (Townson and Ginther 1987, Cuervo-Arango and Newcombe 2012). In order to obtain high pregnancy rates when using fresh or fresh-cooled semen it is optimal to inseminate the mare prior to, but close (12-24 hours prior) to, ovulattion in order to use the stallion more efficiently and to minimize possible uterine problems that may arise from mating (McCue et al. 2007, McKinnon and McCue 2011). Therefore, previous studies conducted on mares have ensured mating at certain hours after ovulation monitoring, but no study has been found which has examined the changes in ovarian tissue when ovulation is missed. The present study was carried out both to evaluate the echotexture of the ovarian image taken at certain hours after ovulation and to determine the optimal mating time after ovulation, as an aid to the use of ultrasound in cases where the ovulation formation time is unknown.

Previous researchers in this field have reported different values regarding the pre-ovulatory follicle diameters of mares. The pre-ovulatory follicle diameter has been measured as 30-70 mm (Bozkurt 2007) and 34-70 mm (Ginther 1995) within 24 hours prior to ovulation. In the current study, the average pre-ovulatory follicle diameter of all the mares was 47.81 ± 3.76 mm (42-54 mm), which was seen to be compatible with the literature results.

Various opinions have been proposed by researchers regarding the distribution of ovulation with respect to the right and left ovaries. While some researchers have suggested that ovulation mostly occurs in the left ovary (Ginther 1972, Ginther 1992, Bozkurt 2007), others have observed equal ovulation in the right ovary (Watson et al. 1994), and some in both ovaries (Davies Morel and O'Sullivan 2001). In the current study, ovulation occurred predominantly in the left ovary (57.5% left, 42.5% right ovary). These findings support the common view that ovulation occurs mostly in the left ovary.

There are a number of conditions in mares that affect pregnancy rates. It has been reported that endometrial fluid accumulation, found in 11-39% of mares in different periods of the estrus cycle, has no impact on the pregnancy rates (Watson 2000, Barbacini et al. 2003). Some researchers have suggested that endometrial fluid accumulation negatively affects pregnancy rates (Pycock and Newcombe 1996, Watson et al. 2001). Endometrial fluid accumulation was not observed in the routine ultrasonographic examinations in the current study. Similarly, several researchers have reported different findings regarding the incidence of uterine cysts in mares. Increased incidence of uterine cysts with advancing age has been reported (Tannus and Thun 1995). It has been stated that if the structure of uterine cysts is too large and the number is high, it may prevent embryo migration and placental-embryo communication in the uterus (Brinsko et al. 2010). Since the mares in the current study were young (4-8 years old) and had not been mated before (maiden), no uterine cysts were encountered.

Embryonic and fetal losses are costly issues and a cause of financial loss and concern in equine breeding. Although the fertilization rate in healthy mares is quite high (>90% in young mares, 85% in older mares), some of these pregnancies cannot be sustained. It has been reported that 5-30% of embryos are lost within 10-45 days of gestation, and in subfertile mares this loss is even higher (Immegart 1997, Yang and Cho 2007, Vanderwall 2008). The age of the mare has been reported to be a significant factor in conceiving, maintaining the pregnancy and giving birth, with an increase in pregnancy loss seen with advancing maternal age (Bosh et al. 2009, Sharma et al. 2010). Embryonic mortality rates have been reported in 15%-35% of pregnancies, as 3.8% in mares mating for the first time, 12.4% in mares that could not conceive in the previous season and 12.4% in mares with a foal. It has also been stated that the embryonic mortality rate increased to 21.6% in mares aged over 15 years (Morris and Allen 2002). In the current study, no embryonic loss occurred in the pregnancies diagnosed at 15 days of gestation to 45 days. This is not surprising as the embryonic loss rate in this age group, for this time period has been reported as (cite study) that which would be consistent with these findings.

In artificial insemination performed at intervals of 6 hours after ovulation in mares, 100% pregnancy has been reported in 6-12 and 12-18 hours, 40% pregnancy in 18-24 hours, and no pregnancy in 24-30 hours (Koskinen et al. 1990). In the present study, the pregnancy rate was found to be 100% in the 6th hour group, 90% in the 12th hour group, 30% in the 18th hour group, and 10% in the 24th hour group. It is thought that the reason for this situation, which is different from the literature, may be affected by many factors such as stallion sperm viability, oocyte viability and ovulation-related mating time.

In recent years, advances in imaging technologies have led to the development of new systems that can be used in reproductive ultrasonography research. Many digital image processing and analysis systems have been developed and mathematical correlation of images has been provided. Therefore, while only visual analysis could be made of pictures in the past, computer-aided echotexture analysis systems are used in current practice (Pierson and Adams 1995, Küçükaslan et al. 2014).

Today, computer-aided image analysis systems are used in the field of reproduction in veterinary medicine (Schmauder et al. 2008, Kauffold et al. 2010, Küçükaslan 2010, Polat et al. 2015, Erdoğan et al. 2017, Zonturlu et al. 2018). In computer-aided analysis there are three ways of obtaining the image: point measurement, linear-time series analysis and regional surface analysis (Pierson and Adams 1995). When measuring the images obtained in the studies, the target tissue is divided into four equal quadrants according to the point measurement, and a small circular area is selected from these regions, and the average numerical data are created (Herzog et al. 2008, Schmauder et al. 2008, Cengiz et al. 2014, Noseir and Sosa 2015, Polat et al. 2015, Scully et al. 2015, Cengiz et al. 2017). In the current study, all the ultrasonographic images to be analyzed were selected in polygon style and numerical data were created as described by Erdogan et al. (2017) in selecting the entire uterus tissue in dogs in a polygonal style to generate average numerical data and as described by Zonturlu et al. (2018) in the distinction between early pregnancy and uterine cysts in mares. Since all of the relevant tissue is analyzed using this method, it was considered to be more reliable and would yield stronger results.

Of the parameters obtained from ultrasonographic images, the mean gray value (MGV), homogeneity (HOM) and contrast (CON) define the echotexture image changes in the uterus (Cengiz et al. 2014). MGV is used to define the total brightness of the image and relates to the cellular and macromolecular content of tissue (Liu et al. 2007). MGV increases with the increase in density and thickness in the tissue resulting from hypertrophy and hyperplasia (Davies et al. 2006, Liu et al. 2008). Furthermore, while MGV increases with the presence of fibrous tissue and proliferation of cells (Duggavathi et al. 2003), it decreases in the presence of reduced tissue density, edema, and blood vessels in the tissue (Liu et al. 2007). CON represents heterogeneity and reaches higher values as various cell types create hyper-hypoechoic areas (Davies et al. 2006). CON correlates positively with cellular density (Liu et al. 2008). As CON negatively correlates with edema, it is defined as the inverse of HOM, which positively correlates with edema (Liu et al. 2007). HOM exhibits maximum values if the pixel plane in the image has the same or a uniform structure. In other words, in cases where the number of gray areas is very low, the HOM values increase. It ranges from 0 to 1 (Schmauder et al. 2008).

To the best of our knowledge, there are no previous studies on echotexture parameters evaluated after ovulation in mares. In this context, this study provides important information for the literature. In the light of the above information, the MGV was at the minimum level in the ultrasonographic image taken at the 6th hour after ovulation, due to the structure formed by the collapse of the follicle. Corpora hemorrhagica (CH), which appears later in the course and thus increased tissue density increases MGV on a regular basis. The homogeneous structure of the same ovarian tissue at the 6th hour after ovulation, and the presence of different cells with different echogenicity due to increased tissue density in later hours, leads to a more heterogeneous image. As a result of this change, CON values are lowest at the 6th hour and the maximum level of HOM is reached. While the CON value increases continuously in parallel with the enhanced tissue density at the 12th, 18th and 24th hours, the HOM value is measured at the lowest level due to CH formation at the 12th hour and the value increases in the following hours. In the current study, the echotexture parameters evaluated after ovulation were seen to be consistent with the findings in the literature.

Conclusions

It was concluded that the use of ultrasonographic echotexture in mares after ovulation provides very important information and may be an important criterion that should be evaluated especially when deciding the timing of insemination after ovulation in mares. In cases where the time of ovulation was not known, by looking at the values of echotexture parameters, it was seen that the highest pregnancy rates were at the 6th hour and the lowest pregnancy rates were at the 24th hour. As the ecotexture parameters MGV and CON increased, it was determined that pregnancy rates decreased, but there was no relationship between them and the HOM value.

Acknowledgements

The authors would like to thank the Şanlıurfa abrasion station team for all the valuable assistance provided.

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